

Presence of β -glucosidase (*bgl1*) gene in *Phaeosphaeria nodorum* and *Phaeosphaeria avenaria* f.sp. *triticea*

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Abstract: *Phaeosphaeria avenaria* f.sp. *avenaria* (Paa), the causal agent of stagonospora leaf blotch in oats, produces a glycosyl hydrolase family 3 enzyme, β -glucosidase, which is responsible for detoxification of steroidal avenacosides in oat leaves, but is not essential for pathogenicity. For a comparative genetic relatedness study, a Paa-like β -glucosidase gene (*bgl1*) was PCR-amplified from *Phaeosphaeria nodorum* and *P. avenaria* f.sp. *triticea*, and *Phaeosphaeria* spp. from dallis grass (*Paspalum dilatatum*) (isolate S-93-48) and rye (*Secale cereale*) (isolate Sn48-1). Different sizes of *bgl1* gene coding sequences ranging from 3018 to 3023 bp were determined. The *bgl1* gene structure in these *Phaeosphaeria* species was identical to that of Paa and contained four exons and three introns. Nucleotide variations occurring in introns 1 and 2 of the *bgl1* gene divided wheat-biotype *P. nodorum* into four groups. Two 12-bp-long direct sequence repeats (5'-TCA/G ACT GGT TT/CA/G) were found in the promoter region of the *bgl1* gene in *Phaeosphaeria* species; only one repeat was present in the two *P. avenaria* f.sp. *triticea* isolates ATCC26370 and ATCC26377 (Pat2) from foxtail barley (*Hordeum jubatum*) and some homothallic *P. avenaria* f.sp. *triticea* isolates (Pat1). With sequence similarities in the noncoding internal transcribed spacer region of nuclear rDNA, the partial *gpd* gene fragment containing the intron 4, and the full-length *bgl1* gene, five *Phaeosphaeria* isolates (5413, 1919WRS, 1920WRS, 1921WRS, Sa37-2) from oat (*Avena sativa*) were molecularly determined to be Paa. Two oat isolates (Sa38-1 and Sa39-2) from Poland appeared to be Pat1. The results suggest that classification of two *Phaeosphaeria avenaria* formae speciales based on host specificity should be re-evaluated.

Key words: wheat, oat, *Phaeosphaeria*, β -glucosidase gene.

Résumé : Le *Phaeosphaeria avenaria* f.sp. *avenaria* (Paa), agent causal de la tache ovoïde à stagonospora chez l'avoine, produit une enzyme de la famille 3 des hydrolases du glucosyl, la β -glucosidase, laquelle est responsable de la détoxification des avenacosides stéroïdiens dans les feuilles d'avoine, mais n'est pas essentielle pour la pathogénicité. Dans le cadre d'une étude comparative sur la parenté génétique, les auteurs ont amplifié par PCR, le gène de la β -glucosidase de type Paa (*bgl1*) obtenu à partir du *Phaeosphaeria nodorum* et du *P. avenaria* f.sp. *triticea*, et du *Phaeosphaeria* spp. provenant de la digitale à larges feuilles (*Paspalum dilatatum*) (isolat S-93-48) et du seigle (*Secale cereale*) (isolat Sn48-1). Ils ont déterminé des séquences codantes de différentes dimensions du gène *bgl1*, allant de 3018 à 3023 pb. Chez ces espèces de *Phaeosphaeria*, la structure du gène *bgl1* est identique au Paa et contient quatre exons et trois introns. La variation des nucléotides présents chez les introns 1 et 2 du gène *bgl1* permet de diviser le *P. nodorum* du biotype-blé en quatre groupes. On retrouve deux répétitions d'une longue séquence directe de 12 pb (5'-TCA/G ACT GGT TT/CA/G) dans la région du promoteur du gène *bgl1*, chez les espèces du genre *Phaeosphaeria*; une seule répétition est présente chez les deux isolats du *P. avenaria* f.sp. *triticea* (ATCC26370 et ATCC26377 (Pat 2)), provenant de l'orge agréable (*Hordeum jubatum*) et de quelques isolats homothalliques du *P. avenaria* f.sp. *triticea* (Pat 1). À partir des similarités des séquences de la région non-codante de l'espaceur interne transcrit du rADN nucléaire, du fragment partiel du gène *gpd* contenant l'intron 4, et de l'ensemble du gène *bgl1*, on a pu déterminer, au plan moléculaire, que cinq isolats provenant de l'avoine (5413, 1919WRS, 1921WRS, Sa37-2) sont des Paa. Deux isolats de l'avoine (*Avena sativa*) semblent être des Pat1. Les résultats suggèrent que la classification des deux formes spéciales

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du *Phaeosphaeria avenaria*, basée sur la spécificité de l'hôte, devrait être réévaluée.

Mots clés : blé, avoine, *Phaeosphaeria*, gène de la β -glucosidase.

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Introduction

Members of glucosyl hydrolase (GH) family 3 in fungi are tremendously diverse (Henrissat and Bairoch 1993). There are five hypothetical proteins in *Aspergillus nidulans* (Eidam) G. Wint. FGSC A4 (accession Nos. XP_408041, 406965, 408239, 410113, and 410789), eight in *Gibberella zeae* (Schwein.) Petch PH-1 (accession Nos. XP_380342, 383563, 383746, 384034, 385129, 386781, 390791, and 391735) and four in *Yarrowia lipolytica* CLIB99 (accession Nos. CAG78289, 81175, 83128, and 83130), all of which contain structurally similar N and C terminal domains of GH family 3 enzymes (Dujon et al. 2004). Wide difference in sizes and amino acid sequences were found in these enzymes in *Aspergillus nidulans* (737–1203 aa and 30.0%–41.1% identical), *Gibberella zeae* (756–942 aa and 26.7%–45.8%) and *Yarrowia lipolytica* (844–963 aa and 36.5%–60.4%). The functions of GH family 3 enzymes are highly diverse and cannot be predicted from a simple elucidation of substrate ranges in vitro or by the comparison of amino acid sequences (Faure 2000). GH family 3 enzymes are involved not only in biodegradation of various polysaccharides but also in assimilation of exogenous glycosides and recycling of cell wall components (Hung et al. 2001). In addition to modification of the biological activity of glycoconjugates such as plant-derived saponins, GH family 3 proteins functioning with specific antigenic characteristics and regulation of cellulolytic enzymes have been reported (Depe and Gebbons 2001; Mach et al. 1995).

β -glucosidase (BGL1) (EC3.2.1.21) is a member of the GH family 3 enzymes, which specifically hydrolyze terminal, nonreducing β -D-glucose residues with release of a simple sugar. Some BGL1 enzymes also hydrolyze other sugar residues such as β -D-galactosides, β -D-xylosides, and β -D-fucosides. Recently, the β -glucosidase gene (*bgl1*) encoding a BGL1 protein secreted by the stagonospora leaf blotch pathogen, *Phaeosphaeria avenaria* f.sp. *avenaria* (Paa), and found to modify secondary metabolites, saponins, in oat leaves has been identified (Morrissey et al. 2000).

Several cereal leaf blotch diseases of worldwide importance are caused by *Phaeosphaeria avenaria* (G.F. Weber) O.E. Eriksson [= *Leptosphaeria avenaria* G.F. Weber], anamorph: *Stagonospora avenae* (A.B. Frank) Bissett [= *Septoria avenae* A.B. Frank] (Couture 1989; Noble and Montgomerie 1954; Weber 1922a). The identification of these pathogens is based largely on morphology of the anamorph and on host pathogenicity (Cunfer 2000; Cunfer and Ueng 1999; Richardson and Noble 1970). The size of pycnidiospores for *P. avenaria* is 18–61 $\mu\text{m} \times 1.6$ –4.7 μm , whereas for *Phaeosphaeria nodorum* (E. Müller) Hedjaroude (PN) (anamorph: *Stagonospora nodorum* (Berk.) Cast. et Germ.), another important pathogen causing leaf and glume blotch disease in cereals, the size of pycnidiospores is 14.2–32 $\mu\text{m} \times 1.9$ –4.0 μm (Holmes and Colhoun 1970; Johnson 1947; Weber 1922b). *Phaeosphaeria avenaria* have

two formae speciales. The *P. avenaria* f.sp. *avenaria* isolates (Paa) infect oats (*Avena* spp.), but not wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.), barley (*Hordeum vulgare* L.), or several grasses (Weber 1922a). In contrast, no other *Phaeosphaeria* species, including *P. nodorum* and *P. avenaria* (G.F. Weber) O. Eriksson f.sp. *triticea* T. Johnson (PAT) (anamorph: *Stagonospora avenae* Bissett f.sp. *triticea* T. Johnson), are pathogenic to oat (Meehan and Murphy 1949; Shaw 1957).

In recent years, genetic relatedness and differentiation of cereal *Phaeosphaeria* species have been re-examined at the molecular level. Restriction fragment length polymorphism (RFLP) fingerprinting as well as sequence data from the rDNA internal transcribed spacer (ITS), mating type gene (*mat1*) conserved region, and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene have been used to define genetic relationships among the wheat- and barley-biotypes in *P. nodorum* (PN-w and PN-b), three genetically distinct groups (homothallic Pat1, heterothallic Pat2 and Pat3) of *P. avenaria* f.sp. *triticea* (PAT), and *P. avenaria* f.sp. *avenaria* (Paa) (Ueng et al. 1995, 1998, 2003a, 2003b; Ueng and Chen 1994). The molecular data indicated that Paa is more closely related to PN-b, homothallic Pat1, and Pat3 than heterothallic Pat2 and PN-w.

In addition to ribosomal DNA and ITS, sequence data from genes encoding structural and functional proteins have recently been used to broaden the base of molecular characters and play important roles in phylogenetic studies of eukaryotes. One of the objectives of this study was to characterize the Paa-like *bgl1* gene in other cereal *Phaeosphaeria* species by polymerase chain reaction (PCR) amplification and determine if the Paa-like *bgl1* genes and their deduced amino acid sequences in cereal *Phaeosphaeria* species will support the evolutionary conclusions of previous studies based on RFLP fingerprinting, and sequences of ITS, *mat1* conserved regions and *gpd* genes (Ueng et al. 1995, 1998, 2003a, 2003b; Ueng and Chen 1994). Another objective was to use the sequence variation of the *bgl1* gene to develop a rapid assay for important cereal *Phaeosphaeria* species and provide molecular evidence to support species designations. A reliable molecular method for *Phaeosphaeria* identification would help diagnose cereal *Stagonospora* and *Septoria* diseases and identify potential disease control strategies.

Materials and methods

Fungal isolates and DNA isolation

Nine isolates of the wheat-biotype *P. nodorum* (PN-w), five barley-biotype *P. nodorum* (PN-b), eight *P. avenaria* f.sp. *triticea* (PAT) isolates comprising five homothallic isolates (Pat1), two heterothallic isolates from foxtail barley (*Hordeum jubatum* L.) (Pat2), and one isolate from Washington state (Pat3), and eleven *P. avenaria* f.sp. *avenaria* iso-

Table 1. Isolates of *Phaeosphaeria* species and GenBank accession numbers for β -glucosidase (*bgII*) gene sequences used for genetic analysis.

Species	Original host	Year isolated	Geographic location	GenBank accession No.
<i>Phaeosphaeria nodorum</i> (wheat-biotype) (PN-w)				
Sn26-1	Wheat (<i>Triticum aestivum</i> L.)	—	Rzeszów, Poland	AY683618
9074	Wheat	1983	Gallatin County, Montana	(=AY683618)
9076	Wheat	1986	Richland County, Montana	(=AY683618)
8408	Wheat	1986	Mandan, North Dakota	(=AY683618)
9506	Wheat	1987	Mandan, North Dakota	(=AY683618)
S-80-301	Triticale (\times Triticosecale)	1980	Bledsoe, Georgia	(=AY683618)
Sn37-1	Wheat	—	Szelejowo, Poland	AY683619
Sn27-1	Wheat	—	Sieradz, Poland	(=AY683619)
S-74-20A (ATCC200806)	Wheat	1975	Griffin, Georgia	(=AY683619)
<i>Phaeosphaeria nodorum</i> (barley-biotype) (PN-b)				
S-83-2 (ATCC200841)	Barley (<i>Hordeum vulgare</i> L.)	1983	Tifton, Georgia	AY683617
S-82-13 (ATCC200805)	Barley	1982	Senoia, Georgia	(=AY683617)
S-83-7	Barley	1983	Holland, Virginia	(=AY683617)
S-84-2	Barley	1984	Moultrie, Georgia	(=AY683617)
S-92-7	Barley	1992	Raleigh, North Carolina	(=AY683617)
<i>Phaeosphaeria avenaria</i> f.sp. <i>triticea</i> (Pat1)				
Sat24-1	Wheat	—	Warmińsko-Mazurskie, Poland	AY683613
10052-2	Wheat	1988	Langdon, North Dakota	(=AY683613)
12618	Wheat	1995	Dickinson, North Dakota	(=AY683613)
12889	Wheat	1997	Mandan, North Dakota	(=AY683613)
13061	Barley	1998	Morton County, North Dakota	(=AY683613)
<i>Phaeosphaeria avenaria</i> f.sp. <i>triticea</i> (Pat2)				
ATCC26370	Foxtail barley (<i>H. jubatum</i> L.)	—	Minnesota	AY683614
ATCC26377	Foxtail barley	—	Minnesota	(=AY683614)
<i>Phaeosphaeria avenaria</i> f.sp. <i>triticea</i> (Pat3)				
S-81-W10	Wheat	1981	Washington	AY683615
<i>Phaeosphaeria avenaria</i> f.sp. <i>avenaria</i> (Paa)				
ATCC12277	Oat (<i>Avena sativa</i> L.)	—	USA	AY683612
1919WRS	Oat	2002	Manitoba, Canada	(=AY683612)
SAA001NY-85	Oat	1985	New York	AY688370
SAT001NY-84 (ATCC58582)*	Wheat	1984	New York	Not determined in full
SAT002NY-84 (ATCC58583)*	Wheat	1984	New York	AY688371
5413	Oat	1983	Ontario, Canada	AY688369
1920WRS	Oat	2002	Manitoba, Canada	AY688367
1921WRS	Oat	2002	Manitoba, Canada	AY688368
Sa37-2	Oat ('Cacko')	2001	Radzików, Poland	(=AY688368)
Sa38-1	Oat ('Santor')	2001	Radzików, Poland	AY688365
Sa39-2	Oat	2001	Radzików, Poland	AY688366
<i>Phaeosphaeria</i> sp.				
Sn48-1	Winter rye (<i>Secale cereale</i> L.)	1995	Jelenia Góra, Poland	AY683620
S-93-48	Dallis grass (<i>Paspalum dilatatum</i> Poir.)	1993	Griffin, Georgia	AY683616

*Based on pycnidiospore morphology and host specificity, two isolates, SAT001NY-84 (ATCC58582) and SAT002NY-84 (ATCC58583), from wheat were reported to be Pat1 by Luz and Bergstrom (1985). Based on gene sequences analyses (see Table 6), they are Paa.

lates were used for Paa-like *bgII* gene sequence analysis (Table 1). *Phaeosphaeria* sp. isolate S-93-48 from dallis grass (*Paspalum dilatatum* Poir.) and *P. nodorum*-like rye isolate Sn48-1 were included. Procedures for growing fungal cultures in a liquid medium and for isolation of genomic DNA have been described previously (Ueng et al. 1992).

Gene amplification, sequencing, and data analysis

Seven primer sets, Bg4A/Bg4B, Bg11A/Bg11B, Bg2A/Bg2B, Bg3A/Bg3B, Bg10A/Bg10B, Bg32A/Bg31B, and Bg1A/Bg1B, were designed from a *bgII* genomic sequence of Paa WAC1293 (GenBank accession No. AJ276675) for PCR amplification of the same gene from

Table 2. Primers used to amplify the *bgl1* gene in *Phaeosphaeria* species.

Name	Nucleotide positions*	Sequence 5'-3'	Pat					
			Paa	Pat1	Pat2	Pat3	Pn (barley-biotype)	Pn (wheat-biotype)
Bg4A / Bg4B	2520-2538 / 3523-3502	CGGCCCGAGTGTGAGAG / CTGTGCCGGTTGTGAGGTTGAC	X	X	X	X	X	X
Bg11A / Bg11B	3357-3380 / 3770-3751	CCACAGCCGTCTAAGCAACATCA / CCGAGAGGGCCGACGACTGG	X	X		X	X	X
Bg11C / Bg11D	3357-3380 / 3770-3751	CCACAACCCGTCCCAAGAAACACAC / CCAAGCGGGCCGACGACTGG			X			
Bg2A / Bg2B	3666-3687 / 4548-4527	AGCATGGACCGTGGCGAGTGG / GTGCGGGAATGCTGCGGAAGT	X	X	X	X	X	X
Bg3A / Bg3B	4411-4429 / 5184-5162	GGCCCGCAITCCTGTCAAC / TACTCGGCTGGTCGCTACCAAT	X	X	X	X		X
Bg7A / Bg3B	4706-4727 / 5184-5162	GGCTGGGGCTCCGGAACATCTG / TACTCGGCTGGTCGCTACCAAT					X	
Bg9A / Bg9B	4376-4399 / 4827-4807	ATTATGCCCGCCTCTATTATGTC / TGAGTGTATGCCCAGTTGTCCG					X	
Bg10A / Bg10B	4974-4995 / 5529-5510	TTTCCTCGGCTGTGCAACAACAC / TGGGAGGCCGCTTTCAGGTC	X	X	X	X	X	X
Bg1A / Bg1B	5453-5475 / 6267-6244	TGGCCACGGACTTCACTACAT / CCGCGAATTTTCCCACCTCCTCAC	X			X		X
Bg1AA / Bg1B	5359-5380 / 6267-6244	GAAGGCCAACGCTGGGCAATAC / CCGCGAATTTTCCCACCTCCTCAC		X	X		X	
Bg32A / Bg31B	5257-5278 / 5763-5742	CATCGACTACCGTGCTTTTGAC / TCAAAGCCACGGAGGACAATCT	X	X	X	X	X	X

Note: Paa, *Phaeosphaeria avenaria* f.sp. *avenaria*; Pat, *Phaeosphaeria avenaria* f.sp. *triticea*; Pn, *Phaeosphaeria nodorum*.

*Nucleotide positions relative to the β -glucosidase gene (*bgl1*) sequence in *P. nodorum* isolate WAC1293 (accession No. AJ276675).

Table 3. Structure of the β -glucosidase (*bgII*) gene in *Phaeosphaeria* species.

Species	Representative isolate	Size (bp)	Intron size (bp)			No. of nucleotide substitutions	No. of amino acid substitutions
			Intron1	Intron2	Intron3		
<i>P. avenaria</i> f.sp. <i>avenaria</i> (Paa)	WAC1293	3007	166	176	49	61	9
<i>P. avenaria</i> f.sp. <i>avenaria</i> (Paa)	ATCC12277	3018	166	178	49	—	—
<i>P. avenaria</i> f.sp. <i>triticea</i> (Pat1)	Sat24-1	3018	166	178	49	134	12
<i>P. avenaria</i> f.sp. <i>triticea</i> (Pat2)	ATCC26370	3023	166	179	53	344	47
<i>P. avenaria</i> f.sp. <i>triticea</i> (Pat3)	S-81-W10	3018	166	178	49	128	12
<i>P. nodorum</i> (wheat-biotype) (PN-w)	Sn27-1 and Sn37-1	3019	166	178	50	218–224	15
<i>P. nodorum</i> (barley-biotype) (PN-b)	S-83-2	3019	166	179	49	129	11
<i>Phaeosphaeria</i> spp. (from rye)	Sn48-1	3020	166	179	50	225	17
<i>Phaeosphaeria</i> spp. (From dallis grass)	S-93-48	3018	166	178	49	135	14

Pat3, PN-w, Sn48-1, S-93-48 and other Paa isolates (Table 2) (Morrissey et al. 2000). The primer sets produced overlapping fragments that together represent the full-length *bgII*-coding region. In addition, more primer sets were designed to construct the *bgII* gene in Pat1, Pat2, and PN-b (Table 2). The Bg1AA/Bg1B primer set was used to amplify a DNA fragment corresponding to Bg1A/Bg1B amplified product in Paa. The primer set Bg11C/Bg11D, which was similar to Bg11A/Bg11B with nucleotide substitutions, was used in *bgII* gene amplification in Pat2. In PN-b, two DNA fragments produced by primer sets Bg7A/Bg3B and Bg9A/Bg9B would overlap the Bg3A/Bg3B-generated fragment in other *Phaeosphaeria* species. PCR amplification and direct sequencing protocols have been described previously (Ueng et al. 2003b).

To identify seven *Phaeosphaeria* isolates (5413, 1919WRS, 1920WRS, 1921WRS, Sa37-2, Sa38-1, and Sa39-2) from oats (Tables 1 and 6), sequence variations in the rDNA ITS, the partial *gpd* gene fragment containing intron 4, and the full-length *bgII* gene were compared. DNA amplification and sequencing followed the procedures previously described, and ITS and *gpd* gene sequence information from Paa (GenBank accession Nos. U77358 and AY364459) and Pat1 (GenBank accession Nos. U77359, AY196988, and AY364463) isolates were used as references (Ueng et al. 1998, 2003b).

The full-length *bgII* gene sequences and their putative amino acid translations were used for the phylogenetic relationship study of cereal *Phaeosphaeria* species. A BGL1-like hypothetical protein (875 amino acid in length) of *Neurospora crassa* (Accession EAA26868) was used as an outgroup. The nucleotide and amino acid sequences were aligned separately with CLUSTALX (1.83) in a multiple sequence alignment mode (Thompson et al. 1997). From the aligned sequences, 1000 data sets were generated by bootstrap resampling in the “seqboot” program of Phylogeny Inference Package (Phylip) Version 3.6 (alpha2) (<http://evolution.genetics.washington.edu/phylip.html>). The bootstrapped data sets were evaluated by the maximum likelihood method using the “dnaml” program for DNAs and the “proml” program for peptides. Finally, the “consense” program was used to construct a tree (Felsenstein 1989).

Results

Sequence and structure of Paa-like *bgII* gene

Consensus sequences determined from overlapping fragments, amplified using the primer sets described in Table 2, varied in length among *Phaeosphaeria* species for the coding region of the Paa-like *bgII* gene (from 3007 to 3023 bp, see Table 3). The *bgII* gene first identified in Paa isolate WAC1293 (accession No. AJ276675) was shorter (3007bp) than those of PN-w, PN-b, PAT, and other Paa isolates used in this study. A 9-bp long fragment (5'-TCG/A T/ACC CTG), located between nucleotides 12 (T) and 13 (G), that encodes “Ser-Thr-Leu” in Paa and “Ser-Ser-Leu” in other *Phaeosphaeria* isolates was absent in the *bgII* gene of this isolate (Fig. 1).

In general, nucleotide substitutions within a species were fewer in number than between species. In comparison with two Paa isolates ATCC12277/1919WRS, there were 61 nucleotide substitutions in WAC1293, 20 in 1921WRS/Sa37-2, 17 in SAT002NY-84, 15 in SAA001NY-85, 14 in 1920WRS, and 11 in the 5413 *bgII* gene coding region. However, sequence differences between the Paa isolate ATCC12277 and other *Phaeosphaeria* species were 128 to 344 bp (see Table 3).

Sequence variations were mainly found in the Paa-like *bgII* gene fragments amplified by the Bg4A/Bg4B primer set (Table 2). In the Bg4A/Bg4B-amplified gene fragment that includes the promoter region and 5' end coding region, two 12-bp-long direct sequence repeats (5'-TCA/G ACT GGT TT/CA/G) were located in the upstream region (nt 88) of the *bgII* gene coding sequence in *Phaeosphaeria* species; only one repeat was present in heterothallic Pat2 and some homothallic Pat1 isolates (Fig. 2). Upon further analysis of the promoter region of the *bgII* gene in 34 homothallic Pat1 isolates, 24 had two direct repeats and the other 10 had one (Table 4). In 43 PN-w isolates, nucleotide substitutions occurring at 14 positions of the Bg4A/Bg4B-amplified gene fragment (nt -288, -23, 71, 96, 97, 244, 323, 324, 330, 444, 465, 470, 488, and 517 according to the *bgII* gene coding sequence) could divide them into four groups: A (t, c, c, g, c, g, g, a, g, t, a, a, a, g), B (c, a, t, a, a, c, g, c, c, c, g, g, a), C (t, c, c, g, c, c, g, c, c, c, c, g, g, a), and D (c, c, c, a,

Fig. 1. (continued).

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DKARIPVNFDSWSRDTYGFHDHYYGKAGYSQINSHVDVRADHFRSIRRRTAAMSTVLLKNEG      420
.....
.....EV.....
.....LE.F.....GL.Q.....
.....V.....
.....E.....A.....
.....T.....F.....Q.....
.....T.....F.....Q.....

ALPLTGSEKWTAVFGDDAGDQGLGPNGFDPDHGGNNGLTAMGWGSGTSDYPYLVTPLESIK      480
.....E.....
.....E.....
.....N.....E.K.....F.....
.....E.....
.....E.....
.....E.....
.....E.....
.....E.....

ATVAQNGGVVTSVTDNWAYTQIQTLAKQASVAIVFVNADSGEGYITVDGNAGDRNNLTLW      540
.....I.....
.....G.....
.....G.....M.....
.....G.....
.....G.....
.....G.....
.....G.....

QDGDTLIKNVSSLCNNTIVVIHSVGPVLVNSFYDSENVTAILWAGLPGQESGNAIADILY      600
.....
.....V.....
.....
.....
.....V.....
.....V.....

GRHNPGGKLPFTIGSDAAEYGPDLIYEPTNNSSPQDNFEEGVFIDYRAFQKQNVTPPIYE      660
.....
.....N.....I.....
.....
.....
.....

FGFGLSYTKFSYSNLTVKKANAGAYTPATGQSKAAPT LGNFSTDASQYQWPSDFTYVDTF      720
.....I.....
.....N.....T.LT.....NI.....N.....
.....
.....T.....N.....
.....T.....N.....
    
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a, g, a, a, g, t, a, a, a, g), with the majority of wheat-biotype *P. nodorum* isolates belonging to group A (Table 5).

The Paa-like *bgll* gene structure in the *Phaeosphaeria* species examined was identical and contained four exons and three introns (Table 3). Based on the nucleotide sequence of the Paa-like *bgll* gene, isolate S-93-48 from dallis grass was more closely related to Pat3, PN-b, Paa, and

homothallic Pat1 than heterothallic Pat2 from foxtail barley, Sn48-1 rye isolates and PN-w (Fig. 3A).

Analysis of the deduced amino acid sequence

The 2625-bp sequence in four exons encoded an 874-amino-acid protein. Many of the nucleotide changes occurred in introns or in the third position of amino acid

Fig 2. Direct sequence repeats present in the region upstream of the β -glucosidase gene (*bglI*) coding sequence in *Phaeosphaeria* species. *Phaeosphaeria avenaria* f.sp. *avenaria* (Paa), three *P. avenaria* f.sp. *triticea* (homothallic Pat1, heterothallic Pat2 and Pat3), two *P. nodorum* (barley-biotype (PN-b) and wheat-biotype (PN-w)), Sn48-1 isolate from rye, and S-93-48 isolate from dallis grass are included. The numbers in square brackets are fungal isolates. The position of the repeat (nucleotide number in relation to the first translation codon ATG, designated as +1) is in parentheses.

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CGCGGTTCGACTGGTTCATCAACTGGTTTACTTTGGCAGCCAAATCT (-72) Paa
CGCGG-----TCAACTGGTTTACTTTGGCAGCCAAATTT (-72) Pat1 [Sat24-1]
CGCGGTCAACTGGTTTATCAACTGGTTTACTTTGGCAGCCAAATTT (-72) Pat1 [12889]
CGCGGTCAACTGGTTTCTC---TGGT---AGCCAACA-CGAAACCC (-66) Pat2
CGCGGTTCGACTGGTTCATCAACTGGTTTACTTTGGCAGCCAAATCT (-77) Pat3
CGCGGTCAACTGGTTCATCAACTGGTTTGCCTTGATAGCCACATCT (-72) PN-b
CGCGGTCAACTGGTTTACTCAACTGGTTTACTCTGGCAGCCACATTT (-72) PN-w
CGCGGTCAACTGGTTTACTCAACTGGTTTACTCTGGCAGCCACATTT (-72) [Sn48-1]
CGCGGTCAACTGGTTCATCAACTGGTTTACTTTGGCAGCCAAATCT (-72) [S-93-48]

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Table 4. Number of direct sequence repeats (5'-TCAACTGGTTTA) in the upstream (-88 bp) of the β -glucosidase gene (*bglI*) coding sequence in homothallic *Phaeosphaeria avenaria* f.sp. *triticea* (Pat1).

Isolates	Original host	Year	Geographic location	No. of repeats
10052-2*	Durum wheat (<i>Triticum durum</i> Desf.)	1988	Langdon, North Dakota	2
12618*	Wheat (<i>Triticum aestivum</i> L.)	1995	Minot, North Dakota	1
12889*	Wheat	1997	Mandan, North Dakota	2
12939-1	Wheat	1997	Mandan, North Dakota	2
13050-2†	Barley (<i>Hordeum vulgare</i> L.)	1998	Dunn County, North Dakota	1
13061*	Barley	1998	Morton County, North Dakota	2
13070	Barley	1998	Dickinson, North Dakota	2
13077-2	Barley	1998	Towner County, North Dakota	2
13079-1	Barley	1998	Crookston, Minnesota	2
13088-1	Barley	1998	Minot, North Dakota	2
13091-1	Barley	1998	Minot, North Dakota	2
13113	Barley	1998	Mandan, North Dakota	2
13114	Barley	1998	Mandan, North Dakota	2
13181	Wheat	1998	Mandan, North Dakota	2
13188-1	Wheat	1998	Mandan, North Dakota	2
13334	Barley	1999	Mandan, North Dakota	2
13387	Wheat	2000	Mandan, North Dakota	2
13395	Wheat	2000	Mandan, North Dakota	2
13408	Barley	2000	Mandan, North Dakota	2
13413-2	Barley	2000	Mandan, North Dakota	1
13556-1	Switch grass (<i>Panicum virgatum</i> L.)	2000	Mandan, North Dakota	2
13557	Switch grass	2000	Mandan, North Dakota	1
Sat 2-1	—	1988	Warمیńsko-Mazurskie, Poland	1
Sat 7-1	Triticale (\times Triticosecale) 'Grado'	1990	Warمیńsko-Mazurskie, Poland	2
Sat 16-1	Triticale	1995	Mazowieckie, Poland	1
Sat 17-1	Winter wheat 'Almari'	1995	Śląskie, Poland	2
Sat 22-1	Rye W-1	1996	Podkarpackie, Poland	2
Sat 23-2	Spring triticale 'Gabo'	1995	Mazowieckie, Poland	2
Sat 24-1*	Wheat	1995	Warمیńsko-Mazurskie, Poland	1
Sat 29-1	Spring barley	1995	Warمیńsko-Mazurskie, Poland	2
Sat 32-2	Winter wheat 'Panda'	1995	Lubuskie, Poland	1
Sat 36-6	Winter wheat 'Almari'	1995	Warمیńsko-Mazurskie, Poland	1
Sa38-1*†	Oat (<i>Avena sativa</i> L.)	2001	Radzików, Poland	2
Sa39-2*†	Oat	2001	Radzików, Poland	1

*Seven Pat1 isolates were used for full-length *bglI* gene sequence analysis (see Table 1).

†Based on gene sequences analyses (see Table 6), two isolates, Sa38-1 and Sa39-2, from oat were Pat1.

Two saponin-hydrolysing enzymes, BGL1 and α -L-rhamnosidase, were purified from the Paa isolate WAC1293 culture filtrate (Hughes et al. 2004; Morrissey et al. 2000).

The BGL1 enzyme, which hydrolyses the β -D-glucose moiety of steroidal avenacosides in oat leaves, was not closely related to either avenacinasases or tomatinasases based on

Table 5. Grouping of wheat-biotype *Phaeosphaeria nodorum* (PN-w) isolates based on nucleotide substitutions in the Bg4A/Bg4B primer-amplified fragment of β -glucosidase gene (*bgl1*).

Isolate	Original host	Geographic location	Year isolated	Type of partial <i>bgl1</i> gene sequence*
238	Wheat (<i>Triticum aestivum</i> L.)	New York	1994	A
240	Wheat	New York	1994	A
242	Wheat	New York	1994	A
406	Wheat	New York	1994	A
408	Wheat	New York	1994	A
409	Wheat	New York	1994	A
410	Wheat	New York	1994	A
414	Wheat	New York	1994	A
417	Wheat	New York	1994	A
8408 [†]	Wheat	Mandan, North Dakota	1986	A
9074 [†]	Wheat	Gallatin County, Montana	1983	A
9076 [†]	Wheat	Richland County, Montana	1986	A
9506 [†]	Barley (<i>Hordeum vulgare</i> L.)	Mandan, North Dakota	1987	A
IN37	Wheat	Indiana	1993	A
IN38	Wheat	Indiana	1993	A
IN46	Wheat	Indiana	1993	A
IN9011	Wheat	Indiana	1993	A
S-80-301 [†]	Triticale	Williamson, Georgia	1980	A
S-80-402	Triticale	Williamson, Georgia	1980	A
S-80-604	Little barley (<i>H. pusillum</i> Nutt.)	Williamson, Georgia	1980	A
S-80-607	Little barley	Williamson, Georgia	1980	A
S-81-616	Little barley	Griffin, Georgia	1981	A
S-82-1	Little barley	Griffin, Georgia	1982	A
S-82-4	Little barley	Southern Georgia	1982	A
S-82-6	Little barley	Georgia	1982	A
S-82-15B	Little barley	Rehobeth, Georgia	1982	A
S-82-18A	Ryegrass (<i>Lolium perenne</i> L.)	Rehobeth, Georgia	1982	A
S-82-18B	Ryegrass	Rehobeth, Georgia	1982	A
Sn26-1 [†]	Wheat	Rzeszow, Poland		A
237	Wheat	New York	1994	B
403	Wheat	New York	1994	B
405	Wheat	New York	1994	B
IN43	Wheat	Indiana	1993	B
S-74-20A [†]	Wheat	Griffin, Georgia	1975	B
Sn27-1 [†]	Wheat	Sieradz, Poland		B
Sn37-1 [†]	Wheat	Szelejewo, Poland		B
407	Wheat	New York	1994	C
420	Wheat	New York	1994	C
IN15	Wheat	Indiana	1993	C
S-81-B13B	Barley	Williamson, Georgia	1981	C
411	Wheat	New York	1994	D
412	Wheat	New York	1994	D
IN39	Wheat	Indiana	1993	D

*The type of the partial *bgl1* gene sequence that resulted from the nucleotide substitutions at 14 positions of Bg4A/Bg4B-amplified gene fragments (see the Results).

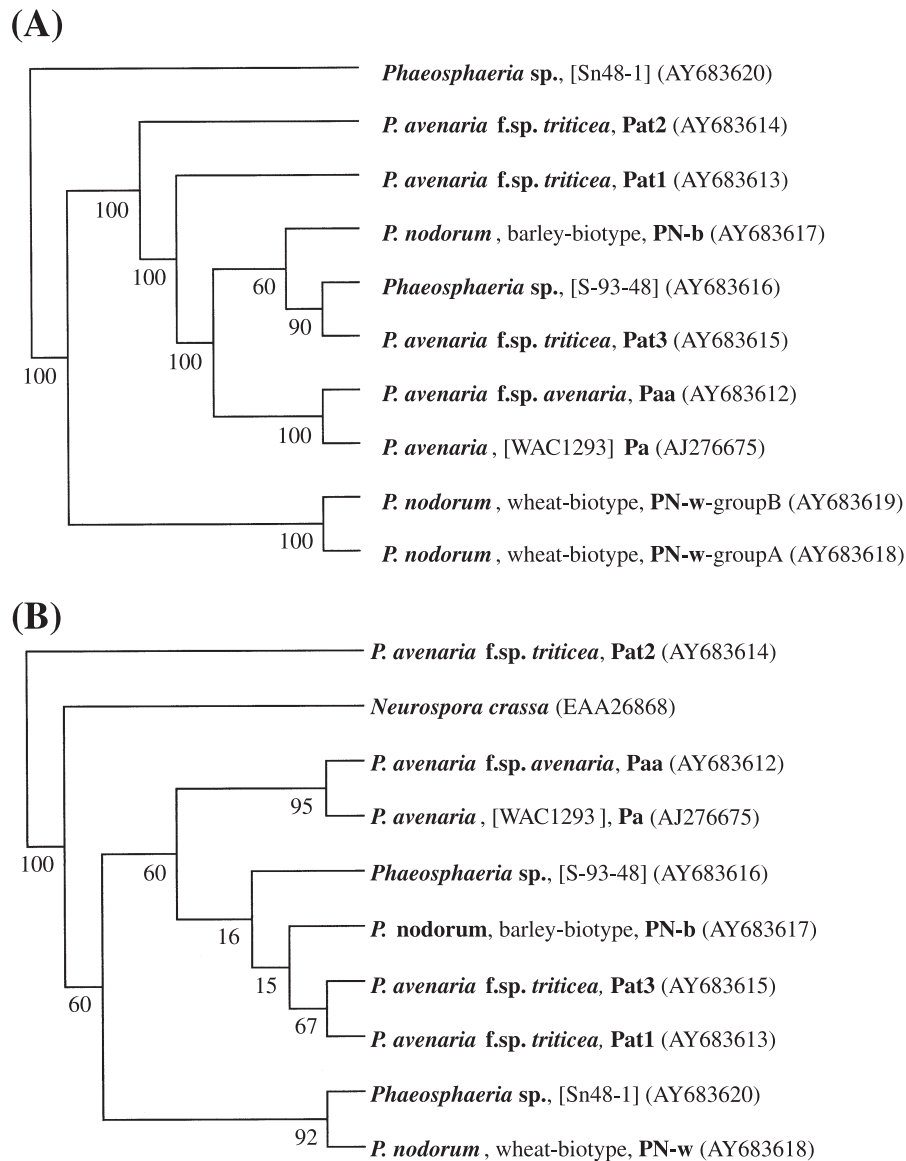
[†]Nine PN-w isolates were used for full-length *bgl1* gene sequence analysis (see Table 1).

phylogenetic analysis of their amino acid sequences (Morrissey et al. 2000; Cournoyer and Faure 2003). Similarity in the protein structures and the conserved domains suggests that these three proteins should share common nonspecific hydrolytic functions acting as cellulose-, cellobiose-, and xylan-degrading enzymes (Henrissat and Bairoch 1993). Nevertheless, unlike the BGL1 protein from

Paa, avenacinase from *G. graminis* var. *avenae* and tomatinase from *Septoria lycopersici*, which are similar in amino acid sequences, are related by pathogenicity and have different substrate specificities (Osborn et al. 1995).

Since the saponin-hydrolyzing BGL1 protein from Paa isolate WAC1293 was not related to fungal pathogenicity (Morrissey et al. 2000), it was not surprising that a Paa-like

Fig 3. Phylogenetic relationships based on nucleotide (A) and deduced amino acid (B) sequences of the β -glucosidase gene (*bgl1*) in *Phaeosphaeria* pathogens. The numbers in parentheses and in square brackets are GenBank accession numbers and fungal isolates, respectively. For nucleotide sequence substitutions in wheat-biotype *P. nodorum* groups A and B, see Table 4 and Results. Bootstrap values (with 1000 replications) of the internal branches are indicated. The relationships within the branches supported by less than 50% were not considered significant.



bgl1 gene was present in other cereal *Phaeosphaeria* species. It might also suggest that the reason other *Phaeosphaeria* species, including PN and PAT, are non-pathogenic to oat is not due to the absence of a saponin-hydrolyzing BGL1 protein. It is unclear if the other saponin-hydrolyzing enzyme α -L-rhamnosidase, which removes the α -L-rhamnose at the C3 position of the steroidal avenacosides, is significantly attributed to Paa infectivity in oats (Hughes et al. 2004). Genetic factors, such as the gene encoding malate synthase, an essential enzyme for lipid catabolism in organisms, are reportedly related to pathogenicity in wheat-biotype *P. nodorum* (Solomon et al. 2004).

Diversity of the *bgl1* gene in cereal *Phaeosphaeria* species appears to follow the same phylogenetic relatedness and support the evolutionary conclusions of previous studies

based on the sequence data from the rDNA ITS, the *mat1* gene conserved region, and the *gpd* gene (Ueng et al. 1998, 2003a, 2003b). The Paa-like *bgl1* genes and their deduced amino acid sequences in cereal *Phaeosphaeria* species would be useful as a new gene system for fungal molecular taxonomy.

Stagonospora and *Septoria* leaf blotch diseases of cereal are a disease complex caused by a number of fungi that are not always closely related (Cunfer and Ueng 1999; Scharen and Sanderson 1985; Sprague 1950). *Septoria* species are classified largely based on fungal morphology and host pathogenicity. *Septoria* species important on cereals are placed in the Loculoascomycetes, with known teleomorphs in *Mycosphaerella* (anamorph *Septoria*) and *Phaeosphaeria* (anamorph *Stagonospora*). Since pycnidiospore size varies

Table 6. Identification of *Phaeosphaeria avenaria* isolated from oats based on DNA sequence data.

Isolate	Host	Gene sequence similarity		
		ITS fragment	<i>gpd</i> gene	<i>bgl1</i> gene
5413	Oat	Paa (U77358)	Paa (AY364459)	Paa (AY688369)
1919WRS	Oat	Paa (U77358)	Paa (AY364459)	Paa (AY6883612)
1920WRS	Oat	Paa (U77358)	Paa (AY364459)	Paa (AY688367)
1921WRS	Oat	Paa (U77358)	Paa (AY364459)	Paa (AY688368)
Sa37-2	Oat	Paa (U77358)	Paa (AY364459)	Paa (AY688368)
ATCC12277	Oat	Paa (U77358)	Paa (AY364459)	Paa (AY683612)
SAA001NY-85	Oat	Paa (U77358)	Paa (AY364459)	Paa1 (AY688370)
Sa38-1	Oat	Pat1 (U77359)	Pat1 (AY364463)	Pat1 (AY688365)
Sa39-2	Oat	Pat1 (AY196988)	Pat1 (AY364463)	Pat1 (AY688366)
SAT001NY-84 [†]	Wheat	Paa (U77358)	Paa (AY364459)	Paa*
SAT002NY-84 [†]	Wheat	Paa (U77358)	Paa (AY364459)	Paa (AY688371)

Note: The ITS region of ribosomal DNA and genes encoding glyceraldehyde-3-phosphate dehydrogenase (*gpd*) and β -glucosidase (*bgl1*) were compared. Sequences from *P. avenaria* f.sp. *avenaria* (Paa) isolate ATCC12277 and homothallic *P. avenaria* f.sp. *triticea* (Pat1) isolates Sat004Cnd-96 and Sat10052-2 were used as reference sequences. Sequences similar to those accession numbers deposited in GenBank are in parentheses.

*Only the DNA fragment amplified by the Bg2A/Bg2B primer set was compared.

[†]Based on pycnidiospore morphology and host specificity, two fungal isolates, SAT001NY-84 and SAT002NY-84, from wheat were reported to be Pat1 by Luz and Bergstrom (1985).

Table 7. Comparison of endonuclease restriction of PCR-amplified β -glucosidase (*bgl1*) gene products in *Phaeosphaeria* leaf pathogens. Bg2A/Bg2B primer set was used to amplify the fragments from *P. avenaria* f.sp. *avenaria* (Paa), *P. avenaria* f.sp. *triticea* (Pat), and *P. nodorum* (PN).

Endonuclease enzyme	Restriction site	Pat				PN-w (wheat-biotype)	PN-b (barley-biotype)
		Paa 883 bp	Pat1 883 bp	Pat2 887 bp	Pat3 883 bp		
<i>Bam</i> HI	426	458, 425	458, 425	—	458, 425	—	458, 425
<i>Cl</i> aI		—	—	—	—	719, 165	—
<i>Eco</i> RI	241	643, 240	—	—	643, 240	—	—
<i>Kpn</i> I	643	642, 241	642, 241	—	642, 241	—	642, 241
<i>Pst</i> I	490	489, 394	—	—	489, 394	—	—
<i>Pvu</i> II	773	771, 112	—	775, 112	—	772, 112	771, 112
<i>Xho</i> I	799	—	—	798, 89	—	—	—

Note: Protocols for PCR amplification were described in a previous study (Ueng et al. 2003b). After PCR reaction, a sample (4 μ L) was used for enzymatic restrictions following the standard procedures (Promega, Madison, Wisconsin, USA). Fragment sizes are given in base pairs (bp). —, not cut by endonuclease enzymes.

within single-spore cultures derived from the same isolate and is influenced by environmental conditions such as culture media and temperature, *Phaeosphaeria* species identification is complicated and difficult (Hooker 1957; Johnson 1952). Application of molecular approaches to study phylogenetic relationships in *Phaeosphaeria* and other ascomycetes may facilitate the identification of *Septoria* complex in cereal leaf blotch diseases.

Recently, PCR-based methods have been used to distinguish plant pathogens in a mixed infection and to detect fungicide resistance and genetic variation in a pathogen population (Fraaije et al. 1999; McCartney et al. 2003; Rohel et al. 2001, 2002). Specific primer sets designed from β -tubulin (*tub*) gene sequences are used to detect and quantify *Mycosphaerella graminicola* [Fuckel] J. Schrt. in Cohn (anamorph: *Septoria tritici* Roberge in Desmaz), *P. nodorum*, and two rust fungi on diseased wheat (Fraaije et

al. 2001). The avenacinase-like gene sequences in cereal take-all fungal pathogens, *G. graminis* var. *avenae*, *G. graminis* (Sacc.) Arx & D. Olivier, and *G. graminis* (Sacc.) Arx & D. Olivier var. *tritici* I. Walker, have also been PCR amplified for molecular identification of the pathogens (Rachdawong et al. 2002). To complement the previous studies using ITS region sequences, the *mat1* gene conserved region, and the partial *gpd* gene to distinguish cereal and other unidentified *Phaeosphaeria* species (Ueng et al. 1998, 2003a, 2003b), fingerprinting of enzymatic restrictions of Paa-like *bgl1* gene fragments amplified with the Bg2A/Bg2B primer set was used (Table 7). The endonuclease restriction sites were deduced from the sequencing data and were experimentally demonstrated by enzymatic restrictions and agarose gel electrophoresis in all isolates listed in Table 1 (P.P. Ueng, unpublished data). Nucleotide substitutions occurring in the amplified *bgl1* gene fragments in

various isolates did not affect the enzymatic restrictions and the banding patterns in agarose gels. We conclude that digestion by *ClaI* and *XhoI* can identify wheat-biotype *P. nodorum* and Pat2 isolates, respectively. Digestion with a combination of five other endonuclease enzymes (*BamHI*, *EcoRI*, *KpnI*, *PstI*, and *PvuII*) can be used to distinguish other cereal *Phaeosphaeria* species.

Our results suggest that separation of morphologically similar *P. avenaria* f.sp. *avenaria* isolates from *P. avenaria* f.sp. *triticea* based simply on host pathogenicity is not a dependable criterion. Based on molecular analyses, two isolates from oats (Sa38-1 and Sa39-2) are likely to belong to Pat1, the homothallic group of *P. avenaria* f.sp. *triticea* (Table 6). On the contrary, two *Phaeosphaeria* isolates from wheat (SAT001NY-84 and SAT002NY-84), considered to be Pat1 by Luz and Bergstrom (1985), should be classified as Paa (Table 6, Ueng et al. 1998, 2003a, 2003b). One of the possible reasons that some Pat1 isolates (Sa38-1 and Sa39-2) were able to infect the oat cultivar ‘Santor’ is that some oat cultivars may contain low levels of antifungal saponins in their leaves (Trojanowska et al. 2001). The presence of other resistance factors in these oat cultivars may affect their host-pathogen interactions (Papadopoulou et al. 1999). Alternatively, isolation of “unusual” fungal pathogens such as Pat1 from oats might be the result of secondary infections. Further inoculation tests are required to establish the true oat-pathogen relationship.

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