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Alternative splicing and genetic diversity of the white collar-1 (*wc-1*) gene in cereal *Phaeosphaeria* pathogens

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Abstract The white collar-1 (*wc-1*) gene encodes an important light-responsive protein (wc-1) that maintains circadian clocks and controls numerous light-dependent reactions including sporulation in ascomycete fungi. The structure and expression of the *wc-1* gene in wheat-biotype *Phaeosphaeria nodorum* (PN-w) was studied. It was shown that the full-size (3,353 bp in length) *wc-1* gene in PN-w contained 4 introns in which introns 1 and 2 were flanked by GC-AG splice borders and were spliced constitutively. However, introns 3 and 4 of the *wc-1* gene were alternatively spliced. As the result of alternative splicing (AS), six transcript variants were identified, encoding different lengths of deduced polypeptides (from 1,044 to 1,065aa). Ratios of the *wc-1* gene transcript variants in the RNA population were the same in the sporulated and non-sporulated PN-w isolate Sn37-1 and the sporulated PN-w isolate S-79-1, grown under light/dark conditions. The AS of the *wc-1* gene may control various light-dependent reactions in PN-w, which leads to diverse morphological, physiological and pathological characters for pathogen infection and spread. Based on the nucleotide and deduced amino acid sequences, the *wc-1* gene in cereal *Phaeosphaeria* pathogens was diverse. It appeared that the deduced

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wc-1 polypeptide sequences of *P. avenaria* f. sp. *avenaria* (Paa), *P. avenaria* f. sp. *triticea* (Pat1 and Pat3) and barley biotype *P. nodorum* (PN-b) were more closely related than PN-w and *Phaeosphaeria* sp. (P-rye) from Poland. Based on the wc-1 deduced polypeptide sequences, *P. avenaria* f. sp. *triticea* (Pat2) from foxtail barley (*Hordeum jubatum* L.) was evolutionary well separated from the other cereal *Phaeosphaeria* pathogens.

Keywords Alternative splicing · *Phaeosphaeria* · White collar-1 · Sporulation · Phylogenetic relationship

Introduction

The white collar-1 (*wc-1*) gene encodes an important light-responsive white collar-1 protein (wc-1) in ascomycete fungi that controls numerous light-dependent reactions and maintains circadian clocks in the organisms (Lee et al. 2003). The best-studied fungal systems in terms of the molecular mechanism of light responses are *Aspergillus nidulans* and *Neurospora crassa*. In response to light, the wc-1 protein acts as a transcription factor to induce gene expression for numerous physiological processes including carotenoid synthesis, photo-adaptation, and spore formation (Ballario et al. 1996). The wc-1 protein forms a heterodimeric photoreceptor white collar complex (WCC) with white collar-2 (wc-2) protein via PAS (Per-Arnt-Sim) domains (Crosthwaite et al. 1997). In response to a light pulse, WCC is phosphorylated and an additional wc-1 molecule is synthesized and joins the WCC, which acts as the positive component for frequency (*frq*) gene induction (Cheng et al. 2003). In *Aspergillus nidulans*, immediately after the induction of wc-1 and frq proteins by light, sequential expressions of the genes including *bristle* (*brlA*), *abacus* (*abaA*), *wet-white conidia* (*wetA*) and *stunted* (*stuA*) establish a linear regulatory pathway required for the transition from vegetative hyphae to conidiophores and conidia (Prade and Timberlake 1993). In genetic and biochemical studies, this group of genes in concert with others has been proven to be the central regulatory pathway to control specific gene expression during asexual spore formation in *A. nidulans* (Fortwendel et al. 2004).

Numerous protein-coding genes of eukaryotes have introns that are removed constitutively from their precursor messenger RNA (pre-mRNA) by splicing. Another mechanism that generates multiple transcription variants from a single gene and miscellaneous proteins is alternative splicing (AS). It was reported that AS affected the expression of nearly 20% and 65% of protein-coding genes in a flowering plant (*Arabidopsis thaliana*) and human (*Homo sapiens*), respectively (Kim et al. 2006).

Regulatory mechanisms of AS for specific gene expression and protein function diversity have recently been reviewed (House and Lynch 2008). AS was commonly reported in mammalian cells and their corresponding viruses. The multiple protein isoforms produced by AS may differ in structure, function and localization (Ogura et al. 2001). The polymorphic proteins encoded by spliced mRNA transcript variants of the human tumor suppressor gene (*ING4*) had different targeting signals, which governed their subcellular localizations and maintained their stability (Tsai et al. 2008). AS of a viral RNA transcript might also provide various proteins with specialized functions, which affected their interactions with other proteins/factors and were important for certain stages of virus developmental cycle (Devireddy and Jones 1998). AS was also recently reported in a plant gene (*JAZ.10*) encoding a jasmonate ZIM-domain protein (JAZ). JAZ are reported to act as repressors of jasmonate signaling in plants (Chini et al. 2007). The *JAZ.10* gene contains 4 introns (accession no. NC_003076), and AS of the *JAZ.10* gene pre-mRNA in *Arabidopsis thaliana* produced three protein isoforms, a regular repressor (JAZ10.3, accession no. NP_974775, 185 aa) and two variants with either a longer C terminus (JAZ10.1, accession no. Q93ZM9, 197 aa) or a shorter one lacking the Jas domain (JAZ10.4, 167 aa) (Chung and Howe 2009). For regulation of plant defence reaction and normal growth development, both variant proteins (JAZ10.1 and JAZ10.4) were antagonistic to JAZ10.3 protein, and became highly resistant to jasmonate-induced JAZ degradation and attenuated jasmonate signaling (Chung and Howe 2009).

Wheat-biotype *Phaeosphaeria nodorum* (PN-w) is one of the important cereal diseases, causing stagonospora nodorum leaf blotch. Spore dissemination during the wheat growing season is an important tool for disease spread and severity. Sporulation in most PN-w isolates, such as Sn37-1, was reportedly activated by the near-ultraviolet light on the culture media (Cooke and Jones 1970). However, a PN-w S-79-1 isolate in B.M. Cunfer's culture collection was unique in that it would produce pycnidiospores in the dark on the culture media, and its sporulation was not affected by blue, green and red light spectra (Personal communication). Since fungal sporulation is probably correlated with the activation of light-responsive genes, we determined to study the expression of the *wc-1* gene in sporulated and non-sporulated PN-w cultures. We report here for the first time that the *wc-1* gene in PN-w is an AS gene. We also show that the *wc-1* gene transcription variants were formed proportionally in the population of both sporulated and un-sporulated cultures grown on the culture media under different light conditions. Also, based on the deduced wc-1 polypeptide sequences, phylogenetic relationships among cereal *Phaeosphaeria* pathogens were investigated.

Materials and methods

Fungal isolates and DNA isolation

Cereal *Phaeosphaeria* species were either collected from Canada, Poland and the USA or purchased from the American Type Culture Collection (ATCC, Manassas, VA). Procedures for maintaining and growing fungal cultures were described previously. The genomic DNA (gDNA) was purified from 9 wheat-biotype *P. nodorum* (PN-w), 5 barley-biotype *P. nodorum* (PN-b), 9 *P. avenaria* f. sp. *avenaria* (Paa), 5 homothallic *P. avenaria* f. sp. *triticea* (*P. a.* f. sp. *t.*, Pat1), 2 heterothallic *P. a.* f. sp. *t.* from foxtail barley (Pat2), 1 *P. a.* f. sp. *t.* from the state of Washington (Pat3) and 2 *Phaeosphaeria* sp. from Polish rye (P-rye) by CsCl gradient ultracentrifugation (Table 1).

Gene amplification and sequencing of genomic DNA

The full-length *wc-1* gene sequence in PN-w isolate SN15 was obtained by a protein BLAST search of the *Stagonospora nodorum* isolate SN15 database (http:www.broad.mit.edu) using the white collar-1 (wc-1) polypeptide sequence from *Neurospora crassa* (accession no Q01371, 1,167aa) as the query (Ballario et al. 1996). The 3,353-bp nucleotide sequence (accession no CH445345, nt235038–238390) encoding a hypothetical protein (SNOG_12044; accession no EAT80456, 1,079aa) was chosen. Six primer sets (8A/5B, 1A/1B, 2A/2B, 3A/3B, 4A/4B and 5A/8B) were designed from the 3,669-bp *wc-1* gene nucleotide sequence of PN-w isolate SN15, which included both 5′ and 3′ flanking-end partial sequences, to amplify the corresponding gene in other PN-w isolates and six other *Phaeosphaeria* pathogens (Table 2). Based on partial sequencing data in PN-b, Paa and Pat1, specific primers were designed to produce the PCR fragments to cover the gaps and complete sequence analysis of the genes (Table 2). PCR amplification was performed in a 50-μl reaction mixture containing 1x colorless GoTaq® flexi buffer (pH8.5), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.5 μM of each primer, 80 ng of genomic DNA and 1.0 unit of GoTaq® DNA polymerase (Promega, Madison, WI). Reaction parameters were: denaturing at 94°C for 3 min, amplifying by 40 cycles of 94°C for 20 s, 55°C for 30 s and 72°C for 1 min, and then extending at 72°C for 10 min. Methods for isolation and direct sequencing of PCR products were reported previously (Ueng et al. 2003).

Gene expression in wheat-biotype *P. nodorum* isolates

To determine the *wc-1* gene expression in two PN-w isolates, Sn37-1 and S-79-1, total RNA was isolated from these cultures grown in the dark and under continuous fluorescent lights at room temperature (20±1°C) on sterile nitrocellulose membranes (BA-S 85, Schleicher & Schuell Inc., Keene, NH), which were layered on V-8 juice agar (18% V8 juice, 0.2% calcium carbonate, and 2% agar). Extraction of total RNA mainly followed the protocols described previously (Wang et al. 2007). The mycelia were harvested and flash-frozen in liquid nitrogen. Total RNA was extracted from mortar/pestle pulverized mycelia using the RNeasy Plant Mini Kit according to the manufacturer's instructions (Qiagen Inc., Valencia, CA) and treated with RNase-Free DNase I enzyme (Roche Diagnostics Corporation, Indianapolis, IN). Lack of residual gDNA in total RNA was evidenced by not being able to amplify the histidinol dehydrogenase (*Hdh1*) gene fragment using the primer set 15A/12-1 (ATGCCGGCAGGACCCAGTGA/CTATCAAG CTACGCCAAGTCGC) (Wang et al. 2007). The first-strand (1x) cDNA was synthesized with random primer $p(dN)_6$ and the First Strand cDNA Synthesis Kit (Roche Diagnostics Corporation, Indianapolis, IN). The thermal cycler settings were 25°C for 10 min, 42°C for 60 min, 99°C for 5 min, and 4°C for 5 min.

Six primer sets for gDNA amplification were also used for PCR production with 1x cDNA as templates. To

Table 1 Isolates of cereal *Phaeosphaeria* species used for analysis of the white collar-1 (*wc-1*) gene

Species	Original host	Year	Geographic location	GenBank accession number
Phaeosphaeria nodorum (wheat-biotype) (PN-w)				
8408	Wheat (*Triticum aestivum* L.)	1986	Mandan, ND, USA	GQ149718
9074	Wheat	1983	Gallatin County, MT, USA	GQ149719
9076	Wheat	1986	Richland County, MT, USA	(=GQ149719)
S-78-13	Wheat	1978	Toluca, Mexico	GQ149720
S-79-1	Triticale (×*Triticosecale* Wittm. ex A. Camus.)	1979	Tifton, GA, USA	GQ149721
S-81-B13B	Barley (*Hordeum vulgare* L.)	1981	Bledsoe, GA, USA	GQ149722
Sn26-1	Wheat	–	Rzeszów, Poland	GQ149723
Sn27-1	Wheat	–	Sieradz, Poland	GQ149724
Sn37-1	Wheat	–	Szelejewo, Poland	GQ149725
Phaeosphaeria sp. (From Poland) (P-rye)				
Sn48-1	Winter rye (*Secale cereal* M.Bieb.)	1995	Jelenia Góra, Poland	GQ149726
Sn23-1	Winter rye	–	Bydgoszcz, Poland	GQ149727
Phaeosphaeria nodorum (barley-biotype) (PN-b)				
S-80-603	Barley	1980	Williamson, GA, USA	GQ149716
S-80-611 (ATCC200842)	Barley	1980	Laurinburg, NC, USA	(=GQ149716)
S-81-B9	Barley	1981	Clayton, GA, USA	(=GQ149716)
S-83-2 (ATCC200841)	Barley	1983	Tifton, GA, USA	(=GQ149716)
S-82-13 (ATCC200805)	Barley	1982	Senoia, GA, USA	GQ149717
Phaeosphaeria avenaria f. sp. *avenaria* (Paa)				
1919WRS	Oat (*Avena sativa* L.)	2002	Manitoba, Canada	GQ149705
1920WRS	Oat	2002	Manitoba, Canada	GQ149706
ATCC12277	Oat	–	USA	GQ149707
1921WRS	Oat	2002	Manitoba, Canada	(=GQ149707)
5413	Oat	1983	Ontario, Canada	GQ149708
ATCC58582	Wheat	1984	New York, USA	GQ149709
ATCC58583	Wheat	1984	New York, USA	(=GQ149709)
Saa001NY-85	Oat	1985	New York, USA	GQ149710
Sat002NY-84	Wheat	1984	New York, USA	GQ149711
Phaeosphaeria avenaria f. sp. *triticea* (Pat1)				
Sat24-1	Wheat	–	Warmińsko-Mazurskie, Poland	GQ149712
10052-2	Wheat	1988	Langdon, ND, USA	(=GQ149712)
12618	Wheat	1995	Dickinson, ND, USA	(=GQ149712)
ATCC26374	Foxtail barley (*Hordeum jubatum* L.)	1972	Minnesota, USA	(=GQ149712)
ATCC26375	Foxtail barley	1972	Minnesota, USA	(=GQ149712)
Phaeosphaeria avenaria f. sp. *triticea* (Pat2)				
ATCC26370	Foxtail barley	1972	Minnesota, USA	GQ149713
ATCC26377	Foxtail barley	1972	Minnesota, USA	GQ149714
Phaeosphaeria avenaria f. sp. *triticea* (Pat3)				
S-81-W10	Wheat	1981	Washington, USA	GQ149715

Table 2 Primer sets used for genomic DNA and 1x cDNA amplifications and sequencings of the white collar-1 (*wc-1*) gene in cereal *Phaeosphaeria* pathogens

Primer sequences (5′→3′)	Nucleotide positions (nt) in the *wc-1* gene (SNOG_12044) of PN-w isolate SN15	Species used[a]						
		PN-w	P-rye	PN-b	Paa	Pat1	Pat2	Pat3
8A/5B[b] (CTCTGCAATGCTCCCAAACACAG/CTG CTCTCTGGCGACATTTC)	−115–−93/293–274	X	X	X	X	X	X	X
1A/1B (ATGAATGGAAACGGCTATCC/ CTTGTGCGACTACCCAATCCAG)	1–20/836–815	X	X	X	X		X	X
VD1-1A/VD1-1B (ATGAATGGAAACGGCTATCC CTAC/CTACAGGTTGATGTGCTTTGAGAG)	1–24/958–935					X		
2A[c]**/2B** (CTCGAGTCTCCTGTAACGCAAG/GTTG GTAGTTGACTCTGTACG)	679–700/1652–1632	X	X		X	X	X	X
VD1-2A/VD1-2BB (GTCTCTGGATTGGGTAGTC GCAC/GATTTCTGAGAATCGTTGAACAC)	811–823/1698–1676			X				
3A/3B (CATGAACTTGCTTACAATGATAC/GTGG TAAATGCCTGCAGCACC)	1458–1480/2418–2398	X	X	X			X	X
3AA/3BA (CATGAACTTGCT**G**ACAATGATAC/GT GGTAAA**G**GCCTGCAGCACC)	1458–1480/2418–2398				X			
VD1-3AA/VD1-3BA (CAGGTGCTATGACAAACA AGAAC/GGGTCTGCCCAACAATGAAGGTGG)	1550–1572/2477–2454					X		
4A[d]**/4B** (GACAAGAATCGAAGGTCCAATTC/TCA TGAACCTGGCTCCGCCACTTC)	2301–2323/3353–3330	X	X	X	X		X	X
VD1-4A/4B (GCATTCTTGAACTTGCAAGATCTG/ TCATGAACCTGGCTCCGCCACTTC)	2328–2351/3353–3330					X		
5A[e]**/8B** (CAGCACCTACAGCAAGGTCG/CAGTCA CCTCTCCAAATGTAAC)	3066–3085/3554–3533	X	X	X	X	X	X	X

[a] PN-w = Wheat-biotype *P. nodorum*; P-rye = *Phaeosphaeria* spp. from Poland; PN-b = Barley-biotype *P. nodorum*; Paa = *P. avenaria* f. sp. *avenaria*; Pat1, Pat2 and Pat3 = *P. avenaria* f. sp. *triticea*. [b] Primer 5B substitution for Pat2 = 5BA (CTG CTT TCT GGC GAC ATT TC); PN-b = 5BB (CTGCTCTCTGGAGACATTTC). [c] Primer 2A substitution for Paa and Pat1 = 2AA (CTCGAATCTCCTGT GACACAAG). [d] Primer 4A substitution for Paa = 4AA (GACAAGAATCGAAAATCCAATTC). [e] Primer 5A substitution for Pat1 = 5AA (GTTAGCACCTACAGCAAGGTCGTG); Pat2 = 5AC (CAGCACTTACAGCAAGGTCG); Pat3 = 5AB (CAGCACCTACAG CAAGGCCG)

confirm the 3′ end sequence in 1x cDNA, three primer sets, 10A/13B (GACAAGAGCAAGAAGAGTCCA TC/GTTGGATCGTGGTTCGTGAG, nt3117–3139/ nt3737–3718), 6A/4B (GTGTTTGACGAACTGAA GACTAC/TCATGAACCTGGCTCCGCCACTTC, nt2756–2778/nt3353–3330) and 7A/4B (GACCATCTG GTAACAGAGACTTG/TCATGAACCTGGCTCCGC CACTTC, nt2958–2980/nt3353–3330) were further used for amplification. Except for the PCR fragments amplified with primer sets 10A/13B, 6A/4B and 7A/4B, the amplified products were isolated and directly sequenced as reported previously (Ueng et al. 2003).

Analysis of the deduced amino acid sequence

Based on the deduced polypeptide sequences of the *wc-1* gene, phylogenetic relationships among 8 cereal *Phaeosphaeria* pathogens were analyzed using Mega Version 4.0 (http://www.megasoftware.net/index.html). The blue-light regulator 1 protein sequence of *Bipolaris oryzae* (BAF35570) was used as the out-group in analysis. The amino acid sequences were aligned with complete deletion option, which eliminated the positions containing gaps. The bootstrap consensus tree was inferred from 10,000 replicates with the neighbor-joining method to represent the evolutionary history (Saitou and Nei 1987).

Results

Expression of the *wc-1* gene in wheat-biotype *P. nodorum* (PN-w)

Five exons and four introns (nt127–177, nt1577–1625, nt3014–3068 and nt3223–3273) were determined in the full-length (3,353 bp) *wc-1* gene of PN-w isolate Sn37-1 (Fig. 1). In PN-w isolates, it was unique that introns 1 and 2 were flanked by a GC-AG splice border, instead of GT-AG (Table 3). Similar splice borders were found in the first two introns of the *wc-1* gene in P-rye, which is closely related to PN-w. In the other five cereal *Phaeosphaeria* pathogens, only intron 1 of the *wc-1* gene contained the GC-AG splice border (Table 3).

AS was found for introns 3 and 4 of the *wc-1* gene in PN-w (Fig. 2). With either the 6A/4B or the 7A/4B primer set, there were two smaller PCR products amplified from the 1x cDNA than the product from the gDNA (Fig. 1). When the 10A/13B primer set was used, two PCR products were amplified when the 1x cDNA was used as template. However, size of one of these PCR products was the same as the amplified product from the gDNA. The PCR products amplified with primer set 7A/4B, which included both introns 3 and 4, were purified with the PCR advanced™ PCR clean up mini prep system (Viogene BioTek, Taipei, Taiwan), cloned with the pGEM®-T Easy vector system (Promega, Madison, WI) and sequenced. The size of spliced nucleotides in intron 3 was either 55 or 64 bp. For intron 4, the region was either retained in the RNA transcript or spliced. The size of spliced nucleotides in intron 4 was either 51 or 54 bp. Through AS at the post-transcriptional level, six *wc-1* gene transcription variants (1–6) were identified in PN-w (Fig. 2A).

Duncan's multiple range test was used to study the significance of difference among transcript variants and the isolates (SAS Version 9.1, 2003).Variance analysis showed that a significant difference of the clone numbers was observed in the randomly cloned population among six transcript variants, but not among two PN-w isolates, Sn37-1 and S-79-1, grown under different light conditions (Table 5). The analysis showed that the means of the number of clones were not

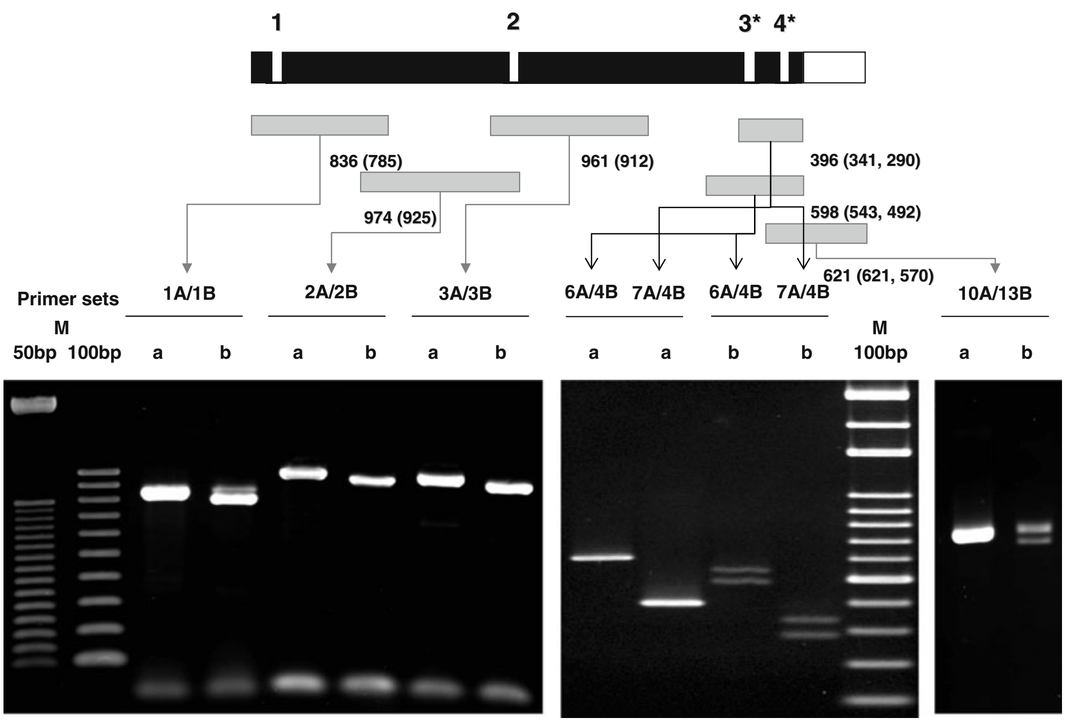

Fig. 1 Amplification of the white collar-1 (*wc-1*) gene in wheat biotype *Phaeosphaeria nodorum* isolate Sn37-1. Five translated exons of the *wc1* gene are shown as *black boxes*, and the non-coding region at the 3′ end as an *open box*. Four introns are indicated as the *thin lines*. The alternative splicing of the introns 3 and 4 are marked with *asterisks* "*". The *wc1* gene was amplified with 6 primer sets (1A/1B, 2A/2B, 3A/3B, 6A/4B, 7A/4B and 10A/13B). The numbers below the *grey boxes* are the fragment sizes in base pairs (bp) produced from genomic DNA (gDNA); the numbers in *parentheses* the fragment sizes from 1x cDNA prepared from the fungal culture grown under fluorescent light. a = Bands produced from genomic DNA; b = bands produced from cDNA. M = Molecular markers of 50 and 100 bp ladders

Table 3 Nucleotide sequences of 5′ and 3′ end exon-intron boundaries in 4 introns of the white collar-1 (*wc-1*) genes in cereal *Phaeosphaeria* pathogens[a]

Species[b]	Intron 1		Intron 2		Intron 3		Intron 4	
	5' end	3' end	5' end	3' end	5' end	3' end	5' end	3' end
PN-w	CAG|gcaagt	cag|A	CAG|gcaagt	tag|A	CAG|gtatgt	cag|CACCTACAG|| C[c]	CGA|gtaaga	aag|CAG|| C[c]
P-rye	CAG|gcaagt	cag|A	CAG|gcaagt	tag|A	CAG|gtatgt	cag|CACCTACAG|| C	CGA|gtaaga	aag|CAG|| C
PN-b	CAG|gcaagt	cag|A	CTG|gttagt	tag|A	CAG|gtatgt	cag|CACCTACAG|| C	CAA|gtaaga	aag|CAG|| C
Paa	CAG|gcaagt	cag|A	CTG|gtaagt	tag|A	CAG|gtatgt	cag|CACCTACAG|| C	CGA|gtaaga	aag|CAG|| C
Pat1	CAG|gcaagt	cag|A	CGG|gtaagt	tag|A	CAG|gtacgt	tag|CACCTACAG|| C	CAA|gtaaga	aag|CAG|| C
Pat2	CAG|gcaagt	cag|A	CAG|gtgagt	tag|A	CAG|gtgcgt	cag|CACCTACAG|| C	CGA|gtaaga	aag|CAG|| C
Pat3	CAG|gcaagt	cag|A	CTG|gttagt	tag|A	CAG|gtatgt	cag|CACCTACAG|| C	CAA|gtaaga	aag|CAG|| C

[a] Lowercase letters indicate the intron nucleotides spliced, and uppercase letters were the adjacent nucleotides in the exons. "|" represented the splice cut points of the introns, in which the surrounding nucleotide positions were always -1 and +1. Exonic nucleotide positions in 5' donor sites were negative numbers in order (-3, -2 and -1), and those in 3' acceptor sites were positive (+1). The CAG|| C in introns 3 and 4 were the alternative 3' end acceptor splicing sites. [b] Cereal *Phaeosphaeria* pathogens included wheat-biotype *P. nodorum* (PN-w), *Phaeosphaeria* sp. from rye (P-rye), barley-biotype *P. nodorum* (PN-b), *P. avenaria* f. sp. *avenaria* (Paa), and *P. avenaria* f. sp. *triticea* (Pat1, Pat2 and Pat3). [c] Shaded nucleotides in introns 3 and 4 had low frequencies in the corresponding nucleotide positions in the 5' and 3' ends exon-intron boundaries of the mammalian genes.

statistically different among transcript variants 1, 2, 3, 5 and 6, but variants 1, 2 and 3 were significantly more common than transcript variant 4 based on the Duncan test at 5% significance level (Table 5).

Six lengths of deduced protein isoforms (from 1,044 to 1,065 aa) could be obtained through the regulation of AS in the *wc-1* gene of the PN-w pathogen (accession no GU322809–GU322813 and GQ149725) (Fig. 2B). Based on the polypeptide sequence deduced from the protein isoform 1 (accession no GU322809), deletions and substitutions of amino acids occurred between aa971 and 1042.

Structure of the deduced wc-1 polypeptide in wheat-biotype *P. nodorum* (PN-w)

SMART sequence analysis (http://smart.embl-heidelberg.de) revealed that the deduced wc-1 protein isoform 3 in PN-w contained 3 Per-Arnt-Sim (PAS) motifs (PAS 1 = aa370–499, PAS A = aa565–678 and PAS B = aa679–750) and a single putative GATA-type zinc finger (Znf) domain (aa933–985) (Fig. 3). When compared to the PAS motif in the wc-2 protein of PN-w isolate SN15 (accession no. EAT78433), the identity of the PAS 1, A and B motifs of wc-1 protein in PN-w was 19%, 33% and 18%, respectively. The PAS 1 also contained a conserved 8 amino acids light-oxygen-voltage (LOV) domain (GRNCRFLQ, aa415–422), which included a reactive cysteine (C) that formed a covalent bond with the C(4a) carbon of a flavin on photoexcitation (Ballario et al. 1998). The Znf domain, which recognizes and binds to DNA consensus sequences (A/T)GATA(A/G) of other genes for transcriptional activation, was well conserved and belonged to zinc finger type IVb (C-x_2-C-x_{18}-C-x_2-C) (Teakle and Gilmartin 1998). Like the wc-1 protein in *Neurospora crassa*, a putative nuclear targeting sequence (KKKRK) upstream (aa923–927) and 2 potentially phosphorylated serine (S) residues (aa993 and 995) downstream of the Znf domain were identified (Fig. 3).

Diversity of the *wc-1* genes and their deduced polypeptides in cereal *Phaeosphaeria* pathogens

The *wc-1* gene coding sequences amplified from cereal *Phaeosphaeria* pathogens differed in nucleotide length, from 3,334 bp in Paa to 3,361 bp in Pat2 (Table 4). Due to the intron size discrepancy, the protein isoform 3 deduced from transcription variant 3 were from 1,043 to 1,050 aa (Table 4). There were 2–57 amino acid substitutions in wc-1 protein isoform 3 of the examined 6 *Phaeosphaeria* pathogens as compared with PN-w isolate Sn37-1 (Table 4). Based on the wc-1 protein isoform 1 sequence in PN-w, most amino acid divergences were present in the N terminus (aa1–330), between PAS B and Znf domains (aa800–900) and the carboxy terminus (after aa990) among cereal *Phaeosphaeria* pathogens (Fig. 3).

Phylogenetic relationships based on the deduced wc-1 protein isoform 3 sequence showed that Pat1 isolates were more closely related to Paa and other *Phaeosphaeria* pathogens than PN-w and P-rye (Fig. 4). All *Phaeosphaeria* pathogens appeared to form a single clade, with the exception of Pat2, which is from foxtail barley (*Hordeum jubatum* L.).

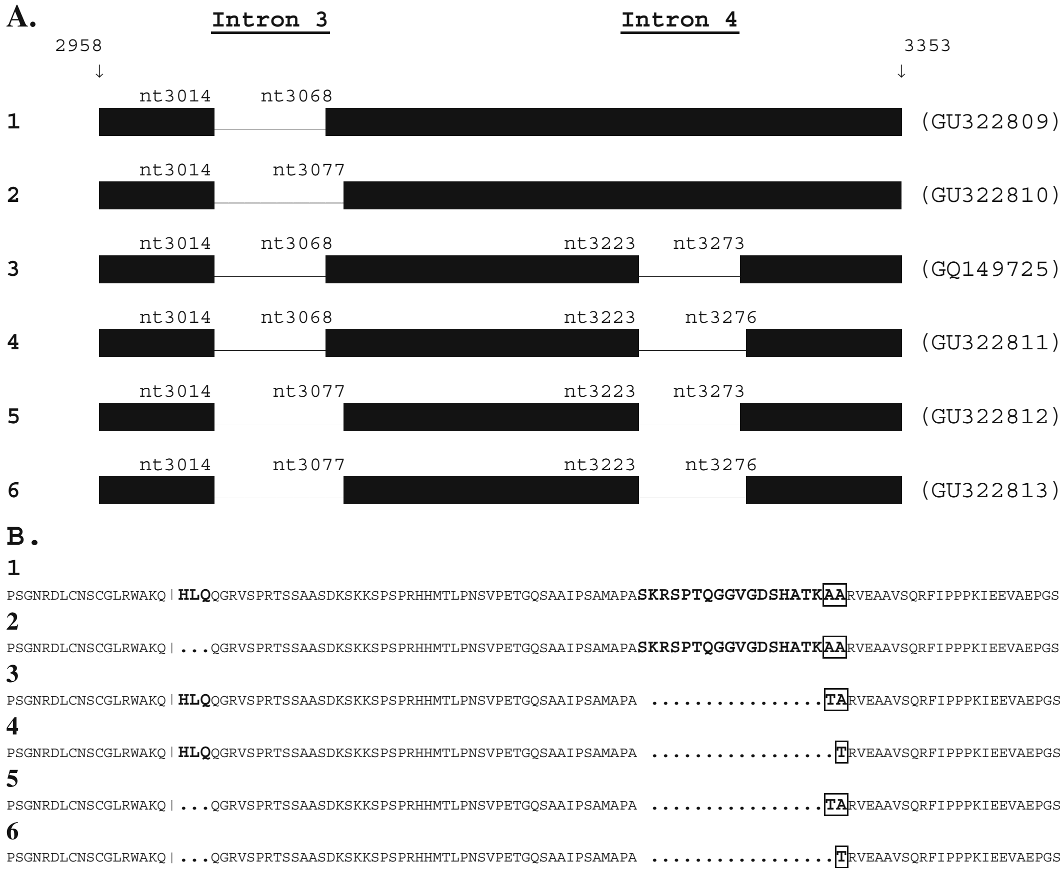

Fig. 2 Alternative splicings in introns 3 and 4 of the white collar-1 (*wc-1*) gene and their deduced polypeptide sequences in wheat-biotype *Phaeosphaeria nodorum* isolate Sn37-1. With the primer set 7A/4B (GACCATCTGGTAACAGAGACTTG/TCATGAACCTGGCTCCGCCACTTC) and 1x cDNA as the template, partial nucleotide sequences (nt2958–3353, see "↓") of the amplified products represented 6 transcript variants (**A**); their deduced polypeptide isoforms (**B**) (from 1 to 6) are presented. In A, the coding sequences are *dark boxed*, and the spliced introns are indicated by *thin lines* with their nucleotide positions (nt) following the genomic sequence of the *wc-1* gene (GQ149725). The GenBank accession numbers are in *parentheses*. In B, the splicing positions of 6 transcript variants are indicated in their polypeptide isoform sequences as "|". Amino acids involved in deletions (.....) and substitutions (*boxed*) are in *bold*

Discussion

The automated annotations of fungal genome sequences sometimes incorrectly identify intron boundaries in the deduced genes. Two introns, at nt1577–1625 and nt3014–3068, were hypothesized in the *wc-1* gene of PN-w isolate SN15 by the Broad Institute (SNOG_12044). When the *wc-1* genomic sequence was analyzed by Softberry (http://linux1.softberry.com/berry.phtml) with *Aspergillus* as the organism parameter, the same two introns but with a 9-bp longer splicing sequence in the second intron (nt3014–3077) were reported. Therefore, the deduced WC-1 polypeptide (1,076 aa) analyzed by Softberry was three amino acids fewer as compared to the analysis from the Broad Institute. These two introns predicted in the *wc-1* gene by both annotations corresponded to introns 2 and 3 when the RNA transcripts were determined by sequencing in this study.

Two AS patterns were found in the *wc-1* gene of PN-w (Fig. 2). One was the retention and excision of the intron from the pre-mRNA, and the other was the alternative 3′ splice sites that shortened the downstream exon (Black 2003). Since the intron sizes in the *wc-1* gene were relatively small (ca. 50 bp) as compared to the exons, regulation of AS was suggested to be an intron definition (Romfo et al. 2000).

Consensus nucleotide sequences positioned at intron/exon borders were essential for precise pre-

```
                                                         (1)

  1 MNGNGYPYPMRSFPSSRQNMMDNMDSGMGGFEDMNQGQSLDQ|IVSQNDKAENIRRQSMPVYASNGTNNNRTQPME   75

 76 MSPESRRLSMLNFGDPGDAGDFQFDMHAAGMNDMMRNNPSFPRASGDMSQHNRNSANNMGLNTQFQNQNQNPPYS   150

151 TMAAPGSAYASPLHPSAPLDLDMSPYPNGMNMSMDLDDSLNMMSGDMNMFSNNQFNTPMLESPVTQEFIGPMPAP   225

226 NQDNSMSAIPQGHFKRPSLSNTPETRSGVSGLGSRTSSQDQNSAPSQSRPQSEQRSSSKNNLPTQMSLSSLKAHQ   300

                                                                        PAS 1

301 PVALDPAQDLPEEKMNQLKDYRTAWKPPAGGFPSTMHSNPHQKTQFKDAYSSTGFDMLGVLMRVATRPD[PEIDIG  375

376 SVDLSCAFVVCDAEMDDIPIVYCSENFERLTGYTRHMILGRNCRFLQSPDGKVEAGIKRKYVDDDSVLYLKNMIN   450

                                                                  (2)

451 TRAEAQISLINYRRGGQPFMNLLTMIPIAWEPGGPMKFIVGFQVDLVEQ]PGAMTNKNSD|GSYRVNYQRGMNMPSY 525

                                                  PAS A

526 VFNDSQKPQSEQGQTISKDEVSNVLATYTGSGDSEITRR[IWDKVLLENTDDVVHVLSLKGLFLYLSPSSSRILEY  600

601 EPSELIGTALSSVCHPSDIVPVTRELKETTNGGSVNVVFRIRRKKSGYMWFEGHGSLHTEQGKGRKCIILVGRER   675

    PAS B

676 PVY][TLSKTIVRESGGVGDNELWTKMSTSGMFLFVSSNVRQLLDKQPEDLVGTSIQALMRQESKVQFGRILELARS]750

751 GRKGEVKHEMINKRGQVLQAFTTIYPGDATEGQKPTFIVGQTRLLKYSRNSANHRPSMYTNKERLGGGSTHSIPT   825

826 QNTLNSPYPGSVQDHTGSNTPITSSTDGRFVTTETNATTHAGHNGLQLGHQDQSLASDDNVFDELKTTRSTSWQY   900

                                            ZnF                               (3)

901 ELRQMEKRNRYLAEEVQTLLAAKKKRKRRKGA[GQMQKDCANCHTRTTPEWRRGPSGNRDLCNSCGLRWAKQ|HLQ 974

                                                               (4)

975 QGRVSPRTSSA]ASDKSKKSPSPRHHMTLPNSVPETGQSAAIPSAMAPAT|ARVEAAVSQRFIPPPKIEEVAEPGS 1048
```

Fig. 3 Deduced polypeptide isoform 3 sequence of the white collar-1 (*wc-1*) gene in wheat-biotype *Phaeosphaeria nodorum* isolate Sn37-1. The numbers 1–1,048 on both side columns represent amino acid sequence. The polypeptide isoform 3 sequence was deduced from transcript variant 3. PAS motifs (PAS 1 = aa370–499, PAS A = aa565–678 and PAS B = aa679–750) and 1 GATA-type zinc finger (Znf) domain (aa933–985) are in *bold* and *bracketed*. The conserved amino acids in PAS 1 and Znf domains in *Phaeosphaeria* and other ascomycetes are *dark boxed*. The 8 amino acids (aa415–422) in the *gray area* of the PAS 1 domain are the conserved residues of the light-oxygen-voltage (LOV) domain. A putative nuclear targeting sequence (KKKRK) is *double underlined* (aa923–927), and two potential phosphorylation sites at serine (S) (aa993 and aa995) are *single underlined*

mRNA splicing. On the other hand, AS was a consequence of 5′/3′ splice sites with weak binding potential for spliceosome components. In GT-AG-type introns, they were ($^{C}/_{A}$)AGIGT($^{a}/_{g}$)agt and ($^{t}/_{c}$)$_{n}$n ($^{c}/_{t}$)AGIG, where “I” indicated the cut positions, at 5′ donor and 3′ acceptor sites, respectively (Mount 1982). In a study of known gene splice sites from five eukaryote species (*Homo sapiens*, *Mus musculus*, *Drosophila melanogaster*, *Caenorhabditis elegans* and *Arabidopsis thaliana*) and ascomycetes, more than 98% of them belonged to the canonical GT-AG-type, and only 0.74–1.0% of introns had non-canonical GC-AG splicing sites (Kupfer et al. 2004). Non-canonical GC-AG-type introns could be spliced constitutively if nucleotides in other positions around the 5′ and 3′ exon-intron borders were as well conserved as those in high-strength GT-AG-type introns (Aebi et al. 1987). It was shown that a GT-AG-type intron with a point mutation at the +2 position of the 5′ donor site from “t”→“c” would retain some 5′-splice signal and produce less of the normal mRNA (Iida 1990). It appeared that the presence of consensus nucleotide sequences CAGIgcaagt, except for the “c” at the +2 position, in the 5′ donor site of intron 1 and

Table 4 Structure of the white collar-1 (*wc-1*) gene in cereal *Phaeosphaeria* species[a]

Species/ Isolates	Gene size (bp)[b]	# of nucleotide substitutions[c]	Transcription variant 3 size (bp)[b]	Deduced protein isoform 3 size (aa)[b]	# of amino acid substitutions[c]
Phaeosphaeria nodorum (wheat-biotype) (PN-w)					
Sn37-1	3353	–	3147	1048	–
Phaeosphaeria sp. (from Poland) (P-rye)					
Sn23-1	3353	13	3147	1048	2
Phaeosphaeria nodorum (barley-biotype) (PN-b)					
S-80-603	3338	278	3132	1043	37
Phaeosphaeria avenaria f. sp. *avenaria* (Paa)					
ATCC12277	3334	276	3132	1043	37
Phaeosphaeria avenaria f. sp. *triticea* (Pat1)					
Sat24-1	3342	297	3135	1044	48
Phaeosphaeria avenaria f. sp. *triticea* (Pat2)					
ATCC26370	3361	344	3153	1050	57
Phaeosphaeria avenaria f. sp. *triticea* (Pat3)					
S-81-W10	3340	272	3132	1043	42

[a] Based on the hypothetical protein gene SNOG_12044 in the *Stagonospora nodorum* data base of the Broad Institute (www.broad.mit.edu/annotation/genome). [b] bp = base pairs; aa = amino acids. [c] Substitutions as compared with PN-w isolate Sn37-1

part of intron 2 in the *wc-1* gene of cereal *Phaeosphaeria* pathogens would ensure their constitutive splicing in pre-mRNA (Table 3).

The presence of weakly conserved nucleotides surrounding the intron splice sites would allow AS to occur. A nucleotide "a", instead of "t" found at the +6 position of the 5′ donor site "gtaagt" of intron 4 in the *wc-1* gene might not affect splicing efficiency since a mutation from "t"→"c" at this position in a human gene reported almost normal splicing (Table 3, Iida 1990). However, a low frequency of nucleotide "A" at the −1 position of the 5′ donor site in intron 4 of the *wc-1* gene as compared to mammalian genes might make the splicing activity vulnerable (Table 3). Mutation at the −1 position of the 5′ donor site from "G"→"C" affected the splicing of human β-globin pre-mRNA (Vidaud et al. 1989). It was possible that relaxation of the 5′ splice site recognition gave ca. 50% of intron 4 retention and excision in the *wc-1* gene of *Phaeosphaeria* pathogens (Table 3).

In comparison to the conservation in nucleotides surrounding the 3′ acceptor site in mammalian genes (Lopez 1998), a low frequency of "C" at the +1 position of the 3′ acceptor site in introns 3 and 4 of the

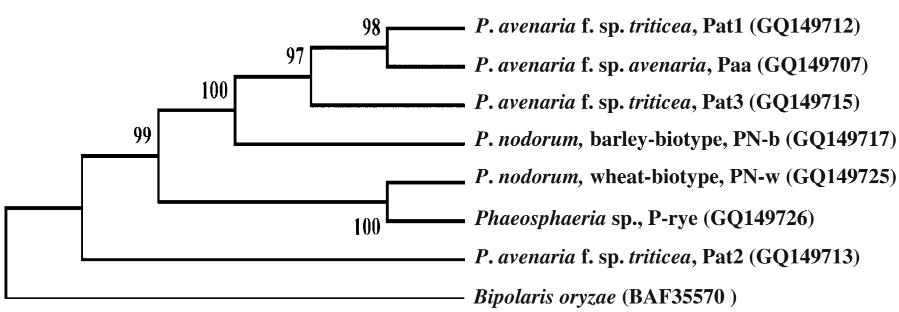

Fig. 4 Phylogenetic relationships based on the deduced polypeptide isoform 3 sequences of the white collar-1 (*wc-1*) gene in cereal *Phaeosphaeria* pathogens. The polypeptide sequences deduced from the *wc-1* transcript variant 3 of *Phaeosphaeria* pathogens were aligned and analyzed. The blue-light regulator 1 protein sequence of *Bipolaris oryzae* (BAF35570) was used as the out-group in analysis. GenBank accession numbers for nucleotide sequences in *parentheses* were used for *Phaeosphaeria* pathogens. Bootstrap values with 10,000 replications of the internal branches are indicated

wc-1 gene in cereal *Phaeosphaeria* pathogens might furnish weak splicing activities and produce multiple isoforms of mRNA transcripts (Fig. 2). It was reported that "(c/t)ag" consensus nucleotides were commonly found at −3 to −1 positions of the 3′ splice site in fungal genes (Kupfer et al. 2004). The presence of a low frequency of "a" at the −3 position of the 3′ acceptor site in intron 4 would affect the splicing activity of the *wc-1* gene in *Phaeosphaeria* pathogens.

Regulation of AS in *Phaeosphaeria* pathogens is unknown. Discrete repeated sequence elements in numerous genes in eukaryotes were reported to act as exonic splicing enhancers, which positively affect the accuracy and efficiency of splicing of upstream introns (Webb et al. 2005). In a search of tandem repeats (http://tandem.bu.edu), two 14-bp continuous consensus repeats, TGGGACACACGACT and TGGGCACACGAGCT, were found at nt1163 downstream of the stop codon (TGA) of the *wc-1* gene pre-mRNA (accession no CH445345, nt239553–239580). These repeats are purine rich, and their potential splicing mechanism needs further study.

In *Neurospora crassa*, expression of the *wc-1* gene was regulated by AS at the 5′ end, which produced transcriptional isoforms in various environments. Under different light conditions, two separate transcription initiation sites located upstream of the *wc-1* gene-coding sequence and the one within the gene open reading frame were detected (Káldi et al. 2006). Therefore, two similar wc-1 polypeptides and one truncated molecule were produced in *N. crassa*. Generation of 6 AS transcription variants from the *wc-1* gene in PN-w might not result from near-UV light damage, since isolate S-79-1 produced the same transcription variants in the dark (Table 5, Muñoz et al. 2009).

Functions of gene activation by the 6 wc-1 protein isoforms in PN-w were not clear and needed further study. One possibility was that all 6 wc-1 protein isoforms might confer only one function, which was to activate the downstream *frq* gene. However, the significance of 25 amino acid residues carboxy-terminal to the Znf domain of the AREA protein, which mediated nitrogen metabolite repression in *Aspergillus nidulans*, in gene expressions was reported (Stankovich et al. 1993). Mutations in this region would result in enhancing, de-repressing and impairing various gene expressions (Stankovich et al. 1993). The amino acid substitutions in particular motifs of the carboxy-terminal of transcription factor Pdr3p in yeast (*Saccharomyces cerevisiae*) also was reported to affect gene activations and induce multi-drug resistance (Nourani et al. 1997). Deletions, retentions and substitutions of amino acid residues occurred between aa971 and aa1042 in the carboxy terminal of the wc-1 polypeptide in PN-w and might have diverse gene-activation functions (Fig. 2B). The abundance of six different AS transcripts during sporulation *in planta* might give a fascinating insight into gene regulation during asexual sporulation on the natural host rather than artificially *in vitro* conditions.

It appeared that the *wc-1* gene did not lead to direct activation of the gene(s) associated with asexual

Table 5 Variance analysis of six transcription variants of the white collar-1 (*wc-1*) gene in sporulated (S) and non-sporulated (NS) cultures of wheat-biotype *Phaeosphaeria nodorum* (PN-w) isolates

Transcription variants	Introns spliced	Fragment sizes[a]	Isolates				Total	Average[b]
			Sn37-1		S-79-1			
			Dark (NS)	Light (S)	Dark (S)	Light (S)		
1	3	341	7	13	6	14	40	10.0 a
2	3	332	6	13	10	5	34	08.5 a
3	3 and 4	290	3	6	17	12	38	09.5 a
4	3 and 4	287	1	1	2	4	8	02.0 bc
5	3 and 4	281	8	7	8	6	29	07.3 ab
6	3 and 4	278	5	4	4	4	17	04.3 ab
No. of clones			30	44	47	45	166	

[a] Fragment sizes in base pairs (bp) were based on the full length PCR products amplified with primer set 7A/4B and 1x cDNA as templates. [b] Based on Duncan test at 5% significant level

sporulation in PN-w (Table 5). Inactivation of the mannitol-1-phosphate dehydrogenase gene (*Mpd1*) in PN-w is reported to prevent the pathogen from sporulating on infected detached leaves (Solomon et al. 2005). Mannitol-1-phosphate dehydrogenase is one of the important enzymes for mannitol synthesis in fungi, and mannitol sugar is reported to be a major carbon and energy source for sporulation and spore germination. However, addition of mannitol to culture medium did not restore sporulation in naturally derived non-sporulated PN-w culture suggesting that the expression of the *Mpd1* and other sporulation genes was regulated by other upstream activator gene(s).

Based on phylogenetic analyses with nucleotide and deduced amino acid sequences of numerous genes, it appeared that genes in closely related cereal *Phaeosphaeria* pathogens had a common ancestor and evolved independently. In the glyceraldehyde-3-phosphate dehydrogenase (*gpd*), β-glucosidase (*bgl1*) and *wc-1* genes, Pat1 was grouped together with Paa, Pat3 and PN-b as a single clade, while the RNA polymerase II (*RPB2*) gene of Pat1 was closely related to PN-w (Reszka et al. 2005). In the β-tubulin (*tubA*) and trifunctional histidine biosynthesis (*his*) genes, Pat1 was phylogenetically separated from all these *Phaeosphaeria* pathogens (Malkus et al. 2005; Wang et al. 2007). Of the studied *wc-1* and other gene nucleotides and their deduced polypeptide sequences, the Pat2 appeared to be the most diversified, and evolved separately from all other cereal *Phaeosphaeria* species.

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