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Short sequence-paper

Cloning and expression studies during vegetative and sexual development of *Pbs1*, a septin gene homologue from *Pyrenopeziza brassicae*

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Abstract

A septin gene homologue designated *Pyrenopeziza brassicae septin 1* (*Pbs1*) has been identified and cloned from the plant pathogenic fungus *Pyrenopeziza brassicae* and its expression analysed. *Pbs1* is present in both mating types and in a single copy within each genome and is transcribed in proportionate levels during both vegetative and sexual growth.

Keywords: Molecular plant pathology; Sexual development; Septin; Fungus

Pyrenopeziza brassicae is the causal organism of light leaf spot disease of oilseed rape and other brassicas [1,2]. The fungus is a discomycete member of the Ascomycotina and is heterothallic with two mating types designated *MAT-1* and *MAT-2* [3,4], which regulate a complex yet co-ordinated pathway of development leading to fruiting body formation [2,4,5]. Septins are proteins thought to have roles in cytokinesis and other developmental processes [6]. They were first identified in the yeast *Saccharomyces cerevisiae* (reviewed in [6]); however, more recently, homologues have been identified in organisms as diverse as fruit flies and mice (reviewed in [6,7]). The septins are similar in both structure and function, with all containing a P-loop nucleotide-binding motif [8] and most also containing a coiled coil domain that may be involved in protein–protein interactions [9]. In fungi, septins have been reported in the dimorphic yeast *Candida albicans* as well as in the budding yeast *S. cerevisiae* and the fission yeast *Schizosaccharomyces pombe* (reviewed in [6]) and, more recently, in the filamentous ascomycete *Aspergillus nidulans* [10]. In a search for genes involved in sexual development of *P. brassicae*, a number of molecular approaches were adopted including a heterologous screening strategy using the MAT A idiomorph from *Neurospora crassa* [11]. Whilst screening a cDNA library of *P. brassicae* JH26 (*MAT-1*) generated from mycelium that had been grown vegetatively and was reproducing asexually, with p2A4 containing the MAT A idiomorph from *N. crassa* ([11]; a gift from Dr R.L. Metzenberg), a gene encoding a *P. brassicae* septin was identified. In order that the role of this septin could be studied during *P. brassicae* development, the *P. brassicae* septin gene, *Pbs1*, was cloned and sequenced and its expression analysed during both vegetative growth and sexual morphogenesis. This is the first report of the cloning, sequence and expression analysis of a septin gene from a discomycete fungus.

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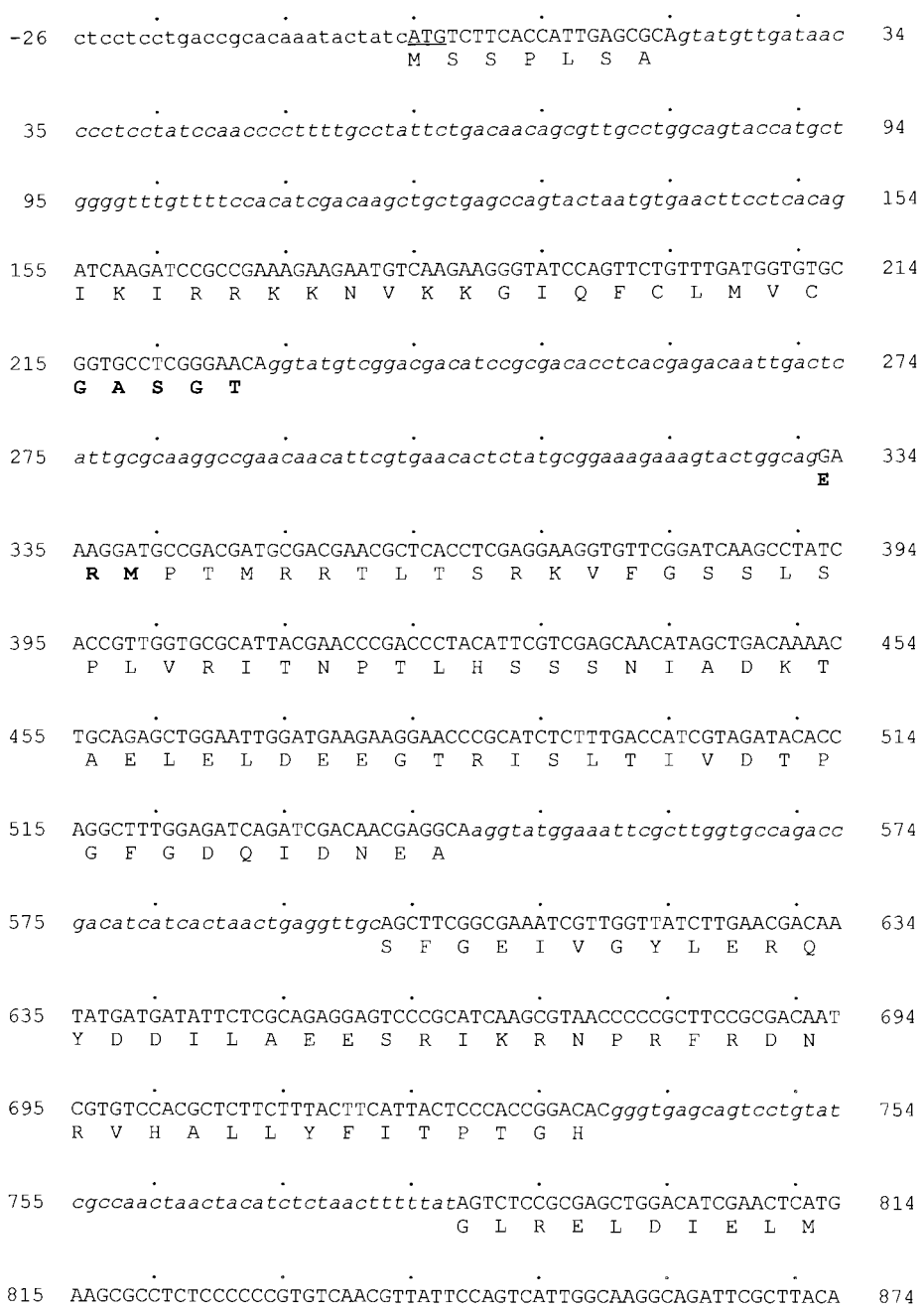

Fig. 1. Full length DNA sequence of the *P. brassicae* septin gene *Pbs1* and the conceptual translated sequence. The predicted translational start codon is underlined. An asterisk denotes the translational stop codon. The introns are written in lower case italic. 5′ and 3′ UTRs are written in lower case. The predicted P-loop motif is in bold. One of the two *Hin*dIII sites which generate the 924 bp product seen in Fig. 3 is denoted with a line above the sequence (at bp 899–905). The second site is at bp 1823–1828 (data not shown). The full sequence of *Pbs1* is deposited within the EMBL database under accession number AJ132791.

A 1.3 kb *Eco*RI–*Xho*I fragment excised from the cDNA clone pGS2 [12], which contained a partial cDNA sequence corresponding to *Pbs1*, was randomly labelled (Random Prime-II kit, Stratagene) and used as a probe against a *P. brassicae* genomic library from isolate CRB (*MAT-2*) constructed in pBK-CMV (Stratagene). The resulting positive clone containing the full length genomic sequence of the

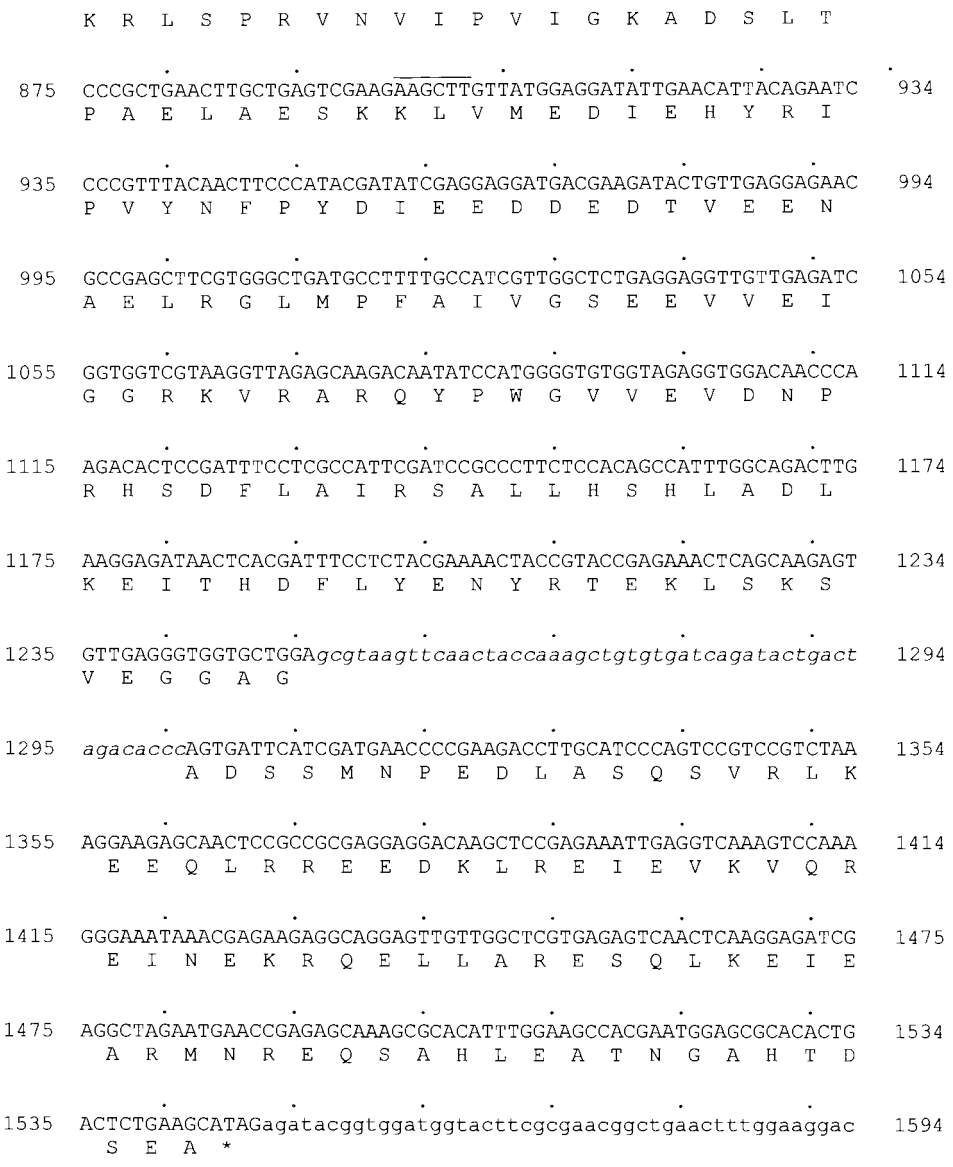

Fig. 1 (*continued*).

septin gene was designated pGS39 [12]. Both pGS2 and pGS39 were sequenced in both orientations to establish the correct sequence of the *P. brassicae* septin gene and to determine precisely the positions of introns. Double-stranded DNA sequencing was performed in an ABI Prism 377 automated sequencer using the facility within the Department of Biochemistry, University of Cambridge. Clones were sequenced from flanking T3 and T7 sites using the corresponding primers and primers designed from derived sequences from pGS2 and pGS39. RT-PCR was performed on total RNA extracted from *P. brassicae* mycelium which was grown vegetatively, using primers derived from the full length genomic sequence, in order to confirm the extent of the cDNA sequence. The nucleotide sequence of *Pbs1* is shown in Fig. 1. The predicted open reading frame (1161 bp) encodes a polypeptide of 387 amino acids (Figs. 1 and 2) with a predicted molecular weight of approximately 43.8 kDa and a theoretical p*I* of 5.31. The transcription start sites were not identified; however, there do not appear to be typical CAAT and TATA elements within the 5′ untranslated region

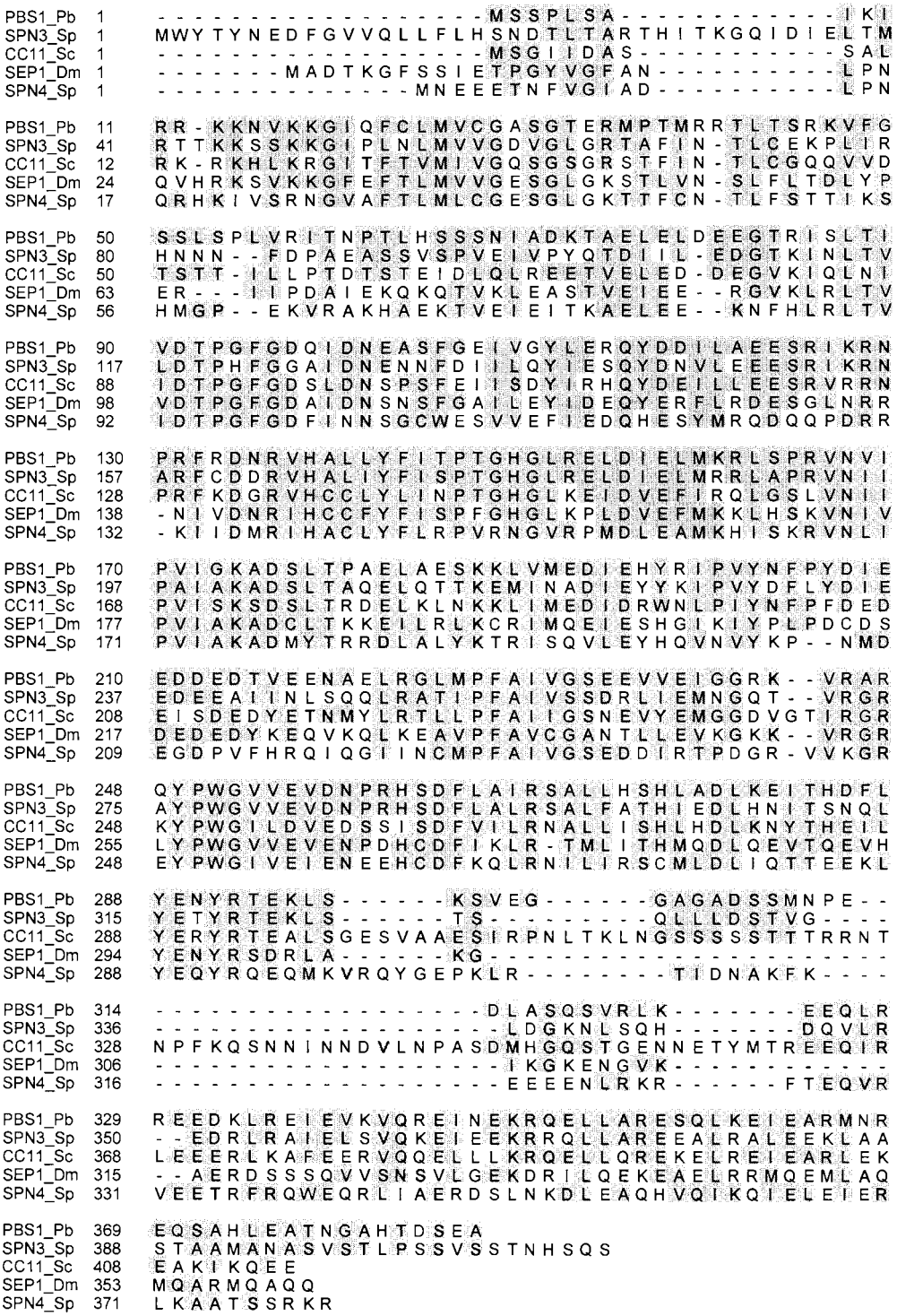

Fig. 2. Amino acid alignment of *P. brassicae* Pbs1 with other septin proteins. SPN3 from *S. pombe* (accession number P48008), CC11 (Cdc 11p) from *S. cerevisiae* (accession number P32458), SEP1 from *D. melanogaster* (accession number P42207) and SPN4 from *S. pombe* (accession number P48009). Gaps that were introduced for optimal alignment are marked with dashes. Shaded amino acids indicate similarity. The pile up was created using the basic local alignment search tool (BLAST X [13,15]) and SeqVu version 1.1 (Garvan Institute, Sydney, Australia) following the default settings within the program.

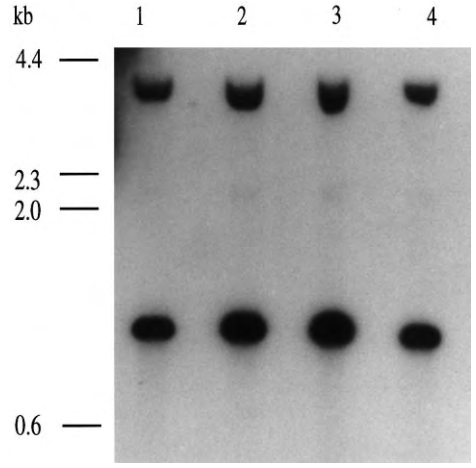

Fig. 3. Gel blot hybridisation of genomic DNA of *P. brassicae* isolates CRB (*MAT-2*, lanes 1 and 2) and JH26 (*MAT-1*, lanes 3 and 4), digested with *Hin*dIII and probed with the 1.3 kb insert from pGS2, corresponding to a partial cDNA fragment from *Pbs1*. The 924 bp hybridising band arises from a *Hin*dIII site present within the coding sequence and one present within the 3′ UTR (refer to Fig. 1).

(UTR) sequence. From a comparison of the cDNA and genomic sequences it was determined that *Pbs1* has five introns (Fig. 1). The first intron (132 bp) is from bp 22 to 154, the second (102 bp) is from bp 230 to 332, the third (52 bp) is from bp 546 to 598, the fourth (47 bp) is from bp 737 to 784 and the fifth (49 bp) is from 1253 to 1302 (Fig. 1). The intron border sequences follow the consensus for fungal 5′ and 3′ splice sequences [14].

Significant sequence homology over the whole sequence was observed between Pbs1 and previously sequenced septin gene products (Fig. 2). The highest homologies, determined using the BLAST X program [13,15], were with Spn 3p from *S. pombe* (63% similarity, 54% identity over 370 aa), Cdc 11p from *S. cerevisiae* (65% similarity, 51% identity over 401 aa), Sep 1 from *Drosophila melanogaster* (56% similarity, 41% identity over 343 aa) and Spn 4p from *S. pombe* (52% similarity, 36% identity over 367 aa). The Pbs1 protein appears to possess a putative P-loop motif ([A/G]X4GK[S/T]; [8]) between residues 28 and 35, with six out of eight residues within the predicted motif sharing some similarity (Fig. 2). Pbs1 does not appear to possess a coiled coil region [9] or other elements required for GTP binding [8], which are present within nearly all of the known septins.

Fungal genomic DNA was extracted as described previously [16], and 5 μg was digested with *Hin*dIII, separated on a 0.8% TBE agarose gel, and blotted onto a Gene Screen membrane (Dupont) according to the manufacturer's instructions. Gel blot hybridisations were performed as described by Sambrook et al. [17] and confirmed that *Pbs1* was present in a single copy within the genome of each mating type (Fig. 3).

The expression of *Pbs1* during vegetative (asexual) growth and during sexual development was studied by Northern blot analysis. 2×10^5 conidia from PC1 (*MAT-2*) and PC12 (*MAT-1*) and from a 50:50 mixture of the two mating types were inoculated onto cellophane discs laid on 90 mm petri dishes containing 20 ml of 3% malt extract agar. Plates were incubated at 18°C in the dark. Mycelium for RNA extraction was collected from vegetatively grown cultures on day 7 and day 14 post inoculation, whereas cultures crossed sexually were harvested at 7, 14, 21 and 28 days post inoculation, by which time the cultures were producing fertile apothecia (data not shown). RNA was extracted from 50–100 mg mycelium using an Ultraspec RNA isolation kit (Biotecx) according to the manufacturer's instructions. The concentration of RNA in each sample was calculated using a Gene-Quant kit (Pharmacia). Approximately

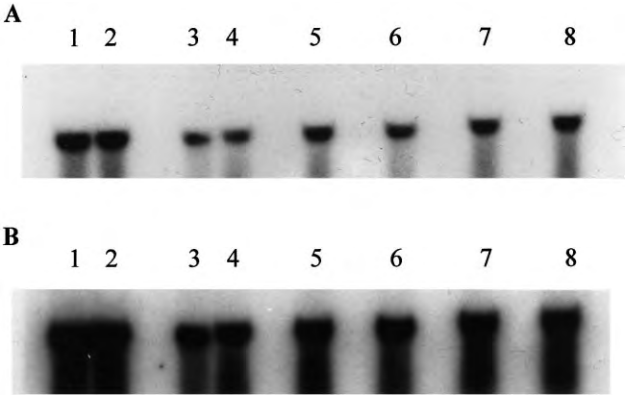

Fig. 4. Northern analysis of *Pbs1* during vegetative growth and during sexual development. Total RNA was extracted from 7 and 14 day old mycelium of isolate PC1 (*MAT-2*; lanes 1 and 2), PC12 (*MAT-1*; lanes 3 and 4) and 7, 14, 21 and 28 day old material from a cross between PC1 and PC12 (lanes 5, 6, 7 and 8 respectively). The RNA blot was probed with (A) the 1.3 kb insert from pGS2 and (B) a 0.6 kb fragment from pGS3 (containing a partial cDNA of the *P. brassicae* GAPDH gene).

20 μg of RNA was loaded per track and was separated under denaturing conditions, blotted onto a nylon membrane (Hybond), and probed with the 1.3 kb *Eco*RI–*Xho*I fragment from pGS2. A signal was observed following exposure to X-ray film (Kodak) for 7 days at −70°C. To ensure that the loading of RNA samples for each treatment was equivalent, the Northern blot was probed with a randomly labelled (Random Prime-II kit, Stratagene) 0.6 kb *Eco*RI–*Xho*I fragment from pGS3 [12] containing a partial cDNA sequence from the *P. brassicae* glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH). Northern hybridisation analysis revealed that the *Pbs1* transcript was expressed proportionately throughout both vegetative growth and asexual development and during sexual morphogenesis (Fig. 4). The constitutively expressed GAPDH gene from *P. brassicae* gave the same profile of expression when analysed under the same experimental conditions and was therefore a useful internal control for RNA loading (Fig. 4). Unfortunately, the cloning of *Pbs1* and analysis of its expression during vegetative asexual growth and sexual development did not shed light on the role that Pbs1 has in *P. brassicae* development. We were interested to determine whether *Pbs1* was a gene involved in sexual morphogenesis, particularly as the gene was isolated following a screen using the MAT A idiomorph from *N. crassa*. However, the results obtained from Northern blot analysis which showed no preferential expression of *Pbs1* during *P. brassicae* development, as well as the gel blot results which showed that the gene was not mating type determined, suggest that it is unlikely that *Pbs1* plays any specific role in the mating process. Gene knockout analysis of the *Pbs1* gene resulted in ectopic integration events within viable transformants with no integration events within the resident *Pbs1* gene ([12]; data not shown). This result implies that an integration within this gene may be detrimental for growth of the fungus; however, further analysis is now required to confirm this result.

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