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Short sequence-paper

# ppt-1, a Neurospora crassa PPT/PP5 subfamily serine/threonine protein phosphatase [1]

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**Abstract**

We isolated a *N. crassa* cDNA clone encoding a novel-type serine/threonine phosphatase. The gene (mapped to LGVR), designated *ppt-1*, encodes a 479 amino acid putative polypeptide which contains a conserved tetratricopeptide repeat (TPR) motif. *ppt-1* transcript levels are abundant in conidia and decrease during germination, indicating that *ppt-1* is developmentally regulated. © 1997 Elsevier Science B.V.

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Serine/threonine protein phosphatases have been subgrouped to two structurally related gene families, the PP1/PP2A/PP2B family and the PP2C family [1,2]. Recently, additional members of this gene family have been identified on the basis of molecular cloning and by genetic analysis. Among them is the PPT/PP5 gene family, which is quiet distinct from the other members of the serine/threonine phosphatase family [3–5].

PPTs have been shown to harbor either three or four tetratricopeptide repeat (TPR) motifs arranged in tandem, as a single domain [3,4]. This domain has previously been found in a variety of unrelated proteins, such as mitochondrial and peroxisomal import receptor complexes, transcription repression complex and the anaphase promoting complex, all of which are involved in different aspects of cellular functions [6]. Structural considerations suggest that each TPR motif may form two amphipathic $\alpha$-helical regions [7,8].

PP5 and PPT1 were isolated from human and *Saccharomyces cerevisiae* genomes, respectively [4]. The human protein phosphatase has been predominantly localized to the nucleus. The *S. cerevisiae PPT1* gene is not essential for growth, as *PPT1* null mutants are viable. *ppt* transcripts analyzed in rat [3], were found to be differentially expressed in various tissues, suggesting that both temporal and spatial regulation of *ppt* expression occurs in higher eukaryotes.

In a previous study *pph-1*, a type 2A phosphatase from *N. crassa* was isolated and analyzed [9]. In order to determine if additional genes encoding for PP2A-like phosphatases are present in the filamentous ascomycete *N. crassa*, we screened the M.

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[1] The DNA sequence reported in this paper has been submitted to GenBank and has received the accession number U89985.


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Sachs conidial germination cDNA library (Fungal Genetics Stock Center) with a fragment of the *pph-1* gene. *E. coli* BB4 and XL1 blue were used as hosts for phage. They were grown in TB medium [10] supplemented with 50 $\mu$g ml$^{-1}$ tetracycline when required. Plasmids and phage $\lambda$ were propagated by standard techniques [10]. The library was probed with a [$\alpha^{32}$P]dCTP hexamer-labeled (Prime-A-Gene, Promega) 570bp *Eco*RV *pph-1* fragment, in the presence of 6 × SSC, 5 × Denhardt's solution, 50 mM sodium phosphate, 10% dextran sulphate, 100 $\mu$g ml$^{-1}$ salmon sperm DNA and 20% formamide at 42°C for hybridization and 0.1 × SSC/0.25% SDS at 42°C for the most stringent wash. Two positive clones containing overlapping cDNA fragments, yet differing in length and in restriction pattern from *pph-1*, were isolated. pEY43, containing the longer (2051 bp) insert, was sequenced completely on both strands (Fig. 1). The other clone, designated pEY44, is truncated at the 5′ end and initiated at nucleotide 504 of pEY43. Sequencing was carried out by the dideoxy chain termination method [11] using the Taq DyeDeoxy Terminator Cycle Sequencing kit and an automated sequencer (Applied Biosystems, 373A). Sequence analyses were carried out with the aid of the GCG program [12].

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   1  CCCTTCTGGCATATCAGACTACATATTTCTCTTTCTTTCTTGCTGAATACTTTTGTTTACACTGTAGCATATTTTCTTATCTAACCCCATCTCTCATACG  100
 101  TTTTCGCCCACTGGCCCCGTGTGCCTGACTTATAAAAAGCGAAGAACGGCCATCCTTGTCCGGTTCCAGTTGTTTCCTTTGGACTCTTTCTTTCTTTGGG  200
 201  TAATCCAAATTCTTTTTCCGCATGTTGGCGCGCCCGAGTCCAAGGTAGCACCCACAGTATCACCCGGATACACACCCAGGACACCCACCCATCGCCCACA  300
 301  TAAACCAGAGACAATGACGACACCCCAAGAGCAGGCCATCGCCTTCAAGAACGAGGGCAACAAGGCCTTCGCCGCCCACGACTGGCCCAAGGCCATCGAG  400
                M  T  T  P  Q  E  Q  A  I  A  F  K  N  E  G  N  K  A  F  A  A  H  D  W  P  K  A  I  E
 401  TTTTACGACAAGGCCATCGAGCTCAACGATAAGGAACCTACGTTCTGGTCCAACAGAGCCCAGGCCCATCTCAAAACAGAAGCATACGGATATGCTATTC  500
      F  Y  D  K  A  I  E  L  N  D  K  E  P  T  F  W  S  N  R  A  Q  A  H  L  K  T  E  A  Y  G  Y  A  I  R
 501  GCGATGCCACAAAAGCCATTGAGCTCAACCCGGGCTTCGTCAAGGCCTACTACCGTCGCGCTACCGCCTACGCCGCCATCCTGAACCCCAAGGAAGCCGT  600
        D  A  T  K  A  I  E  L  N  P  G  F  V  K  A  Y  Y  R  R  A  T  A  Y  A  A  I  L  N  P  K  E  A  V
 601  CAAGGATTTCAAGACTTGCGTCAAGATTGCCCCCGATAACAAGGATGCCAAGCTCAAGCTTGTCGAGTGCGAGAAGATTGTGCGCCAGCTCGCCTTCTTT  700
        K  D  F  K  T  C  V  K  I  A  P  D  N  K  D  A  K  L  K  L  V  E  C  E  K  I  V  R  Q  L  A  F  F
 701  GCCGCTATCGAAGTCGGGGATGAGCTGTCTGCTGCTGAGGGCCTCGATGTCGAGTCCATGGCAGTGGATGCTTCTTACGACGGCGTGCGCCTTGAAGGGA  800
      A  A  I  E  V  G  D  E  L  S  A  A  E  G  L  D  V  E  S  M  A  V  D  A  S  Y  D  G  V  R  L  E  G  N
 801  ATGAGATGACACAGGAGTTTATCGACGACATGATTGAGCGCTTCAAGCGCGGCAAACTGATCCACAAGAAATACGTTTACCAAATCATTATCGCCGTCAG  900
        E  M  T  Q  E  F  I  D  D  M  I  E  R  F  K  R  G  K  L  I  H  K  K  Y  V  Y  Q  I  I  I  A  V  R
 901  GAACATTGTATATAACGAGCCCACCATGGTTGAGGTGGACATCCCTGAGGATGTGCAGTTGACAGTTTGCGGTGATACCCACGGCCAATATTTCGATCTG 1000
        N  I  V  Y  N  E  P  T  M  V  E  V  D  I  P  E  D  V  Q  L  T  V  C  G  D  T  H  G  Q  Y  F  D  L
1001  ATGGAGCTCTTCAGGTTGAACGGTTTCCCTAGCGATAAGCACTACTACTTGTTTAACGGCGACTTCGTTGACAGAGGTTCCTGGTCGACCGAAATTGCCC 1100
        M  E  L  F  R  L  N  G  F  P  S  D  K  H  Y  Y  L  F  N  G  D  F  V  D  R  G  S  W  S  T  E  I  A  L
1101  TCCTCCTTTATGCCTACAAGTGGTTAAGGCCAAACGGCTTCTTCATCAACCGCGGAAACCACGAGACAGACGACATGAACAGGGTGTATGGCTTCGAGGG 1200
        L  L  Y  A  Y  K  W  L  R  P  N  G  F  F  I  N  R  G  N  H  E  T  D  D  M  N  R  V  Y  G  F  E  G
1201  CGAGTGCAAGCACAAGTACAATGAGAGAACCTACAAGCTCTTTTCGGAAAGTTTCTCGGCCCTCCCGCTCGCCACGTTGATTGGCAAGAAGTTCCTCGTC 1300
        E  C  K  H  K  Y  N  E  R  T  Y  K  L  F  S  E  S  F  S  A  L  P  L  A  T  L  I  G  K  K  F  L  V
1301  CTCCACGGCGGTCTCTTCTCGGACGACAACGTCACGCTCGACGACATCCGCAAGCTCGACCGTCACAAGCAGAAGCAGCCCGGTCAGGCTGGCCTCATGA 1400
      L  H  G  G  L  F  S  D  D  N  V  T  L  D  D  I  R  K  L  D  R  H  K  Q  K  Q  P  G  Q  A  G  L  M  M
1401  TGGAGATGCTCTGGACAGATCCTCAGCCGTTCCCTGGTCGCGGCCCCAGCAAGCGCGGTGTTGGCATGCAGTTCGGTCCCGACGTTACCAAGCGTTTCTG 1500
        E  M  L  W  T  D  P  Q  P  F  P  G  R  G  P  S  K  R  G  V  G  M  Q  F  G  P  D  V  T  K  R  F  C
1501  CGACAAGAACGGTTTGGAGGCCATCATCCGCAGTCACGAGGTTCGCATGGACGGTTACGAGGAGGAGCACGACGGAAAGTGCATCACCGTCTTCTCGGCG 1600
        D  K  N  G  L  E  A  I  I  R  S  H  E  V  R  M  D  G  Y  E  E  E  H  D  G  K  C  I  T  V  F  S  A
1601  CCCAAGTACTGCGACATGACGGAGAACAAGGGCGCTTACATCAACATTGGTCCCGATTACAAGTTGAAGTTCTCGCAGTTCGATGCCGTGCCGCATCCCA 1700
      P  K  Y  C  D  M  T  E  N  K  G  A  Y  I  N  I  G  P  D  Y  K  L  K  F  S  Q  F  D  A  V  P  H  P  N
1701  ATATAAAGCCCATGGCATATGCGCAAAGCTCCGTCATGTCGTCGCTGATGTAAAAGTTGGATGTATTTGTATAGCACCACAGAAAATAAAACGGCGTGAA 1800
        I  K  P  M  A  Y  A  Q  S  S  V  M  S  S  L  M  *
1801  TACCTCGGGATGCTGTGTAGATCGGGCATGTTTTGCAAGGAGAATGGTTCTGTTTTGGCTTTCGATTGCATGTGATTAGGCGTCGCGCGCAGGAGTATCG 1900
1901  GGGTTATGGTGTTAGGGTTCTTCTTCTTCTTGTCTTGATGATATATATTCCTTCTGGTGTTATTTGGATTCCATAGTGGTGATATCTTCTAGTCTGCCAA 2000
2001  TCGACAATCTCGATGTTGCCTCTCTCCCAAAAAAAAAAAAGAAAAGGAATC  2051
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Fig. 1. The nucleotide sequence of the pEY43 *N. crassa ppt-1* cDNA clone, and deduced amino acid sequence of PPT1.

The isolated gene, designated *ppt-1*, encodes for a predicted polypeptide 479 amino acids in length. The calculated molecular mass and isoelectric point of PPT1 are 55 kDa and 6.1, respectively. The predicted molecular mass of PPT1 is very similar to that calculated for PP5 [4].

Alignment of PPT1 with other phosphatases (Fig. 2) indicates that this polypeptide belongs to the PPT/PP5 novel-type phosphatase family. The predicted amino acid sequence shows a high level of identity to novel-type serine/threonine protein phosphatase polypeptides from human (49%), rat (50%) and budding yeast (47%). These comparisons were made on the basis of amino acid sequences ranging from 472–477 residues in length (covering almost the entire length of the analyzed polypeptides). A three-repeat TPR motif, characteristic of some PPT/PP5 phosphatases [3], is present in PPT1 (see boxed region in Fig. 2). The major differences in polypeptide sequence can be found at the regions which are linked to the TPR motif. In addition to the high degree of similarity to PPT/PP5 phosphatases, PPT1 also shares sequence similarity (40% identity) with the *N. crassa* PPH1 polypeptide, a type 2A phosphatase. This finding was expected, as a fragment of *pph-1* was used as a probe for the isolation of *ppt-1*. The major structural differences between PPT1 and PPH1 reside in the N-terminal region of the polypeptides (not shown), as PPH1 lacks the conserved TPR motif.

We mapped the *N. crassa ppt-1* gene on the basis of restriction fragment length polymorphism (RFLP) analysis. Among 32 restriction endonucleases used, only *Mbo*I, *Mph*I and *Nco*I revealed polymorphic pattern in the vicinity of *ppt-1* in *N. crassa* of Oak Ridge and Mauriceville backgrounds. *Nco*I was used to digest DNA from progeny of the 'small cross' [13]. A hexamer-labeled 1.3 Kb *Eco*RV/*Sma*I fragment of pEY43 was used as a probe. Based on the polymorphic pattern (Fig. 3), which is identical to that of the *inl* gene, *ppt-1* is located between *al-3* and *pab-1* on the right arm of linkage group V [14]. Hybridization patterns observed during RFLP analyses provided evidence that *ppt-1* is a single-copy gene.

In order to determine if *ppt-1* is differentially expressed during phases of conidial germination of *N. crassa*, Northern analysis was performed. RNA

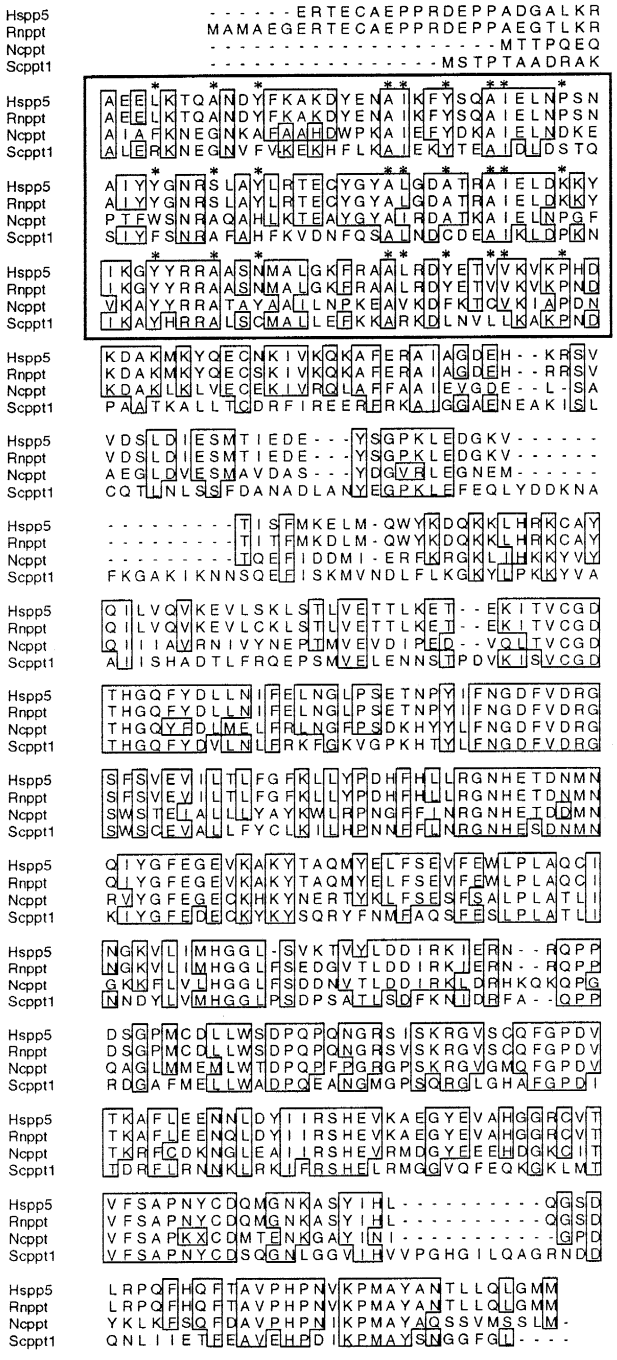


Fig. 2. Amino acid sequence comparison of the predicted PPT1 polypeptide with the PPT/PP5 subfamily of phosphatases deduced from genes isolated from other organisms. The amino acid sequences were aligned using the PILEUP program available in the GCG package. The aligned sequences are as follows: Ncppt - *N. crassa*, Hspp5 - *Homo sapiens* [4], Rtppt - *Rattus norvegicus* [3], Scppt1 - *Saccharomyces cerevisiae* [4]. Identical amino acids conserved in at least three of the four sequences are boxed. The TPR domain is boxed, asterisks indicate the positions (in each line) of the three components of the TPR motif [8].

was extracted from a wild-type strain of *N. crassa*. Conidia from cultures grown on 50 ml solid Vogel's minimal medium [15] were collected and filtered through cheesecloth. Conidia ($10^7$ per ml) were grown (34°C, 120 rpm) for different time periods in liquid medium. Germlings were harvested by sieving on Whatman number 1 filter paper and frozen in liquid nitrogen. Total RNA was prepared from frozen germling samples (25 mg) with TRI REAGENT (Sigma). Subsequently, polyA + RNA were isolated with the Poly A Tract mRNA Isolation Kit (Promega) and resolved on a formaldehyde agarose gel [10]. The RNA was then transferred to a Magnacharge NT Nylon membrane (MSI). A *ppt-1* RNA antisense probe was generated from *Hin*dIII-predigested pEY44, using phage T3 RNA polymerase as described by Sambrook et al. [10], with [$\alpha$-$^{32}$P]UTP as the label. The blot was also probed with an antisense RNA *act-1* (actin) probe [16] to verify loading and transfer of similar quantities of RNA. Northern hybridization was performed in the presence of 100 mM pipes (pH 6.8), 10 × Denhardt's solution, 500 mM NaCl, 0.2% SDS, 250 $\mu$g ml$^{-1}$ yeast tRNA and 20% formamide at 68°C. The most stringent washes were carried out at 68°C with 0.2 × SSC/0.1% SDS. *ppt-1* transcripts were detected on Northern blots prepared from *N. crassa* RNA extracted from various early phases of growth and development (Fig. 4). The transcript size (approx. 2 kb in length) is in agreement with the length of the longer cDNA clone isolated, suggesting that pEY43 contains a full-length *ppt-1* cDNA. As can be seen by Northern analysis, both *ppt*-1 and the *act-1* control probes detect more than one transcript. The presence of multiple (usually two) transcripts in *N. crassa* is not rare and has been

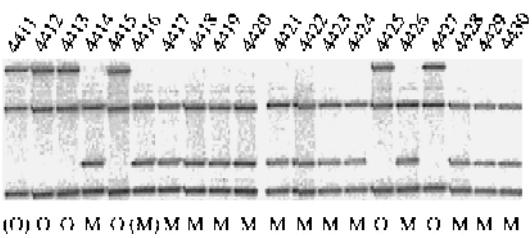


Fig. 3. RFLP analysis of the *ppt-1* gene: Southern blot analysis of 18 progeny of the standard cross of Mauriceville-1c-A(FGSC 4416) (M) with the Oak-Ridge (FGSC 4411) (O). The segregation pattern of the *ppt-1* gene is marked at the bottom of the blot.

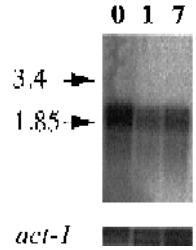


Fig. 4. Northern blot analysis of *ppt-1* transcript levels during conidial germination (0, 1 and 7 h from germination initiation). *act-1* transcript probing (bottom panel) was used to verify similar loading of RNA.

demonstrated in the case of several genes [17]. Abundant levels of transcript were present in dormant conidia. *ppt-1* transcript levels declined shortly after initiation of germination. This pattern of expression is different from that determined for *pph-1* [9], which is only slightly expressed in conidia, and exhibits an increase in transcript levels several hours after germination. Similar results were obtained in RT-PCR assays performed on RNA samples prepared from various phases of *N. crassa* conidial germination (data not shown). Our results indicate that different serine/threonine protein phosphatases are required at different stages of fungal development and that *ppt-1* activity is transcriptionally regulated. *ppt-1* may be involved in events occurring at the onset of conidial germination, after which the required levels of *ppt-1* are reduced. Alternatively, it is possible that *ppt-1* plays a role in conidial maintenance, and its required presence following germination is diminished. As it has now been demonstrated that a novel-type protein phosphatase is present in *N. crassa*, it is conceivable that other novel-type phosphatases are present in this and other filamentous fungi.

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