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Short sequence-paper

pzl-1 encodes a novel protein phosphatase-Z-like Ser/Thr protein phosphatase in *Neurospora crassa*[1]

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Abstract

The gene and cDNA of a novel protein phosphatase were cloned from *Neurospora crassa*. The *pzl-1* gene encompasses three introns and is localized to the left arm of chromosome I between *cyt-21* and *Fsr-12*. It encodes a protein of 58.3 kDa containing a Ser/Pro rich N-terminal segment, and a C-terminal domain that is similar to the catalytic subunit of type 1 protein phosphatases. The first 51 amino acid residues, including a potential *N*-myristoylation site, as well as the C-terminal domain (about 300 residues) have a high level of sequence identity with yeast PPZ phosphatases. However, residues 52–208 do not share high similarity with other proteins. The mRNA of *pzl-1* was detected in all phases of asexual development of the filamentous fungus.

Keywords: Ser/Thr protein phosphatase; PPZ-like protein; Fungal development; (*Neurospora crassa*)

Protein kinases and phosphatases cooperate in interlocking regulatory cycles to control the phosphorylation state of target proteins in eukaryotic cells. They regulate practically all aspects of cellular functions through this reversible postsynthetic modification. Protein kinases evolved from a common ancestor, while protein phosphatases (PP) belong to several distinct superfamilies [1]. In the PP1–PP2A–PP2B family of Ser/Thr protein phosphatases, besides the biochemically well characterized members there are some less abundant representatives whose existence has been revealed by molecular cloning [2].

One group of these novel phosphatases, termed PPZ, was found in budding and fission yeast only. *Saccharomyces cerevisiae* has two related genes, *PPZ1* and *PPZ2*, whose products *ppz1* and *ppz2* contain a C-terminal domain that resembles the catalytic subunit of PP1 [3–5]. Disruption of these genes resulted in a caffeine and temperature sensitive cell lysis [5,6]. Genetic evidence suggested that *PPZ1* and *PPZ2* were involved in the osmotic stability of the cells [4–6], and interacted with the *ENA1* P-type ATPase [7] and the *PKC1* protein kinase C-mediated signaling pathway [8]. An unusual feature of the PPZ enzymes is their less conserved Ser-rich N-terminal domain. Although the C-terminal domain of *ppz1*

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[1] The DNA sequences reported in this publication have been deposited in the EMBL database and have been given the accession numbers AF071751 and AF071752.

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was shown to be sufficient for catalytic activity, the presence of the N-terminal segment was essential for the full physiological function [9]. The homologue of PPZ termed Pzh1 has recently been cloned from *Schizosaccharomyces pombe* [10]. The C-terminal domain of this protein is similar to that of *ppz1* and *ppz2*; however, its N-terminal part, with the exception of about 40 amino acids, is unrelated to the *S. cerevisiae* counterparts. Like the baker's yeast enzymes, the fission yeast *pzh1* affects salt tolerance [7,10] but has additional distinct functions as well [10]. In the present communication we demonstrate that a PPZ-like gene is present not only in yeast but also in *N. crassa*, a filamentous fungus.

Previously we identified the activity of PP1 in *N. crassa* extracts and characterized the biochemical properties of the purified PP1 catalytic subunit [11]. In order to clone its gene we screened the *N. crassa* pSV50 cosmid genomic library [12] with a 0.68 kb *Drosophila PP1(87B)* cDNA fragment [13] labeled by [α-^{32}P]dCTP and the Megaprime random primed labeling kit (Amersham). Seven positive clones were isolated by colony hybridization. Restriction analysis revealed that two pairs of strongly hybridizing clones were identical. The representatives of the two pairs (termed P5A1 and P29) were selected for further analysis. The *Eco*RI–*Hin*dIII fragments of 5.2 kb and 4.0 kb were isolated from P5A1 and P29, respectively. They were subcloned into Bluescript pSK$^+$ plasmid (Stratagene) and were sequenced by the dideoxy chain termination method [14] using Sequenase 2.0 (USB), [α-^{35}S]dATP (Amersham) and synthetic oligonucleotide primers (Fig. 1). Initial sequence analysis revealed that P29 represented a truncated form of P5A1, hence the latter was used in the subsequent studies.

3401 nucleotides of the P5A1 clone were sequenced and its 2.1 kb *Xba*I–*Eco*RI fragment (nucleotides −138 to 2029) was utilized as a probe in screening a λ ZAPII cDNA library prepared from conidiating cultures of *N. crassa* (FGSC). A single positive clone was identified under stringent hybridization conditions. The pSK$^+$ vector was excised from the phage and its 3.0 kb insert was sequenced (Fig. 1). Excluding the three intron regions, the cDNA and genomic sequences were identical. The first intron (nucleotides −718 to −432) is in the 5′-noncoding region while the second (nucleotides 1414 to 1470) and the third (nucleotides 1624 to 1678) introns are at the 3′-end of the coding region. All of the introns contain the *Neurospora* consensus sequences of splice sites (GTRNGY at the 5′-end and NYAG at the 3′-end) as well as the internal lariat sequence (RCTRAC) close to the 3′-end [14]. In intron 1 there are two putative lariat sequences separated by 20 and 12 nucleotides from the 3′-end consensus. The 5′-noncoding sequence is unusually long (1566 nucleotides) and contains a CAAT box (starting at nucleotide −1537), six overlapping TATA box-like motifs (starting at nucleotide −1480) followed by three so-called +1 sequences (TCATCATC starting at nucleotide −1427) that are characteristic to the region around the transcription start site in *N. crassa* [15]. The 3′-noncoding region is rather short and is truncated in both sequences because it terminates in an *Eco*RI site that was used in the construction of the P5A1 clone as well as the cDNA library. The longest open reading frame in the cDNA (1593 bp) encodes a protein of 531 amino acids. The putative translational initiation site is the first ATG in the cDNA; 9 out of 13 nucleotides in this region match the Kozak's consensus for *N. crassa* [16]. The predicted amino acid sequence consists of two parts (Fig. 2). The N-terminal 208-amino-acid long stretch is rich in Ser and Pro residues. The very first 51 residues are reminiscent to the N-terminal end of the yeast PPZ protein phosphatases (41–45% identity), including Gly-2 within a recognition consensus for myristoylation [9,10] (Fig. 2B). The myristoylation of this residue was proven in *S. cerevisiae ppz1*, and its essential role in the protein's physiological function was demonstrated by site-directed mutagenesis [9]. The C-terminal portion of the protein spanning about 300 amino acids is similar to type-1 Ser/Thr protein

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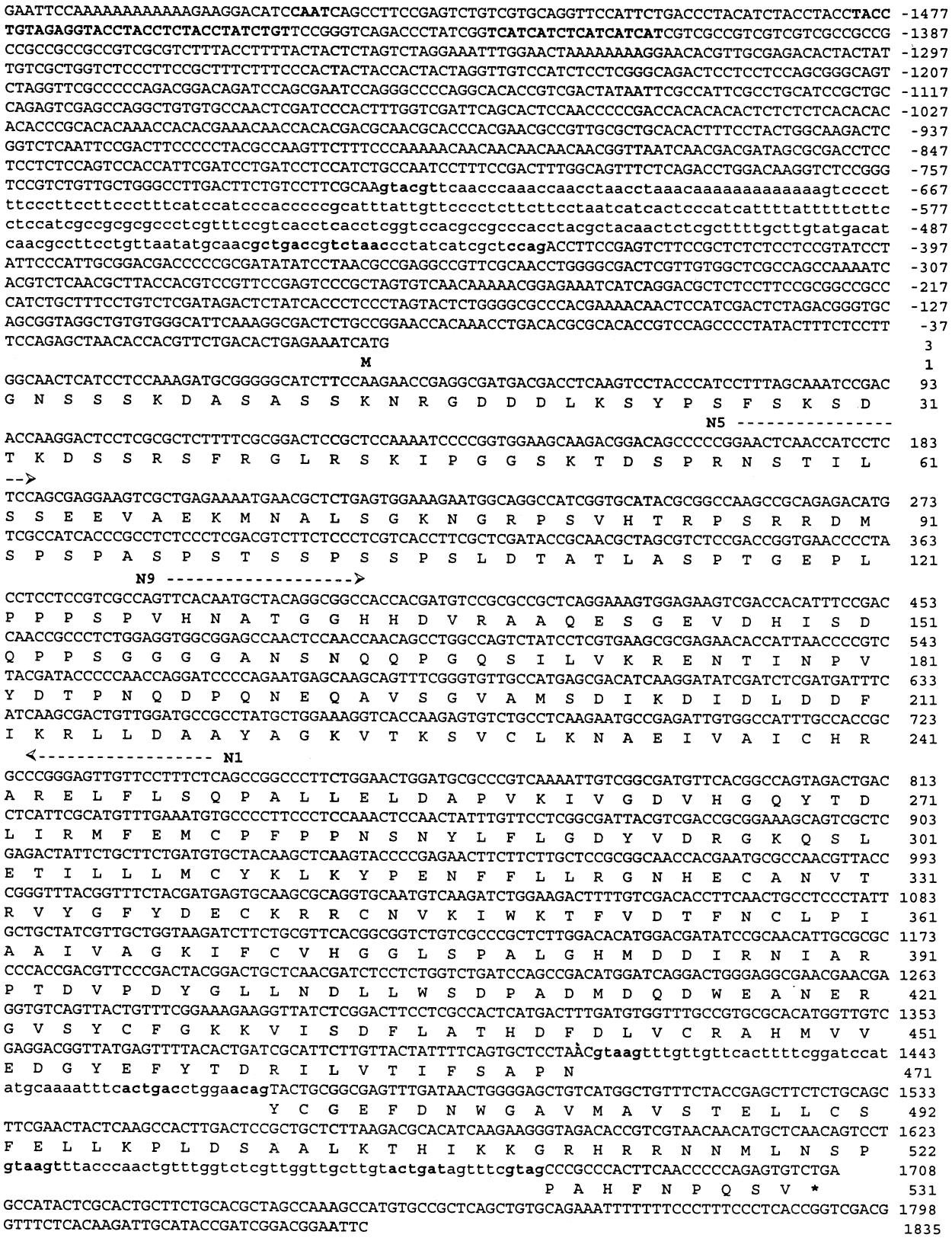
Fig. 1. Nucleotide and amino acid sequences of *N. crassa pzl-1* gene and cDNA. In the nucleotide sequence introns are shown in lower case with splice sites and internal consensus sequences highlighted in boldface. The initiating methionine as well as putative CAAT, +1 sequences and the TATA box-like motifs are shown in bold capitals. The stop codon is marked by an asterisk. The designation, position and direction of oligonucleotide primers used in RT–PCR are shown above the nucleotide sequence. Amino acid residues and nucleotides are numbered on the right of each line. The first 68 nucleotides were sequenced in the cDNA only.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AGCGGTAGGCTGTGTGGGCATTCAAAGGCGACTCTGCCGGAACCACAAACCTGACACGCGCACACCGTCCAGCCCCTATACTTTCTCCTT -37
TCCAGAGCTAACACCACGTTCTGACACTGAGAAATCATG 3
M 1
GGCAACTCATCCTCCAAAGATGCGGGGGCATCTTCCAAGAACCGAGGCGATGACGACCTCAAGTCCTACCCATCCTTTAGCAAATCCGAC 93
G N S S S K D A S A S S K N R G D D D L K S Y P S F S K S D 31
N5 ----------
ACCAAGGACTCCTCGCGCTCTTTTCGCGGACTCCGCTCCAAAATCCCCGGTGGAAGCAAGACGGACAGCCCCCGGAACTCAACCATCCTC 183
T K D S S R S F R G L R S K I P G G S K T D S P R N S T I L 61
-->
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S S E E V A E K M N A L S G K N G R P S V H T R P S R R D M 91
TCGCCATCACCCGCCTCTCCCTCGACGTCTTCTCCCTCGTCACCTTCGCTCGATACCGCAACGCTAGCGTCTCCGACCGGTGAACCCCTA 363
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N9 ---------->
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Q P P S G G G G A N S N Q Q P G Q S I L V K R E N T I N P V 181
TACGATACCCCCAACCAGGATCCCCAGAATGAGCAAGCAGTTTCGGGTGTTGCCATGAGCGACATCAAGGATATCGATCTCGATGATTTC 633
Y D T P N Q D P Q N E Q A V S G V A M S D I K D I D L D D F 211
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I K R L L D A A Y A G K V T K S V C L K N A E I V A I C H R 241
<---------- N1
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R V Y G F Y D E C K R R C N V K I W K T F V D T F N C L P I 361
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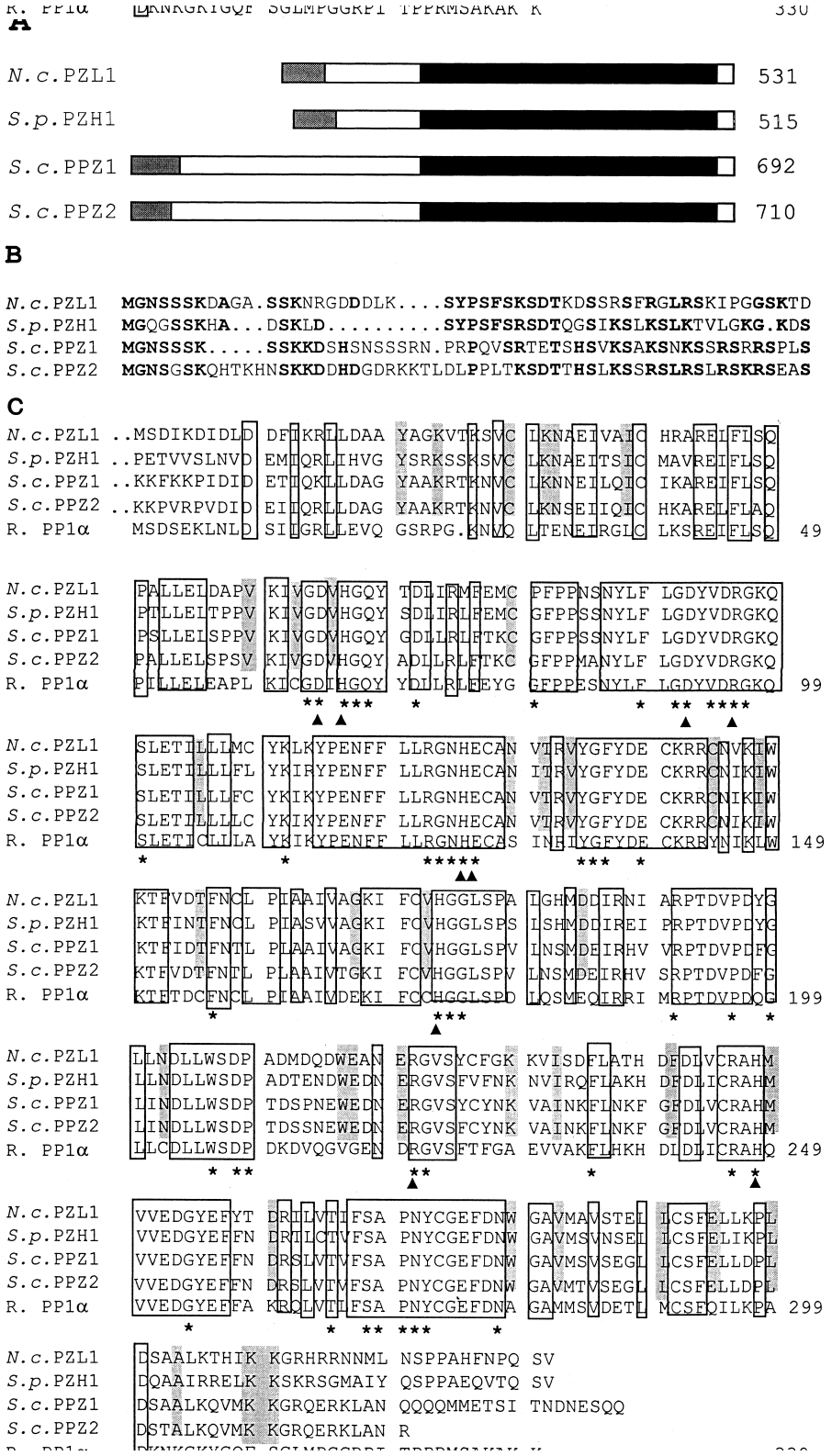
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S.c.PPZ2 MGNSGSKQHTKHNSKKDDHDGDRKKTLDLPPLTKSDTTHSLKSSRSLRSLRSKRSEAS
C
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S.c.PPZ2 ..KKPVRPVDID EIIQRLLDAG YAAKRTKNVC LKNSEIIQIC HKARELFLAQ
R. PP1α MSDSEKLNLD SIIGRLLEVQ GSRPG.KNVQ LTENEIRGIC LKSREIFLSQ 49
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S.c.PPZ2 PALLELSPSV KIVGDVHGQY ADLLRLFTKC GFPPMANYLF LGDYVDRGKQ
R. PP1α PILLELEAPL KICGDIHGQY YDILRLFEYG GFPPESNYLF LGDYVDRGKQ 99
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S.c.PPZ1 SLETILLLFC YKIKYPENFF LLRGNHECAN VTRVYGFYDE CKRRCNIKIW
S.c.PPZ2 SLETILLLLC YKIKYPENFF LLRGNHECAN VTRVYGFYDE CKRRCNIKIW
R. PP1α SLETICLLLA YKIKYPENFF LLRGNHECAS INEIYGFYDE CKRRYNIKLW 149
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S.c.PPZ2 LINDLLWSDP TDSSNEWEDN ERGVSFCYNK VAINKFLNKF GFDLVCRAHM
R. PP1α LLCDLLWSDP DKDVQGVGEN DRGVSFTFGA EVVAKFLHKH DLDLICRAHQ 249
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R. PP1α VVEDGYEFFA KRQLVTLFSA PNYCGEFDNA GAMMSVDETL MCSFQILKPA 299
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S.c.PPZ1 DSAALKQVMK KGRQERKLAN QQQQMMETSI TNDNESQQ
S.c.PPZ2 DSTALKQVMK KGRQERKLAN R

Fig. 2. Comparison of *N. crassa* PZL1 (*N.c.*PZL1), *S. pombe* Pzh1 (*S.p.*PZH1) [10], *S. cerevisiae* ppz1 (*S.c.*PPZ1) [4], and *ppz2* (*S.c.*PPZ2) [5,6] protein sequences. (A) Schematic representation of the fungal proteins. The conserved catalytic domain is shown in black, the similar N-terminal end is shown in gray. The number of amino acids in each protein is given on the right. (B) Comparison of the N-terminal end of the fungal PPZ-like proteins. Amino acid residues identical in at least two sequences are presented in bold. Gaps indicated by dots were introduced to maximize identity. Each sequence starts with the initiating methionine. (C) The catalytic domains of the fungal PPZ-like proteins are compared to each other and to the catalytic subunit of rabbit muscle PP1α (R. PP1α) [25]. Amino acid residues conserved in all PP1–PP2A–PP2B superfamily members are marked by asterisk and residues found in the catalytic center of PP1 by X-ray diffraction are labeled by arrowheads. Residues identical in type 1 protein phosphatases are boxed. Residues specific for the PPZ-like enzymes are shown in a shaded background. Dots indicate that all PPZ forms have an additional N-terminal domain. One gap (.) was introduced into the PP1α sequence to ensure maximum homology. The amino acid residues of rabbit PP1α are numbered for reference on the right of the figure.
←

phosphatases (Fig. 2C). The highest level of identity was found with *S. cerevisiae ppz1* (75%), *ppz2* (74%) and *S. pombe* Pzh1 (76%) catalytic domains. Much smaller identity values were obtained with *A. nidulans bimG* (59%), a PP1 phosphatase from another filamentous fungus [17], and the known type-2 protein phosphatases of *N. crassa pph-1* (39%), *CaMPrP* (29%) and *ppt-1* (26%) [18–20]. All the residues characteristic of the PP1–PP2A–PP2B family members [21], as well as the residues implicated in the formation of the catalytic cleft in rabbit and human PP1 [22,23], are conserved in the *N. crassa* protein. Besides the conserved residues characteristic of type 1 protein phosphatases, we found 38 amino acids that were identical in all known PPZ forms but not in PP1. These signature motifs (Fig. 2) strongly suggest that the novel gene isolated in our laboratories encodes a PPZ-like protein, thus it was designated *pzl-1*. However, the similarity to PPZs is restricted to the C-terminal catalytic domain and a short N-terminal segment. Residues 52–230 of *N. crassa* PZL1 exhibit only 26–28% identity to the corresponding regions of *S. pombe* or *S. cerevisiae* PPZ homologues, and has a similarly low level of identity to several unrelated Ser- and Pro-rich protein segments in the SwissProt database (release 35.0). This region may have a specific function characteristic to PZL1. The last 20 amino acids are also dissimilar. The predicted molecular mass of PZL1 (58.3 kDa) is smaller than that of *S. cerevisiae ppz1* (77.5 kDa), *ppz2* (78.5 kDa), and is close to that of *S. pombe* Pzh1 (57 kDa) [4–10].

The *pzl-1* gene was mapped by RFLP analysis. *N. crassa* was cultured and harvested as described earlier [11]. Oak Ridge (FGSC4411), Mauriceville-1C-A (FGSC4416) strains and the progeny of their 'small cross' were used for the gene mapping [24]. Two of the tested restriction enzymes, *Bst*EII and *Acc*I, yielded polymorphic patterns with the parental strains when analyzed with the random primer labeled insert of P5A1 genomic clone as a probe. The hybridization pattern obtained with *Bst*EII revealed that the gene is located at the left arm of chromosome I between *cyt-21* and *Fsr-12* (Fig. 3).

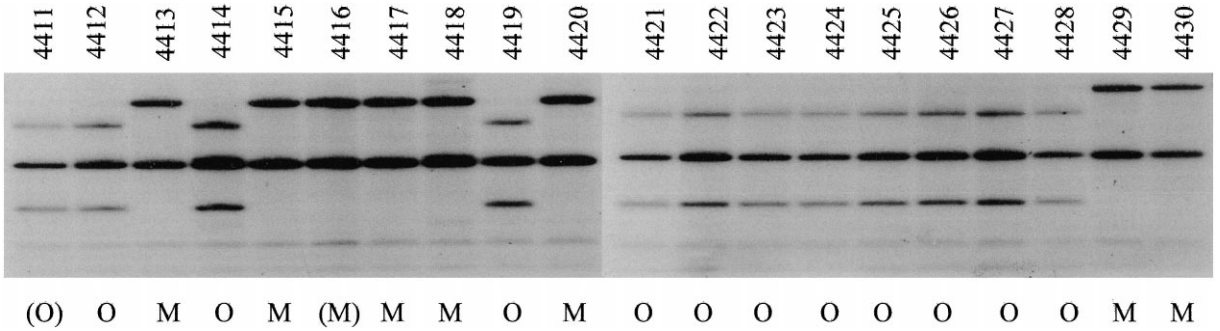

Fig. 3. Chromosomal localization of the *N. crassa pzl-1* gene by RFLP. Southern blot analysis was carried out after *Bst*EII digestion of 18 progeny, originating from the 'small cross' as well as the parental Oak Ridge (O) and Mauriceville-1C-A (M) strains. Fungal Genetics Stock Center numbers are shown above the lanes. The segregation pattern of *pzl-1* among the progeny of the cross is indicated in capital letters.

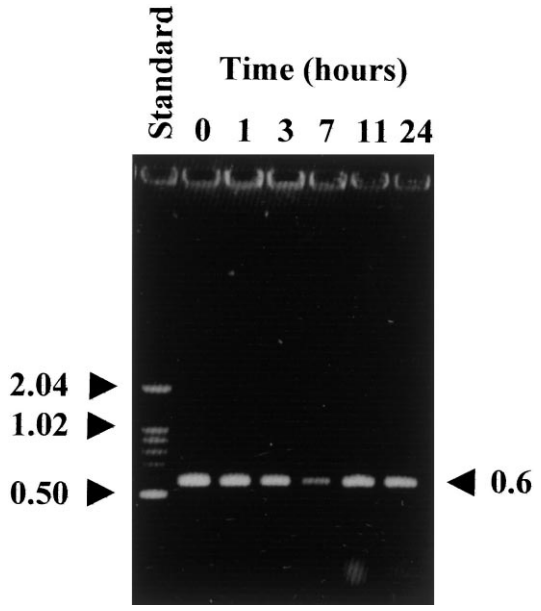

Fig. 4. Detection of *pzl-1* expression during asexual development of *N. crassa*. The N1–N5 primer pair (see Fig. 1) and DNase-treated RNA extracted from *N. crassa* cultures harvested from different developmental phases was used in RT–PCR. The 0.6 kb amplified fragment (stained with ethidium bromide) indicates the presence of the *pzl-1* transcript. Numbers above the picture show the hours after the initiation of conidial germination. *Hin*dIII digested λ phage was used as size standard in the experiment. A representative of three independent reactions is shown.

Results obtained with *Acc*I-digested DNA confirmed the chromosomal localization of *pzl-1* (data not shown).

Although the cDNA of the gene had been cloned we were unable to detect the *pzl-1*-specific mRNA by Northern blotting, most probably due to its low abundance. We therefore used the reverse transcriptase–polymerase chain reaction (RT–PCR) to detect *pzl-1* transcripts. Total RNA was isolated and equal quantities (2 μg) of DNase-treated RNA were analyzed by the Promega access RT–PCR kit using the N1–N9 and N1–N5 primer combinations (see Fig. 1). The expected 0.36 and 0.6 kb fragments were amplified (not documented). Since the sizes of PCR products obtained from the RNA and DNA preparations was identical, the efficiency of the DNase treatment was tested with *rgb-1*-specific oligonucleotides (*rgb-1* encodes the B regulatory subunit of *N. crassa* PP2A; Yatzkan and Yarden, unpublished results). The latter span an intron region in *rgb-1* and yield in a larger PCR fragment with the genomic DNA preparation. Only the smaller fragment was obtained in the DNase-treated RNA preparations, indicating that the *pzl-1* RT–PCR products were not generated from contaminating genomic DNA. Using synchronized *N. crassa* cultures [20], we noticed that the *pzl-1* gene was expressed in all stages of the asexual development (Fig. 4).

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