Biosci. Biotechnol. Biochem., **66** (4), 790–800, 2002

Gene Expression during P_i Deficiency in *Pholiota nameko*: Accumulation of mRNAs for Two Transporters

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Received October 1, 2001; Accepted November 29, 2001

The effects of P_i deficiency on gene expression in *Pholiota nameko* were examined. A cDNA library was constructed from poly(A)$^+$ RNA isolated from mycelia cultured in P_i-depleted (P^-) media, and 150 clones corresponding to *P*$_i$ *d*eficiency-*i*nducible (*pdi*) genes were selected by differential hybridization with probes prepared from poly(A)$^+$ RNAs from the mycelia cultured in the P_i-supplied (P^+) and P^- media. These clones were considered to derive from 31 genes by cross-hybridization. Northern blot analysis showed that these *pdi* genes were expressed in various patterns during P_i deficiency. Among the clones, the DNA sequences of *pdi85* and *pdi343* were analyzed. The deduced amino acid sequences indicated that they have structural similarities to P_i and metabolite transporters.

Key words: gene expression; metabolite transporter; *Pholiota nameko*; P_i deficiency; P_i transporter

Phosphorus (P) is an essential nutrient for all organisms. However, because plants and microorganisms can directly absorb P only as P_i, P often becomes a limiting element for their growth. Especially, P_i deficiency is a serious problem in the cultivation of crops. To cope with P_i-depleted conditions, most plants and microorganisms have developed various adaptive mechanisms, although varying the adaptive abilities. The adaptive mechanisms include the expression of some genes in response to P_i deficiency. In *Saccharomyces cerevisiae*, the adaptive mechanism was named the *PHO* regulon, which constitutes a complex family of genes and regulators.[1,2] Particularly, the expression regulatory system of *PHO5* encoding a repressible acid phosphatase has been vigorously studied as a model for the adaptive responses to environments, and some negative and positive regulatory factors have been identified.[3–8] Recently, it has been reported that genes encoding acid phosphatases,[9–11] ribonucleases,[12,13] P_i transporters,[14–18] β-glucosidase,[19,20] enolase,[21] pyruvate formate-lyase,[21] and probable regulatory proteins[22–24] were specifically expressed in response to P_i deficiency in some plants and microorganisms, and their corresponding proteins would cause structural and metabolic changes to adapt to P_i deficiency or be involved in regulating the expression of other P_i deficiency-inducible genes. This suggests that in various plants and microorganisms a coordinated system of gene expression such as the *PHO* regulon in *S. cerevisiae* may be present. However, little is known about the whole picture of regulation and function of genes expressed under P_i-deficient conditions.

We found previously that during P_i deficiency, the mycelia of *Pholiota nameko* (T. Ito) S. Ito et Imai in Imai grow relatively well and increase the amount of soluble protein and activities of acid phosphatase and RNA-degrading enzyme in the mycelia and culture filtrate.[25–28] Especially, the increase in acid phosphatase activity was greater than in other organisms.[25,28] Also, the increases in amounts of intracellular and extracellular protein were characteristic of *P. nameko*, since it has been reported that they were decreased by P_i deficiency in many organisms.[26,28] These findings suggest that the mycelia of *P. nameko* may have an excellent adaptive mechanism for P_i deficiency. Therefore, the elucidation of the adaptive mechanism will provide some information to resolve problems caused by P_i deficiency in the cultivation of crops.

We further analyzed *in vivo* labeled mycelial proteins by two dimensional polyacrylamide gel electrophoresis. The result showed that many proteins containing acid phosphatases were specifically synthesized *de novo* under P_i-deficient conditions, suggesting that the genes encoding these proteins are expressed at these times.[29] However, we have not understood the functions of proteins induced by P_i deficiency except for acid phosphatase and ribonuclease as yet. In this study, to reveal the effect of P_i deficiency on gene expression in the mycelia of *P. nameko*, we first isolated cDNA clones corresponding to genes that are specifically expressed under P_i-deficient conditions and examined changes in

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the levels of expression during the P_i-depleted (P^-) and P_i-supplied (P^+) cultures by northern blot analysis. Next, to discover the function of genes corresponding to the clones, we analyzed the DNA sequences and found that the deduced amino acid sequences of two clones were similar to sequences of P_i and metabolite transporters.

Materials and Methods

Strain and culture conditions. The strain N2 of *P. nameko* (Onuki Kinjin, Utsunomiya, Japan) was used in this study. The mycelia were cultured in the P^+ and P^- media containing phosphate at the concentrations of 550 and 50 mg/l as KH_2PO_4 as previously described.[28]

Preparation of RNA and construction of a cDNA library. Total RNA was isolated by the phenol-SDS method[30] with modifications. Poly(A)$^+$ RNA was purified using a Oligotex-dT30 〈Super〉 mRNA Purification Kit (Takara, Kyoto, Japan). About 3 μg of poly(A)$^+$ RNA was used to synthesize a double-stranded cDNA library with a cDNA Synthesis Kit (Amersham Pharmacia Biotech, Buckinghamshire, England). The resulting double-stranded cDNAs were cloned into *Eco*RI-digested, dephosphorylated λgt10 Arms (Takara) and packaged with a Packagene Lambda DNA Packaging System (Promega, Madison, WI, USA).

Differential screening of cDNA library. Probes were labeled by adding [α-^{32}P]dCTP to the reaction mixture for first strand cDNA synthesis from poly(A)$^+$ RNAs isolated from the mycelia cultured in the P^+ or P^- media for 20 d. About 2,000 plaques per plate were plated and the phage DNAs were transferred to nylon membranes (Hybond-N+; Amersham Pharmacia Biotech). The membranes were hybridized at 65°C overnight with 10 ng/ml of the ^{32}P-labeled probes. After the hybridization, the membranes were finally washed in 0.2×SSC containing 0.1% (w/v) SDS at 65°C. Plaques that hybridized more strongly with the probes from the mycelia cultured in the P^- media were selected.

DNA dot blot analysis. For the second differential screening, 1 μg of phage DNA from the clones isolated by the first differential screening was dotted onto nylon membranes. Hybridization and washing in the second differential screening were done as described in the first screening, and clones that hybridized more strongly with the probes from the mycelia of the P^- culture were further selected. Next, for classification of positive clones by cross-hybridization, 1 μg of phage DNA from the positive clones was dotted onto nylon membranes. The cDNA inserts of the positive clones were amplified by PCR using λgt10 forward and reverse primers (Takara) and *Taq* DNA Polymerase (Promega), and the products were digested with *Eco*RI and labeled with ^{32}P using a Megaprime DNA Labelling System (Amersham Pharmacia Biotech). The membranes were hybridized at 65°C overnight with the probes from each of the other positive clones and washed as described in the differential screening of cDNA library. The clones that hybridized with the probe were regarded as the same clones and classified into the same group.

Northern blot analysis. Twenty micrograms of total RNA per lane was put onto 1.2% agarose gel containing formaldehyde, separated by electrophoresis, and then blotted onto a nylon membrane using a vacuum transfer apparatus (Bio Craft, Tokyo, Japan). The membrane was hybridized at 42°C overnight with a probe prepared from the positive clones as described in the DNA dot blot analysis. After the hybridization, the membrane was washed for 30 min each in 6×SSC, 0.1% (w/v) SDS at room temperature, 2×SSC, 0.1% (w/v) SDS at room temperature, 0.2×SSC, 0.1% (w/v) SDS at 50°C, and 0.1×SSC, 0.1% (w/v) SDS at 65°C. The size of the detected transcript was estimated using a Perfect RNA Marker set, 0.2–10 kb (Novagen, Madison, WI, USA).

DNA sequencing and computer analysis. The *Eco*RI fragment of cDNA inserts was prepared and subcloned into pUC118 (Takara). The plasmid DNA was prepared using a Quantum Prep Plasmid Miniprep Kit (Bio-Rad, Hercules, CA, USA). The DNA sequence was identified using a Li-Cor 4000 Automated DNA Sequencer (Li-cor, Lincoln, NE, USA) with a M13 Primer (Nisshinbo, Tokyo, Japan) and a SequiTherm EXCEL II DNA Sequencing Kit-LC (Epicentre Technologies, Madison, WI, USA) and analyzed with a Base Image IR software ver.4.0 (Li-cor). The DNA and deduced amino acid sequences were analyzed with a GENETYX-MAC software system (Software Development Co., Tokyo, Japan). A similarity search was done with the BLAST program.[31] The membrane-spanning region and consensus site of the deduced amino acid sequence were predicted by the MEMSAT[32] and the PROSITE[33] programs, respectively.

Amplification of 5′-end of pdi343 cDNA. By 5′ rapid amplification of cDNA end (RACE) technique, the 5′-end of *pdi343* cDNA was amplified with total RNA isolated from the mycelia cultured in the P^- medium for 20 d and 5′-Full RACE Core Set (Takara) according to the recommended protocol. Primers were designed from the known DNA sequence of *pdi343* clone: reverse transcription primer (5′-GAGGAGTAGATTCCG-3′); 1st PCR primers, (5′-ATGCTTGTACTTCGCCGCTA-3′ and 5′-GATGCGGGCATGTTTCATGG-3′); and 2nd PCR

Table 1. Classification and Expression Pattern of *pdi* Clones

Clone	cDNA size (bp)	mRNA size (b)	Number of clones	Expression pattern	Clone	cDNA size (bp)	mRNA size (b)	Number of clones	Expression pattern
pdi310	450	650	31	D	*pdi370*	1400	1400	3	B(P^+)
pdi63	800	800	13	C(P^+)	*pdi201*	400	700	2	B
pdi5	1200	1200	11	F(P^+)	*pdi205*	700	2000	2	J
pdi36	700	700	9	H(P^+)	*pdi251*	500	500	2	J(P^+)
pdi85	2000	2000	8	B	*pdi298*	200	700	2	J
pdi244	550	600	8	G(P^+)	*pdi99*	1000	1100	1	A(P^+)
pdi26	750	1250	6	A(P^+)	*pdi252*	200	800	1	I(P^+)
pdi228	400	450	6	F(P^+)	*pdi259*	400	800	1	I(P^+)
pdi230	900	1300	5	C(P^+)	*pdi284*	200	500	1	J(P^+)
pdi263	400	500	5	J(P^+)	*pdi301*	200	750	1	L(P^+)
pdi315	500	500	5	D	*pdi308*	550	900	1	D(P^+)
pdi319	700	700	5	G(P^+)	*pdi348*	1400	1400	1	K(P^+)
pdi343	1350	1600	5	A(P^+)	*pdi351*	750	800	1	L(P^+)
pdi88	650	1100	4	B(P^+)	*pdi371*	1100	1100	1	E(P^+)
pdi110	750	800	4	B(P^+)	*pdi372*	1100	1100	1	E(P^+)
pdi373	1350	1350	4	E(P^+)					

pdi clones are classified by cross-hybridization. cDNA and mRNA sizes are estimated from PCR products with λgt10 primers and northern hybridization, respectively. Alphabets in the expression pattern correspond to those in Fig. 1.

P^+ in parentheses shows that mRNA corresponding to the clone was detected in the mycelia of P^+ culture.

primers, (5′-CCGAATTCGGCAACCTGGTT CTT-GT-3′ and 5′-GGGAATTCTG GTAGCGAATC-GGTTC-3′).

Southern blot analysis. High molecular mass genomic DNA was isolated from the mycelia cultured for 7 d in the P^+ medium using the cetyltrimethylammonium bromide method according to Wagner *et al.*[34] Ten micrograms of genomic DNA was digested with *Eco*RI, *Bam*HI, *Hind*III, or *Sac*I, electrophoretically separated through 0.8% agarose gel, and transferred to a nylon membrane. The hybridization and washing conditions were the same as those described in northern blot analysis.

Results

Isolation of P_i deficiency-inducible cDNA clones

A cDNA library was constructed from poly(A)$^+$ RNA isolated from the mycelia cultured in the P^- medium for 20 d. Duplicate blots containing about 20,000 recombinant clones of the library were screened by differential hybridization with the probes from the mycelia on day 20 of the P^+ or P^- cultures, and 292 clones displaying a stronger autoradiographic signal with the probe from the mycelia of P^- culture were isolated. Additionally, 150 reproducible clones were selected among them by a second differential screening on dot blots. These clones corresponding to P_i *d*eficiency-*i*nducible genes were designated *pdi* clones. Next, the classification of 150 clones was done by cross-hybridization and they were classified into 31 groups. A representative clone and the number of *pdi* clones obtained in each group are summarized in Table 1. The result shows that there are differences in the number of *pdi* clones in each group, namely from 1 to 31 clones.

Expression of pdi genes under P_i deficiency

The levels of mRNA corresponding to 31 *pdi* clones in the mycelia during the P^+ and P^- cultures were examined by northern blot analysis. On day 20 of cultivation, the transcripts of 6 *pdi* clones could not be detected in the P^+ cultures, such as *pdi85* and *pdi310* (Fig. 1 and Table 1). On the other hand, the transcripts of 25 clones could be detected in the P^+ culture, but the band strengths were weaker in the P^+ culture than in the P^- culture, such as *pdi343* and *pdi5*. These results indicate that all the genes corresponding to the isolated clones are specifically induced by P_i deficiency.

Changes in the levels of transcripts of *pdi* genes during the P^- culture were found to vary considerably with the clones and divided roughly into 12 patterns. Representative patterns are shown in Fig. 1. The transcripts of *pdi343* and *pdi85* increased gradually as the cultivation progressed, but only that of *pdi85* decreased after 25 d. Conversely, that of *pdi63* was the largest on day 7 of the early time of cultivation and then gradually decreased. The transcripts of *pdi310* and *pdi371* were particularly abundant on day 20 and 25 d, respectively. The expression level of *pdi5* remained nearly constant throughout the cultivation. The transcripts of *pdi244*, *pdi36*, *pdi259*, *pdi298*, *pdi348*, and *pdi351* increased in a two-step process. The accumulation of transcripts of *pdi244* and *pdi36* reached a maximum on day 10, then decreased and increased again on day 30 and 25, respectively, and thereafter only the transcript of *pdi36* decreased again. The transcript of *pdi259* temporarily increased at 20 d, while that of *pdi298* increased gradually up to 20 d, and thereafter both the transcripts decreased

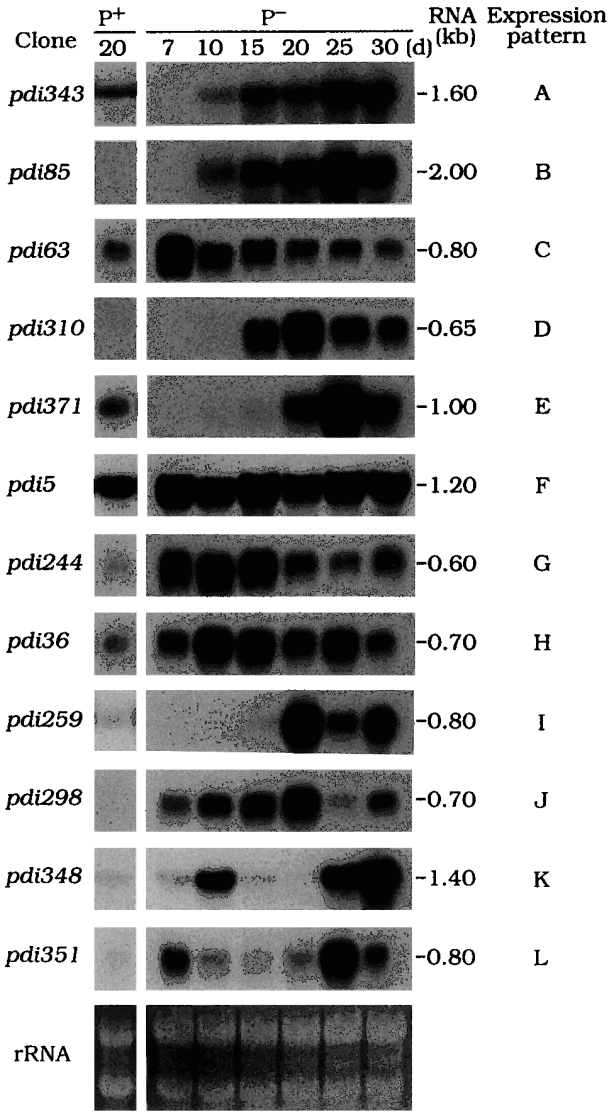

Fig. 1. Northern Blot Analysis of the Expression of *pdi* Genes.
Twenty micrograms of total RNA from mycelia of *P. nameko* cultured in the P^+ medium for 20 d and in the P^- medium for indicated periods were separated by agarose gel electrophoresis. Following transfer to a nylon membrane, the blot was hybridized with labeled cDNAs of *pdi* clones. The rRNA bands stained with ethidium bromide in the bottom panels indicate equivalent loading of total RNA on the gels. Estimated sizes of mRNAs corresponding to the respective *pdi* clones are indicated to the right of each panel.

on day 25 and then increased again. Those of *pdi348* and *pdi351* temporarily accumulated on day 10 and 7, respectively, and then increased again after 20 d, but only that of *pdi351* decreased again. The expression patterns, the presence of transcript under P^+ conditions, and the transcript size of all the *pdi* clones are summarized in Table 1.

DNA sequence analysis of pdi85 and pdi343

The DNA sequences of two *pdi* clones were analyzed. The cDNA of *pdi85* is 1,975 bp long and contains an open reading frame of 1,653 nucleotides encoding 551 amino acid residues with a molecular mass of approximately 59,900 (Fig. 2). Analysis of the deduced amino acid sequence using the PROSITE program showed that it contains 4 N-glycosylation sites displaying the N-{P}-[ST]-{P} consensus motif, 8 putative protein kinase C phosphorylation sites displaying the [ST]-x-[RK] consensus motif, and 6 casein kinase II phosphorylation sites displaying the [ST]-x(2)-[DE] consensus motif.

When the deduced amino acid sequence of *pdi85* was compared with the sequences in SWISS-PROT, PIR, PDB, and GenPept databases using the BLAST program, it shares 56, 47, and 37% identity with P_i transporters of the mycorrhizal fungus *Glomus versiforme* (*GvPT*),[15] the yeast *Saccharomyces cerevisiae* (*PHO84*),[14] and the higher plant *Catharanthus roseus* (PIT1),[16] respectively (Fig. 3A). An N-glycosylation site, a protein kinase C phosphorylation site, and a casein kinase II phosphorylation site are conserved among the four proteins.

An analysis using the MEMSAT program found that the predicted protein of *pdi85* contains 12 putative membrane-spanning regions (Fig. 3A). The hydropathy profile also showed high hydrophobicity and the existence of the 12 putative membrane-spanning regions (Fig. 3B). These membrane-spanning regions consist of 2 groups of 6 membrane-spanning regions separated by a central large hydrophilic loop, and the position and spacing of the membrane-spanning regions are very similar to those of 3 P_i transporters.

The DNA sequence of the *pdi343* was analyzed. As presumed from Table 1, in which the cDNA size of the *pdi343* clone was shorter than the corresponding mRNA size, the *pdi343* clone lacked the 5′ end of the full-length cDNA. Therefore, the region corresponding to the 5′ end of the mRNA was amplified by the 5′ RACE technique and sequenced. The full-length cDNA of *pdi343* is 1,588 bp long and contains an open reading frame of 1,458 nucleotides encoding 486 amino acid residues with a molecular mass of approximately 52,300 (Fig. 4). The deduced amino acid sequence contains an N-glycosylation site, 5 protein kinase C phosphorylation sites, 7 casein kinase II phosphorylation sites, and a tyrosine kinase phosphorylation site.

A similarity search of the amino acid sequence in SWISS-PROT, PIR, PDB, and GenPept databases showed that the deduced amino acid sequence of *pdi343* shares 45, 33, and 29% identity with metabolite transporters from the yeasts *Schizosaccharomyces pombe* (metabolite transporter, PIR: accession no. T39345) and *S. cerevisiae* (GIT1)[36] and the mold *Penicillium paxilli* (*paxT*),[37] respectively (Fig. 5A). The sequence of *pdi343* also has a similarity of 25% with P_i transporters from the higher plants *C. roseus* (PIT1)[16] and *Arabidopsis thaliana* (*PHT6*)[18] and has 23% with that of *pdi85*.

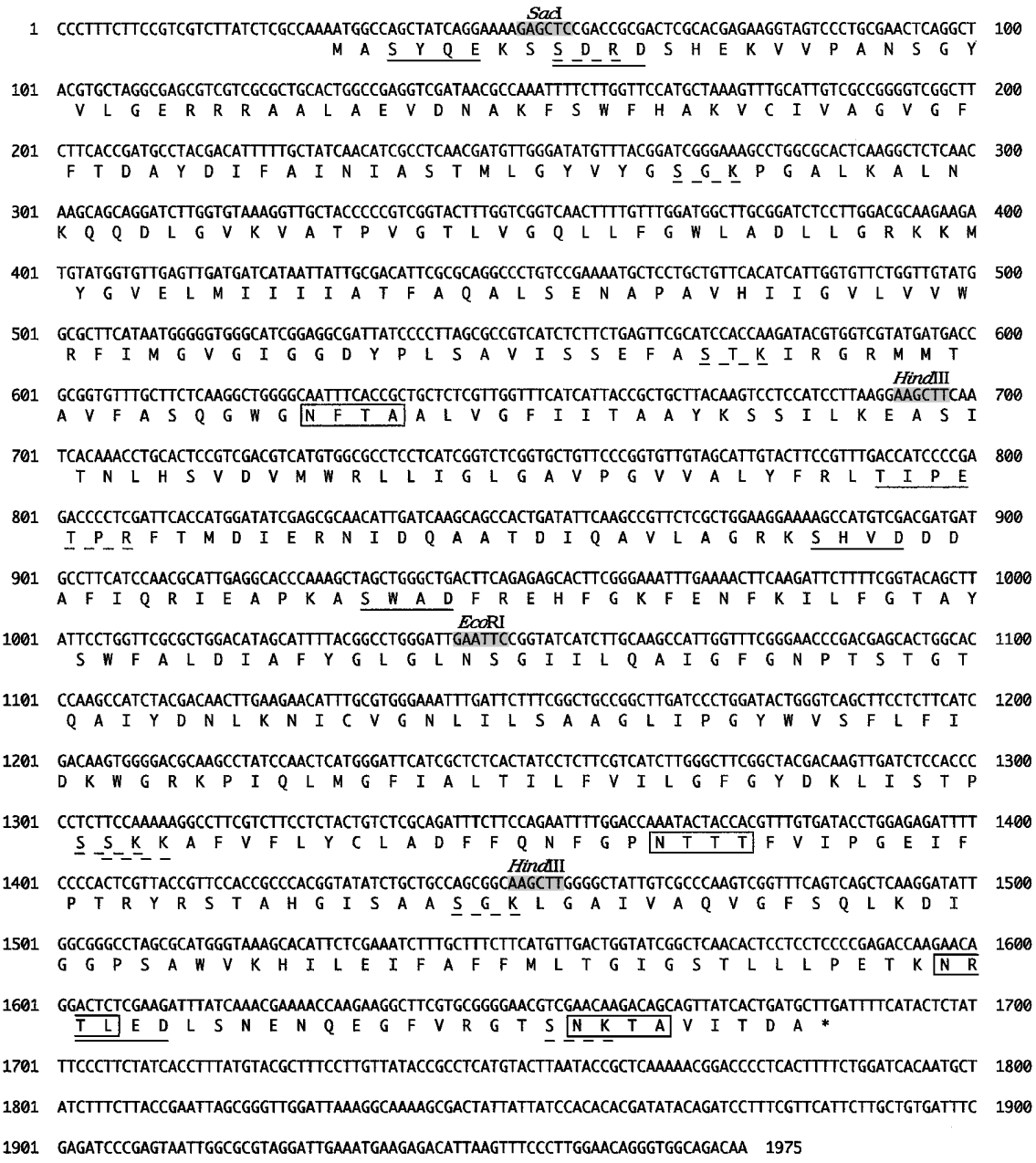

Fig. 2. The DNA Sequence and Deduced Amino Acid Sequence of *pdi85* Clone.

The translation stop is marked by an asterisk. Putative N-glycosylation sites are boxed. Putative phosphorylation sites by protein kinase C and casein kinase II are dot-underlined and underlined, respectively. Restriction enzyme sites are shaded. The DNA sequence data reported in this paper has been submitted to the DDBJ nucleotide sequence database under the accession number AB060641.

The analysis using the MEMSAT program found that the predicted protein of *pdi343* contains 12 putative membrane-spanning regions (Fig. 5A). The hydropathy profile also showed highly hydrophobicity and the existence of the 12 putative membrane-spanning regions (Fig. 5B). The spacing of the membrane-spanning regions is similar to those of P_i transporters. However, in *pdi343*, there was no central large hydrophilic loop like those found in *pdi85* and P_i transporters.

Southern blot analysis of pdi85 and pdi343

Genomic DNA was isolated from the mycelia of *P. nameko* and Southern blot analysis was done using the full-length cDNA probes of *pdi85* and *pdi343*. In *pdi85*, several major bands were detected after the digestion with *Eco*RI and *Hind*III (data not shown). Because the numbers of bands corresponded with those of each restriction enzyme site in the cDNA, the result suggests that the *pdi85* gene may be present in a single copy in the genome of *P. nameko*. In *pdi343*, several major bands were detected after the digestion with *Eco*RI, *Hind*III, and *Sac*I in spite of

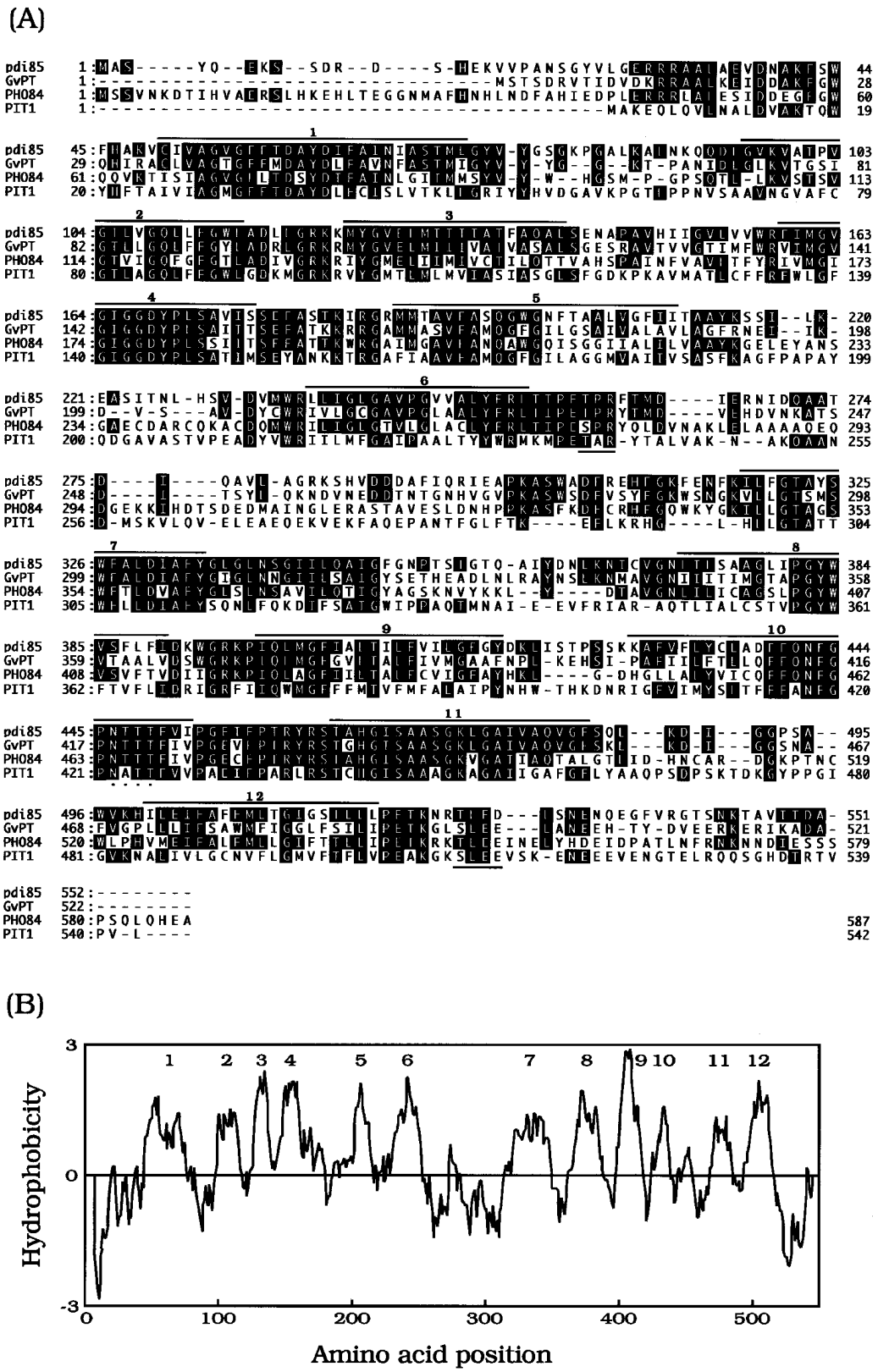

Fig. 3. (A) Alignment of the Deduced Amino Acid Sequence of *pdi85* with Those of *G. versiforme GvPT*,[15] *S. cerevisiae PHO84*,[14] and *C. roseus* PIT1.[16]

The amino acids identical with that of *pdi85* are highlighted. The positions of 12 membrane-spanning regions of *pdi85* predicted by the MEMSAT program are overlined and indicated by numbers. The sequences indicated by dots and underlines are consensus sites for putative N-glycosylation and phosphorylation sites by protein kinase C and casein kinase II among the four proteins, respectively.

(B) Hydropathy Profile Calculated from the Deduced Amino Acid Sequence of *pdi85*.

Hydropathy values were calculated by the method of Kyte and Doolittle[35] with a window size of 13 amino acids. Hydrophobic regions are given by a positive index. The numbers refer to putative membrane-spanning regions.

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1    CTCCCCGGCACTGCGAGCTTTTCTTCGTCATGTCGACCAAGCTCTCTCTCAAGGAACGCATCAAGAGTGTCTCTCTCATATTCGCTTGTGGGACAGCGTT  100
                                M  S  T  K  L  S  L  K  E  R  I  K  S  V  S  L  I  F  A  C  G  T  A  L

101  GTTCTCTGATGGATATGCCAACGGTGTCATTGGCAATGTCAACACATTGCTTACTAGGGTATACGGAGCTGACAAACTCGACCAGCATAATTACAGCAGG  200
      F  S  D  G  Y  A  N  G  V  I  G  N  V  N  T  L  L  T  R  V  Y  G  A  D  K  L  D  Q  H  N  Y  S  R

201  ACGCTCAGCAGTTTTGGTTTTGCGGGGACTGTCGTAGGAATGCTGGTCTTTGGATATCTGTCCGATAAAATTGGAAGGAAATTCGGGATGATGACTGCTG  300
      T  L  S  S  F  G  F  A  G  T  V  V  G  M  L  V  F  G  Y  L  S  D  K  I  G  R  K  F  G  M  M  T  A  A

301  CTGGCATAGTTACCCTATTCTCCGGGCTGTCGGCCGCGTCTTCTGGTGCACATGGAAGCGTTGGTGGATTGTTGAGCATGCTCAGTGCGATGCGCTTCCT  400
       G  I  V  T  L  F  S  G  L  S  A  A  S  S  G  A  H  G  S  V  G  G  L  L  S  M  L  S  A  M  R  F  L

401  CCTTGGCATCGGTGTAGGTGCAGAATATCCTTGTGGGTCTGTCTCTGCGTCAGAGCAATCGGAAGAGCCTGGTATCAGCAAGGGCGCCCAAAACCGCTGG  500
       L  G  I  G  V  G  A  E  Y  P  C  G  S  V  S  A  S  E  Q  S  E  E  P  G  I  S  K  G  A  Q  N  R  W

501  TTTGCGTTGGCGACAAATTCAATGATTGATTTTGGCTTTGTCGTCTCTTCGTTCGTTCCTCTGGTGTTATTCTGGATTTTCGGCAACGATCACCTCCGCG  600
       F  A  L  A  T  N  S  M  I  D  F  G  F  V  V  S  S  F  V  P  L  V  L  F  W  I  F  G  N  D  H  L  R  A

601  CTGTCTGGCGTCTCTCGCTTGGCTTGGGCGTTGTCCCCGCTGCCGCCGTTTTCATTTGGCGAATCAACATGAAAGAACCGATTCGCTACCAGAAGGATTC  700
       V  W  R  L  S  L  G  L  G  V  V  P  A  A  A  V  F  I  W  R  I  N  M  K  E  P  I  R  Y  Q  K  D  S

701  CATGAAACATGCCCGCATCCCTTACATGCTTGTACTTCGCCGCTACGGAGGGCGTCTAGCAGCTATTTCGGCAACCTGGTTCTTGTACGACATTATCGTT  800
      M  K  H  A  R  I  P  Y  M  L  V  L  R  R  Y  G  G  R  L  A  A  I  S  A  T  W  F  L  Y  D  I  I  V

801  TATCCTTTCGGAATCTACTCCTCCATCATCGTCGATCGTATCACTGGCGGAAAATCTGACCTTGCCGTCGTATTCGGCTGGAATGTTATCATCAATTTGT  900
       Y  P  F  G  I  Y  S  S  I  I  V  D  R  I  T  G  G  K  S  D  L  A  V  V  F  G  W  N  V  I  I  N  L  F

901  TTTACATGCCAGGAACTCTGGGCGGAGCATTCGTCGTTGACAGACTAGGCCCGAAGAACACGATGATCACTGGACTCCTACTCCAAGCAGTTATTGGATT  1000
       Y  M  P  G  T  L  G  G  A  F  V  V  D  R  L  G  P  K  N  T  M  I  T  G  L  L  L  Q  A  V  I  G  F

                                                                    HindIII
1001 CATTATGAGCGGTGCCTACGTCAGACTCACCAACAGCATTGCGGCTTTCGCCGTTGTGTATGGTATCTTCTTAAGCTTCGGAGAGCTTGGTCCTGGAAAT  1100
       I  M  S  G  A  Y  V  R  L  T  N  S  I  A  A  F  A  V  V  V  Y  G  I  F  L  S  F  G  E  L  G  P  G  N

1101 TGCCTGGGATTGCTGGCAAGTAAGAGTAGCCCAACTGCCATCCGCGGTCAATTCTACGGTACCGCGGCAGCCATCGGAAAAATTGGCGCCTTCATTGGCA  1200
       C  L  G  L  L  A  S  K  S  S  P  T  A  I  R  G  Q  F  Y  G  T  A  A  A  I  G  K  I  G  A  F  I  G  T

1201 CATGGATATTCCCTCCTATTATCGACGCTTTCGGTGGACCAAAAAGCAACAAGGGAAACACAGGTCCCTTCTGGATCGGCAGTGGTTTGGCCGTATTGAG  1300
       W  I  F  P  P  I  I  D  A  F  G  G  P  K  S  N  K  G  N  T  G  P  F  W  I  G  S  G  L  A  V  L  S

1301 CGCAATCATCACTTTCATGTGGATCAAGCCATTGACCACGGACGGTATGATTGAAGAGGACAGGAAGTTCCGCGAGTACCTAGAAGCTAATGGTTACGAC  1400
       A  I  I  T  F  M  W  I  K  P  L  T  T  D  G  M  I  E  E  D  R  K  F  R  E  Y  L  E  A  N  G  Y  D

1401 ACAAGCGTAATGGGATTGCCTGAGACCCAAAGCGAAGTAGAGACCGAGTCGGTGTCCGTTGACGAGAAACACGAGAAGGTTGATGTCTAGATATGTTATG  1500
       T  S  V  M  G  L  P  E  T  Q  S  E  V  E  T  E  S  V  S  V  D  E  K  H  E  K  V  D  V  *

1501 ATCTTTAATGAGGAGTGCAATGAACGCCGTCCTGTATCCTTTCAAGTAATCTTTCAAGTACTTATACCATGCATTCTGTATTTCAAAA  1588
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Fig. 4. The DNA Sequence and Deduced Amino Acid Sequence of *pdi343* Clone.

The translation stop is marked by an asterisk. Putative N-glycosylation site is boxed. Putative phosphorylation sites by protein kinase C, casein kinase II, and tyrosine kinase are dot-underlined, underlined, and double-underlined, respectively. Restriction enzyme site is shaded. The DNA sequence data reported in this paper has been submitted to the DDBJ nucleotide sequence database under the accession number AB060656.

the numbers of their restriction enzyme sites in the cDNA. Therefore, *pdi343* gene is probably a member of a small gene family.

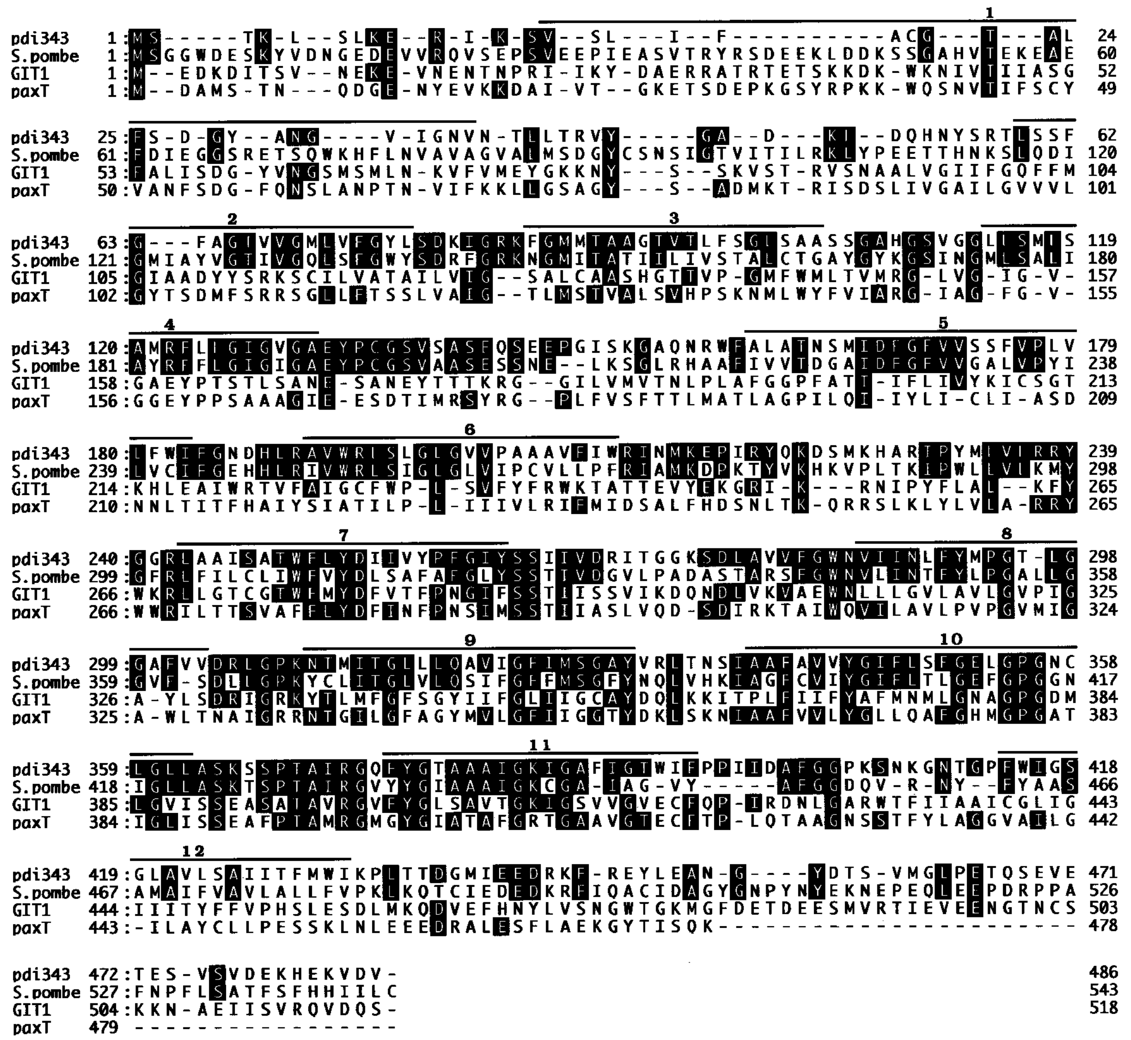
Discussion

In this study, we isolated cDNA clones corresponding to genes that are specifically expressed under P_i-deficient conditions. In differential screening of the cDNA library, 150 clones among about 20,000 recombinant clones screened, 0.75% of the library, were shown to be expressed in relative abundance under P_i-deficient conditions and they were considered to derive from 31 genes by cross-hybridization (Table 1). Attempts to isolate *pdi* genes have been done in some plants and microorganisms so far. In those studies, *pdi* genes encoding proteins such as acid phosphatases, ribonucleases, and probable regulatory proteins have been obtained.[9,13,17,22] However, the *pdi* genes identified in one organism are few and the genes isolated from *P. nameko* in this study are the most, to our knowledge. The analysis of these *pdi* genes will give important information about the adaptive mechanism for P_i deficiency.

When the *pdi* clones were classified by cross-hybridization, there are differences among the numbers of *pdi* clones in each group (Table 1). Because they generally represent the relative amount of transcripts of *pdi* genes, the more the clone number, the gene may code a functionally more important protein to adapt for P_i deficiency.

Northern blot analysis showed that the expression of all the *pdi* genes is controlled at the level of transcription in response to P_i deficiency. The expression of 31 *pdi* genes during the P^- culture was roughly divided into 12 patterns, suggesting that multiple genes showing the same expression pattern may be controlled by the same regulation mechanism. In the

(A)



(B)

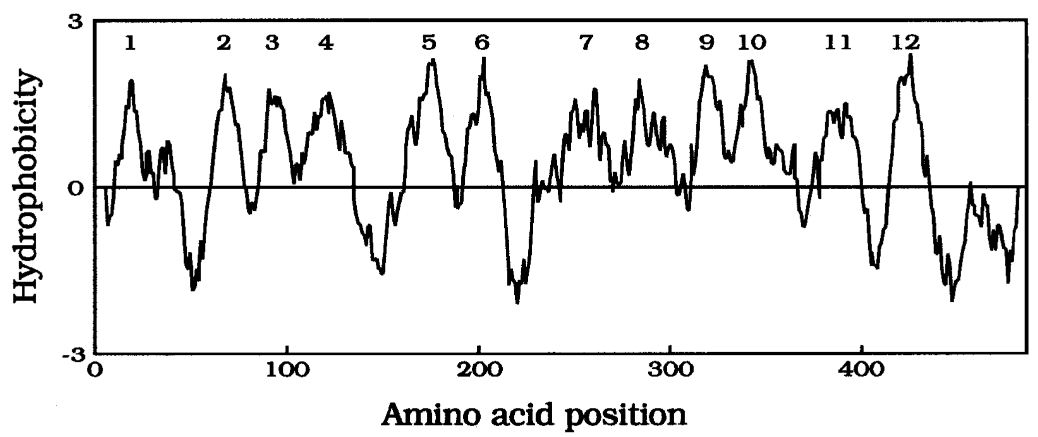


Fig. 5. (A) Alignment of the Deduced Amino Acid Sequence of *pdi343* with Those of the *S. pombe* Metabolite Transporter Gene, *S. cerevisiae* GIT1[36] and *P. paxilli paxT*.[37]

The amino acids identical with that of *pdi343* are highlighted. The positions of the 12 membrane-spanning regions of *pdi343* predicted by the MEMSAT program are overlined and indicated by numbers.

(B) Hydropathy Profile Calculated from the Deduced Amino Acid Sequence of *pdi343*.

Hydropathy values were calculated by the method of Kyte and Doolittle[35] with a window size of 13 amino acids. Hydrophobic regions are given by a positive index. The numbers refer to putative membrane-spanning regions.

expression patterns C, F, G, and H, the transcripts were already abundant on day 7, an early time for the P^- culture, at which time the mycelial growth was not depressed in spite of the depletion of P_i in the culture filtrate.[28] Psr1 gene in *Chlamydomonas*, which code for a regulator of phosphorus metabolism, has been reported to be induced rapidly during P_i deficiency.[24] Therefore, the *pdi* genes showing these expression patterns may code for regulatory proteins in the same manner as the Psr1 gene. On the other hand, in the expression patterns A and B, the transcripts accumulated gradually during the P^- culture. It has been reported that in the tomato, the genes encoding acid phosphatase[9] and P_i transporter[17] are likewise expressed gradually during the course of P_i deficiency. The genes showing the expression patterns A and B probably code for proteins directly involved in metabolism and incorporation of P_i under P_i-deficient conditions like acid phosphatase and the P_i transporter. The transcripts of genes showing the expression patterns D and E accumulated abundantly at the late time of cultivation, that is, their expressions were induced after the mycelial growth was depressed by the depletion of P_i in the culture filtrate.[28] This suggests that the genes may be expressed not necessarily due to the induction by P_i deficiency but due to a secondary response to it. The transcripts in the expression patterns G, H, I, J, K, and L were increased in a two-step process by P_i deficiency. According to Yamaguchi-Shinozaki *et al.*,[38] the expression of RD29, which is expressed in a two-step process upon desiccation of *A. thaliana*, is controlled by two regulatory systems: one is mediated by abscisic acid and the other is not. Although the expression of the corresponding *pdi* genes has not been analyzed in detail yet, the genes may be controlled by two different regulatory systems in a similar manner to the RD29 gene. Thus, the *pdi* genes are considered to show the expression patterns corresponded to their roles. However, the roles of proteins encoded by the genes remain unclear. Therefore, we tried to analyze the DNA sequences and discover the functions of *pdi* genes.

The deduced amino acid sequence of *pdi85* had significant similarity with those of known P_i transporters (Fig. 3A). P_i transporters belong to subfamily 9 of the Major Facilitator Superfamily and share significant structural characteristics.[39] They are internal membrane proteins consisting of 12 membrane-spanning regions that are separated into 2 groups of 6 membrane-spanning regions by a central large hydrophilic loop. The molecular sizes of P_i transporters are similar in various organisms; 518 to 587 amino acids, and the amino acid sequences conserve consensus sites for N-glycosylation, protein kinase C phosphorylation, and casein kinase II phosphorylation.[40] As shown in Fig. 3A and B, these structural characteristics were also found in the predicted protein of *pdi85*, suggesting that the *pdi85* gene encodes a P_i transporter.

The presence of two systems for uptake of P_i through cytoplasmic membranes has been suggested in some plants[41,42] and microorgamisms:[43,44] one system has a low-affinity for external P_i and the other has a high-affinity. It has been found that the low-affinity system is expressed constitutively and operates in the presence of P_i at high concentrations. However, the mechanism of the system is still unclear. On the other hand, because the high-affinity system is induced by P_i deficiency and operates in the presence of P_i at low concentrations, it is considered to be one of adaptive mechanisms for P_i deficiency. In the system, the P_i transporter plays an important role and the genes encoding it have been isolated in some plants and microorganisms.[14–18] In this study, a cDNA clone showing high similarity with the P_i transporter was isolated as the *pdi* gene, suggesting the presence of the high-affinity system for uptake of P_i in *P. nameko*.

The deduced amino acid sequence of *pdi343* showed 45–29% similarity with those of known metabolite transporters (Fig. 5A). Metabolite transporter is a general term for transport proteins of various metabolites such as organic acid and ion, that is, the P_i transporter is also one of metabolite transporters. The deduced amino acid sequence of *pdi343* showed similarity to those of P_i transporters. It contains 12 membrane-spanning regions. Also, no transporter other than the P_i transporter has been reported to be induced by P_i deficiency. These suggest that the *pdi343* gene may encode a P_i transporter as well as the *pdi85* gene. However, the predicted protein of *pdi343* does not have a central large hydrophilic loop found in P_i transporters. Moreover, although the amino acid sequence of *pdi343* contains an N-glycosylation site and several phosphorylation sites by protein kinase C and casein kinase II, the sites do not correspond to those conserved in P_i transporters. Therefore, the *pdi343* gene may encode some other transporter.

In general, it has been considered that the adaptive mechanism for P_i deficiency include increasing the availability of external P and the efficiency of uptake and intracellular use of P_i. In the mycelia of *P. nameko*, acid phosphatase and ribonuclease have already been shown to be induced and secreted by P_i deficiency.[25,27,28] From the result in this study that the gene encoding the P_i transporter is specifically expressed upon P_i deficiency, we propose the following adaptive mechanism for P_i deficiency: under P_i-deficient conditions, secreted ribonuclease hydrolyzes extracellular RNAs into nucleotides, which are subsequently decomposed by secreted acid phosphatase, and the resulting P_i is taken up into the cell by the P_i transporter. This is probably only a portion of the adaptive mechanism for P_i deficiency. In order to un-

derstand the whole picture, characterization of other *pdi* genes is now in progress.

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