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ORIGINAL PAPER

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Characterization of three PDI-like genes in *Physcomitrella patens* and construction of knock-out mutants

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Abstract Plant genomes typically contain several sequences homologous to protein disulfide isomerase (PDI). PDI was first identified as an abundant enzyme in the endoplasmic reticulum, where it catalyzes the formation, reduction, and isomerization of disulfide bonds during protein folding. PDI-like proteins have also been implicated in a variety of other functions, such as the regulation of cell adhesion, and may act as elicitors of the autoimmune response in mammals. A PDI-like protein (RB60) was recently shown to be imported into chloroplasts in the unicellular green alga *Chlamydomonas reinhardtii* and a higher plant, *Pisum sativum*, where it associates with thylakoid membranes. This suggests that the different PDI-like proteins in plant and animals may have diverse biological roles. To begin to elucidate the roles of PDI-like proteins, we have cloned, characterized, and generated knock-out mutants for three PDI-like genes that have high, medium, and low levels of expression, respectively, in the moss *Physcomitrella patens*. Phylogenetic analysis indicates that the three PDI-like proteins cluster with RB60 and four proteins from *Arabidopsis thaliana*. They are typified by an N-terminal domain rich in negatively charged residues. The knock-out mutants, which are the first knockouts available for PDI-like proteins in a multicellular organism, were found to be viable, indicating that the function of each single gene is dispensable, and suggesting that they may be functionally complementary.

Keywords PDI-like proteins · Knock out mutants · Targeted gene disruption · *Physcomitrella patens*.

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Introduction

Protein disulfide isomerase (PDI) is a highly abundant oxidoreductase protein known to assist in the folding of newly synthesized proteins in the endoplasmic reticulum (ER; Freedman et al. 1994). PDI is a member of the superfamily of thioredoxin-like proteins, which are characterized by a thioredoxin fold domain containing a redox active site with two vicinal thiols (Åslund and Beckwith 1999). PDI-like proteins contain either one, two, or three redox-active thioredoxin domains. Detailed structure analyses of PDI-like proteins has identified four types of key domains, *a*, *b*, *c*, and *D* (Ferrari and Soling 1999). Both the *a* and *b* domains have a thioredoxin fold of the $\beta\alpha\beta\alpha\beta\alpha\beta\beta\alpha$ form, where β denotes a β sheet and α an α-helix. The *a* domain is typified by a highly conserved redox active site, whereas the *b* domain lacks a redox active site. A second *a* or *b* domain in a PDI-like protein is denoted, respectively, as an *a'* or *b'* domain. The *c* domain is a unstructured acidic domain of 11–82 amino acids in length, which is suggested to serve as a calcium-binding domain (Lucero and Kaminer 1999). The *d* domain is a conserved 110-amino α helical domain found in some, but not all, PDI-like proteins (Ferrari and Soling 1999). For example, the ER PDI has four thioredoxin folds, two of which (*a* and *a'*) contain a redox active site while the other two (*b* and *b'*) do not, and a *c* domain at the C-terminus. The ER PDI does not contain a *D* domain. It has been suggested that all the PDI-like proteins originated from the same ancestral protein containing either one (McArthur et al. 2001) or two thioredoxin domains (Kanai et al. 1998).

PDI-like proteins typically catalyze the formation, reduction, and isomerization of disulfide bonds during protein folding in the endoplasmic reticulum (ER). However, in addition to their role in the ER, PDI-like proteins also participate in diverse cellular functions. They have been found to be indispensable subunits in protein complexes such as prolyl hydroxylase and triacyl-glycerol transfer protein (Freedman et al. 1994). PDI forms the β subunit of P4H, a $\beta 2\alpha 2$ tetramer enzyme that catalyzes the formation of 4-hydroxyproline residues in collagens (Pihlajaniemi et al. 1987). The assembly of P4H was found to be redox-dependent (John et al. 1993), and the formation of an intra-disulfide bond in the α-subunit was found to be essential for formation of the α-β complex (John and Bulleid 1994). Co-expression of PDI and the α-subunit of P4H in insect cells has demonstrated that the major function of PDI subunit is to maintain the α-subunit in a catalytically active conformation (Vuori et al. 1992). A PDI-like protein has recently been implicated in the regulation of E2A transcription factor dimerization and in the development of the B lymphocyte lineage (Markus and Benezra 1999). Members of the PDI-like protein family have also been found in the nuclei of maturing spermatids, where they may play a role in the redox-dependent condensation of spermatid chromatin (Perreault et al. 1984; Ohtani et al. 1993; Fornes and Bustos-Obregon 1994). A novel function of PDI, as a redox-regulated unfoldase, was identified recently. In its reduced state PDI binds and unfolds the A1 chain of cholera toxin, whereas in its oxidized state it cannot bind this substrate (Tsai et al. 2001). In addition, functions such as calcium storage (Lucero and Kaminer 1999), thyroid-hormone binding (Cheng et al. 1987), and form I phosphoinositide-specific phospholipase C activity (Bennett et al. 1988) have also been ascribed to PDI-like proteins.

PDI-like proteins have been identified in mitochondria and chloroplasts (Kim and Mayfield 1997; Rigobello et al. 2001; Trebitsh et al. 2001). The chloroplast protein RB60, a PDI-like protein, is a major component of the *psbA* mRNA-binding protein complex that is implicated in the redox-responsive regulation of translation in *Chlamydomonas reinhardtii* chloroplasts (Danon and Mayfield 1994; Kim and Mayfield 1997; Trebitsh et al. 2000). RB60 is imported into chloroplasts, where it is partitioned between the soluble stroma and the thylakoid membranes (Trebitsh et al. 2001). Collectively, these findings suggest that PDI-like proteins are used also as regulators in other cell compartments.

The identification of coding sequences for at least 11 PDI-like proteins in the genome of *Arabidopsis thaliana* argues in favor of this hypothesis. The isolation of mutants for each PDI-like gene should help clarify whether the different PDI-like proteins have distinct functions. However, the multiplicity of PDI-like genes in plant genomes hinders a genetic approach to the study of the specific functions of each gene, because other family members may compensate for a mutant one. Lately, the development of an efficient protocol for targeted gene disruption via homologous recombination in the moss *Physcomitrella patens* has been shown to allow the production of gene knockouts of a specific allele in a small gene family (Schaefer and Zryd 1997; Hofmann et al. 1999). The systematic generation of knock-out mutants should help to determine whether the different PDI-likes proteins in this organism have unique functions.

We therefore initiated a screen for PDI-like genes in *P. patens*. Using a PCR-based approach, we have isolated three genomic DNA fragments encoding polypeptides with high homology to PDI. Interestingly, the predicted ORF of one of the *P. patens* PDI-like clones codes for two tyrosine residues in place of the conserved cysteines of the C-terminal redox active site, a unique feature of this putative protein. Moreover, we found that the three PDI-like genes are expressed at different levels, suggesting that each has a unique function. To begin to discern the function of these proteins, a specific knock-out mutant for each of the three PDI-like genes was isolated. These are the first knock-outs available for PDI-like proteins in a multicellular organism.

Materials and methods

Plant growth and protoplasts isolation

P. patens B.S.G. was grown on solid minimal NH_4 medium (Ashton and Cove 1977) in culture room at 25°C. Light was provided from above by fluorescent tubes (cool white 40WT12) under a regime of 16 h of light and 8 h of darkness. Plants were subcultured every 7 days.

Protoplasts were isolated from 5- to 6-day-old protonemal cultures by incubation for 30 min in 1% Driselase (Fluka 44585) dissolved in 0.48 M mannitol. The suspension was filtered through a 100 µm stainless steel sieve, left for 15 min at room temperature to allow for complete digestion of the cell walls, and filtered again through a 50 µm sieve. Protoplasts were sedimented by low-speed centrifugation (500 rpm for 5 min) and washed twice in 0.48 M mannitol. Protoplasts were then resuspended at 1.2×10^6 protoplasts/ml in MMM solution (8.8% mannitol, 15 mM $MgCl_2$, and 0.1% MES pH 5.6). The PDI-H, PDI-M, PDI-L KO cassettes were amplified by PCR, the linear DNA was purified using Gibco-BRL columns (Concert rapid PCR purification system), resuspended in sterile water at a concentration of 0.5 mg/ml, and prepared for transformation (see below).

Isolation of PDI-like genomic and cDNA clones

Three degenerate primers were designed based on homology to RB60 (Accession No. AAC49896): forward primer 1 (5′-GTNCARGGNTAYCC-3′; degeneracy of 64), forward primer 2 (5′-GGNTGGGTNAARAA-3′; degeneracy of 32), and reverse primer 3 (5′-TCCATYTTNGCDAT-3′; degeneracy of 24). Using a nested PCR protocol, we isolated two different PDI-like clones, PDI-H and PDI-L (GenBank Accession No. AF430646). The PDI-M clone was isolated from a PCR with degenerate primer 2 and a primer specific to a *P. patens* EST (GenBank Accession No. AW145074; 5′-GCTCATCTTTACTG-3′). The PCR clones of PDI-H and PDI-M were then used as a radiolabeled probe to isolate the PDI-H (1895 bp; GenBank Accession No. AF430644) and PDI-M (1745 bp; GenBank Accession No. AF430645) cDNAs from a *P. patens* λZAPII cDNA library prepared from RNA extracted from protonemal tissue. For DNA hybridization assays, the

5′ PDI-H, 5′ PDI-L, and 5′ PDI-M probes were amplified in a PCR with the primer pairs H-forward (5′-CCGCGGCTGGGTGAAG AAGAAAT-3′) and H-reverse (5′-GGATCCTTAACATACCC TTC zAG-3′), primers L-forward (5′-CCGCGGTACCCTACTA CTATGTTGTT-3′) and L-reverse (5′-GGATCCTCAGTGCCTG CAAATAG-3′), and primers M-forward (5′-GCGGCCGCGGTG AAGAAGAAAT-3′), and M-reverse (5′-GGATCCACTGAGA-GAGAAA-3′), respectively.

Phylogenetic comparisons

The amino acid sequences of 51 PDI-like proteins were aligned using the computer program CLUSTAL W (v 1.8; Thompson et al. 1994). Phylogenetic trees were constructed by the ML (Maximum Likelihood) method (Felsenstein 1981; Hasegawa et al. 1991). Studies were done with the computer program packages PHYLIP (Felsenstein 1993) and MOLPHY (Adachi and Hasegawa 1995) under the JTT substitution model (Jones et al. 1992). The PDI-like sequences that were analyzed are closely related to the three PDI-like sequences from *P. patens* isolated in this study, and (apart from PAt-4 and Acb) can be found in the dbEST. The corresponding Accession Nos. are listed in Table 1.

Transformation of *P. patens* and generation of knock-out mutants

The knockout (KO) cassettes contained 429 bp, 500 bp and 543 bp of PDI-H, PDI-M, and PDI-L coding sequence, respectively, followed by the neomycin phosphotransferase (neo) gene, the expression of which was driven by the cauliflower mosaic virus (CaMV) 35S promoter, followed by 416 bp, 498 bp and 470 bp of PDI-H, PDI-M, and PDI-L coding sequence, respectively. The linear PDI-H, PDI-M, PDI-L KO cassettes were amplified by PCR, purified using Gibco-BRL columns (Concert Rapid PCR Purification System), and resuspended in sterile water at a concentration of 0.5 mg/ml.

For transformation of *P. patens*, 300 μl of a protoplast suspension was added to 30 μl of the PCR product. After gentle mixing, 300 μl of solution containing 40% polyethylene glycol, 0.1 M $CaNO_3$, 0.38 M mannitol, and 10 mM TRIS-HCl, (pH 8.0) was added, and the suspension was incubated with occasional mixing for 5 min at 42°C, and for 10 min at room temperature. The protoplast suspension was diluted to final volume of 7.5 ml with liquid NH_4 medium (Ashton and Cove 1977) supplemented with 6.8% mannitol, and incubated for 18 h in the dark. After 3 days of culture in a 16 h light/8 h dark regime, the protoplasts were plated on solid NH_4M medium covered with cellophane. After incubation for a further 4 days, the cellophane overlays were transferred to NH_4 medium supplemented with 50 mg/l G418 (GibcoBRL). Stable antibiotic-resistant clones were selected by a second round of growth of fragmented plants on NH_4 medium containing the antibiotic.

From 0.36×10^6 protoplasts derived from haploid protonema, 25, 36, and 55 neomycin-resistant colonies appeared after transformation with the PDI-H, PDI-M, and PDI-L KO constructs, respectively.

Analysis of transgenic moss plants

To screen for instances of simple integration of each KO cassette, the transformants were assayed for the incorporation of the Neo cassette into each PDI locus by PCR. Pairs of primers specific for the 5′ junction [either H-1 (5′-CTGGCGGTGGGGCTTCT-3′) or M-1 (5′-GAGGGAAAGCAGAAG-3′) with neo-2 (5’-TCCACCATGTTGACG-3′)], and the 3′ junction [either H-3 (5′-GTGCTACATTTGGGG-3′) or M-3 (5′-GCTCATCTTTACTG-3′) with neo-4 (5′-GGTTTCGCTCATGTG-3′)] were used. We used primers specific for the Neo sequence (neo-2 and neo-4) in PCR assays to identify and select against transformants that contained several integrated copies of the KO cassette.

Results

Cloning and characterization of three PDI-like genes from *P. patens*

Efficient gene knockout by homologous recombination requires the isolation of a genomic fragment of about

Table 1 List of PDI-like genes

Gene	Species	Accession No.	Name	Species	Accession No.
Pat-1	*Arabidopsis thaliana*	AL049655	Ace-2	*Caenorhabditis elegans*	ACC47065
PAt-2	*A. thaliana*	BAB09837	Ace-3	*C. elegans*	AAC69237
PAt-3	*A. thaliana*	AC037424	Ace-4	*C. elegans*	CAA85491
PAt-4	*A. thaliana*	BAB02677	AGg-1	*Gallus gallus*	ISCHSS
PAt-5	*A. thaliana*	AAD41430	AGg-2	*G. gallus*	A47300
PAt-6	*A. thaliana*	AAF07798	ADm	*Drosophila melanogaster*	AAA86480
PAt-7	*A. thaliana*	CAB38836	ARn-1	*Rattus norvegicus*	P38659
PAt-8	*A. thaliana*	AAK62431	ARn-2	*R. norvegicus*	P04785
PAt-9	*A. thaliana*	AAB91984	AHs-1	*Homo sapiens*	AAA58460
PAt-10	*A. thaliana*	AAF40463	AHs-2	*H. sapiens*	NP_005733
PAt-11	*A. thaliana*	AAC62863	AHs-3	*H. sapiens*	NP_000909
PDg	*D. glomerata*	AF131223	AHs-4	*H. sapiens*	AAC50401
PHv	*Hordeum vulgare*	L33251	AMm-1	*Mus musculus*	AAA39907
PMs-1	*Medicago sativa*	Z11499	AMm-2	*M. musculus*	CAA29759
PMs-2	*M. sativa*	T09614	AOc	*Oryctolagus cuniculus*	P21195
PNt	*Nicotiana tabacum*	Y11209	ABt	*Bos taurus*	P05307
PTa	*Triticum aestivum*	CAC21228	ASp	*Strongylocentrotus purpuratus*	A54757
PRc	*Ricinus communis*	U41385	AOv	*Onchocerca volvulus*	AAA85099
PZm	*Zea mays*	L39014	ASm	*Schistosoma mansoni*	CAA80520
PVc	*Volvox carteri*	AF110784	ACb	*Caenorhabditis briggsae*	CAB40200
POs	*Oryza sativa*	BAA81871	FAo	*Aspergillus oryzae*	Q00248
RB60	*Chlamydomonas reinharditii*	AF027727	FHi	*Humicola insolens*	P55059
rPDI-H	*Physcomitrella patens*	AAA27952	FPp	*Pichia pastoris*	CAC33587
PDI-M	*P. patens*	AF430644	FSc-1	*Saccharomyces cerevisiae*	NP_009887
PDI-L	*P. patens*	AF430645	FSc-2	*S. cerevisiae*	NP_010806
Ace-1	*Caenorhabditis elegans*	AF430646			

1 kb in length. Thus, we designed primers for the direct cloning of fragments of genes encoding PDI-like proteins by PCR in *P. patens*. Three degenerate primers were designed based on conserved regions of PDI-like proteins, and were used in nested PCR to isolate potential clones (Fig. 1). Using this approach, we purified two DNA fragments with high homology to PDI (PDI-H and PDI-L). In parallel, we identified a third gene, PDI-M, by homology searches of the *P. patens* EST database. A genomic fragment of PDI-M was cloned by PCR using a combination of a degenerate primer (No. 2) and a primer specific for the EST. The three genomic clones were then used to construct three KO cassettes that were subsequently employed to transform *P. patens* protoplasts and to generate knock-out mutants.

To characterize the ORF of each cloned gene, the first 500 bp of each of the genomic clones was used as a probe to isolate the corresponding cDNAs from a *P. patens* cDNA library. A full-length cDNA for PDI-H and a near full-length cDNA for PDI-M were isolated (Fig. 1). Due to the low abundance of the PDI-L transcript, we have yet to isolate the PDI-L cDNA. The PDI-H and PDI-M proteins both contain fully conserved N- and C-terminal redox active sites (-Cys-Gly-His-Cys-) typical of PDI-like proteins (Fig. 1). The

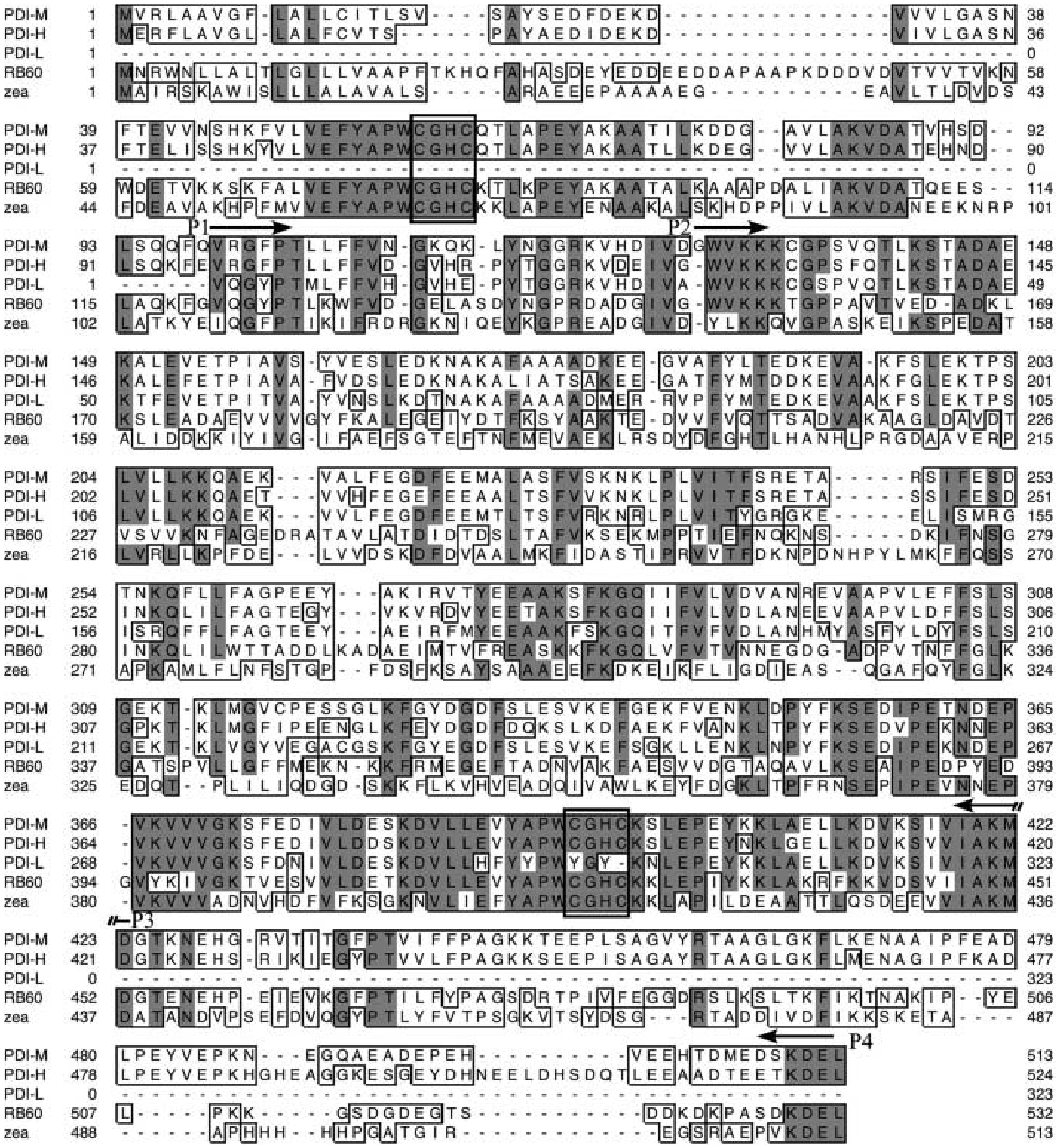

Fig. 1 Comparison of the deducted amino acid sequences of three cloned *P. patens* PDI-like proteins with the *Chlamydomonas reinhardtii* RB60 protein, and a *Zea mays* ortholog. The multiple alignment was generated using the computer program Clustal-W (Thompson et al. 1994). The conserved redox active sites are indicated by the *heavily outlined boxes*. P1, P2, P3, and P4 denote the locations of the four degenerate forward and reverse primers used to amplify the PDI-like clones

available genomic sequence of the PDI-L gene contains a potential short intron in the sequence encoding the putative C-terminal redox site (data not shown), and simulated excision of this predicted intron suggests that the redox site is not conserved. The ORFs for PDI-H and PDI-M both terminates with the -Lys-Asp-Glu-Leu sequence which is suggested to function as an endoplasmic reticulum retention signal.

A comparison of the deduced amino acid sequences of the three gene products with PDI-like proteins revealed a high level of conservation (Fig. 1). PDI-H, PDI-M, and PDI-L showed 57%, 60%, and 53% similarity, respectively, with the chloroplast RB60 protein from the unicellular green alga *C. reinhardtii* (Table 2). In order to determine whether the three *P. patens* clones are closely related to RB60, or whether they belong to different classes of PDI-like proteins, a phylogenetic tree was constructed using the maximum-likelihood method (Felsenstein 1981; Hasegawa et al. 1991). In order to exclude pseudogenes, we chose, for this analysis, sequences that were deposited in dbEST. All eleven PDI-like genes in the *A. thaliana* genome were included, as well as sequences from other plants, fungi, and animals. The sequences formed six clusters differing in primary structure (Fig. 2). The *P. patens* clones grouped with the *C. reinhardtii*, *Volvox catrteri*, *Oryza sativa*, and four of the *A. thaliana* sequences (Fig. 2, Cluster 1). Interestingly, all proteins in this cluster contain an N-terminal acidic domain (Fig. 2) reminiscent of the *c* domain found near the C-terminus of homologs of the ER PDI, and in the N-terminus of homologs of Erp72 (Ferrari and Soling 1999). This domain is implicated in binding of calcium (Lucero and Kaminer 1999). The clustering of the *P. patens* clones with only four of the eleven *A. thaliana* sequences suggests the existence of additional PDI-like genes in the *P. patens* genome. We are currently using new sets of degenerate PCR primers, designed based on conserved regions unique to proteins of the other clades, to determine whether similar orthologs are present in the *P. patens* genome.

Table 2 Degree of sequence homology of PDI-like protein sequences from *P. patens* with each other and with RB60 from *C. reinhardtii*

Protein	PDI-H	PDI-M	PDI-L	RB60
PDI-H	100 (100)	75 (83)	66 (77)	41 (57)
PDI-M		100 (100)	74 (82)	45 (60)
PDI-L			100 (100)	34 (53)
RB60				100(100)

[a] The values indicate sequence identity and similarity scores for the indicated pairwise comparisons

Southern analysis confirmed the authenticity of the PDI-H, PDI-M, and PDI-L clones (Fig. 3). Each of the three yielded a unique hybridization pattern, showing that the clones were derived from different loci. The detection of faint bands, in addition to the major hybridization signals, in the blots probed with PDI-H, PDI-M and PDI-L suggested the existence of additional closely related PDI-like genes. To determine whether the three genes are expressed, and to discriminate against pseudogenes, we hybridized radiolabeled probes for each

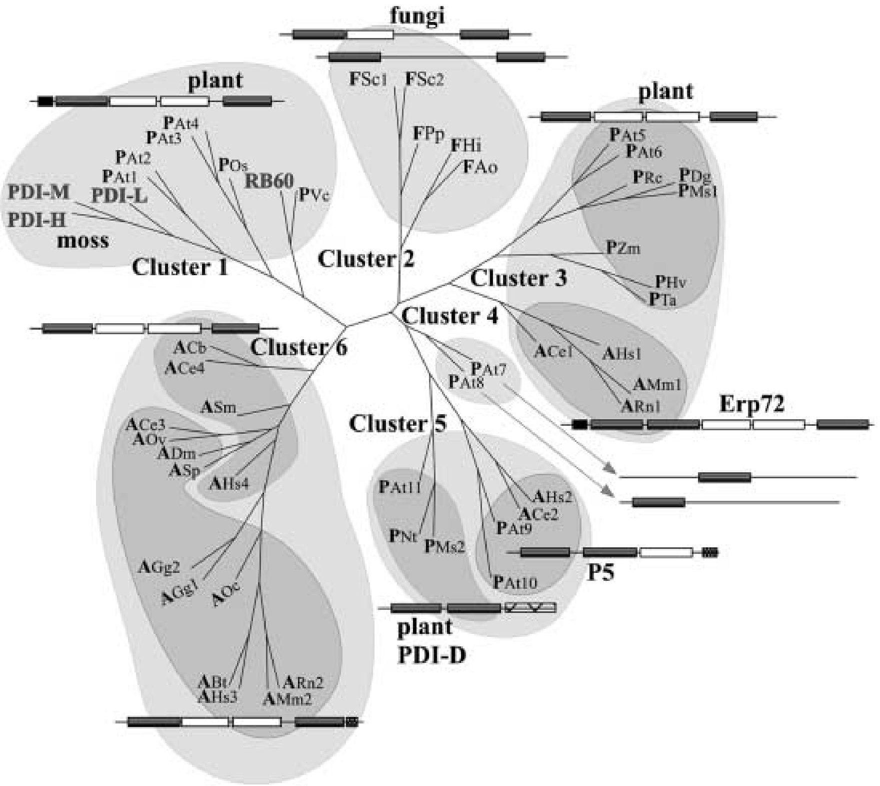

Fig. 2 An unrooted phylogenetic tree based on 51 PDI-like protein sequences (for complete list see Table 1) was constructed using the Maximum Likelihood method. The sequences of animal (prefix A), plant (prefix P) and fungal (prefix F) PDI-like proteins formed six main clusters. The *second and third letters* of each sequence name indicate the species of origin. The number denotes the paralog. The corresponding Accession Nos. are listed in Table 1. A schematic representation of the primary structure of the PDI-like proteins is shown for each cluster. The *black boxes* denote the *a* and *a'* redox-active thioredoxin domains, the *open boxes* the *b* and *b*' thioredoxin domains, the *hatched boxes* denote the highly acidic *c* domains, and the *cross-hatched boxes* denote the *D* domains

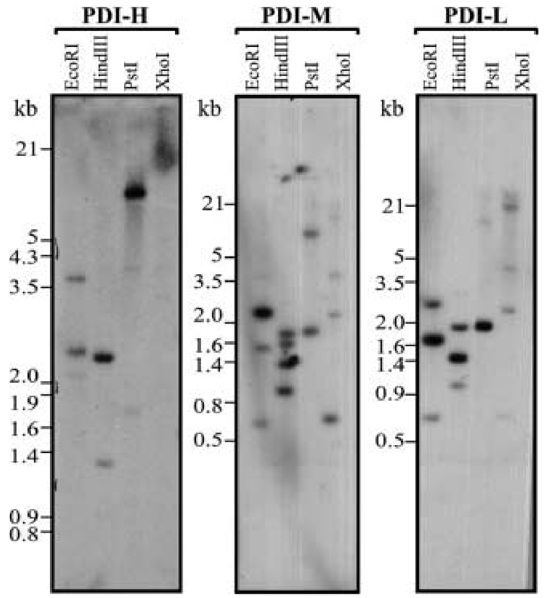

Fig. 3 Southern analysis of wild-type *P. patens* genomic DNA using the three PDI-like clones as probes. Wild-type genomic *P. patens* DNA was digested with the indicated restriction enzymes, fractionated by agarose gel electrophoresis, blotted onto nylon membranes, probed with probes derived from the PDI-H, PDI-M, or PDI-L clone, and washed at high stringency. The three blots show unique hybridization patterns with each PDI-like probe, indicating that each probe is derived from a different gene. The positions of molecular-weight markers (kb) are indicated on the *left* of each panel

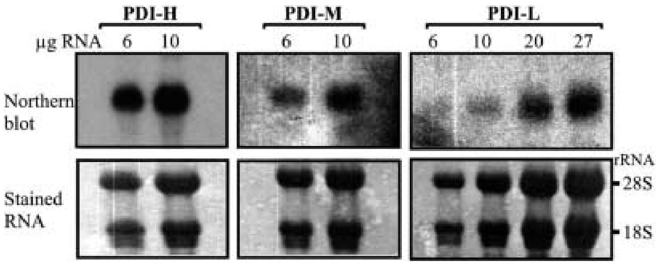

Fig. 4 Hybridization analysis of RNA from wild-type plants with DNA probes for the three PDI-like clones (*upper panels*). The lower panels (Stained RNA) show the stained membrane. The amount of total RNA loaded is indicated (in μg RNA) *above* each lane. PDI-H mRNA is highly abundant, the level of PDI-M mRNA is intermediate, and PDI-L mRNA is least abundant. These results suggest that each gene may carry out a unique function

of the genes to total RNA isolated from protonema cells (Fig. 4). This analysis showed that each of the PDI-like genes encodes an mRNA of approximately 1.7 kb. Interestingly, significant differences were found in the steady-state levels of the three types of transcripts. PDI-H showed the highest level of expression, PDI-M exhibited an intermediate level, whereas PDI-L gave rise to the lowest steady state level of mRNA (Fig. 4). These results are consistent with a potentially unique function for each identified gene.

Construction and characterization of knock-out mutants

Knock-out mutants for the PDI-H, PDI-M, and PDI-L genes were generated by transforming *P. patens* protoplasts with a linear DNA fragment containing the neomycin phosphotransferase (neo) gene flanked by two, approximately 500-bp genomic fragments of the same PDI-like gene (KO cassette, Fig. 5A). Plating of 3.6×10^5 protoplasts transformed with the PDI-H, PDI-M, and PDI-L KO constructs on kanamycin-containing medium yielded 25, 36, and 55 antibiotic resistant plants, respectively.

To identify knock-out mutants of the PDI-H and PDI-M genes with a single insertion of the KO cassette that resulted from a precise double recombination event we conducted two sequential screens by PCR. In the first screen, we identified integration events mediated by homologous recombination occurring within both 5′- and 3′-ends of the KO cassette by a PCR screen of genomic DNA with two sets of primers (Fig. 5A). The first set included a gene-specific forward primer that anneals upstream of the KO cassette and a reverse primer for the Neo gene. PCR of PDI-H DNA with this set of primers is expected to yield a 900-bp DNA fragment only from a PDI-H gene that has been disrupted by a precise insertion of the 5′-end of the KO cassette (Fig. 5B). The second primer pair was designed analogously to verify the integration of the 3′-end of the KO cassette, and was expected to generate a 930-bp fragment from the PDI-H gene disrupted by precise integration of the 3′-end of the KO cassette (Fig. 5C). The results of a similar screen of KO mutants of the PDI-M gene are shown in Fig. 5D and E. Because we did not have flanking sequences upstream and downstream of the KO cassette of PDI-L, we did not apply this screen to the PDI-L KO mutants.

In the second PCR-based screen, we sought KO mutants with a single insertion of the KO cassette. We reasoned that in PCRs containing two Neo primers oriented outwards (tail to tail), only DNA of KO mutants with multiple inserts would yield a PCR fragment, whereas DNA of KO mutants with one copy of KO cassette should not (Fig. 5F). Using this approach, we identified the KO mutants PDI-H-7 and PDI-M-59 as containing one insert (Fig. 5F), whereas PDI-L-8 was found to be the result of multiple integration of the KO cassette into the PDI-L locus (data not shown).

To verify the integration of the Neo gene in the knock-out mutants, we analyzed the change in size of the authentic locus upon integration of the KO cassette, using genomic DNAs from the wild type, PDI-H, PDI-M, and PDI-L mutants (Fig. 6). The ^{32}P-radiolabeled fragment of each PDI-like gene corresponding to the 5′ end of the KO cassette or a ^{32}P-radiolabeled fragment of the Neo gene were used as probes. A comparison of the wild-type PDI-H gene with the three KO mutants showed that only the PDH-H KO mutant contained a PDI-H gene that was disrupted by a single integration event, whereas the other two PDI-like genes

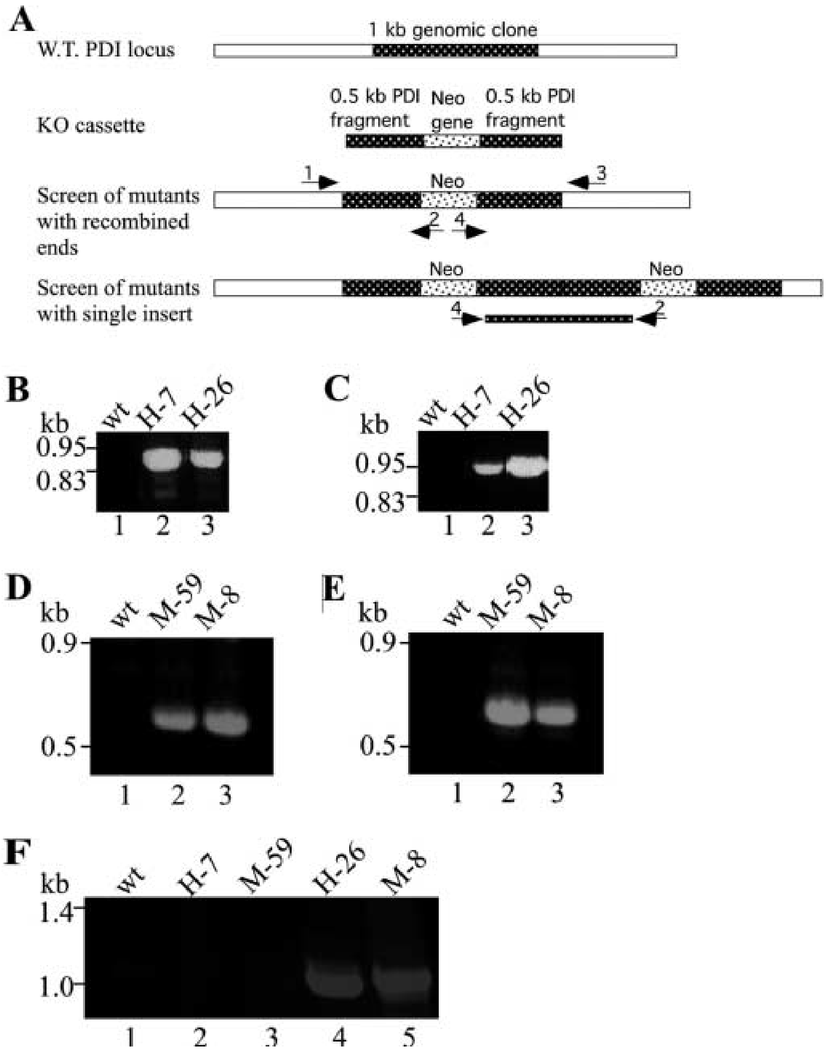

Fig. 5A–F PCR screen for KO mutants that contain a single insert with recombined 5′- and 3′-ends. **A** Schematic representations of the wild-type genomic PDI-like locus (WT PDI locus) and the KO cassette (KO cassette), illustrating the rationale of the PCR screen used to identify mutants containing KO cassettes with recombined 5′- and 3′-ends (Screen of mutants with recombined ends), and mutants containing a single insert (Screen of mutants with single insert). The locations and the orientations of the PDI gene-specific and the Neo gene-specific PCR primers used in each screen are indicated by *numbers and arrows*, respectively. **B** Products of PCRs performed with genomic DNA isolated from wild type (wt), PDI-H-7 (H-7) or PDI-H-26 (H-26) KO mutants as template, and the PDI-1 and Neo-2 PCR primers. Wild-type genomic DNA does not contain an Neo gene insert and therefore does not yield a PCR product (lane 1). A fragment with the anticipated size is amplified from the DNA of each of the PDI-H KO mutants 7 and 26 (lanes 2, 3), indicating that each of the mutants contains a recombined 5′-end of the KO cassette. **C** Results of the screen, using PDI-3 and Neo-4 PCR primers, for recombined 3′-ends in the PDI-H KO mutants 7 and 26, showing that they contain recombined 3′-ends. Panels **D** and **E** are similar to panels **B** and **C**, respectively, except that genomic DNAs of PDI-M-59 and PDI-M-8 KO mutants were used as templates for PCR. **F** Electrophoretic analysis of the products of PCRs performed with genomic DNA isolated from wild-type or PDI-H and PDI-M KO mutants and the Neo-2 and Neo-4 PCR primers, demonstrating that PDI-H-7 and PDI-M-59 each contain a single insert, and PDI-H-26 and PDI-M-8 contain multiple inserts

contained an intact PDI-H locus (Fig. 6, Panel PDI-H). Probing DNA from the PDI-H KO mutant with radiolabeled DNA specific for the two other genes revealed hybridizing DNA bands with similar mobility to wt DNA, indicating that these two loci are intact (Fig. 6, Panels PDI-L, and PDI-M) in the PDI-H KO mutant. Probing of the PDI-H KO mutant DNA with radiolabeled DNA from the Neo gene showed a single DNA band, demonstrating that the KO cassette had not integrated into additional sites (Fig. 6, Panel Neo). Similar results were obtained for the PDI-M KO mutant (Fig. 6, Panel PDI-M), whereas the PDI-L KO mutant probably results from the integration of three copies of the KO cassette into the PDI-L locus (Fig. 6, Panel PDI-L). In order to verify that the integration of the KO cassette into each of the three genes indeed abrogated expression of the cognate mRNA, we compared the steady-state amounts of each mRNA in the wild type and the three KO mutants. Clearly, the disruption of the three PDI-like genes by the integration of the KO cassette resulted in the disappearance of the corresponding transcript in each of the KO mutants, PDI-H, PDI-M, and PDI-L (Fig. 7).

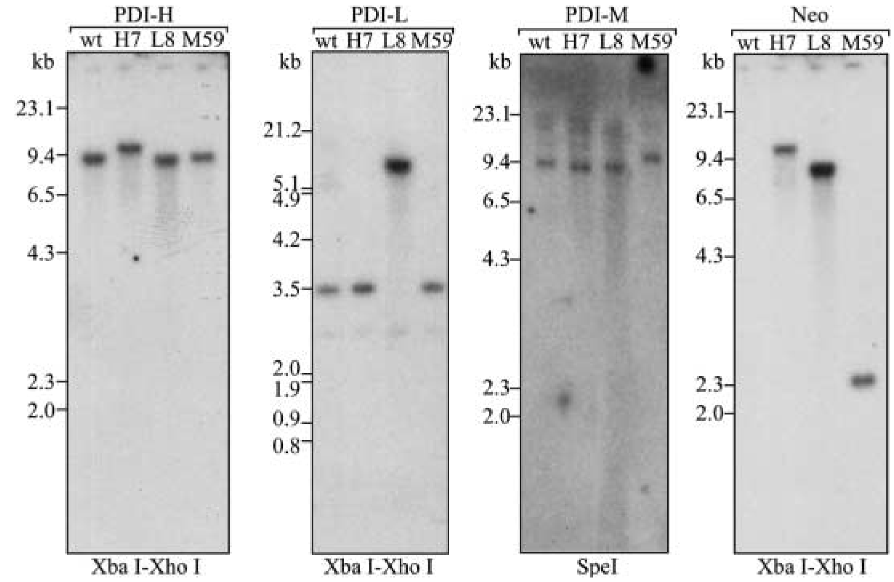

Fig. 6 Southern analysis of KO mutants. Each of the three KO mutants PDI-H-7, PDI-L-8, and PDI-M-59 was assayed for insertion of the specific KO cassette into the corresponding PDI locus. DNA isolated from wild-type plants (wt) or from each of the three KO mutants (H7, L8, M59, as indicated *above* the lanes) was digested with restriction enzymes (indicated at the *bottom* of each panel) and subjected to Southern analysis with ^{32}P-labeled DNA from the three PDI-like clones (PDI-H, PDI-L, PDI-M, indicated *above* each panel) and the neomycin gene (Neo). The positions of molecular weight markers (kb) are marked on the *left* of each panel

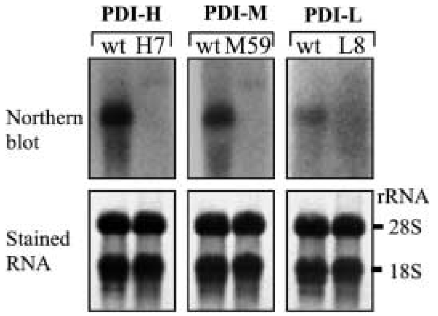

Fig. 7 Hybridization analysis of RNA from wild-type (wt) and three KO mutant plants, PDI-H7, PDI-M59, PDI-L8, using DNA probes isolated from the three PDI-like clones (*upper panel*). The *lower panel* (Stained RNA) shows the stained membrane. Each of the KO mutants lacks the corresponding wild-type band, indicating knock-out of expression

The three types of knock-out mutants were found to be viable, indicating that none of these three PDI-like genes is essential. In addition, the three KO mutants appeared to develop similarly to wild-type plants grown under normal autotrophic conditions, suggesting that at least some of the PDI-like proteins can functionally complement each other. Initial tests suggest that in comparison with wild-plants all three types of knock-out mutants show slight growth retardation. We are currently testing the response of the three types of mutants to adverse conditions.

Discussion

PDI is a multifunctional enzyme that is most abundant in the ER, where it is thought to be active in the formation and isomerization of disulfide bonds in folding proteins (Freedman et al. 1994). Most genomes studied so far were found to contain several genes with high homology with PDI; however, their specific functions are as yet unknown. Recently, numerous studies have identified additional functions of PDI-like proteins, and detected their presence, although at lower abundance, in various cellular compartments besides the ER (Honscha et al. 1993; Couet et al. 1996; Lucero and Kaminer 1999; Rigobello et al. 2001; Trebitsh et al. 2001). The identification of a PDI-like protein as a redox regulatory subunit of a mRNA-binding protein complex in the chloroplast (Kim and Mayfield 1997; Trebitsh et al. 2001) suggests that PDI-like proteins may also function as regulators of gene expression.

The high abundance of PDI enzymes in the ER hinders biochemical studies of the unique roles of each enzyme species. In order to study the function of PDI-like proteins in other cell compartments, they have to be purified away from ER vesicles. One way to eleucidate the diverse functions of PDI-like proteins is via reverse genetics. However, the high degree of homology and the multiplicity of PDI-like genes identified in a single genome make this approach highly laborious. The recent development of an efficient targeted gene knockout system in the moss *P. patens* is an advance that will help alleviate the difficulties imposed by the multiplicity of genes (Schaefer 2001). The ability to produce single gene knockouts in a small gene family suggested that a similar approach should be applicable to a larger family consisting of highly homologous genes, such as the PDI family (Hofmann et al. 1999). To test this, we have started to create knock-out mutations in each of the of PDI-like genes in *P. patens*.

To date, we have cloned three PDI-like genes from *P. patens*. Their characterization showed that they are all expressed, eliminating the possibility that any one of them is a pseudogene. We found that while the three PDI-like genes share a high degree of homology, they differ in several other aspects. PDI-H, PDI-M and PDI-L are expressed at high, intermediate and low steady-state levels, respectively (Fig. 4). Differences were also found in unique domains among the three genes, the major one being the apparent lack of a redox active site in the C-terminal thioredoxin domain of PDI-L (Fig. 1). These results are consistent with a potentially unique function for each identified gene.

The multiplicity of PDI-like genes raises an additional potential hurdle to the application of targeted gene disruption: sequence similarity may prevent gene-specific targeting and functional redundancy may mask the phenotypic effects of specific knock-out mutations. The expression of an active-site mutant form of a protein in place of the wild-type protein is a potential option that could circumvent functional redundancy. The replacement of the wild-type gene with one expressing a mutant protein requires an intermediate step in which a single insertion of the disrupting Neo gene into the targeted PDI-like gene has occurred. With this in mind, we devised a protocol to identify and screen for this type of mutant. Using a PCR-based approach (Fig. 7), we isolated specific knock-out mutants for each of the three PDI-like genes. The PDI-H and PDI-M mutants each contain a single insertion of a selectable marker which disrupts the authentic PDI-like gene and results from a clean double recombination event (Fig. 5). Currently, we are analyzing the phenotypes of each of the single insertion knock-out mutants under different environmental conditions, using several biochemical and physiological tests.

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