Two minD Genes in Physcomitrella patens Are Functionally Redundant

Ryuichi D. Itoh1,*, Katsuaki Takechi2, Akihiro Hayashida2, Shin-ichiro Katsura1 and Hiroyoshi Takano2

1 Department of Chemistry, Biology and Marine Science, Faculty of Science, University of the Ryukyus, Okinawa 903–0213, Japan
2 Graduate School of Science and Technology, Kumamoto University, Kumamoto 860–8555, Japan

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Summary We investigated two genes that encode a homolog of the bacterial cell division site determinent MinD, PpMinD1 and PpMinD2, which were previously identified in the moss Physcomitrella patens. Southern analysis suggested that the P. patens genome does not contain minD genes other than PpMinD1 and PpMinD2. Molecular phylogenetic analysis and the results of a transient expression assay using MinD: green fluorescent protein (GFP) fusions implied that PpMinD1 and PpMinD2 are cyanobacterium-derived, closely related genes encoding chloroplast-targeted MinD proteins. Disruption of the genomic PpMinD1 or PpMinD2 locus had no detectable effects on plastid division. This implies that there is functional redundancy in the two minD genes in P. patens.

Key words Chloroplast division, gene targeting, knockout, minD, moss, Physcomitrella patens.

The genes essential for plastid division have been identified in Arabidopsis thaliana, and most encode proteins that are homologous to bacterial cell division proteins such as FtsZ, ARC11/MinD, MinE, ARTEMIS, ARC6/Ftn2 (for review, see Osteryoung and Nunnari 2003), and GC1/SulA (Maple et al. 2004, Raynaud et al. 2004). The involvement of prokaryote-type cell division factors in plastid division is quite reasonable in view of the endosymbiotic theory, which holds that plastids arose from cyanobacteria engulfed by endocytosis.

In Escherichia coli, correct placement of the cell division site requires the MinC, MinD, and MinE proteins (de Boer et al. 1989). MinD in association with MinC inhibits formation of the cyto-kinetic FtsZ ring at all potential sites except a central division site. This is because MinE sweeps the FtsZ ring-inhibitory MinCD complex from the central division site (Fu et al. 2001, Hale et al. 2001).

To date, minD homologues have been found in the chloroplast genomes of the algae Chlorella vulgaris, Nephroselmis olivacea, Mesostigma viride, and Prototheca wickerhamii (for review, see Itoh 2003). By contrast, known minD homologues in higher land plants, such as A. thaliana and rice (Oryza sativa), are nuclear-encoded (Colletti et al. 2000, Kanamaru et al. 2000).

To gain detailed insight into the evolution of the plastid division mechanism, it is necessary to characterize the minD genes in lower land plants. In a previous study, we isolated two nuclear-encoded minD genes, PpMinD1 and PpMinD2, from the moss Physcomitrella patens (Hayashida et al. 2005). This organism is now widely used in functional genomic studies of plants (e.g., Nishiyama et al. 2003) because this plant exhibits extraordinarily high rates of gene targeting (Schaefer 2001). Taking advantage of this feature, we investigated the cellular functions of the MinD proteins in P. patens by generating minD-knockout plants.

* Corresponding author, e-mail: ryuitoh@sci.u-ryukyu.ac.jp
Materials and methods

Plant material

Protonemata of *Physcomitrella patens* (Hedw.) Bruch & Schimp subsp. *patens* Tan were grown on BCDAT medium solidified with 0.8% agar in a regulated chamber at 25°C under continuous light (35 μmol m⁻² s⁻¹; Nishiyama et al. 2000).

Genomic Southern hybridization

*Physcomitrella patens* genomic DNA was extracted by the CTAB method (Murray and Thompson 1980). For Southern analysis, 6 μg of genomic DNA was digested with *Eco*RI (Nippongene, Japan) or *Hin*dIII (Nippongene), subjected to 1% (w/v) agarose gel electrophoresis, transferred to a nylon membrane (Biodyne A; Pall, USA), and cross-linked to the membrane using UV light. A *PpMinD1* probe fragment of 1299 bp was amplified with the PCR DIG probe synthesis kit (Roche, Germany) using the PMD1-forward (5'-ATAGTTGGAGCCGGATTTGGTGCCGGTGCT-3') and PMD1-reverse (5'-AGGACATTGAAGTCCAGGTCACATTCGCGA-3') primers with *P. patens* genomic DNA as a template. Hybridization was performed in DIG Easy Hyb buffer (Roche) according to the manufacturer’s instructions.

Molecular phylogenetic analysis

The MinD amino acid sequences from various organisms were obtained from the DDBJ/EMBL/GenBank databases and were aligned using Clustal X (Jeanmougin et al. 1998). A phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei 1987) in Clustal X. The accession numbers of the amino acid sequences used for the tree construction are as follows: NP_442592 (*Synechocystis*), NP_682807 (*Thermosynechocystis*), ZP_00071109 (*Trichodesmium*), NP_487496 (*Nostoc*), NC_002186 (*Mesostigma*), NC_000927 (*Nephroselmis*), AJ245645 (*Prototheca*), NC_001865 (*Chlorella*), BAD18007 (*PpMinD1*), BAD18008 (*PpMinD2*), NM_122307 (*Arabidopsis*), NM_185331 (*Oryza*), BAC49477 (*Bradyrhizobium*), NP_357356 (*Agrobacterium*), NP_251934 (*Pseudomonas*), AF345908 (*Neisseria*), NP_231594 (*Vibrio*), P57411 (*Buchnera*), NP_45629 (*Versinia*), J03153 (*Escherichia*), NP_347881 (*Clostridium*), NP_622555 (*Thermoanaerobacter*), NP_470915 (*Listeria*), M95582 (*Bacillus*), AAB98539 (*Methanococcus*), NP_276946 (*Methanothermobacter*), and NP_069530 (*Archaeoglobus*).

Expression and visualization of MinD: GFP fusions in living plant cells

A *PpMinD1* DNA fragment corresponding to the 130 amino-terminal amino acids of MinD1 protein was amplified by PCR from a clone produced by rapid amplification of cDNA 5'-ends (5'-RACE) (Hayashida et al. 2005), with the primers PpMinD1-GFPSal (5’-GGGTCGACCATG-GCAGCGTGGGGATAGTTTTGCT-3’) and PpMinD1-GFBsp (5’-CCTCATGACTTTGAGGTCA-GACGCGA-3’). This fragment was cloned into the pT7Blue T-vector (Novagen, USA) by means of the TA cloning method and was recovered from the vector by SalI/BspHI double digestion (the SalI and BspHI restriction sites in the primer sequences are underlined above). A *PpMinD2* DNA fragment corresponding to the 147 amino-terminal amino acids of the MinD2 protein was amplified by PCR from *P. patens* genomic DNA with the primers PMD2TP-forward (5’-ATAGTTGGAGCCGGATTTGGTGCCGGTGCT-3’) and PMD2TP-reverse (5’-TAGGAATCTCCCCCACAAGGACC-3’). The 5’ termini of the obtained fragments were phosphorylated using the BKL kit (TAKARA BIO, Japan). The MinD1 and MinD2 fragments were subcloned into the SalI/Ncol restriction site and a blunted SalI restriction site, respectively, of the CaMV35S-sGFP(S65T)-nos3’ vector (a generous gift from Dr. Yasuo Niwa, University of Shizuoka, Japan; Niwa et al. 1999) such that the MinD polypeptides were fused in-frame to the amino-terminus of sGFP(S65T), a brighter GFP variant optimized for plant cells. With these constructs, the fusion proteins could be constitutively expressed in plant cells.
under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The constructs were introduced into *P. patens* protoplasts by the polyethylene glycol (PEG)-mediated transformation method described by Nishiyama *et al.* (2000). The fluorescence of transiently expressed MinD: GFP in living *P. patens* protoplasts was observed with an Axioskop2 plus fluorescence microscope (Zeiss, Germany).

**Knockout of minD genes**

A 1299-bp *PpMinD1* fragment was amplified from *P. patens* genomic DNA by PCR using the PMD1-forward and PMD1-reverse primers and was cloned into the pT7Blue T-vector by means of the TA cloning method. The cloned *PpMinD1* fragment was inserted at the unique *ClaI* restriction site of the selection marker gene *nptII*, which confers kanamycin resistance in plants. The resultant construct was used as a *PpMinD1* knockout vector. A 1768-bp *PpMinD2* fragment of was amplified from *P. patens* genomic DNA by PCR using the PMD2-forward (5’-ACGATGGCAATCTGCAATCCAGCTCTG-3’) and PMD2-reverse (5’-GGTCGAGTGATATGAAGTATACAATTCTAC-3’) primers and was cloned into the pT7Blue T-vector. The cloned *PpMinD2* fragment was inserted at the unique *EcoT221* site of the selection marker gene *hpt* (a generous gift from Dr. Hirokazu Tsukaya, National Institute for Basic Biology, Japan), which confers hygromycin resistance in plants. The resultant construct was used as a *PpMinD2* knockout vector. The procedures for PEG-mediated protoplast transformation and the primary selection of antibiotic-resistant lines were those of Nishiyama *et al.* (2000). To confirm specific gene disruption in the candidate lines, these lines were subjected to genomic PCR with the following primers: MinD-MT2 (5’-CTTGAAGCAAATCAACCCGA-3’) and MinD-MT3 (5’-TGAGGGGCAACCTCATTTCA-3’) for *PpMinD1* and PMD2-forward and PpMinD2-TAIL-R1 (5’-AGCCTCCTCGTCAAGAATCTG-3’) for *PpMinD2*.  

**Results and discussion**

**Copy number and molecular phylogeny of minD genes in *P. patens***

The copy number and phylogenetic relationship of *minD* genes in the genome were determined as prerequisites for the knockout analysis of *P. patens minD*. Genomic Southern hybridization using the genomic fragment of *PpMinD1* as a probe produced two obvious bands in both the *EcoRI*– and *HindIII*-digests (Fig. 1), suggesting that the *P. patens* genome contains no more *minD* genes than the two previously isolated, *PpMinD1* and *PpMinD2* (Hayashida *et al.* 2005). In a molecular phylogenetic tree constructed using the deduced amino acid sequences of prokaryotic and eukaryotic *minD* genes, the two *P. patens* MinD proteins form a group that is a sister to the nuclear-encoded MinD proteins of angiosperms (Fig. 2). Green-algal MinD proteins also form a monophyletic cluster. The tree implies that *PpMinD1* and *PpMinD2* are closely related cyanobacterium-derived genes. In prokaryotes, MinD is a peripheral membrane ATPase. Like other MinD proteins, PpMinD1 and PpMinD2 each possess a canonical ATP/GTP-binding site motif A (P-
loop), GKKGKGKT. This implies that both PpMinD1 and PpMinD2 have nucleotide-binding ability.

Subcellular localization of MinD: GFP fusion proteins

Both the PpMinD1: sGFP(S65T) and PpMinD2: sGFP(S65T) fusion proteins, which were transiently expressed in living P. patens protoplast cells under the control of the CaMV 35S promoter, were localized exclusively in chloroplasts (Fig. 3), whereas non-fused sGFP(S65T) expressed in P. patens is known to be located within the cell nucleus and cytoplasm (Kabeya et al. 2002).

Knockout of minD genes in P. patens

To elucidate the functional divergence of the P. patens minD genes, the genomic PpMinD1 and PpMinD2 loci were knocked out using gene targeting (Fig. 4). Two PpMinD1-knockout moss lines were successfully obtained from the 22 transformants, and no identifiable phenotype, including chloroplast number and morphology, was discernable. Similarly, three PpMinD2-knockout lines were obtained from the 16 transformants, and no identifiable phenotype was discernable. From these and the sequencing and localization analyses, it follows that the two minD genes in P. patens are functionally redundant. Currently, the significance of the minD gene redundancy in P. patens is obscure. The difference in the expression profiles of the two minD genes could not be investigated because no signal was detected with Northern hybridization, possibly owing to the scarcity of transcripts (data not shown).

Finally, it is notable that PpFtsZ2-1 and PpFtsZ2-2 of P. patens, which belong to the FtsZ2 subfamily of land plant ftsZ genes and are highly conserved in terms of nucleotide and deduced amino acid sequences (Kiessling et al. 2000), are not functionally redundant, as a single knockout of the PpFtsZ2-1 locus is sufficient to disrupt plastid division in the moss (Strepp et al. 1998). The generation and characterization of double gene-knockout lines of P. patens would provide a useful tool to further elucidate the functional divergence of plastid division genes in lower land plants.

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Fig. 3. Subcellular localization of MinD: GFP fusion proteins. Fluorescence images of GFP (A and D; green), chlorophyll autofluorescence (B and E; red), and the merged images (C and F) for Pp-MinD1: GFP (A–C) or PpMinD2: GFP (D–F) are shown for a living P. patens protoplast cell. Scale bar represents 10 µm.

Fig. 4. Gene knockout of PpMinD1 and PpMinD2. (A) Protonemata of the wild-type (left), PpMinD1-knockout (line no. 15; center), and PpMinD2-knockout (line no. 10; right) plants are shown. The bar indicates 20 µm. (B) Verification of the knockout using PCR analysis. Genomic DNA templates from wild-type plants (lanes 1, 3), a PpMinD1-knockout line (line no. 15; lane 2), and a PpMinD2-knockout line (line no. 10; lane 4) were subjected to PCR with the primers indicated in panels C and D. The PCR fragment amplified from the PpMinD1- or PpMinD2-knockout line was longer than that from the wild type by comparison with nptII (2.0 kbp) or hpt (2.3 kbp). λ-DNA digested with HindIII (for lanes 1, 2) and a 1-kb DNA ladder (for lanes 3, 4) were used as size markers. (C) Comparison of the PpMinD1 genomic regions in the wild-type (WT; upper part) and PpMinD1-knockout (line no. 15; lower) plants. (D) Comparison of the PpMinD2 genomic regions in the wild-type (WT; upper part) and PpMinD2-knockout (line no. 10; lower) plants. For panels C and D, the region for the gene-targeting construct is shown using shadowed rectangles. Thin lines indicate outer genomic regions. The insert site of the nptII or hpt gene is shown with a white rectangle. Arrowheads indicate the locations of primers. One of the primers is located in the outer genomic region.
References


