The Peptidoglycan Biosynthesis Genes MurA and MraY are Related to Chloroplast Division in the Moss Physcomitrella patens

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Abbreviations:  C_{55}-PP, undecaprenyl-pyrophosphate; CaMV35S, cauliflower mosaic virus 35S; EST, expressed sequence tag; GFP, green fluorescent protein; GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid; PBP, penicillin-binding protein; PEP, phosphoenolpyruvate; TP, transit peptide; WT, wild type.

In the moss Physcomitrella patens, 10 Mur genes involved in peptidoglycan biosynthesis were found, and the MurE and Pbp genes are related to plastid division. Although the MraY and MurG genes were missing in our previous expressed sequence tag screening, they were discovered in the P. patens genome in this study, indicating that P. patens has a full set of genes capable of synthesizing peptidoglycan. In addition, a second MurA gene (PpMurA2) was found. Whereas Northern analyses indicated that PpMurA1, PpMurG and PpMraY were expressed, transcripts of PpMurA2 were detected only when RT–PCR was employed. Whereas GFP fusion proteins with either PpMurA1 or PpMraY were detected in chloroplasts, the PpMurA2 fusion proteins were located in the cytoplasm. Protonema cells in the wild-type plants had an average of 46 chloroplasts. PpMurA1 gene-disrupted lines had <10 chloroplasts, whereas approximately 30 chloroplasts existed in the PpMurA2 knockout lines. The PpMurA1/A2 double-knockout lines had only a few macrochloroplasts, suggesting a redundant function for these two genes. Disruption of the PpMraY gene in P. patens resulted in the appearance of macrochloroplasts. Anabaena MraY fused to the N-terminal region of PpMraY and A. thaliana MraY could complement the macrochloroplast phenotype in the PpMraY knockout line. Electron microscopic observations showed no obvious differences in the shape or stacking of thylakoid membranes between all knockout transformants and wild-type plants, suggesting that these Mur genes are related only to plastid division in moss.

Keywords: Moss • MurA • MraY • Peptidoglycan • Plastid division • Physcomitrella patens.

Introduction

Plastid division is a fundamental mechanism in plant cells because plastids do not arise de novo (reviewed in Glynn et al. 2007, Maple and Möller 2007). Because all plastids are believed to be derived from a single cyanobacterial ancestor (Rodríguez-Ezpeleta et al. 2005), it is thought that a common mechanism is used for plastid division, with species-specific variations. One important variation is a division of the cyanelle of glaucocystophytes. The cyanelle is a peptidoglycan-armed plastid and uses peptidoglycan for its division, similarly to bacterial cell division (Iino and Hashimoto 2003). It was thought that the cyanelle is a unique system and that other plants, except glaucocystophytes, did not use peptidoglycan in plastids. However, we found that homologs of bacterial peptidoglycan synthesis genes (Mur genes) affect the number of plastids per cell (plastid number) and their morphology in the moss Physcomitrella patens (Machida et al. 2006).
Bacterial peptidoglycan is synthesized in several steps (Fig. 1). In the first stage (I), two enzymes, MurA and MurB, catalyze the formation of UDP-N-acetylmuramic acid (UDP-MurNAc) from UDP-N-acetylglucosamine (UDP-GlcNAc) and phosphoenolpyruvate (PEP). Then, MurC, D, E and F mediate the formation of UDP-MurNAc-pentapeptide (stage II). Next, MraY transferase yields undecaprenyl-pyrophosphoryl (C55-PP)-MurNAc-pentapeptide (lipid I). Thereafter, MurG transferase creates C55-PP-MurNAc-pentapeptide (lipid II), which is transferred to the periplasm (stage III). Finally, the disaccharide pentapeptide monomer unit is cross-linked to pre-existing peptidoglycan by penicillin-binding proteins (PBPs) to form the sacculus surrounding the bacterial cell membrane (stage IV).

In a previous paper (Machida et al. 2006), we searched the full-length expressed sequence tag (EST) library of P. patens (Nishiyama et al. 2003) in order to isolate plant homologs that are related to bacterial peptidoglycan synthesis. We found 10 genes for peptidoglycan biosynthesis: MurA–F and Pbp genes, two genes for d-Ala–d-Ala ligase (Ddl) and DD carboxypeptidase. DD carboxypeptidase is a monofunctional Pbp (Goffin and Ghuysen 1998). When we searched the P. patens EST library, however, we failed to detect genes for MraY and MurG, which are essential for peptidoglycan biosynthesis in bacteria. Analysis of GFP fusion proteins containing the N terminus of PpMurE or PpPbp have suggested that these proteins are located in the chloroplasts of moss, as expected from computer predictions. A gene-targeting technique has already been established in P. patens (Schaefer and Zryd 1997), and gene disruption of the PpMurE or PpPbp gene in P. patens resulted in the appearance of a few macrochloroplasts in each cell, whereas non-transformed cells had about 50 chloroplasts. Cross-species complementation assays with the PpMurE knockout line showed that Anabaena MurE (AnaMurE) fused to the plastid-targeting signal of PpMurE can restore normal chloroplast phenotype, suggesting that PpMurE and AnaMurE have similar functions (Garcia et al. 2008).

Because no peptidoglycan layer is present in animal cells, the peptidoglycan synthesis pathway is a major target for antibiotics (Barreteau et al. 2008, Vollmer and Bertsche 2008). Fosfomycin is an analog of PEP and inhibits MurA enzyme function. ß-Lactam antibiotics, including penicillin and ampicillin, form covalent complexes with the PBPs and destroy bacterial cell walls by interfering with PBP function. We examined the effects of these antibiotics on plastid division in P. patens. In our results, ampicillin caused a rapid decrease in plastid number, and fosfomycin caused a decrease in plastid number in half of the cells (Katayama et al. 2003). In contrast, flowering plants are unresponsive to ß-lactams (Kasten and Reski 1997, Glynn et al. 2007), and fosfomycin did not affect chloroplast division in leaf disk cultures of Brassica rapa var. peruviridis (Izumi et al. 2008). The Arabidopsis thaliana genome (Arabidopsis Genome Initiative 2000) contains five genes homologous to bacterial Mur genes: MurE, MraY, MurG and two Ddl genes (Machida et al., 2006). We investigated the function of the AtMurE gene in A. thaliana using four T-DNA tagged lines, and found that AtMurE acts in chloroplast development, not in plastid division (Garcia et al. 2008), suggesting that the peptidoglycan biosynthesis pathway is not present in flowering plants.

These results suggest that the bacterial peptidoglycan synthesis pathway is conserved in basal plants in their plastid division. This idea is inconsistent with our previous EST screening, which failed to detect MraY and MurG. However, the sequence of the P. patens genome recently became available (Rensing et al. 2008), facilitating a more comprehensive survey of the genes.

**Results**

**Isolation of MraY, MurG and a second MurA gene from P. patens**

To isolate the P. patens MraY and MurG genes, the genome sequence of P. patens (Rensing et al. 2008) was searched using tBlastN and the amino acid sequences of MraY and MurG from A. thaliana. We found a single copy of these two missing genes in the P. patens genome. In addition, a second MurA gene (PpMurA2) was found, and we renamed the
PpMurA gene (Machida et al. 2006) PpMurA1. The cDNAs of PpMraY, PpMurG and PpMurA2 were cloned and sequenced. Except for the putative transit peptides (TPs), the MurA1 and MurA2 proteins showed high similarity to bacterial MurA proteins, including many conserved residues reported by Yoon et al. (2008) (Supplementary Fig. 1). In eukaryotic plants, the MurA gene is also found in the spikemoss Selaginella moellendorffi (Wang et al. 2005, http://genome.jgi-psf.org/), but no MurA gene exists in the A. thaliana genome. Phylogenetic analysis showed that the PpMurA proteins from P. patens and that of S. moellendorffi are monophyletic with cyanobacterial MurA proteins (Supplementary Fig. 2).

A homolog of the bacterial MraY genes (AtMraY) was also found in the A. thaliana genome (Arabidopsis Genome Initiative 2000, Mondego et al. 2003). Among the 36 invariant amino acid residues in the bacterial MraY proteins (Bouhss et al. 2008), the PpMraY and AtMraY proteins had two and six amino acid substitutions, respectively (Supplementary Fig. 3). MraY from Escherichia coli is an integral membrane protein, spanning the cytoplasmic membrane 10 times (Bouhss et al. 1999). Hydrophobicity plot analysis suggested that PpMraY and AtMraY are also membrane proteins (Fig. 2). Phylogenetic analysis showed that MraY in eukaryotic plants is monophyletic and closely related to cyanobacterial MraY proteins (Supplementary Fig. 4).

The domain structure of bacterial MurG is conserved in PpMurG (Supplementary Fig. 5). Phylogenetic analysis showed that MurG from eukaryotic plants is monophyletic and distinct from other bacterial MurG, including cyanobacterial MurG (Supplementary Fig. 6).

These results indicate that P. patens has all of the genes necessary to synthesize peptidoglycan. Whereas Northern analyses indicated that PpMurA1, PpMurG and PpMraY were expressed, transcripts of PpMurA2 were detected only when RT–PCR was employed (Fig. 3). For detailed analysis, we selected genes for MurA, which catalyzes the first committed step, and for MraY, which catalyzes the first membrane step in peptidoglycan biosynthesis.

Subcellular localization of PpMurA1, PpMurA2 and PpMraY

While both MurA1 and MurA2 are predicted to encode plastid-targeting sequences (possibilities of 0.92 and 0.62, respectively) by the TargetP program, the PpMraY protein had a putative mitochondrial-targeting sequence (possibility of 0.92 by TargetP). To analyze the subcellular localization of PpMurA and PpMraY, we constructed plasmids directing the expression of the putative TP fused to GFP from its native promoter. Polyethylene glycol (PEG)-mediated transformation with the generated plasmids for expression of GFP fused to the putative TP of PpMurA1 showed that GFP fluorescence was observed in chloroplasts of P. patens,

Fig. 2 Hydrophobicity plots for MraY proteins from E. coli, Anabaena sp., P. patens and A. thaliana. The hydrophobicity plot was determined using Genetyx software.

![Fig. 3 Expression of P. patens Mur genes in wild-type and knockout lines. (A) Northern blot analysis with each gene as a probe. Ethidium bromide staining of rRNA bands is shown as a control. (B) The expression of each gene in both the wild-type and knockout plants was determined by RT–PCR. The PpEF1 gene was used as an internal control.](image)
corroborating the computer prediction (Fig. 4). In contrast, fluorescence of the PpMurA2(TP)–GFP fusion protein was observed in the cytosol, contrary to the prediction. Although the prediction suggested a mitochondrial localization for PpMraY, the GFP fusion protein with the N-terminal region of PpMraY, was observed in the chloroplasts. Next, we constructed plasmids that express GFP fused to a full-length PpMurA1 or PpMurA2 from its native promoter. The results were similar to those for the experiments with GFP fused to the TP of each MurA (Fig. 4).

**Phenotypes of PpMurA knockout lines in P. patens**

We constructed plasmids for the generation of each PpMurA knockout transformant (Supplementary Fig. 7). The 5′ and 3′ genomic regions of the PpMurA1 or A2 genes were amplified by using genomic PCR and cloned. The zeocin resistance or the neomycin phosphotransferase (NPTII) gene, driven by the cauliflower mosaic virus (CaMV) 35S promoter and terminated by the CaMV35S polyadenylation signal, was inserted between the 5′ and 3′ genomic regions of PpMurA1 or PpMurA2, respectively. PEG-mediated transformation of P. patens was carried out with the constructed plasmids. To determine the copy number of the inserted DNA, Southern hybridization experiments were performed. For PpMurA1 gene targeting, line #24 showed a single insertion of the zeocin resistance gene (Supplementary Fig. 7). RT–PCR analysis showed that the PpMurA1 transcripts were not detected in this transformant (Fig. 3). Microscopic observation showed that the PpMurA1 knockout transformant had huge chloroplasts (Fig. 5), with an average...
chloroplast number of $9.37 \pm 4.13$ (n = 100), whereas protonemata cells in the wild-type plants had an average of 46 chloroplasts.

Southern hybridization analysis of the PpMurA2 knockout lines was carried out with seven geneticin-resistant transformants, and only one insertion was detected in three transformants (Supplementary Fig. 7). RT-PCR analysis showed that the PpMurA2 transcripts were not detected in the transformants (Fig. 3). The chloroplasts in these transformants were slightly bigger than those in the wild-type plants (Fig. 5), and the chloroplast number was $33.08 \pm 5.64$ (n = 100) in PpMurA2 transformant line #57. These results suggested that both PpMurA genes are related to chloroplast division in P. patens. Given that Northern analysis indicated that PpMurA1 gene transcripts were more abundant than were those for PpMurA2 (Fig. 3), the chloroplast numbers may depend on the amount of PpMurA transcripts in the disrupted lines.

Gene disruption of the PpMurE gene in P. patens results in the appearance of a few macrochloroplasts (Machida et al. 2006). To analyze the redundancy of the PpMurA genes, we generated PpMurA1/A2 double-knockout lines. We disrupted the PpMurA1 gene in PpMurA2 knockout line #57. Southern hybridization showed disruption in both PpMurA1 and PpMurA2 (Supplementary Fig. 7). This double-knockout plant had macrochloroplasts similar to those in the PpMur1 gene-disrupted lines (Fig. 5), indicating that the function of the two PpMurA genes is redundant. Under an electron microscope, no obvious differences in the shape or stacking of thylakoid membranes were observed among the giant chloroplasts in the PpMurA1 or A2 single knockout lines, PpMurA1/A2 double-knockout transformants or small chloroplasts in wild-type plants (Fig. 7; data not shown for PpMurA1 and PpMurA2 single knockout lines).

Characterization of the PpMraY gene in P. patens

MraY transferase catalyzes the first membrane step in bacterial peptidoglycan biosynthesis, and genes for MraY exist in the genome of other land plants, including A. thaliana. The knockout lines for the PpMraY gene were generated in P. patens by the same method as used for disrupting the PpMurA2 gene (Supplementary Fig. 8). We obtained three transformants with macrochloroplasts in the protonema and leaf cells (Fig. 6). These three transformants had no other insertions of the vector sequence in their genomes (Supplementary Fig. 8). RT-PCR analysis showed that PpMraY transcripts were not detected in the transformants (Fig. 3). Electron microscopic observations showed that the phenotype of the PpMraY-disrupted line was similar to that of the PpMurA1/A2 double-knockout line (Fig. 7). These results suggest that PpMraY is also related to chloroplast division in moss.

To compare the functions of the MraY genes of P. patens and cyanobacteria, stable transformants expressing Anabaena MraY (AnaMraY) in chloroplasts were generated using the P. patens PpMraY knockout line. First, a genomic region corresponding to the promoter of the PpMraY gene and the coding region for the plastid-targeting sequence of PpMraY was placed just before the cloned AnaMraY gene to direct the plastid localization of AnaMraY. Then, the constructed region was inserted into the cloned PpDRPSB-2 gene, because its disruption shows no effect in P. patens (Sakaguchi, E., Takechi, K., Yamada, T., Sato, H., Takio, S. and Takano, H. manuscript in preparation). PEG-mediated
transformation was carried out with the PpMraY knockout line, and stable transformants were generated. Microscopic observations indicated that P. patens cells showed normal chloroplast phenotypes (Fig. 6), suggesting that the PpMraY function is the same as that of cyanobacterial MraY.

To compare the function of A. thaliana MraY with that of PpMraY, a plasmid expressing AtMraY in P. patens was constructed. PEG-mediated DNA transfection into the protoplasts of the PpMraY knockout line was carried out, and transient transformants were identified by GFP fluorescence under microscopic observation. When transformation was performed with plasmids containing the AtMraY gene, chloroplast numbers were recovered similar to the phenotype in cells transformed with the normal PpMraY gene (Fig. 6). These results suggest that A. thaliana MraY is functional, although many Mur genes are not present in the A. thaliana genome.

Discussion

In this study, we found two novel genes, PpMraY and PpMurG, in the P. patens genome, indicating that P. patens has the complete set of genes required for peptidoglycan biosynthesis. Double knockouts of the PpMurA genes or knockouts of PpMraY resulted in the appearance of a few macrochloroplasts. Moreover, the phenotype observed in chloroplasts of the PpMurA double-knockout lines was mimicked by applying fosfomycin to wild-type cells as previously reported, although the plastid number decreased in only half of the cells (Katayama et al. 2003). Because all cells of the PpMurA1/A2 double-knockout lines had a macrochloroplast phenotype, the different phenotypes among fosfomycin-treated cells may depend on unknown conditions in the cells. Gene knockouts for PpMurE, PpPBP and PpMraY, and the double-knockout line for PpMurA have the same macrochloroplast phenotype. These results strongly suggest that the moss P. patens contains a plastid peptidoglycan and uses it for plastid division. However, electron microscopy of moss tissues fixed with glutaraldehyde and OsO$_4$ revealed no rigid structures such as those found in bacteria and between the inner and outer envelopes of cyanobacteria in glaucocystophytes (Fig. 7; Machida et al. 2006). Therefore, we still can not conclude the existence of plastid peptidoglycan structures in moss. Purification and biochemical characterization using mass spectrometry may be useful in answering this question.

Results from the PpMurA1–GFP fusion proteins suggest that PpMurA1 functions in the stroma. Although PpMurA2 has the long N-terminal regions that were predicted to be plastid-targeting sequences, GFP fusion proteins were observed in the cytosol. These results suggest that the first step in plastid peptidoglycan biosynthesis with MurA is catalyzed in plastids and in the cytosol of P. patens cells. Since Northern analysis suggested that the expression of PpMurA1 is more abundant than that of PpMurA2, the plastid pathway is thought to be the main pathway for peptidoglycan biosynthesis. If the PpMurA2 proteins are in the cytosol, intermediates in the cytosol should be transferred
into the stroma because PpMurE is localized in the stroma. The identity of the transporter for these intermediates is unknown.

The decrease in chloroplast number is more severe in the PpMurA1 knockout lines than in the PpMurA2 knockout lines. The difference may depend on the amount of MurA transcript remaining in the knockout lines. Another possibility is that the MurA proteins localized in the stroma are only functional. Although the PpMurA2–GFP fusion proteins were observed in the cytosol, we cannot exclude the possibility that a small amount of PpMurA2 is localized in plastids. The different phenotypes between the knockout lines for PpMurA1 and PpMurA2 genes may depend on the amount of MurA protein in the stroma. Histological or Western blot analysis with the proper antibodies against PpMurA proteins may be needed to determine the actual localization of PpMurA proteins.

The PpMraY protein is related to chloroplast division, similarly to other Mur genes in P. patens. While P. patens has all of the genes for peptidoglycan biosynthesis, only five homologous genes exist in the A. thaliana genome (Arabidopsis Genome Initiative 2000). Results from the cross-species complementation assay for PpMraY suggest that the P. patens and A. thaliana MraY proteins have the same function as cyanobacterial MraY. AtMraY was found to be one of the genes that were differentially expressed during late flower bud development using cDNA-fragment length polymorphism (AFLP) (Mondego et al. 2003). These authors postulated that AtMraY is a kind of GlcNAc transferase involved in N-glycan biosynthesis. Although GFP fused to the N-terminus of PpMraY was localized in plastids, localization of AtMraY was not determined. Among Mur-related proteins in A. thaliana, AtMurE was shown to have acquired a divergent function in chloroplast development rather than chloroplast division (Garcia et al. 2008). Although AtMraY can complement the function of PpMraY, we cannot eliminate the possibility that AtMraY has a different function in A. thaliana.

The results of this study show that P. patens uses Mur genes for plastid division. Chloroplast phenotype in the disrupted lines is similar to that observed in P. patens cells treated with antibiotics. Furthermore, plastid division in the liverwort Marchantia polymorpha and the lycophyte Selaginella nipponica is inhibited by treatment with β-lactam antibiotics (Tounou et al. 2002, Izumi et al. 2003, Izumi et al. 2008). Ampicillin did not affect plastid division in three species of moniliformopses, including Adiantum capillus-veneris, Ceratopteris richardii and Equisetum arvense, whereas fosfomycin inhibited plastid division in these pteridophytes (moniliformopses and lycophytes) (Izumi et al. 2008). These results suggest that plastid division systems are related to the peptidoglycan biosynthesis pathway in many basal plants. Antibiotic experiments on other species are now in progress as part of a comprehensive survey of the peptidoglycan-dependent plastid division system in plants.

Materials and Methods

Plant culture

Protonemata and gametophores of the moss P. patens subsp. patens (Gransden Wood strain) were grown on BCDAT medium solidified with 0.8% agar in a regulated chamber at 25°C under continuous light (35 μmol photon m⁻² s⁻¹; Nishiyama et al. 2000).

Characterization of PpMurA1, PpMurA2 and PpMraY genes

The genome sequence of P. patens (Rensing et al. 2008) was searched using tBLASTN and the amino acid sequences of MraY and MurG from A. thaliana. In addition, the PpMurA2 gene was found by searching the P. patens genome sequence with the PpMurA1 amino acid sequence. RNA was isolated from the wild-type and transformant protonemata by a previously described method (Machida et al. 2006). Each cDNA was amplified by RT–PCR, cloned and sequenced. The full-length cDNA sequence of each gene was determined by using 5’ and 3’ RACE.

Probes for Northern hybridization were generated with the PCR DIG Probe kit (Roche Diagnostics, Mannheim, Germany) with the appropriate primer sets. Primers used in this study are listed in Supplementary Table 1. For RT–PCR, we isolated RNA from each disrupted line and wild-type plants, treated it with DNase I and used it for the generation of cDNA from oligo(dt) primers. RT–PCR was performed with the appropriate primer set. Annealing temperatures and cycles were 58°C and 35 cycles, 56°C and 35 cycles, 60°C and 35 cycles, 58°C and 42 cycles, and 60°C and 25 cycles for the PpMurA1, PpMurA2, PpMraY, PpMurG and PpEF1 genes, respectively.

Analysis of the nucleotide sequences and of the hydrophobicity plots were performed using Genetyx software (Genetyx Corporation, Tokyo, Japan). Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007). The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987).


Computer predictions of the subcellular localizations of proteins were carried out using the TargetP program (Emanuelsson et al. 2000). To construct the PpMurA1(TP)–GFP plasmid, which fuses GFP to the N-terminus of PpMurA1 from its native promoter, we used a DNA fragment that included the putative promoter sequence of 1871 bp from -1871 to 1 (A of ATG start codon as 1), the coding sequence...
corresponding to the N-terminal 160 amino acid residues and the first intron, amplified by genomic PCR with the ppMurA1/F1 and ppMurA1/R1-NcoI primers. Genomic DNA was isolated from the protonemata of P. patens using the CTAB method (Machida et al. 2006). Amplified DNA was subjected to blunting and kination using the Takara BKL Kit (Takara Bio, Shiga, Japan), digested with NcoI to cut the restriction site on the primer, and then inserted into the Smal/Ncol-digested sGFP(S65T) plasmid (Chiu et al. 1996).

To construct the ppMurA2(5′′)–GFP plasmid, which fuses GFP to the N-terminus of ppMurA2 from its native promoter, we used a DNA fragment that included the putative promoter sequence of 2525 bp and the coding sequence corresponding to the 274 N-terminal amino acid residues, amplified by genomic PCR using the ppMurA2/FS and ppMurA2/R5 primers. Amplified DNA was subjected to blunting and kination, and then inserted into the Smal-digested sGFP(S65T) plasmid. P. patens was transformed as previously described (Machida et al. 2006).

To construct the ppMurA1(full)–GFP plasmid, which fuses GFP to the full-length ppMurA1 from its native promoter, the DNA fragment was amplified from the ppMurA1/TP–GFP plasmid by PCR using the sGFP/F0-Met and ppMurA1/R9 primers to exclude the sequence corresponding to the N-terminus of the ppMurA1 protein from this plasmid. The coding sequence for the full-length ppMurA1 protein was amplified from the cDNA clone by PCR using the ppMurA1/F0 and ppMurA1/R8 primers. The amplified sequences were then ligated. To construct the ppMurA2(full)–GFP plasmid, which fuses GFP to the full-length ppMurA2 from its native promoter, the DNA fragment was amplified from the ppMurA2/TP–GFP plasmid by PCR using the sGFP/F0-Met and ppMurA2/R7 primers. The coding sequence for the full-length ppMurA2 protein was amplified from the cDNA clone by PCR using the ppMurA2/F0 and ppMurA2/R6 primers. The amplified sequences were then ligated.

To construct the ppMraY(5′′)–GFP plasmid, which fuses GFP to the N-terminus of ppMraY from its native promoter, the DNA fragment including the putative promoter sequence of 3167 bp and the coding sequence corresponding to the N-terminal 108 amino acid residues was amplified by genomic PCR using the ppMraY/F7 and ppMraY/R8-NcoI-2 primers. Amplified DNA was subjected to blunting and kination, and then inserted into the Smal-digested sGFP(S65T) plasmid.

**Generation of the knockout lines**

The p35S-Zeo plasmid was a generous gift from Dr Mitsuyasu Hasebe (National Institute for Basic Biology, Japan) (Supplementary Fig. 7). The 5′′ region of the ppMurA1 gene was amplified from genomic DNA by PCR using the ppMurA1/F1 and ppMurA1/R1-NcoI primers, digested with EcoRI, and cloned into the EcoRI site located upstream of the zeocin resistance gene on the p35S-Zeo plasmid. Then, the 3′ region was PCR-amplified with the ppMurA1/F2 and ppMurA1/R2 primers, subjected to blunting and kination, and inserted into the blunted XbaI site located downstream of the zeocin resistance gene. The constructed plasmid was linearized by digestion with SalI and used to transform P. patens. To determine additional insertions of the transformed DNA into the P. patens genome, Southern hybridization analysis was performed (Supplementary Fig. 7).

The pTN3 plasmid carrying the NPTII gene (Nishiyama et al. 2000) was used to target gene ppMurA2 (Supplementary Fig. 7). The 3′ region of ppMurA2 was amplified by genomic PCR with the ppMurA2/F3 and ppMurA2/R4 primers, subjected to blunting and kination, and inserted into the blunted BamHI site of the pTN3 plasmid. Then, the 5′ region of the ppMurA2 gene was amplified with the ppMurA2/F0 and ppMurA2/R1 (‘RACE’ primers, subjected to blunting and kination, and inserted into the blunted EcoRI site. The constructed plasmid was linearized by digestion with KpnI and used to transform P. patens. We selected ppMurA2 knockout line #57, which has only one insertion of the antibiotic resistance gene in the ppMurA2 genomic region (Supplementary Fig. 7). The constructed plasmid for the knockout of the ppMurA1 gene was transformed to generate the ppMurA1/A2 double-knockout lines. The copy number of the inserted DNA was determined by Southern hybridization (Supplementary Fig. 7).

The pTN3 plasmid was also used to target the ppMraY gene (Supplementary Fig. 8). The 3′ region of ppMraY was amplified by genomic PCR with the ppMraY/F3 and ppMraY/R10 primers, digested with HindIII, and inserted into the HindIII site of the pTN3 plasmid. Then, the 5′ region of ppMraY was amplified with the ppMraY/F6 and ppMraY/R9 primers, subjected to blunting and kination, and inserted into the blunted BamHI site. The constructed plasmid was linearized by digestion with SalI and used to transform P. patens.

**Cross-species complementation assay**

We initially performed the complementation test of the ppMraY knockout lines by using the transient expression assay of the normal ppMraY gene. The ppMraY cDNA was cut by digestion with the appropriate restriction enzymes, blunted with a Takara Blunting kit and inserted into the Smal site between the rice actin promoter and rbcS gene terminator of the pTFH 22.4 plasmid (Fujita et al. 2004), including the GFP gene, which is driven by the CaMV 35S promoter. At 4 d after transformation, P. patens cells with GFP fluorescence were observed under microscopy.

For the cross-species complementation test, we used the MraY gene of Anabaena sp. PCC7120. First, the nopaline synthase (Nos′) terminator was extracted from the sGFP(S65T) plasmid (Chiu et al. 1996) by digestion with...
PstI and EcoRI and inserted into the PstI–EcoRI site of the pBluescript vector (Stratagene, La Jolla, CA, USA). The *Anabaena MraY* (*AnaMraY*) gene was amplified by PCR from *Anabaena* genomic DNA with the AnaMraY/F0-Met and AnaMraY/R0 primers, subjected to blunting and kination, and inserted into the Smal site upstream of the Nos region. The DNA fragment corresponding to the putative promoter of the *PpMraY* gene, and to the coding region for the *PpMraY* TP (amino acids 1–108), was amplified from the *PpMraY*(TP)–GFP plasmid with the *PpMraY*/F7 and *PpMraY*/R8–4 primers, subjected to blunting and kination, and inserted into the blunted *BamHI* site upstream of the *AnaMraY* gene on the constructed plasmid. The DNA region containing the promoter and TP-coding region of *PpMraY* fused to the *AnaMraY* gene with Nos was extracted from the constructed plasmid, subjected to blunting and kination, and inserted into the *Nhel* site of the cloned PpDRPSB-2 gene with the hygromycin resistance gene. Disruption of the *PpDRPSB-2* gene did not affect *P. patens* (data not shown).

The *AtMraY* cDNA was amplified by RT–PCR from the DNase I-treated total RNA of *A. thaliana* with the AtMraY/F1 and AtMraY/R1 primers and cloned into the pBluescript vector. The cDNA region was extracted by digestion with SpeI and PstI, blunted and inserted into the Smal site of the pTFH 22.4 plasmid. At 4 d after transformation, *P. patens* cells with GFP fluorescence were observed under microscopy.

**Microscopic observations**

Bright-field and epifluorescent cell images were recorded with a CCD camera (Nikon DXM1200 or Zeiss Axiocam) under microscopy (Olympus BX60 or Zeiss Axioskop 2 plus). For the electron microscopic observations, samples were fixed in 2% glutaraldehyde buffered with 20 mM sodium cacodylate (pH 7.2) and a 1% osmium tetroxide aqueous solution including 0.1% potassium hexacyanoferrate (II), dehydrated through an ethanol series and embedded in Quetol-651 resin. Thin sections were cut and stained with uranyl acetate and lead citrate and then observed with a JEM-1200EX electron microscope (JEOL, Tokyo, Japan).

**Supplementary data**

Supplementary data are available at PCP online.

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**References**


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