Both the transglycosylase and transpeptidase functions in plastid penicillin-binding protein are essential for plastid division in *Physcomitrella patens*

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Abstract: Class A penicillin-binding proteins (PBPs) are active in the final step of bacterial peptidoglycan biosynthesis. They possess a transglycosylase (TG) domain to polymerize the glycan chains and a transpeptidase (TP) domain to catalyze peptide cross-linking. We reported that knockout of the *Pbp* gene in the moss *Physcomitrella patens*(*ΔPpPbp*) results in a macrochloroplast phenotype by affecting plastid division. Here, expression of PpPBP-GFP in *ΔPpPbp* restored the wild-type phenotype and GFP fluorescence was observed mainly in the periphery of each chloroplast. Stable transformants expressing *Anabaena* PBP with the plastid-targeting sequence, or PpPBP replacing the *Anabaena* TP domain exhibited partial recovery, while chloroplast number was recovered to that of wild-type plants in the transformant expressing PpPBP replacing the *Anabaena* TG domain. Transient expression experiments with site-directed mutagenized PpPBP showed that mutations in the conserved amino acids in both domains interfered with phenotype recovery. These results suggest that both TG and TP functions are essential for function of PpPBP in moss chloroplast division.

Keywords: chloroplast division, penicillin-binding protein, *Physcomitrella patens*, transglycosylase, transpeptidase

Introduction

Peptidoglycan (or murein) forms a mesh-like sacculus surrounding the cytoplasmic membrane of almost all eubacteria.1) Bacterial peptidoglycan maintains turgor pressure and cell shape in addition to functioning in cell division. The primary glycan chain structure of peptidoglycan consists of alternating β-1,4-linked N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues. Neighbor-

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(LMM) PBPs are monofunctional and related to cell separation, peptidoglycan maturation, and its recycling.\textsuperscript{2)\textsuperscript{2}}

It is now widely accepted that an endosymbiotic cyanobacterium evolved into the plastids of green plants. Although free-living bacteria typically have peptidoglycan in their cell walls, it is believed that the plastids of green plants lost peptidoglycan during evolution. However, in the genome of the moss Physcomitrella patens,\textsuperscript{3)\textsuperscript{3}} we were able to identify 10 Mur genes: MurA to G, MraY, D-Ala:D-Ala ligase (Ddl), and Pbp, which are all of the genes necessary for the primary peptidoglycan biosynthetic pathway.\textsuperscript{4)\textsuperscript{4}} Treatment with three different \beta-lactam antibiotics, which interfere with PBP activity, resulted in the appearance of macrochloroplasts in P. patens cells.\textsuperscript{5)\textsuperscript{5}} In addition to \beta-lactams, treatments with an inhibitor of Ddl, D-cycloserine, or an inhibitor of MurA, fosfomycin, also resulted in macrochloroplast phenotypes.\textsuperscript{5)\textsuperscript{5}} Consistent with the antibiotic treatments, gene disruption of the P. patens (Pp) MurA, PpMurE, PpMraY, PpDdl, or PpPbp genes resulted in the appearance of a few macrochloroplasts in each protonemal cell, in contrast to wild-type plant cells, which have approximately 50 chloroplasts, suggesting that these Mur genes, including Ddl and Pbp, are related to plastid division in moss.\textsuperscript{6)\textsuperscript{6}}–\textsuperscript{8)\textsuperscript{8}} Recently, using a sensitive metabolic labeling method for peptidoglycan with a D-Ala:D-Ala dipeptide probe and click chemistry, we found plastid peptidoglycan fully surrounding the chloroplasts of moss.\textsuperscript{8)\textsuperscript{8}}

We found only one gene for class A PBP (accession number, BAE45868: Pp3c11.25910 in the P. patens genome ver. 3.3) in the P. patens genome.\textsuperscript{6)\textsuperscript{6}} As described above, a gene-targeting experiment suggested that PpPBP is essential for moss plastid division.\textsuperscript{6)\textsuperscript{6}} Computer prediction and domain searches of the 936-amino acid PpPBP suggested that it consists of a plastid-targeting sequence at the N-terminus (1–55 amino acids), and TG (205–358 amino acids) and TP (558–828 amino acids) domains (Fig. 2a). If PpPBP functions in the final step of peptidoglycan synthesis in moss plastids, similar to that in bacteria, PpPBP must localize to the intermembrane space of the plastid envelope.

For the PpMurE-knockout line (∆PpMurE) with a macrochloroplast phenotype, expression of the PpMurE plastid-targeting sequence fused to Anabaena MurE restored the wild-type chloroplast phenotype.\textsuperscript{9)\textsuperscript{9}} The macrochloroplast phenotype of the PpMraY-knockout line (∆PpMraY) was also suppressed by Anabaena MraY expression in chloroplasts.\textsuperscript{7)\textsuperscript{7}} These results suggest that MurE and MraY of P. patens have similar functions to their bacterial counterparts. In this study, we investigate whether two domains in PpPBP have similar functions to those in bacterial class A PBPs using cross-species complementation assays with cyanobacterial PBP in the PpPbp-knockout line (∆PpPbp). Based on the results of the site-directed mutagenized PpPBP, we conclude that the final step in peptidoglycan synthesis by PBP in moss plastids is similar to that in cyanobacteria.

Materials and methods

Materials. Protonemata of the moss Physcomitrella patens subsp. patens (Gransden Wood strain)\textsuperscript{10)\textsuperscript{10}} were grown on BCDAT medium solidified with 0.8% agar in a regulated chamber equipped with Biolux lamps (NEC Lighting, Ltd. Tokyo, Japan) at 25°C under continuous white light (100 µmol photons/m²/s).\textsuperscript{8)\textsuperscript{8}} The PpPbp knockout line (∆PpPbp) was generated in a previous study.\textsuperscript{6)\textsuperscript{6}}

Complementation of ∆PpPbp with the PpPBP-GFP fusion protein. PpPbp cDNA was amplified from total RNA of wild-type plants by RT-PCR using ExTaq DNA polymerase (Takara Bio Inc., Otsu, Japan) with the PpPbp-Met-F and PpPbp-R primers. The primers used in this study are listed in Table 1. The PCR product was cloned into the pT7BlueT-vector (Novagen, Madison, WI, U.S.A.) and then subcloned into a pBluescript SK+ (Stratagene, La Jolla, CA, U.S.A.)-based plasmid (pBS-PpPbp plasmid). For the construction of the plasmid containing the whole PpPbp gene fused to the GFP gene (PpPbp-GFP), the coding sequence of PpPbp was amplified by PCR from the pBS-PpPbp plasmid with the PpPbp(GFP)-F-SalI and PpPbp(GFP)-R2-SalI primers. Amplified DNA was digested with SalI and inserted into the SalI-digested sGFP(S65T) plasmid\textsuperscript{11)\textsuperscript{11}} to construct a plasmid containing the PpPbp-GFP fusion gene with a cauliflower mosaic virus (CaMV) 35S RNA promoter and a nopaline synthase terminator (35S-PpPbp-GFP plasmid). We used the P. patens dynamin-related protein 5B-2 (PpDRP5B-2) genomic region to generate the PpPBP-GFP expression line in ∆PpPbp (Fig. 1) because disruption of the PpDRP5B-2 gene did not visibly affect P. patens.\textsuperscript{12)\textsuperscript{12}} The hygromycin phosphotransferase (HPT) gene was inserted into the EcoRV fragment region of the cloned PpDRP5B-2 gene.\textsuperscript{7)\textsuperscript{7}} DNA containing the CaMV 35S promoter, the
coding sequence for PpPBP-GFP, and the nopaline synthase terminator was excised from the 35S-PpPbp-GFP plasmid by SphI and EcoRI digestion, subjected to blunting and kination, and inserted into the blunted NheI site of the cloned PpDRP5B-2 gene with the HPT gene to generate the PpDRP5B-2::PpPbp-GFP plasmid. The protein coding regions in the plasmids used for transformation were sequenced to confirm no PCR errors. The constructed plasmid was linearized by digestion with KpnI before transformation. Polyethylene glycol (PEG)-mediated transformation of the "PpPbp" line was carried out as described previously.8) Bright-field and GFP epifluorescence cell images were recorded with a CCD camera (Zeiss Axiocam, Zeiss, Germany) under a microscope (Zeiss Axioskop 2 plus).

For Southern hybridization, genomic DNA was isolated from protonemal cells of P. patens using the cetyltrimethyl ammonium bromide (CTAB) method.6) Ten micrograms of genomic DNA were digested by the appropriate restriction enzymes and subjected to electrophoresis on a 0.8% agarose gel. The gel was blotted onto a Biodyne Plus nylon membrane (PALL Gelman Laboratory, Michigan, U.S.A.). Probes for Southern hybridization were generated using the PCR DIG Probe kit (Roche Diagnostics, Mannheim, Germany) with PpDRP5B-2-F0 and PpDRP5B-2-R8 primers. Hybridization was performed at 50 °C for at least 14 h in a hybridization incubator. After a stringency wash, the membranes were subjected to immunological detection using anti-DIG antibody conjugated to alkaline phosphatase followed by CDP-Star (Roche). Chemiluminescence images were obtained using X-ray film.

Table 1. Primers used in this study

| Primer name | Sequence (5’–3’) | Application |
|-------------|------------------|-------------|
| PpPbp-Met-F | ATGGAGTGCATTGTGCTCGCGTC | PpPbp cloning, AnaPbp complementation, Domain swapping |
| PpPbp-R | TTCTGCACATAGGAGGGGTGATGCCAGT | PpPbp cloning |
| PpPbp(GFP)-F-SalI | TTGTGCAACATAGGAGGGGTGATGCCAGT | PpPbp-GFP transgenic line |
| PpPbp(GFP)-R2-SalI | AGGAAAATGCTCCCTTTGAGAGCC | PpPbp-GFP transgenic line, Southern |
| PpDRP5B-2-F0 | GGTGATCCATCTTTCGCTTCA | PpPbp-GFP transgenic line, Southern |
| PpDRP5B-2-R8 | GGTGATCCATCTTTCGCTTCA | PpPbp-GFP transgenic line, Southern |
| AnaPbp1A-F0-Met | AATGCTCTCATTAAAGCAGATTGGA | AnaPbp complementation |
| AnaPbp1A-R1-2 | CTATTATTTTTTATCGCCAGG | AnaPbp complementation |
| AnaPbp1B-R6 | CCACACGTGTGGACGTGGTTG | Domain swapping |
| rbcS-ter-F0 | CACATTACGTCCGTCCGCCA | Domain swapping |
| PpPbp-15F | GGATTTGAATGAGCGATGAG | Domain swapping |
| PpPbp-13R-SalI | TTGTGCAACATAGGAGGGGTGATGCCAGT | Domain swapping |
| PpPbp(355-559)R-SalI | TTGTGCAACATAGGAGGGGTGATGCCAGT | Domain swapping |
| AnaPbp-F5-SalI | TTGTGCAACATAGGAGGGGTGATGCCAGT | Domain swapping |
| AnaPbp-R4-SphI | TTTCTGCCATTTAAAAGCAGATTGGA | Domain swapping |
| PpPbp-15F-SphI | TTGTGCAACATAGGAGGGGTGATGCCAGT | Domain swapping |
| PpPbp-13R-SphI | TTGTGCAACATAGGAGGGGTGATGCCAGT | Domain swapping |
| PpPbp-16F-mt | GCTCAACTGGTCTGATCGAAAT | Site-directed mutagenesis |
| PpPbp-14R-mt | GATGATAATCCCTCCTCCATCTTGAAGCCAG | Site-directed mutagenesis |
| PpPbp-TG5/5-int-F | TTGAGATCGAATGCGAAGTGGA | Site-directed mutagenesis |
| PpPbp-TG5/5-int-R | TTGAGATCGAATGCGAAGTGGA | Site-directed mutagenesis |
| PpPbp-T5/5-int-F | TATTCGTACTCCTCCATCTTGAAGCCAG | Site-directed mutagenesis |
| PpPbp-T5/5-int-R | TATTCGTACTCCTCCATCTTGAAGCCAG | Site-directed mutagenesis |
| PpPbp-T3/3-int-R2 | GTGGCCACAAATCAAATCCT | Site-directed mutagenesis |
| PpPbp-Tp1/1F-mt | AATGGATGCGGATGCGGAGTG | Site-directed mutagenesis |
| PpPbp-Tp1/1F-mt | AATGGATGCGGATGCGGAGTG | Site-directed mutagenesis |
| PpPbp-TP1/1F-mt | CACAGCATACATGGTGGAGTG | Site-directed mutagenesis |

Complementation of ΔPpPbp with Anabaena PBP and the plastid-targeting sequence of PpPBP. For the cross-species complementation test, we selected a PBP gene from Anabaena sp. PCC7120 (AnaPbp, alr5324) that encodes a class A PBP protein (AnaPBP) of 643 residues. A coding
sequence corresponding to the plastid-targeting sequence of PpPBP was combined with the AnaPBP gene. Because the PpPbp cDNA has two BstXI sites at 462 bp and 760 bp (A of the ATG start codon defined as 1), the coding sequence corresponding to the middle region of the PpPBP protein (154–253 amino acids) was removed from the pBS-PpPbp plasmid by BstXI digestion, and then the digested plasmid with the sequence coding the N-terminal 153 residues, including a putative plastid-targeting sequence (1–55 amino acids), was blunted with a Takara Blunting kit (Takara Bio). The whole AnaPbp gene was amplified by PCR from Anabaena genomic DNA with the AnaPbp1A-F0-Met and AnaPbp1A-R1-2 primers, subjected to blunting and kination, and inserted into the blunted BstXI site of the pBS-PpPbp plasmid to generate the sequence coding the AnaPBP protein (1–643 amino acids) with the plastid (chloroplast)-targeting sequence of PpPBP (153 amino acids) at the N terminus (CP-AnaPBP). The DNA fragment corresponding to CP-AnaPBP was amplified by PCR using the PpPbp-Met-F and AnaPbp1A-R1-2 primers, subjected to blunting and kination, and inserted into the Smal site between the rice actin promoter and rbcS gene terminator of the pTKM1 vector13) to construct the pTKM1-CP-AnaPBP plasmid. For complementation analysis, we also used the PpDRP5B-2 genomic region. DNA containing the rice actin promoter, coding sequence for CP-AnaPBP, and rbcS gene terminator was excised from the pTKM1-CP-AnaPBP plasmid by KpnI and XbaI digestion, subjected to blunting and kination, and inserted into the NotI site of the cloned PpDRP5B-2 genomic DNA with the HPT gene to generate the PpDRP5B-2::CP-AnaPBP plasmid for the complementation experiment. The constructed plasmid without PCR errors was linearized by digestion with KpnI before transformation. Transformation of the ΔPpPbp line was carried out as described above. Insertion of the plasmid DNA into the PpDRP5B-2 genomic DNA region in the antibiotic resistant transformants was confirmed by genomic PCR (data not shown).

**Domain swapping of PpPBP with Anabaena PBP.** We performed domain-swapping experiments for the TG and TP domains between PpPBP and AnaPBP. For TG domain swapping, we used the PpDRP5B-2::CP-AnaPBP plasmid as the starting material. The coding sequence corresponding to the C-terminal region of AnaPBP (335–643 amino acids), including the TP domain (349–618 amino acids), was removed from the plasmid by inverse PCR using PrimeStar GXL DNA polymerase (Takara Bio) with AnaPbp1B-R6 and rbcS-ter-F0 primers. The coding sequence for the plastid-targeting sequence of PpPBP fused to the N-terminal region of AnaPBP (1–334 amino acids), including the transmembrane region (34–56 amino acids) and TG domain (81–256 amino acids), remained in the inverse PCR product. Then, the coding sequence corresponding to the C-terminal region of PpPBP (440–936 amino acids), including the TP domain (558–828 amino acids), was amplified by PCR from the cloned PpPbp cDNA using the PpPbp-15F and PpPbp-13R-SalI primers, subjected to kination, and inserted into the PpDRP5B-2::CP-AnaPBP plasmid without the coding region for the TP domain of AnaPBP to generate the PpDRP5B-2::PpPBP-AnaTG plasmid.

To construct the plasmid for the TP domain swapping experiment, the coding sequence corresponding to the N-terminal region (1–559 amino acids) of PpPBP, including the targeting sequence (1–55 amino acids) and TG domain (205–358), was amplified by PCR from the cloned PpPbp cDNA using the PpPbp-Met-F and PpPbp(356-559)R-SalI primers and subcloned into the pT7blue T-vector (pT7blue-PpPBP(CP-TG) plasmid). This plasmid was then digested by SalI and SphI. The coding sequence corresponding to the AnaPBP region (259–617 amino acids) including the TP domain (349–618 amino acids) was amplified by PCR using the AnaPbp-F5-SalI and AnaPbp-R4-SphI primers from the cloned AnaPbp gene, digested with SalI and SphI and then ligated to the SalI/SphI sites in the pT7blue-PpPBP(CP-TG) plasmid to generate the pT7blue-PpPBP(CP-TG)-AnaPBP(TP) plasmid. During cloning, the SalI recognition sequence GTGCAG, encoding Val and Asp, was inserted between the coding sequences of PpPBP(CP-TG) and AnaPBP(TP). Then, the coding sequence corresponding to the C-terminal region of PpPBP (829–936 amino acids) was amplified from the cloned PpPbp cDNA using the PpPbp-15F-SphI and PpPbp-13R-SphI primers, digested by SphI and ligated to the SphI-digested pT7blue-PpPBP(CP-TG)-AnaPBP(TP)-PpPBP(C) plasmid. In this cloning, the SphI recognition sequence, GCATGC, coding for Ala and Cys, was inserted between the coding sequences of AnaPBP(TP) and PpPBP(C). The DNA corresponding to PpPBP(CP-TG)-AnaPBP(TP)-PpPBP(C), called PpPBP-AnaTP, was amplified from the plasmid using...
the PpPbp-Mt-F and PpPbp-13R-SphI primers, subjected to blunting and kination, and inserted into the Smal site between the rice actin promoter and rbcS gene terminator of the pTKM1 vector. The DNA region containing the rice actin promoter, coding sequence for PpPBP-AnaTP, and rbcS gene terminator was excised by KpnI and XbaI digestion, subjected to blunting and kination, and inserted into the NheI site of the cloned PpDRP5B-2 gene with the HPT gene to generate the PpDRP5B-2::PpPBP-AnaTP plasmid for the TP domain swapping experiment. The constructed plasmids without PCR errors were linearized by digestion with KpnI before transformation. Transformation of the ΔPpPbp line was carried out as described above. Insertion of each plasmid DNA into the PpDRP5B-2 genomic DNA region of the antibiotic resistant transformant was confirmed by genomic PCR (data not shown).

**Complementation of ΔPpPbp with site-directed mutagenized PpPBP.** The cloned PpPbp cDNA in the pT7Blue T-vector was excised by digestion with EcoRI and SalI, each of which has one cutting site in the multi-cloning region of the vector, blunted using the Takara Blunting kit (Takara Bio), and inserted into the Smal site between the rice actin promoter and rbcS gene terminator of the pTFH 22.4 plasmid.14 Including the GFP gene, which is independently driven by the CaMV 35S promoter.

For the site-directed mutagenesis assay of the PpPbp gene, we constructed four pTFH22.4-PpPbp plasmids with point mutations in the PpPbp cDNA. We performed inverse PCR using PrimeStar max DNA polymerase (Takara Bio) and the following primers: PpPbp-16F-mt and PpPbp-14R-mt for T249I mutation, PpPbp-TG5/mt-F and PpPbp-TG5/mt-R for R281M mutation, PpPbp-TP3/mt-F and PpPbp-TP3/mt-R for S649F and N651I mutations, and PpPbp-TP1/mt-F and PpPbp-TP1/mt-R for Y622P and S623A mutations, then the obtained PCR products were self-ligated. Each point mutation was confirmed by sequencing each plasmid. Transformation of the ΔPpPbp line was carried out as described above. Four days after transformation, the chloroplast numbers in P. patens cells with GFP fluorescence were counted under a microscope.

**Results**

**Complementation of ΔPpPbp with the PpPBP-GFP fusion protein.** PpPBP was predicted to have a plastid-targeting sequence of 55 amino acids (score of 0.71) in the TargetP program.15 In a previous study, we showed that the N-terminal 106 amino acids of PpPBP, fused to GFP, targeted the protein to the chloroplast.6 Although, from an evolutionary viewpoint, PpPBP must localize in the intermembrane space of the plastid envelope, GFP fluorescence was observed in the whole chloroplast.6 Therefore, we do not think that the exact localization of PpPBP was determined, because a truncated PpPBP protein was used. In this study, we constructed a plasmid expressing the whole PpPbp gene fused to the GFP gene. DNA containing the CaMV 35S promoter, PpPbp-GFP gene, and nopaline synthase terminator was inserted into the cloned P. patens dynamin-related protein 5B-2 (PpDRP5B-2) genomic region (Fig. 1a) to construct a plasmid for transformation, because disruption of the PpDRP5B-2 gene did not visibly affect P. patens.12 Gene disruption of the PpPbp gene resulted in the appearance of a few macrochloroplasts in each protonemal cell (Fig. 1c).6 If the PpPbp-GFP fusion protein is functional in the correct target area, transformation with the PpPBP-GFP plasmid should complement the macrochloroplast phenotype of ΔPpPbp. Southern hybridization analysis of the PpPbp-GFP transformants suggested that two (#5 and #95) had a single insertion of the PpPbp-GFP gene in the PpDRP5B-2 gene region (Fig. 1b). Microscopic observations indicated that the cells in these transformants had normal chloroplast phenotypes (Fig. 1d) and that GFP fluorescence was observed in the entire chloroplast, especially in the periphery, with intense fluorescence (Fig. 1d). Localization of PpPBP-GFP fluorescence is inconsistent with that of peptidoglycan observed by the metabolic labeling method in our previous paper.8

**Complementation of ΔPpPbp with Anabaena PBP.** To compare the functions of PBP in P. patens chloroplasts to those of cyanobacteria (Anabaena sp. PCC7120), we generated stable transformants of ΔPpPbp expressing Anabaena PBP (AnaPBP) in the chloroplast. While PpPBP (936 amino acids) consists of a plastid-targeting sequence at the N-terminus (1–55 amino acids) and the TG (205–358 amino acids) and TP (558–828 amino acids) domains, AnaPBP (643 amino acids) consists of a short cytoplasmic tail, a transmembrane (TM) anchor (34–56 amino acids), and domains for TG (81–256 amino acids) and TP (349–618 amino acids) (Fig. 2a). To localize AnaPBP in chloroplasts, the coding sequence corresponding to the plastid (chloroplast)-targeting sequence (CP) of PpPBP was fused just upstream of the cloned whole AnaPbp gene (CP-AnaPbp). The
rice actin promoter and rbcs gene terminator were added to the CP-AnaPbp gene and inserted into the cloned PpDRP5B-2 gene to construct a plasmid to express the CP-AnaPBP protein in P. patens. ΔPpPbp was transformed by the PEG method with this plasmid to express the CP-AnaPBP protein.

These transformants showed suppression of the macrochloroplast phenotype under microscopic observation (Fig. 2b). The average number of chloroplasts in subapical cells of CP-AnaPBP transformant line #28 was 10.3 ± 3.2 compared to 1.7 ± 0.9 in ΔPpPbp and 47.0 ± 7.9 in wild-type plants (n = 50), suggesting partial recovery of the wild-type phenotype (Fig. 2c). These results suggest that cyanobacterial AnaPBP can complement PpPBP functions.

**Domain swapping experiments of PpPBP with Anabaena PBP.** To examine whether the functions of the TG and TP domains were conserved between PpPBP and AnaPBP, we performed domain swapping experiments for each domain. For the TG swapping experiment, a plasmid containing a gene for PpPBP-AnaTG (Fig. 2a) in the cloned PpDRP5B-2 gene was constructed. Using this plasmid and ΔPpPbp, we obtained five stable transformants (Fig. 2b). The subapical cells of generated stable transformant #89 had a chloroplast number...
of 43.6 ± 5.8 (n = 50), suggesting complete recovery compared to wild-type (44.0 ± 6.4) (Fig. 2c). For the TP swapping experiment, a plasmid containing the gene for PpPBP-AnaTP (Fig. 2a) in the cloned PpDRP5B-2 gene was constructed. After transformation using this plasmid and ΔPpPbp, we obtained three stable transformants (Fig. 2b). The cells of generated stable transformant #22 had a chloroplast number of 21.5 ± 6.6 (n = 50), which was approximately half that of wild-type plants (Fig. 2c). These results suggest that both of these conserved domains in PpPBP have similar functions to those of AnaPBP.

**Complementation of ΔPpPbp with site-directed mutagenized PpPBP.** Alignment of the TG domains of many bacterial PBPs revealed five conserved motifs: motif 1, EDxxFxxH; motif 2, GxSTxxQQxxK; motif 3, RKxxE; motif 4, KxxxYxxN; and motif 5, RxxxxL.2) While PpPBP possesses the 1st–4th motifs with no amino-acid changes to the conserved sequence, the amino-acid sequence of motif 5 is KxxxxxL. Because this arginine residue is partially conserved in bacterial PBPs and because the KxxxxL sequence is found in several bacterial PBPs, this change may not affect the function of PBP. For TP, the active site of class A PBPs contains motif 6 (SxxK), motif 7 (SxN), and motif 8 (K(T/S)G).17) PpPBP possesses these motifs without any changes. To examine the functions of the putative TG and TP domains in PpPBP, complementation experiments of ΔPpPbp were performed with site-directed mutagenized PpPBP. We constructed four plasmids containing the mutagenized PpPbp gene cloned into the pTFH22.4 vector containing the GFP gene, which was expressed independently from the PpPbp gene. For the TG domain, the pTFH22.4-PpPbp (T249I) plasmid possesses a mutation in the conserved residue of PpPBP motif 2, which is thought to play an important role in substrate recognition,2) while

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**Fig. 2.** Complementation of ΔPpPbp with Anabaena PBP. (a) Schematic representations of PpPBP, AnaPBP, the N-terminus of PpPBP fused to Anabaena PBP (CP-AnaPBP), and domain swapped PpPBP. Boxes with horizontal lines or light green or light blue fill in PpPBP indicate putative plastid (chloroplast)-targeting sequence (CP), transglycosylase (TG), and transpeptidase (TP) domains, respectively. Anabaena PBP is shown as a gray box with the transmembrane region (red), and TG (dark green) and TP (dark blue) domains. AnaPBP with the plastid-targeting sequence (CP-AnaPBP) and domain swapped PpPBP (PpPBP-AnaTG and PpPBP-AnaTP) are also shown. The plasmids expressing these Pbp genes were transformed into ΔPpPbp. (b) Protonemal cells of the CP-AnaPBP #28, PpPBP-AnaTG #89, and PpPBP-AnaTP #22transformants are shown. (c) The number of chloroplasts in subapical cells was counted.
A mutation was inserted into the non-conserved amino-acid residues between motifs 3 and 4 of the TG domain in the pTFH22.4-PpPbp (R281M) plasmid (Fig. 3c). For the TP domain, the pTFH22.4-PpPbp (S649F and N651I) plasmid had two mutation sites in motif 7, which is thought to function in the correct positioning of substrates or contribute to deacylation activity,\(^\text{17}\) while two mutations were inserted into the non-conserved amino-acid residues between motifs 6 and 7 of the TP domain in the pTFH22.4-PpPbp (Y622P and S623A) plasmid (Fig. 3c). These plasmids were introduced into the proplastids of \(\Delta PpPbp\) using the PEG method, and transformed cells were identified by GFP fluorescence under microscopic observation. When the plasmid containing the normal PpPbp gene without mutations was expressed in \(\Delta PpPbp\), the chloroplast number was restored to a similar level as in wild-type plants (Fig. 3). Similarly, PpPBP containing mutations in non-conserved regions complemented the macrochloroplast phenotype (Fig. 3). In contrast, PpPBP with mutations in conserved amino-acid residues within the TG or TP domains could not restore the wild-type phenotype (Fig. 3). These results suggest that PpPBP has both TG and TP activities, similar to bacterial homologs, and these activities are essential in \(P.\ patens\) chloroplast division.

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**Fig. 3.** Complementation of \(\Delta PpPbp\) with the transient expression of site-directed mutagenized PpPBP. The PpPbp gene was cloned into a vector containing the GFP gene, which was expressed independently of the PpPbp gene. From this plasmid, four plasmids with the site-directed mutagenized PpPbp gene: T249I, R281M, S649F&N651I, and Y622P&S623A were created (e). Symbols and colors in (e) are the same as those in Fig. 2a. These plasmids were introduced into the proplastids of \(\Delta PpPbp\) using the PEG method. Four days after transformation, cells with plasmid DNA were selected by GFP fluorescence under a microscope. Micrographs of the proplastids are shown for (a) wild type, (b) \(\Delta PpPbp\) (\(\Delta Ppbp\)), and \(\Delta PpPbp\) complemented with the wild-type PpPbp gene (+PpPBP) and (c and d) \(\Delta PpPbp\) complemented with PpPbps with mutagenized TG (c) and TP (d) domains. (f) The number of chloroplasts in regenerated protonemal cells was counted (\(n = 50\)).
Discussion

Complementation of $\Delta PpPbp$ with the PpPBP-GFP fusion protein. PpPBP-GFP complemented the macrochloroplast phenotype of $\Delta PpPbp$ (Fig. 1), suggesting that PpPBP-GFP functions in a similar manner to PpPBP in vivo. GFP fluorescence was especially strong in the peripheral region of each chloroplast (Fig. 1d). Based on the origin of plastids from an endosymbiotic cyanobacterium, PpPBP must localize to the intermembrane space where peptidoglycan is present, because PBPs function in the periplasm where new peptidoglycan monomer units join pre-existing peptidoglycans in bacteria. These results are consistent with the observation of plastid peptidoglycan fully surrounding chloroplasts in $P. patens$ cells.\(^8\) The precise localization of peptidoglycan and PpPBP in the plastid remains unclear, because standard electron microscopy showed no wall-like structure between the inner and outer envelopes of $P. patens$ chloroplasts.\(^9\) Cryo-transmission electron microscopy and metabolic labeling methods for peptidoglycan may resolve this issue in the future.\(^8\)

Complementation assays for $\Delta PpPbp$. The generation of three types of stable transformants expressing the plastid-targeting sequence of PpPBP fused to AnaPBP (CP-AnaPBP), PpPBP replacing the TG domain of AnaPBP (PpPBP-AnaTG), or PpPBP replacing the Anabaena TP domain (PpPBP-AnaTP) showed that all the tested combinations complemented the macrochloroplast phenotype of $\Delta PpPbp$. These results suggest that both the TG and TP domains of PpPBP function in a similar manner to their bacterial counterparts. While PpPBP-AnaTG could complement the macrochloroplast phenotype completely, only partial complementation was observed with CP-AnaPBP and PpPBP-AnaTP. The reason for the differences in the amount of complementation is unclear. Both CP-AnaPBP and PpPBP-AnaTP contain the Anabaena PBP TP domain and this domain may have weak activity in moss plastids. If different regions for these domains (for example, wider region including TP domain) are substituted, the amount of complementation may change. Among the PpPBP-AnaTG transformants, #101 had a chloroplast number of 12.6 ± 3.8, which was smaller than that of #89 (43.6 ± 5.8). One possibility is that expression of this protein is increased in the PpPBP-AnaTG transformant #89. It may due to several integrations of the transformed plasmid.

Transient expression experiments showed that mutations in the amino acids conserved in class A-type PBPs affected the function of PpPBP. These conserved amino acids in both TG and TP are essential and both domains have similar functions to those in bacterial PBPs. The composition and sequence of the peptides and of cross-linking in the peptidoglycan varies among bacterial species.\(^18\) These results suggest that both TG and TP activities are essential and function to produce a similar structure to cyanobacterial peptidoglycan where glycan chains are interlinked by short peptides.

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