Effects of Antibiotics That Inhibit Bacterial Peptidoglycan Synthesis on Plastid Division in Pteridophytes

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Summary We investigated the effect of antibiotics that specifically inhibit peptidoglycan synthesis on plastid division in pteridophytes by comparing the numbers of plastids in normal and antibiotic-treated pteridophyte cells. Plastid numbers were unaffected in Moniliformopses cells but decreased in Lycophytina cells after treatment with the β-lactam antibiotic ampicillin. However, cells of all four pteridophyte species exhibited decreased plastid numbers when treated with the antibiotic fosfomycin. Together, these results suggest that the Mur genes may be related to plastid division in pteridophytes, as demonstrated for certain bryophytes.

Key words Ampicillin, Fosfomycin, Peptidoglycan, Plastid division, Pteridophyte.

Peptidoglycans are continuous covalent macromolecules found on the outside of the cytoplasmic membranes of nearly all eubacteria. The bacterial sacculus, a bag-shaped structure formed from peptidoglycans, is generated in several steps (Fig. 1. van Heijenoort 2001, Bramhill 1997, Bugg and Walsh 1992). β-Lactam antibiotics, including ampicillin and penicillin, form covalent complexes with bacterial penicillin-binding proteins (PBPs), thereby interfering with their ability to synthesize a peptidoglycan cell wall and causing cell death. It is now widely accepted that a single cyanobacterial ancestor evolved into the chloroplasts present in glaucocystophyte algae, red algae, and green plants (Cavalier-Smith 2000).

Kasten and Reski (1997) reported that β-lactam antibiotics inhibited plastid division in the moss Physcomitrella patens, but not for tomato (Lycopersicon esculentum) cell suspension cultures. We examined the effects of antibiotics that inhibit peptidoglycan synthesis on plastid division in P. patens and found the following: (1) ampicillin and D-cycloserine caused a rapid decrease in the number of plastids per cell (defined as the plastid number), and (2) fosfomycin caused a decrease in the plastid number in half of the cells (Katayama et al. 2003). Furthermore, we identified the homologous genes related to peptidoglycan biosynthesis in the P. patens genome and reported that disruption of the Php gene in P. patens produced macrochloroplasts (Machida et al. 2006). Although eukaryotic peptidoglycans have not been found in plastids other than cyanelles in glaucocystophyte algae, these results suggest that the bacterial peptidoglycan synthesis pathway has been retained for chloroplast biogenesis of basal plants.

We also reported that ampicillin inhibited plastid division in the cultured cells of Selaginella nipponica, a type of Lycophytina belonging to pteridophytes (Izumi et al. 2003). In the same report,
we noted that ampicillin treatment produced macroplastids in guard cells of *S. nipponica* sporophytes, although no quantitative data were obtained. Furthermore, the effects of other antibiotics (fosfomycin and D-cycloserine) on plastids of *S. nipponica* have not been examined. Pteridophyte is considered to be a polyphyletic taxon comprising Lycophytina and Moniliformopses (Pryer et al. 2001), but no reports have addressed the effects of peptidoglycan synthesis-inhibiting antibiotics on plastid division in Moniliformopses.

Therefore, we selected one species of Lycophytina (*S. nipponica*) and three species of Moniliformopses (*Adiantum capillus-veneris*, *Ceratopteris richardii*, *Equisetum arvense*) to study the effects of inhibition of peptidoglycan synthesis produced by antibacterial ampicillin, fosfomycin, and D-cycloserine on the number of plastids in cells of these four pteridophyte species. Because plastids are easily observed in gametophytes, we used a gametophyte for *A. capillus-veneris*, *C. richardii*, and *E. arvense*, and because the gametophytes of *S. nipponica* mature into spores, we used a sporophyte for the *S. nipponica* experiments. The antibacterial compounds ampicillin, fosfomycin, and D-cycloserine were chosen because of their strong influence on plastid division in cells of *P. patens*.

### Materials and methods

Spores of *A. capillus-veneris* and *E. arvense* were collected from field-grown plants at Oita University. Spores of *C. richardii* were kindly provided by Prof. M. Hasebe (National Institute for Basic Biology, Japan). Sporophytes of *S. nipponica* from our laboratory were used. Seeds of *Brassica rapa* var. *peruviridis* were purchased from Takii Shubyo (Kyoto, Japan) and were grown on nutrient soil at 22°C under continuous light. The antibiotics used in this study were ampicillin sodium, fosfomycin sodium, and D-cycloserine (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Spores of *A. capillus-veneris*, *C. richardii*, and *E. arvense* were cultured on 0.5×Knop medium (Knop 1865) supplemented with 1% agar at 25°C under continuous light. The culture medium containing the appropriate antibiotic (3 ml) was solidified in a plastic petri dish with a 60 mm diameter, and 1 ml of the liquid medium containing spores and the appropriate antibiotic was then added to it. To culture *C. richardii*, 1 ml of the liquid medium containing the appropriate antibiotic was added to the culture medium every 7 d. To culture *A. capillus-veneris* and *E. arvense*, the appropriate antibiotic was added to the medium every 10 d. The sporophytes of *S. nipponica* had been cultured on 0.5×Knop medium supplemented with microelements of MS medium (Murashige and Skoog 1962) and 1% agar at 25°C under continuous light. The culture medium containing the appropriate antibiotic (50 ml) was solidified in an Erlenmeyer flask, and subsequently 1 ml of the liq-
uid medium containing the appropriate antibiotic was added to it. The *S. nipponica* sporophytes were subcultured at intervals of 10 d.

The plastid number of *C. richardii* was determined by light microscopy (Olympus AX70) after 14 d of culturing. In the other plants, the plastid numbers were determined after 30 d of culturing. The leaves of *S. nipponica* contain several kinds of cells. To determine the plasmid number, we used only the guard cells in the leaves that grew up to approximately 5 mm in length. This was done to ensure uniform conditions during the experiment. More than 100 cells were examined for each count.

Leaf disks from *B. rapa* var. *peruviridis* were cultured on MS medium containing 1% agar, 5% sucrose, and 0.5 mg l\(^{-1}\) N6-benzyladenine as described by Yagisawa *et al.* (2003). Leaf disks were transferred to fresh medium every week. To investigate the effects of fosfomycin on cell growth and chloroplast division, ~30–50 \(\mu\)l of 0.1 mM fosfomycin solution were dropped onto each leaf disk once every 3 d. For observation of chloroplasts, the leaf disks were cut into 2 mm\(\times\)2 mm squares and treated with an enzyme solution of 0.1% pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo, Japan), 1% cellulase onozuka RS (Yakult Pharmaceutical, Tokyo, Japan), 0.5% potassium dextran sulfate, and 10.93% mannitol, pH 5.5. Protoplasts were observed with an epifluorescence microscope BX60 (Olympus, Tokyo, Japan) under ultraviolet excitation, and photographs were taken with a Nikon DXM1200 digital camera.

**Results and discussion**

Gametophytes of *A. capillus-veneris*, *C. richardii*, and *E. arvense* cultured on medium without antibiotics exhibited many ovoid or dumbbell-like plastids that are dividing (Fig. 2). Average plastid numbers were 58.6±21.3 for *A. capillus-veneris* (Figs. 2A1, 3A), 131.1±36.8 for *C. richardii* (Figs. 2B1, 3B), and 79.5±15.2 for *E. arvense* (Figs. 2C1, 3C). In *S. nipponica* sporophytes cultured without antibiotics, 77% of the guard cells had 3 to 4 ovoid plastids, and the average plastid number was 3.22±0.82 (Figs. 2D1, 3D).

Guard cells of *S. nipponica* cultured on medium containing 1 mM ampicillin exhibited an average plastid number of 2.07±0.69 (Fig. 3D) with many colorless plastids having no chlorophyll of greater size relative to untreated cells (Fig. 2D2). Many guard cells exhibited colorless plastids, but all other leaf cells exhibited green plastids (Fig. 2D2). Steel’s test (\(\alpha=0.01\)), used for multiple-range testing of nonparametric data, revealed a significant difference in plastid numbers between ampicillin-treated and untreated *S. nipponica* cells. This suggests that ampicillin decreases the plastid number of Lycophytina. On the other hand, Steel’s test (\(\alpha=0.05\)) revealed no significant differences in gametophyte plastid numbers in *A. capillus-veneris*, *C. richardii*, and *E. arvense* cultured on medium containing 1 mM ampicillin relative to no ampicillin. The average plastid numbers were 58.9±21.6 (Figs. 2A2, 3A) for *A. capillus-veneris*, 132.2±27.9 (Figs. 2B2, 3B) for *C. richardii*, and 79.0±14.4 (Figs. 2C2, 3C) for *E. arvense*. These results suggest that ampicillin does not affect the plastid numbers of Moniliformosposes.

Fosfomycin is an inhibitor of MurA transferase that is encoded by the MurA gene (Fig. 1). Sporophytes of *S. nipponica* treated with and without 1 mM fosfomycin were approximately of equal size, and etiolation was observed in the leaves (data not shown). However, sporophytes treated with 0.5 mM fosfomycin grew similarly to the untreated sporophytes (data not shown). Therefore, 0.5 mM fosfomycin medium was used for the *S. nipponica* experiments. In experiments of other pteridophytes, 1 mM fosfomycin medium was used.

Relative to untreated cells, fosfomycin-treated cells of all four pteridophytes exhibited fewer plastids (Fig. 3). Additionally, the plastids in the fosfomycin-treated cells were larger than those in the untreated cells (Figs. 2A3, B3, C3, D3). While the plastids of *C. richardii* and *S. nipponica* were ovoid (Figs. 2B3, D3), the plastids of *A. capillus-veneris* and *E. arvense* displayed irregular mor-
Fig. 2. Representative micrographs of the antibiotic-treated cells of pteridophytes. A1–A3 are micrographs of *A. capillus-veneris* at the same magnifications (Bar=50 μm). B1–B3 are micrographs of *C. richardii* at the same magnification (Bar=50 μm). C1–C3 are micrographs of *E. arvense* at the same magnification (Bar=50 μm). D1–D3 are micrographs of the *S. nipponica* guard cells at the same magnification (Bar=20 μm). A1, B1, C1, and D1 are micrographs of the untreated cells. A2, B2, C2, and D2 are micrographs of the ampicillin-treated cells. A3, B3, C3, and D3 are micrographs of the fosfomycin-treated cells.

Fig. 3. Effects of antibiotics that inhibit bacterial peptidoglycan synthesis on the number of plastids in pteridophytes (A, *A. capillus-veneris*; B, *C. richardii*; C, *E. arvense*; and D, *S. nipponica*). Cont, Amp, and Fos indicate the untreated, ampicillin-treated, and fosfomycin-treated cells, respectively.
phologies (Figs. 2A3, C3). The average plastid numbers of the fosfomycin-treated cells of *A. capillus-veneris*, *C. richardii*, *E. arvense*, and *S. nipponica* were 12.7±12.3, 43.6±6.6, 19.0±4.7, and 1.87±0.52, respectively (Fig. 3). Steel's test (α=0.01) revealed significant differences in plastid numbers between the fosfomycin-treated and -untreated cells for all four pteridophytes. It is thought that the decrease in plastid numbers was due to the inhibition of plastid division by antibiotics, as observed in the case of *P. patens*.

Fosfomycin produced a colorless plastid phenotype in many of the antibiotic-treated guard cells of *S. nipponica* similar to results observed with ampicillin treatment (Figs. 2D2, D3). Although no colorless plastid phenotype was observed in the antibiotic-treated cells of *P. patens* (Katayama *et al.* 2003), we reported that one of the Mur genes, MurE, was associated with the development of chloroplasts from proplastids in *Arabidopsis thaliana* (Garcia *et al.* 2008). Thus, enzymes related to peptidoglycan synthesis might function in chloroplast development in Lycophytina.

The MurA gene is lacking in *A. thaliana* (Machida *et al.* 2006). To observe fosfomycin effects in cells from seed plants, a leaf disk culture was used. Because leaf disk cultures of *A. thaliana* have not been successful (Yagisawa *et al.* 2003), we selected *Brassica rapa* var. *peruviridis*, also a member of the family Brassicaceae with *A. thaliana*. Leaf disks treated with 0.1 mM fosfomycin grew to about 5 mm in diameter (Fig. 4), while leaf disks treated with 0.5 mM fosfomycin did not grow and died (data not shown). Mújica-Jiménez *et al.* (1998) reported that fosfomycin acts as a potent, reversible, and nonessential activator of phosphoenolpyruvate carboxylase from C4 maize leaves by binding to the same allosteric site as glucose-6-phosphate. This suggests that in eukaryotic plant cells, although fosfomycin does not inhibit MurA, it is able to inhibit the enzyme activities of some other enzymes. The growth inhibition of leaf disks may be due to a different effect of fosfomycin. Therefore, we used the concentration of 0.1 mM for fosfomycin treatments. No differences in cell volume or chloroplast number were observed between cells treated with and without 0.1 mM fosfomycin (Fig. 4). These results suggest that fosfomycin does not inhibit plastid division in seed plants lacking the MurA gene.

D-Cycloserine is an inhibitor of D-alanine:D-alanine ligase activity (Fig. 1). When spores of *A. capillus-veneris*, *C. richardii*, and *E. arvense* were cultured on a medium containing 1 mM D-cycloserine, no germination occurred (Fig. 5). The sporophytes of *S. nipponica* grew slightly and died when they were treated with 1 mM D-cycloserine (Fig. 5). Noteworthy inhibition of spore germination or sporophyte growth was observed only during D-cycloserine treatment. Hirase and Molin (2001) reported that cycloserine inhibited cysteine synthase activity and shoot elongation in the barnyard grass *Echinochloa crus-galli*. D-Cycloserine is also a well-known transaminase inhibitor with an action mechanism attributed to binding to pyridoxal-5'-phosphate, the coenzyme of many enzymes involved in amino acid metabolism (Churchich 1967). Because the metabolism of amino acids such as serine and cysteine in plants could be inhibited by D-cycloserine, the spore germination or sporophyte growth of pteridophytes could also be inhibited.

It is generally believed that Lycophytina branched off from other vascular plants first, and then Moniliformopses and seed plants branched off from the common ancestor of those (Pryer *et al.* 2001). In this study, ampicillin and fosfomycin decreased the plastid number of the Lycophytina *S. nipponica* as in *P. patens*. These results suggest that the peptidoglycan biosynthetic pathway, and not just a specific penicillin-binding protein or MurA, is tied to chloroplast division in Lycophytina.

The genome sequence of *Selaginella moellendorffii* is being determined by the U.S. Department of Energy’s Joint Genome Institute (Wang *et al.* 2005, http://genome.jgi-psf.org/), and the database can be searched using TBlastN for the amino-acid sequences of peptidoglycan biosynthetic enzymes. From searching the database, we found that parts of all the genes are related to peptidoglycan biosynthesis, suggesting that all of the genes necessary for peptidoglycan synthesis exist in *S. moellendorffii*. These results may that the chloroplasts in Lycophytina have plastid peptidogly-
Fig. 4. Effects of fosfomycin on cell growth and chloroplast division of B. rapa var. peruviridis. Leaf disks at 0 d (A, D) and 16 d (B, E) on medium without (A, B) and with (D, E) fosfomycin are shown. A, B, D and E are the same magnification (Bar=1 cm). Protoplasts from leaf disks at 10 d of culture without (C) and with (F) fosfomycin are present. C and F are the same magnification (Bar=10 μm). No differences in cell volume or chloroplast numbers were found between cells not treated with antibiotic and those treated with fosfomycin.

Fig. 5. Representative micrographs of D-cycloserine-treated pteridophytes. A and E are micrographs of A. capillus-veneris. B and F are micrographs of C. richardii. C and G are micrographs of E. arvense. D and H are micrographs of S. nipponica. A–D are micrographs of the untreated gametophytes or a sporophyte. E–H are micrographs of D-cycloserine-treated spores or a sporophyte. A–C and E–G are the same magnification (Bar=0.1 mm). D and H are the same magnification (Bar=5 mm).
cans. However, no wall-like structure has been detected between the inner and outer membrane in moss and Lycophytina, and further study will be necessary to determine the existence of peptidoglycan walls in plastids.

While fosfomycin decreased the plastid numbers of all Moniliformopses, ampicillin did not inhibit plastid division. Why is the plastid division of Moniliformopses inhibited by fosfomycin? Because the genome of Moniliformopses has not yet been sequenced, we cannot use genetic information to answer this question, but we present three potential hypotheses as follows: (1) Similar to Lycophytina and moss, Moniliformopses may have all the necessary genes, including Pbp, for peptidoglycan biosynthesis. In this regard, PBP of Moniliformopses could be insensitive to ampicillin under the condition used. (2) If Moniliformopses have no Pbp genes, the question can be rephrased. Why is the plastid division of Moniliformopses inhibited by fosfomycin even though a complete peptidoglycan synthetic pathway does not exist in Moniliformopses? In this case, the MurA protein of Moniliformopses could be expected to be directly related to plastid division, although we cannot determine the relationship between the original transferase function of MurA and plastid division. (3) Fosfomycin could inhibit an enzyme other than MurA protein that is related to plastid division only in Moniliformopses. Since fosfomycin did not inhibit plastid division in B. rapa var. peruviridis leaf disk culture, we believe that the possibility of hypothesis (3) is low. In the future, if the Moniliformopses genome is analyzed and the existence or nonexistence of Mur genes becomes clear, the answer to the above question will be found.

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References