Inhibition of Plastid Division by Ampicillin in the Pteridophyte Selaginella nipponica Fr. et Sav.

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We investigated the effect of the β-lactam antibiotic, ampicillin, on plastid division in the pteridophyte Selaginella nipponica. Guard cells of plantlets treated with 1 mM ampicillin only often had one plastid, whereas guard cells of untreated plantlets had two to four plastids. We generated a S. nipponica cell culture system and used it to investigate the effects of ampicillin. Treatment with 1 mM ampicillin had no effect on cell division in culture. We classified cultured cells into four types based on the number of plastids they contained: one (Type I), two (Type II), three or four (Type III) and more than five (Type IV). After 3 d in culture, the percentage of each cell type (I–IV) was 29.5, 46.7, 20.9, and 1.9%, respectively. Subsequently, the percentage of Types III and IV increased gradually, reaching 61.9 and 11.4%, respectively, after 15 d in culture in the absence of ampicillin. When 1 mM ampicillin was added, there was a minimal increase in the number of Type III and IV cells, with high percentages of Type I and II cells (32.4 and 45.7%, respectively) after 15 d. These results suggest that ampicillin inhibits plastid division in S. nipponica.

Keywords: Ampicillin — Plastid division — Pteridophyte — Selaginella nipponica — β-Lactam antibiotic.

Abbreviations: Fts, filamentous temperature sensitive; PBP, penicillin-binding protein; DAPI, 4',6-diamidino-2-phenylindole; 2,4-D, 2,4-dichlorophenoxyacetic acid.

Introduction

It is now widely accepted that a single cyanobacterial ancestor evolved through a cyanelle-like intermediate to become the chloroplasts of red algae and green plants (Cavalier-Smith 2000). Cyanelles are peptidoglycan-armored plastids of glaucocystophyte algae with close morphological and biochemical resemblances to endosymbiotic cyanobacteria (Steiner and Löffelhardt 2002, Löffelhardt et al. 1997). Eukaryotic peptidoglycan has been found in cyanelles, but it is not found in mitochondria or other plastid types (Aitken and Stanier 1979, Löffelhardt et al. 1997). β-Lactam antibiotics, including ampicillin and penicillin, form covalent complexes with bacterial penicillin-binding proteins (PBPs), thereby killing the bacteria by interfering with their ability to synthesize a peptidoglycan cell wall. Kies (1988) reported that penicillin inhibited cyanelle divisions of the glaucocystophytes, Cyanophora, Gloeochaete and Glaucocystis. Seven PBPs ranging in size from 110 to 35 kDa have been identified in the cyanelle envelope of Cyanophora (Berenguer et al. 1987). Thus, it is thought that penicillin prevents cyanelle division by inhibiting PBP peptidoglycan synthesis activity.

Kasten and Reski (1997) reported that β-lactam antibiotics inhibited plastid division in the moss Physcomitrella patens, but not in tomato cell suspension culture. This result suggests that a part of the peptidoglycan synthetic pathway derived from cyanobacteria is associated with plastid division, at least in moss, although chloroplastic peptidoglycan walls were not found. Despite the use of β-lactam antibiotics as an antibiotic during Agrobacterium-mediated transformation in higher plants, there have been no reports of its inhibiting plastid division, confirming that peptidoglycan is not involved in plastid biogenesis in higher plants.

Like bryophytes, pteridophytes are cryptogamous plants that do not form flowers or seeds. However, like angiosperms, they are vascular plants with distinct differentiated organs, such as roots, stems and leaves. Therefore, the study of the effect of β-lactam antibiotics on plastid division in pteridophytes may be significant in respect to plastid evolution. In this paper, we describe the inhibition of plastid division by ampicillin in the pteridophyte, Selaginella nipponica.

Results

The effect of ampicillin on plastid division in S. nipponica plantlets

Fig. 1 depicts S. nipponica guard cells after 14 d in culture. In plantlets cultured without ampicillin, guard cells had two to four plastids, and no guard cells were found that contained only one plastid (Fig. 1A). In contrast, some guard cells of plantlets treated with 1 mM ampicillin had only one or two plastids (Fig. 1B). This suggested that ampicillin inhibited plastid division in S. nipponica. Detailed investigation of the relationship between cell growth and plastid multiplication is difficult in S. nipponica plantlets. Therefore, we generated a cell...
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Effects of ampicillin on cell growth and plastid number in *S. nipponica* cell culture

To determine the effect of ampicillin on the growth of *S. nipponica* suspension cells, cell dry weight was measured after 12 d in culture media containing 0, 0.01, 0.1 and 1 mM ampicillin. Dry weight per flask ranged from 0.45 to 0.5 g at day 12, with little difference based on ampicillin concentration (data not shown). Dry weight in 1 mM ampicillin medium was measured over a period of time. Fig. 2 shows the growth curves and mitotic indexes of suspension cells cultured in 0 (control) or 1 mM ampicillin. In both cases, cells grew logarithmically from the start and reached a stationary phase after about 12 d, final dry weight per flask was about 0.5 g, and mitotic indexes peaked after 4 d, then declined.

To determine the morphology and number of plastids per cell, protoplasts of the suspension cells were isolated, fixed with glutaraldehyde, stained with the DNA-fluorescent dye DAPI (4',6-diamidino-2-phenylindole), and observed by fluorescence microscopy. Fig. 3 shows the distribution of plastids and their nucleoids in the protoplasts after 12 d of culture without ampicillin. Most cells contained one to six plastids (Fig. 3A–E), although cells containing 10 or more plastids were occasionally observed (Fig. 3F). Plastid nucleoids were scattered along the periphery of the plastids. In contrast, the majority (~90%) of cells grown in 1 mM ampicillin contained one to three plastids after 12 d in culture (Fig. 4). Cells with more than four plastids were rarely observed. In many cells, macroplastids were observed, although their plastid nucleoids were scattered along the periphery, as in control cells. In the normal condition, large cells had many plastids (Fig. 3D–F), while few, larger plastids were observed in large cells treated with ampicillin (Fig. 4C, D).

To understand how plastid numbers changed over time, we classified suspension cells into four types based on the number of plastids they contain. The first type contains only one plastid (Type I; Fig. 3A, 4A), the second contains two (Type II; Fig. 3B, 4B, C), the third contains three or four (Type III; Fig. 3C, D, 4D), and the fourth contains more than five plastids (Type IV; Fig. 3E, F). Fig. 5 shows the frequency of each cell type after 12 d in culture. In the control medium lacking ampicillin, Types I, II, III and IV cells were observed at frequencies of 11.5, 33.1, 44.6 and 10.8%, respectively. In

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**Fig. 1** Plastids (arrowheads) in guard cells (arrows) of *S. nipponica* after 14 d in culture. Cells of plantlets that were cultured in media without (A) and with (B) 1 mM ampicillin are shown. Bar = 50 μm.

**Fig. 2** Growth curves and the mitotic indexes of control cells and 1 mM ampicillin-treated cells. In both cultures, cells grew logarithmically from the start and reached a stationary phase after between 9 and 12 d. Mitotic indexes peaked after day 4 then declined.

**Fig. 3** Representative light (A1, B1, C1, D1, E1, F1) and epifluorescence (A2, B2, C2, D2, E2, F2) photomicrographs of plastids (large arrows), plastid nucleoids (small arrows) and cell nuclei (arrowheads) of *S. nipponica* DAPI-stained protoplasts isolated from control cells after 12 d in culture. (A) Type I cell, containing one plastid; (B) Type II cell, with two plastids; (C) Type III cell, containing three plastids; (D) Type III cell, containing four plastids; (E) Type IV cell, containing six plastids; (F) Type IV cell, with many plastids. Bar = 20 μm.
Fig. 3
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In a medium containing 0.01 mM ampicillin, cell type frequencies were similar to those in the control culture [10.8 (I), 34.5 (II), 47.5 (III) and 7.2% (IV)]. However, the frequency of Type I cells was higher in 1 mM ampicillin medium (34.8%) than in the control. The frequency of Type I cells in 0.1 mM ampicillin-containing medium was approximately halfway between those of control and 1 mM ampicillin media. These results suggest that ampicillin does affect plastid number in cells.

We then monitored the number of plastids per cell over time in culture (Fig. 6). After 3 d in culture without ampicillin, the frequency of each cell type was 29.5 (I), 46.7 (II), 20.9 (III) and 1.9% (IV). Subsequently, Type III and IV cells increased gradually, reaching 61.9 and 11.4%, respectively (Fig. 6A). The frequency of each cell type in cells growing in 1 mM ampicillin was similar to that in the control medium [31.4 (I), 49.5 (II), 15.2 (III) and 3.8% (IV)]. However, unlike in the control, these frequencies did not change significantly throughout the culture period (Fig. 6B). Fig. 7 shows the changes over time in the frequencies of cells containing more than three plastids. In control and 1 mM ampicillin media, the number of cells containing more than three plastids increased gradually, finally reaching 73.3% of the total cell number. In contrast, the percentage of cells growing in ampicillin medium containing three or more plastids remained around 20% throughout the culture period.
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These results indicate that ampicillin inhibited plastid duplication in *S. nipponica* cells in the late stages of culture.

**Discussion**

Under normal culture conditions, *Selaginella nipponica* dry weight increased logarithmically from the start of culture until day 12. The mitotic index peaked at day 4 and then declined. These results suggest that suspension cells divide actively during the early phase of culture, then slow cell division and begin to grow during later phases, similar to what we have observed in a peony cell suspension line (Ono and Harashima 1983). We saw few differences between growth in the control and ampicillin-treated cultures, indicating that ampicillin does not affect growth or cell division in *S. nipponica* cell culture.

In the absence of ampicillin, the frequency of cells with more than three plastids increased during the late phase of the culture. In the sporophytes of lycopods, the apical meristematic cells have one or two plastids, whereas mature cells contain many plastids (Dunlop 1949, Brown and Lemmon 1990). This difference has been attributed to the several plastid divisions that occur after cell division ceases. As in mature sporophytic cells, the plastids of suspension cells must divide during the cell growth phase. In contrast to early stages of culture, ampicillin affected the number of plastids after 6 d, at which point the frequency of cells with more than three plastids did not increase in the ampicillin-grown culture. Thus, ampicillin inhibited plastid division to form large plastids in *S. nipponica* suspension cells.

From the start of the subculture (15 d after inoculation) through the early phase (the first 3 d), the frequency of cells with more than three plastids decreased rapidly, while the number of cells with one or two plastids increased (Fig. 6). This effect was seen in control and in ampicillin-treated cultures, suggesting that plastids do not divide during this early stage. Therefore, frequency changes may be a result of cell division in the absence of plastid division. Alternatively, plastids may have fused during this early stage, or some plastids were destroyed for some unknown reason. Plastid behavior during this early period and the relationship between cell volume and total volume of plastids remain to be investigated further.

β-Lactam antibiotics are inhibitors of the peptidase activity of PBPs in bacteria. It is known that *Escherichia coli* has nine PBPs (Blattner et al. 1997). Six PBPs have been identified in the genome of the cyanobacterium *Synechocystis*

![Fig. 6](image-url)

**Fig. 6** Changes in the frequencies of the four cell types (Types I–IV) in suspension cells grown without ampicillin (A) or with 1 mM ampicillin (B).

![Fig. 7](image-url)

**Fig. 7** Changes in the frequencies of cells containing more than three plastids during subculture in control or 1 mM ampicillin.
Although the cyanelle is surrounded by a peptidoglycan layer, whose synthesis is affected by β-lactams, no PBPs have been found in the cyanelle genome of C. paradoxa (Löffelhardt et al. 1997), suggesting that these genes are located in the nuclear genome in glaucocystophytes. Recently, PBP3 genes have been identified in the plastid genomes of the two earliest-diverging green algae, Nephroelmis olivacea and Mesostigma viride (Tunnel et al. 1999, Lemieux et al. 2000). PBP3, which is encoded by the Pts/PBP3 gene, is involved in cell division in prokaryotes (Nishimura et al. 1977, Spratt 1975, Spratt 1977), suggesting that a PBP3-involved system operates in green plants. However, no PBP genes have been identified in other chloroplast genomes (Sugiura 1995). The Arabidopsis genome has no PBP genes (Arabidopsis Genome Initiative 2000), suggesting that the plastid PBP system has been lost in higher plants. In this study, we showed that ampicillin inhibited plastid division of S. nipponica cultured cells. This suggests that the PBP system is functional, not only in green algae and moss, but also in pteridophytes. It has been thought that Pteridophytes is a polyphyletic taxon containing lycopsids, horsetails and ferns (Pryer et al. 2001). In the future, we will test the effects of antibiotics on plastid division in horsetails, ferns and angiosperms to detect plastid PBP systems.

The function of PBPs in plastids remains to be resolved. Studies with E. coli indicate that septum assembly is mediated by a large number of proteins that localize to the division site, where they are postulated to form a multiprotein complex (for a review, see Bramhill 1997). Among these proteins, it is known that eukaryotic homologs of Fts (filamentous temperature sensitive) Z, the key component of bacterial division, are related to plastid division in algae, moss and higher plants (Strepp et al. 1998, Osteryoung 2001, Miyagishima et al. 2001). The function of PBPs may be to support FtsZ in the division process, as some PSFs are related to eukaryotic photoautotroph sensitive to β-lactam antibiotics. Whereas PBPs may operate in green plants. However, no PBP3-involving system has been observed beyond the glaucocystophytes. At present, these hypotheses are only speculative. The isolation of PBP genes from S. nipponica may be a key step in elucidating their functions.

Materials and Methods

Plant materials

Selaginella nipponica Fr. et Sav. plants were collected from field-grown sporophytes at Oita University. Calluses were induced by culturing on solid MS medium (Murashige and Skoog 1962) with 1 μM 2,4-D (2,4-dichlorophenoxyacetic acid), 4% glucose, 0.1% CaCO3 and 0.3% Gellan Gum (Wako Pure Chemical Industries, Ltd.). Induced calluses were cultured in liquid ASMS medium (Takio et al. 1986) with 1 μM 2,4-D, 4% glucose and 20 mM sodium succinate on a gyratory shaker at 130 rpm and 25°C in continuous light (2,000 lux). Media pH was adjusted to 6.0 before autoclaving. Suspension cells were routinely subcultured at 2-week intervals by resuspending 3-ml cell pellets in 50 ml of fresh medium in a 125-ml Erlenmeyer flask.

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Plantlets of field-grown sporophytes were cultured in 0.5× Knop I medium (Bopp 1952) plus 0, 0.01, 0.1 or 1 mM ampicillin. Microscopy (AX–70; Olympus Optical Co., Ltd.) was performed at day 14. The media of suspension cells contained 2% glucose and 0, 0.01, 0.1 or 1 mM ampicillin. After the pH was adjusted to 6.0, media were sterilized by filtration through 0.2-μm filters.

Growth measurements of suspension cells

To measure cell growth, suspension cell dry weights were measured after 12 d in culture. For suspension cells that were cultured in 0 or 1 mM ampicillin, dry weights were measured at 3-d intervals. All cells contained in a flask were harvested onto a dried, pre-weighed filter paper by vacuum filtration. To determine dry weight, collected cells were dried at 80°C overnight and then weighed. For each measurement, three flasks were weighed, and dry weight calculated as an average of the three.

Plastid numbers and mitotic index measurements

Plastid number per cell and mitotic index were determined in isolated protoplasts from suspension cells. Protoplast isolation buffer consisted of 2% Cellulase Onozuka RS (Yakult Pharmaceutical Ind. Co., Ltd.), 0.3% Pectolyase Y-23 (Seishin Co.), 0.5 M glucose, 3 mM morpholinoethanesulfonic acid, 6 mM CaCl2·6H2O, and 0.7 mM KH2PO4, pH 5.7. Suspension cells were treated in the solution at 32°C for 4 h. Isolated protoplasts were fixed in 2% glutaraldehyde dissolved in buffer NS (0.25 M sucrose, 1 mM EDTA, 0.1 M CaCl2, 0.1 mM MgCl2, 0.8 mM phenylmethylsulfonyl fluoride, 0.05% mercaptoethanol, Tris-HCl at pH 7.6; Kuroiwa et al. 1981) containing 0.5 M glucose at 4°C overnight. Protoplasts were stained with 0.25 mg liter−1 DAPI in buffer NS at room temperature for 5 min. Stained samples were observed under UV-light using an epifluorescence microscope (BH2-RFC; Olympus Optical Co., Ltd.), and plastid numbers per cell were determined. Plastid counting was performed after 12 d in culture. For suspension cells cultured in 0 or 1 mM ampicillin, counts were made every 3 d. More than 100 cells were examined for each count. Mitotic indexes were measured at 1, 2, 3, 4, 5, 6, 9 and 15 d in culture. For each measurement, more than 500 cells were examined.

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References


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