*Journal of Plant Biology*, September 2000, 43(3) : 179-182 **REPORT**

# Antibiotics Resistance of a Red Alga, *Griffithsia japonica*

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**To identify antibiotics suitable for stable transformation, we tested the resistance of a red alga, *Griffithsia japonica* Okamura, to four commonly used antibiotics. Very young germlings, with 1~3 cells, that germinated from the tetraspores were cultured for 40 d in a half PES medium containing kanamycin, streptomycin, hygromycin B, or phleomycin. *G. japonica* was highly sensitive to 1 μg $mL^{-1}$ of phleomycin and 50 μg $mL^{-1}$ of hygromycin B. However, it was resistant to kanamycin and low levels of streptomycin and hygromycin B. These results suggest that resistance genes for phleomycin or hygromycin B can be used as selectable markers for transformation of *G. japonica*.**

*Keywords*: antibiotics, *Griffithsia japonica*, red alga, transformation

Algae comprise a multitude of diverse organisms that inhabit the ocean, freshwater, soil, snow, rocks, trees, and air. Although they are photosynthetic, their relatively simple vegetative structures distinguish them from higher plants. Algae have increased in economic importance as model systems in biological research and also as raw materials for food, fodder, fertilizer, pharmaceuticals, biochemicals, and industrial sources (Waaland, 1981; Fogg, 1989). Genetic engineering technology, either to control individual genes within the organism or to introduce novel genes into the genome, would greatly advance their biological study and industrial application, but genetic manipulation of algae has been very restricted (Stevens and Purton, 1997).

Stable genetic transformation has been accomplished in only a few green algae such as *Chlamydomonas* (Kindle, 1990), *Volvox* (Gruber et al., 1996), and some diatoms (Dunahay et al., 1995; Apt et al., 1996). A suitable DNA marker must be chosen that will enable selection for rare transformant cells. Several genes encoding for antibiotic- or herbicide-resistance proteins can be used for transformation as selection markers. This approach, however, has been hindered because of the natural resistance of algae to many commonly used antibiotics (Apt et al., 1996). In this preliminary study, we tested the resistance of a red alga, *Griffithsia japonica* Okamura, to several commonly used antibiotics to find suitable compounds for red algal transformation.

Tetrasporophytes of *G. japonica* were collected from Dolsan Island on the southern coast of Korea on April 1992 and were cultured at 20°C, 15 μmol $m^{-2}$ $s^{-1}$, and 16:8 h L:D, as described by Lee et al. (1995). Very young germlings, with only 1~3 cells, that germinated from these tetraspores were cultured for 40 d in a half PES medium containing kanamycin, streptomycin, hygromycin B, or phleomycin. The concentrations of kanamycin, streptomycin, and hygromycin B were 5, 10, and 50 μg $mL^{-1}$, those for phleomycin were 1, 5, and 10 μg $mL^{-1}$. We observed morphology of the developing germlings with an Olympus SZH stereomicroscope and an Olympus BH-2 microscope (Olympus, Japan). Nuclei were stained with DNA-specific fluorochrome DAPI (4',6-diamidino-2-phenylindole), via the microwave-fixation method, then observed with an Olympus BX50 fluorescence microscope (Goff and Coleman, 1987). Photographs were taken with Kodak Gold color film and Ektachrome color slide film (Kodak, USA).

In the control PES medium (without any antibiotics), the germlings developed into normal adult thalli, each having 10 axial cells. The average branch number was 3.4 after 40 d of laboratory culture (Figs. 1 and 2). Vegetative cells of the adult thalli were 110 ~ 140 μm wide and 350~700 μm long (Fig. 3, A and B), slightly thinner than the thalli collected in the field (Kim, 1988). Almost 50% of the thalli produced male or female reproductive structures, with normal morphology and position. A female reproductive structure, the procarp, developed on an apical cell, surrounded by 2~4 sterile lateral branches (Fig. 3B). Male reproductive structures, the spermatangia, developed on a subapical cell as surrounded by 10~20 involucral cells. In the DAPI-stained cells, white

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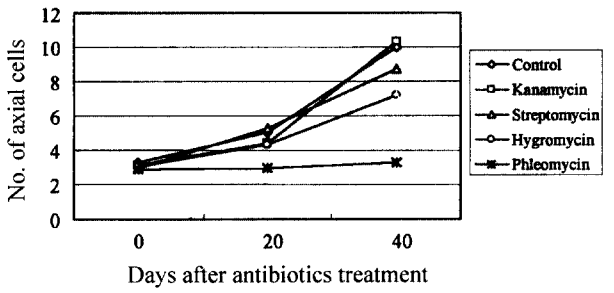



**Figure 1.** The number of axial cells of *G. japonica* that cultured in a half PES media containing 50 μg $mL^{-1}$ kanamycin, 50 μg $mL^{-1}$ streptomycin, 50 μg $mL^{-1}$ hygromycin B, or 1 μg $mL^{-1}$ phleomycin, over 40 days.

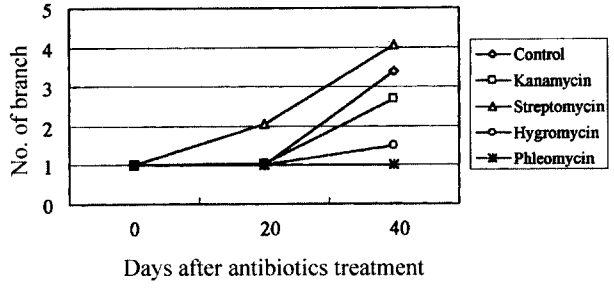


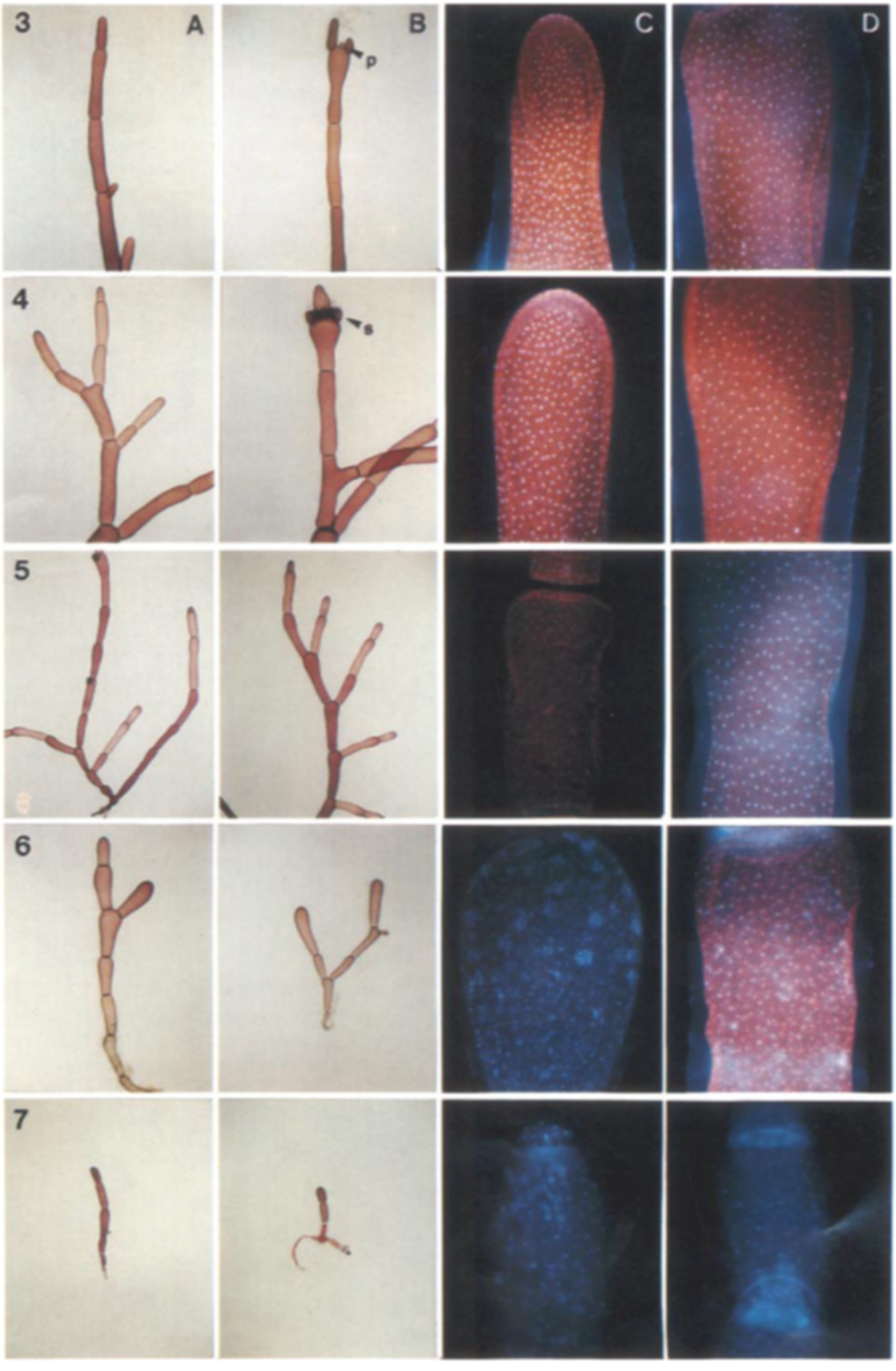

**Figure 2.** The number of branches of *G. japonica* that cultured in a half PES media containing 50 μg $mL^{-1}$ kanamycin, 50 μg $mL^{-1}$ streptomycin, 50 μg $mL^{-1}$ hygromycin B, or 1 μg $mL^{-1}$ phleomycin, over 40 days.

fluorescent spots indicated nuclei while the chloroplasts emitted red auto-fluorescence under UV light (Fig. 3, C and D). The size and shape of the nuclei were normal and their pattern of distribution was regular in all vegetative cells (Fig. 3C). Size, shape, and density of chloroplasts in the vegetative cells were also normal (Fig. 3D).

Kanamycin is a deoxystreptamin aminoglycoside that binds to ribosomal components and inhibits protein synthesis. The neomycin phosphotransferase II (*nptII*) gene is an effective selectable marker for plant transformation. However, *G. japonica* was insensitive to kanamycin at all the concentrations examined, with germlings developing into normal thalli. Thallus habit and size were very similar to those of the control. After 40 d of culture in the PES medium containing 50 μg $mL^{-1}$ kanamycin, the number of axial cells was 10.3 and the average branch number was 2.7 (Figs. 1 and 2). The branch number was slightly lower than that of the control, but not significantly. Size, shape, and color of the vegetative cells were the same as those of the control (Fig. 4, A and B). Almost 50% of the germlings produced morphologically normal reproductive structures, which was similar to the case of the control (Fig. 4B). The size, shape, and distribution pattern of nuclei and chloroplasts of the vegetative cells were also normal (Fig. 4, C and D). Kanamycin apparently could not affect the ribosomal components of the red alga.

Streptomycin acts by inhibiting protein synthesis and damaging cell membranes in susceptible organisms. Because the aminoglycoside-3'-adenyltransferase (*addA*) gene confers resistance to streptomycin, it has been used as a selectable marker in plant transformation. Development of *G. japonica* was affected by 50 μg $mL^{-1}$ of streptomycin. Thalli were smaller than in the control (Fig. 5, A and B). The number of axial cells was 8.7 (Fig. 1), which was slightly lower than that of the control. The smaller thalli resulted from the smaller size of each vegetative cell: vegetative cells were very short (~350 μm) and narrow (~70 μm). The average number of branches, however, was higher than that of the control (Fig. 2). The development of rhizoids was inhibited, so that many germlings could not attach to the bottom of the culture flask (Fig. 5A). Size and distribution pattern of the nuclei were normal (Fig. 5, C and D). When the germlings were cultured in streptomycin-containing media at concentrations of 5 and 10 μg $mL^{-1}$, they developed normally, forming reproductive structures in several thalli.

Hygromycin B is an aminoglycoside antibiotic that kills bacteria, fungi, and higher eukaryotic cells by inhibiting their protein synthesis. The hygromycin phosphotransferase (*hpt*) gene is used as a selectable marker in plant transformation. Germlings of *G. japonica* cultured with hygromycin B concentrations of 5 and 10 μg $mL^{-1}$ developed normally. Reproductive structures were also observed in several thalli. At a concentration of 50 μg $mL^{-1}$, however, only about 50% of the germlings survived after 20 d; more than 95% of the thalli were dead after 40 d. Live thalli were morphologically abnormal. The number of axial cells was 7.2 and the branch number was 1.5, both dramatically lower than those of the control germlings (Figs. 1 and 2). Normal apical cells are usually short with a thin club-shape, but the apical cells of the hygromycin-treated thalli were long and abnormally large, and were broadly obovate (Fig. 6, A and B). The nuclei in apical cells were irregularly distributed, forming patches (Fig. 6C). Chloroplast density was low in the apical cells, making them appear blue rather than red (Fig. 6C). The large apical cells and the irregular distribution pattern of the nuclei proba-




**Figures 3-7. A and B.** Morphology of *Griffithsia japonica*. **C.** DAPI-stained apical cells showing white fluorescent nuclei and red auto-fluorescent chloroplasts. **D.** DAPI-stained vegetative cells showing fluorescent nuclei and chloroplasts. Algae were cultured in a control medium for 40 days (Fig. 3), 50 μg $mL^{-1}$ kanamycin medium for 40 days (Fig. 4), 50 μg $mL^{-1}$ streptomycin medium for 40 days (Fig. 5), 50 μg $mL^{-1}$ hygromycin B medium for 40 days (Fig. 6), or 1 μg $mL^{-1}$ phleomycin medium for 20 days (Fig. 7). p = procarp, s = spermatangia.

bly resulted from inhibited cell division. Distribution of nuclei and chloroplasts in the other vegetative cells was normal (Fig. 6D).

Phleomycin, a member of the bleomycin family, exhibits antibiotic activity against various prokaryotic and eukaryotic organisms by breaking down their DNA (Hallmann and Rappel, 1999). The Ble protein can inhibit phleomycin. *Ble* has been used as a selectable marker in algal transformation, such as with diatoms (Dunahay et al., 1995; Apt et al., 1996) and *Chlamydomonas* (Stevens et al., 1996). In the current study, *G. japonica* was sensitive to low concentrations of phleomycin. In fact, most of the germlings cultured in media containing 5 or 10 μg $mL^{-1}$ were dead within the first 5 d. At a concentration of 1 μg $mL^{-1}$, the cells of some germlings also died within 5 d. Most germlings were dead after 20 d, and even the surviving germlings possessed dead or dying cells (Fig. 7, A and B). Nuclei were larger or smaller than those of normal cells, and their distribution pattern was irregular in all vegetative cells (Fig. 7, C and D). Cells appeared blue because chloroplasts were distributed sparsely in the vegetative cells (Fig. 7, C and D). All germlings were dead and colorless after 40 d. The average number of axial cells was 3.3 and the branch number of germlings was 1.0 after 40 d of phleomycin treatment (Figs. 1 and 2).

In summary, *G. japonica* was highly sensitive to 1 μg $mL^{-1}$ of phleomycin and 50 μg $mL^{-1}$ of hygromycin B. However, the red alga was resistant to a high level of kanamycin as well as to low levels of streptomycin and hygromycin B. Therefore, the resistance genes for phleomycin and hygromycin B can be used as selection markers in the transformation of *G. japonica*.

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