INTRODUCTION

Colonization of host plants by microbial symbionts and avirulent parasites often elicits physiological modifications such as localized and systemic disease resistance and drought or dehydration tolerance. Among such microbes, soil-inhabiting actinomycetes have attracted much attention as potential biocontrol agents in agriculture because of their ability to produce a variety of antibiotics. Recently, however, the usefulness of endophytic actinomycetes for the same purpose was identified after Matsukuma et al. and Okazaki et al. isolated a variety of actinomycetes inhabiting a wide range of plants as either symbionts or parasites. Igarashi et al. discovered novel antibiotics and growth regulators of plants and animals from several species of actinomycetes of plant origin, showing the physiological potential of endophytic actinomycetes in aiding development of novel technology to enhance human life.

Based on these earlier reports, Shimizu et al. developed unique biocontrol methods for endophytic actinomycetes. They successfully produced disease-resistant rhododendron seedlings by isolating Streptomyces galbus strain R-5, which has broad antimicrobial activities, from field-grown rhododendron and added it to flasks in which tissue-cultured seedlings were growing. Subsequently, Nishimura et al. isolated an antifungal strain of S. padanus, AOK-30, from potted mountain laurel (Kalmia latifolia L.) and induced disease resistance in tissue-cultured seedlings. We further found that colonization with AOK-30 rendered tissue-cultured seedlings drought tolerant and enhanced osmotic pressure in seedling cells. We also prepared protoplasts from AOK-30-treated and -untreated seedlings and found that those from untreated seedlings were released readily with cellulase, but an additional xylanase was required to prepare those from treated seedlings. These findings led us to postulate that AOK-30 colonization induces structural cell wall modifications in tissue-cultured seedlings of mountain laurel and that these modifications might be associated with AOK-30-induced drought tolerance.

MATERIALS AND METHODS

Plant and actinomycetes

Tissue-cultured seedlings of mountain laurel (Kalmia latifolia L., cultivar Ostbo Red) growing in flasks were treated with strain AOK-30 of Streptomyces padanus as described previously. Briefly, a spore suspension (500 µl) of AOK-30 in 10% glycerol supplemented with dimethyl sulfoxide (DMSO) was transferred from a stock culture to 100 ml of IMA-2 liquid medium and incubated on a rotary shaker at 100 rpm at 30°C for 24 hr. One ml of a mycelial suspension (3–4 × 10⁶ cfu/ml) prepared from this culture was then spread over the surface of the rooting medium in the glass flasks supporting mountain lau-
rel seedlings obtained from tissue culture. As an untreated control, 1 ml of IMA-2 liquid medium was spread on the medium surface. All seedlings were then grown in an incubation room with 12 hr light at 25°C for 10 days.

**Extraction and analysis of callose in AOK-30-treated and -untreated seedlings**

Callose was extracted from AOK-30-treated and -untreated seedlings according to the method of Kohler et al. with slight modifications. Ten days after AOK-30 treatment about 0.5 g of seedling sample was immersed in 100% ethanol in 20-ml plastic tubes. The tubes were shaken slowly on a rotary shaker overnight. The ethanol was changed several times until decoloration was observed. The descolorized seedlings were homogenized with a small amount of 100% ethanol with an ice-cold mortar and pestle then the homogenate was centrifuged at 12,000 × g for 20 min and the ethanol was carefully decanted from the pellet. The pellet was dissolved in 300–350 μl DMSO in an Eppendorf tube and boiled for 30 min in a commercial pressure cooker. After cooling to room temperature, the samples were centrifuged at 12,000 × g for 5 min in a microcentrifuge then the supernatant was transferred to a fresh tube. For quantitative determination of extracted callose, 100 ml of supernatant was supplemented with 200 μl 1 M NaOH and 1.2 ml aniline blue loading mixture [400 μl 0.1% aniline blue; 50 μl 1 M glycine/NaOH (pH 9.5); 210 μl 1 M HCl]. After mixing vigorously, the samples were incubated in a water bath at 35°C for 20 min and cooled to room temperature for about 30 min.

Total fluorescence of the samples, representing both autofluorescence and fluorescence/sirofluor complexes, was determined with a fluorescence spectrophotometer (Hitachi F-2000) at 393 nm excitation and 479 nm emission. For accurate callose determination, a parallel assay was run in which aniline blue was omitted from the loading mixture. Autofluorescence of these samples was then determined and subtracted from the total fluorescence of the corresponding parallel samples. Fluorescence was quantified for three samples prepared from the treated and untreated samples, respectively.

**Extraction and analysis of proteins in cell walls of AOK-30-treated and -untreated seedlings**

Cell wall fractions from AOK-30-treated and -untreated seedlings were prepared according to the method of Hayashi and Ohsumi and Kiba et al. with slight modifications. Ten days after AOK-30 treatment, about 0.6 g of seedling sample was powdered in liquid nitrogen in a mortar and pestle. The powder was treated with 1.5 ml of 25 mM Tris/HCl buffer (pH 7.4) containing 0.1% Nonidet P-40 and EDTA-free (1 tablet/50 ml) that had been warmed previously at 50°C. The homogenate was transferred to a 2-ml Eppendorf tube and centrifuged at 14,000 × g at 5°C for 10–15 min. The pellet was suspended in 0.7 ml of the above buffer with a vortex followed by centrifugation as before. The pellet was washed at least 10 times in this way. The final pellet was suspended in 0.3 ml of buffer and cooled in an icebox for 2 hr. The suspension was then centrifuged as before and the supernatant was collected as the protein fraction.

For analysis of the proteins in this fraction, 4 μl of 6 × SDS-PAGE sample buffer [1.2 g SDS, 6 ml glycerol and 5 mg bromophenol blue (BPB) in 1 ml 1.25 M Tris/HCl (pH 6.8)] was added to 20 μl of protein fraction, followed by warming until dissolution of glycerol. The total volume was adjusted to 10 ml by addition of distilled water. The fraction was heated in boiling water for 3–4 min then immediately cooled in an icebox. Electrophoresis was carried out on 5–20% gel (Atto Sci. Inst., PAGEL) at 20 mV then the gel was washed three times with sterilized water for 5 min each, followed by staining with CBB (Biolad, Bio-Safe TM Coomassie No. 161–0786) on a shaker for 1 hr. After washing several times to remove the background stain, the bands of AOK-30-treated and -untreated samples were compared. A band of ca. 35 kDa was more intensely observed in the treated than the untreated sample. These bands were cut from gels of both treated and untreated seedlings and sent to the Institute of Life Science, Apro Science Co. Ltd. (Naruto-city, Tokushima prefecture) for sequencing of amino acids. Briefly, the bands were treated in Tris buffer (pH 8.0) containing trypsin at 35°C for 20 hr. The total solution was fractionated into peptide fragments by reverse-phase high performance liquid chromatography (HPLC). As a control, bandless parts of the gels were removed and treated in the same way. The N-terminal amino acid sequence of detected peaks was analyzed using Precise 494 cLC protein sequencing system.

**Microscopic observation of lignified cell walls in AOK-30-treated and -untreated seedlings**

To compare lignification of cell walls, AOK-30-treated and -untreated seedlings with 18–20 leaves were removed from the flasks 10 days after treatment. Thin transverse sections of the stems (ca. 15 μm thick) were prepared from fresh specimens with a plant microtome (Nippon Medical & Chemical Inst. Co. Ltd.; Type MTH-1) then stained with Wiesner reagent (5 ml 99.5% ethanol containing 0.1 g phloroglucide mixed with 2.5 ml HCl before use) for 5 min before observation with a light microscope.

**RESULTS AND DISCUSSION**

Callose fractions were prepared from cell walls isolated from both AOK-30-treated and -untreated seedlings. The total average fluorescence of the fractions from treated and untreated seedlings at 479 nm were ca. 4.2 and 2.6, respectively, and this difference was significantly different (P<0.01) (Fig. 1). Thus, AOK-30 treatment seemed to accelerate accumulation of callose, a heterogeneous β-1,3-
glucan involved in a variety of plant developmental processes such as cell division, ripening of pollen mother cells\textsuperscript{22}) and deposition of wall appositions\textsuperscript{25}). As reported by Kohler et al.\textsuperscript{22}), callose biosynthesis is induced in plants treated with various compounds of microbial origin, and callose deposition often serves as a reliable biochemical marker for activation of the plant defense response. Aist\textsuperscript{25}) distinguished the papillae that form upon microbial attack from wound plugs which serve as structural barriers against external physical or chemical stimuli. Russo and Bushnell\textsuperscript{26}) demonstrated that wall appositions composed of callose were deposited in barley leaf cells mechanically damaged with a microneedle. Moreover, Tsuzuki et al.\textsuperscript{27}) showed chemical induction of wall appositions in barley leaf cells sprayed with Tween 20. Papillae and wall appositions are promptly formed in the space between the inner surface of the cell wall and plasma membrane at the attack site by deposition of various components including callose, efficiently blocking penetration by microbes beyond these barriers. Suzuki et al.\textsuperscript{28,29}) cytochemically demonstrated that β-1,3-glucan is transported from the Golgi apparatus and deposited in papillae in cells of Japanese pear leaves attacked by Alternaria alternata or treated with its toxin. Papillae are also induced in cells of plants inhabited by endophytic actinomycetes, which likely act as parasites or symbionts. Endophytic actinomycetes are thought to enter plant tissues via stomata\textsuperscript{30,31}) then grow within in-

![Fig. 1. Callose content in cell walls of AOK-30-treated and -untreated seedlings, expressed as the fluorescence intensity at 479 nm. Vertical lines on each bar represent the standard error.](image)

![Fig. 2. An intense protein band of ca. 35 kDa (arrow) detected from cell walls of AOK-30-treated seedlings by SDS-PAGE. Marker: BIO-RAD, prestained protein marker.](image)

![Fig. 3. Lignified cell walls of sieve cells in AOK-30-treated seedlings detected by a phloroglucine-HCl reaction. (A) No response was seen in sieve cells (arrows) of AOK-30-untreated seedlings. (B) Pinkish, positive responses were seen in sieve cells (arrows) of treated seedlings. C: cortical cells, M: meristem, S: sieve cells, X: xylem](image)
tercellular spaces\(^{31,32}\). According to Suzuki \textit{et al.}\(^{31}\), when tissue-cultured rhododendron seedlings were inoculated with \textit{S. galbus}, this endophytic \textit{Ericaceae} species\(^{8}\) colonized the substomatal spaces then wall appositions formed in epidermal and mesophyll cells with mycelia attached to their outer surfaces. Wall appositions indicate that the host cells have recognized the presence of the actinomycete, perhaps responding to this stimulus with the deposition of callose. Considering that callose accumulates near the cell surface\(^{28,29,33,34}\), this accumulation probably strengthens the cell framework interfering with water movement through the cell walls. This phenomenon might therefore account, at least partially, for the drought tolerance observed in AOK-30-treated seedlings although more analytical evidence is required to support this conclusion.

An intense band of ca. 35 kDa was detected in the protein extract from cell walls of AOK-30-treated seedlings after SDS-PAGE (Fig. 2). A weak band with a similar molecular weight was also found in the samples from untreated seedlings. After enzymatic digestion of the band, the total solution was fractionated into peptide fragments by reverse-phase HPLC. Seven peaks were detected in fractions with retention times of 55–70 min. Analysis of the amino acid sequences of all these fractions revealed that one with a retention time of 63 min had a fragmented peptide composed of Lys-Leu-Phe-Gly-Val-Thr-Met-Leu-Asp-Val, which showed 100% homology with the known inner sequence of putative mitochondrial NAD-dependent malate dehydrogenase in \textit{Solanum tuberosum}. Malate dehydrogenase is also known to localize in the apoplasts of barley and oat\(^{35}\) and in the cell walls of \textit{Nicotiana tabacum}\(^{16}\). Gross \textit{et al.}\(^{37}\) reported that malate dehydrogenase in horse radish cell walls mediates production of NADH from malate, while the resultant \(\text{H}_2\text{O}_2\) enhances peroxidase activity and eventually lignification of cell walls. Furthermore, Kärkönen \textit{et al.}\(^{38}\) extracted and identified malate dehydrogenase from covalently and ionically bound cell wall proteins of \textit{Picea abies} as a lignification-associated enzyme. They postulated that the activity of this enzyme might be involved in the regeneration of NADH in the apoplast, which is needed for peroxidase-catalyzed \(\text{H}_2\text{O}_2\) production. In addition, the involvement of cell wall proteins such as apoplast peroxidases in lignification and disease resistance has also been shown\(^{39,40}\). Considering this involvement, AOK-30 treatment of seedlings might enhance the activity of NAD-dependent malate dehydrogenase, eventually accelerating lignification in cell walls. Ten days after AOK-30 treatment, the possible lignification of cell walls was examined histochemically in stem sections using phloroglucinol. As illustrated in Fig. 3B, cell walls of sieve cells surrounding the stele stained pinkish in AOK-30-treated seedlings, a positive lignification response, while those of untreated seedlings did not react positively (Fig. 3A). Lignification of sieve cells was therefore accelerated after AOK-30 treatment of seedlings in flasks.

Although the current results insufficiently explain the mechanism underlying drought tolerance in AOK-30-treated seedlings\(^{21}\), the acceleration of lignification in sieve cells is thought to be associated with water preservation. Callose deposition might interfere with water loss through cell walls, in turn, enhancing osmotic pressure in cells of AOK-30-treated seedlings. Further investigations will focus on clarifying these associations.

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**REFERENCES**


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