NaCl dependent production of coniferin in *Alluaudiopsis marnieriana* suspension cultured cells

Takuma Yoshioka¹, Yunosuke Itagaki¹, Yutaka Abe¹², Nobuo Kawahara², Yukihiro Goda³, Yoshihiro Ozeki¹, Akiyo Yamada¹*

¹Department of Biotechnology and Life Science, Faculty of Technology, Tokyo University of Agriculture and Technology, 2-24-16 Naka-cho, Koganei, Tokyo 184-8588, Japan; ²National Institutes of Biomedical Innovation, Health and Nutrition, 1-2 Hachimandai, Tsukuba, Ibaraki 305-0843, Japan; ³National Institute of Health Sciences, 3-25-26 Tonomachi, Kawasaki-ku, Kawasaki, Kanagawa 210-9501, Japan

*E-mail: yamaden@cc.tuat.ac.jp  Tel & Fax: +81-42-388-7383

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**Abstract**  A stable salt-tolerant cell-suspension culture of *Alluaudiopsis marnieriana* was established, and intracellular compounds that accumulated under salt-stress conditions were investigated. HPLC/MS, and NMR analyses indicated that enhanced accumulation of coniferin was found during the growth phase in medium containing 150 mM NaCl. Coniferin or its derivatives may play an important role in salt-tolerance mechanisms in this plant.

**Key words:** *Alluaudiopsis marnieriana*, cell-suspension culture, coniferin, salt stress.

Salinity is known to affect many physiological and metabolic processes, and causes reductions in plant growth and productivity (Jia et al. 2015). However, some plant species, especially halophytes, have evolved a variety of mechanisms to deal with salt stress (Yamada et al. 2002a, b, 2003). Analysis of the mechanisms that enable halophytes to survive under high levels of NaCl will be useful in enhancing agricultural productivity in salinized regions.

*Alluaudiopsis marnieriana* is a member of the Didiereaceae, a family of arboreal fleshy plants that grow in dry salty soils in southwestern districts of Madagascar (Rowley 1992). Although there is little information about their mechanisms for salt tolerance, it can be postulated that this plant species has unique mechanisms for stress resistance. In this paper, stable cell-suspension cultures of *A. marnieriana* were established and the compounds that accumulated under salt-stress conditions were investigated to examine salt tolerance mechanisms in this species at the cellular level.

Leaves of *A. marnieriana* were surface-sterilized, cut into pieces, and placed on Murashige and Skoog’s agar plates (Murashige and Skoog 1962) supplemented with 10⁻⁶M 2,4-dichlorophenoxyacetic acid, 10⁻⁶M benzylaminopurine, 20 g l⁻¹ sucrose, and 0.8% agar at pH 5.6 to generate callus material. The plates were incubated at 25°C for 16 h light/8 h dark conditions. After one month of cultivation, yellowish-white calli were generated. The calli were transferred to Erlenmeyer flasks (300 ml) containing 50 ml of the liquid Murashige and Skoog’s medium and shaken at 80 rpm in the dark at 25°C. After growth was initiated, the suspension-cultured cells were reinoculated in the liquid Murashige and Skoog’s medium. Inoculation was repeated every two weeks for one year, and stable suspension-cultured cells were obtained.

Figure 1 shows a microscopic observation of the suspension-cultured cells. On the 8th day after the start of culture, the wet and dry weights of the cells were 12.2 ± 0.00 g and 0.45 ± 0.09 g, respectively, under 0 mM NaCl condition, and the water content was 96.3 ± 0.07% whereas under 150 mM NaCl condition, the wet and dry weights were 6.2 ± 0.00 g and 0.25 ± 0.02 g, respectively, and the water content was 95.0 ± 0.03%. Which formed small clusters consisting of many ellipsoidal cells in the absence of NaCl (Figure 1A). In contrast, increased spherical cells were found when the cells were cultured in the presence of 150 mM NaCl (Figure 1B). Enhancement of cell division under salt conditions may affect cell morphology. Similar cells were observed in medium containing 500 mM sorbitol [9.11% (w/v)] (date not shown). It can be postulated that these events were dependant on osmotic stress.

We focused on the soluble fraction, considering the possibility of compatible solutes peculiar to this plant. The intracellular compounds that accumulated...
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in the suspension-cultured cells under salt-stressed conditions were investigated using high-performance liquid chromatography (HPLC, LaChrom Elite, Hitachi High-Technologies co., Tokyo, Japan; see Figure 2). Cells that grew in medium containing 0 or 150 mM NaCl for 8 days were filtered onto Whatman No. 1 filter papers and freeze-dried for 3 days using a freeze-dry system (Model Free Zone 18; LABCONCO, MO, USA). Distilled water (500 µl) was added to 10 mg dry cells, which were incubated at 4°C for 24 h. Each sample was then centrifuged at 20,000×g for 30 min and the upper phase (50 µl) used for HPLC analyses. Figure 2A and 2B show the chromatogram of each cell extract cultured without NaCl (A) or with 150 mM NaCl [0.877% (v/v)] (B). The five major peaks in each chromatogram were numbered on the basis of retention time, and the spectra were monitored using diode array detector (DAD) five peaks in Figure 2A and 2B were compared, and significant differences were found in peak no. 5. Absorption spectrum (200–350 nm) of peak no. 5 recorded online during HPLC/DAD analyses showed a characteristic spectrum with absorption maxima at 210, 258, and 296 nm (Figure 2C).

The structure of the unusual compound 5 was identified by purifying the extract of cells grown in the presence of 150 mM NaCl under the following conditions. Distilled water (5 l) was added to approximately 100 g of freeze-dried cells and extracted at 4°C for 24 h. The extract was prefiltered using Advantec No. 3 filter paper, and then filtered with a Millipore membrane filter (HVLP04700). Following filtration, the extract was applied to an 8 cm×3 cm column of Wakosil 25C18 (Wako Pure Chemical Industries, Ltd., Osaka, Japan). After washing with 10% aqueous acetonitrile, acetonitrile:water 10:90 [% (v/v)], the 15% aqueous acetonitrile fraction was collected, dried, and resuspended in 50 ml of distilled water. This fraction was applied to a 3 cm×3 cm column of Dowex Marathon WBA (Sigma-Aldrich, MO, USA) to remove the anionic compounds, and the aqueous wash was collected. The collected fraction was then used with DOWEX 50WX8-100 (Sigma-Aldrich), and the aqueous wash was collected, dried, and resuspended in 8 ml of distilled water to remove the cationic compounds. Finally, compound 5 in the partially purified sample was fractionated using HPLC under the following conditions: Developol RPAQUEOUS-AR-5 (250 mm×4.6 mm i.d.; Nomura Chemical Co., Ltd., Aichi, Japan); column temperature, 30°C; solvent, 12.16% (v/v) aqueous acetonitrile (9 min); flow rate, 1 ml/min.

Using positive ion electron-mass spectrometry (MS), the purified compound 5 produced a quasimolecular ion at m/z 365.12 [M+Na]+. The 1H-nuclear magnetic resonance (NMR) spectrum (CD3OD) showed characteristics of a coniferyl alcohol moiety: δ 3.87 (s, 3H), 4.2 (d, J=6.9, 2H), 6.28 (dt, J=19.5, 5.7, 1H), 6.54 (d, J=19.5, 1H), 6.94 (dd, J=8.3, 1.9, 1H), 7.07 (d, J=2.3, 1H), and 7.1 (d, J=8.3, 1H). The 13C NMR spectrum of compound 5 showed an oxygenated methylene carbon (δ 62.5) and five oxygenated methine carbons (δ 71.4, 75.0, 77.9, 78.3, and 102.8) (Daubresse et al. 1998). This suggested the presence of a hexosyl moiety in the molecule. Using enzymatic hydrolysis, the hexosyl moiety in compound 5 was determined to be a β-glucopyranoside. The heteronuclear multiple bond
correlation observed between an anomeric proton at δ 4.88 (d, J = 8.7, 1H) and the quaternary carbon signal at δ 147.7 indicated that glucose was attached at the C-4 position of coniferyl alcohol. Therefore, the structure of compound 5 was identified as coniferin (Greca et al. 1998).

The relationship between cell growth and coniferin accumulation in the suspension-cultured cells was investigated by culturing them in medium containing 0, 50, 150, and 250 mM NaCl. Coniferin contents were then determined. Growth curves are shown in Figure 3A and 3B, and growth was estimated using fresh weight (FW; Figure 3A) and dry weight (DW; Figure 3B). Reduced biomass of these cells can be thought to depend on cell death, as well as Arabidopsis thaliana suspension-cultured cells after the exponential growth phase (Kobae et al. 2006). The suspension-cultured cells were able to grow in the presence of 250 mM NaCl. Approximately 0.1–0.2 mg/g DW of coniferin was detected in cells cultured with 0 and 50 mM NaCl (Figure 3C). Eight days after inoculation, a maximum coniferin content of 0.9 mg/g DW was observed in cells cultured with 150 mM NaCl. The content gradually decreased after this time (Figure 3C). The coniferin content was less than 0.1 mg/g DW in the presence of 250 mM NaCl (Figure 3C).

Coniferin, a coniferyl alcohol-β-D-glycoside, is an important precursor of the cell-wall constituent, lignin (Freudenberg 1965). It is widely spread in various plant species including gymnosperms (Terasawa et al. 1984a) and angiosperms (Terasawa et al. 1984b). However, there is little information regarding the relationship between coniferin production and salt stress in plants. However, it has recently been shown that coniferin accumulation is increased in Arabidopsis callus-suspended cells adapted to high salinity compared with control cells. (Chun et al. 2019). Sánchez-Aguayo et al. showed that tomato plants under salinity stress undergo xylem lignification (Sánchez-Aguayo et al. 2004). In the next step, it is necessary to investigate the relationship between salt stress and lignification in these cultured cells.

Coniferin, although incorporated at the coniferyl-alcohol level, is also known as an efficient precursor of podophyllotoxin, an anticancer agent. To enhance the production of podophyllotoxin, coniferin was added to cell cultures of Podophyllum hexandrum (Woerdenbag et al. 1990). This was because coniferin synthesis is the rate-determining step in the production of podophyllotoxin. The NaCl-dependent production of coniferin shown in this work will be useful in enhancing the production of coniferin and its derivatives.

In this paper, a salt-tolerant stable cell-suspension culture of A. marnieriana was established, and an intracellular compound that accumulated under salt-stress conditions was identified as coniferin by HPLC/MS, and NMR analyses. The discovery of NaCl-dependent coniferin synthesis will contribute to an elucidation of the salt tolerance mechanisms of members of the family Didiereaceae.

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References

Chun HJ, Baek D, Cho HM, Jeong HS, Jeong MS, Jung WH, Choi CW, Lee SH, Jin BJ, Park MS, et al. (2019) Metabolic adjustment of Arabidopsis root suspension cells during adaptation to salt stress and mitotic stress memory. Plant Cell Physiol 60: 612–625
Daubresse N, Francesch C, Mhamdi F, Rolando C (1998) Coniferin and derivatives: A fast and easy synthesis via the aldehyde series using phase-transfer catalysis. Synthesis 2: 157–161
Freudenberg K (1965) Lignin: Its constitution and formation from p-hydroxyxycinamyl alcohols: Lignin is duplicated by dehydrogenation of these alcohols; intermediates explain formation and structure. Science 148: 595–600
Greca MD, Ferrara M, Fiorentino A, Monaco P, Previtera L (1998) Antialgal compounds from Zantedeschia aethiopica. Phytochemistry 49: 1299–1304
Jia H, Shao M, He Y, Guan R, Chu P, Jiang H (2015) Proteome
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dynamics and physiological responses to short-term salt stress in *Brassica napus* leaves. *PLoS One* 10: e0144808

Kobae Y, Mizutani M, Segami S, Maeshima M (2006) Immunochemical analysis of aquaporin isoforms in *Arabidopsis* suspension-cultured cells. *Biosci Biotechnol Biochem* 70: 980–987

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiol Plant* 15: 473–497

Rowley GD (1992) *Didiereaceae: Cacti of the Old World*. The British Cactus and Succulent Society, Richmond

Sánchez-Aguayo I, Rodríguez-Galán JM, García R, Torreblanca J, Pardo JM (2004) Salt stress enhances xylem development and expression of S-adenosyl-L-methionine synthase in lignifying tissues of tomato plants. *Planta* 220: 278–285

Terasawa M, Okuyama H, Miyake M (1984a) Phenolic compounds in living tissues of woods: I. Phenolic β-glucosidases of 4-hydroxycinnamyl alcohol derivatives in the cambial sap of woods. *Mokuzai Gakkaishi* 30: 322–328 (in Japanese)

Terasawa M, Okuyama H, Miyake M (1984b) Isolation of coniferin and syringin from the cambial tissue and inner-bark of some angiospermous woods. *Mokuzai Gakkaishi* 30: 409–412 (in Japanese)

Woerdenbag HJ, Van Uden W, Frijlink HW, Lerk CF, Pras N, Malingré TM (1990) Increased podophyllotoxin production in *Podophyllum hexandrum* cell suspension cultures after feeding coniferyl alcohol as a β-cyclodextrin complex. *Plant Cell Rep* 9: 97–100

Yamada A, Saitoh T, Mimura T, Ozeki Y (2002a) Expression of mangrove allene oxide cyclase enhances salt tolerance in *Escherichia coli*, yeast, and tobacco cells. *Plant Cell Physiol* 43: 903–910

Yamada A, Sekiguchi M, Mimura T, Ozeki Y (2002b) The role of plant CCTα in salt- and osmotic-stress tolerance. *Plant Cell Physiol* 43: 1043–1048

Yamada A, Tsutsumi K, Tanimoto S, Ozeki Y (2003) Plant RelA/SpoT homolog confers salt tolerance in *Escherichia coli* and *Saccharomyces cerevisiae*. *Plant Cell Physiol* 44: 3–9