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Article

Stimulatory and inhibitory effects of abscisic acid on cell growth in protoplast cultures and the relation to its endogenous levels in Avicenniaceae mangrove cells

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Abstract: Effects of 0.1-100 μM of abscisic acid (ABA) was investigated in protoplast cultures of mangrove plants generated from a suspension culture of *Avicennia alba* and *A. marina* cotyledons at 30°C. Inhibition of cell divisions by ABA was observed in *A. alba*, however, stimulation was observed in *A. marina*. Sterile protoplast culture of *A. marina* cotyledons was first possible after long period (1-3 months) of seed imbibitions in tap water. Endogenous levels of ABA in protoplasts were measured by enzyme-linked immunosorbent assay after small-scale extraction and purification steps with thin layer chromatography. Endogenous level of ABA in cotyledon protoplasts of *A. marina* was very low compared to that of suspension cells of *A. alba*. High and low endogenous content of ABA in each *Avicennia* protoplast might be related to the inhibition and stimulation of colony formation by ABA in the culture medium.

Keywords: ABA content, *Avicennia*, Mangroves, Plant growth regulators, Protoplasts

Introduction

Mangrove plants tolerate high salty conditions as well as different osmotic conditions between river and sea water, in tropical and subtropical brackish water. Elucidation of the unique salt-tolerance mechanisms of mangrove plants and introducing these characteristics to tree-crops, such as poplar through cell fusion (Sasamoto *et al.,* 2006) would be a promising tool of biotechnological breeding. As yearly fruiting is not stable and long term storage of (crypto) viviparous seeds is not possible, the development of in vitro culture system would be useful to augment whole plant studies in the investigation of mechanisms in this group of plants (Kawana *et al.,* 2008). Axenic cell and tissue cultures would also be a useful tool for the maintenance and micropropagation of mangrove plants for conservation of such natural resources and environment (Ogita *et al.,* 2004). In mangrove forests more than 100 species from different families can be found in or near the coastal areas in the world (Tomlinson, 1986). Leaf protoplast isolation is possible from 8 different species of three different families including Avicenniaceae mangroves (Kawana *et al.,* 2004). However, mangrove plants are very recalcitrant in cell culture with the exception of a few species in the families Rhizophoraceae (*Bruguiera sexangula* (Lour.) Poir.) (Mimura *et al.,* 1997; Kura-Hotta *et al.,* 2001) and Sonneratiaceae (*Sonneratia alba* J. Smith and *S. caseolaris* (L.) Engl.) (Kawana *et al.,* 2007; Yamamoto *et al.,* 2009) which are amenable to suspension culture studies. Callus formation from protoplasts of *B. sexangula* suspension cells was successful and tolerant or halophilic nature to sea salts of them were shown (Fukumoto *et al.,* 2004).

Avicennia alba Blume and *A. marina* (Forssk.) Vierh. are two dominant species in Avicenniaceae in a mangrove forest at the coast in Myanmar or Thailand. The latter is also found in the Iriomote island, Okinawa, Japan. These species can grow in the most seaside areas and are highly salt tolerant. Recently, we succeeded in the induction of suspension culture from cotyledons of *A. alba* using a combination of 2,4-dichlorophenoxyacetic acid (2,4-D) and thidiazuron (TDZ) as plant hormones and found halophilic nature and characteristics of tolerance of these cells to sea salts (Hayashi *et al.,* 2009). However, *A. marina* has been difficult for obtaining sub-culturable callus or the generation of a suspension culture using similar combinations of plant hormones.

Abscisic acid (ABA) is one of the plant hormones

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which plays important roles in seed development and in stress tolerance (Clipson *et al.*, 1988). We found that one of the causes of recalcitrancy in protoplast regeneration of a mangrove, *Kandelia obovata* Sheue, H.Y. Liu & J.W.H. Yong (Rhizophoraceae) is the high endogenous ABA level in leaf protoplasts comparing to the regenerable poplar leaf protoplasts (Kaai *et al.*, 2008). Information of endogenous ABA levels enables further optimization of culture conditions which were useful for the regeneration of plants from protoplasts of a recalcitrant broad leaved tree, *Betula platyphylla* Sukatchev var. japonica Hara (Sasamoto *et al.*, 2002).

The purpose of this study was to investigate whether the endogenous levels of ABA plays a role in protoplast cultures of Avicenniaceae mangrove plants. Since another group of plant hormone, gibberellins, is known to antagonize ABA effect in poplar protoplast cultures (Sasamoto et al. 1995), the effects of gibberellins were also investigated. Protoplasts were first isolated from suspension cells of *A. alba* and cotyledons of *A. marina*. The effects of ABA and gibberellic acid (GA_3) on these protoplast cultures were studied and the endogenous levels of ABA in their protoplasts were compared.

Materials and methods

Materials

Seeds of *A. marina* were collected on Iriomote-island, Okinawa Japan. Fresh and clean seeds were selected and imbibed with tap water changing once a day. Or after surface sterilization by successive treatment with neutral detergent for 1 min, and with 2% NaClO for 40 min, they were imbibed with pure water (Millipore Elix3) after sterilization (autoclaved at 121°C, 15 min) or with tap water, changing water occasionally, during 3 months at room temperature before protoplast isolation. Suspension culture of *A. alba* was induced and sub-cultured as previously described (Hayashi et al. 2009). Briefly, seeds were collected in Thailand and a suspension culture was induced from cotyledons of two seeds and sub-cultured at 3-4 week intervals in mAA basal medium, in which the glycine content was decreased to 1/10 of AA medium (Mimura *et al.*, 1997; Thompson *et al.*, 1986), containing 2 μM each of 2,4-D and TDZ and 3% sucrose in 100 ml flasks. They were cultured at 30°C, on a rotary shaker in the dark at 100 rpm speed and sub-cultured at least for 1 year before use.

Protoplast isolation

After imbibition of seeds, cotyledons of *A. marina* were re-sterilized with neutral detergent for 1 min and 1% NaClO for 45 min and washed three times with sterilized water. Cut sections were treated with 2% each of Cellulase RS and Driselase 20 (provided by Kyowa Hakko Co. Ltd.) in 1.3-1.4 M sorbitol solution as described previously (Sasamoto *et al.*, 1997). Incubation time at room temperature or at 30°C was prolonged from 24 to 67 hrs. Protoplasts were purified by passing through nylon mesh (95 μm size) and centrifuged at 100g speed for 4 min. They were washed three times with sorbitol solution by centrifugation. Viability of protoplasts was determined under fluorescent microscope (Olympus CK40) by staining with fluorescein diacetate (final 0.01%).

For protoplast isolation from suspension cells of *A. alba*, the 24-well culture plate method (Sasamoto *et al.*, 1997) was used to optimize the various combinations of cell wall degrading enzymes needed for wall digestion. Six different kinds of enzymes were tested, i.e. 1% each of Cellulase R10, Cellulase RS, Hemicellulase, Driselase 20, Macerozyme R10 and 0.25% of Pectolyase Y23. The final optimized solution was consisted of a 1.2 M sorbitol solution with 1% each of Cellulase RS and Driselase 20. Suspension cells were filtered with 40 μm nylon mesh and were treated for 3-13 hr at 30°C with the enzyme solution. Protoplasts were purified similar to *A. marina*. The protoplasts were used at once for culture or stored at -80°C after freezing with liquid nitrogen before ABA extraction.

Protoplast culture

Multi-well (96-well) culture plates (Falcon No.3705) were used to culture the protoplasts and to determine the effects of ABA and GA_3 on protoplast growth. The 5 μL of isolated protoplast suspension were put in 50 μL liquid mAA medium containing 3% sucrose and 1.2 M sorbitol in each well. Hormonal conditions were 2 μM each of 2,4-D and TDZ in *A. alba* and 1 μM each in *A. marina.* 100 μl of ultra pure water was supplied between wells. Culture was incubated at 30°C in a CO_2-incubator for humidity maintenance without the supply of CO_2 gas. Cell density was adjusted to 2-10 x 10^4 mL^{-1}. When cited in the text, 0, 0.1, 1, 10 μM of ABA and GA_3 were added. Culture of *A. marina* was also incubated at 28°C. Culture was observed under an inverted microscope (Olympus CK40) and numbers of enlarged cells and divided cells were counted. The

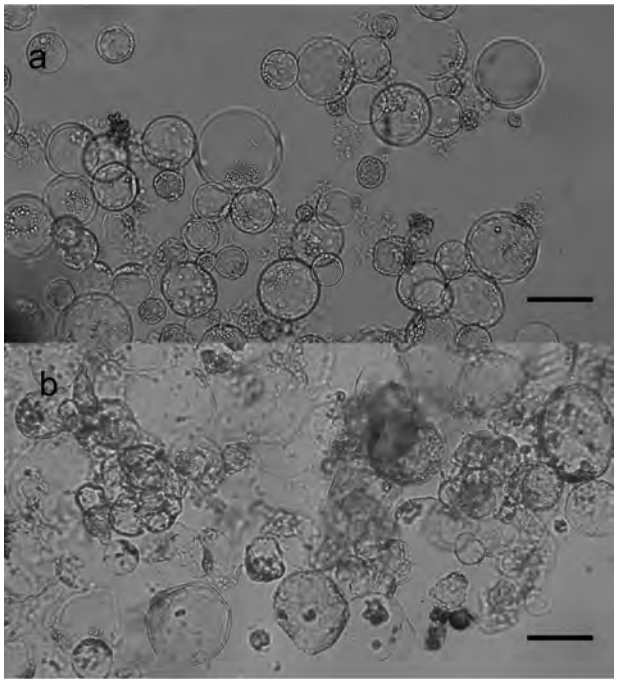

Figure. 1 Colony formation in protoplast culture of suspension cells of *A. alba*. Photographed at 0 day (**a**) and after 46 days (**b**) of culture. Basal medium was mAA containing 1.2 M sorbitol and 3% sucrose in combination with 2 μM each of 2,4-D and TDZ. Bar=50 μm

percentage of reacted cells was described as the total numbers of enlarged cells and cell colonies divided by the initial numbers of protoplasts plated in a well.

Micro-scale extraction and measurements of endogenous ABA in protoplasts

The extraction procedures were carried out as previously described (Sasamoto *et al.*, 2002). Protoplasts were extracted with 0.5 ml of 80% methanol at 4°C for 18 h. After centrifugation at 1600g, the precipitate was re-extracted for 4 h with 0.3 ml of cold 80% methanol. After further centrifugation, the combined extracts were dried in vacuo at 38°C using a Vacuum centrifugal evaporator (CVE-3100, EYELA, Tokyo, Japan) with a glass cold trap (Uni trap UT-1000, EYELA). The residual precipitate was dried at 80°C for 18 h for dry weight measurement. After addition of 0.6 ml or 0.5 ml of H_2O to the extract, the pH was adjusted to 2.5 with 6 N HCl and extracted three times with 1/3 volume of methylene chloride. The combined solvent fraction was dried in vacuo and stored at -80°C before purification by TLC. Purification by TLC was performed after elution of the dried samples with 10 to 20 μl of 80% methanol, with a solvent system of *iso*-propanol: ammonia (28%): water (10:1:1) or iso-propanol: 2M ammonia in 2-propanol (015-19841 Wako pure chemical Industries Ltd.): water (3:7:2), using Silica gel K6 (Whatman) plates (5 x 10 cm). The 0.8 cm band of 3 cm width at the Rf value of standard ABA was eluted twice with a total of 300 μl of 80% methanol. The extract was dried in vacuo and frozen at -80°C before the ELISA test was performed.

ELISA test: ABA was re-eluted with 200 μl of 25 mM Tris buffered saline (0.125 M NaCl, 2.6 mM $MgCl_2$, pH 7.4). The ELISA test, using monoclonal antibody for cis-(+)-ABA (ABA Immunoassay detection kit, Sigma PGR-1) (Weiler, 1982), was performed as instructed by the manufacturer. The amount of ABA was calculated from at least three different concentration measurements using a linear Log-Logit standard curve.

Results

Effects of ABA and GA_3 in protoplast cultures of A. alba suspension cells

Fig. 1a shows isolated protoplasts of the *A. alba* suspension cells. Colony formation (Fig. 1b) was observed in the protoplast culture in mAA medium containing 1.2 M sorbitol and 3% sucrose in combination with 2 μM each of 2,4-D and TDA. When 19 day-old *suspension cells* were used as the source of protoplasts, ABA inhibited the growth of *A. alba* protoplasts at all concen-

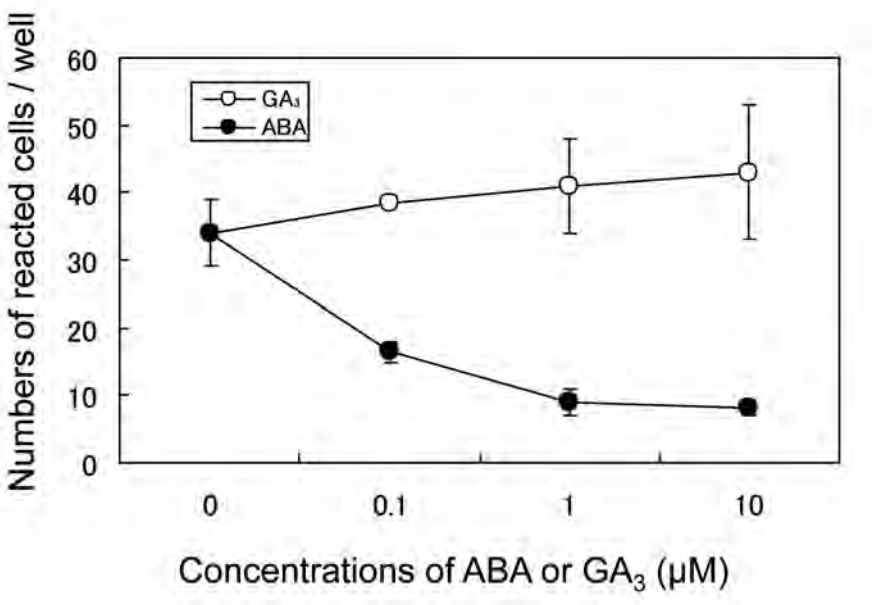

Figure. 2 Effects of ABA and GA_3 on protoplast cultures of 19 day-old *A. alba* suspension cells. Basal medium was the same as in Fig. 1. Cell density was $5x10^4$ mL^{-1} (2500 protoplasts in each well). Days of culture were 19 days. Data were average of numbers of non-circular enlarged cells and divided cells in two wells.

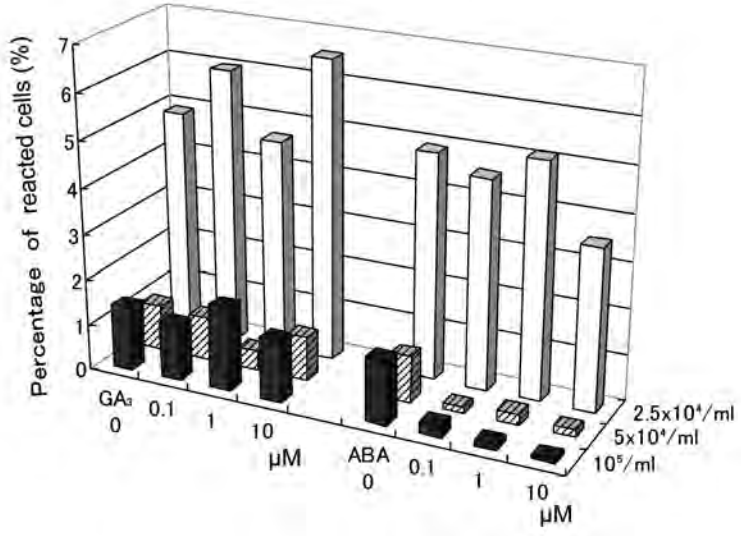

Figure. 3 Effects of ABA and GA_3 on percentage of reacted protoplasts in protoplast cultures of 30 day-old *A. alba* suspension cells. Basal medium was the same as in Fig. 1. Cell densities were 2.5, 5, 10 x $10^4 mL^{-1}$. Days of culture were 17 days. Data were % of enlarged and divided cells.

tration tested, while GA_3 had no inhibitory nor stimulatory effect at a cell density of 5 x 10^4 mL^{-1} (Fig. 2). As shown in Fig. 3 when 30 day-old suspension cells were used, similar effects of ABA and GA_3 were observed at 5-10 x 10^4 mL^{-1} when 55 day-old cells were used (data not shown). At a low cell density, $2.5x10^4$ mL^{-1}, ABA was inhibitory only at 10 μM, while GA_3 slightly stimulated cell growth at 0.1 and 10 μM. Percentage of reacted cells was higher at low cell density than at high cell density.

Effect of imbibing condition on protoplast isolation of A. marina cotyledons

Viable protoplasts of two different sizes, larger than

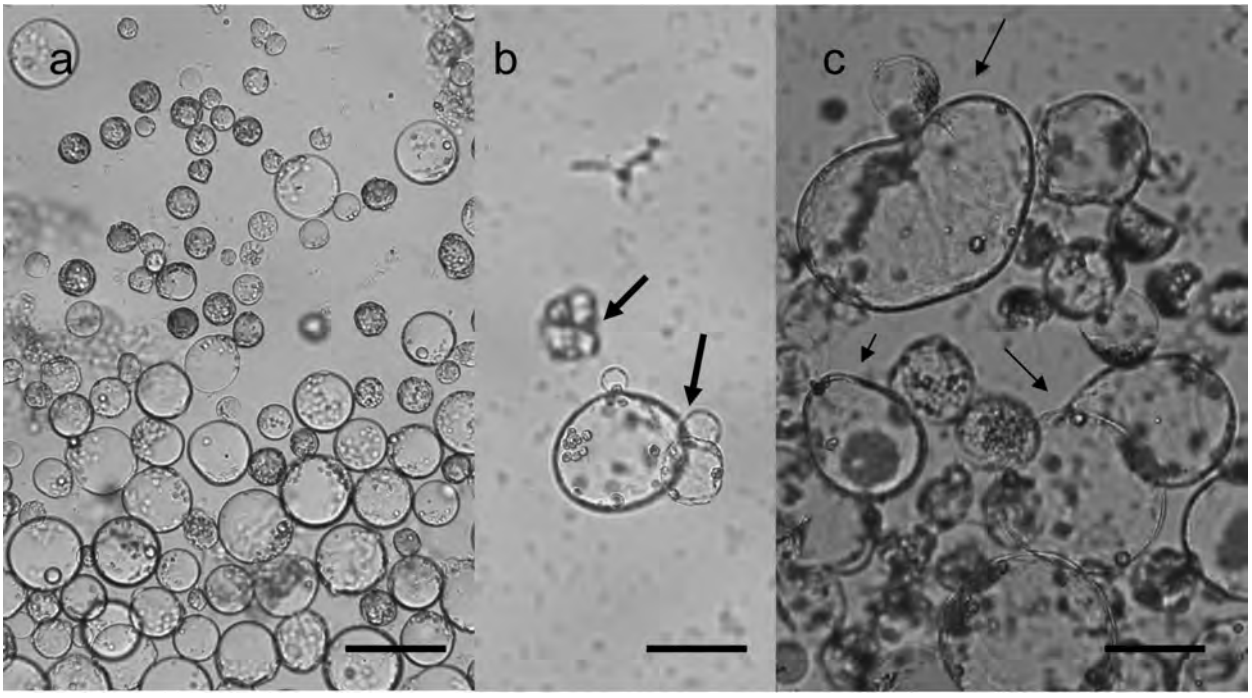

Figure. 4 Cell divisions in protoplast culture of *A. marina* cotyledons. **a**, isolated protoplasts at 0 day. **b**, divided protoplast (arrow) after 5 day at 1 μM of ABA. **c**, non-circular enlarged protoplasts (arrow) after 7 day at 100 μM of ABA. Basal medium is mAA containing 1.2 M sorbitol and 3% sucrose in combination with 1 μM each of 2,4-D and TDZ. Bar=50 μm. Seeds were imbibed for 1 month (a, b), and1.5 month (c).

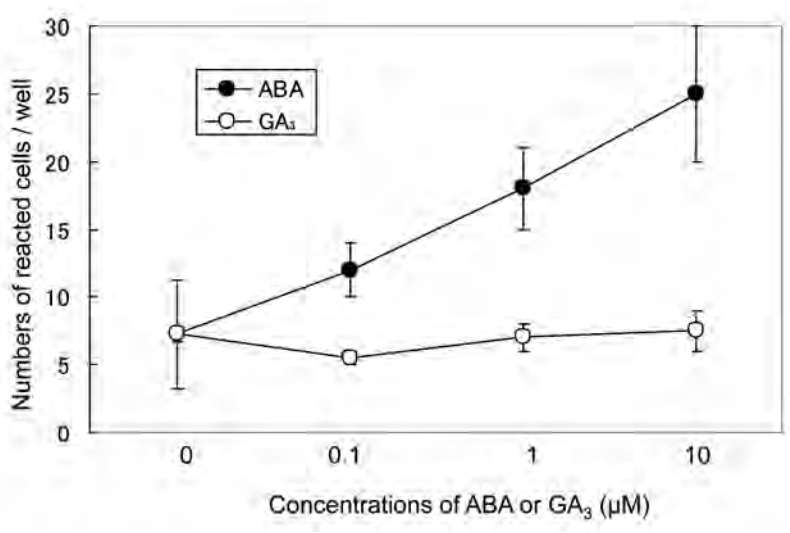

Figure. 5 Effects of ABA and GA_3 on cotyledon protoplast cultures of *A. marina*. Basal medium was the same as in Fig. 4. Cell density was $5x10^4$ mL^{-1} (2500 protoplasts in each well). Days of culture were 9 days. Numbers of protoplasts enlarged more than 70 μm diameter and divided cells were counted. Seeds were imbibed for 1 month.

25 μm and smaller than 20 μm, were obtained (Fig. 4a). Imbibing seeds for less than one month was not enough for efficient protoplast isolation from axenic cotyledons of *A. marina*. When autoclaved pure water was used for imbibition, seeds were browned and not successful for protoplast isolation. Sterile protoplast

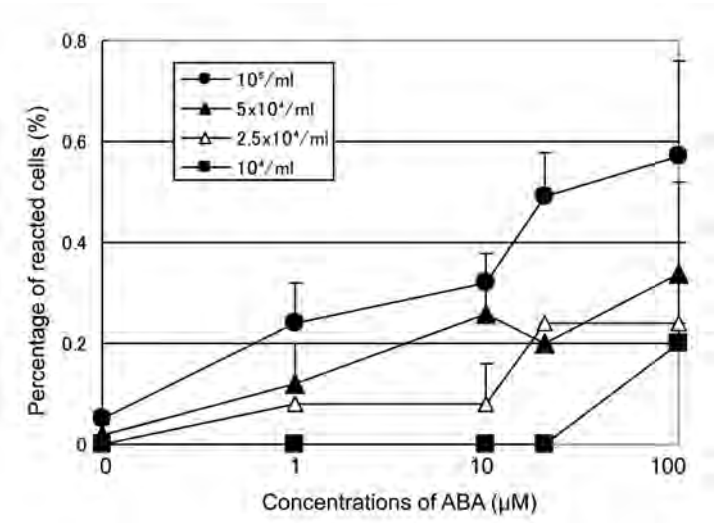

Figure. 6 Effects of ABA concentrations and cell densities on percentage of reacted protoplasts in protoplast culture of *A. marina* cotyledons. Basal medium is the same as of Fig. 4. Days of culture were 13 days. Data were averages of % of enlarged cells (larger than 100 μm diameter) and divided cells of two wells. Seeds were imbibed for 1.5 months.

Table. 1 Endogenous levels of ABA in protoplasts isolated from suspension cells of *A. alba* and from cotyledons of *A. marina*

ABA content	pmoles / $6x10^6$ protoplasts	pmoles / mg dry weight
A. alba suspension cells[a]	3.74±1.42	0.866±0.433
A. marina cotyledons[b]	0.625±0.066	0.059±0.006

[a]Average of four samples (22 to 35 days old cells) with standard error.
[b]Average of two samples (imbibed in tap water for 1.5 month)

cultures were obtained when fresh clean seeds were imbibed with tap water for up to a 3 months period. During the imbibition period, the color of the cotyledons remained green. It is important to note that it took three times longer incubation time for protoplast isolation than in previous work, which was less than one day, at the same high concentrations (2%) of Cellulase RS and Driselase (Sasamoto *et al.*, 1997).

Stimulatory effect of ABA in protoplast culture of A. marina cotyledon

As shown in Fig. 5, ABA stimulated cell enlargement and divisions in protoplast culture of *A. marina* cotyledons, which were imbibed for 1 month in tap water though no increase was observed at any concentrations of GA_3 tested. Fig. 4b shows the cell division of *A. marina* cotyledon protoplast photographed after 5 days of culture at 30°C in 1 μM of ABA. The seed had been imbibed in tap water for 1.5 months. No cell division was observed when cultured at 28°C.

When 1.5 months-imbibed seeds were used, stimulation of cell growth by ABA was repeatedly observed (Fig. 6). 100 μM of ABA had a stronger stimulatory effect on cell divisions and enlargement in protoplasts larger than 100 μm diameter (Fig. 4c). No difference in number was found among protoplasts smaller than 50 μm diameter. The percentage of reacted cells was higher at higher cell densities. When seeds were imbibed for 3 months (data not shown), the number of non-circular enlarged-cells increased by the addition of 1 and 10 μM of ABA at cell density of 2 and 5 x 10^4 mL^{-1}; however, cell divisions were not readily observed in cultures.

Edogenous levels of ABA in protoplasts of A. alba suspension cells and of A. marina cotyledons

As shown in Table 1, high ABA contents in protoplasts of *A. alba* suspension cells were obtained during

culture from 22 to 35 days. In the old culture (49-day) a much high ABA content (42.7±10.1 pmoles/6x10^6 cells) was obtained.

On the contrary, after 1.5 months of imbibition, a much lower (1/6) endogenous level of ABA was obtained in protoplasts from cotyledonary cells of *A. marina* (Table 1).

Discussion

Contamination is a common problem in mangrove research especially with cryptoviviparous *Avicennia* seeds (Anguelova-Merhar *et al.,* 2003). Though, a long imbibition period is needed to obtain viable protoplasts and the need of a high temperature, 30°C, in culture maintenance, axenic protoplast culture was successful for *A. marina* cotyledons by using fresh and clean seeds and tap water-imbibition. Tap water contains low concentrations of each ions; Na^+, Mg^{2+}, Ca^{2+}, Cl^- (Yokohama Waterworks Bureau, 2009). The need of tap water for imbibition of *Avicennia* seeds might reflect the halophilic nature of these mangrove plants.

It is known that ABA antagonize the effect of plant growth hormone, GA_3, and inhibit cell divisions in protoplast cultures (Sasamoto *et al.,* 2002). In this study, ABA application elicited a difference in response between *A. marina* and *A. alba* protoplasts, while GA_3 application had not much effect. Clear stimulation of cell divisions by ABA was found only in the former. This difference could be explained by the difference in the endogenous contents of ABA. Stimulation of cell divisions at up to 100 μM of ABA in *A. marina* cotyledons protoplast culture might be related to the low content of ABA after long imbibition period. On the contrary, in *A. alba* suspension cells, because of the high endogenous ABA content, further addition of exogenous ABA to the culture medium resulted in the inhibition of growth. The high endogenous ABA content might be related to the salt tolerance or halophilic nature of *A. alba* suspension cells (Hayashi *et al.,* 2009). Percentage of reacted cells was improved at lowest cell density in *A. alba* protoplasat culture. Such a phenomenon was also observed in the cotyledon protoplast cultures of a conifer, *Chamaecyparis obtusa,* which showed much high ABA content (Sasamoto and Ogita, 2001). The inhibition of growth by ABA was also found in leaf protoplast cultures of another mangrove, *Kandelia obovata,* in which cell enlargement was prevented. This is most likely caused by the high endogenous ABA level (Kaai *et al.,* 2008).

On the other hand, stimulation of cell divisions by ABA was recently found in another mangrove protoplast culture of *S. alba,* where plants are grown at the most seaside area of a mangrove forest. In *S. alba* (Kawana *et al.,* 2009), the ABA content was high similar to *A. alba* suspension protoplasts. In the *K. obovata* and *S. alba,* exogenously supplied cytokinins inhibits cell proliferation from these mangrove tissues (Kawana *et al.,* 2007; Kaai *et al.,* 2008). This observation is very different from *A. alba,* in which a strong cytokinin, TDZ is needed for growth of suspension cells (Hayashi *et al.,* 2009). In *A. marina,* a weak cytokinin, benzyladenine at high concentration, 10 μM, in MS medium was useful in the maintenance of leaf cultures (Hayashi et al. 2009). In preliminary experiments with cotyledon culture of *A. marina,* 1 μM of TDZ in combination with 1 μM of 2,4-D, in mAA medium, showed positive results in callus induction (unpublished results). This hormonal condition was used in this present study for protoplast culture of *A. marina* cotyledons. Therefore, different mechanism in effects of plant growth regulators between cytokinins and ABA might be considered for stimulation of cell divisions of *S. alba* by ABA.

In this study, by optimizing the ABA level endogenously and exogenous application, colony formation from protoplast cultures of *Avicennia* mangrove can be achieved. Using this protoplast culture system, effects of sea salts can be investigated quantitatively and the mechanisms of salts tolerance in Avicenniaceae mangrove cells can be investigated. Such a work is under way.

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References

Anguelova-Merhar V.S., Calistru C., Berjak P. (2003) A Study of Some Biochemical and Histopathological Responses of Wet-stored Recalcitrant Seeds of *Avi-*

cennia marina infected by *Fusarium moniliforme*. Ann Bot, 92, 401-408.

Clipson N.J.W., Lanchno D.R., Flowers T.J. (1988) Salt tolerance in the halophyte *Suaeda maritime* L. Dum: Abscisic acid concentrations in response to constant and altered salinity. J Exp Bot, 39,1381-1388.

Fukumoto T., Nakamura T., Suzuki M., Ogita S., Mimura T., Sasamoto H. (2004) Different effects of four salts and pHs on protoplast cultures of a mangrove, *Bruguiera sexangula* suspension cells, *Populus alba* leaves and tobacco BY-2 cells. Plant Biotechnol, 21, 177-182.

Hayashi S., Kuriyama S., Kawana Y., Hasegawa A., Kurita A., Minagawa R., Sasamoto H. (2009) Stimulatory effects of sea salts on cell growth in liquid culture of Avicenniaceae mangrove. Plant Biotechnol, 26, 561-564.

Kaai F., Kawana Y., Sasamoto H. (2008) The relation between recalcitrancy of a mangrove plant, *Kandelia obovata*, and high endogenous level of abscisic acid. Plant Cell Tiss Organ Cult, 94, 125-130.

Kawana Y., Sasamoto H., Mochida Y., Suzuki K. (2004) Leaf protoplast isolation from eight mangrove species of three different families; Avicenniaceae, Rhizophoraceae and Sonneratiaceae. Mangrove Sci, 3, 25-31.

Kawana Y., Sasamoto H., Ashihara H. (2008) Mechanism of salt tolerancein mangrove plants. Bull Soc Sea Water Sci Jpn, 62, 207-214 (in Japanese).

Kawana Y., Yamamoto R., Mochida Y., Suzuki K., Baba S., Sasamoto H. (2007) Generation and maintenance of suspension cultures from cotyledons and their organogenic potential of two mangrove species, *Sonneratia alba* and *S. caseolaris*. Plant Biotechnol Rep, 1, 219-226.

Kawana Y., Kaai F., Sasamoto H. (2009) Abscisic acid stimulates cell divisions in cultures of protoplasts isolated from cotyledons and suspension cells of a mangrove plant, *Sonneratia alba:* Small-scale measurements of abscisic acid and gibberellins in protoplasts. Mangrove Sci, 6, 9-15.

Kura-Hotta M., Mimura M., Tsujimura T., Washitani-Nemoto S., Mimura T. (2001) High salt-treatment-induced Na+ extrusion and low salt-treatment-induced Na+ accumulation in suspension cultured cells of the mangrove plant *Bruguiera sexangula*. Plant Cell Environ, 24, 1105-1112.

Mimura T., Mimura M., Washitani-Nemoto S., Sakano K., Shimmen T., Siripatanadilok S. (1997) Efficient callus initiation from leaf of mangrove plant, *Bruguiera sexangula* in amino acid medium: Effect of NaCl on callus initiation. J Plant Res, 110, 25-29.

Ogita S., Yeung E.C., Sasamoto H. (2004) Histological analysis in shoot organogenesis from hypocotyls explant of *Kandelia candel* (Rhizophoraceae). J Plant Res, 117, 457-464.

Sasamoto H., Ogita S. (2001) Endogenous plant hormones in protoplasts of embryogenic cells of conifers. In: Molecular Breeding of Woody Plants, 279-288, Elsevier Science, Tokyo.

Sasamoto H., Hosoi H., Koshioka M. (1995) Endogenous levels of four plant hormones may affect the culture conditions of poplar protoplasts to regenerate plants. In: Current Issues in Plant Molecular and Cellular Biology, pp481-486, Kluwer Academic Publishers.

Sasamoto H., Wakita Y., Baba S. (1997) Effect of high sorbitol concentration on protoplast isolation from cotyledons of mangroves, *Avicennia marina* and *A. lanata*. Plant Biotechnol, 14, 101-104.

Sasamoto H., Ogita S., Wakita Y., Fukui M. (2002) Endogenous levels of abscisic acid and gibberellins in leaf protoplasts competent for plant regeneration in *Betula platyphylla* and *Populus alba*. Plant Growth Regul, 38, 195-201.

Sasamoto H., Wakita Y., Yokota S., Yoshizawa N., Katsuki T., Nishiyama Y., Yokoyama T., Fukui M. (2006) Effects of electric cell fusion treatment among leaf protoplasts of *Populus alba* and *Alnus firma* on growth, leaf morphology, and RAPD pattern of eleven acclimatized plants. In Vitro Cell Dev Biol Plant, 42, 174-178.

Thompson J.A., Abdullah R., Cocking E.C., (1986): Protoplast culture of Rice (*Oryza sativa* L.) using media solidified with agarose. Plant Sci, 47, 123-133.

Tomlinson P.B. (1986) The Botany of Mangroves. Cambridge University Press, USA, 419pp.

Weiler E.W. (1982) An enzyme-immunoassay for cis-(+)- abscisic acid. Physiol Plant, 54, 510-514.

Yamamoto R., Kawana Y., Minagawa R., Sasamoto H. (2009) Effects of carbon and nitrogen sources on induction of cell proliferation in tissue cultures of a mangrove plant, *Sonneratia caseolaris*. Mangrove Sci, 6, 1-6.

Yokohama Waterworks Bureau (2009): http://www.city.yokohama.jp/me/suidou/os/suidou-suishitsu/suidou/suishitsu-kekka.html