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Responses of Poplar Cells to Low Temperature Stress Treatment
— In Vitro Poplar Callus Formation —

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Abstract: To elucidate the behavior of the extracellular APase from poplar cells cultured under low temperature (4°C), authors prepared suspension-cultured poplar (*Populus alba* L.) cells called cell line KT-1. The poplar cells were cultured in 40 m*l* of Murashige and Skoog's medium with 3% sucrose and 1 ppm 2,4-D in dark at 4°C during 7 days of the culture period on a rotary shaker operating continuously at 124 rev per minute. The poplar cells ceased their growth under low temperature as one of the chilling effects. It is the first observation that the surface of the cell wall of the cells became fragile during the culture period at 4°C.

Key Words: *Poplus alba*, callus induction, cell suspension culture, low temperature

INTRODUCTION

A common feature of many plant species exposed to low non-freezing temperatures, from 0 to 15°C, is the phenomenon of chilling stress.

For plants adapted to temperate climates, exposure to low temperatures induces several physiological changes that allow them to withstand this stress[1]. The positive effects of these changes induced by chilling stress are referred to as cold acclimation. Plants are able to acclimate very rapidly in response to low, non freezing temperatures and thus survive transient cold periods during their growing season[2]. Cold acclimation involves a reduction or cessation of growth, the production of reactive oxygen species[3], a reduction of water tissue content[4] and a modification of gene expression[5–8].

To investigate the direct effect of stress on plants the cell suspension culture is a suitable experimental system. It has been reported that poplar suspension cell culture was used to investigate the mechanism of some secondary metabolites production elicited by pectinase in cells[9, 10]. Recently, expression of a copper chaperone was reported using poplar cell suspension culture in response to various abiotic stresses[11]. Expression of a peroxidase gene from poplar cell suspension culture was, moreover, investigated in response to stress[12].

We have been interested in some function of both intracellular and extracellular acid phosphatases (APase) of poplar cells when exposed to low temperature using poplar cell culture, because several either intracellular or extracellular APases were likey to function to help cells cultured in liquid medium survive under stress conditions such as dark-starvation, high osmotic pressure and phosphate starvation[13–16]. To elucidate the behavior of the extracellular APase from cells cultured under low temperature, the cell suspension culture is favorable material, therefore, we tried to induce poplar cell suspension from poplar plants.

Suspension-cultured poplar (*Populus alba* L) cells were prepared from poplar leaves or seed, to characterize endo-1,4-β-glucanases which was released in the extracellular culture medium[17], however, the cell line used by the experiment has not been already supplied,

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therefore, in this study we made a fresh start to establish another cell line from the leaves from poplar plant.

MATERIAL AND METHODS

Material

Seedlings of poplar (*Populus alba* L.) were kindly donated by Dr. Hayashi T (Kyoto University) (Fig. 1, by courtesy of Dr. Nishiguchi M and Kinoshita I).

Methods

Callus induction

The preparation of callus from poplar leave was according to the method previously described by Ohmiya *et al.*[17] with modifications. Popular seedlings were cultured on 100 m*l* of 0.8% agar medium containing Murashige-Skoog (MS)[18] basal medium, 100 mg/*l* myoinositol, 3% sucrose, 0.2 μM N-(2-Chloro-4-pyridyl)-N'-phenylurea (4-PU-30, Sigma) 0.5 mg/*l* indole-3-butyric acid (Sigma) in a glass bottle (580 mm diameter × 155 mm height) under sterile conditions in a growth chamber that was provided with about 2,000 lux of fluorescent light under a 16 hr photoperiod at 25°C. A shoot was snipped off at the second stem from the plant by a sterilized sharp razor blade. After the cutting was cut at a 30 degrees angle leaving the cutting with a point, it was stripped off the leaves. Every one month the cutting of the poplar plant was aseptically transplanted on a new agar medium.

Fig. 1. Poplar seedlings (by courtesy of Drs. Nishiguchi M and Kinoshita I)

For the purpose of inducing poplar callus 5～6 leaves were cut into pieces about 5 mm square by a sterilized sharp razor blade and these pieces of the poplar leaves were incubated aseptically in a 100 m*l*-Erlenmeyer flask containing 40 m*l* of MS basal medium, 3% sucrose, and 1 mg/*l* 2,4-D on a reciprocal shaker at 120 rpm at 25°C in the dark. The detached callus from the cut ends were observed by eye measurement. Five m*l* of the detached callus were collected and transferred into new medium.

Suspension cell culture

Poplar cell line KT-1 cells was cultured in MS basal medium with modification supplemented with 1 mg/*l* 2,4-D and 3% sucrose on a reciprocal shaker at 125 rpm, in the dark at 25°C. Every 7 days 3-m*l* of the poplar cell suspension cultured at 25°C was transferred into 40 m*l* of new medium and cultured at 4°C on a rotary shaker at 124 rev per minute during 7 days. Every 24 or 12 hr during the culture period, aliquots of the cells were harvested to measure the growth. Samples of light microscopy were collected at 24 hr intervals during the culture period.

Measurement of fresh weight and dry weight of the cells and count of the number of the cells with septum

After the suspension culture was harvested and 5 m*l* of the suspension was transferred into a Buchner's funnel, the cells were filtered off on a filter paper. The fresh weight of the cells remaining on a filter paper was measured. The cells remaining on a filter paper was dried overnight at room temperature with about 35% of relative humidity. Every 24 hr after the transfer of the cells into a new medium, the aliquots the cells were collected to observe the cell aspects and to count the number of the cells with a septum for the purpose of judging the cell growth under microscopy (BX-50, Olympus).

Electron microscopy

Cells were immersed in fixative solution consisting of 2% (w/v) glutarldehyde in 50 mmol/*l* Cacodylate buffer (pH 7.2). They were fixed for 2 h at 4°C and then post-fixed in 1% (w/v) osmium tetroxide in 50 mmol/*l* Cacodylate buffer (pH 7.2) for 2 h at 4°C. After dehydration in a grade ethanol series, the specimens were embedded in Spurr resin (Polysciences, Inc., USA) and polymerized overnight at 70°C. For electron microscope observation, ultra-thin sections were sequentially

stained with ulanyl acetate and lead citrate. After drying, the sections were examined with an electron microscopy (JEM-1200 EXS: JEOL. Japan) operating at 80 kV.

RESULTS AND DISCUSSIONS

Callus induction

The effect of concentration of 2,4-D on primary culture of detaching callus from the cut ends of the pieces about 5 mm square of poplar leaves was compared between 1.0 ppm and 2.0 ppm. After 4 weeks of aseptical incubation of the pieces in a 100 m*l*-Erlenmeyer flasks containing 40 m*l* of MS basal medium with modification, 100 mg/m*l* myo-inositol, 3% sucrose and 2,4-D, it revealed not so significant difference in growth between two concentrations of 2,4-D on the callus. Therefore, the concentration of 1.0 ppm 2,4-D was used for further experiments.

The callus formation on agar medium was also attempted (according to the method by Dr. Park, personal communication, 2006). Three-mm rim of a young leaf of a poplar plant about 4 weeks after transplanted on a new agar medium was cut by a sterilized sharp razor blade and the remaining leaf was divided into 4 pieces. Each piece was put aseptically on a 13 m*l* of 0.8% Gelrite gellan gum (Sigma) medium containing MS basal medium, 100 mg/*l* myoinositol, 3% sucrose and 0.2 μM 4-PU-30 (Sigma) in a plate (ϕ=9 cm) under sterile conditions in a growth chamber that was provided with about 2,000 lux of fluorescent light under a 16 hr photoperiod at 25°C. After 4 weeks the primary culture of the small brilliant callus was observed, then it was as first culture transferred to a new 0.8% Gelrite gellan gum medium containing MS basal medium, 100 mg/*l* myoinositol, 3% sucrose, and 1 ppm (or 2 ppm) 2,4-D and was incubated at 25°C in the dark. However, the amount of the callus obtained from the 3-mm rim of a leaf was far less than that from the pieces of 5 mm square of a leaf, therefore, we attempted further to derive the cells suspension culture from the callus obtained by the latter.

Suspension culture

Previoursly the suspension-cultured poplar cells were prepared from poplar leaves and seeds in the presence of 1 ppm 2,4-D. The poplar cells were cultured in 40 m*l* of MS medium with 3% sucrose and 0.1 ppm 2,4-D in dark at 27°C[17]. According to the report we used 2,4-D to culture the poplar cells. To determine the inoculum volume of the cells and the concentration of 2,4-D in the medium to enable the secondary and the third cell culture, the effect of the volume of the callus transferred to a new medium on the growth of the cells by counting the cells with septum was compared. In the case of the medium containing 1 ppm of 2,4-D, the number of the cells with septum in the culture inoculated 3-m*l* of the callus for 30 days was approximately 1.3 times that inoculated 5-m*l* (Table 1, Exp. I ～III, data not shown). For the next 14 days of the culture, each inoculum volume of 3, 4 and 5-m*l* was separately transferred, however, the stable growth was only shown in the cells inoculated 3-m*l* (Table 1, Exp. I and II, data not shown). In the case of the medium containing 2 ppm of 2,4-D, although the growth t of the culture inoculated 4-m*l* increased, that inoculated 2-m*l* of the callus within 30 days stopped (Table 1, Exp. IV and V, data not shown). After 30 days of the

Table 1. Concentration of 2,4-D and the inoculum vol. of cell suspension

Inoculum volume of cell suspension (m*l*)	Experiment Conc. of 2,4-D	I 1	II 1	III 1	IV 2	V 2	(ppm)
	30 days after 1st transfer	5.0	5.0	3.0	4.0	2.0	
	14 days after 2nd transfer	4.0	5.0	3.0	4.0	—	
	10 days after 3rd transfer	—	—	3.0	4.0	—	

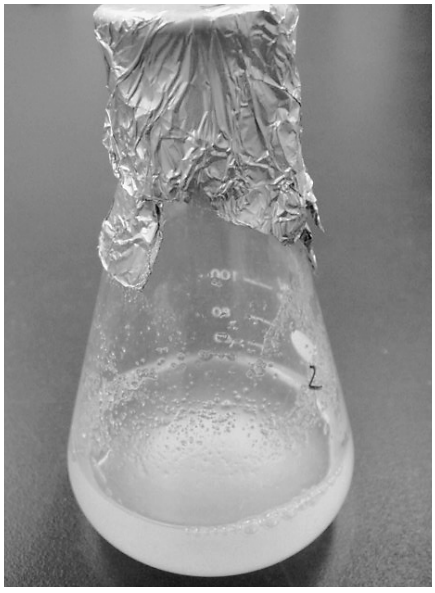

Fig. 2. Poplar cell suspension culture

culture, the culture of EXP, Ⅳ (Table 1) was transferred to new medium with another 4-m*l* inoculation and cultured for 14 days, the growth had continued. The growth in the cultures of EXP, Ⅲ was likely good condition in comparison to the culture of EXP. Ⅳ (Table 1), therefore, for the routine culture the concentration of 2,4-D was determined as 1 ppm and the inoculum volume of the cells as 3 m*l* (Fig. 2). The cell culture was also grown as callus culture on agar medium containing 0.8% agar (Fig. 3).

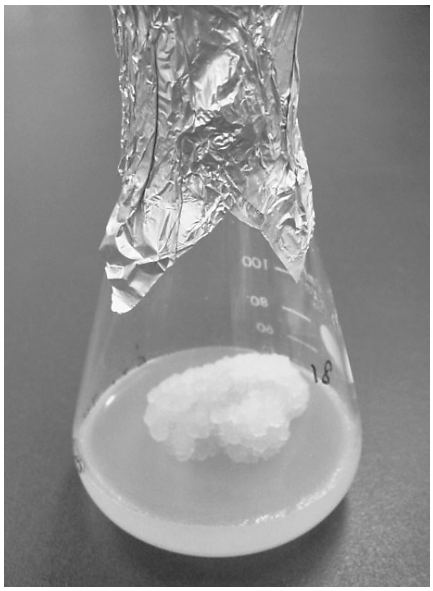

Fig. 3. Poplar callus culture

Cell growth

Changes in the fresh weight and dry weight of cells under low temperature during 7 days of culture period were shown in Fig. 4. Neither fresh weight (Fr.W) nor dry weight (Dr.W) of the cells grown under low temperature increased and exceeded the inoculums weight during the 7 days of the culture period. Fr.W and Dr.W, however, increased approximately linearly from 2 to 7 days at 25°C during the culture period (Fig. 4). Therefore, it is likely that low temperature treatment caused a substantial reductions in both Fr.W and Dr.W of cells.

The cellular aspects of the cells under low temperature during the culture period were shown in Fig. 5a. As shown in the Fig. 5a, it was likely that the cellular

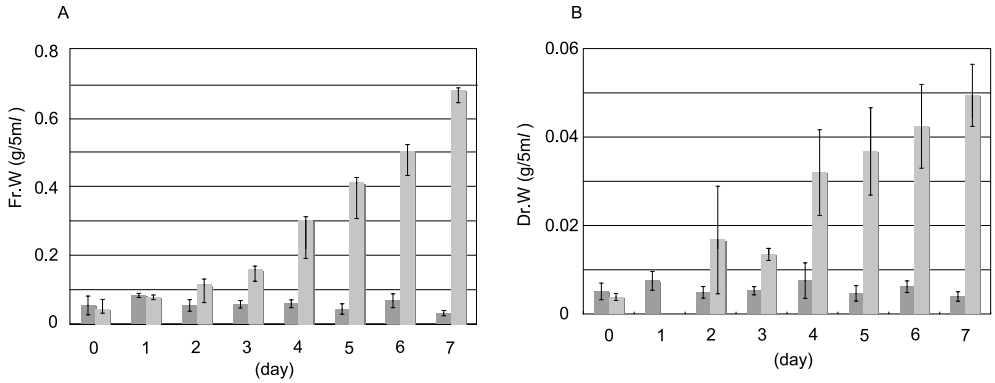

Fig. 4. Changes in fresh weight (Fr.W) and dry weight (Dr.W) of the cells cultured at 4°C and 25°C during the culture period. Five ml of the aliquots of the cell suspension was harvested every 1 day from the culture. Then fresh weight and dry weight of the cells was measured respectively.

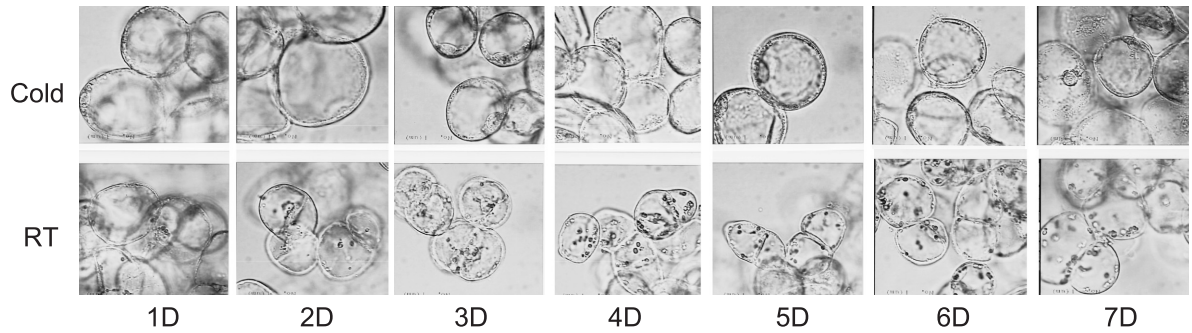

Fig. 5a. Cellular aspects of the cells cultured at 4°C and 25°C during the culture period. The cells cultured at 4°C are shown at the top of the figure indicated as Cold. The cells cultured at 25°C are shown at the bottom of the figure indicated as RT.

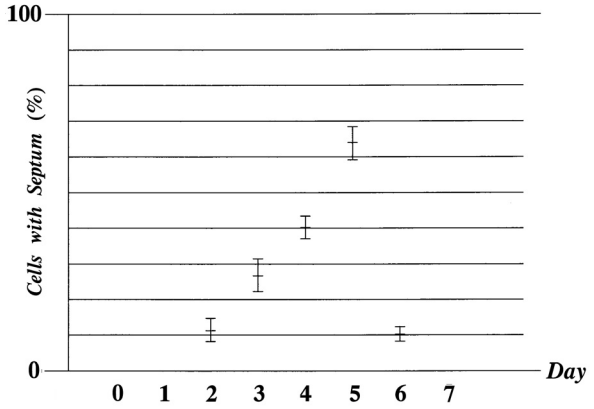

Fig. 5b. Change in the number of cells with septum cultured at 25°C during the culture period. The aliquots of the cell suspension were harvested every 1 day from the culture. Then the number of cells with septum and of without septum were counted respectively.

aspects of the cells cultured for 1 day after the transfer to the new medium was unchanged during the culture period. Within 2～3 days after the transfer of the cells cultured at 25°C the cells with septum near the center of them were observed. The cells with septum increased almost linearly from 1 day through 5 days during the culture period. Within 5 days about 65% of the cells had septums (Fig. 5b). These results suggest that the poplar cells cease their growth under low temperature as a chilling effects on poplar plants described previously[1].

Ultrastructural cellular aspects of the cells cultured under low temperature

It was characteristics of the cells cultured under low temperature that the large starch granules accumulated

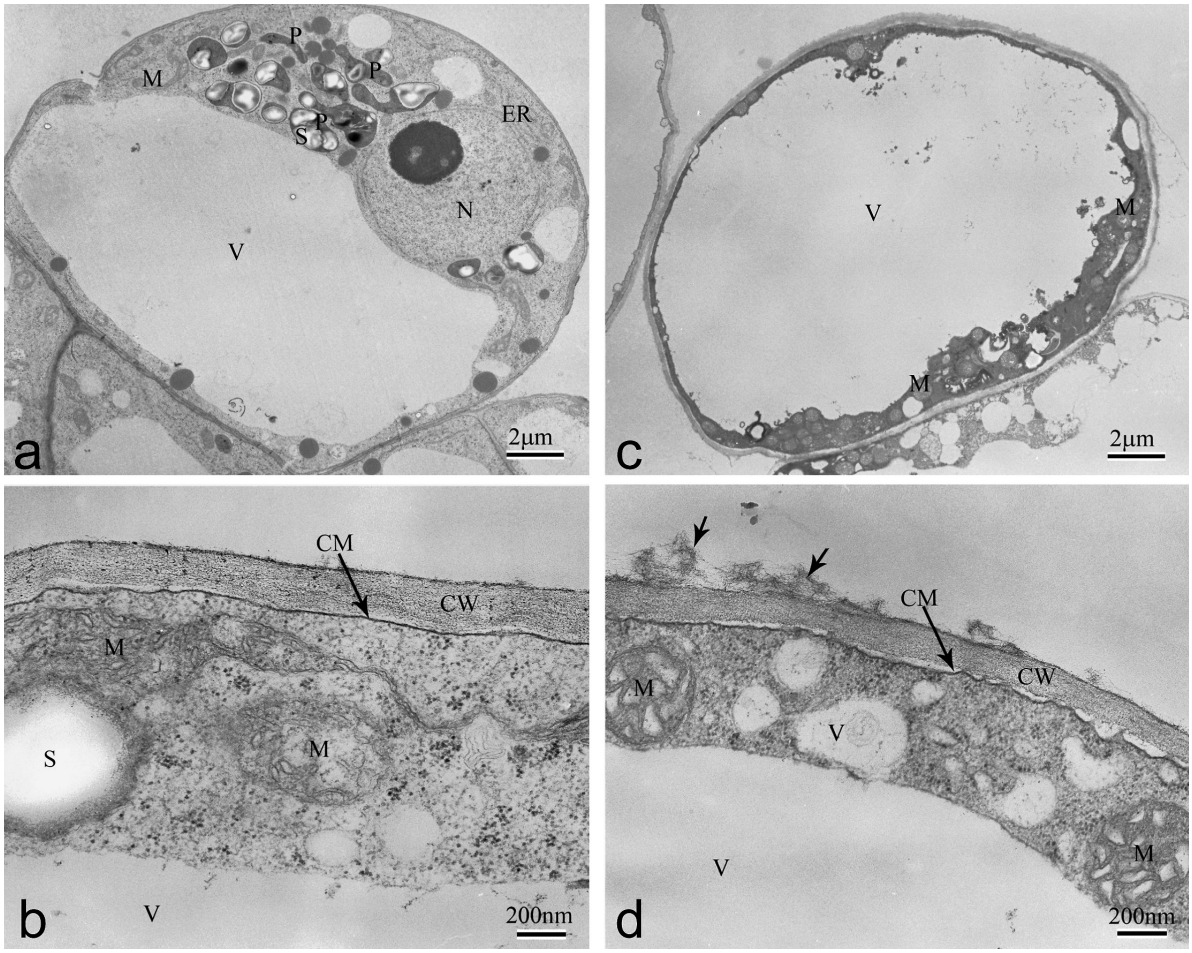

Fig. 6. Ultrastructural cellular aspects of the cells cultured at 4°C and 25°C within 4 day-culture.

A portion of the poplar cells cultured at 25°C within 4 days-culture.
a : V, vacuole; M, mitochondrion; N, nucleus; ER, endoplasmic reticulum; P, Plastid.
b : V, vacuole; M, mitochondrion; CM, cell membrane; CW, cell wall; S, starch granule.
A portion of the poplar cells cultured at 4°C within 4 days-culture.
c : V, vacuole; M, mitochondrion.
d : V, vacuole; M, mitochondrion; CM, cell membrane; CW, cell wall.

in plastids disappeared and that the vacuoles became large during the culture (Fig. 6a, c). It was interesting that the shapes of mitochondria which was eggplant-shape at 25°C had become circular (Fig. 6b, d). It is the first observation that the surface of the cell wall became fragile and that the gap between cell membrane and cell wall became large (Fig. 6b, d).

Conclusion

In this study we succeeded in establishing poplar cell line KT-1 from poplar leaves of the seedling (*Populus alba* L). To elucidate the behavior of the extracellular acid phosphatase (APase) from poplar cells cultured under low temperature (4°C), we then prepared suspension-cultured poplar cells. It revealed that the poplar cells survived at 4°C during the culture period. Therefore, we would like to study the physiological function of the extracellular APase for poplar cells to survive at 4°C on the basis of the behavior of the APase during the culture period at 4°C.

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