Isolation and Culture of Asparagus Microspore Protoplasts

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Microspore protoplasts were isolated enzymatically from uninucleate microspores to pre-anthesis binucleate pollen grains of asparagus (*Asparagus officinalis* L.). These protoplasts were uniform in diameter at each developmental stage and had dense cytoplasm. Mannitol (0.6 M) and CaCl₂ (10 mM) concentrations in the enzyme solution were important elements for stabilizing cell membrane. The frequency of protoplast isolation was closely related to the developmental stage of pollen. The highest frequency of protoplast isolation (approximately 90%) was obtained from immature uninucleate microspores (bud length; 1.0~2.0 mm). Protoplasts isolated from uninucleate microspores were purified and cultured. After 2 days of culture, first cell division (2~3% of protoplasts) was observed.

KEY WORDS: *Asparagus officinalis* L., asparagus, protoplast, pollen, microspore

Introduction

Protoplasts isolated from somatic cells of higher plants have been used extensively in physiology, biochemistry, and genetics, particularly somatic cell genetics. Pollen protoplasts which differ from somatic protoplasts by having a haploid genome and might be an alternative experimental system for cell fusion, genetic transformation and mutation studies. High yields of protoplasts have been obtained from microsporocytes at premeiotic interphase (MAEDA *et al.* 1979) and at all meiotic stages (ITO, 1973, BAJAJ, 1974) and from pollen tetrad (BAJAJ, 1974, BHOJWANI and COCKING 1972, RAJASEKHAR, 1973). However, microspore and pollen grain protoplasts had been considered difficult to isolate due to the existence of pollen exine containing chemically resistant sporopollenin.

Recently, TANAKA *et al.* (1987) obtained high yields of protoplasts from immature binucleate pollen grains in *Lilium longiflorum* by an enzymatic method. ZHOU (1989a,b) also reported that mature pollen protoplasts were efficiently isolated in *Iris tectorum* Maxim (Iridaceae), *Zephyranthes grandiflora* Lindl (Amaryllidaceae) and *Hemerocallis fulva* L. (Liliaceae) and that cell division was induced in culture of *Hemerocallis* pollen protoplasts. Furthermore, UEDA *et al.* (1990) reported that high frequency cell fusion between pollen protoplasts and generative cell protoplasts of *Lilium longiflorum* were induced by using an electric pulse.

In asparagus (*Asparagus officinalis* L., Liliaceae), plant regeneration from somatic protoplasts has been reported by BUI DANG HA (1973, 1975), KONG and CHIN (1988), ELMER *et al.* (1989) and KUNITAKE and MII (1990). However, there has been no report on the pollen protoplast isolation and culture of asparagus. In this report, we describe the enzymatic conditions and

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developmental stage of pollen necessary for the isolation of pollen protoplasts and the initial stages of culture of protoplast isolated from uninucleate microspores of asparagus.

Materials and Methods

Plant Material.

The source of experimental material was asparagus (*Asparagus officinalis* L. cv. 'Mary Washington'. Five to 6-year old mature plants grown in the green house were used as the source of microspore and pollen.

Evaluation of developmental stage of pollen

In order to identify the relationship between developmental stage of pollen and bud length, flower buds of various length (0.5~5.0 mm) were collected from plants. After collection, developmental stages of pollen were examined by DAPI (4,6-diamidine-2-phenylindole dihydrochloride) staining (KUROIWA *et al.* 1985).

Isolation of microspore and pollen protoplasts

Anthers were removed from flower buds with forceps and put into an enzyme solution in a sterilized petri plastic dishes (15 mm in diameter). Anthers were, then, squashed gently with a glass stick to release pollen into the enzyme solution. All dishes were sealed with Parafilm and incubated under stationary condition for 30 min at 25°C in the dark. The enzyme solution contained 1% (w/v) Cellulase Onozuka R-10 (Yakult Pharmaceutical Co. Ltd., Japan), 0.5% (w/v) Macerozyme R-10 (Yakult Pharmaceutical Co. Ltd., Japan), 0.5% (w/v) potassium dextran sulfate, 10 mM CaCl₂, 0.6 M mannitol, at pH 5.7. Throughout this experiment, 1 ml of the enzyme solution was used for releasing protoplasts from pollen contained in each flower bud (6 anthers). The effects of mannitol and CaCl₂ concentrations on production of pollen protoplasts were examined using immature binucleate pollen (bud length: 2~3 mm). Concentrations of mannitol and CaCl₂ were 0.1~0.8 M and 1~50 mM, respectively.

Effect of developmental stage of pollen on the production of microspore and pollen protoplasts was also examined using flower buds of different developmental stages. Flower buds were classified into 6 groups according to the bud length; 1.0~1.5, 1.5~2.0, 2.0~3.0, 3.0~4.0, 4.0~5.0 and 5.0<mm, respectively.

Microscopical observations

Efficiency of microspore protoplast production in the enzyme solution was determined quantitatively using an inverted microscope (Olympus model FLM). The efficiency was represented as a percentage of intact protoplasts out of the treated microspores. More than 500 microspores were counted for each treatment. The data were recorded after 30 min of incubation and expressed as the means of 2 experiments, each with 10~20 replicates. After isolation, microspore protoplasts were stained for 10 min with 0.1% Calcofluor White ST (SIGMA Chemical Company) dissolved in 0.6 M mannitol solution, washed twice with 0.6 M mannitol solution, and observed under a fluorescent microscope (Olympus model FLM) to check the removal of cell walls. Viability of microspore protoplasts was assessed using fluorescein diacetate (FDA) (WIDHOLM 1972).

Purification and culture of microspore protoplasts

For the culture of microspore protoplasts, uninucleate microspores (bud length: approximate-

ly 1.5 mm) were used. After incubation with enzyme solution, microspore protoplasts were collected by filtration through a nylon sieve (60 μ m) and harvested by centrifugation at $100 \times g$ for 3 min. The pellet was resuspended in 0.65 M sucrose solution and centrifugated at $100 \times g$ for 5 min. The floating microspore protoplasts were collected by using pasteur pipette and washed twice by centrifugation with 0.6 M mannitol solution. Yield of microspore protoplast was calculated with a hemocytometer, and the culture density of protoplasts was adjusted before plating. Microspore protoplasts were embedded in 1/2 MURASHIGE and SKOOG (1962) medium containing 1 mg/l 1-naphthaleneacetic acid (NAA), 0.5 mg/l zeatin, 1g/l L-glutamine, 0.6 M glucose and 0.1% Gellan Gum (KUNITAKE and MII 1990) and cultured at a density of 5×10^4 cells/ml in 30 $\times$ 15 mm Petri dishes. For embedding the protoplasts in Gellan Gum medium, liquid medium containing microspore protoplasts was mixed with an equal volume of Gellan Gum solution. The density of microspore protoplasts and the concentrations of culture medium and Gellan Gum were each adjusted to twice as much as the final value before mixing. All dishes were sealed with Parafilm and maintained at 25°C in the dark.

Results and discussion

Cytological observation with DAPI staining revealed that the developmental stage of pollen (from the tetrads to mature binucleate pollen) roughly correlated to the bud length in asparagus. Tetrads and uninucleate microspores were observed in flower buds of 0.6 to 1.0 mm, and 1.0~2.0 mm in length, respectively. Flower buds of more than 2.0 mm mostly contained binucleate pollen (Fig. 1-a). These results are in good agreement with those by FALAVIGNA *et al.* (1982) who reported that the length of flower buds containing uninucleate microspore in asparagus was 1.6~2.1 mm.

For the efficient isolation of microspore and pollen grain protoplasts, we examined several factors in enzyme solution using binucleate pollen (bud length; 2.0~3.0 mm). Among them, mannitol and CaCl₂ concentrations in the enzyme solution were important for the isolation of pollen protoplasts. The highest yield was obtained with 0.6 M mannitol (Fig. 2) and 10 mM CaCl₂ respectively (Table 1). In other concentrations, most pollen bursted before releasing pollen protoplasts from the exine. Particularly, CaCl₂ seemed to play an important role for stabilizing cell membrane when pollen protoplasts extruded from the germinal furrow. In the previous reports, TANAKA *et al.* (1987) and ZHOU (1989a) used the modified White's medium (WHITE, 1963) and K₃ medium (NAGY and MALIGA, 1976) respectively as mineral salts in the enzyme solution for pollen protoplast isolation. It is possible that calcium and/or magnesium ions involved in these media might also be playing a role for stabilizing the membrane in these species. In the previous reports, high concentrations of CaCl₂ and MgCl₂ have been reported to increase stability of protoplasts (GAMBORG, 1977, EVANS and BRAVO, 1983). The results shown in the present experiment also confirmed the importance of Calcium ions.

By using this enzymatic technique, microspore and pollen protoplasts of asparagus could be obtained in a large quantity. After 15 min of incubation in the enzyme solution, microspore and pollen enlarged due to hydration and partially extruded at the germinal furrow (Fig. 1-b, c). After 30 min of incubation, many protoplasts were released from the exine of

Fig. 1. a) Binucleate pollen (bud length: 3.0 mm) observed by DAPI staining. G: generative nucleus V: vegetative nucleus. (bar = 50 μ m)
 b) Release of microspore protoplasts from exine after 15 min of incubation in enzyme solution. (bar = 50 μ m)
 c) Microspore protoplast releasing through the aperture after 15 min of incubation in enzyme solution. (bar = 50 μ m)
 d) Release of microspore protoplast from exine after 30 min of incubation in enzyme solution. (bar = 50 μ m)
 e) Freshly isolated microspore protoplasts after purification. (bar = 50 μ m)
 f) First cell division after 2 days of culture. (bar = 50 μ m)

microspores and pollen grains and became spherical (Fig. 1-d). These protoplasts were uniform in diameter (ex; 40 μ m for uninucleate pollen) and had a dense cytoplasm. Fluorescence of cell wall was not detected by Calcofluor White staining after 10 min of incubation. Staining with fluorescein diacetate also revealed that they were viable at the time of isolation.

Table 1. Effect of calcium chloride concentration in enzyme solution on the frequency of protoplast isolation from binucleate pollen of asparagus (bud length: 2~3 mm).

Calcium chloride concentration (mM)	Frequency of protoplast isolation ¹⁾ (% $\pm$ standard error)
0	0.2 $\pm$ 0.42
1	0.3 $\pm$ 0.64
5	3.7 $\pm$ 3.15
10	25.0 $\pm$ 5.45
50	10.5 $\pm$ 2.36

The data were recorded after 30 min of incubation and expressed as the means of 2 experiments, each with 10~20 replicates. The enzyme solution contained 1.0% Cellulase Onozuka R-10, 0.5% Macerozyme R-10, 0.5% potassium dextran sulfate and 0.6 M mannitol.¹⁾ Frequency of protoplast isolation = Number of protoplasts $\times$ 100/examined binucleate pollen.

Fig. 2. Effect of mannitol concentration in enzyme solution on the frequency of protoplast isolation from binucleate pollen of asparagus (bud length; 2.0~3.0 mm). Production of pollen protoplasts in enzyme solution was determined quantitatively using an inverted microscope. Frequency of protoplast isolation was expressed as the percentage of intact pollen protoplasts out of the treated pollen (more than 500). The data were recorded after 30 min of incubation and represent the means of 2 experiments, each with 10~20 replicates. Vertical bars give standard error. The enzyme solution contained 1.0% Cellulase Onozuka R-10, 0.5% Macerozyme R-10, 0.5% potassium dextran sulfate and 10 mM CaCl_2 .

In the initial studies on pollen protoplast isolation from several angiosperm species, relatively complex mixtures of enzyme solution and long period of incubation (12 h) were required (BAJAJ, 1983). However, TANAKA *et al.* (1987) reported that protoplasts could be isolated much more rapidly from immature pollen grains of *Lilium* by the enzyme treatment for 1.5 h. ZHOU (1989a) obtained mature pollen protoplasts of *Hemerocallis*, *Iris* and *Zephyranthes* by enzyme treatment for 2 h. In the present study, microspore and pollen grain protoplasts of asparagus could be isolated by enzyme treatment for 30 min. The difference in time of enzyme treatment between asparagus and other monocotyledonous species previously reported may attribute to the difference in diameter of pollen, difference in size of germinal furrow, constituent elements in the pollen wall, and the composition of enzyme solution.

The frequency of protoplast isolation was closely related to the developmental stage of pollen (Fig. 3). High frequency

Fig. 3. Effect of bud length on the frequency of protoplast isolation from asparagus pollen. Developmental stages of treated pollen were identified by DAPI staining and expressed as horizontal bars with arrows on the top of columns. The data were recorded after 30 min of incubation and expressed as the means of 2 experiments, each with 10 replicates. Vertical bars give standard error. The enzyme solution contained 1.0% Cellulase Onozuka R-10, 0.5% potassium dextran sulfate, 10 mM CaCl_2 and 0.6 M mannitol.

of protoplast isolation (ca. 90%) was obtained from uninucleate microspores (bud length: 1.0~2.0 mm). However, the yield decreased with increasing bud length and no protoplasts were isolated from flowers after anthesis.

TANAKA *et al.* (1987) reported that high frequency of protoplast isolation (more than 80%) was obtained from mature binucleate pollen (bud length: 130~170 mm) of *Lilium*, but not from uninucleate microspores (bud length: 25~60 mm). In the present study, approximately 90% of uninucleate microspores released protoplasts. Generally, the developmental stage of pollen is a key factor for the induction of pollen plantlets by pollen and anther culture. According to FALAVIGNA *et al.* (1982), the maximum androgenic potential of asparagus was expressed in flower buds of 1.6~2.1 mm (uninucleate microspore stage). Therefore,

protoplasts isolated from uninucleate microspores of asparagus seemed to have a high possibility to form haploid plants. In fact, ZHOU (1989b) reported that pollen protoplasts isolated from late-uninucleate microspores led to the formation of multicellular clusters in *Hemerocallis*.

After 30 min of maceration with enzyme treatment, microspore protoplasts were purified by centrifugation (Fig. 1-e). Most microspore protoplasts floated in the upper part of the sucrose solution and the debris precipitated at the bottom. The protoplasts collected from the upper part were uniform in size and relatively clean but partially contaminated with some exines (less than 10%) and undigested pollen (less than 1%). First cell division (2~3% of microspore protoplasts) and budding were observed after 2 days of culture (Fig. 1-f). Nuclear division of microspore protoplasts were confirmed by aceto-carmin staining. However, further division was not observed and these cells soon browned and died.

Studies are now underway to sustain cell division in asparagus microspore protoplasts.

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Literature Cited

- BAJAJ, Y. P. S., 1974. Induction of repeated cell division in isolated pollen mother cells of *Atropa belladonna*. *Plant Sci. Lett.* 3: 309~312.

- , 1983. Haploid Protoplasts. In "Plant protoplasts", GILES, K. L. (ed), Academic Press, New York, 113~141.
- BHOJWANI, S. S. and COCKING E. C. 1972. Isolation of protoplasts from pollen tetrads. *Nature New Biol.* **239**: 29~50.
- BUI DANG HA, D. and I. A. MACKENZIE. 1973. The division of protoplasts from *Asparagus officinalis* L. and their growth and differentiation. *Protoplasma* **78**: 215~221.
- , B. NORREEL, and A. MASSET. 1975. Regeneration of *Asparagus officinalis* L. through callus cultures derived from protoplasts. *Exp. Bot.* **26**: 263~270.
- ELMER, W. H., T. BALL, M. VOLOKITA, C. T. STEPHENS and K. C. SINK. 1989. Plant regeneration from callus-derived protoplasts of asparagus. *J. Amer. Soc. Hort. Sci.* **114** (6): 1019~1024.
- EVANS, D. A. and J. E. BRAVO. 1983. Plant protoplast isolation and culture. In "Plant Protoplasts", GILES, K. L. (ed), Academic Press, New York, 33~35.
- FALAVIGNA, A, E. E. CHIAPPERINI, G. P. SORESSI. 1982. Influenza de fattori genetici ed ambientali sull'androgenesi in vitro di asparago (*Asparagus officinalis* L.). *Genetica Agraria* **36**: 21~29.
- GAMBORG, O. L. 1977. Culture media for plant protoplasts. In: *Handbook of Nutrition and Food*. RECHCIGL, M. (ed.) CRC Press, Cleveland, 415~422.
- ITO, M. 1973. Studies on the behavior of meiotic protoplasts I. Isolation from microsporocytes of liliaceous plants. *Bot. Mag. Tokyo* **86**: 133~141.
- KUNITAKE, H. and M. MII. 1990. Somatic embryogenesis and plant regeneration from protoplasts of asparagus (*Asparagus officinalis* L.). *Plant Cell Reports* **8**: 706~710.
- KUROIWA, T., S. NAKAMURA, C. SATO and Y. TSUBO. 1985. Epifluorescent microscopic studies on the mechanism of preferential destruction of chloroplast nucleoids of male origin in young zygotes of *Chlamydomonas reinhardtii*. *Protoplasma* **125**: 43~52.
- KONG, Y. and C. K. CHIN. 1988. Culture of asparagus protoplasts on porous polypropylene membrane. *Plant Cell Reports* **7**: 67~69.
- MAEDA, M., M. YOSHIOKA and M. ITO 1979. Studies on the behavior of meiotic protoplasts IV. Protoplasts isolated from microsporocytes of Liliaceous plants. *Bot. Mag. Tokyo* **92**: 111~119.
- MURASHIGE, T. and F. SKOOG. 1962. T A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* **15**: 67~76.
- NAGY, I. J. and P. MARIGA. 1976. Callus induction and plant regeneration mesophyll protoplasts. *Pflanzenphysiol.* **78**: 453~455.
- RAJASEKHAR, E. W. 1973. Nuclear divisions in protoplasts isolated from pollen tetrads of *Datura metel*. *Nature* **246**: 223~224.
- TANAKA, I., C. KITAZUME and M. ITO. 1987. The isolation and culture of lily pollen protoplasts. *Plant Science* **50**: 205~211.
- UEDA, K., Y. MIYAMOTO and M. ITO. 1990. Fusion studies of pollen protoplasts and generative cell protoplasts in *Lilium longiflorum*. *Plant Science* **72**: 259~266.
- WHITE, P. R. 1963. *The Cultivation of Animal and Plant Cells*, 2nd (ed) Ronald Press, New York, 60.
- WIDHOLM, J. M. 1972. The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. *Stain. Tech.* **47** (4): 189~194.
- ZHOU, C. 1989a. A study on isolation and culture of pollen protoplasts. *Plant Science* **59**: 101~109.
- 1989b. Cell divisions in pollen protoplast culture of *Hemerocallis fulva* L. *Plant Science* **62**: 229~235.

アスパラガス (*Asparagus officinalis* L.) の小胞子プロトプラストの単離・培養國武久登¹⁾・神戸敏成²⁾・三位正洋³⁾¹⁾現住所 佐賀県農業試験研究センター, 佐賀県佐賀郡南里, 〒840-23²⁾現住所 味の素(株) 中央研究所, 神奈川県川崎市, 〒210³⁾千葉大学園芸学部, 千葉県松戸市松戸, 〒271)

小胞子または花粉プロトプラストは細胞融合による半数性ゲノムの導入に有効な材料である。本研究では、アスパラガスの小胞子プロトプラストの単離・培養について検討した。

材料には、アスパラガス (*Asparagus officinalis* L.) 品種‘メリーワシントン’を供試した。小胞子プロトプラストの単離条件を明らかにするために、酵素組成と花粉の発達ステージについて検討した。その結果、1%セルラーゼオノズカR-10, 0.5%マセロザイムオノズカR-10, 0.5%デキストラン硫酸カリウム, 10 mM塩化カルシウム, 0.6 Mマンニトールを添加した酵素液で25℃, 暗黒条件下で30分間処理することによりプロトプラストを単離することができた。プロトプラストは発芽孔から遊離され、30分後には完全に花粉壁と分離した。これらのプロトプラストはそれぞれの花粉の発達ステージで直径が均一であり、豊富な細胞質を有していた。プロトプラスト単離のために酵素液中のマンニトールと塩化カルシウムは重要な要因であり、マンニトール濃度は0.6 M, 塩化カルシウムは10 mMで最も効率的に単離された。また、プロトプラストの単離は花粉の発達ステージと密接な関係があり、一核期の小胞子(つばみの長さ: 1.0-2.0 mm)から約90%の最も高い単離効率を得られた。単離した小胞子プロトプラストは、0.65 Mショ糖による遠心分離法で精製した。プロトプラストは、ショ糖の上層に集まり、一方、花粉壁と未消化花粉は沈澱した。精製したプロトプラスト中には10%以下で花粉壁が混入したものの、未消化花粉は1%以下程度の混入であった。

単離効率が最も高い一核期小胞子からプロトプラストを単離・精製し、小胞子プロトプラスト培養について検討した。上記の方法により精製したプロトプラストの密度を $5 \times 10^4/\text{ml}$ に調整し、1 mg/l 1-naphthaleneacetic acid (NAA), 0.5 mg/l zeatin, 1 g/l L-glutamineを添加した1/2 Murashige and Skoog (MS) 培地に懸濁した。さらに、0.1%ゲルライトにより包埋し、25℃, 暗黒条件下で培養した。培養2日後、2-3%の頻度で第一分裂が観察されたが、それ以上の分裂は観察されなかった。

以上のように、アスパラガス小胞子からのプロトプラストの単離・精製法を確立した。