Plant Regeneration from Mesophyll Protoplasts of Japanese Butterbur (*Petasites japonicus* Fr. SCHMIDT)

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Mesophyll protoplasts of butterbur (Fuki) were prepared in the enzyme solution containing 0.1% Pectolyase Y 23, 0.5% Cellulase Onozuka RS, 0.5% Driselase and 0.5 M mannitol at 27°C for 120 min with shaking at the rate of 80 str/min. Purified protoplasts were cultured in the medium containing diluted MURASHIGE & SKOOG's (MS) salts, vitamins, and various concentration of auxins and cytokinins at 1×10^5 cells/ml. The culture was kept at 25°C. After about one or one and a half months, cell colonies were transplanted onto the agar medium containing 1/4 MS salts, 1/2 MS vitamins, 1 mg/l NAA, 0.5 mg/l BA, 1% sucrose and 0.8% agar. Calluses of 2~5 mm in diameter were transferred on the agar medium of MS with 3% sucrose and 0.8% agar supplemented with various concentration of NAA and BA to induce shoot. After 6 month-culture, whole plants were obtained.

KEY WORDS : *Petasites japonicus*, butterbur, mesophyll protoplasts, protoplast culture, plant regeneration.

Introduction

Butterbur (Fuki) is a perennial composite and widely distributed from Hokkaido through Kyushu Districts, and has been cultivated for its succulent petioles as vegetables since 10th century in Japan. Major cultivars, 'Aichi-wase-buki', 'Mizu-buki' and 'Akita-Oo buki' were the selections from naturally grown wild butterbur (IMAZU, 1961).

Although butterbur is a dioecious plant, it is very difficult to find a male plant in the cultivated varieties (IMAZU and FUJISHITA, 1961). Major cultivars of butterbur are triploid ($2n=87$, $X=29$) and usually propagated vegetatively by rhizomes (IMAZU and FUJISHITA, 1962 a, b). These facts show the difficulties of applying cross breeding for butterbur. We are seeking the way to utilize cell and tissue culture in butterbur breeding

In the present experiment, we tried to isolate mesophyll protoplasts and bring them in culture for plant regeneration. Optimal culture conditions were also investigated. The possibility of protoplast approach in butterbur breeding is discussed.

Materials and Methods

'Aichi-wase-buki' was selected as an experimental material, because it is widely grown in Aichi Prefecture. The rhizomes were taken for dissecting the shoot tips, surface-sterilized with 70% ethanol for 60 sec and sodium hypochlorite solution (0.5% available chlorine) for 10 min, and rinsed twice with sterile water. The shoot tips were placed on agar media in the plastic box (Plant box, $6 \times 6 \times 9.5$ cm).

Young leaves, 3~5 cm in length, of aseptic plants were cut into small pieces (1 mm width) and incubated in the enzyme solution ; 0.1% Pectolyase Y 23, 0.5% Cellulase Onozuka RS, 0.5% Driselase and 0.5 M mannitol at 27°C for 120 min with

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shaking at 80 strokes/min. Protoplasts liberated were washed twice with CPW salt solution (MORGAN and COCKING, 1982) containing 0.5 M mannitol, by the centrifugation ($100\times g$) for 2 min.

Culture media tested were composed of inorganic salts of B5 (GAMBORG *et al.*, 1968) or MS (MURASHIGE and SKOOG, 1962) and vitamins of 8p (KAO and MICHAYLUK, 1975) or MS, and supplemented with various kinds and amounts of auxins and cytokinins as shown in Table 1.

Protoplast density of initial culture was ca. 1×10^5 cells/ml. The culture was kept in 6cm disposable plastic petri dishes at 25°C, under dim light. After one or one and a half months, all colonies formed were transferred onto an agar medium containing 1/4 MS minerals, 1/2 MS vitamins, 1 mg/l NAA, 0.5 mg/l BA, 1% sucrose and 0.8% agar. Calluses of 2~5 mm in diameter were transplanted on an agar medium: MS salts, 3% sucrose and 0.8% agar with various concentration of NAA and BA, to induce shoot.

To test the effect of hormones on the resumption of divisions from protoplasts, the medium containing one fourth dilution of inorganic salts of MS and the half vitamins of MS containing 1% sucrose and 0.5 M mannitol at pH 5.7, was supplemented with various concentrations of NAA (0.2, 0.5, 1, 2, 5, and 10 mg/l) and BA (0.2, 0.5, 1, and 2 mg/l) (Table 2).

To clarify the effects of ammonium salts and MS salts on protoplast division, the combinations of various dilutions of the MS salts and NH_4NO_3 were tested, where 1 mg/l NAA, 0.5 mg/l BA, 1% sucrose and 0.5 M mannitol at pH 5.7 were kept constant (Table 3).

The effect of hormone balance on the shoot regeneration from calluses was investigated in various combinations of NAA and BA.

Results

The size of mesophyll protoplasts of butterbur varied between 30 to 50 μ in diameter (Fig.1 A) and some of them possessed anthocyanin pigments. The media of one fourth MS salts with one half MS vitamins supplemented with hormones induced protoplast division within 7 days (Fig.1 B), while no protoplast division was observed in the media of B5 or MS salts with 8p vitamins. In the medium supplemented with 1 mg/l NAA and 0.5 mg/l BA, colony formation was observed (Table 1, Figs.1 C and 1 D). These colonies were transferred onto an agar medium containing 1/4 MS

Table 1. Medium for Japanese butterbur protoplast culture (0.5 M mannitol, pH 5.7)

Minerals	Vitamins	Sucrose	Glucose	NAA	2,4-D	BA	Zeatin	Protoplast division ¹⁾
B5	8p	%	%	mg/l	mg/l	mg/l	mg/l	No division
1/4 MS	1/2 MS	1	0.5	0.5	0.5	0.5	0.5	Colony formation
1/4 MS	1/2 MS	1		1	1	0.5		1st division
1/4 MS	8p	1	1	1	0.25		0.5	No division
1/4 MS	8p	1	1	1	1		0.5	No division

¹⁾ Cultured for 43 days

salts and 1/2 MS vitamins, 1% sucrose and 0.8% agar. With 3 month-culture, calluses of 2~5 mm diameter were formed on the agar medium. Shoot regeneration occurred about one month after transplanting the calluses onto MS medium containing 3% sucrose, 1 mg/l BA and 0.8% agar (Fig.1 E). Figure 1 F shows one of the regenerated

Fig.1. Plant regeneration from mesophyll protoplasts of butterbur.

A : Isolated protoplasts from mesophyll cells ($\times 200$). B : First cell division ($\times 200$).
C : Cell cluster ($\times 200$). D : Cell colony ($\times 100$). E : Shoot regeneration from calluses.
F : Plants regenerated in a plant box.

Table 2. Effect of NAA and BA concentration on protoplast division¹⁾

BA (mg/l)	NAA (mg/l)					
	0.2	0.5	1	2	5	10
0.2	—	—	—	≡	+	+
0.5	—	—	+	≡	+	—
1	—	—	—	+	—	—
2	—	—	—	+	—	—

1) — : no division, + : 2~5 cells, ≡ : 6~10 cells, ≡≡ : 11~15 cells.

Cultured for 22 days in the medium containing 1/4 MS minerals, 1/2 MS vitamins, 1% sucrose, 0.5 M mannitol, pH 5.7 and various concentrations of NAA and BA.

Table 3. Effect of NH_4NO_3 and other MS mineral concentration on protoplast division¹⁾

Dilution of MS salts ²⁾	NH_4NO_3 (mg/l)					
	0	200	400	800	1,600	3,200
1/1	—	—	—	—	—	—
1/2	+	≡	≡	≡	+	—
1/4	≡	≡	≡	≡	+	—
1/8	≡	≡	≡	+	+	+

1) — : no division, + : 2~5 cells, ≡ : 6~10 cells, ≡≡ : 11~15 cells, ≡≡≡ : 16~20 cells, Cultured for 22 days.

2) MS basic medium with various concentrations of NH_4NO_3 , 1/2 vitamins of MS, 1 mg/l NAA, 0.5 mg/l BA, 1% sucrose, 0.5 M mannitol, pH 5.7.

Table 4. The effect of hormone balance on the shoot regeneration from calluses

Medium ¹⁾ No.	NAA	BA	Number of calluses transplanted	Number of shoot regenerated calluses	Percentage of regenerated calluses
	mg/l	mg/l			
S-1	0	1	64	0	0
S-2	0	2	28	4	14.3
S-3	0	4	28	4	14.3
S-4	0.02	1	35	2	5.7
S-5	0.02	2	28	1	3.6
S-6	0.02	4	28	9	32.1
S-7	0.2	1	55	2	3.6
S-8	0.2	2	28	5	17.9
S-9	0.2	4	28	7	25.0

1) Basic medium ; MS salts, MS vitamins, 3% sucrose, 0.8% agar.

plants from mesophyll protoplasts of butterbur at 6th month protoplast culture.

To find out an optimum hormone balance for protoplast division, various concentration of NAA and BA were tested. Table 2 shows the results that the combination of 2 mg/l NAA and 0.2~0.5 mg/l BA were the most favourable. Ammonium salt was favoured in diluted condition for protoplast division (Table 3). Although original MS medium contains 1650 mg/l NH_4NO_3 , the modified MS media with 200 mg/l NH_4NO_3 was diluted to one fourth and induced good results in protoplast division.

Shoot regeneration rate was compared between media with various concentration of NAA and BA. The higher the concentration of BA with a little amount of NAA in the media, the higher the rate of shoot regeneration (Table 4). The media containing 0.02 mg/l NAA and 4 mg/l BA gave the best results. The medium with 1 mg/l BA did not induce shoot in this experiment.

Discussion

Protoplast culture has been conducted on asexually propagated vegetables by Oosawa and Takayanagi (1984) but no plant regeneration was reported so far.

Shoot tip culture for virus elimination (MATSUBARA and MITSUNOBE, 1978), and organ culture for callus induction and plant regeneration (MORISHITA, KAGI and YAMADA, 1980; YABE *et al.*, 1985) have been done, but no trial of protoplast culture was conducted in butterbur.

For preparation of protoplast, enzyme mixtures of Pectolyase and Cellulase were effective in taro, garlic, scallions and wakegi (OOSAWA and TAKAYANAGI, 1984) but were not effective in butterbur. OOSAWA and TAKAYANAGI (1984) used Driselase in addition to Pectolyase and Cellulase RS for young seedlings of strawberry grown in a test-tube. We tried in the present experiment, to use axenic plants of butterbur and to apply one step digestion method with the enzyme mixture of 0.1% Pectolyase Y 23, 0.5% Cellulase Onozuka RS and 0.5% Driselase and succeeded in preparing active protoplasts.

For protoplast culture, we successfully used MS medium as a basic one, which has been frequently used for shoot tip culture (MATSUBARA and MITSUNOBE, 1978) and organ culture (MORISHITA, KAGI and YAMADA, 1980; YABE *et al.*, 1985). Supplemented auxin and cytokinin were 2 mg/l NAA and 0.2~0.5 mg/l BA which were good for callus formation and proliferation in leaf blade culture of butterbur (YABE *et al.*, 1985).

In protoplast culture, it is said that concentration of ammonium salt in the MS are too high for protoplast to continue to survive (KAO *et al.*, 1973; MEYER, 1974; UCHIMIYA and MURASHIGE, 1976). We used modified MS medium with diluted NH_4NO_3 . Preparing modified MS media with various concentration of ammonium nitrate (0, 200, 400, 800, 1600, and 3200 mg/l) containing the same amount of vitamins (1/2 of MS), 1 mg/l NAA, 0.5 mg/l BA, 1% sucrose and 0.5 M mannitol, the effect of dilution of the media on protoplast division was investigated (Table 3). The most favourite was one fourth dilution of MS salts with 200 mg/l NH_4NO_3 , equivalent for 1/33 NH_4NO_3 concentration (50 mg/l) of the original MS medium (1650 mg/l NH_4NO_3). Osmotic regulation during protoplast culture was so simple by using 0.5 M mannitol at initial culture and transferring in mannitol free media after that (one to one and a half months culture).

Plant regeneration was attained after 5 to 6 month-culture as standard callus culture.

Successful protoplast culture and plant regeneration are prerequisite for protoplast fusion, induction and selection of useful mutants, and gene transfer. For the vegetatively propagated crops such as butterbur, effective induction and selection of useful characters through protoplast culture may be highly useful. Present study may contribute to such biotechnological oriented works in vegetatively propagated crops.

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Literature Cited

GAMBORG, O. L., R. A. MILLER and K. OJIMA 1968. Nutrient requirements of suspension cultures of

- soybean root cells. Exp. Cell Res. **50** : 151~158.
- IMAZU, T. 1961. Morphological, ecological, and cytological studies on cultivated and wild butterburs, *Petasites japonicus* MAXIM. I On the morphology of leaf, sprouting- and flowering habits, and germination of seed. Jour. Jap. Soc. Hort. Sci. **30**(3) : 233~240.
- and N. FUJISHITA 1961. ——— II. On the morphology of flower, sex ratio and secondary sex characters. Jour. Jap. Soc. Hort. Sci. **30**(4) : 281~298.
- and ——— 1962 a. ——— III. On the pollen and seed fertilities. Jour. Jap. Soc. Hort. Sci. **31**(1) : 23~29.
- and ——— 1962 b. ——— IV. On the chromosome numbers. Jour. Jap. Soc. Hort. Sci. **31**(4) : 1~10.
- KAO, K. N. and M. R. MICHAYLUK 1975. Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at very low protoplast density in liquid media. Planta **126** : 105~110.
- O. L. GAMBORG, M. R. MICHAYLUK, W. A. KELLER, and R. A. MILLER 1973. The effect of sugar and inorganic salts on cell regeneration and sustained division in plant protoplasts. Coll. Intern. Cent. Natl. Rech. Sci. **212** : 207~213.
- MATSUBARA, S. and T. MITSUNOBE 1978. Meristem culture of Japanese butterbur. 1978 Abstr. Japan. Soc. Hort. Sci. Spring Meet. : 208~209.
- MEYER, Y. 1974. Isolation and culture of tobacco mesophyll protoplasts using a saline medium. Protoplasma **81** : 363~372.
- MORGAN, N. A. and E. C. COCKING 1982. Plant regeneration from protoplasts of *Lycopersicon esculentum* Mill. Z. Pflanzenphysiol. **106** : 97~104.
- MORISHITA, M., T. KAGI and K. YAMADA 1980. Mass production of virus free butterbur plants (*Petasites japonicus* Maxim.) from callus divided from pith tissue of the scape and petiole *in vitro*. Bull. Osaka Agric. Res. Cent. **17** : 1~6.
- MURASHIGE, T. and F. SKOOG 1962. A revised medium for rapid growth and bioassays with tobacco tissues. Physiol. Plant. **15** : 473~479.
- OOSAWA, K. and K. TAKAYANAGI 1984. Protoplast approach in vegetable breeding I. Isolation and culture conditions of protoplasts from mesophyll cells of vegetable crops. Bull. Veg. and Ornamtl. Crops Res. Sta. Japan, A-12 : 9~28.
- UCHIMIYA, H. and T. MURASHIGE 1976. Influence of the nutrient medium on the recovery of dividing cell from tobacco protoplasts. Plant Physiol. **57** : 424~429.
- YABE, K., Y. SAKURAI, T. IIDA and S. WASHIDA 1985. Leaf and leaf petiole culture of Japanese butterbur. 1985 Abstr. Japan. Soc. Hort. Sci. Autumn Meeting : 172~173.

フキ葉肉プロトプラストからの植物体再生

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フキは雌雄異株植物であり, 栽培株には雌株しかみられないため, 繁殖には地下茎を利用している. さらに, 栽培種は3倍体 ($2n=87$, $X=29$) で不稔性のため交雑育種が極めて困難である. そこで, フキの葉肉プロトプラストの単離と培養を試み, 育種への利用の可能性を検討した.

供試品種“愛知早生ブキ”の15~20 cmに伸長した地下茎を採取し, 70% エチルアルコールに60秒間, 活性塩素濃度0.5%のアンチホルミンに10分間浸漬して表面殺菌後, 顕微鏡下で葉原基を含む0.5~1.1 mmの大きさの茎頂を摘出し培地に置床した. 培地は, MS培地にBAを2 mg/l 加えたものを用い, 茎葉分化後BAを1 mg/l 含むMS培地に分割移植し無菌植物を得た. プロトプラストの単離に供試した葉は, この無菌植物から採取した. 0.1% ベクトリアーゼ Y 23+0.5% セルラーゼオノズカ RS+0.5% ドリセラゼ +0.5 M マンニトールの酵素液に, CPW 塩を含む0.5 M マンニトール液中で細断した葉を入れ, 27°C, 80回/分で120分間振とうすることにより活性の高いプロトプラストが得られた. プロトプラストの培養は, 培養温度25°C, 培養密度 1×10^5 個/mlの条件で行い, 基本培地の種類, 濃度及びホルモン濃度について検討した. その結果, 基本培地としては無機塩, ビタミン類とも希釈したMS培地が適することが明らかになった. また, MS培地の無機塩の中で硝酸アンモニウム濃度とその他の無機塩濃度について検討した結果, 硝酸アンモニウムを200 mg/l としたMS無機塩を1/4濃度に希釈した培地で最も分裂が進むことが認められた. 更に, 無機塩をMS培地の1/4濃度, ビタミン類を同じく1/2濃度に希釈した培地でホルモンの濃度を検討した結果, オーキシシンにNAA, サイトカイニンにはBAを用い, それぞれ2 mg/l 及び0.2~0.5 mg/l の濃度としたものがプロトプラストの分裂に最も適することが明らかとなった.

以上のプロトプラスト培養において, 多数のコロニー形成が認められたが, これらのコロニーが径2~5 mmの大きさのカルスに生育した段階で, ショ糖濃度3%, 寒天濃度0.8%のMS培地に移植し, 25°C, 2,000 luxの培養条件で茎葉分化に適するホルモン条件を検討した結果, NAA 0.02 mg/l, BA 4 mg/l を含むMS培地が最も適していた. 再分化した茎葉を, ホルモンを含まないMS寒天培地に移植し幼植物体を得た. 以上の培養実験により, 愛知早生ブキの葉肉プロトプラストの単離条件と, プロトプラストから植物体を再生するための基本的な培地条件が明らかとなった. 今後, 細胞レベルでの選抜や細胞融合による品種間雑種の育成に利用できるものと期待される.

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