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Plant Regeneration from Callus and Protoplasts of Sweet Potato, *Ipomoea batatas* (L.) Lam.

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Introduction

Plant regeneration from callus and protoplasts of sweet potato, *Ipomoea batatas* (L.) Lam., is difficult and genotype-dependent. There have been a number of reports on plant regeneration from callus of sweet potato, but in most cases the frequency of plant regeneration was very low[1-3,9,12,13,15-17].

Few studies related to plant regeneration from sweet potato protoplasts have been done. Murata *et al.*[10] and Sihachakr and Decreux[14] obtained regenerated plants from sweet potato protoplasts at very low frequencies. Murata *et al.*[8] reported low frequency somatic embryogenesis in mesophyll protoplast culture of sweet potato. We have reported root development from sweet potato protoplasts[6]. In a further study, bud differentiation from protoplast-derived callus was observed (unpublished results).

Sweet potato cv. Kokei No. 14 is the most important cultivar for table use in Japan. Plant regeneration from its protoplasts has not been reported. In this paper we report plant regeneration from callus and protoplasts of Kokei No.14.

Materials and Methods

1. Plant materials

Plants *in vitro* grown from shoot tips of sweet potato cv. Kokei No.14 were used as the source of explants and protoplasts in this study.

The stems were grown in pots in a greenhouse (15–32°C). The plants were fertilized weekly with dilute solution of hyponex (Murakamibussan Co., Tokyo), 2 ml/l. About 5 mm-long shoot tips excised from the plants were surface-sterilized with 70% ethanol for 10 s and soaked in 2% sodium hypochlorite solution for 15 min. They were immediately rinsed three times with sterile distilled water. Sterilized shoot tips were inoculated on Murashige and Skoog[7] (MS) medium containing 3.0% (w/v) sucrose and 0.8% (w/v) agar but no plant growth regulators, i.e. basal medium. The pH was adjusted to 5.8 with 1N sodium hydroxide before autoclaving for 20 min at 120°C. The culture was carried out under 13 h day-light at 3000 lux and 27±1°C. The shoots were subcultured every 3 to 4 weeks.

2. Callus induction and plant regeneration

Three-week-old shoots were rinsed with sterile distilled water. The young petioles were cut into 5 mm-long segments, and well-expanded leaves were cut into 5 mm-long, 2 mm-wide strips. All explants were incubated on MS medium supplemented with 0.05 mg/l 2,4-

dichlorophenoxyacetic acid (2,4-D), 0.5 mg/l kinetin, 3.0% (w/v) sucrose and 0.8% (w/v) agar, pH 5.8, in the dark at 27°C.

Six weeks after incubation, the obtained calli were transferred onto MS medium supplemented with 3-indoleacetic acid (IAA; 0, 0.1, 0.5 mg/l), 6-benzylaminopurine (BAP; 0.1, 1.0, 2.0 mg/l), 3.0% (w/v) sucrose and 0.8% (w/v) agar, pH 5.8, (regeneration medium), and cultured for 7 weeks under 13 h day-light at 3000 lux and 27±1°C. The calli formed adventitious roots and shoots. The non-shoot forming calli were further cultured for 7 weeks on the basal medium under 13 h day-light at 3000 lux and 27±1°C. Regenerated shoots were transferred onto fresh basal medium to induce roots. Regenerated plantlets were transplanted in pots with vermiculite and grew to maturity.

3. Protoplast isolation and culture

Protoplasts were isolated from young petioles of Kokei No.14 as described previously[5].

For conventional plating methods protoplasts were cultured in a modified MS medium (mMS), which was composed of 1/2MS inorganic salts (minus NH_4NO_3), MS vitamins, 50.0 mg/l casein hydrolysate, 0.6 M D-mannitol, 0.05 mg/l 2,4-D, 0.5 mg/l kinetin and 1.0% (w/v) sucrose, pH 5.8, at a density of $1\text{–}2\times10^4$ protoplasts/ml in the dark at 27°C. Three weeks after plating, the cultures were incubated in mMS medium in which D-mannitol concentration was reduced to 0.3 M and sucrose concentration was increased to 2.0% (w/v) in the dark at 27°C. Five weeks after plating, protoplast-derived colonies/calli (p-colonies/calli) were cultured at a low density in MS medium containing 0.05 mg/l 2,4-D, 0.5 mg/l kinetin and 3.0% (w/v) sucrose, pH 5.8, in the dark at 27°C.

4. Plant regeneration from protoplast-derived callus

Eight weeks after plating, p-calli up to 2–3 mm in diameter were cultured on MS medium supplemented with 0.05 mg/l 2,4-D, 0.5 mg/l kinetin, 3.0% (w/v) sucrose and 0.8% (w/v) agar, pH 5.8, in the dark at 27°C. After 3 weeks, p-calli up to 7–8 mm in diameter were transferred onto MS medium supplemented with IAA (0, 0.1, 0.5 mg/l), BAP (0.1, 1.0, 2.0 mg/l), 3.0% (w/v) sucrose and 0.8% (w/v) agar, pH 5.8 (regeneration medium) and cultured under 13 h day-light at 3000 lux and 27±1°C. Seven weeks after transfer, adventitious roots formed. And then p-calli or the thick roots (over 1 mm in diameter) excised from p-calli were further transferred onto the basal medium under 13 h day-light at 3000 lux and 27±1°C.

Results and Discussion

1. Callus induction and plant regeneration

Five days after incubation, petiole and leaf explants started to produce white calli from their cuts on MS medium supplemented with 2,4-D and kinetin. Six weeks after incubation, the callus was light yellow in color, hard and compact in texture and about 10 mm in diameter (Fig. 1A), and different from those described by Otani and Shimada[11] and Liu *et al.*[4]. The frequency of callus induction reached 100.0%.

Petiole- and leaf-derived calli were transferred onto the regeneration medium. Two weeks after transfer, they started to form adventitious roots. Adventitious shoots were regenerated from petiole-derived calli at 7 weeks after transfer (Fig. 1B, Table 1). Leaf-derived calli formed only roots but not shoots on the regeneration medium. The calli that did not form shoots on the

Table 1. Shoot regeneration from petiole- and leaf-derived callus of Kokei No. 14 on MS medium supplemented with IAA and BAP and the basal medium

Explant	Regeneration medium IAA (mg/l)	BAP (mg/l)	No. of callus cultured on regeneration medium	Callus forming shoots on regeneration medium No.	%	Callus forming shoots on basal medium No.	%*	Total % of callus forming shoots
Petiole	0	0.1	20	0	0	1	5.0	5.0
	0	1.0	20	0	0	2	10.0	10.0
	0	2.0	20	0	0	1	5.0	5.0
	0.1	0.1	20	0	0	0	0	0
	0.1	1.0	20	0	0	0	0	0
	0.1	2.0	20	1	5.0	1	5.0	10.0
	0.5	0.1	20	1	5.0	2	10.0	15.0
	0.5	1.0	20	0	0	0	0	0
	0.5	2.0	20	1	5.0	0	0	5.0
Leaf	0	0.1	18	0	0	1	5.6	5.6
	0	1.0	18	0	0	2	11.1	11.1
	0	2.0	8	0	0	0	0	0
	0.1	0.1	20	0	0	0	0	0
	0.1	1.0	23	0	0	0	0	0
	0.1	2.0	30	0	0	0	0	0
	0.5	0.1	27	0	0	2	7.4	7.4
	0.5	1.0	22	0	0	0	0	0
	0.5	2.0	20	0	0	1	5.0	5.0

* Percentage to number of callus cultured on regeneration medium.

regeneration medium were further transferred onto the basal medium and regenerated shoots (Table 1).

In this experiment, when petiole- and leaf-derived calli were cultured first on the regeneration medium with 0.5 mg/l IAA+0.1 mg/l BAP and only 1.0 mg/l BAP, respectively, and then on the basal medium, they gave the maximum frequencies of shoot regeneration up to 15.0% and 11.1%, respectively.

Transfer of regenerated shoots onto fresh basal medium resulted in development of whole plantlets (Fig. 1C). Regenerated plantlets were transplanted in pots with vermiculite and vigorously grew to maturity (Fig. 1D).

The similar culture conditions were also used in tissue cultures of *I. triloba* L. and gave a very high frequency of plant regeneration[4]. In these two experiments, two kinds of callus completely different in texture were formed from Kokei No. 14 and *I. triloba* L., respectively. The callus of Kokei No. 14 was hard and compact, whereas the callus of *I. triloba* L. was soft and friable. Therefore, hard and compact callus is probably difficult to regenerate plants. In a further experiment, when callus induction medium contained only 2,4-D, no plant regeneration was observed from Kokei No. 14 callus. These results have demonstrated that genotypes markedly affect plant regeneration in sweet potato and its related species.

Fig. 1A-D. Callus formation and plant regeneration in tissue cultures of Kokei No. 14. A: Calli formed on MS medium supplemented with 2,4-D and kinetin at 6 weeks after incubation. B: Shoots regenerated from a petiole-derived callus at 7 weeks after transfer onto MS medium supplemented with IAA and BAP. C: Plantlet developed from a regenerated shoot on the basal medium. D: Maturing plants regenerated from Kokei No. 14 callus in a pot with vermiculite.

Table 2. Adventitious root and plantlet formation from protoplast-derived callus of Kokei No. 14 on MS medium supplemented with IAA and BAP and the basal medium

Regeneration medium IAA (mg/l)	Regeneration medium BAP (mg/l)	No. of callus cultured on regeneration medium	Callus forming roots on regeneration medium No.	Callus forming roots on regeneration medium %	Callus forming plantlets on basal medium No.	Callus forming plantlets on basal medium %*
0	0.1	28	2	7.1	—**	
0	1.0	27	5	18.5	—	
0.1	0.1	24	7	29.2	1	4.2
0.1	1.0	25	3	12.0	—	
0.1	2.0	25	4	16.0	—	
0.5	0.1	23	4	17.4	—	
0.5	1.0	26	1	3.8	—	
0.5	2.0	18	1	5.6	—	

* Percentage to number of callus cultured on regeneration medium.

** not tested

2. Protoplast culture and plant regeneration

Large numbers of protoplasts were isolated from *in vitro* grown plants of Kokei No. 14 and cultured in mMS medium (Fig. 2A). Plated protoplasts responded rapidly to the culture conditions. First cell division was observed within 2–3 days (Fig. 2B). Two weeks after plating, protoplast plating efficiency was over 70%, and in this time p-colonies were up to about 0.3 mm in

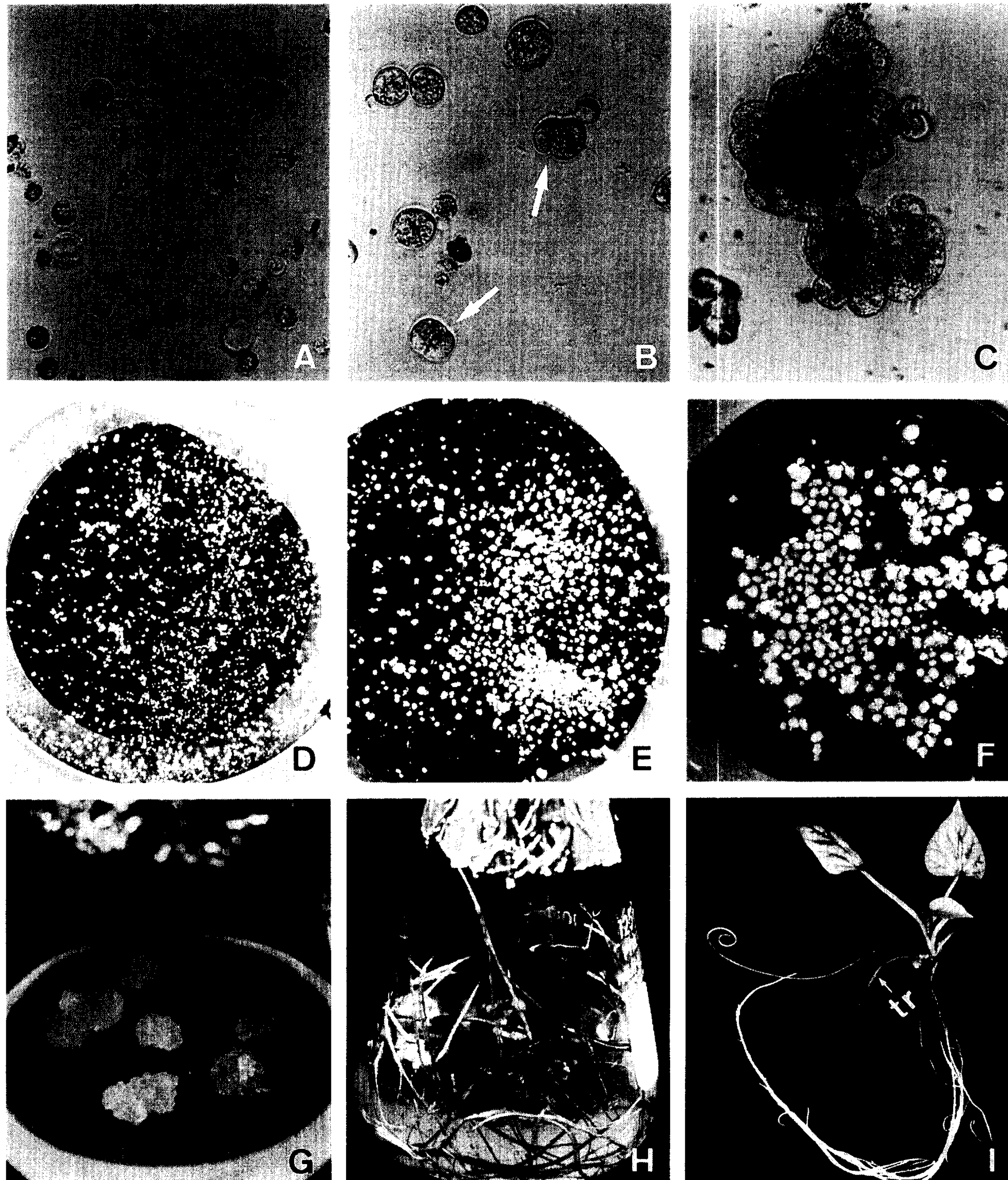

Fig. 2A-I. Callus formation and plant regeneration from Kokei No. 14 protoplasts. A: Freshly isolated protoplasts. B: First cell division within 2–3 days (arrows). C: Colonies derived from protoplasts at 2 weeks after plating (about 0.3 mm in diameter). D: High frequency colony formation from protoplasts. E-F: Small calli derived from protoplasts at 6 weeks and 8 weeks, respectively, after plating (1–2 mm and 2–3 mm, respectively, in diameter). G: Calli rapidly proliferating on MS medium supplemented with 2,4-D and kinetin (7–8 mm in diameter). H: Adventitious roots formed from p-calli on MS medium supplemented with IAA and BAP. I: Plantlet regenerated from the thick root formed from p-callus (tr=thick root).

diameter (Fig. 2C). In this experiment, a high frequency of colony formation was obtained (Fig. 2D). And then p-colonies rapidly developed into small p-calli (Fig. 2E). Eight weeks after plating, p-calli were up to 2–3 mm in diameter (Fig. 2F).

Transfer of p-calli onto MS medium supplemented with 0.05 mg/l 2,4-D and 0.5 mg/l kinetin resulted in rapid callus proliferation (Fig. 2G). Three weeks after transfer, the obtained calli up to 7–8 mm in diameter were further transferred onto the regeneration medium supplemented with IAA and BAP, and after about 2 weeks started to form adventitious roots (Fig. 2H). Adventitious root formation at 7 weeks after transfer onto the regeneration medium was shown in Table 2. Of the adventitious roots formed, some were over 1mm in diameter, called thick root. The maximum frequency of thick root formation was obtained on the regeneration medium supplemented with 0.1 mg/l IAA and 0.1 mg/l BAP.

When the calli with/without adventitious roots were cultured on the basal medium, no shoot regeneration was observed. However, when the thick roots were excised from the callus cultured on the regeneration medium with 0.1 mg/l IAA and 0.1mg/l BAP and then cultured on the basal medium, of 8 thick roots cultured, 1 thick root produced a plantlet (Fig. 2I, Table 2).

In this experiment, high protoplast plating efficiency and high callus formation frequency were obtained, but the frequency of plant regeneration was very low (4.2%). So far, Murata *et al.*[10], Sihachakr and Ducreux[14], and Murata *et al.*[8] have reported plant regeneration from sweet potato protoplasts, but the regeneration frequencies were all very low. Therefore, the difficulty in plant regeneration from sweet potato protoplasts has been the single most important barrier to the application of somatic cell hybridization for the improvement of this species.

Summary

Plant regeneration from callus and protoplasts of sweet potato cv. Kokei No. 14 was studied.

Petiole and leaf explants of Kokei No. 14 gave rise to the callus light yellow in color and hard and compact in texture on MS medium supplemented with 0.05 mg/l 2,4-D and 0.5 mg/l kinetin. When transferred onto MS medium supplemented with 0 to 0.5 mg/l IAA and 0.1 to 2.0 mg/l BAP, some calli formed adventitious roots and shoots. Further transfer of the non-shoot forming callus onto the basal medium resulted in additional shoot regeneration. After regenerated shoots were transferred onto fresh basal medium, they developed into whole plantlets which were grown to maturity in pots with vermiculite.

Protoplasts isolated from *in vitro* grown plants of Kokei No. 14 were cultured in a modified MS medium containing 0.05 mg/l 2,4-D and 0.5 mg/l kinetin. First cell division occurred within 2–3 days. Two weeks after plating, a protoplast plating efficiency over 70% was obtained. High frequency callus formation from protoplasts was observed. Eight weeks after plating, p-calli up to 2–3 mm in diameter were transferred onto MS medium supplemented with 0.05 mg/l 2,4-D and 0.5 mg/l kinetin. When the obtained p-calli were further transferred onto MS medium supplemented with 0 to 0.5 mg/l IAA and 0.1 to 2.0 mg/l BAP, some of them formed the thick roots. The thick root regenerated a plantlet on the basal medium.

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