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Plant Regeneration from Protoplasts of Satsuma Mandarin (Citrus unshiu Marc.) via Somatic Embryogenesis

Hisato KUNITAKE, Hiroshi KAGAMI* and Masahiro MII
Department of Horticulture, Chiba University (Matsudo, Chiba, 271 Japan)

Abstract
Protoplasts were isolated from the embryogenic calli of satsuma mandarin (Citrus unshiu Marc.) cv. Tokumori-Wase and cultured in the Gellan Gum-embedded Murashige and Tucker medium containing 40 mg/l adenine, 0.15M sucrose and 0.45M mannitol. The percentage of colony formation (plating efficiency) in 40 days of culture was 46%, which was much higher than that in MT medium without adenine. Most of the colonies grew to the size of about 1 mm in 2 months of culture and they were successfully transferred onto the MT medium containing 5% lactose for embryoid formation. Five percent of these embryoids germinated normally and grew to plantlets in 30-60 days after transplanting to the MT medium containing 1 mg/l gibberellic acid (GA3), 3% sucrose and 0.2% Gellan Gum. Protoplasts obtained in the culture of other two selections, Miyagawa-Wase and Okitsu-Wase also regenerated plants by using the same method as above.

Discipline: Biotechnology
Additional key words: embryoid induction, lactose, plating efficiency, tissue culture

Introduction

Satsuma mandarin (*Citrus unshiu* Marc.) is one of the most important fruit crops in Japan and occupies 70% of the citrus planting in Japan[11]. Programs of satsuma mandarin improvement have long employed a method of mutation breeding, in which selections of bud sports and chance seedlings from nucellar tissue have been undertaken because of the difficulties in cross-breeding caused by nucellar embryony and male sterility. Despite of those difficulties in cross-breeding, several varieties have been developed through the crossing of satsuma mandarin with other citrus species by using bud pollination[15,16]. However, such a conventional method for breeding is not applicable to a remote hybridization with distantly related species having desirable characters for the crop improvement.

In recent years, inter- and intra-generic somatic hybrid plants have been produced between sexually compatible species such as *Citrus sinensis* and *Poncirus trifoliata*[3,17], *C. sinensis* and *C. unshiu*[8], *C. sinensis* and *C. paradisi*[18] as well as between sexually incompatible species, *C. sinensis* and *Severinia disticha*[4] and *C. reticulata* and *Citropsis gilletiana*[5]. These successful hybridizations were based on the use of *C. sinensis* calli which had high potential to regenerate plants via somatic embryogenesis. In expanding applicability of the somatic hybridization techniques to other species belonging to the family Rutaceae, it is necessary to establish an adequate method of plant regeneration in each species. In *C. sinensis*, embryogenic calli have successfully been induced from nucellar tissues by culturing immature ovules[6,7,9], while in satsuma mandarin, embryogenic calli from immature ovules have never been induced. However, Ling et al.[13] have recently succeeded in inducing embryogenic calli by culturing undeveloped ovules excised from mature fruits. They regenerated

* Present address: Shizuoka Prefectural Citrus Experiment Station (Komagoe-nishi, Shimizu, Shizuoka, 424 Japan)

whole plants from protoplasts isolated from those embryogenic calli. However, the efficiency of producing protoplasts in their studies was not high enough to practically utilize somatic hybridization and genetic transformation.

This report attempts to propose an effective use of adenine in protoplast culture, which should be followed by the use of lactose in regenerating whole plants via somatic embryogenesis, in selected varieties of satsuma mandarin.

Materials and methods

1) Protoplast isolation and culture

Embryogenic calli of selected cultivars of satsuma mandarin, i.e. Tokumori-Wase, Miyagawa-Wase and Okitsu-Wase, were induced after the method of Ling et al.[13]. For protoplast isolation, the embryogenic calli produced were pretreated for a 2–4 week period in Murashige and Tucker[14](MT) medium containing 5% lactose without any growth regulators. The pretreated calli (1 g fresh weight) were gently squashed and incubated with 10 m*l* of filter-sterilized (Millipore, 0.45 μm pore size) enzyme solution, which contained 0.3% Cellulase Onozuka R-10, 0.3% Macerozyme R-10, (both, Yakult Pharmaceutical Co. Ltd., Japan), 0.1% Driselase (Kyowa Hakko Kogyo Co., Ltd. Japan), half strength of MT major mineral salts and 0.7M sorbitol, with the pH adjusted to 5.7 before filter sterilization. The mixture was incubated on a rotary shaker (60 rpm) for 12–14 hr at 25°C to liberate protoplasts. Protoplasts were collected by filtration through a nylon sieve (60 μm) and washed twice with 0.7M mannitol solution after centrifugation (100 × g for 5 min). Protoplast yield was calculated with a hemocytometer and the culture density of protoplasts was adjusted before plating. Protoplasts were embedded in 0.1% Gellan Gum-solidified MT medium containing 0.15M sucrose and 0.45M mannitol, various growth regulators and cultured at a density of 1×10^5 cells/m*l* in 60 × 15 mm plastic petri dishes containing 3 m*l* of culture medium. All the dishes were sealed with parafilm and maintained in the dark at 25°C. Plating efficiency was expressed in percentage of the plated protoplasts which formed colonies after 40 days of culture.

2) Embryoid induction

Effects of various types of sugars on somatic embryogenesis of protoplast-derived calli in the three selections were subjected to investigations. The calli after one-month subculture were used in this experiment. Two hundred mg of calli were transferred to each plate of MT media containing 0.2M sugars and 0.8% agar, and cultured under the same culture conditions. Among the sugars, sucrose, lactose, glucose, fructose and galactose were tested. Two independent experiments each with ten replicates were conducted. The numbers of embryoids up to 0.5 mm in diameter were counted after two-month culture and the average numbers of embryoid formation were compared.

In relation to the process of somatic embryogenesis, a histological observation was undertaken. The cultures were fixed in acetic alcohol (1 acetic acid and 3 ethanol v/v), dehydrated in an ethanol and tertiary buthanol series, embedded in paraffin, sectioned to 20–25 μm thickness, and stained with safranin and fast-green FCF.

3) Plant regeneration from embryoids

Heart-shaped embryoids (1–2 mm) derived from protoplast were transferred to half strength of MT medium containing 1 mg/*l* gibberellic acid (GA_3), 1% sucrose and 0.2% Gellan Gum. All the cultures were kept at 25°C, with continuous lightening at 38 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Regenerated plantlets were potted in vermiculite and kept in an incubator for acclimatization for one month, and then they were transferred to a greenhouse.

Results and discussion

Protoplasts could not be directly isolated from the callus cultures because of incomplete maceration of cell walls and burst of the isolated protoplasts. Therefore, the protoplasts were isolated after pretreatment of culturing the calli in MT medium containing 5% lactose for 2 weeks. The effects of pretreatment on the protoplast isolation from citrus embryogenic calli were earlier reported by Ohgawara et al.[17] and Ling et al.[12]. Ling et al. isolated viable protoplasts by decreasing the level of endogenous growth substances in callus tissue under that pretreatment[12]. Protoplast yields generally ranged between 1×10^6 and 5×10^6 cells per gram of callus.

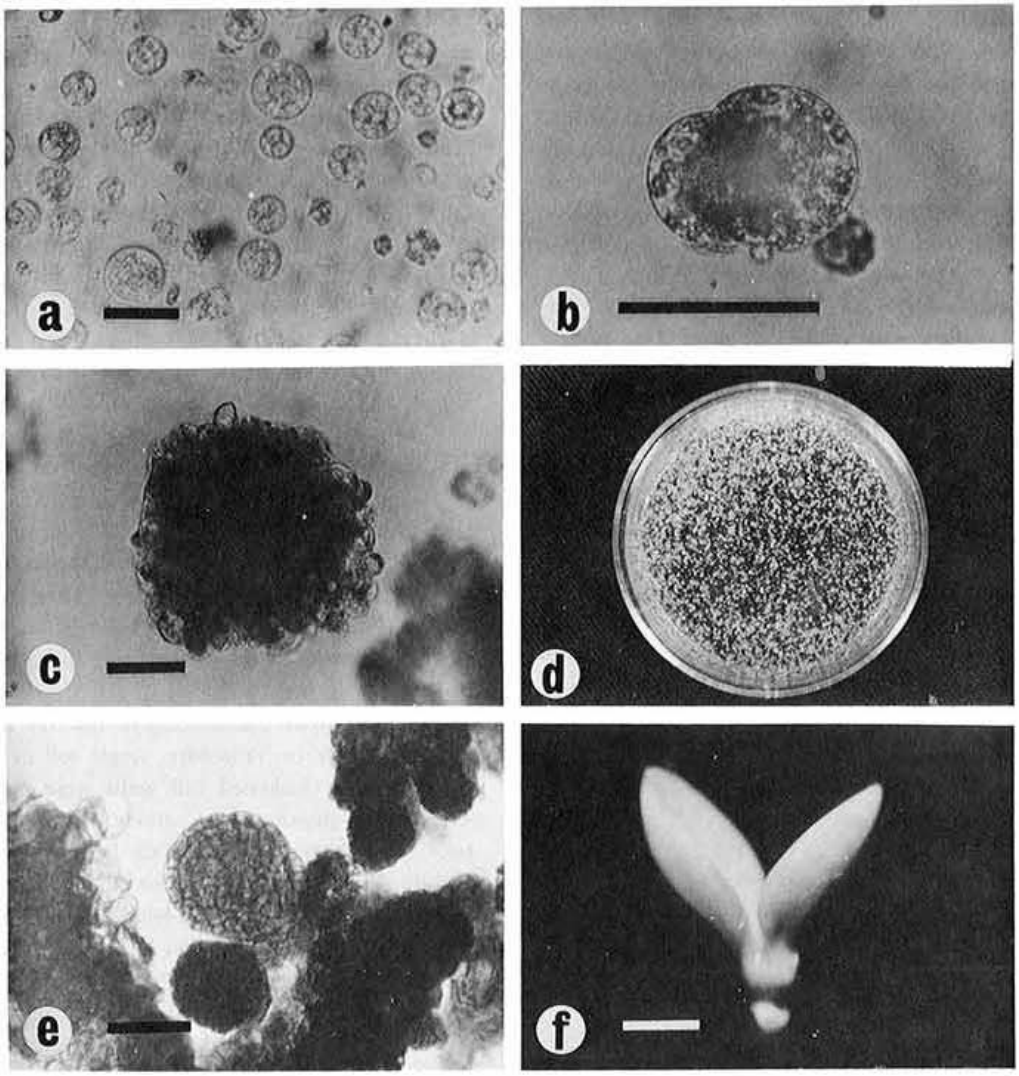

Plate 1.
(a) Protoplasts isolated from embryogenic callus (Bar = 50 μm)
(b) First division of protoplast in 7 days of culture (Bar = 50 μm)
(c) Colony derived from protoplast in 40 days of culture (Bar = 100 μm)
(d) Efficient colony formation on MT medium containing adenine
(e) Globular proembryoid formation in 3 weeks of transferring to MT medium containing 5% lactose (Bar = 100 μm)
(f) Cotyledonary embryoid 2 weeks after transplanting to the regeneration medium (Bar = 2 mm)

More than 90% of the pretreated protoplasts showed strong fluorescence under UV light after staining with fluorescein diacetate, indicating a high level of viability immediately after isolation. Average size of the protoplasts isolated from pretreated callus was approximately 30 μm in diameter (Plate 1-a).

The first mitotic cell division took place in 7–10 days of culture (Plate 1-b). Individual colonies which consisted of as many as 50–100 cells were observed in 30–40 days of culture (Plate 1-C). Plating efficiency obtained in 40 days of culture was strongly affected by adenine and slightly by malt

extract (Table 1). The plating efficiency in Tokumori-Wase was 27.6% without these two ingredients, whereas it was 45.6% by adding 20 mg/*l* adenine (Plate 1-d). The similar effect of adenine was also shown in another satsuma mandarin selection, Okitsu-Wase, which indicated 19.1% of plating efficiency on MT medium containing adenine, whereas 12.5% on adenine free-MT medium. However, in selection Miyagawa-Wase, no improvement of plating efficiency was observed by adding adenine: only 3% of the protoplasts formed colonies. Ling et al.[13] reported approximately 10% of plating efficiency on hormone free-MT medium in 40 days of culture. However, the present study showed much higher plating efficiency than that by using adenine.

In two months of culture, the colonies produced were transferred onto MT medium containing 500 mg/*l* malt extract, 40 mg/*l* adenine, 1 mg/*l* GA_3, 5% sucrose and 0.2% Gellan Gum. These colonies grew rapidly to friable and nodular calli that closely resembled the original calli. Effects of various types of sugars on somatic embryogenesis were investigated by using these protoplast derived-calli (Table 2). It was earlier reported that the somatic embryogenesis of nucellar callus of sweet orange could be stimulated when the sucrose in the medium was replaced by certain other sugars or sugar alcohols such as galactose, lactose, raffinose, maltose and glycerol[1,2,10]. However, in the three satsuma mandarin cultivars under study, embryoids could be induced only when lactose was added as a carbon source and no embryoid formation was observed in MT medium containing the sugars such as sucrose, glucose, fluctose and galactose. However, less embryoids per culture, i.e. below 50, were formed against expectation because the calli of satsuma mandarin used for protoplast culture had almost the same productivity of somatic embryos. Therefore, the ability of somatic embryogenesis in satsuma mandarin calli seemed to decrease during the process of protoplast culture.

One week after transferring to the MT medium containing 5% (w/v) lactose, single cell or groups of cells with thickened cell walls were observed. These cells continued to divide themselves and became globular proembryoids in 3-4 weeks of culture (Plate 1-e). One to two months after transplanting of the callus derived-heart shaped embryoids to the regeneration medium, 5% of them showed normal development and regenerated plantlets via cotyledonary embryoid (Plate 1-f). Fifty to 60% of them, however, showed abnormal growth with the production of secondary embryoids on their surfaces. Five regenerated plants (2 Tokumori-Wase, 2 Okitsu-Wase and 1 Miyagawa-Wase) were transplanted to the soil and grown in a greenhouse (Plate 2). No differences were recognized in growth habits

Table 1. Effects of malt extract, adenine and GA_3 on plating efficiency of Tokumori-Wase protoplasts[a]

Malt extract (mg/*l*)	Adenine (mg/*l*)	GA_3 (mg/*l*)	Plating efficiency (%)[b]
			27.6
500			34.0
	40		45.6
		1	29.3
500	40	1	44.3

a): Embryogenic calli were pretreated for 2 weeks before protoplast isolation by suspending in MT medium containing 5% lactose.

b): Percentage of colonies obtained from the cultured protoplasts. The data were recorded in 30 days of culture, indicating the means of 2 experiments, each with 5 replicates.

Table 2. Effect of sugars on somatic embryogenesis in selections of satsuma mandarin

Cultivars	No. of embryoids[a]				
	Sucrose[b]	Lactose	Glucose	Fructose	Galactose
Tokumori-Wase	0	23.2	0	0	0
Okitsu-Wase	0	42.4	0	0	0
Miyagawa-Wase	0	12.1	0	0	0

a): Embryoids developed into globular or more advanced stages were recorded in 2 months of culture. The data indicate the means of 2 experiments, each with 10 replicates.

b): Concentration of each sugar was 0.2M.

Plate 2. A growing plant in 6 months after being transplanted to soil

and leaf characters as well such as shape, thickness and color between the protoplast-derived plants and the nucellar seedlings.

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