Organ Formation from Calli and Protoplast Isolation, Culture, and Fusion in Apple, *Malus pumila* Mill.

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Summary

Regeneration from nucellus calli was investigated to obtain basic data for somatic cell hybridization in apple *Malus pumila* Mill. In addition, isolation, culture and fusion of protoplasts derived from nucellus calli and leaves of the cultured shoots *in vitro* were investigated. The results are summarized as follows: 1. Calli derived from nucellus 50 days after anthesis of a cultivar 'Fuji' and a rootstock 'CG 80' produced adventitious embryos. Some of the embryos germinated and formed shoots after they were placed at 0°C in the dark for 6 months and then transferred to 25°C in the light. 2. The enzyme solutions containing 2% Cellulase Onozuka R-10 and 0.1% Pectolyase Y-23 or 2% Cellulase Onozuka RS and 0.1% Pectolyase Y-23 were the most effective for protoplast isolation from the nucellus calli and the leaves of shoots. The yield of protoplasts was the highest from the calli and the leaves of shoots cultured for 16~20 days after transfer onto the new medium, except for a rootstock 'M. 26' where the highest yield was with 21~30 days of culture. The effective period of enzyme treatment on protoplast yield was 3.0~4.0 h. Colony formation from protoplasts derived from nucellus calli was observed in all media used, except for the medium without growth regulators. 3. Somatic hybrid cells were produced by fusion of protoplasts isolated from the nucellus calli and the leaves of cultured shoots. Most of the hybrid protoplasts were identified by larger green protoplasts than the single green leaf protoplasts. The hybrid protoplasts and transparent nucellus callus protoplasts divided within 7~10 days of culture, while the single leaf protoplasts and the fused homokaryotic leaf protoplasts could not divide. The hybrid cells divided several times, and still had green chloroplasts. Successive cell division gave rise to cell colonies, and the green chloroplasts disappeared gradually. The colonies grew into calli which are, however, not identified as to whether they are hybrids.

Introduction

Most improvements in apple, *Malus pumila* Mill., have been achieved by cross breeding. In this method, the genotypes of trees in F₁ generation may segregate because of the high heterozygosity of their parents. Therefore, combinations of agriculturally useful traits of both parents are very rare in the F₁ trees. Thus, the selection of the F₁ having the useful traits of both parents is very difficult and requires a large population. In addition, a long time is needed for selection of favourable F₁ trees because of their long juvenile period.

Somatic cell hybridization in apple may overcome the above problems because in this procedure no genotypic segregation of both parents occurs. Furthermore, there is the possibility for introduction of agriculturally useful genes from wild species or rootstocks. In order to make a success of somatic cell hybridization, isolation of protoplasts from appropriate organs or cultured tissues, fusion of genetically different protoplasts and regeneration from the hybrid protoplasts are required. In fruit trees, however, research on regeneration from cultured tissues and cells is very rare. Only in pear (24), peach (3) and *Citrus* (9), has the regeneration from calli derived from the immature

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embryo and nucellus been reported. In apple, there is a considerable number of reports of adventitious embryo or shoot regeneration directly from cultured organs such as leaf(4, 22), nucellus(2, 5), immature embryo(10, 12, 13, 21), seedling(14) and root(15), while there are very few reports of regeneration from calli or cultured cells(11, 17). In addition, there is a considerable number of reports concerning the isolation and culture of protoplasts derived from cultured tissues and cells(1, 7, 11, 20) or leaf(6, 16, 23). However, in somatic cell hybridization in apple, not only the methods of protoplast isolation and culture but also of the regeneration from the cultured tissues or cells have not been completely established. We report here the experimental results concerning organ formation from nucellus calli, and protoplast isolation, culture and fusion, by using nucellus calli and leaves of cultured shoots *in vitro* of several cultivars and rootstocks. This may be the first report of somatic cell fusion between cultivars, or cultivars and rootstocks of *Malus pumila*.

Materials and Methods

Regeneration from nucellus calli

In a preliminary examination, organ regeneration from nucellus calli was attempted by using a cultivar 'Fuji' and a rootstock 'CG 80'. Immature seeds 50 and 100 days after anthesis were sterilized by 70 % ethanol for 5 min and then sodium hypochlorite (Cl concentration ; 0.5 %) for 5 min. Both sterilizing

solutions contained 0.1 % Tween 20. The seeds were then washed 5 times with sterilized distilled water. Nucelli were taken out from the seeds and then cultured on the media indicated in Table 1. All cultures were kept at 25°C under dark conditions. After 60 days of culture, frequencies of the formed calli were examined. For the induction of adventitious embryos, the calli were then transferred onto the MS basic medium(19) supplemented with 0.1 mg/l indole-3-acetic acid (IAA) and 0.1 mg/l kinetin.

Isolation and culture of protoplasts derived from nucellus calli and leaves of cultured shoots *in vitro*

The effect of enzyme composition and concentration on protoplast isolation was investigated. The enzyme solutions used are indicated in Table 2. All solutions contained 0.7 M mannitol and were adjusted to pH 5.8. The calli used for protoplast isolation were nucellus calli of a rootstock 'CG 80' cultured on MS basic medium containing 4 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and leaves of cultured shoots of a cultivar 'Oorin' on the MS basic medium containing 3 mg/l 6-benzyl aminopurine (BAP). Both materials 20 days after culture on the new media were used for protoplast isolation and 0.2~0.3 g of nucellus calli and 0.5 g of leaves of the cultured shoots cut into small pieces were treated with 18 ml of enzyme solution at 28°C, 60 shakes per min for 3.5 h. Then, the obtained protoplasts were isolated by 8 sheets of gauze, and washed 4 times with 0.7 M mannitol solution adjusted to pH 5.8 under centrifugation at 80×g. The yield of protoplasts was determined by protoplasts resuspended in 0.5 ml of the culture medium.

The effect of culture period of calli and shoots on protoplast isolation was also investigated. Nucellus calli of the cultivar 'Fuji' and the rootstock 'CG 80' and cultured shoots

Table 1. Medium compositions used for callus formation from nucellus.

Medium type	Medium composition ²
Na	MS
Nb	MS+0.5 mg/l 2,4-D
Nc	MS+1.0 mg/l NAA
Nd	MS+2.0 mg/l 2,4-D+0.5 mg/l BAP
Ne	MS+2.0 mg/l NAA+0.5 mg/l BAP
Nf	MS+2.0 mg/l IBP +0.5 mg/l BAP
Ng	MS+3.0 mg/l NAA + 10 mg/l BAP

² MS: Basic medium of Murashige and Skog (1962).
2,4-D: 2,4-dichlorophenoxyacetic acid.
NAA: 1-naphthaleneacetic acid.
IBA: Indole-3-butyric acid.
BAP: 6-benzylaminopurine.
All media contained 30 g/l sucrose and were adjusted to pH 5.8.

Table 2. Enzyme compositions and concentrations for protoplast isolation.

Enzyme	Enzyme concentration (%)			
	E-1	E-2	E-3	E-4
Cellulase Onozuka RS	—	—	2.0	—
Cellulase Onozuka R-10	2.0	2.0	—	4.0
Pectolyase Y-23	0.1	0.4	0.1	0.1

Table 3. Compositions and concentrations of growth regulators for protoplast culture.

Concentration of BAP (mg/l)	Concentrations of IBA (mg/l)		
	0.0	1.0	2.0
0.0	A ^z	D	G
0.5	B	E	H
1.0	C	F	I

^z A~I: Type of medium.

of cultivars 'Oorin', 'Hokuto' and 'Starking', and rootstock 'M. 26' were used. Culture period was 10~15, 16~20 and 21~30 days after transferring the calli and shoots onto the new media. In addition, period of enzyme treatment was examined by using the same materials. The enzyme solution used in these examinations was E-1 (Table 2) which contains 2% Cellulase Onozuka R-10 and 0.1% Pectolyase Y-23. Procedures of protoplast isolation and yield determination were the same as described above.

The effect of medium composition on colony formation from protoplasts was also investigated. In this experiment, protoplasts isolated

from nucellus calli of the cultivar 'Fuji' were used. The basic medium was 8p(8) and growth regulators were combined as shown in Table 3. Falcon plastic dishes (35×10 mm) were used for all protoplast cultures. The dishes were sealed with parafilm and placed in a plastic moisture box at 25°C in the dark. After one week of culture, fresh medium was added to the dishes which were then moved into the light. After 60 days, colony formation was examined by the naked eye.

Protoplast fusion

Protoplasts isolated from nucellus calli of the cultivar 'Fuji' and from the leaves of cultured shoots of cultivars 'Oorin', 'Senshu' and 'Starking' were fused by the method of PEG-DMSO (PEG; polyethylene glycol, DMSO; dimethylsulfoxide)(18). Concentrations of PEG 1540 were 25% or 50% and 10% DMSO was added to the PEG solution. After 10 min of fusion treatment, PEG was washed 3 times by 0.7 M mannitol with 0.05 M CaCl₂·2H₂O, then washed 4 times by cultured medium. Culture conditions were the same as those of protoplasts derived from nucellus calli, but the medium used was 8p basal con-

Table 4. Callus formation from nucelli of an apple cultivar and a rootstock, and adventitious embryo regeneration from the calli.

Cultivar or rootstock	Stage of fruits ^z	Callus formation from nucelli			Adventitious embryo formation from the calli ^w	
		medium type ^y	No. of nucelli used	No. of nucelli formed callus	No. of calli used	No. of calli formed embryo
Fuji	50	Na	30	0 (0) ^x	—	—
		Nb	30	12 (40)	10	0 (0) ^x
		Nc	30	12 (40)	10	0 (0)
		Nd	30	16 (53)	10	1 (10)
		Ne	30	14 (47)	10	0 (0)
		Nf	30	18 (60)	10	0 (0)
		Ng	30	12 (40)	10	1 (10)
		Na	20	6 (30)	6	0 (0)
	100	Nd	30	24 (80)	10	0 (0)
		Ne	30	20 (67)	10	0 (0)
		Nf	18	16 (89)	10	0 (0)
		Ng	30	18 (60)	10	0 (0)
CG 80	50	Na	30	0 (0)	—	—
		Nb	30	12 (40)	10	0 (0)
		Ng	30	14 (47)	10	1 (10)

^z Stage of fruits: No. of days after anthesis.

^y Medium type corresponds to Table 1.

^x (): Percentage.

^w For adventitious embryo formation, the calli were cultured on MS basic medium supplemented with 0.1 mg/l IAA and 0.1 mg/l kinetin.

Fig. 1. Adventitious embryo formation from the nucellus-derived callus and germination of the embryo in a cultivar 'Fuji'.

A: A formed adventitious embryo indicated by an arrow.

B: A germinated embryo indicated by an arrow.

taining 0.2 mg/l 2,4-D, 1.0 mg/l 1-naphthalenacetic acid (NAA) and 0.5 mg/l BAP. Formed colonies were transplanted onto the MS basic medium containing 0.1 mg/l IAA and 0.1 mg/l kinetin and solidified by 0.8 % agar.

Results and Discussion

Regeneration from nucellus calli

Callus formation from the nucelli 50 and 100 days after anthesis of the cultivar 'Fuji' and 50 days after anthesis of the rootstock 'CG 80', and the regeneration of adventitious embryos from the calli are indicated in Table 4. Na medium with no growth regulator did not form any callus derived from the nucellus on 50 days after anthesis, in both 'Fuji' and 'CG 80'. On the other media with growth regulators shown in Table 1, 40~60 % of nucelli formed calli. With 'Fuji', nuceli 100 days after anthesis showed considerably higher callus formation than the 50-day nucelli. Indeed, the frequencies of callus formation were about 60~90 %, and even on the medium without growth regulator 30 % of nucelli formed calli. Adventitious embryo formation was, however, observed only on the calli derived from nucelli 50 days after anthesis, although the frequencies of adventitious embryos were not so high (Fig. 1-A). This may support the results of Hammerschlag *et al.* (3) in which regeneration required callus induced from juvenile tissues or organs.

The adventitious embryos formed were morphologically similar to those indicated by

Table 5. Effect of enzyme compositions and concentrations on the yield of protoplasts.

Source materials	Enzyme composition ²	Yield of protoplasts ^y	Test frequency
Nucellus calli (CG 80)	E-1	++	3
	E-2	±	3
	E-3	++	3
	E-4	+	3
Leaves of cultured shootsE (Oorin)	E-1	+	3
	E-2	±	3
	E-3	+	3
	E-4	+	4

² E-1~E-4 correspond to Table 2.

^y ++: More than 10⁶/ml.

+: 10⁶~10⁵/ml.

±: Less than 10⁵/ml.

—: Not isolated protoplast.

The yield of protoplasts was determined by protoplasts resuspended in 0.5 ml of the cultured medium.

Ishihara and Ooya (5) and Eichholtz *et al.* (2). Some of the embryos germinated and formed shoots when they were placed at 0°C in the dark for 6 months and then transferred to 25°C in the light (Fig. 1-B).

Protoplast isolation and culture

Table 5 shows the effect of enzyme composition on protoplast isolation. From about 0.2~0.3 g of nucellus calli, a high yield of intact protoplasts, more than 10⁶/ml, was obtained by enzyme solution E-1 and E-3, which contain 2 % Cellulase Onozuka R-10 and 0.1 % Pectolyase Y-23, and 2 % Cellulase Onozuka RS and 0.1 % Pectolyase Y-23. The use of E-2 and E-4 enzyme solutions decreased

intact protoplasts. These results seem to be due to high concentrations of enzymes such as 0.4 % Pectolyase Y-23 in the E-2 and 4.0 % Cellulase Onozuka R-10 in the E-4, which might injure the protoplasts in the process of protoplast isolation. From leaves of cultured shoots, the yield of intact protoplasts was fairly low such as $10^5 \sim 10^6$ /ml by the E-1, E-3 and E-4, and less than 10^5 /ml by the E-2. From the results mentioned above, the E-1 and E-3 enzyme solutions were comparably better among 4 enzyme solutions for protoplast isolation from both nucellus calli and leaves of cultured shoots, while they were not completely satisfactory in the case of the cultured shoots, because a considerable number of non-intact protoplasts occurred. The relation between yield of protoplasts and culture period of source materials for protoplasts after transferring to the new medium is indicated in Table 6. A high yield of protoplasts was obtained from the nucellus calli cultured for 16 ~ 20 days. The yield of protoplasts was also higher in the case of the leaves of shoots in the cultivars 'Oorin', 'Hokuto', 'Senshu' and 'Starking' cultured for 16~20 days than for other shorter or longer periods, while the highest yield of protoplasts was produced in shoots of the rootstock 'M. 26' cultured for 21 ~ 30 days. This is likely to be the result of very slow shoot growth in rootstock 'M. 26' compared with the other cultivars.

Effect of period of E-1 enzyme treatment on the yield of protoplasts is shown in Table 7. The highest protoplast isolation, 10^6 /ml, from nucellus calli was obtained by 3.5 h treatment. Protoplast isolation from the leaves of cultured shoots was also the highest under treatment of 3.0~4.0 h.

Colony formation from nucellus protoplasts in various media used is shown in Table 8. There was no conspicuous difference among media except for the medium without growth regulator which did not form a colony. On the other hand, cell division and colony formation from the leaf protoplasts could not be observed in any media used.

Fusion of protoplasts and culture

Fused protoplasts were obtained between the protoplasts from nucellus calli of the cultivar 'Fuji' and those from the leaves of cultured

Table 6. Effect of culture period on protoplast yield.

Source of protoplasts	Cultivars and rootstocks	Yield of protoplasts ^z		
		Culture duration (days)		
		10~15	16~20	21~30
Nucellus calli	'Fuji'	±	++	+
	'CG 80'	±	++	+
Leaves of cultured shoots	'Oorin'	+	++	+
	'Hokuto'	+	++	+
	'Senshu'	+	++	+
	'Starking'	+	++	+
	'M. 26'	±	+	++

^z ++, +, ±: The same as Table 5.

Table 7. Effect of period of enzyme treatment on protoplast yield.

Source of protoplasts	Period of enzyme treatment (h)	Protoplast yield ^z	Test frequency
Nucellus calli (CG 80)	1.0	—	2
	2.5	+	2
	3.5	++	3
	4.5	+	3
Leaves of cultured shoots (Oorin)	1.0	—	3
	2.5	±	3
	3.0—4.0	++	3
	5.0—6.0	+	2

^z ++, +, ±, —: The same as Table 5.

Table 8. Effect of medium compositions on colony formation from nucellus callus protoplasts.

Medium type ^z	No. of culture dishes	No. of culture dishes formed colony
A	5	0
B	5	2
C	5	3
D	5	3
E	5	3
F	5	3
G	5	3
H	5	2
I	5	2

^z A~I correspond to Table 3.

shoots of cultivars 'Oorin', 'Senshu' and 'Starking'. The protoplasts derived from nucellus calli were transparent with large vacuoles and had no green chloroplasts (Fig. 2-A). Sizes of the protoplasts were $12 \sim 65 \mu\text{m}$ with a mean size of $32 \mu\text{m}$. On the other hand, the protoplasts derived from the leaves of cultured

Fig. 2. Protoplast isolation, fusion and culture.

A: Protoplasts isolated from the nucellus calli of a cultivar 'Fuji'. B: Mesophyll protoplasts isolated from the leaves of cultured shoots of a cultivar 'Oorin'. C: A fused heterokaryotic protoplast indicated by an arrow. D: A divided hybrid cell (indicated by an arrow) which maintained green chloroplasts. E: A formed cell cluster in which the green chloroplasts disappeared after 30 days. F: Formed colonies in which the hybrids were not identified.

shoots had green chloroplasts (Fig. 2-B). Sizes of the protoplasts were 9–26 μm , with a mean size of 14 μm . Therefore, after fusion treatment, most of the hybrid protoplasts were identified by a size of more than 26 μm with green chloroplasts (Fig. 2-C). The large fused homokaryotic leaf protoplasts, which could not be identified as to whether they were hybrids, were excluded because the leaf protoplasts lacked ability to divide in any

medium. For protoplast fusion 50 % PEG was more effective than the 25 % PEG, but it is unknown whether the high concentration of PEG is harmful to the viable protoplasts.

The hybrid protoplasts and nucellus protoplasts divided within 7–10 days of culture in the 8p medium containing 0.2 mg/l 2,4-D, 1.0 mg/l NAA and 0.5 mg/l BAP, while the single leaf protoplasts and fused homokaryotic leaf protoplasts could not divide. The divided

hybrid cells were identified because they had the green chloroplasts (Fig. 2-D). However, during continuous cell division, the green chloroplasts disappeared (Fig. 2-E) and at the stage of colonies the hybrids were not identified at all (Fig. 2-F).

The colonies were then transferred and formed calli on MS medium supplemented with 0.1 mg/l each of IAA and kinetin. We are now trying to analyse by isozymes whether the calli are hybrids. If we can find the hybrid calli, those can be expected to regenerate because of nucellus-derived calli keeping their embryogenetic ability. This, however, has not yet been realized.

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リンゴのカルスからの器官形成及びプロトプラストの単離、培養及び細胞融合

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摘 要

リンゴ, *Malus pumila* Mill. の珠心由来カルスの再分化条件について検討した。さらに、リンゴの細胞融合による体細胞雑種作出のために、珠心カルス及び培養シュートの葉からプロトプラストを単離、培養し、両者の細胞融合を行った。その結果の概要を以下に示した。

1. 満開後 50 日に採取した品種 'ふじ', 台木 'CG 80' の珠心由来カルスから低頻度であるが、不定胚が形成された。これらの不定胚は 0℃ の暗所で 6 カ月間処理後、25℃ の明所に移したところ、発芽シュートを形成した。

2. 珠心カルス及び培養シュートの葉からプロトプラストを単離するのに最適な酵素液組成は 2% Cellulase Onozuka R-10 + 0.1% Pectolyase Y-23 又は、2% Cellulase Onozuka RS + 0.1% Pectolyase Y-23 であった。

プロトプラスト収量は珠心カルス、培養シュートともに、新鮮培地に移植後 16~20 日のもので最大となった。

しかし、台木 'M. 26' の培養シュートでは培養後 21~30 日のものの方が収量が多かった。

プロトプラストの単離に適した酵素処理時間は 3~4 時間であった。

単離されたプロトプラストは植物生長調節物質を含む 8 種の培地で、培養 60 日後にはコロニー形成した。

3. 珠心カルスと培養シュートの葉から単離したプロトプラストを融合した。雑種プロトプラストは緑色で、大きさは 40~50μm であった。雑種プロトプラストと透明な珠心カルスプロトプラストは培養後 7~10 日に細胞分裂を開始したが、葉肉プロトプラストは細胞分裂しなかった。雑種プロトプラストは数回分裂した時点では、葉肉プロトプラスト由来の緑色の葉緑体が確認できた。しかし、コロニー形成の過程で、緑色の葉緑体は徐々に消失した。コロニーはカルスまで生長したが、雑種かどうか判定できなかった。