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Plant regeneration from meristem-derived callus protoplasts of apple (*Malus* × *domestica* cv. 'Fuji')

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Abstract A procedure has been established for regeneration from meristem-derived callus protoplasts of scion cultivars of apple that have been difficult to regenerate from leaf protoplasts. Calli were induced from the meristem of apples, *Malus*×*domestica* cvs 'Fuji' and 'Jonagold' and *Malus prunifolia* var 'ringo Asami Mo84-A', cultured on MS medium (2 mg/l 2,4-D, 1 mg/l BA, 0.8% agar) and subcultured in a liquid medium. The ability to regenerate plants from suspension calli was studied under eight different combinations with respect to IAA, ABA, and TDZ concentrations. With the materials studied here, two combinations, one with 0.1 mg/l IAA, 0.1 mg/l ABA, and 2.0 mg/l TDZ and another with 0.1 mg/l IAA, 1.0 mg/l ABA, and 2.0 mg/l TDZ, were effective for plant regeneration. Protoplasts were isolated from the above suspension cultures and then cultured in KM8P medium containing IBA (2 mg/l), BA (1 mg/l), 2,4-D (0.4 mg/l), and MES (5 mM, pH 5.7). Shoot formation of protoplast-derived calli was studied in the above-mentioned regeneration media. The high concentration of Gelrite (0.5% and 0.7%) was also shown to be important for shoot formation of protoplast-derived calli. Shoot primordia were formed in the medium containing IAA (0.1 mg/l), ABA (1.0 mg/l), and TDZ (2.0 mg/l). Ultimately, five regenerants of 'Fuji' protoplasts were obtained from 200 protoplast-derived calli.

Key words Apple · *Malus*×*domestica* · Plant regeneration · Protoplast culture

Abbreviations *ABA* Abscisic acid · *BA* 6-benzyladenine · *2,4-D* 2,4-dichlorophenoxyacetic acid · *IAA* 3-indole acetic acid · *IBA* indole butyric acid · *MES* 2-morpholinoethanesulfonic acid monohydrate · *TDZ* thidiazuron (N-phenyl-N′,2,3-thidiazol-5-ylurea) · *KM8P* Kao and Michayluk (1975) · *MS* Murashige and Skoog medium (1962)

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Introduction

Improvements in apple production, such as in fruit quality, yield, and disease resistance, have been generally achieved through conventional cross-breeding. However, F_1 individuals containing the desirable traits of both parents are not easily obtained because apples are highly heterozygous and have a long juvenile period. New possibilities for improving apples may be provided by protoplast technologies involving somatic hybridization, somaclonal variation, and transformation. There are only a limited number of reports on apple regeneration from protoplasts, and these are restricted to a few varieties. To date, there have been reports of successful regeneration from the leaf protoplasts of two rootstocks and one scion genotype (Patat-Ochatt et al. 1988) and another columnar scion genotype (Wallin and Johansson 1989), and from the protoplasts of seedling leaves from four scion varieties (Perales and Schieder 1993). Patat-Ochatt et al. (1993) also produced haploid clones from stem protoplasts of 'Golden Delicious' apple. However, the availability of methods using leaves or stems are very limited for special genotypes in apples. Previously, we tried to regenerate plants from protoplasts of 'Fuji', the most popular apple cultivar in Japan, using leaves or stems as starting materials; however, we failed to obtain regenerants from them.

In the case of cereal and legume plants, calli from immature embryos or seeds are generally used as materials for regeneration from protoplasts, since regeneration from leaf protoplasts is very difficult in these plants (Ozias-Atkins and Vasil 1982; Luo and Jia, 1998). Besides cereals and legume plants, regeneration from leaf protoplasts is limited to special genotypes in some plant species. In woody plants, regeneration from protoplasts of non-hybrid species of poplar was only successful using suspension calli of seeds (Qiao et al. 1998), although regeneration from

leaf mesophyll protoplasts of hybrid species had been achieved earlier (Russell and McCown 1986). Recently, Ai-Ping et al. (1995) reported regeneration from protoplasts derived from cotyledons and ovule-derived suspension cells of apple. While calli derived from immature embryos and seeds are good materials for regeneration from protoplasts, apple breeding is problematic in that regenerants from seed calli, as in the case of conventional cross breedings, no longer carry traits identical with those of the parents. An alternative material seems to be the meristems of shoot apices. Apical buds and nodal segments have been demonstrated to be useful materials for mass propagation in woody plants, since such explants have a high regenerative ability (Deshpande et al. 1998; Kaur et al. 1998; Kumar et al. 1998; Quraishi and Mishra 1998). In apple, apical meristems have also demonstrated a high regeneration rate (Saito et al. 1989, 1997), and their traits are identical with parents. Moreover, calli derived from meristem maintained a high regeneration activity for several months. As for regeneration from protoplasts of lawngrass, calli from the apical meristem were recently used instead of seed-derived calli (Inokuma et al. 1998). In this report, we present the successful regeneration of protoplasts of calli induced from the meristem of scion cultivars of apple.

Materials and methods

Plant materials and callus induction

Three different scion cultivars (*Malus*×*domestica* cvs 'Fuji' and 'Jonagold') and two rootstocks (*Malus prunifolia* var 'ringo Asami Mo84-A' and M.9) were used in this experiment. Meristem tissue from the shoot apex was axenically isolated and cultured on the callus induction medium [MS; 2 mg/l 2,4-D, 1 mg/l BA, 0.8% Bacto-Agar (Difco), pH 5.7] at 25°C in darkness. Calli were induced from the tissue during a 60-day culture. Induced calli were subcultured in liquid medium (MS; 0.4 mg/l 2,4-D, 2 mg/l IBA, 1 mg/l BA) at 25 °C in the light. The suspended calli were occasionally filtrated with approximately 1 mm diameter stainless mesh to collect active young cells (Tsugawa et al. 1998) and were subcultured every 2 weeks.

Regeneration from meristem-derived calli

Regeneration capability of the calli was studied. Ten calli per petri dish were transferred to new media containing various combinations of plant growth regulators and gelling agents. Standard conditions were as follows: calli were transferred to shoot regeneration media (MS; 0.1–1.0 mg/l IAA, 0.1–1.0 mg/l ABA, 0.2–2.0 mg/l TDZ, 0.3% Gelrite, pH 5.7) at 25 °C in the light. Adventitious buds appeared during the 60-day culture period, and these calli were then cultured for 1 month more in the same medium. The calli were subsequently transferred to shoot proliferation medium (MS; 1 mg/l IBA, 0.8% Bacto-Agar, pH 5.7) at 25 °C in the light for 1–2 months. Proliferated shoots were transferred to rooting medium (MS; 1 mg/l IBA, 0.8% Bacto-Agar, pH 5.7). Plantlets were regenerated after 2–3 months followed by acclimatization. Mature plants were cultivated in pots containing vermiculite and soil.

Protoplast isolation and culture

Protoplasts were isolated from a suspension culture of cv 'Fuji'. After the calli were washed with new medium, 0.5 g of the calli was incubated in an enzyme solution (pH 5.7) containing 2% Cellulase Onozuka RS, 0.1% Pectolyase Y-23, 0.7 M mannitol at 25 °C with gentle shaking (120 rev/min) for 2 h. Protoplasts were washed with 0.7 M mannitol containing 5 mM MES (pH 5.7); thereafter, adequate amounts of protoplasts (10^6/ml) were cultured in KM8P medium (pH 5.7) containing 2,4-D (0.4 mg/l), IBA (2 mg/l), and BA (1 mg/l) at 25 °C in darkness for 7 days. New medium was then added to the protoplasts, which were subsequently cultured in the light. After a 60-day culture period, protoplast-developed micro-calli were transferred to shoot regeneration medium. Regeneration was carried out as described above.

Table 1 Shoot regeneration from meristem-derived calli under different plant growth regulator conditions

Plant growth regulator concentration (mg/l)[a]			Frequency of calli forming adventitious buds[b,c] (%)		
IAA	ABA	TDZ	Apple cultivar		
			Fuji	Jonagold	Mo84-A
0.1	0.1	0.2	0[a]	0[a]	0[a]
0.1	0.1	2.0	5[a]	5[a]	55[b]
0.1	1.0	0.2	0[a]	0[a]	0[a]
0.1	1.0	2.0	5[a]	15[b]	60[b]
1.0	0.1	0.2	0[a]	0[a]	0[a]
1.0	0.1	2.0	0[a]	0[a]	5[a]
1.0	1.0	0.2	0[a]	0[a]	0[a]
1.0	1.0	2.0	0[a]	0[a]	15[a]

[a] MS medium containing the above combinations and concentrations of plant growth regulators supplemented with 3% sucrose and 0.3% Gelrite was used

[b] Twenty calli were transferred to each medium

[c] Means followed by the same letter are not significantly different from each other at the 5% level. Each value is the mean of two replicates

Results and discussion

Regeneration of meristem-derived calli

The regeneration of calli derived from the meristem of two scion cultivars ('Fuji', 'Jonagold') and one rootstock cultivar ('Mo84-A') was studied prior to protoplast regeneration. The calli were transferred to shoot regeneration media under eight different conditions with respect to combinations of plant growth regulators (Table 1). Regenerants were obtained from these apple calli with the combinations of (1) IAA (0.1 mg/l), ABA (0.1 mg/l), and TDZ (2.0 mg/l) and (2) IAA (0.1 mg/l), ABA (1.0 mg/l), and TDZ (2.0 mg/l). Regeneration ability was highest in 'Mo84-A', but a high regeneration rate was not observed in the scion cultivars of 'Fuji' and 'Jonagold'. The addition of 2.0 mg/l TDZ to the culture medium was very effective for shoot formation but, in contrast, no shoot formation occurred at a low concentration of TDZ (0.2 mg/l) in any of the apple calli tested (Table 1). Other cytokinins such as BA could not induce adventitious bud formation (data not shown). The results are in good agreement with those obtained with apple leaf protoplasts, where TDZ induced the promotion of protoplast division (Wallin and Jo-

Table 2 Differentiation of bud-like structures from protoplast-derived calli under different plant growth regulator conditions (scion cv 'Fuji')

Plant growth regulator concentration (mg/l)[a]			Frequency of calli with greenspots[b,c] (%)	Frequency of calli forming adventitious bud-like structures[b,c] (%)
IAA	ABA	TDZ		
0	0	0	8.0[a]	0[a]
0.1	0.1	0.2	12.5[a]	0[a]
0.1	0.1	2.0	33.0[c]	11[c]
0.1	1.0	0.2	12.0[a]	0[a]
0.1	1.0	2.0	30.5[c]	3[b]
1.0	0.1	0.2	16.0[a]	0[a]
1.0	0.1	2.0	20.5[b]	0[a]
1.0	1.0	0.2	24.0[b]	0[a]
1.0	1.0	2.0	27.5[c]	0[a]

[a] MS medium containing the above combinations and concentrations of plant growth regulators supplemented with 3% sucrose and 0.3% Gelrite was used
[b] Two hundred calli were transferred to each medium
[c] Means followed by the same letter are not significantly different from each other at the 5% level. Each value is the mean of ten replicates

hansson 1989) and a high frequency of shoot formation (Perales and Schieder 1993). When a high concentration of IAA was added to the medium, there were very few regenerants even in 'Mo84-A'.

Differentiation from protoplasts of the scion cultivar 'Fuji'

Scion cultivars were more difficult to regenerate than rootstock cultivars (Table 1). Moreover, the regenerative activity from calli differed among cultivars (Perales and Schieder 1993). We had tried earlier to regenerate leaf protoplasts of the scion cultivar 'Fuji' but failed to achieve regeneration, although we did obtain a quantity of viable protoplasts. Ai-Ping et al. (1995) reported that in vivo-produced leaves are the worst protoplast source irrespective of genotype, and they used cotyledon-derived suspension calli instead. Cotyledon, however, is one of the seed-derived tissues, that shows a genetic background different from that of the parents. In this work we used meristem-derived calli as the source material of protoplasts to avoid such a problem. We were able to isolate a high yield of viable protoplasts (approximately 1×10^7 protoplasts/g FW) from scion cultivars of 'Fuji'. Thus, we tried to regenerate protoplast-derived calli using the conditions that were optimum for the regeneration of meristem-derived calli. Protoplast culture was carried out in KM8P medium because it had been reported to be the best for the stimulation of protoplast growth and microcalli formation of apple protoplasts (Ai-Ping et al. 1995). Although many greenspots were observed with the addition of 2.0 mg/l TDZ, complete adventitious buds did not appear among them. However, the formation of adventitious bud-like structures was observed within 2 months with the combination of 0.1 mg/l IAA, 0.1 mg/l ABA, 2.0 mg/l TDZ and the combination of 0.1 mg/l IAA, 1.0 mg/l ABA, 2.0 mg/l TDZ (Table 2). As for culture temperature, little difference was observed at 20°, 25°, and 30 °C (data not shown); so we continued to culture protoplasts and calli at 25 °C. However, further development of the adventitious bud-like structures into shoots was not observed under these conditions. Improvement of other factors was still necessary for the further differentiation of adventitious bud-like structure.

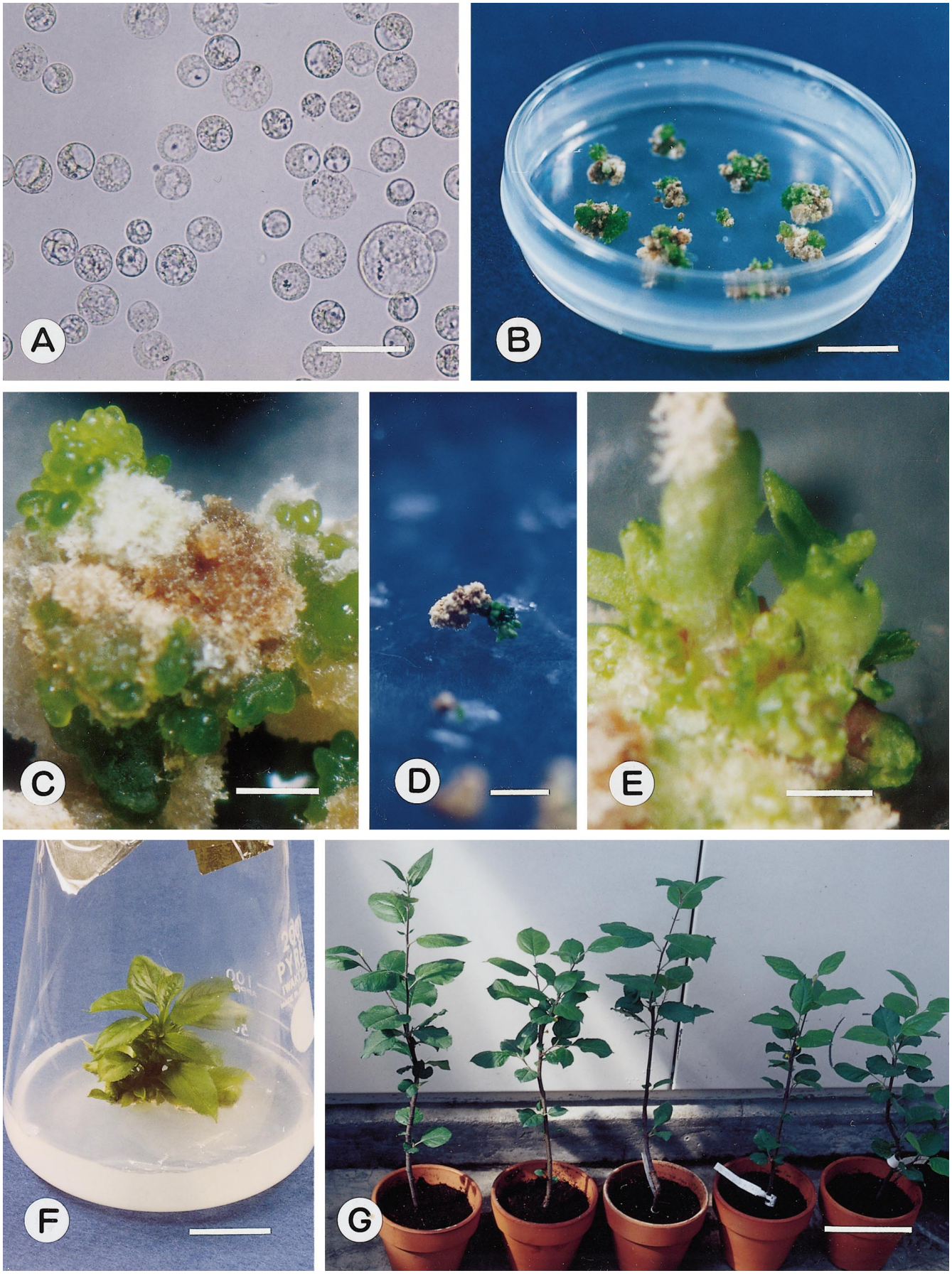
Table 3 Effect of gelling agents and their concentrations on regeneration from protoplast-derived calli (scion cv 'Fuji')

Gelling agents[a]	Concentration (%)	Frequency of calli regenerated[b,c]
Gelrite	0.3	0[a]
	0.5	2[a]
	0.7	3[a]
	1.0	0[a]
Bacto-Agar	0.8	0[a]

[a] MS medium containing 0.1 mg/l IAA, 1.0 mg/l ABA, 2.0 mg/l TDZ, 3% sucrose, and supplemented with the above concentrations of gelling agents was used
[b] One hundred calli were transferred to each medium
[c] Means followed by the same letter are not significantly different from each other at the 5% level. Each value is the mean of ten replicates

Regeneration from protoplasts of the scion cultivar 'Fuji'

During the several trials that we ran to improve the culture conditions, we found that a slight desiccation of the calli on the medium was very effective for the differentiation of adventitious bud-like structures. Thus, we investigated the concentration of gelling agents. Preliminary results showed that a high concentration of Gelrite was effective in inducing calli differentiation (Saito et al. 1997). The Gelrite concentration was also found to be important for further differentiation of protoplast-derived calli (Table 3). After adventitious buds were induced in shoot regeneration media containing 0.3–1% of Gelrite or 0.8% Bacto-Agar, they were transferred to shoot proliferation medium and cultured for 1 month. In this study, we also compared two concentrations (0.1 and 1 mg/l) of ABA for their effects on regeneration from apple calli and protoplast-derived calli. The frequency of regeneration from meristem-derived calli did not differ largely with concentrations between 0.1 and 1 mg/l ABA (Table 1), while 0.1 mg/l ABA induced more adventitious bud-like structures than did 1 mg/l ABA in the protoplast-derived calli (Table 2). However, some of the calli induced with 0.1 mg/l ABA did not develop to complete adventitious buds compared with those induced with 1.0 mg/l ABA. From adventitious buds induced with the combination of 0.1 mg/l IAA, 1.0 mg/l ABA, and 2.0 mg/l TDZ, three and two regenerants (Fig. 1) were ultimately obtained from each group of 100 calli cultured on the media containing 0.7% and 0.5% Gelrite, respectively. In contrast, the bud-like structures ceased to grow any further on the medium supplemented with 0.3% Gelrite. Bacto-Agar, a different type of gelling agent,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could not induce regeneration of protoplast-derived calli of apple even at the high concentration (0.8%). As for another scion cultivar, 'Hokuto', we were able to obtain regenerants from protoplast-derived calli by means of the same procedure (data not shown).

We found the presence and concentration of ABA in the medium to be significant for regeneration. Pretreatment of the leaves in ABA has been reported to stimulate the initial protoplast division (Wallin and Johansson, 1989). In the case of 'Mo84-A', the regeneration frequency was three- to fourfold higher in the medium containing 1 mg/l ABA than in that lacking ABA (Saito et al. 1997). ABA is known to play an important role in physiological desiccation (Meurs et al.,1992) and seed formation from embryos (Hein et al. 1984). In producing regenerants from protoplast-derived calli of apple, there seems to be a correlation between the desiccation of the medium and the presence of ABA, both of which were indispensable for regeneration from apple protoplasts. Ai-Ping et al. (1995) reported that one-third of the differentiated shoots rooted. In our case, all differentiated shoots produced roots and grew to plantlets. Regenerated plants were transferred into soil and cultivated. So far, no differences have been observed in phenotypic appearance among the regenerated plants and the original apple plants.

Perales and Schieder (1993) observed embryo-like structures from apple protoplasts which regenerated whole plants. On the other hand, three groups (Patat-Ochatt et al. 1988; Wallin and Johansson 1989; Ai-Ping et al. 1995) reported that plant regeneration from apple protoplasts took place through adventitious shoot formation followed by root formation. No proembryo-like structures were ever observed during the culture in this experiment. The difference in the pathway of plant regeneration might be due to a difference in culture conditions rather than a difference in scion cultivar because the same scion cultivar was used for two groups, and different pathways of regeneration were observed (Patat-Ochatt et al. 1993; Perales and Schieder 1993).

We have presented here a successful protocol for regenerating apple plants from meristem-derived calli that maintain the genetic background of the original plants and for overcoming the difficulties formerly faced in attempting to regenerate plants from leaf protoplasts. This procedure opens a way for protoplast fusion between apples which could not be crossed each other. At present we actually do have fused calli of *Malus prunifolia* var 'ringo Asami Mo84-A' and dwarf rootstock M.9.

Fig. 1A–G Plant regeneration from protoplast-derived calli (cv 'Fuji'). **A** Isolated protoplasts from cell suspension (*bar:* 20 μm), **B** transferred protoplast-derived calli on regeneration medium after 1 month of culture (*bar:* 2 cm), **C** adventitious bud-like structure formed from protoplast-derived calli after 1–2 months of culture (*bar* 1 mm), **D** whole appearance of adventitious bud-like structures in callus (green parts) (*bar:* 1 cm), **E** adventitious buds formed from protoplast-derived calli after 2 months in culture (*bar:* 2 mm), **F** shoot proliferation from adventitious buds after 1–2 months of culture on shoot-proliferation medium(*bar:* 2 cm), **G** regenerated plants 2 years after protoplast isolation (*bar:* 15 cm)

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