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Regeneration of Fertile Barley Plants from Mechanically Isolated Protoplasts of the Fertilized Egg Cell

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A simple procedure is described for the mechanical isolation of protoplasts of unfertilized and fertilized barley egg cells from dissected ovules. Viable protoplasts were isolated from ~75% of the dissected ovules. Unfertilized protoplasts did not divide, whereas almost all fertilized protoplasts developed into microcalli. These degenerated when grown in medium only. When cocultivated with barley microspores undergoing microspore embryogenesis, the protoplasts of the fertilized egg cells developed into embryo-like structures that gave rise to fully fertile plants. On average, 75% of cocultivated protoplasts of fertilized egg cells developed into embryo-like structures. Fully fertile plants were regenerated from ~50% of the embryo-like structures. The isolation-regeneration techniques may be largely genotype independent, because similar frequencies were obtained in two different barley varieties with very different performance in anther and microspore culture. Protoplasts of unfertilized and fertilized eggs of wheat were isolated by the same procedure, and a fully fertile wheat plant was regenerated by cocultivation with barley microspores.

INTRODUCTION

The egg cell of the higher plants resides in the female gametophyte, the embryo sac. The embryo sac is surrounded by ovule and ovary tissues, and access to plant egg cells is therefore difficult in comparison to egg cells in animals. Embryo sac formation, fertilization, and early embryogenesis in the higher plants have been characterized in great detail at the structural and cytochemical level (for review, see Huang and Russell, 1992). Significant progress has also been obtained by identifying several mutations affecting embryo development in plants, such as Arabidopsis (Mayer et al., 1991, 1993), rice (Kitano et al., 1993), and maize (Clark and Sheridan, 1991). Studies on fertilization and zygote development in the brown alga *Fucus* (for review, see Goodner and Quatrano, 1993) may also prove to be very relevant for plant experimental embryology.

The lack of techniques for isolating the viable unfertilized and fertilized eggs has been a major restriction for an experimental approach to fertilization and zygote development in plants. Likewise, experimental studies on the early studies of embryogenesis have not been possible due to inadequate culture techniques for the isolated egg cells and young embryos. In the last few years, major progress has been made in the isolation of plant gametes (reviewed in Dumas and Mogensen, 1993). Simple and efficient techniques have been developed for the isolation of viable sperm cells from pollen of a variety of plant species (reviewed in Chaboud and Perez, 1992). Several studies have also addressed the isolation of the embryo sac and its individual cells by enzyme maceration and micromanipulation (Zhou and Yang, 1983; Hu et al., 1985; reviewed in Theunis et al., 1991; Huang and Russell, 1992). However, in several of these cases, embryo sac isolations were restricted to fixed material, allowing for only structural or cytochemical and immunochemical observation. Prolonged enzymatic treatments of ovules and embryo sacs have allowed for the liberation of protoplasts of the individual cells of the embryo sac in a few species (Hu et al., 1985; Huang and Russell, 1989; van Went and Kwee, 1990; Huang et al., 1992; van der Maas et al., 1993). In general, the viability of these cells was reported to be poor, and prolonged enzyme treatment apparently had a negative effect on the viability of the cells.

Maize constitutes the first plant in which an in vitro fertilization system has been established that functions well. Highly viable protoplasts of the unfertilized egg cell were isolated by micromanipulation from enzymatically digested ovules (Kranz et al., 1991; Faure et al., 1992). The protoplasts could be electrofused to isolated sperm cells with a high frequency (Kranz et al., 1991; Faure et al., 1993), and the fusion products were regenerated to plants by cocultivation with a nonmorphogenic maize cell suspension (Kranz and Lörz, 1993). The hybrid nature of the regenerants was verified by genetic analysis.

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This study presents efficient techniques for the isolation and regeneration of barley egg cells. We showed that highly viable protoplasts of the unfertilized and the fertilized egg cell can be isolated with a high frequency by a simple dissection of the ovule. Although unfertilized egg protoplasts did not develop, healthy plantlets could be regenerated from fertilized egg protoplasts by cocultivation with barley microspores undergoing embryogenesis and grown into fully fertile plants.

RESULTS

Isolation of Protoplasts

The morphology of the ovule and the egg apparatus, as seen in transmitted light through a dissection microscope, is shown in Figures 1A to 1C. In ovules where both integuments were present, the egg apparatus was difficult to recognize, but it could easily be identified if the two integuments were removed from the ovule tip (P.B. Holm, S. Knudsen, P. Mouritzen, D. Negri, F.L. Olsen, and C. Roué, manuscript submitted). The polar nuclei, which are associated with the unfertilized egg, could also be identified. As described by Engell (1989), the polar nuclei move away from the egg a few minutes after the fertilization of the egg; this provides an excellent internal control indicating that fertilization has taken place. It was also possible under these conditions to see if the egg had collapsed, which occasionally occurred during the processing of the ovary.

The initial step in the isolation of the protoplasts was to puncture the central cell's giant vacuole by inserting one fine-pointed leg of a pair of forceps. This resulted in a partial collapse of the central cell. The persistent synergid and particularly the fertilized egg cell are pyriform and associated with their narrow end to the nucellus cell layers in the micropyle region (Figure 1D). The sudden change in pressure in the central cell caused the egg cell and often the persistent synergid to loosen their adhesion to the nucellus layer and their own cell wall and become spherical (Figure 1E). Generally, the micropylar end of the ovule was gently tabbed by the forceps to further ease the liberation of the protoplasts. In situ, the egg cell showed a distinct polarity, with the nucleus being located in the bulbous part of the egg facing the chalaza, whereas the vacuoles were primarily found at the other end facing the micropyle (Figure 1D). This polarity was also apparent in egg protoplasts isolated immediately after the puncturing of the vacuole. In general, the dissected ovules were placed for 30 min on Kao 90 (see Methods) droplets to stabilize the protoplasts. After this period, the polarity of the egg was less pronounced because the nucleus tended to be more centrally located in the cell while the vacuoles were placed in the outer layer of the cytoplasm (Figure 1E). The organelles were primarily located around the nucleus. After removing the few nucellus cell layers of the micropylar region, the egg protoplasts could be extruded by applying gentle pressure on the ovule with the forceps (Figures 1C and 1F). Occasionally, synergid protoplasts were also isolated, in particular from unfertilized ovaries. These were readily distinguishable from the egg protoplasts due to their small peripherally located nuclei and very vacuolated cytoplasm (Figure 1D). In a few cases, the central cell was also extruded as a protoplast.

Protoplasts of unfertilized and fertilized eggs were isolated with the same frequencies, and there was no difference with respect to the protoplast isolation frequencies in the two cultivars used. Approximately 20 ovaries were dissected per spike, and on average protoplasts could be isolated from 75% of these, that is, ~15 protoplasts were isolated per spike. The entire sterilization, dissection, and isolation procedure took ~90 min per spike. Protoplasts of the fertilized egg cell could be isolated until shortly before the first division of the zygote. Occasionally, protoplasts containing nuclei in mitotic prophase were isolated, but after the division of the zygote, it was no longer possible to obtain protoplasts. In a few cases, dyads with intact walls were isolated. These structures degenerated rapidly.

The extruded cells appeared to be genuine protoplasts. Thus, it was not possible to identify any fluorescent wall material around the protoplasts after staining with Calcofluor white (Poly-Sciences, Inc.). Occasionally, a few patches of fluorescent materials were identified at the periphery of the cells, but these appeared to reflect a secondary adhesion of wall fragments from other cells binding to the surface of the protoplast after the isolation. Preliminary observations using transmission electron microscopy have also failed to reveal a wall or wall remnants around the egg protoplasts (H.L. Mogensen and P.B. Holm, unpublished observation).

The protoplasts of fertilized and unfertilized eggs were remarkable because of their stability at low osmotic pressure. The osmotic pressure of Kao 90 was 350 to 375 mosmol/kg H_2O, and at this pressure, the protoplast diameter remained approximately constant. Minor variations were encountered and depended on, for example, the condition of the spike or the plant or the time of watering of the plant relative to the harvest of the spike. Viable and regenerable protoplasts could also be isolated in Kao 60 (60 g/L maltose, 250 mosmol/kg H_2O) and Kao 120 (120 g/L maltose, 525 mosmol/kg H_2O), although they responded to the changes in osmolarity by a slight swelling in the Kao 60 medium and a slight shrinkage in the Kao 120 medium. The divalent cation concentration, in particular the Ca^{2+} concentration, was essential for the stability of the egg protoplasts. In Kao 90 medium without Ca^{2+} and Mg^{2+}, protoplasts could still be isolated, but they appeared to lack a plasmalemma. Under the interference contrast optics, we detected no distinct boundary between the cell and the surrounding medium. These cells were readily perforated by a microinjection needle, whereas protoplasts isolated in Kao 90 medium (Ca^{2+}, 3 mM; Mg^{2+}, 1.5 mM) often showed considerable resistance to perforation. The presumed membraneless cells degenerated shortly after isolation. An increase in the Ca^{2+} concentration of the Kao 90 medium to 5 mM changed the morphology of the protoplast to a more opaque and apparently more compact form. Under all isolation conditions

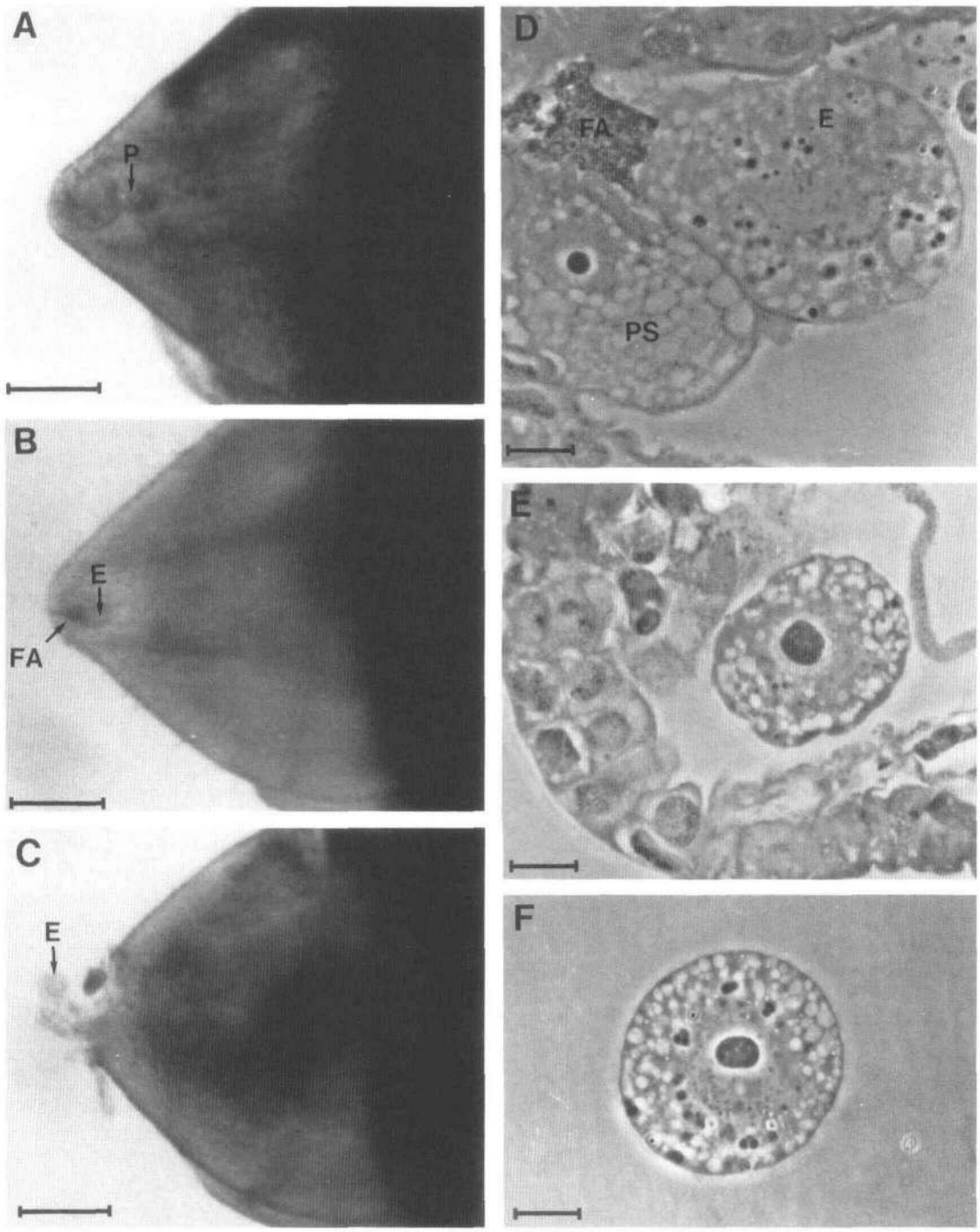

Figure 1. Isolation of Egg Protoplasts from Barley Ovules.

(A) Dissected ovary showing the tip of an unfertilized ovule. P, polar nuclei. Bar = 100 μm.
(B) Fertilized ovule. E, egg cell; FA, filiform apparatus. Bar = 100 μm.
(C) Ovule with extruded egg protoplast (E). Bar = 100 μm.
(D) Section of egg cell (E) in intact ovule. PS, persistent synergid; FA, filiform apparatus. Bar = 10 μm.
(E) Section of egg protoplast liberated by puncturing the central cell. Bar = 10 μm.
(F) Section of extruded egg protoplast. Bar = 10 μm.

tested, the egg protoplasts were sticky and adhered to untreated plastic and glass surfaces. Adhesion was less strong to plastic dishes treated for protoplast culture or coverslips (see Methods) that had not been cleaned by organic solvents, but only sterilized by autoclaving. The only way to completely prevent adhesion to the surface was to embed the protoplasts in agarose. Under these conditions, the protoplasts could also be readily microinjected. Preliminary data using the technique of Ansorge (1982) showed that ~70% of the protoplasts survived the injection, and of these, more than 50% continued their development beyond the microcallus stage.

Cultivation of Protoplasts in Medium Only

Protoplasts of fertilized eggs cultured in medium only divided a few times and formed a spherical microcallus, after which the cells developed large vacuoles and degenerated. In one experiment, ~40 protoplasts were isolated from the barley cultivar Igri 8 and 16 hr after pollination, then cultured on coverslips in 1 mL of Kao 90 medium. All the protoplasts divided a couple of times over the next 6 to 8 days and formed a microcallus, after which they degenerated. A similar experiment was performed using agarose-embedded protoplasts. In this instance, the cells were also unable to pass the microcallus stage. In several other control experiments, in which protoplasts were isolated at different intervals after pollination and cultured in Kao 60 or Kao 90, the microcalli degenerated after a few days. In some cases, the medium was supplemented with 1 mg/L 2,4-D.

In a similar series of experiments, protoplasts of unfertilized eggs were cultured in liquid medium or embedded in agarose, and their development was monitored at regular intervals. In no case did protoplasts of unfertilized eggs divide. They remained unicellular and degenerated after a few days in culture.

Regeneration by Cocultivation with Microspores

Regeneration of protoplasts of fertilized barley egg cells into plants was achieved by cocultivation with microspores undergoing embryogenesis. In all experiments performed, the egg protoplasts were placed in Transwell inserts (see Methods) that were submersed in the liquid microspore culture.

Under the cocultivation conditions described below, the protoplasts of the fertilized barley egg developed readily. Three to 4 days after isolation, multicellular globular microcalli had formed (Figure 2B); over the next few days these microcalli developed into bipolar structures (Figure 2C). After 2 to 3 weeks, the protoplast-derived structures often attained an embryo-like shape with a sheet-like scutellum. The outer cell layers consisted of dense cells filled with cytoplasm, whereas more vacuolated cells constituted the inner parts of the structure (Figure 2D). Sometimes the scutellum-like structures were very irregular, and globular proembryos often formed along the rim of the scutellum; in particular, this occurred when culturing the protoplasts in Kao medium supplied with 1 mg/L 2,4-D. In other cases, more torpedo-like or trumpet-like structures formed. Both types of embryo-like structures germinated readily into plantlets that developed into completely normal and fully fertile plants. Structures that were less than 1 mm in diameter after cocultivation for 30 days (Figure 3A) rarely developed further when transferred to regeneration medium or formed calli with a poor regeneration capacity.

The compiled results from five experimental series are given in Table 1. The data show that for both cultivars, Igri and Alexis, ~75% of the protoplasts developed beyond the microcallus stage, and plantlets were regenerated in numbers corresponding to the number of developing structures. In Igri, the regeneration frequency appeared to be slightly higher than in the spring barley cultivar Alexis. In some experiments, all plantlets grew into plants, whereas in other experiments the frequencies were lower. On average, plants formed from ~50% of the plantlets.

Evaluation of Parameters to Develop an Optimal Cocultivation Method

Cocultivation Method

In method A, a 12-mm Transwell insert (No. 3405; Costar Corporation, Cambridge, MA) with the protoplasts was placed inside a 24.5-mm Transwell insert (No. 3418) containing the microspores; the membrane of the 12-mm Transwell insert rested directly on the membrane of the 24.5-mm Transwell insert. In this cocultivation set up, some protoplasts developed, but plants were rarely regenerated (Table 2). In contrast, protoplasts cultured according to method B developed into plantlets with a frequency of 70%. In this case, the 12-mm Transwell insert was immersed in the liquid microspore preparation because it was hanging on the rim of the well in the cluster well plate. This ensured an unimpeded transport of nutrients and other compounds from the microspore preparation to the protoplasts. Protoplasts embedded in 0.75% agarose and cocultivated (method C) developed nearly as fast as protoplasts in liquid medium; however, embedding in 1.5% agarose appeared to impede growth. It is not known if this reflects a mechanical restriction of the development that is imposed by the more compact agarose, or an impediment of the free diffusion of microspore-derived compounds to the protoplast.

Effect of the Length of the Cocultivation Period

Protoplasts of the fertilized egg were isolated from three spikes of Igri 6 hr after pollination and cocultivated according to method B for different periods in Kao 90 plus 1 mg/L 2,4-D. A 5-day-old microspore culture was used. Thereafter, the Transwell inserts with the developing structures were transferred

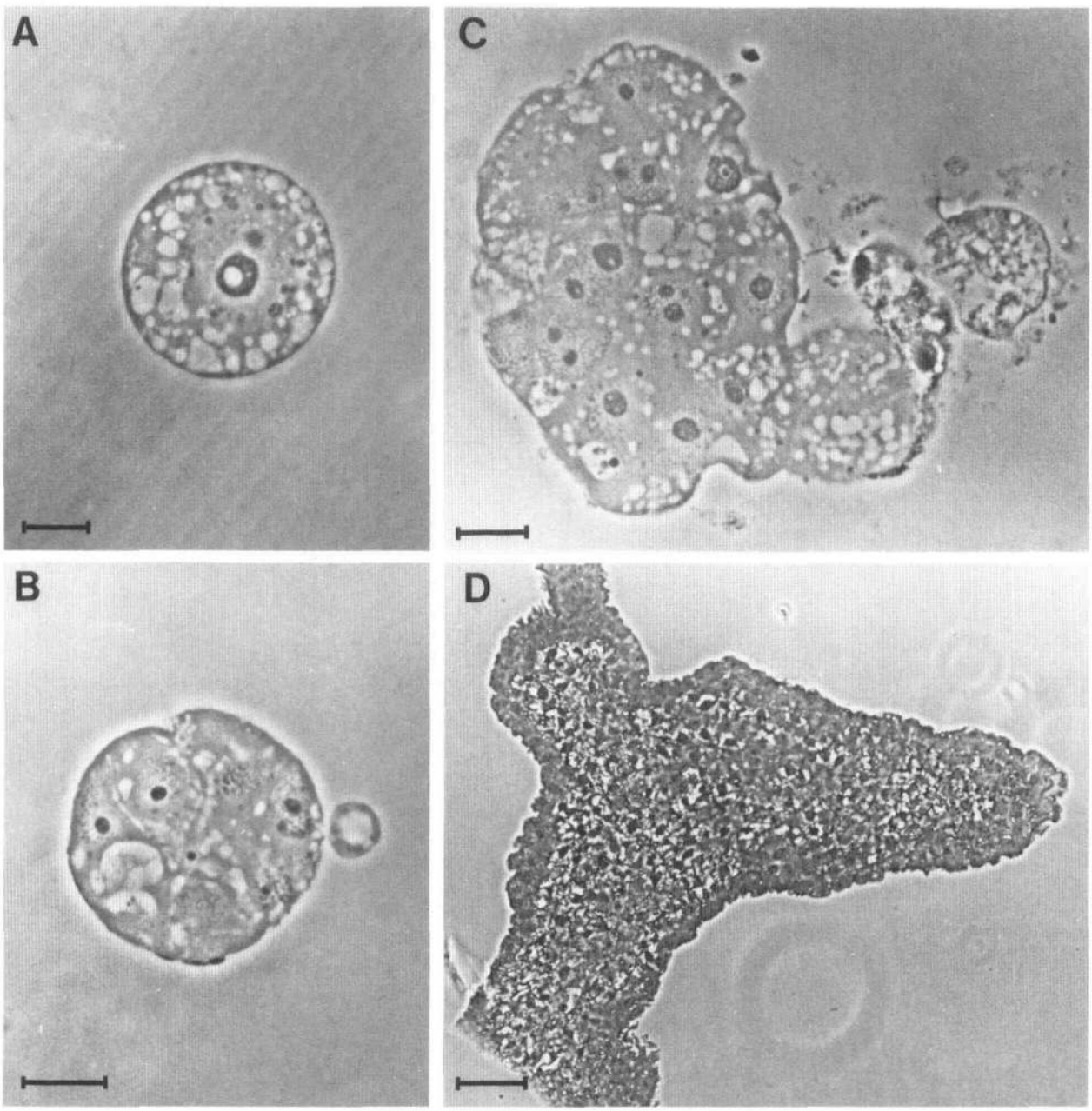

Figure 2. Phase Contrast Photographs of Sections Illustrating Growth and Development of Protoplasts of the Fertilized Egg.

(A) Freshly isolated protoplast. Bar = 10 μm.
(B) Globular protoplast-derived microcallus after 4 days of cocultivation. Bar = 10 μm.
(C) Bipolar protoplast-derived structure after 7 days of cocultivation. Bar = 10 μm.
(D) Developing structure after 18 days of cocultivation. Bar = 500 μm.

to Kao 90 plus 1 mg/L 2,4-D. Throughout this period, the cultures were inspected and photographed at regular intervals. When the structures were ~2 mm in length, they were transferred to Kao 90-agarose for further development and germination (Table 3). Cocultivation for 9 days resulted in very limited growth and development of protoplasts, whereas protoplasts cocultivated for 16 days developed into small, loose callus structures. Cocultivation for 23 and 30 days resulted

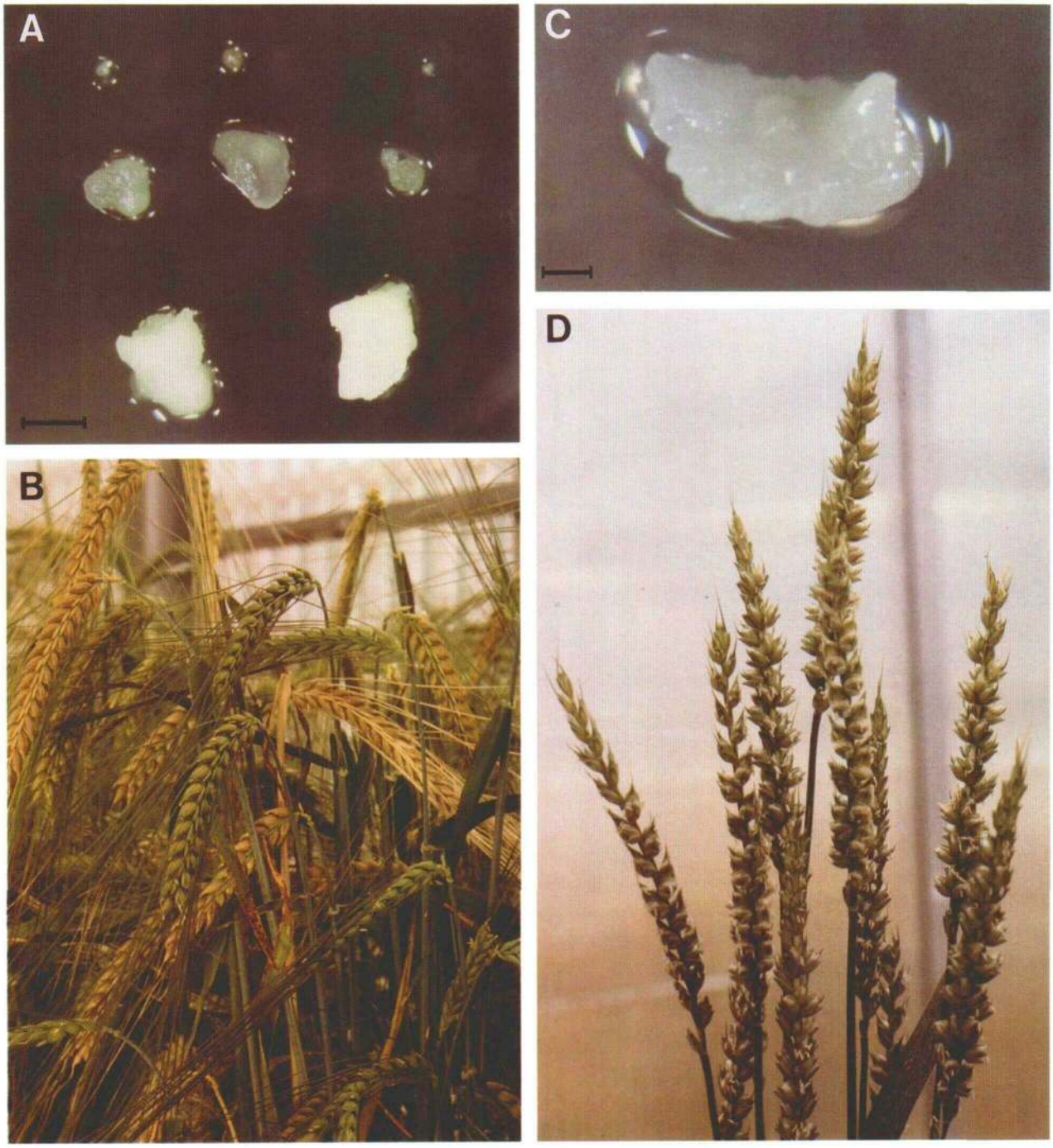

Figure 3. Embryo-like Structures of Barley, Wheat, and Regenerated Plants.

(A) Embryo-like structures of barley. The three globular structures in the upper row were not regenerable. Bar = 1 mm.
(B) Regenerated Igri plants.
(C) Embryo-like structure of wheat. Bar = 1 mm.
(D) Spikes of a regenerated wheat plant.

Table 1. Regeneration Frequencies for Protoplasts of Fertilized Eggs of the Barley Cultivars Igri and Alexis

	Proto-plasts	Developing Structures		Plantlets	
Cultivars	No.	No.	%	No.	%
Igri	56	44	79	46	105
Alexis	40	29	73	22	76

in a high frequency of plant regeneration, but the structures germinated faster into plantlets with 30 days of cocultivation.

Effect of the Age of the Microspore Preparation

Egg protoplasts were isolated from Alexis 8 hr after pollination, transferred to 12-mm Transwell inserts, and cocultured with microspore cultures of different ages (method B). Five to 33 protoplasts were cultured for each time interval. Microspore-derived embryoid cultures older than 21 days were diluted to half the density. On average, 78% of the protoplasts developed beyond the microcallus stage when cocultivated with microspore preparations ranging from freshly isolated microspores to 6-week-old cultures in which embryoids had formed (Figure 4). Most microspore cultures supported the development of the protoplasts to nearly the same extent, but a slightly lower frequency of developing structures was observed for the 1- to 3-day-old microspore cultures.

The morphology of the protoplast-derived structures was, however, very dependent on the age of the microspore culture. When cocultivated with up to 3-day-old microspore cultures, the protoplasts developed into small, loose aggregates in which multiple globular proembryo-like structures formed (Figure 5A). Cocultivation with 5- to 19-day-old microspore preparations resulted in the formation of embryo-like structures with an often irregular scutellum-like sheet of cells and a central axis of cells resembling a coleoptile (Figure 5B). At a later stage, there was also frequent formation of secondary, globular proembryo-like structures budding off from the periphery of the scutellum-like structures. Microspore cultures older than 21 days supported a completely different type of growth; in these cultures, the protoplasts developed into spherical structures (Figure 5C).

The size of these structures also related to the age of the microspore preparation; cocultivations with microspore preparation of increasing age resulted in a reduction of the size of the regenerating structures. These changes in morphology of the protoplast-derived structures correlated to their regeneration capacity, and it was not possible to regenerate plantlets from egg protoplasts in acceptable frequencies using microspore preparations older than 21 days. Plantlets were regenerated in normal frequencies from the cocultivations with microspores younger than 21 days. In most experiments, microspore preparations younger than 14 days were used, but it remains to be seen if the age of the microspore culture within this interval affects the regeneration frequency.

Effect of the Stage of the Zygote

During this study, egg protoplasts were isolated at stages from immediately after fertilization until shortly before the first division of the zygote. By monitoring the development of several hundred cocultivated protoplasts, we determined that the stage of the zygote, that is, the time of isolation after fertilization, did not affect the frequency of developing structures from the protoplasts. There was also no evidence that further development into embryo-like structures and the regeneration capacity of these structures were affected.

Effect of 2,4-D on the Regeneration of Egg Protoplasts

Protoplasts were isolated from cultivars Igri and Alexis 5 to 20 hr after pollination and cocultivated with 3- to 10-day-old microspore preparations in Kao 90 only, or Kao 90 with 1 mg/L 2,4-D. The addition of 2,4-D appeared to promote the formation of globular proembryos and the formation of secondary globular proembryos from the scutellum-like structures. The data in Table 4 show that there was an increase in the number of developing structures when protoplasts were grown in 1 mg/L 2,4-D for both Igri and Alexis. In the latter cultivar, there were also indications that more plantlets may be regenerated when using 2,4-D.

Fertility of the Regenerated Plants

The regenerated plants were indistinguishable from control plants of the same varieties grown from seeds. Albino plants were never observed. The fertility of the plants was measured by counting the number of grains in the first 15 spikes formed

Table 2. Regeneration of Protoplasts of the Fertilized Egg of the Barley Cultivar Igri Using Two Different Cocultivation Principles

Cultivar	Cocultivation Method	Age of Microspore (Days)	Hours after Pollination	No. of Protoplasts	No. of Developing Structures	No. of Plantlets
Igri	A	10	7	10	5	0
Igri	B	10	7	10	8	7

Table 3. Effect of the Length of Cocultivation on the Growth and Regeneration of Protoplasts of the Fertilized Barley Egg (Cultivar Igri)

Cocultivation (Days)	No. of Protoplasts	No. of Developing Structures after 21 Days	No. of Plantlets after 57 Days	No. of Plantlets after 73 Days
9	5	4	0	0
16	5	5	0	0
23	5	5	0	6
30	6	5	7	7

from each plant. In 55 regenerants of Igri, 35% of the spikes had a 100% fertility, 41% had a fertility of 75 to 100%, and 8, 10, and 5% had a fertility of 50 to 75%, 25 to 50%, and 0 to 25%, respectively. For Alexis, the corresponding frequencies from 11 regenerants were 0, 75, 11, 10, and 4%. The fertility of both cultivars was very similar to that observed in seed-grown material, which was cultured at the same time and under the same conditions.

Regeneration of Wheat Protoplast

A feasibility study of wheat egg protoplasts was conducted to assess if the isolation–cocultivation techniques described above could also be applied to other species. Plants of the wheat variety Walter were grown as described for barley. The ovaries were isolated 8 to 24 hr after pollination, sterilized, and further processed as was described for barley. Protoplasts of the unfertilized wheat egg were readily obtained, but it was more difficult to obtain protoplasts of the fertilized egg. The diameter of these protoplasts isolated in Kao 90 exceeded that of barley by a factor of two to three. As for barley, the unfertilized protoplasts were unable to divide, whereas protoplasts of the fertilized egg divided a few times and formed spherical multicellular structures that subsequently degenerated. Ten protoplasts of the fertilized egg were cocultivated with barley microspores as described above. Three of these developed into torpedo-like multicellular structures, whereas a typical wheat embryo was regenerated from the fourth (Figure 3C), which germinated and produced a completely normal, fully fertile wheat plant (Figure 3D).

DISCUSSION

The study showed that it is possible by simple dissections to mechanically isolate protoplasts of the fertilized and the unfertilized egg cell of barley. The same isolation procedure also permitted us to isolate egg protoplasts from wheat. The ease with which the protoplasts could be isolated probably resulted from the fact that the angiosperm egg cell, at least before fertilization, is only partly surrounded by a cell wall. It is thus a general observation from light and electron microscopical analyses of plant egg cells and embryo sacs that a prominent wall is present only between the egg and the synergids in the micropylar region. Toward the chalazal region, the wall gradually becomes thinner, and the bulbous upper part of the egg is only separated from the central cell and the synergids by the plasmalemma (reviewed in Kapil and Bhatnagar, 1981; Huang and Russell, 1992).

Staining with the periodic acid–Schiff's reagent (PAS) has in a few cases revealed material enclosing the individual cells of the egg apparatus. In *Capsella*, the wall extends over the entire cell, but at the chalazal end it is honeycombed with large portions in which no wall is present (Schulz and Jensen, 1968). In barley, Cass and Jensen (1970) reported that when performing the staining on Epon sections, all three cells of the egg apparatus were surrounded by PAS positive material. However, when Engell (1989) used PAS staining of glycol methacrylate-embedded material, stained wall material was found only along the lower two-thirds of the interface between the egg and the synergids. Likewise, in studies on the ultrastructure of fertilization of barley, Mogensen (1982, 1988) observed that only the plasmalemma separated the egg from the degenerated synergid in the region where the sperm nucleus entered the egg.

On the other hand, it is also evident that the developing embryos are surrounded by a complete wall in barley (Norstog, 1972). In a recent study of tobacco in which propane-jet-frozen embryo sacs were analyzed at the ultrastructural level (Huang et al., 1993), a distinct wall was observed around the zygote. However, in tobacco the zygote remains undivided 6 to 7 days after pollination. Together, these observations suggest that in general the unfertilized egg is only delimited in the chalazal region by its plasmalemma. This may be essential for

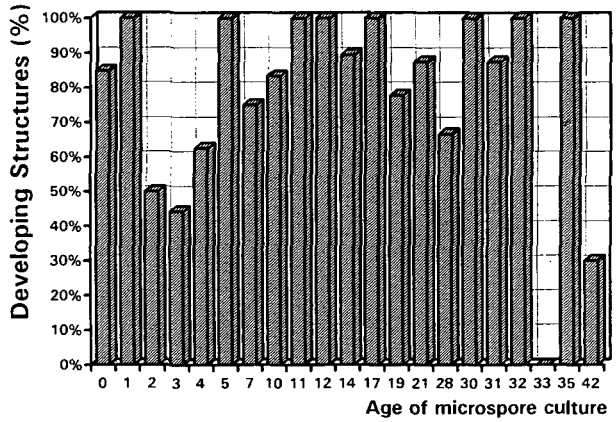

Figure 4. Histogram of the Frequency of Developing Structures.

The histogram illustrates the frequency of egg protoplasts developing beyond the microcallus stage when cocultured with microspores of different age (days after microspore isolation).

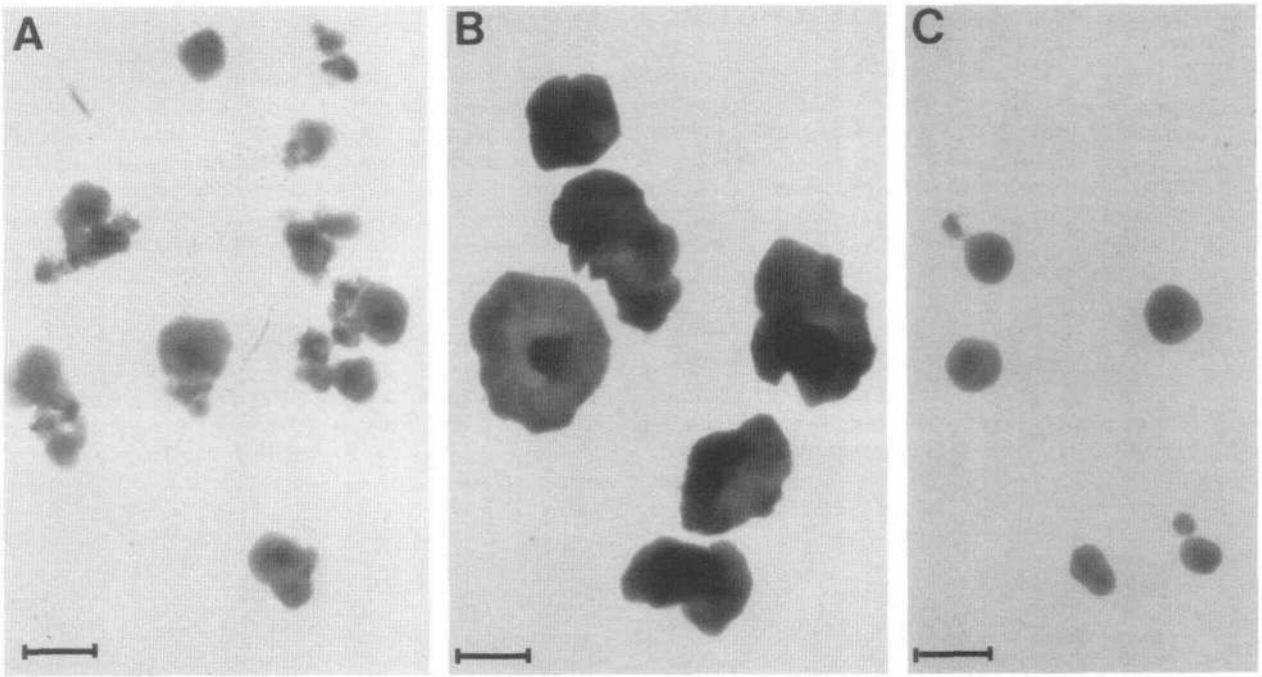

Figure 5. Morphology of Egg Protoplast-Derived Structures as Seen in Transmitted Light in the Dissecting Microscope after 27 Days of Cocultivation.

(A) Cocultivation with freshly isolated microspores. Bar = 500 μm.
(B) Cocultivation with a 14-day-old microspore preparation. Bar = 500 μm.
(C) Cocultivation with a 28-day-old microspore preparation. Bar = 500 μm.

penetration of the sperm nucleus into the egg and perhaps also for sperm and egg cell interaction and recognition. Wall formation in the chalazal part of the egg is accordingly a postfertilization event that may be triggered by the fertilization process itself.

A lack of plasmodesmata between the egg and the synergid and the egg and the nucellus tissue may be a second factor essential for the mechanical isolation of undamaged egg protoplasts. Norstog (1972) reported that plasmodesmata were absent between the barley zygote and the central cell. In other plants investigated, there appears to be no general rule as to the plamodesmata connection between the individual cells of the egg apparatus as well as between these cells and the central cell. In some species, plasmodesmata are present in all walls except the external embryo sac wall, and in others, plasmodesmata are entirely lacking or confined to the walls between synergids (reviewed in Huang and Russell, 1992).

The unfertilized and the fertilized eggs in situ are accordingly largely protoplasts with a pyriform shape. They have few or no plasmodesmata connections to the neighboring cells, and wall materials are confined to the narrow micropylar part of the cell, forming a socket for the protoplast. The sudden change in the turgor pressure of the central cell, achieved by puncturing the vacuole, together with gentle tapping on the micropylar end of the ovule cause the egg to lose its weak adhesion to the socket of wall material; it is then liberated into the central cell as a spherical protoplast.

In vivo and in vitro, the egg cell protoplast is stable at an osmotic pressure only half of that required for stabilizing enzymatically released protoplasts from other tissues. The reasons for this stability are not known but may be attributed to an elaborate cytoskeleton structure that in vivo primarily stabilizes the bulbous upper part of the pyriform egg cell. This may also explain why the egg can be isolated under conditions where it appears to be stripped of its plasmalemma.

In this study, there were no indications for a strong genotype effect on the regeneration of plants from protoplasts of the fertilized egg cell. Likewise, protoplasts isolated immediately after fertilization until the first division appeared to have

Table 4. Effect of 1 mg/L 2,4-D on the Regeneration of Protoplasts from the Fertilized Barley Egg

Cultivar	2,4-D[a]	No. of Proto-plasts	Developing Structures		Frequency of Plantlets per Proto-plast (%)
			No.	%	
Alexis	–	20	12	60	25
Igri	–	30	23	77	67
Alexis	+	20	17	85	85
Igri	+	16	21	131	63

[a] –, absent; +, present.

the same regeneration potential. These conclusions are supported by more detailed analyses of regeneration of the barley zygote in situ using ovule culture (P.B. Holm, S. Knudsen, P. Mouritzen, D. Negri, F.L. Olsen, and C. Roué, manuscript submitted). In these studies, there appeared to be a very limited effect of the zygote stage on the regeneration ability in both Igri and Alexis. In Igri, more ovules responded with the formation of embryo-like structures; this was partly offset by a higher frequency of polyembryony in Alexis. A similar tendency may be seen in our study in which Alexis appeared to respond with a higher frequency of secondary embryo-like structures when cultured in the presence of 2,4-D.

The results obtained in this study are remarkably similar to those reported for the isolation of unfertilized egg cells of maize by Kranz and coworkers (Kranz et al., 1991; Faure et al., 1993; Kranz and Lörz, 1993). The maize protoplasts were isolated at a higher osmotic pressure (540 mosmol/kg H_2O) than the barley protoplasts (350 to 375 mosmol/kg H_2O), but it remains to be determined if this reflects a difference in the stability of the egg. In both species, unfertilized egg protoplasts do not divide. In barley, 75% of the fertilized egg protoplasts developed beyond the microcallus stage, and most of the developing structures continued their development into plantlets. In some cases, more than one plantlet was regenerated from a developing structure. Fifty percent of the plantlets grew into plants.

In maize, 85% of the egg protoplasts fertilized in vitro developed into microcalli, and in a sample of 170 fusion products, 41% were capable of further growth when cocultivated with a nonmorphogenic maize suspension. In a sample of 28 fusion products, 15 developed into globular structures and transition phase embryos, from which 11 plants were regenerated (Kranz and Lörz, 1993). This suggests that the brief enzymatic digestion (30 min) of the maize ovule may not have a major adverse effect on the viability and regeneration capacity of the egg. It is a general observation, though, that prolonged enzymatic digestion for isolating embryo sacs or protoplasts of the individual cells of the embryo sac has a negative effect on cell viability (reviewed in Huang and Russell, 1992). It is also considered likely that mechanically isolated egg protoplasts are superior if the objective is to study gamete surface receptors or other plasmalemma factors essential for gamete recognition and fusion. Ideally, in vitro fertilization should be as close to the in vivo situation as possible, that is, the gametes fuse spontaneously with exclusion of most or all paternal cytoplasm.

For a priori reasons, microspores undergoing embryogenesis may be considered optimal for supporting protoplast regeneration and embryogenesis due to their synchronous embryogenic development. Thus, this study showed that the protoplasts require cocultivation until embryo-like structures have formed. The morphology of the protoplast-derived structures also related directly to the age of the microspore culture when starting the cocultivation. Formation of embryo-like structures with a high regeneration capacity required cocultivation with microspores that were less than 19 days old, while cocultivation with older structures gave rise to globular nonregenerable structures. One possible explanation is that the microspore cultures at all stages excrete compounds promoting a basic type of undifferentiated growth, whereas embryogenesis in the protoplast-derived structures requires an additional set of stimuli from the microspores at an early stage of embryogenesis.

The conclusion that embryogenic microspore cultures of barley are optimal for cocultivation was further supported by experiments in which fertilized egg protoplasts were cocultured with a nonmorphogenic and a morphogenic barley cell suspension. The nonmorphogenic suspension did not support protoplast development, whereas developing structures formed from nearly all protoplasts if cocultivated with the morphogenic suspension. Plantlets were regenerated from these but with a frequency of only one-fifth of that obtained when using microspores (C. Roué and P.B. Holm, unpublished observation). Microspore cocultivations have also allowed regeneration of fertile barley plants from protoplasts isolated from 36-day-old microspore-derived embryoids and promoted development of microspores undergoing embryogenesis from other barley cultivars (F.L. Olsen, K.A. Nielsen, P. Mouritzen, and P.B. Holm, unpublished observation). These studies are in agreement with previous investigations of tomato that show that cocultivation with anthers has a positive effect on callus formation from microspores (Sharp et al., 1972). Culture medium, conditioned by cultivation of anthers of oat, wheat, maize, and tobacco, has also been shown to support callus formation from microspores in barley anther culture (Xu et al., 1981).

However, as shown by Kranz and Lörz (1993), a nonmorphogenic maize suspension could also support embryogenesis in in vitro fertilized maize eggs. Different cell cultures may thus be used for cocultivation of egg protoplasts, but it is considered a major advantage that microspore cultures undergo a synchronous embryogenic development from which particular developmental stages can be used for cocultivation. An additional advantage is that embryo-like development of egg protoplasts is supported in different barley varieties, as well as in wheat. In maize, recent studies showed that regeneration of maize protoplasts derived from suspension cells was only possible when using particular combinations of protoplasts and suspension cell genotypes. In each case the optimal combination had to be established (Petersen et al., 1992).

In summary, we conclude that efficient techniques are now available for the nonenzymatic isolation of protoplasts of the unfertilized and fertilized barley egg and for the regeneration of in vivo fertilized egg protoplasts into normal plants. This may allow experimental studies on fertilization and early embryogenesis in barley and other plants. Cultivation of egg protoplasts may also be used as a very sensitive bioassay for the identification of embryogenesis-promoting compounds, secreted by cell cultures, such as microspores, that are undergoing embryogenesis. We would also like to draw attention to the prospects of these techniques for biotechnology. The techniques outlined above may facilitate the generation of hybrids

or cybrids that cannot be created by conventional crossing or somatic cell fusions. Protoplasts of the egg, fertilized in vivo or in vitro, are also ideal targets for transformation by microinjection. In a variety of animals, very high transformation frequencies have been obtained by microinjection of DNA into the nuclei of the eggs (Brinster et al., 1985; for review, see Palmiter and Brinster, 1986; Gordon, 1989). Transformation by microinjection, thus, does not require the use of reporter genes or selectable genes. This may be of importance when generating transgenic plants for human consumption.

METHODS

Plant Material

Barley plants of the winter barley cultivar Igri and the spring barley cultivar Alexis were grown in growth cabinets at 15°C/10°C day/night temperature using a 16-hr light period at a light intensity of ~350 μE m^{-2} sec^{-1}. The florets were emasculated 3 to 4 days before anthesis and pollinated by hand using a fine-tipped brush.

Media

For cultivation of microspores, a modified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) was used as described by Olsen (1987). In this medium, the NH_4NO_3 concentration is reduced to 165 mg/L, while 750 mg/L of glutamine is added. The medium also contained 1 mg/L 6-benzylaminopurine (BAP) and maltose (60 g/L), which was used as the only carbohydrate source. For the cultivation of egg protoplasts, a medium referred to as Kao 90 medium was used. This medium consisted of the modified MS medium supplemented with the vitamins and organic acids from the Kao medium (Kao and Michayluk, 1975). The vitamins were purchased as a ready-to-use mixture (No. K3129; Sigma). Vitamin-free casamino acids (No. 2238; Merck, Darmstadt, Germany) were added at a concentration of 250 mg/L together with 20 mL/L coconut water (No. 570-5180AJ; Gibco Laboratories) and 90 g/L maltose. The medium was made in double concentration, the pH adjusted to pH 5.8 with 1 N NaOH, and the solution sterilized by filtration (0.22 μm pore size). Solid Kao 90 medium was made by mixing the double concentrated Kao 90 medium with 0.8% autoclaved SeaPlaque agarose (FMC BioProducts, Rockland, ME) in a 1:1 ratio. For regeneration, the MS medium described above was used but containing only 0.4 mg/L 6-benzylaminopurine and 30 g/L maltose. The medium was also solidified with SeaPlaque agarose as described above. Unless otherwise stated, all chemicals were from Merck or Sigma.

Isolation and Cultivation of Microspores

The cultivation of donor plants and the isolation and culture of microspores were performed as described by Olsen (1991) and can be summarized as follows. The spikes were isolated when the microspores had reached the late uninucleate stage; the anthers were removed and placed on 0.3 M mannitol for 3 days at 25°C in the darkness. Microspore isolation was performed by microblending the anthers. The microblended suspension was filtered and washed by centrifugation, and the microspores were resuspended in MS medium. After additional centrifugation, the microspores were resuspended in 5 mL of MS medium. A sample of the microspore preparation was counted in a Burker-Türk hemocytometer, and the preparation was adjusted to a density of 2 to 3 × 10^3 microspores per mL. The microspore preparation was transferred to 24.5-mm Transwell inserts (No. 3418; Costar Corporation, Cambridge, MA; 1 mL per insert) that were placed in Costar 6 well cluster dishes (No. 3406; Costar Corporation) in 2 mL of MS medium. Alternatively, the microspores were pipetted into Costar 12 well cluster dishes for 12-mm Transwells (No. 3405; Costar Corporation; 0.5 mL per well), and MS medium was added to ensure that the liquid microspore culture just covered the bottom of the well. The Costar dishes were sealed with Nescofilm (Nippon Shoji Kaisha Ltd., Osaka, Japan) and incubated at 25°C in the darkness.

Isolation of Protoplasts

The spikes were harvested 1 to 24 hr after pollination and the ovaries carefully removed from the florets and placed on Kao 90 medium. Ovaries from one spike were sterilized for 10 min in 5 mL 10% sodium hypochlorite (No. 23039; BDH, Ltd., Poole, England) with 50 μL of 10% Tween 20 (No. 23039; Merck, Schuchardt, Germany). After two washes in sterile water, the ovaries were transferred to Kao 90 and dissected under a dissection microscope (magnification ×40, transmitted light). The ovaries were placed with the placental side facing downward, and the pedicel and the two lodicules were removed by using a pair of fine-tipped forceps. A cross-section through the ovarian tissue was made with the tip of the forceps at a median position and along the one side down to the micropylar end of the ovary. The tip of the ovary was removed, exposing the micropylar end of the ovule, and the two integuments removed.

The protoplasts were isolated as described in Results. Each protoplast was sucked up in a sterile Gilson pipette tip with 3 μL of Kao 90, and either transferred to liquid Kao 90 medium in a 12- or 24.5-mm Transwell insert or extruded into a droplet of 15 μL of 1.5% ultrapure, low-melting point (LMP) agarose (No. 5517UB; Bethesda Research Laboratories) in Kao 90 on a sterile cover slip. The agarose–Kao 90 solution was maintained at a temperature of 35 to 40°C before being used for the embedding of the protoplasts. When the agarose bead had solidified, a few microliters of additional low-melting point agarose–Kao 90 were in some experiments added to the top of the agarose drop. The cover slip with the agarose beads was placed in a Falcon Primaria organ tissue culture dish (No. 3037; Becton Dickinson, Lincoln Park, NJ) and sealed with Nescofilm. Water was added to the outer chamber to prevent desiccation of the cultures. In such preparations, the growth of embedded protoplasts could be monitored over the following couple of days. Unfertilized egg protoplasts were isolated from unpollinated, emasculated spikes in the same way. In some cases, the protoplasts were also cultured in dishes treated for protoplast culture (Falcon Primaria; No. 3802; Beckton Dickinson) or cover slips (Menzel-gläser; Gerhard Menzel, Braunschweig, Germany) that had not been cleaned by organic solvents but only sterilized by autoclaving.

Cocultivation and Regeneration

Microspore preparations to be used for cocultivation were left in the 24.5-mm Transwell insert, and a 12-mm Transwell insert, containing

the protoplasts, was placed inside (method A). Alternatively, the microspores were transferred to cluster dishes, in which either the entire microspore population of a 24.5-mm Transwell insert was moved to a single well of a six-well cluster dish, or the preparation was divided between the two wells of a 12-well cluster dish. The Transwell inserts containing the isolated protoplasts in Kao 90 were then placed in the culture well (method B). In method C, the protoplasts embedded in agarose were washed down into a 24.5-mm Transwell insert, and the insert was immersed into a liquid microspore culture in a six-well cluster dish. Extra Kao 90 medium was added to ensure that the protoplasts were covered by about 2 mm of medium. The dishes were sealed with Parafilm, wrapped in aluminum foil, and incubated at 23°C. The plates were opened at weekly intervals, and fresh Kao 90 medium was added if the medium level in the inserted Transwell insert was below 2 mm. When the protoplast-derived structures had reached a diameter of 1 to 2 mm, the cocultivation was terminated and they were transferred to 2.5-cm filter paper discs (No. 1004025; Whatman International, Maidstone, England), placed on solid Kao 90 medium in 50-mm Petri dishes (Bibby Sterilin Ltd., Stone, Staffs, U.K.), or cultured in the Transwell inserts in liquid Kao 90 for an additional 1 to 2 weeks. The dishes with the developing structures that were growing on the solid medium were sealed with Nescofilm, wrapped in aluminum foil, and incubated at 23°C. Germinating structures were thereafter transferred to MS regeneration medium and cultured under low-light conditions at 23°C. When additional growth had occurred, the plantlets were moved to fresh regeneration medium in 10-cm-high cylindrical containers (Greiner; Bibby Sterilin Ltd., Stone, Staffs, U.K.). For the winter barley cultivar Igri, the plantlets were transferred to 4°C for 8 weeks for vernalization when large plantlets had formed, filling the container. Thereafter, the plantlets were transferred to soil and cultured in a greenhouse equipped with artificial light and heating/cooling facilities (15/10°C, 16-hr light period).

Light Microscopy

Samples of protoplasts were stained with Calcofluor white (Cellufluor; Polysciences Inc., Warrington, PA) at a concentration of 0.005% in Kao 90 and analyzed for the presence of wall material using a microscope (Axiovert; Carl Zeiss, Oberkochen, Germany) equipped with a XBO 75 lamp, a G365 excitation filter, the FT 395 dichromatic beam splitter, and a LP 420 barrier filter. For the analysis of protoplast development, the egg protoplasts and their derived structures were fixed in 4% glutaraldehyde in 50 mM phosphate buffer, pH 7.0, for 90 min. After three washes in the same buffer, the material was stored in buffer at 4°C overnight and the next day postfixed for 2 hr in 1% OsO_4 in phosphate buffer. After a wash for 15 min in buffer, the cells were dehydrated in a graded ethanol series (30 min in each concentration), washed twice in absolute ethanol, once in propylene oxide, and thereafter infiltrated with Spurr's low-viscosity epoxy resin (Spurr, 1969). The resin was polymerized at 70°C overnight in flat embedding molds. The material was sectioned at 2 to 5 μm thickness with a glass knife, using an ultramicrotome (OmU3; Reichert, Leica AG, St. Gallen, Switzerland). The sections were placed on water droplets on the slides that were baked on a hot plate. Permanent slides were made by mounting in Eukitt (acrylic resin in xylene, O. Kindler GmbH & Co., Freiburg, Germany). The sections were analyzed under phase contrast optics in a microscope (Axioplan; Carl Zeiss, Inc.).

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