Colony formation from mesophyll protoplasts of a cereal, oat

(Avena sativa/cell division/dedifferentiation/mitosis)

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ABSTRACT Differentiated leaf cells of gramineous plants, among them the cereals with their immense importance for human nutrition, are considered extremely recalcitrant to, if not incapable of, reentering the cell cycle. This recalcitrance is related to the poor wound response of the monocots—in contrast to most dicots—and the difficulties encountered in monocot tissue culture. We report here the highly reproducible induction of sustained divisions at high frequency (up to 95%) and colony formation from mesophyll protoplasts of a cereal, oat, demonstrating that—albeit to most earlier evidence—mesophyll cells of a gramineous plant have not irreversibly lost their potential for cell division.

Ever since the beginnings of plant tissue culture, the cereals have received much attention because of their important for human nutrition. Unfortunately, however, species of the Gramineae family are far less amenable to tissue culture than are many species of dicotyledonous plants, especially those of the Solanaceae family. Nevertheless, in spite of many difficulties, almost all of the main approaches used in plant tissue culture have been now shown to be feasible for cereals (1). This includes callus induction from various, not all, parts of the plant and regeneration of plants after establishment of embryogenic callus and suspension cultures. Recently, even the regeneration of mature and transformed plants from protoplasts obtained from embryogenic suspension cultures has been reported (2). However, mesophyll protoplasts of cereals generally have been considered to be incapable of frequent and sustained division (3). This is unfortunate because mesophyll protoplasts—i.e., green leaf cells devoid of their cell walls—represent one of the tools best suited to genetic engineering of plants because of their morphological and genetic uniformity, their availability in high numbers, and the relative absence of mutations compared to callus or suspension cultures (4). Therefore, it is not surprising that the culture of cereal mesophyll protoplasts has been the object of much attention. However, after 15 years of intensive effort (which has seen the screening of numerous species and varieties, protoplast isolation procedures, and culture media, e.g., ref. 5), technical improvements concerning mesophyll protoplast culture of cereals have been few in number. The conclusion accepted by most workers was that mesophyll cells of cereals most probably have lost their totipotency. Here we present results showing that, in contrast to this prevailing view, mesophyll protoplasts of oat are capable of dedifferentiation and of reproducibly reentering the cell cycle. Such cultures can even give rise to small colonies.

MATERIALS AND METHODS

Plant Material. Avena sativa cv. Peniarth was provided by the Institut National de la Recherche Agronomique (INRA), Colmar, France; cv. Alfred was provided by H. Lörz, Max-Planck-Institut für Züchtungsforschung, Cologne, F.R.G.; cvv. Phoenix, Major, Alfred, and Adano were provided by Belljoy Semences, Estrées-St.-Denis, France. Seeds of Alfred thus originated from two different sources. Suspension cultures of Triticum monococcum (6), and rice [Oryza sativa SRA-1 (7)] were maintained as previously published (6, 7).

Protoplast Isolation. Seeds were placed in Vermiculite that had been thoroughly rinsed with water and were allowed to germinate at 70% humidity in a cycle of 16 hr of light at 23°C and 8 hr of darkness at 18°C. Light was provided by two 15-W cool fluorescent tubes at a mean distance of 30 cm (corresponding to 1000 lux at soil level). The basal 4 cm of the leaf blades of the first leaves of 10- to 12-day-old plants was excised and surface-sterilized in 70% ethanol for 30 sec and in 0.06% sodium hypochlorite for 15 min. The tissue was then washed and cut into slices of 0.5–1 mm and incubated for 3 hr at 23°C in 1% cellulase RS and 0.4% Macerozyme R 10 (both Onozuka, from Kinki Yakult, Nishinomiya, Japan) that had been dissolved in 9 volumes of 0.5 M mannitol/0.005 M potassium citrate, pH 5.6, and 1 volume of protoplast culture medium. Protoplasts were released by pipetting them several times into a wide-mouth pipet. The suspension was filtered through a loose 1-cm layer of hydrophilic cotton, and centrifuged for 7 min at 70 × g, and the pellet of protoplasts was washed twice with the same solution that was used to dissolve the enzymes.

Protoplast Culture. Protoplasts were cultured in the dark at 25°C at a density of about 50,000 per ml in a completely defined medium. The culture medium for oat mesophyll protoplasts was modified as described by Imbrie-Milligan and Hodges (8). It contained (in mg/liter): Ca(NO3)2·4H2O (500), KNO3 (125), MgSO4·7H2O (325), KH2PO4 (125), Na2EDTA (7.45), FeSO4·7H2O (5.56), glycine (2), nicotinic acid (5), pyridoxine hydrochloride (0.5), thiamine hydrochloride (0.5), biotin (0.05), folic acid (0.05), myo-inositol (100), ascorbic acid (125), fructose (125), cellobiose (125), mannose (125), ribose (125), xylose (125), sucrose (20,000), (2,4-dichlorophenoxy)acetic acid (2), and MES micromolar of ref. 9; pH was adjusted to 5.6 with KOH. Mannitol was added as osmotic stabilizer up to 650 millimosem/kg of H2O (ca. 0.5 M). The following supplements were also tested: coconut water (GIBCO, 20 ml/liter), peptone (Serva, 200 mg/liter), and proline (Sigma, 700 mg/liter).

Feeder Cultures. Suspension culture cells of T. monococcum or rice were suspended in protoplast culture medium containing 0.4% molten agarose (Sigma type VII) but only half-strength mannitol and were poured in the form of a ring around the wall of a 35-mm Petri dish, thus leaving a well in the middle for culture and observation of the developing protoplasts. In an alternative setup, the embedded feeder cells covered the whole bottom of the Petri dish. A membrane filter (nylon, 0.22-μm pore size; or cellulose nitrate, 0.45-μm pore size) was placed on top of the feeder cells (10), and the oat protoplasts at various stages of development were spread

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onto the filter. This second setup allows no close microscopic observation and thus no estimation of division rates.

**RESULTS**

**Dedifferentiation and Early Development.** An essential prerequisite for obtaining divisions of mesophyll protoplasts of oat (*Avena sativa*, cv. Peniarth) was establishing appropriate strictly controlled growth conditions for the protoplast donor plants. Once these growth conditions were established (for description see *Material and Methods*), sustained divisions at high frequency were obtained in all of the 22 repetitions of the protoplast isolation and culture experiment.

The oat mesophyll protoplasts isolated under these conditions (Fig. 1 a and b) were stable, not undergoing the phenomenon of lysis that is often encountered with meso-

![Fig. 1](image-url)
phyll protoplasts of cereals (12, 13). Of the protoplasts, 75–95% always survived the first 24 hr, and the further daily loss was no more than ca. 1%. The initial development of oat mesophyll protoplasts was similar to that of mesophyll protoplasts of other species (Fig. 1 c–e): First, the chloroplasts gathered around the nucleus, and the protoplast increased in size. A new cell wall was synthesized. Subsequently, cytoplasmic strands were formed, and the nucleus migrated to the center of the cell. Around day 11 of culture, the chloroplasts began to dedifferentiate visibly, as judged by their loss of chlorophyll, and the first incompletely divided cells appeared, which completed division in the following 2 days (Fig. 1f).

These cell plates were frequently undulating and did not appear to be compact. Typically, the two daughter cells tended to separate from each other soon after division. Therefore, the division frequency inevitably will be underestimated as, at a given time, two newly formed daughter cells may already have lost contact with each other, thus being undistinguishable from undivided cells. Around day 13 of culture, as many as 5–33% of the living cells had visibly just undergone a division [ca. 70% of the initially plated protoplasts still being alive at this age of culture (Fig. 1g)]. In a few cases, cells were observed to have undergone a second division before the daughter cells of the first division were completely detached from each other.

Colony Formation and Feeder Cultures. After about 2 weeks of culture, the frequency of septum formation decreased regardless of whether the cultures were left undisturbed, supplied with fresh medium, or embedded in agarose. Since daughter cells still tended to separate quickly from each other, the overall number of recognizably divided cells rapidly declined. The cultures stayed alive for many more weeks, and the chloroplasts continued to dedifferentiate until—months later—they could no longer be identified by light microscopy. After 10 weeks, the cells assumed their final size and appearance: irregular in shape, large (often >300 μm in diameter compared with 30–40 μm for the freshly isolated protoplasts), and surrounded by a thick cell wall (Fig. 1h).

This development towards a final, nonmitotic state could be overcome if the developing protoplasts were brought into contact with a feeder culture of Triticum monococcum or of Oryza sativa (SRA-1). When the transfer to the feeder cultures was performed with 7- to 21-day-old protoplasts, a rapid increase in the density of the cytoplasm and a high frequency of cell divisions were induced. For example, a protoplast culture that was brought into contact with such a feeder culture on day 9 had a division rate of 52% of all living cells on day 14, whereas in the control (not feeder-nursed), only 15% of the cells showed divisions. This low value in the control may be due in part to the separation of divided cells from each other as described above, which seemed to be less pronounced in feeder-nursed cultures.

The inductive effect of the feeder became even more evident when protoplast cultures were brought into contact with a feeder culture after an extended preculture period. Thus, in a protoplast culture that had been transferred to a feeder culture after complete cessation of cell divisions on day 21, 20% of the cells showed freshly induced divisions 6 days later. The control (no feeder) remained totally inactive. The highest division rate obtained so far in feeder-nursed cultures was 95% of the surviving cells (Fig. 2a). Since feeder cultures exert a detrimental as well as beneficial effect (see below), their dose must be correctly balanced. Thus, feeder conditions yielding such a high division frequency do not normally permit further development into colonies.

In these feeder-nursed cultures, repeated cell divisions were regularly observed (Fig. 2b). In contrast to the small and evenly shaped Triticum and rice cells, the cells of the developing oat colonies were large and exhibited diverse and often bizarre shapes, allowing a clear distinction between the two species. The growing oat callus was extremely friable, reflecting the earlier observation that daughter cells often separate after cell division has been completed. Protoplast

![Fig. 2. Further development of protoplast cultures in the presence of T. monococcum feeder cultures.](image)
viability in liquid feeder-nursed cultures gradually declined, whereas feederless control cultures remained viable for up to 4 months (but without division activity, as noted above). Obviously, the feeder cells exerted some toxic effect in addition to their beneficial inductive effect. This was not due to exhaustion of the feeder culture, as it was still actively growing at this point. Furthermore, exchange of the feeder culture did not prevent necrosis, nor were divisions in the oat cells sustained upon removal from the feeder. Other feeders—e.g., sunflower and maize suspension cells—were not inductive for divisions. Additions to the culture medium or procedures that have been reported to be beneficial for cereal protoplasts—e.g., coconut water, casein hydrolysate, spermidine, ascorbate, or dilution or replacement of the medium—did not prevent necrosis of the oat cells in the presence of the feeder cells.

So far, small clones of about 12 cells have been obtained. These microcalli are extremely friable. Thus possibly, the total number of divisions of the initial protoplast might have been even higher. When such a culture is transferred to a membrane filter with a feeder culture underneath, macroscopically visible colonies develop. However, their development is partly due to cell enlargement rather than cell proliferation beyond that observed in liquid cultures (Fig. 2c and d).

Other Genotypes. Is the genotype of cv. Peniarth an exceptionally responsive one? Four other randomly chosen spring oat varieties were tested (Adamo, Alfred, Major, Phoenix). Without any adaptation of the described protocol to possible specific demands of these other varieties, first and second divisions could be obtained in all of them, though at different rates. The developmental pattern in all cultivars was similar to that of cv. Peniarth, with formation of colonies composed of four or more cells. Therefore, we feel that the genotype is of minor importance for the capacity of mesophyll protoplasts to dedifferentiate and divide, although there is certainly a modulation of the response level caused by genotypic differences.

Plant age and developmental state of the donor tissue do not appear to be extremely critical, as protoplasts isolated from older plants (the second leaves of 19-day-old oat plants) yielded cultures with a comparable division frequency.

DISCUSSION

An obvious question arises from our work. What, in view of the past failures, is the key to our success in obtaining reproducible division of cereal mesophyll protoplasts? At present we cannot fully answer this question. We can exclude the possibility that our cultures contained protoplasts other than from mesophyll at any significant frequency. This is assured by the fact that the part of the leaf used for protoplast isolation produced homogeneous cultures without other contaminant cell types, as judged by microscopical observation. We have not used any unusual ingredients for our culture medium, nor did we apply any novel protoplast isolation or culture technique. Plant growth conditions are certainly of extreme importance, but other factors may also have contributed synergistically to our success. As Potrykus et al. (5), who have devoted much effort to the culture of cereal mesophyll protoplasts, put it in 1976: "We are still optimistic that some day somebody, possibly just by chance, will overcome the present difficulties. . . ."

It is possible that the oat varieties used here are simply much more amenable to protoplast isolation and culture than those used for instance by Potrykus et al. (5) or Galston and coworkers (14), who worked with cvv. Arnold, Flämingskrone, and Tiger; and cvv. Victory and Garry, respectively. However, our choice was made absolutely at random. The probability is low that this has resulted in a selection of particularly responsive genotypes.

Concerning the culture medium, the rate of divisions was much higher in the absence of coconut water than in its presence, but otherwise we feel that the composition of the culture medium is not crucial. No additions such as spermidine, arginine (15), ascorbate (13), or n-propylgallate (12) that have been described to improve cereal mesophyll protoplast viability were necessary in our medium. More than a decade ago, some rare divisions in oat mesophyll protoplasts were observed by Galston and colleagues (14, 16). Our observations concerning oat mesophyll protoplast development and its requirements are in line with their results—e.g., observations that divided cells may separate and that the addition of fresh medium does not improve the cultures in any way. However, the frequency of divisions that we achieved exceeds by orders of magnitude those reported earlier and are, in our hands, absolutely reproducible. Furthermore, our cultures could be regularly induced to multiple divisions, giving rise to small clones. This demonstrates that cereal mesophyll cells are not inherently incapable of dedifferentiation and of reentering the cell cycle.

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