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Computer-Assisted Identification of Protoplasts Responsible for Rare Division Events Reveals Guard-Cell Totipotency

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With the use of a computer-controlled microscope system to assist in the positioning and rapid relocation of large numbers of cultured cells, we were able to identify those protoplasts with the capacity to divide within a highly recalcitrant culture in which only a tiny fraction of the total population proceeds to produce viable microcalli. In the cultures used, comprising *Beta vulgaris* L. (sugar beet) leaf protoplasts, it was confirmed that these cells can be recognized solely on the basis of morphological characters. Therefore, a direct link exists between competence for cell division in vitro and cell type. Divergent callus morphologies and totipotent potential could also be ascribed to distinct protoplast types and hence to cells with a specific origin. The progenitors of the totipotent protoplasts in these cultures have been confirmed as being stomatal guard cells. Consequently, in plants even the most highly adapted living cells clearly retain and can reactivate all of the functional genetic information necessary to recreate the whole organism; an extreme degree of cytodifferentiation is, therefore, no hindrance to expressing totipotent potential. In addition to the considerable practical value of these findings, their implications concerning our understanding of both the control of gene expression and plant cell differentiation and its reversibility are of fundamental significance.

Cell differentiation is the morphological realization of genetic commitment, as exemplified by qualitative changes in cell form and function (Wareing and Phillips, 1970). It represents the fundamental process underlying the biological complexity necessary in all multicellular systems. In higher plants, such cell diversification results primarily from differences in gene expression rather than physical alterations to the genetic information (Wareing and Phillips, 1970). This permits a degree of phenotypic plasticity (Jennings and Trewavas, 1986), which is of critical importance in plants in which de novo organ formation is a necessary requirement for the survival of immobile organisms in a constantly changing and hostile environment (Walbot and Cullis, 1985). That certain individual plant cells could actually be totipotent was finally confirmed unequivocally in 1958 when Steward obtained whole plants from suspension cells of wild carrot (Steward, 1958). Since that time, plants have also been obtained from a range of other cell types. However, varied responses or the complete failure to demonstrate totipotency in many systems lead to the consideration that, even in living plant cells, totipotent potential may be (partially) lost as a result of genetic changes accompanying cytodifferentiation (Tran Thanh Van, 1981; Binding, 1986).

In cells of higher animals such phenomena are commonplace (Blau, 1989). Furthermore, cellular responses in general are notoriously heterogeneous in in vitro plant cultures. In terms of metabolic activity, cell division capacity, or regeneration potential, even cells directly isolated from whole plants (e.g. protoplasts) show extensive intercellular variation (Spangenberg et al., 1985; Hall and Yeoman, 1987). Our understanding of this heterogeneity is extremely limited, although links with tissue type, physiological state, position in the cell cycle, etc. have previously been speculated on (Magnien et al., 1982; Bergounioux et al., 1988a, 1988b; Jones et al., 1989; Siminis et al., 1993). Improved insight into this phenomenon would be extremely valuable, because not only would a better understanding of the processes regulating cell (de)differentiation be gained but also predictions could be made concerning the subsequent responses of particular cell types in culture.

Guard cells of the plant stomatal complex are one of the most highly differentiated of all living cell types in higher plants. They play a pivotal role in the maintenance of plant homeostasis by regulating internal conditions in accordance with external environmental fluctuations (Assmann, 1993). To perform this function, guard cells have both a morphology and a physiology that are unique within the plant (Sack, 1987; Mansfield et al., 1990). Stomatal movement is possible only through the modified form and composition of the cell wall and is under the control of a cytoplasm with a highly specialized physiology. Altered starch metabolism, built-in sensitivity to factors in the immediate environment such as pollution, light, and humidity and cell signaling mechanisms specifically allow guard cells to respond rapidly to unstable climatic conditions. This extreme degree of differentiation and the repeated failure to observe cell division in these cells led to the suggestion that guard cells might be nullipotent (Tran Thanh Van, 1981; Pillai et al., 1992).

In this paper results are presented from an investigation in which a unique computer-driven microscopic system has been applied in the successful search for the totipotent cells within a highly recalcitrant protoplast culture system of a commercially important crop, sugar beet (*Beta vul-*

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Abbreviations: CSLM, confocal scanning laser microscopy; EtBr, ethidium bromide; FCM, flow cytometry; PE, plating efficiency.

garis). Leaf protoplasts of this species divide at frequencies generally lower than 1% and produce both compact and friable callus types (Krens et al., 1990; Hall et al., 1993). Only the latter appears capable of producing plants, with regeneration frequencies of ≤30% being observed (Hall et al., 1993). The results presented here illustrate the potential value of a semi-automated microscope system for individual cell studies; also the identification of the totipotent protoplasts as having originated from stomatal guard cells not only has a unique applied value but is an observation of fundamental importance to our understanding of cell differentiation and plant development.

MATERIALS AND METHODS

Plant Material

Shoot cultures of male fertile *Beta vulgaris* (sugar beet) line Bv-NF were used (Krens et al., 1990). Initiation and maintenance of the cultures were as described previously (Hall et al., 1993).

Protoplast Isolation and Culture

Protoplasts from leaf material were isolated and purified by the modified method of Krens et al. (1990) as described by Hall et al. (1993). Protoplasts were cultured at low density (62,500 protoplasts/6-cm Petri dish; 15,600 protoplasts/mL) after having been embedded in 1% (w/v) calcium alginate (Hall et al., 1993). Modified K8p medium was used with or without a supplement of 25% (v/v) conditioned medium, obtained as described by Hall et al. (1993).

The Cell Finder System and Analysis of Cellular Development

To be able to follow the development of selected individual protoplasts during the initial 14 d of culture, a computer-controlled, motor-driven cell finder system was used. This system is similar to that used by Golds et al. (1992) but has significant advancements as detailed by Toonen et al. (1994). Initial modifications to the culture system were required and are illustrated in Figure 1. To ensure that the cells remained in fixed positions the alginate-embedded cells were firmly adhered to a sterile glass coverslip (24 × 40 mm) using 1.6% (w/v) molten agarose. In addition, two gold electron microscope grids were attached to the coverslip, the positions of the centers of which were recorded in the computer for use as fixed reference points. Cell positions (x,y) were recorded and stored on a disc. At a later date, after re-entering the positions of the fixed reference points, the computer could then relocate the recorded cells to an accuracy of ±1 μm by controlling two stepper motors (x,y) attached to the microscope stage. In general, the positions of 100 cells per Petri dish were recorded and automatically received an identification number. Notes concerning size and morphology were made on each cell and development was followed and recorded, usually every 2nd d for 10 to 14 d.

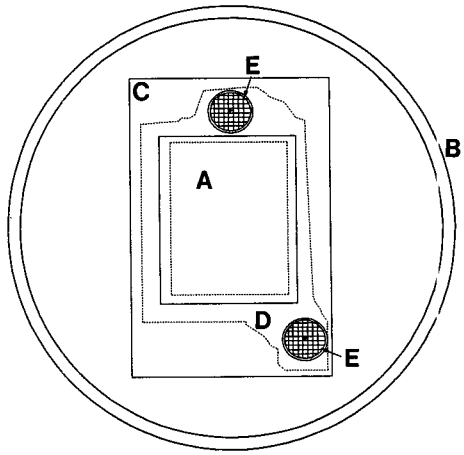

Figure 1. The culture system for use with the semi-automated cell relocation system. Protoplasts were first immobilized in a 1-mm-thick Ca alginate layer, of which a 10 × 20 mm piece (A) was then cultured in a 6-cm Petri dish (B) after having been firmly attached to an agarose-precoated 24- × 40-mm sterile coverslip (C) using a ring of 1.6% (w/v) agarose (D). Two gold electron microscope grids (E) were also adhered to the coverslip, in the same way, for use as the fixed reference points.

Cell Typing

Starch/Nuclear Content

Immobilized cells or protoplasts of interest at known locations were first stained to localize the nuclei with a solution of 20 μg/mL Hoechst 33242 in ethanol:culture medium (1:4, v/v). After the sample was observed under a UV microscope the same preparation was then stained with Lugol's solution to reveal the presence of starch.

CSLM

To examine nuclear structure and to determine the DNA content of individual cells, a Bio-Rad MRC 500 CSLM, fitted with an Argon ion laser (model 5425; Ion Laser Technology, Salt Lake City, UT), was used in combination with EtBr staining. After the positions of approximately 50 cells of each type were fixed, the culture medium was removed and supplemented with Triton X-100 and EtBr to give final concentrations of 0.1% (v/v) and 10 mg/L, respectively. This solution was then returned to the culture dish and staining was allowed to reach equilibrium by incubating for 16 h (overnight) at 4°C in the dark. All cells were then relocated and images of each, after careful focusing on the center of the nucleus, were stored on an optical disc for subsequent examination and analysis. Relative estimates of nuclear DNA content were determined by measuring the level of nuclear fluorescence using the standard software package. In all cases, background measurements were also made for subtraction from the nuclear value.

RESULTS

Characterization of the Protoplast Population

Even the most rudimentary examination of a leaf protoplast suspension revealed substantial differences in cell

size and morphology (Fig. 2A). Cell size ranged from 15 to 100 μm in diameter and mesophyll protoplasts predominated with, typically, 90% of all protoplasts having well-developed chloroplasts (Fig. 2B). However, even within this green cell population, differences were clearly evident in terms of chloroplast size, number, relative volume (to cell), etc. Nongreen cell populations were also heterogeneous, ranging from cells consisting almost entirely of a vacuole to cytoplasm-rich cells with many subcellular structures and a distinct subcellular organization. Preliminary experiments involved recording the positions of cells of all sizes and morphological types and then following their development. A summary of some of the results is presented in Table I. It could be clearly identified that cells with the capacity to divide occurred exclusively in the nongreen subpopulation. Although the majority of chloroplast-containing cells remained viable, grew extensively, and gained a centralized nucleus within 8 d of culture, development then stopped (Fig. 3A). In all subsequent experiments no green leaf protoplast was ever observed to divide. Consequently, further detailed experiments concentrated exclusively on the minority of cells present in the isolates that lacked well-developed chloroplasts.

Table I. *Summation of the results of the first three experiments wherein the development of individual sugar beet leaf protoplasts, specifically chosen on the basis of morphology, was followed for 14 d in culture*

Results are presented in relation to the presence or absence of well-developed chloroplasts on d 0.

Cell Type	Total	Died	Divided (%)
With chloroplasts	265	39	0 (0)
Without chloroplasts	163	34	28 (17)

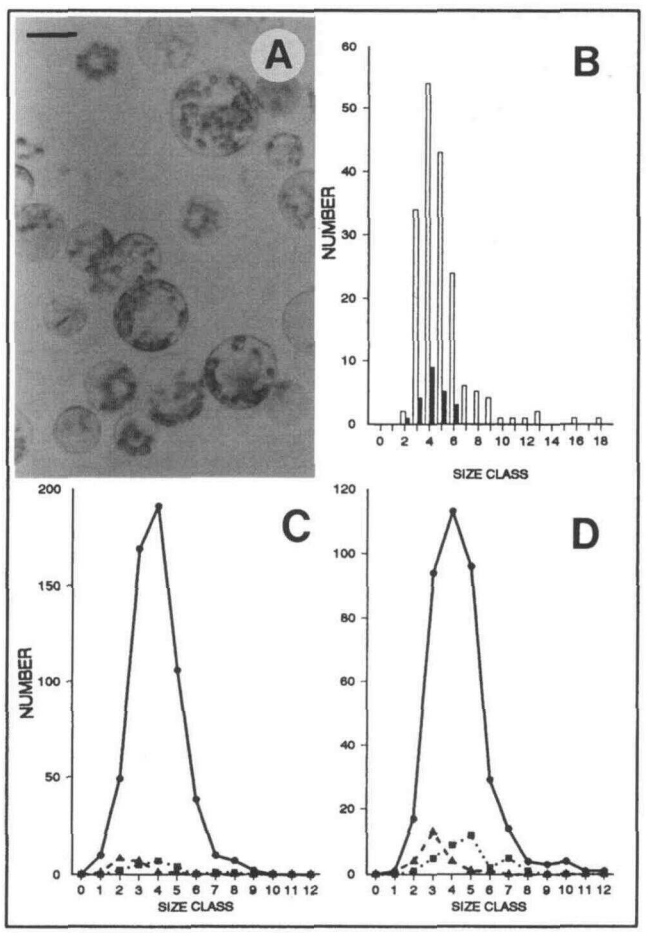

Figure 2. A, Leaf protoplasts isolated from sugar beet (*B. vulgaris* L.) shoot cultures. Bar = 40 μm. B, Analysis of a random population (n = 200) of protoplasts from A. Cells were grouped into size classes, beginning at 1 (15–20 μm) and increasing in increments of 5 μm up to 18 (95–100 μm). Open bars, Cells with well-developed chloroplasts; solid bars, cells lacking obvious chloroplasts. C, Analysis of cell division capacity of randomly located cells lacking chloroplasts (n = 592) cultured under standard culture conditions. ●, Total number of cells followed per size class; ■, cells that gave rise to compact callus; ▲, cells that gave rise to friable, totipotent callus. D, Like C but using 25% conditioned medium to stimulate PE (n = 386). In both C and D cells were followed for 10 to 14 d after plating and in each case results from three experiments have been combined.

Characterization of the Nonphotosynthetic Cell Population

The locations of cells lacking chloroplasts, found at random, were recorded in a number of experiments and the development of these cells was followed with time. Initial cell size was noted along with details of cell morphology relating to the presence of amyloplasts, amount of cytoplasm, location of the nucleus, etc. The results of these experiments have been combined and are presented in Figure 2C.

Under standard culture conditions (normally supporting a PE of approximately 0.2–0.3%) few dividing cells were, as expected, found among those studied. Nevertheless, there was an indication that the smaller cells tended to give rise to friable callus and larger cells tended to give rise to the harder, nonregenerable callus. This observation was supported by the appearance of distinct twin peaks when conditioned medium was used to enhance the overall PE to approximately 0.8% (Fig. 2D). For friable callus, maximum division frequencies occurred in cells with an initial diameter of 22.5 to 25 μm, whereas for compact calli this was 32.5 to 35 μm. Retrospective examination of the cell descriptions revealed that not only size but also cell morphology could be used as an indicator of divisional capacity.

Typical cells and their development are represented in Figure 3. In every case, protoplasts giving rise to compact callus were initially rich in a fine granular cytoplasm and a nucleus positioned centrally in the cell (Fig. 3D). The first division in these cells occurred very early, usually within 2 to 3 d, and proceeded at an exceptionally rapid rate, yielding colonies of approximately 200 cells after 8 d of culture. In contrast, protoplasts giving rise to friable callus were not only distinctly smaller (Fig. 3C) but the starch content of these cells was also distinctive (see below), and under light microscopy starch grains were the only subcellular features that could be observed. Division in these cells occurred late, generally only after 7 to 8 d, and proceeded at a slower rate than for the previous type, giving rise to colonies of 100 cells after 15 to 17 d.

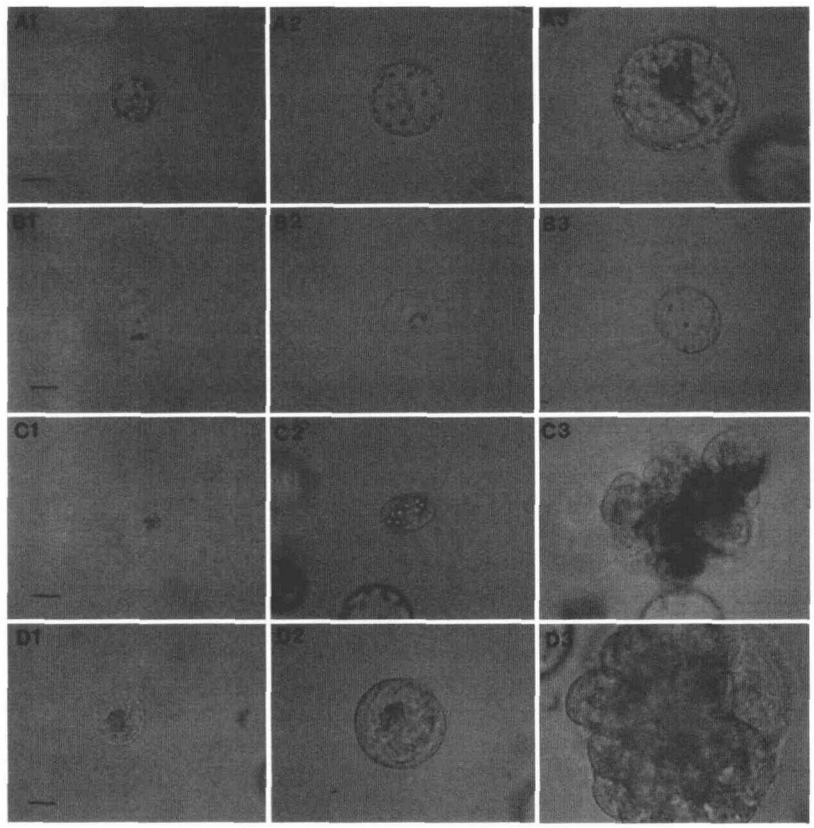

Figure 3. *Stages in the development of four different protoplast types from sugar beet leaves as distinguished on the basis of cell morphology. Photos were taken on d 0, d 3, and d 8 (A1–A3, B1–B3, D1–D3) or on d 0, d 8, and d 17 (C1–C3). A, Mesophyll cell. B, Highly vacuolate (epidermal?) cell. C, Cell type that gives rise to friable callus of the totipotent type. D, Cytoplasm-rich cell that gives rise to compact callus. Bars = 30 μm.*

On the basis of these observations the usual number of these two cell types in the protoplast isolates was determined. In both cases the proportion of each cell type was extremely low, never exceeding 1% of the total protoplast population. In addition, a predictability study was made on the callus-forming capacities of these two subpopulations. In subsequent experiments the positions of those protoplasts which fell within the desired size range and had the precise characteristics typical of cells that give rise to either compact or friable callus were noted separately and, at the end of the experiment, the success of the predictions was determined. From the results presented in Table II, it is evident that prediction of callus type can be made with 100% certainty. Every cell that divided produced the expected callus type and the chance that selected cells indeed proceeded to form colonies, either compact or friable (i.e the PE of the selected subpopulation) was approximately 60%.

Table II. *Determination of the fate of selected sugar beet leaf protoplasts predicted on d 1 to form either friable (totipotent) or compact (nontotipotent) callus*

Results are the summations from three experiments. Final examinations were made on d 14.

Cell Type	Total	Died	Grew	Divided (%)	Callus type
Friable	86	25	14	47 (56)	100% friable
Compact	77	21	9	47 (61)	100% compact

Characterization of the Dividing Subpopulations

Starch

After the position of the nucleus was located with 4′,6-diamidino-2-phenylindole dihydrochloride, Lugol's solution was used to stain for the presence of starch. Cells destined to produce friable callus were found to be rich in starch. Characteristically, this was located in a small number (usually 8–10) of large, regularly shaped grains equal in size to the nucleus. Although starch was also present in the protoplasts that give rise to compact calli, the grains in these cells were more numerous, considerably smaller, and irregularly shaped.

Nuclear Structure and Cellular DNA Content

Examination of the nuclear structure of the different cell types revealed that both mesophyll protoplasts and protoplasts that yield friable calli had small and compact nuclei (Fig. 4, D and E). In contrast, cells that produce compact calli had much larger nuclei with an open structure. Many had a distinctly lobed or multistructural organization (Fig. 4F). Examination of the friable cell type after 5 d of culture revealed that the nuclei had switched from having a compact structure to having an open form of structure. The cross-sectional area of the center of the nuclei, which can be taken as an approximate indication of nuclear size, had increased during this period from 34 to 58 μm^2, which is

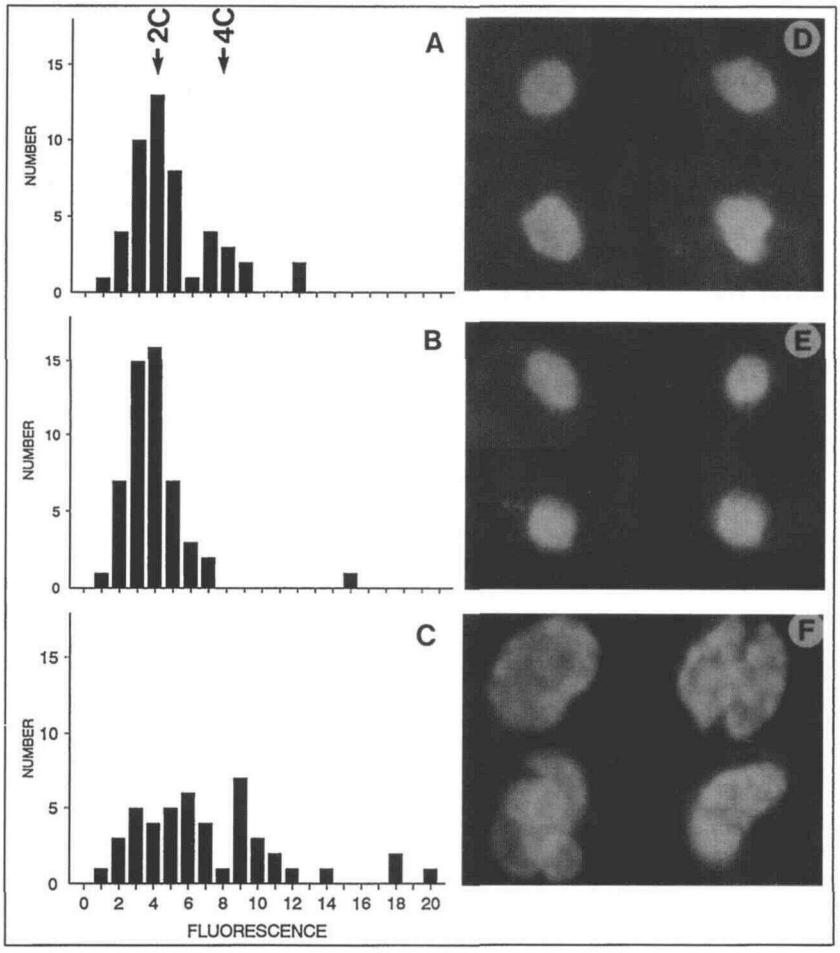

Figure 4. CSLM analysis of nuclear DNA content (A–C) and nuclear morphology (D–F) of three subpopulations of sugar beet leaf protoplasts, as measured on d 0, after staining with EtBr. A, Mesophyll protoplasts (see Fig. 3A). Arrows indicate positions of the 2C and 4C peaks. B, Protoplasts that give rise to friable callus (see Fig. 3C). C, Protoplasts that give rise to compact callus (see Fig. 3D). In each case $n = 45$. Nuclear DNA content/cell (fluorescence) is presented in arbitrary units after correcting for individual background readings. D to F, Structure of typical stained nuclei of cells from A to C, respectively. All photographs were taken at the same magnification.

equivalent to the size of the nuclei in protoplasts destined to produce compact calli, as measured on d 1.

To estimate the relative DNA content of individual cells, the degree of fluorescence of the nuclei was measured and corrected for background using the CSLM software package. Because approximately 90% of the cells contained chloroplasts and because it had been previously determined, by FCM, that the great majority of protoplasts had a diploid 2C nucleus, it was concluded that most green cells contained a 2C DNA content and could thus be used as a reference standard. Results have indicated that, despite the relatively small sample size (approximately 45 protoplasts/cell type), it was still possible, using green cells, to obtain a histogram similar to that produced using many thousands of cells by FCM (Fig. 4A). Peaks equivalent to 2C, 4C, and possibly 8C nuclei could be identified. From this pattern it was possible to determine that protoplasts that give rise to friable callus had a single peak coinciding with the 2C peak of the mesophyll cells (Fig. 4B). Homogeneity in both cell size and plastid number in these cells, therefore, also extends to nuclear structure and ploidy level. In contrast, cells destined to produce compact callus had no distinct peak and contained nuclei with a wide range of DNA levels, apparently even occasionally exceeding that equivalent to a 4C DNA content (Fig. 4C). Analysis of cells derived from the friable callus type 5 d after plating (i.e. generally before the first cell division) revealed a pattern similar to that for compact callus-producing protoplasts directly after isolation (results not presented).

Identification of the Totipotent Cell Type

A number of key features of these protoplasts, such as recurrent (fixed) numbers of large starch grains and rare sightings of reniform cells of similar appearance, led to the consideration that these protoplasts were derived from stomatal guard cells. The apparent absence of Chl (which is usually present in guard cells) was clarified when this was indeed confirmed to be present in the plastids using enhanced fluorescence microscopy (results not presented). The enigma that guard cells in vivo looked quite different from the protoplasts was clarified when guard cells were observed to take on a modified appearance during isolation, which resulted from enhanced starch accumulation in darkness (Fig. 5, A and B). The release of the totipotent protoplast type from guard cells was finally confirmed by direct observation of enzymic digestions of isolated epidermal strips (Fig. 5C).

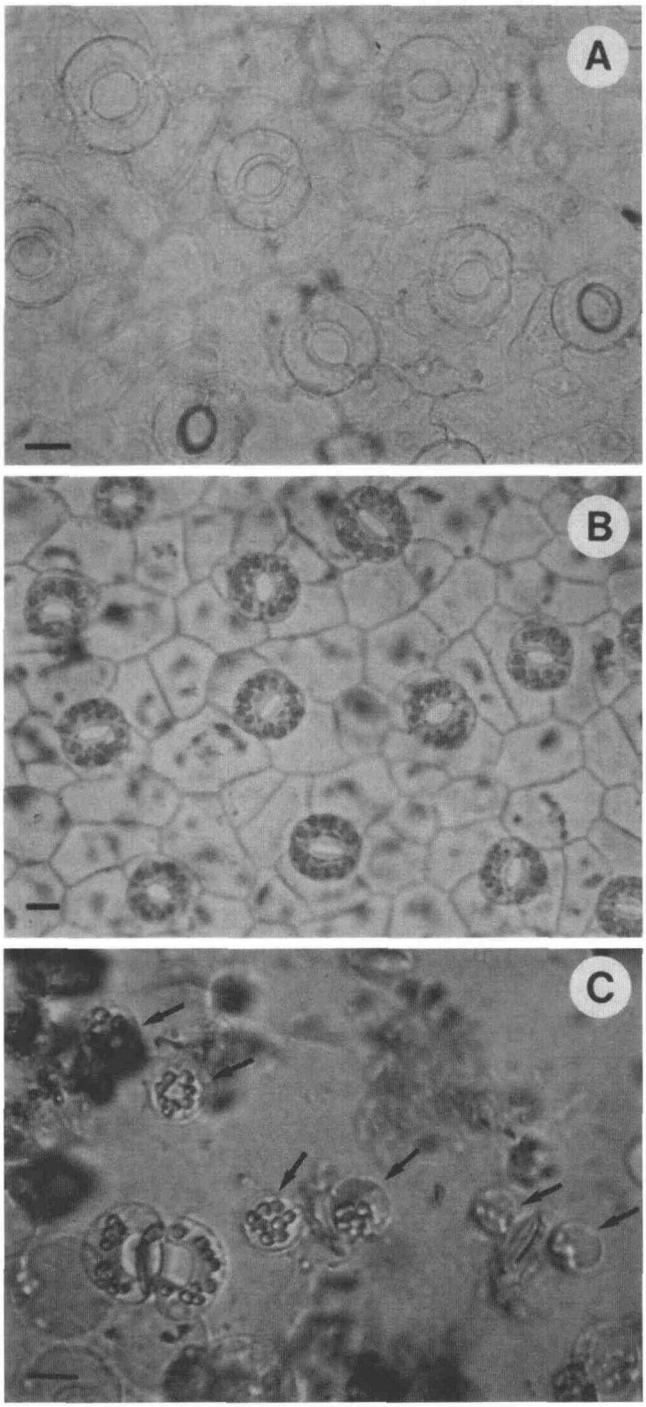

Figure 5. A comparison of sugar beet guard-cell morphology in isolated epidermal strips before (A) and after (B) overnight incubation in protoplast isolation medium lacking enzymes. Note the increased prominence of the plastids, confirmed to be due to increased starch content (results not presented). C, Following the enzymatic digestion of isolated sugar beet epidermis strips revealed directly the release of pairs of guard-cell protoplasts (arrows, separated by the stoma) with a size and morphology identical with protoplasts of the totipotent type (see Fig. 3C). Bar = 30 μm.

DISCUSSION

It has become feasible to identify the specific cells involved in rare cell division events in a highly recalcitrant culture system by using a computer-controlled microscope to follow the development of a large number of individual cells in time. The results reported here therefore represent the realization of the potential of semi-automated microscopy as proposed previously for both plant and animal cell systems (Golds et al., 1992). However, the ability to identify the dividing cells and also to distinguish different subpopulations, both solely on the basis of morphology, is remarkable. As a consequence of these findings, a number of issues are raised concerning the factors determining the response of isolated plant cells in vitro and also concerning our fundamental understanding of plant cell *(de)differentiation.*

Recalcitrance of sugar beet protoplasts is particularly well documented (Schlangstedt et al., 1992; Hall et al., 1994). The existence of clear morphological heterogeneity within these protoplasts augured well for the use of such features as a means to identify distinct subpopulations that might have the capacity to divide. The search was also assisted by the observation that mesophyll cells, which constitute approximately 90% of the total population, appear to be incapable of cell division in these cultures. Although the majority remained viable and appeared to proceeded through all of the stages typical of precytokinetic development (Nagata and Yamaki, 1973; Gigot, 1975), cell division never occurred. The reason for this is as yet unclear. Because we know from FCM studies (R.D. Hall, H.A. Verhoeven, and F.A. Krens, unpublished observations) that most mesophyll cells have diploid 2C nuclei and are therefore in the G_1 (or G_0) stage of the cell cycle, blockage of cell division could occur before or after the S phase. Further research is therefore necessary before the causative factors can be identified that prevent division in this cell type, which is frequently observed to divide in other nonrecalcitrant species (Durr et al., 1993).

Despite the likely occurrence of a high degree of "unseen" heterogeneity (physiological, cytogenetic) in these cells, it has become evident from this investigation that in vitro response is directly correlated with a distinct cellular morphology and hence with cells derived from a specific tissue within the source material. Indeed, this response is so specific that the previously reported appearance of two divergent callus types from beet protoplasts (Krens et al., 1990; Pedersen et al., 1993), often within the same culture (Hall et al., 1993), can now be clearly associated with two distinctly different protoplast types. This would strongly imply that some aspect of temporal or spatial epigenetic heterogeneity, established in vivo, predisposes these cells to follow specific but different developmental pathways in vitro under the same culture conditions. Furthermore, the extremely high division capacity of these two subpopulations would suggest either that cellular origin was the overriding factor determining subsequent cellular response in vitro or that these cells were highly homogeneous regarding other characteristics such as physiological state and position in the cell cycle. In this regard the totipotent

protoplasts were indeed remarkably homogeneous in terms of cell size, structure, and ploidy level. Further determination of the nature of these epigenetic differences could bring us much closer to understanding and predicting responses of cells in vitro, particularly within recalcitrant cultures.

Identifying the tissues of origin of the two division-competent cell types was of primary importance. Although both were rich in starch, those cells destined to produce compact calli had numerous starch grains of varying size and morphology. Qualitative and quantitative analysis of individual nuclei from these cells revealed an open and frequently bilobial structure with DNA contents predominantly varying between 2C and 4C. Micronuclei were observed in approximately 30% of the cells (results not presented). These features suggest that such cells were derived from a tissue that was already dividing in vivo (Hagège et al., 1992; Ramulu et al., 1993) Furthermore, the observed changes in nuclear structure of the totipotent protoplast type, prior to cell division, would clearly support this conclusion. With the progenitors of compact callus also being cytoplasm rich and poorly vacuolated and having no chloroplasts, our present working hypothesis is that they are derived from cambial initials. Additional support for this possibility comes from experiments on protoplasts isolated from beet petioles (Pedersen et al., 1993; Schlangstedt et al., 1994). These had a higher proportion of vascular tissue and yielded significantly enriched populations (approximately 10%) of compact callus protoplasts that had a correspondingly higher PE (approximately 8%, R.D. Hall, unpublished observations).

The confirmation that the protoplasts giving rise to totipotent, friable callus in this culture system were derived from stomatal guard cells was very surprising. Only recently, Sahgal et al. (1994) reported the first demonstration of guard-cell totipotency using tobacco. The results detailed here consequently support these findings and indicate that, even in a species much less amenable to plant cell culture, guard cells retain the ability to regenerate into plants. In practical terms, with this knowledge it is now possible to modify the isolation and culture protocols for this species in ways specifically geared toward enhancing the isolation and division frequency of this single totipotent cell type. Therefore, we have now gained the knowledge to enable us to overcome the recalcitrance of this system, even making it feasible to consider single-cell studies with sugar beet, something that was previously quite impractical. Indeed, preliminary enzymic digestions of isolated epidermal strips have resulted in enhancement of the frequency of guard-cell protoplasts from 0.5 to 80%. With a concomitant increase in PE also being observed this provides further confirmation of our conclusions regarding totipotent cell origin. However, because overall protoplast yields are severely limited using this method, mass guard-cell protoplast isolation techniques (Weyers and Meidner, 1990) are also under investigation to enable more widespread application of the technique.

Anatomy is clearly not destiny in living plant cells. Demonstration of guard-cell totipotency represents a clear indication that, in plants, an extreme degree of cytodifferentiation is not "terminal" as appears to be the case with many animal cells (Blau, 1989). The changes in gene expression that translate cell competence via cell determination into the ultimate differentiated state are thus fully reversible. With a PE of approximately 60% it would also appear that competence for cell division is a general feature of guard cells and is not limited to a specific (immature/meristematic) subpopulation (Cupples et al., 1991). Furthermore, our initial consideration that the process of guard-cell protoplast isolation possibly instigated a genetic "reprogramming" of the cell, initiating cell division, proved incorrect because it has since been possible to induce cell division directly in guard cells at high frequency in cultured epidermal strips (R.D. Hall, H.A. Verhoeven, and F.A. Krens, unpublished data).

Although it is interesting to speculate why, extraordinarily, guard-cell protoplasts have been found to be the first to respond in this highly recalcitrant sugar beet system, the fundamental scientific and practical implications of these findings deserve more immediate consideration. The unique biological complexity and functional versatility of guard cells make this system an ideal choice for investigating ontogeny and the dedifferentiation and redifferentiation of plant cells. Exploiting their many specific physiological properties by, for example, studying the loss of responses to ABA (Mansfield et al., 1990; McAinsh et al., 1990) and blue light (Assmann et al., 1985) could not only assist in studies of temporal and spatial expression of gene function but also could prove to be significant to current searches for guard-cell-specific genes and promoters (Müller-Röber et al., 1994). Broadening our understanding of stomatal physiology is not only of immediate value in pollution studies (ozone, SO_2, CO_2) but also is important in regard to current interest in decentralizing world agriculture to include marginal cultivation. In this context maximum exploitation of stomatal characteristics could be of paramount importance in relevant plant-breeding strategies.

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