Controlling cellular development in a single cell system of *Nicotiana*

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ABSTRACT Cells regenerated from freshly isolated leaf mesophyll protoplasts can be induced to develop along very different pathways. Depending on the hormone content and the mechanical characteristics of the environment, cells choose between division and expansion. In the former case they form microcolonies of isodiametric cells, while in the latter they exhibit a typical tubular growth or increase their volume considerably by bulging and budding. A culture method was developed which allows a careful control of the experimental environment and non-destructive high resolution monitoring of development of individual cells.

KEY WORDS: *cell development, Nicotiana, CSLM, plant hormones*

Introduction

Unlike animals, plants continue to develop new organs throughout their lifetime (Lyndon, 1990). During embryonic development the basic structure of the plant-body is formed. It is further elaborated by an iterative growth pattern, during the vegetative as well as the generative development. Perhaps the best known example of organs produced in a repetitive way are the leaves.

During the development of plant organs, cells go first through a period of intensive division and then proceed through a period of expansion and differentiation. This is also the case for leaves, where the change from division to expansion occurs nearly simultaneously throughout the whole cell population (Verbelen and De Greef, 1979) or moves along a gradient through the leaf (Maksymowych, 1973). For tobacco the basic features of leaf development were already described by Avery (1933). The principal shape and the basic architecture of plants is genetically defined. In some cases the cellular basis of pattern formation and organ development is reasonably well described and predictable (Lindenmayer, 1984; Green, 1985). Being sessile organisms, plants however adapt their habitus to the environment they live in. Both chemical and physical factors in the environment affect the final shape of the plant and its organs.

It is known that plant hormones play a crucial role in both the basic developmental history of plants and in the typical morphogenetic reactions to external stimuli (Davies, 1987), but there is not a single plant hormone for which a typical and unique function is known (Palme *et al.*, 1991). The physiological response to hormones is strongly dependent on the age, developmental status and complexity of the target organ or tissue. In the search for explanations accounting for the role of hormones in plant morphogenesis, model systems were developed and used successfully. Some of these models were very simple in composition and contained only a limited number of different cell types (Tran Thanh Van, 1981). They still incorporated a very typical feature of plant structure: tissue structure was maintained, including cell-to-cell contact through plasmodesma.

To increase the simplicity of the model and the unambiguity of the observations a further step is taken in developing model systems based on a homogeneous population of single cells.

Isolated protoplasts have been used with success in studies of primary reactions to light (Bossen *et al.*, 1988) as well as to hormones (Ephritikhine *et al.*, 1987). For research in developmental biology, remarkable results were obtained with isolated cells in culture (Takeuchi and Komamine, 1982; Hazezawa and Syono, 1983). The Zinnia system in which isolated mesophyll cells develop rather homogeneously into xylem cells is perhaps the best documented example (Fukuda and Komamine, 1980).

When cultures are initiated with protoplasts the dedifferentiation of the starting material is taken a step further.

In optimal conditions, protoplasts will regenerate a cell wall and will soon start dividing. However interesting data have been published that show that these young cells have other morphogenetic potentials whose expression can be triggered experimentally. Typical tubular cell expansion (Lloyd and Barlow, 1982; Hasezawa and Syono, 1983) as well as cell budding (Bawa and Torey, 1971; Meyer and

Abbreviations used in this paper: BAP, benzylaminopurine; CSLM, Confocal scanning laser; microscope; NAA, Naphtalene acetic acid; SEM, Scanning electron microscope.

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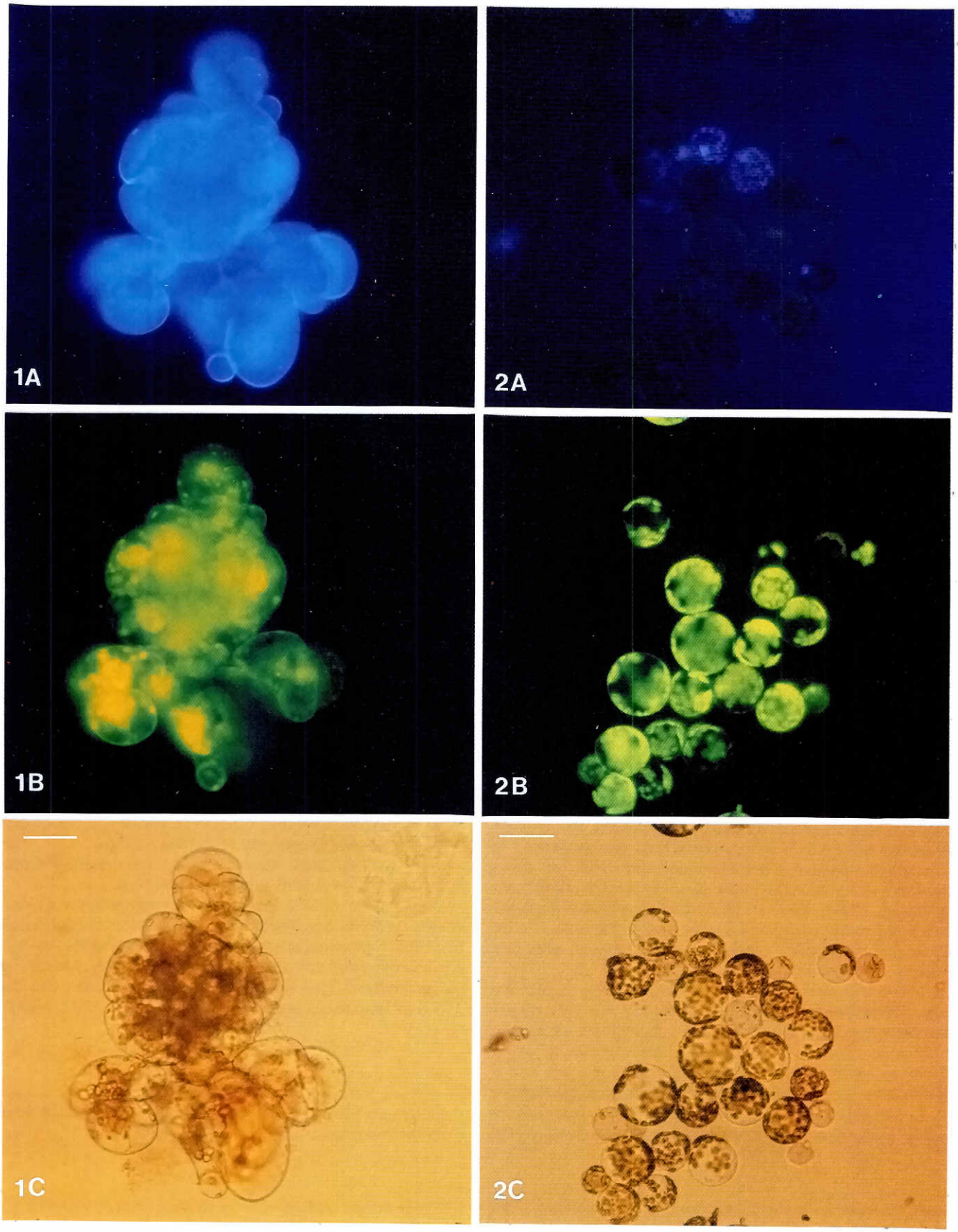
0214-6282/92/$03.00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Abel, 1975; Lörz and Potrykus, 1980) were reported. Mostly these data refer to experiments with a precise but limited goal in the developmental study of the system.

For our studies we used and modified a standard cell culture method for tobacco (Potrykus and Shillito, 1986), which starts with freshly isolated mesophyll protoplasts. We report here on the first results in trying to control the development of single immobilized cells in culture.

Protoplasts of tobacco mesophyll cells were isolated and brought in culture in K3A medium. They were either kept in suspension in, or immobilized on an agarose gel. Four different hormone compositions of the medium were used.

Results

Freshly isolated protoplasts form a rather homogeneous population of vacuolated, spherical cells with numerous well developed chloroplasts, the average cell diameter being 40 µm (Fig. 1). The cell wall is well removed as staining for cellulose gives a totally negative result. The nucleus has a peripheral position. After 1 day cells have regenerated a stainable cell wall. They have a similar appearance in the different culture conditions used.

After 3 to 4 days of culture, differences in cellular behavior between the 4 experimental series could be seen. Each hormonal condition used forced the cells into a specific developmental pathway, leading to dramatic differences in cell shape and cell activity after 5 to 10 days of culture.

K3A with auxin (NAA) and cytokinin (BAP)

The first cell division is visible after 4 days of culture. Division activity goes on during 10 days and as a consequence each 'mother-cell' produces a micro-colony (Fig. 2). Reducing the total hormone concentration by 50% does not affect this development. The resulting cells have a diameter of 25-30 µm. The nucleus has a central position. Cellular development is similar in both liquid and agarose gel cultures.

K3A with auxin (NAA) only

In general cells do not divide. Instead cell expansion obviously starts after 5 days and continues until the end of the experiment (Fig.3). The majority of cells are polarized in their growth, since they expand into huge tubular cells; these can reach lengths of 600 µm and have a diameter of 40 µm. The nucleus remains in a central position in the cell lumen and is suspended by well-developed cytoplasmic threads. Cellular development is similar in both liquid and agarose gel cultures.

K3A with cytokinin (BAP) only

Cells do not divide. Instead the cell volume increases considerably by bulging from day 3 on. As a result, after 5 days most cells look as if they have budded, and the buds themselves have budded again, and so on. This activity stops after 10 days of culture. Both in suspension culture and with cells embedded in agarose, this budding or bulging has a random orientation. However cells embedded at the upper surface of the agarose layer will develop typically into the supernatant liquid medium and form typical rows of «buds». These complex cell structures remain in fact unicellular, having one nucleus which keeps a peripheral position generally in the original cell body. The cytoplasmic layer and the cell wall of these cells are considerably thinner than in cells of the K3A + auxin series. The «buds» form a cell wall which stains with calcofluor white. Studies with the SEM show that the wall of these buds is indeed continuous (Fig. 5a). Observation of these cells in three dimensions with the CSLM makes clear that buds do not separate and that the cytoplasm is continuous throughout the constrictions between them (Fig. 5b).

K3A without hormones

Cells do not divide. From day 2 on they form bulges much like the cells of the K3A + cytokinin series, but the development stops sooner. In general also fewer «buds» per cell are formed. Cells were not subcultured and experiments were stopped after 20 days of culture.

Discussion

During normal leaf growth, the developmental history of the cells is well known and even predictable. After a period of meristematic activity cell division stops and cells expand considerably. The mechanism of expansion is similar for all cells: the existing cell walls gain in plasticity and the turgescence of the cell's vacuole acts as the motor of cell growth. However, epidermal cells expand in the dimensions parallel to the leaf blade, the palisade mesophyll cells elongate to tubular cells in a direction perpendicular to the leaf blade and spongy mesophyll cells expand in three dimensions into cells with irregular branched shape (Maksymowych, 1973). It is not known by which rules these and other cell expansion activities are governed, and it is remarkable how much more we know about cell division and its control (Lyndon, 1990).

If cells are isolated from an organ in which they were already defined and (partially) differentiated, they can be rejuvenated and made to start dividing again. In our experiments this clearly happens in the K_3A medium supplied with auxin and cytokinin (Fig. 2). If the resulting microcolonies are subcultured they give rise to cell clusters in which later cell heterogeneity develops. Ultimately new meristems can be formed and plants can be regenerated in this way (Firoozabady, 1986). However we stopped our experiments after 20 days of culture, keeping the experimental system deliberately simple. Cell division is induced only if both auxin and cytokinin are added to the culture medium. Omitting the cytokinins makes the cells expand considerably, with most of them elongating into long tubular cells (Fig. 3). This rather typical cell morphogenesis was already described (Lloyd and Barlow, 1982; Hasezawa and Syono, 1983). The culture conditions used by these authors were however

Fig. 1. Freshly isolated protoplasts immobilized on an agarose containing medium. (a) *Bright field;* **(b)** *FDA fluorescence. Note that two protoplasts in the lower middle part of the picture do not fluoresce; they are not viable.* **(c)** *Calcofluor white fluorescence; cell wall is absent from all protoplasts. The two dead cells (see 1b) have accumulated stain inside the cell membrane. Bar, 50 µm.*

Fig. 2. Microcolony of cells formed on an agarose medium containing NAA + BAP, after 15 days in culture. (a) *Bright field.* **(b)** *FDA fluorescence, all cells are viable, intense fluorescence is due to accumulation of stain in the chloroplasts.* **(c)** *Calcofluor white fluorescence. Bar, 50 µm.*


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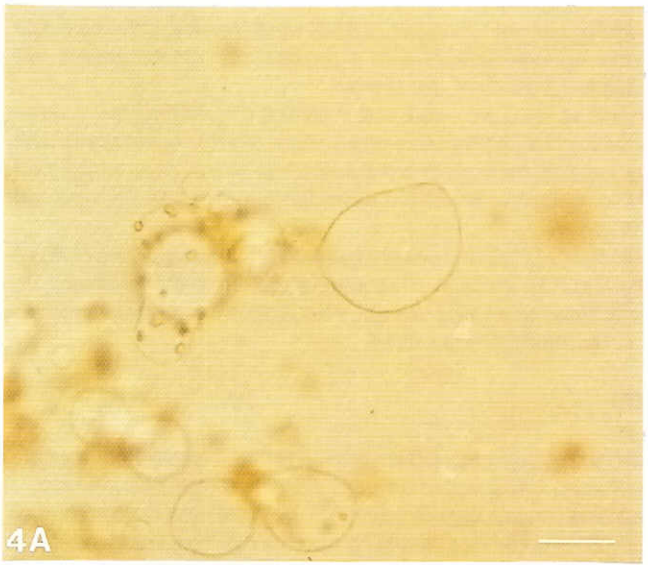
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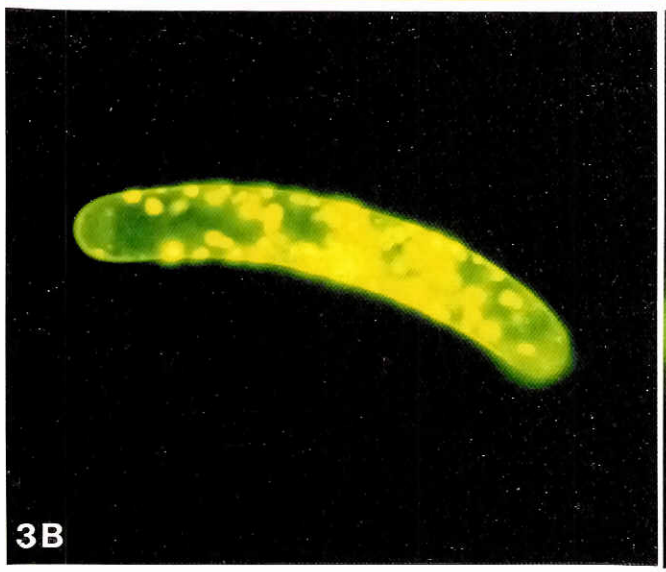
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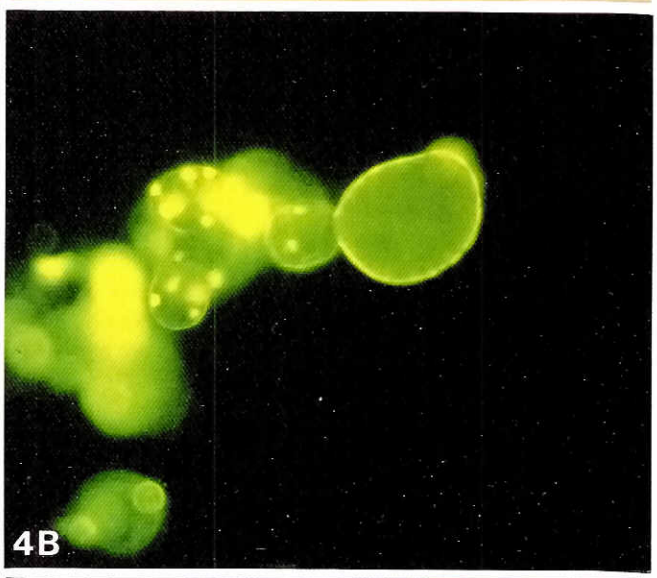
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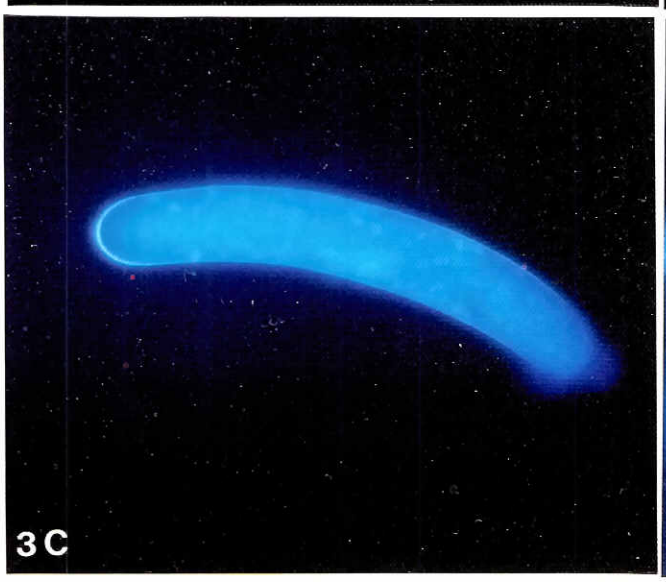
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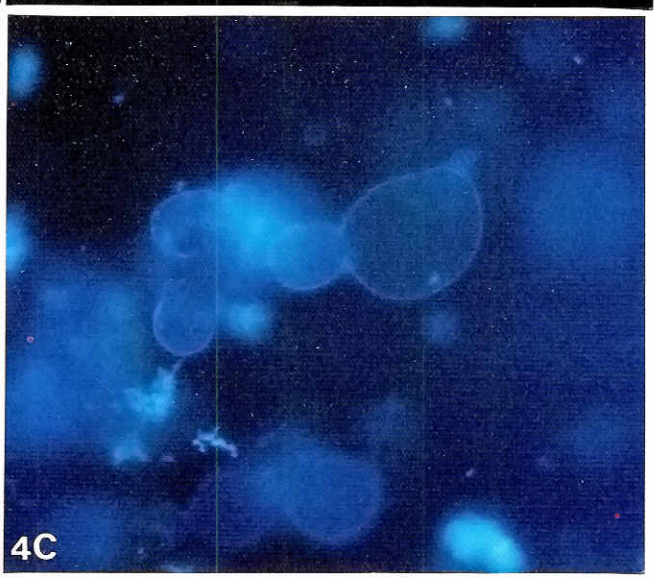
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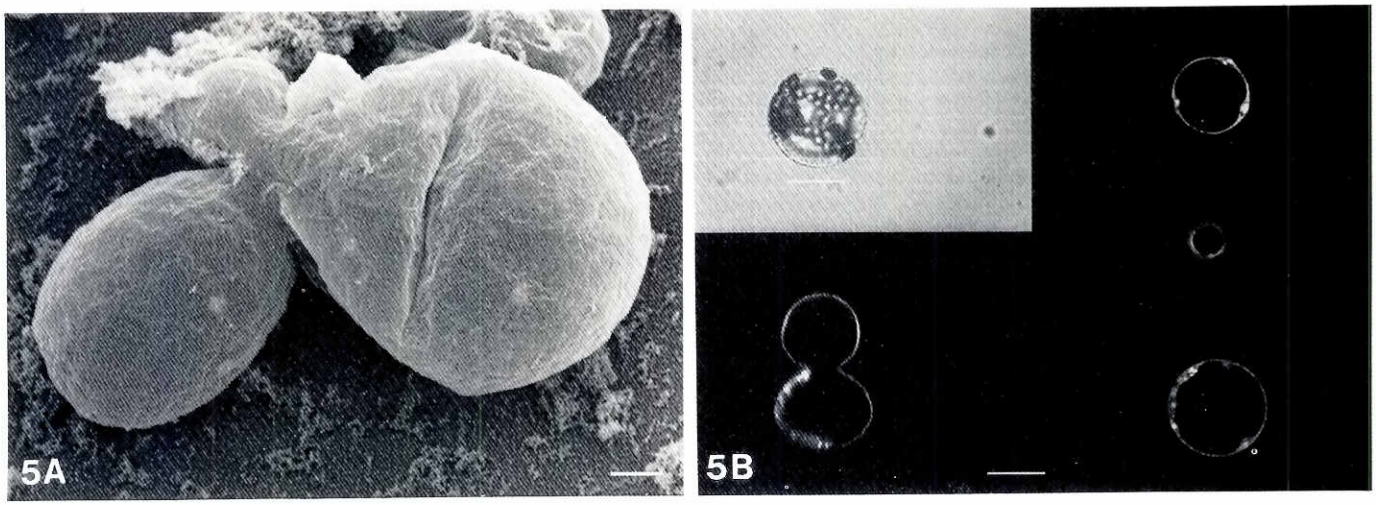

Fig. 5. Budding cells during culture on a medium containing only BAP. (a) *SEM pictures. The cell wall is continuous over the whole cell construct. The furrow on the right part of the cell is a preparation artifact. Bar, 10 μm.* **(b)** *CSLM picture after FDA staining. Upper left: bright field image of the cell. Upper middle and lower right: confocal sections in the XY plane through, respectively, the budding part, the constriction and the original cell part. Lower left: XZ scan of the same cell, showing its shape in a plane perpendicular to the plane of the former pictures. Bar, 25 μm.*

different from ours: the absence or low levels of auxin induced tubular growth; if the concentration of auxin was increased above a critical level, cells started dividing. Whereas protoplasts and dividing cells do not show any consistent morphological signs of polarity, the elongating cells are clearly polarized in their development: for days they grow along one axis. Data published by Goldsworthy and Mina (1991) confirm this opinion: tubular cells exhibit transcellular currents, resembling very much the well-documented cases of polarized growth in normal unicellular and multicellular organisms (Weisenseel and Kicherer, 1981). When cells are cultured in the absence of auxin but with a standard supply of cytokinin the cells do not divide but expand a lot in volume. The mechanism of volume increase is quite different from the tubular growth described before: cells bulge and form buds with a size comparable to the original cell body (Figs. 4, 5). This budding is repeated many times, leading to cells with a pearl necklace appearance. Compared to the two former types of development these cells have a less prominent cell wall. This suggests a less rigid and eventually a heterogeneous cell wall structure; the turgescent protoplast would then bulge out at weak points. A similar cellular morphogenesis is found in normal plants during tylosis in older parts of the xylem tissue. The orientation of this bulging activity can be directed easily by simple physical means. If at the beginning of the experiment cells are embedded in the surface of an agarose layer, all consecutive bulging and budding will occur in a direction perpendicular to the agarose layer and pointing upwards. The growth is thus directed by the buoyancy of the cell's content and by the change in mechanical strength of the cell's immediate environment on the border between the agarose gel and the liquid medium. We thus believe that cells going through this kind of growth are not necessarily polarized in their development. The fact that the direction of bulging is oriented at random in a homogeneous medium (liquid or agarose) confirms this point of view.

Budding of cells in culture has been reported before (Meyer and Abel, 1975; Lörz and Potrykus, 1980). The pictures published indeed very much resemble the cell constructs we obtained after a few days of culturing without hormones or with only cytokinins present in the medium. In some cases budding was described in relation to a very specific way of cell division by cleavage (Herth and Meyer, 1978). In our experiments the cells clearly do not divide. Also nuclear divisions were not observed. The cytokinin-induced budding cells survived for 15 days.

This paper reports on the first results of experiments aimed at controlling the development of single cells in culture. Just by varying the hormonal conditions of the cell's environment we are able to make the cells choose between division, tubular elongation and growth by budding. More experiments are needed to define in the first place dose-response curves and temporal patterns of sensitivity and reactivity of the cell system used.

Although we are aware of the very artifactual nature of the experimental subject, we are convinced that an approach using isolated and cultured cells will bring new insights into the understanding of the developmental biology of plants and of their different organs.

Fig. 3. Typical elongated cell after 20 days of culture on a medium containing only NAA. (a) *Bright field.* **(b)** *FDA fluorescence.* **(c)** *Calcofluor white fluorescence: note the heavy staining of the cell wall. Bar, 50 μm.*

Fig. 4. Typical cell budding on a medium containing only BAP after 15 days in culture. *The structure in the middle of the picture represents one cell.* **(a)** *Bright field.* **(b)** *FDA fluorescence: all parts of the branched cell contain viable cytoplasm.* **(c)** *Calcofluor white fluorescence: cellulose is detected in the wall of the whole cell construct. Bar, 50 μm.*

Materials and Methods

Plants of *Nicotiana tabacum* L. cv. Petite Havana were grown in sterile culture on a Murashige and Skoog medium without hormones (4.7 g/l, Flow Laboratories), supplemented with 10 g/l sucrose and solidified with 8 g/l agar, pH 5.7.

Only healthy leaves were used in the experiments. Protoplasts were isolated from the leaves basically following the method of Potrykus and Shillito (1986), using 2% cellulase Onozuka R10 and 0.2% macerozyme R10 (Yakult Honsha Co, Ltd.) After a short vacuum infiltration, leaves were incubated for 90 min at 26°C. Living protoplasts were isolated from cell debris by filtration and centrifugation (at 60 g).

At the end the purified protoplasts were either suspended in K3A liquid culture medium (Potrykus and Shillito, 1986), embedded in the same medium containing 0.6% Sea Plaque agarose (FMC BioProducts) or immobilized on the surface of such an agarose layer. Four different culture conditions were used: the complete K3A medium with 1 mg/l NAA (an auxin) and 0.2 mg/l BAP (a cytokinin); a medium with only NAA; a medium with only BAP and a medium without hormones. They were kept in culture for 20 days at 22°C in a 16 h photoperiod at 2000 lux (Philips tlm 65W/33).

Cell development was followed using a Nikon TMD inverted microscope equipped for epifluorescence.

Acridine orange (BDH Chemicals Ltd), (C)FDA (Carboxy) fluorescein diacetate (Serva) and calcofluor white (Fluorescent Brightener 28, Sigma) were used as standard fluorochromic stains for nuclei, cell viability and cell wall cellulose. Observations on crucial stages in cell development were confirmed or documented with scanning electron microscopy (Jeol 220 A) or confocal scanning laser microscopy (Biorad-MRC 600).

Acknowledgments

This research was supported by grants of the Belgian National Fund for Scientific research (FKFO and FGWO). D. Stickens is the recipient of a grant from the Flemish Community. W. Tao is the recipient of grants from the Belgian State Research Programme (87/92-119) and from the Chinese Government. The authors acknowledge the invaluable help of S. Foubert.

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