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Rapid Communication

Stomatal Guard Cells Are Totipotent[1]

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It has been successfully demonstrated, using epidermis explants of sugar beet (*Beta vulgaris* L.), that stomatal guard cells retain full totipotent capacity. Despite having one of the highest degrees of morphological adaptation and a unique physiological specialization, it is possible to induce a re-expression of full (embryogenic) genetic potential in these cells in situ by reversing their highly differentiated nature to produce regenerated plants via a callus stage. The importance of these findings both to stomatal research and to our understanding of cytodifferentiation in plants is discussed.

Delegation of cellular function is a universal feature of all higher plants and animals and is essential for the complexity necessary for the coordinated functioning of multicellular organisms. In plants the resultant heterogeneity between cells is the product of a continuous process of cell differentiation (Wareing and Phillips, 1970). It arises through specific patterns of differential gene expression, realizing not only visible changes but also a myriad of "unseen" cellular modifications concerning metabolic processes, membrane function, the cell cycle, etc. Whereas nonliving cells (e.g. xylem vessels) are clearly irreversibly differentiated, it has long been a subject of conjecture to what extent living plant cells become terminally specialized (Tran Thanh Van, 1981; Binding, 1986).

The totipotency of many plant cells and tissues has been regularly demonstrated in vitro (Binding, 1986). However, despite our rapidly expanding knowledge, it has often proven to be impossible to reinitiate cell division in certain highly specialized cell types. Furthermore, even when cell division is obtained, the subsequent morphogenic response can be distinctly limited. Such findings have led to the use of terms such as "unipotent" and "nullipotent" to describe cells with limited or total loss of developmental potential (Tran Thanh Van, 1981).

Stomatal guard cells, in terms of their morphological specialization (Sack, 1987) and unique metabolic organization (Assmann, 1993), are considered to be one of the most highly differentiated living cell types in plants (Zeiger, 1983). Previous attempts to induce division in guard cells failed entirely (Thielman, 1925; Dehnel, 1960; Pillai et al., 1992), and they have also been described as being nullipotent (Tran Thanh Van, 1981). Failure to demonstrate plant cell totipotency can be due to one of two possibilities: the cells may indeed be irreversibly differentiated, presumably through permanent physical changes to the DNA, or alternatively, the cells simply may not yet have been placed under the environmental conditions necessary to release them from the restraining factors that maintain their morphological integrity in vivo. In this short paper we demonstrate that the latter is indeed the true cause in stomatal guard cells in situ and that, despite their highly differentiated nature, full totipotent potential is retained. As a result, a novel model system has been identified that can be used for studying stomatal physiology, gene expression, and fundamental aspects of cytodifferentiation in plants.

MATERIALS AND METHODS

Leaves harvested from shoot cultures of the sugar beet (*Beta vulgaris* L.) line Bv NF were used (for details of maintenance, see Hall et al., 1993).

Epidermis Isolation

Under sterile conditions, epidermis was manually peeled from the abaxial surface of leaves taken from 3- to 4-week-old shoot cultures using fine forceps. Fragments of varying sizes (dimensions varying from 1 to 5 mm) were removed from regions of leaf lamina between the veins. Care was taken to avoid the inclusion of fragments of mesophyll or vascular tissue. Although free chloroplasts were observed to adhere to the explants, the absence of intact mesophyll cells could be confirmed using fluorescence microscopy. The flattest leaves with the least prominent veins proved to be the easiest to use. Upon removal, each epidermal fragment was immediately floated on liquid culture medium with the cuticle side upward.

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Abbreviation: FDA, fluorescein diacetate.

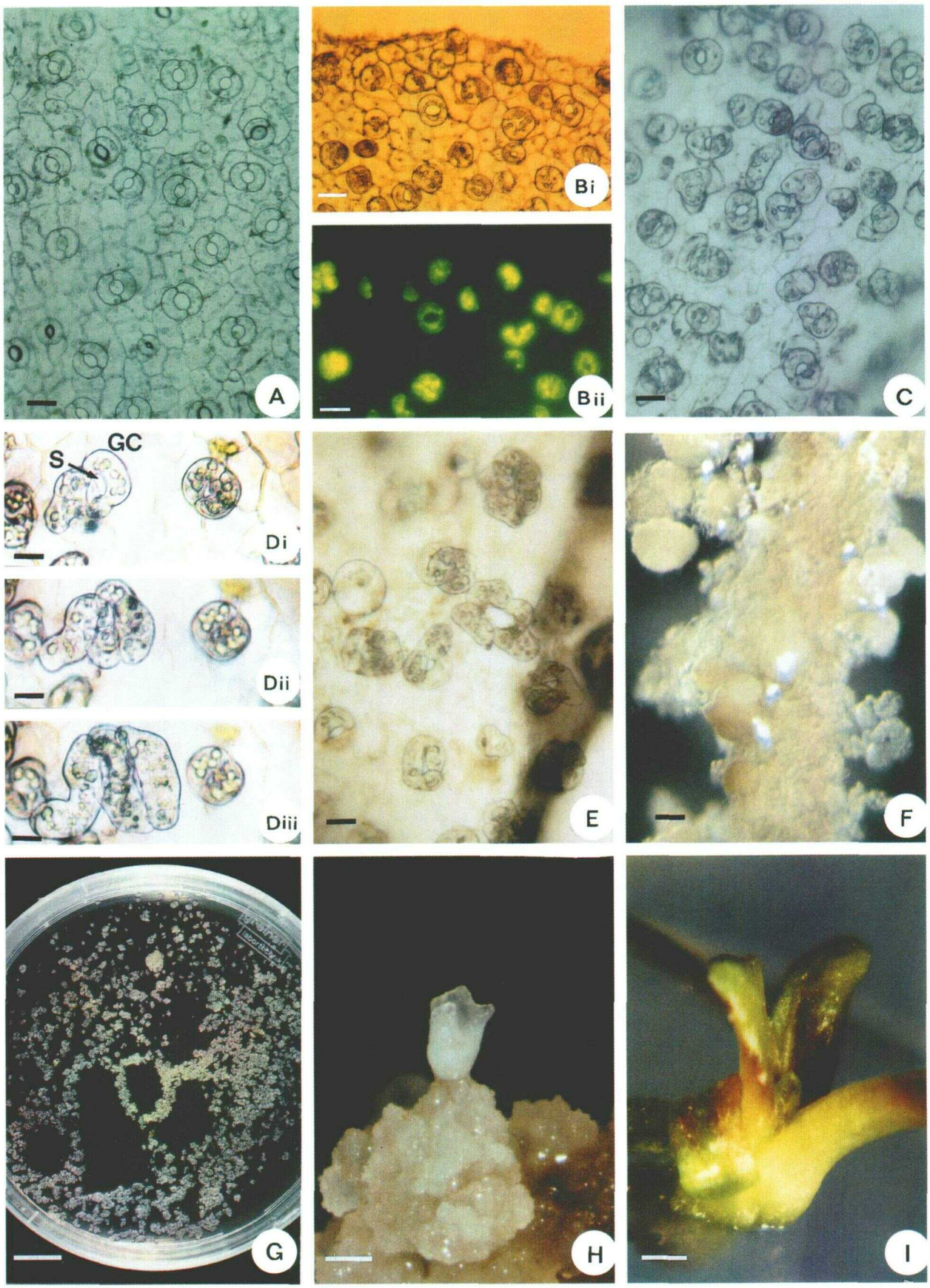

Figure 1. (Legend appears on facing page.)

Culture Conditions

Epidermal fragments with a surface area totaling approximately 50 mm^2 were cultured in 2 mL of medium in 3-cm Petri dishes (Greiner, Frickenhausen, Germany, tissue culture grade) in darkness at 28°C. The medium used was a modified K8p medium, identical to that used for sugar beet protoplast culture (Hall et al., 1993). FDA staining was performed as described by Widholm (1972). After the formation of microcalli, 1-mL aliquots of the cultures were transferred to 20 mL of PGo medium (de Greef and Jacobs, 1979) supplemented with 0.9% (w/v) agarose (Seaplaque, Duchefa, Haarlem, The Netherlands), 3% (w/v) Suc, and 1 μM benzylaminopurine in 9-cm Petri dishes. Individual calli were subcultured every 2 weeks in darkness until the first appearance of regeneration, at which time the dishes were transferred to the light (2000 lux, 16 h light, 8 h dark; 25°C).

RESULTS

Although sugar beet epidermis is not easy to isolate, it was nevertheless possible to obtain sufficient "clean" strips for experimentation (Fig. 1A). Older, more mature leaves proved to be the best starting material, whereas it was virtually impossible to obtain mesophyll-free strips from younger leaves.

After 24 h in culture the only visible change in the epidermal explants was the increased prominence of the plastids as a result of enhanced starch accumulation (data not shown). After 3 to 4 d, FDA staining revealed that essentially only guard cells had survived the isolation procedure (Fig. 1, Bi and Bii). After 1 week extensive modifications to the guard cells had occurred, typified by a general swelling of the cells, which became irregular in shape, often entailing a disappearance of the stomatal pore (Fig. 1 C). After approximately 10 d the first guard cell divisions were observed (Fig. 1, Di-Diii). Divisions were either symmetrical or asymmetrical and the paired cells of each stomatal complex acted as individuals. Sugar beet stomata do not have subsidiary cells and no surviving epidermal (pavement) cells were ever observed to divide. Guard cell divisions proceeded as distinct localized islands at a rapid rate (Fig. 1E), and after 24 d the entire epidermal strip had disappeared under a mass of microcalli (Fig. 1F). Many cells were shed onto the base of the Petri dish, where they continued to grow. The callus produced was distinctly friable and consisted of cells that were densely cytoplasmic and contained numerous large and small starch grains. Loosening this culture using a pipette and plating it out onto solid medium resulted in extensive colony formation (Fig. 1G). The calli continued to grow and after 2 to 4 weeks embryo formation was observed (Fig. 1H). In two experiments the average regeneration frequency was determined, using 100 calli in each case and was found to be 20%. The plants obtained developed normally (Fig. 1I) and could be transferred to soil after rooting on PGo medium containing 20 μM indole butyric acid.

Some heterogeneity was observed between explants. Although in most hand-peeled epidermal strips the guard cell response approached 100% (Fig. 1E), division in others was almost or completely absent. This was observed even in cultures initiated from a single leaf. The amount of epidermis per dish had no apparent influence on the frequency of cell division. However, the presence of adhering vascular fragments resulted in a rapid production of compact callus, which significantly (or totally) inhibited guard cell division.

DISCUSSION

The results presented here demonstrate that, when using the correct environmental conditions, sugar beet guard cells can be induced to divide in situ and that regenerable callus is produced. Therefore, an extreme degree of cytodifferentiation in plants does not necessarily entail irreversible genetic modification or loss of totipotent capacity.

In a previous publication (Hall et al., 1995), we showed, using computer-assisted microscopy, that in recalcitrant sugar beet cultures, surprisingly, guard cell protoplasts appeared to be capable of dividing to produce totipotent callus. Totipotency of protoplasts from guard cells of tobacco has also recently been reported (Sahgal et al., 1994). However, from both of these papers it is unclear as to what potential role the physical process of protoplast isolation played in this phenomenon. The results presented here clearly indicate that protoplast isolation, which, for example, could have instigated a complete genetic "reprogramming" of the guard cells, is not a prerequisite for the realization of their totipotent potential. We show that intact guard cells retain an intrinsic ability to re-enter the cell division cycle and produce regenerable callus. This appears at least in sugar beet to be a general phenomenon.

Figure 1. (Figure appears on facing page.) Time course of the isolation, dedifferentiation, and plant regeneration from stomatal guard cells of sugar beet. A, Leaf epidermis of sugar beet after hand peeling (bar = 50 μm). Bi and ii, Epidermal strip after 4 d in culture, stained with FDA to reveal the nonviability of cells other than those of the stomatal complexes (bar = 40 μm). C, Epidermal strip of sugar beet after floating for 7 d on culture medium, showing clear swelling of the stomatal guard cells, which begin to take on an aberrant form (bar = 50 μm). D, Early divisions in a stomatal complex as seen on d 8 (i), d 9 (ii), and d 10 (iii). On d 8 one guard cell has already divided, whereas the other (GC) is still clearly recognizable. The stoma (S) is still present. After division of the second guard cell (ii), the stoma disappears and a microcolony is formed (bar = 20 μm). E, Guard cell divisions result in the formation of individual microcallus islands on top of an otherwise collapsed epidermis (bar = 50 μm). F, After approximately 4 weeks in culture a floating epidermal strip has become covered in hundreds of guard cell callus colonies (bar = 300 μm). G, Individual guard cell callus colonies developed 1 week after plating onto solid medium (bar = 1 cm). H, Embryo formation on guard cell-derived callus 3 weeks after transfer to solid medium (bar = 1 mm). I, Developing sugar beet plantlet derived from a single guard cell ready for rooting and transfer to soil (bar = 1 mm).

Although the results presented concern just a single genotype, we have also been successful in inducing guard cell division in a wide range of other genotypes (data not shown). Furthermore, it has also been possible to obtain guard cell callus from strips taken from greenhouse-grown plants (R.D. Hall, T. Riksen-Bruinsma, unpublished observations). However, sterility problems prevented the continuation of these experiments to the regeneration phase.

The results reported here show remarkable parallels with responses observed using leaf protoplasts (Hall et al., 1995), in which guard cell protoplasts were also observed to have a high cytokinetic capacity (approximately 60%), whereas epidermal protoplasts were found only to undergo extensive swelling. The observance of heterogeneity in response between epidermal explants in this study requires further investigation. Although it is possible that the detrimental influence of vascular fragments may be the causal factor, the possible influence of explant location on the source leaf cannot be excluded. Heterogeneity of in vivo stomatal response across a leaf lamina is a recently recognized phenomenon (Mansfield et al., 1990). The intriguing possibility that these two observations could be related is worthy of detailed attention.

Guard cells differentiate early in the development of leaves to form a unique and relatively uniform population of cells that subsequently undergo little further modification (Sack, 1987). In diploid plants, guard cells remain diploid and do not appear to undergo endopolyploidization, as is typical of many somatic (e.g. epidermal) cells (Melaragno et al., 1993). Consequently, the ability to obtain normal regenerated plants from these cells is an indication that their unique specialization in terms of a distinct morphology and membrane physiology and a modified plastid metabolism, etc., is solely the result of differential gene expression and is not maintained by any permanent loss of genetic integrity through physical modifications within the cell genome.

This system, in addition to having potential value as a new experimental tool for studying aspects of guard cell physiology, could also be exploited to extend our understanding of the processes of cytodifferentiation in plants. The unique properties of guard cells in terms of physiology (Fitzsimons and Weyers, 1987) or membrane structure (Marten et al., 1992) could be readily used as markers of differentiation. The influence of culture conditions, chosen to stimulate dedifferentiation and its possible reversal, could then be followed. It is interesting that the presence of large amyloplasts in guard cells, which is atypical compared with the other cells in a sugar beet leaf, is carried over into the callus phase. This suggests that some degree of cell differentiation may be retained after the reinitiation of cell division. It would therefore be valuable to use this system to determine whether and for how long other more specific guard cell features are also retained. The rapid and essentially synchronous onset of the dedifferentiation process in these explants also offers intriguing possibilities for using sugar beet guard cells in gene expression studies, to assist in the identification of proteins essential to stomatal function, and in the quest for guard cell-specific genes and promoters associated, for example, with signaling pathways (Schroeder, 1992; Taylor et al., 1995).

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LITERATURE CITED

Assmann SM (1993) Signal transduction in guard cells. Annu Rev Cell Biol **9:** 345–375

Binding H (1986) Regeneration from protoplasts. *In* IK Vasil, ed, Cell Culture and Somatic Cell Genetics, Vol 3. Academic Press, Orlando, FL, pp 259–274

de Greef W, Jacobs M (1979) In vitro culture of the sugarbeet: description of a cell line with high regeneration capacity. Plant Sci Lett **17:** 55–61

Dehnel GS (1960) Response of stomata to wounding. Bot Gaz **122:** 124–130

Fitzsimons PJ, Weyers JDB (1987) Responses of *Commelina communis* guard cell protoplasts to abscisic acid. J Exp Bot **38:** 992–1001

Hall RD, Pedersen C, Krens FA (1993) Improvement of protoplast culture protocols for *Beta vulgaris* L. (sugarbeet). Plant Cell Rep **12:** 339–342

Hall RD, Verhoeven HA, Krens FA (1995) Computer-assisted identification of protoplasts responsible for rare division events reveals guard cell totipotency. Plant Physiol **107:** 1379–1386

Mansfield TA, Hetherington AM, Atkinson CJ (1990) Some current aspects of stomatal physiology. Annu Rev Plant Physiol Plant Mol Biol **41:** 55–75

Marten I, Zeilinger C, Redhead C, Landry DW, Al-Awqati Q, Hedrich R (1992) Identification and modulation of a voltage-dependent anion channel in the plasma membrane of guard cells by high-affinity ligands. EMBO J **11:** 3569–3575

Melaragno JE, Mehrotra B, Coleman AW (1993) Relationship between endopolypoidy and cell size in epidermal tissue of *Arabidopsis*. Plant Cell **5:** 1661–1668

Pillai KG, Rao U, Rao IVR, Mohan Ram HY (1992) Induction of division and differentiation of somatic embryos in the leaf epidermis of *Gaillardia picta*. Plant Cell Rep **10:** 599–603

Sack FD (1987) Development and structure of stomata. *In* E Zeiger, GD Farquhar, IR Cowan, eds, Stomatal Function. Stanford University Press, Stanford, CA, pp 59–89

Sahgal P, Martinez GV, Roberts C, Tallman G (1994) Regeneration of plants from cultured guard cell protoplasts of *Nicotiana glauca*. Plant Sci **97:** 199–208

Schroeder JI (1992) Plasma membrane ion channel regulation during abscisic acid-induced closing of stomata. Philos Trans R Soc Lond-Biol Sci **338:** 83–89

Taylor JE, Renwick KF, Webb AAR, McAinsch MR, Furini A, Bartels D, Quatrano RS, Marcotte WR, Hetherington AM (1995) ABA-regulated promoter activity in stomatal guard cells. Plant J **7:** 129–134

Thielman M (1925) Über Kulturversuche mit Spaltöffnungszellen. Arch Exp Zellforsch **1:** 66–108

Tran Thanh Van KM (1981) Control of morphogenesis in *in vitro* cultures Annu Rev Plant Physiol **32:** 291–311

Wareing PF, Phillips IDJ (1970) The Control of Growth and Differentiation in Plants. Pergamon, Oxford, UK

Widholm JM (1972) The use of fluorescein diacetate and phenosafranine for determining viability of cultured cells. Stain Technol **47:** 189–194

Zeiger E (1983) The biology of stomatal guard cells. Annu Rev Plant Physiol **34:** 441–475