Production of Transgenic Plants by Electrofusion of Single Plant Protoplasts

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The possibility of producing transgenic plants which incorporate genetic attributes from different plant species and/or genetic attributes from specific plant cell tissue, presents an exciting prospect. However, there are many technical difficulties which have limited progress in producing such transgenic plants. Here we discuss a new technique, based on somatic fusion of individual plant protoplasts using radio frequency electric fields, which has the potential to overcome many of these obstacles.

INTRODUCTION

The introduction of new and desirable genetic features into plant cells can be achieved to varying degrees using methods ranging from standard cross-fertilisation breeding to highly sophisticated DNA manipulation using molecular-biology-based techniques. Whilst these approaches have been (and are) used with considerable success, they also have limitations. For this reason the possibility of enhancing genetic variability using somatic fusion is an attractive alternative. The difficulties of inducing somatic fusion between cells, the problems associated with identifying and selecting individual cells for fusion and the isolation of the desired hybrid-fusing product has limited progress in this area. The production of transgenic plants by somatic fusion of cells requires the regeneration of the whole plant from a single hybrid cell. Until now the regeneration efforts were restricted largely to wild sunflower species in in-vitro culture or sunflower genotypes which were thought to have contributions from wild type genomes (Krasnyanski and Menczel, 1993).

Regeneration difficulties present a major obstacle to somatic hybridisation although recently a reproducible shoot and root regeneration protocol was developed for genotypes of *Helianthus annuus,* which had no wild type contributions (Wingender et al., 1996).

We have perfected a technique to fuse a pair of single, individually selected, protoplasts using radio frequency electric fields to manipulate and selectively fuse the protoplasts. The technique overcomes the problems of isolation and identification of hybrid fusates which are encountered in other commonly used methods to induce somatic cell fusion. Further, the fusates so produced have a high division potential and will proliferate in culture from a single cell without the need for feeder cells or conditioned media. We have used this technique to produce hybrid cells from broad bean guard cells and hypocotyl cells from sunflower. Guard cells have the C4-directed enzymatic apparatus which is known to be coupled with a reduction in photorespiration resulting in a higher survival rate and yield potential of crops. The possibility to enhance the genetic variability of agriculturally important plants using somatic hybridisation between a C4-specific guard cell protoplast and a C3-

equipped hypocotyl protoplast with a high division potential, has considerable appeal.

MATERIALS AND METHODS

Hypocotyl protoplasts were isolated from young plants of *H. annuus* L. grown under sterile conditions from seeds (cv. Sunshine, No.4, Royston Petrie Seeds Ltd. Kenthust, Australia) by a micro-isolation process (Schmitz and Schnabl, 1989). Protoplasts which showed good cytoplasmic streaming were picked up by drawing them individually into a micropipette/microsyringe from a mixture of debris and protoplasts. Guard-cell protoplasts from *Vicia faba L.* were isolated in a similar fashion.

When plant cells are exposed to alternating (AC) electric fields, the cells will undergo translation, rotation on their own axis, and will elongate. Each of these electro-mechanical responses is dependent in a complex manner on the frequency of the AC electric field, the gradients in this field, and the particular electrical properties of the cells (Mahaworasilpa et al., 1994). By choosing the field frequency and adjusting the field gradients it is possible to selectively manipulate individual cells in this manner. Cell rotation (on its own axis) occurs over two narrow bands of frequencies (Mahaworasilpa et al., 1996) and can be utilised to orient cells relative to each other prior to fusion. The translation effects can be used to bring two different cell species together and appress them tightly for subsequent fusion. The fusion of the two cells held together in tight contact by the AC field can be induced by the application of a very short-pulsed, intense, electric field. The latter, if of sufficient magnitude causes electrical breakdown of the (plasma) membrane envelopes of plant cells, at the point of contact of the two cells (Coster and Zimmermann 1975, 1976). This causes the cells to fuse.

For our experiments two vertical nickel alloy wire electrodes, 180 μm in diameter were lowered into the solution using micromanipulators; the two protoplasts being positioned approximately in the central region between the electrodes. These electrodes were used to generate radio frequency electric fields. The protoplasts were then positioned more precisely between the two electrodes by a combination of the application of electric fields of appropriate frequency and manipulation of the electrodes themselves. These operations were performed under an inverted microscope (Zeiss Telaval 31). The alignment of protoplasts was achieved by application of an AC field at a frequency of 0.5 to 1MHz and variable field strength. Prior to alignment the protoplasts were also subjected to electro-rotation by application of an AC field at frequencies of either around 1 kHz or 10 MHz to both obtain a relative cell orientation that produced good cell-to-cell contact as well as to evaluate cell viability (Arnold and Zimmermann, 1984; Mahaworasilpa et al., 1996). Fusion of selected protoplast pairs was induced by a DC pulse (100 μs, 50 to 100 kV/m, see Table 1) following alignment of the cells.

RESULTS

A sequence of the fusion of a pair of two different protoplasts (experiment 1) is shown in Figures 1a-1d. The time required for the fusion product to become spheroidal after the fusion pulse was typically 2 min (see Figures 1c to 1d). Immediately after fusion the fusate, which was ~35 μm in diameter (Figure 1d) was transferred, under the microscope using the micropipette system, from the mannitol solution into a liquid

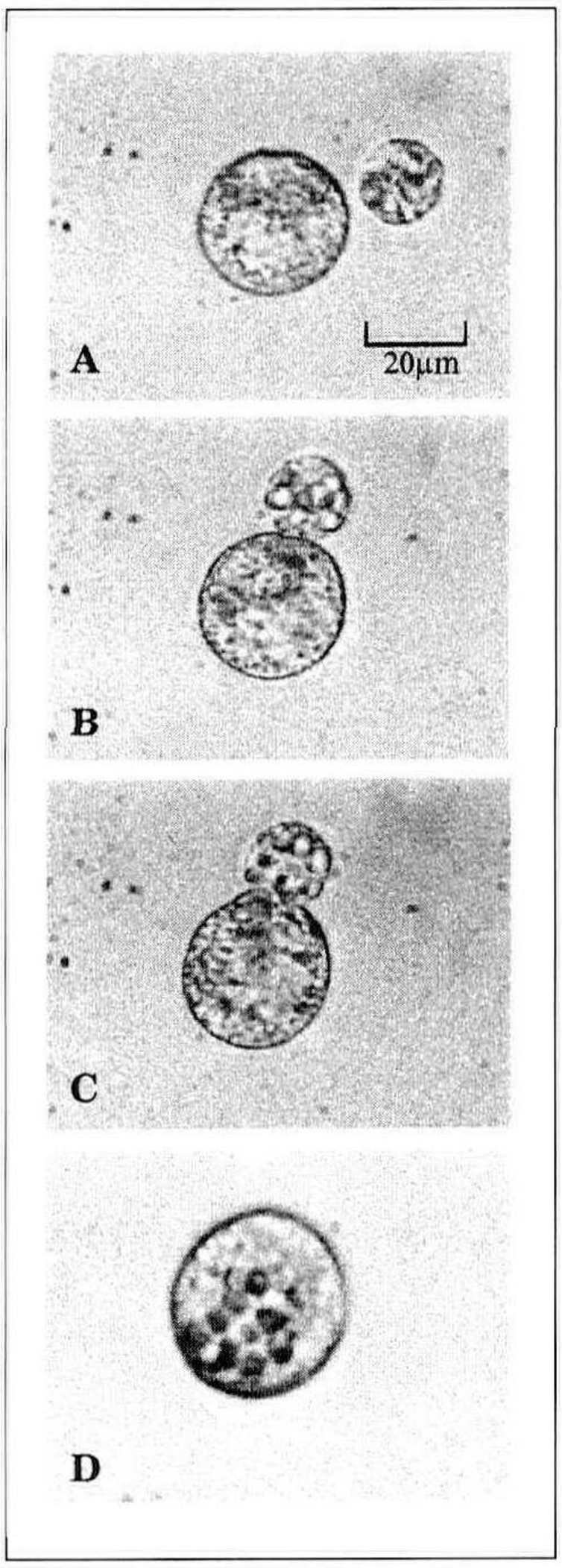

Figure 1. Fusion of two different protoplasts.

nutrient medium (Wingender et al., 1995) in a 300-µl microwell. The protoplasts were cultivated in the dark at 25C for 9 days without using feeder cells ("nursing culture") or conditioned medium.

The sequence of the formation of a microcolony (microcallus) over the first 9 days of cultivation is shown in Figures 2a - 2d. Between 30% and 60% of the hybrid fusates divided (Table 1), depending on the combination of parameters required to achieve fusion. The hybrid product budded for the first time after 3 days in culture (Figure 2a); On day 6 after the fusion event an identical arrangement of the starch-containing guard-cell plastids became visible in the daughter cell (Figure 2b). These large plastids (approximately 3 to 5 µm) play an important role as starch reservoirs during the volume regulation of guard cells (Schnabl et al., 1982; Schnabl, 1985; Schnabl, 1992). Due to their characteristic appearance (numbers, morphology, and arrangement) the plastids provided an excellent means of following the progress of fusion and formation of the colony of cells. Duplication of the nuclear materials from the mother to daughter cell occurred simultaneously with the appearance of the plastids (Figure 2b). On Day 7 a second budding was formed, but without the characteristic stomatal plastids (Figure 2c). Figure 2d shows the microcolonies of 5 cells which had developed (after a cultivation of 8 days); a day later (after 9 days in total) the microcallus consisted of eight cells showing the characteristic plastid equipment of guard cells (not shown).

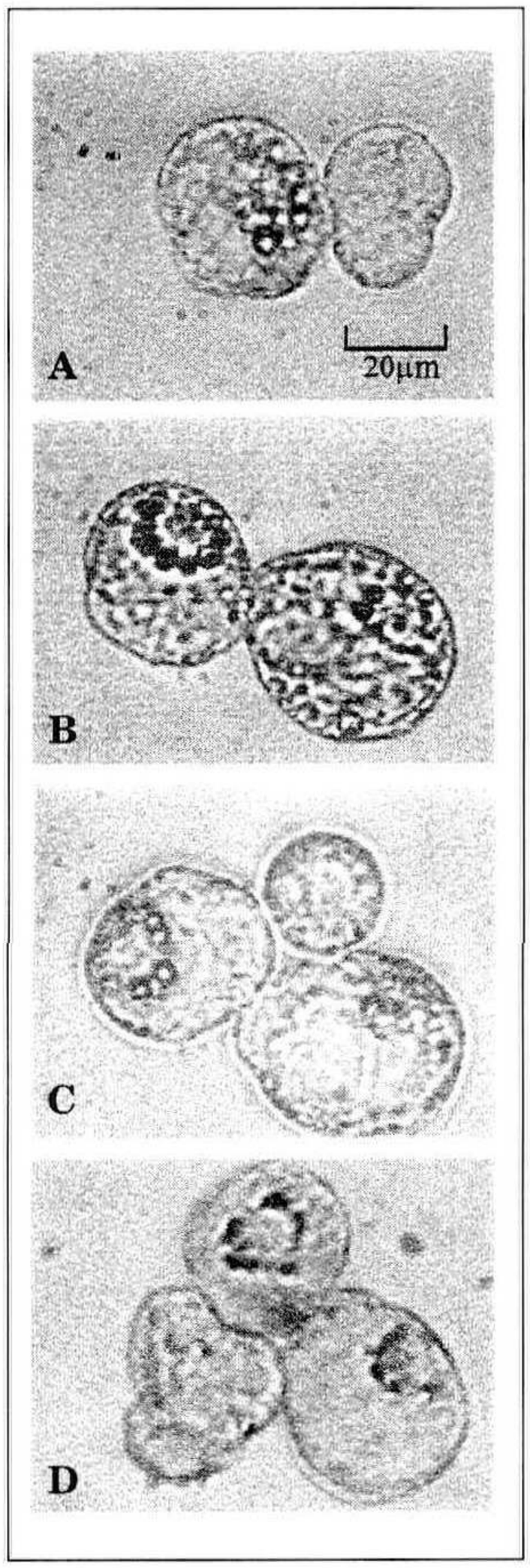

Figure 2. Formation of microcallus.

DISCUSSION

We have successfully produced, for the first time, microcolonies of a hybrid of two different plant species (sunflower and broad bean) and of two differ-

ent tissues with different types of CO_2 fixation pathways; a C3-equipped hypocotyl protoplast of sunflower and a C4-equipped guard cell protoplast of broad bean. The hybrid products proliferated individually, or from groups of 2 or 3 cells, in a liquid nutrient medium; without feeder cells ("nursing") or conditioning. In contrast, single unfused hypocotyl and guard-cell protoplasts did not divide under these conditions but died.

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