

Non-enzymatic access to the plasma membrane of *Medicago* root hairs by laser microsurgery

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SUMMARY

Using UV laser microsurgery, the cell walls of root hairs from *Medicago sativa* (alfalfa) were perforated under plasmolysing conditions, giving direct access to the plasma membrane without enzyme treatment. The opening in the cell wall of a few μm in diameter results in immediate movement of the protoplasm and partial or complete extrusion of the cell contents. The movement of the protoplasm is retarded by increases in calcium concentration. The calcium-dependency of the movement of the protoplasm allows us to obtain preferentially the extrusion of protoplasm, or to gain access to a small area of plasma membrane in situ. The complete protoplasm can be expelled, to form a protoplast. Fluorescein diacetate staining indicated esterase activity and membrane integrity of the protoplasts. Microscopic

examination revealed organelle movement and the presence of a nucleus. The plasma membrane was free from cell wall fragments, as shown by Tinopal staining. Conditions for obtaining plasmolysis without disturbing the physiology of the root hairs too much were achieved by slow, stepwise and reversible plasmolysis. Cytoplasmic streaming in root hairs was maintained during plasmolysis and laser microperforation. This laser technique should be suitable for the performance of electrophysiological studies using the patch-clamp technique on plasma membrane from non-enzyme-treated cells.

Key words: laser microsurgery, fluorescence microscopy, plant cells, protoplasts, root hairs, *Medicago sativa*

INTRODUCTION

Until recently, enzymatic digestion has been the preferred technique for the removal of plant cell walls prior to protoplast preparation (Galun, 1981). However, there is increasing evidence indicating that enzymes have deleterious effects on membrane properties (Morris et al., 1981, and references therein; Ishii, 1987; Browse et al., 1988; Hahn and Lörz, 1988; Dugas et al., 1989) and that they induce new physiological properties in protoplasts, such as defence reactions (Chen and Boss, 1990) or the generation of binding sites for lectins (Sun et al., 1992). To overcome these problems, a new technique has recently been developed with the aim of reducing the time of tissue incubation in enzyme solution; the release of protoplasts is then induced by transferring the tissues into a low osmotic medium (Elzenga et al., 1991). In addition to the problems reported above, there are some restrictions in the use of enzymes for some plant material such as cells of the giant alga *Chara*, which are refractory to enzyme action (Laver, 1991). Finally, the membrane surface of protoplasts released from enzyme-treated cells is often not 'clean'

enough for electrophysiological studies; for example, in patch-clamping where a clean membrane surface is necessary to obtain a high-resistance seal between the patch pipette and the membrane. This is the case for protoplasts isolated from corn cells in suspension culture (Fairley and Walker, 1989), root hairs of *Medicago* and mesophyll cells of tobacco.

Before the enzymatic methods had been developed (Cocking, 1960), protoplasts were isolated by mechanical methods (Klercker, 1892). Their release was generally achieved by plasmolysing the tissue and slicing it with a sharp blade (Cocking, 1965). This basic mechanical method has been modified to adapt it to the type of plant material being studied (see references cited by Sun et al., 1992). As the yield of protoplasts is generally low, these methods have not been used extensively, but they have attracted new interest because of the increasing number of drawbacks reported in the literature on the use of enzymes. In this context, an improved mechanical method consisting of the use of an electrically driven cutter allows the isolation of a consistent number of protoplasts and subprotoplasts (Sun et al., 1992). Access to the plasma membrane in situ, without

enzyme treatment, can also be achieved by surgical removal of the cell wall. This technique has successfully been applied by Laver (1991) to *Chara* internodal cells, which have a diameter of about 1 mm.

A UV laser microbeam (Wiegand et al., 1987) can be used to induce precisely localized submicrometer perforations on the surface of cells of various sizes. This contact-free technique has been used to open plant cell walls for the introduction and expression of DNA (Weber et al., 1990) and for the micromanipulation of chloroplasts (Weber et al., 1989). Recently, the removal of the cell wall in the tip region of *Fucus* rhizoid cells has been performed using a UV laser beam, and the presence of ion channels in the plasma membrane could be demonstrated in these conditions (Taylor and Brownlee, 1992). We report here the use of the UV laser micro-irradiation technique on the root hair cells of a higher plant (alfalfa) to gain access to a piece of plasma membrane, and for the microscopically controlled formation of subprotoplasts or protoplasts under plasmolysing conditions.

MATERIALS AND METHODS

Plant material

Seeds of *Medicago sativa* L. cv. Milefeuill (alfalfa) were disinfected (45 min) with ethanol (95%). The seeds were then rinsed 5 to 6 times with sterile distilled water. They were grown for 48 hours at 21°C in the dark in 5 mM 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer adjusted to pH 6.0 with KOH, and solidified with Bacto agar (Difco; 10 g l⁻¹). For laser experiments, whole seedlings were deposited on a coverslip in a drop (150 µl) of the buffer containing sorbitol or glycine (0.35 M) to plasmolyse the root hairs. Alternatively, the seedlings were plasmolysed by gradually increasing the concentration of the osmoticum in steps from 0.2 M via 0.3 M to 0.35 M, with incubation for 5 minutes at each step. The seedlings were then covered with a second coverslip.

For control of growth and viability, the seedlings were deplasmolysed by incubation for a few minutes in buffer containing no osmoticum and then deposited for 24 hours in buffer (MES-Tris, 5 mM, pH 6.0, containing KCl, NaCl, CaCl₂ at 100 µM), solidified with Bacto agar (Difco; 10 g l⁻¹) and grown in the conditions described above. After 24 hours, roots were checked for the presence of hairs and the lengths of control and treated plants were measured.

For the experiments on the effect of calcium, the plants were deposited in a 3.5 cm diameter Petri dish (Petriperm, Heraeus) and submerged in 5 ml of medium to prevent evaporation.

The laser microbeam apparatus

A pulsed UV nitrogen laser (VSL 337 ND, Laser Science Inc. Cambridge, USA) or an excimer pumped dye laser (EMG 102 MSC with FL 2002, Lambda Physik, Göttingen, Germany) was coupled with an inverted microscope (IM 35 or Axiovert, Zeiss, Oberkochen, Germany) via the epifluorescence illumination path. The wavelength was about 340 nm, the typical pulse energy at a repetition rate of 20 Hz was 200 µJ; the pulse duration of such a laser is a few nanoseconds. A variable attenuator was used to modulate the laser power. According to the basic laws of light focussing the laser beam had first to be expanded by a telescope system before it could be focussed to the diffraction limit through the microscope objective (Zeiss Ultrafluor ×100, Glyc, NA, 1.25). The microscope image was recorded with a video camera and read

into an image-processing system (C 1966, Hamamatsu, Herrsching, Germany). For a detailed description of the laser microbeam system see, for example, Wiegand et al. (1987), Greulich and Weber (1992) and Weber and Greulich (1992).

Fluorescence microscopy

For staining the nucleus, Hoechst 33342 (Riedel-de Haën) was used as a vital dye at a final concentration of 1 µg ml⁻¹ (Arndt-Jovin and Jovin, 1977). Whole seedlings were incubated for 1 hour in the dye solution (MES buffer, pH 6.0) before the laser treatment. For cell wall staining, the seedlings were incubated with Tinopal ABP (Ciba-Geigy, Basel; Bilkey and Cocking, 1982), at a final concentration of 0.5%, diluted from a commercial stock solution a few minutes before laser treatment. Fluorescein diacetate (FDA; Larkin, 1976) was used as a probe for the viability of the protoplasts. The dye was prepared as a stock solution in acetone (2 mg ml⁻¹) and diluted to a final concentration of 0.1 mg ml⁻¹.

RESULTS

The dynamics of the formation of a protoplast from a previously plasmolysed root hair perforated at its tip with a UV laser microbeam is reported in Fig. 1 (A-D). A few seconds after the opening of the hair tip, the protoplasm swells and tends to fill the apical plasmolytic space, which had been formed between the cell wall and the plasma membrane during plasmolysis. We have studied the influence of calcium on the movement of protoplasm, since this ion is currently used to improve the yield and quality of protoplasts (Clint, 1985). The results reported in Table 1 indicate that the speed of displacement of the protoplasm is decreased as the calcium concentration in the medium is increased. In the absence of calcium, the extrusion of the protoplasm from the cell envelope is so fast that the plasma membrane ruptures in more than 50% of the hairs. This percentage decreased to about 18% in the presence of 1 mM CaCl₂ and the movement was completely arrested at 10 mM CaCl₂. The calcium-dependency of the process allowed us to obtain preferentially an opened root hair (Fig. 2A), the formation of a subprotoplast (Fig. 1A-D) or the formation of a complete protoplast (Fig. 2C). During the extrusion of the plasma membrane a bud is formed, which enlarges and fills up with the organelles expelled from the root hair cell. Generally, a cytoplasmic bridge, which can be less than 1 µm in diameter and several µm long, connects the protoplasm remaining in the root hair to the protoplasm that is being expelled (Fig. 2B). When the hairs are rather short (up to 50 µm long), their whole content can be released, forming one protoplast containing the nucleus, as seen by microscopic observation (Fig. 2C) and staining with the dye Hoechst 33342 (Fig. 2C, insert). In some cases, the nucleus remains in the hair cell (Fig. 2D). The protoplasts showed organelle movement and fluorescein fluorescence 2 to 3 minutes after the addition of fluorescein diacetate (Fig. 2E). After incubation with Tinopal the root hairs showed a blue fluorescence of their cell wall that was of higher intensity at the tip of the cell. In contrast, the protoplasts prepared by laser treatment did not fluoresce, indicating that they are not surrounded by visible traces of cell wall material (Fig. 2F).

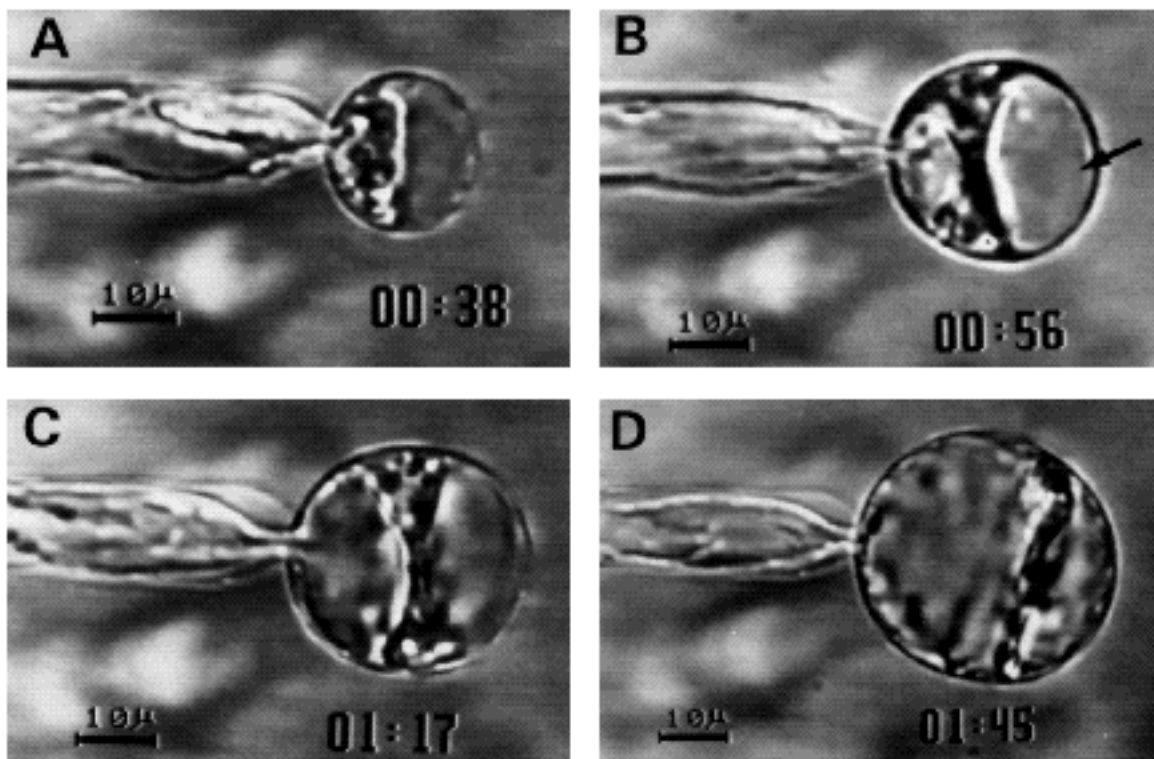


Fig. 1. Time course of the evolution of protoplast formation from a root hair cell after laser microsurgery. (A-D) Flow of protoplasm out of the root hair, filling a bleb of plasma membrane and forming a protoplast. The time course is indicated in minutes and seconds. The arrow points to the vacuole. $\mu = \mu\text{m}$.

Experiments have been performed to determine which conditions of plasmolysis were minimally disturbing for root hair growth after deplasmolysis. Good results have been achieved using glycine as an alternative to sorbitol as an osmoticum. Plasmolysis of the seedlings with gradually increasing glycine concentrations (0.2 M, 0.3 M, 0.35 M for 5 min each) did not impair root hair development. Immediate treatment of the seedlings with 0.35 M glycine is more drastic, i.e. only a few root hairs were able to grow after deplasmolysis.

As a visible sign of the viability of the hairs during plasmolysis, cytoplasmic streaming has been followed for a number of young root hairs. Here again, a stepwise increase in glycine concentration decreases the speed of cytoplas-

mic streaming only slightly during plasmolysis. After a complete round of plasmolysis and deplasmolysis, these hairs showed no staining with Erythrosin, indicating that they were alive.

Finally, we checked that the roots grow normally after the seedlings have been plasmolysed and deplasmolysed. The results of one experiment run in triplicate indicate that the difference in the length of the roots for treated seedlings (mean length $3.97 \text{ cm} \pm 0.43 \text{ cm}$, $n=10$) compared to the controls (mean length $4.59 \text{ cm} \pm 0.53 \text{ cm}$, $n=10$) is not significant.

DISCUSSION

The experiments described here have shown that protoplasm from root hair cells can be extruded under microscopic control after laser microperforation of the cell wall and, under suitable osmotic and ionic conditions, can form protoplasts. The characterization of the protoplasts by fluorescent dyes indicates that: (i) their plasma membrane is free from cell wall fragments, contrary to most protoplasts prepared by enzyme digestion; (ii) they accumulate fluorescein, indicating esterase activity and membrane integrity.

One apparent drawback of the laser method is the fact that plasmolysis can create osmotic stress by decreasing membrane fluidity (Morris et al., 1981; Furtula et al., 1990), modifying the membrane potential (Göring et al., 1979), and disturbing the elongation of tip-growing cells (Schnepf et al., 1986; Schnepf, 1988). Regular methods of plas-

Table 1. Movement of the protoplasm of root hair cells of *Medicago sativa* after the microperforation of the cell wall by a UV laser beam

CaCl ₂ (mM)	Speed of movement ($\mu\text{m s}^{-1}$)	Burst protoplast (%)
0	$7.2 \pm 3.3^*$ ($n=11$)	50 ($n=47$)
0.1	1.3 ± 0.6 ($n=11$)	18 ($n=17$)
1.0	0.5 ± 0.1 ($n=17$)	15 ($n=64$)

*Mean \pm s.d.; n , number of tested root hairs.

A video recording has been made of the movement of the protoplasm of a number of root hairs taken from seedlings incubated in the presence or absence of calcium chloride. The calcium chloride was added at two concentrations at the time of the experiments. The speed of the movement ($\mu\text{m s}^{-1}$) was calculated from these video recordings.

molysing cells for protoplast preparations induce a large decrease in the surface of the plasma membrane and its detachment from the cell wall. The situation for root hairs prepared for laser microsurgery is quite different, since these cells plasmolyse preferentially at their tip. In addition, plasmolysis is gentle enough to allow the normal development of root hairs and seedlings after deplasmolysis.

The immediate movement of the protoplasm after laser microperforation can be interpreted as being due to an increase in the osmotic pressure in the root hair.

The effect of calcium ions in slowing down the movement of the protoplasm is probably due to non-specific interactions of the ion with negatively charged phospholipids on surface membranes, thus stabilizing the membranes (Brasitus and Dudeja, 1988, and references therein; Curtain et al., 1988). In the case of *Fucus* rhizoid cells (Taylor and Brownlee, 1992), the experiments were performed in a medium (sea water) containing a relatively high concentration of calcium (about 10 mM). In these conditions, the emergence of membrane-bound cytoplasm was

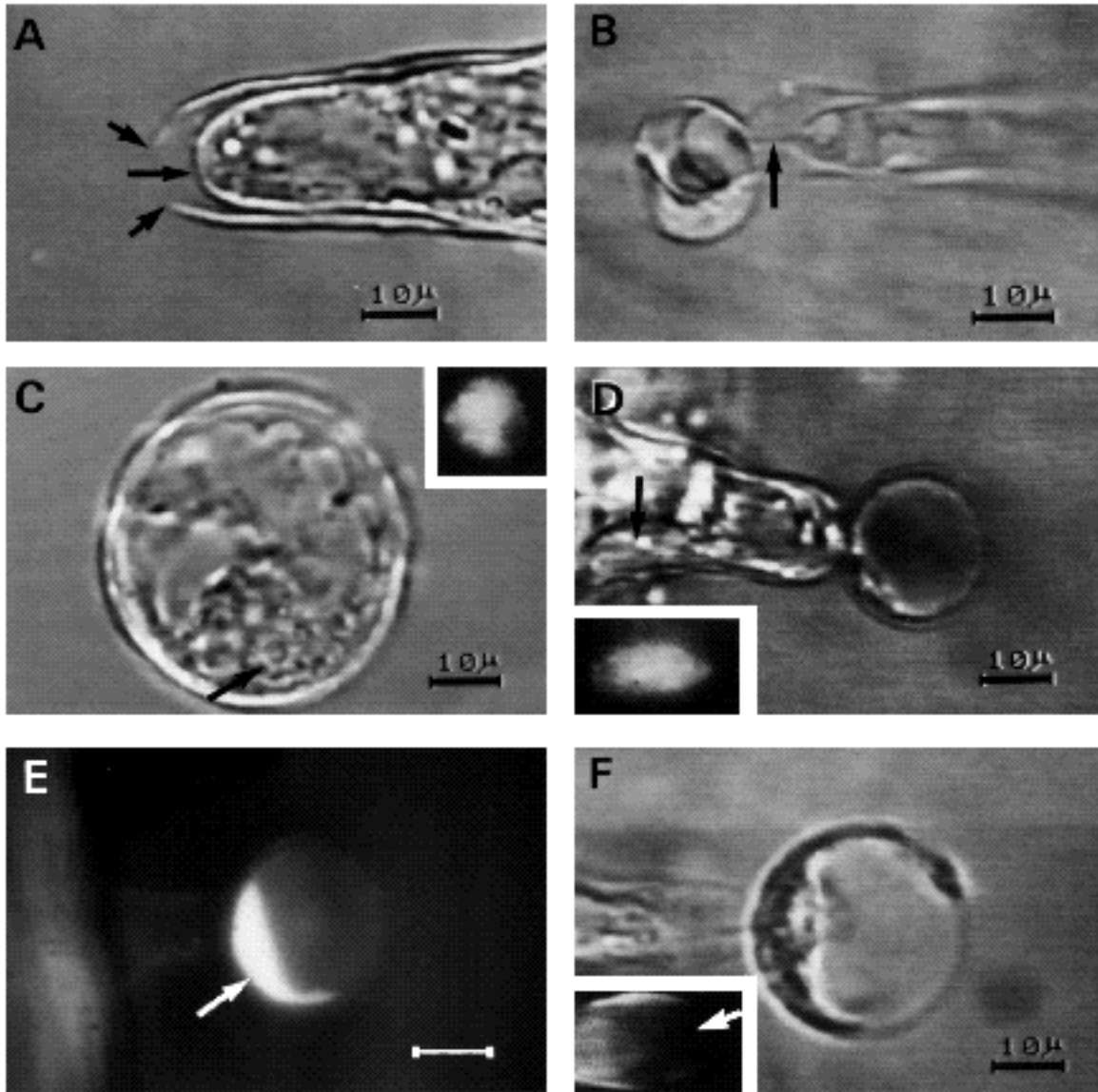


Fig. 2. Transmitted light and fluorescence micrographs of root hairs and protoplasts after laser microsurgery. (A) The cell wall at the tip of the root hair has been destroyed by the laser microbeam (short arrows); the plasma membrane (long arrow) is now in direct contact with the external medium. (B) A protoplast that is being formed. A cytoplasmic filament (arrow) still connects protoplast to protoplast. (C) Light micrograph of a protoplast. The arrow points to the nucleus. Inset: corresponding image after staining with the DNA-specific dye Hoechst 33342, showing bright fluorescence of the nucleus. (D) Light micrograph of a short hair with a forming protoplast. Inset: corresponding image of the hair stained with Hoechst 33342, showing bright fluorescence of the nucleus, which remains in the hair (arrow). (E) Protoplast stained with fluorescein diacetate. Fluorescence in the cytoplasm (arrow) indicates esterase, i.e. metabolic activity. Bar, 10 μ m. (F) Light micrograph of a protoplast. Inset: corresponding image of the hair stained with Tinopal, a dye that stains the cell wall. The cell wall of the hair is fluorescent except for the tip (arrow), which has been destroyed by the laser microbeam. The forming protoplast does not show any fluorescence. $\mu = \mu$ m.

carefully monitored by modifying the osmotic pressure of the medium. In addition to the stabilizing effect of calcium on membranes, it has been shown that these ions are involved in the control of tip growth in other tip-growing cells such as pollen tubes and moss protonema (Schnepf, 1988; Steer and Steer, 1989; Herth et al., 1990). The mechanical properties of the cytoskeleton that sustain growth depend on the calcium concentration in the cytoplasm (Takagi et al., 1990). For applied laser microperforation, the addition of calcium greatly increased the yield of successfully opened root hairs, but the detailed mechanism of this stabilizing action of calcium is not clear.

Since after laser microperforation the orientation of the cells is maintained, it should be possible to study the distribution of ion channels in the plasma membrane in relation to the polarity of cells such as root hairs. In addition, the finding that cell to cell interactions are not disrupted is of great interest for the study of ion channels in conditions close to those existing in situ, where plasmodesmata provide pathways for the passage of nutrients, hormones and electrical stimuli via the symplast (Gunning and Robards, 1976; Robards, 1976; Robards and Lucas, 1990; Clarkson, 1991).

The microperforation of plant walls from various tissues (embryonic tissue, epidermal cells, hypocotyl tissue) with a UV laser beam has previously been reported (see references cited by Weber and Greulich, 1992). This indicates that the step of wall opening is not a limiting factor in the application of the technique to cells other than tip-growing cells. However, some adaptations would have to be made for determining the concentration of the osmoticum needed for plasmolysis and for monitoring (using calcium ions or by decreasing the osmoticum) the movement of protoplasm when the cell wall has been opened.

Laser microperforation of root hair cells is a single-cell technique that allows precise and gentle micromanipulation of plant cells, giving direct access to the plasma membrane for the study of membrane functions.

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