Electro-fusion of mesophyll protoplasts of *Avena sativa*

Determination of the cellular adenylate-level of hybrids and its influence on the fusion process

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Abstract. In order to assay the viability of electrically fused mesophyll protoplasts of *Avena sativa* a technique was developed to determine adenylate levels in single protoplasts and fusion products. The results demonstrate that the intracellular ATP/ADP ratios are identical before and after fusion (values between 1.4 and 1.8) and that the time of the rounding up process is directly related to the ATP level of the hybrid. This was shown by the manipulation of the intracellular ATP/ADP ratio in the light using different effectors. Hybrids with an ATP/ADP ratio of 2.3 needed 54 s to round up completely; in the presence of antimycin (inhibition of both oxidative and light-dependent cyclic electron flow: ATP/ADP = 1.1) or dibromothymoquinone (plastoquinone antagonist: ATP/ADP = 1.0) the time for rounding up was slightly increased (64 s and 76 s respectively), whereas after preincubation with antimycin, dichlorophenyl-dimethylurea (inhibition of oxidative and light-dependent electron flow) or uncouplers (ATP/ADP = 0.19–0.32) this process needed 128–153 s for completion. These results are discussed in relation to the viability of electrically induced fusion products and to energy-dependent events involved in the process of fusion.

Key words: Adenylate – *Avena* cell fusion – Cell, single cell analysis – Cell fusion, electric-field induced.

Introduction

Fusion between membranes is an important step in many cellular processes such as secretion, endocytosis, fertilization, and mitosis. Membrane fusion can also be induced by virus (Volsky and Loyter 1978), polyethylene glycol (Lucy 1982), calcium (Volsky and Loyter 1978; Zakai et al. 1977; Majumdar et al. 1980; Wilschut and Papahadjopoulos 1979), and freezing and thawing (Yu and Branton 1976). Zimmermann and coworkers (see reviews: Zimmermann et al. 1981; Zimmermann and Vienken 1982; Zimmermann 1982) demonstrated recently, that plant protoplasts, yeast protoplasts, and mammalian cells can be fused by an electric field. Close membrane contact between cells is achieved by the presence of an inhomogenous alternating field (so-called dielectrophoresis) and fusion is initiated by the application of a field pulse of high intensity and short duration (µs-range) which leads to a local perturbation of the membrane structure in the contact zone of adhering cells (so-called electrical breakdown). The electro-fusion technique can be applied to all membrane-surrounded compartments no matter of what origin and leads to a high yield of fused products. In particular, the fusion process can be followed under the microscope and the parameters determining the establishment of membrane contact and the subsequent fusion process can be predicted for each cellular or artifical membrane system because the underlying mechanism is understood.

With respect to the wide field of important technological applications of fusion, especially of electro-fusion, the determination of parameters of the viability of fused cells is an important factor for further steps in the direction of somatic hybrids. A highly sensitive parameter of cellular integrity is the ability to preserve a certain cytosolic energy state. Using isolated mesophyll protoplasts of *Avena* it could be demonstrated that there is a very sensitive regulation of the cytosolic ATP/ADP ratio by the coordination of oxidative and light-dependent phosphorylation (Hampp et al. 1982; Goller et al. 1982). Interference with cellular
compartmentation (i.e. damage of membranes) or electron-transport reactions by the addition of specific effectors causes significant changes in the cytosolic energy state, i.e., a severe reduction of the ATP/ADP ratio in this system (Goller et al. 1982). Thus, it was the aim of this investigation to compare the energy state of *Avena* protoplasts before and after electric-field-induced fusion. Using a method that enables the determination of adenylates in a single protoplast, the results indicate that the fusion process does not impair the cellular energy state.

By manipulating the cellular ATP/ADP ratio before fusion we show in addition that it is not the intermingling process itself, but the time required for the rounding up of the hybrid protoplast which is directly dependent on the cellular energy state for ATP/ADP ratios between 0.2 and 2.3.

**Material and methods**

*Isolation of protoplasts.* Primary leaves from 7-d-old oat seedlings (*Avena sativa* L., cv. Arnold), grown in a growth chamber at 25 °C for 5 d in the dark and then transferred to a greenhouse for 2 d, were sliced into segments of 0.5–1 mm width and incubated in 2% Cellulysin (Calbiochem, Frankfurt, FRG), 0.6 M mannitol, 1 mM CaCl₂, 0.5% bovine serum albumin (BSA) and 5 mM 2-(N-morpholin) ethane sulfonic acid (Mes)-KOH (pH 5.6) for 2 h at 30 °C, as described previously (Hampp and Ziegler 1980). The resulting protoplast suspension was purified twice by flotation on a sucrose-sorbitol (0.5 M each) step gradient (Hampp and Ziegler 1980) and then resuspended in 0.5 M mannitol.

*Incubation of protoplasts.* The protoplast suspension was preincubated for 2 min in the dark. When testing the influence of effectors these compounds were added at the start of the dark period in order to find the following final concentrations: Antimycin 3 × 10⁻⁶ M, carbonyl cyanide m-trifluoro-methoxyphenylhydrazone (FCCP) 10⁻⁵ M, carbonyl cyanide m-chlorophenylhydrazone (CCCP) 10⁻⁴ M, 3-(3',4'-dichlorophenyl)-1,1-dimethyl urea (DCMU) 10⁻³ M, dibromothymoquinone (2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone) (DBMIB) 10⁻³ M. Protoplasts (30 μl; 10⁴ ml⁻¹) were pipetted into the fusion chamber (Zimmermann and Scheurich 1981) and illuminated (2 W m⁻²) for 5 min under the microscope before the fusion process was started. Care was taken to avoid evaporation and an increase in temperature. Due to the very low number of protoplasts, changes in oxygen content during incubation were kept to a minimum.

**Electrical-field-induced fusion of protoplasts.** The experimental set up for the fusion of protoplasts by an electric-field pulse technique is given elsewhere (Zimmermann and Scheurich 1981). Briefly, two platinum wires (diameter 0.2 mm) were mounted on a perspex slide in parallel to each other (gap separation: 200 μm) and connected to a function generator (Type 7404 P, Toelner Electronic G. Werner, Frankfurt, FRG) which was used as voltage source for the generation of an alternating electric field. A pulse generator (Type 214 B, Hewlett Packard, Boeblingen, FRG) was linked in series for the injection of a square pulse into the protoplast suspension. At the end of the incubation, protoplasts were collected by dielectrophoresis using an alternating electric field (field strength 200 V cm⁻¹, frequency 1 MHz) and a square pulse of high intensity (750 V cm⁻¹) and short duration (50 μs) was applied, resulting in electrical breakdown of the outer protoplast membranes, followed by protoplast fusion. The duration of the rounding up process after releasing the alternating electric field, was documented by and exactly determined from a series of photographs.

**Determination of adenylates (ATP, ADP).** Single protoplasts or fusion products were picked up from the suspension with the aid of a self-made capillary pipette (tip diameter 40 μm, length of the tip 5 mm) into 10 μl of 0.1 N HCl to stop the metabolic reactions. Adenylates were determined from neutralised extracts (5 μl 0.2 N NaOH) by the luciferin-luciferase method after enzymatic interconversion and internal standardisation (Wirtz et al. 1980; Hampp et al. 1982). The resulting luminescence (Lumit PM, Abimed, FRG) was integrated over 30 s (Biolumat, Lab. Professor Berthold, Wildbad, FRG). To correct for adenylates set free from broken protoplasts and thus contained in the transferred volume, identical aliquots of the suspension medium (minus protoplasts or hybrids) were assayed (compare Table 1).

**Results**

In Table 1 a typical series of determinations of levels of adenylates within single protoplasts is given. The values clearly demonstrate that adenylate levels in single protoplast preparations are well above the background, i.e., the same aliquot of the incubation system but without a protoplast. A comparison of ATP/ADP ratios obtained from the values given in Table 1 are in the same range as routinely observed with macroassays (Goller et al. 1982).

A measure of the viability of cells is their energy state (Chapman and Atkinson 1971; Schimz and Holzer 1979; Akkerman and Gorter 1980). In order to find out whether electric field induced fusion of single protoplasts alters their ability to retain a given ATP/ADP ratio, we compared adenylate levels within both single protoplasts and fusion products. The results of these determinations are given in Table 2 and the data can be summarized in the following way:

(a) adenylate levels and ATP/ADP ratios are identical before and after complete fusion, when calculated on a per cell basis;

(b) adenylate levels within a single fusion product are directly related to the number of protoplasts that formed a hybrid: i.e. a hybrid out of two protoplasts contains twice, that of three protoplasts about thrice the adenylates contained in the unfused protoplast;

(c) if protoplasts are osmotically lysed (=decompartmentation) the ATP/ADP ratio significantly differs from both intact protoplasts either before or after fusion.

To obtain information about the process of membrane fusion itself and the process of rounding up...