Plant Cell Reports (1986) 5:252–255

Plant Cell Reports

The effect of fluorescent labeling on calcium-induced fusion of fusogenic carrot protoplasts

Kamnoon Kanchanapoom and Wendy F. Boss

Department of Botany, North Carolina State University, NC 27695-7612, USA

Received December 18, 1985 / Revised version received May 2, 1986 – Communicated by J. M. Widholm

Abstract

Various fluorescent compounds - carboxyfluorescein, scopoletin, fluorescein isothiocyanate (FITC), rhodamine B isothiocyanate (RITC), rhodamine 123, and rhodamine B ethyl ester - were used to study their effects on calcium-induced fusion of fusogenic carrot (Daucus carota L.) protoplasts. These protoplasts normally fused at a high percentage (50-60%) in response to 10 mM calcium, pH 6.0; however, if cells had been labeled with scopoletin, FITC, or RITC, fusion was greatly reduced. In contrast, labeling with carboxyfluorescein, rhodamine 123, or rhodamine B ethyl ester had no detectable effect on calcium-induced fusion. The two rhodamine dyes are shown to be localized in mitochondria.

Abbreviations: EGTA = ethyleneglycol-bis-(β-aminoethyl ether) N',N'-tetraacetic acid; FITC = fluorescein isothiocyanate; RITC = rhodamine isothiocyanate; PE = phosphatidylethanolamine

Introduction

Fusion permissive or fusogenic protoplasts have been isolated from wild carrot cells (Boss et al., 1984). These protoplasts fuse readily in 1 to 10 mM calcium at pH 6.0. Since the fusion process is an important tool for crop improvement, several methods for selecting fusion products have been developed including the use of fluorescent dyes. For example, carboxyfluorescein and scopoletin (Kanchanapoom et al., 1985) and FITC and RITC (Galbraith and Mauch 1980) have been reported to label protoplasts during chemically-induced fusion. The mitochondrial specific dyes, rhodamine 123 and rhodamine B ethyl ester, have not been used previously for these purposes. In this paper, we describe the use of rhodamine dyes and the effects of all the above mentioned fluorescent dyes on calcium-induced fusion of fusogenic protoplasts.

Materials and Methods

Stock cell cultures: The methods for obtaining embryogenic suspension cultures and fusogenic cultures of wild carrot have been published elsewhere (Boss et al., 1984).

Protoplast isolation: Protoplasts were isolated from cells in fusion inducing medium 4 days after transfer. Cells were incubated 2 h in a solution of 2% (w/v) Driselase (Plenum Sci. Lot No. KY 115) dissolved in 0.4 molal sorbitol, 2.5 mM EGTA, and 1 mmolal MES at pH 4.8. For monitoring fusion, the protoplasts were suspended in 0.45 molal sorbitol, 1 mmolal MES at pH 6.0.

Protoplast culture: For analysis of growth, aliquots of 4×10^5 labeled and non-labeled protoplasts were cultured in liquid medium (Kanchanapoom et al., 1985) and incubated at room temperature in dim light without shaking. After 2 to 3 days they were transferred to a rotary shaker at 60 rpm until harvested for fresh weight and dry weight measurements.

Fluorescent labeling: Carboxyfluorescein and scopoletin labeling of protoplasts was carried out as described by Kanchanapoom et al. (1985). Solutions of FITC and RITC were prepared as described by Galbraith (1984). Two methods of FITC and RITC labeling were used: (1) Dye solutions were added to cell suspension cultures at the concentration of 1 µl of stock solution (5 mg/ml in absolute ethanol) per ml medium and incubated overnight. Labeled cells were harvested on filter paper (Whatman No. 5 paper; 11.0 cm diameter) and washed in fresh medium to remove excess dye. (2) Dye solutions were added directly to the enzyme solution during cell wall digestion using a concentration of 1 µl of stock solution per ml enzyme solution. After 2 h incubation, protoplasts were washed several times in fresh medium. Rhodamine 123 and rhodamine B ethyl ester were added directly to isolated washed protoplasts. For rhodamine 123, 0.25 µl of stock (1 µl/ml in distilled water), and for rhodamine B ethyl ester, 0.25 µl of stock (1 µg/ml in absolute ethanol) were added to 4×10^5 protoplasts. Protoplasts were labeled with these fluorochromes for 30 min and washed three times by centrifugation in 0.45 molal sorbitol. Control experiments using the same volumes of ethanol or water showed no effect of the solvents on protoplast fusion. All dye concentrations and incubation times were optimized for maximum visualization of fluorescence. Carboxyfluorescein was obtained from Kodak, NY, USA; FITC, RITC, rhodamine 123 and rhodamine B were obtained from Sigma Chemical Co., St. Louis, MO, USA. Rhodamine B ethyl ester was synthesized using the procedure of Boss et al. (1975). The reaction was stopped by adding water and the unreacted rhodamine B was removed by repeated extraction with ethyl

Offprint requests to: W. F. Boss

acetate. Purity of rhodamine B ethyl ester was checked by thin-layer chromatography using silica gel G plates eluted with pyridine : water (1:4, v/v).

Fluorescence microscopy: For light microscopy, a Zeiss inverted microscope IM 35 equipped with neofluar 63X lens for observing bright field, differential interference contrast (DIC), and epi-fluorescence optic systems was used. For fluorescein the filter combinations were exciter filter BP 450-490, beam splitter FT 510, and barrier filter BP 520-560. For rhodamine the filter combinations were exciter filter BP 510-560, beam splitter FT 580, and barrier filter LP 590. Photographs were made with Kodak Ektachrome ASA 400 film and developed with Kodak E6 processing.

Results and Discussion

The use of rhodamine dyes for labeling protoplasts: Rhodamine 123 is a known mitochondrial marker and has been well established for use with a variety of animal cell lines (Johnson et al., 1980). With plant protoplasts rhodamine 123 or rhodamine B ethyl ester fluorescence was superimposable with mitochondria as observed by differential interference contrast optics (photographs not shown). To further substantiate the localization of rhodamine fluorochromes, labeled cells were homogenized and fluorescent labeled mitochondria were isolated using standard differential centrifugation procedures. Maximum cytochrome c oxidase activity corresponded to the intensely fluorescent-labeled membrane fraction, which with rhodamine B ethyl ester was also visibly pink.

Optimum labeling and localization were achieved by adding the rhodamine fluorochromes for 30 min after protoplast isolation. Addition of these fluorochromes for 30 min during wall digestion did not provide sufficient labeling, and fluorescence was found throughout the cytoplasm. Therefore, for these studies we used the 30 min labeling procedure after protoplast isolation whereby the dyes were localized in the mitochondria (Fig. 1a, b). Rhodamine 123 could be excited by two wavelengths (Johnson et al., 1980) but rhodamine B ethyl ester could be excited only with standard rhodamine excitation filter (546 nm) (Fig. 1a, b). Plastids and nuclei were not labeled by either dye. It should be noted that

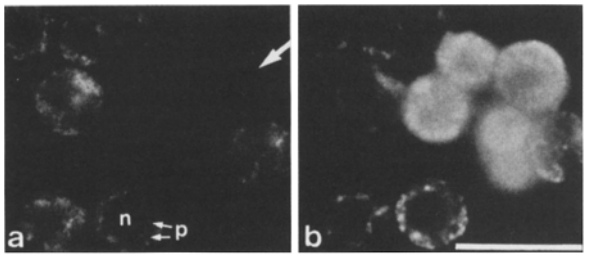

Fig. 1. Mixed population of fusogenic protoplasts labeled with either rhodamine 123 or rhodamine B ethyl ester were photographed with 485 nm excitation (a) and 546 nm excitation (b). Rhodamine 123 can be visualized at either fluorescein excitation (485 nm) as in (a) or rhodamine exciation (546 nm) as in (b) but rhodamine B ethyl ester can be excited only at 546 nm as in (b). Arrows in (a) indicate rhodamine B ethyl ester-labeled protoplasts which cannot be seen at 485 nm excitation. (n = nucleus, p = plastid). Bar = 20 um.

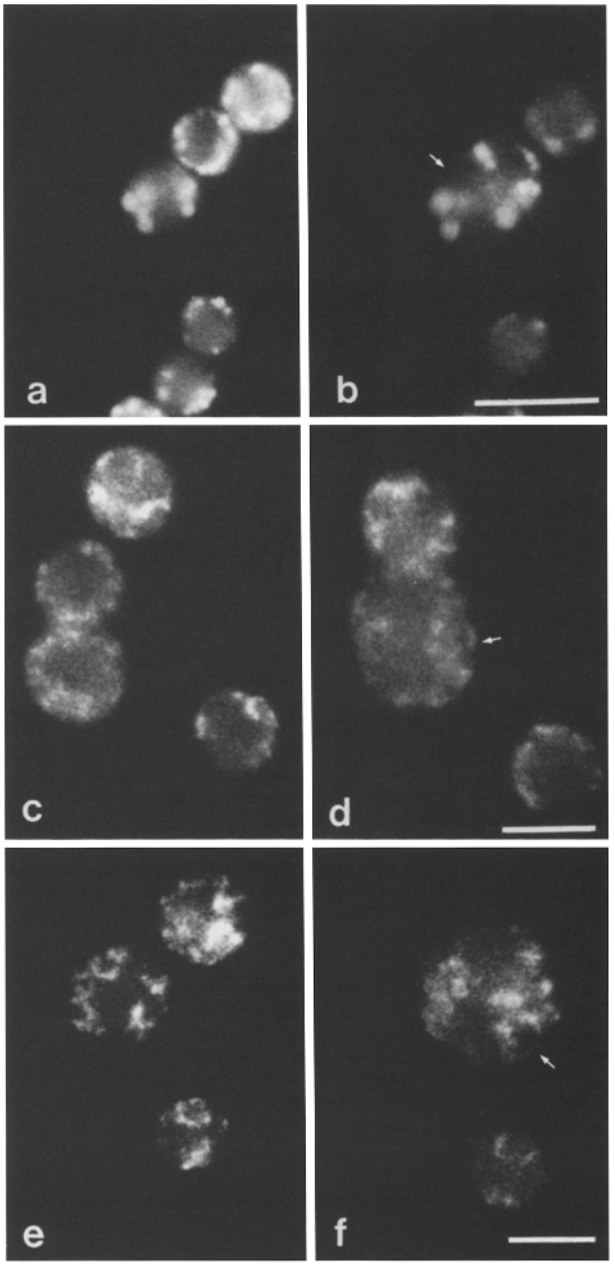

Fig. 2. Fusogenic protoplasts labeled with different fluorescent dyes were induced to fuse with 10 mM calcium in 0.45 molal sorbitol. (a) carboxyfluorescein-labeled protoplasts prior to fusion. (b) The same protoplasts as in (a), a few seconds later. (c) rhodamine 123-labeled protoplasts. (d) Same as (c), a few seconds later. (e) rhodamine B ethyl ester-labeled protoplasts. (f) Same as (e). Arrows indicate fusion products. Bar = 20 um.

the free acid, rhodamine B, did not localize in the mitochondria. This indicated that rhodamine B ethyl ester was not hydrolyzed by the protoplasts.

Effect of fluorescent labeling on protoplast fusion:

Fusogenic protoplasts could be induced to fuse by 10 mM calcium when protoplasts were labeled with carboxyfluorescein, rhodamine 123, or rhodamine B ethyl ester (Fig. 2) but not when labeled with

Table 1. Summary of the properties of the fluorescent dyes used in these studies.

Fluorescent dyes	Labeling time (h)	Localization	Conc. used (µl)	Effects on fusion	Effects on growth	Remarks
Carboxyfluorescein	>2	vacuole	200/flask (stock 0.1 mg/ml d.H_2O)	none	none	Overnight incubation gives best localization.
Scopoletin	>2	vacuole	200/flask (stock 1 mg/ml d.H_2O)	decreased	none	Overnight incubation gives best localization.
Fluorescein isothiocyanate (FITC)	2	cytoplasm, vacuole	1/ml enzyme solution (stock 5 mg/ml ethanol)	decreased if labeled overnight	none	Labeling during the period of protoplast release decreased cross fusion with RITC-labeled protoplasts but not with other FITC-labeled protoplasts.
Rhodamine B isothiocyanate (RITC)	2	cytoplasm, vacuole	same as FITC	same as FITC	none	Same as FITC.
Rhodamine 123	0.5	mitochondria	0.25/4x10^5 protoplasts (stock 1 µg/ml d.H_2O)	none	none	Can be visualized at 485 and 546 nm.
Rhodamine B ethyl ester	0.5	mitochondria	0.25/4x10^5 protoplasts (stock 1 µg/ml ethanol)	none	none	Can be visualized only at 546 nm.

scopoletin, FITC, or RITC (photographs not shown). The latter three dyes were added to the cells overnight and may have affected the biogenesis of the fusion permissive membrane. Scopoletin has been shown to block secretion of Golgi-derived vesicles in plant cells (Deutscher et al., 1981). FITC and RITC, on the other hand, react readily with free amine groups of proteins and PE to form stable FITC- or RITC-protein and RITC- or RITC-PE complexes which could affect membrane biogenesis during overnight incubation. When shorter labeling times (2 h during cell wall digestion) were employed which did effectively label the protoplasts, calcium-induced fusion was not significantly affected compared to the non-labeled controls. However, cross fusion of labeled and non-labeled protoplasts or of protoplasts with two different dyes was never observed. These data indicated that even during short exposures, association of these dyes was affecting some membrane proteins or lipids, and thus the fusion permissive state of the plasma membrane. The properties of the dyes are summarized in Table 1.

With all the fluorochromes used, the intensity of the fluorescence varied among the labeled protoplasts. This variation may be due to different stages of cell growth, to variation in vacuolar pH or membrane potential, or to photobleaching. For example, the fluorescence of scopoletin and carboxyfluorescein (Kanchanapoom et al., 1985), which localize in the vacuoles, is pH sensitive. Rhodamine 123 uptake is dependent upon the membrane potential of both the mitochondria and the plasma membrane. Changes in membrane potential induced by the wall digestion solution may explain why the dyes did not localize in mitochondria if cells were labeled during wall digestion. Once the protoplasts were labeled, the intensity of the fluorescence should have reflected the potential of the mitochondrial membrane in the carrot protoplasts as has been reported in a number of animal cell lines (Johnson et al., 1981).

Effect of fluorescent labeling and EGTA on protoplast growth: We have reported previously that scopoletin and carboxyfluorescein did not affect cell or protoplast growth (Kanchanapoom et al., 1985). Galbraith and Mauch (1980) showed that protoplast viability was not affected by FITC and RITC. Similarly, rhodamine 123 and rhodamine B ethyl ester caused no decrease in viability of labeled protoplasts as determined by callus growth (Table 2). The calcium chelator, EGTA, which reversibly inhibits fusion of fusogenic protoplasts was used routinely at a concentration of 2.5 mM to decrease the fusion that would normally occur due to the low calcium levels present in the wall digestion solution. It was important, therefore, to determine whether EGTA at the concentration used (2.5 mM) decreased protoplast viability. As shown in Table 3, EGTA had no effect on viability of the isolated protoplasts except at higher concentrations (10mM).

In summary, several fluorescent dyes have been evaluated with regard to their effect on calcium-induced fusion. Fusion was not affected by carboxyfluorescein, rhodamine 123, and rhodamine B ethyl ester at the concentrations used. In addition, these fluorochromes do not affect the growth and viability of protoplasts, so they can be used for selecting viable fusion products. The advantages of rhodamine fluorochromes for labeling plant protoplasts are: 1. they are specific to mitochondria, 2. they are sensitive to the mitochondrial membrane potential and do not stain cellular debris, 3. they are non-toxic to protoplasts at the effective concentrations, 4. unlike Hoechst 33258, a nuclear stain (Meadows and Potrykus 1981), rhodamine 123

Table 2. Effects of rhodamine 123 and rhodamine B ethyl ester staining on subsequent protoplast growth

Treatment	Mean fresh weight (mg) exp. 1	exp. 2	Mean dry weight (mg) exp. 1	exp. 2
Control	81.8(±1.6)*	67.6(±3.6)	22.3(±1.2)	30.5(±0.7)
Rhodamine 123	81.3(±3.0)	64.6(±2.9)	21.9(±1.0)	29.9(±0.7)
Rhodamine B ethyl ester	83.4(±2.6)	62.6(±3.3)	19.9(±1.0)	30.9(±1.4)

*Values represent the mean weight (± standard deviation) of 3 cell cultures harvested on 2 different days approximately 2 weeks after plating $4x10^5$ protoplasts in liquid medium. Student's t-test shows no significant difference between treatments at the 95% confidence level.

Table 3. Effect of EGTA on protoplast growth

Treatment	Mean fresh weight (mg) exp. 1	exp. 2	Mean dry weight (mg) exp. 1	exp. 2
Control	20.2(±1.3)*	22.4(±1.5)	17.7(±1.4)	16.9(±1.1)
1.0 mM EGTA	18.5(±2.0)	22.5(±2.0)	16.4(±2.2)	17.5(±0.5)
2.5 MM EGTA	18.5(±2.4)	20.7(±1.3)	16.0(±2.0)	16.4(±1.8)
5.0 mM EGTA	18.6(±1.0)	19.8(±2.1)	15.3(±2.1)	15.5(±2.0)
10 mM EGTA	15.0(±1.0)	15.9(±1.3)	13.4(±1.0)	13.3(±1.2)

*Values represent the mean weight (± standard deviation) of 3 cell cultures harvested on 2 different days approximately 2 weeks after plating $4x10^5$ protoplasts in liquid medium. Student's t-test shows no significant difference at the 95% confidence level only on 10 mM EGTA treatment.

and rhodamine B ethyl ester can be used without detergent solubilization, and 5. they can be used with the same filter package which has both fluorescein and rhodamine excitation. This is convenient for light microscopy, and yet for cell sorting purposes, the fluorescent wavelength maxima are distinct from fluorescein and each other so that one can use the rhodamine dyes to select for mitochondrial localized stains in hybrid fusion products.

Acknowledgements

This work was supported by Pioneer Hi-Bred International, Inc. and the USDA competitive grant # 83-CRCR-1-1276. This is paper number 10156 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh, N.C. 27695-7601.

References

Boss, WF, Kelly CJ, Landsberger FR (1975) Anal Biol 64: 289-292

Boss, WF, Grimes HD, Brightman AO (1984) Protoplasma 120: 209-215

Deutscher SL, Creek KE, Morre DJ (1981) J Cell Biol 25: 324-330

Galbraith DW (1984) In: Vasil IK (ed) Cell Culture and Somatic Cell Genetics of Plants, Vol 1, Academic Press, NY, pp 433-447

Galbraith DW, Mauch TJ (1980) Z Pflanzenphysiol 98: 129-140

Johnson, LV, Walsh ML, Chen LB (1980) Proc Nat Acad Sci 77: 990-994

Johnson, LB, Walsh ML, Bockus BJ, Chen LB (1981) J Cell Biol 88: 526-535

Kanchanapoom K, Brightman AO, Grimes HD, Boss WF (1985) Protoplasma 124: 65-70

Meadows MG, Potrykus I (1981) Plant Cell Rep 1: 77-79