Bioelectromagnetics 29:296–301 (2008)

Pulsed Electric Field Reduces the Permeability of Potato Cell Wall

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The effect of the application of pulsed electric fields to potato tissue on the diffusion of the fluorescent dye FM1-43 through the cell wall was studied. Potato tissue was subjected to field strengths ranging from 30 to 500 V/cm, with one 1 ms rectangular pulse, before application of FM1-43 and microscopic examination. Our results show a slower diffusion of FM1-43 in the electropulsed tissue when compared with that in the non-pulsed tissue, suggesting that the electric field decreased the cell wall permeability. This is a fast response that is already detected within 30 s after the delivery of the electric field. This response was mimicked by exogenous H_2O_2 and blocked by sodium azide, an inhibitor of the production of H_2O_2 by peroxidases. Bioelectromagnetics, 29:296–301, 2008.

Key words: cell wall; FM1-43; electric fields; stress; oxidative cross-linking

INTRODUCTION

The application of pulsed electric fields (PEFs) to living cells causes a transient increase in the transmembrane potential difference, modifying the plasma membrane organization and bringing it locally to a permeable state [Sabri et al., 1996]. Irreversible permeabilization is inter alia studied as a cancer therapy [Mir, 2001; Rols, 2006] and as a food processing method [Fincan et al., 2004]. By strict control of the electropulsation parameters, the permeabilization may evade affecting cell viability. This is, under the name electrotransformation, routinely used for gene transfer with walled as well as wall-less systems [Ganeva et al., 1995].

Interestingly, it has been suggested that in walled cells such as yeasts, the electric field affects the cell wall organization and, therefore, the electrotransformation procedure [Ganeva et al., 1995]. Hence, the molecular mechanisms affecting cell wall structure need to be understood to elucidate the optimal parameters for loading molecules into permeabilized cells.

The composition and structure of the plant cell wall can be markedly altered by environmental stimuli, notably in response to biological stresses [Gómez Galindo et al., 2004]. Thus, mechanical wounding, infection or elicitors obtained from microbial cell walls or culture fluids stimulate the synthesis of lignin in peripheral tissues and cause the accumulation of cell wall proteins [Lamb et al., 1989]. Emerging evidence indicates that elicitors as well as mechanical wounding cause a rapid, H_2O_2-mediated oxidative cross-linking of preexisting cell wall structural proteins that precedes the activation of transcription-dependent defenses

Grant sponsors: Portuguese Foundation of Science and Technology; Swedish Foundation for International Co-operation on Research and Higher Education; USA Air Force Office of Scientific Research.

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Received for review 2 March 2007; Final revision received 6 September 2007

DOI 10.1002/bem.20394
Published online 28 December 2007 in Wiley InterScience (www.interscience.wiley.com).

[Bradley et al., 1992]. This stimulus-dependent cross-linking, initiated within 1 min [Apostol et al., 1989], provides a rapid defense response as a protection against environmental stresses.

Stress resistance, reactive oxygen species (ROS) metabolism and signaling, cell survival and redox homeostasis are some of the complex processes associated with the carbon and oxygen metabolism of plant cells of which there is limited understanding [Gómez Galindo et al., 2007]. Procedures such as PEF application are known to induce stress and influence metabolic processes [Gabriel and Teissié, 1994; Sabri et al., 1996] in the surviving cells.

We suggest here that PEFs may cause a rapid H_2O_2-mediated decrease on cell wall porosity in potato tissue, apparently mimicking the stress response upon wounding or elicitor detection. This decrease was assessed through kinetic studies of the labeling of plasma membrane with the fluorescent dye FM1-43. This amphipathic styryl dye has a divalent cationic head group and a lipophilic tail, and it reversibly partitions into the outer leaflet of the cell membrane. FM1-43 fluoresces weakly in an aqueous environment, and its quantum yield increases by two orders of magnitude on interaction in the lipid membrane [Schote and Seeling, 1998].

MATERIALS AND METHODS

Potato Tissue Preparation

Potatoes (*Solanum tuberosum* cv. white rose) purchased in the local market (Los Angeles, CA) were used. Potatoes were manually washed and peeled. One slice, 15 mm thick was obtained from the phloem parenchyma tissue of a single tuber. The slice was oriented perpendicular to the major tuber axis. A rectangular cross-section sample, 15 mm long and 7.0 mm wide, was obtained from the phloem parenchyma tissue of the slice using a pair of parallel sharp blades separated 7 mm. Immediately after cutting, the sample was rinsed with distilled water and gently blotted with medical wipes to remove the excess water from the sample surface. The potato samples were humid enough to provide electrical contact between the specimen and the electrodes. The rectangular samples, one per tuber, were subjected to different treatments described in detail below. After the treatment, an individual rectangular sample was held, for a defined time that did not exceed 3 min, in humid medical wipes to avoid desiccation. After the holding time, a 1 mm thick slice was cut from the center of the rectangle and used immediately for FM1-43 treatment and microscopic examination.

Treatments

The rectangular samples were either treated with 25 mM solution of H_2O_2 for 5 min, PEF treated, or pre-treated with 10 mM solution of sodium azide in 5 mM KCl for 15 min before PEF treatment. Control samples were neither chemically nor PEF treated.

Electric pulses were delivered to the rectangular samples through 2 parallel, flat stainless steel electrodes (20 mm long and 9 mm wide) separated 7 mm. A pulse generator designed and assembled by the department of Electrical Engineering and Electrophysics, University of Southern California was used. This pulse generator uses a voltage supply (XHR 600-1.7; 0-600 V, Xantrex Technology Inc., Vancouver British Columbia, Canada) connected in series to a switch consisting of a MOSFET IRF840 transistor and 2 optoisolators. A digital oscilloscope (Tektronix, TDS 5104, Oregon) was connected to the system to monitor the delivery of the pulse to the sample. Samples were treated at varying voltages (21, 70, 140, 210, 280, and 350 V, which corresponds to the electric field strength in air of 30, 100, 200, 300, 400, and 500 V/cm) with a single 1 ms rectangular pulse. The pulse had a rise time of 4 μs and a fall time of 20 μs (Fig. 1).

Application of FM1-43 and Microscopic Examination

Slices sampled from the treated rectangular tissues 30 s to 3 min after the delivery of the electric pulse were dipped in a 2 μM solution of FM1-43 (Molecular Probes, Eugene, OR; $\lambda_{ex} = 480$ nm, $\lambda_{em} = 535$ nm) in 5 mM KCl for 2 min before microscopic examination.

Observations on the stained slice were made with a Zeiss Axiovert 200 epifluorescence microscope (Carl Zeiss Microimaging Inc., New York, NY) at a magnification of 10X. Images were captured and analyzed with a Zeiss Axio camera (AxioCam monochrome, New York, NY) and software (Zeiss Axiovision 3.1). Five slices from five different tubers were examined at each experimental condition.

RESULTS

Effect of the Incubation of Potato Tissue in H_2O_2 on the Diffusion of FM1-43

Kinetic studies on plant cells have shown that after application of FM1-43, staining of the plasma membrane is immediate and the cell wall does not act as a barrier that can significantly slow the staining process [Bolte et al., 2004]. Therefore, the potential use of FM1-43 as a marker for assessing cell wall permeability changes was tested by incubating the

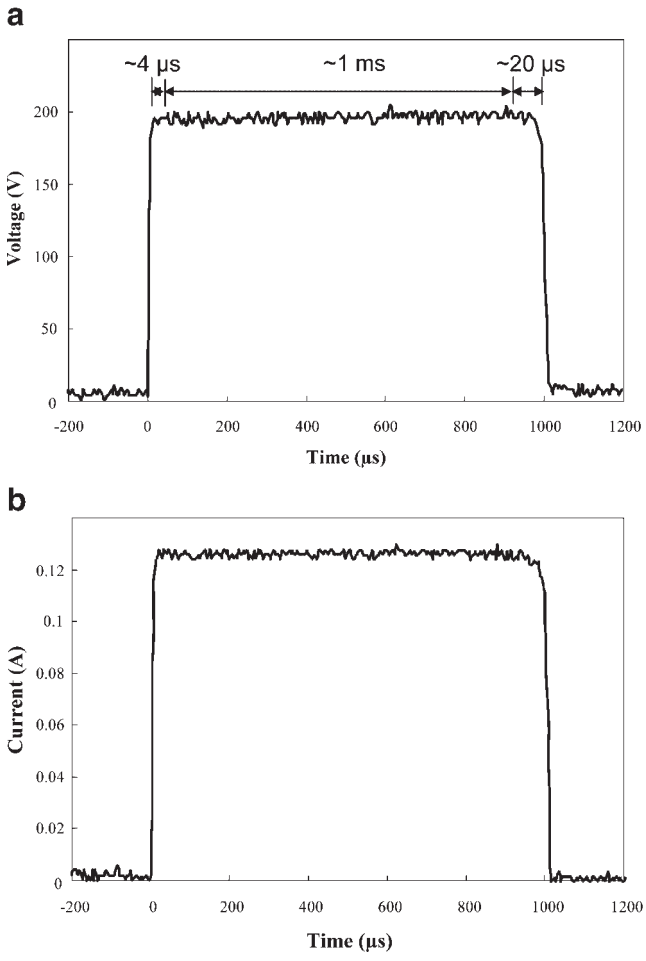

Fig. 1. Characteristics of the applied electric pulse. **a**: Typical voltage waveform as a function of time. The pulse width is 1 ms, rise and fall times are shown. **b**: Typical current waveform as function of time.

potato sample in H_2O_2, known to induce oxidative cross-linking of cell wall proteins [Bradley et al., 1992], prior to applying the dye. In Figure 2a–e, typical microscopic observations of the fluorescent interaction of the dye with the cell membrane are shown. Figure 2a,b shows a dramatic decrease of the fluorescence intensity of the H_2O_2-treated sample in comparison with that of the control. The fluorescence intensity is progressively restored when increasing the dipping time of the potato slice in FM1-43 from 2 to 5 min (Fig. 2c–e), showing that incubation of the tissue with H_2O_2 has affected the rate of diffusion of the dye through the extracellular space, slowing the staining process. Preliminary experiments confirmed that H_2O_2 by itself did not affect the fluorescence properties of FM1-43 and that no measurable photo-bleaching of the dye is taking place during the image acquisition process (not shown). This observation suggests that FM1-43 can be used to monitor changes in cell wall permeability.

Electropulsation Slows the Diffusion of FM1-43 in Potato Tissue

Figure 3 shows the variation of the fluorescence intensity of FM1-43-stained potato tissue with time after electropulsation at various field intensities. Interestingly, in the range of 200–500 V/cm, a reduction of the FM1-43 fluorescent in the plasma membrane was already noticeable 30 s after the pulse, suggesting a rapid effect of the electric field on the dye diffusion through the extracellular space. A 60% reduction in FM1-43 fluorescence intensity relative to control samples was observed 3 min after exposure to pulses from 200 to 500 V/cm. When the field strength was 100 V/cm, the reduction in FM1-43 uptake with time was less pronounced and there was no noticeable effect when a field of 30 V/cm was applied.

To verify that the diffusion of the dye was slower in the extracellular space after electropulsation, two of the working field strengths (300 and 400 V/cm) were selected and, after electropulsation, the potato slice was dipped for longer times in the FM1-43. Figure 4 shows the progressive increase of the fluorescence intensity with dipping time. These results suggest that, after electropulsation, the extracellular space has slowed the diffusion of the FM1-43 towards the cell membrane.

Changes in the Extracellular Space Upon Electropulsation May Involve H_2O_2 Produced by Cell Wall-Associated Peroxidases

After pre-treatment with sodium azide, a strong inhibitor of cell wall-associated peroxidases [Bestwick et al., 1997; Razem and Bernards, 2003], the diffusion of FM1-43 in the tissue was not affected by electropulsation (Fig. 5). This result strongly suggests the involvement of H_2O_2 production in the slow diffusion of FM1-43 towards the cell membrane after electropulsation.

DISCUSSION

The results presented here provide evidence that electropulsation, in the conditions used in the present study, slows the diffusion of FM1-43 through the extracellular space of potato tissue.

The molecular mechanism for this decrease in the FM1-43 diffusion may involve H_2O_2 produced by cell wall-associated peroxidases. In support of this idea, we show that the response could be mimicked by exogenous H_2O_2 and blocked by a reagent that inhibits the production of H_2O_2 by peroxidases, such as sodium azide. The rapid decrease of fluorescence intensity, detected within 30 s after the delivery of the pulse, is in good agreement with the rapid burst of H_2O_2 production

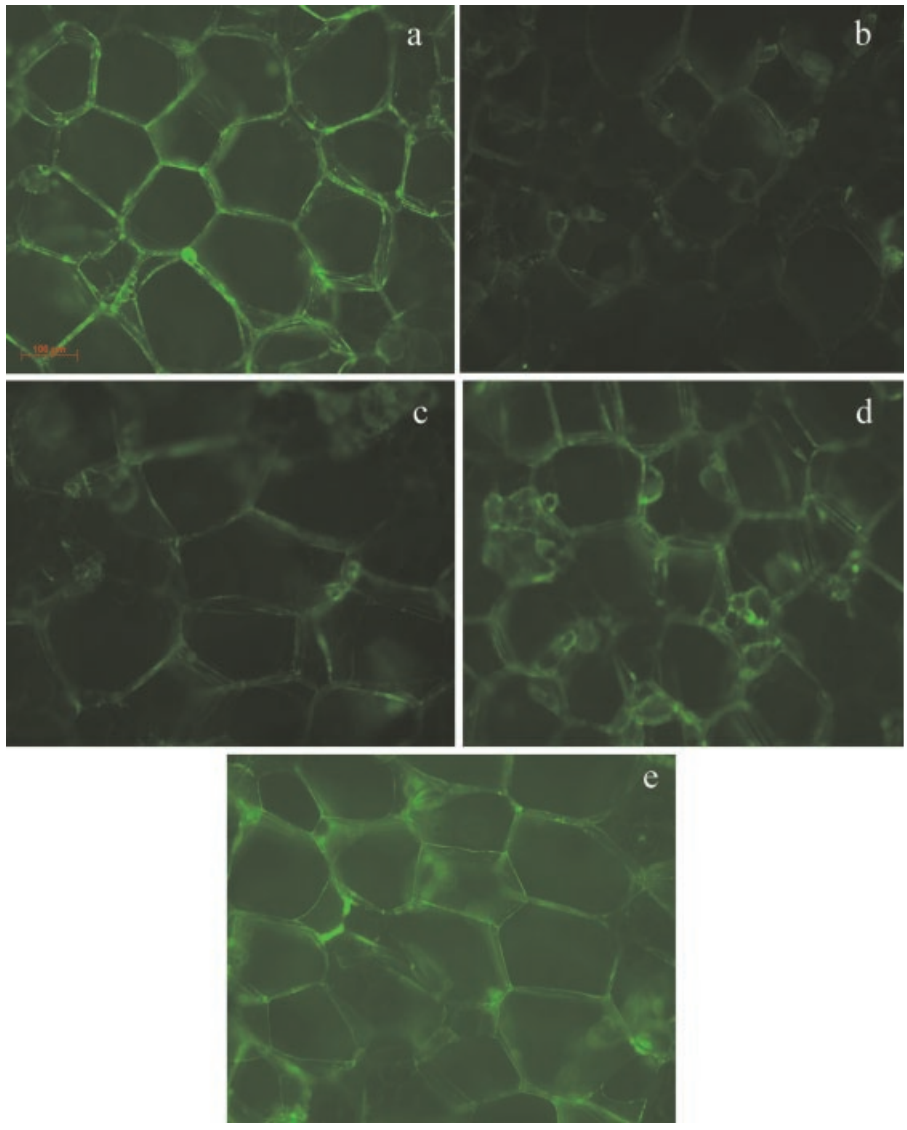

Fig. 2. Typical experimental results from the effect of the incubation of potato tissue in hydrogen peroxide on the diffusion of FM1-43. Potato tissue was treated with H_2O_2 prior to application of FM1-43 for different times, as detailed in Materials and Methods Section. (**a**) Potato tissue untreated with H_2O_2 (control). Following images: potato treated with H_2O_2 and dipped in FM1-43 for (**b**) 2 min, (**c**) 3 min, (**d**) 4 min, (**e**) 5 min. [The color figure for this article is available online at www.interscience.wiley.com.]

reported to take place in plant cells upon elicitor treatment [Brisson et al., 1994; Bolwell et al., 1998] or wounding reaction [Razem and Bernards, 2003], providing substrate at the appropriate time for peroxidase-mediated oxidative cross-linking reactions. Upon wounding stress in potatoes, another cell H_2O_2-producing system such as the plasma membrane NADPH [Wojtaszek, 1997] showed not to be associated to this first, rapid production of H_2O_2 [Razem and Bernards, 2003]. Interestingly, in the range from 200 to 500 V/cm the FM1-43 fluorescence intensity after electropulsation was independent of field strength, indicating saturation of the underlying mechanism at field strengths above 100 V/cm (Fig. 3). More extensive characterization might be expected to reveal that different combinations of pulse width, intensity and number can lead to different response patterns.

The diameter of cell wall pores is expected to be in the range of 3.5–5.5 nm [Gekas et al., 2001]. Oxidative cross-linking is likely to reduce the pore dimensions, restricting the movement of particles through the apoplast. It may appear surprising that the diffusion of a small molecule such as FM1-43 (molecular weight 451 Da) was retarded by the putative cross-linking. The dye's ability to pass through pores is determined by the diameter of its ethyl and butyl end groups

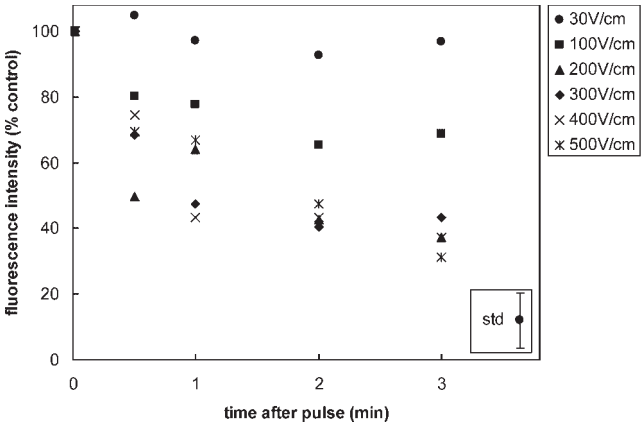

Fig. 3. Effect of electropulsation on the fluorescence intensity of FM1-43. Potato tissue was treated with field strengths varying from 30 to 500 V/cm (one single 1 ms rectangular pulse) and FM1-43 was applied from 30 s to 3 min after the delivery of the pulse. Each point represents the average of 5 measurements except for the control which is the average of 83 measurements. The error bar represents the pooled standard deviation of the mean.

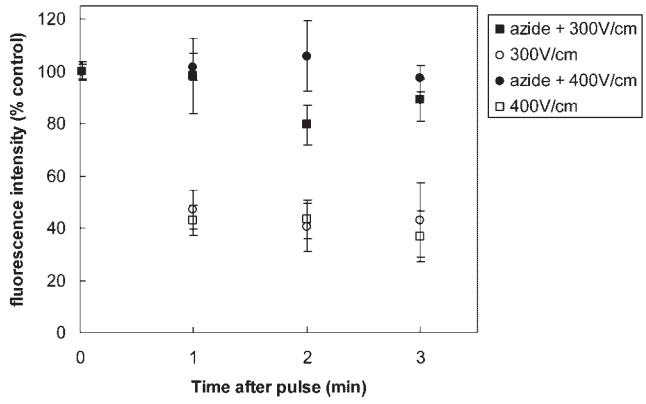

Fig. 5. Effect of pre-treatment with sodium azide prior to electropulsation on the fluorescence intensity of FM1-43. Potato tissue with or without azide pre-treatment was treated with field strengths of 300 and 400 V/cm (one 1 ms rectangular pulse) and FM1-43 was applied from 30 s to 3 min after the delivery of the pulse. Each point represents the average of 5 measurements except for the control which is the average of 30 measurements. Error bars are the standard deviation of the mean.

(~0.78 nm × 0.5 nm [Gale et al., 2001]); however, the average rate of transport is given by its hydrodynamic radius which is determined by its rod-like form with major axis of around 2.3 nm according to the model of Schote and Seeling [1998]. Electrostatic interactions may also play a role in the diffusion of a molecule through the cell wall.

The diffusion of another small molecule, sucrose (with a molecular weight of 342 Da and molecular diameter of 0.9 nm) [Bhakdi et al., 1986], has been reported to be significantly depressed during osmotic

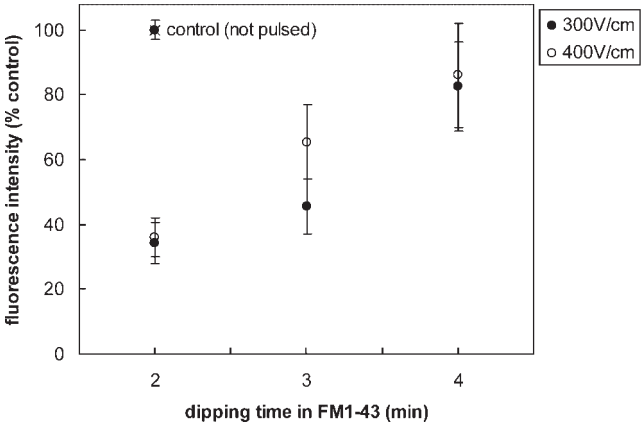

Fig. 4. Effect of electropulsation on the diffusion of FM1-43. Potato tissue was treated with field strengths of 300 and 400 V/cm (one 1 ms rectangular pulse) prior to the application of FM1-43 from 2 to 4 min. Each point represents the average of 5 measurements except for the control which is the average of 83 measurements. Error bars are the standard deviation of the mean.

dehydration by cold acclimated, cross-linked carrot cell wall [Paredes Escobar et al., 2007], suggesting significant changes in the functional architecture of the cell wall upon stress.

From a biological point of view, industrial treatment of plant tissue will mimic stress. For example, slicing, chilling, drying, and thermal shock will induce metabolic responses that mimic those from environmental stress. The production of ROS, among them H_2O_2, is a phenomenon common to stress conditions [Gómez Galindo et al., 2007]. Little is known, however, about how plant tissue responds to processing stresses that are not encountered in nature, such as PEFs. In this study we have shown that the effects of PEFs on plant tissues are similar to those observed in response to other stress conditions, and we have developed a method for characterizing these responses that can be used in subsequent, more intensive investigations.

CONCLUSIONS

Electropulsation, in the range of 100–500 V/cm (one 1 ms rectangular pulse), slows the diffusion of the dye FM1-43 through the cell wall of potato tissue, suggesting a significant decrease in cell wall permeability at nanometer scale. Experimental evidence links those changes with the production of H_2O_2 by cell wall associated peroxidases. This is a fast response that is already detected within 30 s after the delivery of the electric field.

To our knowledge, such findings have not been reported before for plant tissues. The observed

phenomena may affect the processing properties and composition of plant products extracted or otherwise processed using PEFs.

ACKNOWLEDGMENTS

The authors would like to thank Hao Jessica Chen from Electrical Engineering and Electrophysics, University of Southern California for designing and assembling the pulse generator used in this study.

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