Electric Gene Expression in Single-cells of Rice Protoplast via Ca\textsuperscript{2+} Entering the Cell

Hideaki Matsuoka,\textsuperscript{a,b} * Tamu Komazaki,\textsuperscript{a} Yoshiko Mukai,\textsuperscript{a} and Mikako Saito\textsuperscript{a,b}

\textsuperscript{a}Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology (Koganei, Tokyo 184-8588, Japan)
\textsuperscript{b}CREST (Core Research for Evolutional Science and Technology), Japan Science and Technology Agency (5, Sanbancho, Chiyoda-ku, Tokyo 102-0075, Japan)

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Spatial distribution of intracellular Ca\textsuperscript{2+} concentration was measured with a spectro-imaging system composed of an image slicer (10 \times 10 channels), a grism, and a high sensitive CCD camera. The Ca\textsuperscript{2+} concentration of each single protoplast was different from others both at a steady state (10-100 nmol dm\textsuperscript{-3}) and after electric stimulation. Therefore traceable observation of each single-cell was essential, which contrasted conventional methods dealing with an average of many cells. When a pulsing electric stimulation was applied to single-cells of rice protoplast, the transient variation of intracellular Ca\textsuperscript{2+} concentration was observed and chitinase gene expression followed (16 out of 36 cells, 44.4\%). Its significance level was estimated as 90% by \chi\textsuperscript{2}-test.

Key Words: Electric Gene Expression, Cross Membrane Potential, Plant Cell, Ca\textsuperscript{2+} Response, Single-cell Experiment

1 Introduction

The role of an electric signal in animal cells is well described in the relevance to the voltage-gated Ca\textsuperscript{2+} channels in the nerve terminal. The action potential triggers the entry of Ca\textsuperscript{2+} into the presynaptic terminal. The Ca\textsuperscript{2+} then induces the fusion of synaptic vesicles with a presynaptic membrane and, consequently, causes the release of neurotransmitters. In contrast, the role of the electric signal in plant cells is still unclear. About a decade ago, Weldon \textit{et al.} showed a possible involvement of an electric signal in the signal translocation from the wound site of a tomato leaf to a remote site of the same plant. They observed that an electric signal traveled along the stem then the proteinase inhibitor gene was activated in another leaf at a remote site.\textsuperscript{11} Later, Stancovic \textit{et al.} applied an electric stimulus directly to the stem of a tomato plant and observed the expression of the same gene at another remote site.\textsuperscript{2,3} These results suggested that an electric signal was effective in the induction of the gene expression. However, it still remained at a phenomenological stage.

As far as an electric signal of stimulus is concerned, the quantitative discussion about its effective intensity is essential. Because of the complicated structure of plant leaf cells, the estimation of the effective intensity that each cell should receive is difficult. Only the single-cell experiment\textsuperscript{4,7} was expected to conquer this difficulty. The single-cell experiment is composed of a systematic procedure of the selection of sample single-cells and their storage in micro wells, the microinjection of a gene into each cell, the transient assay of its gene expression, and associated micromanipulation. According to the protocol of this single-cell experiment, we intended to show a possible role of an electric signal in the gene expression in plant.

Considering a proper cell size and easy preparation, we selected rice protoplasts as plant cell material. Because of the availability of the promoter and its preliminary study results, we selected one clone of the rice chitinase gene family, RCC1 (CHI\textsubscript{RCC1}).\textsuperscript{2,5} RCC1 codes one of the isozymes belonging to class I chitinases and can be induced by the action of a chemical elicitor such as N-acetylglucosamine (AC8). Rice cells are thought to recognize AC8 as chitin of the cell wall of pathogenic fungi. Chitinases hydrolyze the internal \beta,1,4-glycosidic linkages of chitin and are expected to be involved in the defense against these pathogens.

Changes in cytosolic free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{c}) are essential for the transduction of various abiotic and biotic signals in plants.\textsuperscript{10,15} We found that the Ca\textsuperscript{2+} entry was induced by AC8 action\textsuperscript{7} and that this Ca\textsuperscript{2+} entry was essential for the CHI\textsubscript{RCC1} gene expression. Moreover, the microinjection of Ca\textsuperscript{2+} alone could induce the gene expression without an elicitor.\textsuperscript{56} Thus we suspected that the Ca\textsuperscript{2+} entry could induce the CHI\textsubscript{RCC1} gene expression irrespective of the means of Ca\textsuperscript{2+} entry; chemical, physical, or electrical. In this study, a pulsing electric stimulation was adopted. Under a proper condition of electric field, Ca\textsuperscript{2+} could be introduced into protoplast via voltage-dependent Ca\textsuperscript{2+} channel.\textsuperscript{17,18}

2 Experimental

2.1 Plant material

Rice cells (\textit{Oryza sativa} L. japonica cv. Nipponbare) were cultured in an N6 medium\textsuperscript{39} containing 300 mg dm\textsuperscript{-3} casein acid hydrolysate, 10 mmol dm\textsuperscript{-3} proline, and 1 mg dm\textsuperscript{-3} 2,4-Dichlorophenoxyacetic acid (2,4-D). The cells were cultured in 24 ml of this culture medium in a 100 cm\textsuperscript{3} Erlenmeyer flask at 25°C with 100 rev min\textsuperscript{-1}
shaking in the dark, and maintained by a regular transfer of 10 cm³ of 7-day-old calli into a 14 cm³ fresh medium.

2. 2 Chemicals

AC8 was obtained from Yaizu Suisan Co. (Yaizu, Japan) and further purified and used as the elicitor to induce CHI₅₅ gene expression. Fluo-3-AM and Fura-2-AM were obtained from Dojin Co., Ltd. (Kumamoto, Japan). Verapamil and EGTA were from Sigma (St Louis, MO, USA). Pectolyase Y-23 and Cellulase Onozuka RS were from Kikkoman Co., Ltd. (Tokyo, Japan) and Yakult Honsha Co., Ltd. (Tokyo, Japan), respectively. Other reagents were of commercially available analytical grade.

2. 3 Preparation of rice protoplasts

After the last transfer, 4-day-old calli were collected to prepare rice protoplasts. One gram of filtered calli was suspended in a 10 cm³ enzyme solution consisting of 0.05% (w/v) Pectolyase Y-23, 2% (w/v) Cellulase Onozuka RS, 0.01% (w/v) CaCl₂, 0.1% (w/v) K-dextran sulfate, and 9% (w/v) mannitol. After the incubation for 1 h on a shaker (30 rpm) at 30°C and for a successive 2 h without shaking at 30°C, the suspension was passed through a 30 μm mesh sieve to remove callus debris. Then, the protoplasts were collected by centrifugation (900 rpm, 2 min), washed twice with a 9% mannitol solution, and finally suspended in a protoplast medium (PP medium) containing 9% mannitol, 1 mmol dm⁻³ CaCl₂·2H₂O, and other components.³⁰

2. 4 Detection of the gene expression

The expression of CHI₅₅ gene was detected by a means of a green fluorescent protein (GFP) reporter gene. A plasmid composed of a cauliflower mosaic virus 35S promoter and a GFP gene (p35S-GFP) was obtained from Clontech Laboratories, Inc. (Palo Alto, CA, USA). The sequence of the 35S promoter was replaced by the CHI₅₅ gene promoter sequence to generate a plasmid pCHI-GFP (CHI₅₅ gene promoter, GFP reporter gene). The pCHI-GFP was introduced into single-cells by the microinjection. After that, the single-cells were incubated at 25°C for 20 h and the generation of GFP was examined with a fluorescent microscope equipped with a spectro-imaging system.²¹ When the fluorescent intensity of a test cell was three times higher than that of the control cell, we judged that the gene expression occurred in the test cell.

2. 5 Application of an electric signal to single-cell of rice protoplasts

A reaction dish was placed on an automatic stage of a microscope (Fig. 1 (a)). A comb plate depicted in Fig. 1 (b) was placed at the center of the dish bottom. A micropipette (tip diameter 300 μm) containing rice protoplasts was moved along a trench of the comb and protoplasts were ejected slowly one by one so that they were placed in line at the middle of the trench. The protoplasts were stood still for 12 h at 25°C until they adhered to the bottom glass of the reaction dish.

A stimulating electrode was composed of a pair of Pt/Ir wires (diameter 200 μm) (Fig. 1 (c)). The distance between the electrodes was 250 μm. This electrode was manipulated and placed so that a test cell is situated at the mid point between the electrodes. Then a pulsing electric signal was applied to the cell.

2. 6 Measurement of Ca²⁺ concentration

Fura-2 measurement was done to determine the absolute value of Ca²⁺ concentration using a fluorescent

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Fig. 1  Experimental set for the electric signal application. (a) Automatic stage control system and electric signal supplying system. (b) Comb plate for the cell line-up. (c) Electric stimulation electrode.
microscope equipped with a spectro-imaging system and a 2-wavelength (340 nm, 380 nm) excitation light source. The spectro-imaging system was composed of an image slicer, a grism, and a CCD camera (LN/CCD-576, Roper Scientific Inc., Trenton, NJ, USA). The image slicer was composed of a bundle of 100 optical fibers. In the image slicer, optical fibers were arranged in 10 × 10 array at the entrance (light-reception) surface, while in 100 × 1 array at the exit (light-emission) surface. A line of the optical signals of 100 channels was dispersed through a grism to obtain the respective spectra and projected on the CCD (576 × 151 pixels, about half area of the CCD). On the CCD, the x-axis (576 pixels) is the channel number (1-100), while the y-axis indicates the spectral data from 400 nm to 800 nm by 5 nm step. A spatial data at 340 nm or at 380 nm can be displayed on a TV monitor as a 10 × 10 honeycomb pattern.

A calibration curve for the present system was made using a thin liquid layer (40-50 µm thick) of commercially available standard calcium buffer solutions (Calcium calibration buffer kit, Invitrogen Japan K.K., Tokyo, Japan). The kit was composed of 11 components containing various concentrations of free Ca2+.

2.7 Single-cell gene expression experiment

The single-cell experiment was done according to the following items from (1) to (6) (Fig. 2).

(1) Cell selection and placement: A 100-200 mm3 aliquot of protoplast suspension was added to a reaction dish (diameter 30 mm). Cells with a proper size (diameter 30-50 µm) were selected and sucked in a micropipette. Then the cells were placed in line as depicted in Fig. 1 (c).

(2) Cell address registration: The reaction dish was set on the automatic stage of microscope. The automatic stage was driven with a joystick so that an arbitrarily selected cell came to the center of the microscope view. Then the head switch installed on the joystick (or a foot switch) was clicked. Thus the XY-address of the cell (No. 1) was registered and displayed on a TV monitor. Then the automatic stage was driven so that another cell came to the center of the microscope view. This cell was registered as the No. 2 cell by clicking the head switch. In the same way, the XY-addresses of 50 cells in total were registered per dish.

(3) Microinjection of pCHI-GFP: When the XY-address of No. 1 cell or the cell number “1” was input in the keyboard of the stage controller, the stage moved automatically so that the No. 1 cell came to the center of the microscope view. A micropipette for injection (tip diameter < 1.0 µm) that was loaded with pCHI-GFP beforehand was inserted into the cell. This insertion was done very carefully and smoothly. The micropipette tip should be precisely positioned in the cytosol and not in the vacuole or other compartments. In the present case of the rice protoplast, it was difficult to distinguish the cytosol from the vacuole only by the microscopic observation. Therefore, the insertion was done so that the tip position was located as near as possible to the inside surface of the cell membrane. The plasmid solution was forced out of the micropipette by applying the pressure using a Pneumatic Pico Pump (PV800, World Precision Instruments Inc., Sarasota, FL, USA). Then the micropipette was pulled off the cell. When the head switch of the joystick was clicked, the microscope stage was automatically driven so that the next No. 2 cell came to the center of the microscope view. The microinjection was performed in the same way. Thus the automatic stage driving and microinjection were performed nonstop for 50 cells. After the microinjection, the reaction dishes were incubated at 25°C for 20 h.

(4) Fura-2 loading and Ca2+ transient measurement: Fura-2-AM (4 µmol dm−3) was added to the reaction dish in order to introduce Fura-2 into the plasmid-loaded cells. After incubation for 1 h at 30°C, the reaction dish was placed on the microscope stage. An electric signal was applied to the No. 1 cell with an electric stimulation electrode (Fig. 1 (c)) and the Ca2+ transient pattern of the cell was measured with a spectro-imaging system. In the same way, the automatic stage driving and the Ca2+ transient measurement were performed nonstop for 50 cells. Then the reaction dishes were incubated at 25°C for 20 h.

(5) GFP expression measurement: The CHIgene expression was checked from the GFP generation. The fluorescent spectrum of each cell was measured with a spectro-imaging system. When the spectrum showed the typical profile of GFP and its fluorescent intensity was 3 times higher than that of the control cells, we decided that GFP was generated in the cell.

(6) Application of AC8: When GFP was not generated in the cell, AC8 was applied to the cells in order to check if the cells maintained the gene expression activity. For this purpose, the reaction dishes were incubated at 25°C for another 20 h. Those cells that showed no GFP fluorescence even at this step were regarded as the cases of unsuccessful microinjection.

3 Results and Discussion

3.1 Cross membrane potential (Vcm)

The effect of an electric signal on a target single-cell may be concentrated on the cell membrane and therefore Vcm may be a proper indicator of its effective intensity. In order to evaluate Vcm, the electric potential gradient at the cell location was estimated.

![Fig. 2 Time course of single-cell gene expression experiment.](image-url)
Electric pulses were applied to the Pt/Ir stimulating electrodes and the potential distribution was measured by scanning a measuring microelectrode (Ag/AgCl) at each 50 μm step (Fig. 3 (a)). The reference microelectrode (Ag/AgCl) was set near the mid point between the electrodes. When the electric voltage applied to the electrode terminal (V<sub>ET</sub>) was 5-20 V, the electric potential gradient ranged 0.45-1.43 mV μm⁻¹ (Fig. 3 (b)). When V<sub>ET</sub> was 20 V, for example, V<sub>CMP</sub> for a cell with diameter of 30-50 μm was calculated as 22-36 mV based on a simple shell model (Fig. 3 (c)).

3.2 Applicability of Fura-2 measurement to a micro space

It was necessary to check whether Fura-2 measurement was applicable even to such a micro space as a single-cell size. A thin liquid layer (40-50 μm thick) of each standard buffer solution was placed on the microscope stage and its fluorescent spectra were obtained under the excitation both at 340 nm and 380 nm. The ratio of the microscopic fluorescent intensities (FI<sub>EX340</sub>/FI<sub>EX380</sub>) was calculated and plotted versus the Ca<sup>2+</sup> concentration (Fig. 4). Another calibration curve was made using a drop (2 mm thick) of each standard buffer solution. Thus obtained 2 calibration curves well coincided with each other. Therefore the accurate Fura-2 measurement was possible in the present measurement system.

Figure 5 shows a spatial distribution of fluorescent Fura-2 image analyzed with an image slicer system. The average concentration of intracellular Ca<sup>2+</sup> in the cell was obtained from the FI<sub>EX340</sub>/FI<sub>EX380</sub> values at the channels within the cell. Temporal changes of the intracellular Ca<sup>2+</sup> concentration of 23 cells were measured at every 30 s (Fig. 6). The Ca<sup>2+</sup> concentrations in these cells were steady for 240 s, but their levels were so different among them, ranging from 10 to 100 nmol dm⁻³.

3.3 Ca<sup>2+</sup> concentration changes in response to electric stimulation

Since the steady levels of intracellular Ca<sup>2+</sup> concentration were so different among test cells, their relative changes were used for the evaluation of the response intensity to an electric stimulation. As an approximation, the relative change of Ca<sup>2+</sup> concentration was defined as follows:

\[ \Delta[Ca^{2+}] = \Delta(FI_{EX340}/FI_{EX380}) = \frac{(FI_{EX340}/FI_{EX380}(t) - FI_{EX340}/FI_{EX380}(0))}{(FI_{EX340}/FI_{EX380}(0))} \times 100 \%
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**Fig. 3** Evaluation of V<sub>CMP</sub> generated by the electric pulse application. (a) Schematic diagram of potential gradient estimation. (b) Potential profiles obtained under the condition V<sub>ET</sub> = 5 V(○), 10 V(□), 15 V(△), 20 V(○). (c) Simple shell model for V<sub>CMP</sub> calculation.

**Fig. 4** Calibration curves for Ca<sup>2+</sup> concentration in a micro space by Fura-2 measurement.
where $F_{I_{EX340}}(t)$ is the value measured at time $t$ (s). The change of $F_{I_{EX340}}(t)$ was relatively small and therefore $F_{I_{EX380}}(t)$ was approximated to be same as $F_{I_{EX380}}(0)$. Thus the above formula became a simpler form:

$$\left(\frac{F_{I_{EX340}}(t)-F_{I_{EX340}}(0)}{F_{I_{EX340}}(0)}\right) \times 100\% = \Delta F_{I_{EX340}}(t)$$

where $F_{I_{EX340}}(t)$ is the value measured at time $t$ (s). $\Delta F_{I_{EX340}}(t)$'s of 35 cells are plotted in Fig. 7. The value of $\Delta F_{I_{EX340}}(t)$ ranged mostly from 0 to ~10%. From these results, we defined the criteria of Ca$^{2+}$ response as: $|\Delta F_{I_{EX340}}(t)| > 15\%$.

Then electric stimulation was applied to the cell. The pulse duration was 10 ms and pulse height ($V_{ET}$) was 5-20 V. When $V_{ET}$ was 15 V or higher, more than 50% of cells were killed which was ascertained by staining with propidium iodide (PI). When $V_{ET}$ was 5 V, $|\Delta F_{I_{EX340}}(t)|$ was small as investigated separately. Therefore $V_{ET}$ was fixed 10 V hereafter. Under this condition, $\Delta F_{I_{EX340}}(t)$'s obtained with 40 cells ranged mostly from ~30% to 50%. At the highest, $\Delta F_{I_{EX340}}(t)$ became 148%. On the other hand, in the presence of 100 µmol dm$^{-3}$ verapamil, $\Delta F_{I_{EX340}}(t)$'s obtained with 24 cells were within the range from ~21% to 3%. This suggests that the Ca$^{2+}$ response occurred via voltage-gated Ca$^{2+}$ channel. On the other hand, the Ca$^{2+}$ concentration decrease seemed not to be effective to the gene expression.

3. 4 Ca$^{2+}$ transient change and chitinase gene expression by electric stimulation

According to the protocol (Fig. 2), an electric stimulus was applied to each protoplast and its response was measured. Following the Fura-2 loading, $F_{I_{EX340}}$ was measured at 0, 30, 60, 90 s. Shortly after that an electric stimulus ($V_{ET}$: 10 V, pulse width: 10 ms) was applied to each protoplast. Then $F_{I_{EX340}}$ was measured successively at every 30 s. $\Delta F_{I_{EX340}}(t)$ calculated from the measured values of $F_{I_{EX340}}$ was shown in Fig. 8. The solid green lines indicate the cases that exceeded the Ca$^{2+}$ response criteria, while the broken blue lines indicate the cases of no Ca$^{2+}$ response.

After incubation, GFP expression was investigated and the results are summarized in Table 1. The electric gene expression via Ca$^{2+}$ response was observed with 16 out of 36 cells (44.4%). This result should include the influence of mechanical wound due to the microinjection and/or the electric stimulation induced mechanical wound. In fact, the electric gene expression without

![Fig. 5](image_url) Spatial distribution of Fura-2 fluorescence intensity in a single-cell of rice protoplast. (a) Phase contrast image. (b) Fluorescent image at 340 nm detected and analyzed with a spectro-imaging system. Microscopic view is projected on 10 × 10 optical fibers. The optical signal of each fiber is resolved into spectral data. The light intensity pattern at any wavelength can be displayed as a 10 × 10 honeycomb image on a TV monitor.

![Fig. 6](image_url) Temporal changes of intracellular Ca$^{2+}$ concentration in protoplasts. Ca$^{2+}$ concentrations of honeycomb units located in the protoplast were averaged and its temporal change was measured. Such a temporal change of Ca$^{2+}$ concentration was measured with 35 protoplasts. Each line indicates the result of each protoplast.

![Fig. 7](image_url) Relative temporal changes of intracellular Ca$^{2+}$ concentration in protoplasts. n = 35.

![Fig. 8](image_url) Ca$^{2+}$ response patterns obtained by an electric stimulation. A pulsing electric stimulus was applied at the arrow. Ca$^{2+}$ response: observed (green lines), not observed (blue lines).
involvement of Ca\(^{2+}\) response was also observed with 3 out of 17 cells (17.8\%). The gene expression was also observed under the condition of no electric stimulation nor Ca\(^{2+}\) response (6/22 = 27.3\%). Despite this Ca\(^{2+}\) independent gene expressions, statistical analysis described below indicates that electric stimulation can induce the RCC1 gene expression via Ca\(^{2+}\) dependent pathway in a similar manner to other stress response genes.

Significance of the results was evaluated by \(\chi^2\)-test for the pairs of electric stimulation versus Ca\(^{2+}\) response and Ca\(^{2+}\) response versus GFP expression. Cross-tabulation for respective pairs was made from Table 1. In the case of electric stimulation versus Ca\(^{2+}\) response, \(\chi^2\) was calculated as 260. Since \(\chi^2_{0.05}\) (DOF = 1) is 3.84, both were dependent upon each other at 95% probability level. In the latter case, \(\chi^2\) was calculated as 3.50. Therefore Ca\(^{2+}\) response and GFP expression correlation was less significant. Their significance level was 90%.

### 4 Conclusion

In the evaluation of electric effects on biological samples, quantitative discussion is important. In this study, effective intensity of cross membrane potential was estimated, though its model was a simple shell model. Consequently a proper condition for protoplasts could be determined (\(V_{ef} 10\) V, Pulse width 10 ms). A more important point is the quantitative analysis of Ca\(^{2+}\) transient variation. Though its spatio-temporal variation was much greater than expected, we could propose a proper method for the estimation of the intracellular Ca\(^{2+}\) mobility. In conclusion, the electric gene expression in plant cells could be demonstrated for the first time at single-cell level and the involvement of Ca\(^{2+}\) in the gene expression was presented in a quantitative manner.

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### References