Short report

A simple measurement of the pH of root apoplast by the fluorescence ratio method

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Abstract: We present a simple procedure for measuring the pH in the apoplast of the rice root, using the fluorescent ratio method and Oregon Green 488 (OG). Staining with 5 μM OG for 3 min followed by 5-min washing with calcium solution was an effective method for measuring the pH of the apoplast at the root surface. Diffusion of OG from the apoplast could be prevented by circulating a low concentration of OG (0.02μM) in the root vessel. However, aluminum ions disturbed the pH measurement. In the presence of 50μM $AlCl_3$, the pH value was inaccurate at values higher than pH 5.0.

Keywords: Aluminum, apoplastic pH, fluorescence ratio method, Oregon Green, rice root.

Abbreviations: OG, Oregon Green 488

Introduction

The apoplastic pH of root cells is very important because it strongly affects the process of nutrient uptake in higher plants (Grignon and Sentenac 1991). Recently, the direct measurement methods of apoplastic pH have been investigated and the results have indicated the physiological importance of apoplastic pH. For example, the response of apoplastic pH to a change in the environmental pH, such as the pH change of a hydroponic culture, is very small. When the pH of a culture solution rapidly increased from 5.0 to 8.5, the increase in the apoplastic pH was only 0.25 (Kosegarten et al. 1999). On the other hand, the apoplastic pH changes greatly in response to gravity (Fasano et al. 2001, Taylor et al. 1996) or by the addition of ions, such as NH_4^+ or NO_3^- (Kosegarten et al. 1999).

Currently direct measurement of apoplastic pH is limited to the fluorescence ratio method and a method which uses a pH-sensitive microelectrode. Peters et al. (1999) measured the apoplastic pH of *Zea Mays* by a microelectrode, and found a correlation between the apoplastic pH and the regulation of growth. Toulon et al. (1992), Felle (1998), and Degenhardt et al. (1998) also developed a pH measurement device with a microelectrode. However, the direct method for measuring the pH, using a microelectrode, can damage the microscopic root surface. In addition, it is also difficult to perform continuous measurements at the same position, since the application of the microprobe changes the micro-environmental condition at the point of contact. Therefore, the use of the fluorescence ratio method is preferable in order to avoid damaging the root surface and thus changing the micro-environmental conditions. In the case of the fluorescence ratio method, the calculation of the ratio of the fluorescence intensity, as measured at two different excitation wavelengths, provides a two-dimensional pH profile of a root. To measure the apoplastic pH of the root, a pH-sensitive boronic acid solution was prepared as a fluorescent dye by Kosegarten et al. (1999). Taylor et al. (1996) used 4-[2-chloro-6-(ethylamino)-7-methyl-3-oxo-3H-xanthen-9-yl]-1,3-benzenedicarboxylic acid (Cl-NERF) to measure the epidermal apoplastic pH using a scanning conforcal microscope. However, these ratio methods also present problems regarding the high cost of instrumentation, or the need for very precise techniques in order to appropriately induce the fluorescent dye into the apoplast.

This paper describes a simple method for measuring the apoplastic pH of the rice root using Oregon Green 488 (OG) dye, which is widely used in the pH range of 4.2 to 5.7, and a conventional fluorescent microscope by means of a simple staining method.

Materials and Methods

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Plant preparation

Japonica type rice, *Oryza sativa* cv. Nipponbare, was used in this study. Seeds were soaked in deionized water for two days and then germinated on a sheet of mesh net floating on 0.5 mM $CaCl_2$ solution (pH 4.5). The solution was renewed every day. After 4 days of culture at 25 ºC, the seedling roots were harvested for the experiment.

Fluorescence ratio measurement

To calculate the pH from the fluorescent ratio, a calibration curve was obtained using OG dye (Oregon Green 488, O-6146, Invitrogen, Co.) solutions adjusted to different pH values, ranging from pH 2.0 to pH 7.5. Thereafter, the rice root was stained with a 5 µM OG solution as described later, and was then observed under a fluorescent microscope (IMT-2, Olympus Co.). The rice root was placed in an acrylic vessel (10 mm × 120 mm × 1.0 mm) wherein a 0.5 mM $CaCl_2$ solution was introduced at a rate of 0.3 m min^{-1} and maintained at a constant volume (600 µL) of solution. Next, images of the area 1 cm distal from the root tip were taken by the CCD camera (C4742-95-12ER, Hamamatsu Photonics, Co.) by applying two excitation wavelengths, namely 440nm and 490nm.

Results and Discussion

Simple staining method of the epidermal apoplast

The accurate calculation of the apoplastic pH requires the following two points when the apoplast is dyed by fluorescent probes: (1) It must be confirmed that the areas which emit fluorescence are the apoplastic part of the plant. (2) A clear difference in fluorescence must exist between the apoplast area and the rest of the root tissue. Generally, the use of a microinjection system makes it possible to directly inject the fluorescent probe into the appropriate point in the root tissue. In our study we investigated another method of introducing the fluorescent probe by studying how the fluorescent probes gradually infiltrate the root from the surface to the center. As a result of our investigations, we found that staining with a 5 µM OG solution for 3 minutes and then washing for 5 minutes with a 0.5 mM $CaCl_2$ solution at 25ºC was sufficient to remove the OG on the surface of the root, while providing a good contrast image of the epidermal apoplast. The observations with a scanning confocal microscope confirmed the image of fluorescence derived from the radial and the horizontal walls of the outer most cell layers (data not shown). An overview of this staining process is shown in Fig.1. It was

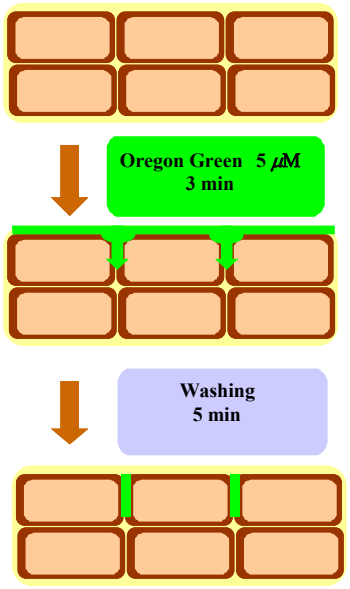

Fig. 1. The staining process of the epidermal apoplast of the root by fluorescent dye**.** Staining for 3 minutes and washing for 5 minutes was sufficient to remove the OG on the surface of the root, and also gave a good contrast image of the epidermal apoplast. Green: Fluorescent dye, Yellow: apoplast, Skin color: cell, Blown: cell wall.

impossible to detect the apoplast clearly without washing for 5 minutes with a 0.5 mM $CaCl_2$ solution, because almost all areas of the root epidermis were stained and emitted a strong degree of fluorescence. In addition, staining longer than three minutes tended to hinder the clear identification of the apoplast region due to the deep staining of the second and third cell layers. Such staining conditions have been optimized for the present plant material (rice roots), and some

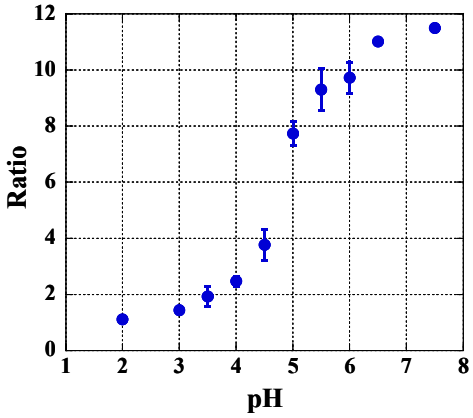

Fig. 2. A calibration curve of pH and the ratio value (R) for Oregon Green 488. The ratio value of the fluorescence intensity through the 530 nm bandpath filter, excited by 490 nm and 440 nm wavelength, was investigated three times with each pH solution containing OG.

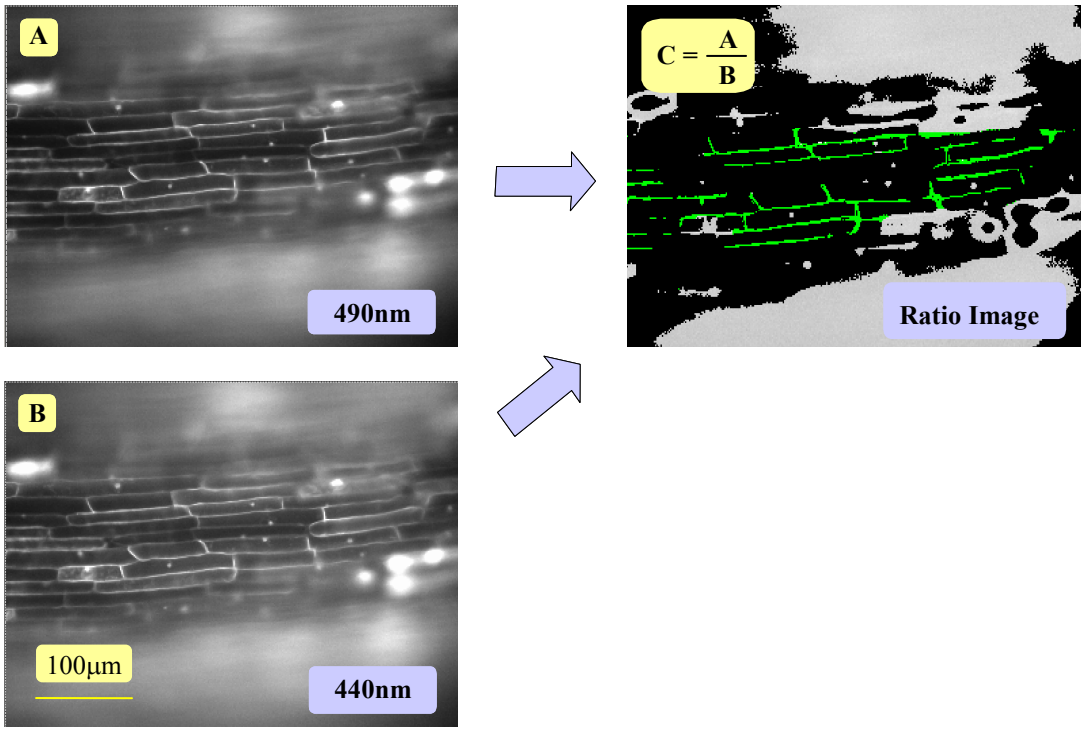

Fig. 3. The fluorescence ratio method. The fluorescent images through the 530 nm bandpath filter, excited by 490 nm and 440 nm wavelength, are shown in Fig. 3A. and 3B. respectively. The pH profile of the apoplast was obtained as shown in Fig. 3C., namely the ratio value of the fluorescence intensity of 3A and 3B at each pixel.

modifications would thus be necessary for other plant materials.

Fluorescent ratio measurement

Figure 2 shows the relationship between the solution pH and the ratio value of the fluorescence intensity through the 530 nm bandpath filter, as excited by the 490 nm and the 440 nm wavelength, using the OG solutions. The calibration formula to determine the pH is shown below, and uses the maximum and minimum ratios (R_{max} and R_{min}) shown in Fig. 2

$$pH = pKa - \log \frac{1.45(R_{max} - R)}{(R - R_{min})} \qquad (1)$$

where pKa is a dissociation coefficient of OG (4.7), R is the ratio obtained at the target point in a root image and 1.45 is the OG specific coefficient obtained from Fig. 2.

The fluorescent images obtained through the 530 nm bandpath filter, excited by the 490 nm and the 440 nm wavelength, are shown in Fig. 3A. and 3B. respectively. The pH profile of the apoplast was obtained as shown in Fig. 3C., from the ratio value of the fluorescence intensity of 3A/3B at each pixel. The pH value was calculated by Equation (1) at each pixel. The apoplastic area demonstrated in Fig. 3C, as indicated by the green color, was taken out from the image, and the apoplastic pH was calculated from the mean of all the pixel values. The apoplastic pH from this method is shown in Fig. 4. Diffusion of OG from the apoplast, was prevented by circulating a low concentration of OG (0.02 µM) in the root vessel.

Effect of Al ions in the ratio measurement

When investigating the correlation between the

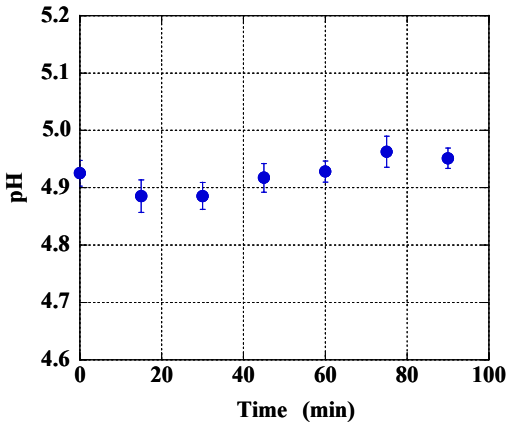

Fig. 4. Time course of the apoplastic pH value as measured by this fluorescence ratio method.

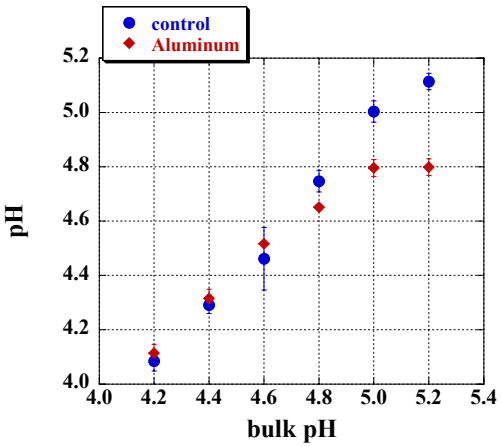

Fig. 5. The relationship between the pH of the OG solution and the calculated pH with (red) or without (blue) 50 μM $AlCl_3$.

apoplastic pH and its ionic condition, any effect of ions on the ratio method is very important. Since aluminum is a toxic element found in soil, we tested the effect of $AlCl_3$ on the linearity of the calculated pH value vs the real pH value in vitro. To study the effect of Al ions on the fluorescence ratio method, the pH measurement was performed for a 5 μM OG solution containing 50 μM of $AlCl_3$. Figure 5 shows that in the presence of Al ions the pH value decreased when the pH value was higher than 4.6. This result suggests that Al ions may inhibit the accurate measurement of the apoplastic pH.

The features of our new fluorescent ratio method

The main advantage of this method is its simplicity and low cost. Normally in order to measure pH values by the fluorescence ratio method it is necessary to use, a microinjection system and a confocal microscope. However, our method needs only conventional fluorescence microscopy with a filter set. In addition, this method enables the users to introduce the fluorescent probe quickly into the apoplast of the first layer of the root (around 15 minutes) without any special instruments. This method also enables the measurement of solute density, such as Ca^{2+}, in the apoplast by using the fluorescent probe specific to the solute.

Conclusion

We herein describe a simple pH measurement method using a conventional fluorescent microscope. Our conclusions are as follows:

1. Staining for three minutes with 5 μM of OG, followed by washing for 5 minutes with 0.5 mM $CaCl_2$ solution was found to be an effective method for measuring the pH of the apoplast at the root surface.
2. Diffusion of OG from the apoplast of the root could be presented by, circulating a low concentration of OG (0.02 μM) in the root vessel.
3. Although OG is widely used to measure pH in the range of 4.2 to 5.7, the presence of Al ions made it difficult to obtain accurate pH measurements.

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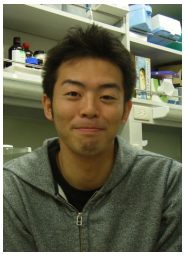

Hiroki Nishiyama's research interest is the mechanism of acid soil tolerance in grain crops, imaging techniques by using microscope or radioisotope, and analysis of chemical compounds from plant root.