Disposable Na⁺-ion-sensing Device
for Research on Salt-tolerant Plants

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A disposable microfabricated Na^+-ion-sensing device was developed for the direct on-site measurement of the Na^+-ion concentration in live plants. To conduct reliable measurements, silver chloride (AgCl) in thin-film Ag/AgCl electrodes within the ion-selective electrode (ISE) and the reference electrode (RE) was regenerated prior to measurements using platinum electrodes formed in the containers of the electrolyte solutions. Distinct responses were observed, and the relationship between the potential of the ISE and Na^+-ion concentration agreed with that expected from the Nernst equation. Welsh onion (*Allium fistulosum*) was used as a model plant, and the internal Na^+-ion concentration was measured using the ISE. The tendency observed using the device agreed well with those obtained using conventional glass-capillary ISE and RE.

1. Introduction

One of the most critical issues in the world is to increase food production. To this end, high salinity is an obstacle. Every year, approximately two million hectares or 1% of agricultural land in the world is lost due to high salinity caused by heavy irrigation, rapid water evaporation, poor agricultural management, and previous exposure to seawater.[1] It is estimated that 50% of cultivable land will disappear by the middle of the twenty-first century.[1] Therefore, there is an urgent need to develop new plants that can grow even under high-salinity conditions.

In the development, salt-tolerant plants were evaluated in terms of phenotypic features including plant growth rate,[2] biomass weight,[3,4] shoot weight,[5] leaf Na^+-ion concentration and the ratio of shoot Na^+/K^+ ions,[6] and leaf area.[7,8] However, phenotypic screening is highly time-consuming. Meanwhile, compounds related to salt tolerance were identified. These include antioxidants,[9] ions,[10] proteins,[11] reactive oxygen species,[12,13] and sugars.[14] Among them, Na^+ ions are directly related to the salt tolerance of plants and can be used for screening.[10]

To measure Na^+-ion concentration in plants, atomic absorption spectrophotometry,[10] flame photometry,[15] plasma spectrometry,[16] and ^{23}Na-NMR microscopy[17] are used. However, these techniques are invasive and require expensive instruments and expertise. On the other

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hand, as a simple method carried out using an inexpensive portable instrument, conductivity measurement is often performed. However, this method is not specific because all ionic species in the sample solution contribute to the conductivity. Also, fluorescence measurement is often attempted in basic research.[18] However, fluorescent dyes are chemically invasive.

To solve these problems, glass-capillary ion-selective electrodes (ISEs) were used for the measurement of Na^+-ion concentration in plant cells.[19–21] However, a problem for researchers in related fields is that the preparation of the ISEs requires various processes such as the formation of an ion-selective membrane, a Ag/AgCl electrode, and a liquid junction for the reference electrode (RE), which requires expertise, collection of materials and reagents, and special instruments. If there is a completed inexpensive disposable device used to measure Na^+-ion concentration and the user can start measurement immediately, the device will surely be of great help and accelerate research in these fields.

The objective of this study was to realize such a Na^+-ion-sensing device. In considering such practical applications, stability during storage and reliability of measurements are critical. In microfabricated electrochemical sensing devices of this kind, a deterministic factor for lifespan is the Ag/AgCl electrodes used to measure potential difference. A thin-film Ag/AgCl structure with a thin AgCl layer on top of a silver layer is very easily damaged.[22] Meanwhile, from our experience, screen-printed Ag/AgCl electrodes cannot provide sufficiently reliable potentials for potentiometric applications. To solve this problem, we used a thin-film Ag/AgCl electrode structure with a protective layer along with an additional electrode to grow AgCl *in situ* before the measurement.[23] This technique improved the long-term stability and reliable measurement considerably. In this study, we used the Na^+-ion-sensing device and demonstrated the direct measurement of Na^+-ion concentration using Welsh onion as a model plant. A comparison of data with those obtained using glass-capillary ISEs demonstrated that our device can be used for research on salt-tolerant plants.

2. Experimental Procedure

2.1 Reagents and materials

Materials and reagents used for the fabrication and characterization of the devices were purchased from the following commercial sources: a polyimide sheet (130 μm thick) from JMT Corporation (Osaka, Japan); a polyvinylchloride (PVC) sheet (120 μm thick) from As One Corporation (Osaka, Japan); a positive photoresist (S-1818G) from Dow Chemical (Midland, MI, USA); soda lime glass capillaries (external diameter: ~1.8 mm, internal diameter: ~1.5 mm) from Asahi Glass (Tokyo, Japan); bis(12-crown-4) and 2-nitrophenyloctyl ether (NPOE) from Dojindo (Kumamoto, Japan); sodium tetraphenylborate from Sigma Aldrich (Buchs, Switzerland); agarose S from Nippon Gene (Toyama, Japan); a silver wire (diameter: 1 mm) from The Nilaco Corporation (Tokyo, Japan); an adhesive, Aron Alpha®, from Toagosei (Tokyo, Japan). PVC powder, tetrahydrofuran (THF), and other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan). The Welsh onion was obtained from a local supermarket. Solutions were prepared with Milli-Q water (Millipore, Tokyo, Japan).

2.2   Microfabrication of Na$^+$-ion-sensing device

The microfabricated Na$^+$-ion-sensing device consisted of a Na$^+$ ISE and a RE. The device was constructed with polyimide and PVC layers with electrodes and solution reservoirs (Fig. 1). We used plastics considering the fabrication cost and the necessity of the deformation of the device when it is placed and fixed on a part of a plant. Two sets of circular Ag/AgCl electrodes and concentric platinum electrodes were formed on the polyimide base layer. The Ag/AgCl electrodes were used to measure the potential of the ISE, whereas the concentric platinum electrodes were used to grow AgCl additionally *in situ* after the device was completed. Reservoirs for the electrolyte solutions of the ISE and the RE were formed by making through-holes in the second polyimide layer, which was bound to the base layer using adhesive. The top PVC layer with a Na$^+$-ion-selective membrane and the through-hole for the liquid junction of the RE was bound to the second polyimide layer.

The electrodes were formed by a thin-film process. A 30-nm-thick chromium layer and a 300-nm-thick platinum layer were sputter-deposited in this order on the polyimide base layer
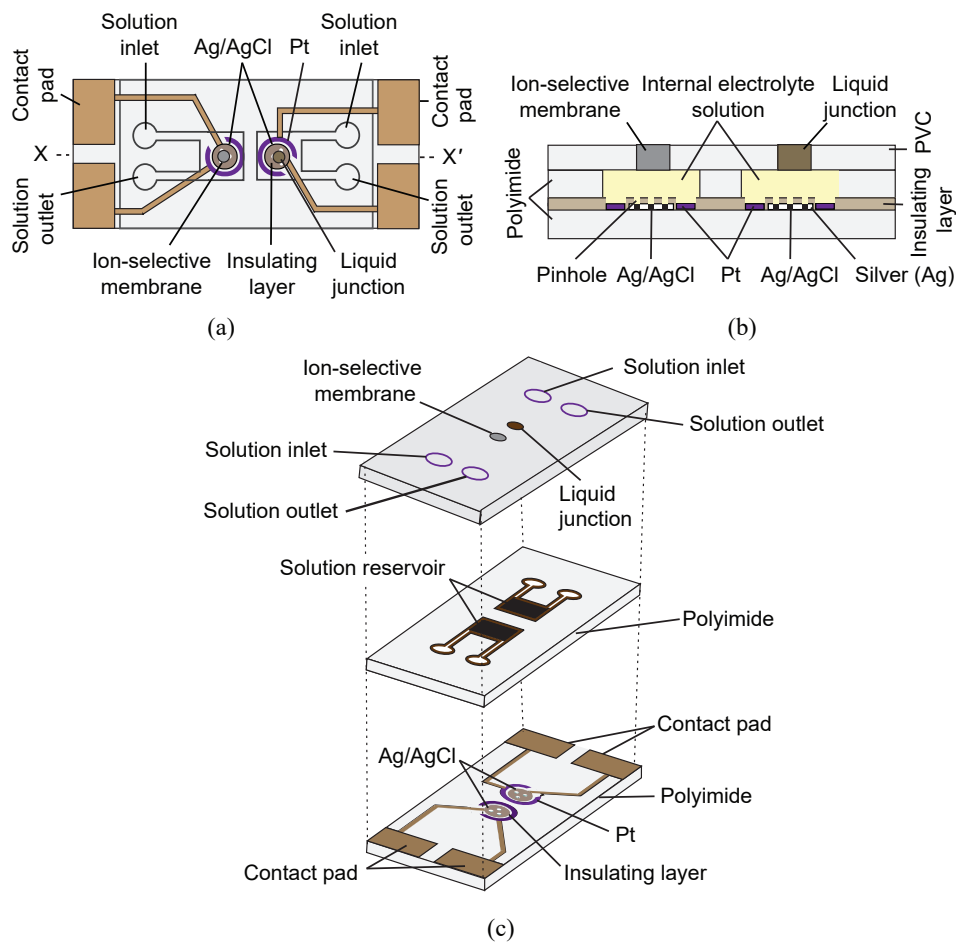

Fig. 1.   (Color online) Construction of the microfabricated Na$^+$-ion-sensing device. (a) Top view. (b) Cross section of the ISE and RE regions along the line X–X′. The thicknesses are not to scale. (c) Exploded view.

after the formation of positive photoresist patterns, and the electrode patterns were formed by lift-off. The chromium layer was used to promote the adhesion of the platinum layer to the base layer. Then, 600-nm-thick silver patterns were formed only on the two circular platinum areas by lift-off in the same manner.

The positive photoresist was also used for insulation, and three pinholes of 40 μm diameter were formed on each circular silver electrode. The thickness of the insulating layer was 2.7 μm. Next, a 100 mM KCl solution was placed on the silver pattern and the concentric platinum pattern, and AgCl was grown from the pinholes into the silver layer by applying a constant current (50 nA) for 10 min using a galvanostat (HA-151, Hokuto Denko, Japan).[23] After the device was completed and used for experiments, AgCl was additionally grown using the internal electrolyte solution (1.0 M KCl) by applying 50 nA for 5 min in the same manner to guarantee the stable potential of the Ag/AgCl electrode.

For the Na^+ ion-selective membrane, crown ether is a representative ionophore.[24] To form the membrane, 101 mg of PVC powder was dissolved in 3 mL of THF. Then, 200 μL of NPOE, 10 mg of bis(12-crown-4), and 6 mg of sodium tetraphenylborate were added. The mixture was thoroughly stirred and 2 μL of the solution was injected into one of the through-holes (diameter: 1 mm) formed in the PVC layer before adhering it to the underlying polyimide layer. Before the injection, the bottom of the through-hole was closed with sticky note paper, which was removed after the membrane was formed. The PVC substrate with an ion-selective membrane was stored at room temperature for 24 h and was fixed to the polyimide layer using an adhesive.

To make the liquid junction for the RE, a 2% agarose gel solution was prepared with a 1.0 M KCl solution. The solution was heated on a hot plate for 30 min at 90 °C and was injected into the other through-hole of the PVC layer without the ion-selective membrane after bonding the PVC layer to the second polyimide layer.

To complete the Na^+-ion-sensing device, a 1.0 M KCl solution was injected into the reservoirs for the ISE and the RE as internal electrolyte solutions using a micropipette. The solutions were replenished with fresh solutions every time before conducting the new experiment.

2.3   Fabrication of glass-capillary ISE and RE

A Na^+ ISE was also made with a glass capillary by an established technique and used for comparison as a reliable device. It consisted of a soda lime glass capillary, an ion-selective membrane formed at one end, a Ag/AgCl electrode, and an internal filling solution. One end of the glass capillaries was made thinner (inner diameter: ~1 mm) using a puller (Narishige, Japan).

In forming the ISE, the glass capillary was washed with distilled water and dried overnight in a covered plastic box. The materials used for the ion-selective membrane were the same as those used for the microfabricated ISE. To form the ion-selective membrane, the end of the glass capillary was immersed in the mixture containing the ionophore and was removed. After that, the capillary was kept in a plastic box overnight. The Ag/AgCl electrode was made by immersing a silver wire in a 100 mM KCl solution and growing AgCl on the surface by applying 250 μA for 10 min using the galvanostat. The AgCl area was 0.8 cm long. Finally, the

glass capillary with the ion-selective membrane was filled with a 1.0 M KCl solution, and the Ag/AgCl wire was inserted there so that only the part covered with AgCl made contact with the solution.

The RE for the glass-capillary ISE was fabricated in the same manner. It consisted of a glass capillary with a liquid junction and a Ag/AgCl electrode immersed in an electrolyte solution. The glass capillary with a smaller diameter at the end was prepared as the one used for the ISE. An agarose gel (2%) containing 1.0 M KCl was used for the liquid junction. The agarose solution was heated on the hot plate for 30 min at 90 ºC, and the end of the glass capillary was filled with an electrolyte gel by dipping the end into the solution. Finally, the Ag/AgCl wire was inserted into the capillary, and a 1.0 M KCl solution was introduced into the glass capillary to complete the RE. When not in use, the glass-capillary ISE and RE were stored in a plastic box after gently cleaning them with distilled water and wiping with tissue paper.

2.4   Characterization of devices

The response profile of the microfabricated ISE was obtained with NaCl standard solutions. One of the solutions was placed on the chip to cover the ion-selective membrane and the liquid junction of the RE. After the potential was stabilized, the solution was discarded and the ion-selective membrane and the liquid junction were cleaned with distilled water and dried with tissue paper. Then, the next solution was placed there. The same steps were repeated for all solutions.

The stability of the microfabricated Ag/AgCl electrode was checked in a 1.0 M KCl solution in a beaker. A commercial Ag/AgCl electrode (2060A, Horiba, Kyoto, Japan) was immersed there. A 3.3 M KCl solution was used for the internal solution of the commercial electrode. The potential of the microfabricated Ag/AgCl electrode was measured with respect to the commercial electrode.

2.5   Measurement of Na$^+$-ion concentration in Welsh onion

The potential of both types of the ISE was measured with respect to the corresponding RE using an electrometer (AutoLab PGSTAT12, Eco Chemie, Utrecht, Netherland). For preconditioning, the ISE was immersed in a 100 mM NaCl solution for 30 min. The ion-selective membrane and the liquid junction were cleaned with distilled water before and after the experiments. To use it on other days, the microfabricated device was rinsed with water, gently dried with tissue paper, and kept in a plastic box with a cover. Between measurements with the same Welsh onion sample, the ion-selective membrane on the device was immersed in 100 mM NaCl solution.

The roots of Welsh onions were immersed continuously in a 100 mM NaCl solution in a beaker and the solution was replaced with fresh solution every 2 days. The Na$^+$-ion concentration was measured at several locations, indicated by distance from the bulb of the Welsh onion [Fig. 2(a)]. Both sides of the same Welsh onion were used for the measurements using both microfabricated and glass-capillary ISEs. For the microfabricated ISE, a part of
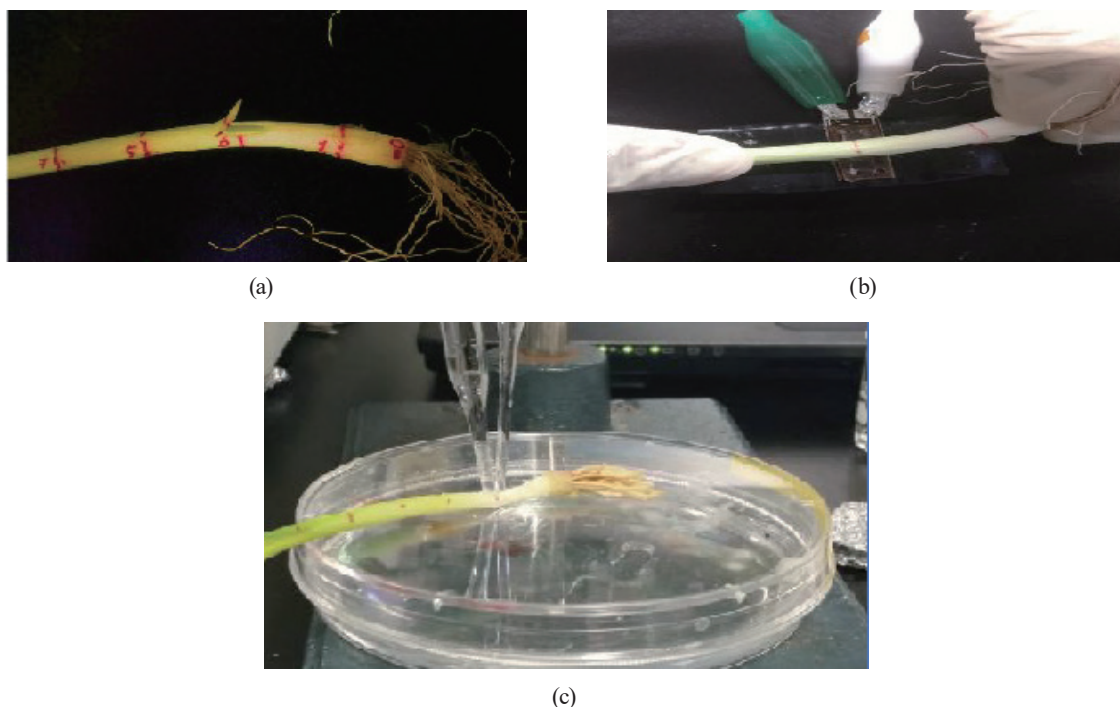
(a)

(b)

(c)

Fig. 2.   (Color online) Measurement of Na^+-ion concentration. (a) A predetermined part of the Welsh onion is peeled off for the measurement using the microfabricated Na^+-ion-sensing device. (b) A part of the Welsh onion is placed on the ion-selective membrane and liquid junction of the microfabricated Na^+-ion-sensing device and slight pressure is applied. (c) For the glass-capillary ISE and RE, a part of the Welsh onion is scratched, and the electrodes are inserted there.

the stem surface of the Welsh onion was peeled off [Fig. 2(a)], and the ISE and RE areas were attached and fixed there by applying slight pressure by hand [Fig. 2(b)].

The areas used for the measurement for the microfabricated ISE and RE were closed with polyimide tape and the locations used for the glass-capillary ISE and RE were prepared on the other side of the same Welsh onion by scratching the surface slightly. The glass-capillary ISE and RE were inserted there [Fig. 2(c)].

Welsh onions were discarded every time after measurement at all locations. The Na^+-ion concentration was calculated on the basis of the calibration plot obtained with NaCl standard solutions. The internal solutions of ISE and RE for both the microfabricated device and the glass capillary electrodes were replenished with fresh solution every time prior to measurement. The experiments were conducted at 25 °C.

3.   Results and Discussion

3.1   Measurement of Na^+-ion concentration in Welsh onion

The adhesion of the platinum and silver electrodes to the polyimide base layer was sufficient even after immersion in a 1.0 M KCl solution for at least 8 days. The stability of the potential

of the thin-film Ag/AgCl electrode was checked in the same 1.0 M KCl solution as that used for the internal electrolyte solutions. The potential settled at approximately 30 mV, which is a value expected from the Nernst equation. The potential could be maintained for at least 2.5 h accompanying a gradual drift in the negative direction (–2 mV). Therefore, by reproducing AgCl prior to each measurement, reliable measurements can be conducted.

Figure 3(a) shows the response profile of the microfabricated ISE when the standard NaCl solution on the chip was changed sequentially. The potential was shifted in the positive direction with the increase in Na^+-ion concentration. Figure 3(b) shows the relationship between the measured potential and the Na^+-ion concentration of the microfabricated and glass-capillary ISEs. As anticipated from the Nernst equation, a linear relationship was observed for both cases in the examined concentration range. The slopes of the plots for the microfabricated ISE and the glass capillary ISE were +55.1 and +55.4 mV/decade, respectively, which are slightly smaller than the expected value at 25 °C (+59.2 mV/decade). The values obtained with the two types of ISE were close, demonstrating that the microfabricated ISE along with the integrated RE can be used like the conventional ISE and RE made with glass capillaries.

3.2   Measurement of Na^+-ion concentration in stem of Welsh Onion

To demonstrate the applicability of our device, the Na^+-ion concentration at several locations on the Welsh onion was directly measured. Although the device worked normally for measurements over several days, we used a new one when we judged that the output became unstable. The performance of the devices used was essentially the same as that described in Sect. 3.1 and no significant difference was observed. The root of the Welsh onion was exposed to 100 mM NaCl and the change in Na^+-ion concentration was monitored (Figs. 4 and 5).

(a)   (b)
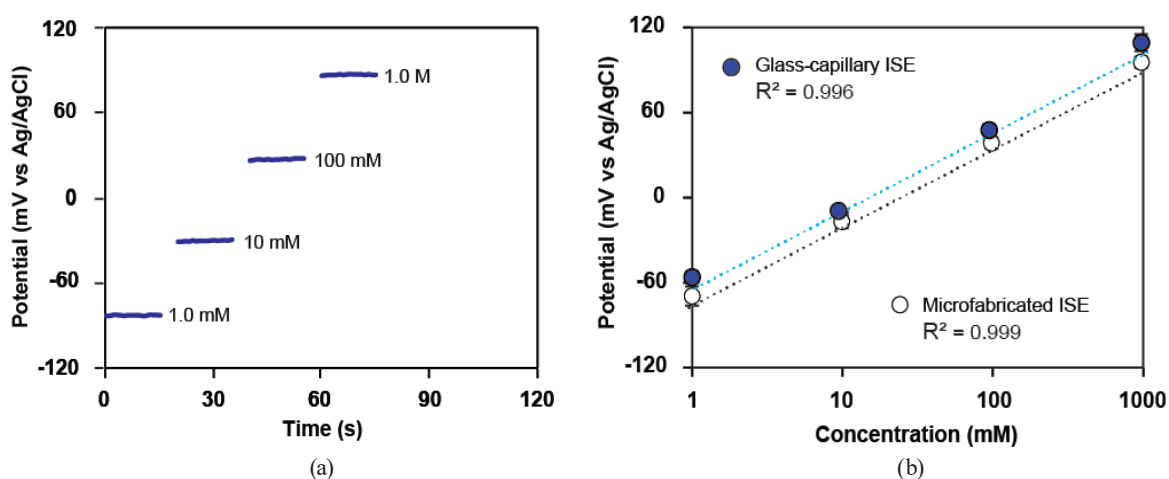
Fig. 3.   (Color online) Response of the microfabricated ISE obtained with NaCl standard solutions. (a) Response profile recorded when the standard solution on the sensitive area was changed. The parts while exchanging the solution are omitted. (b) Dependence of the potential of the ISE on the concentration of Na^+ ions. For reference, the calibration plot for the glass capillary ISE is also shown. Averages and standard deviations are shown ($n = 5$), although most of the error bars are smaller than the symbols.
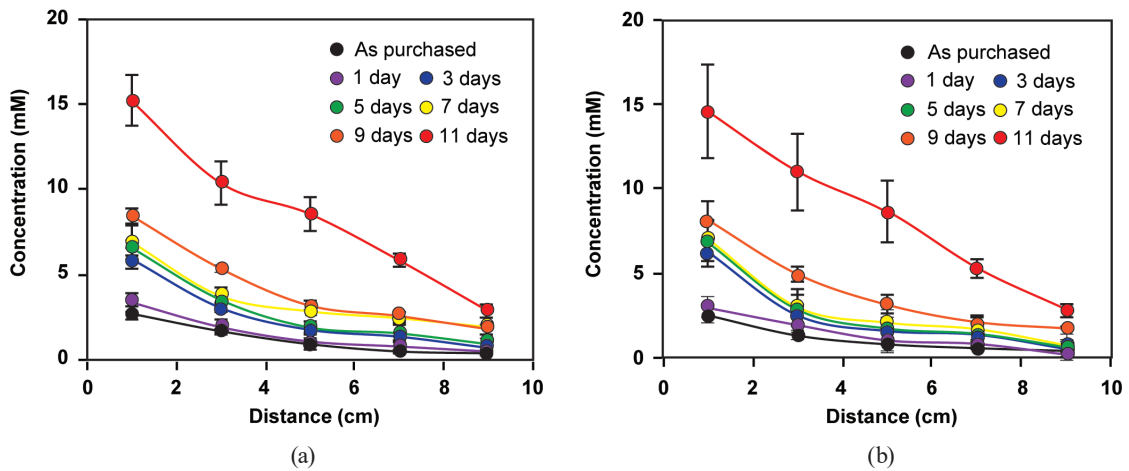
Fig. 4.   (Color online) Distribution of Na⁺-ion concentartion in Welsh onion after immersing the root in 100 mM NaCl solution. (a) Changes obtained using the microfabricated ISE. (b) Changes obtained using the glass capillary ISE. In (a) and (b), the lines are guides for the eyes.
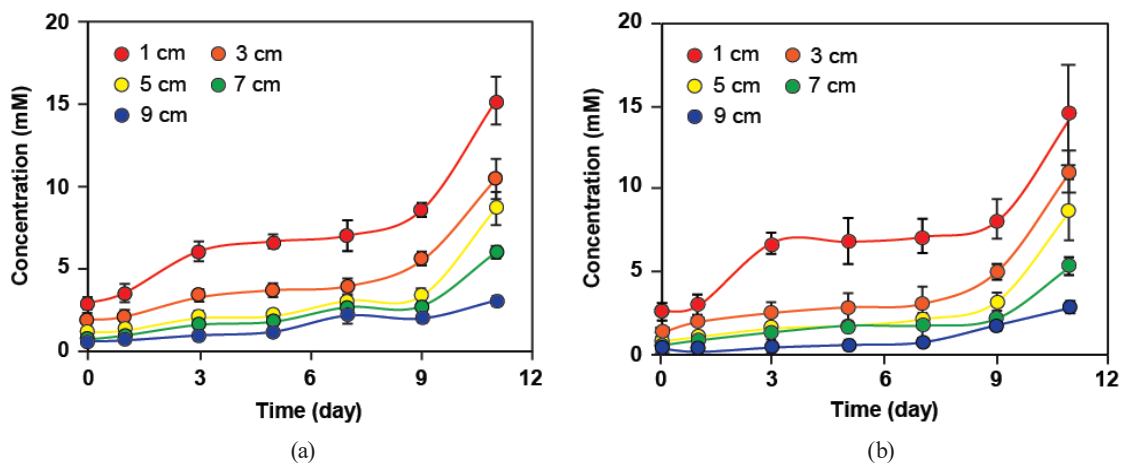

Fig. 5.   (Color online) Time courses of Na⁺-ion concentration measured at five locations on Welsh onion. The root of the Welsh Onion was immersed in 100 mM NaCl solution continuously. (a) Changes obtained using the microfabricated ISE. (b) Changes obtained using the glass capillary ISE. In (a) and (b), the lines are guides for the eyes.

The Na⁺-ion concentration increased in all locations on the Welsh onion with time, but decreased as the location become more distant from the root. The measured Na⁺-ion concentration and observed tendency were very close between the microfabricated device and the glass-capillary device. This tendency agrees with that observed in similar experiments conducted with rice,[25] onion,[26] and sorghum.[27]   The results demonstrate that our microfabricated ISE could be used for the direct measurement of Na⁺-ion concentration in live plants.

4. Conclusions

The microfabricated ISE is applicable to the direct measurement of Na$^+$-ion concentration without any pretreatment. The thin-film Ag/AgCl electrodes have a structure that includes AgCl grown from a pinhole formed in the insulating layer. The AgCl can be grown repeatedly prior to measurements using a platinum electrode formed within the same solution reservoir even after the device is completed. The electrodes actually provided the potential expected as a Ag/AgCl electrode for the sufficiently long duration needed for measurements. The Na$^+$-ion concentration measured using the microfabricated ISEs agreed well with those obtained using the conventional ISEs made with glass capillaries. Changes at different times and locations on the Welsh onion could be measured clearly. On the basis of this technique, ion-sensing devices for other ions can also be realized. This device will be a useful tool for the efficient screening of salt-tolerant plants.

Acknowledgments

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