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SHORT REPORT

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## A rapid quantification method for tissue Na<sup>+</sup> and K<sup>+</sup> concentrations in salt-tolerant and susceptible accessions in *Vigna vexillata* (L.) A. Rich.

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### ABSTRACT

A rapid quantification method for leaf sodium (Na<sup>+</sup>) and potassium (K<sup>+</sup>) concentrations was developed using a compact ion meter. Leaf ion concentrations were evaluated for species of *Vigna vexillata* (L.) A. Rich. after two weeks of treatment with 0–200 mM of sodium chloride. To compare the ion extraction efficiency, extraction solutions of distilled water and ammonium acetate were tested. The ion concentrations of extracts obtained by both solutions were measured using an ion meter, and the values were validated using ion chromatography. For both extraction solutions, the ion meter values were highly correlated with those of the ion chromatograph. However, correlations between ion meter and chromatograph values were largely different for Na<sup>+</sup> and K<sup>+</sup>. The rapid quantification of ion concentrations using an ion meter developed in this study was successfully utilized for evaluating differences in leaf Na<sup>+</sup> concentrations, K<sup>+</sup> concentrations, and K<sup>+</sup>/Na<sup>+</sup> ratio in species of *V. vexillata*.

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### CLASSIFICATION

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### Introduction

The concentrations of sodium (Na<sup>+</sup>) and potassium (K<sup>+</sup>) in plant tissues are important determinants of salt stress tolerance. A high leaf Na<sup>+</sup> concentration inhibits photosynthetic enzymes and carbohydrate metabolism, which can induce oxidative damage and cell death (Chaves et al., 2009; Wang et al., 2003). Leaf Na<sup>+</sup> concentrations also correlate with pollen sterility (Pushpavalli et al., 2016). To avoid these effects, plants have developed salt tolerance mechanisms that can exclude Na<sup>+</sup> from roots to reduce uptake and sequester Na<sup>+</sup> into vacuoles to separate them from cytosolic enzymes (Munns & Tester, 2008). Inclusion mechanisms also control Na<sup>+</sup> concentrations in the cytosol and maintain a high cytosolic K<sup>+</sup>/Na<sup>+</sup> ratio, indicating that the maintenance of a high cytosolic K<sup>+</sup>/Na<sup>+</sup> ratio is important for plant growth under salt stress (Yamaguchi & Blumwald, 2005). As such, measuring the Na<sup>+</sup> and K<sup>+</sup> concentrations in leaves and roots is crucial not only to understanding salt tolerance mechanisms but also to selecting tolerant genes for breeding purposes (Dionisio-Sese & Tobita, 1998; Lin et al., 2004; Munns & James, 2003).

The quantification of Na<sup>+</sup> and K<sup>+</sup> concentrations in plant tissues is widely performed using flame photometer, atomic absorption spectrophotometry, or ion chromatography

(Vera-Estrella et al., 2005; Wang et al., 2012; Yoshida et al., 2016). These methods are accurate and reliable, even for samples with low ion concentrations. However, expensive instrumentation and complex sample-preparation procedures, such as grinding, ashing, extraction, and filtration, can be a hindrance to research studies. In addition, the use of dangerous reagents, such as sulfuric acid, nitric acid, hydrogen chloride, or hydrogen peroxide, in the procedures also makes the application of these methods difficult. A rapid and easy method of tissue ion measurement is needed, especially for large numbers of samples.

In the present study, we used a compact ion meter to easily measuring leaf Na<sup>+</sup> and K<sup>+</sup> concentrations. In comparison with other methods, the ion meter is inexpensive, consists of a simple procedure, and does not use dangerous reagents, and thus, is suitable for measuring large numbers of samples. Previous studies have used the ion meter for measurements of tissue Na<sup>+</sup> or K<sup>+</sup> concentrations in several plant species (Akita & Cabuslay, 1990; Sone et al., 2011; Trifilo et al., 2013). However, the ion concentrations obtained through the ion meter have not been verified using other, more common methods, and thus, the accuracy of this method is not well known. Here, we used a compact ion meter that enabled us to measure Na<sup>+</sup> or K<sup>+</sup>

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concentrations, even for small liquid samples. The leaf ion concentrations were determined for accessions of *Vigna vexillata*, which are promising gene donors for cowpea (*V. unguiculata*) breeding (Tomooka et al., 2011, 2014). To compare the extraction efficiency of the ion meter, two different solutions were tested, and the accuracy of obtained values was checked with the ion chromatograph method.

## Materials and methods

### Plant materials and growth conditions

Two accessions of *V. vexillata* (L.) A. Rich. (JP202334:V1 and JP235869:V5) and an accession of *V. vexillata* var. *macrosperma* (L.) A. Rich. var. *macrosperma* Maréchal, Mascherpa & Stainier (JP235905:macro) were used in this study. V1 and V5 are known as salt-tolerant and susceptible accessions, respectively (Marubodee et al., 2015). All of the accessions were provided by the Genebank, National Agriculture and Food Research Organization, Tsukuba, Japan. Seeds were sown in a granular culture soil with high water permeability in a plastic pot 10 cm high and 5.5 cm in diameter. For each accession, 16 pots (1 plant per pot) were prepared. All plants were grown in a growth chamber. The growth conditions were 12-h photoperiods, 1200  $\mu\text{mol photon m}^{-2} \text{s}^{-1}$  PPFD of irradiance, and 30 °C/25 °C (day/night) temperatures. Sodium chloride (NaCl) treatments were initiated two weeks after sowing, when the second trifoliolate leaves were fully expanded in all accessions, by soaking the bottom half of each pot in the NaCl solution. For each accession, four pots were used for each of the 0 mM (control), 50, 100, and 200 NaCl treatments. During the NaCl treatments, light and temperature conditions were the same as before the treatments. After a week of treatments, the topmost, fully expanded leaves were sampled and dried for one day at 70 °C. All dried samples were ground using mortar and pestle, and used for Na<sup>+</sup> and K<sup>+</sup> tissue analyses. Among the 48 samples (four salt treatments  $\times$  three accessions  $\times$  four replications), 24 samples (two samples per accession) were used for both the ion meter and the ion chromatograph methods to obtain calibration equations.

### Ion chromatograph

Ground leaf samples (>1.0 g) were weighed and reduced to ash with an electric muffle furnace. Subsequently, 100  $\mu\text{L}$  of 1 N HNO<sub>3</sub> was added for cation extraction. The leaf Na<sup>+</sup> and K<sup>+</sup> concentrations were determined using an ion chromatograph with a conductivity detector (Shimadzu, Japan) and in accordance with our previous study (Yoshida et al., 2016). Oxalic acid (3.3 mM) was used as the mobile phase. The mobile phase was degassed by the degasser (DGU-12A) and pumped with a liquid chromatograph

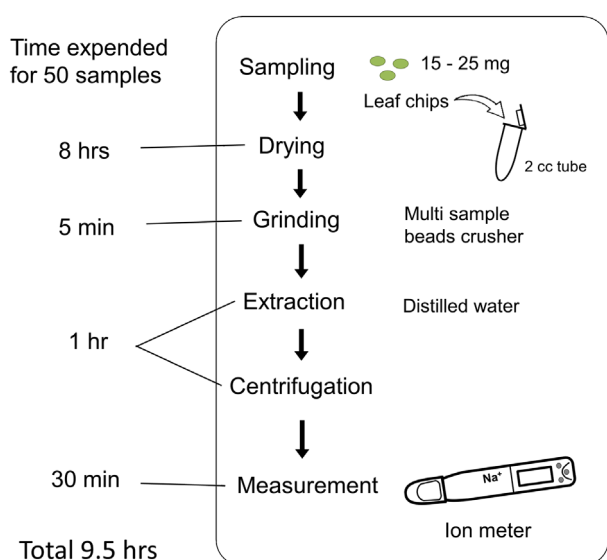
pump (LC-9A) at a speed of 1  $\mu\text{L min}^{-1}$ . This mobile phase was flown to the auto injector (SIL-6B) and mixed with 10  $\mu\text{L}$  of leaf sample solutions to be homogenized, which was controlled by the system controller (SCL-6B). The leaf Na<sup>+</sup> and K<sup>+</sup> concentrations were detected through the analytical column (IC-C3) in the column oven (CTO-10A) at 40 °C, and the results were printed by a chromatopac (C-R6A). The standard Na<sup>+</sup> and K<sup>+</sup> solutions (100% was equated with 2 ppm) were used for calibration and for calculation of the correct ion concentrations of the leaf sample solutions.

### Ion meter

All 48 samples were analyzed using the ion meter. To compare the ion extraction efficiency, different extraction solutions of distilled water (DW) and 1 N ammonium acetate (AA) were used. Approximately 15–25 mg of ground leaf samples was weighed and collected into 2-cc tubes, and 500  $\mu\text{L}$  of the extraction solution (DW or AA) was added. Tubes were shaken for an hour with metal beads for cation extraction. After centrifuging the samples at 7000 rpm for 5 min, 200  $\mu\text{L}$  of the supernatant was analyzed using the Na<sup>+</sup> and K<sup>+</sup> meters (LAQUAtwin, Horiba, Japan). The samples were first analyzed with the Na<sup>+</sup> meter, and then the same sample was transferred to the K<sup>+</sup> meter. Before taking the measurements, both ion meters were calibrated using the corresponding calibration solution. All procedures were conducted at room temperature.

## Results and discussion

A rapid quantification method for plant tissue Na<sup>+</sup> and K<sup>+</sup> concentrations was developed using a compact ion meter. Figure 1 shows the approximate times necessary for each step of the recommended ion meter procedure. This method comprises fewer steps in comparison with atomic absorption spectrophotometry or ion chromatography. In the ion chromatograph method, ashing and filtration were applied before analyzing samples because impure liquid samples could not be applied to the column. These steps took a long time and used dangerous reagents, such as nitric acid. In addition, ion chromatography needed a large amount of leaf sample (>1 g leaf dry weight), limiting the number of potential samples. In contrast, the ion meter used a small amount of liquid sample (200–500  $\mu\text{L}$ ), which was derived from 15–25 mg of leaf dry weight. This small sample size reduced the time needed for drying, grinding, and extraction. In addition, if a multi sample bead crusher is available, many samples can be ground at once (30 s is sufficient). Total expected time for analyzing 50 samples is 9.5 h, although in this study, mortar and pestle were used because the ground samples were analyzed using both of the ion meter and ion chromatograph. This time is



**Figure 1.** Recommended procedure of the ion meter method. Time needed to analyze 50 samples using the ion meter method is shown. In this study, a multi-bead shocker was not used because the ground samples were analyzed using both the ion meter and the ion chromatograph.

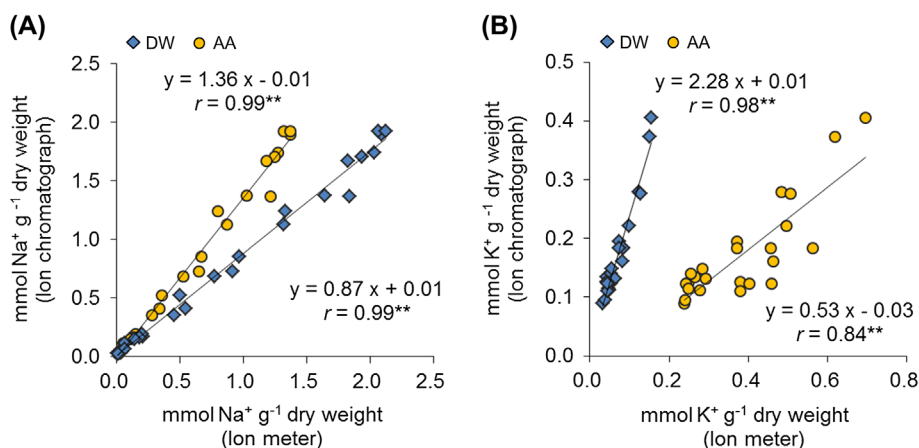
much shorter than the time needed for the ion chromatograph (>40 h). Thus, the ion meter method is simple, rapid, inexpensive, and suitable for quantifying tissue  $\text{Na}^+$  and  $\text{K}^+$  concentration in many samples.

In this study, the tissue ion concentrations were determined from ground samples of whole leaves. In contrast, when leaf tips are used, as shown in Figure 1, the ion localization in a leaf should be considered (Makino Nakanishi & Matsumoto, 1991). If the ion concentration is not uniform in a target leaf, results may differ depending on the

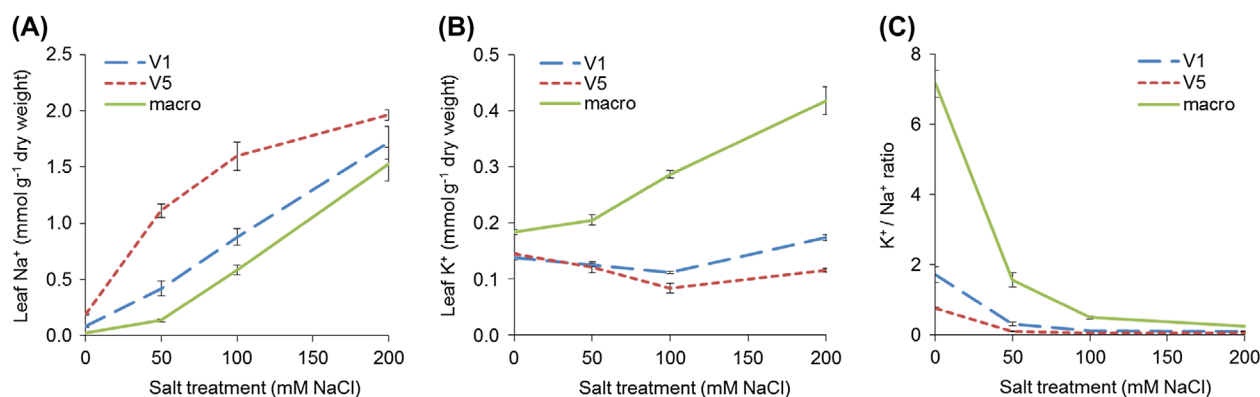
sampling part. In this case, leaf tips should be taken from same part of all leaf samples.

In the ion meter method, we used AA extraction solutions in addition to the commonly used DW extraction solutions because AA extractions are widely used in soil cation analyses (Simard, 1993). In Figure 2, ion concentrations determined from the ion chromatograph method with nitric acid extraction were compared with those determined from the ion meter method with DW or AA extractions. In both the DW and AA extractions, high correlations ( $r = 0.99$ ) were observed in leaf  $\text{Na}^+$  concentrations (Figure 2(A)). The DW extraction showed some overestimation in comparison with the AA extraction. For the leaf  $\text{K}^+$  concentrations, the correlation coefficient of the AA extraction was lower ( $r = 0.84$ ) than that of the DW extraction ( $r = 0.98$ ) (Figure 2(B)). The values of DW extraction were underestimated in comparison with those of the AA extraction. The correlation coefficients of the DW extraction were not largely different from those of the AA extraction, and thus, the DW extraction was preferred over the AA extraction because it did not need any reagent. Over- and underestimation of leaf  $\text{Na}^+$  and  $\text{K}^+$  concentrations do not present a problem when examining the relative order of ion concentrations between treatments. In contrast, when the values of  $\text{Na}^+$  and  $\text{K}^+$  concentrations are compared, calibrations are needed in each experiment, because correlation equations are different depending on the target ions.

Although the cause of leaf ion concentration differences between the ion meter and ion chromatograph methods could not be determined in this study, it is conceivable that the differences were dependent on extraction methods and measurement error in the ion meter. Using an electric



**Figure 2.** Relationships between the leaf ion concentrations determined by the ion meter method and the ion chromatograph method. The relationships of (A) leaf  $\text{Na}^+$  concentrations and (B) leaf  $\text{K}^+$  concentrations are shown. Squares and circles represent the samples extracted using distilled water (DW) and ammonium acetate (AA) in the ion meter method, respectively. The same ground samples were used for ion extraction in both the ion meter and ion chromatograph methods, independently. For the ion chromatograph method, samples were extracted using  $\text{HNO}_3$  solution.



**Figure 3.** Response in (A) leaf Na<sup>+</sup> concentration, (B) leaf K<sup>+</sup> concentration, and (C) leaf K<sup>+</sup>/Na<sup>+</sup> ratio against different salt treatments in three accessions of *V. vexillata*. All the values were estimated using ion meter methods with DW extraction. For the estimation of ion concentrations, the calibration equations obtained from 24 samples were applied for all 48 samples. Each data point is an average of four replications. Bars represent the standard deviations.

muffle furnace and nitric acid extractions in ion chromatography reduces variability among the samples and is the most reliable way to extract ions. Meanwhile, in the ion meter method, extraction efficiency may be affected by sample conditions such as plant tissue hardness or ion form in tissues. In addition, ion meter values are thought to be affected by other cations in plant tissues. To confirm the cause of leaf ion concentration differences, ion concentrations of the same extraction solutions should be compared between ion meter and ion chromatograph values in further studies.

Figure 3 showed the estimated leaf Na<sup>+</sup> concentrations, K<sup>+</sup> concentrations, and K<sup>+</sup>/Na<sup>+</sup> ratio using the ion meter and DW extraction in the *V. vexillata* accessions under various NaCl stress treatments. To estimate ion concentrations, calibration equations obtained from the 24 ion chromatograph samples were applied to all 48 ion meter samples. The leaf Na<sup>+</sup> concentrations were highest in V5 (susceptible) accession compared to V1 (tolerant) and macro (*V. vexillata* var. *macrosperma*) accessions under all NaCl stress treatments. In contrast, significant increases in leaf K<sup>+</sup> concentrations were observed only in macro accessions with increasing NaCl concentrations, while K<sup>+</sup> concentrations of V1 and V5 were not changed. These results suggest that macro accessions had high selectivity for K<sup>+</sup> rather than Na<sup>+</sup> concentrations, reducing internal Na<sup>+</sup> concentrations. A high K<sup>+</sup>/Na<sup>+</sup> ratio is a common trait of salt tolerance (Munns & Tester, 2008). The relationship between leaf K<sup>+</sup>/Na<sup>+</sup> ratio and salt tolerance was also observed in genus *Triticum* and genus *Aegilops* (Gorham, 1990; Gorham et al., 1991). Therefore, the macro accession would be a genetic resource for improving salt tolerance in the relative crop species cowpea (*V. unguiculata*). The ion meter method could be successfully used for evaluating the physiological traits of macro accessions.

In conclusion, the ion meter method enables the rapid evaluation of tissue ion concentrations, even for large sample numbers comprising several treatments, genotypes, and replications. High correlations were found between values obtained from ion chromatograph and ion meter methods, especially for samples extracted using DW. Therefore, the ion meter method can be applied without any calibrations in plant evaluation cases where the order among tested plants is important but not absolute values. However, for evaluating the K<sup>+</sup>/Na<sup>+</sup> ratio, calibration is needed because correlation equations between values obtained by the ion chromatograph and ion meter methods were largely different for Na<sup>+</sup> and K<sup>+</sup>. In the case of evaluating large sample numbers and reducing the time required for analyses, a portion of samples should be calibrated using both the ion meter and the established ion concentration methods, whereas the rest of the samples should be analyzed using only the ion meter method.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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