Starch-dependent sodium accumulation in the leaves of Vigna riukiuensis.

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Research Article

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Abstract

This research provides insight into a unique salt tolerance mechanism of *Vigna riukiuensis*. *V. riukiuensis* is one of the great genetic resources of salt tolerance. We have previously reported that *V. riukiuensis* accumulates a higher amount of sodium in the leaves, whereas *V. nakashimae*, a close relative of *V. riukiuensis*, suppresses sodium allocation to the leaves. We first suspected that *V. riukiuensis* would have developed vacuoles for sodium sequestration, but there were no differences compared to a salt-sensitive accession. However, many starch granules were observed in the chloroplasts of *V. riukiuensis*. In addition, forced degradation of leaf starch by shading treatment resulted in no radio-Na (^{22}Na) accumulation in the leaves. We performed SEM-EDX to locate Na in leaf sections and detected Na in chloroplasts of *V. riukiuensis*, especially around the starch granules but not in the middle of those. Our results could provide the second evidence of Na-trapping system by starch granules, following the case of common reed that accumulates starch granule at the shoot base for binding Na.

Introduction

Salt tolerance has been one of the most important issues in plant science as soil salinity is a major constraint of crop production. In addition, given a rapid depletion of ground water plus a rapid growth of global population, there is a concern that demands for drinking water may compete with those for food production (Wada et al., 2010). Thus, there is a growing demand for salt-tolerant crops, which can be grown with salinized water (Panta et al., 2014).

To this goal, we have screened genetic resources of wild Vigna species and identified several species that are highly tolerant to salt stress (Iseki et al., 2016, Yoshida et al., 2016). The genus *Vigna* is a reservoir of diversity and many species are adapted to harsh environments, such as marine beach, desert, limestone karsts and marshes (Tomooka et al., 2014, van Zonneveld et al., 2020). As expected, the species collected from coastal areas presented high tolerance to salt (Iseki et al., 2016)

The following studies on sodium allocation have revealed that *V. riukiuensis* accumulates relatively higher amount of sodium in the leaves, whereas *V. nakashimae*, a close relative of *V. riukiuensis*, suppresses sodium accumulation to the leaves (Yoshida et al., 2016, Noda et al., 2022). Thus, we have hypothesized that *V. riukiuensis* has a mechanism of salt-includer, which isolates sodium into vacuoles to lower sodium concentration in cytoplasm.

To confirm this hypothesis, in this study, we first observed vacuoles of the leaf cells to see whether there are any structural differences between *V. riukiuensis* and other species. However, we could not find any characteristic features in the vacuoles. Instead, we found lots of starch granules in the chloroplast of *V. riukiuensis* leaves. Since there has been a report that common reed accumulates starch granules that bind Na at the shoot base (Kanai et al., 2007), we were intrigued to test whether the starch granules in *V. riukiuensis* also has ability to bind Na. To do so, we performed further experiments including element mapping.

Materials And Methods

Plant material and growth conditions

All plant seeds were provided by the NARO Genebank in Tsukuba, Japan (https://www.gene.affrc.go.jp/index_en.php). Seeds were germinated on Seramis clay (Westland Deutschland GmbH, Mogendorf, Germany) for 1 week and then transferred to hydroponic solution in a growth chamber (Light: 28°C for 14 h and Dark: 25°C for 10 h. Light intensity 500 μM^{-1} s^{-1} m^{-2}) until autoradiographic imaging. Hydroponic solution contained diluted nutrient solution of a 1:1 ratio of OAT House No.1 (1.5 g L^{-1}) : OAT House No.2 (1 g L^{-1}) (Otsuka Chemical Co, Japan), which contained 18.6 mEq L^{-1} N, 5.1 mE1 L^{-1} P, 8.6 mEq L^{-1} K, 8.2 mEq L^{-1} Ca and 3.0 mEq L^{-1} Mg.

Visualization of ^{22}Na

Pre-cultured plant was transplanted to new hydroponic solution containing 5 kBq ^{22}Na (PerkinElmer, USA) with non-radioactive 100 mM $^{23}NaCl$. After adding the radio-isotope, plants were incubated again in a long-day condition (Light: 28 ℃ for 14 h and Dark: 25 ℃ for 10 h. Light intensity 200 μmol s^{-1} m^{-2}) for 3 days. After incubation, we carefully washed the roots and then enclosed the whole plant body into a plastic bag, and exposed it to a Storage Phosper Screen (BAS-IP-MS-2025E, GE Healthcare, UK) in Amersham exposure cassettes (GE Healthcare, UK) for 24 h. We then scanned the exposed screen with a laser imaging scanner Typhoon FLA-9500 (GE Healthcare, UK). To arrange radioactive intensity equally at each image, photo-stimulated luminescence and contrast were equalized by Multi Gauge version 3.0 (Fujifilm, Japan). All the experiments were independently done on more than three biological replicates.

ICP-MS

We germinated the seeds on Seramis clay, cultivated for 1 week and then transferred 4 plants of each species to hydroponic solution (as described above) in a growth chamber (Light: 28℃ for 14 h and Dark: 24℃ for10 h). When the 3rd leaves have fully expanded, we transferred the plants to hydroponic culture with 100 mM NaCl for 2 days. After incubation, we separately collected the 1st and the 2nd leaves and dried at 50°C for 3 days. The leaves were digested with 200 μL 69% HNO_3 at 90°C for 0.5 h. The digestate was diluted 1-in-140 with Milli-Q water and inductively coupled plasma-mass spectrometry (ICP-MS, NexION 350S, PerkinElmer, Waltham, MA, USA) determined the contents of Na. The Tukey HSD of statistical analysis was used to compare differences in the measured variables of leaf Na and K concentration, respectively. Differences were significant when $p < 0.05$.

Starch staining and correlation between starch and Na accumulation with shading in V. riukiuensis

Iodine staining

At the beginning of the treatment, we grew *V. angularis, V. nakashimae* and *V. riukiuensis* in hydroponic solution for 7 days, 14 days and 21 days, respectively. Plants were grown in 100 mM NaCl hydroponic solution or non-NaCl solution for 3 days. After incubation, we harvested fresh leaves, immediately immersed in hot water for 10 min and decolorized in 99.5% ethanol for 5 minutes. Finally, samples were immersed 1/50 iodine solution (20 g l^{-1} KI, 10 g Iodine) for 10 minutes.

Shading experiment

V. riukiuensis plants were grown in hydroponic solution for 21 days. The middle leaflets of the 1st, 2nd and 3rd leaves were masked with aluminum foil (shaded) or cling film (non-shaded control). Plants were transferred to 100 mM ^{22}NaCl solution (5 kBq) for 3 days under long-day condition. After ^{22}Na treatment, ^{22}Na localization was visualized using the method described above.

Electron microscopy and SEM-EDX

We treated *V. angularis*, *V. nakashimae* and *V. riukiuensis* with 100 mM NaCl for 3 days. We cut the leaves into 5x5 mm^2 pieces, and the samples were fixed with 4% (w/v) paraformaldehyde and 2% (v/v) glutaraldehyde in 50 mM cacodylate buffer for 2.5 h at room temperature. The samples were washed six times by the same buffer and postfixed with 1% osmium tetroxide in 50 mM cacodylate buffer for 2 h. After washing by the double distilled water, the samples were dehydrated in methanol series (25, 50, 75, 90, 100%) and substituted to methanol:propylene oxide (1:1) to 100% propylene oxide. Next, the samples were substituted in propylene oxide:Epon812 series (3:1, 1:1, 1:3) and finally embedded in 100% Epon812 resin (TAAB, UK). Semi-thin sections (1 or 2 μm) were cut with a diamond knife on an ultramicrotome (EM UC7, Leica Microsystems, Germany). To test whether there are any structural differences between each species, 1 μm thickness sections were observed by a field-emission scanning electron microscope (FE-SEM). Sections were dried on glass slides and stained with 0.4% uranyl acetate and lead citrate solution. Sections were coated with osmium tetroxide with an osmium coater (HPC-1SW, Vacuum device, Japan), then observed by FE-SEM (SU8220, Hitachi High-Tech, Japan) at accelerating voltage 5 kV with an yttrium aluminum garnet backscattered electron detector. For scanning electron microscope-energy dispersive x-ray spectrometry (SEM-EDX), 2 μm thickness sections were dried on carbon tape at 60°C and set on scanning electron microscope (SEM) stub. The Na imaging condition was high vacuum and acceleration voltage mode as follows: Acceleration voltage, 15 kV; Spot intensity, 80; Beam exposure time, 1 hour. As beam irradiation causes a slight elongation of the sections, electron microscopy images were acquired after beam irradiation in low vacuum and acceleration voltages. Spectral acquisition of Na and electron microscope images were acquired by EMAX Evolution (version EMAX 2.2 SP2, HORIBA, Japan) and SU3500 (Hitachi High-Tech, Japan), respectively. Spectral data was converted into Na mapping image by ImageJ version 1.51j8 (National Institutes of Health, USA). We checked intensity of Na signal in energy dispersive X-ray spectroscopy (EDX) images by quantitative analysis provided in the EMAX Evolution. Sodium mapping and intensity of signal in these species were tested by more than three biological replicates.

Results

Sodium allocation in V. riukiuensis *and its relatives*

To confirm that *V. riukiuensis* accumulates higher amount of Na^+ in the leaves, we evaluate Na allocation in *V. angularis*, *V. nakashimae* and *V. riukiuensis* by autoradiograph and mass spectrometry. The results reproduced our previous observations (Yoshida et al., 2016, Noda et al., 2022), where Na allocation to the leaves were low in *V. nakashimae* but high in *V. riukiuensis* (Fig. 1). *V. angularis*, which is sensitive to salt stress, allocated more Na in the lower leaves than in the higher leaves.

Electron microscope image of leaf cells

Because *V. riukiuensis* accumulated higher amount of Na^+ in the leaves, we considered that it isolated Na^+ to the vacuoles. To test whether any specific features are observable in the vacuoles of *V. riukiuensis*, we obtained electron microscope images on the leaf cells of *V. angularis* and *V. riukiuensis*.

However, we could not find any specific features in vacuoles of *V. riukiuensis*. Regardless of salt stress, the vacuoles were fully developed and occupied most of the cell compartments in both the species (Fig. 2). The only difference we found between the two species was in that *V. riukiuensis* contained well-developed starch granules in the chloroplasts whereas *V. angularis* did not (Fig. 2).

Iodine-starch staining

To confirm that *V. riukiuensis* accumulates starch granules in the leaf cells, we did iodine-starch staining on the leaves of *V. angularis, V. nakashimae* and *V. riukiuensis* (Fig. 3). As a result, we observed strong staining in the leaves of *V. riukiuensis* but not in those of *V. angularis*, confirming high starch content in *V. riukiuensis* but low in *V. angularis.* However, we also observed strong staining in the leaves of *V. nakashimae*. Thus, it did not seem that the Na^+ allocation to the leaves in *V. riukiuensis* was because of the higher content of starch.

Suppressed Na^+ *allocation to shaded leaves of* V. riukiuensis

However, we still suspected that starch granules in *V. riukiuensis* positively affected Na^+ allocation to the leaves. To test the hypothesis, we shaded some leaflets of *V. riukiuensis* plants for 24h to have all the starch granules degraded (Fig. 4). We then fed the plants with ^{22}Na and took autoradiograph to visualize Na allocation. As a control, we wrapped the leaflets of the same positions with cling film to exclude a possibility that “covering” had negatively affected Na accumulation by decreasing transpiration.

The results were remarkable. The autoradiography after ^{22}Na treatment clearly showed that the shaded leaflets did not accumulate ^{22}Na, while all other leaves (including “wrapped” leaflets in control) exhibited strong indication of ^{22}Na allocation (Fig. 5).

Scanning electron microscope-energy dispersive x-ray spectrometry (SEM-EDX)

As the results motivated us to test whether the starch granules have capability of binding Na^+ in *V. riukiuensis*. To do so, we performed SEM-EDX to locate Na in leaf sections (Fig. 6). We should note that the process of preparing leaf sections (including fixation) washes free Na^+ ions away from vacuoles, cytoplasm and apoplastic spaces. Thus, SEM-EDX can detect only Na bound to cellular components.

As expected from the results of iodine-starch staining experiments (Fig. 3), the SEM image showed that there were few starch granules in the leaf sections of *V. angularis* whereas there were a lot in those of *V. nakashimae* and *V. riukiuensis* (Fig. 6).

The following EDX analysis detected few Na in the sections of *V. angularis* and *V. nakashimae* but detected Na in the sections of *V. riukiuensis*, especially in chloroplasts (Fig. 6). Moreover, Na was not in the middle of starch granules but was enriched around them (Fig. 6). As such, in the leaves of *V. riukiuensis*, Na was isolated in chloroplasts, especially around starch granules.

Discussion

In this study, we demonstrated that *V.riukiuensis* accumulates starch granules that bind Na, which could be the second evidence of Na-binding starch granules in plants. The only report before this study was of common reed, which forms Na-binding starch granules in the shoot base in response to salt stress (Kanai et al., 2007). Kanai et al. have demonstrated co-localization of Na with starch granules and the isolated starch granules contained higher amounts of Na than other parts of the shoot base did (Kanai et al., 2007). Although *V. riukiuensis* forms Na-binding starch granules in the leaves not in the shoot base (Figs. 1–6), these facts suggest that the Na-trapping system by starch granules had independently evolved in multiple salt-tolerant species.

Since we previously revealed that *V. riukiuensis* allocate relatively higher amount of Na to the leaves, we have considered it has an includer-type mechanism, which sequesters excess Na^+ into vacuoles (Yoshida et al., 2016, Noda et al., 2022). However, recent studies argue that vacuole is not an ideal organelle for Na^+ sequestration, as tonoplast is permeable to Na^+ and thus allows back-leak to cytosol (Shabala et al., 2019). Thus, for halophytic species including *V. riukiuensis*, it would be better to have a mechanism other than, or in addition to, the one using vacuole.

What is complicating in our results is in that *V. nakashimae* also accumulates lots of starch granules in the chloroplasts but does not allocate Na to the leaves. One possibility is that *V. nakashimae* have supreme ability to exclude Na^+ out of the leaves. If Na^+ does not enter leaf cells, it can never reach chloroplasts where starch granules are formed. The other possibility is that the starch granules in *V. nakashimae* lacks Na-binding ability. Although pure amylose chains do not have any ability to bind cations including Na^+, modification of hydroxyl groups such as phosphorylation turns them into cation

exchangers (Matsumoto et al., 1998). Given amylose chains are extended from the surface of starch granules (Goren et al., 2018), V. *riukiuensis* and common reed may have higher enzymatic activity in modifying those chains.

It should be noted that *V. riukiuensis* may also have ability to exclude Na out of the leaves, at least when the leaves have run out of starch granules (Fig. 5). One may argue that the shading leads to stomatal closure and reduces xylem flow that transports Na to the leaves. However, the results of the control plants do not support such arguments. We also covered the leaflets of the control plants with transparent film to minimize the effect of transpiration, but sodium allocation did not seem to be reduced compared to other leaves or leaflets (Fig. 5). In addition, as observed in Fig. 5, ^{22}Na have entered into veins of the shaded leaves but not into the mesophylls. Thus, there should be a mechanism to suppress Na^+ transport from xylem to mesophyll cells.

One limitation in this study is that we do not know how much the starch granules contribute to salt tolerance in *V. riukiuensis*. However, this is a testable issue because *V. riukiuensis* can be crossed with *V. angularis.* We have already crossed them and will perform genetic analyses to see if there is any correlation between starch content in leaves and salt tolerance.

To conclude, we have demonstrated common reed is not the only species that have evolved Na-binding starch granules. As it is effective in multiple plant taxa, we are intrigued to apply this system for developing salt-tolerant crops. By combining other mechanisms of salt tolerance, it would bring synergistic effects on salt tolerance.

Declarations

Funding

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Conflict of interest: The authors have no conflicts of interest to declare that are relevant to the content of this article.

Author contributions

YN, AH, KT, NK, JF, KT and KN planned the research.

YN, AH, MA, MS, MW and KT performed experiments.

YN, AH, MS and KN analyzed data.

AH, MS, KT and JF tested the results.

YN and KN wrote the paper.

References

1. Goren A, Ashlock D, Tetlow IJ (2018) Starch formation inside plastids of higher plants. Protoplasma 255:1855–1876
2. Iseki K, Takahashi Y, Muto C, Naito K, Tomooka N (2016) Diversity and Evolution of Salt Tolerance in the Genus *Vigna*. PLoS ONE 11:e0164711
3. Kanai M, Higuchi K, Hagihara T, Konishi T, Ishii T, Fujita N, Nakamura Y, Maeda Y, Yoshiba M, Tadano T (2007) Common reed produces starch granules at the shoot base in response to salt stress. New Phytol 176:572–580
4. Matsumoto K, Hirayama C, Motozato Y (1998) Preparation of Bead-shaped Starch Ion Exchangers and Their Properties. Nippon Kagaku Kaishi 10:657–663
5. Noda Y, Sugita R, Hirose A, Kawachi N, Tanoi K, Furukawa J, Naito K (2022) Diversity of Na^+ allocation in salt-tolerant species of the genus *Vigna*. Breed Sci 72:326–331
6. Panta S, Flowers T, Lane P, Doyle R, Haros G, Shabala S (2014) Halophyte agriculture: Success stories. Environ Exp Bot 107:71–83
7. Shabala S, Chen G, Chen ZH, Pottosin I (2020) The energy cost of the tonoplast futile sodium leak. New Phytol 225:1105–1110
8. Tomooka N, Naito K, Kaga A et al (2014) Evolution, domestication and neo-domestication of the genus *Vigna*. Plant Genetic Resources 12:168–171
9. Wada Y (2010) Global depletion of groundwater resources. Geophys Res Lett 37:L20402**van** Kempen, C.M.**et al**
10. Yoshida Y, Marubodee R, Ogiso-Tanaka E et al (2016) Salt tolerance in wild relatives of adzuki bean, *Vigna angularis* (Willd.) Ohwi et Ohashi. Genet Resour Crop Evol 63:627–637
11. van Zonneveld M (2020) Mapping patterns of abiotic and biotic stress resilience uncovers conservation gaps and breeding potential of *Vigna* wild relatives. Sci Rep 10:2111**et al**.

Figures

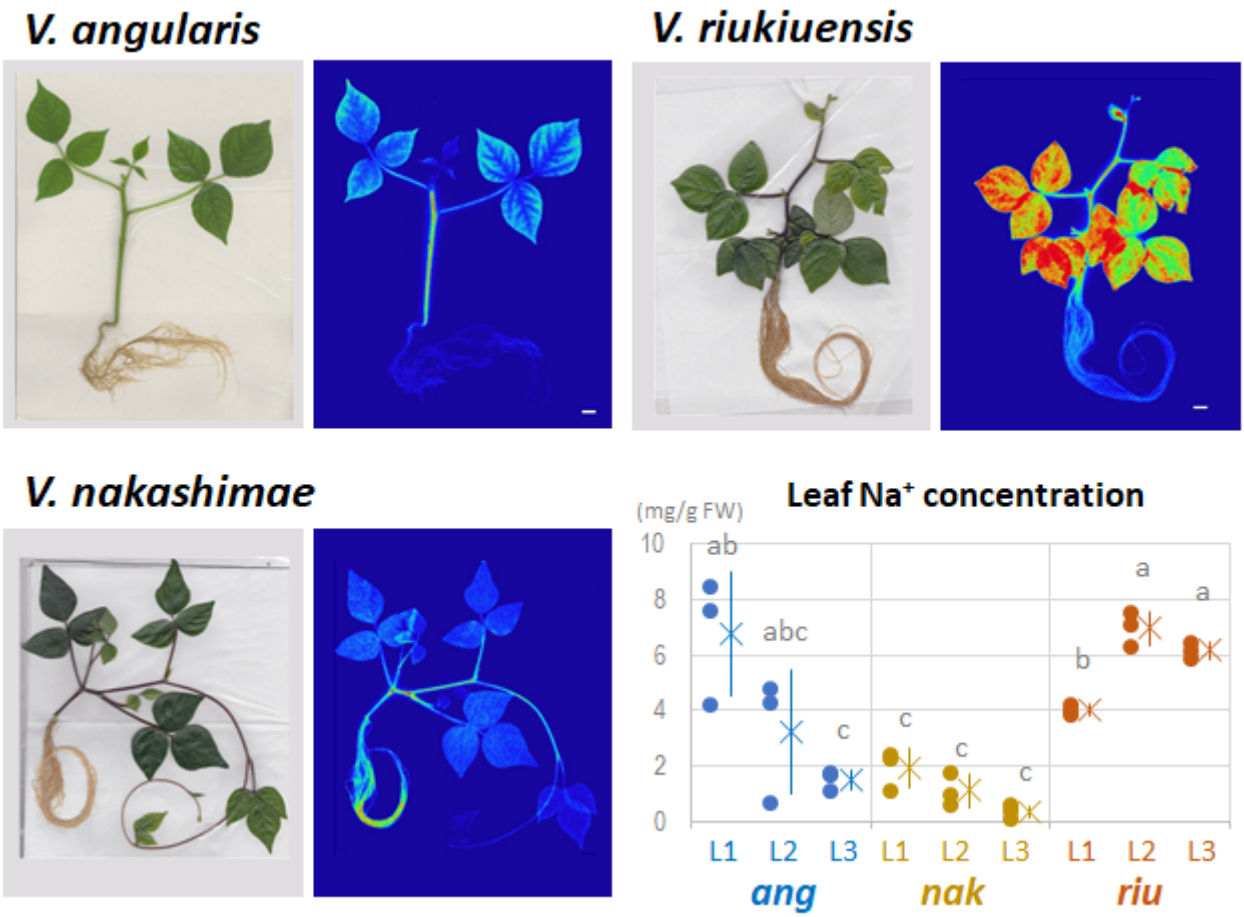

Figure 1

Na allocation in the plants. a-c. Bright field photograph (left) and autoradiography (right) of the plants fed with ^{22}Na. The luminance of ^{22}Na distribution was standardized for each accession. Color change from blue to red indicates ^{22}Na accumulation. White bars indicate 1 cm. d. Na concentrations in each leaf. Circles, X and error bars indicate values of each replicate, means and standard deviation, respectively. L1, L2 and L3 indicate the 1st, 2nd and 3rd leaves, respectively. ang, nak and riu indicate *V. angularis*, *V. nakashimae* and *V. riukiuensis*, respectively. Means not sharing the same alphabet are significantly different (Tukey HSD $p < 0.05$).

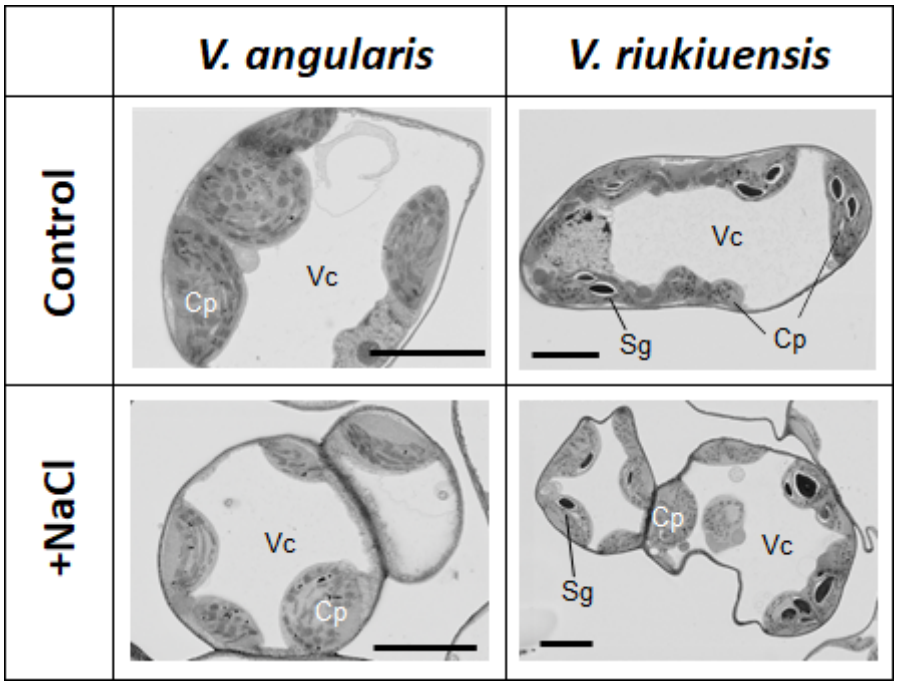

Figure 2

Electron microscopy images of leaf cells. Cp. Sg and Vc indicate chloroplast, vacuole and starch granules, respectively. Black bars indicate 5 μm.

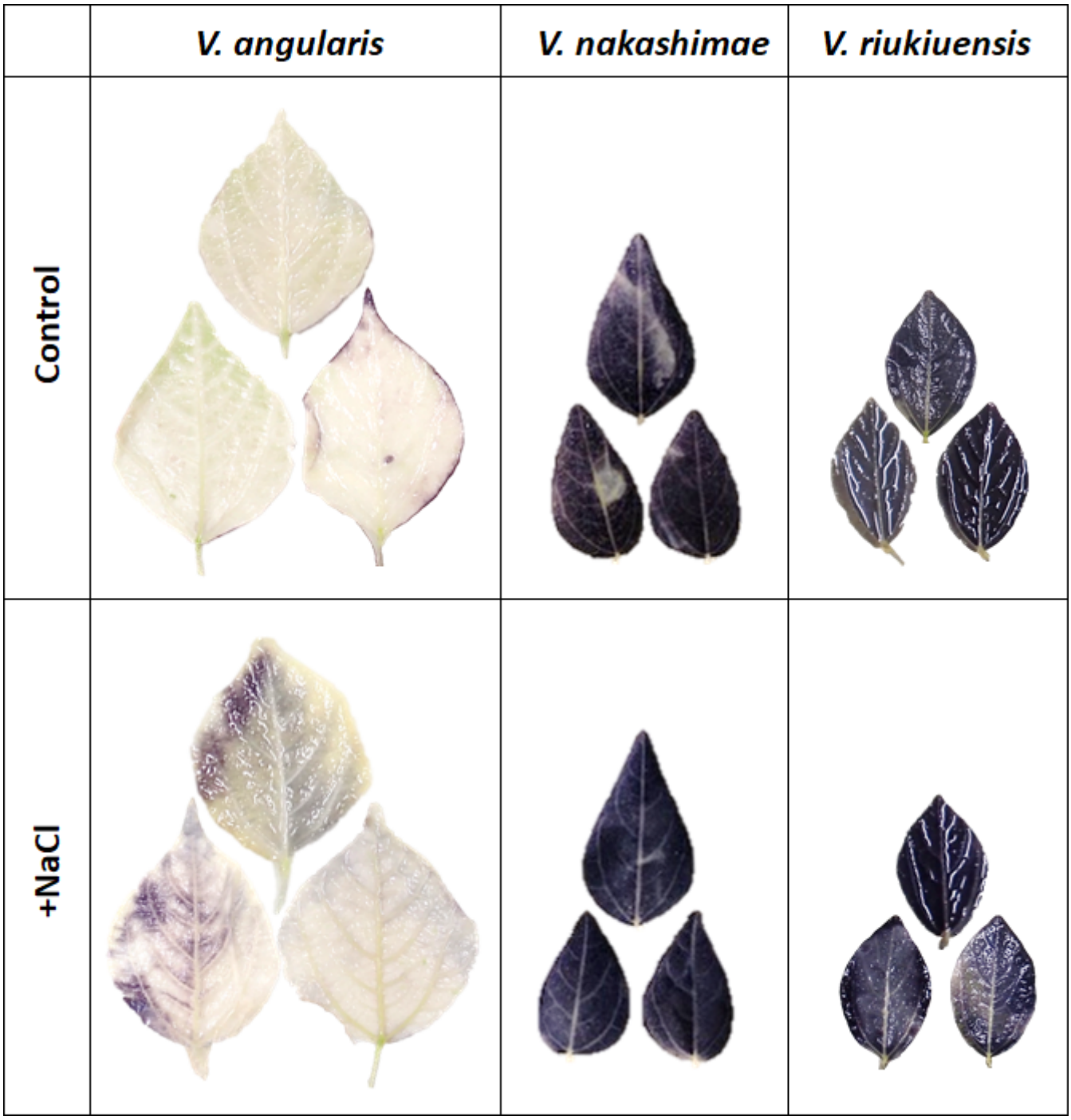

Figure 3

Iodine staining of the leaves. The 3rd leaves of non-stressed or salt-stressed plants were stained with iodine.

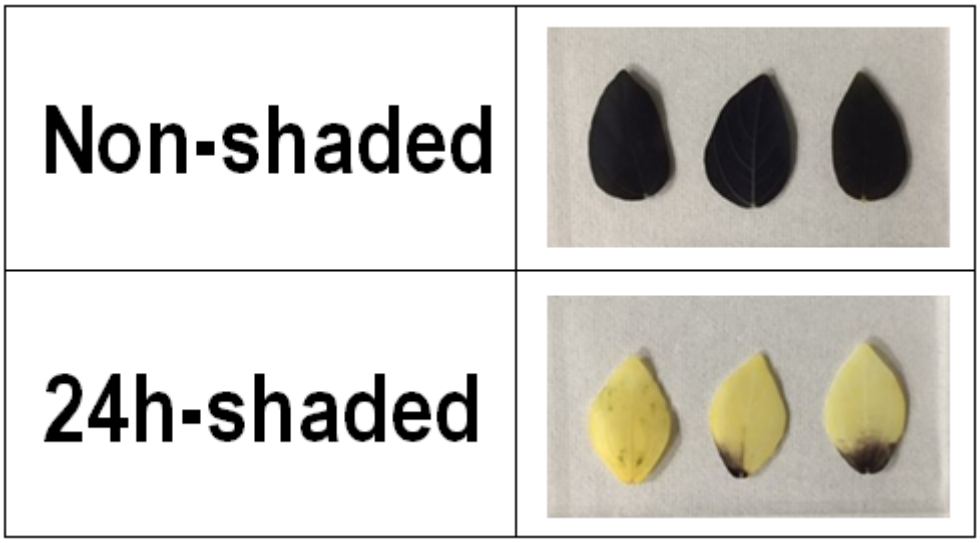

Figure 4

Effect of 24-hour-shading on starch content in the leaves. Shaded and non-shaded leaflets were stained with iodine.

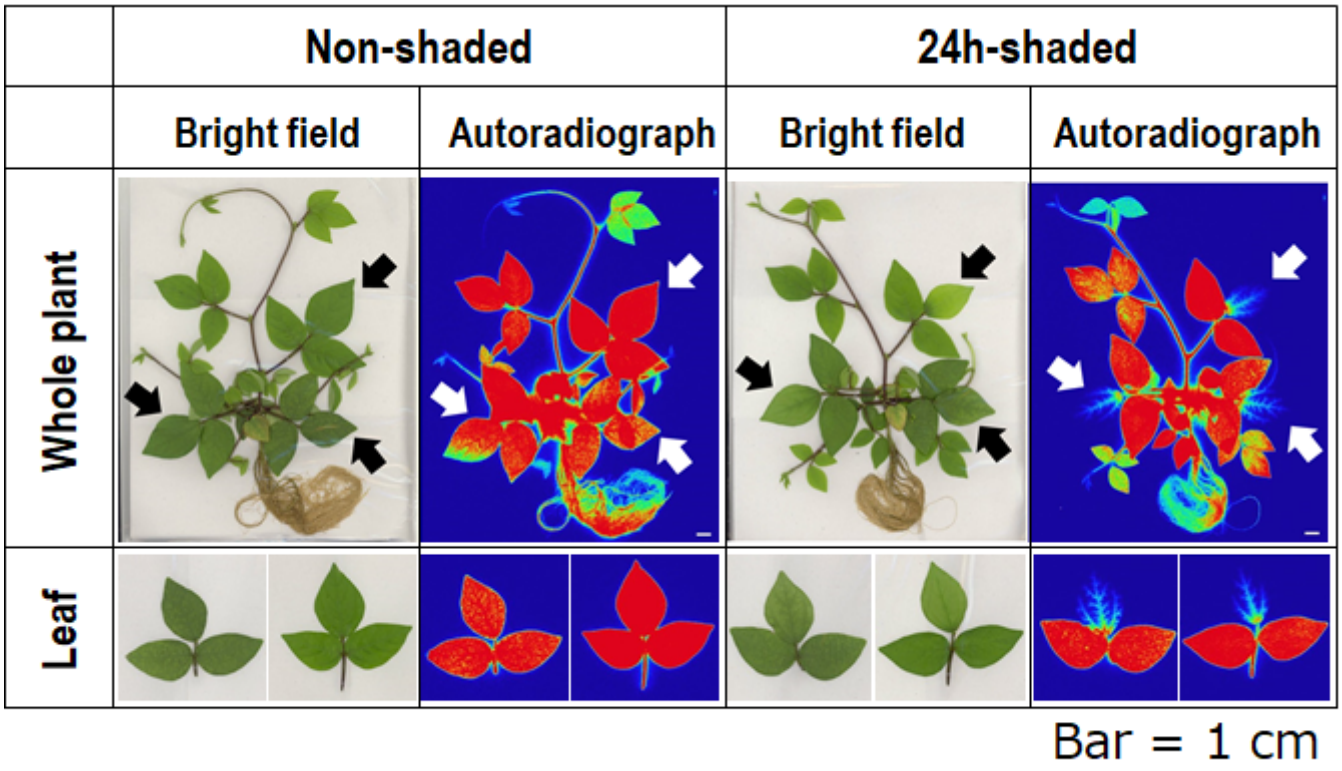

Figure 5

^{22}Na accumulation in shaded and non-shaded leaves of *V. riukiuensis.* Black and white arrows indicate leaves that were wrapped with cling film (non-shaded) or foil (24h-shaded). The luminance of each autoradiography was standardized. White bars indicate 1 cm.

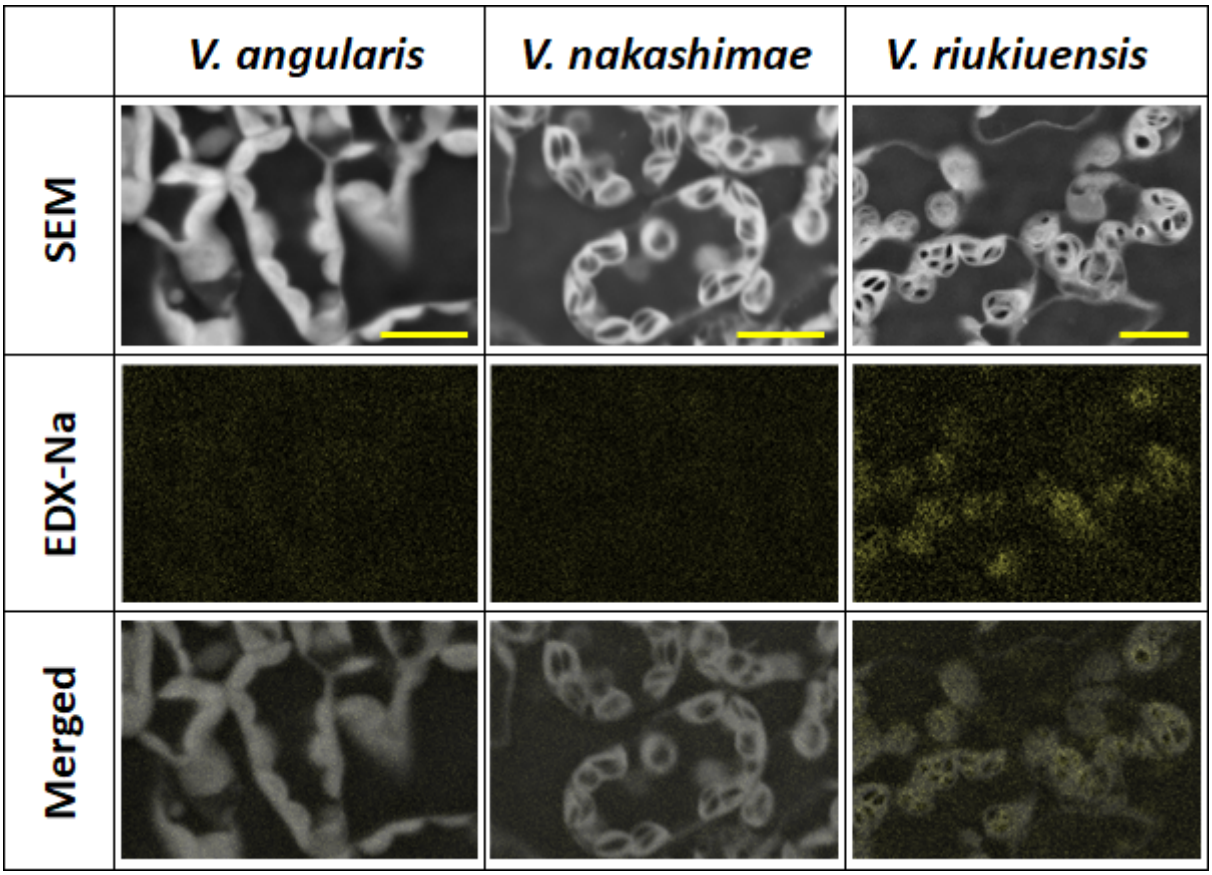

Figure 6

Electron microscopy (SEM), EDX (EDX-Na), and merged images of leaf cells. In the SEM images, the white structures in the cells are chloroplasts and the black objects inside them are starch granules. The yellow dots in EDX-Na images indicate the detected Na signals. Yellow bars indicate 1 μm.