Diurnal regulation of SOS Pathway and Sodium Excretion Underlying Salinity Tolerance of *Vigna marina*

Yusaku Noda[1], Fanmiao Wang[2], Sompong Chankaew[3,4], Hirotaka Ariga[2], Chiaki Muto[2], Yurie Iki[5], Haruko Ohashi[5], Yu Takahashi[3], Hiroaki Sakai[6], Kohtaro Iseki[7], Eri Ogiso-Tanaka[8], Nobuo Suzui[1], Yong-Gen Yin[1], Yuta Miyoshi[1], Kazuyuki Enomoto[1], Naoki Kawachi[1], Prakit Somta[4], Jun Furukawa[9], Norihiko Tomooka[2], Ken Naito[2*]

[1] Takasaki Institute for Advanced Quantum Science, National Institutes for Quantum Science and Technology (QST), Gunma 370-1292, Japan

[2] Research Center of Genetic Resources, National Agriculture and Food Research Organization, Ibaraki 305-8602, Japan

[3] Department of Agronomy, Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand

[4] Department of Agronomy, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand

[5] Graduate School of Frontier Sciences, The University of Tokyo, Chiba 277-8562, Japan

[6] Research Center of Advanced Analysis, National Agriculture and Food Research Organization, Ibaraki 305-8518, Japan

[7] Japan International Research Center for Agricultural Sciences, Ibarkai 305-8686, Japan

[8] Center for Molecular Biodiversity Research, National Museum of Nature & Science, Ibaraki 305-0005, Japan

[9] Faculty of Life and Environmental Sciences, University of Tsukuba, Ibaraki 305-8572, Japan

Corresponding author:

K. Naito. Research Center of Genetic Resources, National Agriculture and Food Research Organization, 2-1-2 Kannondai, Tsukuba, Ibaraki, 305-8602, Japan. knaito@affrc.go.jp

Keyword: Genus Vigna, salt tolerance, sodium 22, positron-emitting tracer imaging system, RNA-seq, QTL analysis

Abstract:
Vigna marina (Barm.) Merr. is adapted to tropical marine beaches and has an outstanding tolerance to salt stress. Given there are growing demands for cultivating crops in saline soil or with saline water, it is important to understand how halophytic species are adapted to the saline environments. Here we sequenced the whole genome of *V. marina* with longreads, and performed a forward genetic study to identify QTLs involved in salt tolerance. As the QTL region harbored *SOS1*, encoding plasma membrane Na^+/H^+ antiporter, we traced the sodium dynamics in *V. marina* using the positron emitting tracer imaging system (PETIS) and revealed that it actively excretes sodium from the root. In addition, the sodium excretion was faster in the light period and slower in the dark period, indicating it is under diurnal regulation. The following comparative transcriptome analyses revealed that the SOS pathway plays a key role in the diurnal regulation of sodium excretion. Furthermore, we demonstrated that, under a condition of mild salt stress, the plants with the diurnally regulated SOS pathway outperformed those with the constitutively activated one.

Introduction

Vigna marina (Barm.) Merr. is the most salt-tolerant species in the genus *Vigna* (Iseki et al., 2016). It is an oceanic dispersal species and dominates the vegetation of tropical and subtropical beaches across the Pacific and Indian Ocean (Tomooka 2002). Given the increasing problems of soil salinization and fresh water shortage, it is important to understand its mechanisms of salt tolerance and the underlying genetics for developing practical salt-tolerant crops.

The preceding studies have revealed the outstanding performance of *V. marina* under salt stress. It survives 500 mM NaCl for more than 8 weeks (Yoshida et al., 2020), maintains root growth in 200 mM NaCl (Wang et al., 2023), and shows higher photosynthetic rate and stomatal conductance in 150 mM NaCl than in control (Yoshida et al., 2020). The following study of ours, by tracer experiment with radioactive sodium (^{22}Na), revealed *V. marina* allocates the least amount of sodium in leaves, stems and roots compared to other species (Noda et al., 2022). Thus, the ability of sodium exclusion underlies its extraordinary tolerance to salt stress.

However, still unknown is whether *V. marina* simply suppresses sodium uptake from the root, or actively excretes sodium that is once loaded to xylem. Moreover, genetic factors responsible for the sodium exclusion must be identified to fully understand how *V. marina* is adapted to saline environments such as marine beaches.

Thus, in this study, we reconstructed a new reference genome of *V. marina* and performed a forward genetic study to identify genetic loci involved in salt tolerance. Then we adopted the positron emitting tracer imaging system (PETIS) (Uchida et al., 2004; Fujimaki et al., 2015), which enables real-time mapping of radio isotopes, to elucidate the dynamics of sodium ions in a living plant of *V. marina* and other accessions with various degrees of salt tolerance (Table 1) (Yoshida et al., 2016) (Chankaew et al., 2014, Yoshida et al., 2020). Finally, we performed comparative transcriptome analyses to identify genes that could be responsible for the ability of sodium exclusion in *V. marina*. The knowledge obtained in this study will provide insights regarding how to use the known genes to make a plant adapted to saline environments.

Table 1. Plant materials tested in this study.

Species	Accession Number	Degree of salt tolerance
Vigna marina	JP247202	tolerant to 500 mM NaCl
Vigna luteola	JP233389	tolerant to ~350 mM NaCl
Vignaluteola	JP235855	tolerant to <150 mM NaCl
Vigna angularis	cv. 'Shumari'	tolerant to <50 mM NaCl

Materials and Methods

Plant materials and growth condition

All plant seeds were provided by the NARO Genebank in Tsukuba, Japan (https://www.gene.affrc.go.jp/index_en.php) (Table 1). Seeds were sterilized by shaking in 70% ethanol for 5 min and then shaking another 5 min in 0.5% sodium hypochlorite. The sterilized 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, Dark: 25°C for 10 h. Light intensity 500 µM-1 s-1 m-2). Each plant was cultivated for the following number of days: *V. angularis* 10 days, *V. luteola* (river) 14 days, *V. luteola* (beach) 14 days, *V. marina* 17 days. The hydroponic solution contained a diluted nutrient solution of OAT House No.1 (1.5 g L-1):

OAT House No.2 (1 g L-1) (Otsuka Chemical Co, Japan) in a 1:1 ratio with concentrations of 18.6 mEq L-1 nitrogen, 5.1 mEq L-1 phosphorus, 8.6 mEq L-1 potassium, 8.2 mEq L-1 calcium and 3.0 mEq L-1 magnesium.

Visualization of ^{22}Na allocation

To visualize ^{22}Na allocation, pre-cultured plants were transferred to the Center for Research in Radiation, Isotopes and Earth System Sciences at the University of Tsukuba, Japan. The plants were transplanted into a new hydroponic solution containing 5 kBq ^{22}Na (PerkinElmer, USA) with non-radioactive 100 mM ^{23}NaCl. After the addition of the radioisotope, the plants were again incubated under long-day conditions (Light: 28°C for 14 h, Dark: 25°C for 10 h. Light intensity 200 µmol s-1 m-2) for 3 days. After incubation, the roots were carefully washed and the whole plant body was placed in a plastic bag and exposed to a storage phosphor screen (BAS-IP-MS-2025E, GE Healthcare, UK) in Amersham exposure cassettes (GE Healthcare, UK) for 24 h. The exposed screen was then scanned with a laser imaging scanner Typhoon FLA-9500 (GE Healthcare, UK). All experiments were performed independently on more than three biological replicates. To arrange radioactive intensity equally at each image, photo-stimulated luminescence and contrast were equalized by Multi Gauge version 3.0 (Fujifilm, Japan). In an experiment to visualize ^{22}Na allocation in *V. marina* under different salt stresses, plants were transplanted into 100, 200 and 300 mM ^{23}NaCl hydroponic solutions containing 5 kBq ^{22}Na. Subsequent procedures were as described above.

DNA extraction, sequencing and assembly of V. marina genome

As described by Naito (2023), we extracted genomic DNA from the unexpanded leaves with Nucleobond HMW DNA kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany), size-selected the DNA with Short Read Eliminatior XL kit (Pacific Biosciences of California, Inc., California,USA), and prepared 3 libraries for nanopore sequencing with SQK-LSK109 (Oxford Nanopore Technologies KK, Tokyo, Japan). Each library was then loaded to MinION R9.4.1 Flowcell (Oxford Nanopore Technologies KK, Tokyo, Japan) and was run for 72 h. The obtained raw data was transformed into fastq format with bonito-0.2.1. We also obtained short-reads with Hiseq 4000 (Illumina, San Diego, USA), which was provided as a customer service by GeneBay, Inc. (Yokohama, Japan).

For draft assembly, we used necat-0.0.1 (Chen et al., 2021) with default parameters except “PREP_OUTPUT_COVERAGE=60” and “CNS_OUTPUT_COVERAGE=40”. The contigs were polished twice with racon-1.4.3 (Vaser et al., 2017) and once with medaka-1.0.3 (https://github.com/nanoporetech/medaka).

The polished contigs were then scaffolded into pseudomolecules by anchoring to the linkage map (see below). When we found controversies between contigs and the linkage map, we manually fixed the misassemlies as described by Sakai et al. (2015). The pseudo molecules were then annotated with ORFs exactly as described in our previous study (Naito et al., 2021).

QTL analysis

We crossed *V. marina* and *V. luteola* (beach), selfed the F1 plant, and obtained F2 seeds. To generate replicates, the F2 plants were cultivated in hydroponic culture as described in Yoshida et al. (2016) for 8 weeks, and were clonally propagated by cutting (Supplementary Fig 1). For phenotyping, three clones of each F2 plant were cultivated in hydroponic culture with 50 mM NaCl for a week, transferred to the new culture containing 350 mM NaCl for 8 weeks, and then evaluated for 3 traits, “wilt score”, ”shoot generation score” and ”stay green score”. For wilt score, the F2 clones were scored as 1, 3, 5, 7, or 9 according to the salt damage as shown in Supplementary Fig 1. For shoot generation score, the clones were scored as 3 if they kept growing and generating new branches, or 1 if otherwise. For stay green score, the clones were scored as 3 if all the leaves stayed green during the experiment, or 1 if otherwise. For genotyping, genomic DNA was extracted from young leaves of each F2 plant with CTAB method. The extracted DNA was then used for preparing RAD-seq libraries as described by Matsumura et al. (2014), except *Bam*HI and *Mbo*I were used for DNA digestion. The prepared library was sequenced with Hiseq2000 (Illumina), which was provided as a customer service by GeneBay Inc. (Yokohama, Japan). The obtained sequence data were mapped to the *V. marina* genome with bwa (Li et al., 2010) and then processed with Stacks-1.19 (Catchen et al., 2013) to obtain genotypes. The obtained genotype data was then analyzed for linkage with onemap (Taniguti et al., 2023) to construct a linkage map, which was also used for scaffolding contigs (see above). The association between the phenotype and the genotypes were then estimated with R/qtl2 (Broman et al., 2019).

PETIS imaging and analysis

The visualization of sodium excretion from plant roots was carried out using PETIS in Takasaki Institute for Advanced Quantum Science, Japan. Initially, a polytetrafluoroethylene sheet with holes was placed in an 8 cm high acrylic cell as a partition to enable quantification of ^{22}Na radioactivity from hydroponic culture. A silicone tube was then installed for injecting the ^{22}Na solution into the acrylic cell, which was attached to an acrylic board. The plants of 2 weeks old were inserted into the acrylic cell and stabilized with black urethane sponge. The plants were transferred to a growth chamber equipped with the PETIS detector. Prior to treatment with ^{22}Na, the plants were pretreated with hydroponic solution containing 100 mM of non-radioactive NaCl for 1 hour. After 1 hour, the hydroponic solution was exchanged with fresh solution containing 100 mM of non-radioactive NaCl and 500 kBq ^{22}Na to feed the plants for 24 hours. Then, the ^{22}NaCl hydroponic solution was exchanged twice with ice-cold 10 mM $CaCl_2$ solution to wash the roots. After washing, the plants were transplanted to back to 100 mM NaCl hydroponic solution without ^{22}Na and the data collection by PETIS was started. During PETIS imaging, the surface of the hydroponic solution was maintained by a siphon pump, and roots were shaded with aluminum foil. Growth conditions during imaging were as follows: Light: 28°C for 14 h, Dark: 25°C for 10 h; light intensity: 200 μmol s-1 m-2.

PETIS collected data every minute for 48 or 72 hours. The original images acquired every minute were then re-integrated into montage images at 1 hour intervals. Quantification of ^{22}Na in the hydroponic solution, root and shoot was done by defining a region of interest (ROI) with ImageJ (National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih. gov/ij/). Time-course analysis of ^{22}Na radioactivity within each ROI was performed by generating a time-activity curve (TAC) from signal intensities using ImageJ.

All the experiments were independently performed more than 3 times with 3 or 4 biological replicates.

Transcriptome analysis

1st experiment:

Before initiation of salt stress, plants of *V. marina* were grown in hydroponic culture for 2 weeks and pre-treated with 50 mM NaCl for 3 days. Then, at 11 am, we transferred the plants to hydroponic culture containing 200 mM NaCl.

After the initiation of salt stress, we collected leaves and roots of 3 plants at 24, 36, 48 and 60 hours. The collected leaves and roots were immediately wrapped with foil and frozen in liquid nitrogen. From the collected samples, total RNA was extracted with RNeasy Plant Mini Kit (Qiagen KK, Tokyo, Japan). From the extracted RNA we prepared libraries for 3'mRNA-seq with Collibri 3'mRNA Library Preparation Kit for Illumina Systems (Thermo Fisher Scientific K.K., Tokyo, Japan). The prepared libraries were sequenced with Hiseq4000 as a customer service of GeneBay Inc. (Yokohama, Japan). After sequencing, we estimated read counts of each gene of *V. marina* by kallisto-0.45.2 (Bray et al., 2016). With edgeR (Chen et al., 2016), we separately analyzed leaf dataset and root dataset. We first normalized the read counts and extracted differentially expressed genes, which were not only significant as ANOVA, but also at least one of the data points had 2-fold or greater difference compared to the average. The extracted gene sets were then clustered with SOM-clustering (Wehrens et al., 2007).

2nd experiment:

V. angularis, V. luteola (river), *V. luteola* (beach) and *V. marina* were grown, salt-stressed, sampled, sequenced and analyzed as described above.

Transformation

First, we acquired *sos1-1* and *sos2-1* mutant lines from ABRC, crossed them and obtained *sos1/sos2* double mutant lines. Second, we subcloned coding regions of DNA of *AtSOS1* and *AtSOS2* and the promoter region of *PRR9* (2.4 kbp upstream of *AtPRR9* transcription start site) from Col-0 into pENTR/D-TOPO cloning vector. Third, we transferred the cloned sequences to pRI201-AN vector to construct binary vectors that harbor *35S:SOS1* and *35S:SOS2* or *35S:SOS1* and *proPRR9:SOS2* (Supplementary Fig 1). Fourth, the binary vectors were transferred to *Rhizobium radiobactor* strain EHA105. Finally, *sos1/sos2* double mutant was transformed by the floral dip method.

Salinity tolerance test on Arabidopsis thaliana

We first planted seeds of Arabidopsis ecotype Col-0, *sos1/sos2* double mutant, *35S:SOS1/proPRR9:SOS2* and *35S:SOS1/35S:SOS2* transgenic plant on horticultural soil (Tachikawa Heiwa Nouen Co.Ltd, Japan) and treated them with vernalization for 3 days at 4°C. The plants were then transferred to a growth chamber set at 24°C, light 10 h, dark 14 h, light intensity 150 µM-1 s-1 m-2 and

were incubated for 14 days. After that, the plants were transplanted to hydroponic culture containing 1/2 Murashige & Skoog (MS) solution and were incubated for 21 days. For salt stress, plants were pretreated with hydroponic culture with 5 mM NaCl for 3 days, and then treated with 25 mM NaCl for 1 week. After treatment, the shoots and roots were collected separately, dried at 60°C for 3 days and measured for dry weights.

Results

^{22}Na- allocation in V. marina and its relatives

In addition to our previous study that revealed the ^{22}Na allocation in *V. marina, V. luteola* (beach) and *V. angularis* (Noda et al., 2022), we visualized that in *V. luteola* (river) in the condition of 100 mM NaCl (Fig 1A). The result of *V. luteola* (river) showed a typical pattern of glycophytes, which was similar to *V. angularis*, presenting high ^{22}Na allocation to leaves and low allocation to roots. In contrast, *V. luteola* (beach) allocated more ^{22}Na to roots and less to leaves and stems, while *V. marina* highly restricted ^{22}Na allocation even in roots.

Whole genome sequence by nanopore and QTL analysis by RAD-seq

To elucidate the genetic factors underlying the salt tolerance of *V. marina*, we crossed *V. marina* with *V. luteola* (beach), obtained 286 F2 plants and evaluated the degree of salt tolerance on each plant (wilt score, shoot generation score and stay green score) (Supplementary Fig 1, Supplementary Table 1). Next, we sequenced the whole genome of *V. marina* with Oxford Nanopore sequencer and reconstructed the reference genome (v2.3), which was dramatically improved compared to v1.0 (Naito et al., 2022) (Supplementary Tables 2, 3). Third, we genotyped the F_2 plants by RAD-seq and obtained genotypes of 1,205 loci (Supplementary Tables 4-6). Finally, we performed a genome scan to detect genomic regions that were associated with salt tolerance.

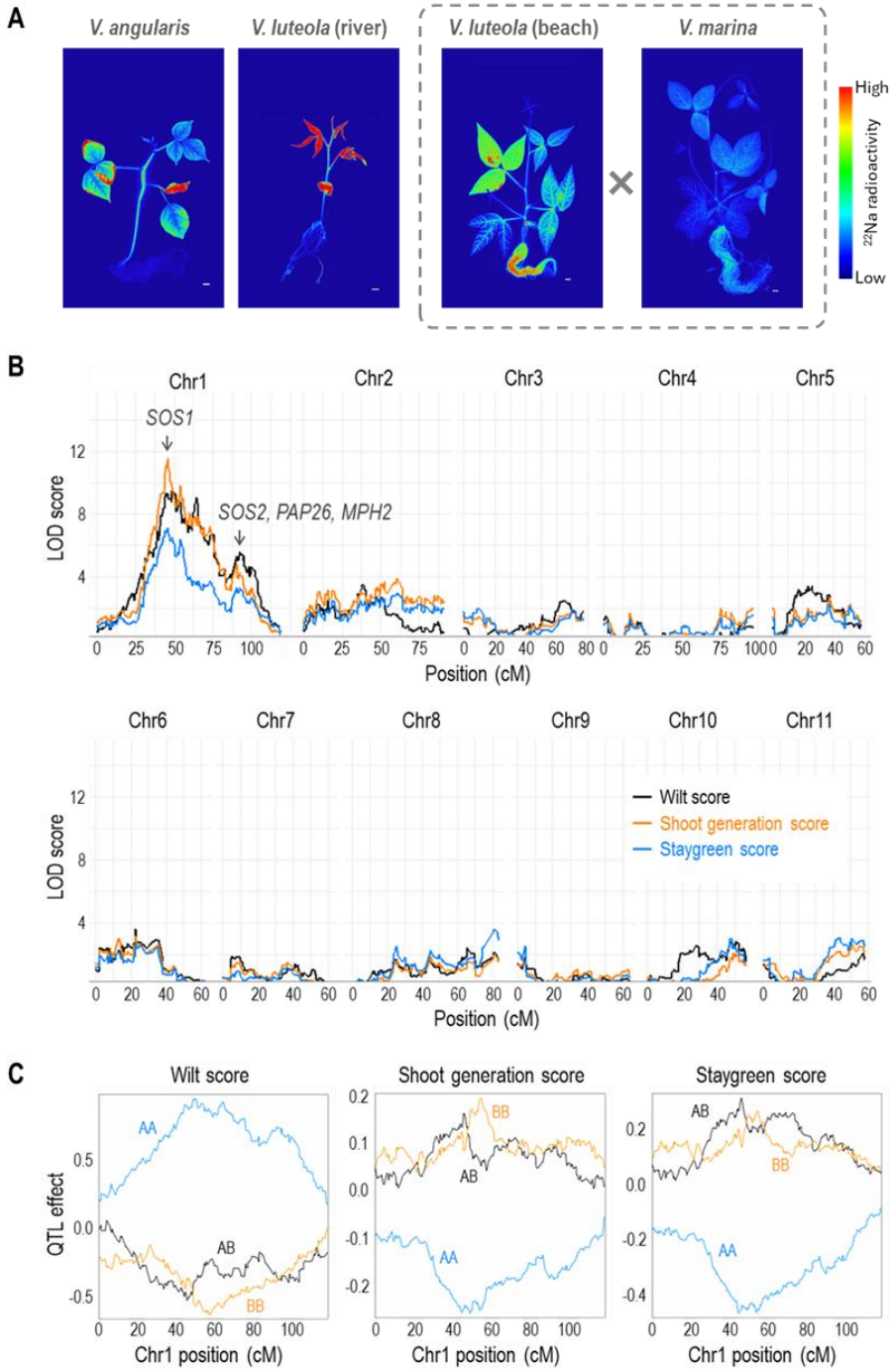

Figure 1. Sodium allocation in Vigna plants and association of the genotypes and salt tolerance-related traits in the F2 plants derived from *V. marina* and *V. luteola* (beach). A. Autoradiographs of Vigna plants fed with ^{22}Na. The two accessions surrounded by dashed lines are the parental lines used for QTL analysis. The white lines indicate 1 cm. B. QTL plot representing LOD scores calculated by genome scan. C. Estimated QTL effects along Chr1. AA, AB and BB indicate genotype, where the A allele is of *V. luteola* type and B of *V. marina* type. Higher wilt score indicates lower tolerance to salt stress, whereas higher shoot generation and staygreen scores indicate higher tolerance.

As a result, we identified two QTL peaks, which were both located in chr1 (Fig 1B, Supplementary Table 7). Of the two, one located in 44.1-47.6 cM (17.5-32.0 Mbp) was associated with all the three traits related to salt tolerance and exhibited the highest LOD score (7.1-11.5), explaining 10-24% of phenotypic variance. The other in 88.0-94.7 cM (64.3-67.6 Mbp) was associated with wilt score and shoot generation score, with LOD scores of 4.8-5.6, explaining 7.4-8.6% of phenotypic variance. In both QTLs, alleles of *V. marina* type were dominant to those of *V. luteola* type (Fig 1C, Supplementary Table 7).

Though the QTL peaks covered a broad range of the genome regions, there were a limited number of genes that were potentially involved in salt tolerance (Supplementary Table 8). Those within the first QTL region were *Vigma.01G148800.01* (*VmSOS1*), *Vigma.01G153100.01* (*VmT5PTASE9*), and *Vigma.01G182100.01* (*VmABF4*). Those within the second QTL were *Vigma.01G349000.01* (*VmSOS2*), *Vigma.01G349500.01* (*VmMPH2*), and *Vigma.01G353500.01* (*VmPAP26*).

***Sodium excretion and culture alkalization by* V. marina**

To elucidate to which intensity of salt stress *V. marina* is able to keep sodium out of the plant, we fed the plants with ^{22}Na in conditions of 100, 200 and 300 mM NaCl (Fig 2A). The results turned out that *V. marina* kept sodium allocation low even under 300 mM NaCl condition. Instead, we found traces of ^{22}Na around the root of the plants treated with 200 mM NaCl, and more traces of ^{22}Na in those treated with 300 mM NaCl.

During the experiment, we noticed that the hydroponic culture containing NaCl turned cloudy after plant cultivation for 72 h (Fig 2B). The culture with 100 mM NaCl formed a little precipitation, while that of 200 or 300 mM NaCl formed more precipitation.

As we suspected that the precipitation was the nutrient salts because the activity of Na^+/H^+ antiporters could alkalize the hydroponic culture by depriving proton, we measured the pH of the hydroponic culture before and after cultivating the plants of *V. marina* (Fig 2C). As a result, with 0 mM NaCl, the pH of the culture medium slightly declined after cultivation, while it increased by ~1 with 100 mM NaCl, and by more than 3 with 200 or 300 mM NaCl.

***Dynamics of ^{22}Na in a living plant body of* V. marina**

To observe sodium dynamics in a plant of *V. marina*, we performed real-time mapping of ^{22}Na using PETIS. The real-time images revealed that, with time, the ^{22}Na decreased in the root while increased in the hydroponic culture (Fig. 3A). From the images, we did not notice that the amount of ^{22}Na in the shoot changed during the experiment (Fig. 3A).

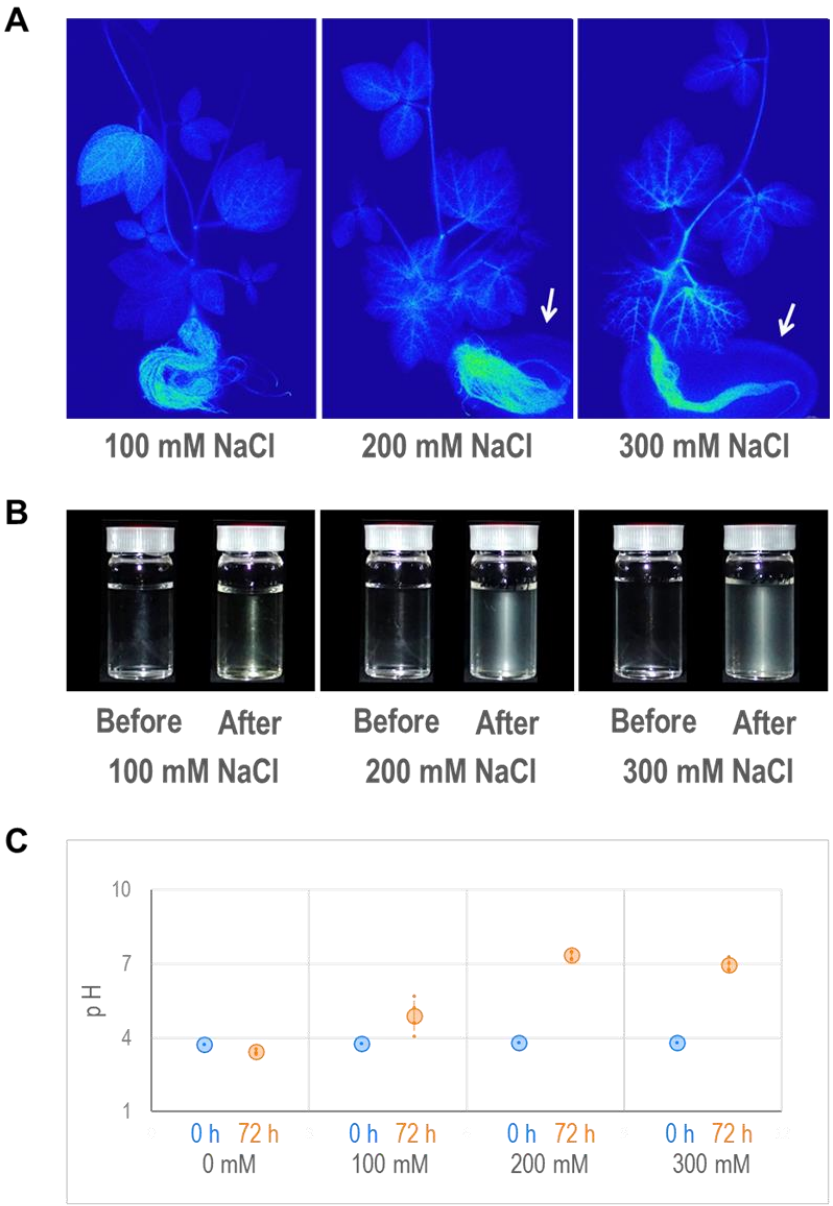

Figure 2. Sodium excretion and culture alkalization by *V. marina*. A. Autoradiographs of *V. marina* plants fed with 100, 200 or 300 mM NaCl containing ^{22}Na. White arrows indicate traces of exudated ^{22}Na from the root. B. Hydroponic culture solution before and after cultivation of *V. marina*. C. pH of hydroponic culture solution before (0h) and after (72h) cultivation of *V. marina*. Small dots indicate values of biological replicates, while large plots and error bars indicate mean ± S.D.

However, the following quantitative analysis revealed there was a diurnal rhythm in sodium dynamics in *V. marina.* In the shoot, the amount of ^{22}Na increased during the light period and decreased during the dark period (Fig 3B), while that in the root kept simply decreasing but faster in the light period and slower in the dark period (Fig 3B). In contrast to the root, the amount of ^{22}Na in the hydroponic culture increased faster during the light period and slower or even stopped during the dark period (Fig 3B). Notably, the rate of ^{22}Na increase in the shoot and in the hydroponic culture were maximized in the first 6 h of the light period, indicating *V. marina* actively excreted sodium against the water flow from the root to the leaf.

***Diurnally-regulated genes in* V. marina**

As *V. marina* alkalized the hydroponic culture when exposed to salt stress, we suspected that the SOS-related genes were involved in sodium excretion. The diurnal regulation of sodium excretion as observed above further intrigued us to test whether the SOS-related genes were also diurnally regulated or not. Thus, we performed RNA-seq on the leaf and the root sampled in the light period (11 am) and in the dark period (11 pm). The following clustering identified 4 clusters from each dataset, which were diurnally up-regulated or down-regulated at least under the condition of salt stress (Supplementary Fig 3).

Although the following GO-enrichment analysis did not provide informative output (Supplementary Tables 9-16), we found that *VmSOS2* was, both in the leaf and the root, was one of the diurnally-regulated genes that were up-regulated in the light period and down-regulated in the dark period (Supplementary Fig 3). In addition, *VmSOS1* was abundantly transcribed in the light period on the 2nd day in the root under salt stress (Supplementary Fig. 3).

***Dynamics of ^{22}Na excretion in* V. marina*,* V. luteola *and* V. angularis**

To test whether the diurnal regulation of sodium excretion was a unique feature of *V. marina*, we further performed PETIS experiments on *V. angularis*, *V. luteola* (river), and *V. luteola* (beach), and again on *V. marina*. As in the former PETIS experiment, we fed the plants with ^{22}Na for 24 h and then transferred to non-^{22}Na containing culture.

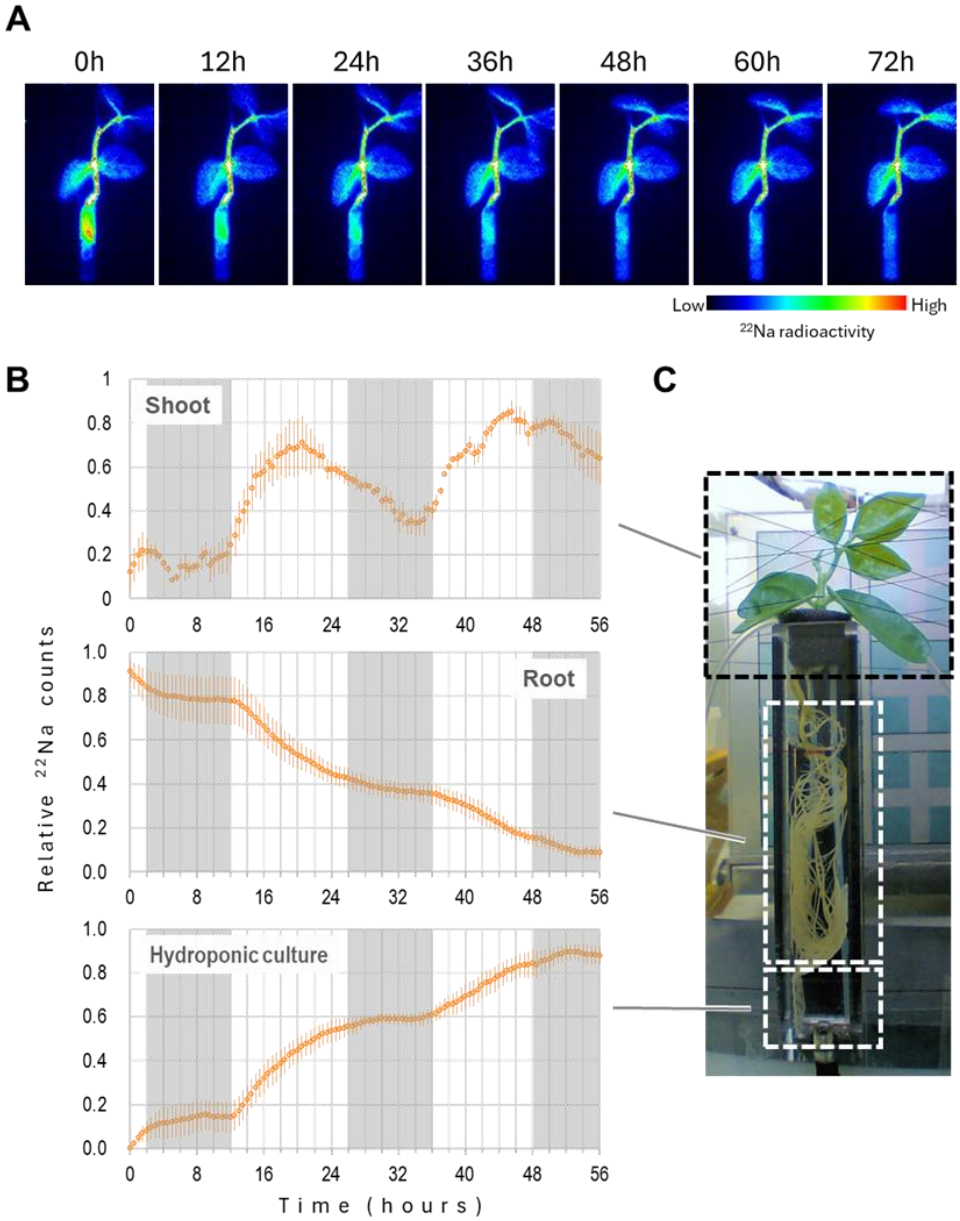

Figure 3. Dynamics of sodium transport in *V. marina*. A. Time-lapse images of gamma irradiation mapped by PETIS. A plant of *V. marina* was fed with ^{22}Na for 24h and then time-lapsed. B. Indexed amount of irradiation across time in the shoot, the root and the hydroponic culture media. Light and dark periods are indicated as white and gray in the plot, respectively. Error bars indicate standard errors. C. Setup of PETIS.

The real-time images by PETIS revealed a great variation across the 4 accessions (Fig 4A). *V. angularis* and *V. luteola* (river) retained little ^{22}Na in the root, indicating most of ^{22}Na had passed through the root and was transported to the shoot (see also Fig. 1A). In contrast, *V. luteola* (beach) allocated the

highest amount of ^{22}Na in the root. *V. marina* also allocated a high amount of ^{22}Na but the amount was lower than *V. luteola* (beach). With time, we observed in all the 4 accessions that the amount of ^{22}Na in the root decreased while that in hydroponic culture increased (Fig. 4A).

The following quantification of PETIS data revealed that, interestingly, the diurnal pattern of ^{22}Na excretion was observed in the both accessions of *V. luteola* as in *V. marina* (Fig 4B). In contrast, *V. angularis* did not show any sign of diurnal pattern (Fig 4B).

However, there were still differences between the accessions of *V. marina* and *V. luteola*. First, *V. luteola* (beach) and *V. marina* excreted more ^{22}Na than *V. luteola* (river) did (Fig 4B). Second, the difference between the light and dark periods was greater in *V. mariina* than that in *V. luteola* (beach) (Fig 4B). That is, the net amount of sodium excretions was the highest in *V. marina* during the light period, but it became lower in *V. marina* than in *V. luteola* (beach) during the dark period.

Transcriptome of the candidate genes

To identify differentially expressed genes (DEGs) within the QTL region, we performed another transcriptome analysis on the 4 accessions (Table 1). In this analysis, we started salt stress at 11:00 am on day0 and sampled 11:00 am (24h) and 11:00 pm (36h) on day1, and 11:00 am (48h) and 11:00 pm (60h) on day2. For *V. angularis,* we sampled only on day1 (24 and 36h).

Of the candidate genes within the first QTL, only *SOS1* showed positive correlation between the salt tolerance and the transcript abundance in the root (Fig. 5). In *V. angularis,* the transcript of *SOS1* was the least in the control condition and was not induced by salt stress. In *V. luteola* (river), the transcript of *SOS1* was ~3 times higher than that of *V. angularis* in the control condition and was slightly increased in response to salt stress. In *V. luteola* (beach), it was similar to that of *V. luteola* (river) in the control condition, but more strongly induced in response to salt stress. In *V. marina*, the transcript abundance of *SOS1* in the control was already as high as that of *V. luteola* (beach) under salt stress, and even increased in response to salt stress. The expression pattern of *SOS1* in the leaf was similar to that in the root, except it was dramatically induced in *V. luteola* (river) in response to salt stress (Fig. 5). Further, the independent qPCR analysis confirmed the expression profile of *SOS1* (Supplementary Fig 4). In contrast, the transcript abundance of *T5PTASE9* was

negatively correlated with the salt tolerance (Supplementary Fig 5). That of *ABF4* was similar across the accessions, except it was highly up-regulated on day2 under salt stress in the root of *V. marina* (Supplementary Fig 5).

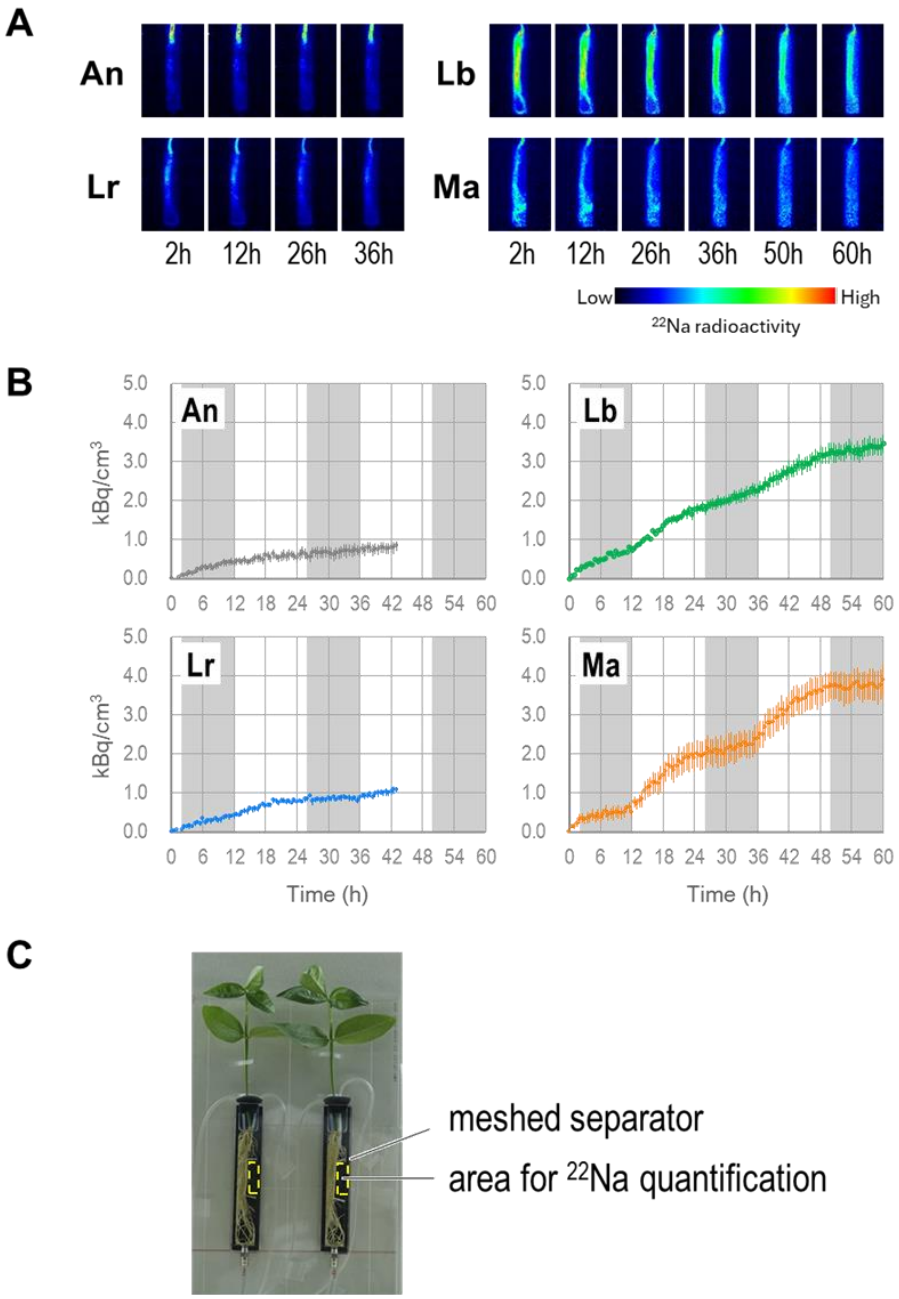

Figure 4. Dynamics of sodium excretion in *V. marina* and other accessions. A. Time-lapse of ^{22}Na mapping. An, Lr, Lb and Ma indicate *V. angularis, V. luteola* (river), *V. luteola* (beach), and *V. marina*, respectively. B. Relationships between time and the estimated concentration of ^{22}Na in the hydroponic culture. Each plot is labeled in the same way as in A. C. Set up of the PETIS. The area enclosed by the yellow line indicates the area where the separator prevented the roots from interfering with collecting data of hydroponic solution.

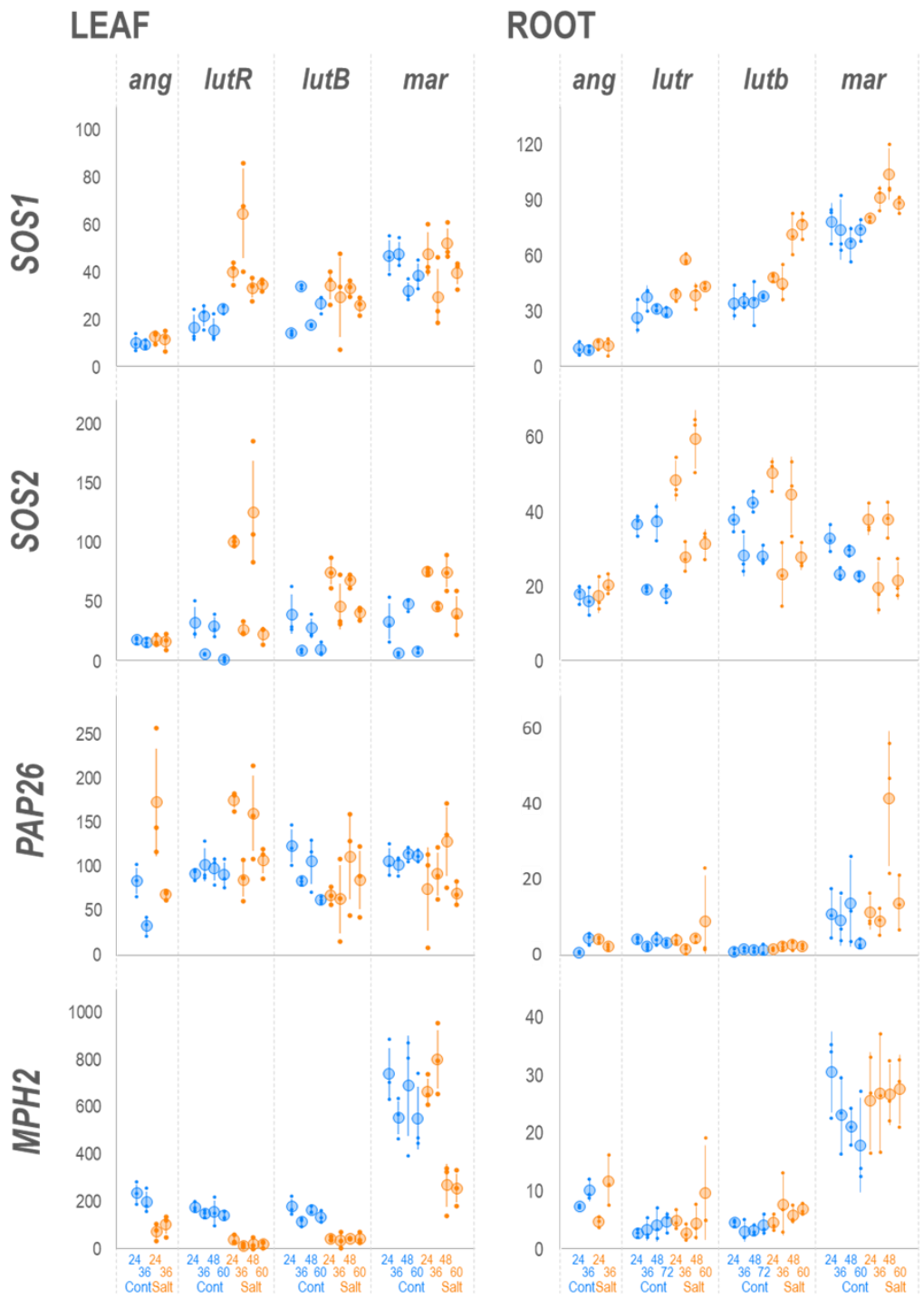

Figure 5. Expression patterns of the candidate genes for salt tolerance in the 4 accessions. *ang, lutr, lutb* and *mar* indicate *V. angularis, V. luteola* (river), *V. luteola* (beach) and *V. marina*, respectively. Numbers below the plots indicate hours after initiation of salt stress. Cont (blue) and Salt (orange) indicate the control and salt-stressed conditions, respectively. Y-axes indicate the abundance of transcripts in count per million (CPM). Small plots indicate values of each replicate while larger ones and error bars indicate mean ± S.D.

Of those within the second QTL, *SOS2* was again up-regulated in the light period and down-regulated in the dark period, not only in *V. marina* but also in the both accessions of *V. luteola* (Fig. 5). However, it was not different between the parental lines, *V. marina* and *V. luteola* (beach). In contrast, *PAP26* and *MPH2* were much more transcribed in *V. marina* than in *V. luteola* or *V. angularis* (Fig. 5).

In addition, the transcript abundance of *SOS3* did not correlate with salt tolerance across the 4 accessions (Supplementary Fig. 4).

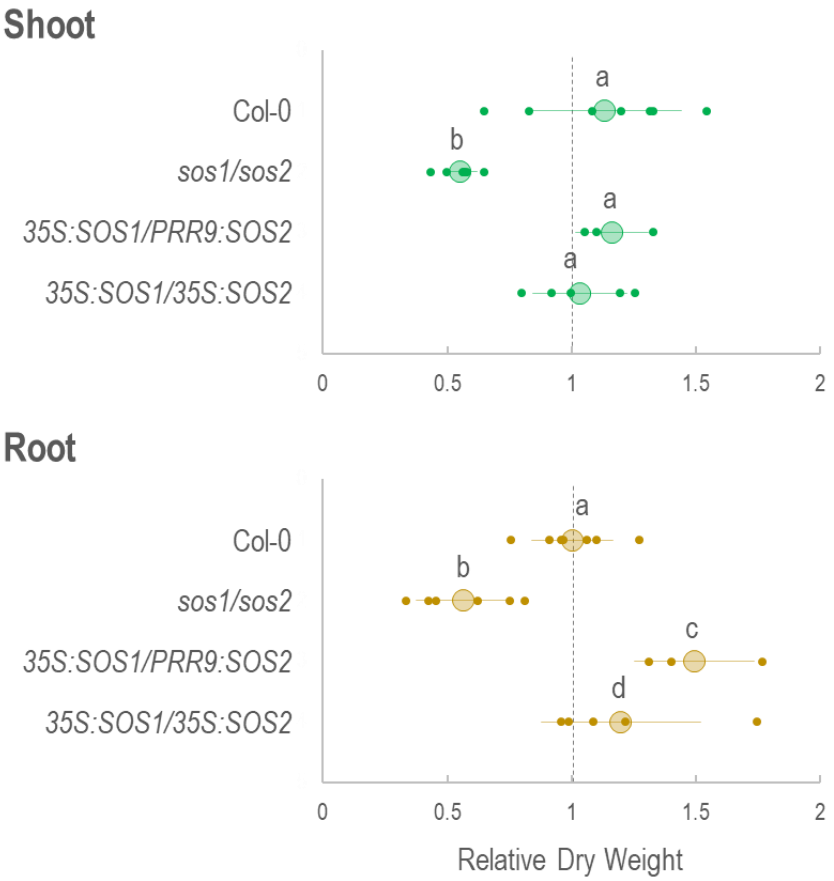

Figure 6. Relative dry weight of shoots and roots of various *SOS1/SOS2* expression lines. While small plots indicate the values of each replicate, large plots and error bars indicate means ±S.D. Different alphabets above the plots indicate significant difference by Tukey-Kramer test ($p<0.05$).

SOS2 *overexpression vs.* SOS2 *diurnal expression*

As we were surprised to find the conservation of the diurnally regulated sodium excretion in *V. marina* and *V. luteola*, we wondered if it has any adaptive

aspect. To elucidate this query, we reproduced the *V. marina*'s expression profile of *SOS1* and *SOS2* in *Arabidopsis thaliana* by introducing *35S:SOS1* and *proPRR9:SOS2* into *sos1/sos2* double mutant, as the *proPRR9* promotes transcription in the light period but not in the dark period (Ito et al., 2007) (Supplementary Fig 1). To compare, we also tested Col-0, *sos1/sos2* double mutant and *35S:SOS1/35S:SOS2* double overexpression (Supplementary Fig 1). In the experiment, we cultivated the plants in hydroponic culture with or without salt stress, measured the dry weight of shoots and roots separately, and calculated the relative dry weight (RDW).

The results revealed a significant difference in the root across different patterns of *SOS1/SOS2* expression (Fig. 6). While the shoot RDW was significantly lower only in the *sos1/sos2* double mutant, the root RDW was significantly higher in the *35S:SOS1/proPRR9:SOS2* line and the *35S:SOS1/35S:SOS2* double overexpression line. Notably, the *35S:SOS1/proPRR9:SOS2* line showed even higher RDW values than the *35S:SOS1/35S:SOS2* line.

Discussion

Here we revealed not only that *V. marina* has greater ability to excrete sodium out of the root, but that the sodium excretion is under diurnal regulation. Although the sodium excretion occurs against the direction of transpiration-dependent water flow, the rate of sodium excretion reached the maximum in the earlier time of light period, which is the time of the highest water uptake. In addition, the forward genetic and transcriptome analyses indicate that *SOS1* could determine the maximum rate of sodium excretion and *SOS2* could regulate its diurnal manner. Finally, we demonstrated that the diurnal expression of *SOS2* significantly confers salt tolerance and attenuates the negative effect of the SOS pathway on root growth.

Diurnal regulation of sodium excretion

It was surprising to discover by PETIS that *V. marina* and *V. luteola* excrete sodium in a diurnally-regulated manner: more in the light period and less in the dark period (Figs 3, 4). It was reasonable to observe the diurnal pattern in the shoot, especially for the rapid increase in the earlier time of the light period, as plants open stomata and absorb sodium with water in a transpiration-dependent manner (Dinneny, 2015). As stomata close in the later time, the transpiration-

dependent sodium uptake slows down (Fig 3). In the dark period, the sodium allocated to leaves is transported back to stems and roots presumably *via* phloem. We also observed that root sodium rapidly decreased in the earlier time of the light period, which could partly be due to transpiration-dependent transport to the shoot. However, as the sodium concentration in hydroponic culture also rapidly increased in the light period, the decrease in the root was also due to excretion of sodium. If the outward transport were a passive diffusion, it should have slowed down in the light period and speeded up in the dark period, given the transport must occur against the transpiration-dependent water uptake, which should be larger during the light period. Given the result was opposite (Fig 3), *V. marina* should actively excrete sodium out of the root during the light period.

Role of* SOS1, SOS2 *and other genes in salt tolerance

As the sodium excretion was accompanied by alkalization of hydroponic culture (Fig 2), we consider the Na^+/H^+ antiporter activity of *SOS1* (Ismail and Horie, 2017) plays the key role in excreting sodium out of the root. This hypothesis is also supported by our results that *SOS1* is located in the first QTL of salt tolerance (Fig 1B) and that *SOS1* transcription was highly correlated not only with salt tolerance (Table 1) but the ability of sodium excretion (Figs 4,5). In addition, the pattern of *SOS1* expression also well explains the manner of salt allocation observed in this study (Fig 1A) and our previous study (Noda et al., 2022): The lower expression of SOS1 in the root of *V. angularis* and *V. luteola* (river) cannot suppress sodium loading to xylem and allow high allocation to the shoot. In contrast, the higher expression of SOS1 in the root of *V. marina*, even before salt stress, enables immediate excretion of sodium after initiation of salt stress. Although the expression of SOS1 in the root of *V. luteola* (beach) is strongly induced, the lower expression in the control condition might allow sodium to accumulate in the root in the first ~24 h of salt stress.

The diurnally-regulated transcription of *SOS2*, encoding calcineurin binding protein-like (CBL) interacting protein kinase (CIPK) that directly phosphorylates and activates SOS1 (Liu et al., 2000), also explains the observed pattern of sodium excretion. As *V. marina* has expressed SOS1 before exposure to salt stress, the up-regulation of *SOS2* in the light period should initiate effective excretion of sodium, while its down-regulation in the dark period turns down the SOS1 activity and thus slows down the sodium

excretion. However, although *VmSOS2* was also located within the QTL region in the genome of *V. marina*, it could not explain the difference of salt tolerance between *V. marina* and *V. luteola* (beach) given its transcriptional pattern was not different (Fig 5). Thus, although *VmSOS2* is not responsible for the difference of salt tolerance between *V. marina* and *V. luteola,* it could be responsible for the difference between the SOS1 activity in the light period and that in the dark period (Figs 3-5).

Instead, *PAP26*, *MPH2,* or both could be responsible for the difference of salt tolerance between *V. marina* and *V. luteola* (beach). *PAP26* is a member of purple acid phosphatase and enhances salt tolerance when overexpressed (Abbasi-Vineh et al., 2021). It does so by enhancing ion homeostasis *via* the SOS pathway, ROS scavenging ability, and acid phosphatase activity required for increasing available P storage. As *PAP26* is more abundantly transcribed in the root of *V. marina* (Fig 5), it could further enhance the salt tolerance of this species. *MPH2* is required for repairing photosystem II (PSII) under fluctuating light conditions or photoinhibitory light (Liu and Last, 2017). As PSII is particularly vulnerable to abiotic stresses including salt stress (Jajoo, 2013), the higher expression of *MPH2* (Fig 5) could help acclimation of *V. marina* to saline environments.

Positive and negative effect of the SOS pathway

It was first puzzling to notice that the diurnal regulation of SOS-dependent sodium excretion is conserved across species, given high and low tides do not occur in 24 h cycle, or extreme high tides by typhoons could occur at any time of a day. However, if the SOS pathway is energetically costly, the diurnal down-regulation could contribute to saving energy and to keeping growth.

Supporting our hypothesis, the Arabidopsis line transformed with *35S:SOS1/proPRR9:SOS2* showed the best performance under 25 mM NaCl condition (Fig 7). Although the double overexpression line (*35S:SOS1/35S:SOS2*) showed better performance than Col-0 and the *sos1/sos2* double mutant, it underperformed the *35S:SOS1/proPRR9:SOS2* line at least in the condition of 25 mM NaCl. In addition, in the control condition, the *sos1/sos2* double mutant line grew even bigger than both of the transformant lines (data not shown). Thus, though the SOS pathway is essential in salt tolerance, it could also negatively affect growth. The diurnal regulation seems to be a reasonable compromise to confer a certain level of salt tolerance with

minimized loss of growth. It could be even more reasonable to activate the SOS pathway in the light period, given it is the time of the highest transpiration-dependent sodium uptake (Fig 3).

Limitations

Though we have demonstrated the importance of *SOS1* and *SOS2* in *V. marina* and *V. luteola*, there must be other mechanisms than the SOS pathway. It is because the QTLs explained only ~20% of phenotypic variance (Supplementary Table 7) and because even the overexpression of *SOS1/SOS2* did not make the Arabidopsis plants as salt-tolerant as *V. marina*. We are now working on the follow-up studies that further enforce salt tolerance and provide further insights regarding how coastal species adapt themselves to saline environments.

In addition, although this study provides insights to the mechanisms of salt tolerance in *V. marina*, it is not easy to explain how *V. luteola* (beach) keeps excreting sodium even in the dark period (Fig 4). One possible explanation is that it could be passive diffusion along the cline of ^{22}Na concentration across the root and the hydroponic culture, as *V. luteola* (beach) accumulates more sodium in the root (Figs 1, 5) (Noda et al., 2021, Wang et al., 2023). However, it is also possible that other mechanisms than the SOS pathway that enable active excretion of sodium for 24 h. In any case, further studies are needed to elucidate the query.

Conclusion

We revealed at least one aspect of the mechanism of salt tolerance in *V. marina*, which lives in marine beach and is the most salt-tolerant species in the genus. It has higher potential of sodium excretion from the root, presumably through the elevated expression of *VmSOS1.* In addition, the sodium excretion is diurnally regulated, *via* diurnal transcriptional regulation of *VmSOS2*, which could save the energy cost of the SOS pathway and maximize growth under saline environment. This knowledge will be valuable information for future crop development especially for cropping with saline water and soil.

Data availability

All the sequence data are available from the Sequence Read Archive of the NCBI website. The BioProject ID is PRJNA1080052 and BioSamples accessions SAMN40099256-99737.

Funding

This study was financially supported by JST PRESTO Grant Number 11103610, Moonshot R&D Program for Agriculture, Forestry and Fisheries by Cabinet Office, Government of Japan (JPJ009237), JSPS KAKENHI (JP18H02182), NARO Innovation Program, Environmental Radioactivity Research Network Center (Y-19-05) and Interdisciplinary Project on Environmental Transfer of Radionuclides (No. Y-1).

Acknowledgements

The corresponding author is grateful to Dr. Akira Isogai, Dr. Yasunobu Ohkawa, Dr. Naoko Nishizawa, Dr. Kazuo Shinozaki and other committee members of the PRESTO program for their valuable advice and extraordinary support, especially when he was involved in an incident in his affiliation.

Author contributions

KN, NS, JF and NT conceived the study.
KN, HO and HS sequenced, assembled and annotated the *V. marina* genome.
SC, CM, EOT, YT, PS, YT, NT and KN developed the mapping population, performed phenotyping, genotyping, and QTL analysis.
YN, NS, YGY, YM, KE, NK and JF performed the tracer experiments and PETIS.
HO, YN, CM and KN collected the RNA samples and performed the sequencing.
FW, HO and KN analyzed the transcriptome data.
KN and YN wrote the manuscript.

Disclosures

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

References

Abbasi-Vineh MA, Sabet MS, Karimzadeh G. 2021. Identification and Functional Analysis of Two Purple Acid Phosphatases AtPAP17 and AtPAP26 Involved in Salt Tolerance in *Arabidopsis thaliana* Plant. *Frontiers in Plant Science* **11**: DOI:10.3389/fpls.2020.618716

Bray NL, Pimentel H, Melsted P, Pachter L. 2016. Near-optimal probabilistic RNA-seq quantification. *Nature Biotechnology* **34**: 525-527.

Broman KW, Gatti DM, Simecek P, Furlotte NA, Prins P, Sen Ś, Yandell BS, Churchill GA. 2019. R/qtl2: Software for Mapping Quantitative Trait Loci with High-Dimensional Data and Multiparent Populations. *Genetics* **211**: 495-502.

Catchen J, Hohenlohe PA, Bassham S, Amores A, Cresko WA. 2013. Stacks: an analysis tool set for population genomics. *Molecular Ecology* **22**: 3124-3140.

Chen Y, Lun A, Smyth G. 2016. From reads to genes to pathways: differential expression analysis of RNA-Seq experiments using Rsubread and the edgeR quasi-likelihood pipeline. *F1000Research* **5**: doi:10.12688/f1000research.8987.2.

Chen Y, Nie F, Xie S-Q, Zheng Y-F, Dai Q, Bray T, Wang Y-X, Xing J-F, Huang Z-J, Wang D-P, et al. 2021. Efficient assembly of nanopore reads via highly accurate and intact error correction. *Nature Communications* 12: doi.org/10.1038/s41467-020-20236-7.

Dinneny JR. 2015. Traversing organizational scales in plant salt-stress responses. *Current Opinion in Plant Biology* **23**: 70-75.

Fujimaki S, Maruyama T, Suzui N, Kawachi N, Miwa E, Higuchi K. 2015. Base to tip and long-distance transport of sodium in the root of common reed (*Phragmites australis* (Cav.) trin. ex steud.) at steady state under constant high-salt conditions. *Plant and Cell Physiology* **56**: 943-950.

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.

Ismail AM, Horie T. 2017. Genomics, Physiology, and Molecular Breeding Approaches for Improving Salt Tolerance. *Annual Review of Plant Biology* **68**: 405-434.

Ito S, Nakamichi N, Kiba T, Yamashino T, Mizuno T. 2007. Rhythmic and Light-Inducible Appearance of Clock-Associated Pseudo-Response Regulator Protein PRR9 Through Programmed Degradation in the Dark in *Arabidopsis thaliana*. *Plant and Cell Physiology* **48**: 1644-1651.

Jajoo A 2013. Changes in Photosystem II in Response to Salt Stress. In: Ahmad P, Azooz MM, Prasad MNV eds. *Ecophysiology and Responses of Plants under Salt Stress.* 149-168.

Li H, Durbin R. 2010. Fast and accurate long-read alignment with Burrows–Wheeler transform. *Bioinformatics* **26**: 589-595.

Liu J, Ishitani M, Halfter U, Kim C-S, Zhu J-K. 2000. The *Arabidopsis thaliana SOS2* gene encodes a protein kinase that is required for salt tolerance. *Proceedings of the National Academy of Sciences* **97**: 3730-3734.

Liu J, Last RL. 2017. A chloroplast thylakoid lumen protein is required for proper photosynthetic acclimation of plants under fluctuating light environments. *Proceedings of the National Academy of Sciences* **114**: E8110-E8117.

Matsumura H, Miyagi N, Taniai N, Fukushima M, Tarora K, Shudo A, Urasaki N. 2014. Mapping of the Gynoecy in Bitter Gourd (Momordica charantia) Using RAD-Seq Analysis. *PLOS ONE* **9**: e87138.

Naito K, Wakatake T, Shibata TF, Iseki K, Shigenobu S, Takahashi Y, Ogiso-Tanaka E, Muto C, Teruya K, Shiroma A, et al. 2022. Genome sequence of 12 *Vigna* species as a knowledge base of stress tolerance and resistance. *bioRxiv* https://doi.org/10.1101/2022.03.28.486085

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*. *Breeding Science* **72**: 326-331.

Ritu K, Joshua C, Wing-Kin S. 2019. HyPo: Super Fast & Accurate Polisher for Long Read Genome Assemblies. *bioRxiv* https://doi.org/10.1101/2019.12.19.882506

Sakai H, Naito K, Ogiso-Tanaka E, Takahashi Y, Iseki K, Muto C, Satou K, Teruya K, Shiroma A, Shimoji M, et al. 2015. The power of single molecule real-time sequencing technology in the *de novo* assembly of a eukaryotic genome. Scientific Reports **5**: 16780.

Shi H, Quintero FJ, Pardo JM, Zhu J-K. 2002. The Putative Plasma Membrane Na^+/H^+ Antiporter SOS1 Controls Long-Distance Na^+ Transport in Plants. *The Plant Cell* **14**: 465-477.

Taniguti CH, Taniguti LM, Amadeu RR, Lau J, Gesteira GdS, Oliveira TdP, Ferreira GC, Pereira GdS, Byrne D, Mollinari M, et al. 2023. Developing best practices for genotyping-by-sequencing analysis in the construction of linkage maps. *GigaScience* **12**: 1-19

Tomooka N. 2002. The Asian Vigna: genus Vigna subgenus ceratotropis genetic resources: *Springer Science & Business Media*.

Uchida H, Okamoto T, Ohmura T, Shimizu K, Satoh N, Koike T, Yamashita T. 2004. A compact planar positron imaging system. *Nuclear Instruments and Methods in Physics Research Section A: Accelerators, Spectrometers, Detectors and Associated Equipment* **516**: 564-574.

Vaser R, Sovic I, Nagarajan N, Sikic M. 2017. Fast and accurate de novo genome assembly from long uncorrected reads. *Genome Research* **27**: 737-746.

Wang F, Iki Y, Tanoi K, Naito K. 2023. Phenotypic responses in the root of salt-tolerant accessions of *Vigna marina* and *Vigna luteola* under salt stress. *Genetic Resources and Crop Evolution* https://doi.org/10.1007/s10722-023-01794-3

Wehrens R, Buydens LMC. 2007. Self- and Super-organizing Maps in R: The kohonen Package. *Journal of Statistical Software* **21**: 1-19.

Yoshida J, Tomooka N, Mishima T, Matsuda Y, Ehara H. 2021. Comparison of Growth and Ion Absorption with Compatible Solute Accumulation of Three Wild *Vigna* Species under Salt Stress. *Tropical Agriculture and Development* **65**: 195-205.

Yoshida J, Tomooka N, Yee Khaing T, Shantha PGS, Naito H, Matsuda Y, Ehara H. 2020. Unique responses of three highly salt-tolerant wild *Vigna* species against salt stress. *Plant Production Science* **23**: 114-128.

Legends to figures

Figure 1.

Sodium allocation in Vigna plants and association of the genotypes and salt tolerance-related traits in the F2 plants derived from *V. marina* and *V. luteola* (beach). A. Autoradiographs of Vigna plants fed with ^{22}Na. The two accessions surrounded by dashed lines are the parental lines used for QTL analysis. The white lines indicate 1 cm. B. QTL plot representing LOD scores calculated by genome scan. C. Estimated QTL effects along Chr1. AA, AB and BB indicate genotype, where the “A” allele is of *V. luteola* type and the “B” of *V. marina* type. Higher wilt score indicates lower tolerance to salt stress, whereas higher shoot generation and stay green scores indicate higher tolerance.

Figure 2.

Sodium excretion and culture alkalization by *V. marina.* A. Autoradiographs of *V. marina* plants fed with 100, 200 or 300 mM NaCl containing ^{22}Na. White arrows indicate traces of exudated ^{22}Na from the root. B. Hydroponic culture solution before and after cultivation of *V. marina*. C. pH of hydroponic culture solution before (0h) and after (72h) cultivation of *V. marina*. Small dots indicate values of biological replicates, while large plots and error bars indicate mean ± S.D.

Figure 3.

Dynamics of sodium transport in *V. marina*. A. Time-lapse images of gamma irradiation mapped by PETIS. A plant of *V. marina* was fed with ^{22}Na for 24h and then time-lapsed. B. Indexed amount of irradiation across time in the shoot, the root and the hydroponic culture media. Light and dark periods are indicated as white and gray in the plot, respectively. Error bars indicate standard errors. C. Setup of PETIS.

Figure 4.

Dynamics of sodium excretion in *V. marina* and other accessions. A. Time-lapse of ^{22}Na mapping. An, Lr, Lb and Ma indicate *V. angularis, V. luteola* (river), *V. luteola* (beach), and *V. marina*, respectively. B. Relationships between time and the estimated concentration of ^{22}Na in the hydroponic culture. Each plot is labeled in the same way as in A. C. Set up of the PETIS. The area enclosed by the yellow line indicates the area of the hydroponic solution where the separator prevented the roots from interfering with collecting data of hydroponic solution.

Figure 5.

Expression patterns of the candidate genes for salt tolerance in the 4 accessions. *ang, lutr, lutb* and *mar* indicate *V. angularis, V. luteola* (river), *V. luteola* (beach) and *V. marina*, respectively. Numbers below the plots indicate hours after initiation of salt stress. Cont (blue) and Salt (orange) indicate the control and salt-stressed conditions, respectively. Y-axes indicate the abundance of transcripts in count per million (CPM). Small plots indicate values of each replicate while larger ones and error bars indicate mean ± S.D.

Figure 6.

Figure 6. Relative dry weight of shoots and roots in various *SOS1/SOS2* expression lines. While small plots indicate the values of each replicate, large plots and error bars indicate means ±S.D. Different alphabets above the plots indicate significant difference by Tukey-Kramer test ($p<0.05$).