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Ky-2, a Histone Deacetylase Inhibitor, Enhances High-Salinity Stress Tolerance in *Arabidopsis thaliana*

Kaori Sako[1], Jong-Myong Kim[1], Akihiro Matsui[1], Kotaro Nakamura[2], Maho Tanaka[1], Makoto Kobayashi[3], Kazuki Saito[3,4], Norikazu Nishino[5], Miyako Kusano[3,6], Teruaki Taji[2], Minoru Yoshida[7] and Motoaki Seki[1,8,9,*]

[1]Plant Genomic Network Research Team, RIKEN Center for Sustainable Resource Science, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa, 230-0045 Japan
[2]Faculty of Applied Bioscience, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo, 156-8502 Japan
[3]Metabolomics Research Group, RIKEN Center for Sustainable Resource Science, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa, 230-0045 Japan
[4]Graduate School of Pharmaceutical Sciences, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba, 260-8522 Japan.
[5]Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology, Kitakyushu, 808-0196 Japan
[6]Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki, 305-8572 Japan
[7]Chemical Genomics Research Group, RIKEN Center for Sustainable Resource Science, 2-1 Hirosawa, Wako, Saitama, 351-0198 Japan
[8]Kihara Institute for Biological Research, Yokohama City University, 641-12 Maioka-cho, Totsuka-ku, Yokohama, Kanagawa, 244-0813 Japan
[9]Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology (JST), 4-1-8 Honcho, Kawaguchi, Saitama, 332-0012 Japan
*Corresponding author: E-mail, motoaki.seki@riken.jp; Fax, +81-45-503-9584.
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Adaptation to environmental stress requires genome-wide changes in gene expression. Histone modifications are involved in gene regulation, but the role of histone modifications under environmental stress is not well understood. To reveal the relationship between histone modification and environmental stress, we assessed the effects of inhibitors of histone modification enzymes during salinity stress. Treatment with Ky-2, a histone deacetylase inhibitor, enhanced high-salinity stress tolerance in Arabidopsis. We confirmed that Ky-2 possessed inhibition activity towards histone deacetylases by immunoblot analysis. To investigate how Ky-2 improved salt stress tolerance, we performed transcriptome and metabolome analysis. These data showed that the expression of salt-responsive genes and salt stress-related metabolites were increased by Ky-2 treatment under salinity stress. A mutant deficient in *AtSOS1* (*Arabidopis thaliana SALT OVERLY SENSITIVE 1*), which encodes an Na^+/H^+ antiporter and was among the up-regulated genes, lost the salinity stress tolerance conferred by Ky-2. We confirmed that acetylation of histone H4 at *AtSOS1* was increased by Ky-2 treatment. Moreover, Ky-2 treatment decreased the intracellular Na^+ accumulation under salinity stress, suggesting that enhancement of SOS1-dependent Na^+ efflux contributes to increased high-salinity stress tolerance caused by Ky-2 treatment.

Keywords: Chemical biology • HDAC inhibitor • Histone deacetylase • Salinity stress.

Abbreviations: ChIP, chromatin immunoprecipitation; DMSO, dimethylsulfoxide; HDAC, histone deacetylase; MS, Murashige and Skoog; P5CS1, DELTA1-PYRROLINE-5-CARBOXYLATE SYNTHASE 1; qRT-PCR, quantitative real-time PCR; SOS1, SALT OVERLY SENSITIVE 1; TSA, trichostatin A.

Introduction

Salinization is a growing problem for agriculture worldwide. More than 800 Mha of global land are affected by salinity stress (Munns and Tester 2008). High salt concentrations make it harder for roots to absorb water, and ionic Na^+ and Cl^- prevent plant growth by impairing metabolic processes and decreasing photosynthesis (Deinlein et al. 2014). To feed the burgeoning population, the elucidation of salt responses in plants and improvement of resistance to salinity stress are urgent necessities. The SALT OVERLY SENSITIVE (SOS) pathway mainly composed of SOS1, SOS2 and SOS3 is a well-characterized antiporter system that excludes Na^+ from the cytosol. *Arabidopsis thaliana SOS1* (*AtSOS1*) encodes a plasma membrane Na^+/H^+ antiporter that plays a crucial role in Na^+ efflux (Shi et al. 2000). AtSOS1 is phosphorylated and activated by an AtSOS2/AtSOS3 kinase complex (Qiu et al. 2002). *AtSOS2* encoding a Ser/Thr protein kinase (Liu et al. 2000) interacts with *SOS3* encoding an EF-hand Ca^{2+}-binding protein. AtSOS3 functions as a calcium sensor for salt tolerance (Halfer et al. 2000) and activates AtSOS2. Overexpressing *AtSOS1*, *Thellungiella salsuginea SOS1* and rice *OsSOS1* genes in plants successfully enhanced salinity stress tolerance (Shi et al. 2003, Martínez-Atienza et al. 2007, Oh et al. 2009, Yang et al. 2009). Therefore, enhancement of SOS1-dependent Na^+ exclusion from the cytosol is important for improvement of salt stress tolerance.

Histone modifications can dynamically affect chromatin conformation and gene expression, so that an apparently identical gene exhibits different patterns in a temporally and spatially dependent manner. Recent studies have indicated that chromatin regulation plays a pivotal role in environmental stress responses (Kim et al. 2015). Histone H3 Ser10 phosphorylation and histone H4 acetylation have been shown to increase in response to high salinity and cold stresses in tobacco BY2 and

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Arabidopsis T87 cells (Sokol et al. 2007). The up-regulation of cell wall-related genes in maize, such as *ZmEXPB2* and *ZmXET1*, is accompanied by elevated H3 Lys9 acetylation in the promoter and coding regions, which is thought to be necessary for high-salinity responses (Li et al. 2014). However, to elucidate the molecular mechanism of chromatin regulation in abiotic stress responses, the difficulty caused by the fact that many epigenetic regulatory factors show functional redundancy needs to be overcome. For example, Arabidopsis histone acetyltransferases HAM1 and HAM2 are redundantly required for cell division during gametogenesis (Latrasse et al. 2008). To overcome these problems, we used a chemical biological approach. In this study, we showed that the class I histone deacetylase (HDAC) inhibitor Cyclo(-L-2-amino-8-hydroxamido-suberoyl-aminoisobutylyl-L-phenylalanyl-D-prolyl-), called Ky-2, enhanced salinity stress tolerance in Arabidopsis. Ky-2 was designed and synthesized as a HDAC inhibitor based on the structure and HDAC inhibitory activity of chlamydocin and trichostatin A (TSA) (Nishino et al. 2004). The HDAC inhibitory activity of Ky-2 has been verified in vivo and in mammals. Here, we confirmed the HDAC inhibitory activity of Ky-2 in plants. Ky-2 treatment induced changes in the accumulation of metabolites, such as proline and polyamines, and in the transcription of many salt-responsive genes. Moreover, increased acetylation by Ky-2 caused up-regulation of *AtSOS1* leading to a decrease in Na^+ accumulation under salinity stress. Thus, the enhancement of *SOS1* transcription contributes to increased high-salinity stress caused by Ky-2 treatment.

Results

A histone deacetylase inhibitor enhances salinity stress tolerance

To analyze the role of epigenetic regulation under salinity stress, inhibitors of histone modification enzymes were screened to identify compounds involved in salinity stress responses (**Supplementary Fig. S1**). Wild-type Columbia plants grown in liquid culture medium were administered inhibitors for 24 h and then treated with 100 mM NaCl, as described in the Materials and Methods. The plants treated with Ky-2, a class I HDAC inhibitor, showed green cotyledons under salinity stress conditions (**Fig. 1A**) and almost 100% of the Ky-2-treated plants survived (**Fig. 1B**). We confirmed that Ky-2-treated plants had higher Chl contents than non-treated plants (**Fig. 1C**), suggesting that Ky-2 enhanced salinity stress tolerance in Arabidopsis.

Ky-2 exhibits HDAC inhibitory activity in Arabidopsis

Although Ky-2 has been established as an HDAC inhibitor in mammals (Nishino et al. 2004, Fujii et al. 2013), it was still not clear that Ky-2 was also functional in plants. To confirm the HDAC inhibitory activity of Ky-2 in Arabidopsis, we performed an immunoblot assay. Histone H4 acetylation levels were increased by Ky-2 treatment in a dose-dependent manner (**Fig. 2A, B**). Increased histone H4 acetylation by Ky-2 treatment was maintained at a high level under salinity stress conditions (**Supplementary Fig. S2**). These results suggested that Ky-2 works as an HDAC inhibitor in Arabidopsis.

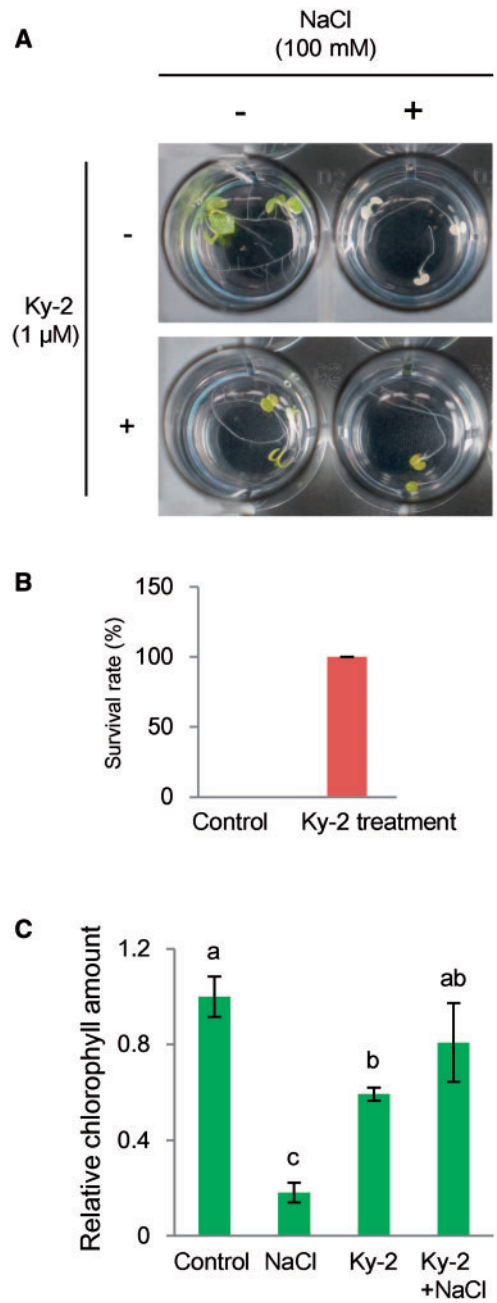

Fig. 1 Phenotype of seedlings with Ky-2 treatment under salinity stress. (A) Morphology of seedlings treated with 1 μM Ky-2 for 24 h and/or 100 mM sodium chloride for 4 d. DMSO was used as a negative control. (B) Survival rate under salinity stress conditions with/without Ky-2. Error bars represent the means ± SD. Three independent experiments were performed (10 plants per experiment). (C) Chl contents of non-treated plants and plants treated with 100 mM NaCl, 1 μM Ky-2 or both 1 μM Ky-2 and 100 mM NaCl. Error bars represent the means ± SD ($n = 3$). Statistical significance was determined by ANOVA, followed by post-hoc Tukey's tests. Means that differed significantly ($P < 0.05$) are indicated by different letters.

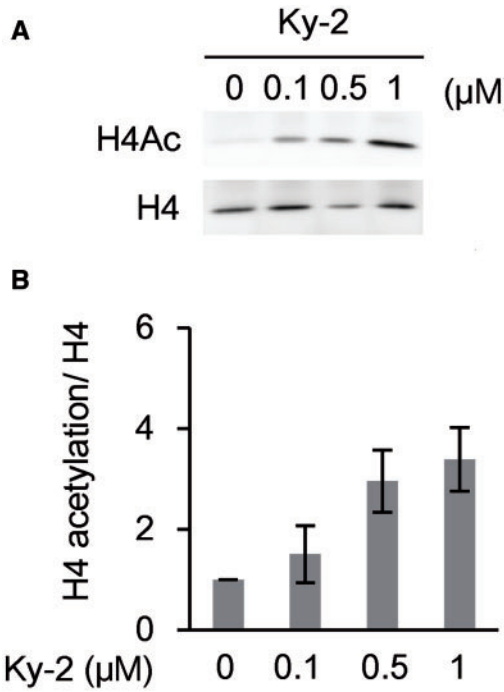

Fig. 2 Changes in histone H4 acetylation levels during Ky-2 treatment. (A) Total proteins were extracted from seedlings treated with 0, 0.1, 0.5 and 1 μM Ky-2 for 6 h. DMSO was used as a negative control. Immunoblot analysis was performed using an anti-histone H4 or an anti-acetylated histone H4 antibody. (B) The signal intensity of acetylation of histone H4 in each sample was normalized against histone H4. The acetylation of histone H4 without Ky-2 treatment was taken as 1.

Salt-responsive genes are up-regulated by Ky-2 treatment under salinity stress

Histone acetylation is generally correlated with gene activation. We showed that Ky-2 treatment elevated the global histone acetylation level. Thus, we hypothesized that the increased histone acetylation caused by Ky-2 treatment must lead to up-regulation of salt stress tolerance-related genes. To identify and characterize the genes with increased expression in response to Ky-2 treatment and salinity stress, we performed global gene expression analysis with a microarray. Based on the criteria (2-fold difference in expression, Student's *t*-test $q < 0.05$), we identified 1,725 genes (**Supplementary Table S1**) whose expression was up-regulated by 24 h Ky-2 treatment and 1,870 genes (**Supplementary Table S2**) whose expression was up-regulated by 2 h salt treatment in the presence of Ky-2 (**Supplementary Table S2**). In a comparison of these up-regulated genes, 1,257 genes overlapped, i.e. the expression of these genes was induced by Ky-2 pre-treatment and the high expression level was maintained under salinity stress (**Fig. 3A**). Forty genes among the 1,257 genes up-regulated by both Ky-2 and salt stress treatment were annotated as salinity stress signaling-related genes by Gene Ontology (GO) analysis at TAIR10 (**Table 1**). To confirm the validity of the microarray data, the expression profiles of *AtSOS1*, *AtP5CS1*, *AtRAP2.6L* and *AtCIPK6* were analyzed by quantitative real-time PCR (qRT-PCR) (**Fig. 3B**). The expression of these salinity stress-responsive

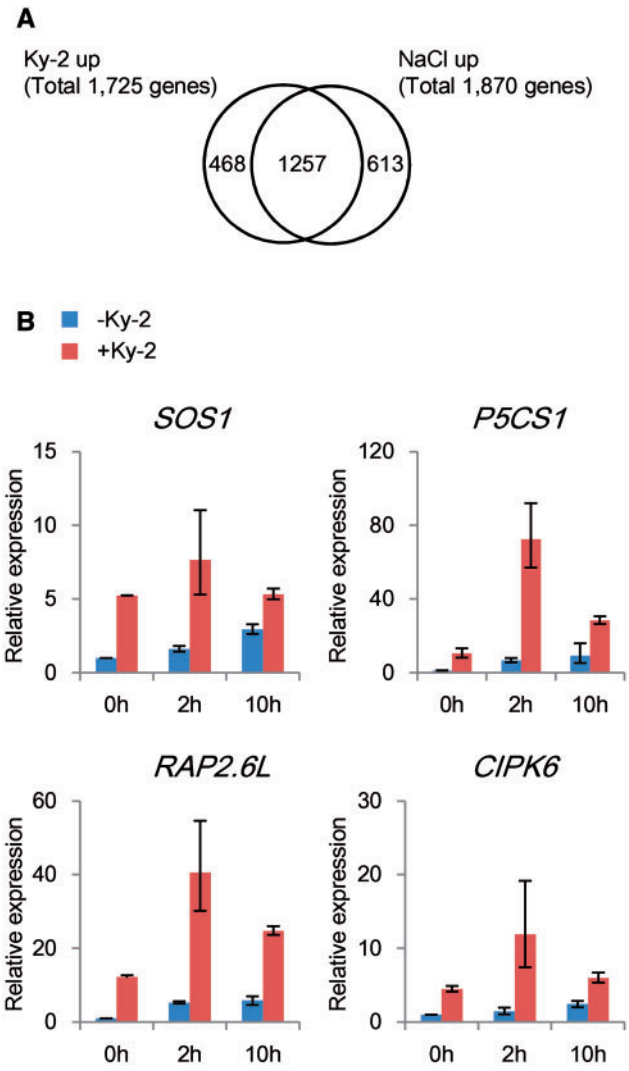

Fig. 3 Expression profile of the genes up-regulated by both Ky-2 treatment and salinity stress. (A) Venn diagram representation of 1,725 genes with increased expression in Ky-2-treated seedlings and 1,870 genes with increased expression in Ky-2-treated seedlings subjected to salt stress conditions. The expression of 1,257 genes was up-regulated by both Ky-2 and subsequent salinity stress treatment. (B) Relative expression levels of the *AtSOS1*, *AtP5CS1*, *AtRAP2.6L* and *AtCIPK6* genes during salinity stress treatment for 0, 2 and 10 h with or without 1 μM Ky-2. The expression level of the plants treated with DMSO was set as 1. *18S rRNA* was used as an internal standard. Error bars represent the means ± SD ($n = 3$).

genes was up-regulated by Ky-2 treatment, and the high level of expression was also observed during high-salinity stress treatment. These results suggested the tolerance conferred by Ky-2 might be caused by up-regulation of salt-responsive genes.

Proline and polyamines are accumulated under Ky-2 treatment

In addition to microarray analysis, a metabolite profiling was performed to investigate the molecular mechanism of the salt tolerance increased by Ky-2. First, we compared seedlings treated with Ky-2 for 24 h with non-treated seedlings. Proline,

Table 1 List of 40 salinity stress signaling-related genes up-regulated by both Ky-2 and NaCl

AGI code	Function	Ky-2/no treatment[a]	*q*-value	Ky-2/no treatment under salt stress[b]	*q*-value
AT1G01520	ALTERED SEED GERMINATION 4; AtASG4	1.480	0.004	1.515	0.002
AT1G10170	NF-X-LIKE 1; AtNFXL1	1.179	0.001	1.110	0.001
AT1G10370	GLUTATHIONE S-TRANSFERASE U17; AtGSTU17	2.021	0.001	1.783	0.004
AT1G11910	ASPARTIC PROTEINASE A1; AtAPA1	1.131	0.001	1.077	0.002
AT1G29660	GDSL-like Lipase/Acylhydrolase superfamily protein	1.396	0.004	1.138	0.001
AT1G48420	1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID DEAMINASE 1; ATACD1	1.765	0.002	1.511	0.001
AT1G54100	ALDEHYDE DEHYDROGENASE 7B4; ALDH7B4	1.195	0.001	1.282	0.003
AT1G69310	WRKY57	1.191	0.001	1.268	0.001
AT1G73500	MAP KINASE KINASE 9; AtMKK9	2.277	0.020	2.357	0.002
AT1G78380	GLUTATHIONE S-TRANSFERASE TAU 19; ATGSTU19	1.180	0.007	1.015	0.005
AT2G01980	ARABIDOPSIS NA+/H+ ANTIPORTER 7; SOS1	1.311	0.001	1.158	0.000
AT2G22430	HOMEOBOX PROTEIN 6; AtHB6	1.039	0.001	1.259	0.002
AT2G37770	ALDO-KETO REDUCTASE FAMILY 4 MEMBER C9; AKR4C9	1.684	0.004	1.663	0.006
AT2G39800	delta1-pyrroline-5-carboxylate synthase 1; P5CS1	1.797	0.010	1.507	0.007
AT2G41430	Hydrophilic protein lacking Cys residuese	1.197	0.001	1.145	0.001
AT2G47000	ARABIDOPSIS P-GLYCOPROTEIN 4; AtABCB4	1.084	0.001	1.322	0.001
AT2G47180	Galactinol synthase 1; AtGolS1	1.956	0.003	1.768	0.001
AT2G47770	OUTER MEMBRANE TRYPTOPHAN-RICH SENSORY PROTEIN RELATED; AtTSPO	4.505	0.000	1.676	0.002
AT3G03250	UDP-GLUCOSE PYROPHOSPHORYLASE 1; AtUGP1	1.008	0.000	1.014	0.001
AT3G55980	Salt-inducible zinc finger 1; AtSZF1	1.375	0.039	1.093	0.022
AT3G61890	ARABIDOPSIS THALIANA HOMEOBOX 12; AtHB12	1.245	0.001	1.465	0.002
AT4G12110	STEROL-4ALPHA-METHYL OXIDASE 1–1; AtSMO1-1	1.172	0.002	1.072	0.002
AT4G17260	Lactate/malate dehydrogenase family protein	1.052	0.002	1.247	0.001
AT4G26400	RING/U-box superfamily protein	1.302	0.002	1.235	0.002
AT4G26630	DEK-DOMAIN CONTAINING PROTEIN 3; AtDEK3	1.094	0.002	1.155	0.001
AT4G30650	Low temperature and salt responsive protein family	1.030	0.002	1.568	0.009
AT4G30960	CBL-interacting protein kinase 6; AtCIPK6.	1.176	0.000	1.838	0.002
AT4G40010	SNF1-RELATED PROTEIN KINASE 2.7; AtSNRK2-7	1.307	0.001	2.098	0.004
AT5G03740	HISTONE DEACETYLASE 2C; AtHD2C	1.510	0.002	1.153	0.001
AT5G10220	ANNEXIN ARABIDOPSIS THALIANA 6; ANNAT6	3.220	0.000	2.945	0.001
AT5G12380	Annexin 8; ANNAT8	2.005	0.001	1.629	0.005
AT5G13330	RELATED TO AP2 6L; AtRAP2.6L	1.157	0.003	1.220	0.004
AT5G15970	COLD-RESPONSIVE 6.6; KIN2	2.306	0.000	1.223	0.004
AT5G17300	REVEILLE 1; RVE1	2.103	0.002	1.105	0.001
AT5G17310	UDP-glucose pyrophosphorylase 2; AtUGP2	2.188	0.001	1.580	0.003
AT5G22500	FATTY ACID REDUCTASE 1; AtFAR1	1.793	0.002	1.307	0.006
AT5G39610	A NAC-domain transcription factor; ANAC092	2.281	0.001	1.022	0.003
AT5G45550	MOB1-LIKE	1.237	0.001	1.157	0.000
AT5G50720	ARABIDOPSIS THALIANA HVA22 HOMOLOGUE E; AtHVA22E	1.023	0.001	1.028	0.009
AT5G58670	ARABIDOPSIS THALIANA PHOSPHOLIPASE C; AtPLC1	2.592	0.001	1.999	0.001
AT5G63320	Nuclear Protein X1; NPX1	1.257	0.001	1.441	0.001

These genes were selected from 1,257 genes up-regulated by both Ky-2 and NaCl treatments based on the GO category (stress signaling).

[a] The value represents the $\log_2$ ratio (plants treated with Ky-2 for 24 h/plants treated with DMSO for 24 h) >1, $q < 0.05$.

[b] The value represents the $\log_2$ ratio (plants treated with Ky-2 for 24 h followed by 2 h NaCl treatment/plants treated with DMSO for 24 h followed by 2 h NaCl treatment) > 1, $q < 0.05$.

Table 2 List of metabolites that were increased in Ky-2-treated plants compared with non-treated plants

Metabolite	Ky-2/non-treated[a]	*P*-value
Proline	3.87	0.000
5-Hydroxytryptamine	2.43	0.004
Phenylalanine	1.94	0.000
Serine	1.70	0.002
Tyrosine	1.67	0.007
Threonic acid	1.13	0.008
Threonine	1.07	0.002

[a] The value represents the log2 ratio (plants treated with Ky-2 for 24 h/plants treated with DMSO for 24 h) > 1, $P < 0.05$.

Table 3 List of metabolites that were increased in plants treated with NaCl for 10 h after Ky-2 treatment compared with plants treated with NaCl for 10 h without Ky-2

Metabolite	Ky-2 treatment/no treatment under salt stress[a]	*P*-value
Proline	4.52	0.000
Galacturonic acid	3.59	0.019
Spermidine	2.52	0.015
Glycine	1.76	0.027
(3*R*)-3-Methyl-1,4-bis(trimethylsilyl) piperazine-2,5-dione	1.69	0.009
Valine	1.68	0.002
3-Aminopiperidin-2-one	1.46	0.026
Putrescine	1.42	0.002
Alanine	1.40	0.003
Threonine	1.30	0.017
Asparagine	1.24	0.002
Arginine	1.23	0.004
2-Aminobutyric acid	1.22	0.015
Phenylalanine	1.18	0.001
Glucose-6-phosphate	1.10	0.009
3-Cyanoalanine	1.08	0.006

[a] The value represents the $\log_2$ ratio (plants treated with Ky-2 for 24 h followed by 10 h NaCl treatment/plants treated with DMSO for 24 h followed by 10 h NaCl treatment) > 1, $P < 0.05$.

which is known as a compatible solute, was highly accumulated under Ky-2 treatment (**Table 2**). Secondly, metabolite profiling was performed on seedlings treated with 100 mM NaCl for 10 h with or without Ky-2 pre-treatment (**Table 3**). The accumulation of proline was enhanced under salinity stress conditions. This result was consistent with the expression pattern of *AtP5CS1* in microarray analysis and qRT-PCR. The expression of *AtP5CS1*, which is a rate-limiting enzyme in the proline biosynthetic pathway (Yoshiba et al. 1995), was increased by Ky-2 treatment, and this increase was greater after salinity stress. Polyamines such as spermidine and putrescine were also accumulated under salinity stress treatment with Ky-2 pre-treatment. Polyamines are accumulated during salinity stress and thought to be important for stress tolerance as direct protective compounds and signaling molecules (Pál et al. 2015). Actually, *AtADC2*, which is a rate-limiting enzyme catalyzing the first step of polyamine biosynthesis (Urano et al. 2004), was up-regulated under salt stress with Ky-2 pre-treatment but not by Ky-2 treatment alone. These metabolite changes following Ky-2 treatment might improve salt stress tolerance. Although proline was accumulated after Ky-2 treatment, two knock-out mutant lines of *AtP5CS1* survived under salinity stress with Ky-2 treatment (**Supplementary Fig. S3**). This result suggested that the accumulation of proline alone could not explain why Ky-2 treatment enhanced salinity stress tolerance.

Salinity stress tolerance conferred by Ky-2 is caused by decreased accumulation of Na^+ through enhancement of the SOS pathway

Next, we focused on the up-regulation of *AtSOS1* by Ky-2 treatment (**Table 1**; **Fig. 3**), because *AtSOS1* has been reported to play a critical role in Na^+ efflux and *AtSOS1*-overexpressing plants exhibit tolerance to salinity stress. We examined the *atsos1* mutant phenotype under salinity stress after Ky-2 treatment. *atsos1-1* and *atsos1-14*, two *sos1* mutant alleles, were unable to survive under salinity stress even with Ky-2 treatment (**Fig. 4A, B**), suggesting that increased transcription of *AtSOS1* is important for the salinity stress tolerance conferred by Ky-2. In addition to the *atsos1* mutants, *atsos2-1* and *atsos3-1* mutants were also hypersensitive to salt stress even with Ky-2 treatment (**Supplementary Fig. S4A**). *AtSOS3* was highly expressed under Ky-2 treatment; on the other hand, *AtSOS2* was not up-regulated by Ky-2 (**Supplementary Fig. S4A**). We analyzed histone acetylation levels by using chromatin immunoprecipitation (ChIP) assay. Acetylation levels of histone H4 were significantly increased at *AtSOS1* and *AtSOS3* genes by Ky-2 treatment (**Fig. 4C**; **Supplementary Fig. S5**), suggesting that the increased acetylation by Ky-2 induces up-regulation of *AtSOS1* and *AtSOS3*. The SOS signaling pathway is essential to maintain a low level of intracellular Na^+ under salt stress. Thus, we compared the Na^+ contents of seedlings treated or not with Ky-2. Seedlings treated with Ky-2 accumulated less Na^+ than non-treated seedlings (**Fig. 4D**). These results indicated that strengthening the SOS signaling pathway by Ky-2 treatment contributed to the exclusion of Na^+, leading to salinity stress tolerance.

Discussion

In this study, we found that Ky-2, a class I HDAC inhibitor, enhanced salinity stress tolerance in *A. thaliana*. Ky-2 increased global histone acetylation and the transcription of many genes by inhibiting HDAC. Because acetylation of histone H4 was increased at the *AtSOS1* gene encoding a Na^+/H^+ antiporter and the *AtSOS3* gene encoding an EF-hand Ca^{2+}-binding protein by Ky-2 treatment, and the accumulation of Na^+ was decreased by Ky-2 treatment, the up-regulated expression of *AtSOS1* and the *AtSOS3* was the leading cause of salt stress

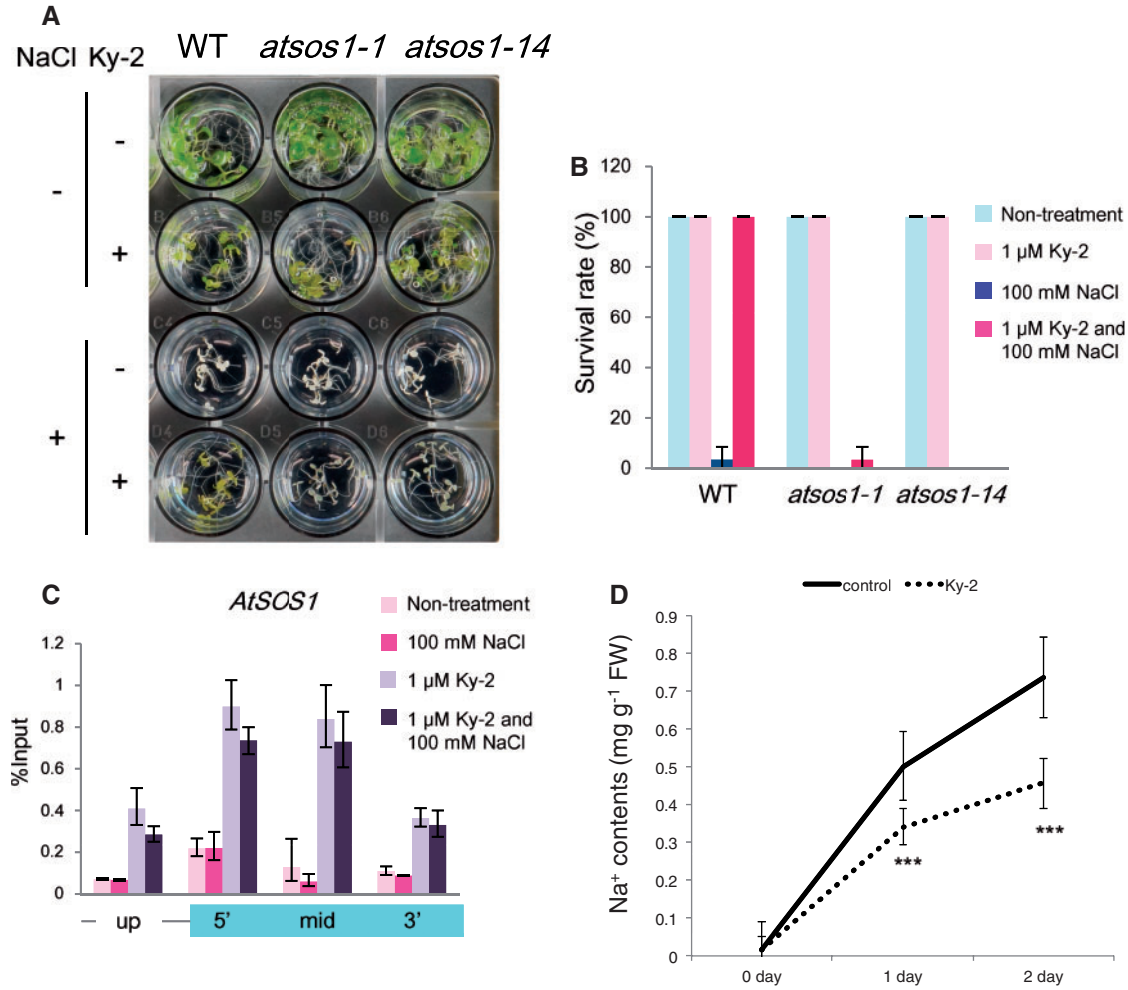

Fig. 4 Phenotype of *sos1* mutants and Na^+contents under salinity stress conditions with Ky-2. (A) Morphology of the *atsos1-1* and *atsos1-14* mutants treated with 1 μM Ky-2 for 24 h and/or 100 mM sodium chloride for 4 d. (B) Survival rates of *atsos1-1* and *atsos1-14* mutants under salinity stress conditions with/without Ky-2. Three independent experiments were performed (10 plants per experiment). (C) ChIP assay for histone H4 acetylation at the *AtSOS1* gene in seedlings treated with 1 μM Ky-2 for 24 h and/or 100 mM sodium chloride for 2 h. The position of qPCR-amplified fragments is indicated on the *x*-axis. (D) Na^+ contents in seedlings under salinity stress. Values represent the means ± SD from six individual experiments. Five seedlings were used in each experiment. Differences between seedlings treated with/without Ky-2 were analyzed by Student's *t*-test; $^{***}P < 0.001$.

tolerance. Our results are consistent with previous research showing that Arabidopsis plants overexpressing *AtSOS1* and *AtSOS3* genes exhibited increased salt stress tolerance following reduction of Na^+ (Yang et al. 2009). Ky-2 treatment also induced metabolite changes including increased accumulation of proline and polyamines through the up-regulation of rate-limiting biosynthetic pathway enzyme genes for these metabolites. The *atp5cs1* mutant showed tolerance to salt stress under Ky-2 treatment; thus, proline did not contribute greatly to the tolerance conferred by Ky-2.

Histone acetylation is regulated by the antagonistic balance between histone acetyltransferase and HDAC proteins. Under salinity stress conditions, the expression of a large proportion of HDAC genes (i.e. *OsHDA701*, *OsHDA702*, *OsHDA704*, *OsHDA705*, *OsHDA706*, *OsHDA712*, *OsHDA714*, *OsHDA716*, *OsHDT701* and *OsHDT702*) was down-regulated in rice (Hu et al. 2009). In maize, two histone acetyltransferases (ZmHATB and ZmGCN5) increased histone H3 Lys9 acetylation under salinity stress, which was accompanied by the up-regulation of cell wall-related genes, resulting in cell enlargement and root swelling to adapt to a high concentration of salt (Li et al. 2014). In accordance with previous reports, we found that increased histone acetylation has an important role in the response to salinity stress. Ky-2 treatment increased the global histone acetylation level and up-regulated salt-responsive genes under normal conditions. In particular, we found that histone acetylation of *AtSOS1* and *AtSOS3* was increased by Ky-2 treatment. Therefore, the increased histone acetylation by Ky-2 treatment might contribute to chromatin structure shifting into the open, relaxed conformation ready for transcription. Plants could then enhance their responsiveness to salinity stress, resulting in the acquisition of salt tolerance. From our results, we speculate that some class I HDACs may repress the transcription of salt-responsive genes under normal conditions. This suppression presumably protects normal plant growth, because overexpression of stress-responsive genes in

plants sometimes prevents plant growth (Todaka et al. 2012). Mutants deficient in HDA6 and HDA19 have been shown to be hypersensitive to salinity stress (Chen and Wu. 2010, Chen et al. 2010), suggesting that Ky-2 may inhibit class I HDACs except for HDA6 and HDA19, although further experiments are necessary to confirm this hypothesis.

Ky-2 was designed and synthesized based on TSA, which is a well-known HDAC inhibitor (Nishino et al. 2004). Chang and Pikkard (2005) performed a microarray analysis using Arabidopsis seedlings treated with TSA, although the growth stage of their plants was different from ours. Their data showed that small numbers of genes were changed by TSA treatment and these genes were not up-regulated by our Ky-2 treatment. Each HDAC inhibitor affects a different set of genes despite sharing HDAC inhibition activity; thus, analyses using various inhibitors will promote a better understanding of epigenetic mechanisms during stress responses.

Materials and Methods

Plant materials and growth conditions

Seeds of *A. thaliana* (ecotype Columbia-0) were surface-sterilized and placed in 24-well plates on half-strength Murashige and Skoog (MS) liquid medium supplemented with 1% sucrose and 0.1% agar. After cold treatment for 2 d to synchronize germination, the seeds were transferred to 22°C and 50% relative humidity conditions under a 16/8 h light/dark cycle. For the experiments, the plants were grown for 4 d and treated with inhibitors for 24 h, and then given 100 mM sodium chloride. Survival rates were calculated using 10 seedlings at 4 d after the treatments. Three independent experiments were performed. To identify the function of Ky-2, we used two *atp5cs1* mutant lines, *atp5cs1-1* (SALK_058000) and *atp5cs1-4* (SALK_063517), and four *atsos* mutant lines, *atsos1-1*, *atsos1-14* (CS859742), *atsos2-1* and *atsos3-1*.

Compounds

Cyproheptadine, nicotinamide and UNC0638 were purchased from Sigma-Aldrich. S2101 and SIRT1 inhibitor III were purchased from Merck. 3-Deazaneplanocin A was purchased from Cayman Chemical. Ky-2 is a synthetic cyclic tetrapeptide HDAC inhibitor containing a hydroxamic acid, previously described in Nishino et al. (2004).

Measurement of Chl contents

To measure Chl contents, 10 seedlings were used for each treatment. The Chl content was determined as described previously (Maekawa et al. 2015). Three independent experiments were performed.

Protein extraction and immunoblot analysis

Seedlings were pulverized in liquid nitrogen with a mortar and pestle, and SDS sample buffer was added. After centrifugation at 2,000×*g* for 10 min at 4°C, the supernatants were transferred into clean tubes. Immunoblotting was performed as described previously (Sako et al. 2014) using anti-histone H4 (Ab10158, abcam plc) and anti-acetyl-histone H4 (06-866, Millipore).

RNA extraction

Total RNA was extracted from 5-day-old seedlings that were treated with 100 mM sodium chloride for 2 or 10 h after 24 h treatment with 1 μM Ky-2. Dimethylsulfoxide (DMSO) was used as a negative control. For qRT-PCR, total RNA was extracted as described previously (Nguyen et al. 2015). For microarray analysis, total RNA was extracted with an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions (Qiagen).

Microarray analysis

Agilent Arabidopsis custom microarrays (GEO array platform: GPL19830) were used in this study. The microarray analysis was carried out as described previously (Nguyen et al. 2015). The microarray data are available on the GEO web site (GEO ID: GSE71855). Genes with an expression ratio >2.0-fold (*t*-test analysis; Benjamini and Hochberg false discovery rate: 0.05) were identified as up-regulated.

RT-PCR analysis

First-strand cDNA synthesis was performed with a PrimeScript™ RT reagent Kit (TAKARA) using random hexamer primers. qRT-PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems) according to the protocol for the StepOnePlus (Applied Biosystems) instrument. The sequences of the primers used for RT-PCR are shown in **Supplementary Table S3**.

Metabolite profiling

Four-day-old seedlings were treated with 1 μM Ky-2 for 24 h, 100 mM sodium chloride was added, and they were kept for 10 h. Subsequently, 18–35 seedlings (equivalent of 13–29 mg FW) were used for metabolite profiling by using GC-TOF/MS (Kusano et al. 2007). The metabolite level was calculated by dividing by the number of seedlings. Three independent experiments were performed.

ChIP assay

The ChIP assay was performed according to the method for the *A. thaliana* ChIP assay as previously described (Kim et al. 2014). Four-day-old seedlings were treated with 1 μM Ky-2 for 24 h, 100 mM sodium chloride was added, and they were kept for 2 h. A 500 mg aliquot of seedlings per each treatment was used for ChIP assay. Anti-acetyl-histone H4 antibody (Millipore 06-866) was used in this study. Three independent experiments were performed. The precipitates were analyzed with qPCR. The primers used are listed in **Supplementary Table S3**.

Measurement of Na^+

Four-day-old seedlings grown on nylon mesh on half-strength MS liquid medium were treated with 1 μM Ky-2 for 24 h and then transferred to half-strength MS liquid medium supplemented with 100 mM NaCl and grown for 3 d. The seedlings were harvested at 0, 1 and 2 d after transfer and soaked in 5 ml of sterile distilled water for 5 s twice. The solution with the plants was then boiled for 15 min, passed through a 0.2 μm filter (Toyo Roshi Kaisha, Ltd.) and analyzed for Na^+ using an IonPac CS12A column on a Dionex ICS-900 system (Thermo Scientific™ Dionex™).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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