

Note

Salinity stress-responsive transcription factors in the liverwort *Marchantia polymorpha*

Hiroyuki Tanaka^{1,†}, Rin Suzuki^{2,†}, Nanako Okabe², Tomohiro Suzuki¹,
Yutaka Kodama^{1,*}

¹Center for Bioscience Research and Education, Utsunomiya University, Utsunomiya, Tochigi 321-8505, Japan; ²Yokohama Science Frontier High School, Yokohama, Kanagawa 230-0046, Japan

*E-mail: kodama@cc.utsunomiya-u.ac.jp Tel: +81-28-649-8154 Fax: +81-28-649-8651

Received February 25, 2018; accepted May 1, 2018 (Edited by K. Suzuki)

Abstract Salinity stress limits plant growth and productivity. To cope with this limitation, the expression patterns of numerous genes are altered in response to salt stress; however, the regulatory mechanisms involved in these changes are unclear. In the present study, we investigated the regulation of the salinity stress response in the liverwort *Marchantia polymorpha*. The growth of *M. polymorpha* gemmalings was severely inhibited by NaCl, and RNA-sequencing and quantitative RT-PCR analyses revealed that the expression of several transcription factor gene families was induced by salinity stress. This work provides insight into the molecular mechanisms underlying the salinity stress response in *M. polymorpha*.

Key words: abiotic stress, bryophyte, RNA sequencing, salinity, salt, transcription factor.

Salinity stress limits plant growth by causing osmotic stress, ionic stress, and a series of secondary stresses, such as oxidative stress (You and Chan 2015). These stresses interfere with the absorption of mineral nutrients and water, resulting in cellular damage and growth retardation (Qadir et al. 2014). Many genes are differentially expressed in response to salinity stress, conferring a level of tolerance to the plants. In the model plant *Arabidopsis thaliana*, a number of salinity stress-responsive genes have been identified (Seki et al. 2002), including those encoding transcription factors (TFs) in the bZIP, AP2/ERF, WRKY, NAC, bHLH, and MYB families, which are upregulated to promote the expression of groups of genes that confer salt tolerance (Gollack et al. 2011). However, the signalling network underlying the salinity response is unclear.

Liverworts occupy a basal position in the phylogeny of land plants, and are believed to be key species for addressing a wide variety of questions in plant biology (Bowman et al. 2017; Qiu et al. 2006). *Marchantia polymorpha* is a dioecious liverwort species, recently established as a model liverwort. Compared with the sequenced genomes of other land plants, the *M. polymorpha* genome lacks much of the redundancy in many regulatory genes. For example, *A. thaliana* has

approximately 2000 TFs, whereas *M. polymorpha* has only 398 TF genes that classified into 47 families, which are all represented in the genomes of other land plants (Bowman et al. 2017). In the present study, we identified salinity stress-responsive TFs in *M. polymorpha*.

A male strain (Tak-1) of *M. polymorpha* was cultured on half-strength Gamborg's B5 medium containing 1% agar (1/2 B5 solid medium), then asexually maintained in a culture room under continuous white light (approximately 70 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$; FL40SW, NEC Corporation), as described previously (Ogasawara et al. 2013). Immature thalli of two-week-old gemmalings obtained from approximately one-month-old thalli were used for all experiments.

To investigate the salinity tolerance of *M. polymorpha*, a growth test was performed. The gemmalings were grown on 1/2 B5 solid medium in the presence (50 mM or 250 mM) or absence (0 mM) of NaCl. Gemmaling growth was inhibited after two weeks of culture on the 50 mM NaCl medium, and completely repressed on the 250 mM NaCl medium (Figure 1A). To quantify the inhibitory effect of 50 mM NaCl, we measured the fresh and dry weights of the gemmalings, and found them to be approximately one-third less than those of the control gemmalings (Figure 1B, 1C). These results demonstrated

Abbreviations: DEGs, differentially expressed genes; ds cDNA, double-stranded cDNA; GO, gene ontology; RNA-Seq, RNA sequencing; TFs, transcription factors.

[†]These authors contributed equally to this work.

This article can be found at <http://www.jspcmb.jp/>

Published online July 24, 2018

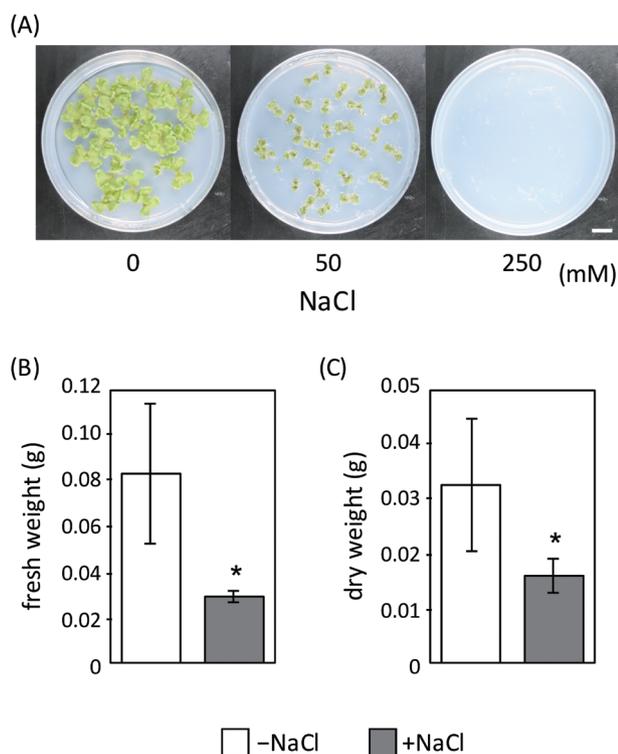


Figure 1. Effect of NaCl on *Marchantia polymorpha* growth. Gemmalings were grown for two weeks on half-strength Gamborg's B5 medium containing 1% agar ($\frac{1}{2}$ B5 solid medium) and either 0 mM (control), 50 mM, or 250 mM NaCl. (A) Photographs of representative gemmalings under the three salinity conditions. Scale bar=1 cm. (B) Fresh and (C) dry weights of gemmalings in the presence (+NaCl, 50 mM) and absence (-NaCl, 0 mM) of NaCl. Data are means \pm SD for four individual experiments ($n=4$) using independently grown gemmalings. Significant differences were determined by the *t*-test. * $p<0.05$.

the sensitivity of *M. polymorpha* to salinity stress.

To identify the TFs responsible for the response to salinity stress, we next conducted an RNA-sequencing (RNA-Seq) analysis using gemmalings grown in the absence (0 mM) or presence (50 mM) of NaCl. Total RNA was extracted from two-week-old gemmalings using TRIZOL Reagent (Thermo Fisher Scientific), purified using an RNeasy Plant Mini Kit (Qiagen), and treated with DNase I (Qiagen) to prevent contamination with genomic DNA. The RNA was quantified using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific), and its integrity was evaluated by electrophoresis on a 2% agarose gel stained with ethidium bromide and quantified using an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies). A 200 ng aliquot of RNA from each of three biological replicates was used for library preparation and RNA-Seq analysis.

Libraries for RNA-Seq analysis were constructed using a SureSelect Strand-Specific RNA Library Prep Kit (Agilent Technologies), according to the manufacturer's instructions. Normalized and pooled libraries were sent

to the sequencing facility at Utsunomiya University, Japan, for cluster generation on a MiSeq Sequencer (Illumina), and sequenced following the protocol of the Illumina MiSeq Reagent Kits v3 (2×76 cycles, paired-end reads). Three replicates of the samples grown on 0 mM and 50 mM NaCl were sequenced. The resulting RNA-Seq reads are available from the DDBJ Sequence Read Archive (DRA) under the accession number of DRA004674. After sequencing, the raw reads were processed using cutadapt version 1.8.1 to remove the adaptor sequences, the low-quality sequences ($< QV30$), and reads shorter than 50 bp. More than 1.9 million clean reads for each library were generated from the RNA-Seq (Supplementary Table S1). Of these, 90.0–93.1% of the sequences from the plants grown on 0 mM NaCl and 90.0–92.0% of sequences from plants grown on 50 mM NaCl could be mapped to the *M. polymorpha* genome (JGI Ver. 3.1; Bowman et al. 2017) using HISAT software (Kim et al. 2015) (Supplementary Table S1). StringTie was then used to assemble the aligned reads into transcripts and estimate their abundance (Pertea et al. 2015), and a differential expression analysis was performed using the ballgown R package (Frazer et al. 2015).

We identified genes that were differentially expressed between plants grown in the 0 mM and 50 mM NaCl media (Supplementary Table S2). A total of 662 significant differentially expressed genes (DEGs) with a false discovery rate (FDR) <0.05 were identified (gene names, log fold changes (logFC), and *p*-values for the up- and downregulated DEGs in each treatment are listed in Supplementary Table S3). The distributions of the DEGs were further analysed using the edgeR package and visualized as an MA plot (log ratio versus abundance plot) (Supplementary Figure S1). Among the 662 DEGs, 302 were upregulated in plants under salinity stress, while 360 were downregulated.

The DEGs were annotated by querying their consensus sequences against the Swiss Institute of Bioinformatics (Swiss-Prot) database (Bairoch and Boeckmann 1991) using BLASTx (E-value cut-off was set at $1e^{-5}$ to isolate the maximum number of similar genes) (Supplementary Table S3). These genes were further annotated with Gene Ontology (GO) terms using InterProScan and the GO database and informatics resource (Harris et al. 2004). More detailed TF family annotations were obtained from the Plant Transcription Factor Database (Plant TFDB) (Jin et al. 2014).

After the annotation and classification of the DEGs, we performed a GO enrichment analysis by the previously described method (Kim and Volsky 2005). The result showed that two GO terms were upregulated and seven were downregulated in plants grown in 50 mM NaCl. The most enriched GO terms were "peroxidase activity" (GO: 0004601) in the molecular functions category

and “response to oxidative stress” (GO: 0006979) in biological processes, but no cellular component GO terms were enriched (Supplementary Figure S2). Moreover, “heme binding” (GO: 0020037), “inorganic phosphate transmembrane transporter activity” (GO: 0005315), “transporter activity” (GO: 0005215), “cation binding” (GO: 0043169), “phosphate ion transport” (GO: 0006817), “oxidation-reduction process” (GO: 0055114), and “DNA replication” (GO: 0006260) were all found to be significantly enriched in the salt-stress DEGs. As aquaporin (included in the “transporter activity” group) and alpha-amylase (included in the “cation binding” group) were previously reported to be salinity stress-responsive genes (Boursiac et al. 2005; Yokotani et al. 2009), the presence of these GO terms in our DEGs confirmed that the salinity stress response in *M. polymorpha* is similar to those in angiosperms.

The Plant TFDB revealed five differentially expressed TFs under the salinity stress condition, four of which were upregulated (Table 1, Supplementary Table S4). The differentially expressed TFs were members of the MYB, bHLH, B3, and bZIP families (Table 1), and their gene symbols were obtained from the *M. polymorpha* genome database (MarpolBase: <http://marchantia.info/>). To validate reliability of the expression profiles obtained by RNA-Seq analysis, we performed quantitative RT-PCR (qRT-PCR) with a LightCycler® 96 System (Roche) and FastStart Universal SYBR Green Master (Roche) (Roche). Total RNA for qRT-PCR was extracted from two-week-old gemmalings, and the first-strand cDNAs were synthesized using PrimeScript™ RT Master Mix (Perfect Real Time) (Takara Bio). Primers for qRT-PCR were designed using Primer3plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>). The primer sequences are shown in Supplementary Table S5. *MpEF1α* mRNA was used as an internal standard in all experiments. When qRT-PCR was performed, expression patterns of the three TF genes (*MpABI3A*, *MpABI5B* and *MpR2R3-MYB17*) were similar with the results of RNA-Seq analysis (Figure 2). However, the qRT-PCR results of the other two TF genes (*MpBHLH2* and *MpRR-MYB1*) were inconsistent with the results of RNA-Seq (Table 1 and Figure 2). In the RNA-Seq data, FDR values of *MpBHLH2* and *MpRR-MYB1* genes (0.0365 and 0.0405,

respectively) were higher than those of *MpABI3A*, *MpABI5B* and *MpR2R3-MYB17* genes (0.0054, 0.0161, and 0.0115, respectively) (Table 1). Our cut-off criteria (FDR < 0.05) to identify significantly DEGs might not be adaptable in our RNA-Seq data. Nevertheless, the identified *M. polymorpha* TF families involved in salinity stress were similar to the TF families previously reported to be involved in the salinity stress response and salinity tolerance in other plant species (Lippold et al. 2009; Richardt et al. 2010; Sohn et al. 2006; Uno et al. 2000; Yanhui et al. 2006). Given that the liverworts are related to all other land plants (Qiu et al. 2006), the TFs in *M. polymorpha* may reflect the ancestral TFs involved in the

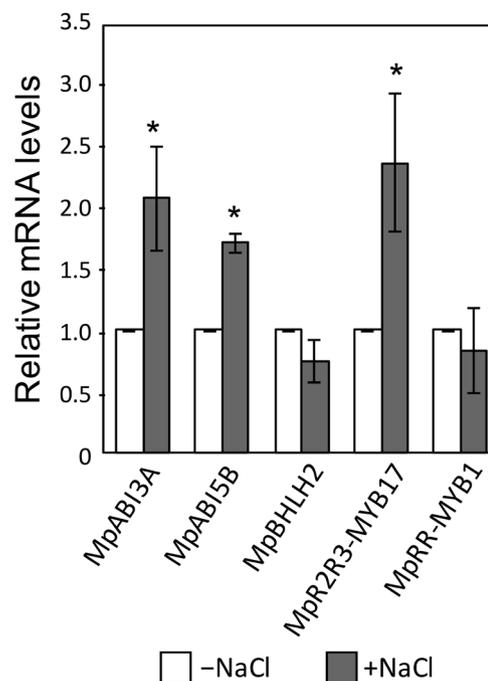


Figure 2. Validation of TF genes' expression in response to NaCl by quantitative RT-PCR. Gemmalings were grown for two weeks on half-strength Gamborg's B5 medium containing 1% agar (1/2 B5 solid medium) presence (+NaCl, 50 mM) or absence (-NaCl, 0 mM) of NaCl. Quantitative RT-PCR was performed using total RNAs from 2-weeks-old gemmalings as templates with specific primers for the TFs and *MpEF1α* genes. The equal loading of each amplified cDNA was determined by amount of *MpEF1α* PCR product as a control. Data are means ± SD for three individual experiments ($n=3$) using independently grown gemmalings. Significant differences were determined by the *t*-test. * $p < 0.05$.

Table 1. Salinity stress-responsive TF genes in *Marchantia polymorpha* from RNA-seq data.

Gene ID	Gene symbol ^a	LogFC	FDR	TF family ^b	Best hit in <i>Arabidopsis thaliana</i> ^b	e-value ^b	Common name ^b
Mapoly0028s0062	MpBHLH2	4.24	0.0365	bHLH	AT4G09820.1	4e ⁻¹⁴	AtTT8, BHLH42, EN32, F17A8.170, TT8
Mapoly0072s0050	MpABI5B	1.57	0.0161	bZIP	AT1G45249.1	4e ⁻³²	ABF2, AREB1, AtABF2, ATAREB1, BZIP36, T2P3
Mapoly0086s0035	MpABI3A	1.37	0.0054	B3	AT3G24650.1	3e ⁻³⁷	ABI3, MSD24.2, SIS10
Mapoly0096s0058	MpR2R3-MYB17	1.12	0.0115	MYB	AT5G15310.1	2e ⁻⁸¹	ATMIXTA, ATMYB16, F8M21_200, MYB16
Mapoly0001s0480	MpRR-MYB1	-4.86	0.0405	MYB	AT3G52250.1	9e ⁻³⁹	Not annotated

^aMarpolBase (<http://marchantia.info/>). ^bPlantTFDB (<http://planttfdb.cbi.pku.edu.cn/>).

salinity-stress response and salinity tolerance in the land plants.

In the present study, we showed that the TF families mediating the salinity stress response in *M. polymorpha* are similar to those reported in other plant species. Given that *M. polymorpha* contains conserved TF families, only with fewer members and therefore a lower level of redundancy than those found in other plants. The TF-mediated signalling pathways for the salinity-stress response in this liverwort may represent the minimal network model required in all land plants. Further knockout and overexpression experiments are required to elucidate the detailed functions of the salinity-stress responsive TFs in *M. polymorpha*. The genetic modification techniques established for *M. polymorpha*, including transformation and genome editing (e.g., Ishizaki et al. 2008; Sugano et al. 2014; Tsuboyama and Kodama 2014), will enable these experiments to be performed in the near future.

Acknowledgements

We thank Dr. Takayuki Kohchi (Kyoto University) for providing the Tak-1 strain. We also thank Dr. Masaru Matsuda (Utsunomiya University), Mr. Shigeki Yabe (Yokohama Science Frontier High School) and Ms. Lee Kien Yong (Utsunomiya University) for their valuable discussions and comments. This work was supported by the JST-GSC Incubation Program for Innovative Students at Utsunomiya University (iP-U). A post-doctoral researcher (H.T.) and two high school students (R.S. and N.O.) were also supported by iP-U.

References

- Bairoch A, Boeckmann B (1991) The SWISS-PROT protein sequence data bank. *Nucleic Acids Res* 19(Suppl): 2247–2249
- Boursiac Y, Chen S, Luu DT, Sorieul M, van den Dries N, Maurel C (2005) Early effects of salinity on water transport in Arabidopsis roots: Molecular and cellular features of aquaporin expression. *Plant Physiol* 139: 790–805
- Bowman JL, Kohchi T, Yamato KT, Jenkins J, Shu S, Ishizaki K, Yamaoka S, Nishihama R, Nakamura Y, Berger F, et al. (2017) Insights into land plant evolution garnered from the *Marchantia polymorpha* genome. *Cell* 171: 287–304
- Frazee AC, Pertea G, Jaffe AE, Langmead B, Salzberg SL, Leek JT (2015) Ballgown bridges the gap between transcriptome assembly and expression analysis. *Nat Biotechnol* 33: 243–246
- Golldack D, Lüking I, Yang O (2011) Plant tolerance to drought and salinity: Stress regulating transcription factors and their functional significance in the cellular transcriptional network. *Plant Cell Rep* 30: 1383–1391
- Harris MA, Clark J, Ireland A, Lomax J, Ashburner M, Foulger R, Eilbeck K, Lewis S, Marshall B, Mungall C, et al.; Gene Ontology Consortium (2004) The Gene Ontology (GO) database and informatics resource. *Nucleic Acids Res* 32: D258–D261
- Ishizaki K, Chiyoda S, Yamato KT, Kohchi T (2008) *Agrobacterium*-mediated transformation of the haploid liverwort *Marchantia polymorpha* L., an emerging model for plant biology. *Plant Cell Physiol* 49: 1084–1091
- Jin J, Zhang H, Kong L, Gao G, Luo J (2014) PlantTFDB3.0: A portal for the functional and evolutionary study of plant transcription factors. *Nucleic Acids Res* 42(D1): D1182–D1187
- Kim D, Langmead B, Salzberg SL (2015) HISAT: A fast spliced aligner with low memory requirements. *Nat Methods* 12: 357–360
- Kim SY, Volsky DJ (2005) PAGE: Parametric analysis of gene set enrichment. *BMC Bioinformatics* 6: 144
- Lippold F, Sanchez DH, Musialak M, Schlereth A, Scheible WR, Hinch DK, Udvardi MK (2009) AtMyb41 regulates transcriptional and metabolic responses to osmotic stress in *Arabidopsis*. *Plant Physiol* 149: 1761–1772
- Ogasawara Y, Ishizaki K, Kohchi T, Kodama Y (2013) Cold-induced organelle relocation in the liverwort *Marchantia polymorpha* L. *Plant Cell Environ* 36: 1520–1528
- Pertea M, Pertea GM, Antonescu CM, Chang TC, Mendell JT, Salzberg SL (2015) StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nat Biotechnol* 33: 290–295
- Qadir M, Quillérou E, Nangia V, Murtaza G, Singh M, Thomas RJ, Drechsel P, Noble AD (2014) Economics of salt-induced land degradation and restoration. *Nat Resour Forum* 38: 282–295
- Qiu YL, Li L, Wang B, Chen Z, Knoop V, Groth-Maloney M, Dombrowska O, Lee J, Kent L, Rest J, et al. (2006) The deepest divergences in land plants inferred from phylogenomic evidence. *Proc Natl Acad Sci USA* 103: 15511–15516
- Richardt S, Timmerhaus G, Lang D, Qudeimat E, Corrêa LG, Reski R, Rensing SA, Frank W (2010) Microarray analysis of the moss *Physcomitrella patens* reveals evolutionarily conserved transcriptional regulation of salt stress and abscisic acid signalling. *Plant Mol Biol* 72: 27–45
- Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y, Kamiya A, Nakajima M, Enju A, Sakurai T, et al. (2002) Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. *Plant J* 31: 279–292
- Sohn KH, Lee SC, Jung HW, Hong JK, Hwang BK (2006) Expression and functional roles of the pepper pathogen-induced transcription factor RAV1 in bacterial disease resistance, and drought and salt stress tolerance. *Plant Mol Biol* 61: 897–915
- Sugano SS, Shirakawa M, Takagi J, Matsuda Y, Shimada T, Hara-Nishimura I, Kohchi T (2014) CRISPR/Cas9-mediated targeted mutagenesis in the liverwort *Marchantia polymorpha* L. *Plant Cell Physiol* 55: 475–481
- Tsuboyama S, Kodama Y (2014) AgarTrap: A simplified *Agrobacterium*-mediated transformation method for sporelings of the liverwort *Marchantia polymorpha* L. *Plant Cell Physiol* 55: 229–236
- Uno Y, Furihata T, Abe H, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K (2000) Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. *Proc Natl Acad Sci USA* 97: 11632–11637
- Yanhui C, Xiaoyuan Y, Kun H, Meihua L, Jigang L, Zhaofeng G, Zhiqiang L, Yunfei Z, Xiaoxiao W, Xiaoming Q, et al. (2006) The MYB transcription factor superfamily of Arabidopsis: Expression analysis and phylogenetic comparison with the rice MYB family. *Plant Mol Biol* 60: 107–124
- Yokotani N, Ichikawa T, Kondou Y, Matsui M, Hirochika H, Iwabuchi M, Oda K (2009) Tolerance to various environmental stresses conferred by the salt-responsive rice gene ONAC063 in transgenic Arabidopsis. *Planta* 229: 1065–1075
- You J, Chan Z (2015) ROS regulation during abiotic stress responses in crop plants. *Front Plant Sci* 6: 1092