Accession difference in leaf photosynthesis, root hydraulic conductance and gene expression of root aquaporins under salt stress in barley seedlings

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ABSTRACT
Soil salinity causes considerable losses of crop productivity. Barley (Hordeum vulgare) is one of the most salt-tolerant Gramineae crops. Previously, we found that net photosynthetic rate \( A_n \) was kept remarkably higher in the salt-tolerant barley accession OUE812 than in the salt-sensitive accession OUC613 after heading under salt stress due to the low level of salt accumulation in leaves. Here we grew seedlings in Hoagland solution with 100 mM NaCl (salt treatment) or without added NaCl (control), and compared \( A_n \), stomatal conductance \( g_s \), salt accumulation in leaves, root hydraulic conductance and gene expression of root aquaporins between the accessions under salt stress for a few days. \( A_n \), \( g_s \) and root hydraulic conductance of the plants with salt treatment decreased significantly in OUC613 compared to OUE812 with no accession difference in salt accumulation in leaves at 2 days after the onset of treatment (DAT). The reduction in root hydraulic conductance in OUC613 was caused by the reduction of the root hydraulic conductivity \( L_p \). Salt treatment also decreased the transcript levels of some plasma membrane intrinsic aquaporin genes \( (HvP IPs) \) in OUC613 and, on the contrary, increased those of some HvPIP \( s \) in OUE812, resulting in a large difference between OUC613 and OUE812 in the transcript levels at 2 DAT. The accession difference in HvPIP \( s \) expression and thus \( L_p \) was closely associated with the accession difference in \( A_n \) and \( g_s \) under the short-term salt stress.

1. Introduction
Soil salinity causes considerable losses of crop productivity. Salt tolerance differs significantly among crop species. Among Gramineae crops, barley (Hordeum vulgare) is the most salt tolerant, whereas rice (Oryza sativa) is the most susceptible (Munns et al., 2006; Munns & Tester, 2008; Rawson et al., 1988). In barley, wheat (Triticum aestivum), and rice, salt tolerance differs among cultivars (Mano, 2007; Rawson et al., 1988;
Richards et al., 1987; Royo & Aragues, 1999; Slavich et al., 1990; Yamanouchi, 1989). To adopt gene mining and molecular breeding for the improvement of crop salt-stress tolerance, it would be important to clarify natural variations in salt-stress tolerance and its characteristics in each crop species.

In most cases, crops grow under salinity conditions from seed germination to harvest, and thus investigation of the response to salt stress over the whole life of the plants is needed for characterization of their salt tolerance. The harmful effects of soil salinity on plant growth and yield are caused by two main factors: osmotic stress and ion toxicity (Horie et al., 2012; Munns & Tester, 2008). Plant salt tolerance can be characterized from these viewpoints.

Long-term salinity increases Na⁺ and Cl⁻ concentrations in plants, which causes ion toxicities (Munns & Tester, 2008). Differences in ion exclusion capacity underlie cultivar differences in salt-stress tolerance in rice (Tsuchiya et al., 1994), wheat (Colmer et al., 2006), and barley (Shabala et al., 2010; Zhu et al., 2016). HvNax3 and HvNax4 were identified in barley as QTLs related to Na⁺ exclusion (Rivandi et al., 2011; Shavrukov et al., 2010). A wheat cultivar carrying a Na⁺ transporter gene, TmHKT1;5-A, has decreased leaf Na⁺ concentration and increased grain yield under salinity (Munns et al., 2012).

Soil salinity leads to a decrease in water uptake and, consequently, water potential in plants. The osmotic and water-deficit-inducing effects of salinity lead to reduced leaf photosynthesis and growth (Munns & Tester, 2008). Salinity also affect hydraulic conductivity of roots (LPᵣ; root hydraulic conductance per unit root surface area) (Aroca, Porcel & Ruiz-Lozano, 2012; Afzal et al., 2016; Chaumont & Tyerman, 2014; Javot & Maurel, 2002). Changes in LPᵣ in turn, affect water potential in plants through changing plant hydraulic conductance (Hirasawa, 2018). LPᵣ is highly affected by root aquaporins, by which water transport across cell membranes is facilitated (Chaumont & Tyerman, 2014; Maurel et al., 2015) and the transcriptional regulation of root aquaporins and accession difference in aquaporins expression under salt stress were reported (Boursiac et al., 2005; Horie et al., 2011; Katsuahara et al., 2011; Kirch et al., 2000; Martens et al., 2015). However, the studies on the association of the accession difference in aquaporin expression with salt tolerance are very limited (Katsuahara et al., 2011; Kresseis et al., 2020).

Among approximately 6000 accessions of barley from the collection at Okayama University, Mano (2007) identified several accessions as salt tolerant and several accessions as salt sensitive. We selected OUE812 as a particularly salt tolerant and OUC613 as a particularly salt sensitive accession and grew them under long-term salt stress from 30 days after sowing to maturity for approximately 5 months (Hirasawa et al., 2017). OUE812 produced higher dry matter yield at maturity than did OUC613. OUE812 maintained significantly higher net photosynthetic rate (Aₑ) than did OUC613 during ripening, with no difference in leaf water potential (LWP) between the accessions. OUE812 maintained lower concentrations of Na⁺ and Cl⁻ and a higher concentration of K⁺ in leaves than did OUC613, suggesting that the difference in Aₑ between the accessions was caused by the differences in ion accumulation in leaves (Hirasawa et al., 2017).

Plants grow under the conditions of salt stress from the seedling stage. However, we have not examined the accession difference in physiological responses to salt stress at the seedling stage. In the current study, to investigate the accession difference in salt-stress tolerance at the seedling stage and the causal mechanisms, we compared Aₑ, stomatal conductance (gₛ), LWP, ion accumulation in leaves, root hydraulic conductance, Lpᵣ, and gene expression of root aquaporins between OUE812 and OUC613 seedlings under salt stress for a few days.

2. Materials and methods

2.1. Plant materials

Seeds of cultivated barley (Hordeum vulgare L.) accessions OUC613 (a two-rowed landrace collected in China) and OUE812 (a six-rowed landrace collected in Ethiopia) were sterilized with 10% H₂O₂ for 10 min and immersed in distilled water with aeration for 1 day. Germinated seeds were transplanted on a seeding plate with 61 holes for planting in 3.5-L pots filled with 0.25 mM CaSO₄ and were hydroponically cultured with aeration for 2 days at 25°C in the dark. Plants were then grown in half-strength Hoagland solution in a growth chamber (KG-50HLA, Koito Manufacturing, Yokohama, Japan) at 14 h day/10 h night, 25°C, 60% relative humidity, and approximately 600 μmol m⁻² s⁻¹ photosynthetic photon flux density (PPFD) at the leaf surface. Plants were thinned periodically for preventing mutual shading.

The full-strength Hoagland solution contained 4.0 mM Ca(NO₃)₂, 4.0 mM KNO₃, 1.0 mM MgSO₄, 1.0 mM NH₄H₂PO₄, 1.0 mM (NH₄)₂HPO₄, 1.0 mM NaCl, 36 μM FeNaEDTA, 12.5 μM H₂BO₃, 0.25 μM CuSO₄, 1.0 μM MnSO₄, 1.0 μM ZnCl₂, and 0.4 μM NaMoO₄.

2.2. Salt treatment

The treatment started 8 days (for OUC613) or 10 days (for OUE812) after sowing, when the first leaf had fully expanded. Plants were grown in half-strength Hoagland solution without additional NaCl (control) or with 100 mM
NaCl (salt treatment). The solution was renewed every 2 days. Each treatment consisted of three or five replications. For the measurements of $A_{nr}$, $g_s$, LWP, leaf ion concentration, and root aquaporin expression, plants grown in the same pot were grouped as one replication. For the measurements of root hydraulic conductance, plants sown on the same day were grouped as one replication.

2.3. Measurements of $A_{nr}$, $g_s$, and LWP

Measurements of $A_{nr}$, $g_s$, and LWP were started 4 to 5 h after the onset of the light period using fully expanded leaves. $A_{nr}$ and $g_s$ were determined with a portable open-flow gas-exchange system (LI-6400; LI-COR, Lincoln, NE, USA) at the center of a leaf. During measurements, leaf temperature, the leaf–air vapor-pressure difference and the ambient CO$_2$ concentration were controlled at 25°C, approximately 1.5 kPa, and 370 µmol mol$^{-1}$, respectively. PPFD was controlled at 2000 µmol m$^{-2}$ s$^{-1}$ to obtain light-saturated $A_{nr}$ as no detectable photoinhibition was observed during the measurements.

LWP was measured with a thermocouple psychrometer by the dew-point method. Leaf discs (6 mm in diameter) were excised at the center of a leaf so that they did not include mid-rib tissues, and were loaded immediately into the sample chamber of a psychrometer (C-52; Wescor Inc., Logan, UT, USA). After equilibration for 3 h at 25°C, the dew point was measured with a microvoltmeter (HR-33 T; Wescor Inc.).

Two or three independent experiments were conducted for the comparison of $A_{nr}$, $g_s$, and LWP between the accessions.

2.4. Determination of ion concentrations

Fully expanded leaves were collected, oven-dried at 80°C, and powdered with a ball mill (85,200; Qiagen, Haan, Germany). Ions were extracted from the powdered material with water at 80°C for 3 h, and their concentration was measured by high-performance liquid chromatography on an HIC-6A instrument (Shimadzu Co., Kyoto, Japan) equipped with an electrochemical detector (CDD-6A; Shimadzu) on a cation exchange column (Shim-pack IC-C4; Shimadzu) or an anion exchange column (Shim-pack IC-A3; Shimadzu).

2.5. Measurements of root hydraulic conductance, root surface area and $L_{p}$

Root hydraulic conductance was estimated according to Fiscus (1975). Plants were cut at the base of the stem. Roots were placed in a pressure chamber (3005, Soil Moisture Equipment, Santa Barbara, CA, USA) containing Hoagland solution without added NaCl. Pressure of 0.2 MPa was applied to the solution containing the roots for 10 min. Then the pressure was changed stepwise to 0.2, 0.35, 0.25, and 0.3 MPa. The exuded xylem sap was collected for 10 min at each pressure by attaching sanitary cotton at the cut end. The cotton weighed before and after exudate collection to determine the mass of the exudate. Root hydraulic conductance ($m^3 s^{-1} MPa^{-1}$) was calculated as the slope of the line fitted by linear regression of the exudation rate against applied pressure (Figure S1) (Miyamoto et al., 2001). At the end of the measurements, pressure of 0.2 MPa was applied again for 10 min to confirm the exudation rate at 0.2 MPa doesn’t change from that of the first measurement (Figure S1).

Upon completion of the exudation measurements, root systems were removed from the chamber and stored in 70% ethanol. Root surface area was measured with an image analysis system (Win-RHIZO; Regent Instruments, Quebec, Canada). The ratio of root hydraulic conductance to root surface area was calculated for $L_{p}$ (m s$^{-1}$ MPa$^{-1}$) (Miyamoto et al., 2001). Root hydraulic conductance and $L_{p}$ values of three to five plants per replicate were measured and averaged.

The root exudation rate was almost constant for at least 100 min after a given pressure was applied to the solution containing the root system (Figure S2). In the plants with salt treatment, the exudation rate was small, and showed no increase or decrease for approximately 100 min after air pressure was applied to the solution without additional NaCl (Figure S2). $L_{p}$ was kept constant during the time from 10:00 to 20:00 in a day (Figure S3). Based on these results, each measurement was taken between 10:00 and 20:00 within 100 min.

2.6. Extraction of RNA and quantitative PCR

Whole root tissues were collected and immediately frozen in liquid nitrogen. Total RNA was extracted from 100 mg root tissue using an RNaseasy Plant Mini Kit (Qiagen). First-strand cDNA was synthesized using a High Capacity cDNA Reverse Transcript Kit (Applied Biosystems, Waltham, MA, USA). PCR mixtures were prepared using Power SYBR Green PCR Master Mix (Applied Biosystems), and PCR was performed in a 7300 Real Time PCR System (Applied Biosystems) under the following conditions: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. As PIPs are the main aquaporins mediating water transport in root cells (Horie et al., 2011; Javot & Maurel, 2002), the transcript levels of HvPIPs were observed. PCR primer sets are listed in Table S1. Serial dilution of a known copy number of each cRNA was reverse transcribed, and produced cDNA was amplified as a standard for absolute quantification (Katsuhara et al.,...
Two and four independent experiments were conducted for the comparison between the accessions at 1 DAT and 2 DAT, respectively.

### 2.7. Statistical analysis

Accession differences and effects of salt treatment were tested using Tukey’s HSD test. Differences between treatments (T) and accessions (G) and T × G interactions were tested by two-way analysis of variance (ANOVA). All statistical analyses were performed using JMP v.13 software (SAS Institute, Cary, NC, USA).

### 3. Results

#### 3.1. \( A_n \), \( g_s \) and LWP under salt stress

At all days after the onset of treatment (DAT) analyzed, significant difference in \( A_n \), \( g_s \), and intercellular CO\(_2\) concentration (\( C_i \)) was not observed between accessions in the control. Salt treatment decreased \( A_n \), \( g_s \), and \( C_i \) in all measurements except for \( A_n \) and \( C_i \) of OUC613 at 1 DAT (Figure 1). In salt-treated plants, there was not significant accession difference in \( A_n \) and \( g_s \) at 1 DAT although they tended to be lower in OUE812 than in OUC613. \( A_n \) and \( g_s \) became significantly higher in OUE812 than in OUC613 at 2 DAT (Figure 1). Under salt stress, \( C_i \) was significantly lower in OUE812 than in OUC613 at 1 DAT but became significantly higher at 2 DAT (Figure 1). There was no significant difference in \( A_n/ C_i \) between OUC613 and OUE812 at 1 DAT (0.068 ± 0.003 and 0.066 ± 0.009 mol m\(^{-2}\) s\(^{-1}\), respectively) and at 2 DAT (0.067 ± 0.01 and 0.072 ± 0.005 mol m\(^{-2}\) s\(^{-1}\), respectively). The higher \( A_n \), \( g_s \), and \( C_i \) in OUE812 tended to be kept under salt stress at 7 DAT and 14 DAT (Figure S4).

LWP was approximately −0.3 to −0.4 MPa in the control in both accessions (Figure 1). At 1 DAT, salt treatment decreased LWP to approximately −0.6 MPa in both accessions. At 2 DAT, the salt treatment also decreased LWP in both accessions and LWP tended to be higher in OUE812 than in OUC613 in the plants with and without treatment. The reduction of LWP by the salt treatment (T) was significant at 1 and 2 DAT and the accession (G) difference was not significant at 1 DAT but significant at 2 DAT (Table 1). T × G interactions was not significant at 1 and 2 DAT.

#### 3.2. Ion concentrations in leaves under salt stress

Salt treatment markedly increased the leaf concentration of Na\(^+\), with no significant differences between the accessions either in the control or salt treatment at any DAT (Figure 2). The leaf concentration of Cl\(^-\) was significantly higher in OUC613 than in OUE812 in the control and salt treatment at all DAT (Figure 2). The concentration slightly but significantly increased in salt-treated plants, but no difference in the increase was observed between the two accessions. Salt treatment gradually decreased the leaf concentration of K\(^+\) and markedly decreased the ratio of K\(^+\) concentration to Na\(^+\)

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**Table 1. Analysis of variance (ANOVA) of leaf water potential between salt treatments (T) and accessions (G), and T × G interactions.**

|          | T     | G     | T × G |
|----------|-------|-------|-------|
| 1 DAT    | ***   | ns    | ns    |
| 2 DAT    | **    | *     | ns    |

*P<0.05; **P<0.01; ***P<0.001; ns, not significant (two-way ANOVA).
3.3. Root hydraulic conductance and \( L_p \), under salt stress

At 1 DAT, salt treatment did not affect the root hydraulic conductance of both accessions; there was no difference between the accessions either in the control or salt treatment (Figure 3). At 2 DAT, no significant difference in root hydraulic conductance was observed between OUC613 and OUE812 in the control. Salt treatment did not affect root hydraulic conductance in OUE812 but decreased it significantly in OUC613 (Figure 3). At 1 and 2 DAT, difference in root surface area was not significant between the accessions in either the control or salt treatment (Figure 3). At 1 DAT, no significant difference in \( L_p \) was observed between the accessions in either the control or salt treatment (Figure 3). At 2 DAT, salt treatment did not decrease \( L_p \) in OUE812 but decreased it in OUC613, although the reduction was not significant, resulting in a significant difference in \( L_p \) between the accessions with salt treatment (Figure 3). These results indicate that the significant reduction of root hydraulic conductance in OUC613 under salt stress at 2 DAT was resulted from the reduction in \( L_p \).

3.4. Expression of aquaporins under salt stress

The transcript levels of \( \text{Hv} \text{PIP1;2}, \text{Hv} \text{PIP1;3}, \text{Hv} \text{PIP1;4}, \) and \( \text{Hv} \text{PIP2;1} \) were higher than those of other \( \text{Hv} \text{PIPs} \) at 1 DAT concentration, with no difference between the accessions (Figure 2).
and 2 DAT, and those of HvPIP1;2, HvPIP2;1, and HvPIP2;5 increased at 2 DAT in both accessions regardless of salt treatment (Figure 4). At 1 and 2 DAT, there was no difference in the transcript levels between the two accessions for all HvPIPs in the control except for HvPIP2;3 (Figure 4), and no accession difference was observed in the total transcript level (Figure S5). Salt treatment had no significant effects on the HvPIP transcript levels in either accession at 1 DAT. Salt treatment affected the transcript levels of HvPIPs differently between OUC613 and OUE812 at 2 DAT (Figure 4). The transcript levels of HvPIP1;2, HvPIP1;3, and HvPIP2;1 decreased significantly or tended to decrease in salt-treated OUC613. In contrast, a large and significant increase in the transcript levels of HvPIP1;3, HvPIP1;4, and HvPIP2;1 was observed in salt-treated OUE812 in comparison with the control. In the salt treatment, the transcript levels of HvPIP1;2, HvPIP1;3, HvPIP1;4, and HvPIP2;1 were higher in OUE812 than in OUC613 (Figure 4) and the total transcript level was significantly higher in OUE812 than that in OUC613 at 2 DAT (Figure S5).

4. Discussion

Under salt stress, A_n and g_s decreased in both of the salt-tolerant accession OUE812 and the salt-sensitive accession OUC613 (Figure 1). A_n and g_s did not differ between the accessions at 1 DAT, but became higher in OUE812 than OUC613 at 2 DAT. Physiological mechanisms underlying the accession difference in A_n and g_s under salt stress for 2 days were discussed.

![Figure 4](image_url)

*Figure 4*. Representative transcript levels of aquaporin genes in OUC613 and OUE812 at 1 and 2 days after the onset of treatment (DAT). Error bars represent SD (n = 3). The same letters represent no significant difference at the 5% level by Tukey’s HSD test.
4.1. Ion accumulation in leaves was not a cause of the accession difference in $A_n$ under salt stress at 2 DAT

Most plants let in only 2% of Na$^+$ or Cl$^-$ in the soil solution owing to their salt exclusion mechanisms (Munns, 2005). In the current study, leaf Na$^+$ and Cl$^-$ concentration increased in plants with salt treatment, but the rate of the increase per day was a few % or less of the estimated value for the plants without salt exclusion mechanisms. Previously we have shown that OUE812 maintained high $A_n$ in comparison with OUC613 under long-term salt stress, and OUE812 kept low leaf Na$^+$ and high leaf K$^+$/Na$^+$ ratio (Hirasawa et al., 2017) probably due to a high ion exclusion capacity compared with OUC613 (Rivandi et al., 2011; Shavrukov et al., 2010). Compared with OUC613, OUE812 showed high $g_s$, i.e., high transpiration rate under salt treatment (Figure 1), but showed no large increase in the concentration of Na$^+$ or Cl$^-$ in leaves (Figure 2). This suggests that OUE812 has a high ion excluding capacity even at the seedling stage. $A_n$ was kept higher in OUE812 than in OUC613 at 2 DAT under salt stress (Figure 1). However, there was no difference in the increase of leaf Na$^+$ and Cl$^-$ concentrations and in the decrease of leaf K$^+$/Na$^+$ ratio between the accessions (Figure 2). These results suggest that the accession difference in $A_n$ under salt stress at 2 DAT was not caused by the difference in ion accumulation in leaves.

4.2. Stomatal limitation of photosynthesis was a major cause of the accession difference in $A_n$ under salt stress at 2 DAT

$C_i$ is determined by the diffusion rate of CO$_2$ into the substomatal cavity from the atmosphere through stomata and the rate of CO$_2$ fixation in mesophyll cells. We can estimate that the major limitation of photosynthesis is the decreased CO$_2$ diffusion into the substomatal cavity by the stomatal closure (stomatal limitation of photosynthesis) for the leaf where $C_i$ decreases along with the reduction in $A_n$, and is the inhibited photosynthesis metabolism in mesophyll cells (biochemical limitation of photosynthesis) for the leaf where $C_i$ increases along with the reduction in $A_n$ (Hirasawa et al., 1989; Kramer & Boyer, 1995). In the salt treatment, $C_i$ decreased along with the reduction in $A_n$, and the reduction in both $A_n$ and $C_i$ was larger in OUC613 than in OUE812 at 2 DAT with no accession difference in $A_n/C_i$ (Figure 1). These results indicate that the reduction in $A_n$ under salt stress in both accessions and the larger reduction of $A_n$ in OUC613 were caused mainly by the reduction in $g_s$ (stomatal limitation of photosynthesis), not by biochemical limitation of photosynthesis in the mesophyll. The mechanism involved in the accession difference in $A_n$ reduction differs in the current study from that of the previous study under long-term salt stress where the main cause of the accession difference in $A_n$ was biochemical limitation in the mesophyll due to salt accumulation (Hirasawa et al., 2017).

4.3. Difference in root hydraulic conductance is a probable cause of the accession difference in $A_n$ and $g_s$ under salt stress at 2 DAT

$A_n$ and $g_s$ decreased both in OUE812 and OUC613 under salt stress. OUE812 kept high root hydraulic conductance even under salt stress at 2 DAT and maintained high $A_n$ and $g_s$ compared with OUC613 in which root hydraulic conductance decreased (Figures 1 and Figures 3). It is well known that $A_n$ and $g_s$ decrease in the plants under water stress and the reduction is remarkable in the plants with low hydraulic conductance compared to those with high hydraulic conductance (Christmann et al., 2013; Comstock, 2002; Hirasawa, 2018). The results of the current study were consistent with the results of many previous studies, and it was suggested that the difference in root hydraulic conductance was a probable cause of the accession difference in $A_n$ and $g_s$ under salt stress at 2 DAT, and keeping high root hydraulic conductance under salt stress might be a key trait for salt tolerance.

Physiological mechanisms underlying the relations between $g_s$ (or $A_n$) and root hydraulic conductance have not been uncovered sufficiently yet. Plants with high hydraulic conductance could keep high leaf water potential compared to plants with low hydraulic conductance when transpiration rate is same in both plants (Hirasawa, 2018). Many studies showed the decrease of $A_n$ and $g_s$ with no clear reduction of LWP under soil moisture stress and close correlations of $A_n$ or $g_s$ with the plant hydraulic conductance (Christmann et al., 2013; Comstock, 2002; Hirasawa, 2018; Hubbard et al., 2001; Meinzer, 2002; Salindra et al., 1995). It is suggested that $A_n$ and $g_s$ are primarily modulated by hydraulic signals (Christmann et al., 2013, 2007; Tombesi et al., 2015). In the current study, the accession difference in $A_n$ and $g_s$ at 2 DAT in salt treatment was consistent with the accession difference in root hydraulic conductance, but not with the difference in LWP (Figures 1 and Figures 3). Hydraulic signals or other signals might underlie the relations between $g_s$ ($A_n$) and root hydraulic conductance in the current study (Christmann et al., 2013, 2007; McAdam & Brodribb, 2014). The reduction in $g_s$ suppresses the reduction in LWP and this might make it difficult to find a close correlation of $A_n$ and $g_s$ with LWP in the plants especially
under mild osmotic stress like in the current study. LWP tended to be lower in OUC613 than in OUE812 (Figure 1, Table 1). LWP might decrease beyond the critical value of stomatal closure in the plants with salt treatment and cause the larger reduction in $g_s$ and $A_n$ in OUC613 (Figure 1).

4.4. *Regulation of aquaporin genes expression is a probable cause of the accession differences in $L_p$, root hydraulic conductance and $A_n$ under salt stress at 2 DAT*

The accession difference in root hydraulic conductance was caused by the accession difference of $L_p$, in response to salt stress (Figure 3). Changes in aquaporin gene expression corresponded to the accession difference in $L_p$ in the salt treatment at 2 DAT (Figures 3 and Figures 4, S5). Among the PIPs of barley, HvPIP1;2, HvPIP1;3, HvPIP2;1, HvPIP2;2, HvPIP2;4, and HvPIP2;5 are relatively highly expressed (Horie et al., 2011; Katsuhara et al., 2011). In line with these reports, we found relatively high transcript levels of HvPIP1;2, HvPIP1;3, HvPIP1;4, and HvPIP2;1 in both accessions regardless of salt treatment (Figure 4). In Arabidopsis, the reduction in expression of aquaporin genes under salt treatment is larger for highly expressed genes than for genes expressed at low levels (Boursiac et al., 2005). In the current study, changes in transcript levels in response to salt treatment also tended to be large for highly expressed genes.

Salt stress decreases the expression of root aquaporins in Arabidopsis and barley (Afzal et al., 2016; Boursiac et al., 2005; Horie et al., 2011; Katsuhara et al., 2011), but increases it in ice plant (Kirch et al., 2000) and sweet orange (Martins et al., 2015). The response of aquaporin expression to salinity depends on the duration of salt stress (Aroka et al., 2012). Transcription of PIPs was down-regulated within 1 day after the onset of salinity (Boursiac et al., 2005; Horie et al., 2011; Katsuhara et al., 2011); it was up-regulated under salinity for 14 days (Kirch et al., 2000) or 20 days (Martins et al., 2015). In our study, the expression of aquaporin genes was not affected by salt treatment at 1 DAT in either accession and the expression of some HvPIP genes increased in OUE812 at 2 DAT, but decreased in OUC613 (Figure 4). Water flow into roots at a given water potential difference between soil (culture solution) and roots would be increased by the up-regulation of root aquaporin genes. The effects of salt stress are reportedly mitigated by the overexpression of aquaporin genes (Hu et al., 2012; Sreedharan et al., 2013). The increase in the expression of HvPIP genes might keep high water uptake by maintaining high $L_p$, and high root hydraulic conductance in OUE812 under salt treatment at 2 DAT, and, on the contrary, water uptake might decrease markedly in OUC613 under salt treatment by the suppression of HvPIPs expression and the decreased $L_p$, at 2 DAT (Figures 3 and Figures 4, S5). Some accessions of Arabidopsis do not decrease hydraulic conductivity of a root cell ($L_p$) and probably $L_p$, in salt treatment (Sutka et al., 2011). However, how the difference in $L_p$ is associated with the accession difference in salt tolerance was unclear. Recently, the higher expression of root aquaporin genes and larger $L_p$, have been observed in salt stress tolerant wild barley compared with cultivated barley (Kreszies et al., 2020). Our results suggest that changes in the expression of HvPPIPs in response to salt stress cause the difference in $A_n$, and $g_s$, between the cultivated barley accessions under salt stress through changes in $L_p$, and root hydraulic conductance.

Both the amount and activity of root aquaporins affect $L_p$, (Horie et al., 2011; Johansson et al., 1998; Kaneko et al., 2015; Maurel et al., 1995; Van Wilder et al., 2008). Cultivar difference in aquaporin activity was assumed in barley within 24 h after the onset of salt stress treatment (Kaneko et al., 2015). It was reported recently that osmotic stress decreases $L_p$, by suberization in barley (Kreszies et al., 2020, 2019). Although we did not examine the connection of aquaporin activity and suberization with the accession difference in $L_p$, our results reveal that changes in the expression of aquaporin genes induced by salt treatment cause the accession difference in $L_p$, which is associated with the accession difference in $A_n$ and $g_s$. Mapping populations derived from OUE812 and OUC613 developed by Kodama et al. (2018) can be a useful material to identify quantitative trait loci (loci) regulating the HvPIP expression level under salt stress. It would provide the beneficial knowledge for the future breeding to improve the salt tolerance in barley.

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References

Afzal, Z., Howton, T. C., Sun, Y., & Mukhtar, M. S. (2016). The roles of aquaporins in plant stress responses. Journal of Developmental Botany, 4(1), 9. https://doi.org/10.3390/jdb4010009

Aroca, R., Porcel, R., & Ruiz-Lozano, J. M. (2012). Regulation of root water uptake under abiotic stress conditions. Journal of Experimental Botany, 63(1), 43–57. https://doi.org/10.1093/jxb/err266

Boursiac, Y., Chen, S., Luu, D.-T., Sorieu, 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 Physiology, 139(2), 790–805. https://doi.org/10.1104/pp.105.065029

Chaumont, F., & Tyerman, S. D. (2014). Aquaporins: Highly regulated channels controlling plant water relations. Plant Physiology, 164(4), 1600–1618. https://doi.org/10.1104/pp.113.233791

Christmann, A., Grill, E., & Huang, J. (2013). Hydraulic signals in long-distance signaling. Current Opinion in Plant Biology, 16 (3), 293–300. https://doi.org/10.1016/j.pbi.2013.02.011

Christmann, A., Weiler, E. W., Steudle, E., & Grill, E. (2007). A hydraulic signal in root-to-shoot signaling of water shortage. The Plant Journal, 52(1), 167–174. https://doi.org/10.1111/j.1365-313X.2007.03234.x

Colmer, T. C., Flowers, T. J., & Munns, R. (2006). Use of wild relatives to improve salt tolerance in wheat. Journal of Experimental Botany, 57(5), 1059–1078. https://doi.org/10.1093/jxb/erj124

Comstock, J. P. (2002). Hydraulic and chemical signaling in the control of stomatal conductance and transpiration. Journal of Experimental Botany, 53(367), 195–200. https://doi.org/10.1093/jexbot/53.367.195

Fiscus, E. L. (1975). The interaction between osmotic- and pressure-induced water flow in plant roots. Plant Physiology, 55(5), 917–922. https://doi.org/10.1104/pp.55.5.917

Hirasawa, T. (2018). Leaf photosynthesis of upland and lowland crops grown under moisture-rich conditions. In W. W. Adams III & I. Terashima (Eds.), The leaf: A platform for performing photosynthesis (pp. 345–369). Springer Nature

Hirasawa, T., Iida, Y., & Ishihara, K. (1989). Dominant factors in reduction of photosynthetic rate affected by air humidity and leaf water potential in rice plants. Japanese Journal of Crop Science, 58(3), 383–389. https://doi.org/10.1626/jcs.58.383

Hirasawa, T., Sato, K., Yamaguchi, M., Narita, R., Kodama, A., Adachi, S., & Sato, K. (2017). Differences in dry matter production, grain production, and photosynthetic rate in barley cultivars under long-term salinity. Plant Production Science, 20(3), 288–299. https://doi.org/10.1080/1343943X.2017.1343647

Horie, T., Kaneko, T., Sugimoto, G., Sakano, S., Pandra, S. K., Shibasaka, M., & Katsuhara, M. (2011). Mechanisms of water transport mediated by PIP aquaporins and their regulation via phosphorylation events under salinity stress in barley roots. Plant & Cell Physiology, 52(4), 663–675. https://doi.org/10.1093/pcp/pcr027

Horie, T., Karahara, I., & Katsuhara, M. (2012). Salinity tolerance mechanisms in glycoproteins: An overview with the central focus on rice plants. Rice, 5(1), 11. https://doi.org/10.1186/1939-8433-5-11

Hu, W., Yuan, Q., Wang, Y., Cai, R., Deng, X., Wang, J., & He, G. (2012). Overexpression of a wheat aquaporin gene, TaAQPB, enhances salt stress tolerance in transgenic tobacco. Plant & Cell Physiology, 53(12), 2127–2141. https://doi.org/10.1093/pcp/pcs154

Hubbard, R. M., Ryan, M. G., Stiller, V., & Sperry, J. S. (2001). Stomatal conductance and photosynthesis vary linearly with plant hydraulic conductance in ponderosa pine. Plant, Cell & Environment, 24(1), 113–121. https://doi.org/10.1046/j.1365-3040.2001.00660.x

Javot, H., & Maurel, C. (2002). The role of aquaporins in root water uptake. Annals of Botany, 90(3), 301–313. https://doi.org/10.1093/aob/mcf199

Johansson, I., Karlsson, M., Shukla, V. K., Chrispeels, M. J., Larsson, C., & Kjellborn, P. (1998). Water transport activity of the plasma membrane aquaporin PM28A is regulated by phosphorylation. The Plant Cell, 10(3), 451–459. https://doi.org/10.1105/tpc.10.3.451

Kaneko, T., Horie, T., Nakahara, Y., Tsuij, N., Shibaoka, M., & Katsuhara, M. (2015). Dynamic regulation of the root hydraulic conductivity of barley plants in response to salinity/ osmotic stress. Plant & Cell Physiology, 56(5), 875–882. https://doi.org/10.1093/pcp/pcv013

Katsuhara, M., Phee, J. Y., Sugimoto, G., & Chung, G. C. (2011). Early response in water relations influenced by NaCl reflects tolerance or sensitivity of barley plants to salinity stress via aquaporins. Soil Science and Plant Nutrition, 57(1), 50–60. https://doi.org/10.1080/00380768.2010.541870

Kirch, H. H., Vera-Estrella, R., Golldack, D., Quigley, F., Michalowski, C. B., Barkla, B. J., & Bohnert, H. J. (2000). Expression of water channel proteins in mesembryanthemum crystallinum. Plant Physiology, 123(1), 111–124. https://doi.org/10.1104/pp.123.1.111

Kodama, A., Narita, R., Yamaguchi, M., Hisano, H., Adachi, S., Takagi, H., Okawa, T., Sato, K., & Hirasawa, T. (2018). QTLs maintaining grain fertility under salt stress detected by exome QTL-seq and interval mapping in barley. Breeding Science, 68(5), 561–570. https://doi.org/10.1270/jsbbs.18082

Kramer, P. J., & Boyer, J. S. (1995). Water relations of plants and soils. Academic Press

Kreszies, T., Eggles, S., Kreszies, V., Osthoff, A., Shellakkutti, N., Baldauf, J. A., Zeiser-Diehl, V. V., Hochholding, F., Ranathunge, K. & Schreiber, L. (2020). Seminal roots of wild and cultivated barley differentially respond to osmotic stress in gene expression, suberization, and hydraulic conductivity. Plant, Cell & Environment, 43(2), 344–357. https://doi.org/10.1111/pce.13675

Kreszies, T., Shellakkutti, N., Osthoff, A., Yu, P., Baldauf, J. A., Zeiser-Diehl, V. V., Ranathunge, K., Hochholding, F., & Schreiber, L. (2019). Osmotic stress enhances suberization of apoplastic barriers in barley seminal roots: Analysis of chemical,
transcriptomic and physiological responses. *New Phytologist*, 227(1), 180–194. https://doi.org/10.1111/nph.15351

Mano, Y. (2007). Studies on the breeding and evaluation of germplasm for salt tolerance in barley: Special report of the barley germplasm center. Research Institute for Bioresources, Okayama University.

Martins, C. P. S., Pedrosa, A. M., Du, D., Gonçalves, L. P., Yu, Q., Gmitter, F. G., Jr., & Costa, M. G. C. (2015). Genome-wide characterization and expression analysis of major intrinsic proteins during abiotic and biotic stresses in sweet orange (*Citrus sinensis* L. Osb.). *PLoS One*, 10(9), e0138786. https://doi.org/10.1371/journal.pone.0138786

Maurel, C., Boursiac, Y., Luu, D.-T., Santoni, V., Shahzad, Z., & Verdoucq, L. (2015). Aquaporins in plants. *Physiological Reviews*, 95(4), 1321–1358. https://doi.org/10.1152/physrev.00008.2015

Maurel, C., Kado, R. T., Guern, J., & Chrispeels, M. J. (1995). Phosphorylation regulates the water channel activity of the seed-specific aquaporin α-TIP. *The EMBO Journal*, 14(13), 3028–3035. https://doi.org/10.1002/j.1460-2075.1995.tb07305.x

McAdam, S. A. M., & Brodribb, T. J. (2014). Separating active and passive influences on stomatal control of transpiration [OPEN]. *Plant Physiology*, 164(4), 1578–1586. https://doi.org/10.1104/pp.113.231944

Meinzer, F. C. (2002). Co-ordination of vapour and liquid phase water transport properties in plants. *Plant, Cell & Environment*, 25(2), 265–274. https://doi.org/10.1046/j.1365-3040.2002.00781.x

Miyamoto, N., Steudle, E., Hirasawa, T., & Lafitte, R. (2001). Hydraulic conductivity of rice roots. *Journal of Experimental Botany*, 52(362), 1835–1846. https://doi.org/10.1093/jexbot/52.362.1835

Munns, R. (2005). Genes and salt tolerance: Bringing them together. *New Phytologist*, 167(3), 645–663. https://doi.org/10.1111/j.1469-8137.2005.01487.x

Munns, R., James, R. A., & Lauchli, A. (2006). Approaches to increasing the salt tolerance of wheat and other cereals. *Journal of Experimental Botany*, 57(S), 1025–1043. https://doi.org/10.1093/jxb/erj100

Munns, R., James, R. A., Xu, B., Athman, A., Conn, S. J., Jordans, C., & Gillham, M. (2012). Wheat grain yield on saline soils is improved by an ancestral Na⁺ transporter gene. *Nature Biotechnology*, 30(4), 360–364. https://doi.org/10.1038/nbt.2120

Munns, R., & Tester, M. (2008). Mechanisms of salinity tolerance. *Annual Review of Plant Biology*, 59(1), 651–681. https://doi.org/10.1146/annurev.arplant.59.032607.092911

Rawson, H. M., Richards, R. A., & Munns, R. (1988). An examination of selection criteria for salt tolerance in wheat, barley and triticale genotypes. *Australian Journal of Agricultural Research*, 39(5), 759–772. https://doi.org/10.1071/AR9880759

Richards, R. A., Dennett, C. W., Quaisel, C. O., Epstein, E., Norlyn, J. D., & Winslow, M. D. (1987). Variation in yield of grain and biomass in wheat, barley, and triticale in a salt-affected field. *Field Crops Research*, 15(3–4), 277–287. https://doi.org/10.1016/0378-4290(87)90017-7

Rivandi, J., Miyazaki, J., Hrmova, M., Pallotta, M., Tester, M., & Collins, N. C. (2011). A SOS3 homologue maps to HvNAX4, a barley locus controlling an environmentally sensitive Na⁺ exclusion trait. *Journal of Experimental Botany*, 62(3), 1201–1216. https://doi.org/10.1093/jxb/erq346

Royo, A., & Aragues, R. (1999). Salinity-yield response functions of barley genotypes assessed with a triple line source sprinkler system. *Plant and Soil*, 209(1), 9–20. https://doi.org/10.1023/A:1004549927123

Saliendra, N. Z., Sperry, J. S., & Comstock, J. P. (1995). Influence of leaf water status on stomatal response to humidity, hydraulic conductance, and soil drought in *Betula occidentalis*. *Planta*, 196(2), 357–366.

Shabala, S., Shabala, S., Cuin, T. A., Pang, J., Perry, W., Chen, Z., & Wegner, L. H. (2010). Xylem ionic relations and salinity tolerance in barley. *The Plant Journal*, 61(5), 839–853. https://doi.org/10.1111/j.1365-313X.2009.04110.x

Shavrukov, Y., Gupta, N. K., Miyazaki, J., Baho, M. N., Chalmers, K. J., Tester, M., Langridge, P., & Collins, N. C. (2010). HvNAX3—a locus controlling shoot sodium exclusion derived from wild barley (*Hordeum vulgare* ssp. *spontaneum*). *Functional & Integrative Genomics*, 10(2), 277–291. https://doi.org/10.1007/s10142-009-0153-8

Slavich, P. G., Read, B. J., & Cullis, B. R. (1990). Yield response of barley germplasm to field variation in salinity quantified using the EM-38. *Australian Journal of Experimental Agriculture*, 30(4), 551–556. https://doi.org/10.1071/EA990551

Sreedharan, S., Skekhwat, U. K. S., & Ganapathi, T. R. (2013). Transgenic banana plants overexpressing a native plasma membrane aquaporin *MusapPIP1* display high tolerance levels to different abiotic stresses. *Plant Biotechnology Journal*, 11(8), 942–952. https://doi.org/10.1111/pbi.12086

Sutka, M., Li, G., & Bouder, J. (2011). Natural variation of root hydraulics in Arabidopsis grown in normal and salt-stressed conditions. *Plant Physiology*, 155(3), 1264–1276. https://doi.org/10.1104/pp.111.163113

Tombesi, S., Nardini, A., Frioni, T., Soccollini, M., Zadrea, C., Farinelli, D., Poni, S. & Pallioti, A. (2015). Stomatal closure is induced by hydraulic signals and maintained by ABA in drought-stressed grapevine. *Scientific Reports*, 5(1), 12449. https://doi.org/10.1038/srep12449

Tsuchiya, M., Miyake, M., & Naito, H. (1994). Physiological response to salinity in rice plant. III. A possible mechanism for Na⁺ exclusion in rice root under NaCl-stress conditions. *Japanese Journal of Crop Science*, 63(2), 326–332. https://doi.org/10.1626/jcs.63.326

Van Wilder, V., Mielcicza, U., Degand, H., Derua, R., Waekens, E., & Chaumont, F. (2008). Maize plasma membrane aquaporins belonging to the PIP1 and PIP2 subgroups are in vivo phosphorylated. *Plant & Cell Physiology*, 49(9), 1364–1377. https://doi.org/10.1093/pcp/pcn112

Yamanouchi, M. (1989). The mechanism of salinity tolerance in rice plants. Relationship between the varietal difference of salinity tolerance and characteristics of absorption and translocation of sodium ion (2). *Japanese Journal of Soil Science and Plant Nutrition*, 60(3), 210–219. https://doi.org/10.20710/doi.jso.60.3.210

Zhu, M., Zhou, M., Shabala, L., & Shabala, S. (2016). Physiological and molecular mechanisms mediating xylem Na⁺ loading in barley in the context of salinity stress tolerance. *Plant & Cell Environment*, 40(7), 1009–1020. https://doi.org/10.1111/pce.12727