Effect of salinity on water relations of wild barley plants differing in salt tolerance

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Abstract

Background and aims

Certain lines of wild barley (Hordeum spontaneum) are more tolerant of salinity than others. The physiological basis of this difference is examined in a comparative study of a saline-tolerant and saline-intolerant line that emphasizes plant water relations.

Methodology

Effects of salt-treatment (75 mM maximum) extending from a few hours to 3 weeks were quantified in 8-day-old seedlings of a saline-sensitive wild barley line ('T-1') and a less saline-sensitive line ('20-45'). Plants were grown in nutrient culture. Levels of mRNA of the HtPIP2;4 aquaporin (AQP) gene were determined together with a range of physiological responses including root hydraulic conductivity, osmotic potential of root xylem sap, transpiration, leaf relative water content, root water content, leaf water potential, leaf sap osmolality, leaf length, leaf area and chlorophyll content.

Principal results

Salt treatment inhibited transpiration and hydraulic conductivity more in salt-tolerant ‘20-45’ plants than in salt-sensitive ‘T-1’. In ‘20-45’, the effect was paralleled by a fast (within a few hours) and persistent (3 days) down-regulation of aquaporin. In salt-sensitive ‘T-1’ plants, aquaporin down-regulation was delayed for up to 24 h. Greater tolerance in ‘20-45’ plants was characterized by less inhibition of leaf area, root fresh weight, leaf water content and chlorophyll concentration. Leaf water potentials were similar in both lines.

Conclusions

(i) Decline in hydraulic conductivity in salt-treated barley plants is important for stomatal closure, (ii) lowered transpiration rate is beneficial for salt tolerance, at least at the seedling stage and (iii) changes in AQP expression are implicated in the control of whole plant hydraulic conductivity and the regulation of shoot water relations.

Introduction

Salinity is an important environmental factor that can severely inhibit plant growth and agricultural productivity. Crop improvement for saline conditions requires an understanding of the mechanisms enabling salt tolerance. In addition to the toxic effects of the
sodium and chloride ions, salinity disturbs the plant’s water relations due to decreased availability of water from soil solution as a result of lowered osmotic potential (Munns, 2005). The stomatal closure often observed in salt-treated plants ameliorates tissue dehydration by limiting water losses (Fricke et al., 2004). It may also limit accumulation of toxic ions in plants since their rate of upward passage in the xylem is largely determined by the rate of transpiration (Kerstiens et al., 2002). Previous comparison of water relations in barley cultivars contrasting in drought resistance showed that, at the beginning of salt action, lower stomatal conductivity and transpiration contributed to higher salt tolerance in terms of improved extension growth and smaller accumulation of toxic ions (Veselov et al., 2008). Stomatal closure in salt-stressed plants may be induced by accumulation of abscisic acid (Mulholland et al., 2003; Fricke et al., 2004; Veselov et al., 2008). However, Tardieu and Simonneau (1998) concluded that the maintenance of water relations may be linked to an interaction between both chemical and hydraulic information. There is mounting evidence that stomatal regulation of vapour loss is exquisitely sensitive to short-term, dynamic perturbations of liquid water transport (Cochard et al., 2002; Meinerz, 2002; Bunce, 2006). These observations emphasize the importance of the study of regulation of stomatal and hydraulic conductivity in salt-stressed plants.

Under transpiring conditions, water is thought to come from the soil to the root xylem mostly along an apoplastic path driven by a hydrostatic pressure gradient. However, this situation changes when transpiration is restricted by stressful conditions such as salinity. Under these circumstances, more of the water follows the cell-to-cell path, flowing across membranes of living cells (Steudle, 2000). Recent results indicate that, in the cell-to-cell path, most of the water is transported by aquaporins (i.e., membrane proteins forming water channels) (Morillon and Chrispeels, 2001). Plasma membrane aquaporins belonging to the PIP2 subfamily are one of the abundant isoforms in roots and display high water channel activity when expressed in heterologous cells (Chaumont et al., 2000; Javot et al., 2003). The expression of maize PIP2;4 aquaporin has been found by Zhu et al. (2005) to be responsive to salt treatment.

Young barley plants are an attractive object for the study of aquaporins in stressed plants, since in their roots the cell-to-cell transport pathway dominates even in highly transpiring plants (Steudle and Jeschke, 1983). The present study investigates how changes in hydraulic conductivity and expression of the aquaporin (AQP) gene may be related to the transpiration rate and consequences for shoot water relations and salt tolerance in terms of improved extension growth. The AQP gene was analysed by quantitative RT–PCR in roots of two wild barley lines which differ in salt resistance defined in terms of their growth responses to salt.

Materials and methods

All experiments were carried out using two wild barley (Hordeum spontaneum) lines (‘T-1’ and ‘20-45’). ‘T-1’ was originally identified in a wild barley population 50 km west of Gaziantep, Turkey (800 m altitude, longitude 37.18 E, latitude 36.82 N) and ‘20-45’ originated from Sede Boqer in Israel (450 m altitude, longitude 34.78 E, latitude 30.78 N) (Nevo et al., 1986). These lines were selected as being morphologically comparable but having contrasting responses to salinity in a preliminary hydroponics trial carried out by M Chechulina, BP Forster and HG Jones (unpubl. res.) that included a diverse collection of 36 H. spontaneum lines from across the ‘Fertile Crescent’ (Forster et al., 1997; Pakniyat et al., 1997) and a small number of Hordeum vulgare cultivars and semi-dwarf mutants that have associations with salt tolerance (Pakniyat et al., 1997).

Plants were grown for 3 days in darkness at 25 °C between glass tubes sealed at the ends, tied together and floated on a 0.1 strength Hoagland–Arnon nutrient solution and then hydroponically in 2 L containers (10 plants per container, 10 containers for each treatment) with full Hoagland–Arnon nutrient solution (5 mM KNO₃, 5 mM Ca(NO₃)₂, 1 mM KH₂PO₄, 1 mM MgSO₄, 5 mM CaSO₄, changed daily) under illumination of 450 μmol photon m⁻² s⁻¹ from mercury arc and sodium vapour lamps with a 14-h photoperiod at 24 °C. Salinity treatments were applied when plants were 8 days old. Plants were exposed to 25 mM NaCl for 24 h, then the concentration was first increased to 50 mM NaCl for 2 days and then to 75 mM NaCl (moderate salinity level, which may be expected in the field according to Munns et al., 2006). Salt concentration was raised in 25 mM increments to diminish any shock effect of a sudden osmotic stress (Flowers, 2004). Some plants remained in 75 mM NaCl for 3 weeks to allow a study of long-term effects of salinity on plant growth and chlorophyll content. All treatments were compared with similar aged control plants maintained in the standard solution.

Changes in leaf length and width of not less than 10 plants grown in different containers were measured frequently after the start of the experiment using a ruler. Estimates of leaf area were made by scanning the leaves and by analysis of the scanned image. Transpiration rates were measured gravimetrically as the rate of water loss over 20 min from containers with 10 plants
placed in 50 mL of nutrient solution (n = 10, one container with 10 plants comprising each replicate). The containers were sealed with aluminium foil and tape to prevent the evaporative loss of water. Water flux, osmotic potential of xylem sap, relative water content (RWC) of leaves and root water content were measured when plants were sampled for final expression measurements (n = 5, 3–10 plants comprising each replicate and the number of plants in a replicate depending on characteristics measured). Water flux from the detached root systems was measured as described by Vysotskaya et al. (2004). In short, the aerial parts of the plant were removed, leaving a cylinder of leaf bases, which were then connected to a thin glass capillary by means of a silicone tube (the weight of each capillary was measured prior to connecting to the root system). The weight of the capillary containing xylem sap was measured again after 1 h. Xylem sap from 10 capillaries was collected for measuring osmotic potential (joint exudates from roots of 10 plants comprising one of five replicates). Hydraulic conductivity (Lp) was calculated according to the equation: hydraulic conductivity = Jv/ΔΨπ, where Jv is the water flux (calculated as volume per unit fresh weight of root) and ΔΨπ is the difference between the osmotic pressure of xylem sap and that of the nutrient medium. Sap samples for the measurement of tissue osmotic potential were obtained after freezing and thawing of all the leaves of 28-day-old plants and expressing sap by squeezing in a syringe. Osmotic potential of xylem sap and leaves was measured with a freezing point depression osmometer (CAMLAB Limited, UK). Relative water content was measured by floating leaf pieces of known fresh weight on distilled water for 13–14 h at 22 °C during 4 h. The turgid weight (TW) was then determined after blotting and the dry weight (DW) determined as the constant weight reached by the samples at 80 °C during 4 h. Relative water content was calculated from RWC = FW–DW/TW–DW. Root water content was measured as the difference between fresh and dry weights expressed as a percentage of FW. Chlorophyll was determined as described by Wintermans and Mots (1965).

Root samples were harvested in triplicate pools (1 g of roots collected from several plants of the same container for each replicate) and flash-frozen in liquid nitrogen. Total RNA was isolated as recommended using the RNeasy Mini Plant Kit (Qiagen), including on-column DNase1 digestion. Purified RNA was QC’d in a Bioanalyzer (Agilent) and 10 μg used for the synthesis of first-strand complementary DNA (cDNA) with random hexamer primers and Ready-To-Go-You-Prime Beads (AP biotech). Primers used for the detection of barley AQP (For: ggccttcgcgtttcatg; Rev: ggccttcgcgtttctatc) and 26S rRNA (For: gaagagccgacatcgaagga; Rev: gaaaggtcccccagggatactg) genes were designed from the respective 3′-untranslated regions. PCR reactions contained 1 × QuantiTect SYBR Green PCR Master Mix (Qiagen), 1 μM each primer and 5 ng of cDNA in a volume of 25 μL. Real-time RT–PCR was carried out on an ABI PRISM7700 (Applied Biosystems), with thermal cycling conditions of 95 °C, 15 min, followed by 40 cycles (95 °C, 15 s; 58 °C, 30 s; 72 °C, 30 s). Relative quantification was calculated using the comparative cycle threshold (Ct) method as described by the manufacturer with 26S rRNA for input RNA normalizations (PE Applied Biosystems (2001) User Bulletin#2).

Results
In control plants (grown without NaCl), transpiration by whole plants was higher in line ‘20-45’ than in ‘T-1’, but since leaf area was greater in ‘20-45’, no difference in transpiration was apparent when transpiration was calculated on a per unit leaf area basis (Table 1). Addition of salt over 3 days to a maximum of 75 mM NaCl decreased transpiration, in both lines. In salt-tolerant ‘20-45’, the effect was statistically significant 24 h after the start of treatment, but only after 72 h in salt-sensitive ‘T-1’ and to a smaller extent (Fig. 1). Most plant water relation parameters were measured 3 h after transfer of the plants to the final maximum concentration of 75 mM NaCl and these are summarized in Table 1. At this time, the relative reduction in xylem flux (measured in detached roots) was substantially greater for line ‘20-45’ (48 %) than for line ‘T-1’ (22 %). This difference in flow rate sensitivity between the lines could not be attributed to differences in the driving force for water flow (ΔΨπ) since this was very similar in both lines and treatments. However, it was more closely related to a change in calculated root hydraulic conductivity (Table 1), which was reduced much more in salt-tolerant ‘20-45’ (by 28.3 %) than in ‘T-1’ (43.1 %). Water content of roots was decreased slightly by salt treatment, the effect being significant only in the case of ‘T-1’ plants. Relative water content of the shoot was reduced by salt treatment by a similar extent (3 %) in both ‘T-1’ and ‘20-45’ plants. At the same time, when all the characteristics presented in Table 1 were measured (i.e. 72 h after the start of the salt treatment and 3 h after transfer of plants to 75 mM NaCl), transpiration from whole plants (Fig. 1) was decreased in salt solution for both ‘T-1’ and ‘20-45’. The inhibition was greater in ‘20-45’, suggesting that the decline in stomatal conductance of the two lines paralleled changes of hydraulic conductance and brought about a similar decline in shoot hydration (RWC) in both lines.
The time course of changes in AQP gene expression in salt-treated plants is summarized for ‘T-1’ and ‘20-45’ in Fig. 2. Expression data are shown relative to untreated controls. This approach was adopted because the transcript level tended to increase with plant age, possibly related to the development of lateral roots. AQP gene expression was slightly down-regulated in ‘20-45’ plants 0.5 h after the initiation of salt treatment. The down-regulation increased and became statistically significant by 2 h ($P < 0.05$) with the effect persisting to the end of the experiment (72 h), by which time the NaCl concentration had been raised to 75 mM. ‘T-1’ was much less sensitive to salt, with down-regulation only apparent after 24 h, with a slight increase in expression.

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Table 1 Comparison of the short-term effects of salt treatment on water relations of lines of wild barley with high ('20-45') or low ('T-1') saline tolerance. Measurements were made 3 h after addition of 75 mM NaCl to the nutrient medium (3 days after the start of salt treatment). Values are means ± SE ($n = 5$; 10 plants taken from different containers were grouped in each replicate). Plants were 8 days old at the start (addition of the first portion of NaCl) and 11 days old when these characteristics were measured.

| Characteristic                        | Control  | NaCl treatment  | Control  | NaCl treatment  |
|----------------------------------------|----------|----------------|----------|----------------|
| Transpiration per plant (mg plant$^{-1}$ h$^{-1}$) | 72 ± 2$^a$ | 63 ± 2$^a$ | 111 ± 5$^b$ | 64 ± 3$^{0.05}$ |
| Transpiration per unit of leaf area (mg cm$^{-2}$ s$^{-1}$) | 4.3 ± 0.2$^b$ | 3.8 ± 0.2$^b$ | 4.4 ± 0.3$^b$ | 2.6 ± 0.1$^{0.05}$ |
| Root water content (%)                 | 92.8 ± 0.4$^a$ | 89.8 ± 0.8$^{0.05}$ | 92.6 ± 0.5$^b$ | 91.9 ± 0.7$^a$ |
| RWC in shoot (%)                       | 92.7 ± 0.8$^a$ | 89.7 ± 1$^{0.05}$ | 92 ± 0.7$^a$ | 89 ± 0.5$^{0.05}$ |
| Xylem flow (mg g$^{-1}$ FW h$^{-1}$)   | 33 ± 2.2$^b$ | 25.8 ± 1.4$^{0.05}$ | 54 ± 5.6$^b$ | 28.2 ± 4.8$^{0.05}$ |
| ΔΨp (MPa)                              | 0.11$^a$ | 0.12$^a$ | 0.12$^a$ | 0.11$^a$ |
| Hydraulic conductivity (mg g$^{-1}$ FW h$^{-1}$ MPa$^{-1}$) | 300 ± 26$^b$ | 215 ± 12$^{0.05}$ | 450 ± 46$^c$ | 256 ± 22$^{0.05}$ |

*Significant difference (t-test, $P < 0.05$) between control and salt-treated plants.
**Significant difference (t-test, $P < 0.01$) between control and salt-treated plants.
Means followed by the same letters are not significantly different at $P < 0.05$ (two-way ANOVA).

Fig. 1 Effect of up to 72 h of salt treatment on transpiration in lines of wild barley with high (‘20-45’) or low (‘T-1’) saline tolerance. Values are means ± SE. Plants were 8 days old at the start and collected for the measurements from several big containers. $n = 10$ (the rate of water losses from 10 small containers with 50 mL of nutrient solution and 10 plants placed in each container). There was no difference in effect of treatment on plant size. Means with the same letters are not significantly different at $P < 0.05$ as assessed by two-way ANOVA.

Fig. 2 Effect of up to 72 h of salt treatment on transcript levels of the HtPIP2;4 aquaporin-gene in the roots of lines of wild barley with high (‘20-45’) or low (‘T-1’) saline tolerance. Values are means ± SD ($n = 3$) and expressed relative to the untreated control. *Means for the two lines are statistically significantly different ($P < 0.05$, t-test). Plants were 8 days old at the start.
at the first sample point (0.5 h). Comparison of changes in gene expression and hydraulic conductivity showed that the greater decline in hydraulic conductivity (Lp) for salt-treated ‘20-45’ compared with that of ‘T-1’ (Table 1) may be related to the greater down-regulation of AQP gene expression. The mean value of the AQP expression in roots of salt-treated plants averaged over the whole experiment was significantly lower than in the control in the case of ‘20-45’ plants (0.48 ± 0.14, n = 12, not different from 1).

Leaf length was measured during 2 weeks following the start of salt treatment, the results being presented as a percentage of untreated control values. Figure 3 shows that third and fourth leaves of salt-treated plants were shorter than in control plants; however, the extent of inhibition was much greater in ‘T-1’ than in ‘20-45’, with ‘T-1’ being inhibited by as much as 30 %, compared with <10 % for ‘20-45’. Statistical analysis showed that the decline in leaf size was significant only in ‘T-1’ and was never statistically significant in ‘20-45’. Slight differences between the two lines in the degree of salt-induced inhibition of second leaf extension were noted within 3 days of the start of salt treatment and became statistically significant 3 days later (Fig. 3).

The final measurements of leaf area and of root weight, total leaf chlorophyll and tissue osmolality after 20 days of salt treatment (Table 2) show a substantially greater decline in leaf area in ‘T-1’ than in ‘20-45’. Leaf area per se was larger in ‘20-45’ than in ‘T-1’ with salt treatment of ‘20-45’ bringing leaf area down to that of ‘T-1’ controls. The size of the root system on a FW basis was inhibited much less by salt than was the leaf area. Nevertheless, ‘T-1’ was again more inhibited than ‘20-45’ (13.2 versus 10 %). Similarly, chlorophyll content was decreased 20 % in ‘T-1’ by salt, but there was no statistically significant decrease in salt-treated ‘20-45’. This represents a particularly important indicator of the greater salt resistance of ‘20-45’, since degradation of chlorophyll may result from the direct effect of toxic ions (Munns et al., 2006) and have long-term consequences for the plants’ later productivity. Osmolality of leaf sap was increased by salinity, indicating osmotic adjustment. The effect was more pronounced in ‘20-45’.

**Discussion**

The two genotypes selected for studies are genetically (Ivandic et al., 2002) and ecologically distinct (Forster...
et al., 1997). It is therefore not surprising that they respond differently to salinity. The responses of the two lines to mild salt stress that are shown here confirm other short- and long-term growth data (M Chechulina, BP Forster and HG Jones, unpubl. res.), all of which indicate ‘T-1’ to be more sensitive to salinity than ‘20-45’. Differences in inhibition of leaf growth appeared within 3 days after the start of salt treatment, which, though more rapid than in the experiments of Munns (2005), agrees with the timescale reported by Cramer (1992).

The initial addition of small amounts of NaCl (25 mM) changed the water potential of the nutrient medium only slightly (by ~0.16 MPa). Although this did not cause a decline in transpiration in ‘T-1’, a decrease was seen the next day in ‘20-45’. At later times and at stronger salt concentrations, osmotically driven water flow from the roots in xylem was decreased by salinity. This was not due to osmotic effects, since osmotic gradients were maintained. Our results indicate that the slower sap flux flow was a result of lowered root hydraulic conductivity. A possible consequence of this could be lowered leaf water potential. This, in turn, will have reduced transpiration in ‘20-45’, probably an outcome of stomatal closure. In a similar way, decreased hydraulic conductivity can close stomata when roots are cooled (Veselova et al., 2005). Decreases in hydraulic conductivity are likely to be the result of the down-regulation of AQP gene expression. This conclusion is supported by a correlation between down-regulation of AQP expression and both transpiration rate and hydraulic conductivity, with greater reductions of both being seen in ‘20-45’. The difference between the lines in the degree of inhibition of transpiration became evident later than the difference in expression of the AQP gene. This is to be expected since it takes time for changes in expression to have a clear physiological effect. Our data are in accordance with those of Ehert et al. (2009), who showed that aquaporin-mediated decline in hydraulic conductivity results in stomatal closure at high transpirational demand.

Osmotic stress has been shown to decrease AQP activity per se by changing the phosphorylation state, thereby lowering the water permeability of cell membranes (Johanson et al., 1996). In contrast, drought has been shown to reduce the hydraulic conductance of plants through diminishing the AQP protein abundance in roots (Aroca et al., 2006). Positive and negative changes in AQP expression have been observed in different experiments following studies of gene expression in salt-stressed plants, which may depend on the different contribution of the cell-to-cell pathway for water transport in plants of different species (Aroca et al., 2006; Fricke et al., 2006; Katsuhara and Shibasaka, 2007; Martinez-Ballesta et al., 2008; Lopez-Perez et al., 2009). The importance of these changes in AQP gene expression for hydraulic conductance and salt tolerance remains unclear. Overexpression of HvPIP2;1 has been reported to render transgenic rice plants more sensitive to 100 mM NaCl (Katsuhara et al., 2003). In maize, the expression of PIP2;4 AQP was transiently induced after addition of NaCl to the nutrient medium (Zhu et al., 2005) but this was not correlated with decreased hydraulic conductance (Steudle, 2000). The contrast with our results possibly arises because, unlike in maize, a greater proportion of the water follows the cell-to-cell path in young barley (Steudle and Peterson, 1998). This may explain the closer correlation between changes in AQP expression and hydraulic conductivity in our experiments.

It might seem puzzling that salt-tolerant plants decrease their root hydraulic conductance more strongly in response to salt since this would increase difficulties in water delivery to the shoot already imposed directly by osmotic stress. It may be that by reducing leaf water potential and hence decreasing stomatal conductances, the decrease in hydraulic conductance reduces inward water flow, leading to smaller amounts of toxic ions delivered to the root surface and into the plant. Recent research has confirmed that stomatal closure at the beginning of salinity may contribute to a decline in the flow of toxic ions within the transpiration stream (Veselov et al., 2008). Other literature on the link between transpiration and salt tolerance is contradictory. An ability of plants to close stomata in response to salinity has been suggested as an important component of salt tolerance (Robinson et al., 1997), although the associated and consequential reduction in carbon assimilation cannot be beneficial in the long term (James et al., 2002).

**Conclusions and forward look**

Changes in stomatal conductance allow plant adaptation to the environment (Jones, 1998). In our experiments, the genotype (‘20-45’) with a higher degree of salt tolerance, in terms of growth and chlorophyll retention, showed a greater reduction in transpiration than the comparatively salt-sensitive genotype (‘T-1’). This result supports the hypothesis that a lowered transpiration rate is beneficial for salt tolerance, at least in small plants. We propose that decreases in root plant hydraulic conductance initiated by down-regulation of AQP gene expression are responsible for this stomatal closure and the observed slowing of transpirational losses. This observation may be important in the search for
strategies to improve salt tolerance of barley plants by identifying increased root hydraulic resistance as a potentially advantageous characteristic.

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Contributions by the authors
All authors contributed to a similar extent overall.

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Conflict of interest statement
None declared.

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