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Oxygen deficiency and salinity affect cell-specific ion concentrations in adventitious roots of barley (*Hordeum vulgare*)

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Summary

- Oxygen deficiency associated with soil waterlogging adversely impacts root respiration and nutrient acquisition. We investigated the effects of O_2 deficiency and salinity (100 mM NaCl) on radial O_2 concentrations and cell-specific ion distributions in adventitious roots of barley (*Hordeum vulgare*).
- Microelectrode profiling measured O_2 concentrations across roots in aerated, aerated saline, stagnant or stagnant saline media. X-ray microanalysis at two positions behind the apex determined the cell-specific elemental concentrations of potassium (K), sodium (Na) and chloride (Cl) across roots.
- Severe O_2 deficiency occurred in the stele and apical regions of roots in stagnant solutions. O_2 deficiency in the stele reduced the concentrations of K, Na and Cl in the pericycle and xylem parenchyma cells at the subapical region. Near the root apex, Na declined across the cortex in roots from the aerated saline solution but was relatively high in all cell types in roots from the stagnant saline solution.
- Oxygen deficiency has a substantial impact on cellular ion concentrations in roots. Both pericycle and xylem parenchyma cells are involved in energy-dependent K loading into the xylem and in controlling radial Na and Cl transport. At root tips, accumulation of Na in the outer cell layers likely contributed to reduction of Na in inner cells of the tips.

Introduction

Soil waterlogging decreases the availability of O_2 to roots and thus adversely impacts respiration and nutrient acquisition. Impaired root growth and functioning in waterlogged soils is a major factor contributing to nutrient deficiency in shoots and, as a consequence, reduced crop productivity (Colmer & Greenway, 2011; Shabala *et al.*, 2014). Large areas of waterlogged agricultural land are often also saline (Barrett-Lennard, 2003). The combined effects of waterlogging and salinity can be especially damaging to plants, substantially reducing the threshold of salinity that causes detrimental effects on plant growth (Drew *et al.*, 1988; Barrett-Lennard, 2003; Malik *et al.*, 2009). Thus, improved combined stress tolerance is needed to sustain future crop production (Colmer *et al.*, 2005).

Waterlogging tolerance is associated with internal gas-phase diffusion of O_2 from shoots to roots through large interconnected air channels (aerenchyma) (Armstrong, 1979; Colmer, 2003; Voesenek & Bailey-Serres, 2015). For roots in anaerobic media receiving O_2 via aerenchyma, O_2 deficiency (hypoxia) may occur in apical regions of roots and inside the stele (Armstrong, 1979), causing energy deficits (for data for *Zea mays*, see Gibbs *et al.*, 1998; Darwent *et al.*, 2003). The condition of hypoxia in these tissues results from their high metabolic demand for O_2, low porosity of tissues and locations at the ends of longitudinal and radial O_2 diffusion pathways (Armstrong *et al.*, 1994). It is unclear how tissue hypoxia, and especially hypoxia in the stele, affects radial solute transport from the epidermis to the xylem. During stelar hypoxia/anoxia, the activity of H^+-ATPases would be restricted by low ATP as a result of low respiration, so the trans-membrane H^+ difference could be diminished and xylem loading would be affected (suggested by Colmer & Greenway, 2011).

Salinity tolerance requires the regulation of ion transport to the shoot (Tester & Davenport, 2003). When soil salinity occurs together with root-zone O_2 deficiency, rates of sodium

†Deceased.

(Na) and chloride (Cl) transfer from roots to shoots increase, whereas potassium (K) transport decreases even further than when exposed to salinity alone (Barrett-Lennard, 2003; Malik *et al.*, 2009). Xylem Na concentration depends on several active transport processes in specific cell types in roots, which in saline conditions act to restrict the amount of Na reaching the shoots (Tester & Davenport, 2003), but this mechanism can be compromised when roots become O_2 deficient (Drew & Läuchli, 1985). Because energy-dependent ion transport mechanisms can be inhibited during root hypoxia (Shabala *et al.*, 2014), it is of importance to evaluate roots and specific cell types for their Na, Cl and K homeostasis during combined salinity and hypoxia.

The aim of this study was to investigate the effects of O_2-deficiency, NaCl-salinity and their combination on radial ion distributions and concentrations in various cell types within roots of barley (*Hordeum vulgare*). This is the first investigation of cell-specific ion distributions across roots in relation to radial O_2 concentrations. So far, ion transport processes in O_2-deficient roots have been considered in short-term experiments on maize or barley seedlings (Drew & Läuchli, 1985; Gibbs *et al.*, 1998; Pang *et al.*, 2006; Zeng *et al.*, 2014) or speculated upon in reviews (Colmer & Greenway, 2011; Barrett-Lennard & Shabala, 2013). Barley was chosen as it is a relatively salt-tolerant cereal (James *et al.*, 2006) but it is sensitive to waterlogging (Garthwaite *et al.*, 2003). Microelectrode profiling was used to measure pO_2 across roots, and X-ray microanalysis was used to determine cell-specific elemental concentrations of K, Na and Cl in root samples from these various treatments, and at two positions behind the apex. The following hypotheses were tested: for barley roots in anaerobic media and receiving O_2 via aerenchyma, O_2 deficiency occurs most severely in the stele and towards the apex, as found in maize roots (Gibbs *et al.*, 1998; Darwent *et al.*, 2003) (albeit with greater diameters than barley roots); hypoxia in the stele reduces the accumulation of K in xylem parenchyma cells and in the xylem; root hypoxia compromises ion selectivity when combined with salinity stress, and thus decreases the K : Na ratio.

Materials and Methods

Plant material and growth conditions

Seeds of *Hordeum vulgare* L. (var. Franklin) were surface-sterilised with 0.4% (w/v) sodium hypochlorite, imbibed in aerated 0.5 mM $CaSO_4$ for 3 h and then grown on floating plastic mesh in Al-covered 4.5-l pots containing aerated nutrient solution as described elsewhere (Malik *et al.*, 2009; see Supporting Information Tables S1 and S2 for details). Plants were grown at 25 : 20°C, day : night conditions in a phytotron in a series of batches (four, each with three or four replicate pots of each treatment) between August and October in Perth (Australia), to provide plants for the various measurements conducted. From day 4, plants were exposed to natural sunlight (PAR value of 700–900 μmol m^{-2} s^{-1} at shoot height at midday). The solution in all pots was renewed weekly.

Treatments

The following treatments in nutrient solution were given to 14-d-old plants: aerated nonsaline (control), aerated saline (100 mM NaCl), stagnant nonsaline and stagnant saline (100 mM NaCl). Saline treatment was administrated in two steps at 12 h intervals (Malik *et al.*, 2009). Before the addition of NaCl, supplemental Ca^{2+} was added ($CaSO_4$) to all nutrient solutions in order to increase the final concentration to 4 mM during the treatment period (Greenway & Munns, 1980; Malik *et al.*, 2009). The hypoxic treatment was imposed by using deoxygenated stagnant 0.1% (w/v) agar nutrient solution as described elsewhere (Malik *et al.*, 2009). Roots were hypoxic pre-treated by flushing the nutrient solution with N_2 gas for 30 min and then left overnight without bubbling before the stagnant treatment was imposed (Malik *et al.*, 2009). Pots were in a completely randomized design and were re-randomized at the time of each nutrient solution renewal.

Sampling procedures

For the batch of plants used for growth measurements, an initial sample of one plant from each pot was taken immediately before treatments were imposed. Forty-eight hours after treatments were imposed, three adventitious roots on each remaining plant were marked with a small dot of xylene-free ink near the root base and the length of these roots was measured every 24 h for 10 d so that the daily root extension was determined. Final sampling was taken after 14 d of treatments. Roots and the stem bases of plants from saline treatments were thoroughly rinsed in 200 mM mannitol + 4 mM $CaSO_4$ solution, and plants from nonsaline treatments were rinsed in 4 mM $CaSO_4$. Various plant parts were separated (shoots, adventitious and seminal roots) and dried at 65°C for 72 h and their dry mass then recorded. Shoot and root relative growth rates (RGR) were calculated from dry mass measured at the initial and final samplings.

Tissue ion (K, Na, Cl) analyses

Oven-dried tissues were ground to a fine powder and extracted in 0.5 M HNO_3 as described elsewhere (Munns *et al.*, 2010). Na and K were determined using a flame photometer (PFP7; Jenway, Dunmow, UK) and Cl using a chloridometer (model 50CL 1-50; SLAMED, Frankfurt, Germany). The reliability of these analyses was confirmed by taking a reference tissue through the same procedures.

Root anatomy

Segments (10 mm long) were excised from adventitious roots (70–100 mm long) of plants after 14 d of treatments at distances of *c.* 10 and 50 mm from the apex, and fixed overnight with 2.5–5% glutaraldehyde in 0.005 M phosphate buffer, pH 7, at room temperature. Samples were then dehydrated, infiltrated and embedded in glycol methacrylate (GMA). A Sorvall microtome equipped with a glass knife was used to cut sections (4 μm thick),

which were transferred into a drop of deionized water on a microscope slide and dried on a hot plate. GMA-embedded sections were stained for 1 min in 0.05% (w/v) Toluidine Blue O in benzoate buffer at pH 4.4, washed and air dried. Sections were viewed under white light and photographed using a Zeiss Axioskop2 Plus with a Zeiss Axiocam digital camera. The percentage of each cross-section area occupied by stele was determined using IMAGE J software (v1.39u; NIH, Bethesda, MD, USA).

Root porosity

Porosity (% gas volume per unit tissue volume) measurements were taken on adventitious roots of plants previously used for pO_2 profile measurements by determining root buoyancy before and after vacuum infiltration of gas spaces in the roots with water, as described by Raskin (1983), using the equations as modified by Thomson *et al.* (1990). Adventitious roots 60–100 mm in length were cut into 30–50-mm segments and subsamples of *c.* 0.6 g fresh mass were used.

Radial profiles of O_2 partial pressure (pO_2) in intact adventitious roots determined by microelectrodes

Roots of 28-d-old barley plants, after treatments described above for the final 14 d, were placed in a horizontal chamber. One adventitious root was secured onto a metal grid in the chamber. The shoot base was fixed at the end of the chamber using Blue-Tac putty (Bostik, Middleton, MA, USA). The chamber was filled with the same nutrient solution where plants had been previously grown so that the roots were submerged and the shoot was in air (except for *c.* 20 mm base of the shoot). The solution was gently bubbled and continuously streamed across the surface with either high-purity N_2 (plants from stagnant treatments) or with air (plants from aerated treatments). The root compartment was covered with plastic plates and a small opening enabled insertion of an O_2 microelectrode. Experiments were conducted in a room at 25°C with 350–400 μmol m^{-2} s^{-1} PAR on the shoots.

Radial pO_2 profiles were measured using Clark-type microelectrodes with a guard cathode and tip diameter of 10 μm (OX-10; Unisense A/S, Aarhus, Denmark) as described elsewhere (Colmer & Pedersen, 2008; Pedersen *et al.*, 2009). Briefly, a microelectrode was secured on a motor-driven manipulator, positioned vertically above the root surface and advanced into the root in steps of 10 μm each 6 s. Root tissue pO_2 radial profile measurements were taken at apical (*c.* 10 mm from the apex) and subapical (*c.* 40 mm from the apex) regions of 70–100 mm-long, intact adventitious roots.

Sample preparation for X-ray microanalysis

Samples of adventitious roots were collected from plants transported in their pots to the laboratory after 14 d in the treatments (plants were 28 d old). Transpiration rates would have been perturbed in plants under laboratory conditions; however, it was not possible to cryoplunge samples in the glasshouse. Root segments were excised at distances of *c.* 10 and 50 mm behind the apex and immediately plunge-frozen into liquid N_2 slush. This took < 20 s. Frozen roots were subsequently freeze-substituted, embedded in resin and analysed using X-ray microanalytical techniques as outlined in detail in Kotula *et al.* (2015). Briefly, freeze-substituted material in resin blocks were planed flat, coated with carbon and analysed using scanning electron microscopy at 15 kV. Analysis and quantification was performed using AZTEC software (Oxford Instruments; Tubney Woods, Abingdon, UK).

Statistical analysis

Data are presented as means ± SE. GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA) was used for one- or two-way ANOVA (with Tukey multiple comparison test) to compare means or to assess the effects of treatment, cell type or treatment × cell type interactions.

Results

Responses of root growth, porosity and anatomy to saline and stagnant treatments

In aerated nonsaline solution the daily root extension rate was 28 ± 2 mm (Fig. 1). Salinity in the aerated solution reduced root growth rate by 15% ($P < 0.05$). Roots in stagnant nonsaline and stagnant saline treatments initially grew at similar rates as roots in both aerated treatments (first 3 d), but then growth slowed and had almost stopped by the 10th day. As a result, the lengths of these roots (mm) after 10 d were: 250 ± 3 in aerated nonsaline, 220 ± 5 in aerated saline, 81 ± 1 in stagnant nonsaline and 72 ± 2 in stagnant saline treatments. The growth parameters of plants from all four treatments are shown in Table S3 and Fig. S1.

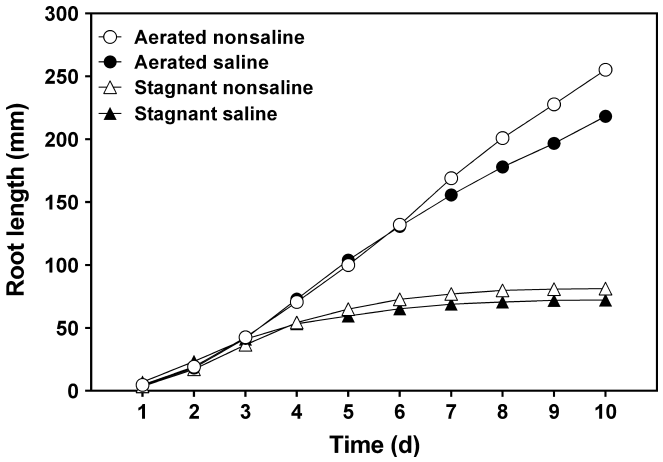

Fig. 1 Cumulative length of adventitious roots of barley (*Hordeum vulgare* var. Franklin) in four treatments: aerated nonsaline, aerated saline (100 mM NaCl), stagnant nonsaline or stagnant saline (100 mM NaCl). Three adventitious roots on each of four plants in each treatment were measured and the data from the three roots of each plant were pooled to provide a plant mean as one replicate. The growth rates were measured for 10 d starting 48 h after treatments were imposed, at 25 : 20°C, day : night temperatures. Data are means ± SE ($n = 4$). Error bars are small and not visible in the figure.

The porosities of adventitious roots were measured as internal O_2 transport is enhanced by increased porosity. In both aerated nonsaline and aerated saline solutions, the adventitious root porosity was 9–10% (Table 1). Imposition of the stagnant nonsaline treatment increased root porosity to $19 \pm 1\%$ ($P < 0.05$). Addition of 100 mM NaCl in stagnant medium resulted in a root porosity of $14 \pm 2\%$. The increased porosity of roots from stagnant treatments resulted from the development of aerenchyma, which can be seen in the cross-sections taken at 50 mm from the apex (Fig. 2g,h).

Radial pO_2 profiles in intact adventitious roots differ between aerated and hypoxic solutions

Because the growth of roots in stagnant solutions had almost ceased by day 10 (Fig. 1), it is likely that the O_2 supply to the root tips was insufficient despite the increased root porosity (Table 1). In the experiments described in this section, we tested the hypothesis that the O_2 deficiency occurs in the apical region and in the stele of roots in stagnant solutions but not in roots in aerated solutions.

In roots in aerated solutions, the pattern of radial pO_2 profiles and the magnitude of pO_2 were similar at both the apical and subapical regions (Fig. 3a,b). pO_2 declined across the boundary layer and the outer root cell layers (epidermis and sub-epidermis) from *c.* 20.6 kPa in air-equilibrium bulk solution to an average of 11.7 ± 0.6 kPa in the cortex. The pO_2 was fairly constant across the cortex but declined again across the endodermis/pericycle to an average of 9.3 ± 0.7 kPa within the stele.

In roots in stagnant solutions, the radial pO_2 profiles showed substantially lower cortical and stellar pO_2 within these roots when compared with those in aerated treatments. For roots in stagnant nonsaline solution, the radial pO_2 profile was approximately flat in the apical region, with the pO_2 in the cortex and stele of *c.* 0.07 ± 0.02 kPa and 0.02 ± 0.003 kPa, respectively (Fig. 3c). The corresponding pO_2 in roots from stagnant saline solution was below the detection limit (< 0.02 kPa) in both the cortex and the stele. When the radial profiles were measured in the sub-apical region, the pO_2 increased above that near the apex and it was higher at any radius of the root. The radial profiles at the sub-apical region in roots in stagnant treatments were characterised by a slight pO_2 increase across the outer root cell layers (epidermis and sub-epidermis) from *c.* 0 kPa in the deoxygenated solution to 0.5 and 0.4 ± 0.2 kPa within the outer tissue layers of these roots in stagnant nonsaline and stagnant saline solutions, respectively. The pO_2 was fairly constant across the cortex but then sharply declined across the endodermis/pericycle to very low pressures in the stele of 0.2 and 0.045 ± 0.045 kPa in roots in the stagnant nonsaline (Fig. 3d) and stagnant saline treatments, respectively.

Table 1 Porosity as a percentage of tissue volume of adventitious roots of barley (*Hordeum vulgare* var. Franklin) grown in aerated nonsaline, aerated saline (100 mM NaCl), stagnant nonsaline or stagnant saline (100 mM NaCl) nutrient solutions

Treatment	Porosity (% gas volume per unit tissue volume)
Aerated nonsaline	9.0 ± 1.8^{a}
Aerated saline	9.9 ± 0.7^{a}
Stagnant nonsaline	18.9 ± 1.0^{b}
Stagnant saline	14.3 ± 2.2^{ab}

Plants were raised in aerated nutrient solution for 14 d before the treatments being imposed for 14 d. Porosity was measured on roots of 60–100 mm in length. Different letters indicate significant differences among treatments ($P < 0.05$; Tukey test). Data are means ± SE ($n = 4$).

Radial K, Na and Cl concentrations in various cell types of adventitious roots

Having established that severe O_2 deficiency occurred in the stele and apical regions of roots in stagnant solutions, we evaluated whether O_2 deficiency in these tissues affects cell-specific K, Na and Cl concentrations. Vacuolar concentrations (cf. Läuchli *et al.*, 2008) of K, Na and Cl were measured in various cell types across adventitious roots (Fig. 2i,j), both at 10 and 50 mm behind the apex. The anatomy of the root, where the radial ion profiles were measured, consisted of an epidermis, a cortex and a stele (Fig. 2i,j). The cortex included a sub-epidermis, six to eight layers of cortical cells and an endodermis (Fig. 2j). The cells studied in the stele included pericycle, xylem parenchyma and xylem vessels. The xylem consisted of several peripheral metaxylem vessels (early metaxylem) and 3–6 central metaxylem (late metaxylem) vessels (Fig. 2i).

Potassium – 10 mm from the apex Potassium concentrations in cells of aerated nonsaline roots were similar in epidermis and cortex with an average of 57 ± 3.5 mmol kg^{-1} equivalent wet mass (Fig. 4a). Potassium was highest in the pericycle and xylem parenchyma (average of 135 ± 6.8 mmol kg^{-1}) and declined towards the xylem vessels, being lowest in the central metaxylem vessels (37 ± 4 mmol kg^{-1}). The K concentrations of cells across aerated saline roots were not statistically different from those of the aerated nonsaline roots ($P = 0.4$). The stagnant treatment resulted in different K profiles across roots than those in aerated treatments, with considerably lower K concentrations in the pericycle in roots from both of the stagnant treatments ($P < 0.0001$). In these hypoxic roots, K tended to increase from the sub-epidermis across the cortex and although it was relatively low in the pericycle (45 ± 3.5 mmol kg^{-1}), K was equally high to the concentrations in aerated roots for the xylem parenchyma (127 ± 7 mmol kg^{-1}) and for the central metaxylem vessels (38 ± 6 mmol kg^{-1}).

Potassium – 50 mm from the apex In aerated nonsaline roots, K concentrations in cells were similar in epidermis, sub-epidermis, outer and middle cortex (average of 36 ± 3 mmol kg^{-1}; Fig. 4b). Potassium concentrations then increased across the inner cortex and endodermis, reaching the highest levels in the pericycle (152 ± 10 mmol kg^{-1}), and declined towards the xylem vessels, reaching the lowest levels in the central metaxylem (29 ± 3 mmol kg^{-1}). The pattern of the corresponding K profile across aerated saline roots was similar to that in the nonsaline

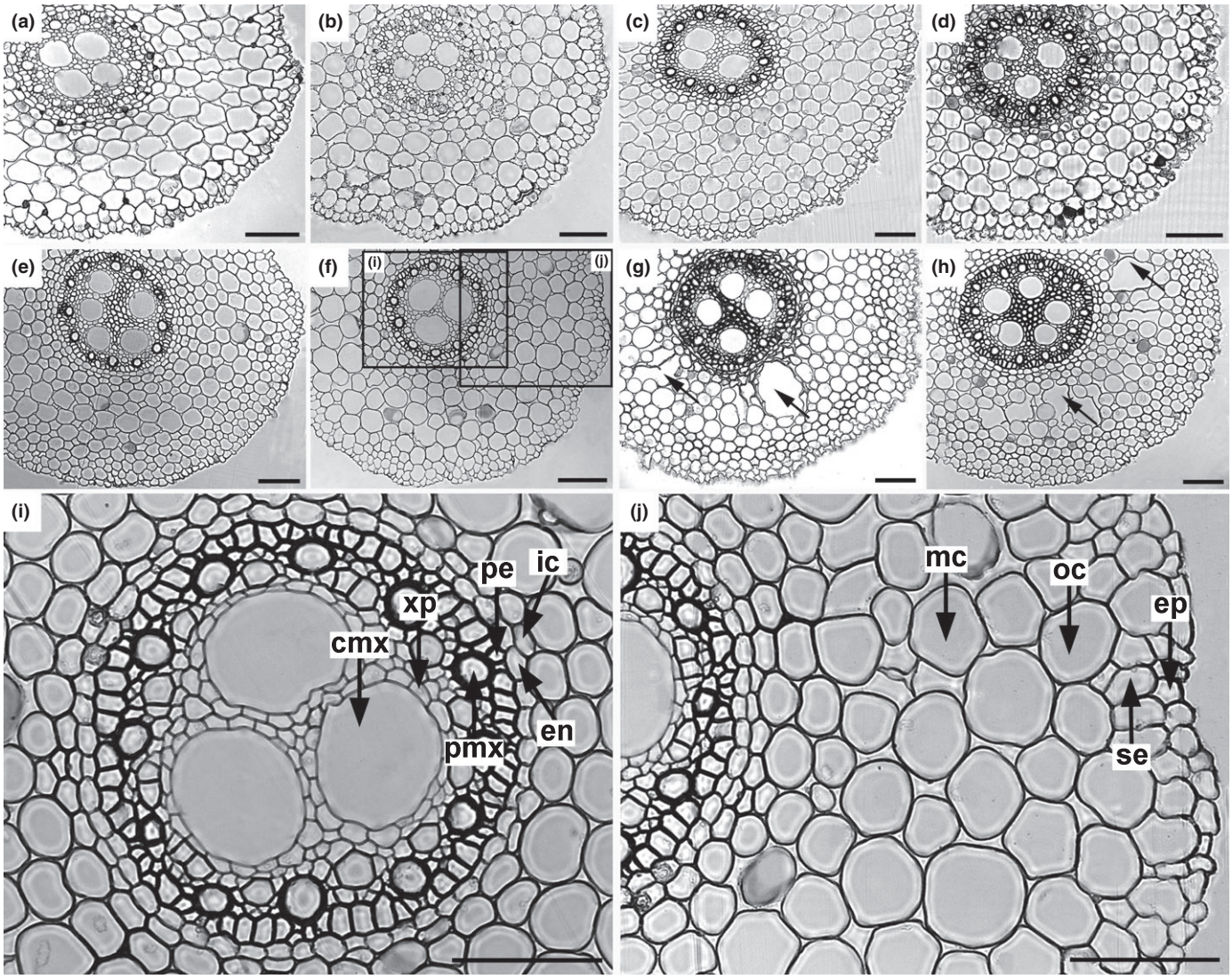

Fig. 2 Anatomical features of adventitious roots of barley (*Hordeum vulgare* var. Franklin) grown in aerated nonsaline (a, e), aerated saline (100 mM NaCl) (b, f), stagnant nonsaline (c, g) or stagnant saline (100 mM NaCl) (d, h) nutrient solution for the final 14 d. Cross-sections were taken at 10 mm (a–d) and 50 mm (e–h) from the root apex of 70–100-mm-long roots by cutting GMA blocks containing a root segment using a microtome. The sections were stained in Toluidine Blue O and viewed with bright field. The photographs are shown in black and white. Examples of the presence of aerenchyma in (g) and (h) are indicated by black arrows. Higher magnification of the marked areas in (f) showing cell types within the stele (i) and cortex (j). Arrows in (i) and (j) indicate typical cells that were analysed to give radial ion profiles (Fig. 4). ep, epidermis; se, sub-epidermis; oc, outer cortex; mc, middle cortex; ic, inner cortex; en, endodermis; pe, pericycle; xp, xylem parenchyma; pmx, peripheral metaxylem vessel; cmx, central metaxylem vessel. Bars, 100 μm.

roots, but the K concentrations in the pericycle, xylem parenchyma and peripheral metaxylem vessels were on average 70% higher in the aerated saline roots than in these cells in aerated nonsaline roots ($P < 0.05$). In roots from both stagnant treatments, K concentrations were similar across the cortex to those in aerated roots, but significantly lower in the pericycle, xylem parenchyma and peripheral metaxylem vessels ($P < 0.0001$).

Xylem K concentrations and comment on xylem maturity The K concentrations in both the peripheral and central metaxylem vessels were relatively high at 10 mm from the apex in all treatments, indicating that these vessels were likely not yet mature and contained cellular contents rather than a xylem stream (Fig. 4a). This is consistent with the study by Huang & Van Steveninck (1988) who reported high concentrations of K in the xylem vessels at 10 mm from the root apex of seminal roots of young barley seedlings grown in aerated nutrient solution. In their study, the K concentration declined in peripheral metaxylem at 50 mm from the apex but remained high in central metaxylem up to a distance of 150 mm from the apex. For roots in the present study, we are uncertain whether xylem vessels were mature at 50 mm from the apex. In adventitious roots (maximum length 75 mm) of 14–17-d-old hydroponically grown barley seedlings, the axial hydraulic resistance decreased with distance from the apex until *c.* 60 mm from the root apex, indicating that not all xylem vessels were mature up to this position (Knipfer & Fricke, 2011). In the present study, the K concentration in the peripheral metaxylem at 50 mm from the apex tended to be

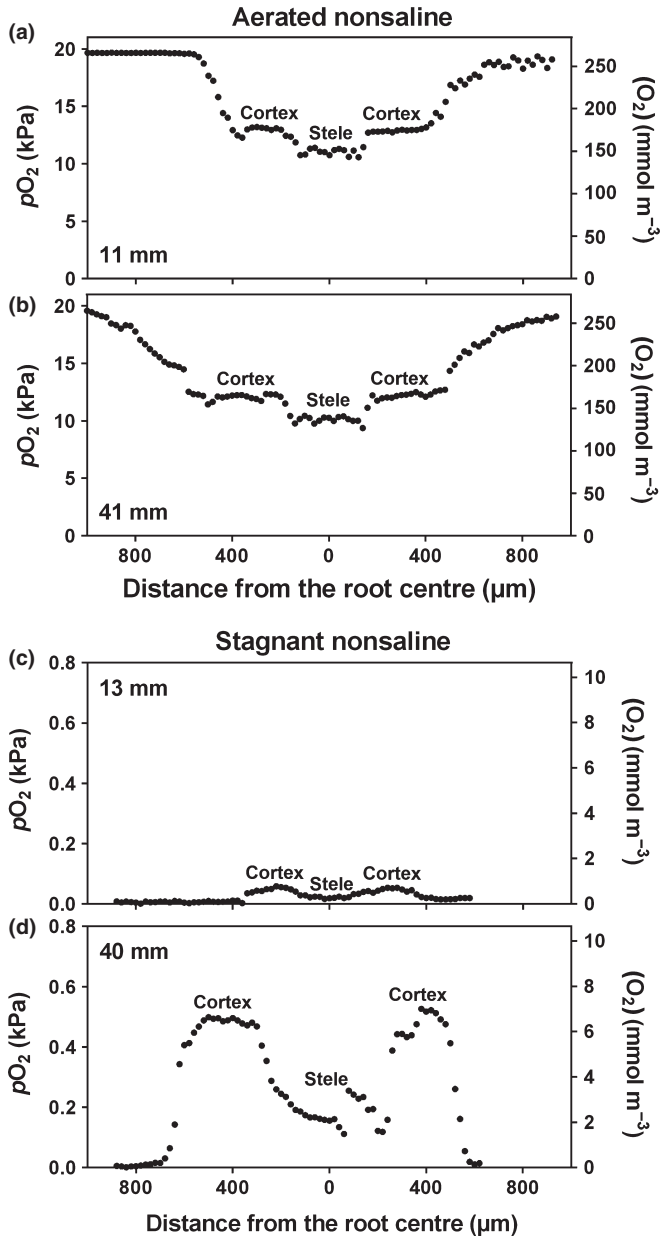

Fig. 3 Radial O_2 profiles taken at different positions along adventitious roots of barley (*Hordeum vulgare* var. Franklin) in aerated nonsaline (a, b) or deoxygenated stagnant nonsaline (c, d) nutrient solutions for the final 14 d. Profiles were taken at apical (a, c) and subapical (b, d) positions of the roots at 25°C. Radial O_2 profiles for roots in the aerated saline solution were similar to those in the aerated nonsaline solution. For roots in stagnant saline solution, at the apical position pO_2 was below the detection limit (< 0.02 kPa) and at the subapical position, the profile pattern was similar to that in (d) but pO_2 in roots in the stagnant saline solution were 0.4 ± 0.2 and 0.045 ± 0.045 kPa in the cortex and stele, respectively. Radial O_2 profiles were taken for roots 70–100 mm in length. The adventitious roots from both stagnant treatments were near the maximum length supported by internal O_2 diffusion in these conditions (see Fig. 1).

higher in roots grown in aerated solutions than in stagnant solutions (Fig. 4b). Differences in ion concentration in xylem vessels at 50 mm from the apex of roots from aerated and stagnant solutions should be interpreted with caution as the slower extension rates of roots in both stagnant treatments (Fig. 1) would mean that tissues at equivalent distances from the apex would be older in the stagnant roots. As a result, xylem vessels could mature closer to the apex in slower-growing roots in stagnant treatments when compared with the faster-growing roots in aerated treatments.

Sodium – 10 mm behind the apex Sodium concentrations in nonsaline roots were low in all cells analysed and did not exceed 12 mmol kg^{-1} for either aerated or stagnant treatments (Fig. 4c). In roots from aerated saline treatment, the Na concentration was greatest in the epidermis, sub-epidermis and outer cortex (average of 103 ± 10 mmol kg^{-1}), and was lower in cells from the middle cortex inwards and in the xylem vessels (average of 20 ± 1.5 mmol kg^{-1}) (Fig. 4c). The corresponding Na profile in stagnant saline roots differed significantly from that in aerated saline roots ($P < 0.0001$). The Na concentrations in the epidermis and the sub-epidermis in stagnant saline roots were on average 47% less than in aerated saline roots, similar in the outer cortex, but on average 300% more than in the other cells from the middle cortex and inwards to the xylem vessels ($P < 0.05$).

Sodium – 50 mm behind the apex Sodium concentrations in nonsaline roots were low in all cells analysed for aerated and stagnant treatments and did not exceed 10 mmol kg^{-1} (Fig. 4d). In aerated saline roots, the Na was low and similar in cells of the epidermis, sub-epidermis, outer and middle cortex (average of 33 ± 1 mmol kg^{-1}), then dramatically increased across the inner cortex and endodermis, reaching the highest concentration in the pericycle (172 ± 13 mmol kg^{-1}), before dramatically declining towards the xylem vessels, falling to the lowest concentration in the central metaxylem (21 ± 3 mmol kg^{-1}). For roots in the stagnant saline treatment, Na concentrations were similar in cells across the root until the xylem parenchyma (average 56 ± 2 mmol kg^{-1}) but fell to 26 ± 0 mmol kg^{-1} in the xylem vessels.

In contrast to aerated saline roots, which showed different patterns of Na profiles at 10 and 50 mm from the apex ($P < 0.0001$; Fig. 4c,d), profiles in stagnant saline roots were not statistically different at 10 and 50 mm from the apex ($P = 0.7$; Fig. 4c,d). Moreover, in stagnant saline roots at both positions from the apex the Na concentration was more uniform across the roots, so that the distinct cell-type differences described above for Na profiles across the aerated roots were not evident for roots in the stagnant saline treatment.

Potassium : sodium ratio – 10 mm behind the apex The K : Na ratio was highest in the aerated nonsaline roots, varying from 2.2 in the epidermis to 23 in the pericycle (Fig. 4e). Both salinity and stagnant treatments resulted in lower K : Na ratios: in cells of aerated saline and stagnant nonsaline roots ratios varied (respectively) from 0.4 and 0.7 in the sub-epidermis to 8.2 and 14.5 in the pericycle, whereas in cells from stagnant saline roots ratios averaged 0.5.

Potassium : sodium ratio – 50 mm behind the apex The K : Na ratio in cells of aerated nonsaline roots was relatively low from

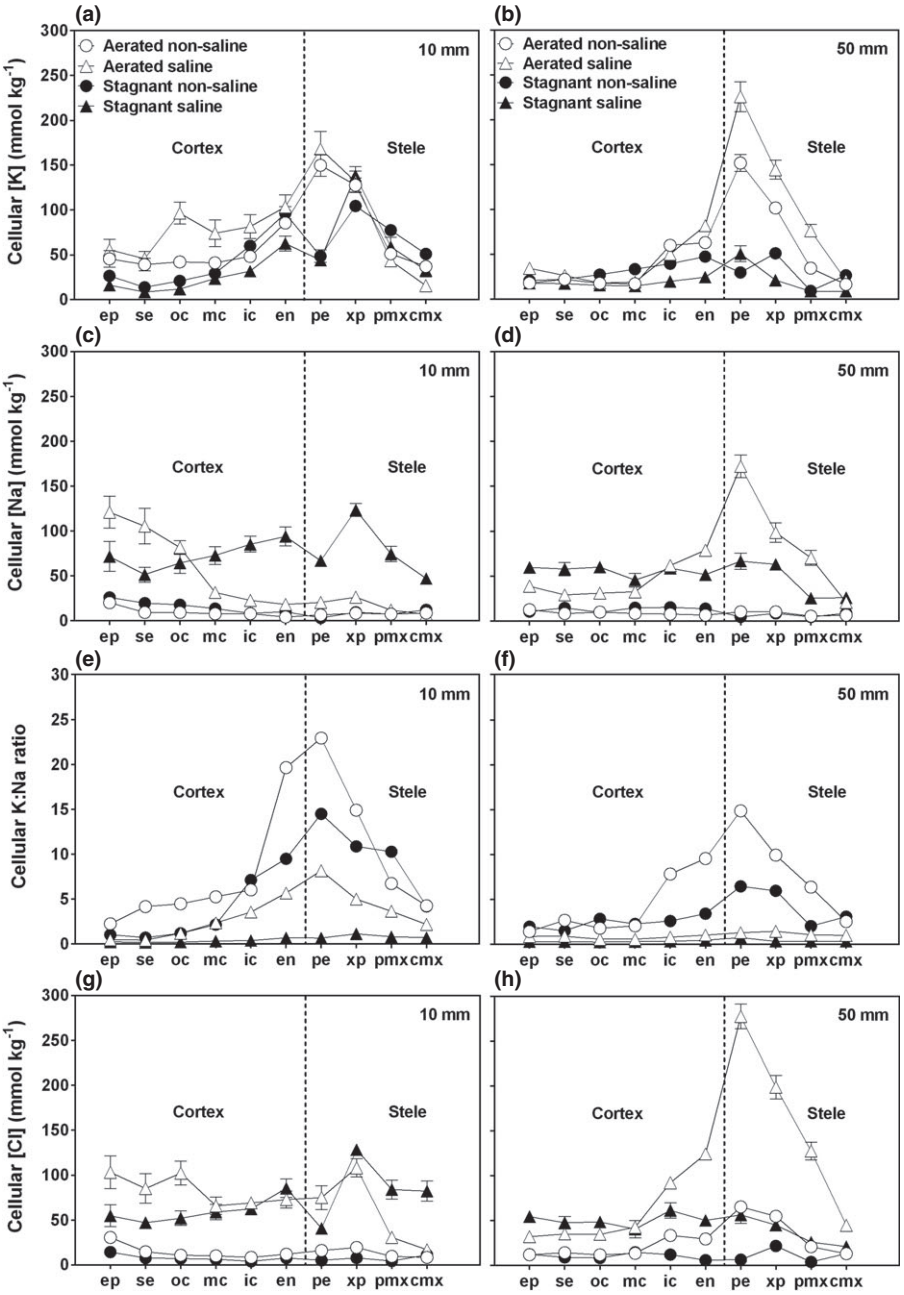

Fig. 4 Concentration profiles of potassium (K) (a, b), sodium (Na) (c, d) and chloride (Cl) (g, h), and K : Na ratio (e, f) in various cell types across adventitious roots at 10 mm (left panels) and 50 mm (right panels) from the root apex for barley (*Hordeum vulgare* var. Franklin) grown in aerated nonsaline, aerated saline (100 mM NaCl), stagnant nonsaline or stagnant saline (100 mM NaCl) nutrient solutions for the final 14 d. Elemental concentrations of K, Na and Cl were measured using X-ray microanalysis of freeze-substituted samples. The concentrations are given in mmol kg^{-1} embedded tissue, which, if the resin fully occupies the space previously occupied by water in the tissue, closely reflects the concentration on a wet weight basis. ep, epidermis; se, sub-epidermis; oc, outer cortex; mc, middle cortex; ic, inner cortex; en, endodermis; pe, pericycle; xp, xylem parenchyma; pmx, peripheral metaxylem vessel; cmx, central metaxylem vessel. There were significant treatment × cell type interactions at $P < 0.0001$ each for K, Na and Cl at both 10 and 50 mm from the apex (two-way ANOVA). Data are means ± SE ($n = 4$–57 cells measured from three to four different roots from each treatment).

the middle cortex outwards, with an average of *c.* 2. It then increased to its highest value in the pericycle (15) and declined towards the xylem vessels, being lowest in the central metaxylem (2.5). In stagnant nonsaline roots, the K : Na ratio was low in the epidermis and cortex with an average of 2.3, increased to *c.* 6 in the pericycle and xylem parenchyma, and declined to *c.* 2.5 in the xylem vessels. The ratio in roots from both aerated saline and stagnant saline treatments was low in all cells analysed, averaging 0.7.

Chloride – 10 mm behind the apex Chloride concentrations in nonsaline roots were low at 14 ± 0.7 and 7 ± 0.5 mmol kg^{-1}, respectively, for aerated and stagnant roots (Fig. 4g). Addition of 100 mM NaCl to the aerated solution resulted in increased Cl in all cells across the roots to an average concentration of 87 ± 4 mmol kg^{-1} ($P < 0.05$), but Cl remained relatively low in the xylem vessels (27 ± 4 mmol kg^{-1}). Corresponding Cl concentrations in stagnant saline roots were *c.* 48% less in the epidermis, sub-epidermis and outer cortex than in aerated saline roots, but they were similar between these two treatments in other cells until the xylem vessels, where Cl was approximately three-fold higher (83 ± 8 mmol kg^{-1}) in the stagnant saline treatment. High concentrations of Cl in the epidermis, sub-epidermis and outer cortex in aerated saline roots would provide a charge balance for the high Na in these cells (Fig. 4c), but unlike Na, the Cl concentrations were relatively high across the entire root radius,

presumably because K was relatively high in the inner cells (Fig. 4a) and Cl only decreased to low values in xylem vessels.

Chloride – 50 mm behind the apex Chloride concentrations in nonsaline roots were low in the epidermis, sub-epidermis, outer and middle cortex (average of 12 ± 0.7 mmol kg^{-1}; Fig. 4h). Chloride remained low in stagnant nonsaline roots, but in aerated nonsaline roots, it increased across the inner cortex and endodermis, reaching the highest concentration in the pericycle (65 ± 3 mmol kg^{-1}), and then declined towards the xylem vessels. In aerated saline roots, the Cl concentration was similar in cells of the epidermis, sub-epidermis, outer and middle cortex (37 ± 2 mmol kg^{-1}). Chloride then dramatically increased across the inner cortex and endodermis, and was highest in the pericycle (278 ± 14 mmol kg^{-1}), and then dramatically declined towards the central metaxylem vessels (45 ± 3 mmol kg^{-1}). For roots in the stagnant saline treatment, the Cl concentration was similar in cells across the root until the xylem parenchyma (average of 50 ± 2.5 mmol kg^{-1}) and was 21 ± 0.8 mmol kg^{-1} in the central metaxylem.

Comment on the effect of reduced transpiration on xylem ion concentrations Samples for the X-ray microanalysis were collected under laboratory conditions, when transpiration would have decreased relative to that in the glasshouse. Transpiration would presumably have had little effect on ion concentrations in the 'hydraulically isolated' root tip zone (10 mm from the apex), where xylem vessels contained cellular contents rather than a transpiration stream (see the Discussion section; Frensch & Steudle, 1989; Melchior & Steudle, 1993). At 50 mm from the apex, where some of the xylem vessels were probably mature (see the section 'Xylem K concentrations and comment on xylem maturity' above), absolute ion concentrations in the xylem were likely higher when transpiration was reduced (Munns, 1985); nevertheless, the treatment comparisons remain informative.

K, Na and Cl whole tissue concentrations in adventitious roots

Whole adventitious root ion concentrations were assayed to provide further context for the cellular concentrations determined using X-ray microanalysis at two specific positions along roots (described above). Tissue ion data for adventitious roots are presented in Fig. 5, and for completeness shoot and seminal root ion data are in Fig. S2.

Adventitious root K concentrations were similar for plants grown in aerated nonsaline, aerated saline and stagnant nonsaline treatments (76–105 mmol l^{-1} tissue water). Growth in the stagnant saline treatment decreased the K concentration in adventitious roots to 34% of its value of the aerated nonsaline control ($P < 0.05$) (Fig. 5).

Adventitious root Na concentrations were similarly low in plants grown in the aerated nonsaline and stagnant nonsaline treatments (22–26 mmol l^{-1}; Fig. 5). Imposition of the aerated saline and stagnant saline treatments increased Na concentrations in adventitious roots 5.1- and 3.5-fold, respectively, compared with the aerated nonsaline control ($P < 0.05$).

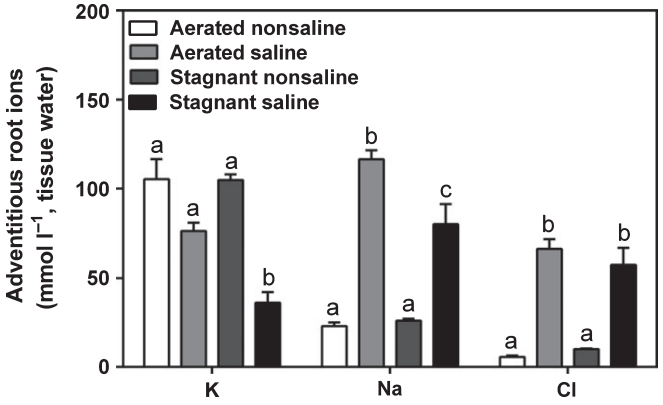

Fig. 5 Whole tissue concentrations of potassium (K), sodium (Na) and chloride (Cl) in adventitious roots of barley (*Hordeum vulgare* var. Franklin) grown in aerated nonsaline, aerated saline (100 mM NaCl), stagnant nonsaline or stagnant saline (100 mM NaCl) nutrient solutions for 14 d. The concentrations are expressed on a tissue water basis. Different letters indicate significant differences among treatments within each of the ions measured ($P < 0.05$; Tukey test). Data are means ± SE ($n = 3$).

The K : Na ratio was highest (4.6) in adventitious roots of plants grown in the aerated nonsaline control (Table 2). Growth in aerated saline, stagnant nonsaline and stagnant saline treatments decreased the ratio in adventitious roots to 0.7, 4.0 and 0.4, respectively, compared with the aerated nonsaline control ($P < 0.05$). Shoot and seminal root K : Na ratios are in Table S4.

Adventitious root Cl concentrations were low and similar in plants grown in the aerated nonsaline and stagnant nonsaline treatments (6–10 mmol l^{-1}; Fig. 5). Growth in the aerated saline and stagnant saline solution increased Cl concentrations by 11.8- and 10.2-fold, respectively, compared with the aerated nonsaline control ($P < 0.05$).

Discussion

Salinity and O_2 deficiency had significant impacts on tissue distributions and cellular concentrations of K, Na and Cl in roots of barley. The most severe root O_2 deficiency, determined by microelectrode profiling (Fig. 3), occurred in the stele and apical regions of adventitious roots in the deoxygenated stagnant solution; these roots are reliant on internal O_2 movement from the shoot via gas-phase diffusion (root porosity ranged from *c.* 14%

Table 2 Potassium : sodium (K : Na) ratio in adventitious roots of barley (*Hordeum vulgare* var. Franklin) grown in aerated nonsaline, aerated saline (100 mM NaCl), stagnant nonsaline or stagnant saline (100 mM NaCl) nutrient solutions for the final 14 d

Treatment	K : Na ratio
Aerated nonsaline	4.6 ± 0.06^{a}
Aerated saline	0.7 ± 0.05^{b}
Stagnant nonsaline	4.0 ± 0.08^{c}
Stagnant saline	0.4 ± 0.03^{b}

Different letters indicate significant differences among treatments ($P < 0.05$; Tukey test). Data are means ± SE ($n = 3$).

to 19%; Table 1). One of the consequences of root O_2 deficiency can be a decrease in the K : Na ratio, as ion selectivity is compromised (maize, Drew & Läuchli, 1985; wheat, Kuiper *et al.*, 1994), which for salinised barley was observed at both whole adventitious root and cellular levels (Table 2; Fig. 4e,f). In stagnant saline roots, where O_2 deficiency would have impeded some of the active transport of ions, the K : Na ratio was very low at 0.4 for the whole roots (Table 2) and 0.2–1.1 in various cell types across these roots (Fig. 4e,f). We initially focus our discussion on the cellular ion relations in roots of barley when exposed to salinity in the aerated solution, and then consider the effect of the combination of hypoxia and salinity on ion regulation in root cells and the xylem.

We found marked differences in Na concentrations at 10 and 50 mm behind the apex of roots grown under aerated saline conditions. At 10 mm behind the apex, cells of the epidermis, sub-epidermis and outer cortex accumulated high amounts of Na, whereas Na concentrations were much lower in the inner cells and in the xylem vessels (Fig. 4c). By contrast, relatively low Na concentrations were observed in the outer layers of aerated saline roots at 50 mm from the apex, but Na accumulated in the pericycle (Fig. 4d). The accumulation of Na in the outer root cell layers as well as in the pericycle has been previously observed at 100 mm from the apex of seminal roots of durum wheat exposed to 50 mM NaCl (Läuchli *et al.*, 2008). In our study, high Na accumulation in the epidermis, sub-epidermis and outer cortex of aerated saline roots was restricted only to the root tip region. In this tip zone, accumulation of Na in the outer cell layers could result in the inner cells being exposed to lower Na concentrations, as xylem vessels are not mature and thus mass flow of water (which would likely occur predominantly through a cell-to-cell pathway in barley roots; Steudle & Jeschke, 1983; Knipfer & Fricke, 2010, 2011) and ions across the tips of roots would be limited (root tips have been described as 'hydraulically isolated' due to high axial hydraulic resistance of immature xylem vessels; e.g. maize, Frensch & Steudle, 1989; onion, Melchior & Steudle, 1993). In subapical regions (50 mm from the apex) of aerated saline roots, Na concentration was the highest in the pericycle (Fig. 4d). In addition, the pericycle cells also contained Cl at even higher concentrations than that of Na (Fig. 4h). The pericycle may be an important component limiting Na and Cl flow to the xylem but given its low storage capacity, it remains to be determined whether there exists outwardly directed efflux of Na towards the epidermis (Läuchli *et al.*, 2008). Consistent with our finding of high Na and Cl in the pericycle of barley roots, in roots of grapevine grown in 25 mM NaCl, the pericycle cells also had the highest Na and Cl concentrations (data for xylem parenchyma and xylem vessels were not presented; Storey *et al.*, 2003). Moreover, the salt-tolerant grapevine genotype (low shoot Cl) had 20% higher Cl in pericycle cells than the salt-sensitive genotype (high shoot Cl). Similarly, a salt-tolerant durum wheat genotype with lower Na in leaves when grown in 50 mM NaCl accumulated *c.* 2-times higher concentration of Na in the pericycle than in the endodermis or the xylem parenchyma cells (Läuchli *et al.*, 2008).

High Na concentrations in the cells of the outer portion of roots in the apical position, or in the pericycle in the subapical position, were observed only in aerated roots. The measured Na concentrations would reflect vacuolar concentrations (see the Results section; cf. Läuchli *et al.*, 2008). Vacuolar compartmentation is mediated by Na^+/H^+ antiporters in the tonoplast, driven by the H^+ gradient established by the vacuolar H^+ATPase and H^+PP_iase; the Na^+/H^+ exchange activity in the tonoplast of barley roots can be activated upon addition of NaCl (Garbarino & DuPont, 1988). Because some of the Na sequestered into vacuoles leaks back to the cytoplasm (Shabala & Mackay, 2011), the energetic cost of vacuolar compartmentation of Na would be significant and likely inhibited in O_2-deficient roots. In contrast to Na accumulation in the outer cell layers or pericycle of aerated saline roots, relatively uniform and large concentrations of Na were observed across the entire radius of roots in stagnant saline conditions in both the apical and subapical regions (Fig. 4c,d). In these roots, the energetically expensive Na vacuolar compartmentation and possible Na efflux out of roots via plasma membrane Na^+/H^+ antiporters encoded by the *SOS1* gene (Blumwald *et al.*, 2000) and energised by the plasmamembrane H^+ATPase, would be diminished; severe hypoxia developed across the entire root radius at 10 mm and in the stele at 50 mm from the apex and likely restricted ATP production via respiration (see next paragraph). Instead, a passive transport of Na across root tissues likely resulted in Na accumulation in the stele (Shabala, 2013) under these O_2-deficient conditions.

Energy-dependent K accumulation in xylem parenchyma cells is essential for subsequent release into the xylem (cf. data for barley, Drew *et al.*, 1990; de Boer & Volkov, 2003) but also pericycle cells might play a role in K transport to the xylem (de Boer & Volkov, 2003). In the present study, high concentrations of K were observed in xylem parenchyma and pericycle cells of roots grown in both nonsaline and saline aerated solutions (Fig. 4a,b), indicating that both cell types do play important roles in K transport to the xylem (discussed further in the next paragraph). By contrast, low concentrations of K were observed in these cells in hypoxic roots at 50 mm behind the apex (Fig. 4b) where O_2 deficiency occurred in the stele (Fig. 3d), providing evidence for the necessity of energetically expensive K accumulation in the pericycle and xylem parenchyma as occurred in aerated roots, but which was inhibited by severe hypoxia. At 10 mm behind the apex, K concentrations in the pericycle cells were much lower in roots from both stagnant treatments than in roots from both aerated treatments, but, unexpectedly, in the xylem parenchyma cells K concentrations were similar in roots from all four treatments (Fig. 4a). As pO_2 was lower at the apical than at the subapical position, the O_2 status alone does not explain K status in the xylem parenchyma cells. Cells in hypoxic tissues would have produced ATP during some, albeit reduced, oxidative phosphorylation when O_2 was above zero, or from glycolysis linked to ethanolic fermentation.

Pericycle and xylem parenchyma cells both contained high K concentrations (Fig. 4a,b), which may be indicative of an accumulation of K in these cells before transport to the xylem (de Boer & Volkov, 2003). As pericycle cells are not in direct contact

with xylem vessels, the K accumulated in these cells would either move via plasmodesmata into xylem parenchyma cells or be released through SKOR channels into the apoplast, and then taken up by xylem parenchyma cells prior to release into the xylem (de Boer & Volkov, 2003). Gene transcripts encoding the SKOR channel were expressed only in pericycle cells and xylem parenchyma cells (Gaymard *et al.*, 1998). The K inward rectifying channels (KIRC) in xylem parenchyma cells would be involved in K uptake from the apoplast in barley roots (Wegner & de Boer, 1997).

Our O_2 microelectrode profiling (Fig. 3) showed that in barley roots in either aerated or stagnant deoxygenated solutions, steep pO_2 gradients developed across roots with pO_2 declining in the outer cell layers (epidermis/sub-epidermis) and again in the stele. Such pO_2 declines indicate high diffusive resistance of tissues of low porosity and/or high respiratory demand for O_2 (Armstrong *et al.*, 1994; Colmer & Greenway, 2011). The high radial resistance to O_2 diffusion in the epidermis/sub-epidermis and stele contributed to the decrease in pO_2 with distance into the root when the O_2 was supplied from the medium (aerated solution). The diffusive boundary layer between the epidermis and outer medium provided additional radial resistance to that across the tissues of the roots, so that the pO_2 declined from 20.6 kPa in the outer medium to between 8.6 and 11.6 kPa in the stele; this O_2 partial pressure should be sufficient for respiratory activity (cf. Atwell *et al.*, 1985; Gibbs *et al.*, 1998; Darwent *et al.*, 2003). This situation for roots in aerated solution is in marked contrast to the findings for roots in stagnant deoxygenated solution and reliant on internal O_2 diffusion via the aerenchyma, in which tissues at *c.* 40 mm behind the tip (and towards the apex) were severely hypoxic and with particularly very low O_2 amounts in the stele (0.045–0.2 kPa) as compared with the cortex (0.4–0.5 kPa). Nearer the root apex, severe hypoxia developed across the entire root radius. A decline of pO_2 with distance from the root–shoot junction and thus low pO_2 at the apex is a feature in roots receiving O_2 by internal longitudinal transport from the shoot (Armstrong, 1979), eventually restricting root growth into an anaerobic medium (Fig. 1, present study; rice, Armstrong & Webb, 1985; wheat, Wiengweera & Greenway, 2004).

The amount of O_2 that reaches the root apex depends on root length, the cortex/stele volume ratio, tissue porosity (increased porosity lowers resistance), metabolic consumption along the diffusion path and rates of radial O_2 loss from basal parts of the roots (Armstrong, 1979; Colmer, 2003). In the present study, adventitious roots of barley raised in stagnant and stagnant saline solutions formed 19% and 14% gas-filled porosity, respectively, which is consistent with previous reports for barley (McDonald *et al.*, 2001). Such porosity is beneficial for internal O_2 diffusion as compared with the lower values in many other dryland species, but is well below the root porosity of many wetland species (Justin & Armstrong, 1987). Moreover, aerenchyma did not occur 10 mm behind the root apex (Fig. 2) which would limit O_2 diffusion to apical positions (cf. for bread wheat, Thomson *et al.*, 1992). Radial O_2 loss (ROL) from basal root zones, although not measured in the present study, can be substantial for barley (McDonald *et al.*, 2001; Garthwaite *et al.*, 2003) and would have reduced internal O_2 reaching the tips. The present results on tissue pO_2 profiles across barley adventitious roots are consistent with those for *Zea mays* seedling roots which showed that internal pO_2 was lowest towards the root apex and declined radially into the stele (Darwent *et al.*, 2003), both as predicted by mathematical modelling (Armstrong *et al.*, 1994). Interestingly, severe stelar hypoxia at *c.* 40 mm from the apex occurred despite the smaller stele radius (*c.* 150 μm; the stele occupied 7.6–10.6% of the whole root cross-section; Table S5) in the thinner barley roots (present study) when compared with the larger stele radius (230–300 μm; the stele occupied *c.* 15–17% of the whole root cross-section) of the thicker maize roots which also showed O_2 deficiency (Darwent *et al.*, 2003).

In conclusion, our results support the hypothesis that severe O_2 deficiency occurred in the stele and apical regions of barley adventitious roots grown in stagnant deoxygenated solutions, which, as hypothesised, decreased the whole-root K : Na ratio and affected K, Na and Cl concentrations in various cell types across the roots. Oxygen deficiency in the stele at a subapical root region reduced the accumulation of K not only in xylem parenchyma cells, as hypothesised, but also in the pericycle, indicating that both of these cell types were involved in energy-dependent K loading into the xylem. When combined with salinity, stelar hypoxia reduced the concentrations of Na and Cl in the pericycle, suggesting that these cells play a role in the energy-dependent control of Na and Cl transport to xylem. Moreover, the Na profiles showed that the accumulation of Na in the outer root cell layers likely contributed to regulation of Na reaching the inner cells near the root tips in aerated conditions, but this regulation was abolished when severe O_2 deficiency occurred in the root tips; the Na accumulation in outer cells was evident at the 'hydraulically-isolated' root tips (no mature xylem) but not further back along the roots where some xylem presumably had matured.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Relative growth rate (RGR) of barley (*Hordeum vulgare* var. Franklin) shoots and roots in plants grown in aerated nonsaline, aerated saline (100 mM NaCl), stagnant nonsaline or stagnant saline (100 mM NaCl) nutrient solutions.

Fig. S2 Concentrations of K, Na and Cl in seminal roots and shoots of barley (*Hordeum vulgare* var. Franklin) grown in aerated nonsaline, aerated saline (100 mM NaCl), stagnant nonsaline or stagnant saline (100 mM NaCl) nutrient solutions.

Table S1 Composition of nutrient solutions used in experiments

Table S2 Strength of nutrient solutions during experiments

Table S3 Growth of barley (*Hordeum vulgare* var. Franklin) in aerated, aerated saline (100 mM NaCl), stagnant or stagnant saline (100 mM NaCl) nutrient solutions

Table S4 K : Na ratio in shoots and seminal roots of barley (*Hordeum vulgare* var. Franklin) grown in aerated nonsaline, aerated saline (100 mM NaCl), stagnant nonsaline or stagnant saline (100 mM NaCl) nutrient solutions

Table S5 Absolute root area and percentage area of stele in cross-sections taken 10 and 50 mm from the root apex of 60–100 mm adventitious roots of barley (*Hordeum vulgare* var. Franklin) grown in aerated, aerated saline (100 mM NaCl), stagnant or stagnant saline (100 mM NaCl) nutrient solutions

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