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Expressing *Arabidopsis thaliana* V-ATPase subunit C in barley (*Hordeum vulgare*) improves plant performance under saline condition by enabling better osmotic adjustment

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Abstract. Salinity is a global problem affecting agriculture that results in an estimated US$27 billion loss in revenue per year. Overexpression of vacuolar ATPase subunits has been shown to be beneficial in improving plant performance under saline conditions. Most studies, however, have not shown whether overexpression of genes encoding ATPase subunits results in improvements in grain yield, and have not investigated the physiological mechanisms behind the improvement in plant growth. In this study, we constitutively expressed *Arabidopsis Vacuolar ATPase subunit C* (*AtVHA-C*) in barley. Transgenic plants were assessed for agronomical and physiological characteristics, such as fresh and dry biomass, leaf pigment content, stomatal conductance, grain yield, and leaf Na^+ and K^+ concentration, when grown in either 0 or 300 mM NaCl. When compared with non-transformed barley, *AtVHA-C* expressing barley lines had a smaller reduction in both biomass and grain yield under salinity stress. The transgenic lines accumulated Na^+ and K^+ in leaves for osmotic adjustment. This in turn saves energy consumed in the synthesis of organic osmolytes that otherwise would be needed for osmotic adjustment.

Additional keywords: organic osmolytes, osmotic adjustment, potassium, salinity stress tolerance, sodium, vacuolar sequestration.

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Introduction

As the world population has been estimated to exceed 9.3 billion people by 2050, there has been a shift towards agricultural production systems on marginal saline lands (Shabala 2013; Panta *et al*. 2014). The problem of salinity is exacerbated by a reduction in precipitation in subtropical areas, resulting in farmers irrigating with brackish and low-quality water, thereby increasing the salinity of soils (Munns and Tester 2008; Barrett-Lennard and Setter 2010). The estimated cost of salinity-induced loss to world crop production is believed to be in excess of US$27 billion per year (Qadir *et al*. 2014). Improving performance of crop plants so that they produce a better yield under saline conditions is of paramount importance for using these marginal lands (Munns 2002; Roy *et al*. 2013).

Excess Na^+ in the cytosol, however, is toxic to cellular processes, regardless of whether plants are halophytes or glycophytes (Flowers and Colmer 2008). Therefore, for Na^+ to be an effective osmolyte it must be sequestered in the vacuole. Three components are essential for the storage of Na^+ in the vacuole: (i) establishment of the electrochemical gradient required to transport Na^+ into the vacuole; (ii) Na^+ transport across the tonoplast into the vacuole using ion transporters; and (iii) retention of Na^+ within the vacuole (Shabala 2013).

Tonoplast Na^+/H^+ antiporters, such as members of the Na^+/H^+ exchanger (NHX) family, have been shown to be important in the loading of Na^+ into the vacuole (Blumwald and Poole 1985; Barkla *et al*. 1995; Gaxiola *et al*. 1999; Apse and Blumwald 2007). NHXs rely on tonoplast localised proton pumps, namely vacuolar ATPases (*VHAs*) (Vera-Estrella *et al*. 1999; Wang *et al*. 2001; Krebs *et al*. 2010) and vacuolar pyrophosphatases (V-PPases) (Parks *et al*. 2002; Vera-Estrella *et al*. 2005; Guo *et al*. 2006) to generate a proton gradient between the vacuole and the cytoplasm. Once sequestered in to the vacuole, Na^+ is prevented from leaking back into the cytosol by the efficient control of and regulation of activity of sodium permeable slow (SV) and fast (FV) vacuolar channels

(Pantoja *et al*. 1989; Shabala and Mackay 2011; Bonales-Alatorre *et al*. 2013*a*, 2013*b*).

There have been many studies in different plant species investigating whether improvements in vacuolar Na^+ sequestration through the enhancement of *NHX* expression can improve plant salinity tolerance. Some studies have found enhanced expression of *NHX* genes can enhance growth under saline conditions (Apse *et al*. 1999; Zhang and Blumwald 2001; Zhang *et al*. 2001; Bayat *et al*. 2011) whereas others have discerned no yield improvements (Adem *et al*. 2015). A better approach to enhance growth under salt may be manipulating the expression of genes encoding proteins which establish the proton gradient necessary for effective Na^+ sequestration in the vacuole, in particular manipulating of the expression of *VHA* and *V-H*$^+$*-PPases*.

Manipulation of genes encoding H^+-PPases has often resulted in enhanced performance under salt, although vacuolar H^+-PPases are often considered a supporting mechanism to VHA in the acidification of the vacuole (Maeshima 2000). Hence, investigating whether greater improvements in vacuolar storage of Na^+, and thus plant salinity tolerance, can be made by enhancing the abundance of VHA is important.

Unlike vacuolar H^+-PPases, vacuolar H^+-V-ATPase (VHA) are made of several protein subunits, which are encoded by different genes. Studies have investigated, however, whether manipulation of the expression of genes encoding specific VHA subunits can improve plant growth under saline conditions. Expressing the *VHA-c-1* subunit from the halophyte grass *Spartina alterniflora* Loisel. improved transgenic rice growth under saline conditions (Baisakh *et al*. 2012). However, the beneficial effect was reported only for vegetative plant growth and not for grain yield. However, *VHA-c-1* expressing rice had early closure of the leaf stomata and reduced stomata density (Baisakh *et al*. 2012) during salt stress. This seems to contradict the finding that transgenic plants had increased biomass, as it is assumed these lines would have a reduced capacity to assimilate CO_2.

More recently, wheat genes encoding individual V-ATPase subunits were expressed in *Arabidopsis* (He *et al*. 2014). Although improvements in the salt tolerance of seedling of transgenic *Arabidopsis* plants were observed, no insights into the mechanisms behind the improved tolerance were reported (He *et al*. 2014). Similar results were reported when wheat *V-ATPase subunit B* (*VHA-B*) (Wang *et al*. 2011) and *subunit E* (*VHA-E*) (Zhao *et al*. 2009) were expressed in *Arabidopsis* and when the *V-ATPase subunit c* (*VHA-c*) from *Limonium bicolor* was expressed in tobacco (Xu *et al*. 2011).

In the present study, we generated lines of barley expressing the *Arabidopsis V-ATPase subunit C* (*VHA-C*) and assessed their physiological and agronomical performance under saline conditions. The V-ATPase subunit C (*VHA-C*) was chosen as it plays a role in the hydrolysis of ATP in the V1 complex of the vacuolar ATPase structure (Sze *et al*. 2002; Krebs *et al*. 2010), and can directly enhance the activity of the V-ATPase. Barley lines expressing the *AtVHA-C* gene were found to have smaller salinity induced reductions both in biomass and grain yield compared with non-transformed wild-type plants. These beneficial effects may be linked to the improved ability of the transgenic lines to maintain higher stomata conductance, resulting from increased Na^+ and K^+ accumulation in the leaf which contributed to osmotic adjustment and made plants less reliant on *de novo* synthesis of organic osmolytes.

Materials and methods

*Generating transgenic barley plants expressing VHA-C (*Arabidopsis V-ATPase Subunit C*)*

For the experiments wild-type barley (accession WI4330) was kindly supplied by the University of Adelaide Barley Breeding Program. The coding sequence of *AtVHA-C* (*At1g12840*) was amplified from the cDNA of *Arabidopsis thaliana* (L.) Heynh. (ecotype Col-0) utilising primer pairs, 5′-ATG ACT TCG AGA TAT TGG GTG-3′ and 5′-TTA AGC AAG GTT GAT AGT GAA G-3′ and high-fidelity DNA polymerase (Elongase, Invitrogen). The amplified sequence was introduced into the Gateway enabled entry vector *pCR8/GW/TOPO TA* (Invitrogen). *AtVHA-C* was recombined into the transformation vector *pMDC32* vector via an LR recombination reaction (Invitrogen) (Curtis and Grossniklaus 2003), under the control of a *CaMV 35S* promoter. The *AtVHA-C pMDC32* vector was transformed into wild-type barley WI4330 via *Agrobacterium tumefaciens*-mediated transformation (Tingay *et al*. 1997; Jacobs *et al*. 2007). After antibiotic selection, the transformed plantlets were regenerated in soil (Singh *et al*. 1997; Jacobs *et al*. 2007) and grown to produce T_1 seed. After one round of seed multiplication the T_2 plants were grown for experiments at the University of Tasmania.

Plant growth conditions

Four *AtVHA-C* expressing (*35S:AtVHA-C*) T_2 lines described here as OE1, OE2, OE3 and OE4, and one non-transformed WI4330 were grown in 2 L pots containing potting mix. The four lines were selected as a reasonable compromise between the reliability of the data and the workload. The potting mix was composed of 70% composted pine bark; 20% coarse sand; 10% sphagnum peat; Limil at 1.8 kg m^{-3}, dolomite at 1.8 kg m^{-3}). The plant nutrient balance was maintained by adding the slow release fertiliser Osmocote Plus (Scotts Australia) at 6 kg m^{-3} and ferrous sulfate (at 500 g m^{-3}) (Bonales-Alatorre *et al*. 2013*a*). The plants were grown in a glasshouse with regulated temperature (24–20°C day/15°C night), 15 h day/8 h night period and artificial light 150 µmol $m^{-2}s^{-1}$ for ~2 weeks after seedling establishment (third leaf stage). Plants were grown in 2 L pots, with three plants per pot and 4–5 pots per treatment/genotype. Salinity treatment was administered by adding 300 mM NaCl irrigation water based on water requirement and uniform to the treatments until maturity. The experiment was conducted between January and April 2014.

DNA extraction and PCR analysis

Barley genomic DNA from the above lines was extracted using the method described by Edwards *et al*. (1991). PCR was performed to confirm the presence of the *AtVHA-C* transgene in the transformed plants using a forward (5′-AGA GAC TCG TAA ACA AGA G-3′) and reverse (5′-CAG CCA TGG CTC CTG CA-3′) primer, which amplified a fragment of 299 bp in size. The *Hv-VRT2* vernalisation gene (GenBank DQ201168) was used as a control gene, and this gene was amplified using *Hv-VRT2* specific forward (5′-CCG AAT GTA CTG CCG TCA TCA CAG-3′) and reverse (5′-TGG CAG AGG AAA ATA TGC GCT

TGA-3′) primers, which amplifies a fragment of 280 bp in size. PCR reactions included 2 × reaction ImmoMix (Bioline), 10 µM of forward and reverse primers, 1 mg mL^{-1} BSA, MilliQ water and 0.5 µL of template DNA. The PCR conditions used were similar for both the *VRT2* and the *VHA-C* (95°C for 10 min, followed by 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, repeated for 35 cycles, with a final extension of 72°C for 10 min). PCR products were visualised by gel electrophoresis using a 2% agarose gel containing Gel Red Nucleic acid stain (Biotium) (5 µl per 100 ml of gel) and a photograph of the gel was obtained (Molecular imager, Gel Doc imaging system, Bio-Rad).

RNA extraction and gene expression analysis

Total RNA was extracted from leaf tissue using Bioline plant Isolate II RNA kit (www.bioline.com/isolate, accessed 11 August 2017). The RNA was diluted to 10 ng µL^{-1} and subjected to RT-PCR with the reaction mix containing 5 × buffer, 10 mM dNTP, water and enzyme mix (*Taq* polymerase and reverse transcriptase), forward and reverse primers for *AtVHA-C* and *HvGAP* (*VHA-C* gene: forward primer 5′-AGA GAC TCG TAA ACA AGA G-3′ and reverse primer 5′-CAG CCA TGG CTC CTG CA-3′, and *Hv-GAP*: forward primer 5′-GTG AGG CTG GTG CTG ATT ACG-3′ and reverse primer 5′-TGG TGC AGC TAG CAT TTG ACA C-3′) and RNA at 10 ng µL^{-1}. Reverse transcription was performed first at 50°C for 30 min, before PCR (initial PCR activation 95°C for 15 min, then 35 cycles of 94°C for 1 min, of 55°C for 1 min and 72°C for 1 min, before a final extension 72°C for 10 min). PCR products were visualised by gel electrophoresis as described above.

Biomass and grain yield

As plants were grown to maturity to collect grain, only the DW of the barley plants (no fresh weights) were recorded at the end of experiment. Plant height was measured at the same period of time as DW. The number of seeds per plant and their total weight were recorded. The sample size for biomass and yield data for the salt treated plants range from eight to 15 plants.

Pigment content and stomatal conductance

The number of chlorotic and necrotic leaves per plant was recorded after 5 weeks of salt treatment. Leave which were partially yellow leaves were deemed as chlorotic leaves. Completely dead and dried leaves were deemed as necrotic. Relative leaf chlorophyll content was measured using a SPAD-502 Chlorophyll Meter (Konica Minolta). Measurements were taken on the third true leaf, at a position about one-quarter of the length of the leaf from the leaf tip. The same leaf/position was used to measure stomatal conductance (g_s) from control and salt treated plants using a Decagon leaf porometer (Decagon Devices Inc.), under constant light conditions (artificial light of 150 µmol m^{-2} s^{-1}) between 1100 and 1500 hours.

Na^+ and K^+ concentration

The third leaf from both salt treated and control plants were excised after 5 weeks of salt treatment for Na^+ and K^+ ion concentration measurement. Leaves were dried in the oven at 65°C for 72 h. A subsample of a known weight (~0.1 g) was digested in a solution consisting of 5 mL of 65–70% HNO_3 and 2 mL of 30% H_2O_2 using a microwave digester (MDS-2000, CEM Corp.). The digested samples were diluted 1 : 5 using distilled water (Skoog *et al*. 2000). The Na^+ and K^+ ion concentration was measured using a flame photometer (Model PFP7-flame photometer, Jenway).

Sap osmolality measurements and calculation of contribution of inorganic ions towards leaf osmotic adjustment

After 5 weeks of treatment with 300 mM NaCl, the third leaf of the barley transgenic and WT plants harvested and stored at −20°C. Leaf sap was extracted using a freeze–thaw method (Tomos *et al*. 1984) and osmolality was measured using the vapour pressure osmometer (Vapro, Wescor Inc.). The sample size for both control and salt treated plants was $n = 6$. The measured Na^+ and K^+ concentrations in the tissue were used to calculate the relative contribution of these ions towards overall sap osmotic potential. Contribution of Cl^- was estimated as 1.2 of that for Na^+, as previously described (James *et al*. 2006; Puniran-Hartley *et al*. 2014).

Estimation of vacuolar and cytosolic pH using fluorescence dyes

In this study, the gradient of the pH between the cytosol and the vacuole was estimated with a qualitative method using one wavelength (488 nm). The gradient of the pH in different cellular compartments in mesophyll cells from *AtVHA-C* expressing and non-transformed WT barley plants were measured using

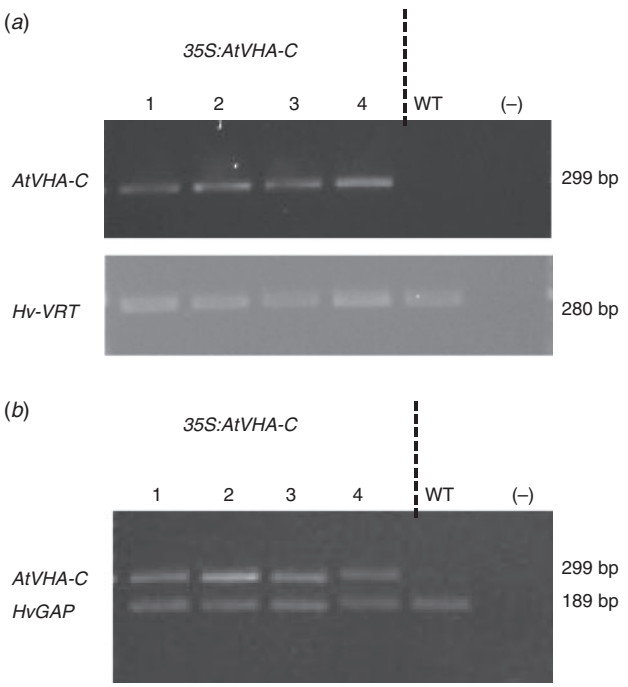

Fig. 1. Genotyping *AtVHA-C* transgenic barley. (*a*) Detection of transgene presence by PCR using specific primers for *AtVHA-C* and *Hv-VRT2* (internal control); (*b*) Expression analysis of the *AtVHA-C* gene using RT-PCR with specific primers for the gene and *HvGAP* specific primers (internal control). Lanes *35S:AtVHA-C*-1, 2, and 3, are transgenic *AtVHA-C* barley lines, WT (non-transformed barley cv. WI4330), (–) negative control (water).

pH sensitive dye (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF)-AM after 2 weeks of 300 mM NaCl treatment (see Figs S1 and S2, available as Supplementary Material to this paper). Dye loading was performed essentially according to Krebs *et al*. (2010). The choice of optimal BCECF-AM dye concentration was based on the published work on *Arabidopsis* (Scheuring *et al*. 2015). False colour images were obtained using a confocal laser-scanning microscope (SP5, Leica) with ×20 and ×40 objectives after excitation with 488 nm and recording emissions between 520 nm and 550 nm. A further validation of the above protocol was conducted using mesophyll samples co-stained with BCECF-AM and N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl) hexatrienyl) pyridinium dibromide (FM4–64), a dye that stains both plasma and vacuole membranes (Wu *et al*. 2015) and allow a better resolution between intra-cellular compartments. After 1 h incubation with 20 µM BCECF-AM, the same mesophyll samples were then incubated together with 20 µM FM4–64 for 30 min to visualise tonoplast. Mesophyll samples were then rinsed with buffer solution (10 mM KCl, 5 mM Ca^{2+}-MES, pH 6.1) for 3 min. For FM4–64 fluorescence, the 488-nm excitation was used and collected with a 615 nm long-pass filter. Experiments were conducted with WT and three OE lines (OE1, OE2 and OE3) in both control conditions and 2 weeks of 300 mM NaCl exposure. Chloroplast fluorescence was detected between 680 and 700 nm in order to separate the autofluorescence of chlorophyll in chloroplasts from the BCECF fluorescence. Images were analysed with LAS AF software (Leica Microsystems), and ImageJ software (National Institutes of Health) which was also used to calculate the corrected total cell fluorescence and estimated fluorescence for cytosol, vacuole and chloroplast after background subtraction (Bonales-Alatorre *et al*. 2013*b*).

Statistical analysis

The data were analysed using Student's *t*-tests. Significant differences were determined and indicated: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ using a statistical software SPSS ver. 22 (IBM SPSS).

Results

Generation of barley plants expressing V-ATPase Subunit C

Four *AtVHA-C* expressing lines, designated as OE1 to OE4 (*35S*:*AtVHA-C*-1 to *35S*:*AtVHA-C*-4), and one non-transformed WI4330 line (WT) were included in this study. The OE4 line did not show a consistent phenotype throughout all experiments, most likely due to a detrimental effect from the transformation process, and was therefore omitted from analysis. The presence of the *VHA-C* gene in the genome of transgenic lines and its absence in the WT were confirmed using transgene specific PCR on genomic DNA extracted from the leaf (Fig. 1*a*). The

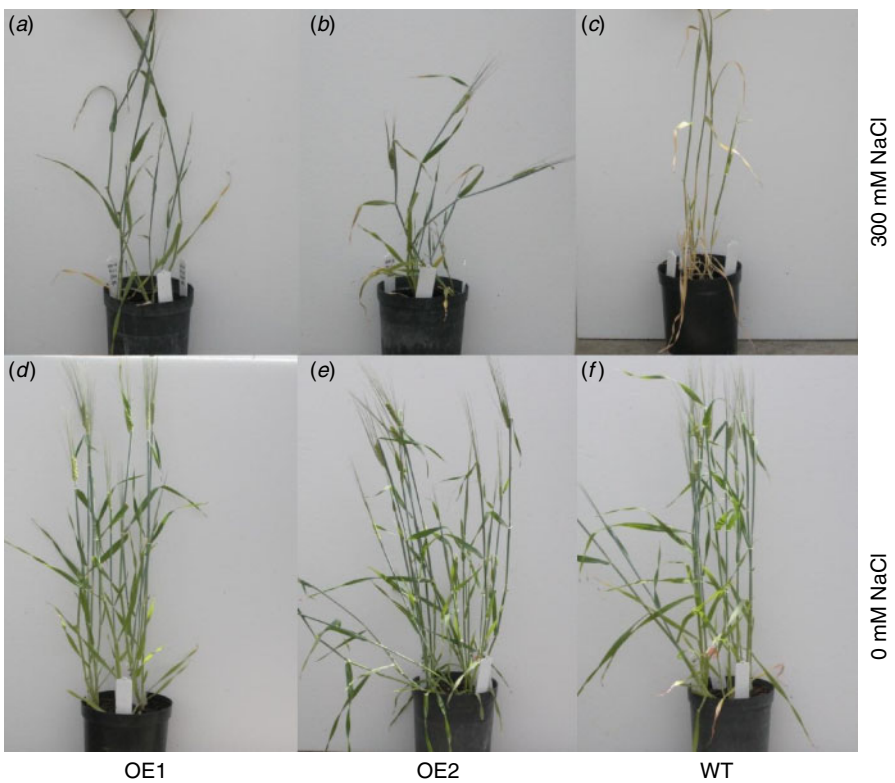

Fig. 2. Phenotypic difference between non-transformed and overexpressing lines. For simplicity, only two (of three) overexpressing (OE) lines are shown. (*a–c*) Plants were treated with 300 mM NaCl for five weeks; (*d–f*) zero NaCl treatment (no salt stress). The photos were taken 8 weeks after sowing.

expression of the *VHA-C* gene in transgenic lines and the absence of its expression in the WT were confirmed by RT-PCR (Fig. 1*b*).

Transgenic barley expressing V-ATPase subunit C has improved overall salinity stress tolerance and grain yield

VHA-C expressing barley produced more tillers and maintained better shoot health when grown on 300 mM NaCl for 5 weeks compared with WT plants, which showed signs of severe senescence and wilting (Fig. 2). The DW of OE lines and WT under control conditions at 0 mM NaCl was not affected (Fig. 3*a*), and an increase in the DW of transgenic plants under salt stressed conditions for OE3 line was observed at 300 mM NaCl (Fig. 3*b*), and resulted in transgenic lines exhibiting an improvement in salt tolerance (as measured by biomass in 300 mM NaCl divided by the biomass in 0 mM NaCl) when compared with WT plants (Fig. 3*c*), 43 to 47% in *AtVHA-C* lines vs 35% in WT.

The number of chlorotic leaves in salt-grown plants did not differ significantly between WT and OE lines (Fig. 4*a*). At the same time, the WT had a significantly ($P \leq 0.001$) higher number of necrotic leaves compared with *AtVHA-C* expressing lines when grown for 5 weeks on 300 mM NaCl (Fig. 4*b*). We noted that there were large differences in stomatal conductance observed between both WT and OE lines under control and salt stressed conditions. While the stomatal conductance of the OE lines was significantly lower than the wild type in 0 mM NaCl (Fig. 5*a*), all three OE lines had significantly higher ($P \leq 0.001$) stomatal conductance than WT when the plants treated with 300 mM NaCl for 5 weeks (Fig. 5*b*).

As expected, the chlorophyll content was not significantly different between OE lines and WT under control conditions (Fig. 5*d*) but WT plants had a significant reduction in chlorophyll content under saline conditions when compared with *AtVHA-C* expressing barley (significant at $P \leq 0.05$; Fig. 5*d*, *e*). Also significantly higher was relative chlorophyll content in transgenic lines compared with WT barley (Fig. 5*f*, significant at $P \leq 0.001$). At harvest, *AtVHA-C* expressing lines (OE) had larger seed size and were significantly ($P \leq 0.001$) heavier in seed weight compared with WT barley plants after growing under 300 mM NaCl stress (Fig. 6; Table 1). Under 0 mM NaCl there was no significant difference ($P \leq 0.05$) between the numbers of seeds per plant between OE lines and WT neither (Fig. 7*a*). However, an increase in the number of seeds per plant in the transgenic lines in 300 mM NaCl, when compared with WT, (Fig. 7*b*) resulted in an increase in salinity tolerance (as measured by yield under 300 mM NaCl divided by the yield under 0 mM NaCl) (Fig. 7*c*). Although the grain yield per plant (g plant^{-1}) was not significantly (at $P \leq 0.05$) different between the OE lines and WT under 0 mM NaCl conditions (Fig. 7*d*), there was significantly higher grain yield in two of three *AtVHA-C* expressing lines under 300 mM (OE-1 and OE-2; significant at

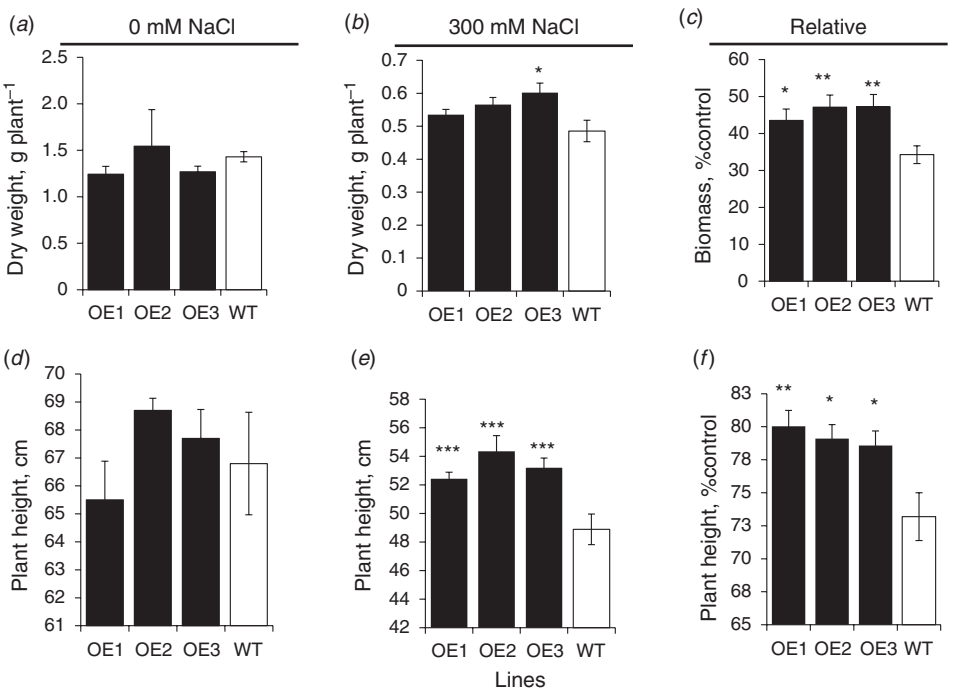

Fig. 3. Comparative analysis of agronomic characteristics between non-transformed and overexpressing (OE) plants. Shoot DW (*a*–*c*) and plant heights (*d*–*f*) are shown for plants grown under 0 mM NaCl (no salt; *a*, *d*) and 300 mM NaCl (saline; *b*, *e*) conditions. Panels (*c*, *f*) show relative shoot dry weight and plant height respectively (calculated for salt-treated plants as % of control). Results are mean ± s.e. ($n = 6$–15). Measurements were performed 12 weeks after sowing. Significant differences (compared with wild type) are indicated: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

$P \leq 0.001$ and $P \leq 0.01$ respectively; Fig. 7*e*). This resulted in a greater maintenance of grain yield in the transgenic lines compared with the control (Fig. 7*f*).

Transgenic barley expressing V-ATPase subunit C has increased leaf Na^+ and K^+ and these ions contributed towards osmotic adjustment

The OE lines had significantly higher leaf Na^+ concentration than WT plants, when grown in both 0 mM NaCl (Fig. 8*a*) and 300 mM NaCl (Fig. 8*b*) ($P \leq 0.001$). However, the relative increase in leaf Na^+ under salt stress was less in OE lines compared with WT (6- to 7-fold vs 9.2-fold in WT; significant at $P \leq 0.001$; Fig. 8*c*). The leaf K^+ concentration for all the OE lines and WT was not significantly different ($P \leq 0.05$) under control conditions (Fig. 8*d*) but all the OE lines showed significantly higher leaf K^+ concentration compared with WT under saline conditions (significant at $P \leq 0.001$; Fig. 8*e*). Two out of three OE lines (OE-1 and OE-2) have significantly increased the overall leaf K^+ concentration (1.5- and 1.4-fold respectively) whereas WT-plants were not able to do this when grown under saline conditions (Fig. 8*f*).

The osmolality of leaf sap showed no differences between OE lines and WT (Table 2) but the measured contribution of inorganic ions in WT plants was lower than in OE lines. We estimated the relative contribution of inorganic and organic osmolytes towards osmotic adjustment in barley leaves. In WT, the overall contribution of three major inorganic osmolytes (Na^+, Cl^-, and K^+) was only ~34%, implying that the major bulk of osmotic adjustment under saline conditions (~66%) was achieved by other means, such as *de novo* synthesis of organic osmolytes (Table 2). In a stark contrast, contribution of organic osmolytes towards osmotic adjustment in OE lines varied between 49 and 52% (Table 1; significant compared with WT at $P \leq 0.01$), e.g. was by ~15% less than in WT.

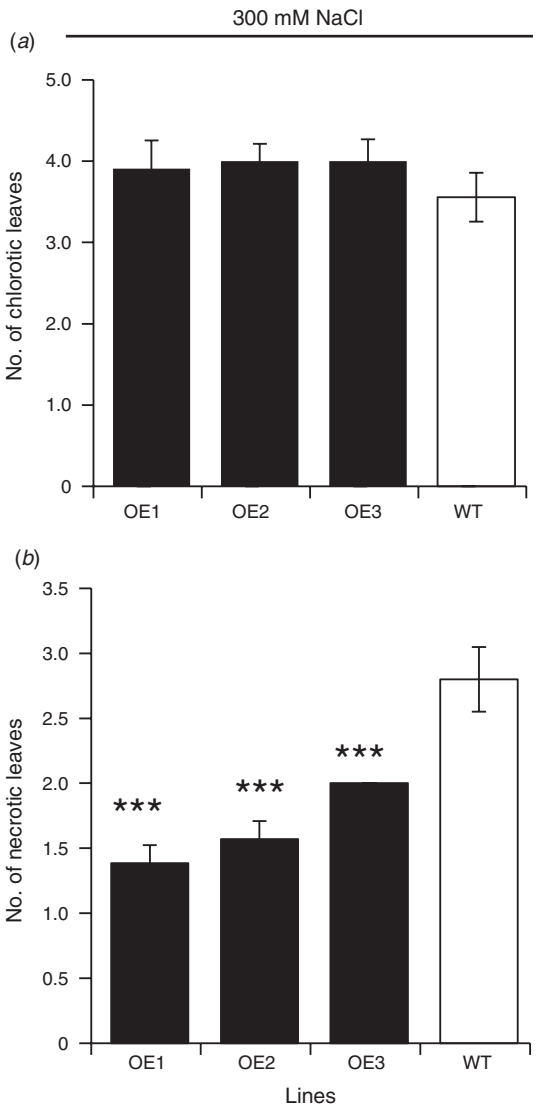

Fig. 4. Difference in the number of chlorotic (*a*) and necrotic (*b*) leaves between OE lines and non-transformed barley grown in 300 mM NaCl. Results are mean ± s.e. ($n = 6$ to 15). Measurements were performed 5 weeks after salt stress. Significant differences (compared with wild type) are indicated: ***, $P \leq 0.001$.

Enhancing Na^+ accumulation transgenic barley may be associated with cellular pH adjustment

We further estimated the relative changes in cytosolic and vacuolar pH in leaf mesophyll cells of WT and OEs for control and 300 mM NaCl treated plants (Fig. 9). As confocal microscopy was not suitable for ratiometric pH quantification, BCECF-AM was used to estimate changes in the relative fluorescent intensity in both the cytosol and vacuole in order to evaluate the H^+ gradients across the tonoplast. We used corrected cell fluorescence by subtracting the background fluorescence similar to our work in Na^+ measurements (Bonales-Alatorre *et al*. 2013*a*, 2013*b*; Wu *et al*. 2015). We also sampled a large number of cells (>200) in order to reduce systematic errors. We first conducted experiments to separate BCECF fluorescence signals between cytosol and vacuole using FM4-64 and auto fluorescence of chloroplasts (Fig. 9), to show intracellular pH distribution in the mesophyll of WT after 2 weeks of 300 mM NaCl. This was achieved by selecting one typical cell possessing a large central vacuole for the region of interest (ROI) analysis (Fig. 9*a–e*). ROI lines were then drawn crossing the cytosol twice and the large vacuole in the cells (Fig. 9*f–i*), and intracellular pH distribution was quantified as relative intensity of BCECF fluorescence in mesophyll cells in the control and salt treatment (Fig. 9*j*, *k*).

A higher H^+ pump activity by the VATPase should cause an acidification in the vacuole and alkalinisation of the cytosol. The proton increase in the vacuole can then be used for Na^+/H^+ antiportation, accumulating Na^+ into the vacuole. Cytosolic pH is usually maintained at around 7.5 (low H^+ concentration) in plant cells, which was indicated by a low BCECF fluorescence intensity (Fig. 9*e*, *j*). In contrast, vacuolar pH is normally between 4.5 and 5.5 (high H^+ concentration), which was clearly shown as much higher by BCECF fluorescence (Scheuring *et al*. 2015) compared with the cytosolic pH (Fig. 9*e*, *k*). Moreover, salt treatment led to no significant (at $P < 0.05$) changes in cytosolic pH changes in mesophyll cells of WT and three OE barley lines (Fig. 9*j*), suggesting cytosolic pH was tightly regulated even under high salinity stress. However, overexpressing *AtVHA-C* resulted

in significant acidification of vacuolar pH especially in mesophyll cells of line OE1 (Fig. 9*k*). The BCECF fluorescence intensity in mesophyll cells of WT and three barley lines overexpressing *AtVHA-C* was significantly reduced by salt stress ($P < 0.001$). In addition, vacuolar pH in mesophyll cells of OE2 showed significantly high BCECF fluorescence intensity after salt tress as compared with WT and other two OE lines (Fig. 9*k*). Again, the overexpressing *AtVHA-C*-induced vacuolar pH changes matched well with the overall high salt tolerance in OE1 and OE2 in contrast to WT and OE3 (Figs 2–8). Therefore, the results demonstrate that the overexpression of V-ATPase subunit C in barley may have resulted in a vacuolar high H^+ pump activity for more NHX-mediated Na^+ sequestration in vacuole, potentially leading to an alkalinisation of the vacuole (e.g. from pH 5 to 6) and slight acidification of cytosol (e.g. from pH 7.5 to 7.2).

Discussion

Transgenic VHA-C barley improved growth and grain yield under saline growth condition

The activity of VHA has been shown to increase under salt stress (Silva *et al*. 2010), accompanied by an upregulation of both *VHA* and *NHX* transcripts under salt stress (Qiu *et al*. 2007). Transgenic plants expressing subunits of *VHA* have shown improvement in growth and performance under saline condition in several species, such as the expression of the *VHA-c-1* subunit in rice (Baisakh *et al*. 2012) and tobacco (Xu *et al*. 2011), as well as the expression of several *VHA* subunits in *Arabidopsis* (Zhao *et al*. 2009; Wang *et al*. 2011; He *et al*. 2014). The possible mechanisms for increased pump activity by overexpressing a certain subunit (subunit C in this work) may be due to the fact that this subunit is functioning in the active centre of the pump that hydrolyses ATP. Hence, having a higher number of subunits during the protein assembly of the pump will increase the probability of having more pumps with a higher catalytic activity. This is especially true for subunit C that is a catalytic subunit and it can be limiting as compared with the other subunits.

To date, the expression of *VHA* has been enhanced in plants which are considered to exclude Na^+ from the shoot and do not normally rely on vacuolar Na^+ sequestration mechanisms (Shabala 2013). To understand the role of VHA in a crop plant, which can accumulate a substantial amount of shoot Na^+ in the shoot, the *VHA-C* gene was successfully cloned from *Arabidopsis* and transformed into barley under the control of the *CaMV 35S* promoter. In this study we show, for the first time, that expressing *VHA-C* from *Arabidopsis* also enhances salinity stress tolerance in a crop plant (barley) which has the ability to accumulate significant levels of shoot Na^+.

The transgenic lines expressing *VHA-C* not only showed significant ($P < 0.05$ and higher) dry weight and plant height when grown under saline conditions (Fig. 3) but also higher grain yield (Fig. 7*c*, *e*). As far as we are aware, this is the first work showing transgenic plants expressing *VHA* subunits have improvements in this important agronomical trait.

Increased stomatal conductance contributes to enhanced salinity tolerance in transgenic plants

Previous reports suggest the beneficial effects of overexpressing VHA transporter subunits were related to either improved plant

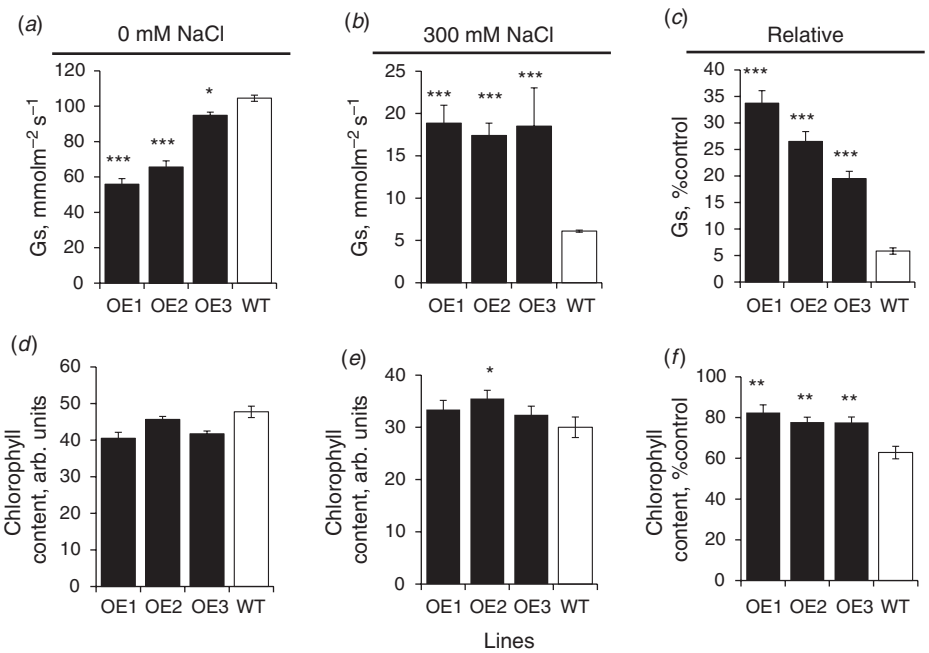

Fig. 5. Stomatal conductance (*a*–*c*) and leaf chlorophyll content (SPAD values; *d*–*f*) of OE lines as compared with non-transformed barley. (*a*, *d*) Treatment with 0 mM NaCl, (*b*, *e*) treatment with 300 mM NaCl, (*c*, *f*) relative values (calculated for salt-treated plants as % of control). Results are mean ± s.e. ($n = 6$–15). Measurements were performed 8 weeks after sowing. Significant differences (compared with wild type) are indicated: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

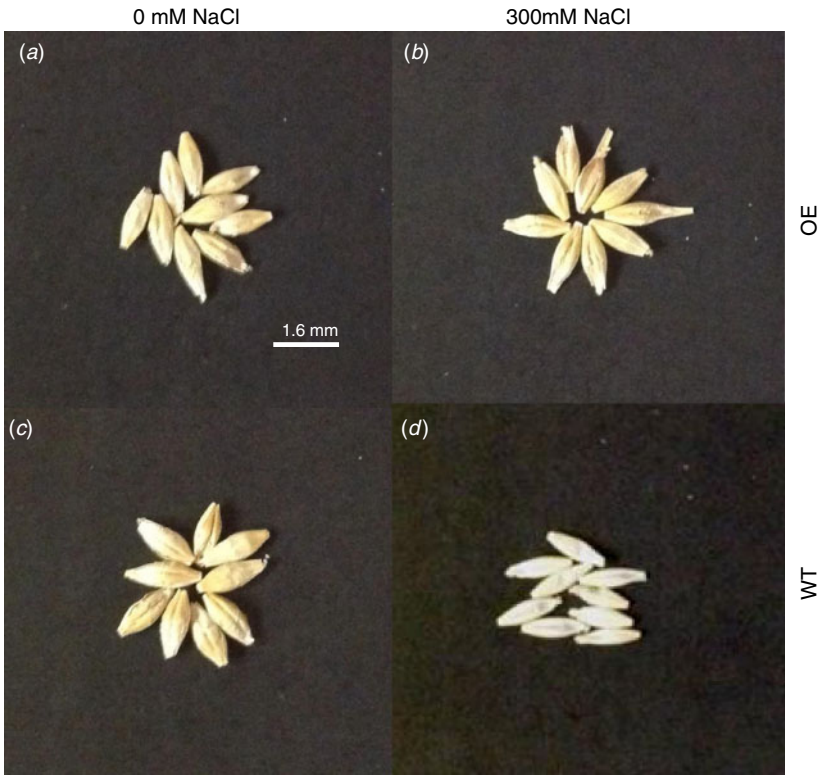

Fig. 6. Seed size of transgenic lines. The difference in seed size is illustrated for one *35S*:*AtVHA-C* overexpressing line (OE3) under no salt (*a*, *c*) and salt stress (*b*, *d*) conditions. These are representative images for the phenotypes observed in the other lines.

water status, resulting from reduced stomatal density and early stomatal closure (Baisakh *et al*. 2012), or from increased activity of enzymatic antioxidants (AO) in transgenic lines (He *et al*. 2014). However, plant biomass (DW) is directly proportional to the ability of the plant to assimilate CO_2 (which relies on gas exchange through open stomates). Therefore reductions in stomatal density and early stomatal closure, as suggested by Baisakh *et al.* (2012), cannot be the sole reason *VHA* expressing plants to have greater salinity tolerance, as it would be expected that CO_2 assimilation would be lower in these plants. In addition, there are many reports showing there is no direct correlation between AO activity and salinity stress tolerance in crops (Maksimović *et al*. 2013). Many salt tolerant plants (such as halophytes) do not require high AO activity as they prevent formation of high levels of reactive oxygen species in the first place (Bose *et al*. 2014). Therefore, there must be other mechanisms by which *VHA* can improve salt tolerance in transgenic plants. From the data reported here, two physiological mechanisms were identified which would lead to improved performance of overexpression lines. The first one was reduced number of necrotic leaves (Fig. 4) and the second one – higher relative stomatal conductance (Fig. 5). The association between expressing *VHA* subunits and stomatal opening and closing has been reported by (Allen *et al*. 2000) using *VHA-C* mutant (*det3*), and also by RNAi knockdown of *OsVHA-A* (Zhang *et al*. 2013). Two to 3-fold higher g_s values in transgenic lines compared with WT (Fig. 5*b*) might account for better CO_2 assimilation, and fewer necrotic leaves increased the overall photosynthetic leaf surface in transgenic place. Both these factors could contribute to increase plant biomass and, ultimately, higher grain yield in OE lines.

Table 1. Seed weight of *AtVHA-C* expressing barley lines and wild type under 0 mM NaCl and 300 mM NaCl growth condition

Significant differences are indicated: ***, $P \leq 0.001$. Genotypes: WT, wild type; OE, overexpressing cultivar. Results are the mean ± s.e. ($n = 35$–50)

Cultivar/treatment	Seed weight (mg)
WT control	68.1 ± 4.0
OE control	58.0 ± 3.0
WT salt	15.0 ± 0.9
OE salt	48.1 ± 3.6***

Transgenic lines expressing VHA-C have better Na^+ sequestration

All the transgenic plants expressing *VHA-C* showed higher leaf Na^+ concentration than the WT when grown under saline condition (Fig. 8*b*). At the same time, the chlorophyll content was not affected (Fig. 4), and the number of necrotic leaves was reduced in OE lines (Fig. 4). Taken together, these facts point out at highly efficient vacuolar Na^+ sequestration in transgenic plants. All OE lines also showed higher Na^+ concentration under control conditions, that can be interpreted as plants using freely

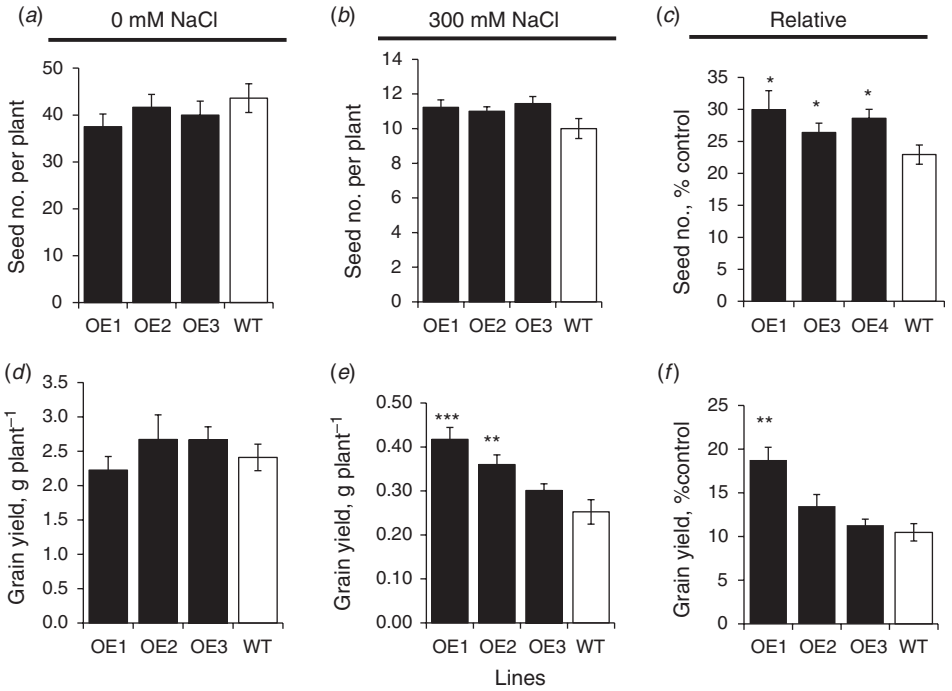

Fig. 7. Seed number (*a–c*) and total grain yield (*d–f*) of OE lines as compared with non-transformed controls: (*a*, *d*) 0 mM NaCl treatment; (*b*, *e*) 300 mM NaCl treatment; (*c*, *f*) relative values (calculated for salt-treated plants as % of control). Results are mean ± s.e. ($n = 6$–15). Measurements were performed 12 weeks after sowing. Significant differences (compared with wild type) are indicated: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

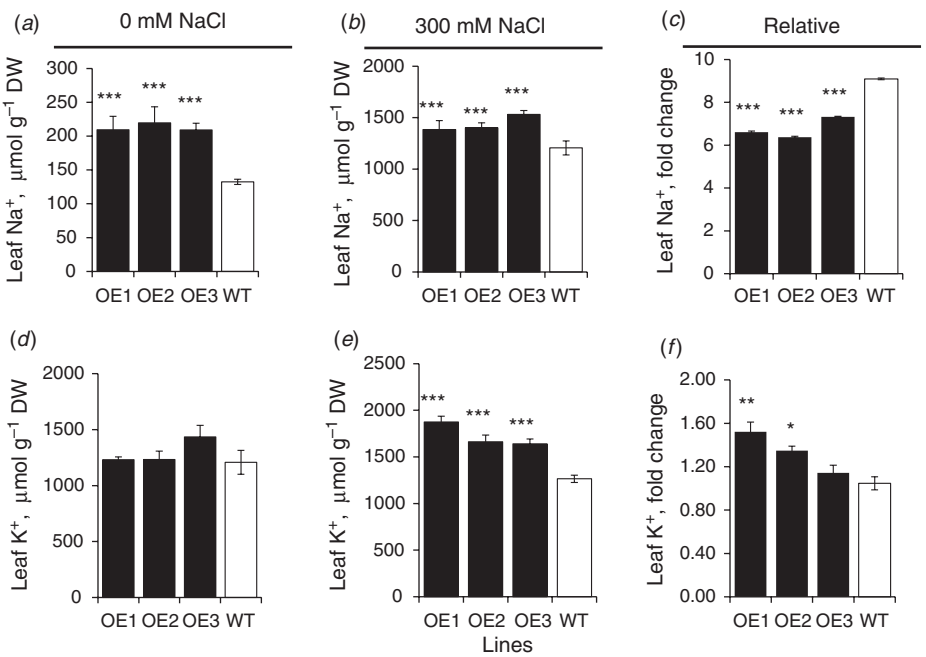

Fig. 8. Leaf Na^+ (*a–c*) and K^+ (*d–f*) concentration of OE lines as compared with non-transformed barley: (*a*, *d*) 0 mM NaCl treatment; (*b*, *e*) 300 mM NaCl treatment; (*c*, *f*) relative values (fold changes in salt-treated plants compared with control). Results are mean ± s.e. ($n = 6$–15). Measurement were performed 5 weeks after salt application. Significant differences (compared with wild type) are indicated: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

Table 2. Relative contribution of Na^+, K^+ and Cl^- towards total shoot sap osmolality in transgenic barley leaves grown under saline growth condition
Barley lines designated as*35S:AtVHA-C*-1 (OE-1), *35S:AtVHA-C*-2 (OE-2), *35S:AtVHA-C*-3 (OE-3) and non-transformed control (WT). Significant differences compared with WT: *, $P \leq 0.01$. The measured Na^+ and K^+ sap concentration values were converted into mOsm to calculate their relative contribution towards osmotic potential. For chlorine estimation see 'Materials and methods'

Lines	Measured osmolality					Contribution	Relative contribution	
	Total organic osmolality (mOsm)	Contribution of inorganic osmolality (mOsm)				of organic ions to total osmolality (mOsm)	Organic %	Inorganic %
		Na^+ (mOsm)	K^+ (mOsm)	Cl^- (estimated) (mOsm)	Total (mOsm)			
OE1	967	138.4	187.4	166.1	492	475.1	49.3	50.9**
OE2	1002	140.1	166.2	168.1	474	527.5	49.1	47.4**
OE3	986	153.2	164.0	183.8	500	485.7	52.6	50.7**
WT	1129	120.6	126.5	144.7	392	737.1	65.3	34.7

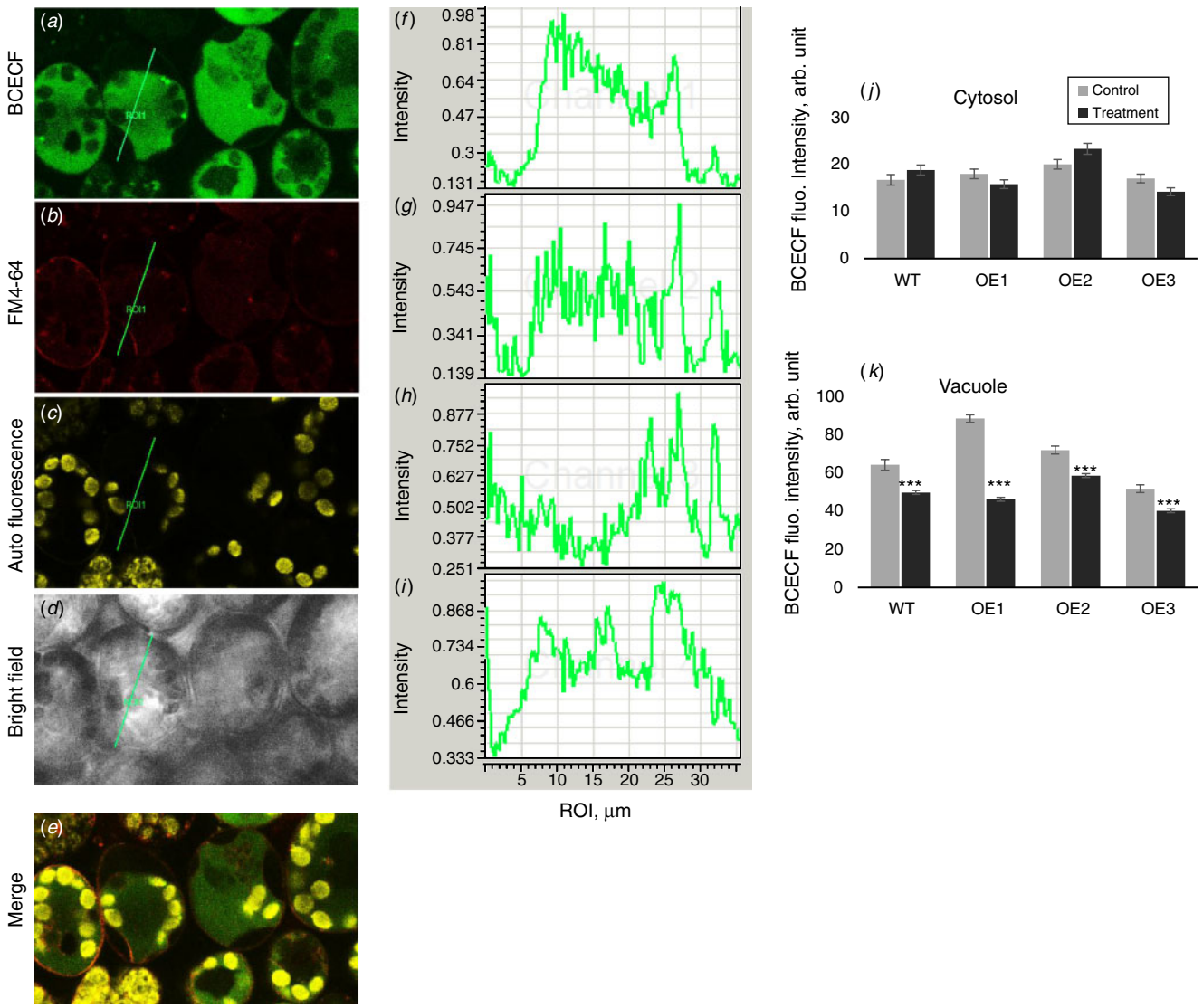

Fig. 9. Effect of overexpressing AtVHA-C on the cytosolic and vacuolar pH in barley. Representative confocal images of mesophyll cells showing fluorescence of BCECF: (*a*), FM4–64 (*b*), chlorophyll auto fluorescence (*c*) and brightfield (*d*) in a salt-treated WT barley plant (300 mM NaCl for 2 weeks) are shown. The BCECF fluorescence distribution between the cytosolic and vacuole vacuolar compartments can be separated by FM4-64 fluorescence at the tonoplast membrane illustrated in a merged image (*e*). Lines are drawn across the region of interest (ROI) in a presentative mesophyll cell of WT for BCECF (*f*), FM 4-64 (*g*), chlorophyll auto fluorescence (*h*), and brightfield (*i*). The mean cytosolic (*j*) and vacuolar (*k*) BCECF fluorescence intensity are calculated for WT and three OE lines in the control and salt treatment. Results are mean ± s.e. ($n = 200$–240 cells from four independent experiments). Significant differences (compared with control) are indicated: ***, $P < 0.001$.

available Na^+ (always present in the soil) as a cheap osmoticum to generate turgor and enable shoot growth.

Na^+/H^+ antiporters, such as members of the NHX family (Blumwald and Poole 1985; Barkla *et al*. 1995; Gaxiola *et al*. 1999; Apse and Blumwald 2007) energised by the proton motive force generated by proton pumps run the Na^+ sequestration process. The improvement in salinity tolerance in the *VHA-C* expressing barley may be due to an improvement in the H^+ gradient between the vacuole and the cytosol, so that NHX transporters could compartmentalise more Na^+. Expression of the subunit C of the VHA, therefore, could be instrumental to increase vacuolar H^+-ATPase phosphorylation (Armbrüster *et al*. 2004) or stability of its operation (Sze *et al*. 2002), therefore better energising the NHX activity and thus assisting Na^+ sequestration process. This is further confirmed in experiments using fluorescent pH dye (Fig. 9). Consistent with this model, transgenic *Arabidopsis* expressing wheat VHA subunit E showed increased Na^+ accumulation after 10 days of 120 mM NaCl but with a lower Na^+ accumulation in the cytosol (Zhao *et al*. 2009). It remains to be seen whether the improved sequestration in the vacuole was due solely to an increase in the activity of Na^+/H^+ antiporter which were already present in the cell or if there is also an upregulation of genes encoding Na^+/H^+ antiporters, resulting in a greater concentration (and more activity) of the transporters.

Plants expressing VHA-C showed higher K^+ retention

The barley plants expressing *VHA-C* showed significantly higher leaf K^+ concentration compared with WT (Fig. 8). This feature correlated with the fewer number of necrotic leaves in salt stressed plants (Fig. 4). This suggests that K^+ retention in the shoot was essential for preventing salt stress-induced senescence, a programmed cell death process (PCD). The loss of cytosolic K^+ in cells under stress induces the activation of caspase-like proteases and endonucleases and, hence causes PCD in plants (Shabala *et al*. 2007; Demidchik *et al*. 2010) and mammalian cells (Hughes and Cidlowski 1998, 1999). Potassium retention in both shoots and roots is an important mechanism in salt tolerance in barley, which was corroborated by K^+ kinetics study on shoots and roots of barley and on roots using electrophysiological studies (Adem *et al*. 2014). Recently, Wu *et al*. (2013) and Wu *et al*. (2014) demonstrated that K^+ retention in the leaf mesophyll is a contributing factor towards salt tolerance in barley, as measured by non-invasive ion flux. Notably, several NHX transporters have been shown to have a higher affinity for K^+ than Na^+ (Rodríguez-Rosales *et al*. 2008; Leidi *et al*. 2010; Bassil *et al*. 2011; Barragan *et al*. 2012) – enhanced proton pumping in *VHA-C* expressing barley may therefore may enable higher accumulation of K^+ in cell vacuoles. This may contribute to increased cell turgor and better osmotic adjustment under saline conditions (as discussed below). Higher K^+ concentration in leaves may be also essential for more efficient stomatal control, given the critical role of this nutrient in stomata movements (Anschütz *et al*. 2014).

Relative contribution of organic and inorganic osmolytes

Synthesis of organic osmolytes is energy expensive, particularly in stressful environments (Raven 1985), and plants with enhanced salt tolerance tend to use Na^+ as a cheap osmoticum for osmotic adjustment (Chen *et al*. 2007). As shown in Table 2, WT plants relied predominantly (65.3% of the cell's osmotic potential) on organic osmolytes for their osmotic adjustment. *AtVHA-C* expressing lines, however, had a large proportion of inorganic osmolytes (specifically, Na^+ and K^+) to achieve ~50% of cell osmotic potential (Table 2). The transgenic lines were therefore likely to expend less energy generating organic osmolytes and make better use of photosynthetic assimilate. Osmotic adjustment using inorganic ions is the predominant mechanism in halophytes (Inan *et al*. 2004), and it appears here that some 'halophytism' was highly beneficial for the transgenic barley plants.

Conclusion

Expressing *Arabidopsis* VHA-C in barley plants has shown better Na^+ and K^+ sequestration, which led to the utilisation of these ions as a cheap osmoticum to improve gas exchange characteristics. This result indicates that the presence of vacuolar H^+-ATPase has a crucial role in enhancing Na^+ and K^+ sequestration in the presence of NHX and thereby, improved seed yield. It is therefore strongly advocated that the concurrent expression of the vacuolar H^+-ATPase and NHX together in one genotype should be done to enhance plant performance under salt stress conditions.

Conflicts of interest

The authors declare no conflicts of interest.

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