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Expression of the *Arabidopsis* vacuolar H^+-pyrophosphatase gene (*AVP1*) improves the shoot biomass of transgenic barley and increases grain yield in a saline field

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Summary

Cereal varieties with improved salinity tolerance are needed to achieve profitable grain yields in saline soils. The expression of *AVP1*, an *Arabidopsis* gene encoding a vacuolar proton pumping pyrophosphatase (H^+-PPase), has been shown to improve the salinity tolerance of transgenic plants in greenhouse conditions. However, the potential for this gene to improve the grain yield of cereal crops in a saline field has yet to be evaluated. Recent advances in high-throughput nondestructive phenotyping technologies also offer an opportunity to quantitatively evaluate the growth of transgenic plants under abiotic stress through time. In this study, the growth of transgenic barley expressing *AVP1* was evaluated under saline conditions in a pot experiment using nondestructive plant imaging and in a saline field trial. Greenhouse-grown transgenic barley expressing *AVP1* produced a larger shoot biomass compared to null segregants, as determined by an increase in projected shoot area, when grown in soil with 150 mM NaCl. This increase in shoot biomass of transgenic *AVP1* barley occurred from an early growth stage and also in nonsaline conditions. In a saline field, the transgenic barley expressing *AVP1* also showed an increase in shoot biomass and, importantly, produced a greater grain yield per plant compared to wild-type plants. Interestingly, the expression of *AVP1* did not alter barley leaf sodium concentrations in either greenhouse- or field-grown plants. This study validates our greenhouse-based experiments and indicates that transgenic barley expressing *AVP1* is a promising option for increasing cereal crop productivity in saline fields.

Introduction

Salinity reduces the grain yield of cereal crops worldwide. Globally, at least 77 million ha of agricultural land is currently affected by salinity (Munns, 2002; Munns and Tester, 2008). The presence of high salt concentrations, particularly sodium chloride (NaCl), causes osmotic stress, ion toxicity and ion deficiencies in cereal crops (Colmer *et al.*, 2005; Munns and Tester, 2008). Consequently, salt stress reduces water uptake and increases leaf senescence, resulting in stunted growth and an overall reduction in tiller number and grain yield (Munns, 2002). Cereal crop varieties with improved salinity tolerance are needed to increase crop productivity in saline soils.

One way to improve plant salinity tolerance is to increase the sequestration of sodium (Na^+) ions into vacuoles by enhancing the activity of vacuolar sodium/proton (Na^+/H^+) antiporters (Apse *et al.*, 1999). This enhanced vacuolar sequestration of Na^+ can reduce Na^+ toxicity in the cytoplasm and facilitate water uptake into plant cells (Blumwald, 2000). The Na^+ pumping activity of vacuolar Na^+/H^+ antiporters is driven by an electrochemical potential difference for H^+ established across the tonoplast by two proton pumps, the vacuolar H^+-pumping ATPase and the vacuolar H^+-pumping pyrophosphatase (H^+-PPase) (Maeshima, 2000; Sze *et al.*, 1992).

The constitutive expression of *AVP1*, an *Arabidopsis* gene encoding a type I vacuolar H^+-pyrophosphatase, has been shown to improve the salinity tolerance of transgenic *Arabidopsis* (Gaxiola *et al.*, 2001), alfalfa (*Medicago sativa*) (Bao *et al.*, 2009), creeping bentgrass (*Agrostis stolonifera*) (Li *et al.*, 2010), cotton (*Gossypium hirsutum*) (Pasapula *et al.*, 2011), peanut (*Arachis hypogaea*) (Qin *et al.*, 2013) and rice (*Oryza sativa*) (Zhao *et al.*, 2006). This improved salinity tolerance of transgenic plants expressing *AVP1* was attributed to an enhanced electrochemical potential difference for H^+ across the tonoplast facilitating Na^+/H^+ antiporter activity and thus increasing the sequestration of Na^+ into vacuoles (Duan *et al.*, 2007; Gaxiola *et al.*, 2001). In support of this hypothesis, the co-expression of the *Suaeda salsa* Na^+/H^+ antiporter (*SsNHX1*) and *AVP1* resulted in greater salinity tolerance in rice than the expression of *SsNHX1* alone (Zhao *et al.*, 2006). Thus, previous studies have shown that the expression of *AVP1* can improve shoot biomass under saline conditions in the greenhouse and that the expression of this gene could potentially

increase the salinity tolerance of other agriculturally important cereal crops, such as barley (*Hordeum vulgare*).

Previous studies phenotyping transgenic plants expressing *AVP1* in saline conditions have been limited to shoot biomass measurements at one time point (Bao *et al.*, 2009; Gaxiola *et al.*, 2001; Li *et al.*, 2010; Lv *et al.*, 2008; Pasapula *et al.*, 2011; Qin *et al.*, 2013; Zhao *et al.*, 2006). Recent advances in high-throughput phenotyping technologies offer the opportunity to nondestructively evaluate plant growth through time, providing accurate measures of relative plant growth rates (Berger *et al.*, 2010; Furbank and Tester, 2011; Rajendran *et al.*, 2009). The use of nondestructive plant imaging has been shown to reveal novel aspects of plant responses to abiotic stresses, such as drought and salinity (Berger *et al.*, 2010; Rajendran *et al.*, 2009; Sirault *et al.*, 2009). By allowing more detailed growth analysis of transgenic plants expressing *AVP1* under salt stress through time, the use of nondestructive imaging technology could provide further insight into the timing and extent of effects from *AVP1* expression on plant growth, including the separation of possible effects on early vigour (Ferjani *et al.*, 2011) from those on later growth stages.

Previous testing of transgenic *AVP1* plants in saline conditions has also been solely greenhouse-based (Bao *et al.*, 2009; Gaxiola *et al.*, 2001; Li *et al.*, 2010; Lv *et al.*, 2008; Pasapula *et al.*, 2011; Qin *et al.*, 2013; Zhao *et al.*, 2006) with a limited focus on evaluating yield traits (Pasapula *et al.*, 2011; Qin *et al.*, 2013). An important component of a salt-tolerant cereal crop is not only the ability to grow in a saline soil but also to produce high grain yields (Flowers, 2004). Saline field trials of transgenic plants are required to measure yield traits and validate greenhouse-based findings of improved salinity tolerance (Flowers, 2004; Plett and Møller, 2010; Roy *et al.*, 2011).

The aim of this study was to evaluate the growth of transgenic barley expressing *AVP1* in saline conditions in the greenhouse using nondestructive plant imaging technology and to test whether these plants have improved grain yield in a saline field.

Results

Generation of transgenic barley expressing *AVP1*

Transgenic barley (cv. Golden Promise) expressing *AVP1* using the *CaMV 35S* promoter was successfully generated via *Agrobacterium*-mediated transformation (Jacobs *et al.*, 2007; Singh *et al.*, 1997). The results for three independent barley transformation events (*35S-AVP1*-1, *35S-AVP1*-2 and *35S-AVP1*-3) with two sibling lines from one transformation event (*35S-AVP1*-1a and *35S-AVP1*-1b) were used in this study. PCR analysis of genomic DNA confirmed the presence of *AVP1* in the transgenic barley (*35S-AVP1*-1a, 1b, 2 or 3) and the absence of *AVP1* in wild-type and null segregants (Nulls 1, 2 & 3) (Figure 1a). Additionally, reverse transcription PCR (RT-PCR) on cDNA confirmed the expression of *AVP1* in the transgenic barley lines and the lack of *AVP1* expression in wild-type and null segregants (Figure 1b).

Transgenic *AVP1* barley has increased shoot biomass in a pot experiment

Nondestructive plant imaging of greenhouse-grown plants showed that three independent transgenic barley lines expressing *AVP1* (*35S-AVP1*-1a, *35S-AVP1*-2 and *35S-AVP1*-3) produced significantly larger (11–33%) projected shoot areas (pixels) compared to null segregants when grown for 47 days in soil with 150 mM NaCl (Figure 2a,b). The sibling *35S-AVP1*-1b, however, showed no significant difference in projected shoot

Figure 1 Molecular characterization of wild-type, null segregants and transgenic barley expressing *AVP1*. (a) Genotyping for the presence or absence of *AVP1* using polymerase chain reaction (PCR) with *AVP1*-specific primers and *HvVRT2*-specific primers (internal control) (b) Expression analysis of *AVP1* using reverse transcription PCR (RT-PCR) with *AVP1*-specific and *HvGAP*-specific primers (internal control) for wild-type (cv. Golden Promise), null segregants and transgenic barley expressing *AVP1*. Lane (–) is a negative control (water). Lane WT is wild-type. Lanes Nulls 1, 2 & 3 are null segregants. Lanes *35S-AVP1* 1a, 1b, 2 & 3 are transgenic *AVP1* barley lines.

area (pixel) under salinity treatment compared to null segregants (Figure 2b). Additionally, no significant difference in the 4th leaf blade Na^+ and potassium (K^+) concentrations was detected between the transgenic *AVP1* barley and null segregants grown under saline conditions (Figure 3a,b).

Relative growth rates derived for *35S-AVP1*-1a plants show that this line had a faster relative growth rate than null segregants during early growth stages between 9–19 days after sowing in soil with 150 mM NaCl (Figure 4a,c). However, this line had relative growth rates similar to null segregants in the later growth stages from 28–47 days after sowing under saline conditions (Figure 4b,c). Notably, *35S-AVP1*-2 and *35S-AVP1*-3 showed a similar relative growth rate as null segregants under saline conditions between 9–19 days and between 28–47 days after sowing (Figure 4c). However, both lines already had a significantly larger projected shoot area than null segregants at 9 days after sowing under saline conditions (Figure 4c). As expected, the relative growth rates of all plants decreased over time (Figure 4c). In nonsaline conditions, transgenic barley expressing *AVP1* also had a larger projected shoot area than null segregants with a trend towards a faster relative growth rate during the early growth stages (9–17 days) and similar relative growth rates to null segregants in the later growth stages (28–47 days) (Table S1).

Characterization of soil properties at a saline field trial site

The soil of the saline field trial site near Kunjin in the central wheatbelt of Western Australia comprised 90% sand, 5% silt and 5% clay and was therefore classified as a sandy soil. An electromagnetic (EM) map of the field site showed a gradient in the apparent soil electrical conductivity (EC_a) from south to north, ranging from areas of low EC_a (41 mS/m) to areas of higher EC_a (199 mS/m) (Figure 5). Soil electrical conductivity ($EC_{1:5}$) measurements (0–10 cm depth) were used to identify suitable low-salinity ($EC_{1:5}$ = 161 ± 11 μS/cm) and high-salinity ($EC_{1:5}$ = 1231 ± 155 μS/cm) areas for the field trial plots (Figure 5). The low-salinity field area is considered nonsaline for cereal crop production in the wheatbelt of Western Australia. The grain yield (g/plant) results from this low-salinity area are also consistent with those obtained for the transgenic *AVP1* barley and wild-type

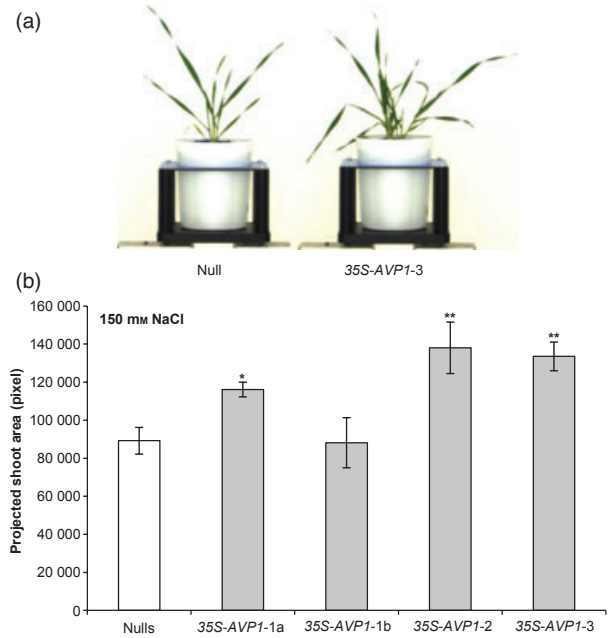


Figure 2 Projected shoot area of salt-treated transgenic barley expressing *AVP1* and null segregants in the greenhouse. (a) High-resolution visible light (RGB) side-view image of a representative null segregant (cv. Golden Promise) and transgenic barley line expressing *AVP1* (*35S-AVP1*-3) 47 days after sowing in soil with 150 mM NaCl (b) Projected shoot area (pixel) derived from visible light (RGB) plant images of null (white bar) and transgenic barley lines *35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 and *35S-AVP1*-3 (grey bars) 47 days after sowing in soil with 150 mM NaCl. Values are the mean ± s.e.m (n = 3–8) with asterisks (* or **) indicating a significant difference (one-way ANOVA, LSD, $P < 0.05$ or <0.01).

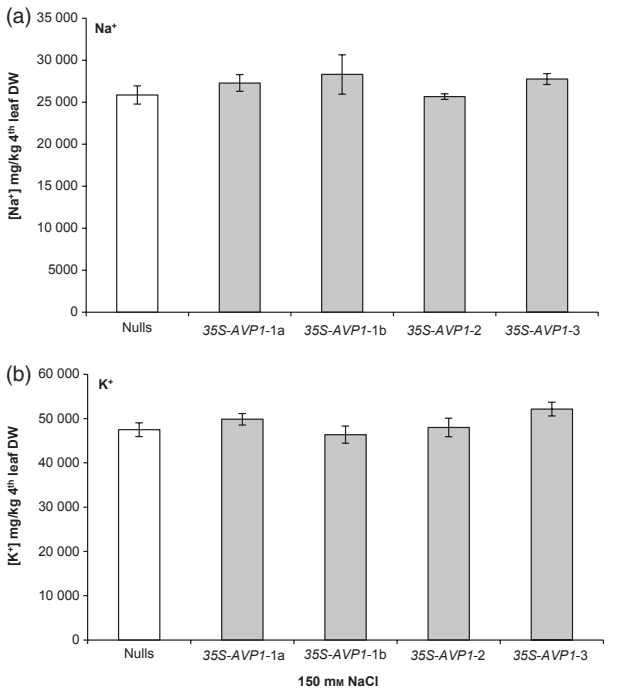


Figure 3 Leaf Na^+ and K^+ concentrations of transgenic barley expressing *AVP1* and null segregants in saline soil. (a) Na^+ and (b) K^+ concentrations (mg/kg DW) of the 4th leaf blade of null segregants (cv. Golden Promise) (white bars) and transgenic barley expressing *AVP1* (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 and *35S-AVP1*-3) (grey bars) 47 days after sowing in soil with 150 mM NaCl. Values are the mean ± s.e.m (n = 3–8).

plants grown at a separate nonsaline field area (Table S2). The soil pH differed slightly between the low-salinity (pH = 6.18 ± 0.03) and high-salinity (pH 7.10 ± 0.04) areas (Figure 5).

Transgenic *AVP1* barley has increased shoot biomass and grain yield in a saline field

Transgenic barley plants expressing *AVP1* (lines identified as *35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 and *35S-AVP1*-3) and wild-type barley (cv. Golden Promise) plants were grown in a saline field trial. In the low-salinity area, the transgenic barley expressing *AVP1* had a significantly greater (17–33%) shoot biomass compared to wild-type plants (Figure 6a). The average grain weight, number of grain heads and grains per plant of transgenic barley expressing *AVP1* were similar to those of wild-type barley in the low-salinity area (Table 1). Nevertheless, two transgenic lines (*35S-AVP1*-1a and *35S-AVP1*-2) had significantly higher (23–34%) grain yield per plant than wild-type plants (Table 1).

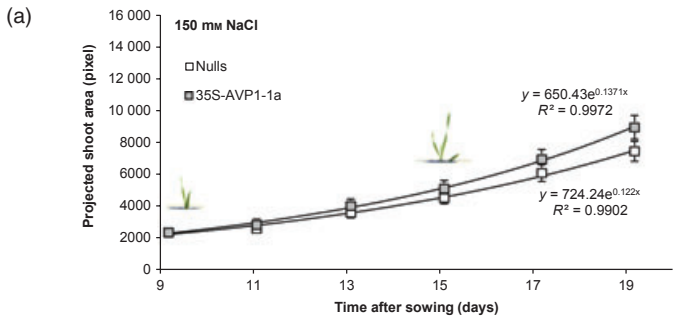
In the high-salinity area, the growth of all plants was greatly reduced (Figure 6a,b). However, the transgenic barley expressing *AVP1* produced a significantly greater (30–42%) shoot biomass and had greater survival in the high-salinity area than the wild-type plants (Figure 6a,b). As with greenhouse-grown plants, there were no significant differences in Na^+ and K^+ concentrations of youngest fully emerged leaf blades between the transgenic barley expressing *AVP1* and wild-type plants (Figure S1). Due to the large growth reduction of wild-type plants in the high-salinity area, the grain yield was only measured on representative plants surviving in each plot. As such, these provide an overestimate of average grains per plant across the whole plot. Nevertheless, the number of heads and grains per plant from transgenic barley expressing *AVP1* was significantly greater (16–58% and 76–85%, respectively) than from wild-type plants in the high-salinity area (Table 1). The average grain weight of transgenic *AVP1* barley plants was also significantly greater (29–43%) than that of wild-type plants (Table 1). Furthermore, the grain yield per plant of the transgenic *AVP1* barley was significantly higher (79–87%) than that of wild-type plants in the high-salinity area (Table 1).

Discussion

Transgenic *AVP1* barley has increased shoot biomass and grain yield under saline conditions

The expression of *AVP1* has previously been shown to improve transgenic plant growth in saline greenhouse conditions (Bao *et al.*, 2009; Gaxiola *et al.*, 2001; Li *et al.*, 2010; Lv *et al.*, 2008; Pasapula *et al.*, 2011; Qin *et al.*, 2013; Zhao *et al.*, 2006). In this study, transgenic barley expressing *AVP1* produced a greater projected shoot area, and therefore shoot biomass, in soil with 150 mM NaCl compared to null segregants (Figures 2 and 4). This result supports previous studies suggesting that *AVP1* contributes to improving shoot biomass under saline conditions.

To further understand the role of *AVP1* in improving plant salinity tolerance, it is important to evaluate the yield of transgenic plants expressing *AVP1* in saline conditions and to validate greenhouse-based findings of improved salinity tolerance in the field. Cotton plants expressing *AVP1* were previously



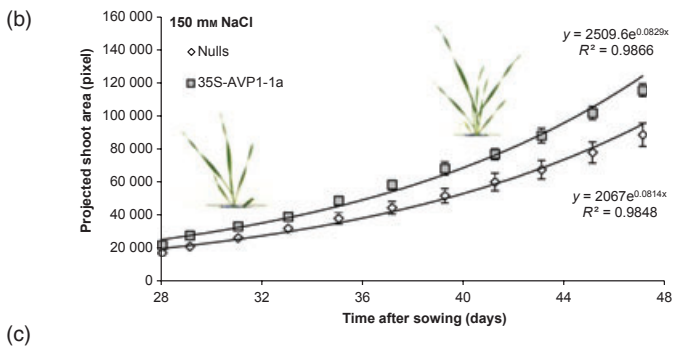


Figure 4 Nondestructive plant imaging of salt-treated transgenic barley expressing *AVP1* and null segregants. The projected shoot area (pixel) of null segregants (white squares) and *35S-AVP1*-1a line (grey squares) between (a) 9–19 days and (b) 28–47 days after sowing in soil with 150 mM NaCl. Representative RGB side-view images of a null plant showing the different growth stages are shown on the graph for selected time points. (c) The projected shoot area (pixel) of null segregants and transgenic *AVP1* barley lines (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 and *35S-AVP1*-3) at 9 and 47 days after sowing in soil with 150 mM NaCl and the relative growth rates (per day) of null segregants and transgenic barley lines (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 and *35S-AVP1*-3) derived from an exponential fitted curve of projected shoot area between 9–19 days and 28–47 days. Values are the mean ± s.e.m (n = 3–8) with asterisks (* or **) indicating a significant difference (one-way ANOVA, LSD, $P < 0.05$ or <0.01).

(c)

Line	Projected shoot area (pixel) at 150 mM NaCl		Relative growth rate (per day) at 150 mM NaCl	
	9 days	47 days	9–19 days	28–47 days
Null segregants	2353 ± 181	89 201 ± 7008	0.1220	0.0814
35S-AVP1-1a	2348 ± 289	11 6129 ± 3880*	0.1371	0.0829
35S-AVP1-1b	2558 ± 359	88 146 ± 13 169	0.1239	0.0794
35S-AVP1-2	4282 ± 381**	13 8041 ± 13543**	0.1033	0.0799
35S-AVP1-3	3794 ± 139**	13 3524 ± 7560**	0.1268	0.0688

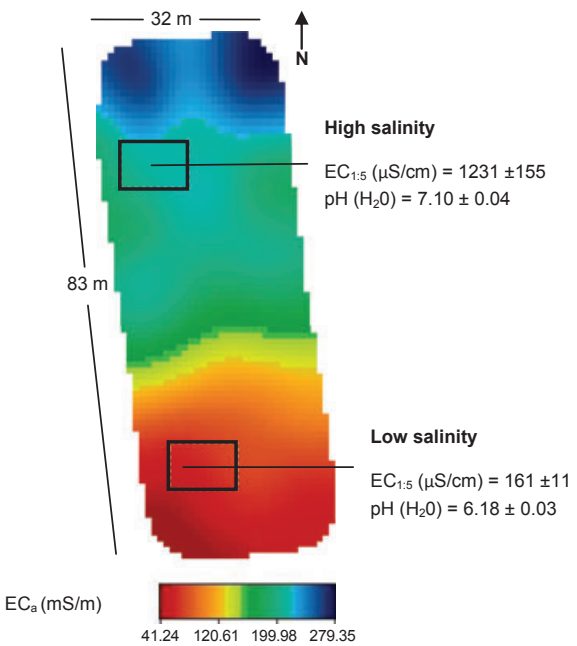


Figure 5 Soil characterization of a saline field trial site. An electromagnetic (EM) map showing the apparent electrical conductivity (EC_a) of a saline field trial site (83 m length × 32 m width, N = north) with red indicating low EC_a (41 mS/m) and blue high EC_a (199 mS/m). Black rectangles indicate the location of trial plots in the low- and high-salinity field areas with corresponding soil electrical conductivity ($EC_{1:5}$) (soil : water) (µS/cm) and pH (H_20) values. Values are the mean ± s.e.m (n = 12–21).

shown to have higher fibre yield compared to wild-type plants at 200 mM NaCl treatment in a greenhouse experiment and at a nonsaline dryland field site (Pasapula *et al.*, 2011). Additionally, transgenic peanuts expressing *AVP1* grown in the field under low and high irrigation treatments had a higher yield than the wild-type (Qin *et al.*, 2013). However, to our knowledge, there are no previous reports on a saline field trial evaluating the growth and yield of a transgenic plant expressing *AVP1*. In this study, the results of a saline field trial show that transgenic barley expressing *AVP1* have a significantly larger shoot biomass when grown in both low- and high-salinity areas compared to the wild-type (Table 1, Figure 6). This increase in shoot biomass supports the pot experiment results presented in this study. Additionally, one transgenic *AVP1* barley line (*35S-AVP1-1b*) had an increase in shoot biomass under field conditions that was not observed in the more controlled greenhouse conditions (Figure 2b and Figure 4c). This highlights the need to phenotype transgenic plants in both greenhouse and field conditions. Importantly, the transgenic barley expressing *AVP1* also produced a higher grain yield per plant in the high-salinity field plots compared to wild-type plants, which comprised more infertile heads and less grains per plant (Table 1). An increase in grain number and grain weight are both contributing towards this increase in grain yield per plant of the transgenic *AVP1* barley lines (Table 1).

Expression of *AVP1* in transgenic barley does not alter leaf Na^+ concentrations

The improved growth of transgenic plants expressing *AVP1* in saline conditions has been previously attributed to AVP1, facilitating an increase in the activity of vacuolar Na^+/H^+ antiporters

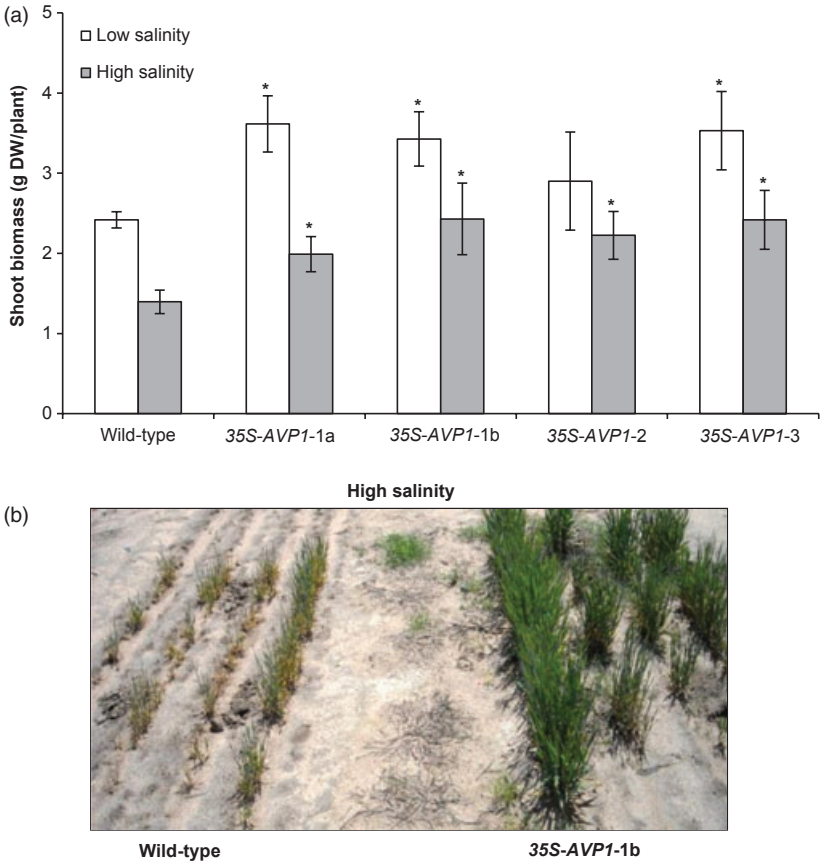


Figure 6 Shoot biomass of wild-type and transgenic barley expressing *AVP1* in a saline field trial (a) Shoot biomass (g DW/plant) of wild-type (cv. Golden Promise) and four transgenic barley lines expressing *AVP1* (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 & *35S-AVP1*-3) after 12 weeks growth in the low- (white bars) and high-salinity (grey bars) field. (b) Image of shoot growth of a representative wild-type (cv. Golden Promise) and transgenic barley expressing *AVP1* (*35S-AVP1*-1b) at high salinity. Values are the mean ± s.e.m (n = 12) with asterisks (*) indicating a significant difference (one-way ANOVA, LSD, $P < 0.05$).

Table 1 The number of heads, number of grains, grain weight and grain yield per plant of transgenic barley expressing *AVP1* (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 & *35S-AVP1*-3) and wild-type (cv. Golden Promise) in a low- and high-salinity field area. Values are the mean ± s.e.m (n = 6–2) with asterisks (* or **) indicating a significant difference (one-way ANOVA, LSD, $P < 0.05$ or <0.01)

Treatment	Line	No. of heads (per plant)	No. of grains (per plant)	Grain weight (mg)	Grain yield (g/plant)
Low salinity	Wild-type	6.0 ± 0.4	107 ± 13	33.2 ± 1.0	3.57 ± 0.49
	35S-AVP1-1a	7.5 ± 0.6	150 ± 16*	36.6 ± 0.8	5.45 ± 0.57*
	35S-AVP1-1b	5.8 ± 0.3	98 ± 6	40.1 ± 3.8	3.77 ± 0.23
	35S-AVP1-2	6.6 ± 0.6	116 ± 12	40.3 ± 1.0	4.66 ± 0.49*
	35S-AVP1-3	6.4 ± 0.5	122 ± 13	36.2 ± 1.5	4.40 ± 0.45
High salinity	Wild-type	2.7 ± 0.3	10 ± 3	24.6 ± 5.7	0.28 ± 0.07
	35S-AVP1-1a	5.4 ± 0.9*	60 ± 13**	32.6 ± 1.8	2.02 ± 0.50**
	35S-AVP1-1b	3.3 ± 0.2	56 ± 4.0**	34.9 ± 2.0*	1.97 ± 0.21**
	35S-AVP1-2	6.4 ± 0.7*	67 ± 11**	41.3 ± 6.6*	2.20 ± 0.34**
	35S-AVP1-3	3.2 ± 0.5	41 ± 14**	42.9 ± 5.5*	1.34 ± 0.38**

and thus greater sequestration of Na^+ into vacuoles (Bao *et al.*, 2009; Gaxiola *et al.*, 2001; Li *et al.*, 2010). This sequestration of Na^+ into vacuoles presumably lessens the toxic effects of Na^+ on cytosolic enzymes and could also facilitate the retention of water in the plant tissues (Gaxiola *et al.*, 2001). In support of this hypothesis, transgenic *Arabidopsis* overexpressing *AVP1* retain more Na^+ in their rosette leaves and have enhanced leaf water content after the treatment with 100 mM NaCl compared to wild-type plants (Gaxiola *et al.*, 2001). An increase in Na^+, and other ions, has also been reported in shoot and root tissue of several other transgenic plants expressing *AVP1* (Bao *et al.*, 2009; Gaxiola *et al.*, 2001; Li *et al.*, 2010). Furthermore, under saline conditions, a decrease in vacuole membrane leakage and an increase in net photosynthesis have been measured in transgenic plants expressing *AVP1* (Bao *et al.*, 2009; Li *et al.*, 2010; Pasapula *et al.*, 2011; Qin *et al.*, 2013). This suggests that transgenic plants expressing *AVP1* have improved tolerance to both the ionic and osmotic effects of NaCl, which may help improve plant growth under saline conditions.

In this study, there were no significant differences in Na^+ or K^+ concentrations in the leaf tissue of barley lines expressing *AVP1* in the pot and field experiments under saline conditions compared to plants without this gene (Figures 3 and S1). This contrasting result to previous studies (Bao *et al.*, 2009; Gaxiola *et al.*, 2001; Li

et al., 2010) could be due to several factors, such as the plant species, plant age, the level and extent of salt treatment, and the type of plant tissue sampled for ion analysis. However, it cannot be ruled out that, although the same amount of Na^+ is present per unit leaf area compared to wild-type (Figures 3 and S1), the subcellular location of Na^+ within the transgenic barley leaf expressing *AVP1* could be different, being potentially higher in the vacuole and lower in the cytoplasm. Nevertheless, the lack of increased Na^+ accumulation in the leaves of the transgenic barley expressing *AVP1* suggests that there may also be other factors, in addition to the accumulation of Na^+ within the vacuole, which contribute to the increased shoot growth.

Transgenic *AVP1* barley has improved shoot growth in nonsaline conditions

The transgenic barley expressing *AVP1* (*35S-AVP1*-1a, *35S-AVP1*-1b and *35S-AVP1*-3) also had improved shoot growth in nonsaline conditions compared to null segregants (Table S1). This is in agreement with previous studies, where transgenic plants expressing *AVP1* developed larger shoot and root biomass in nonsaline conditions compared to plants without this gene (Li *et al.*, 2005, 2010; Vercruyssen *et al.*, 2011; Yang *et al.*, 2007). Additionally, studies on transgenic plants expressing a H^+-PPase from other plant species, including *Thellungiella halophila* (syn. *Eutrema salsugineum*) (*TsVP*) and *Triticum aestivum* (*TVP1*), have reported an increase in shoot biomass under nonsaline conditions (Gouiaa *et al.*, 2012; Lv *et al.*, 2008). Although there are exceptions where no growth differences between nontransgenic and transgenic *AVP1* plants in nonsaline conditions are seen (Bao *et al.*, 2009; Pasapula *et al.*, 2011; Qin *et al.*, 2013), the increase in biomass of transgenic plants expressing *AVP1* in both nonsaline and saline conditions is yet to be fully elucidated.

There are several factors potentially contributing to the improved growth of transgenic barley expressing *AVP1*. A recent study with *AVP1* loss-of-function mutants suggests that the major role of AVP1 is the hydrolysis of inorganic pyrophosphate (PP_i) in the cell cytoplasm rather than vacuolar acidification (Ferjani *et al.*, 2011). This removal of cytosolic PP_i, which at high levels is an inhibitor of gluconeogenesis, may result in improved plant heterotrophic growth (Ferjani *et al.*, 2011). The nondestructive plant imaging in our study shows that transgenic barley expressing *AVP1* had already produced a significantly larger projected shoot area 9 days after sowing in both saline (*35S-AVP1*-2 & *35S-AVP1*-3) and nonsaline soils (*35S-AVP1*-1a, *35S-AVP1*-1b and *35S-AVP1*-3) compared to null segregants (Figure 4c and Table S1). It is possible that the transgenic *AVP1* barley plants are larger at 9 days due to a larger seed weight or a faster relative growth rate prior to imaging at 9 days after sowing. In support of the latter, the relative growth rates of transgenic barley expressing *AVP1* were higher compared to null segregants in the early growth stages (9–19 days after sowing), whilst they were similar to null segregants in the later growth stages (28–47 days after sowing) (Figure 4c and Table S1). The larger shoot biomass of transgenic barley expressing *AVP1* in nonsaline and saline conditions could be due to the enhanced removal of cytosolic PP_i improving seedling vigour.

The improved growth of transgenic barley expressing *AVP1* may also be a result of more efficient sucrose transport-enhancing plant water use or nutrition. Previous studies have demonstrated that transgenic plants expressing *AVP1* or *AVP1D* (the *E229D* gain-of-function mutant) have improved tolerance to low water (Gaxiola *et al.*, 2001; Park *et al.*, 2005; Pasapula *et al.*, 2011), phosphorus (Yang *et al.*, 2007) and nitrate provisions (Paez-Valencia *et al.*, 2013). This has been attributed to an increase in root biomass and rhizosphere acidification, allowing greater exploration of soil and consequently improved water, phosphorus and nitrate uptake (Paez-Valencia *et al.*, 2013; Park *et al.*, 2005; Yang *et al.*, 2007). AVP1 has also been shown to affect auxin-dependent organogenesis and root morphological traits (Li *et al.*, 2005; Yang *et al.*, 2007). Recently, it has been hypothesized that transgenic plants expressing *AVP1* may have more efficient sucrose transport to sink organs enabling improved root growth (Gaxiola *et al.*, 2012; Paez-Valencia *et al.*, 2013). Subtle alterations in nutrient or water availability could therefore allow transgenic *AVP1* plants an advantage over plants without expression of this gene. In this current work, attempts were made to ensure that all factors other than the desired treatment were nonlimiting throughout the experiment duration. However, an increase in nutrient-use efficiency or improved water uptake may explain the observed increase in shoot biomass of transgenic *AVP1* barley plants in nonsaline and saline conditions.

Conclusions

In this study, it is shown that the expression of *AVP1* increases the shoot biomass of barley in saline and nonsaline conditions. Additionally, it is shown that the expression of *AVP1* in transgenic barley improves the grain yield per plant of this cereal crop when grown in a high-salinity field. To our knowledge, this is the first time that such effects of *AVP1* expression in transgenic plants have been validated in a saline field trial. The mechanism for this yield increase is unknown, although detailed nondestructive growth analysis of greenhouse-grown transgenic *AVP1* barley plants is consistent with an effect of *AVP1* expression on early vigour. This study supports the concept that *AVP1* may have additional benefits beyond facilitating increased sequestration of Na^+ ions into vacuoles (Ferjani *et al.*, 2011; Gaxiola *et al.*, 2012). Furthermore, the results of this study indicate that the expression of *AVP1* in transgenic barley could provide a useful option for increasing cereal crop productivity in saline fields.

Experimental procedures

Generation of transgenic barley expressing *AVP1*

The coding sequence of *AVP1* (At1g15690) was amplified from the *Arabidopsis thaliana* ecotype Col-0 cDNA and ligated into a pENTR-D-TOPO (Invitrogen) entry vector, before *AVP1* was recombined into the pMDC32 destination vector using the Gateway® LR recombination reaction (Invitrogen, Carlsbad, CA, USA) (Curtis and Grossniklaus, 2003; Jacobs *et al.*, 2007). Transformation of barley (*Hordeum vulgare* cv. Golden Promise) with the *AVP1* pMDC32 vector was conducted using *Agrobacterium tumefaciens*-mediated transformation, followed by the regeneration of barley plantlets in soil (Jacobs *et al.*, 2007; Singh *et al.*, 1997). A total of seven independent transgenic *AVP1* barley lines were generated. The five T_1 *AVP1* barley lines that produced the most seed were grown for 14 days in nutrient solution containing 50 mM NaCl in a hydroponic system. Four lines showed a significant increase in leaf fresh weight compared to the wild-type (data not shown). Three of these four lines (*35S-AVP1*-1, *35S-AVP1*-2 and *35S-AVP1*-3), which had the largest growth improvement under saline conditions, are described in this study. In addition, two sibling lines from one transformation event (*35S-AVP1*-1a and *35S-AVP1*-1b) were used.

Plant material and greenhouse growth conditions

Seeds of T_3 transgenic barley lines expressing *AVP1* and null segregants were surface-sterilized by a 5-min exposure to ultraviolet light, then germinated at 21 °C for 5 days on moist filter paper in Petri dishes (145 mm diameter), which were placed in polyethylene bags to maintain humidity. Individual uniform size seedlings were transplanted (sowing) to sealed white pots (19.46 cm height × 14.94 cm diameter, Berry Plastics Corporation, Evansville, USA) filled with 3 kg of University of California (UC) mixture (1 : 1 peat : sand) and either 0 or 150 mM NaCl (9 mL of 5 M NaCl) mixed into the UC mixture (1.5 kg) within the bottom half of each pot. To maintain Ca^{2+} activity similar to that of control pots, an additional 3 mM $CaCl_2$ (990 μL of 1 M $CaCl_2$) was added to salt-treated pots. To minimize the loss of soil water via evaporation, the soil surface of each pot was covered in 100 g of blue polypropylene beads (Misc 430C, Plastic's Granulating Service, Kilburn, SA, Australia).

Nondestructive plant imaging and image analysis

Nine days after transplanting, the pots were randomly loaded onto a fully automated conveyor system within a temperature-controlled Smarthouse maintained between 15–27 °C (The Plant Accelerator®, Adelaide, Australia; longitude: 138.639933, latitude: −34.971353). Plants were grown in natural light between the months of June and July in 2010. Every second day, an electronic conveyor system watered each pot using industrial scales (Bizerba, Balingen, Germany) and reverse osmosis (RO) water to maintain the soil water content at field capacity (300 mL water/pot).

Nondestructive measurements of plant growth occurred using a plant image capture and analysis system in The Plant Accelerator® facility (Scanalyzer 3D, LemnaTec, Aachen, Germany). High-resolution visible light (RGB) digital images, including two side and one top view, were obtained for each plant every second day between 9–19 days and between 28–47 days after sowing. The projected shoot area (pixel) of each plant was calculated from the total shoot pixel area derived from the three combined RGB images (Golzarian *et al.*, 2011; Rajendran *et al.*, 2009). A linear correlation between shoot biomass and projected shoot area has been shown to occur in the early stages of plant development (Rajendran *et al.*, 2009). The mean relative growth rate of each line was determined from the slope of an exponential curve fitted to the mean projected shoot area from 9–19 days and 28–47 days after sowing to separate early and late growth stages. Following the final imaging measurements, the 4th leaf blade was sampled for ion analysis and the youngest fully emerged leaf blade for genotyping and gene expression.

DNA extraction and PCR analysis

Genomic DNA was extracted from leaf tissue following the protocol of Edwards *et al.* (1991). The presence or absence of the *AVP1* gene in each plant was determined using PCR amplification from 1 μL of genomic DNA template with an *AVP1*-specific forward primer 5′-TGT TTT GAC CCC TAA AGT TAT C-3′ and reverse primer 5′-TGG CTC TGA ACC CTT TGG TC-3′, which amplified a fragment of 439 bp in size. The PCR conditions used to amplify the *AVP1* fragment was an initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 1 min. The *HvVRT2* vernalization gene (GenBank DQ201168) was used as a control gene for PCR and was amplified using *HvVRT2*-specific forward primer 5′-CCG AAT GTA CTG CCG TCA TCA CAG-3′ and reverse primer 5′-TGG CAG AGG AAA ATA TGC GCT TGA-3′, which amplified a fragment of 280 bp in size. The PCR conditions used to amplify *HvVRT2* were an initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. All PCR mixtures contained 1× Platinum® *Taq* PCR buffer, 2 mM $MgCl_2$, 200 μM each dNTPs and 0.5 U of Platinum® Taq DNA polymerase (Invitrogen). Gel electrophoresis with 2% agarose gel containing 5 μL/100 mL SYBR safe® stain (Invitrogen) and a ChemiScope 2850 imaging system (Clinx Science Instruments, Shanghai, China) was used to visualize PCR products and record gel images.

RNA extraction and gene expression analysis

Total RNA was extracted from the leaf tissue as described by Chomczynski (1993). Extracted RNA was treated with Ambion® DNase-free (Madison, WI, USA) to remove DNA contamination. Superscript III RT kit (Invitrogen) was used to synthesize cDNA using 1 μL volume of DNase-treated RNA. The expression of *AVP1* in each plant was determined using PCR amplification of 1 μL of cDNA template with *AVP1*-specific forward primer 5′-TGT TTT GAC CCC TAA AGT TAT C-3′ and reverse primer 5′-TGG CTC TGA ACC CTT TGG TC-3′. The PCR conditions used to amplify a fragment of the *AVP1* transcript (expected band size of 439 bp) were an initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 1 min. The *HvGAP* gene (GenBank EF409629) was used as a control gene and amplified using *HvGAP*-specific forward primer 5′-GTG AGG CTG GTG CTG ATT ACG-3′ and reverse primer 5′-TGG TGC AGC TAG CAT TTG ACA C-3′. The PCR conditions used to amplify a fragment of *HvGAP* (expected band size of 189 bp) were an initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min. All PCR mixtures contained 1× Platinum® *Taq* PCR buffer, 2 mM $MgCl_2$, 200 μM each dNTPs and 0.5 U of Platinum® Taq DNA polymerase (Invitrogen). Gel electrophoresis with 2% agarose gel containing 5 μL/100 mL SYBR safe® stain (Invitrogen) and a ChemiScope 2850 imaging system was used to check PCR products and record gel images.

Soil characterization of a saline field trial site

The field trial site was located near Kunjin, Western Australia (longitude: 177.73390, latitude: −32.33960). An electromagnetic (EM) map of the field site showing the apparent electrical conductivity was obtained using a vehicle-fitted EM Geonics device (Precision Agronomics Australia, Esperance, Western Australia). Soil was collected from 0 to 10 cm depth using a spade in two field areas identified from the EM map as having low and high salinity. Soil texture (% sand, silt and clay) was determined using the hydrometer method (Day, 1965). Soil electrical conductivity (EC) and pH were measured in a 1 : 5 (soil : water) extract, after shaking on an orbital shaker for 1 h and settling for 30 mins, using a CyberScan PC 510 meter (Eutech Instruments, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Saline field trial of transgenic barley

A field trial of T_4 transgenic barley lines expressing *AVP1* (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 and *35S-AVP1*-3) and wild-type (cv. Golden Promise) was conducted at the saline field site.

The field trial design was completely randomized with 2 plots (1.2 m width × 2 m length) per line in each salt treatment (low- and high-salinity area). Plots were sown in July 2011 at a sowing rate of 160 plants/plot (Kalyx Australia, Perth, Western Australia). Total rainfall during the growing season was 287 mm (Weather Station 010536, Corrigin WA, http://www.bom.gov.au/climate/), with the high-salinity field area prone to waterlogging. Standard agronomic practices were used including weed control using 2 L/ha Sprayseed® (Syngenta), 2 L/ha Treflan® (Nufarm) and 1 L/ha Chlorpyrifos® (Dow AgroSciences) immediately before sowing; pre-emergent deep banding of 80 kg/ha Vigour Atlas® fertilizer containing 10N : 12P : 9K (Summit Fertilizers); and pre- and post-emergent application of 100 kg/ha of urea. Shoot and leaf tissues were sampled and plant tillers counted in October 2011 at the vegetative growth stage Z37 (Zadoks *et al.*, 1974). Shoot material was dried for 3 days in an oven at 70 °C (Contherm Scientific Ltd, Wellington, New Zealand) for biomass measurements. A leaf blade was collected for genotyping, and the youngest fully emerged blade was collected for solute measurements. Grain was sampled from each plot in December 2011, and the number of grain heads, the number of individual grains and grain weight per plant were recorded.

ICP-OES determination of leaf solute concentrations

The 4th leaf blade (greenhouse-grown plants) and the youngest fully emerged blade (field-grown plants) were dried for 3 days in an oven at 70 °C (Contherm Scientific Ltd). Dried leaf tissue was cut into 2- to 5-cm pieces and digested using 70% nitric acid and 30% hydrogen peroxide for inductive coupled plasma optical emission spectrometry (ICP-OES) analysis (Wheal *et al.*, 2011).

Statistical analysis

Data were statistically analysed using a one-way analysis of variance (ANOVA) in Microsoft® Office Excel 2007, and the least significant difference (LSD) was used to identify significantly different means at a probability level of $P < 0.05$ or <0.01.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Leaf Na^+ and K^+ concentrations of wild-type and transgenic *AVP1* barley in a high-salinity field.

Table S1 Projected shoot area and relative growth rates of null segregants and transgenic *AVP1* barley in 0 mM NaCl.

Table S2 Grain yield of wild-type and transgenic *AVP1* barley in a nonsaline field.