Evaluating the abiotic stress tolerance of transgenic barley expressing an Arabidopsis vacuolar proton-pumping pyrophosphatase gene (AVP1)

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List of Abbreviations

3’	three prime, of nucleic acid sequence
5’	five prime, of nucleic acid sequence
~	approximately
ºC	degrees Celsius
Ψ_m	matric potential
(-)	negative control (water)
ACPFG	Australian Centre for Plant Functional Genomics
ANOVA	analysis of variance
AVP1	type- I *Arabidopsis* vacuolar H^+-pyrophosphatase
AVP1D	gain-of-function *AVP1* allele
bp	base pairs, of nucleic acid
bv	between vein
$CaCl_2$	calcium chloride
CaMV	cauliflower mosaic virus
Ca-P	calcium phosphate
cDNA	complementary deoxyribonucleic acid
Cl^-	chloride ion
cm	centimetre(s)
cv.	cultivar
d	day(s)
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dS	deciSiemens
DHA	dehydroascorbic acid
dpi	dots per inch
DW	dry weight
$EC_{1:5}$	electrical conductivity of 1:5 (soil:water) extract
EC_a	apparent soil electrical conductivity
EM	electromagnetic
FAO	Food and Agricultural Organization of the United Nations
FW	fresh weight
g	gram(s)
gDNA	genomic deoxyribonucleic acid
GM	genetically modified
GRDC	Grains Research and Development Corporation
GUS	β-glucuronidase protein
h	hour(s)
H^+	hydrogen ion
H^+-PPase	proton-pumping pyrophosphatase
H^+-ATPase	proton-pumping adenosine 5'-triphosphatase
ha	hectare
H_2O	water
HCl	hydrochloric acid
ICP-OES	Inductive Couple Plasma Optical Emission Spectrometry
K^+	potassium ion
kg	kilogram(s)

kPa	kiloPascal(s)
L	litre(s)
M	molar
mg	milligram(s)
Mg^{2+}	magnesium ion
min	minute(s)
mL	millilitre(s)
mm	millimetre(s)
mM	milliMolar
MPa	megaPascal(s)
n	sample size
Na^{+}	sodium ion
NaCl	sodium chloride
NO_3^-	nitrate
nulls	null segregants
P	phosphorus
PCR	polymerase chain reaction
pH	power of hydrogen
P_i	orthophosphate
PM	plasma membrane
PO_4^{3-}	phosphate
PP_i	inorganic pyrophosphate
ppm	parts per million
RNA	ribonucleic acid
RO	reverse osmosis
RT-PCR	reverse transcription polymerase chain reaction
SA	South Australia
s	seconds
SWP	soil water potential
T_1	1st progeny of primary transformant
T_2 to T_5	2nd, 3rd, 4th and 5th progeny of T_1 plant
uidA	*β*-glucuronidase gene
UV	ultraviolet light
VRT	vernalisation
v/v	volume per volume
WA	Western Australia
WABC	Western Australia Biogeochemistry Centre
WAS	Waite Analytical Services
wk	week(s)
WHC	water holding capacity
WT	wild-type
w/v	weight per volume
X-Gluc	5-bromo-4-chloro-3-indoyl-glucuronide
µg	microgram(s)
µL	microlitre(s)
µm	micrometre(s)
µM	micromolar
µmol	micromole(s)
µS	microSiemens

Abstract

Commercially relevant barley varieties with improved abiotic stress tolerance are needed to increase crop productivity. Previously, transgenic barley with constitutive *CaMV 35S* expression of *AVP1*, a gene encoding the type I *Arabidopsis* vacuolar proton-pumping pyrophosphatase (H^+-PPase), had a larger shoot biomass in non-saline and saline conditions compared to null segregants. However, the growth and grain yield of the transgenic *AVP1* barley was yet to be evaluated in a saline field. It was also yet to be investigated whether the larger shoot biomass of transgenic *AVP1* barley in both non-saline and saline conditions arose from a change in tissue solute accumulation, water use, plant nutrition, carbohydrate metabolism, heterotrophic growth or a combination of these traits. In addition, for this *AVP1* technology to be applicable for barley grain growers, a commercially relevant transgenic *AVP1* barley cultivar with well-regulated control of *AVP1* expression was needed.

The first focus of this project evaluated the growth and grain yield of *35S*:*AVP1* barley (cv. Golden Promise) in a low and high salinity field near Kunjin, Western Australia. Field trial results validated greenhouse-based findings of improved shoot biomass in transgenic *AVP1* barley compared to wild-type. Furthermore, results demonstrated for the first time that transgenic *AVP1* barley had increased grain yield per plant compared to wild-type in a field with high salinity. These findings suggest that transgenic *AVP1* barley is a promising option to help increase the grain yield of cereal crops in a saline field.

The second focus of this project investigated the abiotic stress tolerance and potential factors contributing to the larger shoot biomass of *35S*:*AVP1* barley. At low phosphorus (P) supply, *35S*:*AVP1* barley had a larger shoot biomass, greater root P uptake and increased rhizosphere acidification compared to wild-type. At low nitrate (NO_3^-) supply, two *35S*:*AVP1* barley lines had increased shoot biomass but with no difference in NO_3^- uptake capacity compared to null segregants. The shoot biomass of *35S*:*AVP1* barley was also increased compared to null segregants under low water availability and low water availability concurrent with salinity. Furthermore, an increase in plant biomass from 6 days after seed imbibition, thus seedling vigour, was detectable in *35S*:*AVP1* barley compared to null segregants. Leaf metabolites involved in ascorbic acid synthesis were also significantly altered in the *35S*:*AVP1* barley compared to null segregants. Collectively, these findings suggest that a combination of traits is contributing to the improved growth of transgenic *AVP1* barley.

The third focus of this project evaluated the salt stress inducibility of the *ZmRab17* promoter and investigated the salinity tolerance of commercially relevant barley (cv. WI4330) expressing *AVP1* via the *ZmRab17* and the constitutive *ZmUbi1* promoter. The *ZmRab17* promoter was salt-stress inducible in barley root stelar cells with basal transgene expression in non-saline conditions. However, the shoot and root biomass of *ZmRab17*:*AVP1* and *ZmUbi1*:*AVP1* barley did not differ to wild-type and null segregants in saline conditions. These findings suggest that the type of promoter driving *AVP1* expression in transgenic barley is an important factor.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Rhiannon Kate Schilling and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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*Schilling, R. K., Marschner, P., Shavrukov, Y., Berger, B., Tester, M., Roy, S.J., and Plett, D.C. (2014). "Expression of the *Arabidopsis* vacuolar H^+-pyrophosphatase gene (*AVP1*) improves the shoot biomass of transgenic barley and increases grain yield in a saline field" Plant Biotechnology Journal **12**(3): 378-386.

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R. K. Schilling

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Date

List of Publications

Research Articles

Schilling, R. K., Marschner, P., Shavrukov, Y., Berger, B., Tester, M., Roy, S.J., and Plett, D.C. (2014). "Expression of the *Arabidopsis* vacuolar H^+-pyrophosphatase gene (*AVP1*) improves the shoot biomass of transgenic barley and increases grain yield in a saline field" Plant Biotechnology Journal **12**(3): 378-386. (5-year IF: 6.247)

List of Awards

The following prizes were awarded to Rhiannon K. Schilling during her PhD candidature:

The AgPOGS Prize (2012)

Awarded for the best oral presentation by audience choice at the University of Adelaide, School of Agriculture, Food and Wine Postgraduate Symposium by the Waite Agriculture Postgraduate Society.

The K.P. Barley Prize (2012)

Awarded by the Faculty of Sciences to a postgraduate student within the School of Agriculture, Food & Wine or the School of Earth & Environmental Sciences at The University of Adelaide on the basis of academic merit and research performance.

The Max Tate Prize (2012)

Awarded for the best oral presentation at the University of Adelaide, School of Agriculture, Food and Wine Postgraduate Symposium.

The Royal Society of South Australia Postgraduate Student Prize (2nd)

Awarded runner-up for the best oral presentation by a South Australian postgraduate student delivered at the Royal Society of South Australia meeting in 2012.

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Chapter 1

Literature review and research aims

Background

Need to improve the abiotic stress tolerance of cereal crops

Abiotic stresses, such as drought, salinity and low nutrient availability, reduce the grain yield of cereal crops (Boyer, 1982; Tester and Bacic, 2005). Globally, crop growth is limited by both frequent drought events in arid and semi-arid regions and by salinity in more than 77 million ha of arable land (Boyer, 1982; Munns, 2002). Vast amounts of nitrogen (N) and phosphorus (P) fertiliser used to increase yield of cereal crops is also inefficient and costly, with estimates suggesting $2.3 billion of farm input costs in Australia alone are due to fertiliser use (ABARE, 2012). Furthermore, the impact of abiotic stresses on crop production is predicted to intensify in the future due to increasing land degradation, climate variability, urban expansion, and rising farm input costs (Burke et al., 2006; Tester and Langridge, 2010). With the human population expected to reach 9 billion people by the year 2050 (http://faostat.fao.org), it has been estimated that global food production will need to increase by a further 44 million tons each year above current increases (Tester and Langridge, 2010). The development of cereal crop varieties with improved abiotic stress tolerance is therefore needed to help increase crop productivity (Schroeder et al., 2013; Tester and Langridge, 2010).

Commercially relevant barley varieties with improved abiotic stress tolerance are needed

Barley (*Hordeum vulgare*) is the fourth most cultivated cereal crop in the world (FAO, 2013). Malting and feed barley are important commodities for both the brewing and livestock industries respectively (Baik and Ullrich, 2008). In 2011-2012, the Australian grain harvest alone yielded 8.6 million tons of barley with an export value of $1.8 billion (ABARE, 2012). However, abiotic stresses limit the productivity of barley production (Colmer et al., 2005; Jamieson et al., 1995; Raun and Johnson, 1999). Furthermore, success in breeding new varieties of barley with improved abiotic stress tolerance is limited, due to the complexity of abiotic stress tolerance, variation in the timing and extent of stresses (genotype x environment) influencing plant selection processes, and the lack of desired traits in closely related species (Cushman

and Bohnert, 2000; Richards, 1996; Tester and Bacic, 2005; Vinocur and Altman, 2005). Alternatively, the use of genetic engineering provides an opportunity to advance the development of barley varieties with improved abiotic stress tolerance (Cushman and Bohnert, 2000; Schroeder et al., 2013). The transfer of one or more candidate genes for abiotic stress tolerance into barley, such as genes involved in stress signalling, growth regulation, ion transport or reactive oxygen scavenging, has the potential to help improve the abiotic stress tolerance of this cereal crop (Cushman and Bohnert, 2000; Roy et al., 2011; Schroeder et al., 2013). The development of transgenic barley expressing *AVP1*, a gene encoding a vacuolar proton-pumping pyrophosphatase (H^+-PPase) from *Arabidopsis thaliana*, is one such example with the potential to improve the abiotic stress tolerance of barley.

Arabidopsis vacuolar H^+-pyrophosphatase (*AVP1*)

Role in vacuolar ion sequestration

Vacuolar H^+-PPases (EC. 3.6.1.1) have a number of key roles in plants (Barkla and Pantoja, 1996; Maeshima, 2000; Martinoia et al., 2007; Rea and Poole, 1993; Robinson, 1996). In *Arabidopsis*, three genes (*AVP1*, *AVP2* and *AVP3*) encoding vacuolar H^+-PPases have been identified (Drozdowicz et al., 2000; Sarafian et al., 1992). AVP1 is a type I K^+-dependent vacuolar H^+-PPase and both AVP2 and AVP3 are type II K^+-insensitive vacuolar H^+-PPases (Drozdowicz and Rea, 2001; Sarafian et al., 1992). However, compared to AVP2 and AVP3, much more research has focused on characterising AVP1. AVP1 is a tonoplast bound protein which uses energy derived from the hydrolysis of cytosolic inorganic pyrophosphate (PP_i) to orthophosphate (P_i) to actively pump H^+ from the cytoplasm into vacuoles (Duan et al., 2007; Kim et al., 1994; Zhen et al., 1997). This process (1) reduces the PP_i concentration in the cytoplasm and (2) increases the acidification of vacuoles (Ferjani et al., 2011; Maeshima, 2000). The increased vacuole acidification by vacuolar H^+-PPases (and vacuolar H^+-ATPases (EC. 3.6.1.3) establishes an electrochemical difference for H^+ across the tonoplast (Maeshima, 2000; Sze et al., 1992). This electrochemical difference can be used by other vacuolar transporters, such as sodium/proton

(Na^+/H^+) antiporters, to pump ions into vacuoles (Blumwald, 2000). Hence, vacuolar H^+-PPases have been implicated in facilitating the sequestration of ions into vacuoles, which can enhance cell turgor and reduce the accumulation of toxic ions, such as Na^+, in the cytoplasm (Blumwald, 2000). In addition to facilitating (1) vacuolar ion sequestration, it has also been proposed that AVP1 regulates a number of other traits including (2) auxin abundance and distribution, (3) heterotrophic growth and (4) loading of sucrose into the phloem (Ferjani et al., 2011; Gaxiola et al., 2012; Li et al., 2005).

Role in auxin abundance and distribution

AVP1 is thought to facilitate auxin transport and regulate auxin dependent organogenesis (Li et al., 2005) (Figure 1). *Arabidopsis* mutants without functioning AVP1 (*avp1-1*), due to an insertion of transfer-DNA (t-DNA) in the fifth exon preventing full-length transcription, had poor root, shoot and flower development (Li et al., 2005). The rosette leaf size of *avp1-1* mutants were 20 % smaller than wild-type and root cell elongation was disrupted in mutant plants compared to wild-type (Li et al., 2005). Conversely, transgenic *Arabidopsis* over-expressing *AVP1* had a greater number and size of rosette leaves, due to an increase in cell number and a greater number of plasma membrane (PM) H^+-ATPases and Pinformed 1 (PIN1) auxin efflux facilitator proteins compared to wild-type (Li et al., 2005) (Figure 1). The shoot tissue of *Arabidopsis* over-expressing *AVP1* also had 50 % higher auxin content than wild-type (Gonzalez et al., 2010; Li et al., 2005) and transgenic bentgrass expressing *AVP1* had significantly higher root auxin content than wild-type (Li et al., 2010). However, other loss-of-function *Arabidopsis* mutants (*fugu5*) defective in AVP1 activity due to point mutations lacked an auxin phenotype suggesting AVP1 may not alter auxin fluxes or abundance and that the auxin phenotype of *avp1-1* plants may be allele specific (Ferjani et al., 2011).

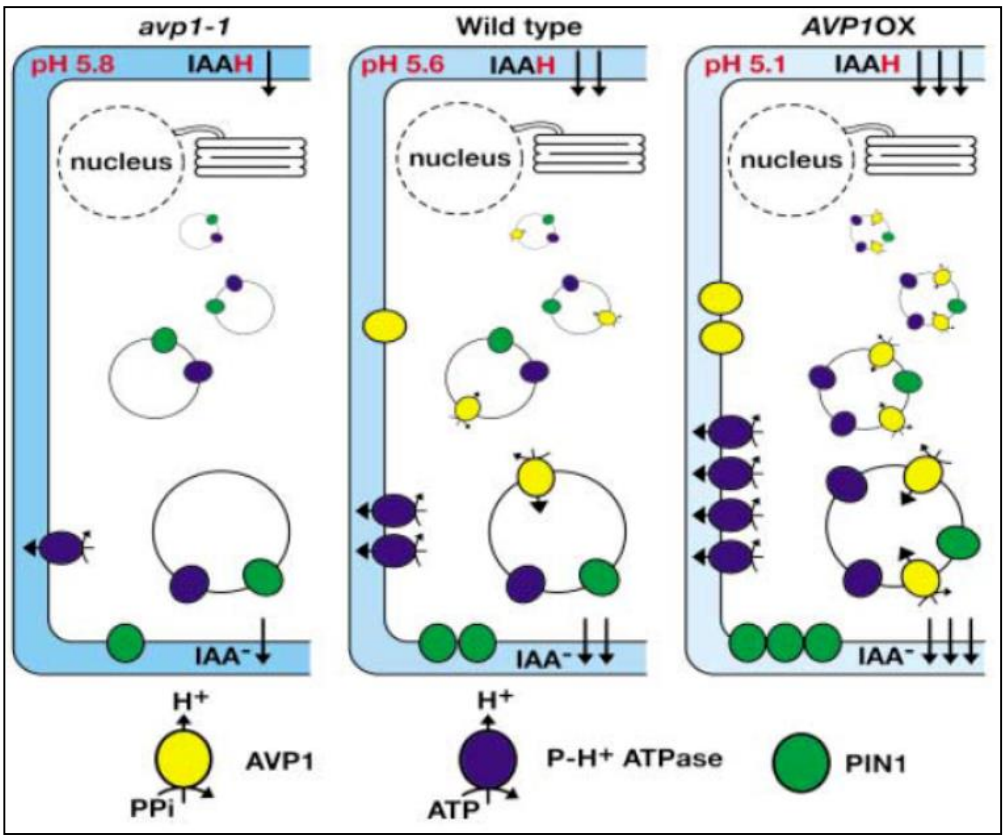

Figure 1 This schematic depicts cells of the recessive *Arabidopsis* loss-of-function *AVP1* mutant (*avp1-1*), wild-type and *Arabidopsis* plant over-expressing *AVP1*. It is suggested that AVP1 may regulate auxin-mediated organ development by altering the distribution and abundance of plasma membrane (PM) H^+-adenosine triphosphatase (H^+-ATPase) and Pinformed 1 auxin efflux facilitator (PIN1). The mutant *avp1-1* plants have no AVP1 at the tonoplast (yellow shaded circle) and decreased PM H^+-ATPase (dark blue shaded circle) and PIN1 (green shaded circle) compared to wild-type. While transgenic plants over-expressing *AVP1* have increased AVP1 at the tonoplast and increased PM H^+-ATPase and PIN1 at the plasma membrane compared to wild-type. Source: Li et al., (2005).

Role in heterotrophic growth

Another proposed role of AVP1 is the hydrolysis of cytosolic PP_i, and thus, the regulation of cytosolic PP_i concentrations (Ferjani et al., 2011). Various metabolic reactions generate PP_i as a by-product including the synthesis of fatty acids, aminoacyl-tRNA, nucleic acids, cellulose, starch and sucrose (Maeshima,

2000). *Arabidopsis fugu5* mutants, which are defective in the *AVP1* gene, had 60 % fewer and 175 % larger cells in the cotyledon than wild-type (Ferjani et al., 2011). In addition, the *fugu5* mutants had 2.5-fold higher PP_i contents per seedling, around 50 % less sucrose per seedling and lacked heterotrophic growth when compared to wild-type (Ferjani et al., 2011). However, the wild-type phenotype was recovered in *fugu5* mutants when either sucrose or glucose was supplied in their growth media or when they were genetically engineered to express *IPP1*, a transgene encoding a cytosolic soluble inorganic pyrophosphatase from yeast (*Saccharomyces cerevisiae*) (Ferjani et al., 2011). The authors suggest that the enhanced removal of cytosolic PP_i, which is an inhibitor of gluconeogenesis at high levels, could enhance gluconeogenesis and thus plant heterotrophic growth (Ferjani et al., 2011).

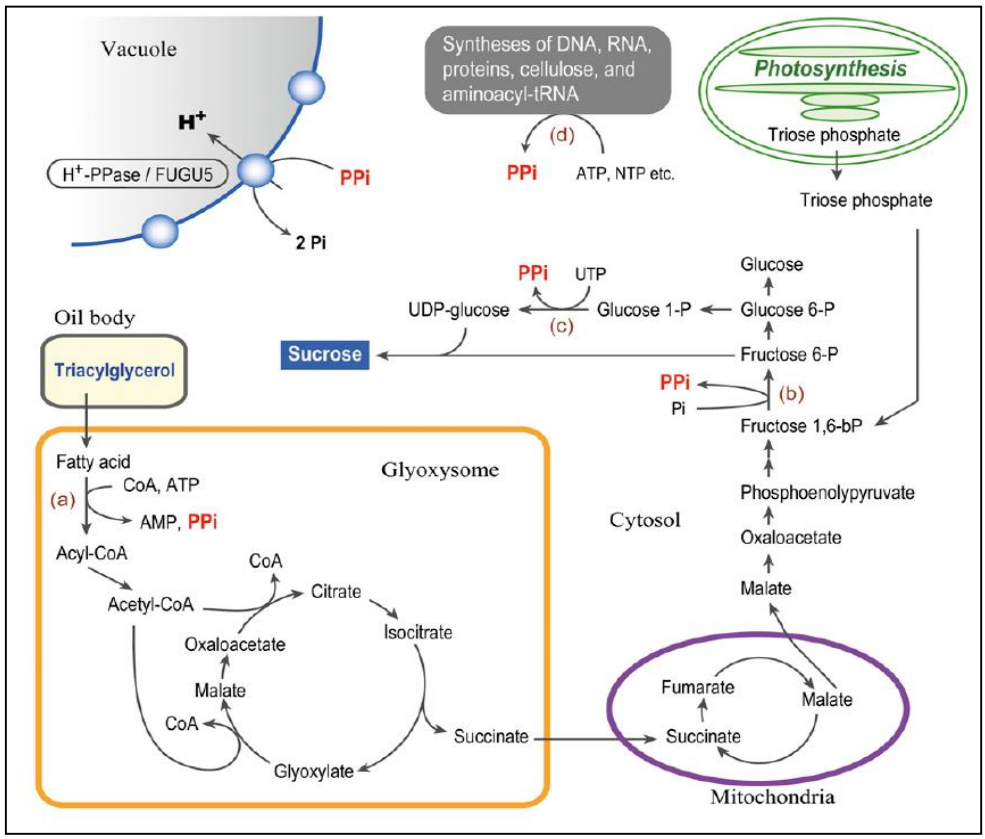

Figure 2 An outline of sucrose synthesis and other metabolic processes that generate inorganic pyrophosphate (PP_i) (outlined in red) including (a) the synthesis of fatty acids, (b) the breakdown of Fructose 1,6-b-P to Fructose 6-P, (c) the breakdown of Glucose-1-P to UDP-glucose and (d) the synthesis of nucleic acids, proteins, cellulose and aminoacyl-tRNA. It has been suggested that the main function of

vacuolar H^+-PPase/FUGU5 is the hydrolysis of cytosolic PP_i enhancing processes, including gluconeogenesis. Source: Ferjani et al., (2011).

Role in loading of sucrose into the phloem

It is also proposed that AVP1 facilitates sucrose phloem-loading, and thus more efficient sucrose transport from source to sink tissues (Gaxiola et al., 2012; Paez-Valencia et al., 2011). It has been hypothesised that AVP1 is localised on the PM in phloem companion cells and can function as a PP_i-synthase (Gaxiola et al., 2012). An increase in PP_i synthesis could increase sucrose respiration, and thus ATP supply, helping to facilitate the activity of PM H^+-ATPases in companion cells (Gaxiola et al., 2012). Greater H^+-ATPase activity helps to maintain an electrochemical potential difference for H^+ across the PM of companion cells, and thus, meditate sucrose phloem-loading (Gaxiola et al., 2012). The authors suggest that greater sucrose transport from leaves (source) to roots (sink) could therefore increase root growth and rhizosphere acidification leading to improved nutrient and water uptake in transgenic plants expressing *AVP1* (Gaxiola et al., 2012). In support of this hypothesis, there is evidence that AVP1 is localised on the PM of sieve-element companion cells in *Arabidopsis* (Paez-Valencia et al., 2011) and that vacuolar H^+-PPases from other plant species are located on the PM of phloem cells (Langhans et al., 2001; Long et al., 1995; Robinson, 1996). However, it cannot be ruled out that the localisation of H^+-PPases at the PM of phloem cells is due to remnants of the tonoplast adhering to the cell surface during sieve-element formation (Long et al., 1995). Nonetheless, it is thermodynamically feasible *in vitro* for H^+-PPases to synthesise PP_i (Baltscheffsky H et al., 1966; Davies et al., 1997; Rocha Façanha and de Meis, 1998; Seufferheld et al., 2004) suggesting it is possible that this 'vacuolar' H^+-PPases could alter sucrose phloem-loading (Gaxiola et al., 2012; Robinson, 1996). Furthermore, genes involved with sucrose transport and metabolism, including the sucrose proton symporter *SUC1*, are up-regulated in *Arabidopsis* over-expressing *AVP1* suggesting AVP1 does influence transporters involved with sucrose phloem-loading (Gaxiola et al., 2012; Gonzalez et al., 2010). Irrespective of the exact function of AVP1, this

vacuolar H^+-PPase has been implicated in altering the phenotype of transgenic plants expressing this transgene (Gaxiola et al., 2001).

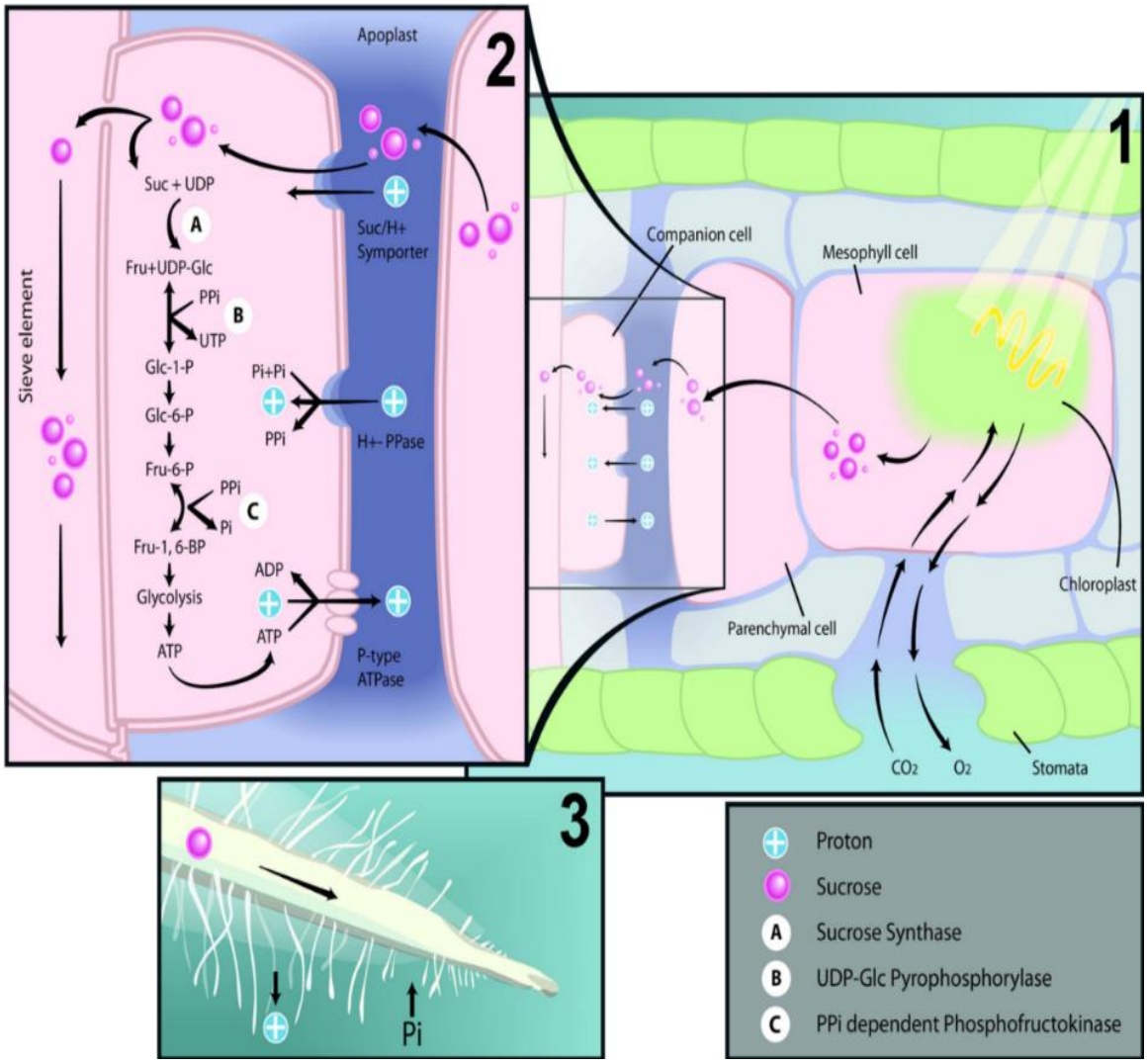

Figure 3 A proposed model suggesting H^+-PPases, such as AVP1, are involved with sucrose (Suc) phloem-loading. It has been hypothesised that in phloem companion cells, AVP1 is localised on the plasma membrane (PM) and acts as a PP_i-synthase. By increasing the concentration of cytosolic PP_i, AVP1 is thought to facilitate sucrose respiration, and thus increase ATP supply, enhancing PM H^+-ATPase activity in companion cells. Enhanced PM H^+-ATPase activity helps to maintain an electrochemical potential difference for H^+ across the PM meditating sucrose phloem-loading. Greater sucrose transport from leaves (source) to roots (sink) may increase root growth and rhizosphere acidification (proton

exudation) leading to improved nutrient and water uptake in transgenic plants expressing *AVP1*. Source: Gaxiola et al., (2012).

Phenotypes of transgenic plants expressing *AVP1*

Salinity

The constitutive expression of *AVP1* has been shown to improve the salinity tolerance of a salt-sensitive yeast (Gaxiola et al., 1999) and the growth of many transgenic plants under saline conditions. Compared to wild-type and/or null segregants under salinity stress, transgenic *Arabidopsis* (Gaxiola et al., 2001), lucerne (*Medicago sativa*) (Bao et al., 2009) and barley (Schilling et al., 2014) expressing *AVP1* had increased shoot biomass, transgenic rice (*Oryza sativa)* expressing *AVP1* had larger total plant biomass (Kim et al., 2013) and transgenic creeping bentgrass (*Agrostis stolonifera*) (Li et al., 2010), cotton (*Gossypium hirsutum*) (Pasapula et al., 2011) and peanuts (*Arachis hypogaea*) (Qin et al., 2013) expressing *AVP1* had increased shoot and root biomass. Furthermore, in a greenhouse-based experiment, transgenic cotton expressing *AVP1* had greater fibre yield compared to wild-type in saline conditions (Pasapula et al., 2011).

The improved growth of transgenic plants expressing *AVP1* in saline conditions has been attributed to AVP1 facilitating an increase in the activity of vacuolar Na^+/H^+ antiporters, and thus greater sequestration of Na^+ into vacuoles (Bao et al., 2009; Gaxiola et al., 2001; Li et al., 2010). It is suggested that the sequestration of Na^+ into vacuoles away from cytosolic enzyme activity lessens the toxic effects of this cation and helps to increase the retention of water in plant tissues (Gaxiola et al., 2001). In support of this concept, transgenic *Arabidopsis* over-expressing *AVP1* had increased leaf Na^+ and a higher leaf water content compared to wild-type after treatment with 100 mM NaCl (Gaxiola et al., 2001). Other transgenic plants expressing *AVP1* also had an increase in shoot and root Na^+ concentrations in saline conditions (Bao et al., 2009; Li et al., 2010). Measurements using the fluorescent indicator sodium green suggest

that the expression of *AVP1* in transgenic tobacco increases Na^+ accumulation within vacuoles (Duan et al., 2007). The co-expression of *AVP1* and *PgNHX1*, a gene encoding a vacuolar Na^+/H^+ antiporter from pearl millet (*Pennisetum glaucum*), in tomato (Bhaskaran and Savithramma, 2011) and the co-expression of *AVP1* and *SsNHX1*, a gene encoding a vacuolar Na^+/H^+ antiporter from the halophyte *Suaeda salsa*, in rice (Zhao et al., 2006) also resulted in larger plant growth and higher leaf Na^+ accumulation in saline conditions than the expression of either gene alone. This further implies that an enhanced electrochemical difference for H^+ across the tonoplast by AVP1 can facilitate greater Na^+ transport into vacuoles by NHX1. Furthermore, transgenic plants expressing *AVP1* had a decrease in vacuole membrane leakage and an increase in net photosynthesis compared to plants without this gene under salinity stress (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 helps to improve plant growth under saline conditions.

However, to date, no studies have evaluated the shoot biomass or yield of a transgenic plant expressing *AVP1* in a field with salinity. An important component of a salt-tolerant crop is not only the ability to grow in a saline soil but to also produce high yields (Flowers, 2004). Given that larger plants may use more water, a larger shoot biomass may not necessarily be favourable in the field and could limit grain-filling by depleting soil water earlier in the growing season, particularly if low rainfall occurs at the end of the season (Richards et al., 2002). It is thus important that greenhouse-based findings of increased growth in saline conditions are validated in a field with salinity and that yield traits of transgenic plants are analysed (Flowers, 2004; Plett and Møller, 2010; Roy et al., 2011). Recently, transgenic barley constitutively expressing *AVP1* was shown to have a larger shoot biomass compared to null segregants in soil with a 150 mM NaCl treatment (Schilling, 2010). However, it is yet to be determined whether these transgenic *AVP1* barley lines have increased shoot biomass or altered grain yield in a field with salinity (Schilling, 2010).

Drought

The constitutive expression of *AVP1* has been shown to improve the drought tolerance of plants. After 10 d of water deprivation, *Arabidopsis* over-expressing *AVP1* had greater survival and retained more solutes and water in leaf tissue than wild-type (Gaxiola et al., 2001). Transgenic lucerne expressing *AVP1* also had a larger shoot biomass, increased leaf water retention and higher photosynthetic rates than wild-type after watering was withheld (Bao et al., 2009). After 13 d of water stress, tomato (*Lycopersicon esculentum* Mill.) plants expressing *AVP1D* had a larger root dry weight, higher leaf water potential and improved recovery of shoot growth upon re-watering compared to null segregants (Park et al., 2005). Transgenic cotton and peanuts expressing *AVP1* also had a larger shoot and root biomass under reduced irrigation conditions in a greenhouse and higher yields in a dryland field compared to wild-type and/or null segregants (Pasapula et al., 2011; Qin et al., 2013). However, transgenic tomato plants expressing *AVP1D* did not have improved drought resistance in the field (Yang et al., 2014). The increase in drought tolerance of transgenic plants expressing *AVP1*, which could not be explained by greater stomata closure, was ascribed to a larger root biomass (Park et al., 2005). A larger root biomass allows increased water uptake from a greater volume of soil and, thus a reduced amount of cell dehydration in transgenic plants expressing *AVP1* compared to those without this gene (Park et al., 2005; Pasapula et al., 2011). However, it is yet to be determined whether transgenic barley expressing *AVP1* has a larger root biomass or altered water use in conditions with low water availability compared to plants without this gene (Schilling, 2010).

Low nutrient availability (P and N)

The expression of *AVP1* has been shown to improve the tolerance of transgenic plants to low P supply. Tomato expressing *AVP1D* had a larger shoot and root biomass and increased fruit dry weight production compared to wild-type in soil supplied with 100 ppm of P (Yang et al., 2007). The tomato expressing *AVP1D* also had 25 % more ripened fruit per plant than wild-type in a field with 22 µg P g^{-1} soil (Yang et al., 2014). In a soil with low P availability (10 µM P/kg soil), *Arabidopsis* over-expressing *AVP1* had a

larger shoot and root biomass, longer roots and a greater number of lateral roots than wild-type (Yang et al., 2007). This altered root morphology, a known plant response to low soil P (Gahoonia and Nielsen, 2004; Lambers et al., 2006), is thought to enable a greater exploration of soil and a larger root surface area for P uptake (Gaxiola et al., 2011). The transgenic *Arabidopsis* over-expressing *AVP1* also had 2.3-fold more shoot biomass than wild-type in soil containing poorly soluble rock phosphate. Furthermore, the transgenic *Arabidopsis* over-expressing *AVP1* had increased root proton exudation compared to wild-type at low P supply (10 µM P), which was attributed to the up-regulated activity of the PM H^+-ATPase (Yang et al., 2007). This increased rhizosphere acidification, another known plant response to low soil P availability (Hinsinger, 2001), can displace P from poorly soluble aluminium, iron or calcium phosphate complexes to increase P availability for plant uptake (Vance et al., 2003). Additionally, a more acidic apoplastic pH in the transgenic *AVP1* plants could help to facilitate greater P movement within plants (Li et al., 2005; Yang et al., 2007). Thus, the expression of *AVP1* in transgenic plants appears to be a useful strategy for increasing the tolerance of plants to low P supply (Gaxiola et al., 2011; Yang et al., 2007). However, it is yet to be determined whether transgenic *AVP1* barley has improved growth, altered rhizosphere acidification or greater P uptake at low P supply compared to plants without this gene (Schilling, 2010).

Transgenic romaine lettuce (*Lactuca sativa*) constitutively expressing *AVP1D* had larger shoot and root biomass compared to wild-type at low nitrate (NO_3^-) supply (Paez-Valencia et al., 2013). The transgenic *AVP1D* lettuce also had more marketable yields per unit of N compared to wild-type in the field (Paez-Valencia et al., 2013). The improved growth of transgenic *AVP1D* lettuce at low NO_3^- supply was attributed to greater NO_3^- uptake, potentially as a result of enhanced rhizosphere acidification and larger root growth (Gaxiola et al., 2012; Paez-Valencia et al., 2013). In support of this, the transgenic *AVP1D* lettuce had a higher shoot N content compared to wild-type at low NO_3^- supply (Paez-Valencia et al., 2013). The expression of a high affinity root NO_3^- transporter gene (*LsNRT2.1*) was also up-regulated in the

transgenic *AVP1D* lettuce compared to wild-type (Paez-Valencia et al., 2013). However, to date, no studies have compared the high- and low-affinity NO_3^- uptake capacity of transgenic plants expressing *AVP1* compared to plants without this gene. Additionally, it is not known if transgenic barley expressing *AVP1* has improved growth at low NO_3^- supply compared to plants without this gene (Schilling, 2010).

Multiple abiotic stresses

To date, most studies have focused on improving the tolerance of cereal crops to a single abiotic stress, such as salinity or drought (Cushman and Bohnert, 2000). However, in a field, multiple concurrent abiotic stresses can influence crop growth throughout the growing season (Mittler, 2006; Mittler and Blumwald, 2010; Suzuki et al., 2014; Tester and Bacic, 2005). In addition, plant responses to combined stresses have been shown to be different to that of either individual stress (Rasmussen et al., 2013; Rivero et al., 2013; Rizhsky et al., 2002; Rizhsky et al., 2004). In *Arabidopsis*, 61 % of transcriptome changes under two combined abiotic stresses could not be predicted from the transcriptomic response to either stress applied individually (Rasmussen et al., 2013). Likewise, when combined drought and heat stress was applied to *Arabidopsis*, 454 transcripts were identified that were specifically expressed under the combined stresses compared to that under either stress alone (Rizhsky et al., 2004). In tobacco, a combined drought and heat stress also induced the expression of specific genes that were not induced when either stress was applied alone (Rizhsky et al., 2002). It has thus been suggested that a combination of one or more abiotic stresses must be considered a new state of abiotic stress (Mittler and Blumwald, 2010) and that more studies are needed to evaluate plant growth responses under combined abiotic stresses (Mittler, 2006; Mittler and Blumwald, 2010; Suzuki et al., 2014).

Considering the improved tolerance of transgenic plants expressing *AVP1* to various individual abiotic stresses (i.e. salinity, drought or low nutrient supply) (Bao et al., 2009; Gaxiola et al., 2001; Li et al., 2010; Park et al., 2005; Pasapula et al., 2011; Qin et al., 2013; Schilling, 2010; Yang et al., 2007; Yang et al.,

2014), it is possible that the expression of *AVP1* may improve plant tolerance to multiple concurrent stresses. In a dryland field, where multiple abiotic stresses can occur, transgenic cotton expressing *AVP1* had a larger shoot biomass and increased fibre yield than wild-type and null segregants (Pasapula et al., 2011). However, to date, the growth of transgenic plants expressing *AVP1* under two or more combined abiotic stresses, such as salinity and low water availability, is yet to be tested in controlled conditions.

Non-stress conditions

Transgenic plants expressing *AVP1* have a larger shoot biomass, and occasionally root biomass, in non-stressed conditions compared to plants without this gene (Gonzalez et al., 2010; Li et al., 2005; Li et al., 2010; Paez-Valencia et al., 2013; Schilling, 2010; Vercruyssen et al., 2011; Yang et al., 2007). In addition, transgenic plants expressing vacuolar H^+-PPases from other species have a larger shoot biomass under non-stressed conditions (Gouiaa et al., 2012; Lv et al., 2008). Although there are some exceptions where no difference in biomass between non-transgenic and transgenic *AVP1* plants in non-stressed conditions occurs (Bao et al., 2009; Pasapula et al., 2011; Qin et al., 2013), an explanation for the larger biomass of transgenic plants expressing *AVP1* in non-stressed conditions is yet to be elucidated. Given the role of AVP1 in improving plant abiotic stress tolerance (Bao et al., 2009; Gaxiola et al., 2001; Li et al., 2010; Park et al., 2005; Pasapula et al., 2011; Qin et al., 2013; Schilling, 2010; Yang et al., 2007; Yang et al., 2014), it is possible that the larger growth of transgenic plants expressing *AVP1* in the 'non-stressed' conditions is due to subtle alterations in nutrient or water availability allowing increasing nutrient or water uptake compared to null segregants. However, it is not known if transgenic *AVP1* barley has improved nutrient or water use compared to null segregants.

Given the proposed role of AVP1 in improving plant heterotrophic growth (Ferjani et al., 2011), it is also possible that transgenic plants expressing *AVP1* have improved seedling vigour. In *Arabidopsis*, *AVP1* appears to be involved with controlling cell proliferation and consequently the final leaf size (Vercruyssen

et al., 2011). Furthermore, the activity of vacuolar H^+-PPases is often highest in young, growing tissues which have actively dividing cells and thus high PP_i and low ATP supplies (Heinonen, 2001; Maeshima, 2000; Nakanishi and Maeshima, 1998; Shiratake et al., 1997). Transgenic *AVP1* barley also had a larger projected shoot area, and thus shoot biomass, compared to null segregants at 9 d after sowing (14 d after seed imbibition) in non-stressed conditions and a faster relative growth rate between 9 to 19 d after sowing (14 to 24 d after seed imbibition) than null segregants (Schilling, 2010). However, it is unknown whether this difference in growth is due to differences in seed weight or relative growth rate prior to imaging at 9 d after sowing (before 14 d after seed imbibition). To date, no studies have investigated the seedling vigour of transgenic plants expressing *AVP1* from seed imbibition and it is not known if improved seedling vigour is contributing to the larger growth of transgenic barley expressing *AVP1* compared to null segregants in non-stressed conditions.

Furthermore, given the proposed role of AVP1 in enhancing sucrose phloem-loading (Gaxiola et al., 2012), it is possible that altered sucrose metabolism is contributing to the larger growth of transgenic plants expressing *AVP1*. Metabolomics on leaf tissue from *Arabidopsis* over-expressing *AVP1* showed a significant increase in 11 amino acids compared to wild-type suggesting AVP1 has a role in nitrogen metabolism (Gonzalez et al., 2010). An increase in the sugar signalling metabolite trehalose-6-phosphate (T6P) was also observed in *Arabidopsis* over-expressing *AVP1* suggesting AVP1 has a role in carbon signalling (Gonzalez et al., 2010). However, the increase in T6P was not statistically significant and the metabolomics analysis was limited to leaf tissue only (Gonzalez et al., 2010). It is thus not clear whether transgenic plants expressing *AVP1*, such as the transgenic *AVP1* barley, have altered carbohydrate metabolism compared to plants without this gene.

Refining the phenotypes of transgenic plants expressing *AVP1*

Refining transgene expression to specific cell types or to specific environmental conditions can help conserve cellular energy (Potenza et al., 2004), which may limit unfavourable growth phenotypes in transgenic plants in non-stress conditions, especially if the transgene of interest is important for plant growth (Morran et al., 2011). Hence, the stress-inducible expression of a transgene can improve transgenic plant growth in both non-stressed and stressed conditions compared to constitutive expression of the same transgene (Kasuga et al., 1999; Kovalchuk et al., 2013; Morran et al., 2011; Su and Wu, 2004; Waterer et al., 2010). To develop a high yielding salt tolerant transgenic crop it may therefore be beneficial to use a salt stress-inducible promoter to activate a transgene only when salinity is present (Roy et al., 2014; Tester and Bacic, 2005).

Previously, the constitutive *CaMV 35S* (Gaxiola et al., 2001; Li et al., 2010; Paez-Valencia et al., 2013; Pasapula et al., 2011; Qin et al., 2013; Schilling et al., 2014; Zhao et al., 2006) and maize *Ubiquitin* (*ZmUbi1*) (Kim et al., 2013) promoters have been used to control the expression of *AVP1* in transgenic plants to improve salinity tolerance. However, given that AVP1 is a H^+-PPases which utilises a high-energy phosphoanhydride bond from the hydrolysis of cytosolic pyrophosphate (PP_i) (Maeshima, 2000), the salt stress-inducible expression of *AVP1* may help to conserve cellular energy in transgenic *AVP1* barley in areas of a field where no salinity occurs. Transgenic barley with salt stress-inducible expression of *AVP1* may also be better perceived by consumers than constitutive expression due to the greater control over the transgene expression (Potenza et al., 2004). However, alternatively given the larger growth of *35S:AVP1* barley in non-stressed conditions (Schilling, 2010), the use of a stress-inducible promoter, which may prevent this increase in growth, might not be beneficial.

Expression of a stress-inducible maize *ZmRab17* promoter has been shown to increase under drought stress (Busk et al., 1997) and preliminary testing of the salt stress inducibility of the *ZmRab17* promoter

has been conducted (Schilling, 2010). However, further work is needed to test the salt stress inducibility of T_2 *ZmRab17*:*uidA* barley in more controlled growth conditions, such as hydroponics (Schilling, 2010). It thus remains not known if the *ZmRab17* promoter is salt stress inducible, and if so, in what tissue(s) and cell type(s) expression occurs. Preliminary testing of T_1 *ZmRab17*:*AVP1* barley (cv. WI4330) in soil with a 75 mM NaCl treatment was previously completed (Schilling, 2010). However, further testing of the T_2 *ZmRab17*:*AVP1* barley (cv. WI4330) at higher concentrations of salinity and in a more controlled set-up, such as hydroponic conditions, is needed to determine whether the salt stress-inducible expression of *AVP1* is advantageous or disadvantageous over constitutive *CaMV 35S* driven expression of *AVP1*.

Transgenic *35S*:*AVP1* barley was previously generated using the barley cultivar Golden Promise. However, Golden Promise is not well-adapted to Australian growing conditions (Forster et al., 1994) and is not suitable for commercial use by Australian grain growers. Thus, for the AVP1 technology to be applicable to Australian grain growers, a high yielding commercially relevant Australian barley cultivar with *AVP1* expression is required. Furthermore, concerns have been raised over the use of the *CaMV 35S* promoter, which is derived from a virus, in transgenic plants (Ho et al., 1999). This suggests that the use of an alternative constitutive promoter derived from a plant, such as the maize *Ubiquitin* (*ZmUbi1*) promoter, to control *AVP1* expression in barley may be better perceived by consumers (Christensen and Quail, 1996). Transgenic rice with *ZmUbi1* driven expression of *AVP1* had improved plant growth compared to wild-type under salinity stress (Kim et al., 2013). Preliminary testing of T_1 *ZmUbi1*:*AVP1* barley (cv. WI4330) in soil with a low salinity treatment was previously conducted (Schilling, 2010). However, further testing of the T_2 *ZmUbi1*:*AVP1* barley (cv. WI4330) at higher concentrations of salinity is needed to determine whether the constitutive *ZmUbi1* driven expression of *AVP1* alters the growth or ion contents of transgenic barley in saline conditions.

Research aims

The focus of this PhD project is to evaluate the abiotic stress tolerance of transgenic barley expressing *AVP1*. The following research aims will be addressed:

1. To evaluate the shoot biomass and grain yield of wild-type and transgenic *AVP1* barley in a field with low and high salinity **(Chapter 2)**

2. To investigate the abiotic stress tolerance and potential factors contributing to the larger shoot biomass of transgenic *AVP1* barley by evaluating:
    a. the growth, rhizosphere acidification and P uptake of transgenic *AVP1* barley at low and sufficient P supply **(Chapter 3)**
    b. the growth and nitrate (NO_3^-) uptake capacity of transgenic *AVP1* barley at low and sufficient NO_3^- supply **(Chapter 4)**
    c. the growth, tissue ion contents and water use of transgenic *AVP1* barley under salinity, low water availability and a combination of the two stresses **(Chapter 5)**
    d. the seedling vigour and carbohydrate metabolism of transgenic *AVP1* barley **(Chapter 6)**

3. To characterise the salt stress inducibility of the *ZmRab17* promoter and to evaluate the growth of commercially relevant barley (cv. WI4330) with *AVP1* expression via the stress-inducible promoter (*ZmRab17*) or the plant-derived constitutive promoter (*ZmUbi1*) in non-saline and saline conditions **(Chapter 7)**

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Chapter 2

Evaluating the salinity tolerance of transgenic *AVP1* barley

Statement of Contributions

The following chapter contains a published research article (see Appendix):

Schilling, R. K., Marschner, P., Shavrukov, Y., Berger, B., Tester, M., Roy, S.J., and Plett, D.C. (2014). "Expression of the *Arabidopsis* vacuolar H^+-pyrophosphatase gene (*AVP1*) improves the shoot biomass of transgenic barley and increases grain yield in a saline field" Plant Biotechnology Journal **12**(3): 378-386. (5-year IF: 6.247)

Results in Figures 1, 2 and 3, and Table S1 and S2 are derived from a greenhouse-based experiment conducted by Rhiannon K. Schilling during her Honours candidature in 2010 at The University of Adelaide.

Results in Table 1 and Figures 4, 5 and 6 and Figure S1 are derived from a field trial experiment conducted by Rhiannon K. Schilling during her PhD candidature in 2011 at The University of Adelaide.

Schilling conducted the experiments, data analysis, interpretation of results and wrote the manuscript

Marschner, Tester, Roy and Plett supervised the project

Roy assisted with conducting the field trials

Plett generated the *AVP1* construct

Berger contributed to the image data analysis

Shavrukov grew the T_2 generation of transgenic *AVP1* barley

All authors contributed to the discussion of the results and revision of the manuscript

By signing this statement of contributions, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis:

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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

Running title: AVP1 improves barley growth in saline conditions

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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 non-destructive 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 non-destructive 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 non-saline 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.

Word count: 246 words

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 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 non-destructively 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 non-destructive 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 non-destructive 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 to also 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 non-destructive 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 & *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

Non-destructive 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 to 33 %) projected shoot areas (pixels) compared to null segregants when grown for 47 d in soil with 150 mM NaCl (Figure 2a & b). The sibling *35S-AVP1*-1b, however, showed no significant difference in projected shoot 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 were 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 to 19 d after sowing in soil with 150 mM NaCl (Figure 4a & c). However, this line had similar relative growth rates to null segregants in the later growth stages from 28 to 47 d 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 to 19 d and between 28 to 47 d after sowing (Figure 4c). However, both lines already had a significantly larger projected shoot area than null segregants at 9 d after sowing under saline conditions (Figure 4c). As expected, the relative growth rates of all plants decreased over time (Figure 4c). In non-saline 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 to 17 d) and similar relative growth rates to null segregants in the later growth stages (28 to 47 d) (Table S1).

Characterisation 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^{-1}) to areas of higher EC_a (199 mS m^{-1}) (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^{-1}) and high salinity ($EC_{1:5}$ = 1231 ± 155 µS cm^{-1}) areas for the field trial plots (Figure 5). The low salinity field area is considered non-saline for cereal crop production in the wheatbelt of Western Australia. The grain yield (g $plant^{-1}$) results from this low salinity area are also consistent with those obtained for the transgenic *AVP1* barley and wild-type plants grown at a separate non-saline field area

(Table S2). The soil pH differed slightly between the low (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 to 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 in the low salinity area (Table 1). Nevertheless, two transgenic lines (*35S-AVP1*-1a & *35S-AVP1*-2) had significantly higher (23 to 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 to 42 %) shoot biomass and had greater survival in the high salinity area than wild-type plants (Figure 6a & b). As with greenhouse grown plants, there was 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 over-estimate of average grains per plant across the whole plot. Nevertheless, the number of heads and grains per plant from transgenic barley expressing *AVP1* were significantly greater (16 to 58 % and 76 to 85 % respectively) than wild-type plants in the high salinity area (Table 1). The average grain weight of transgenic *AVP1* barley plants was also significantly greater (29 to 43 %) than wild-type plants (Table 1). Furthermore, the grain yield per plant of the transgenic *AVP1* barley was significantly higher (79 to 87 %) than 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 (Figure 2 & 4). This result supports previous studies suggesting 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 shown to have higher fibre yield compared to wild-type at 200 mM NaCl treatment in a greenhouse experiment and at a non-saline 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 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 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 & 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 activity of vacuolar Na^+/H^+ antiporters, 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 retention of water in the plant tissues (Gaxiola et al., 2001). In support of this hypothesis, transgenic *Arabidopsis* over-expressing *AVP1* retain more Na^+ in their rosette leaves and have enhanced leaf water content after 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 & 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

& 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 non-saline conditions

The transgenic barley expressing *AVP1* (*35S-AVP1*-1a, *35S-AVP1*-1b & *35S-AVP1*-3) also had improved shoot growth in non-saline 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 non-saline conditions compared to plants without this gene (Li et al., 2005; Li et al., 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 non-saline conditions (Gouiaa et al., 2012; Lv et al., 2008). Although there are exceptions where no growth differences between non-transgenic and transgenic *AVP1* plants in non-saline 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 non-saline 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 non-destructive plant imaging in our study shows that transgenic barley expressing *AVP1* had already produced a significantly larger

projected shoot area 9 d after sowing in both saline (*35S-AVP1*-2 & *35S-AVP1*-3) and non-saline 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 d due to a larger seed weight or a faster relative growth rate prior to imaging at 9 d 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 to 19 d after sowing), whilst they were similar to null segregants in the later growth stages (28 to 47 d after sowing) (Figure 4c and Table S1). The larger shoot biomass of transgenic barley expressing *AVP1* in non-saline and saline conditions could be due to 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 hypothesised 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 all factors other than the desired treatment were non-limiting 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 non-saline and saline conditions.

Conclusions

In this study, it is shown that the expression of *AVP1* increases the shoot biomass of barley in saline and non-saline 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 non-destructive 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 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 d in nutrient solution containing 50 mM NaCl in a hydroponic system. Four lines showed a significant increase in leaf fresh weight compared to wild-type (data not shown). Three of these four lines (*35S-AVP1*-1, *35S-AVP1*-2, *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 sterilised by a 5 min exposure to ultraviolet light, then germinated at 21 °C for 5 d 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 mix (1.5 kg) within the bottom half of each pot. To maintain similar Ca^{2+} activity 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 minimise 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).

Non-destructive 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^{-1}).

Non-destructive 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 to 19 d and between 28 to 47 d 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 to 19 d and 28 to 47 d 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 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* vernalisation gene (GenBank DQ201168) was used as a control gene for PCR reactions 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 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 reactions 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 visualise PCR products and record gel images.

RNA extraction and gene expression analysis

Total RNA was extracted from 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 synthesise 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 reactions 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 characterisation 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 randomised 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 $plot^{-1}$ (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 water-logging. Standard agronomic practices were used including: weed control using 2 L ha^{-1} Sprayseed® (Syngenta), 2 L ha^{-1} Treflan® (Nufarm) and 1 L ha^{-1} Chlorpyrifos® (Dow AgroSciences) immediately before sowing; pre-emergent deep banding of 80 kg ha^{-1} Vigour Atlas® fertiliser containing 10N:12P:9K (Summit Fertilisers); and pre- and post-emergent application of 100 kg ha^{-1} of urea. Shoot and leaf tissue was sampled and plant tillers counted in October 2011 at the vegetative growth stage Z37 (Zadoks et al., 1974). Shoot material was dried for 3 d 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 collected for solute measurements. Grain was sampled from each plot in

December 2011 and the number of grain heads, 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 d 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 was 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 $P \leq 0.05$ or ≤ 0.01.

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Tables

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 = 6-12) with asterisks (* or **) indicating a significant difference (one-way ANOVA, LSD, $P \leq 0.05$ 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 **

Figure Legends

Figure 1 Molecular characterisation 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.

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 d 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 d 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, $P \leq 0.05$ 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^{-1} 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 d after sowing in soil with 150 mM NaCl. Values are the mean ± s.e.m (*n* = 3-8).

Figure 4 Non-destructive 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 to 19 d and (b) 28 to 47 d 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 d after sowing in soil with 150 mM NaCl and the relative growth rates (d^{-1}) 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 to 19 d and 28 to 47 d. Values are the mean ± s.e.m (*n* = 3-8) with asterisks (* or **) indicating a significant difference (one-way ANOVA, LSD, $P \leq 0.05$ or ≤ 0.01).

Figure 5 Soil characterisation 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^{-1}) and blue high EC_a (199 mS m^{-1}). 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^{-1}) and pH (H_2O) values. Values are the mean ± s.e.m (*n* = 12-21).

Figure 6 Shoot biomass of wild-type and transgenic barley expressing *AVP1* in a saline field trial (a) Shoot biomass (g DW $plant^{-1}$) 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, $P \leq 0.05$).

Figures

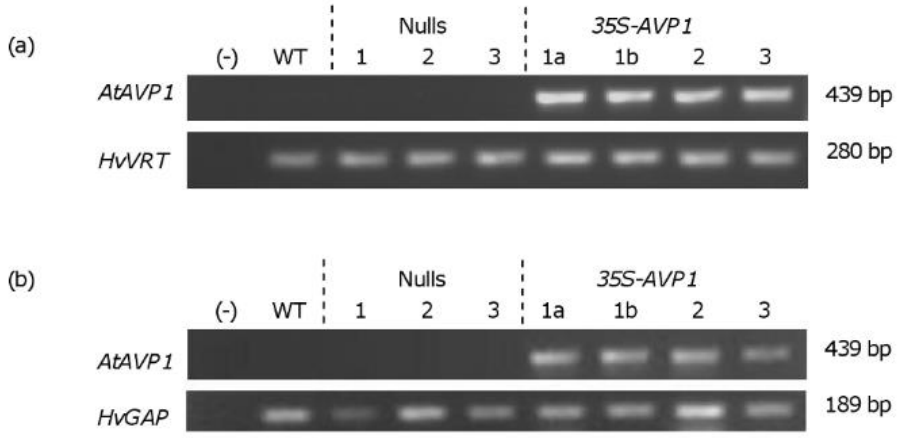


Figure 1

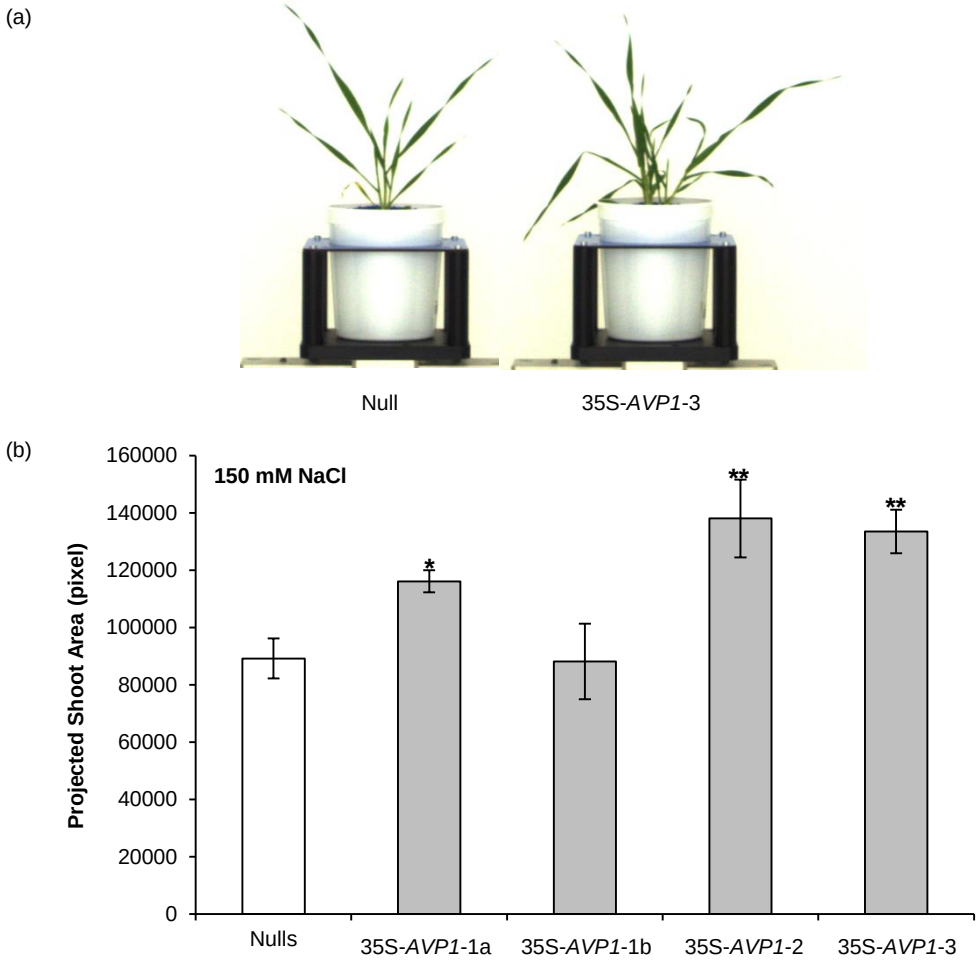

(a)
Null
35S-AVP1-3
(b)
150 mM NaCl
Projected Shoot Area (pixel)
160000
140000
120000
100000
80000
60000
40000
20000
0
*
**
**
Nulls
35S-AVP1-1a
35S-AVP1-1b
35S-AVP1-2
35S-AVP1-3

Figure 2

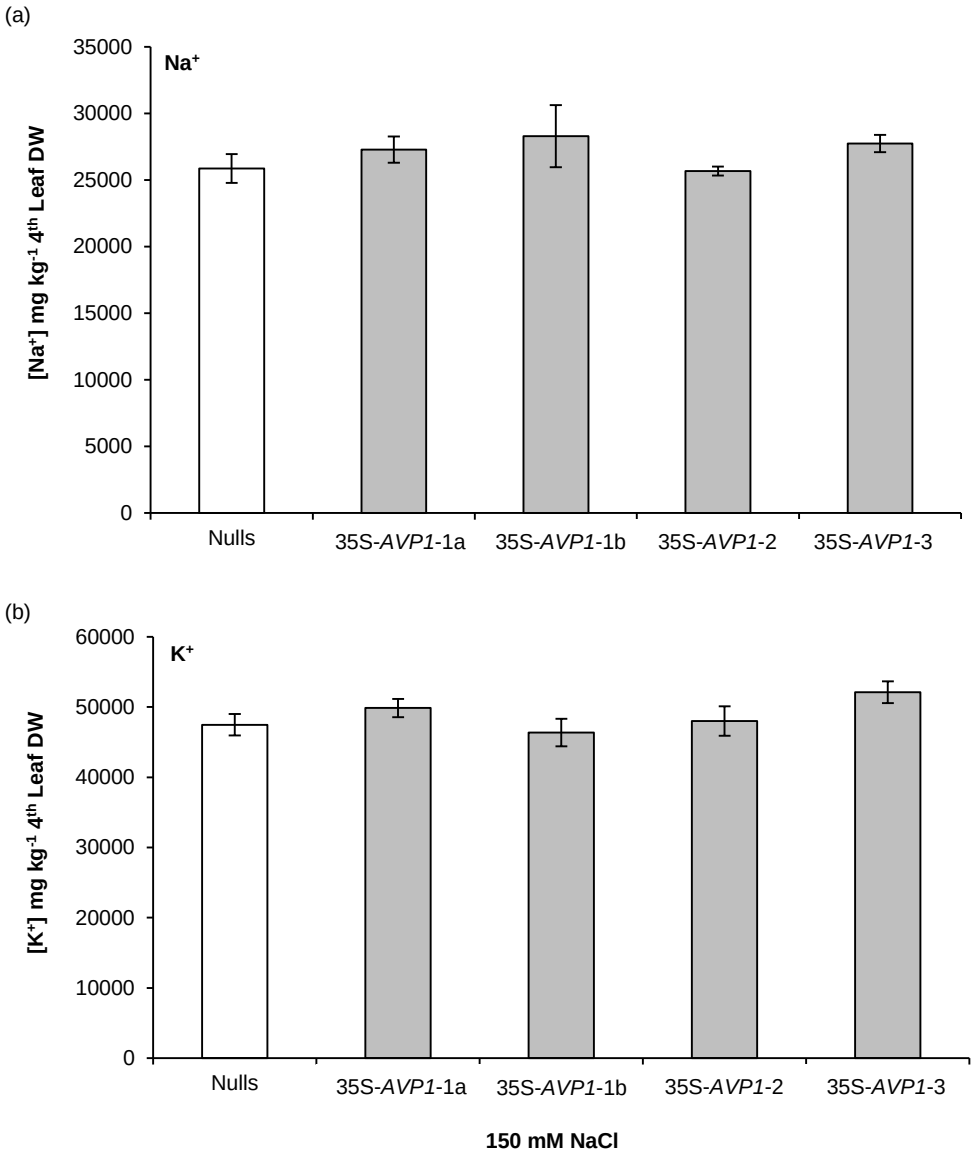
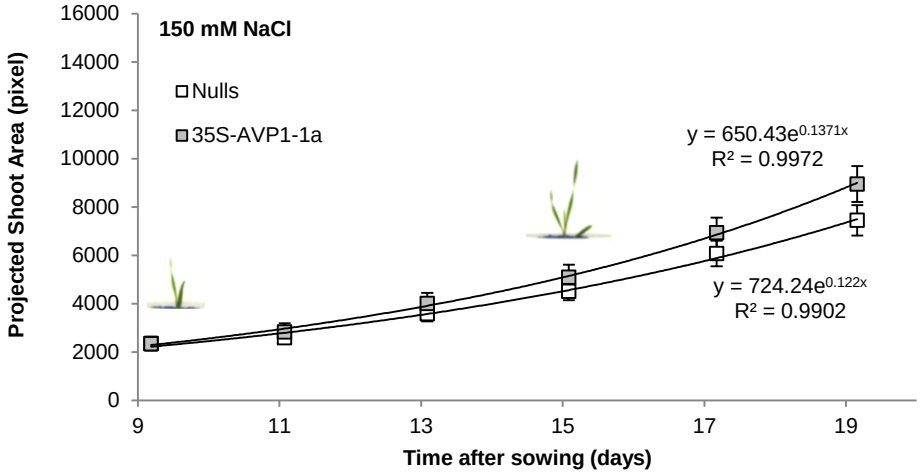
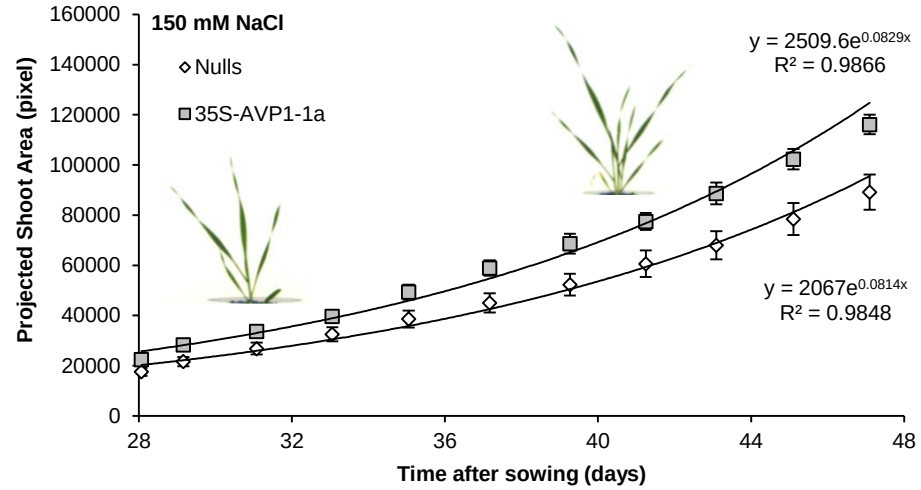

(a)
Na+
[Na+] mg kg-1 4th Leaf DW
35000
30000
25000
20000
15000
10000
5000
0
Nulls
35S-AVP1-1a
35S-AVP1-1b
35S-AVP1-2
35S-AVP1-3
(b)
K+
[K+] mg kg-1 4th Leaf DW
60000
50000
40000
30000
20000
10000
0
Nulls
35S-AVP1-1a
35S-AVP1-1b
35S-AVP1-2
35S-AVP1-3
150 mM NaCl

Figure 3

(a)



(b)



(c)

Line	Projected Shoot Area (pixel) at 150 mM NaCl		Relative Growth Rate (d^{-1}) at 150 mM NaCl	
	9 d	47 d	9 to 19 d	28 to 47 d
Null segregants	2353 ± 181	89201 ± 7008	0.1220	0.0814
35S-*AVP1*-1a	2348 ± 289	116129 ± 3880*	0.1371	0.0829
35S-*AVP1*-1b	2558 ± 359	88146 ± 13169	0.1239	0.0794
35S-*AVP1*-2	4282 ± 381**	138041 ± 13543**	0.1033	0.0799
35S-*AVP1*-3	3794 ± 139**	133524 ± 7560**	0.1268	0.0688

Figure 4

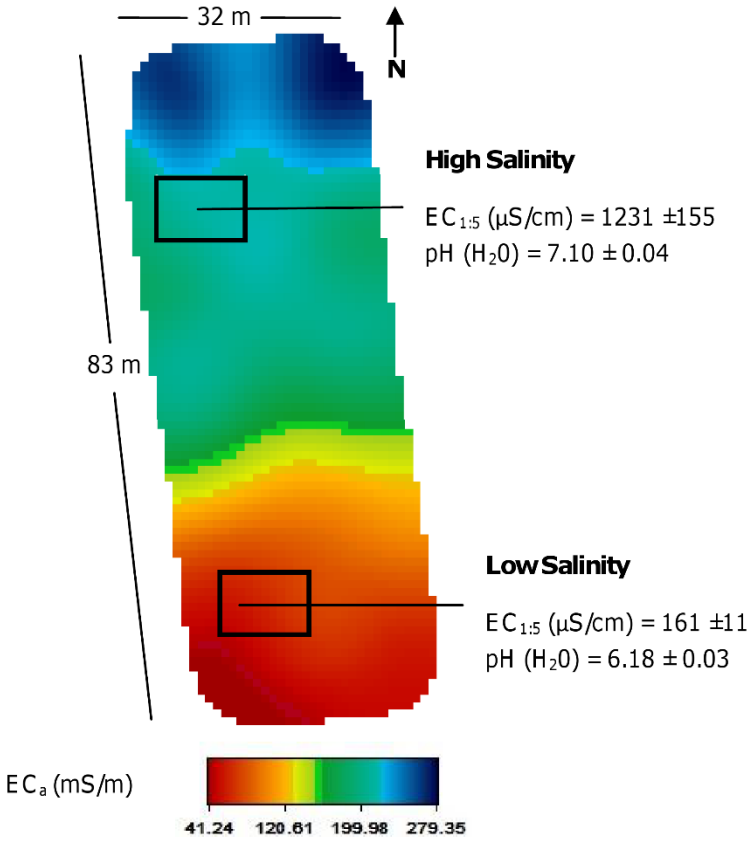
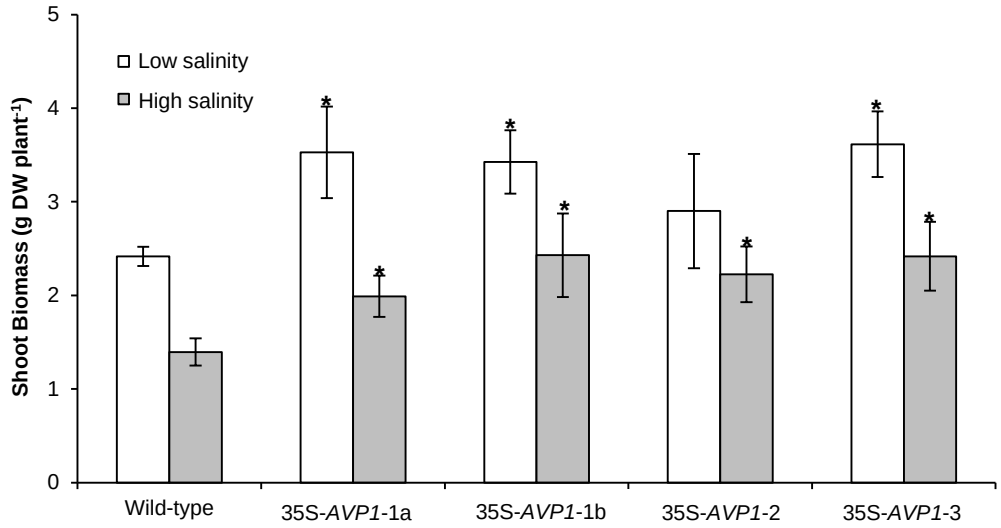

32 m
N
High Salinity
EC1:5 (µS/cm) = 1231 ±155
pH (H20) = 7.10 ± 0.04
83 m
Low Salinity
EC1:5 (µS/cm) = 161 ±11
pH (H20) = 6.18 ± 0.03
ECa (mS/m)
41.24
120.61
199.98
279.35

Figure 5

(a)


Low salinity
High salinity
Shoot Biomass (g DW plant-1)
0
1
2
3
4
5
Wild-type
35S-AVP1-1a
35S-AVP1-1b
35S-AVP1-2
35S-AVP1-3

(b)

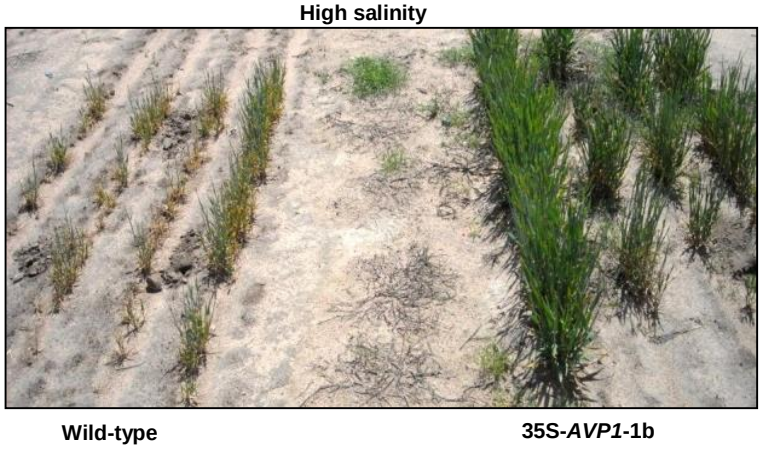

High salinity
Wild-type
35S-AVP1-1b

Figure 6

Supporting Information

Table S1 Transgenic barley expressing *AVP1* in non-saline conditions show a larger projected shoot area compared to null segregants in the greenhouse with a trend towards a faster relative growth rate during the early growth stages (9 to 17 d) and similar relative growth rates to null segregants in the later growth stages (28 to 47 d). 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 d after sowing in soil with 0 mM NaCl. The relative growth rates (per day) of null segregants and transgenic *AVP1* 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 to 19 d and 28 to 47 d after sowing in soil with 0 mM NaCl. Values are the mean ± s.e.m (n = 6-8) with asterisks (* or **) indicating a significant difference (one-way ANOVA, LSD, $P \leq 0.05$ or ≤ 0.01).

Line	Projected Shoot Area (pixel) at 0 mM NaCl		Relative Growth Rate (d^{-1}) at 0 mM NaCl	
	9 d	47 d	9 to 19 d	28 to 47 d
Null segregants	2692 ± 127	178484 ± 12109	0.1355	0.0935
35S-AVP1-1a	4011 ± 197 **	294635 ± 8001 **	0.1462	0.0867
35S-AVP1-1b	4520 ± 351 **	278032 ± 7848 **	0.1419	0.0877
35S-AVP1-2	3006 ± 349	180439 ± 18187	0.1389	0.0918
35S-AVP1-3	3473 ± 361 *	217219 ± 19621 *	0.1352	0.0912

Table S2 Transgenic barley expressing *AVP1* have similar grain yield (g/plant) compared to wild-type in a non-saline field ($EC_{1:5}$ = 114 ± 0.7 µS cm^{-1}) at O'Halloran Hill, South Australia (Longitude: 138.556277, Latitude: -35.057095) in 2010. Plants were grown to increase seed amount for a saline field trial in Kunjin, Western Australia and were sown and harvested by hand. No difference in grain yield (g $plant^{-1}$) was evident between the transgenic *AVP1* barley and wild-type. Values are the mean ± s.e.m (*n* = 42-60).

Line	Grain yield (g $plant^{-1}$)
Wild-type	5.31 ± 0.76
35S-AVP1-1a	5.56 ± 0.47
35S-AVP1-1b	5.11 ± 0.28
35S-AVP1-2	5.42 ± 0.45
35S-AVP1-3	4.86 ± 0.92

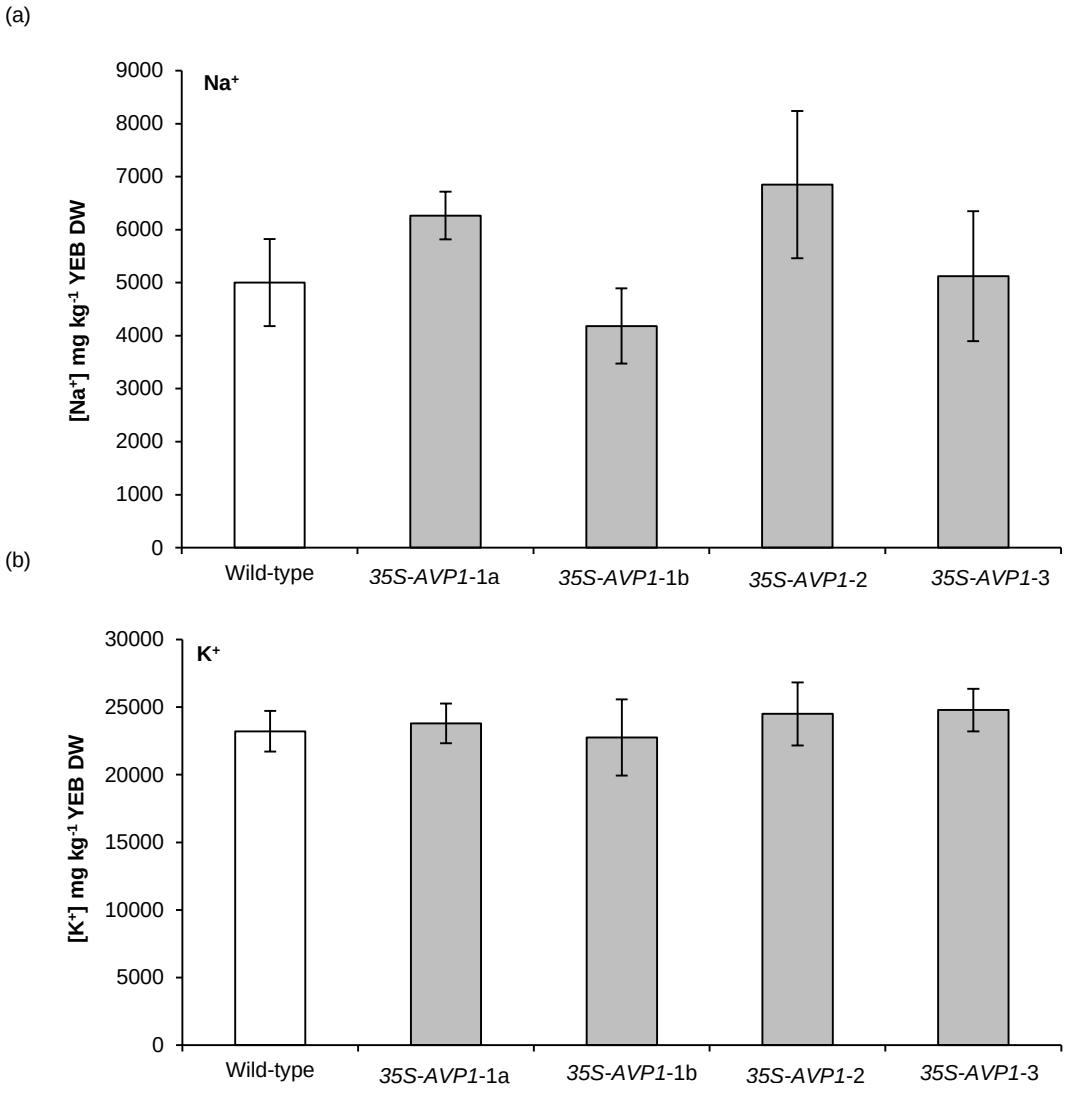


Figure S1 Leaf Na^+ and K^+ concentrations of transgenic barley expressing *AVP1* and wild-type in a saline field. (a) Na^+ and (b) K^+ concentrations (mg kg^{-1} DW) of youngest fully-emerged leaf blades of wild-type (cv. Golden Promise) (white bars) and transgenic barley expressing *AVP1* (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 and *35S-AVP1*-3 (grey bars) at vegetative growth stage Z37 after 12 weeks in a high salinity field. Values are the mean ± s.e.m (*n* = 12).

Additional Information

Subsequent field trials of the *35S:AVP1* barley (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 and *35S-AVP1*-3) were conducted in the low and high salinity field area at Kunjin, Western Australia (WA) in 2012 (Figure A1) and 2013 (Figure A2). The shoot biomass (Figures A3 and A4), leaf Na^+, K^+ and Cl^- concentrations (Figures A5 and A6) and grain yield traits (Table A1 and A2) were measured following methods outlined in Schilling et al (2014). However, in 2012, plant growth was affected by low rainfall conditions in July, August and October (Figure A7a) and, in 2013, plant growth was affected by low rainfall in June and high rainfall in July (waterlogging) (Figure A7b). Variability in salinity also influenced plant establishment between plots in the low (Figure A8a) and high salinity (Figure A8b) areas. Both factors (variability in rainfall and salinity) made interpretation of field trial results difficult due to variation in plant densities within plots. Due to the lack of growth, plants were not sampled from the high salinity field area in 2013. In addition, due to time constraints, grain yield traits in 2013 were limited to total plot grain yields, which are strongly influenced by variation in plot plant densities (i.e. higher plot grain yields occurred for plots with a higher number of plants). Nonetheless, the results of the 2012 and 2013 saline GM field trials at Kunjin, WA have been included in the following section (Figures A1-A6, Tables A1-A2).

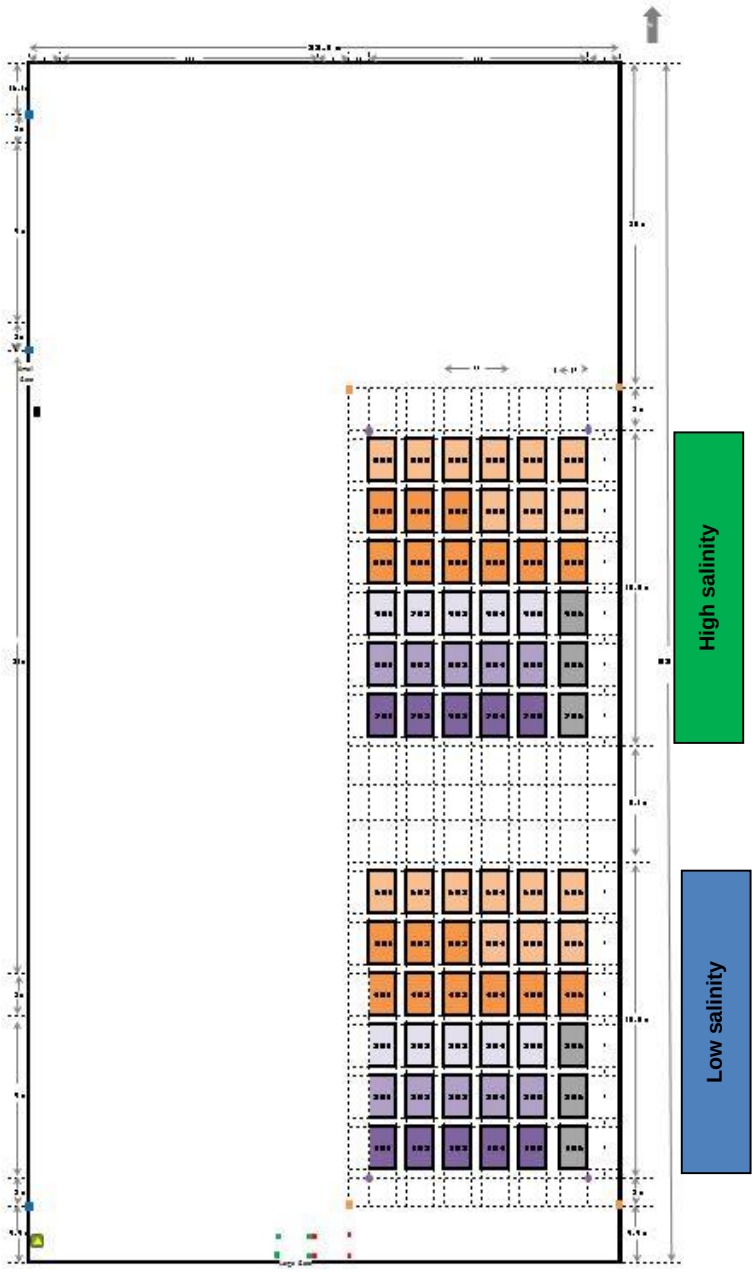


Figure A1 The saline GM field trial design at Kunjin, Western Australia in 2012 with plots (1.2 m × 2 m) in the low salinity area (blue) and high salinity area (green). The purple plots have wild-type (cv. Golden Promise) and T_5 *35S*:*AVP1* barley (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 and *35S-AVP1*-3) in a completely randomised design with blocking (dark purple, purple, light-purple blocks) with 3 replicate plots/line for each treatment. The orange plots contained other transgenic barley lines developed at the ACPFG and grey plots comprised wild-type barley (cv. WI4330) used to balance the plot design.

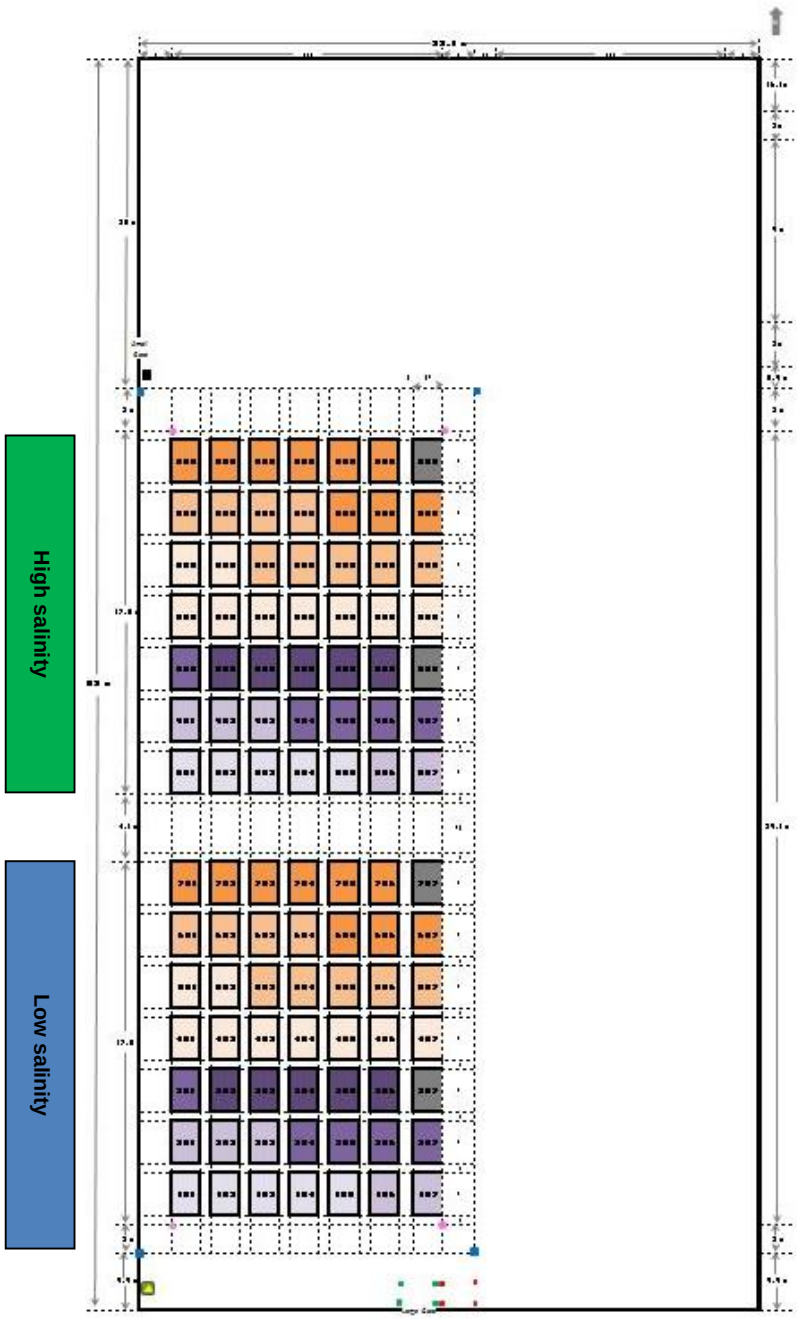


Figure A2 The saline GM field trial design at Kunjin, Western Australia in 2013 with plots (1.2 m × 2 m) in the low salinity area (blue) and high salinity area (green). The purple plots have wild-type (cv. Golden Promise) and T_5 *35S:AVP1* barley (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 and *35S-AVP1*-3) in a completely randomised design with blocking with blocking (dark purple, purple, light-purple blocks) with 4 replicate plots/line for each treatment. The orange plots contained other transgenic barley lines developed at ACPFG and grey plots comprised extra wild-type barley (cv. Golden Promise) used to balance the plot design.

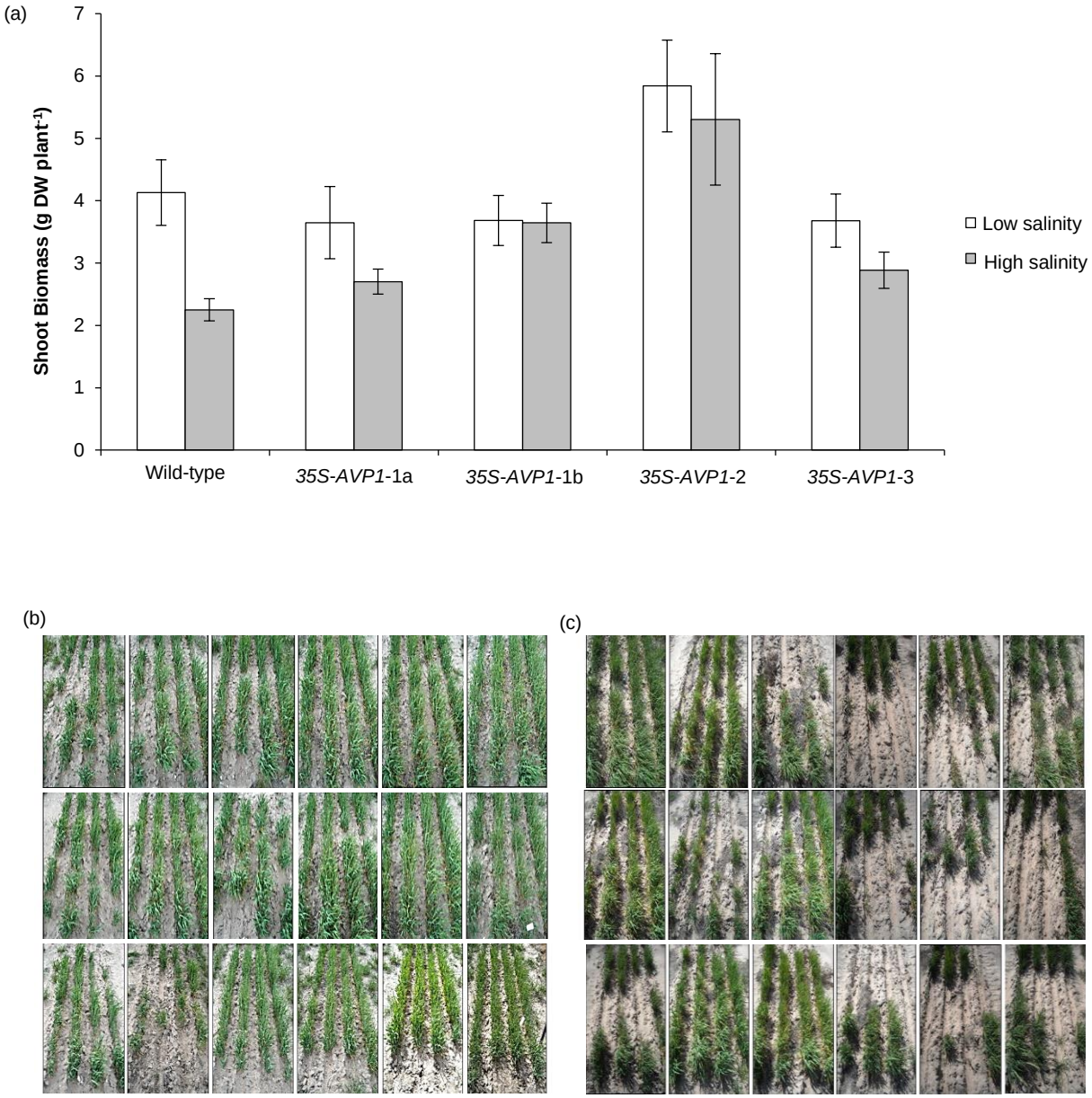


Figure A3 The (a) shoot biomass (g DW plant^{-1}) of wild-type (cv. Golden Promise) and T_5 *35S:AVP1* barley (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 and *35S-AVP1*-3) in the low (white bars) and high salinity (grey bars) field areas at Kunjin, Western Australia in 2012 when sampled at the growth stage of Z47. Values are presented as the mean ± s.e.m (*n* = 3-18). Digital images of the wild-type and transgenic *AVP1* barley plots in (b) the low salinity and (c) high salinity field area at Kunjin, Western Australia in 2012, showing the variation in plant density between plots.

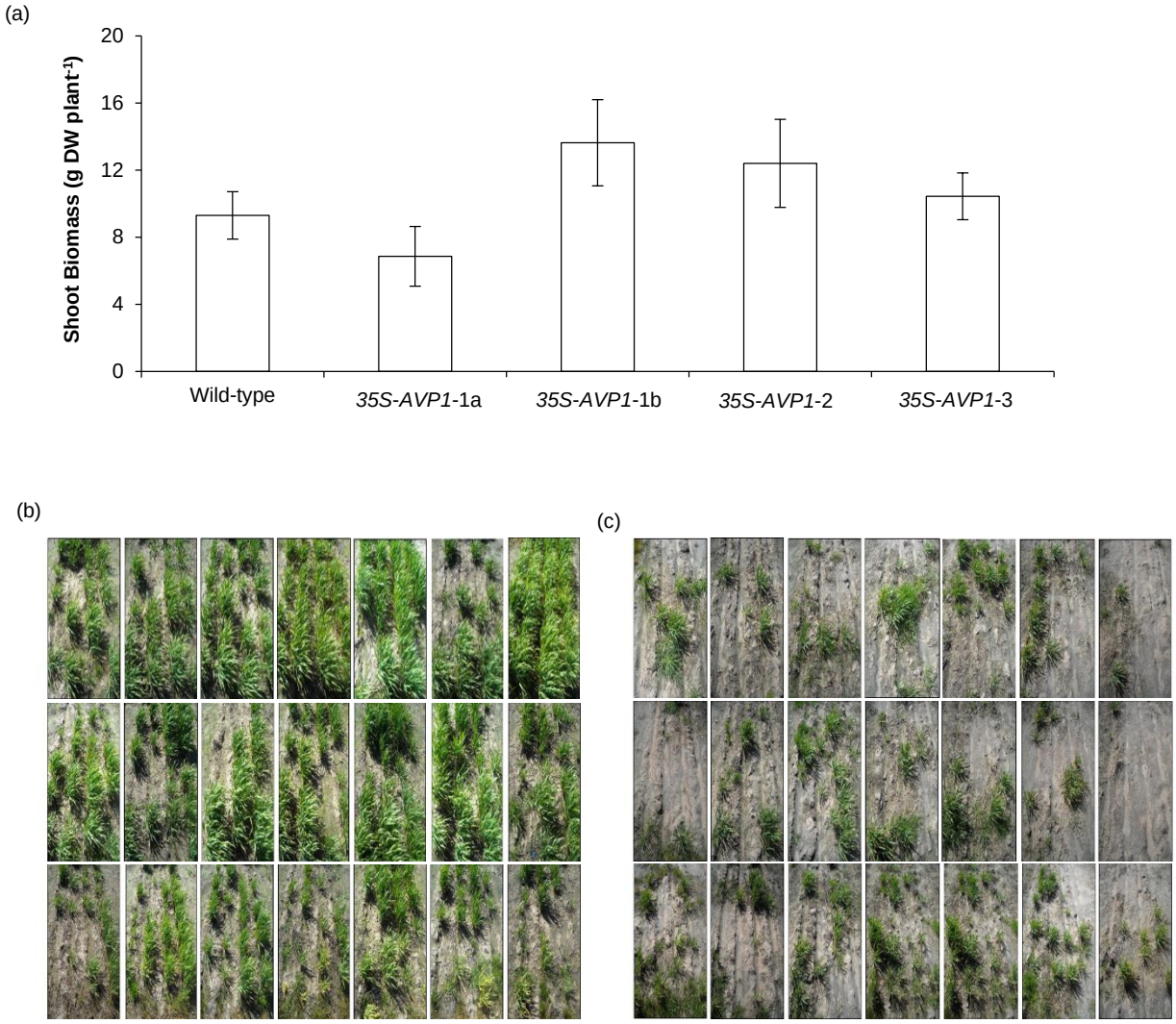


Figure A4 The (a) shoot biomass (g DW plant^{-1}) of wild-type (cv. Golden Promise) and T_5 *35S:AVP1* barley (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 and *35S-AVP1*-3) at the growth stage of Z47 in the low salinity field area at Kunjin, Western Australia in 2013. Values are presented as the mean ± s.e.m (*n* = 8-24). Digital images of the wild-type and transgenic *AVP1* barley plots in (b) the low salinity and (c) high salinity field area at Kunjin, Western Australia in 2013, showing the variation in plant density between plots.

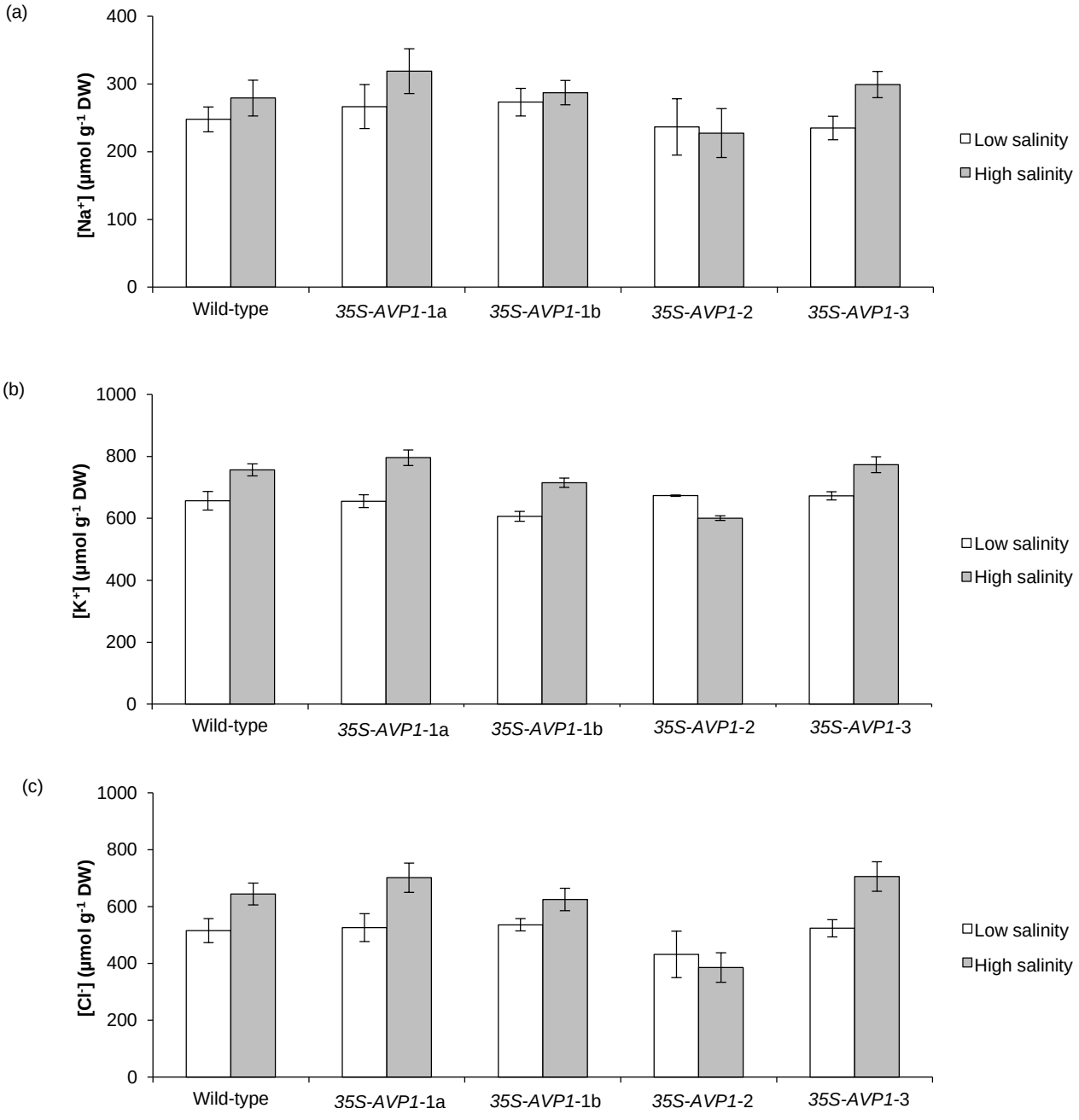


Figure A5 (a) Sodium (Na^+), (b) potassium (K^+) and (c) chloride (Cl^-) (µmol g^{-1} DW) concentration in the youngest fully-emerged leaf blade of wild-type and T_5 *35S:AVP1* barley (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 and *35S-AVP1*-3) in the low (white bars) and high (grey bars) salinity field area at Kunjin, Western Australia in 2012 when sampled at growth stage of Z47. Values are presented as the mean± s.e.m (*n* = 3-18).

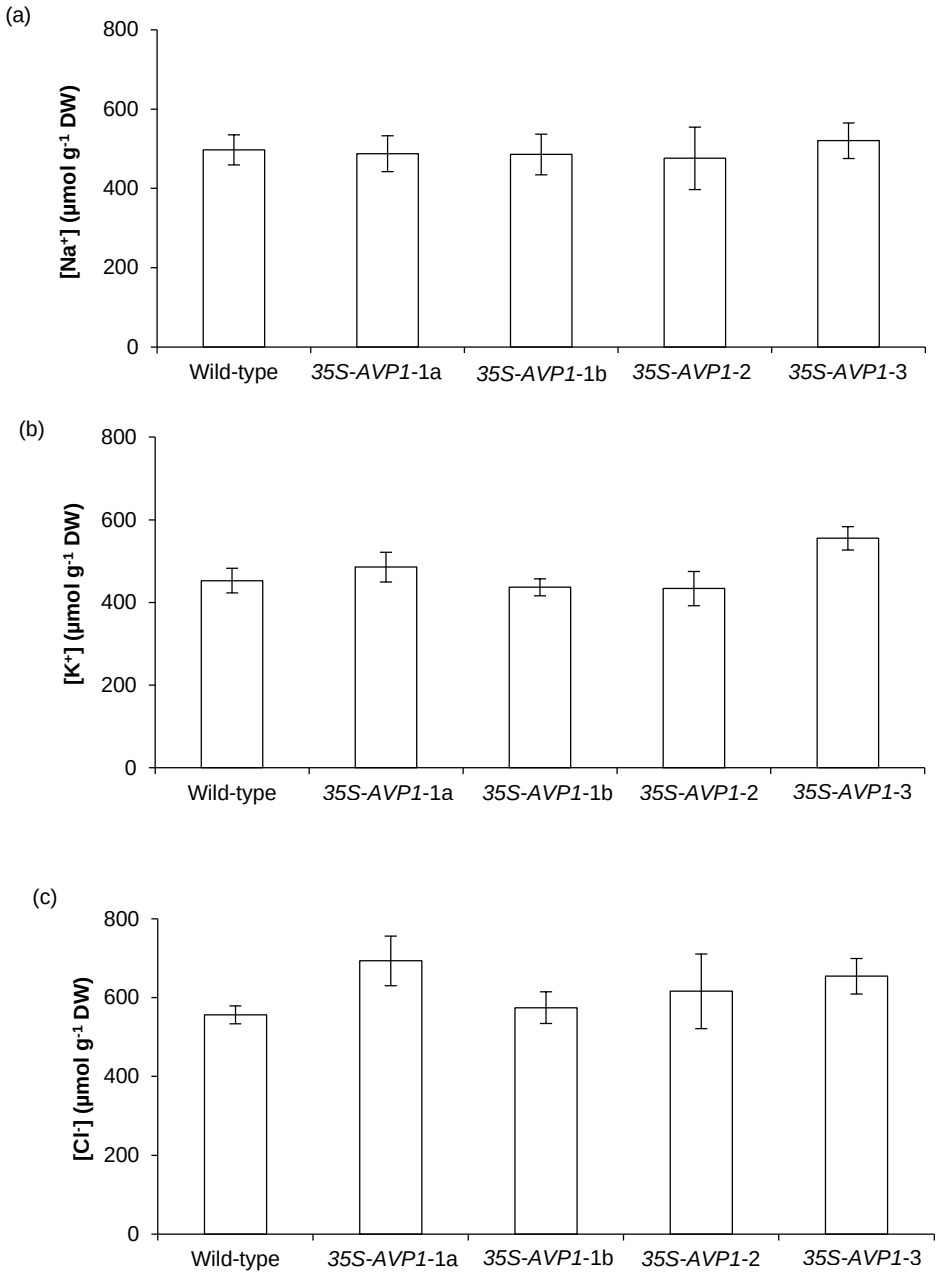


Figure A6 The (a) sodium (Na^+), (b) potassium (K^+) and (c) chloride (Cl^-) concentrations (µmol g^{-1} DW) in the youngest fully-emerged leaf blade of wild-type and T_5 *35S:AVP1* barley (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 and *35S-AVP1*-3) in the low salinity field area at Kunjin, Western Australia at growth stage of Z47. Values are presented as the mean ± s.e.m (*n* = 8-24).

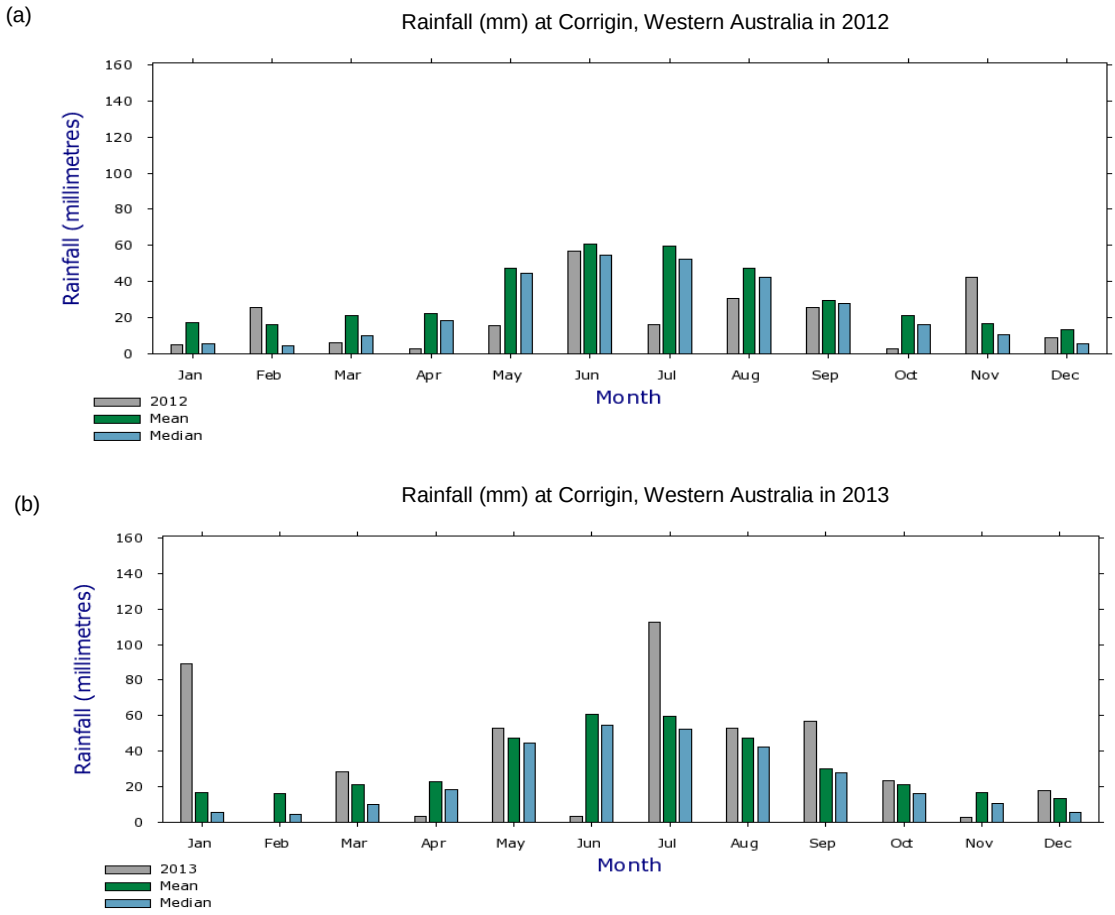


Figure A7 Average rainfall (mm) at Corrigin, Western Australia (near Kunjin) for the year (a) 2012 and (b) 2013 (grey bars), mean monthly rainfall (green bars) and median monthly rainfall (blue bars). Data and graphs were obtained from the weather station 010536 (Bureau of Meteorology, Australian Government, http://www.bom.gov.au). In 2012, sowing of the field trial at Kunjin, Western Australia occurred on the 22nd of June with low rainfall in July and August limiting plant growth. In 2013, sowing of the field trial at Kunjin, Western Australia occurred on the 17th of June with plant growth limited by low rainfall in June and high rainfall in July, August and September.

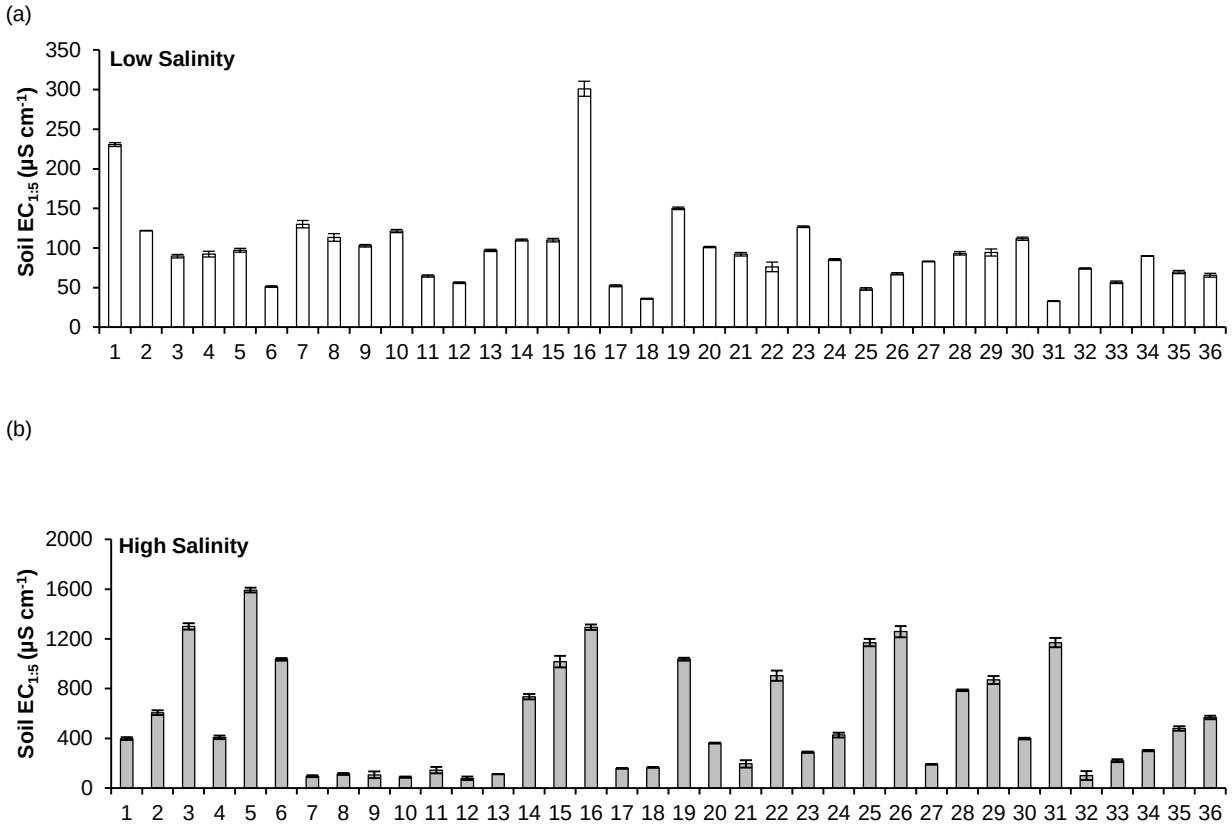


Figure A8 The electrical conductivity ($EC_{1:5}$) (soil:water) (µS cm^{-1}) of soil pooled from three sub-samples collected from different positions within individual plots (labelled as 1 to 36) in the (a) low and (b) high salinity field at Kunjin, Western Australia in September 2012 using a soil core (0-10 cm). Values are presented as the mean ± s.e.m (*n* = 3).

Table A1 The number of tillers (plant^{-1}) at Z47 and the number of grains (plant^{-1}), grain weight (mg), grain yield (g plant^{-1}) and plot grain yield (g) of wild-type (cv. Golden Promise) and T_5 *35S*:*AVP1* barley (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 & *35S-AVP1*-3) from the low and high salinity area at Kunjin, Western Australia in 2012. Values are presented as the mean ± s.e.m (*n* = 15-18 for yield traits and *n* = 3 for plot yields).

Treatment	Line	No of heads (plant^{-1})	No. of grains (plant^{-1})	Grain weight (mg)	Grain yield (g plant^{-1})	Plot Grain Yield (g)
Low salinity	Wild-type	4.9 ± 0.4	89 ± 9	31.2 ± 1.5	2.85 ± 0.33	240 ± 12
	35S-AVP1-1a	6.4 ± 0.6	94 ± 9	31.1 ± 0.7	2.91 ± 0.27	267 ± 49
	35S-AVP1-1b	5.7 ± 0.5	99 ± 9	29.2 ± 0.7	2.92 ± 0.30	295 ± 34
	35S-AVP1-2	6.0 ± 0.7	96 ± 13	31.4 ± 0.9	3.07 ± 0.45	85 ± 35
	35S-AVP1-3	6.3 ± 0.6	101 ± 11	31.0 ± 0.4	3.15 ± 0.36	266 ± 13
High salinity	Wild-type	2.8 ± 0.3	34 ± 10	30.6 ± 1.4	1.03 ± 0.2	90 ± 22
	35S-AVP1-1a	3.6 ± 0.5	29 ± 7	29.4 ± 1.3	0.89 ± 0.2	57 ± 29
	35S-AVP1-1b	4.9 ± 0.5	51 ± 7	27.0 ± 1.1	1.43 ± 0.2	42 ± 23
	35S-AVP1-2	3.6 ± 0.5	43 ± 8	32.0 ± 1.4	0.93 ± 0.2	49 ± 23
	35S-AVP1-3	3.3 ± 0.8	26 ± 8	31.1 ± 0.7	0.91 ± 02	55 ± 31

Table A2 The number of tillers (plant^{-1}) at Z47 and the total plot grain yield (g) of wild-type (cv. Golden Promise) and T_5 *35S:AVP1* barley (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 & *35S-AVP1*-3) from the low salinity field area at Kunjin, Western Australia in 2013. Values are presented as the mean ± s.e.m (*n* = 8-24 for tiller no. and *n* = 4 for plot grain yields).

Line	Tiller No. (plant^{-1})	Plot Grain Yield (g)
Wild-type	8.8 ± 0.5	243.5 ± 33.8
35S-AVP1-1a	8.8 ± 1.1	172.6 ± 54.5
35S-AVP1-1b	11 ± 0.7	195.4 ± 97.7
35S-AVP1-2	9.9 ±1.3	215.0 ± 82.0
35S-AVP1-3	9.6 ± 0.7	299.0 ± 63.4

Chapter 3

Evaluating the low phosphorus tolerance of transgenic *AVP1* barley

Statement of Contributions

The following manuscript in this chapter is formatted for publication in BMC Plant Biology:

Title: Transgenic barley expressing the *Arabidopsis* vacuolar H^+-pyrophosphatase gene (*AVP1*) has increased shoot growth and rhizosphere acidification at low phosphorus supply

Schilling conducted the experiments, data analysis, interpretation of results and wrote the manuscript
Marschner, Tester, Plett and Roy conceived and supervised the experiments
All authors contributed to the discussion of the results

By signing this statement of contributions, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis:

Petra Marschner
Signature: Date: 25/06/2014

Mark Tester
Signature: Date: 25/06/2014

Darren Plett
Signature: Date: 25/06/2014

Stuart Roy
Signature: Date: 25/06/2014

Transgenic barley expressing the *Arabidopsis* vacuolar H^+-pyrophosphatase gene (*AVP1*) has increased shoot growth and rhizosphere acidification at low phosphorus supply

Running title: AVP1 improves transgenic barley growth at low P supply

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Abstract

Background

The use of phosphorus (P) fertilisers to increase cereal crop productivity in soils with low P availability is costly and unsustainable. Cereal varieties with improved P uptake are needed to increase the profitability of crop production in soils with low P availability. Here, we evaluate the growth of transgenic barley with constitutive expression of an *Arabidopsis* vacuolar H^+-pyrophosphatase (*AVP1*) in a soil with low P availability amended with poorly soluble calcium phosphate (25 mg/kg soil) and in hydroponic conditions with low P supply (10 µM P).

Results

The transgenic barley expressing *AVP1* had a larger shoot biomass compared to wild-type in both soil and hydroponic conditions at low P supply. In hydroponic conditions with a low concentration of P, the transgenic *AVP1* barley also had a higher root P concentration compared to wild-type. Furthermore, bromocresol purple staining of transgenic *AVP1* barley plants from hydroponic conditions with low P supply had enhanced rhizosphere acidification compared to wild-type. No difference in root biomass or other root morphological traits was observed in transgenic *AVP1* barley compared to wild-type in both the soil and hydroponic conditions at low P supply.

Conclusions

This study indicates that the expression of *AVP1* increases the shoot growth, P uptake and rhizosphere acidification of transgenic barley under low P supply compared to plants without this gene. It is likely that the enhanced root acidification contributed, at least in part, to the improved P uptake of transgenic *AVP1* barley. Furthermore, this study indicates that transgenic barley expressing *AVP1* could be a useful option for increasing profitable crop production in soils with low P availability.

Keywords: cereal crops, plant nutrition, plant growth, *AVP1*, abiotic stress, rhizosphere acidification

Background

Phosphorus (P) is the second most limiting macronutrient for plant growth and development after nitrogen (Holford, 1997; Vance et al., 2003). It has been estimated that cereal crop yield on 30 to 40 % of agricultural land worldwide is limited by soils with low P availability (Batjes, 1997; Uexküll and Mutert, 1995). Globally, 20 Mt of P fertiliser is applied to soils with low P availability each year to increase cereal crop productivity (Cramer, 2010; Vance et al., 2003). However, this dependence and extensive use of P fertilisers for crop production is unsustainable. Only 10 to 30 % of applied P is taken up by plants in the year of fertiliser application (Bolland and Gilkes, 1998) and excessive fertiliser use causes environmental issues, such as the eutrophication of waterways (Smith et al., 2006). High quality non-renewable rock phosphate reserves are also depleting (Cordell et al., 2009; Dawson and Hilton, 2011). The development of cereal crop varieties with greater uptake of P or increased P utilisation efficiency are therefore needed to achieve sustainable crop production (Vance et al., 2003; Veneklaas et al., 2012).

Plants have developed several strategies for increasing P uptake under low P availability (Vance et al., 2003). Changes in root morphology, such as increased root length, the formation of cluster roots and elongation of lateral roots and root hairs, enable greater exploration of soil and a larger root surface area for P uptake (Gahoonia and Nielsen, 2004; Gamuyao et al., 2012; Lambers et al., 2006). The formation of symbiotic relationships between plants and arbuscular mycorrhiza also enable greater plant P uptake (Bolan, 1991; Smith et al., 2011). Furthermore, the secretion of organic acid anions, such as citrate and malate, and increased rhizosphere acidification can mobilise P from insoluble aluminium, iron or calcium phosphate complexes to increase P availability for plant uptake (Hinsinger, 2001; Vance et al., 2003). The development of crop varieties with one or more of these traits could help to increase P uptake, and thus crop growth, in soils with low P availability (Veneklaas et al., 2012).

Vacuolar H^+-pyrophosphatases (H^+-PPases) are membrane-bound proton pumps, which utilise energy released from the breakdown of inorganic pyrophosphate (PP_i) (Maeshima, 2000). The constitutive over-expression of an *Arabidopsis* vacuolar H^+-PPase gene (*AVP1*) has been shown to increase the root biomass, root length, number of lateral roots and rhizosphere acidification of transgenic *Arabidopsis* under low P supply compared to wild-type (Yang et al., 2007). Transgenic rice (*Oryza sativa*) and tomato (*Lycopersicon esculentum* Mill.) expressing the gain-of-function *AVP1D* allele also had greater root biomass under low P supply compared to wild-type (Yang et al., 2007). The shoot biomass of transgenic *Arabidopsis* over-expressing *AVP1* and transgenic *AVP1D* tomato and rice was also increased under low P supply compared to wild-type (Yang et al., 2007). Furthermore, transgenic *AVP1D* tomato had 25 % more ripened fruit per plant than wild-type in a field with soil containing 22 μg P g^{-1} soil (Yang et al., 2014). It has been hypothesised that AVP1 facilitates more efficient sucrose transport to roots, which increases root growth and rhizosphere acidification, and thus improves water and nutrient uptake by transgenic *AVP1* plants (Gaxiola et al., 2012).

Barley (*Hordeum vulgare*) is the fourth most cultivated cereal crop in the world and is an important commodity for both the brewing and livestock industries (FAO, 2013). However, due to the presence of soils with low P availability, barley production relies extensively on costly P fertiliser applications (Bovill et al., 2013). Potentially, the expression of a vacuolar pyrophosphatase gene, such as *AVP1*, could help to improve the growth of barley in soils with low P availability and help increase the profitability of barley production worldwide (Gaxiola et al., 2011; Gaxiola et al., 2012). However, the potential for *AVP1* expression to improve the low P tolerance of transgenic barley is yet to be tested.

Transgenic barley lines with constitutive expression of *AVP1* have been previously generated (Schilling et al., 2014). The transgenic *AVP1* barley lines had an increase in shoot biomass in saline conditions within both the greenhouse and field, and importantly, had a higher grain yield in a field with high salinity

compared to wild-type (Schilling et al., 2014). Notably, the transgenic *AVP1* barley also had an increase in shoot biomass under non-stressed conditions (Schilling et al., 2014), a trait also observed in other studies involving transgenic plants expressing *AVP1* (Li et al., 2005; Li et al., 2010; Vercruyssen et al., 2011; Yang et al., 2007). This increase in shoot growth of transgenic *AVP1* barley under non-stressed conditions (Schilling et al., 2014) and the hypothesis of larger root systems in transgenic *AVP1* plants (Gaxiola et al., 2012), suggests that the transgenic *AVP1* barley may also have improved P nutrition.

Here, we evaluate the growth of transgenic *AVP1* barley in a soil with low P availability amended with poorly soluble calcium phosphate and in hydroponics with a low concentration of P. We find that transgenic *AVP1* barley plants have a larger shoot biomass and greater P uptake compared to wild-type barley at low P supply. We also show that the transgenic barley expressing *AVP1* is better able to acidify the rhizosphere than wild-type. Our findings suggest that transgenic barley expressing *AVP1* could be a useful option for increasing crop production in soils with low P availability.

Results

Characterisation of a field soil with low P availability

The soil texture of a field soil from Monarto, South Australia with low P availability comprised 75.0 % sand, 16.2 % silt and 8.8 % clay and was therefore classified as a sandy loam. The soil water holding capacity (WHC) at -10 kPa was 17.4 %, the pH (H_2O) was 7.02 ± 0.03 and the electrical conductivity ($EC_{1:5}$) (soil:water) was 0.080 ± 0.001 dS/m. The unamended soil had a resin P concentration of 4.95 ± 0.15 mg P/kg soil and for soil amended with 25 mg and 75 mg $CaHPO_2.2H_2O$/kg soil, the resin P concentration was 11.57 ± 0.40 and 19.71 ± 1.66 mg P/kg soil respectively.

Transgenic *AVP1* barley has increased shoot biomass in soil with low P availability

Wild-type barley (cv. Golden Promise) had lower shoot biomass (27 %) and greater root biomass (6 %) after 21 days in a soil with low P availability amended with 25 mg $CaHPO_2.2H_2O$/kg soil (low P) compared to wild-type barley in the same soil with 75 mg $CaHPO_2.2H_2O$/kg soil (sufficient P) (Figure 1a & S1). In the low P soil, two independent transgenic barley lines expressing *AVP1* (*35S-AVP1*-1 and *35S-AVP1*-2) including two sibling lines (*35S-AVP1*-1a and *35S-AVP1*-1b) had a significantly larger shoot biomass (10 to 24 %) compared to wild-type (Figure 1a & b). One transgenic *AVP1* barley line (*35S-AVP1*-3), however, showed no increase in shoot biomass compared to wild-type in the low P soil (Figure 1a). No significant difference in root biomass was evident between all transgenic *AVP1* barley lines and wild-type in the low P soil (Figure S1). Additionally, the transgenic *AVP1* barley and wild-type had a similar shoot and root biomass in the sufficient P soil (Figure 1a & S1).

Transgenic *AVP1* barley has increased shoot biomass in hydroponics at low P supply

Wild-type had lower shoot biomass (29 %) and greater root biomass (3 %) after 14 days in hydroponic conditions with 10 µM P (low P) compared to wild-type with 100 µM P (sufficient P) (Figure 2a & b). Three independent transgenic barley lines expressing *AVP1* (*35S-AVP1*-1a, *35S-AVP1*-2 and *35S-AVP1*-3) had a significantly larger shoot biomass (9 to 24 %) compared to wild-type in the hydroponic conditions with low P supply (Figure 2a). However, the shoot biomass of one sibling line *35S-AVP1*-1b at low P supply was not significantly different to wild type (Figure 2a). The dry matter root to shoot ratio increased for both wild-type and transgenic *AVP1* barley lines at low P supply (Figure 2c). However, the transgenic *AVP1* barley (*35S-AVP1*-1a, *35S-AVP1*-2 and *35S-AVP1*-3) tended to have a smaller dry matter root to shoot ratio compared to wild-type (Figure 2c). There was no significant difference in root biomass or other root morphology traits, including number of root tips, total root length and average root diameter, between the transgenic *AVP1* barley and wild-type at low P supply (Figure 2b & Table S1).

Expression of *AVP1* increases root P uptake of transgenic barley at low P supply

No significant difference in initial grain P concentrations was evident between wild-type and transgenic *AVP1* barley plants (Figure S2). After 21 days in the low P soil, two transgenic *AVP1* barley lines (*35S-AVP1*-1a and *35S-AVP1*-2) had a significant increase in shoot P concentration (mg/kg DW) compared to wild-type (Figure S3). However, the transgenic *AVP1* barley and wild-type plants did not differ in shoot P concentration in hydroponic conditions with low P supply (Figure 3a.). Nonetheless, all plants in the hydroponic conditions with low P supply had a decrease in shoot (60 to 74 %) and root (60 to 68 %) P contents (mg) compared to those with sufficient P supply, with two transgenic *AVP1* barley lines (*35S-AVP1*-1a and *35S-AVP1*-3) having significantly higher total shoot P content compared to wild-type (Figure S4). Additionally, transgenic *AVP1* barley in the hydroponics conditions with low P supply had a significant increase (14 to 32 %) in root P concentration (mg/kg DW) compared to wild-type plants (Figure 3b).

Transgenic barley expressing *AVP1* has enhanced rhizosphere acidification

The extent of rhizosphere acidification was qualitatively assessed for wild-type (cv. Golden Promise) and transgenic *AVP1* barley from hydroponic conditions with low P supply using the pH indicator bromocresol purple. A colour change from purple to yellow (pH change from 6.5 to 5.2) was observed surrounding the transgenic *AVP1* barley roots and was absent from the roots of wild-type (Figure 4). This intense yellowing (acidification) of the media surrounding transgenic *AVP1* barley roots occurred predominantly near mature roots (Figure 4). Furthermore, quantitative soil pH measurements of rhizosphere soil collected from the whole root system of each plant show a decrease in rhizosphere pH of all transgenic *AVP1* barley lines in the low P soil compared to sufficient P soil, with one transgenic *AVP1* barley line (*35S-AVP1*-3) having a significantly lower rhizosphere pH than wild-type in the low P soil (Figure S5).

Discussion

In both soil and hydroponic conditions at low P availability, transgenic barley expressing *AVP1* had an increase in shoot biomass (Figures 1 & 2) and shoot P content (Figures S3 & S4) compared to wild-type. An increase in root P concentration (mg/kg DW) was also evident in transgenic *AVP1* barley in the hydroponic conditions with low P supply (Figure 3b). This demonstrates that transgenic *AVP1* barley roots were able to take up a greater amount of P under low P supply and increase their shoot biomass accordingly compared to wild-type. This supports the previous study showing a larger shoot biomass and higher total P content (mg) in transgenic *Arabidopsis* over-expressing *AVP1* and transgenic rice and tomato plants expressing *AVP1D* under low P supply (Yang et al., 2007). It also supports the concept that transgenic plants expressing *AVP1* offer the potential to achieve more sustainable crop production in soils with low P availability (Gaxiola et al., 2011; Gaxiola et al., 2012; Yang et al., 2007).

A larger root system in transgenic plants expressing *AVP1* or *AVP1D* has previously been attributed to improving P uptake, and thus increasing shoot biomass, under low P supply (Yang et al., 2007). An increase in root biomass is a known mechanism for improving plant growth at low P availability by enabling greater exploration of soil and a larger root surface area for P uptake (Gahoonia and Nielsen, 2004; Gamuyao et al., 2012; Hermans et al., 2006). The up-regulation of *AVP1* has been hypothesised to facilitate more efficient sucrose transport to roots increasing root growth and rhizosphere acidification leading to improved water and nutrient uptake (Gaxiola et al., 2012). Notably, transgenic *Arabidopsis* over-expressing *AVP1* had longer roots, a greater number of lateral roots and denser root hairs under low P supply compared to wild-type plants (Yang et al., 2007). The expression of a vacuolar H^+-pyrophosphatase gene from *Thellungiella halophila* (syn. *Eutrema salsugineum*), *TsVP*, in transgenic maize (*Zea mays*) also had 37 to 40 % larger root biomass than wild-type under low P supply (Pei et al., 2012).

In this study, there was no significant difference in root biomass between the transgenic *AVP1* barley and wild-type in both soil and hydroponic conditions with low P supply (Figure S1 & 2b). There was also no significant difference in other root morphology traits, including total root length, number of root tips and average root diameter between the transgenic *AVP1* barley and wild-type in hydroponic conditions with low P supply (Table S1). As expected for P deficient plants, the root growth of all plants increased compared to shoot growth in the hydroponic conditions with low P supply (Figure 2c) (Hermans et al., 2006). However, due to their larger shoot biomass, the transgenic *AVP1* barley had a smaller dry matter root to shoot ratio compared to wild-type under low P supply (Figure 2c). The finding that root biomass in the transgenic *AVP1* barley was not increased in the hydroponic conditions at low P supply may be due to the higher availability of P in the nutrient solution compared to soil, reducing the necessity for plant roots to search for P. Although no increase in root biomass of transgenic *AVP1* barley was observed in these experiments, it cannot be ruled out that such effects could occur if the plants had been treated for a longer time-frame or at a more severe low P treatment. However, no increase in root biomass in both the soil and hydroponic conditions suggests this factor may not be responsible, in this study, for the improved shoot growth of transgenic *AVP1* barley plants under low P supply.

Greater rhizosphere acidification in transgenic *AVP1* plants has also been attributed to increased P availability, and thus P uptake, under low P supply (Gaxiola et al., 2007; Gaxiola et al., 2012; Yang et al., 2007). Proton exudation from plant roots has been shown to mobilise P from insoluble calcium phosphate complexes to increase P availability for plant uptake (Vance et al., 2003). Transgenic *Arabidopsis* over-expressing *AVP1* had greater rhizosphere acidification under low P supply compared to wild-type (Yang et al., 2007). This increase in rhizosphere acidification, which was inhibited by vanadate, is thought to arise from AVP1 facilitating an up-regulation of the plasma membrane H^+-ATPase activity (Yang et al., 2007). Transgenic lettuce (*Lactuca sativa*) expressing *AVP1D* also had greater rhizosphere acidification under low nitrate supply (Paez-Valencia et al., 2013). Furthermore, enhanced rhizosphere acidification

was also observed in transgenic maize expressing *TsVP*, under low P supply (Pei et al., 2012). An increase in rhizosphere acidification could therefore enhance nutrient uptake in transgenic plants expressing *AVP1* (Gaxiola et al., 2011; Gaxiola et al., 2012).

In this study, bromocresol purple staining showed qualitatively that transgenic barley expressing *AVP1* has greater rhizosphere acidification than wild-type in hydroponic conditions with low P supply. This acidification (visualised by yellowing of the media) surrounding transgenic *AVP1* barley roots was primarily localised around mature roots (Figure 4). In the low P soil treatment, the transgenic *AVP1* barley had a similar rhizosphere soil pH as wild-type, with only one transgenic *AVP1* barley line (*35S-AVP1*-3) showing a significant decrease in rhizosphere soil pH (Figure S5). It is probable that a decrease in rhizosphere soil pH of all transgenic *AVP1* barley lines was not observed in this study due the use of rhizosphere soil collected from the entire root system and not solely from mature roots. Potentially, the pH of rhizosphere soil directly surrounding the mature roots of transgenic *AVP1* barley may have been more acidic than wild-type (as seen in the bromocresol staining results) or that the pH buffering capacity of the Monarto soil was a limiting factor. Nonetheless, the transgenic *AVP1* barley had enhanced root acidification at low P supply supporting previous observations in transgenic *Arabidopsis* over-expressing *AVP1* (Yang et al., 2007).

It is therefore probable that root acidification contributed, at least in part, to the increased root P uptake, and thus greater shoot biomass of the transgenic *AVP1* barley under low P availability. However, in hydroponic conditions, given the higher availability of P, the aeration of the nutrient solution and that the pH was adjusted when the nutrient solution was regularly replenished, it seems unlikely that root acidification was responsible for improving root P uptake and increasing the shoot biomass of transgenic *AVP1* barley. Other factors, such as enhanced auxin transport (Li et al., 2005; Pei et al., 2012) or improved

seedling vigour (Ferjani et al., 2011) could also be contributing to the larger shoot biomass of the transgenic *AVP1* barley observed under low P supply.

Previously, in a non-saline soil, transgenic *AVP1* barley had a larger shoot biomass than null segregants (Schilling et al., 2014). Other transgenic plants expressing *AVP1* also had an increase in shoot and root biomass under non-stressed conditions (Li et al., 2005; Li et al., 2010; Vercruyssen et al., 2011; Yang et al., 2007). However, in this study, the shoot biomass of transgenic *AVP1* barley was not significantly different to wild-type in both hydroponic and soil conditions with sufficient P supply (Figure 1a & 2a). This inconsistency could be due to differences in experimental design, with the field soil in this study being amended with all basal nutrients to limit any deviations in soil nutrients levels and thus any unintended growth advantage occurring in transgenic barley expressing *AVP1*. Nonetheless, this study highlights the importance of ensuring factors, such as soil nutrient levels, are carefully controlled when phenotyping transgenic plants, particularly transgenic *AVP1* plants which appear to tolerate several different abiotic stresses.

Conclusions

In this study, transgenic barley expressing *AVP1* had a larger shoot biomass compared to wild-type in both soil and hydroponic conditions with low P supply. The transgenic *AVP1* barley also had enhanced rhizosphere acidification and increased root P uptake compared to wild-type at low P supply. No difference in root biomass or other root morphological traits were observed between wild-type and transgenic *AVP1* barley. An explanation for the increase in shoot biomass of transgenic *AVP1* barley at low P supply remains to be fully elucidated, although increased rhizosphere acidification may have partially contributed. Overall, this study demonstrates that the expression of *AVP1* increases the shoot growth, rhizosphere acidification and root P uptake of transgenic barley under low P supply. It also indicates that the

expression of *AVP1* in barley offers the potential to achieve more sustainable cereal crop production in soils with low P availability.

Methods

Characterisation of a field soil with low P availability

A soil with low P availability was collected at a depth of ≤ 20 cm near Monarto, South Australia (Latitude: -35.098631, Longitude: 139.074707). The field soil was air dried and sieved to ≤ 2 mm. 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). The soil texture (% sand, silt and clay) was determined using the hydrometer method (Day, 1965). The soil water holding capacity (WHC) was determined using a sintered glass funnel connected to a 100 cm water column (Ψ_m= -10 kPa) (Setia et al., 2011). The amount of plant available P was determined using resin strips (6 × 2 cm) (BDH # 55164 2S, BDH Laboratory Supplies, Poole, England) following the method of Kouno et al., (1995). The amount of P in resin P extracts was determined colourimetrically at 712 nm using a UV-1601 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) (Murphy and Riley, 1962).

Establishing sufficient and low available P soil treatments

A preliminary soil incubation experiment was conducted to determine the amount of calcium phosphate ($CaHPO_2.2H_2O$) required for amending a soil with low P availability to obtain relevant low and sufficient available soil P treatments. Briefly, different rates of $CaHPO_2.2H_2O$ (0, 50, 100, 200, 300, 400, 500 and 700 mg/kg soil) were evenly mixed into 25 g of air-dry soil. The soil was incubated at 70 % of field capacity by watering to weight each day for 7 days. Following incubation, the soil was collected and resin P measurements were completed to determine plant available P levels (Table S2). The shoot biomass (g DW) of wild-type barley at five rates of $CaHPO_2.2H_2O$ (0, 25, 50, 75 and 150 mg/kg soil) was also tested

and 25 and 75 mg of $CaHPO_2.2H_2O$/kg soil was selected as relevant low and sufficient P treatments respectively (Table S3).

The field soil with low P availability was amended with basal nutrients (excluding P) to ensure all other nutrients were non-limiting. The rate and composition of supplied nutrients were (g/kg soil) 0.92 $Ca(NO_3)_2$, 0.17 K_2SO_4, 0.19 $MgSO_4$ and (mg/kg soil) 0.4 NaFeEDTA, 2.0 $CuSO_4.5H_2O$, 0.6 $MnSO_4.H_2O$, 0.4 $Co(NO_3)_2.6H_2O$, 0.5 H_3BO_3, 0.5 Na_2MoO_4 and 2.2 $ZnSO_4.7H_2O$ (Mat Hassan et al., 2012). All nutrients were mixed evenly in a small proportion of field soil and gradually combined into the bulk soil. Two rates of $CaHPO_2.2H_2O$ at 25 and 75 mg/kg soil were mixed into the bulk soil prior to being distributed into individual pots. The water content of the soil was established at 70 % of field capacity (122 mL RO water/pot).

Plant material and pot experiment using a soil with low P availability

Transgenic barley expressing *AVP1* from three independent transformation events (*35S-AVP1*-1, *35S-AVP1*-2 and *35S-AVP1*-3) and wild-type (cv. Golden Promise) were used in this study (Schilling et al., 2014). Seeds from the T_4 generation were selected by weight (43 mg each) and surface sterilised by a 5 min exposure to ultraviolet light, then germinated at 21 °C for 4 days on moist filter paper in Petri dishes (145 mm diameter) placed in polyethylene bags to maintain humidity. Individual uniform size seedlings were transplanted to a sealed white pot (12 cm × 13 cm) filled with 1 kg of Monarto soil amended with 25 mg $CaHPO_2.2H_2O$/kg soil (low P) or 75 mg $CaHPO_2.2H_2O$/kg soil (sufficient P) treatments. All pots were watered to 70 % of field capacity using RO water and electronic scales every second day. After 21 days, shoot and root tissue from each plant was collected and dry weights recorded after drying in an oven at 70 °C for 3 days. Rhizosphere soil, defined in this study as the soil closely adhering to the plant roots after gently shaking the roots free of soil, was collected using a paintbrush and used for pH measurements as outlined above.

Plant material and hydroponics experiment with a low P concentration

Transgenic barley expressing *AVP1* and wild-type (Schilling et al., 2014) were grown in a small hydroponics system with sufficient or low P supply. Seeds from the T_4 generation were pre-germinated on moist filter paper in large Petri dishes for 3 days. Uniform size seedlings were transplanted to hydroponic containers with 10 L of nutrient solution consisting of RO water with the following (in mM): 0.2 NH_4NO_3, 5.0 KNO_3, 2.0 $Ca(NO_3)_2.4H_2O$, 2.0 $MgSO_4.7H_2O$, 0.1 KH_2PO_4, 0.5 $Na_2Si_3O_7$, 0.05 NaFe(III)EDTA, 0.05 H_3BO_3, 0.005 $MnCl_2.4H_2O$, 0.01 $ZnSO_4.7H_2O$, 0.005 $CuSO_4.5H_2O$ and 0.0001 $Na_2MoO_4.2H_2O$, except the low P treatment with 10 µM KH_2PO_4. The nutrient solutions was changed every 5 days and the pH adjusted to 6.5 using 10 % HCl to ensure all nutrients (except P) were adequately supplied. An aerator stone attached to a Precision SR 7500 aerator (Aqua One, Sydney, Australia) was used to provide continuous aeration to each hydroponic container. After 14 days, root tissue from each plant was analysed using the WinRHIZO, both the shoot and root tissue was oven dried at 70 °C for 3 days and the tissue dry weights were recorded.

Analysis of root traits using WinRHIZO Pro® software

The roots of each plant were detached from the shoot and immediately imaged using an A3 Epson Expression 10000 XL 3.49 scanner (Epson, Sydney, Australia) at a grey scale with 800 dpi. Briefly, the roots of each plant were placed in a tray (20 × 30 cm) containing the same growth nutrient solution (sufficient or low P) and were carefully arranged to ensure all roots were separated for imaging. Images of each plant were analysed using WinRHIZO Pro® 2009 software (Régent Instruments, Quebec, QC, Canada) to determine the total root length, root diameter and number of root tips.

Determination of grain, shoot and root P concentrations

Grain P concentrations in transgenic barley expressing *AVP1* (*35S-AVP1*-1, *35S-AVP1*-2 and *35S-AVP1*-3) and wild-type (cv. Golden Promise) seed used in this experiment were measured in individual grains selected by weight (43 mg each) sourced from plants previously grown in non-stressed conditions. The

grain was digested using 70 % nitric acid and 30 % hydrogen peroxide for Inductive Coupled Plasma Optical Emission Spectrometry (ICP-OES) analysis (Wheal et al., 2011). To determine total shoot (soil and hydroponic) and root P concentrations (hydroponic only), the whole dried shoot or root tissue of each plant was cut into ~10 cm fragments before being digested using 70 % nitric acid and 30 % hydrogen peroxide for ICP-OES analysis (Wheal et al., 2011).

Visualisation of rhizosphere acidification using bromocresol purple staining

The pH indicator bromocresol purple was used to observe root acidification in 13-day-old wild-type and *35S:AVP1* barley from hydroponic conditions with low P supply (as described above) using a method adapted from Heckman and Strick (1996). Briefly, 7.5 g of agarose (Cat. No. BIO-41026, Bioline, London, UK), 0.06 g of bromocresol purple (Lot 29F3712, Sigma-Aldrich Pty. Ltd., Sydney, Australia) and 1000 mL of distilled water were mixed together and adjusted to a pH of 6.5 using 0.5 M NaOH, prior to heating in a microwave until the solution boiled. The cooling liquid bromocresol purple agarose solution, which was stirred to ensure the solution did not settle, was gently poured over the roots of an intact plant carefully placed in a white tray (13 cm × 13 cm) to a final gel thickness of ~ 3 mm (100 mL solution/tray). The solution was allowed to solidify and the extent of acidification (yellowing) surrounding the plant roots (1 plant/gel) was visualised under natural sunlight in a greenhouse after 4 h of staining. The pH of the same bromocresol purple solution at the specific purple (pH = 6.5) and yellow (pH = 5.2) colouring was also measured.

Statistical analysis

Data was 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 compared to wild-type within treatments at a probability level of $P \leq 0.05$.

Competing interests

The authors declare that they have no competing interests.

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Figure legends

Figure 1 - Transgenic *AVP1* barley has increased shoot biomass in a soil with low P availability. (a) Shoot biomass (g DW) of wild-type (cv. Golden Promise) and transgenic *AVP1* barley (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2, *35S-AVP1*-3) after 21 days in a soil with low P availability amended with sufficient P (75 mg $CaHPO_2.2H_2O$/kg soil) or low P (25 mg $CaHPO_2.2H_2O$/kg soil) treatments. Values are the mean ± s.e.m (n = 3-7) with asterisks (*) indicating a significant difference to wild-type (one-way ANOVA, LSD, $P \leq 0.05$). (b) A representative image of wild-type (cv. Golden Promise) and transgenic *35S-AVP1*-1a barley plant growth in the low P soil (25 mg $CaHPO_2.2H_2O$/kg soil) at 17 days of treatment.

Figure 2 - Transgenic barley expressing *AVP1* has increased shoot biomass in hydroponics conditions at low P supply. (a) Shoot and (b) root biomass (g DW) and (c) dry matter root to shoot ratio of wild-type (cv. Golden Promise) and transgenic *AVP1* barley (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2, *35S-AVP1*-3) in hydroponic conditions at sufficient and low P (10 µM P) supply for 14 days. Values are the mean ± s.e.m (n = 4-9) with asterisks (*) indicating a significant difference to wild-type (one-way ANOVA, LSD, $P \leq 0.05$).

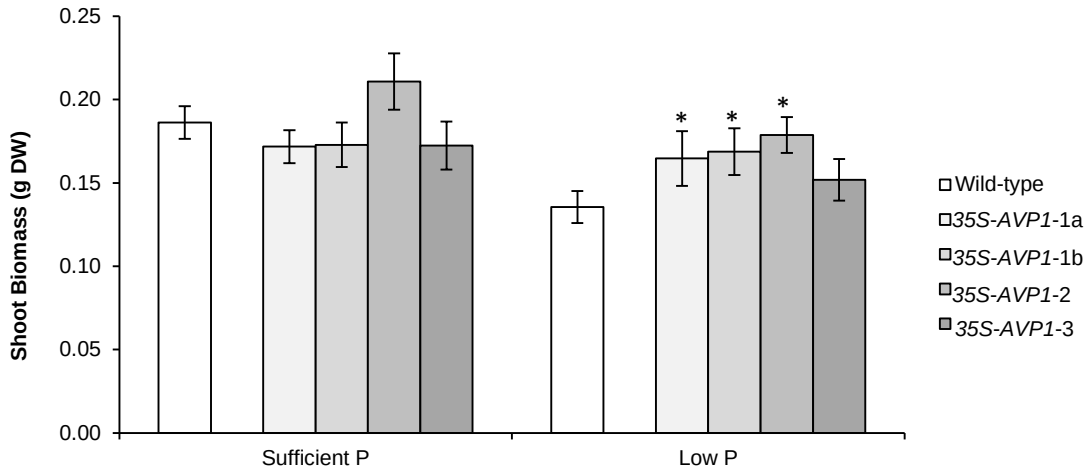
Figure 3 - Transgenic barley expressing *AVP1* has greater root P concentrations in hydroponics conditions at low P supply. (a) Shoot and (b) root P concentrations (mg/kg DW) of wild-type and transgenic *AVP1* barley in hydroponic conditions at low P (10 µM P) supply for 14 days. Values are the mean ± s.e.m (n = 4-9) with an asterisks (*) indicating a significant difference to wild-type (one-way ANOVA, LSD, $P \leq 0.05$).

Figure 4 - Transgenic *AVP1* barley plants have greater rhizosphere acidification than wild-type in hydroponic conditions with low P supply. A representative image of 13-day-old wild-type (cv. Golden Promise) and transgenic *AVP1* barley roots (*35S:AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 and *35S-AVP1*-

3) from hydroponic conditions with low P (10 µM P) supply after staining for 4 h using the pH indicator bromocresol purple. A colour change from purple to yellow indicates acidification (pH change from 6.5 to 5.2 units).

Figures

(a)



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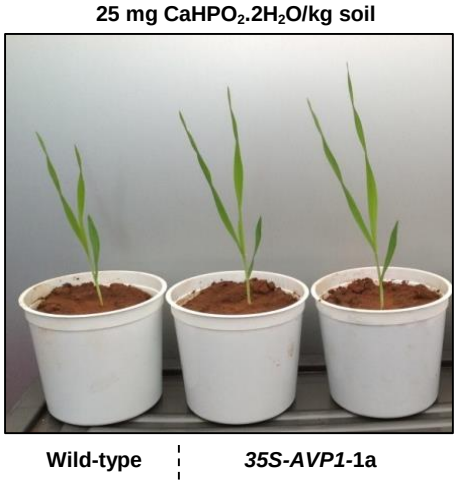

Figure 1

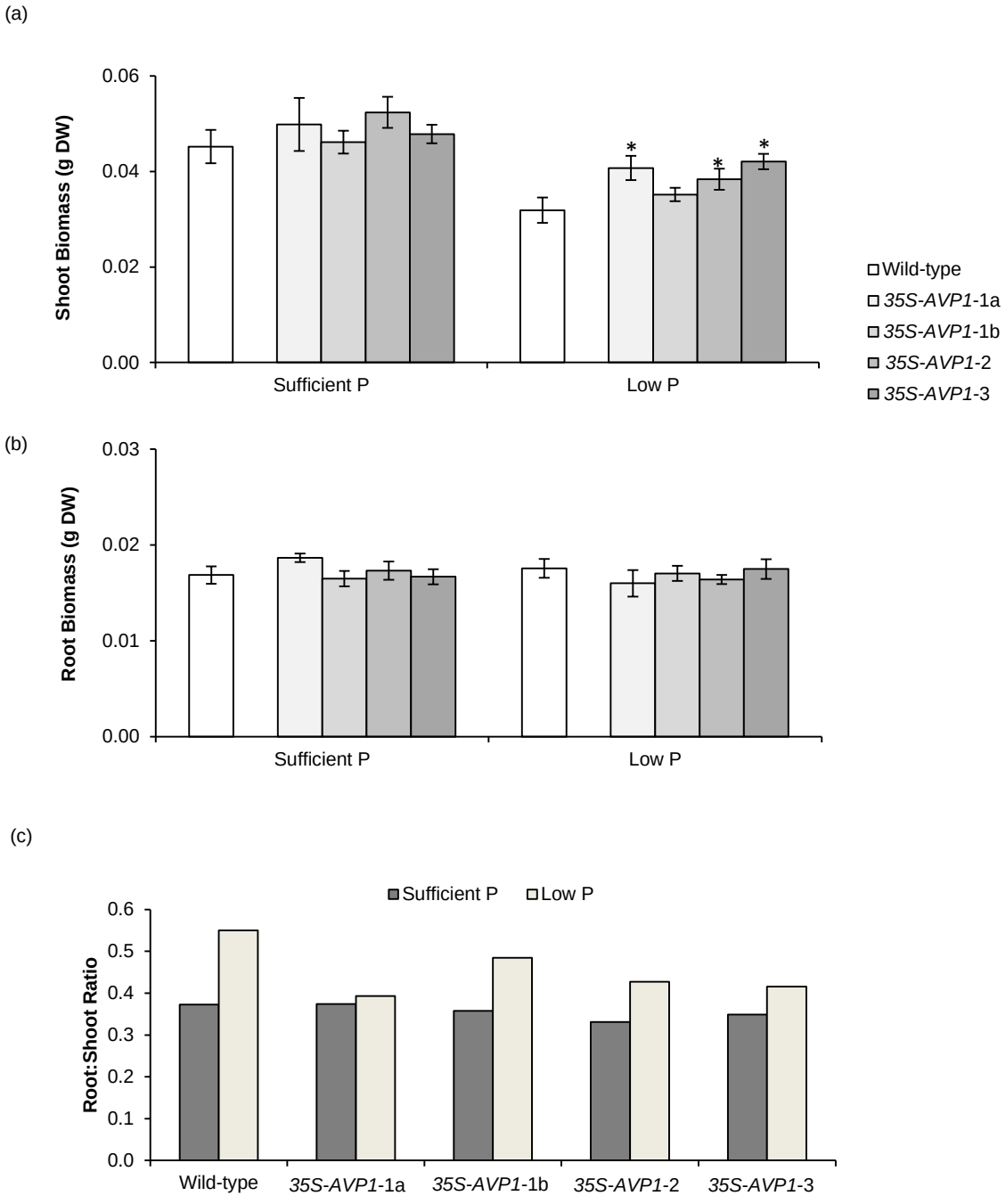
(a)
Shoot Biomass (g DW)
0.06
0.04
0.02
0.00
Sufficient P
Low P
Wild-type
35S-AVP1-1a
35S-AVP1-1b
35S-AVP1-2
35S-AVP1-3
(b)
Root Biomass (g DW)
0.03
0.02
0.01
0.00
Sufficient P
Low P
(c)
Sufficient P
Low P
Root:Shoot Ratio
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Wild-type
35S-AVP1-1a
35S-AVP1-1b
35S-AVP1-2
35S-AVP1-3

Figure 2

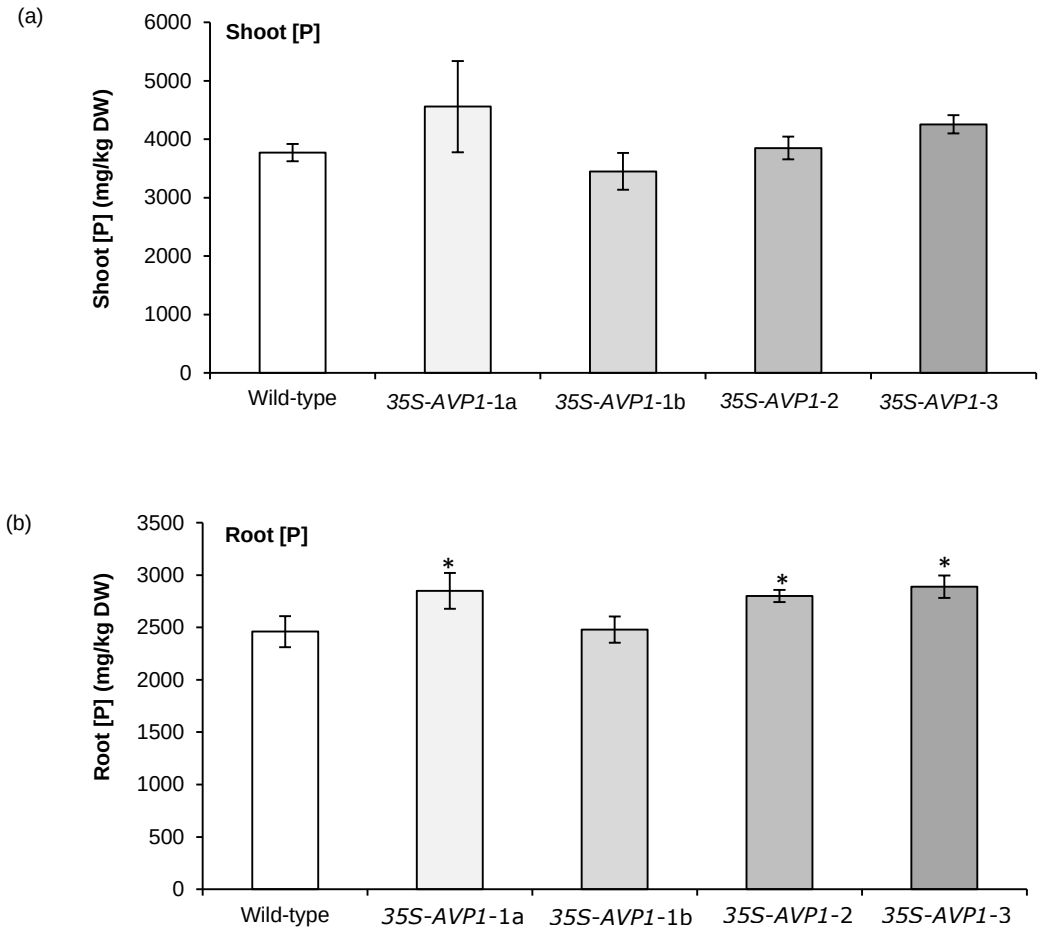
(a)
Shoot [P]
Shoot [P] (mg/kg DW)
6000
5000
4000
3000
2000
1000
0
Wild-type
35S-AVP1-1a
35S-AVP1-1b
35S-AVP1-2
35S-AVP1-3
(b)
Root [P]
Root [P] (mg/kg DW)
3500
3000
2500
2000
1500
1000
500
0
*
*
*
Wild-type
35S-AVP1-1a
35S-AVP1-1b
35S-AVP1-2
35S-AVP1-3

Figure 3

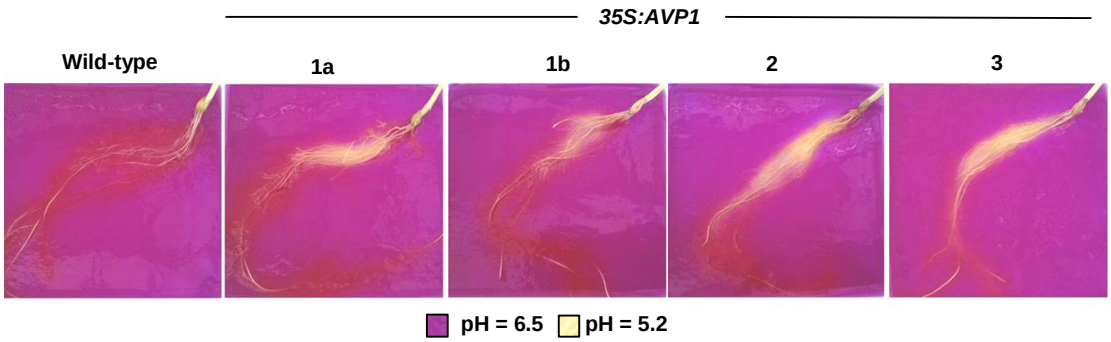

Figure 4

Additional files

Table S1 - No difference in root morphological traits was observed between transgenic *AVP1* barley and wild-type. The number of root tips, total root length (cm) and root diameter (mm) of wild-type (cv. Golden Promise) and transgenic *AVP1* barley (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 & *35S-AVP1*-3) derived from digital root images using WinRHIZO Pro® 2009 software following growth in hydroponic conditions with a low P concentration (10 µm P) for 14 days. Values are presented as the mean ± s.e.m (*n* = 4-9).

Line	Number of Root Tips	Total Root Length (cm)	Root Diameter (mm)
Wild-type	226 ± 30	182 ± 4	0.874 ± 0.03
35S-AVP1-1a	193 ± 14	159 ± 19	0.938 ± 0.06
35S-AVP1-1b	208 ± 20	155 ± 9	0.967 ± 0.04
35S-AVP1-2	214 ± 22	187 ± 16	0.889 ± 0.04
35S-AVP1-3	200 ± 20	188 ± 22	0.892 ± 0.06

Table S2 - The amount of plant available P (resin P) in soil increases with $CaHPO_2.2H_2O$ addition.

Varied levels of $CaHPO_2.2H_2O$ (0, 50, 100, 200, 300, 400, 500 and 700 mg/kg soil) were added to a soil with low P availability from Monarto, South Australia to determine the extent to which plant available P levels are altered. The 25 and 75 mg of $CaHPO_2.2H_2O$/kg soil rates were selected as suitable low and sufficient available P levels for barley growth respectively. Values are the mean ± s.e.m (*n* = 3).

$CaHPO_2.2H_2O$ (mg/kg soil)	Resin P (mg P/kg soil)
0	4.95 ± 0.15
50	11.86 ± 0.47
100	28.14 ± 1.57
200	57.25 ± 6.10
300	84.85 ± 2.20
400	119.88 ± 4.21
500	137.46 ± 7.29
700	210.55 ± 8.67

Table S3 - Shoot biomass of wild-type barley in a soil with low P availability amended with calcium phosphate. The shoot biomass of wild-type barley (g DW) in a soil with low P availability from Monarto, South Australia amended with varied levels of $CaHPO_2.2H_2O$ (0, 25, 50, 75 and 150 mg/kg soil) after 21 days. The 25 and 75 mg of $CaHPO_2.2H_2O$/kg soil rates were identified as suitable low and sufficient P levels for barley growth respectively. Values are the mean ± s.e.m (n = 7).

$CaHPO_2.2H_2O$ (mg/kg soil)	Shoot Biomass (g DW)
0	0.1086 ± 0.008
25	0.1355 ± 0.010
50	0.1666 ± 0.019
75	0.1862 ± 0.010
150	0.2171 ± 0.026

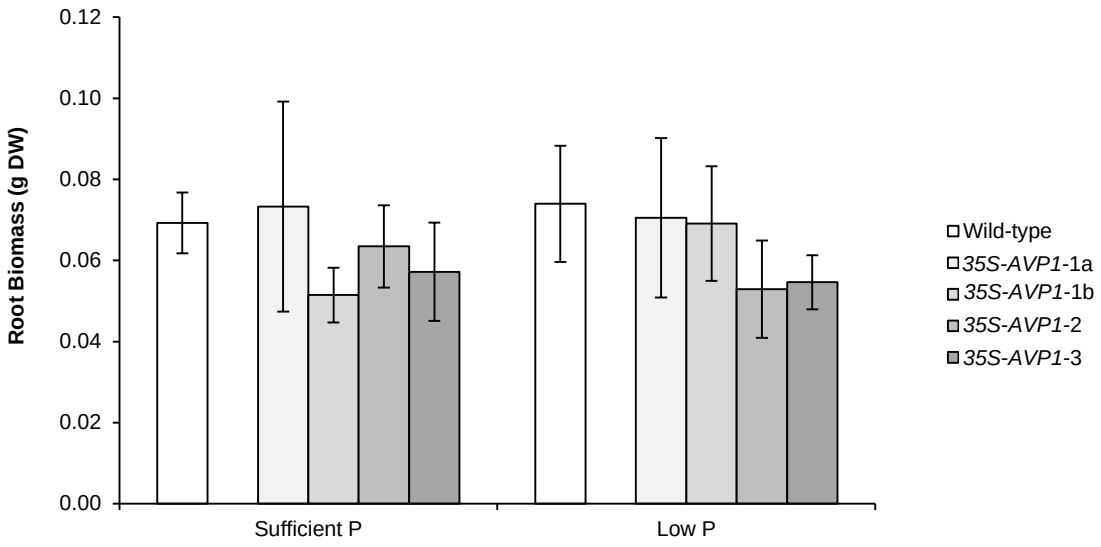

Figure S1 - Root biomass of transgenic *AVP1* barley is similar to wild-type. The root biomass (g DW) of wild-type and transgenic *AVP1* barley (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2, *35S-AVP1*-3) after 21 days of growth in a soil with low P availability amended with sufficient (75 mg of $CaHPO_2.2H_2O$/kg soil) or low P (25 mg of $CaHPO_2.2H_2O$/kg soil). Values are the mean ± s.e.m (*n* = 3-7).

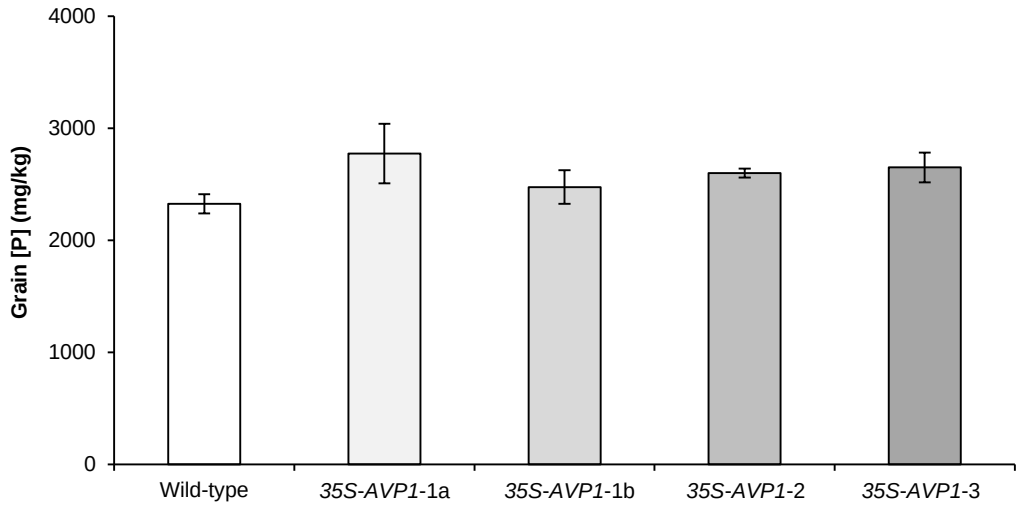

Figure S2 - No difference in grain P concentrations between wild-type and transgenic *AVP1* barley.

Grain P concentrations (mg/kg) of wild-type and transgenic *AVP1* barley (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2, *35S-AVP1*-3) determined using ICP-OES analysis of seed with similar grain weights derived from the seed source used in the soil and hydroponics experiment. Values are the mean ± s.e.m (n = 4).

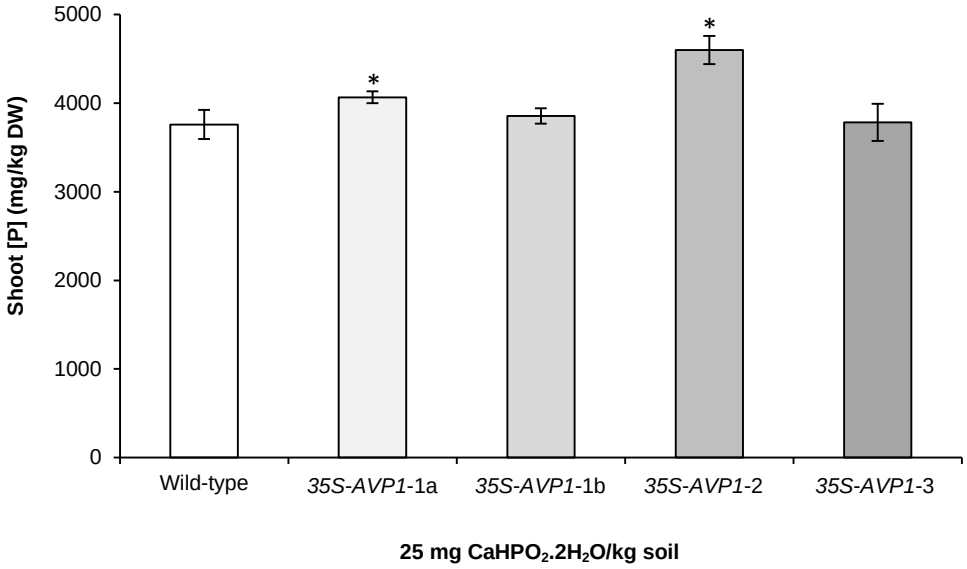

Figure S3 - Transgenic *AVP1* barley has increased shoot P concentrations compared to wild-type.

The concentration of P in shoot tissue (mg/kg DW) of wild-type and transgenic *AVP1* barley (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2, *35S-AVP1*-3) after 21 days in a soil with low P availability amended with 25 mg $CaHPO_2.2H_2O$/kg soil. Values are the mean ± s.e.m (n = 3-7) with an asterisks (*) indicating a significant difference to wild-type (one-way ANOVA, LSD, $P \leq 0.05$).

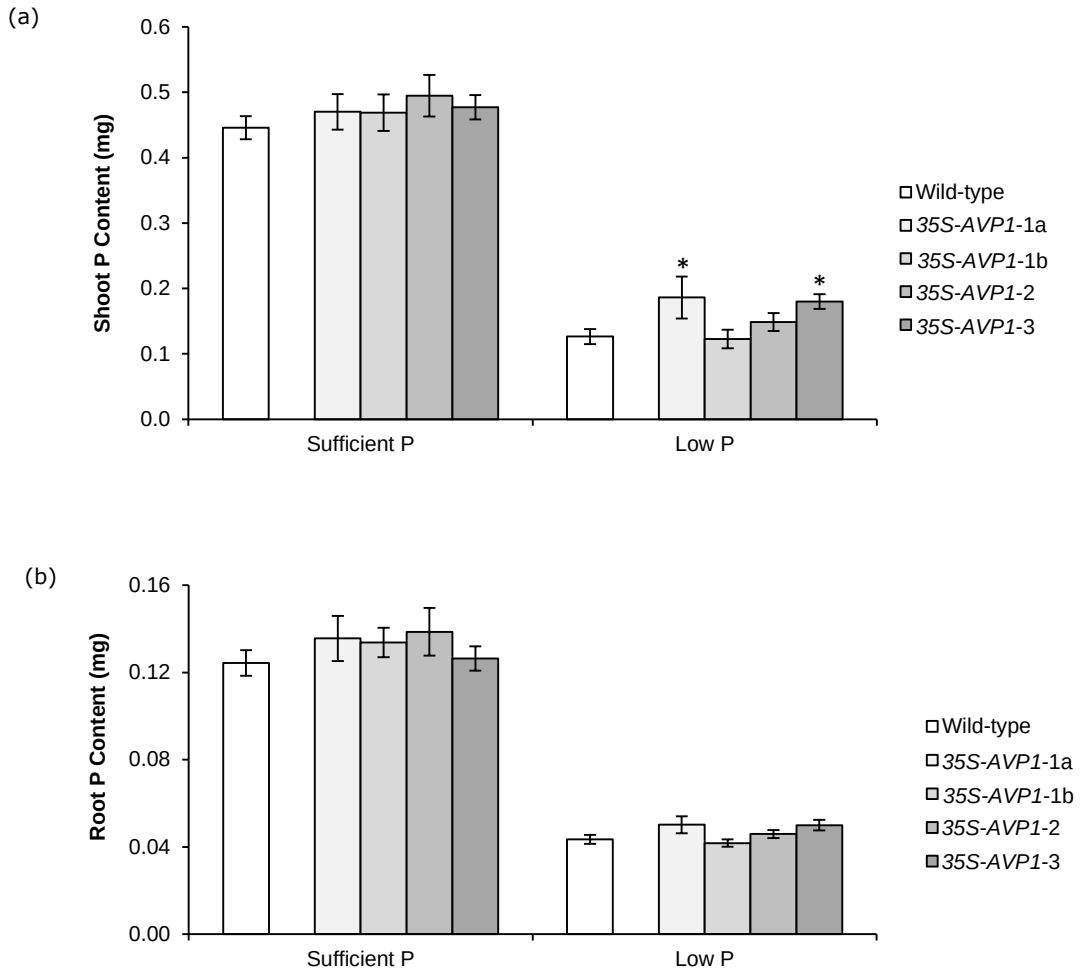

Figure S4 - Transgenic *AVP1* barley has increased shoot P content compared to wild-type at low P supply. (a) Shoot and (b) root P content (mg) of wild-type and transgenic *AVP1* barley (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2, *35S-AVP1*-3) in hydroponic conditions with sufficient and low P (10 µM KH_2PO_4) supply for 14 days. Values are the mean ± s.e.m (n = 3-9) with an asterisks (*) indicating a significant difference to wild-type (one-way ANOVA, LSD, $P \leq 0.05$).

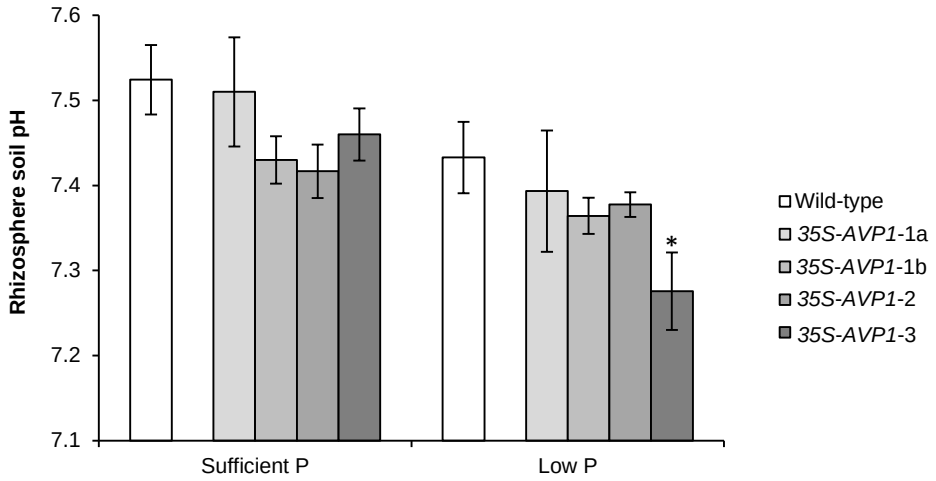

Figure S5 - Low P concentrations decrease the pH of rhizosphere soil in wild-type and transgenic *AVP1* barley. The pH of rhizosphere soil collected from roots of wild-type and transgenic *AVP1* barley (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2, *35S-AVP1*-3) following 21 days of plant growth in a soil with low P availability amended with either sufficient P (75 mg $CaHPO_2.2H_2O$/kg soil) or low P (25 mg $CaHPO_2.2H_2O$/kg soil). Values are the mean ± s.e.m (*n* = 3-7).

Chapter 4

Evaluating the low nitrate tolerance of transgenic *AVP1* barley

Statement of Contributions

The following chapter is formatted as a manuscript. However, it is not intended that this manuscript will be submitted for publication.

Title: Transgenic barley expressing an *Arabidopsis* vacuolar H^+-pyrophosphatase gene (*AVP1*) has improved shoot biomass at low nitrate supply

Schilling conducted the experiments, data analysis, interpretation of results and wrote the manuscript

Marschner, Tester, Plett and Roy supervised the experiments

All authors contributed to the discussion of the results

By signing this statement of contributions, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis:

Petra Marschner

Signature: Date: 25/06/2014

Mark Tester

Signature: Date: 25/06/2014

Stuart Roy

Signature: Date: 25/06/2014

Darren Plett

Signature: Date: 25/06/2014

Transgenic barley expressing an *Arabidopsis* vacuolar H^+-pyrophosphatase gene (*AVP1*) has improved shoot biomass at low nitrate supply

Running title: AVP1 improves transgenic barley growth at low nitrate supply

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Keywords: barley, nitrate, *AVP1*, biomass, ^{15}N, influx, LATS, HATS, *NRT2.1*

Word count:

Abstract

Nitrogen (N) fertilisers are applied to cereal crops each year to increase productivity. However, the use of N fertilisers can be inefficient with only a small proportion of applied N taken up by cereal crops. To increase the profitability of N fertiliser use, crop varieties with increased N fertiliser use efficiency, such as greater root uptake of nitrate (NO_3^-), are needed. Here, we evaluate the growth of transgenic barley with constitutive expression of an *Arabidopsis* vacuolar H^+-pyrophosphatase (*AVP1*) in 0.5 mM (low) and 5 mM NO_3^- (sufficient) treatments. Furthermore, we measure the high- and low-affinity NO_3^- uptake capacity of the transgenic *AVP1* barley. At low NO_3^- supply, two transgenic *AVP1* barley lines (*35S-AVP1*-2 and *35S-AVP1*-3) had a larger shoot biomass, but no significant difference in root NO_3^- uptake capacity compared to null segregants. Whilst at sufficient NO_3^- supply, one transgenic *AVP1* barley line (*35S-AVP1*-2) had a significantly larger shoot and root biomass, higher shoot and root N contents and greater root NO_3^- uptake capacity in the low-affinity range compared to null segregants. These findings suggest that transgenic barley expressing *AVP1* has the potential to be a useful option for increasing the N fertiliser use efficiency of cereal crops.

Background

Nitrogen (N) is an essential macronutrient for plant growth and development (Lam et al., 1996; Marschner and Marschner, 2012). Globally, a vast amount of N fertiliser (> 100 million tons) is applied to crops each year to increase productivity (FAO, 2013). However, the use of N fertilisers is inefficient with on average only 30 to 50 % of applied N taken up by cereal crops (Raun and Johnson, 1999; Sylvester-Bradley and Kindred, 2009). This inefficiency is undesirable given that fertiliser use is a high input cost and can cause environmental issues, such as the production of greenhouse gases and the eutrophication of waterways (Good and Beatty, 2011). Cereal crop varieties with increased biomass or grain yield produced per unit of applied N, and thus improved nitrogen use efficiency (NUE), are needed to improve the efficiency of N fertiliser use (McAllister et al., 2012; Schroeder et al., 2013).

The main form of plant available N in agricultural soils is nitrate (NO_3^-) and thus the development of crop varieties with greater uptake of NO_3^- is one option for improving the NUE of crops (Schroeder et al., 2013; Wang et al., 2012). In plants, there are two NO_3^- uptake systems: (1) the low-affinity transport system (LATS), which operates at high NO_3^- concentrations (>1 mM) and (2) the high-affinity transport system (HATS) (Crawford and Glass, 1998; Glass, 2003; Kronzucker et al., 1995). A proton gradient generated by the plasma membrane H^+-ATPase is thought to facilitate NO_3^-/H^+ uptake into roots by both LATS and HATS (de Angeli et al., 2007; Glass et al., 1992; McClure et al., 1990; Ullrich and Novacky, 1981). Changes in root morphology, such as greater root vigour and greater lateral root density, can also increase plant NO_3^- uptake (Garnett et al., 2009; Liao et al., 2004). The development of cereal crop varieties with enhanced plasma membrane NO_3^-/H^+ symporter activity or a more vigorous root system could therefore help to increase N fertiliser use efficiency of crops.

The type I *Arabidopsis* vacuolar H^+-pyrophosphatase (H^+-PPase) (AVP1) is a membrane-bound H^+-pump that establishes an electrochemical potential difference for H^+ across the tonoplast by hydrolysing

cytosolic pyrophosphate (P_i) to actively pump H^+ into the vacuole (Duan et al., 2007; Kim et al., 1994; Zhen et al., 1997). In many different growth conditions, transgenic plants with the constitutive expression of *AVP1* or the gain-of-function *AVP1D* allele have a larger shoot and root biomass compared to plants without this gene (Bao et al., 2009; Gaxiola et al., 2001; Li et al., 2005; Li et al., 2010; Paez-Valencia et al., 2013; Park et al., 2005; Pasapula et al., 2011; Qin et al., 2013; Schilling et al., 2014; Vercruyssen et al., 2011; Yang et al., 2007; Yang et al., 2014). At low NO_3^- supply, the constitutive expression of *AVP1D* increased the shoot and root biomass of transgenic romaine lettuce (*Lactuca sativa*) compared to wild-type (Paez-Valencia et al., 2013). The transgenic *AVP1D* lettuce also produced more marketable yields per unit of N compared to wild-type in the field (Paez-Valencia et al., 2013). The improved growth of transgenic *AVP1D* lettuce at low NO_3^- supply was attributed to greater NO_3^- uptake as a result of enhanced rhizosphere acidification and larger root growth, potentially due to enhanced sucrose transport from source to sink tissues (Gaxiola et al., 2012; Paez-Valencia et al., 2013). In support of this, the transgenic *AVP1D* lettuce had a higher N content in the aboveground biomass compared to wild-type at low NO_3^- supply (Paez-Valencia et al., 2013). Furthermore, the expression of a gene encoding a HATS root NO_3^- transporter (*LsNRT2.1*) was up-regulated in the transgenic *AVP1D* lettuce roots compared to wild-type (Paez-Valencia et al., 2013). However, to date, no studies have compared the HATS or LATS NO_3^- uptake capacity of transgenic plants expressing *AVP1* or *AVP1D* compared to plants without this gene. It is thus not clear whether the expression of *AVP1* affects NO_3^- uptake in both the HATS and LATS ranges.

Transgenic barley (*Hordeum vulgare*) lines with the constitutive *CaMV 35S* expression of *AVP1* were previously generated (Schilling et al., 2014). In both non-saline and saline conditions the transgenic *AVP1* barley had a larger shoot biomass compared to null segregants (Schilling et al., 2014). Furthermore, at low phosphorus (P) supply, transgenic *AVP1* barley had a larger shoot biomass and enhanced rhizosphere acidification compared to wild-type (Chapter 3). Given this increase in growth and the suggested role of AVP1 in improving nutrient uptake (Gaxiola et al., 2011; Paez-Valencia et al., 2013;

Yang et al., 2007; Yang et al., 2014), transgenic *AVP1* barley may be a useful option to help develop a cereal crop variety with improved N fertiliser use efficiency. However, to date, no studies have evaluated the potential for *AVP1* expression to improve the N fertiliser use efficiency of a monocotyledonous plant, such as barley.

Here, we evaluate the growth and high- and low-affinity NO_3^- uptake capacity of transgenic *AVP1* barley at 0.5 mM NO_3^- (low) and 5 mM NO_3^- (sufficient). We show that two transgenic *AVP1* barley lines (*35S-AVP1*-2 and *35S-AVP1*-3) have a significantly larger shoot biomass at low NO_3^- supply with no difference in root NO_3^- uptake capacity compared to null segregants. Furthermore, we show that at sufficient NO_3^- supply one transgenic *AVP1* barley line (*35S-AVP1*-2) had a significantly larger shoot and root biomass, higher shoot and root N contents and increased LATS NO_3^- uptake capacity compared to null segregants. These findings suggest that transgenic barley expressing *AVP1* has the potential to be a useful option for increasing the N fertiliser use efficiency of cereal crops.

Experimental Design

Plant material and growth conditions

Seeds of wild-type barley (cv. Golden Promise) and three independent T_4 transgenic barley lines with constitutive *CaMV 35S* expression of *AVP1* (*35S-AVP1*-1, *35S-AVP1*-2 and *35S-AVP1*-3) (Schilling et al., 2014) and two sibling lines from one transformation event (*35S-AVP1*-1a and *35S-AVP1*-1b) were surface sterilised by a 5 min exposure to ultra-violet (UV) light and germinated for 4 d on moist filter paper in 145 mm Petri dishes. Seedlings were transplanted to a fully-supported hydroponics set-up (Genc et al., 2007). Briefly, each hydroponic trolley had 42 PVC tubes (4 cm diameter × 28 cm depth) with a mesh collar (3 cm × 0.5 cm) in each tube to support an individual seedling. The PVC tubes were positioned in two individual trays connected to an 80 L tank containing nutrient solution, which cycled every 30 min between nutrient solution and free drainage within the trays (Figure S1a).

The nutrient solution consisted of reverse osmosis (RO) water with the following nutrients (in mM): 2.0 $MgSO_4.7H_2O$, 0.1 KH_2PO_4, 0.5 $Na_2Si_3O_7$, 0.05 NaFe(III)EDTA, 0.05 H_3BO_3, 0.005 $MnCl_2.4H_2O$, 0.01 $ZnSO_4.7H_2O$, 0.0005 $CuSO_4.5H_2O$ and 0.0001 $Na_2MoO_4.2H_2O$ with 2 KNO_3 and 1.5 $Ca(NO_3)_2.4H_2O$ in the sufficient NO_3^- treatment (5 mM NO_3^-) and 0.25 KNO_3 and 0.125 $Ca(NO_3)_2.4H_2O$ in the low NO_3^- treatment (0.5 mM NO_3^-). To maintain similar K^+ and Ca^{2+} levels to the sufficient NO_3^- treatment, the low NO_3^- treatment also comprised (in mM): 0.875 K_2SO_4 and 1.375 $CaCl_2.2H_2O$. The treatments were established from the start of the experiment and nutrient solution was replaced every 10 d to ensure nutrients were not depleted. Following 21 d of treatment, unidirectional NO_3^- root influx capacity measurements were conducted (as described below), SPAD meter readings were taken at the centre of the youngest fully-emerged leaf using a hand-held SPAD 502 meter (Minolta, Osaka, Japan) and the 2nd leaf blade was collected for DNA and RNA extractions to genotype the presence or absence of *AVP1* (data not shown) and determine *AVP1* expression levels (Figure S2a,b). The remaining shoot and root tissue was oven dried at 70 ºC for 3 d and the shoot and root dry weights were recorded.

Unidirectional ^{15}N-labelled root NO_3^- influx capacity measurements

After 21 d, unidirectional root influx capacity measurements were completed (Garnett et al., 2009). Briefly, between 11:00 and 15:00 h plants were transferred from the hydroponics tanks to a 3 L container containing the same nutrient solution (0.5 mM or 5 mM NO_3^-) with continuous aeration provided by a Precision SR 7500 aerator (Aqua One, Sydney, Australia) (Figure S1b). Roots were then given a 5 min rinse in the same nutrient solution, but with either 0.1 or 1 mM NO_3^- (supplied as KNO_3^-) then a 10 min exposure to the same solution, but containing ^{15}N-labelled KNO_3^- (^{15}N 10%) (Cambridge Isotope Laboratories Inc. Andover, MA, USA). The flux timing was selected to minimise possible efflux of ^{15}N-labelled NO_3^- from the roots and to minimise transport of ^{15}N-labelled NO_3^- to the shoots (Kronzucker et al., 1995). Roots were then rinsed for 2 min in the same nutrient solution without ^{15}N-labelled KNO_3^- to remove any adhering ^{15}N-labelled solution on the root surface. Roots were blotted using paper towel and

the roots and shoot separated, weighed and oven dried at 65 °C for 3 d. The root and shoot tissue of null segregants and the two transgenic *AVP1* barley lines (*35S-AVP1*-2 & *35S-AVP1*-3) showing the largest improvement in shoot biomass in the low NO_3^- treatment were analysed for ^{15}N atm% and total N using a continuous flow system consisting of a SERCON 20-22 mass spectrometer connected to an automated nitrogen carbon analyser (Sercon, Crewe, Cheshire, United Kingdom) at the West Australian Biogeochemistry Centre (WABC) (Paul et al., 2007). The root NO_3^- influx value (μmoles g^{-1} DW h^{-1}) was calculated using this measured root ^{15}N content.

Semi-quantitative RT-PCR analysis of *AVP1* expression

Leaf tissue was ground to a fine powder and total RNA was extracted following an established protocol using Trizol (Chomczynski, 1993). Extracted RNA was treated with DNase-free (Ambion, Madison, WI, USA) to remove DNA contamination. DNase-treated RNA (1 μL) was used to synthesize cDNA with a Superscript III RT kit (Invitrogen). Semi-quantitative *AVP1* expression in a subset of plants (*n* =3-4) was determined using reverse transcription PCR (RT-PCR) amplification of 1 μL of cDNA template with *AVP1*-specific forward primer: 5' – GCA GCT CTT AAG ATG GTT GAA – 3' and reverse primer 5' – AGA GGT GTG AGC ATG ACA AGG – 3'. The PCR conditions used to amplify a fragment of the *AVP1* transcript (expected band size of 164 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 reactions contained 1× Platinum® *Taq* PCR buffer, 2 mM $MgCl_2$, 200 μM of each dNTPs, 0.5 U of Platinum® Taq DNA polymerase (Invitrogen) and 10 μM of each primer. Gel electrophoresis with a 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 visualise PCR products and record gel images. To account for differences in initial concentrations of cDNA, the amount of gene expression was determined by normalising the level of PCR product as gel band intensity (minus background intensity) obtained using *HvGAP* relative to that of *AVP1* using GIMP 2.6.11 GNU Image Manipulation Program (www.gimp.org).

Statistical analysis

Data was 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 within treatments compared to null segregants at a probability level of $P \leq 0.05$, except the root NO_3^- influx data which was statistically analysed using a mixed model in GenStat (16th edition, VSN International Ltd., UK) and the LSD was used to identify significantly different means at a probability level of $P \leq 0.05$. No significant difference was observed between wild-type and null segregants in this study, and as such, the transgenic *AVP1* barley lines were compared to null segregants only.

Results

Transgenic *AVP1* barley has increased shoot biomass at low NO_3^- supply

In the sufficient NO_3^- treatment (5 mM NO_3^-), there was no difference in the shoot and root biomass of transgenic *AVP1* barley lines (*35S-AVP1*-1a, *35S-AVP1*-1b and *35S-AVP1*-3) compared to null segregants (Figure 1a,c,d). However, one transgenic *AVP1* barley line (*35S-AVP1*-2) had a significantly greater shoot (14 %) and root (18 %) biomass than null segregants in the sufficient NO_3^- treatment (Figure 1c,d). In the low NO_3^- treatment (0.5 mM NO_3^-), two independent transgenic *AVP1* barley lines (*35S-AVP1*-2 & *35S-AVP1*-3) had significantly greater shoot biomass (9 to 18 %) than null segregants (Figure 1b,c). The shoot biomass of two sibling lines (*35S-AVP1*-1a and *35S-AVP1*-1b) from a third transgenic event, however, was not different to null segregants in the 0.5 mM NO_3^- treatment (Figure 1c). There was

also no significant difference in root biomass between the transgenic *AVP1* barley and null segregants in the 0.5 mM NO_3^- treatment (Figure 1d). The dry weight root to shoot ratio of all plants increased in the 0.5 mM NO_3^- treatment compared to the 5 mM NO_3^- treatment (Figure 2). The SPAD value (indicator of leaf greenness) of the youngest fully-emerged leaf blade did not differ between null segregants and transgenic *AVP1* barley in the 5 mM and 0.5 mM NO_3^- treatments, except for *35S-AVP1*-1a which had a significantly greater SPAD value in the 5 mM NO_3^- treatment than null segregants (Figure 3).

35S-AVP1-2 barley has increased shoot and root N content at sufficient NO_3^- supply

In both 5 mM and 0.5 mM NO_3^- treatments, the total shoot and root N concentration (g kg^{-1} DW) did not differ between null segregants and transgenic *AVP1* barley (*35S-AVP1*-2 and *35S-AVP1*-3) (Table 1). While both transgenic *AVP1* barley lines (*35S-AVP1*-2 and *35S-AVP1*-3) had higher total shoot and root N content (mg $plant^{-1}$) compared to null segregants in both 5 mM and 0.5 mM NO_3^- treatments (Table 2), only the total shoot and root N content of *35S-AVP1*-2 barley in the 5 mM NO_3^- treatment was significantly greater than null segregants (Table 2).

35S-AVP1-2 barley has enhanced root LATS NO_3^- influx capacity at sufficient NO_3^- supply

In both 5 mM and 0.5 mM NO_3^- treatments, the HATS (0.1 mM NO_3^-) NO_3^- influx capacity (µmoles g^{-1} DW h^{-1}) of all plants was less than that in the LATS range (1 mM NO_3^-) (Figure 4). The LATS and HATS root NO_3^- influx capacity was higher in plants from the 0.5 mM NO_3^- treatment than those in the 5 mM NO_3^- treatment (Figure 4). There was no significant difference in the HATS NO_3^- influx capacity of null segregants and transgenic *AVP1* barley (*35S-AVP1*-2 and *35S-AVP1*-3) for both 5 mM and 0.5 mM NO_3^- treatments (Figure 4). In the 5 mM NO_3^- treatment, the *35S-AVP1*-2 barley had significantly higher LATS NO_3^- influx capacity than null segregants (Figure 4). However, there was no significant difference in the LATS NO_3^-influx capacity of *35S-AVP1*-3 and null segregants in the 5 mM NO_3^- treatment (Figure 4). In

the 0.5 mM NO_3^- treatment, the LATS NO_3^- influx capacity of *35S-AVP1*-2 and *35S-AVP1*-3 barley was greater than null segregants, however, this difference was not statistically significant (Figure 4).

Discussion

In this study, two transgenic *AVP1* barley lines (*35S-AVP1*-2 and *35S-AVP1*-3) had a larger shoot biomass in the low NO_3^- treatment (Figure 1b,c) with a corresponding (but not significant) increase in root NO_3^- uptake capacity compared to null segregants (Figure 4, Table 2). This finding demonstrates that the expression of *AVP1* can increase the shoot growth of barley at low NO_3^- supply. One transgenic *AVP1* barley line (*35S-AVP1*-2) also had significantly larger shoot and root biomass (Figure 1 a,c,d), significantly greater LATS NO_3^- uptake capacity (Figure 4) and a significantly higher shoot and root N content (Table 2) compared to null segregants in the sufficient NO_3^- treatment. Overall, this suggests that transgenic barley expressing *AVP1* has the potential to be a useful option for increasing the N fertiliser use efficiency of cereal crops.

35S-AVP1-2 and *35S-AVP1*-3 barley has increased shoot biomass at low NO_3^- supply

Previously, in many different growth conditions, the constitutive expression of *AVP1* or *AVP1D* increased the shoot and root biomass of transgenic plants compared to those without this gene (Bao et al., 2009; Gaxiola et al., 2001; Li et al., 2005; Li et al., 2010; Paez-Valencia et al., 2013; Park et al., 2005; Pasapula et al., 2011; Qin et al., 2013; Schilling et al., 2014; Vercruyssen et al., 2011; Yang et al., 2007; Yang et al., 2014). At low NO_3^- supply, transgenic lettuce expressing *AVP1D* had 2-fold more shoot biomass compared to wild-type (Paez-Valencia et al., 2013). In this study, two transgenic *AVP1* barley lines (*35S-AVP1*-2 and *35S-AVP1*-3) also had a larger shoot biomass compared to null segregants in the low NO_3^- treatment (Figure 1b,c). However, one transgenic line (*35S-AVP1*-1) did not have a significantly larger shoot biomass in the low NO_3^- treatment compared to null segregants (Figure 1c). Nevertheless, these findings are in agreement with the previous study (Paez-Valencia et al., 2013) and show for the first time

that the expression of *AVP1* can increase the shoot growth of barley, a monocotyledonous plant, in low NO_3^- conditions (Figure 1b,c).

The larger shoot biomass of transgenic *AVP1D* lettuce at low NO_3^- supply compared to wild-type was attributed to an increase in root biomass and greater rhizosphere acidification facilitating improved NO_3^- uptake (Paez-Valencia et al., 2013). Furthermore, compared to wild-type at low NO_3^- supply, the transgenic *AVP1D* lettuce had a higher total shoot N and up-regulated expression of *LtNRT2.1* in root tissue (Paez-Valencia et al., 2013). However, in this study, the root biomass (Figure 1d), shoot and root N concentration and content (Table 1 & 2) of the transgenic *AVP1* barley was not significantly different to null segregants in the low NO_3^- treatment. Furthermore, unidirectional root influx measurements using ^{15}N-labelled NO_3^- showed no significant difference in HATS and LATS NO_3^- influx capacities between transgenic *AVP1* barley and null segregants in the low NO_3^- treatment (Figure 4). This suggests that, in this case, an increase in NO_3^- uptake was not contributing to the larger shoot biomass of transgenic *AVP1* barley (*35S-AVP1*-2 and *35S-AVP1*-3) compared to null segregants at low NO_3^- supply.

35S-AVP1-2 barley has increased shoot and root biomass at sufficient NO_3^- supply

Previously, transgenic lettuce expressing *AVP1D* had a larger shoot and root biomass and increased root acidification at sufficient NO_3^- supply (Paez-Valencia et al., 2013). It has been hypothesised that AVP1 may act as a PP_i-synthase helping to facilitate phloem loading of sucrose leading to enhanced sucrose transport from source (leaf) to sink (root) tissues increasing root growth and proton exudation, which as a consequence, improves plant water and nutrient uptake (Gaxiola et al., 2012; Paez-Valencia et al., 2011). In support of this concept, the shoot and root biomass of one transgenic *AVP1* barley line (*35S-AVP1*-2) was significantly larger than null segregants in the sufficient NO_3^- treatment (Figure 1c,d). It is not known why the shoot and root growth of the two other transgenic lines (*35S-AVP1*-1 and *35S-AVP1*-3) was inconsistent with the *35S-AVP1*-2 line (Figure 1c,d, Table 2). However, differences between transgenic

lines, such as *AVP1* copy number or the chromosomal location of the inserted *AVP1* gene (Matzke and Matzke, 1998), may be contributing factors. Nonetheless, the findings suggest that *35S-AVP1*-2 barley has increased shoot and root biomass at sufficient NO_3^- supply compared to null segregants.

***35S-AVP1*-2 barley has increased root influx of NO_3^- in the LATS range at sufficient NO_3^- supply**

To date, no studies have compared the NO_3^- uptake capacity of transgenic *AVP1* or *AVP1D* plants within the HATS or LATS ranges to plants without this gene. In this study, significantly higher root NO_3^- influx was observed in plants in the LATS range (1 mM NO_3^-) compared to HATS (0.1 mM NO_3^-) and in the low NO_3^- treatment (0.5 mM NO_3^-) compared to the sufficient NO_3^- treatment (5 mM NO_3^-) (Figure 4), which are typical plant responses (Garnett et al., 2013). Furthermore, this study showed for the first time that the transgenic *AVP1* barley (*35S-AVP1*-2) had increased root NO_3^- influx capacity compared to null segregants in the sufficient NO_3^- treatment (Figure 4). The concentration of N in the shoot and roots of this transgenic *AVP1* barley line did not differ to null segregants (Table 1). However, the total shoot and root N contents were significant higher in *35S-AVP1*-2 plants than null segregants (Table 2) suggesting this transgenic *AVP1* barley line utilised the greater root NO_3^- uptake to increase shoot and root biomass. Given that the uptake of NO_3^- by roots is thought to occur via plasma membrane NO_3^-/H^+ symporters (de Angeli et al., 2007; Glass et al., 1992; McClure et al., 1990; Ullrich and Novacky, 1981) and that transgenic plants expressing *AVP1* had increased rhizosphere acidification compared to plants without this gene (Chapter 3; Paez-Valencia et al., 2013; Yang et al., 2007) is possible that enhanced root acidification facilitated the higher root NO_3^- influx of the transgenic *AVP1* barley (*35S-AVP1*-2) compared to null segregants.

Previously, 7-fold higher expression of *LsNRT2.1* was observed in transgenic *AVP1D* lettuce roots compared to wild-type (Paez-Valencia et al., 2013). However, the HATS NO_3^- uptake capacity of this transgenic *AVP1D* lettuce was not tested (Paez-Valencia et al., 2013). In the present study, the HATS

NO_3^- uptake capacity of *35S-AVP1*-2 did not differ from null segregants (Figure 4). This suggests that, in this case, the expression of *AVP1* in transgenic barley does not influence root NO_3^- uptake in the HATS range. It is possible that the up-regulated expression of *LsNRT2.1* in the transgenic *AVP1D* lettuce was a pleiotropic effect or that other factors, such as differences between plant species, plant age, the level and extent of the low NO_3^- treatment or the alleles *AVP1* or *AVP1D* are contributing to this inconsistency. It is also possible that there may be transcriptional regulation and post-translational control over NRT2.1 activity, considering that the expression of NO_3^- responsive genes does not necessarily match the NO_3^- uptake capacity of plants (Garnett et al., 2013). In addition, given that the NO_3^- uptake capacity of plants can vary across the lifecycle (Garnett et al., 2013), it may be that the HATS NO_3^- uptake capacity of transgenic *AVP1* plants is up-regulated at specific growth stages. Measurements of HATS NO_3^- uptake capacity in the transgenic *AVP1D* lettuce and the expression of NRT genes in the transgenic *AVP1* barley across the lifecycle is needed to investigate this further. Nonetheless, the findings of *35S-AVP1*-2 barley support the concept that the expression of *AVP1* can improve the NO_3^- uptake of transgenic plants (Gaxiola et al., 2012; Paez-Valencia et al., 2013) and show that at sufficient NO_3^- supply the expression of *AVP1* increases LATS NO_3^- uptake capacity.

Conclusions

In this study, it was shown that expression of *AVP1* can lead to increased shoot biomass of barley, a monocotyledonous plant, at low NO_3^- supply compared to null segregants. It was also shown that one transgenic *AVP1* barley line (*35S-AVP1*-2) had significantly larger shoot and root biomass, higher shoot and root N contents and higher root LATS NO_3^- influx capacity than null segregants at sufficient NO_3^- supply. To our knowledge, this is the first time that increased root NO_3^- influx capacity has been shown in a transgenic plant expressing *AVP1* or *AVP1D*. Overall, the findings of this study support those suggesting that the expression of *AVP1* can improve the growth of transgenic plants at low NO_3^- supply (Gaxiola et

al., 2012; Paez-Valencia et al., 2013) and indicates that transgenic barley expressing *AVP1* has the potential to be a useful option for increasing the N fertiliser use efficiency of cereal crops.

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Tables

Table 1 The total shoot and root N concentration (g kg^{-1} DW) of null segregants and transgenic *AVP1* barley (*35S-AVP1*-2 & *35S-AVP1*-3) in 5 mM NO_3^- (sufficient) or 0.5 mM NO_3^-) (low) treatments for 21 d. Values are the mean ± s.e.m (*n* = 4-15).

Line	Total Shoot N Concentration (g kg^{-1} DW)		Total Root N Concentration (g kg^{-1} DW)	
	5 mM NO_3^-	**0.5 mM NO_3^-**	**5 mM NO_3^-**	**0.5 mM NO_3^-**
Null segregants	56.6 ± 1.8	51.2 ± 1.2	50.5 ± 1.8	51.7 ± 1.5
35S-AVP1-2	61.4 ± 2.4	53.1 ± 3.2	54.9 ± 9.9	48.8 ± 2.0
35S-AVP1-3	55.9 ± 1.3	50.9 ± 1.4	51.0 ± 1.6	50.4 ± 1.4

Table 2 The total shoot and root N content (mg plant^{-1}) of null segregants and transgenic *AVP1* barley (*35S-AVP1*-2 & *35S-AVP1*-3) in 5 mM NO_3^- (sufficient) or 0.5 mM NO_3^- (low) treatments for 21 d. Values are the mean ± s.e.m (*n* = 4-15) with an asterisks (*) indicating a significant difference to null segregants (one-way ANOVA, LSD, $P \leq 0.05$).

Line	Total Shoot N Content (mg plant^{-1})		Total Root N Content (mg plant^{-1})	
	5 mM NO_3^-	**0.5 mM NO_3^-**	**5 mM NO_3^-**	**0.5 mM NO_3^-**
Null segregants	4.26 ± 0.17	2.92 ± 0.15	1.35 ± 0.10	1.73 ± 0.12
35S-AVP1-2	5.65 ± 0.23*	3.80 ± 0.66	1.88 ± 0.12*	1.83 ± 0.24
35S-AVP1-3	4.53 ± 0.48	3.34 ± 0.30	1.60 ± 0.13	1.76 ± 0.09

Figure Legends

Figure 1 A representative image of 25-day-old wild-type and *35S-AVP1*-3 after 21 d in (a) 5 mM NO_3^- and (b) 0.5 mM NO_3^- treatments. The (c) shoot and (d) root biomass (g DW) of null segregants and transgenic *AVP1* barley (*35S-AV*P1-1a, *35S-AVP1*-1b, *35S-AVP1*-2 & *35S-AVP1*-3) at 5 mM and 0.5 mM NO_3^-. Values are the mean ± s.e.m, (*n*= 7-16) with an asterisks (*) indicating a significant difference to null segregants (one-way ANOVA, LSD, $P \leq 0.05$).

Figure 2 The average dry matter root to shoot ratio of null segregants and transgenic *AVP1* barley (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 & *35S-AVP1*-3) at 5 mM NO_3^- (sufficient) (dark-grey) or 0.5 mM NO_3^- (low) (light-grey) treatments at 21 d.

Figure 3 SPAD values of the youngest fully-emerged leaf blade of 25-day-old null segregants and transgenic *AVP1* barley (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 & *35S-AVP1*-3) after 21 d of 5 mM NO_3^- (sufficient) (dark-grey) or 0.5 mM NO_3^- (low) (light-grey) treatments. Values are the mean ± s.e.m (*n* = 7-16) with an asterisks (*) indicating a significant difference to null segregants (one-way ANOVA, LSD, $P \leq 0.05$).

Figure 4 Unidirectional ^{15}N-labelled NO_3^- influx (µmoles g DW^{-1} h^{-1}) into the roots of null segregants and transgenic *AVP1* barley (*35S-AVP1*-2 & *35S-AVP1*-3) at 5 mM NO_3^- (sufficient) or 0.5 mM NO_3^- (low) treatments for 21 d. Root NO_3^- influx was measured over a 10 min influx period with either 0.1 or 1 mM NO_3^-. Values are the mean ± s.e.m (*n* = 4-8), except line *35S-AVP1*-2 at 5 mM NO_3^- in 0.1 mM flux treatment where *n* = 2. A different letter indicates a significant difference between means (LSD, $P \leq 0.05$).

Figures

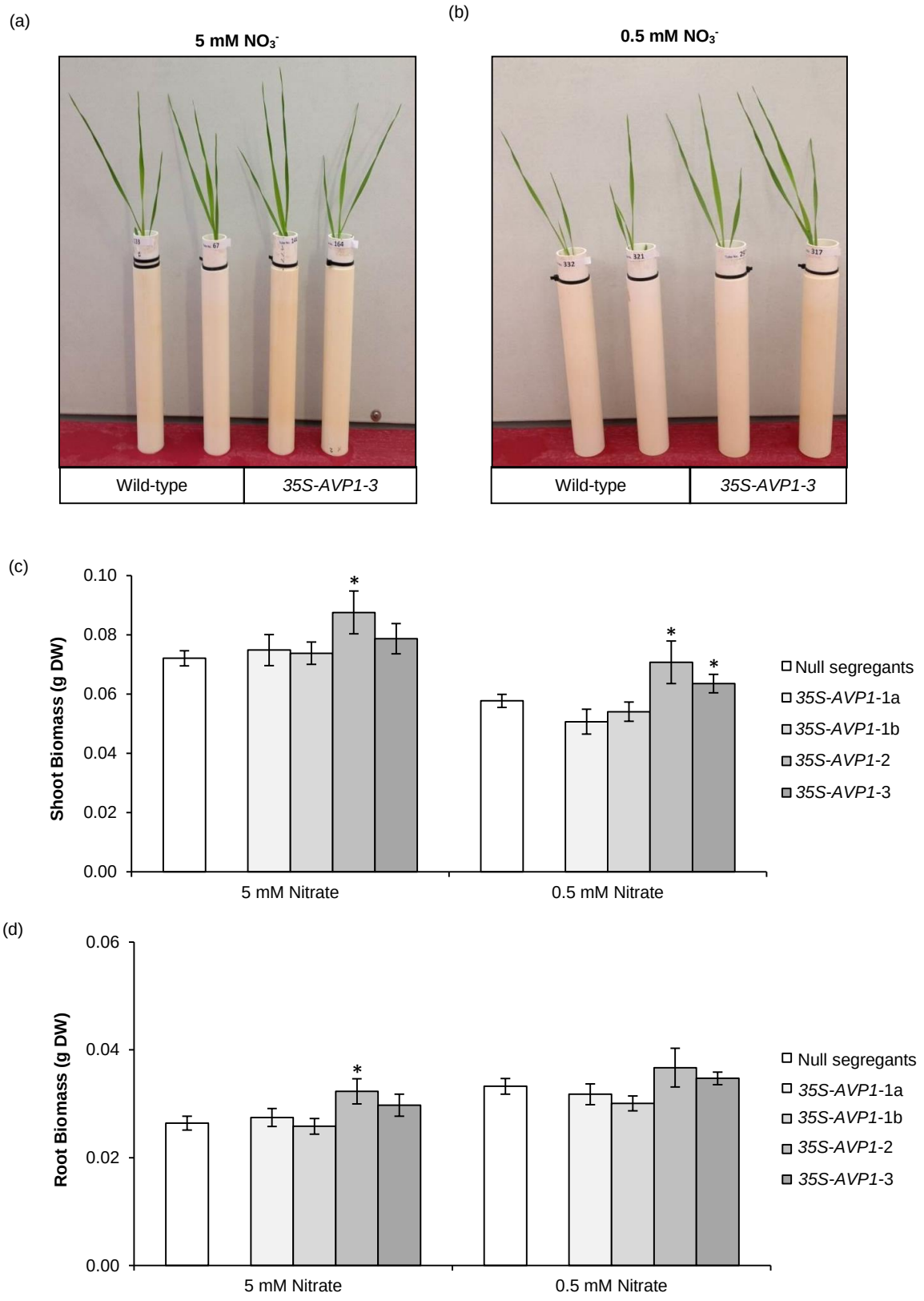

Figure 1

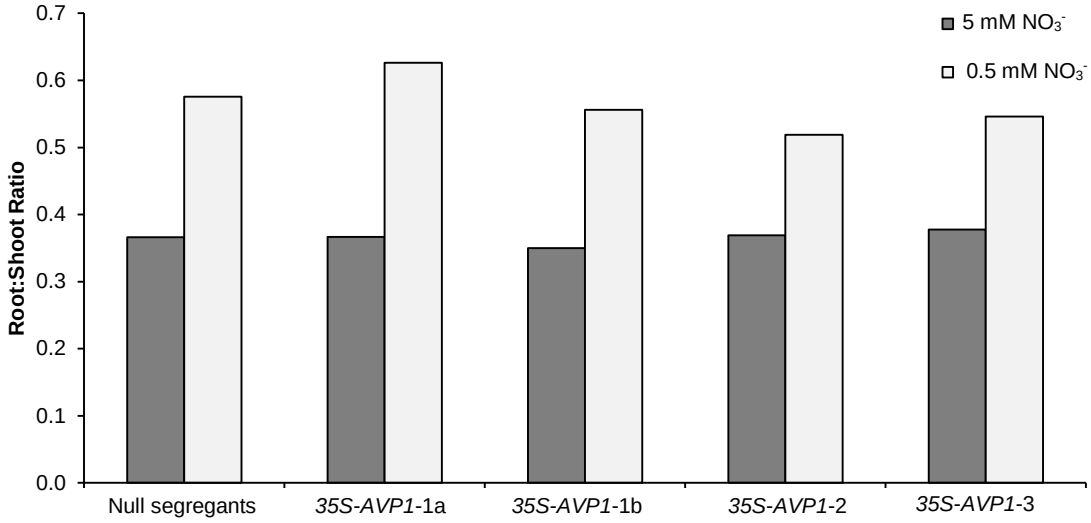
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Root:Shoot Ratio
5 mM NO3-
0.5 mM NO3-
Null segregants
35S-AVP1-1a
35S-AVP1-1b
35S-AVP1-2
35S-AVP1-3

Figure 2

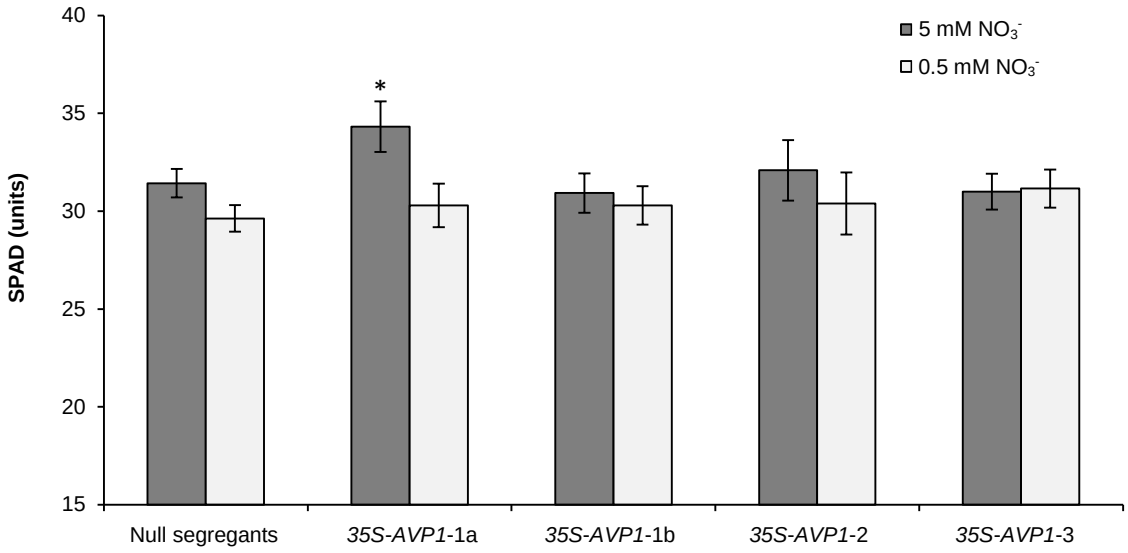
40
35
30
25
20
15
SPAD (units)
5 mM NO_3^-
0.5 mM NO_3^-
*
Null segregants
35S-AVP1-1a
35S-AVP1-1b
35S-AVP1-2
35S-AVP1-3

Figure 3

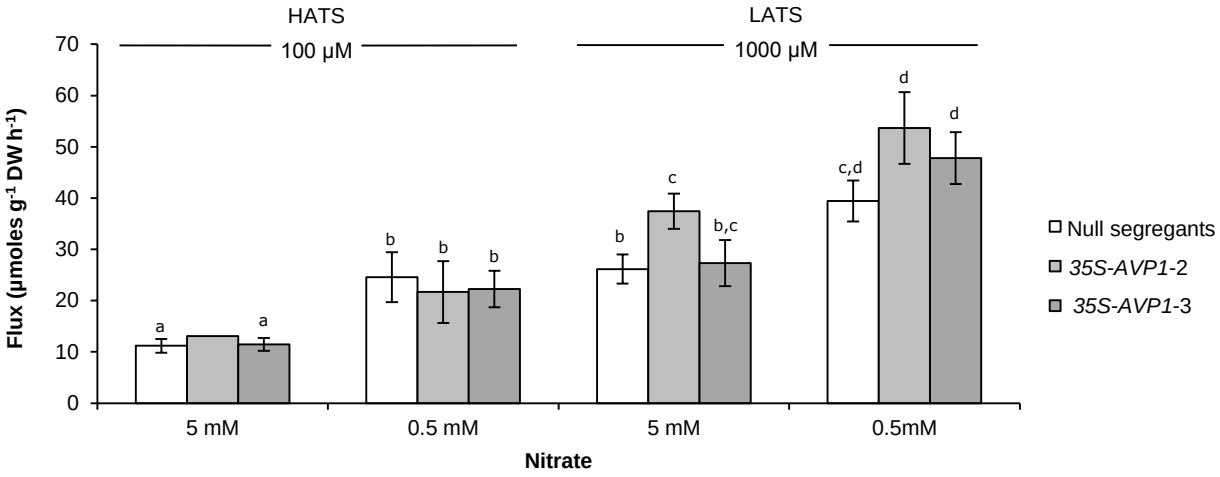
HATS
100 µM
LATS
1000 µM
Flux (µmoles g-1 DW h-1)
70
60
50
40
30
20
10
0
a
a
b
b
b
b
c
b,c
c,d
d
d
5 mM
0.5 mM
5 mM
0.5mM
Nitrate
Null segregants
35S-AVP1-2
35S-AVP1-3

Figure 4

Supplementary Figures

(a) (b)

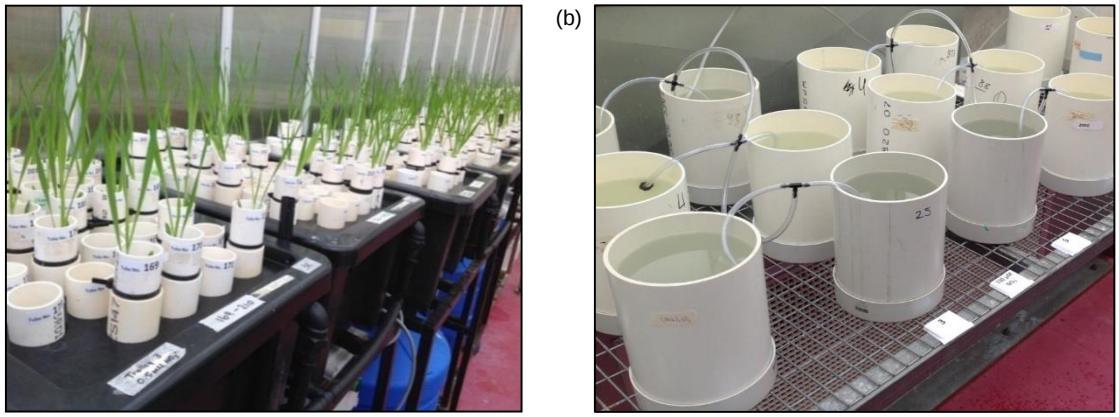

Figure S1 (a) An image of the 80 L hydroponics set-up used in this experiment to evaluate wild-type, null segregants and transgenic *AVP1* barley in 5 mM NO_3^- (sufficient) and 0.5 mM NO_3^- (low) treatments for 21 d and (b) an image of the ^{15}N-labelled NO_3^- flux pots with aerators used for measuring the unidirectional ^{15}N-labelled NO_3^- influx into barley roots.

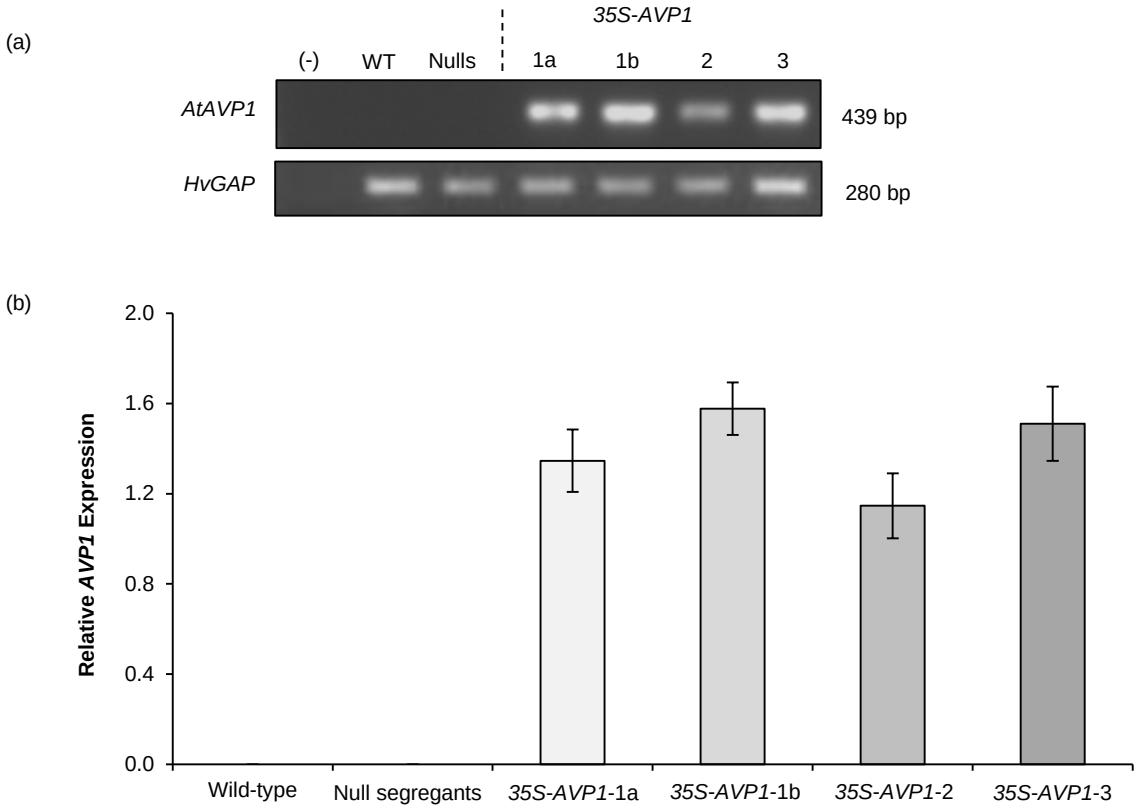

Figure S2 (a) Individual gel images showing reverse-transcription PCR (RT-PCR) band intensity of wild-type (WT), null segregants (Nulls), and transgenic *AVP1* barley (*35S-AVP*1-1a, *35S-AVP1*-1b, *35S-AVP1*-2 & *35S-AVP1*-3) leaf tissue in the 5 mM NO_3^- treatment using *AVP1* and *HvGAP* specific primers (internal control) with (-) a negative water control (b) Relative *AVP1* expression levels (compared to *HvGAP*) in leaf tissue of wild-type null segregants (nulls) and transgenic *AVP1* barley in the 5 mM NO_3^- treatment showing no significant difference in expression levels between the transgenic *AVP1* barley lines. Values are presented as the mean ± s.e.m (*n* = 3-4).

Chapter 5

Evaluating the tolerance of transgenic *AVP1* barley to combined stresses

Statement of Contributions

The following manuscript in this chapter is formatted for future submission:

Title: Evaluating the effect of soil matric and osmotic potentials on the growth of wild-type barley and transgenic barley expressing an *Arabidopsis* vacuolar H^+-pyrophosphatase (*AVP1*)

Schilling conducted the experiments, data analysis, interpretation of results and wrote the manuscript

Marschner, Tester, Plett and Roy supervised the experiments

All authors contributed to the discussion of the results

By signing this statement of contributions, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis:

Darren Plett

Signature: Date: 25/06/2014

Mark Tester

Signature: Date: 25/06/2014

Stuart Roy

Signature: Date: 25/06/2014

Petra Marschner

Signature: Date: 25/06/2014

Evaluating the effect of soil matric and osmotic potentials on the growth of wild-type barley and transgenic barley expressing an *Arabidopsis* vacuolar H^+-pyrophosphatase (*AVP1*)

Running title: Growth of wild-type and transgenic *AVP1* barley at varied soil matric and osmotic potentials

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Keywords: H^+-PPase, drought, salinity, abiotic stress, combined stresses, phenotyping

Abstract

Multiple concurrent abiotic stresses, such as salinity and low water availability, can reduce the growth of cereal crops in field conditions. However, most studies focus on improving the tolerance of cereal crops to a single abiotic stress. To date, little is known about the effects of combined salinity and low water availability on crop growth, particularly the effects from varied osmotic and matric potentials established at an equivalent soil water potential. The development of transgenic plants expressing an *Arabidopsis* vacuolar H^+-pyrophosphatase (*AVP1*) gene, also offers an opportunity to engineer crops with improved growth under multiple concurrent abiotic stress conditions. In this study, a pot experiment was conducted to evaluate the growth of wild-type barley (cv. WI4330 and Golden Promise) and transgenic barley (cv. Golden Promise) expressing *AVP1* at a soil water potential of -0.5 MPa imposed as either matric potential only (low water availability), osmotic potential only (salinity) or combined matric and osmotic potential (low water availability and salinity). The osmotic only treatment reduced the shoot and root biomass of WI4330 to a greater extent than the equivalent matric only or combined treatment. In contrast, all treatments reduced the shoot and root biomass of Golden Promise to a similar extent. This suggests that the combined matric and osmotic treatment does not have an additive effect on barley growth and that the effect of the different potentials on plant growth is dependent on the barley variety. Furthermore, this study confirms that transgenic *AVP1* barley has a greater shoot biomass in the osmotic only treatment compared to null segregants. It also shows in the matric only treatment that the shoot biomass of some transgenic *AVP1* barley lines are larger than null segregants. However, it suggests that a more severe matric only treatment may be needed to further elucidate this growth advantage. Nevertheless, the shoot and root biomass of two transgenic *AVP1* barley lines were larger than null segregants in the combined matric and osmotic treatment. This suggests that transgenic *AVP1* barley is a promising option to improve crop growth under multiple concurrent stresses.

Introduction

Abiotic stresses, such as salinity and drought, are major constraints to crop productivity (Boyer, 1982; Munns and Tester, 2008). Globally, salinity affects more than 77 million hectares of arable land and low water availability frequently reduces crop yields in both arid and semi-arid regions (Boyer, 1982; Munns, 2002). Furthermore, the area of land affected by salinity is increasing and the duration of low rainfall events is predicted to rise in the future (Burke et al., 2006; Munns, 2002). With the human population also expected to reach 9 billion people by 2050 (http://faostat.fao.org), it has been estimated that global food production will need to increase by a further 44 million metric tons each year above current increases (Tester and Langridge, 2010). The development of cereal crop varieties with improved abiotic stress tolerance is therefore needed (Schroeder et al., 2013; Tester and Langridge, 2010).

To date, most studies have focused on improving the tolerance of cereal crops to a single abiotic stress, such as salinity or drought (Cushman and Bohnert, 2000). However, in field conditions, crops often encounter multiple abiotic stresses concurrently throughout the growing season (Mittler, 2006; Mittler and Blumwald, 2010; Schmidhalter and Oertli, 1991; Suzuki et al., 2014). Transcriptomic analysis of *Arabidopsis thaliana* ecotypes also show that 61 % of transcriptome changes in response to two combined stresses are not predicted from the response to either individual stress (Rasmussen et al., 2013). Furthermore, the combined effects of salinity and heat on the growth of tomato (*Solanum lycopersicon*) reduced plant growth to a lesser extent than salinity alone (Rivero et al., 2013) and a combination of drought and heat stress in *Arabidopsis* (Rizhsky et al., 2004) and tobacco (*Nicotiana tabacum*) (Rizhsky et al., 2002) induced the expression of specific transcripts that were not altered under either stress alone. To develop a cereal crop with improved abiotic stress tolerance for field conditions, further research is needed to evaluate plant growth responses under multiple concurrent stresses (Hirayama and Shinozaki, 2010; McCree, 1986; Mittler, 2006; Mittler and Blumwald, 2010; Suzuki et al., 2014).

Very few studies have evaluated the growth of cereal crops, such as barley, with combined salinity and low water availability (Ahmed et al., 2013a; Ahmed et al., 2013b; Hackl et al., 2014; Jensen, 1982). A plant in a saline soil can experience the effects of both salinity and low soil water content simultaneously (Rengasamy, 2010; Shani and Dudley, 2001). However, as a saline soil dries through evaporation and transpiration, the intensity of salinity and low water content stresses also increases (Rengasamy, 2010). The soil water potential (i.e. predominantly the sum of the soil matric and osmotic potentials) is thus an important component influencing plant growth (Campbell, 1988; Wadleigh and Ayers, 1945). In the studies to date, a combined salinity (KCl or NaCl) and low water availability treatment appears to have an additive effect on barley growth, reducing barley shoot and root growth to a greater extent than either stress individually (Ahmed et al., 2013a; Ahmed et al., 2013b; Jensen, 1982). However, the overall soil water potential of the combined salinity and low water availability treatment in these previous studies was not equivalent to that occurring in either individual stress treatments, and, as such, comparisons between the treatments is not possible. It thus remains unknown what effect the overall soil water potential and, importantly, the relative contributions of the soil osmotic and matric potentials, have on barley growth.

Given the complexity of plant abiotic stress tolerance, the use of genetic modification to engineer crop varieties with improved growth under multiple concurrent stresses may be useful (Mittler, 2006; Mittler and Blumwald, 2010). The constitutive expression of the type I *Arabidopsis* vacuolar H^+-pyrophosphatase (*AVP1*) gene has been shown to improve the tolerance of transgenic plants to various abiotic stresses, including salinity, drought and low nutrient availability (Bao et al., 2009; Gaxiola et al., 2001; Li et al., 2010; Paez-Valencia et al., 2013; Park et al., 2005; Pasapula et al., 2011; Qin et al., 2013; Schilling et al., 2014; Yang et al., 2007). This improved tolerance to various individual stresses suggests that this vacuolar H^+-PPase may also improve transgenic plant growth under multiple concurrent abiotic stresses. Promisingly, dryland field trials of transgenic *AVP1* cotton (Pasapula et al., 2011) and a saline field trial of transgenic *AVP1* barley (Schilling et al., 2014) show transgenic *AVP1* plants have a larger shoot

biomass and increased yield compared to those plants without the *AVP1* transgene. However, the growth of transgenic plants expressing *AVP1* under two or more combined stresses, such as salinity and low water availability, is yet to be evaluated in controlled conditions.

This study tested for the first time the growth of two barley varieties (cv. WI4330 and Golden Promise) in salinity, low water availability and combined salinity and low water availability treatments using varied osmotic and matric potentials at an equivalent soil water potential. Furthermore, it evaluated the growth of transgenic *AVP1* barley and null segregants in salinity, low water availability and combined salinity and low water availability treatments to determine whether this transgene has the potential to improve transgenic barley growth under multiple concurrent stresses.

Materials and Methods

Establishing soil water potential treatments with varied osmotic and matric levels

A non-saline sandy loam (sand 75.0 %, silt 16.2 %, clay 8.8 %, pH 7.02, electrical conductivity $(EC)_{1:5}$ (soil:water) 0.05 dS cm^{-1} and water-holding capacity 140 g kg^{-1}) collected from Monarto, South Australia (35°05'S and 139°06'E) was air dried and sieved to ≤ 2 mm. The soil water retention curve was obtained using suction (-10 kPa) and pressure plate analysis (-30 kPa, -100, -500 and -1500 kPa) (Klute, 1986) (Figure S1). The matric potential (Ψ_m) was estimated from the water retention curve following the equation by Hillel (1998), $\Psi_m = \alpha\theta^{-b}$, where α and b are empirical constants and θ is the gravimetric water content (g g^{-1}). Briefly, an exponential curve was fitted to the measured water retention curve and the derived equation ($y = 0.1738x^{-0.131}$) was used to determine the soil matric potential at specific water contents. The osmotic potential (O_s) of the soil solution at specific water contents was estimated using the equation $0_s = -0.036\ EC_{meas}(\theta_{ref}/\theta_{act})$, where the EC_{meas} = the measured EC of the 1:5 (soil:water) extract (dS m^{-1}), θ_{ref} = the reference water content of the 1:5 (soil:water) extract (g g^{-1}) and θ_{act} = the actual water content of the soil (g g^{-1}) (Richards, 1954). To determine the amount of NaCl needed to adjust the Monarto soil to a

desired $EC_{1:5}$ different amounts of NaCl (0, 1.5, 2.9, 5.9, 8.8, 14.6, 29.3 g kg^{-1} soil) were mixed into the soil and incubated at 70 % water holding capacity (11.86 mL RO water pot^{-1}) for 7 d (Figure S2). An overall soil water potential of -0.5 MPa using varied levels of matric and osmotic potentials was selected to establish three treatments: matric only, matric and osmotic (combined) and osmotic only (Table 1) as this level was when the maximum matric potential of the soil was achieved (Figure S1). A control treatment with optimal water content and no added NaCl for an overall soil water potential of -0.01 MPa was also included.

Basal nutrients were mixed into the field soil to ensure all nutrients were non-limiting. The rate and composition of supplied nutrients were (g kg^{-1}) 0.92 $Ca(NO_3)_2$, 0.17 K_2SO_4, 0.19 $MgSO_4$, 75 $CaHPO_2.2H_2O$ and (mg kg^{-1} soil), 0.4 NaFeEDTA, 2.0 $CuSO_4.5H_2O$, 0.6 $MnSO_4.H_2O$, 0.4 $Co(NO_3)_2.6H_2O$, 0.5 Na_2MoO_4 and 2.2 $ZnSO_4.7H_2O$ (Mat Hassan et al., 2012). All nutrients and water were mixed evenly into a small proportion of field soil and gradually combined into the bulk soil. Salinity treatments were established by mixing NaCl into the bulk soil for control (0 g NaCl kg^{-1} soil), matric only (0 g NaCl kg^{-1} soil), combined (0.3 NaCl kg^{-1} soil) and osmotic only (1.2 NaCl kg^{-1} soil) treatments (Table 1).

Plant material and growth conditions at varied soil water potentials

Seeds of two barley wild-types (cv. WI4330 kindly supplied by Jason Eglinton (University of Adelaide), and Golden Promise) and three independent T_4 transgenic barley lines (cv. Golden Promise) with constitutive *CaMV 35S* expression of *AVP1* (*35S-AVP1*-1, *35S-AVP1*-2, *35S-AVP1*-3) and two sibling lines (*35S-AVP1*-1a and *35S-AVP1*-1b) were surface sterilised by a 5 min exposure to ultra-violet (UV) light and germinated in Petri dishes on moist filter paper for 5 d. Individual seedlings were transplanted to a sealed 1 kg pot containing 1 kg of amended field soil (7 replicates $treatment^{-1}$). All pots were watered to field capacity (147 g water kg^{-1} soil) every second day for 8 d to allow seedling establishment. After 8 d, watering of control and osmotic only pots was maintained every second day and watering withheld from

matric only and combined pots until each pot reached the desired matric potential for control (147 g water kg^{-1} soil), matric only (73 g water kg^{-1} soil), combined (80 g water kg^{-1} soil) and osmotic only (147 g water kg^{-1} soil) treatments around 6 d later (Table 1). Each pot was then maintained for a further 13 d by watering to weight before shoots and roots were harvested for biomass and tissue ion measurements (27 d after transplanting).

The cumulative plant water use was determined for each plant using the change in weight of each pot recorded every second day from the desired target pot weight for each treatment. A hand-held SPAD 502 meter was used to measure the greenness of the 4th leaf blade of each plant as an indicator of chlorophyll content (Minolta, Osaka, Japan). All plants were genotyped to confirm the presence or absence of the *AVP1* transgene (data not shown). The expression of *AVP1* was also confirmed in a subset of plants using RNA extracted from leaf tissue (Figure S3a,b).

Determination of leaf Na^+, K^+ & Cl^- concentrations

The fully-expanded 4th leaf of each plant was dried in an oven for 3 d at 70 °C and digested in 1 % nitric acid (v/v) at 95 °C for 5 h in a 54-well HotBlock (Environmental Express, Mount Pleasant, SC, USA). The Na^+ and K^+ concentrations of digested leaf tissue were determined using a flame photometer (Model 420, Sherwood Scientific, Cambridge, UK) (Shavrukov et al., 2010). The concentration of Cl^- in digested leaf tissue was determined using a chloride analyser (Model 926, Sherwood Scientific, Cambridge, UK).

Statistical analysis

Data was statistically analysed using a mixed model in GenStat (16th edition, VSN International Ltd., UK) and the Least Significant Difference (LSD) was used to identify significantly different means at a probability level of $P \leq 0.05$ or 0.001. No statistical difference was evident between wild-type Golden

Promise and null segregants and, as such, the transgenic *AVP1* barley lines were compared to null segregants only.

Results

Shoot and root biomass of WI4330 and Golden Promise

The shoot biomass of Golden Promise was significantly smaller (25 %) than WI4330 in control conditions (Figure 1a & b). Both barley varieties had significantly reduced shoot biomass in matric only, combined and osmotic only treatments compared to control conditions (Figure 1b). The osmotic only treatment decreased the shoot biomass of WI4330 to a greater extent (55 %) than the matric only and combined treatments (36 % and 46 % respectively) when compared to control conditions (Figure 1b). However, all three stress treatments reduced the shoot biomass of Golden Promise to a similar extent (33-44 %) compared to control conditions (Figure 1b).

The root biomass of Golden Promise was significantly smaller than WI4330 in control (53 %), matric only (45 %) and combined (34 %) treatments (Figure 1c). There was no significant difference in root biomass between the control, matric only and combined treatments of both barley varieties (Figure 1c). In the osmotic only treatment, the root biomass of Golden Promise was smaller but not significantly different to control conditions (Figure 1c) but the root biomass of WI4330 was significantly lower (49 %) than control conditions (Figure 1c). The dry matter root to shoot ratio of WI4330 was greater than Golden Promise in control conditions (Figure S4). Both barley varieties also had increased dry matter root to shoot ratios in the matric only and combined treatments compared to the control (Figure S4).

Leaf Na^+, K^+ and Cl^- concentrations of WI4330 and Golden Promise barley

Leaf Na^+ concentrations (mM) did not differ between the control and matric only treatments (Figure 2a). As expected, the leaf Na^+ concentration of WI4330 and Golden Promise increased in the combined (54-

60 %) and osmotic only (93 %) treatments compared to control conditions (Figure 2a). In the combined treatment, both barley varieties had a similar leaf Na^+ concentration (Figure 2a). However, in the osmotic only treatment Golden Promise had a significantly less (18 %) 4th leaf blade Na^+ concentration compared to WI4330 (Figure 2a). No significant difference in SPAD values (or leaf greenness) of the 4th leaf blade was evident between WI4330 and Golden Promise in all treatments (Figure S5).

Compared to control conditions, WI4330 had a significantly higher leaf K^+ concentration (mM) in the matric only treatment and the combined treatment (Figure 2b). However, there was no difference in leaf K^+ concentration of Golden Promise between control and matric only treatments (Figure2b). In contrast, the leaf K^+ concentration of Golden Promise was less than the WI4330 and reduced by 24 % in the combined treatment compared to control conditions (Figure 2b). The concentration of K^+ in the leaf tissue of both WI4330 and Golden Promise was significantly reduced (26 % and 36 % respectively) by the osmotic only treatment compared to control conditions (Figure 2b).

The concentration of Cl^- in the leaf tissue of both WI4330 and Golden Promise was higher in the combined and osmotic only treatments compared to the control and matric only treatments (Figure 2c). Furthermore, leaf Cl^- concentrations of both WI4330 and Golden Promise barley were similar to Na^+ concentrations in the combined treatment (around 100 mM) but less than leaf Na^+ concentrations in the osmotic only treatment (Figure 2a,c). WI4330 had a higher leaf Cl^- concentration in the osmotic only treatment compared to the combined treatment. In contrast, the leaf Cl^- concentration of Golden Promise was not significantly different between the combined and osmotic only treatments (Figure 2c). However, Golden Promise had a significantly higher leaf Cl^- concentration than WI4330 in the combined treatment (Figure 2c).

Cumulative water use of WI4330 and Golden Promise barley

Cumulative water use (g pot^{-1}) of WI4330 and Golden Promise was greatest in the control and matric only treatments (Figure 3). Both varieties had a significant decrease in cumulative water use in the combined and osmotic only treatments compared to control conditions (Figure 3). There was no significant difference in cumulative water use between the two varieties in all treatments (Figure 3).

Shoot and root biomass of transgenic *AVP1* barley

The matric only, combined and osmotic only treatments reduced the shoot biomass of null segregants and transgenic *AVP1* barley lines (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2, *35S-AVP1*-3) compared to control conditions (Figure 4a, b). There was no significant difference in shoot biomass between null segregants and transgenic *AVP1* barley lines under control conditions (Figure 4b). All the transgenic *AVP1* barley lines had greater shoot biomass in the matric only treatment compared to null segregants, however, only two sibling *AVP1* barley lines from one transformation event (*35S-AVP1*-1a and *35S-AVP1*-1b) had a significantly greater shoot biomass (12 to 26 %) (Figure. 4b). In the combined treatment, the transgenic *AVP1* barley tended to have a larger shoot biomass than null segregants, with *35S-AVP1*-1a and *35S-AVP1*-2 having a significantly greater (18 to 27 %) shoot biomass (Figure 4b). Likewise, with the exception of *35S-AVP1*-1b, the transgenic *AVP1* barley tended to have a greater shoot biomass than null segregants, with two transgenic *AVP1* barley lines (*35S-AVP1*-1a and *35S-AVP1*-3) having a significantly greater shoot biomass (22 to 30 %) than null segregants in the osmotic only treatment (Figure 4b).

The root biomass of null segregants and the transgenic *AVP1* barley lines did not differ between the control, matric only and combined treatments (Figure 4c). Notably, the osmotic only treatment significantly reduced the root biomass of null segregants and transgenic *AVP1* barley compared to all other treatments (Figure 4c). Two transgenic *AVP1* barley lines (*35S-AVP1*-1a and *35S-AVP1*-2) had a significantly greater root biomass than null segregants in the combined treatment (Figure 4c). No significant differences in root

biomass of transgenic *AVP1* barley compared to null segregants were evident in all other treatments (Figure 4c).

Leaf Na^+, K^+ and Cl^- concentrations in transgenic *AVP1* barley

For all lines, the leaf Na^+ concentration (mM) in the combined and osmotic only treatments was greater than those in control and matric only treatments (Figure 5a) and the highest leaf Na^+ concentrations occurred in the osmotic only treatment (Figure 5a). However, there was no significant difference in the leaf Na^+ concentration between null segregants and transgenic *AVP1* barley in all treatments (Figure 5a). There was also no significant difference in 4th leaf SPAD meter values (indicator of greenness) between null segregants and transgenic *AVP1* lines in all treatments (Figure S6).

The leaf K^+ concentration (mM) did not differ between null segregants and transgenic *AVP1* barley lines in control and matric only treatments (Figure 5b). Compared to control and matric only treatments, the leaf K^+ concentration was significantly lower in the combined and osmotic only treatments for all lines (Figure 5b).The leaf K^+ concentration was also lowest in the osmotic only treatment for all lines (Figure 5b). However, the K^+ concentration of null segregants and transgenic *AVP1* barley did not differ in the combined and osmotic only treatments (Figure 5b).

Compared to control and matric only treatments, the leaf Cl^- concentration (mM) of null segregants and the transgenic *AVP1* barley was significantly higher in the combined and osmotic only treatments (Figure 5c). The leaf Cl^- concentrations in the combined treatment were higher than those in the osmotic only treatment for all lines (Figure 5c). Compared to null segregants, two transgenic *AVP1* barley lines (*35S-AVP1*-1a and *35S-AVP1*-2) had significantly greater leaf Cl^- concentrations in the combined treatment (Figure 5c). However, the leaf Cl^- concentrations of null segregants and transgenic *AVP1* barley lines did not differ in the osmotic only treatment (Figure 5c).

Cumulative plant water use of null segregants and transgenic *AVP1* barley

Cumulative water use for all lines was highest in the control treatment (Figure 6). There was no significant difference in cumulative water use between null segregants and transgenic *AVP1* barley in the control and matric only treatments, although the transgenic *AVP1* barley tended to have lower cumulative water use compared to null segregants (Figure 6). There was also no significant difference in cumulative water use between null segregants and transgenic *AVP1* barley in the combined and osmotic only treatments (Figure 6).

Discussion

In this study, the growth of two barley varieties (cv. WI4330 and Golden Promise) were tested for the first time in salinity, low water availability and combined salinity and low water availability treatments using varied osmotic and matric potentials at an equivalent soil water potential. The results showed that a combined salinity and low water availability treatment does not have an additive effect on barley growth if the soil water potential is equivalent to that in the individual salinity and low water availability treatments. Furthermore, the response of barley growth to both individual and concurrent salinity and low water availability treatments was found to be dependent on the barley variety. In addition, this study showed that transgenic *AVP1* barley had a larger shoot biomass compared to null segregants in saline conditions, supporting the results of a previous study (Schilling et al., 2014). It also showed that two transgenic *AVP1* barley lines had significantly larger shoot and root biomass compared to null segregants in soil with low water availability. Furthermore, two transgenic *AVP1* barley lines had significantly larger shoot and root biomass than null segregants in a combined salinity and low water availability treatment suggesting that the expression of *AVP1* has the potential to improve transgenic barley growth under multiple concurrent stresses.

Effects of the osmotic only treatment on wild-type WI4330 and Golden Promise

Although a relatively salt tolerant cereal, the shoot and root growth of barley is reduced by saline conditions due to a combination of shoot salt accumulation independent effects, ion toxicities and ion deficiencies (Maas and Hoffman, 1977; Munns and Tester, 2008; Roy et al., 2014). High salinity can increase Na^+ uptake, which can cause leaf chlorosis and reduce root K^+ uptake in barley plants (Lynch and Läuchli, 1984; Rains and Epstein, 1967; Tavakkoli et al., 2011). In support of these findings, this study also shows that both WI4330 and Golden Promise barley varieties have a significant reduction in shoot biomass, greater leaf Na^+ and Cl^- concentrations, lower leaf K^+ concentrations and lower cumulative water use in the osmotic only treatment compared to control conditions (Figure 1,2,3). The lower leaf Cl^- concentration compared to Na^+ concentration in the osmotic only treatment, also suggests that both WI4330 and Golden Promise barley exclude more Cl^- than Na^+ from the shoot (Figure 2a,c). Furthermore, this study suggests that WI4330 barley is more sensitive to salinity than Golden Promise, with WI4330 having a greater reduction in shoot and root biomass in the osmotic only treatment compared to control conditions (Figure 1b). Golden Promise barley is known to have low shoot Na^+ accumulation in saline conditions (Forster et al., 1994; Wei et al., 2003) and, in agreement, the Golden Promise in this study had a significantly lower leaf Na^+ concentration than WI4330 in the osmotic only treatment (Figure 2a). This suggests that greater shoot Na^+ exclusion is contributing to the improved salt tolerance of Golden Promise compared to WI4330.

Effects of the matric only treatment on wild-type WI4330 and Golden Promise

Low water availability is known to reduce barley growth and grain yield by promoting stomata closure, increasing wilting and reducing nutrient uptake (Day et al., 1978; Hu and Schmidhalter, 2005; Legg et al., 1979; Rajala et al., 2011). In this study, both WI4330 and Golden Promise barley had a smaller shoot biomass in the matric only treatment compared to control conditions (Figure 1b). Notably, the WI4330 barley had a significantly larger shoot and root biomass than Golden Promise in the matric only treatment

(Figure 1a,b). Considering a larger and more vigorous growing root system can increase plant uptake of water (Palta et al., 2011; Passioura, 1983), the larger root biomass of WI4330 barley could be contributing to the larger shoot biomass compared to Golden Promise in the matric only treatment (Figure 1). Additionally, the accumulation of K^+ in water stressed plants can improve cellular osmotic adjustment, and thus help to maintain plant turgor and transpiration rates (Hu and Schmidhalter, 2005; Jensen, 1981). In this study, the WI4330 barley had an increase in leaf K^+ concentration in the matric only treatment compared to control conditions suggesting it may also have improved osmotic adjustment (Figure 2b).

A combined matric and osmotic treatment does not have an additive effect on barley growth

In the combined salinity and low water availability treatment, both WI4330 and Golden Promise had reduced shoot growth (Figure 2a,b), greater leaf Na^+ and Cl^- concentrations (Figure 3a,c) and lower cumulative water use (Figure 4) compared to control conditions. However, compared to control conditions, the combined salinity and low water availability treatment reduced the shoot biomass of Golden Promise to a similar extent as the equivalent matric only and osmotic only treatment and did not affect the root biomass (Figure 1a,b). While in contrast, the osmotic only treatment reduced the shoot and root biomass of WI4330 to a greater extent than the equivalent matric only and combined treatments compared to control conditions (Figure 1b). These findings suggest that a combined salinity and low water availability treatment does not have an additive effect on barley growth if the soil water potential is equivalent to that in the individual salinity and low water availability treatments (Figure 1). It further shows that the response of barley plants to individual and concurrent salinity and low water availability stresses is dependent on the barley variety (Figure 1).

In this study, the osmotic only treatment was more detrimental to WI4330 growth, particularly reducing root biomass, compared to the equivalent matric only or combined salinity and low water availability treatments (Figure 1). However, the osmotic potential does not include the ionic effects of salinity on plant

growth (Hackl et al., 2014; Richards, 1954). It is thus likely that both shoot salt accumulation independent effects, such as reduced water uptake (Figure 6), and ionic effects, such as leaf Na^+ or Cl^- toxicity, are also reducing WI4330 growth in the osmotic only treatment. Considering the leaf Cl^- concentrations of WI4330 was similar between the combined and osmotic only treatments plants (Figure 2c) it is unlikely that Cl^- toxicity is responsible for the reduction in root biomass in the osmotic only treatment. However, given the high concentrations of leaf Na^+ observed in the WI4330 in the osmotic only treatment (Figure 2a), it is probable that Na^+ toxicity is contributing to the reduction in shoot and root growth in that treatment. In support of this, the shoot biomass of Golden Promise, which had a lower leaf Na^+ concentration than WI4330 in the osmotic only treatment, was similar between the osmotic only and combined treatments and combined and matric only treatments (Figure 1) suggesting this barley variety was more affected by the overall soil water potential than the ionic effects of NaCl. Overall, the differences observed between WI4330 and Golden Promise in the equivalent matric only, combined and osmotic only treatments suggests there is genetic variation in barley tolerance to combined matric and osmotic stresses. The findings of this study also reiterate the need for more research towards evaluating the response(s) of plants to combined abiotic stresses (Mittler, 2006) and the need to consider the total soil water potential for salinity and low water availability treatments (Hackl et al., 2014; Rengasamy, 2010).

Transgenic *AVP1* barley has improved shoot growth in the osmotic only treatment

Previously, transgenic plants expressing *AVP1* have been shown to have increased plant growth in saline conditions (Gaxiola et al., 2001; Li et al., 2010; Pasapula et al., 2011; Qin et al., 2013; Schilling et al., 2014). Transgenic *AVP1* barley lines (*35S-AVP1*-1a, *35S-AVP1*-2 & *35S-AVP1*-3) had a larger shoot biomass and no significant difference in leaf Na^+ concentrations compared to null segregants in greenhouse-based saline conditions (Schilling et al., 2014). This study supports these findings with the transgenic *AVP1* barley lines (*35S-AVP1*-1a, *35S-AVP1*-2 & *35S-AVP1*-3) having a larger shoot biomass (Figure 4a,b) and no significant difference in leaf Na^+, K^+ or Cl^- concentrations compared to null

segregants in the osmotic only treatment (Figure 5). In addition, a sibling line (*35S-AVP1*-1b) had no increase in shoot biomass compared to null segregants in this study (Figure 4b), which supports previous findings for this line in greenhouse-based saline conditions (Schilling et al., 2014).

A larger root biomass, which can increase plant uptake of water and nutrients, has been observed in transgenic cotton and bentgrass expressing *AVP1* in saline conditions (Li et al., 2010; Pasapula et al., 2011). In this study, all plants had a significantly lower root biomass in the osmotic only treatment compared to control conditions (Figure 4c). However, the transgenic *AVP1* barley lines had no significant difference in root biomass compared to null segregants in the osmotic only treatment (Figure 4c). This lack of a larger root biomass in the transgenic *AVP1* barley could be due to differences between plant species or the level and extent of salinity treatments. It also suggests that the larger shoot biomass of transgenic *AVP1* barley lines (*35S-AVP1*-1a, *35S-AVP1-2* and *35S-AVP1*-3) in the osmotic only treatment, in this case, is not due to a larger root system facilitating improved nutrient or water uptake (Figure 4b,c).

Transgenic *AVP1* barley has improved shoot growth in the matric only treatment

Transgenic plants expressing *AVP1* have previously been shown to have a greater shoot and root biomass at low water availability compared to plants without this gene (Bao et al., 2009; Gaxiola et al., 2001; Park et al., 2005; Pasapula et al., 2011; Qin et al., 2013). This improved growth in transgenic *AVP1* plants was attributed to increased solute accumulation and a larger root biomass enabling enhanced water uptake (Bao et al., 2009; Gaxiola et al., 2001; Park et al., 2005). However, to our knowledge, there are no previous studies evaluating the growth of transgenic *AVP1* barley at low water availability. In this study, the shoot biomass of all plants was reduced in the matric only treatment compared to control conditions (Figure 4a,b). Two siblings of one transgenic *AVP1* barley line (*35S-AVP1*-1a & *35S-AVP1*-1b) had a significantly larger shoot biomass than null segregants in the matric only treatment (Figure

4a,b). However, two other transgenic *AVP1* barley lines (*35S-AVP1*-2 and *35S-AVP1*-3) were larger but had no significant increase in shoot biomass in the matric only treatment compared to null segregants (Figure 4b). There was also no significant difference in root biomass or leaf Na^+, K^+ or Cl^- concentration of transgenic *AVP1* barley compared to null segregants in the matric only treatment (Figure 4c,5).

Previous studies evaluating the drought tolerance of transgenic plants expressing *AVP1* merely withheld water until severe drought symptoms, such as wilting and leaf chlorosis were observed (Bao et al., 2009; Park et al., 2005). However, in this study, the matric only treatment was established at a matric potential of -0.5 MPa for 13 d. Although this matric only treatment reduced shoot biomass compared to control conditions (Figure 4a,b), it did not cause plant wilting (Figure 4a) or leaf chlorosis (Figure S6). This suggests that either a more severe matric only treatment or a matric treatment of a longer duration, which would further reduce plant growth, may be needed. This might enable any effects of *AVP1* expression in transgenic barley, such as potentially larger root growth enhancing plant water uptake (Bao et al., 2009; Park et al., 2005), to become more apparent. Nevertheless, the results of *35S-AVP1*-1a and *35S-AVP1*-1b suggest the expression of *AVP1* has the potential to improve transgenic barley growth in conditions with low water availability (Figure 4a,b).

Transgenic *AVP1* barley has improved shoot and root growth in combined salinity and low water availability treatment

This study also shows that the constitutive expression of *AVP1* can increase the shoot biomass of transgenic barley (*35S-AVP1*-1a & *35S-AVP1*-2) compared to null segregants in a combined salinity and low water availability treatment (Figure 4a,b). To our knowledge, this is the first time that a transgenic plant expressing *AVP1* has been shown to tolerate multiple concurrent stresses in controlled conditions. The majority of transgenic *AVP1* barley lines (*35S-AVP1*-1b, *35S-AVP1*-2 and *35S-AVP1*-3) had no significant differences in leaf Na^+, K^+ and Cl^- concentrations compared to null segregants in the combined

treatment (Figure 5a,b,c), except one line (*35S-AVP1*-1a) which had significantly higher leaf Cl^- concentration compared to null segregants (Figure 5a,c). This suggests that differences in leaf ion accumulation were not contributing to the improved shoot growth of transgenic *AVP1* barley in the combined treatment. Interestingly, the transgenic *AVP1* barley lines (*35S-AVP1*-1a & *35S-AVP1*-2) with the largest shoot biomass in the combined treatment also had the largest root biomass of all lines in the combined treatment (Figure 4b,c). This suggests that a larger root system may have contributed to the improved shoot growth of transgenic *AVP1* barley in the combined stress treatment by potentially facilitating improved water or nutrient uptake (Gaxiola et al., 2011; Park et al., 2005). Although two transgenic *AVP1* barley lines did not have a significant increase in shoot or root growth compared to null segregants (Figure 4b) in the combined treatment, the results indicate that the expression of *AVP1* has the potential to improve the growth of transgenic barley under multiple concurrent stresses.

Conclusions

This study evaluated the effect of the soil water potential and, importantly, the effects of the soil osmotic and matric potentials, on barley growth. It showed that a combined salinity and low water availability treatment does not have an additive effect on barley growth when the combined treatment has the same overall soil water potential as the individual salinity and low water availability treatments. It also showed that the effect of combined salinity and low water availability on barley shoot and root growth, ion concentrations and water use is dependent on the barley variety. Furthermore, this study tested for the first time the growth of transgenic *AVP1* barley under multiple concurrent stresses in controlled conditions. It confirmed that transgenic barley expressing *AVP1* has a larger shoot biomass in saline conditions without altering leaf Na^+ concentrations compared to null segregants (Schilling et al., 2014). It also showed that in conditions with low water availability, the shoot biomass of two transgenic *AVP1* barley lines is larger than null segregants. However, it suggests that a more severe drought treatment may be needed to further elucidate this growth advantage. Nevertheless, the shoot biomass of two transgenic *AVP1*

barley lines was larger than null segregants in conditions with combined salinity and low water availability. Overall, the findings of this study support those suggesting it is imperative that future research considers the effect of combined stresses on plant growth (Mittler, 2006; Mittler and Blumwald, 2010) and suggest that transgenic *AVP1* barley is a promising option to help improve crop growth under multiple concurrent stresses.

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Tables

Table 1 The matric only, combined and osmotic only treatments used with the corresponding amount of NaCl (g kg^{-1} soil) and reverse osmosis (RO) water (g kg^{-1} soil) added to each pot and the calculated matric potential (MPa), osmotic potential (MPa) and soil water potential (SWP) (MPa) with the percentage contribution of matric potential (%) and osmotic potential (%) to the overall soil water potential.

Treatment	Amount of NaCl (g kg^{-1} soil)	Amount of Water (g kg^{-1} soil)	Matric Potential (MPa)	Osmotic Potential (MPa)	Soil Water Potential (MPa)	% of SWP Matric	% of SWP Osmotic
Osmotic Only	1.2	147	-0.01	-0.490	-0.500	2	98
Combined	0.3	80	-0.30	-0.225	-0.525	57	43
Matric Only	0	73	-0.50	-0.000	-0.500	100	0

Figure Legends

Figure 1 (a) Images of wild-type WI4330 (WI) and Golden Promise (GP) barley at 27 d after transplanting in control, matric only, combined and osmotic only treatments (b) Shoot and (c) root biomass of WI4330 (light grey) and Golden Promise (dark grey) in control (-0.01MPa) and matric only, combined and osmotic only treatments (-0.5MPa) at 27 d after transplanting. Values are presented as the mean ± s.e.m (*n* = 6-7) with a different letter indicating a significant difference between means (LSD, $P < 0.001$).

Figure 2 (a) Na^+, (b) K^+ and (c) Cl^- concentration (mM) of the fully-expanded 4th leaf blade of WI4330 (light grey) and Golden Promise (dark grey) barley after 27 d in soil with control (-0.01MPa), matric only, combined and osmotic only (-0.5 MPa) treatments. Values are presented as the mean ± s.e.m (*n* = 6-7) with a different letter indicating a significant difference between means (LSD, $P \leq 0.05$).

Figure 3 Cumulative plant water use (g pot^{-1}) for WI4330 (light grey) and Golden Promise (dark grey) barley after 27 d in soil with control (-0.01 MPa), matric only, combined and osmotic only (-0.5MPa) treatments. Values are presented as the mean ± s.e.m (*n* = 6-7) with a different letter indicating a significant difference between means (LSD, $P \leq 0.05$).

Figure 4 (a) An image of representative wild-type (cv. Golden Promise) and transgenic barley expressing *AVP1* (*35S*:*AVP1*-1a) at 27 d after transplanting in a soil with (1) control, (2) osmotic only, (3) matric only and (4) combined treatments. (b) Shoot and (c) root biomass (g DW) of null segregants and transgenic *AVP1* barley lines (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 and *35S-AVP1*-3) in control (-0.01 MPa), matric only, combined and osmotic only (-0.5 MPa) treatments at 27 d after transplanting. Values are presented as the mean ± s.e.m (*n* = 3-7) with a different letter indicating a significant difference (LSD, $P \leq 0.05$).

Figure 5 (a) Na^+, (b) K^+ and (c) Cl^- concentration (mM) of the fully-expanded 4th leaf blade of null segregants, and transgenic barley expressing *AVP1* (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 and *35S-AVP1*-3) after 27 d in soil with control (-0.01MPa), matric only, combined and osmotic only (-0.5MPa) treatments. Values are presented as the mean ± s.e.m (n = 3-7) with a different letter indicating a significant difference between means (LSD, $P \leq 0.05$).

Figure 6 Cumulative plant water use (g pot^{-1}) for of null segregants, and transgenic barley expressing *AVP1* (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 and *35S-AVP1*-3) after 27 d in soil with control (-0.01 MPa), matric only, combined and osmotic only (-0.5MPa) treatments. Values are presented as the mean ± s.e.m (n = 3-7) with a different letter indicating a significant difference between means (LSD, $P \leq 0.05$).

Figures

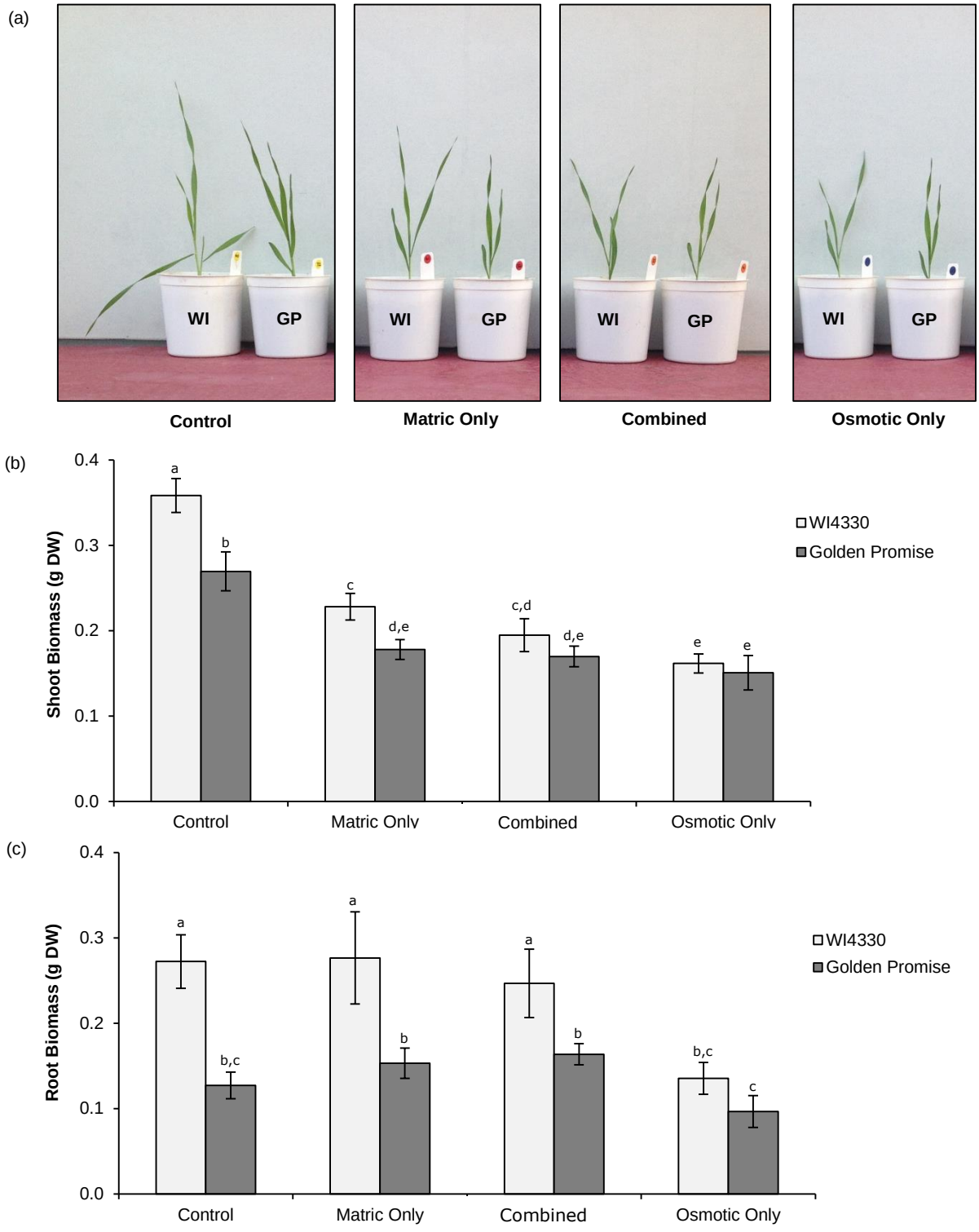


Figure 1

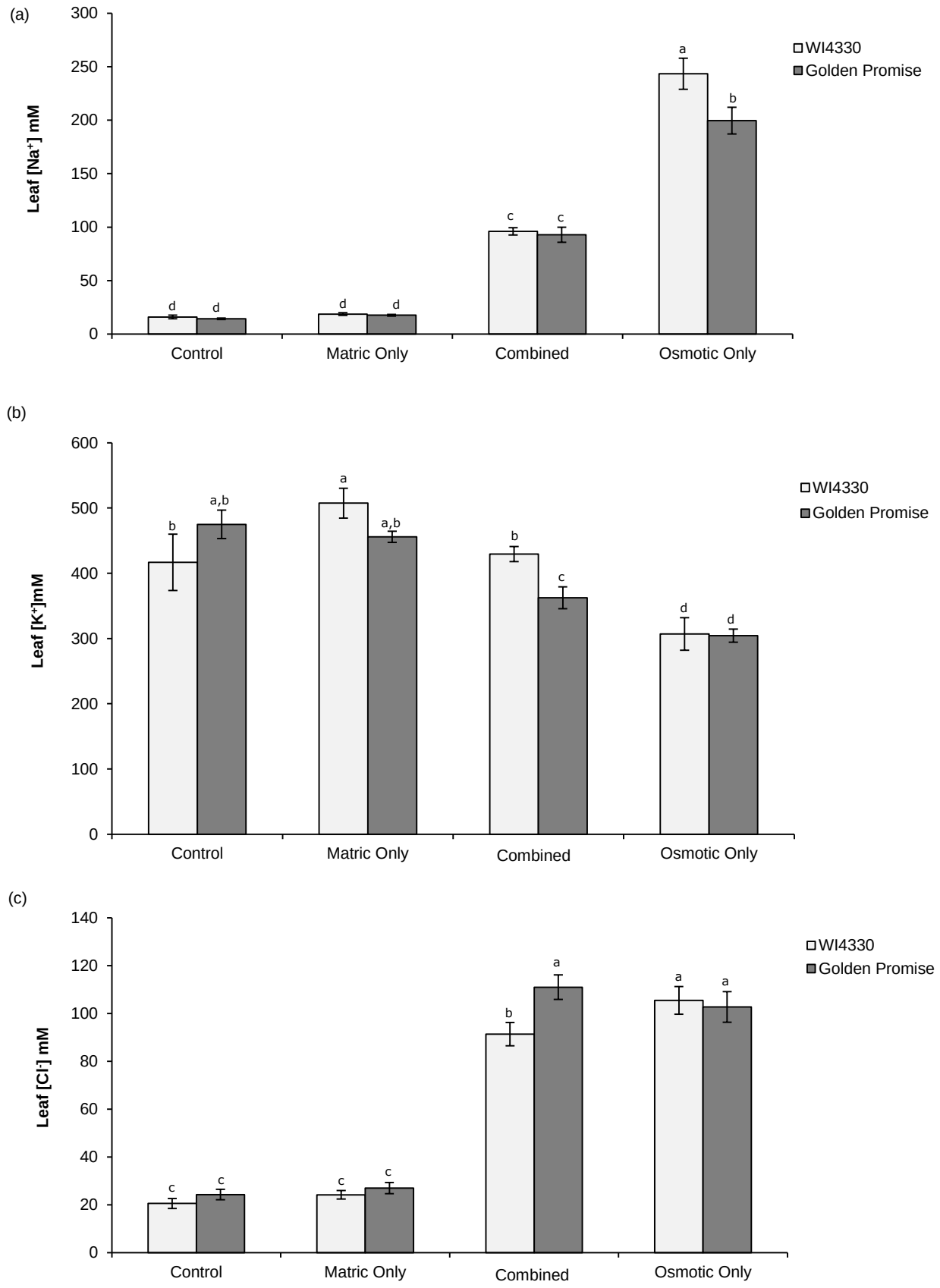

(a)
Leaf [Na+] mM
300
250
200
150
100
50
0
d
d
d
d
c
c
a
b
Control
Matric Only
Combined
Osmotic Only
WI4330
Golden Promise
(b)
Leaf [K+]mM
600
500
400
300
200
100
0
b
a,b
a
a,b
b
c
d
d
Control
Matric Only
Combined
Osmotic Only
WI4330
Golden Promise
(c)
Leaf [Cl-] mM
140
120
100
80
60
40
20
0
c
c
c
c
b
a
a
a
Control
Matric Only
Combined
Osmotic Only
WI4330
Golden Promise

Figure 2

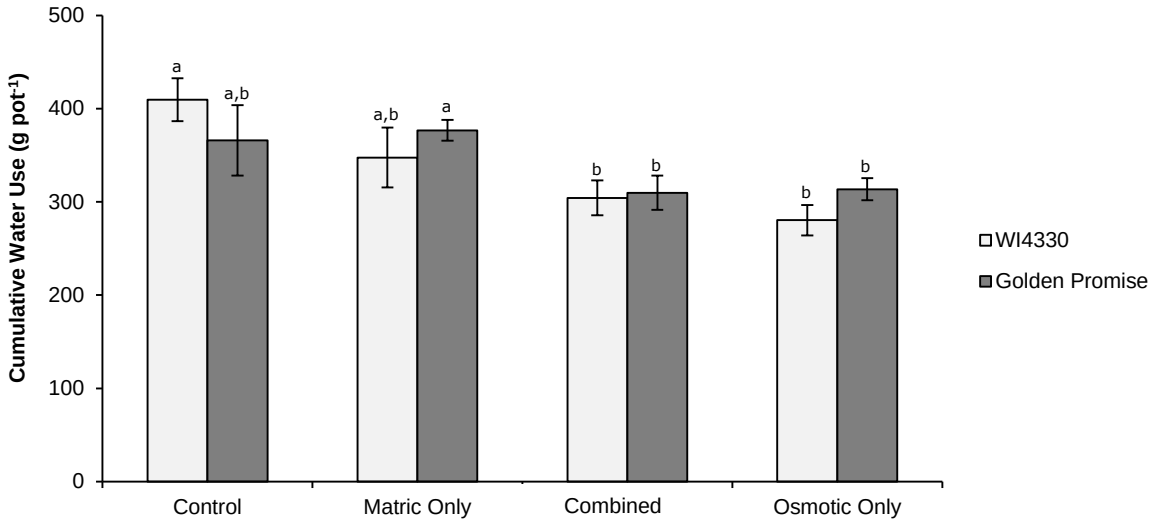
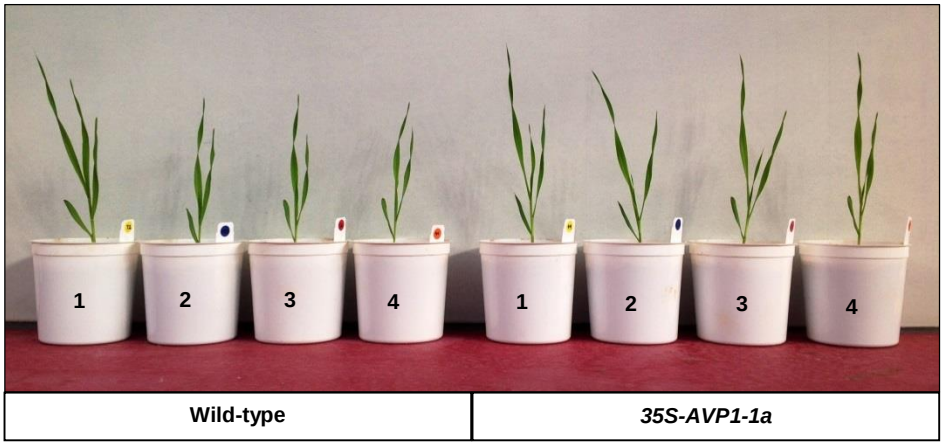
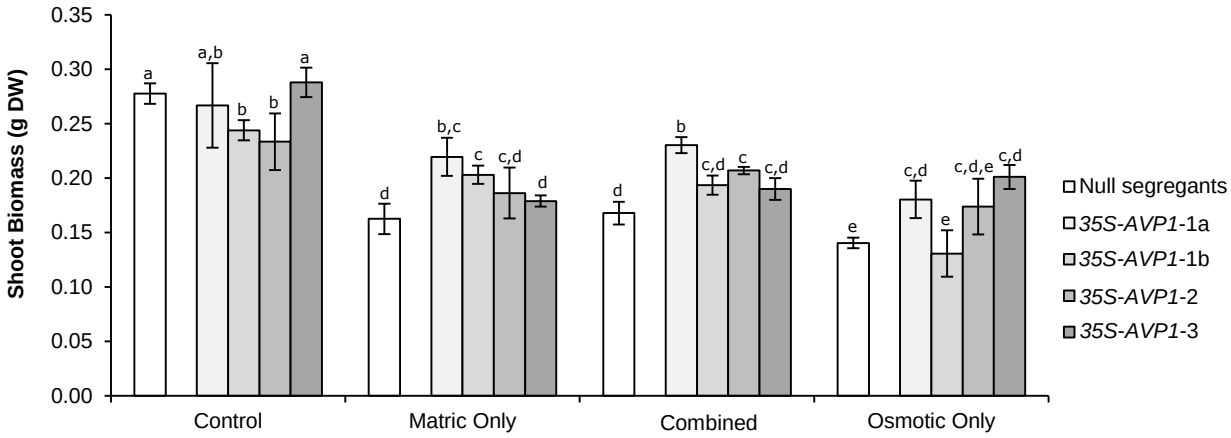

Cumulative Water Use (g pot-1)
500
400
300
200
100
0
a
a,b
a,b
a
b
b
b
b
Control
Matric Only
Combined
Osmotic Only
WI4330
Golden Promise

Figure 3

(a)


1
2
3
4
1
2
3
4
Wild-type
35S-AVP1-1a

(b)


Shoot Biomass (g DW)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Control
Matric Only
Combined
Osmotic Only
Null segregants
35S-AVP1-1a
35S-AVP1-1b
35S-AVP1-2
35S-AVP1-3

(c)

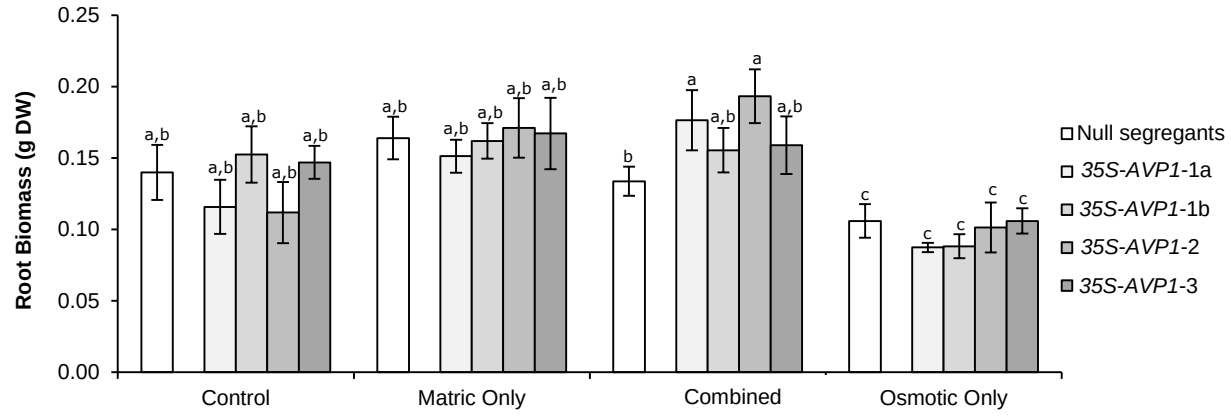

Root Biomass (g DW)
0.25
0.20
0.15
0.10
0.05
0.00
Control
Matric Only
Combined
Osmotic Only
Null segregants
35S-AVP1-1a
35S-AVP1-1b
35S-AVP1-2
35S-AVP1-3

Figure 4

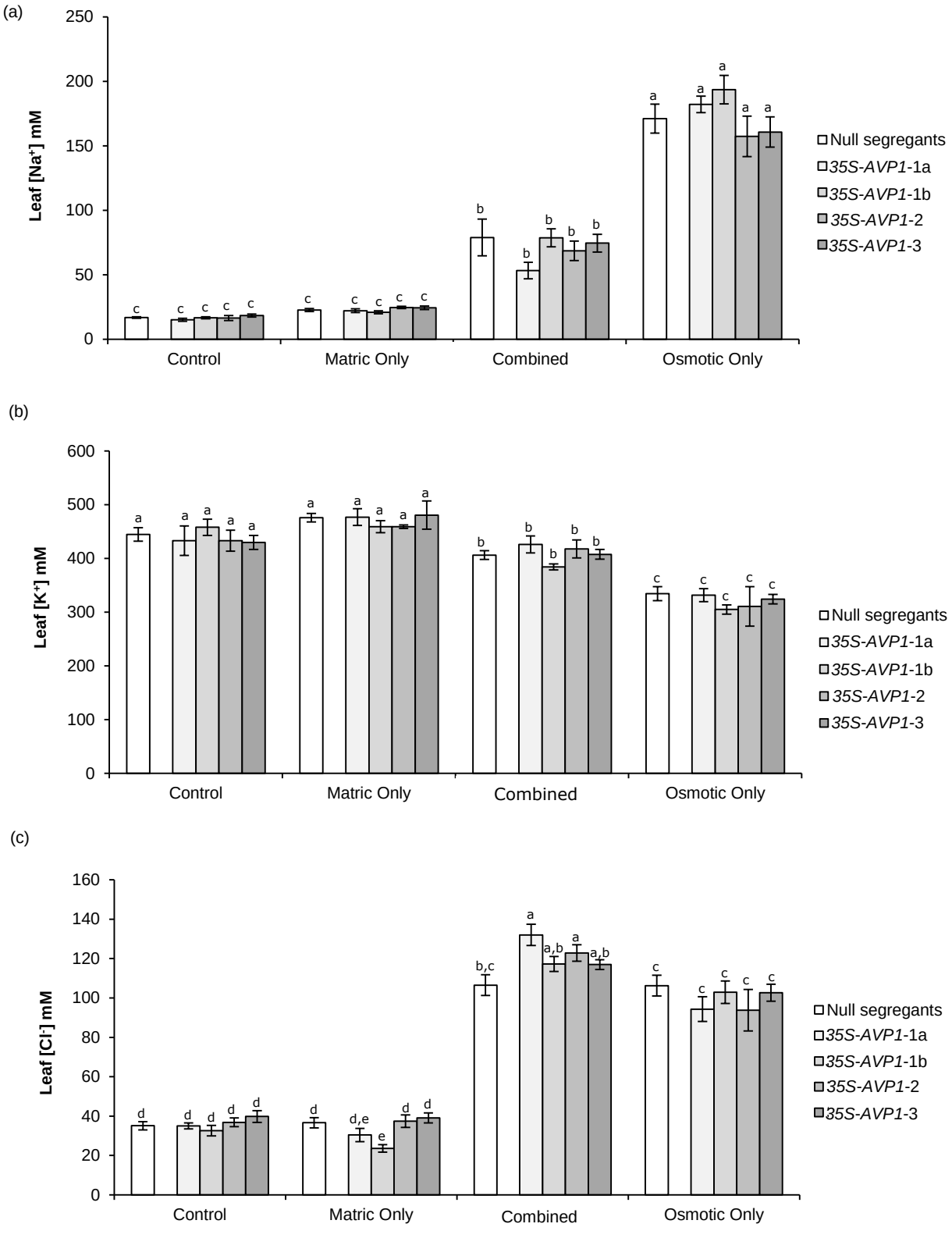

(a)
Leaf [Na+] mM
250
200
150
100
50
0
Control
Matric Only
Combined
Osmotic Only
Null segregants
35S-AVP1-1a
35S-AVP1-1b
35S-AVP1-2
35S-AVP1-3
(b)
Leaf [K+] mM
600
500
400
300
200
100
0
Control
Matric Only
Combined
Osmotic Only
Null segregants
35S-AVP1-1a
35S-AVP1-1b
35S-AVP1-2
35S-AVP1-3
(c)
Leaf [Cl-] mM
160
140
120
100
80
60
40
20
0
Control
Matric Only
Combined
Osmotic Only
Null segregants
35S-AVP1-1a
35S-AVP1-1b
35S-AVP1-2
35S-AVP1-3

Figure 5

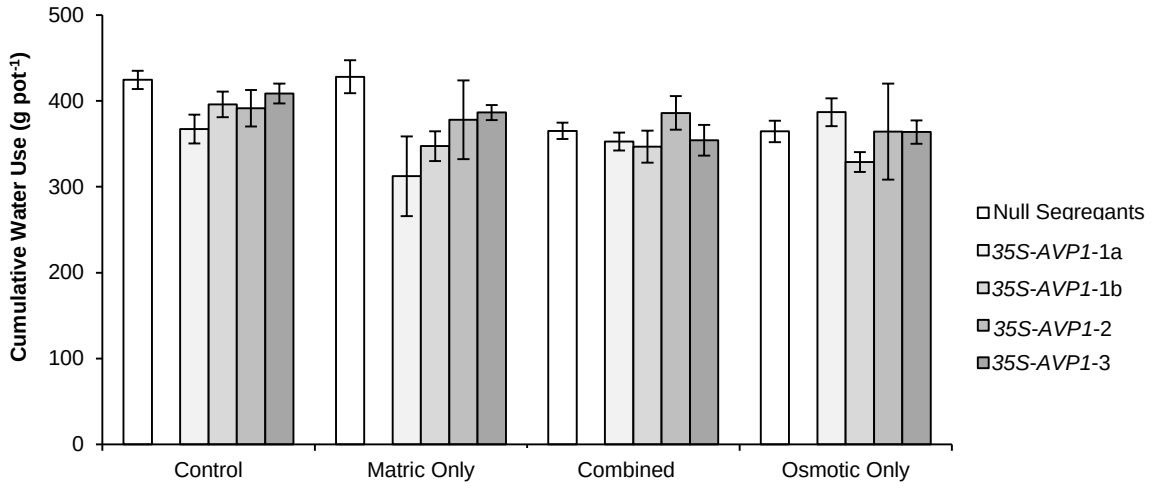

Cumulative Water Use (g pot-1)
500
400
300
200
100
0
Control
Matric Only
Combined
Osmotic Only
Null Segregants
35S-AVP1-1a
35S-AVP1-1b
35S-AVP1-2
35S-AVP1-3

Figure 6

Supplementary Figures

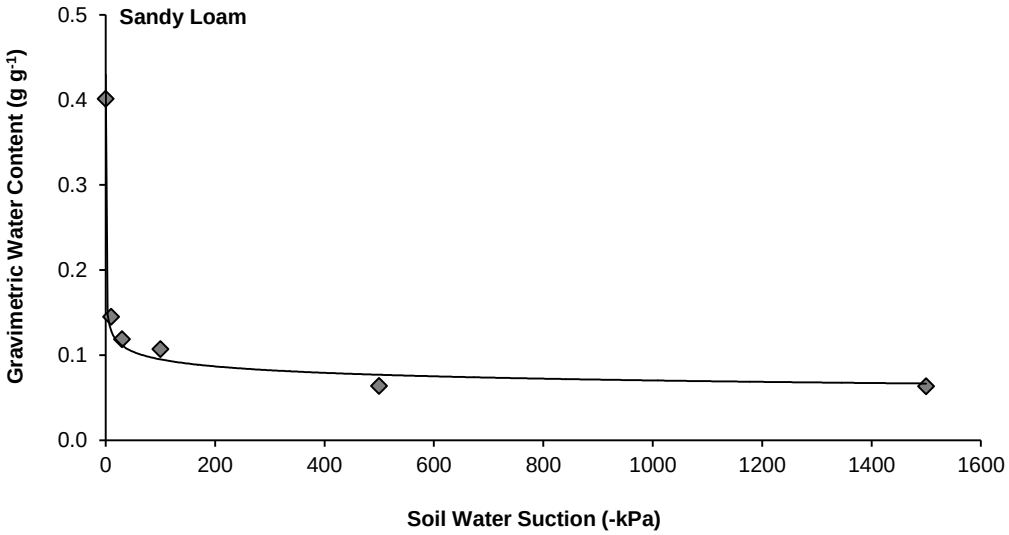


Figure S1 A soil water retention curve of a sandy loam from Monarto, South Australia (35°05'S and 139°06'E), with the soil water suction (-kPa) versus the gravimetric water content (g g^{-1}) at 0, -10, -30, -100, -500 and -1500 kPa fitted with an exponential curve. Values are presented as the mean ± s.e.m (*n* = 3).

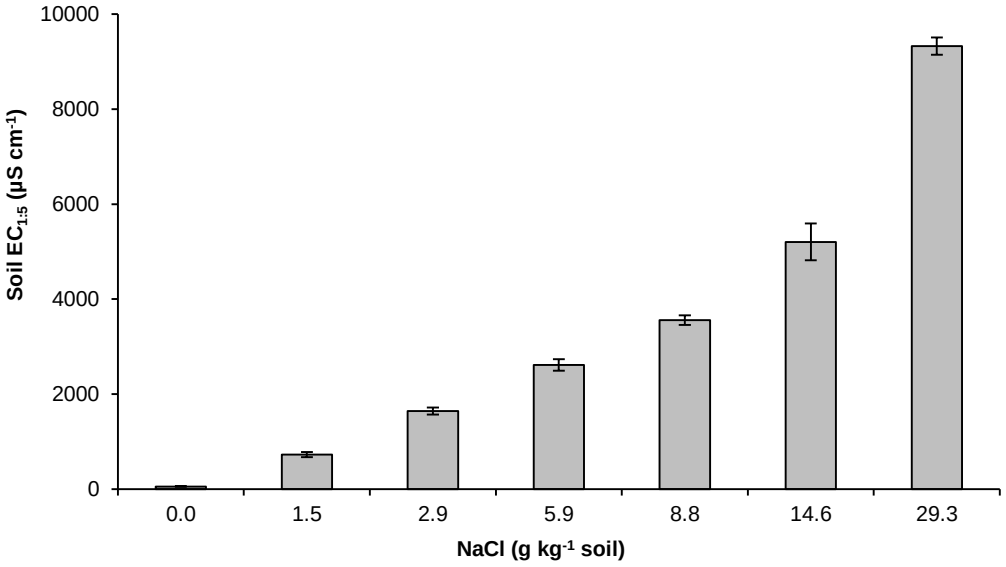


Figure S2 The electrical conductivity (µS cm^{-1}) of a 1:5 (soil:water) extract ($EC_{1:5}$) of soil incubated for 7 d with different rates of sodium chloride (0.0, 1.5, 2.9, 5.9, 8.8, 14.6 and 29.3 g NaCl kg^{-1} soil). Values are presented as the mean ± s.e.m (*n* = 3).

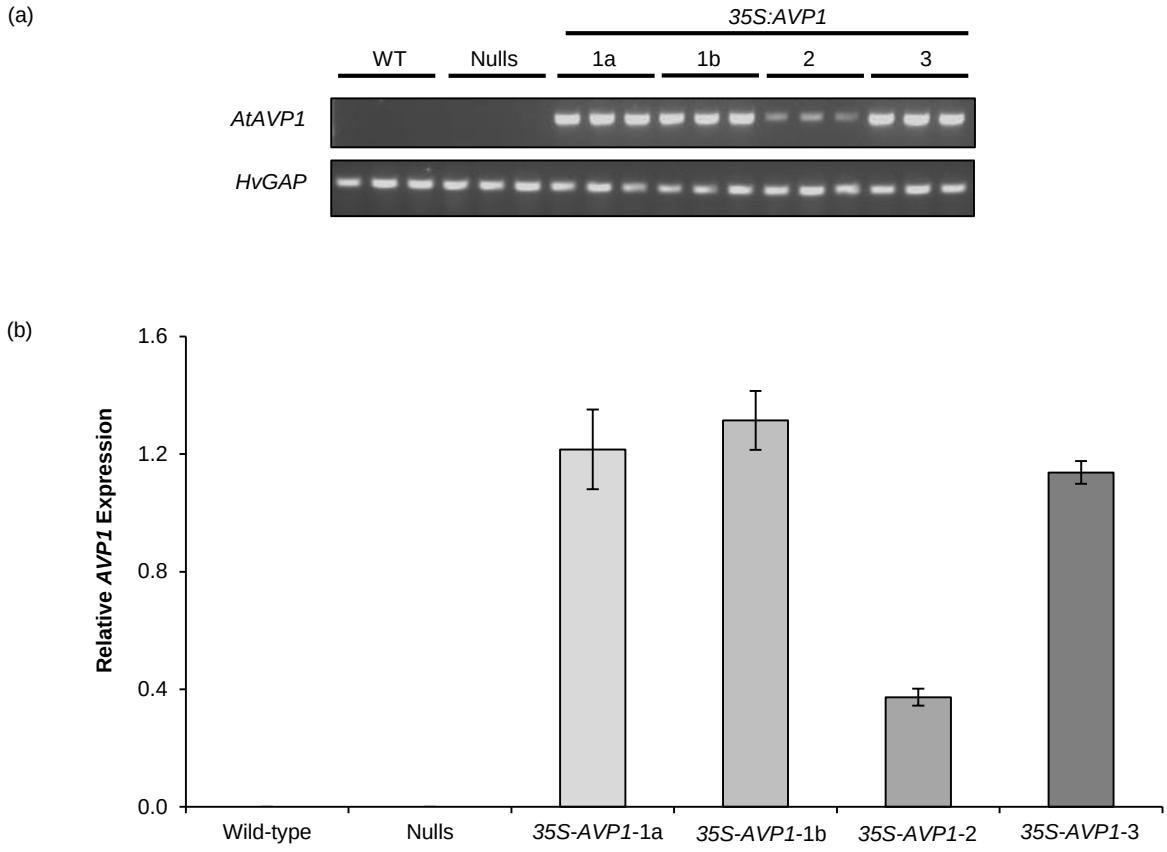


Figure S3 Semi-quantitative expression analysis of *AVP1* relative to *HvGAP* in transgenic *AVP1* barley plants (a) Gel images showing the intensity of PCR products for wild-type (WT), null segregants (Nulls) and transgenic *AVP1* barley (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 and *35S-AVP1*-3) using *AVP1*-specific primers and *HvGAP*-specific primers (internal control) (b) Semi-quantitative relative 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 *AVP1* barley. Values are presented as the mean ± s.e.m (n = 3).

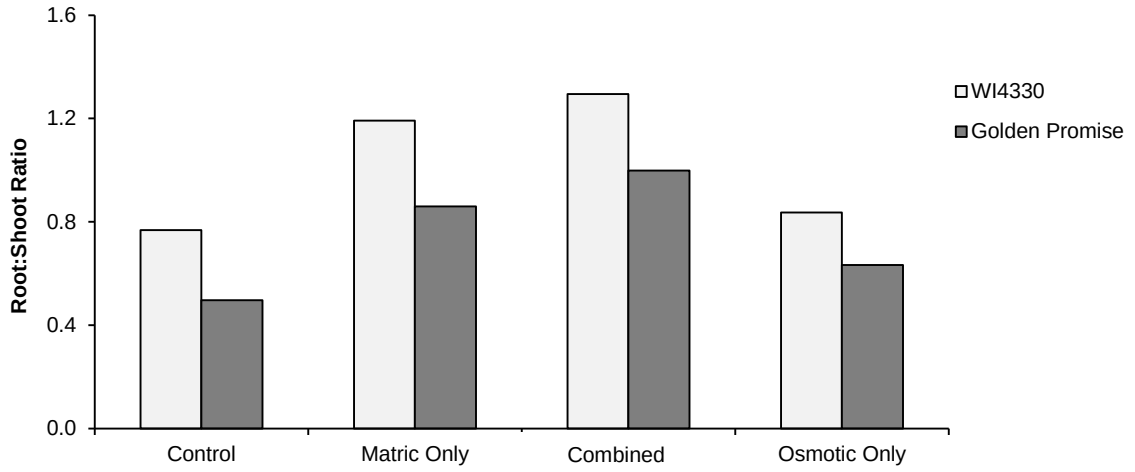


Figure S4 The dry matter root to shoot ratio of wild-type WI4330 (light grey) and Golden Promise (dark grey) barley in control, matric only, combined and osmotic only treatments. Values are presented as the mean ratio for each variety (*n* = 6-7).

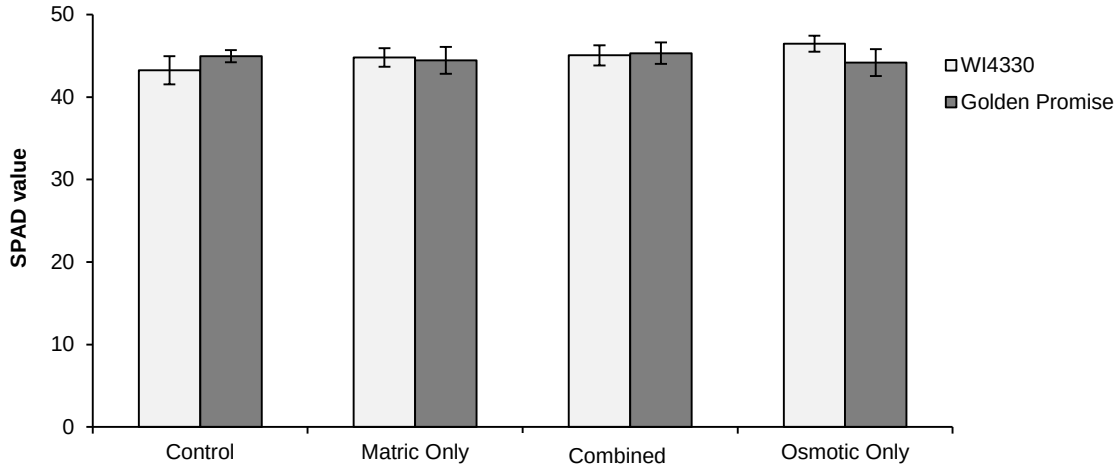


Figure S5 The SPAD value of the 4th leaf blade of WI4330 (light grey) and Golden Promise (dark grey) after 27 d in control, matric only, combined and osmotic only treatments. Values are presented as the mean ± s.e.m (*n* = 6-7).

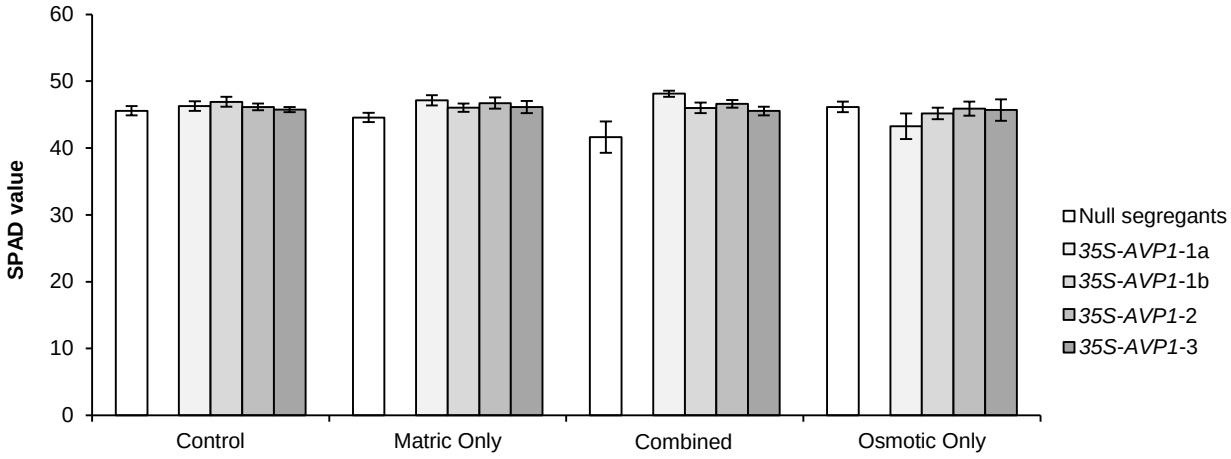


Figure S6 SPAD values of the 4th leaf blade of null segregants (white) and transgenic *AVP1* barley (cv. Golden Promise) lines (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 and *35S-AVP1*-3) (grey bars) plants after 27 d in control, matric only, combined and osmotic only treatments. Values are presented as the mean ± s.e.m (*n* = 3-7).

Chapter 6

Evaluating the seedling vigour of transgenic *AVP1* barley

Statement of Contributions

The following manuscript in this chapter is formatted future submission:

Title: Transgenic barley expressing *AVP1* has improved seedling vigour and increased ascorbic acid synthesis

Schilling conducted the experiments, data analysis, interpretation of results and wrote the manuscript

Marschner, Tester, Plett and Roy supervised the experiments

All authors contributed to the discussion of the results

By signing this statement of contributions, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis:

Petra Marschner

Signature: Date: 25/06/2014

Mark Tester

Signature: Date: 25/06/2014

Darren Plett

Signature: Date: 25/06/2014

Stuart Roy

Signature: Date: 25/06/2014

Transgenic barley expressing *AVP1* has improved seedling vigour and increased ascorbic acid synthesis

Running title: Expression of *AVP1* improves the seedling vigour of transgenic barley

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Keywords: germination, pyrophosphate, gluconeogenesis, sucrose, vitamin C, metabolomics, cell size

Abstract

Transgenic barley expressing the type I *Arabidopsis* vacuolar H^+-pyrophosphatase gene (*AVP1*) have a larger shoot biomass in both non-saline and saline conditions compared to null segregants. However, an explanation for this larger shoot biomass in transgenic *AVP1* barley is yet to be fully elucidated. In this study, the seedling vigour of null segregants and transgenic barley expressing *AVP1* was evaluated at 0 and 100 mM NaCl from 0 to 11 d after seed imbibition using non-destructive plant biomass and WinRHIZO® measurements. Furthermore, GC-MS analysis of leaf and root tissue at 11 d after seed imbibition was conducted to investigate the effects of *AVP1* expression on barley metabolism. Transgenic *AVP1* barley had a larger plant biomass, including an increase in both shoot and root biomass, at 11 d after seed imbibition in 0 mM NaCl compared to null segregants. The larger plant biomass was first detected 6 d after seed imbibition, a result of the transgenic *AVP1* barley having a faster growth rate between 0 to 5 d after seed imbibition compared to null segregants. Furthermore, metabolomic analysis revealed that transgenic *AVP1* barley had significantly lower galactose and significantly higher ascorbic acid and dehydroascorbic acid in leaf tissue compared to null segregants. Overall, the findings suggest that both improved seedling vigour and enhanced ascorbic acid synthesis are contributing to the larger plant biomass of transgenic *AVP1* barley in non-saline conditions.

Introduction

Vacuolar proton-pumping pyrophosphatases (H^+-PPases, EC 3.6.1.1) have a number of key roles in the physiology of plant cells (Barkla and Pantoja, 1996; Maeshima, 2000; Martinoia et al., 2007). By hydrolysing inorganic pyrophosphate (PP_i) to orthophosphate (P_i), these membrane-bound proteins, along with vacuolar H^+-ATPases (Sze et al., 1992), actively pump protons (H^+) from the cytoplasm into vacuoles which establishes an electrochemical potential difference for H^+ across the tonoplast (Duan et al., 2007; Kim et al., 1994; Zhen et al., 1997). Vacuolar membrane transporters, such as sodium/proton (Na^+/H^+) antiporters, use this electrochemical potential difference to actively translocate ions into vacuoles (Apse et al., 1999; Wang et al., 1986). Thus, vacuolar H^+-PPases indirectly facilitate the sequestration of ions into vacuoles, which can enhance cell turgor and reduce the accumulation of toxic ions, such as Na^+, in the cytoplasm (Blumwald, 2000). For this reason vacuolar H^+-PPases have been studied for their role in plant abiotic stress tolerance, particularly salinity tolerance (Colombo and Cerana, 1993; Fukuda et al., 2004; Parks et al., 2002; Queiros et al., 2009; Wang et al., 2000).

Much research has focused on characterising transgenic plants expressing the type I *Arabidopsis* vacuolar H^+-PPase gene (*AVP1*). Transgenic plants expressing *AVP1* or the gain-of-function *AVP1D* allele have a larger shoot and root biomass compared to plants without this gene under various abiotic stresses including drought, salinity and low nutrient availability (Bao et al., 2009; Gaxiola et al., 2001; Li et al., 2010; Park et al., 2005; Pasapula et al., 2011; Qin et al., 2013; Schilling et al., 2014; Yang et al., 2007; Yang et al., 2014). The improved growth of transgenic *AVP1* or *AVP1D* plants in stress conditions has been attributed to increased vacuolar Na^+ sequestration reducing the toxicity of Na^+ ions and increased root growth and rhizosphere acidification enhancing water and nutrient uptake (Gaxiola et al., 2001; Park et al., 2005; Yang et al., 2007).

Transgenic plants expressing *AVP1* also have an increase in shoot and root biomass in non-stressed conditions compared to plants without this gene (Gonzalez et al., 2010; Li et al., 2005; Li et al., 2010; Paez-Valencia et al., 2013; Schilling et al., 2014; Vercruyssen et al., 2011; Yang et al., 2007). An increase in auxin may be contributing to the larger growth of transgenic plants expressing *AVP1* (Li et al., 2005). It has been hypothesised that AVP1 alters the amount and distribution of plasma membrane Pinformed 1 (*PIN1*) auxin efflux facilitators, and thus, regulates auxin dependent organogenesis (Li et al., 2005). In support of this, an *Arabidopsis* mutant defective in AVP1 (*avp1*) due to the insertion of transfer-DNA in the fifth exon had poor shoot and root development compared to wild-type (Li et al., 2005). *Arabidopsis* over-expressing *AVP1* and transgenic bentgrass expressing *AVP1* also had higher leaf and root auxin contents respectively than wild-type (Gonzalez et al., 2010; Li et al., 2005; Li et al., 2010). However, *Arabidopsis fugu5* mutants, which are defective in the *AVP1* gene due to point mutations, did not have an auxin phenotype, suggesting that the auxin phenotype of *avp1* plants may be allele specific and that AVP1 may not alter auxin abundance or distribution (Ferjani et al., 2011). It is thus yet to be fully elucidated what is contributing to the larger shoot biomass of transgenic *AVP1* plants compared to null segregants in non-stressed conditions.

One possible factor contributing to the larger shoot growth of transgenic plants expressing *AVP1* is improved seedling vigour (Ferjani et al., 2011). It was recently suggested that, rather than vacuolar acidification, the major role of AVP1 is the hydrolysis of cytosolic PP_i (Ferjani et al., 2011). In support of this, *Arabidopsis fugu5* mutants had 2.5-fold higher PP_i levels than wild-type and lacked heterotrophic growth (Ferjani et al., 2011). The enhanced removal of cytosolic PP_i, which is a by-product of metabolism and an inhibitor of gluconeogenesis at high concentrations, may therefore improve the heterotrophic growth of transgenic plants expressing *AVP1* (Ferjani et al., 2011). However, to date, no studies have evaluated the seedling growth of transgenic plants expressing *AVP1* in non-stressed conditions from seed imbibition.

Another possible factor contributing to the larger growth of transgenic plants expressing *AVP1* is that AVP1 facilitates sucrose phloem-loading, and thus more efficient sucrose transport from source to sink tissue (Gaxiola et al., 2012). In phloem companion cells, it has been hypothesised that AVP1 is localised to the plasma membrane and functions as a PP_i-synthase (Gaxiola et al., 2012). An increase in PP_i synthesis, which increases sucrose respiration and thus ATP supply, would increase the activity of the plasma membrane H^+-ATPase helping to maintain an electrochemical potential difference for H^+ across the plasma membrane meditating sucrose phloem-loading (Gaxiola et al., 2012). In support of this hypothesis, there is evidence that AVP1 is localised to the PM of sieve-element companion cells in *Arabidopsis* (Paez-Valencia et al., 2011) and that is thermodynamically possible for AVP1 to synthesis PP_i rather than hydrolyse PP_i (Baltscheffsky H et al., 1966; Davies et al., 1997; Rocha Façanha and de Meis, 1998; Seufferheld et al., 2004). Furthermore, genes involved with sucrose transport and metabolism, including the sucrose proton symporter *SUC1*, are up-regulated in *Arabidopsis* over-expressing *AVP1* (Gonzalez et al., 2010). Enhanced sucrose transport from leaves (source) to roots (sink) may increase root growth leading to improved nutrient and water uptake in transgenic plants expressing *AVP1* compared to plants without this gene (Gaxiola et al., 2012; Paez-Valencia et al., 2011). However, to date, information regarding the effects of *AVP1* expression on plant metabolism is limited to one study (Gonzalez et al., 2010) and it is yet to be determined whether transgenic plants expressing *AVP1* have altered carbohydrate metabolism.

Previously, we generated transgenic barley lines with the constitutive expression of *AVP1* (Schilling et al., 2014). In saline conditions, transgenic *AVP1* barley had a larger shoot biomass and increased grain yield per plant compared to plants without this gene (Schilling et al., 2014). No significant increase in leaf Na^+ accumulation was evident in the transgenic *AVP1* barley compared to null segregants (Schilling et al., 2014). Additionally, the transgenic *AVP1* barley had a larger shoot biomass in non-saline conditions compared to null segregants (Schilling et al., 2014). Non-destructive plant imaging through time revealed

that transgenic *AVP1* barley were larger at 14 d after seed imbibition and had a faster relative growth rate in the early growth stages (14 to 24 d after seed imbibition) compared to null segregants (Schilling et al., 2014). However, it is not known whether this large shoot biomass of transgenic barley expressing *AVP1* arises from a larger seed weight, improved seedling vigour or altered carbohydrate metabolism (Schilling et al., 2014).

Here, we evaluate the seedling vigour of transgenic barley expressing *AVP1* and null segregants from 0 to 11 d after seed imbibition in non-saline and saline conditions using non-destructive plant biomass measurements and WinRHIZO® imaging of plants. Furthermore, we compare the leaf and root metabolic profiles of transgenic *AVP1* barley and null segregants to investigate the effects of *AVP1* expression on barley metabolism. Our findings suggest that transgenic *AVP1* barley has improved seedling vigour and altered leaf ascorbic acid synthesis, both of which appear to be contributing to the larger shoot and root biomass of transgenic barley expressing *AVP1* compared to null segregants.

Experimental Design

Plant material and seed source

Transgenic barley with constitutive expression of *AVP1*, driven by the *CaMV 35S* promoter, from three independent transformation events (*35S-AVP1*-1, *35S-AVP1*-2, *35S-AVP1*-3) and null segregants were used in this study (Schilling et al., 2014). All plants were genotyped to confirm the presence or absence of *AVP1* (data not shown). Individual T_4 seeds were sourced from plants previously grown in non-stressed greenhouse conditions and were each selected by weight (~ 43 mg).

Non-destructive growth measurement of barley seedlings

A paper roll method was optimised to non-destructively measure the shoot and root growth of barley seedlings through time (Figure S1). Briefly, white Scott® brand paper towels (27 × 24 cm) (FSC C103572,

Kimberly-Clark Professional, Australia) were cut to 18 cm in length. An individual seed was aligned in the centre of the paper towel (1 cm from the edge) with the seed ventral surface facing upwards and embryo positioned towards the base. The paper towel was carefully rolled into a column and soaked for 30 sec in nutrient solution consisting of RO water with the following nutrients (in mM): 0.2 NH_4NO_3, 5.0 KNO_3, 2.0 $Ca(NO_3)_2.4H_2O$, 2.0 $MgSO_4.7H_2O$, 0.1 KH_2PO_4, 0.5 $Na_2Si_3O_7$, 0.05 NaFe(III)EDTA, 0.05 H_3BO_3, 0.005 $MnCl_2.4H_2O$, 0.01 $ZnSO_4.7H_2O$, 0.005 $CuSO_4.5H_2O$ and 0.0001 $Na_2MoO_4.2H_2O$ without (0 mM NaCl) or with the addition of 100 mM NaCl (29.2 g of NaCl in 5 L) and supplementary $CaCl_2.H_2O$ (0.95 g of NaCl in 5 L). Each roll was then placed vertically into a glass jar (20 cm length, 8 cm diameter) (10 paper rolls/jar) wrapped in aluminium foil to prevent root being exposed to light and containing 400 mL of nutrient solution which by capillary action kept the paper rolls moist. A total of 14 replicates for each line were used with 2 replicates per line in each jar and a total of 7 jars per salinity treatment. An aerator stone attached to a Precision SR 7500 aerator was used to provide continuous aeration to each jar and nutrient solutions were changed every 5 d to ensure all nutrients were adequately supplied. The experiment was conducted in a controlled growth chamber (The Plant Accelerator®, Adelaide, Australia) with the following settings: 12 h day length, temperature between 20 to 23°C, lighting at 800 µmol m^{-2} s^{-1}, carbon dioxide (CO_2) at 300 ppm and relative humidity of 52 %.

Imaging of root and cotyledon tissue using a WinRHIZO Pro®

From 2 d after imbibition, the root and shoot tissue of all intact seedlings were imaged every day for 11 d using an A3 Epson Expression 10000 XL 3.49 (Epson, Sydney, Australia) scanner at a grey scale with resolution of 600 dpi. Briefly, each plant was carefully unwrapped from the paper roll and the total plant biomass (g FW) recorded using electronic scales. Each plant was placed in a clear tray (20 × 30 cm) containing the same growth nutrient solution (supplemented with 0 or 100 mM NaCl as appropriate) with all roots carefully arranged to ensure separation. Images were analysed using WinRHIZO Pro® 2009 software (Régent Instruments, Canada) to determine the projected shoot and root area, total root length

and average root diameter of each plant. The final shoot and root biomass was recorded 11 d after imbibition and the 2nd leaf was collected for genotyping the presence or absence of the *AVP1* transgene (Schilling et al., 2014).

Metabolomics analysis of transgenic *AVP1* barley leaf and root tissue

Eleven days after imbibition, the 1st leaf and total roots of 0 mM NaCl treated plants were excised, immediately weighed using electronic scales and placed into 10 mL tubes frozen in liquid nitrogen. Frozen leaf and root samples of transgenic *AVP1* barley were ground to a fine powder, extracted and analysed by Metabolomics Australia (Melbourne, Australia) using a GC-MS system comprising a AS 3000 autosampler, a Trace Gas Chromatography Ultra and a DSQ quadrupole mass spectrometer (ThermoElectron Cooperation, Austin, USA) with either splitless or split-injection following established protocols (Jacobs et al., 2007; Roessner et al., 2006). The amounts of each metabolite were normalised to the fresh tissue weight used for extraction and final metabolite values are presented as a fold-change compared to null segregants with those in bold having a significant t-test value below $P \leq 0.05$.

Visualisation of embryo size and measurement of intact embryo weight

To visual the embryo size of wild-type and transgenic *AVP1* barley, individual seeds (n = 6) were cut longitudinally using a scalpel and both seed halves were stained using Lugol's solution (5 % w/v iodine and 10 % w/v potassium iodine) for 30. Seeds were blotted dry and embryos were visualised using a Leica MZ FLIII stereo-microscope, equipped with a Leica DC 300F camera and Leica IM50 image manager software (Leica Microsystems Ltd, Heerbrugg, Switzerland).

Intact embryos of wild-type and transgenic *AVP1* barley were dissected and weighed following a protocol adapted from Richards & Lukacs (2002). Briefly, seeds weighing 43 mg each were imbibed in a Petri dish containing RO water at 4 ºC overnight in the dark. The embryo of each seed was dissected using forceps

under a Leica MZ FLIII stereo-microscope. The intact embryos were oven dried at 30 ºC for 48 h and dry weights recorded using AX105 DeltaRange electronic scales (Mettler Toledo Ltd., Port Melbourne, Australia).

Cyanoacrylate adhesive adaxial leaf impressions and cell size measurements

A second paper roll experiment was conducted as described above with only a non-saline treatment. Following 11 d after imbibition, the length and width of the 1st leaf of each plant was recorded using a ruler. Cyanoacrylate adhesive leaf impressions using a method adapted from Dhingra and Sinclair (1995) was conducted to measure adaxial leaf cell size. Briefly, the 1st leaf was cut in half and a thin layer of cyanoacrylate adhesive (Selleys Fix 'n' Go Supa Glue, New South Wales, Australia) was uniformly applied to the middle of the adaxial leaf surface. The glued leaf surface was placed against a microscope slide previously cleaned with 100 % ethanol. A second cleaned microscope slide was placed on top of the non-glued leaf side and moderate pressure was applied using a large bulldog clip. After 3 mins, the two microscope slides were separated and the leaf was carefully peeled off the slide using forceps. The adaxial leaf imprint left on the glue was visualised using a Leica AS-LMD Laser Micro-dissection microscope equipped with a video camera and IM1000 software at 4x objective (Leica Microsystems Ltd.). The number and length of adaxial epidermal between vein (bv) cells in the second cell file from the mid-vein were measured in each plant (Figure S2) (Wenzel et al., 1997).

Statistical analysis

Data was 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 $P \leq 0.05$.

Results

Transgenic *AVP1* barley has improved seedling vigour in non-saline conditions

The shoot and root biomass (g FW) of transgenic *AVP1* barley (*35S-AVP1*-1, *35S-AVP1*-2 & *35S-AVP1*-3) at 11 d after imbibition was significantly larger (22 to 26% and 21 to 23% respectively) than null segregants (Figure 1a,b,c). The seed weight (day 0) and total plant biomass between 2 to 5 d after imbibition of transgenic *AVP1* barley did not differ to null segregants (Figure 2a). However, from 6 d after imbibition, the transgenic *AVP1* barley had a significantly greater total plant biomass (g FW) compared to null segregants (Figure 2a). The growth rate (g FW d^{-1}) of all transgenic *AVP1* barley lines was also greater than null segregants between 0 to 5 d after imbibition (Figure 2b). There was no significant difference in total plant biomass from seed imbibition to 11 d after seed imbibition between null segregants and transgenic *AVP1* barley at 100 mM NaCl (Figure S3). Additionally, there was no difference in shoot or root biomass between null segregants and transgenic *AVP1* barley at 100 mM NaCl (Figure S4a,b).

Non-destructive images of individual plants through time were recorded and analysed using WinRHIZO® software (Figure 3a). The projected shoot area (cm^2) of all plants increased from 0 to 11 d after seed imbibition for the 0 mM NaCl treated plants (Figure 3b). Compared to null segregants, the projected shoot area of transgenic *AVP1* barley was larger, particularly for *35S-AVP1*-1 and *35S-AVP1*-2 between 4 to 6 d and 5 to 6 d after imbibition respectively where the difference was significant (Figure 3b). The projected root area (cm^2) of all three transgenic *AVP1* barley lines was significantly greater (13 to 19%) than null segregants 8 to 11 d after imbibition. However, the projected root area of the transgenic *AVP1* barley and null segregants was not significantly different between 3 to 7 d after imbibition (Figure 3c). The total root length (cm) of all plants increased from 3 to 11 d after imbibition with the transgenic *AVP1* barley tending to have longer roots than null segregants from 8 d after seed imbibition (Figure 4a). However, differences in root lengths were not significant, except at 5 d after imbibition when *35S-AVP1*-3 had a significant decrease in total root length compared to null segregants (Figure 4a). The root diameter of all plants

decreased from 3 to 11 d after imbibition with no significant differences between null segregants and transgenic *AVP1* barley (Figure 4b).

35S-AVP1-3 barley had a larger embryo than wild-type

The embryo size of wild-type and transgenic *AVP1* barley seeds of a similar weight were qualitatively assessed using longitudinal seed cross-sections stained with Lugol's solution (Figure 5a & S5). The embryo size of transgenic *AVP1* barley (*35S-AVP1*-3) appeared consistently larger than wild-type (Figure 5a). Furthermore, the individual embryo dry weight (mg DW) of the transgenic *AVP1* barley lines was larger than wild-type, but only significantly larger in *35S-AVP1*-3 (Figure 5b).

Metabolites involved in an ascorbic acid pathway are altered in transgenic *AVP1* barley compared to null segregants

Differences in the leaf and root metabolite profiles of null segregants and transgenic *AVP1* barley plants were observed 11 d after seed imbibition at 0 mM NaCl (Table 1 and 2). All three transgenic *AVP1* barley lines (*35S-AVP1*-1, 35S-*AVP1*-2 and *35S-AVP1*-3) had significantly lower leaf galactose (0.19 to 0.43-fold) compared to null segregants (Table 1 & Figure 6). An increase in leaf ascorbic acid and dehydroascorbic acid was also observed in all three transgenic *AVP1* barley lines compared to null segregants, with *35S-AVP1*-1 and *35S-AVP1*-2 having significantly higher ascorbic acid (1.9-fold and 3.8-fold respectively) and dehydroascorbic acid dimer (15.0-fold and 13.1-fold respectively) (Table 1 & Figure 6). A significant increase in cellobiose (1.7-fold) and inositol (1.5-fold) was also evident in the *35S-AVP1*-1 barley compared to null segregants (Table 1).

In the roots, *35S-AVP1*-1 had a significant reduction in 2-keto-L-gluconic acid (0.62-fold) and ribonate (0.8-fold) and phosphate (0.5-fold) and *35S-AVP1*-2 had a significant decrease in melibose (0.8-fold), N-acetyl-glucosamine (0.72-fold) and phosphate (0.62-fold) compared to null segregants (Table 2). There

was no significant difference in the metabolite profile of *35S-AVP1*-3 root tissue compared to null segregants (Table 2). The leaf and root sucrose fold-changes of transgenic *AVP1* barley also did not differ to null segregants (Table 1 & 2).

The size and number of bv adaxial epidermal cells are unaltered in transgenic *AVP1* barley

The average length (cm) of the 1st leaf blade of transgenic *AVP1* barley 11 d after seed imbibition was not significantly different to null segregants (Figure 7a). The 1st leaf blade of *35S-AVP1*-2 and *35S-AVP1*-3 was significantly wider (cm) than null segregants (Figure 7b). However, the 1st leaf blade width of *35S-AVP1*-1 barley did not differ to null segregants (Figure 7b). The average length and number of bv adaxial epidermal cells of transgenic *AVP1* barley lines also did not differ to null segregants (Figure 7c,d).

Discussion

In this study, transgenic barley expressing *AVP1* had a larger plant biomass, including an increase in both shoot and root biomass, in non-stressed conditions compared to null segregants at 11 d after seed imbibition (Figure 1). Using non-destructive plant biomass measurements from seed imbibition, the larger plant biomass of transgenic *AVP1* barley compared to null segregants was first detectable 6 d after seed imbibition (Figure 2a). The transgenic *AVP1* barley also had a faster relative plant growth rate between 0 to 5 d after seed imbibition, and thus improved seedling vigour, compared to null segregants in non-stressed conditions (Figure 2b). Furthermore, metabolomic analysis revealed that transgenic *AVP1* barley had significantly lower galactose and significantly higher ascorbic acid and dehydroascorbic acid in leaf tissue compared to null segregants at 11 d after seed imbibition (Table 1, Figure 6). It is thus possible that both improved seedling vigour and enhanced ascorbic acid synthesis are contributing to the larger shoot and root biomass of transgenic *AVP1* barley compared to null segregants in non-stressed conditions (Figure 3c).

Transgenic *AVP1* barley has a larger shoot and root biomass in non-stressed conditions

Previously, 14-day-old transgenic barley expressing *AVP1* had a greater projected shoot area, and therefore shoot biomass, than null segregants in both non-saline and saline conditions (Schilling et al., 2014). Additionally, other studies have shown an increase in shoot and root biomass of transgenic plants expressing *AVP1* (Gonzalez et al., 2010; Li et al., 2005; Li et al., 2010; Paez-Valencia et al., 2013; Vercruyssen et al., 2011; Yang et al., 2007) or a gene encoding a H^+-PPase from another plant species (Gouiaa et al., 2012; Li et al., 2014; Lv et al., 2008) in non-stressed conditions. In agreement with these previous studies, the transgenic *AVP1* barley (*35S-AVP1*-1, *35S-AVP1*-2 and *35S-AVP1*-3) in this study also had a larger shoot and root biomass at 11 d after seed imbibition compared to null segregants in non-stressed conditions (Figure 1a,b,c).

Transgenic *AVP1* barley has improved seedling vigour in non-stressed conditions

A paper roll assay using plant biomass measurements and WinRHIZO® analysis was developed in this study to non-destructively evaluate the growth of null segregants and transgenic *AVP1* barley through time from seed imbibition (Figure S1). Using this assay, this study showed that the larger plant biomass of transgenic *AVP1* barley compared to null segregants, was first detectable from 6 d after seed imbibition (Figure 2a). It also showed that the transgenic *AVP1* barley had a faster relative growth compared to null segregants between 0 to 5 d after seed imbibition (Figure 2b). To our knowledge, this is the first time that improved seedling vigour has been demonstrated in a transgenic plant expressing *AVP1*. There were discrepancies between total plant biomass measurements and the derived projected shoot and root areas in this study and higher than expected root diameter values, which both may be due, in part, to known limitations of WinRHIZO® software (Genc et al., 2007; Wang and Zhang, 2009). Nevertheless, an increase in shoot growth in the early stages after seed imbibition, as seen by an increase in projected shoot area between 4 to 6 d after seed imbibition, and then an increase in root growth, as seen by an increase in projected root area from 8 d after seed imbibition, are both contributing to the larger plant biomass of

transgenic *AVP1* barley compared to null segregants (Figure 3b,c). Given that a larger embryo can improve seedling vigour (Richards and Lukacs, 2002), the larger embryo of *35S-AVP1*-3 barley may explain the improved seedling growth of this line (Figure 5a,b). However, the absence of a larger embryo in the other transgenic *AVP1* barley lines (*35S-AVP1*-1 and *35S-AVP1*-2) suggests other factors are also contributing to the improved seedling growth.

It is well known that during the heterotrophic stage of germination, seedling growth is entirely dependent on seed reserves (Aoki et al., 2006; Edelman et al., 1959; Zhang et al., 2007). Previously, loss-of-function *AVP1* mutants (*fugu5*) had 2.5-fold higher PP_i levels and lacked heterotrophic growth without the addition of either sucrose or glucose to their growth media (Ferjani et al., 2011). The heterologous expression of *IPP1*, a gene encoding a cytosolic soluble inorganic H^+-PPase from yeast (*Saccharomyces cerevisiae*) also restored the wild-type phenotype of *fugu5* mutants (Ferjani et al., 2011). This suggests that the hydrolysis of cytoplasmic PP_i by AVP1 may enhance gluconeogenesis and thus improve plant heterotrophic growth (Ferjani et al., 2011). In this study, the increase in plant biomass of transgenic *AVP1* barley compared to null segregants was detected from 6 d after imbibition, suggesting the improved growth of transgenic *AVP1* barley occurs close to the transition from heterotrophic to autotrophic growth. Greater gluconeogenesis could therefore be contributing to the faster growth rate between 0 to 5 d after imbibition, and thus improved seedling vigour, of transgenic barley expressing *AVP1* compared to null segregants (Figure 2b).

In phloem companion cells, it has been suggested that AVP1 is localised to the plasma membrane and functions as a PP_i-synthase (Gaxiola et al., 2012; Paez-Valencia et al., 2011). An increase in PP_i synthesis, which increases sucrose respiration and thus ATP supply, would increase the activity of the plasma membrane H^+-ATPase helping to increase the electrochemical potential difference for H^+ across the plasma membrane (Gaxiola et al., 2012). This enhanced electrochemical potential across the

companion cell plasma membrane would help to meditate sucrose phloem-loading and thus, sucrose transport from leaves (source) to roots (sink) enabling larger root growth (Gaxiola et al., 2012). In support of this hypothesis, the transgenic *AVP1* barley in this study had a larger root biomass compared to null segregants at 11 d after seed imbibition (Figure 1a,c). However, null segregants and transgenic *AVP1* barley had no significant difference in leaf or root sucrose (Table 1 & 2). Nonetheless, these sucrose measurements are only an indication of the sucrose level at one time-point and do not consider differences in sucrose amounts within different cell-types. It thus possible that the amount of sucrose may vary in specific cell types or at specific stages of the lifecycle in the transgenic *AVP1* barley. Considering the transgenic *AVP1* barley had a large plant biomass compared to null segregants (Figure 1, 2), it is also possible that the transgenic *AVP1* barley utilised any additional sucrose as a carbon source to increase plant biomass.

To support developing shoot and roots during the heterotrophic stage of germination, seedlings depend on seed reserves mobilised from the endosperm and transported in vasculature tissue to the embryo (Aoki et al., 2006). It has been proposed that in germinating wheat seeds, starch is converted to maltose and glucose in the endosperm and this maltose and glucose is then transferred to the scutellum where sucrose is re-synthesised in the epidermis before it is symplastically transported to the scutellum vascular parenchyma and loaded into phloem sieve-element cells by apo- or sym-plastic transport, such as via sucrose/H^+ symporters (SUT), for transport to the embryo (Aoki et al., 2006; Edelman et al., 1959). Given the proposed role of AVP1 in facilitating sucrose phloem-loading from source to sink tissue (Gaxiola et al., 2012), it is possible that AVP1 may have a similar role as a H^+-synthase helping to facilitate sucrose loading from the scutellum vascular parenchyma (source) to phloem sieve-element cells, and thus greater sucrose transport to the embryo (sink). The concentration of sucrose is known to be highest in barley seedlings between 3 to 6 d after germination (James, 1940), which corresponds to the timing (6 d after seed imbibition) in this study when the larger plant biomass was detected in the transgenic *AVP1* barley

compared to null segregants (Figure 2a). An increase in starch, which is converted to sucrose during the night (Geiger and Servaites, 1994; Geiger et al., 2000), was also qualitatively observed in the solution surrounding the roots of transgenic *AVP1* barley seedlings compared to wild-type after starch leaked into the solution from the cotyledon after it was excised from the seed (Figure S6). It is thus plausible that the enhanced seedling vigour of transgenic *AVP1* barley at 6 d after seed imbibition reflects the time-point when greater sucrose transport from the scutellum (source) to the embryo (sink) began to enhance shoot and root growth, and thus improve the seedling vigour, of transgenic *AVP1* barley compared to the null segregants.

Transgenic *AVP1* barley does not have improved growth compared to null segregants when salinity occurs from seed imbibition

Unlike other studies showing an increase in shoot or root biomass of transgenic *AVP1* plants in saline conditions (Bao et al., 2009; Gaxiola et al., 2001; Li et al., 2010; Pasapula et al., 2011; Schilling et al., 2014), in this study there was no significant difference in the total plant biomass, or shoot or root biomass, of transgenic *AVP1* barley at 100 mM NaCl compared to null segregants between 0 to 11 d after seed imbibition (Figure S3,S4). This unexpected result suggests that the improved growth of transgenic plants expressing *AVP1* in saline conditions may be dependent on the timing of salinity stress. To date, all previous studies showing an increase in the shoot or root biomass of transgenic plants expressing *AVP1* in saline conditions have either pre-germinated seeds for at least 5 d or allowed plants to grow in non-saline conditions for 2 to 10 weeks before imposing a salinity treatment during vegetative growth stages (Bao et al., 2009; Gaxiola et al., 2001; Li et al., 2010; Pasapula et al., 2011; Qin et al., 2013; Schilling et al., 2014). In contrast, in this study the 100 mM NaCl treatment was initiated from the very start of seed imbibition (0 d). Given that at 0 mM NaCl, transgenic *AVP1* barley had a faster growth rate between 0 to 5 d after imbibition and a larger plant biomass from 6 d after imbibition compared to null segregants (Figure 2), it is possible that the 100 mM NaCl treatment from seed imbibition prevented any increase in

seedling vigour of the transgenic *AVP1* barley by inhibiting any enhanced gluconeogenesis or sucrose-phloem loading. It is thus possible that the larger plant biomass of transgenic *AVP1* barley compared to null segregants in saline conditions may only occur if the onset of salinity occurs following 6 d after seed imbibition. Alternatively, it is possible that the salinity treatment from the start of seed imbibition did not prevent but rather delayed the seedling vigour of transgenic *AVP1* barley and that more time may be necessary for the transgenic *AVP1* barley to first adjust to the effects of salinity on metabolism prior to any improvement of plant growth.

Transgenic barley expressing *AVP1* has enhanced ascorbic acid synthesis compared to null segregants

This study shows for the first time that transgenic barley expressing *AVP1* has lower glucose-6-P, significantly lower galactose and significantly higher ascorbic acid and dehydroascorbic acid in non-stressed conditions compared to null segregants at 11 d after seed imbibition (Table 1). Interestingly, all four metabolites are involved in the Smirnoff-Wheeler pathway (Wheeler et al., 1998), one of four known metabolic pathways proposed for the synthesis of ascorbic acid in plants (Agius et al., 2003; Jain and Nessler, 2000; Lorence et al., 2004) (Figure 6). During the synthesis of ascorbic acid via the Smirnoff-Wheeler pathway, PP_i is produced during the conversion of D-mannose-1 phosphate to GDP-D-mannose via GDP-mannose pyrophosphorylase (Conklin et al., 1999; Keller et al., 1999; Wheeler et al., 1998) (Figure 6). Given the important role of AVP1 in the hydrolysis of cytosolic PP_i to P_i (Ferjani et al., 2011), it is possible the increased ascorbic acid and dehydroascorbic acid in the transgenic *AVP1* barley compared to null segregants is a result of decreased cytosolic PP_i levels favouring the conversion of D-mannose-1 phosphate to GDP-D-mannose, and thus, the synthesis of ascorbic acid (Osorio et al., 2013). In support of this concept, the ripe fruit of transgenic tomato with fruit-specific expression of *ppa*, a soluble pyrophosphatase gene from *Escherichia coli* (*E. coli*), had lower PP_i, lower galactose and higher ascorbic acid and dehydroascorbic acid compared to wild-type (Osorio et al., 2013). Furthermore, tubers of

transgenic potato expressing the same *E. coli* soluble pyrophosphatase gene (*ppa*) had a higher ascorbate concentration compared to parental lines (Farré et al., 2006). Collectively, this suggests that the transgenic barley expressing *AVP1* in this study may have had decreased cytosolic PP_i levels, which increased ascorbic acid synthesis compared to null segregants. Although, is also cannot be ruled out that the increased ascorbic acid and dehydroascorbic acid in the transgenic *AVP1* barley compared to null segregants is due to the transgenic *AVP1* barley being larger (Figure 1) and healthier, and thus having less metabolic demand for these two metabolites.

Increased ascorbic acid could be contributing to the larger shoot and root growth of transgenic *AVP1* barley compared to null segregants

In plants, ascorbic acid (or vitamin C) has a number of roles including cell protection against oxidation damage, as a co-factor of key enzymes, and in the regulation of cell division and cell expansion, flowering time and the onset of leaf senescence (Gallie, 2013; Horemans et al., 2000; Smirnoff, 1996). Accordingly, plants with more ascorbic acid have a larger shoot and root biomass (Lisko et al., 2013) and improved tolerance to various abiotic stresses including salinity, drought, cold, heat and aluminium toxicity (Eltayeb et al., 2011; Hemavathi et al., 2010; Lisko et al., 2013; Shalata and Neumann, 2001; Yin et al., 2010; Zhang et al., 2011). Plants with a high level of ascorbic acid, which increases plant cell division by shortening the G1 phase and thus the cell cycle duration, also have a greater number of cells and, occasionally, smaller cell size (Liso et al., 1988; Pignocchi and Foyer, 2003). Furthermore, a high level of ascorbic acid induces fruit ripening (Agius et al., 2003; Clutter and Miller, 1961; Mellidou et al., 2012) and monozygotic twinning (Chen and Gallie, 2012).

Interestingly, the phenotype of plants with high ascorbic acid closely resemble those apparent in transgenic plants expressing *AVP1*. Transgenic plants expressing *AVP1* or the gain-of-function *AVP1D* allele had a larger shoot and root biomass and increased tolerance to various abiotic stresses including

drought, salinity and low nutrient availability compared to plants without this gene (Bao et al., 2009; Gaxiola et al., 2001; Li et al., 2010; Paez-Valencia et al., 2013; Park et al., 2005; Pasapula et al., 2011; Qin et al., 2013; Schilling et al., 2014; Yang et al., 2007; Yang et al., 2014). Transgenic *Arabidopsis* over-expressing *AVP1* also had a greater number of epidermal cells with no change in cell size compared to wild-type (Gonzalez et al., 2010). The *Arabidopsis fugu5* mutants without AVP1 also had fewer but larger palisade mesophyll cells compared to wild-type (Ferjani et al., 2011) and other *Arabidopsis* mutants without AVP1 had less mesophyll cells with no change in cell size compared to wild-type (Li et al., 2005). Furthermore, compared to wild-type, transgenic tomato plants expressing AVP1D had more ripe fruit (Yang et al., 2014).

In this study, transgenic barley expressing *AVP1* had a larger shoot and root biomass and higher leaf ascorbic acid and dehydroascorbic acid content compared to null segregants in non-stressed conditions. On one occasion, twinning was also observed in the transgenic *AVP1* barley (*35S-AVP1-2*) (Figure S7). These findings suggests there is a link between ascorbic acid and the larger shoot and root biomass of transgenic plants expressing *AVP1* (Table 1, Figure 1a,b,c). The number and size of adaxial bv epidermal cells in transgenic *AVP1* barley was not statistically different to null segregants, although two lines had approximately 10 % more cells (Figure 7c,d). Nonetheless, it cannot be ruled out that the number and/or size of other cell types, such as mesophyll cells, are unaltered in the transgenic *AVP1* barley, particularly given that two transgenic *AVP1* barley lines had an increase in leaf width (Figure 7b) and a larger shoot and root biomass compared to null segregants (Figure 1b,c). It is thus possible that by increasing cell number the higher ascorbic acid and dehydroascorbic acid content is contributing to the larger shoot and root biomass of transgenic barley expressing *AVP1* in non-stressed conditions compared to null segregants. However, it also cannot be ruled out that a decrease in galactose, which at high levels influences carbon input into barley roots and inhibits cell expansion (Farrar et al., 1994), may also be contributing to the larger shoot and root growth of transgenic *AVP1* barley (Table 1).

Conclusions

In this study, transgenic barley expressing *AVP1* had a larger shoot and root biomass compared to null segregants at 11 d after seed imbibition in non-stressed conditions. The transgenic *AVP1* barley also had a faster relative plant growth rate from 0 to 5 d after seed imbibition and a larger total plant biomass compared to null segregants detectable from 6 d after seed imbibition. Metabolomics analysis revealed significant changes in leaf metabolites involved with ascorbic acid synthesis in transgenic *AVP1* barley compared to null segregants. To our knowledge this is the first time a link between vacuolar H^+-PPases and ascorbic acid metabolism has been identified. Overall, the findings of this study suggest that both improved seedling vigour and enhanced ascorbic acid synthesis are contributing to the larger plant biomass of transgenic *AVP1* barley in non-saline conditions.

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Table Legends

Table 1 Metabolite levels of amino acids and amines, sugars, organic acids and other compounds measured in the 1st leaf of null segregants (nulls) and transgenic *AVP1* barley (*35S-AVP1*-1, *35S-AVP1*-2, *35S-AVP1*-3) at 0 mM NaCl. Values are presented as a fold-change ± s.e.m compared to null segregants (set at 1) and those metabolite values shown in bold have a significant t-test value below $P \leq 0.05$. Blue shaded cells indicate a fold-change lower than null segregants and red shaded cells indicate a fold-change higher than null segregants. Green-shaded cells indicate a metabolite measured using a split-injection, with all other metabolites were measured using a splitless injection.

Table 2 Metabolite levels of amino acids and amines, sugars, organic acids and other compounds measured in the root tissue of null segregants (nulls) and transgenic *AVP1* barley (*35S-AVP1*-1, *35S-AVP1*-2, *35S-AVP1*-3) at 0 mM NaCl. Values are presented as a fold-change ± s.e.m compared to null segregants (set at 1) and those metabolite values shown in bold have a significant t-test value below $P \leq 0.05$. Blue shaded cells indicate a fold-change lower than null segregants and red shaded cells indicate a fold-change higher than null segregants. Green-shaded cells indicate a metabolite measured using a split-injection, all other metabolites were measured using a splitless injection.

Tables

Table 1

Leaf Tissue				
Amino Acids & Amines	**Nulls**	***35S-AVP1-1***	**35S-*AVP1*-2**	**35S-*AVP1*-3**
Aspartate	1.000 ± 0.231	0.928 ± 0.102	0.913 ± 0.102	0.848 ± 0.108
Ethanolamine	1.000 ± 0.302	0.982 ± 0.194	0.963 ± 0.205	0.995 ± 0.212
Homoserine	1.000 ± 0.249	0.831 ± 0.222	0.868 ± 0.092	0.775 ± 0.167
Phenylalanine	1.000 ± 0.304	0.623 ± 0.108	0.644 ± 0.053	0.619 ± 0.101
Putrescine	1.000 ± 0.212	0.884 ± 0.074	0.901 ± 0.158	0.888 ± 0.167
Glutamic acid	1.000 ± 0.227	1.157 ± 0.090	1.299 ± 0.103	1.161 ± 0.092
Glutamine	1.000 ± 0.271	1.109 ± 0.111	1.051 ± 0.127	1.021 ± 0.123
Glycine	1.000 ± 0.192	2.381 ± 0.359	1.602 ± 0.204	2.370 ± 0.227
Threonine	1.000 ± 0.242	1.133 ± 0.197	1.105 ± 0.154	1.019 ± 0.182
Tyrosine	1.000 ± 0.223	1.570 ± 0.154	1.695 ± 0.164	1.498 ± 0.190
β-Alanine	1.000 ± 0.213	1.364 ± 0.147	1.467 ± 0.162	1.110 ± 0.149
Alanine	1.000 ± 0.321	1.007 ± 0.185	0.991 ± 0.172	1.055 ± 0.194
Asparagine	1.000 ± 0.255	2.208 ± 0.412	0.576 ± 0.180	2.066 ± 0.309
GABA	1.000 ± 0.152	0.983 ± 0.130	1.019 ± 0.101	0.951 ± 0.124
Isoleucine	1.000 ± 0.291	1.263 ± 0.274	0.728 ± 0.254	0.847 ± 0.213
Leucine	1.000 ± 0.364	1.212 ± 0.293	0.693 ± 0.315	0.749 ± 0.235
Pyroglutamate	1.000 ± 0.064	1.071 ± 0.050	1.027 ± 0.082	0.979 ± 0.058
Serine	1.000 ± 0.158	1.113 ± 0.138	0.839 ± 0.097	1.009 ± 0.122
Tyramine	1.000 ± 0.090	1.157 ± 0.053	0.937 ± 0.082	1.060 ± 0.110
Valine	1.000 ± 0.211	1.189 ± 0.178	1.040 ± 0.187	0.925 ± 0.137
Sugars	**Nulls**	***35S-AVP1-1***	**35S-*AVP1*-2**	**35S-*AVP1*-3**
Galactose	1.000 ± 0.145	**0.191 ± 0.432**	**0.279 ± 0.281**	**0.431 ± 0.198**
Glucose	1.000 ± 0.326	0.375 ± 0.237	0.463 ± 0.174	0.605 ± 0.191
Glucose-6-P	1.000 ± 0.192	0.770 ± 0.079	0.777 ± 0.152	0.964 ± 0.144
Glycerol-3-phosphate	1.000 ± 0.349	0.594 ± 0.110	0.573 ± 0.115	0.674 ± 0.101
Inositol-1-P	1.000 ± 0.198	0.716 ± 0.043	0.708 ± 0.057	0.794 ± 0.054
Melibiose	1.000 ± 0.127	0.919 ± 0.047	0.973 ± 0.079	0.873 ± 0.083
Cellobiose	1.000 ± 0.168	**1.700 ± 0.094**	1.547 ± 0.170	1.292 ± 0.099
Inositol	1.000 ± 0.182	**1.465 ± 0.053**	1.465 ± 0.096	1.212 ± 0.080
Maltose	1.000 ± 0.136	1.254 ± 0.065	1.269 ± 0.143	1.169 ± 0.093
6-Kestose	1.000 ± 0.255	1.195 ± 0.066	1.277 ± 0.150	0.984 ± 0.162
Fructose	1.000 ± 0.319	0.767 ± 0.169	1.039 ± 0.116	0.711 ± 0.099
Galactinol	1.000 ± 0.102	1.173 ± 0.075	1.174 ± 0.052	0.995 ± 0.055
Raffinose	1.000 ± 0.169	1.268 ± 0.124	1.284 ± 0.198	0.954 ± 0.216
Sucrose	1.000 ± 0.205	0.979 ± 0.151	1.195 ± 0.109	0.947 ± 0.116
Xylitol	1.000 ± 0.057	1.028 ± 0.027	1.074 ± 0.071	0.975 ± 0.066
Xylose	1.000 ± 0.094	0.960 ± 0.074	1.233 ± 0.184	0.979 ± 0.104
Organic Acids	**Nulls**	***35S-AVP1-1***	**35S-*AVP1*-2**	**35S-*AVP1*-3**
Glycolic acid	1.000 ± 0.096	0.979 ± 0.057	0.965 ± 0.059	0.986 ± 0.050
Malic acid	1.000 ± 0.094	0.834 ± 0.063	0.966 ± 0.076	0.957 ± 0.050
Malonic acid	1.000 ± 0.151	0.738 ± 0.084	0.809 ± 0.061	0.919 ± 0.065
Quinate	1.000 ± 0.242	**0.504 ± 0.169**	0.610 ± 0.154	0.730 ± 0.073
Shikimate	1.000 ± 0.272	0.697 ± 0.199	0.781 ± 0.127	0.741 ± 0.067
Aconitate	1.000 ± 0.109	1.002 ± 0.044	1.052 ± 0.030	1.017 ± 0.059
Ascorbic acid	1.000 ± 0.172	**1.904 ± 0.159**	**3.761 ± 0.278**	2.930 ± 0.295
Citrate	1.000 ± 0.159	1.273 ± 0.076	1.208 ± 0.084	1.195 ± 0.082
Dehydroascorbic acid dimer	1.000 ± 0.189	**14.964 ± 0.218**	**13.080 ± 0.259**	7.422 ± 0.269
Galactonate	1.000 ± 0.158	1.121 ± 0.045	1.228 ± 0.051	2.882 ± 0.455
Gluconate	1.000 ± 0.155	1.023 ± 0.028	1.038 ± 0.043	1.004 ± 0.043
Glycerate	1.000 ± 0.108	1.130 ± 0.066	1.300 ± 0.135	1.055 ± 0.088
Gulonic acid	1.000 ± 0.149	1.031 ± 0.029	1.084 ± 0.043	1.013 ± 0.050
Maleate	1.000 ± 0.183	1.057 ± 0.081	1.189 ± 0.074	1.124 ± 0.079
Ribonic acid	1.000 ± 0.179	1.077 ± 0.048	1.233 ± 0.072	1.084 ± 0.056
2-keto-L-gluconic acid	1.000 ± 0.137	0.818 ± 0.082	1.123 ± 0.136	0.876 ± 0.072
2-oxo-Glutarate	1.000 ± 0.156	0.831 ± 0.099	1.001 ± 0.094	0.805 ± 0.103
Fumarate	1.000 ± 0.111	0.783 ± 0.056	0.917 ± 0.033	1.037 ± 0.069
Glucarate	1.000 ± 0.090	1.024 ± 0.048	0.995 ± 0.106	1.014 ± 0.054
Isocitrate	1.000 ± 0.094	1.027 ± 0.060	0.899 ± 0.060	0.919 ± 0.049
Succinate	1.000 ± 0.079	0.961 ± 0.079	1.157 ± 0.087	1.136 ± 0.084
Threonate	1.000 ± 0.094	0.944 ± 0.070	1.015 ± 0.066	0.983 ± 0.064
Other Compounds	**Nulls**	***35S-AVP1-1***	**35S-*AVP1*-2**	**35S-*AVP1*-3**
Campesterol	1.000 ± 0.349	0.700 ± 0.234	0.597 ± 0.245	0.557 ± 0.215
Hexadecanoate	1.000 ± 0.063	0.911 ± 0.042	0.874 ± 0.040	0.899 ± 0.039
Octadecanoate	1.000 ± 0.064	0.893 ± 0.047	0.846 ± 0.058	0.858 ± 0.049
Oleic_acid	1.000 ± 0.164	0.841 ± 0.079	0.837 ± 0.071	0.882 ± 0.077
Phosphate	1.000 ± 0.353	0.580 ± 0.075	**0.528 ± 0.043**	0.635 ± 0.088
Un_156_10.08	1.000 ± 0.215	0.931 ± 0.095	0.823 ± 0.127	0.752 ± 0.116
Un_204_33.73	1.000 ± 0.424	0.513 ± 0.066	0.554 ± 0.035	0.670 ± 0.184
Un_286_28.70	1.000 ± 0.083	0.955 ± 0.063	0.905 ± 0.061	0.873 ± 0.079
Un_308_21.83	1.000 ± 0.202	0.873 ± 0.069	0.910 ± 0.046	0.838 ± 0.104
Digalactosylglycerol	1.000 ± 0.177	1.367 ± 0.093	1.319 ± 0.137	1.317 ± 0.103
Galactosylglycerol	1.000 ± 0.209	**1.499 ± 0.085**	1.501 ± 0.141	1.274 ± 0.111
Un_14.86_191	1.000 ± 0.102	1.161 ± 0.044	1.009 ± 0.123	1.142 ± 0.062
Un_221_35.61	1.000 ± 0.095	1.039 ± 0.150	1.005 ± 0.089	1.182 ± 0.178
Un_231_18.06	1.000 ± 0.209	1.152 ± 0.055	1.208 ± 0.178	1.172 ± 0.058
Un_242_18.38	1.000 ± 0.127	1.247 ± 0.061	1.071 ± 0.115	1.186 ± 0.062
Un_315_11.67	1.000 ± 0.138	1.184 ± 0.056	1.020 ± 0.107	1.103 ± 0.086
Un_380_21.50	1.000 ± 0.205	1.173 ± 0.183	1.113 ± 0.160	1.278 ± 0.134
Un_394_22.16	1.000 ± 0.260	1.266 ± 0.094	1.517 ± 0.139	1.205 ± 0.106
Linoleic_acid	1.000 ± 0.236	1.108 ± 0.155	0.784 ± 0.276	0.946 ± 0.150
Monomethylphosphate	1.000 ± 0.092	1.037 ± 0.055	0.990 ± 0.074	0.995 ± 0.050
N-Acetyl_glucosamine	1.000 ± 0.227	0.943 ± 0.202	1.002 ± 0.226	1.003 ± 0.144
Octadecatrienoic acid	1.000 ± 0.264	1.061 ± 0.170	0.890 ± 0.314	0.955 ± 0.204
Un_241_18.15	1.000 ± 0.271	1.089 ± 0.241	1.213 ± 0.359	0.931 ± 0.168
Un_292_15.53	1.000 ± 0.144	0.943 ± 0.054	1.083 ± 0.077	0.866 ± 0.073

Table 2

Root Tissue				
Amino Acids & Amines	**Nulls**	***35S-AVP1-1***	**35S-*AVP1*-2**	**35S-*AVP1*-3**
Aspartate	1.000 ± 0.058	0.892 ± 0.077	0.961 ± 0.044	0.951 ± 0.051
Glutamate	1.000 ± 0.238	0.920 ± 0.089	0.920 ± 0.135	0.993 ± 0.108
Glutamine	1.000 ± 0.193	0.871 ± 0.140	0.573 ± 0.246	0.717 ± 0.160
Pyroglutamate	1.000 ± 0.115	0.842 ± 0.096	0.910 ± 0.056	0.966 ± 0.092
Alanine	1.000 ± 0.174	1.044 ± 0.071	1.201 ± 0.061	1.115 ± 0.053
Glycine	1.000 ± 0.094	1.010 ± 0.045	1.013 ± 0.032	1.017 ± 0.023
Serine	1.000 ± 0.117	1.133 ± 0.060	1.036 ± 0.074	1.072 ± 0.054
Threonine	1.000 ± 0.119	1.211 ± 0.074	1.131 ± 0.062	1.092 ± 0.068
Tyramine	1.000 ± 0.222	1.107 ± 0.150	1.200 ± 0.157	1.062 ± 0.054
Valine	1.000 ± 0.216	1.039 ± 0.148	1.012 ± 0.095	1.151 ± 0.064
Ethanolamine	1.000 ± 0.134	0.979 ± 0.051	0.979 ± 0.061	1.079 ± 0.040
Isoleucine	1.000 ± 0.201	1.014 ± 0.163	0.533 ± 0.300	0.751 ± 0.226
Leucine	1.000 ± 0.158	1.057 ± 0.093	0.908 ± 0.075	1.001 ± 0.113
Phenylalanine	1.000 ± 0.297	0.927 ± 0.188	1.013 ± 0.197	0.978 ± 0.156
Putrescine	1.000 ± 0.157	0.969 ± 0.060	0.986 ± 0.103	1.035 ± 0.058
Sugars & Sugar Phosphates	**Nulls**	***35S-AVP1-1***	**35S-*AVP1*-2**	**35S-*AVP1*-3**
Galactinol	1.000 ± 0.172	0.874 ± 0.072	0.691 ± 0.109	0.947 ± 0.148
Melibiose	1.000 ± 0.055	0.950 ± 0.055	**0.814 ± 0.054**	0.954 ± 0.043
Ribonate	1.000 ± 0.056	**0.800 ± 0.069**	0.970 ± 0.107	0.858 ± 0.080
Sucrose	1.000 ± 0.067	0.883 ± 0.050	0.828 ± 0.055	0.954 ± 0.062
Fructose	1.000 ± 0.182	1.359 ± 0.174	1.107 ± 0.070	1.353 ± 0.145
Fructose-6-p	1.000 ± 0.150	1.209 ± 0.084	1.115 ± 0.076	1.218 ± 0.070
Galactonate	1.000 ± 0.207	1.033 ± 0.136	1.032 ± 0.174	1.069 ± 0.168
Galactosylglycerol	1.000 ± 0.142	1.193 ± 0.074	1.061 ± 0.065	1.207 ± 0.067
Digalactosylglycerol	1.000 ± 0.148	1.261 ± 0.047	0.931 ± 0.070	1.150 ± 0.084
Glucose	1.000 ± 0.191	1.072 ± 0.096	0.946 ± 0.055	1.217 ± 0.130
Glucose-6-P	1.000 ± 0.170	0.868 ± 0.167	1.027 ± 0.173	0.870 ± 0.208
Inositol	1.000 ± 0.181	1.067 ± 0.066	0.944 ± 0.077	1.040 ± 0.040
Inositol-1-P	1.000 ± 0.101	0.999 ± 0.070	1.018 ± 0.067	0.907 ± 0.075
Maltose	1.000 ± 0.106	0.892 ± 0.064	0.873 ± 0.131	1.136 ± 0.085
Raffinose	1.000 ± 0.140	1.015 ± 0.081	0.892 ± 0.071	1.220 ± 0.113
Trehalose	1.000 ± 0.285	0.699 ± 0.090	0.874 ± 0.089	1.234 ± 0.219
Xylose	1.000 ± 0.163	0.950 ± 0.080	1.002 ± 0.080	0.991 ± 0.086
Organic Acids	**Nulls**	***35S-AVP1-1***	**35S-*AVP1*-2**	**35S-*AVP1*-3**
2-keto-L-gluconic acid	1.000 ± 0.120	**0.624 ± 0.087**	0.822 ± 0.155	0.822 ± 0.156
2-oxo-Glutarate	1.000 ± 0.125	0.846 ± 0.091	0.815 ± 0.084	0.866 ± 0.110
Glycerate	1.000 ± 0.094	0.996 ± 0.073	0.954 ± 0.044	0.997 ± 0.055
Quinate	1.000 ± 0.084	0.894 ± 0.051	0.919 ± 0.063	0.926 ± 0.058
Shikimate	1.000 ± 0.084	0.899 ± 0.051	0.918 ± 0.062	0.927 ± 0.057
Fumarate	1.000 ± 0.150	1.068 ± 0.088	1.018 ± 0.068	1.090 ± 0.079
Aconitate	1.000 ± 0.085	0.951 ± 0.083	1.092 ± 0.068	0.946 ± 0.069
Azelaic acid	1.000 ± 0.043	1.050 ± 0.062	0.972 ± 0.044	1.061 ± 0.057
Citrate	1.000 ± 0.101	1.068 ± 0.046	0.880 ± 0.057	0.996 ± 0.073
Glucarate	1.000 ± 0.043	1.050 ± 0.062	0.972 ± 0.044	1.061 ± 0.057
Gluconate	1.000 ± 0.033	0.979 ± 0.043	0.984 ± 0.052	1.009 ± 0.050
Glycolic acid	1.000 ± 0.071	0.981 ± 0.106	1.002 ± 0.088	0.934 ± 0.085
Gulonic acid	1.000 ± 0.049	0.961 ± 0.036	1.016 ± 0.041	0.993 ± 0.040
Maleate	1.000 ± 0.094	1.093 ± 0.072	0.929 ± 0.075	0.907 ± 0.082
Pyruvic acid	1.000 ± 0.129	0.932 ± 0.088	1.000 ± 0.079	1.040 ± 0.088
Succinate	1.000 ± 0.175	1.193 ± 0.080	0.909 ± 0.036	1.215 ± 0.090
Threonate	1.000 ± 0.094	1.020 ± 0.116	0.969 ± 0.126	1.248 ± 0.084
Other Compounds	**Nulls**	***35S-AVP1-1***	**35S-*AVP1*-2**	**35S-*AVP1*-3**
Monomethylphosphate	1.000 ± 0.223	0.774 ± 0.083	0.688 ± 0.077	0.874 ± 0.123
N-Acetyl_glucosamine	1.000 ± 0.109	0.791 ± 0.066	**0.719 ± 0.067**	0.757 ± 0.088
Phosphate	1.000 ± 0.186	**0.540 ± 0.094**	**0.617 ± 0.061**	0.731 ± 0.147
Un_14.86_191	1.000 ± 0.162	0.892 ± 0.114	0.957 ± 0.042	0.946 ± 0.055
Un_242_18.38	1.000 ± 0.136	0.849 ± 0.103	0.974 ± 0.043	0.953 ± 0.071
Un_380_21.50	1.000 ± 0.142	0.732 ± 0.082	0.712 ± 0.078	0.867 ± 0.140
Urea	1.000 ± 0.319	0.785 ± 0.172	0.944 ± 0.322	0.858 ± 0.272
Linoleic_acid	1.000 ± 0.062	1.198 ± 0.060	1.096 ± 0.080	1.203 ± 0.062
Octadecatrienoic acid	1.000 ± 0.117	1.215 ± 0.063	1.060 ± 0.087	1.194 ± 0.068
Oleic_acid	1.000 ± 0.046	1.139 ± 0.070	1.198 ± 0.089	1.084 ± 0.092
Un_204_33.73	1.000 ± 0.164	1.058 ± 0.097	1.013 ± 0.102	1.092 ± 0.107
Campesterol	1.000 ± 0.137	0.957 ± 0.098	0.764 ± 0.125	1.256 ± 0.151
Hexadecanoate	1.000 ± 0.078	1.045 ± 0.050	1.104 ± 0.026	0.987 ± 0.042
Octadecanoate	1.000 ± 0.087	0.966 ± 0.047	1.050 ± 0.033	1.009 ± 0.042
Un_221_35.61	1.000 ± 0.097	2.715 ± 0.415	**0.719 ± 0.102**	2.432 ± 0.427
Un_231_18.06	1.000 ± 0.069	0.919 ± 0.113	1.079 ± 0.085	0.955 ± 0.093
Un_241_18.15	1.000 ± 0.120	1.101 ± 0.096	0.887 ± 0.174	1.100 ± 0.120
Un_308_21.83	1.000 ± 0.080	1.071 ± 0.049	0.920 ± 0.056	0.849 ± 0.066
Un_357_19.07	1.000 ± 0.173	1.005 ± 0.106	1.158 ± 0.132	0.942 ± 0.118
Uracil	1.000 ± 0.131	1.072 ± 0.143	1.139 ± 0.118	0.926 ± 0.137

Figure Legends

Figure 1 (a) A representative image of two null segregants and two transgenic *AVP1* barley (*35S-AVP1*-3) seedlings 11 d after imbibition at 0 mM NaCl and the (b) shoot and (c) root biomass (g DW) of null segregants (nulls) and transgenic *AVP1* barley (*35S-AVP1*-1, *35S-AVP1*-2 and *35S-AVP1*-3) 11 d after seed imbibition at 0 mM NaCl. Values are presented as the mean ± s.e.m (*n* = 6-14) with an asterisks (*) indicating a significant difference compared to wild-type (LSD, $P \leq 0.05$).

Figure 2 (a) The total plant biomass (g FW) of null segregants (◇) and transgenic *AVP1* barley *35S-AVP1*-1 (●), *35S-AVP1*-2 (■) and *35S-AVP1*-3 (▲) in 0 mM NaCl at 3, 4, 5, 6, 7, 8, 9, 10, and 11 d after imbibition. Values are presented as the mean ± s.e.m (*n* = 6-14) with an asterisks (*) indicating a significant difference compared to wild-type (LSD, $P \leq 0.05$). (b) the plant growth rates (g FW d^{-1}) of null segregants and transgenic *AVP1* barley (*35S-AVP1*-1, *35S-AVP1*-2 and *35S-AVP1*-3) between 0 to 5 d after seed imbibition.

Figure 3 (a) WinRHIZO® derived digital images showing the non-destructive imaging of intact wild-type barley (cv. Golden Promise) seedlings through time from 3 to 9 d after imbibition at 0 mM NaCl (b) the projected shoot area (cm^2) and (c) the projected root area (cm^2) of null segregants and transgenic *AVP1* barley (*35S-AVP1*-1, *35S-AVP1*-2 and *35S-AVP1*-3) at 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 d after imbibition at 0 mM NaCl. Values are presented as the mean ± s.e.m (*n* = 6-14) with an asterisks (*) indicating a significant difference compared to wild-type (LSD, $P \leq 0.05$).

Figure 4 (a) The total root length (cm) and (b) average root diameter of null segregants and transgenic *AVP1* barley (*35S-AVP1*-1, *35S-AVP1*-2 and *35S-AVP1*-3) at 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 d after imbibition at 0 mM NaCl derived from WinRHIZO® software analysis. Values are presented as the mean

± s.e.m (*n* = 6-14) with an asterisks (*) indicating a significant difference compared to wild-type (LSD, *P* ≤ 0.05).

Figure 5 (a) Representative images of two longitudinal cross-sections of wild-type (cv. Golden Promise) and *35S-AVP1*-3 seeds stained with Lugol's solution with the seed coat, endosperm (dark black) and embryo (yellow) labelled. (b) The average dry weight of individual intact embryos of wild-type (white bar) and transgenic *AVP1* barley (*35S-AVP1*-1, *35S-AVP1*-2 and *35S-AVP1*-3) (grey bars) from seeds weighing 43 mg. Values are presented as the mean ± s.e.m (*n* = 6) with an asterisks (*) indicating a significant difference compared to wild-type (LSD, *P* ≤ 0.05).

Figure 6 The fold-changes of glucose-6-P, galactose, ascorbic acid and dehydroascrobic acid measured in the 1st leaf of 11-day-old transgenic *AVP1* barley lines (*35S-AVP1*-1, *35S-AVP1*-2 & *35S-AVP1*-3) relative to null segregants (set at 1, indicated as a line) at 0 mM NaCl in the ascorbic acid pathway proposed by Wheeler et al. (1998). Enzymes involved in the pathway are labelled 1 to 11 including 1. Hexose phosphate isomerase (EC 5.3.1.9); 2, phosphomannose isomerase (EC 5.1.3.1.8); 3, phosphomannose mutase (EC 5.4.2.8); 4, GDP-D-mannose pyrophosphorylase (EC 5.1.3.18); 5, GDP-D-mannose-3',5'-epimerase (EC 5.1.3.18); 6, GDP-galactose phosphorylase (EC 2.7.7.B2); 7, L-galactose-1-phosphate phosphatase; 8, L-galactose dehydrogenase (EC 1.1.1.48); 9, L-galactono-1,4-lactone dehydrogenase (EC 1.3.2.3); 10, monodehydroascorbate reductase (MDHAR) (EC 1.6.5.4); 10, dehydroascorbate reductase (DHAR) (EC 1.8.5.1). Values are presented as the mean ± s.e.m (*n* = 6-14) with an asterisks (*) indicating a significant difference compared to null segregants (t-test, *P* ≤ 0.05).

Figure 7 The (a) length and (b) width (cm) of the 1st leaf and the adaxial epidermal (c) between veins (bv) cell length (μM) and (d) number of bv cells of null segregants (white bar) and transgenic *AVP1* barley (*35S-AVP1*-1, *35S-AVP1*-2 and *35S-AVP1*-3) (grey bars) seedlings at 11 d after imbibition at 0 mM NaCl.

Values are presented as the mean ± s.e.m (*n* = 3-6) with an asterisks (*) indicating a significant difference compared to null segregants (LSD, $P \leq 0.05$).

Figures

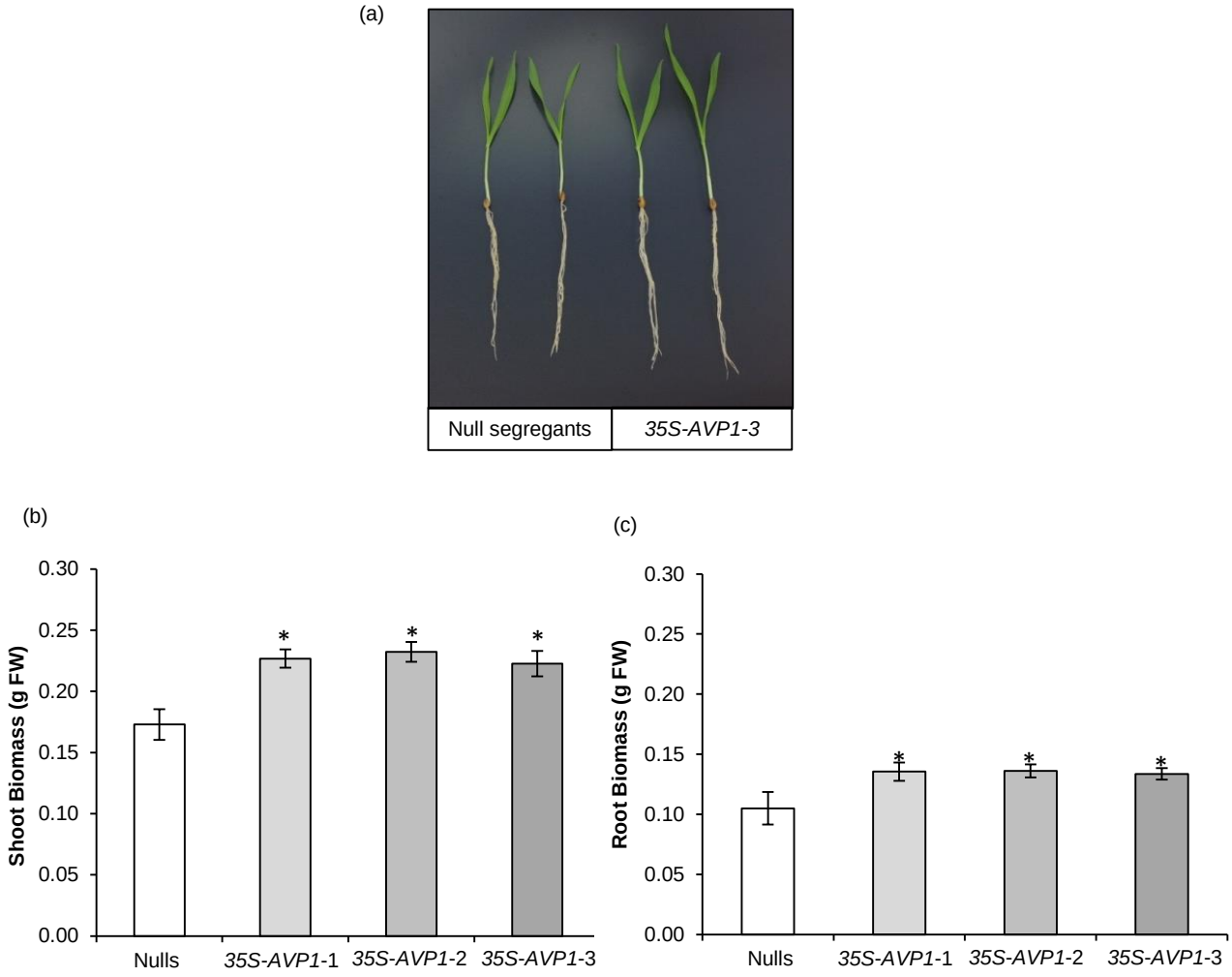
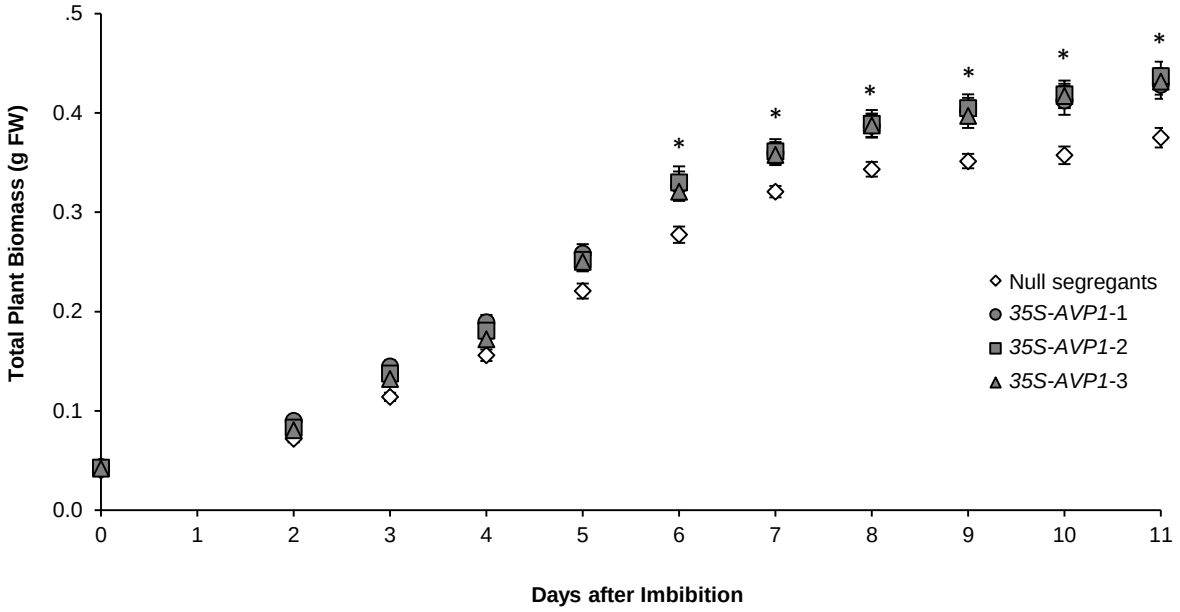


Figure 1

(a)



(b)

Lines	Relative growth rate (g FW d^{-1}) 0 to 5 d
Null segregants	0.3465
35S-AVP1-1	0.3614
35S-AVP1-2	0.3594
35S-AVP1-3	0.3598

Figure 2

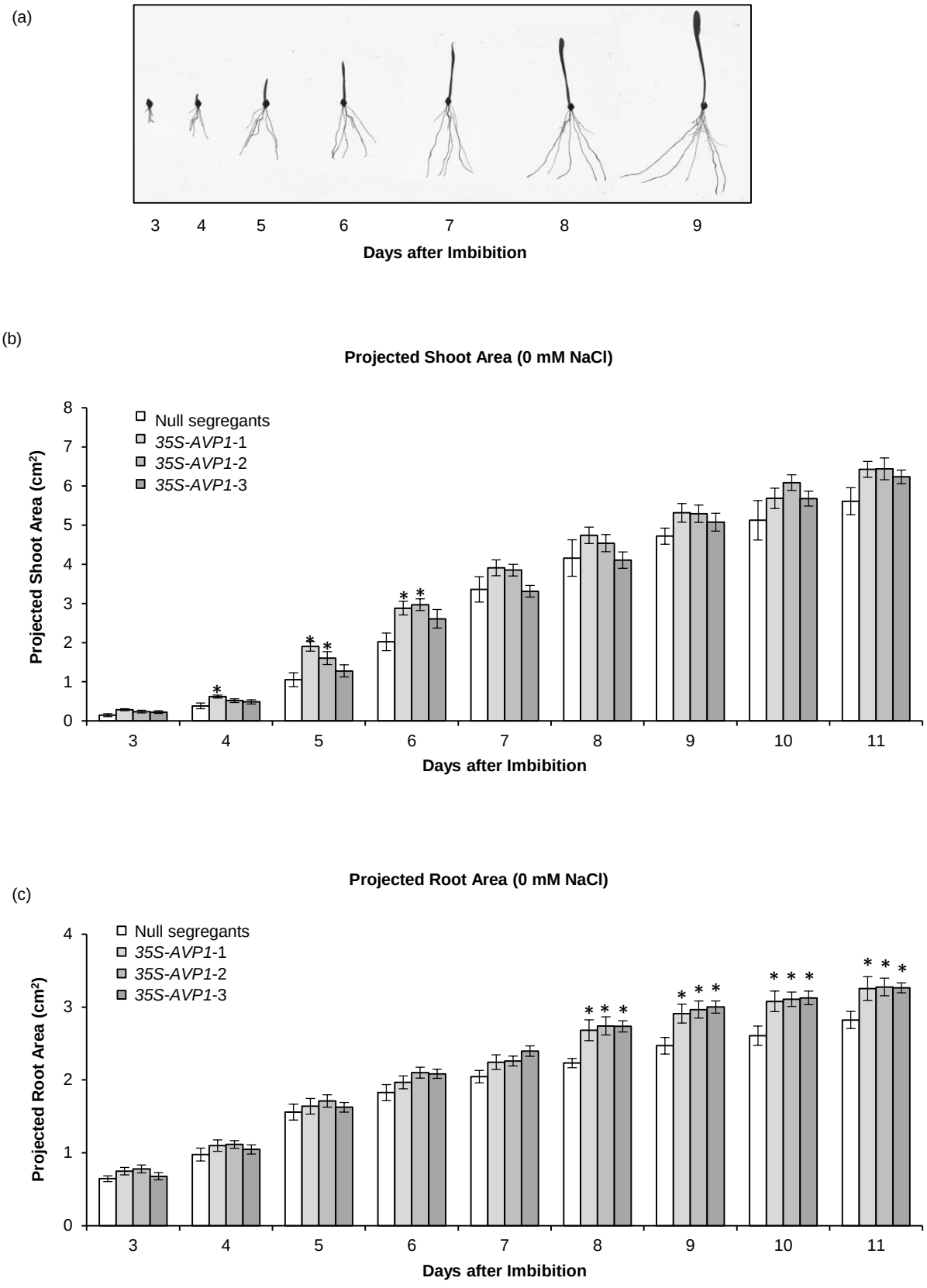
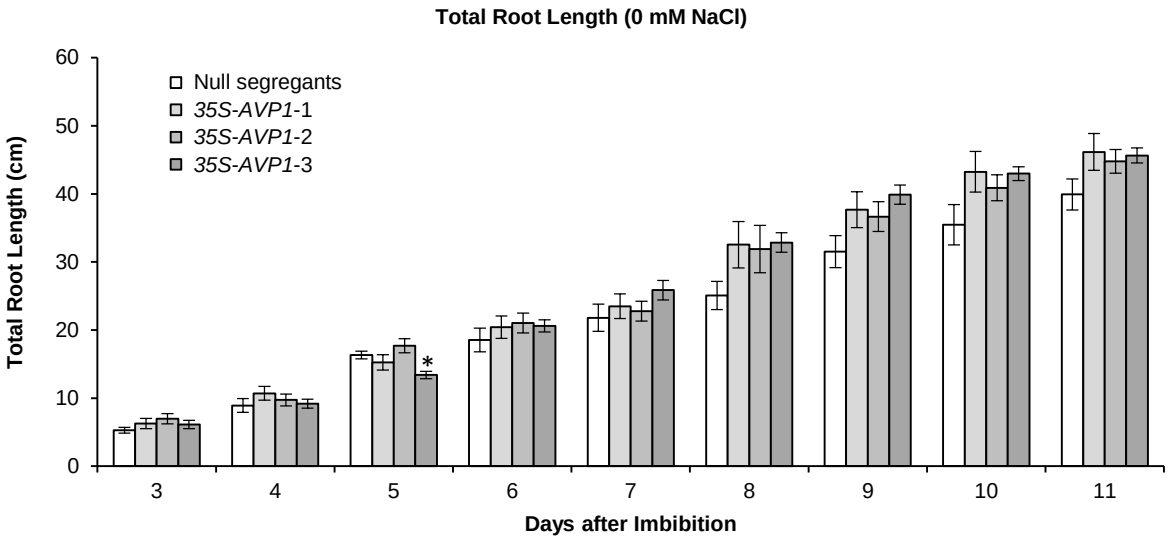

(a)
3
4
5
6
7
8
9
Days after Imbibition
(b)
Projected Shoot Area (0 mM NaCl)
Null segregants
35S-AVP1-1
35S-AVP1-2
35S-AVP1-3
Projected Shoot Area (cm²)
Days after Imbibition
(c)
Projected Root Area (0 mM NaCl)
Null segregants
35S-AVP1-1
35S-AVP1-2
35S-AVP1-3
Projected Root Area (cm²)
Days after Imbibition

Figure 3

(a)


Total Root Length (0 mM NaCl)
Null segregants
35S-AVP1-1
35S-AVP1-2
35S-AVP1-3
Total Root Length (cm)
Days after Imbibition
*

(b)

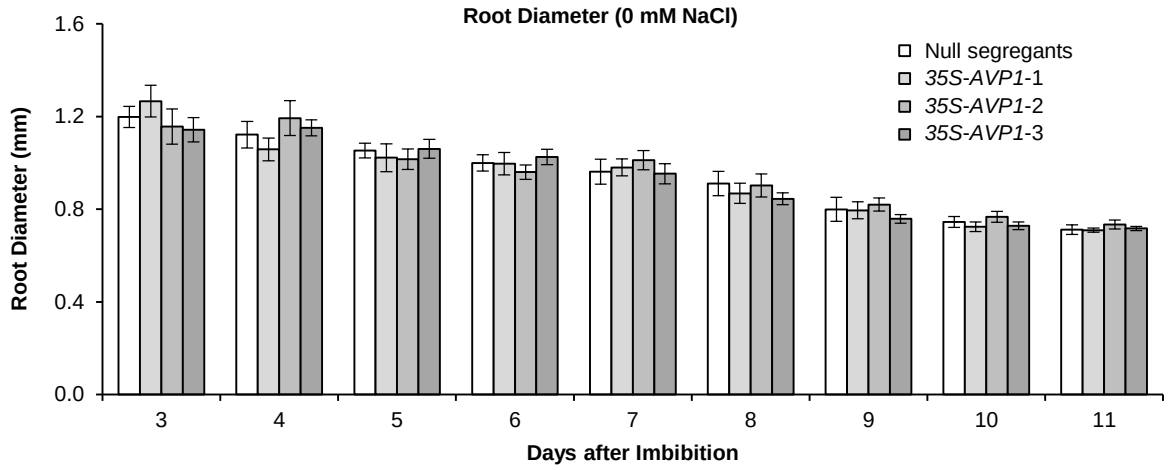

Root Diameter (0 mM NaCl)
Null segregants
35S-AVP1-1
35S-AVP1-2
35S-AVP1-3
Root Diameter (mm)
Days after Imbibition

Figure 4

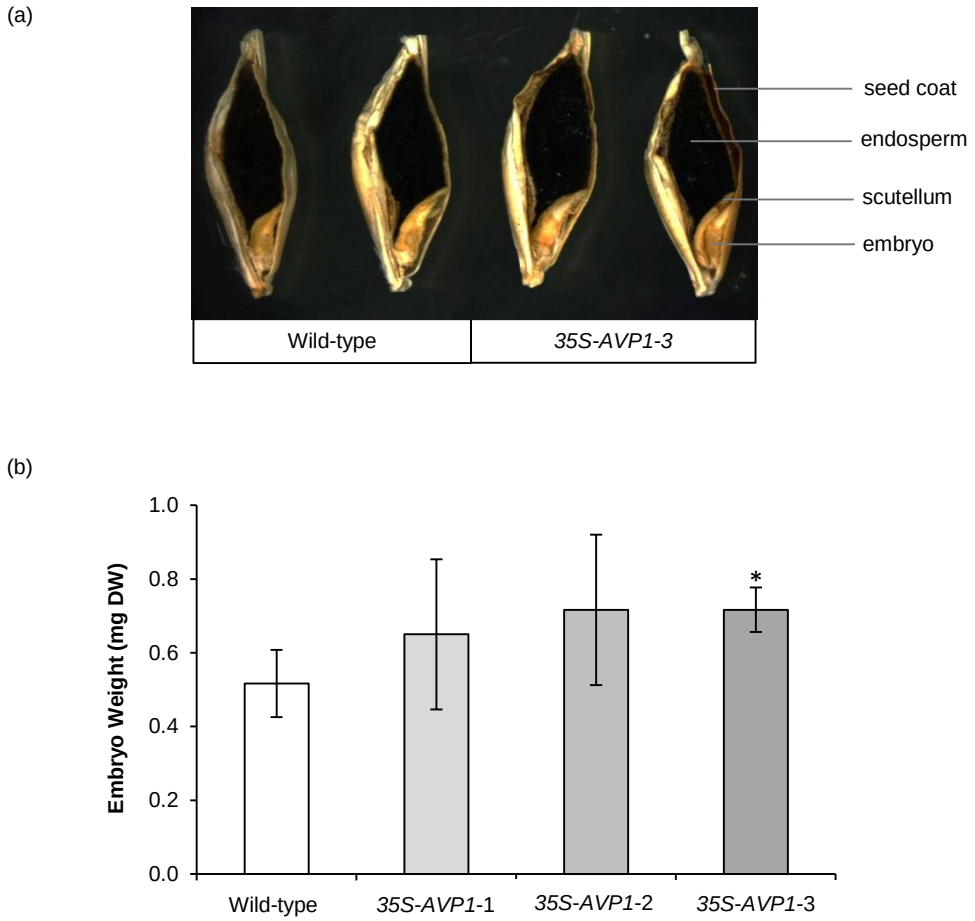

(a)
seed coat
endosperm
scutellum
embryo
Wild-type
35S-AVP1-3
(b)
Embryo Weight (mg DW)
1.0
0.8
0.6
0.4
0.2
0.0
*
Wild-type
35S-AVP1-1
35S-AVP1-2
35S-AVP1-3

Figure 5

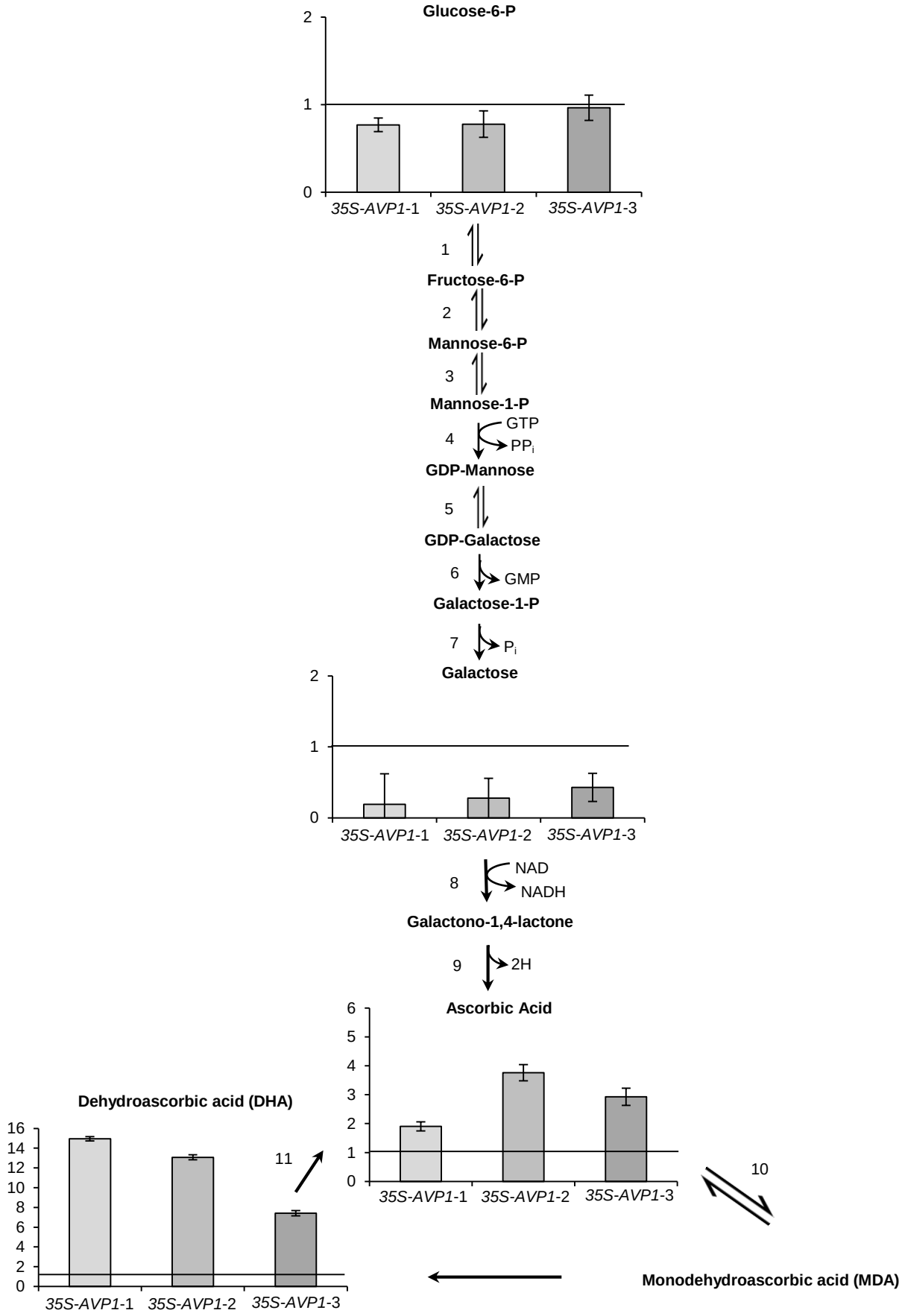

Glucose-6-P
2
1
0
35S-AVP1-1
35S-AVP1-2
35S-AVP1-3
1
Fructose-6-P
2
Mannose-6-P
3
Mannose-1-P
4
GTP
PPi
GDP-Mannose
5
GDP-Galactose
6
GMP
Galactose-1-P
7
Pi
Galactose
2
1
0
35S-AVP1-1
35S-AVP1-2
35S-AVP1-3
8
NAD
NADH
Galactono-1,4-lactone
9
2H
Ascorbic Acid
6
5
4
3
2
1
0
35S-AVP1-1
35S-AVP1-2
35S-AVP1-3
10
Dehydroascorbic acid (DHA)
16
14
12
10
8
6
4
2
0
11
35S-AVP1-1
35S-AVP1-2
35S-AVP1-3
Monodehydroascorbic acid (MDA)

Figure 6

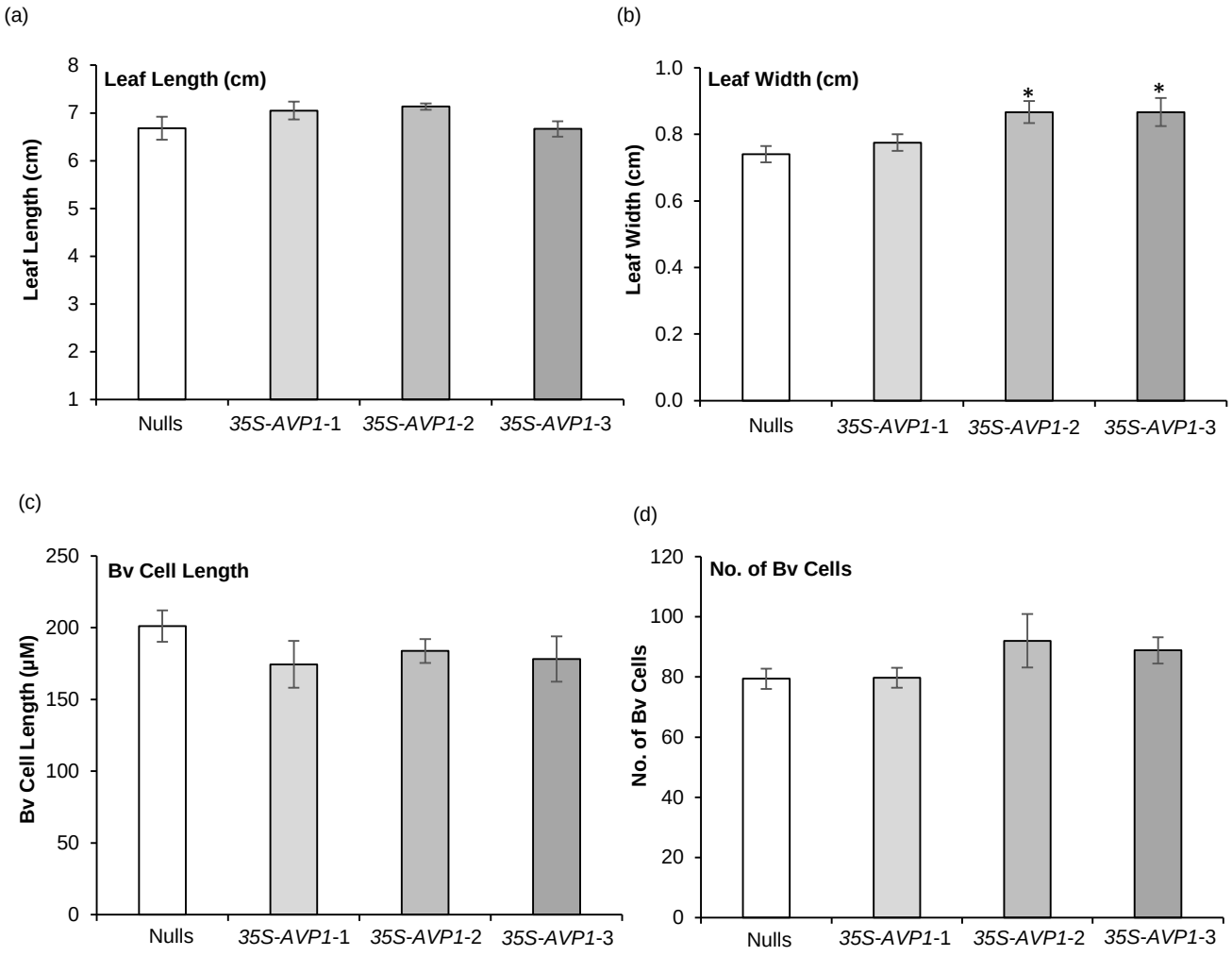

(a)
Leaf Length (cm)
Leaf Length (cm)
8
7
6
5
4
3
2
1
Nulls
35S-AVP1-1
35S-AVP1-2
35S-AVP1-3
(b)
Leaf Width (cm)
Leaf Width (cm)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Nulls
35S-AVP1-1
35S-AVP1-2
35S-AVP1-3
(c)
Bv Cell Length
Bv Cell Length (µM)
250
200
150
100
50
0
Nulls
35S-AVP1-1
35S-AVP1-2
35S-AVP1-3
(d)
No. of Bv Cells
No. of Bv Cells
120
100
80
60
40
20
0
Nulls
35S-AVP1-1
35S-AVP1-2
35S-AVP1-3

Figure 7

Supplementary Figures

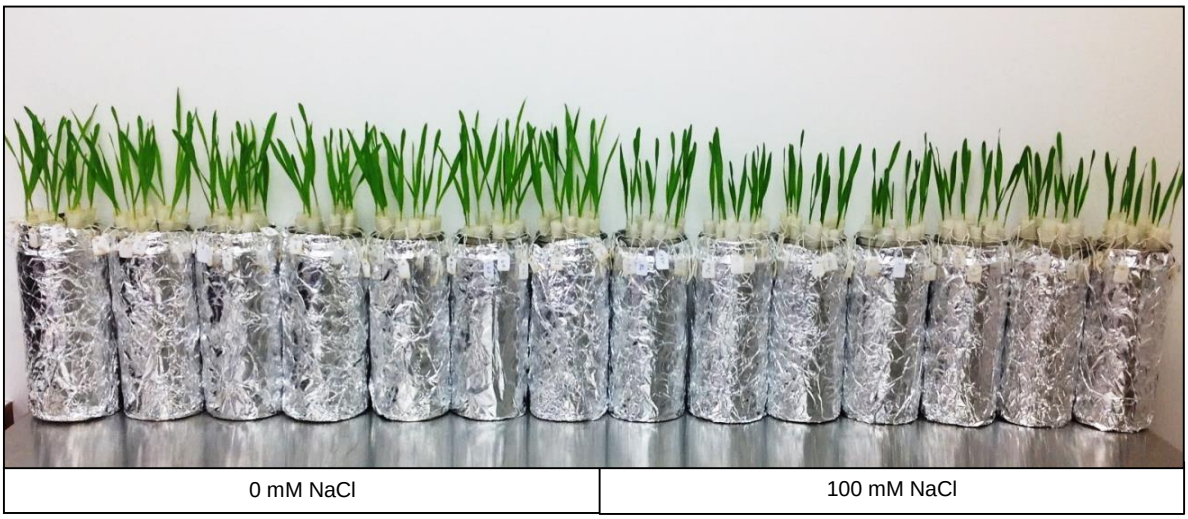


Figure S1 An image of 11-day-old null segregants and transgenic *AVP1* barley (*35S-AVP1*-1, *35S-AVP1*-2 and *35S-AVP1*-3) in a non-destructive paper roll germination assay at 0 mM NaCl (non-saline) and 100 mM NaCl (saline) treatments.

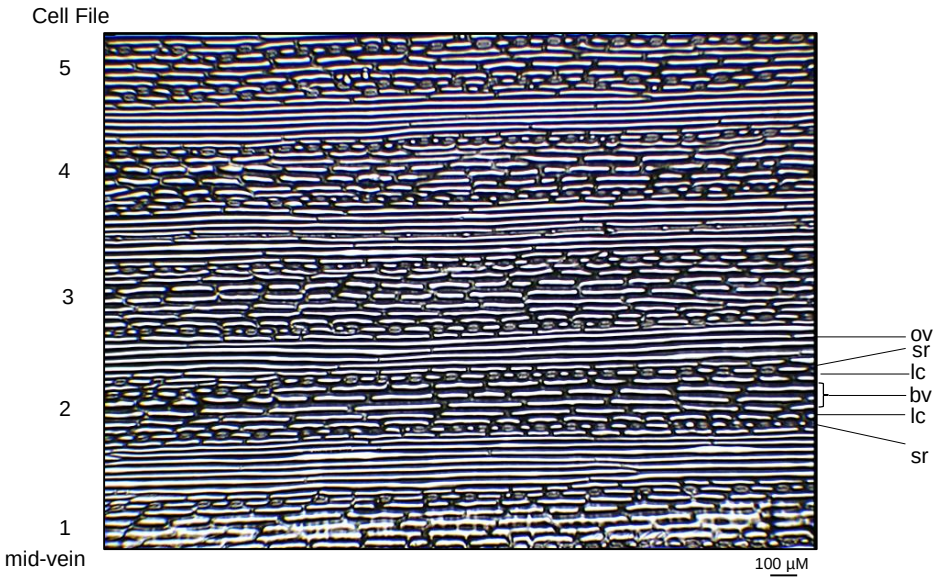


Figure S2 A microscope image of an adaxial leaf imprint of wild-type barley (cv. Golden Promise). The cell files (labelled 1 to 5) are positioned from the mid-vein towards the leaf edge. Stomata row (sr), lateral cells (lc), between veins (bv) cells and over vein (ov) cells were classified according to Wenzel et al. (1997).

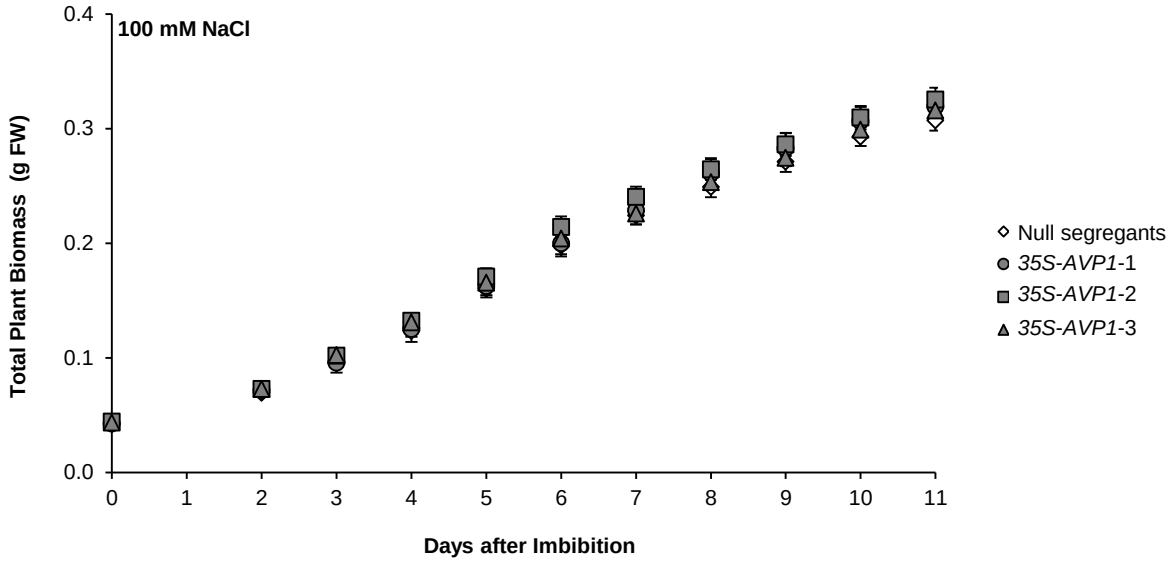


Figure S3 The total plant biomass (g FW) of null segregants and transgenic *AVP1* barley lines (*35S-AVP1*-1, *35S-AVP1*-2 & *35S-AVP1*-3) from 0 d (seed weight) and 2 to 11 d after imbibition in 100 mM NaCl solution. Values are presented as the mean ± s.e.m (*n* = 8-14).

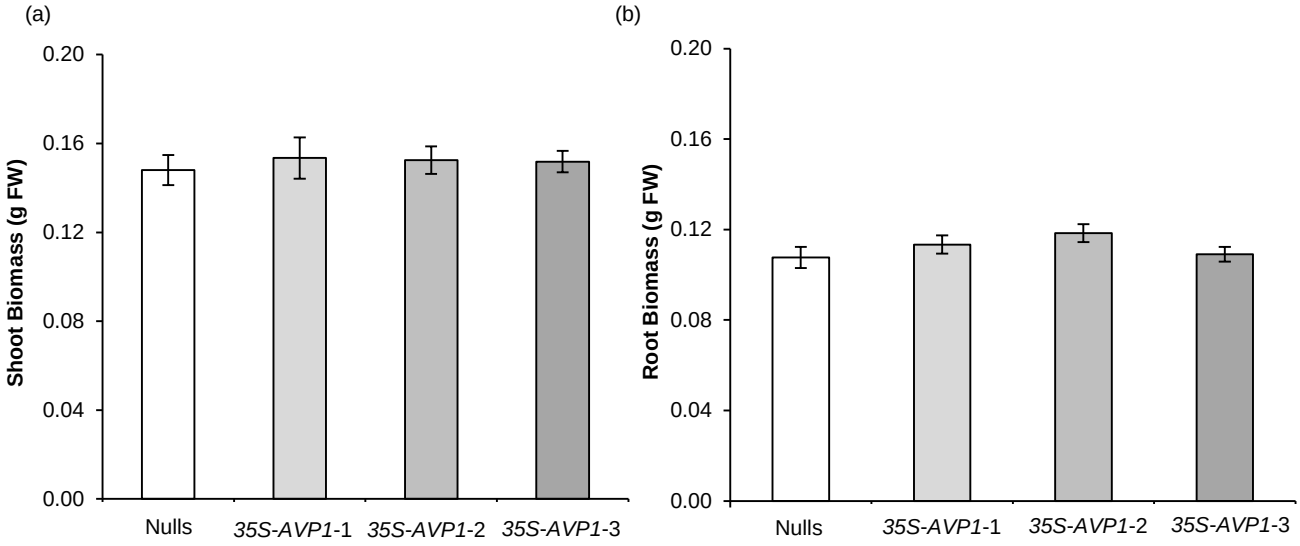


Figure S4 The (a) shoot and (b) root biomass (g FW) of null segregants (nulls) and transgenic *AVP1* barley lines (*35S-AVP1*-1, *35S-AVP1*-2 & *35S-AVP1*-3) at 11 d after imbibition in 100 mM NaCl solution. Values are presented as the mean ± s.e.m (*n* = 8-14).

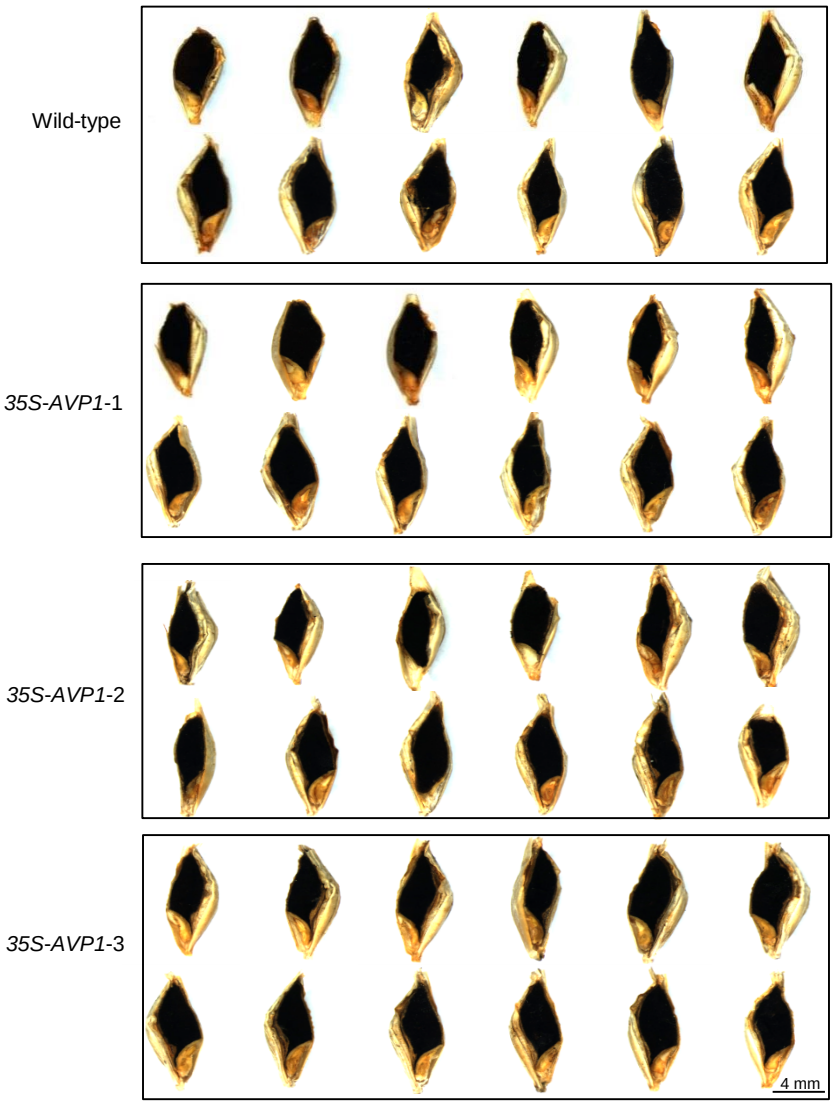


Figure S5 Visual assessment of the transgenic *AVP1* barley embryo size. Images of longitudinal sections of wild-type and transgenic *AVP1* barley lines (*35S-AVP1*-1, *35S-AVP1*-2 and *35S-AVP1*-3) seeds (*n* = 6) of similar weight (~ 43 mg) stained in Lugol's solution to show the endosperm (dark black) and embryo (yellow). Seeds in the bottom row of each rectangle are the corresponding half of the seed directly above in the top row.

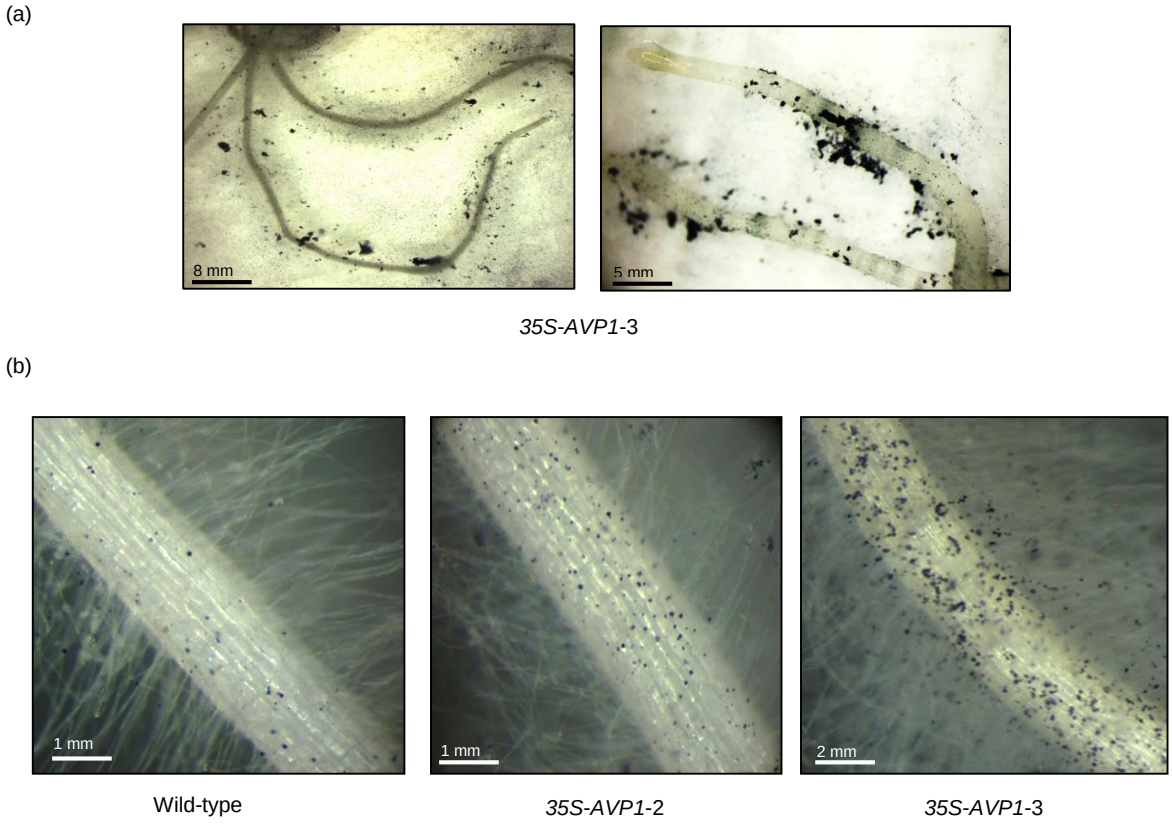


Figure S6 (a) Microscope images showing starch granules in the solution surrounding the root hairs of 8-day-old transgenic *AVP1* barley (*35S-AVP1*-3) after the cotyledon was excised from the seed and the roots and cotyledon stained together in Lugol's solution for 10 mins (b) Close-up microscope images of 8-day-old wild-type (cv. Golden Promise) and transgenic *AVP1* barley (*35S-AVP1*-2 & *35S-AVP1*-3) roots after 10 mins of staining in Lugol's solution showing starch granules in the solution.

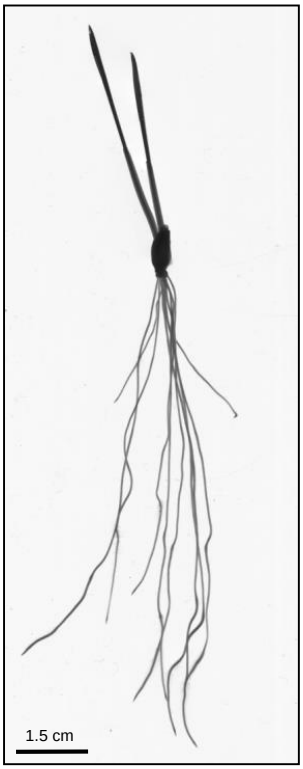


Figure S7 A digital image of a transgenic *AVP1* barley plant (*35S-AVP1*-2) recorded using an Epson scanner 8 d after seed imbibition at 0 mM NaCl showing an observed 'twinning' phenotype.

Chapter 7

Fine-tuning *AVP1* expression in transgenic barley

Statement of Contributions

The following chapter is formatted as a manuscript. However, it is not intended that this manuscript will be submitted for publication.

Title: Evaluating a commercially relevant transgenic barley cultivar (cv. WI4330) with salt stress-inducible (*ZmRab17*) or constitutive (*ZmUbi1*) expression of *AVP1* in saline conditions

Schilling conducted the experiments, data analysis, interpretation of results and wrote the manuscript

Marschner, Tester, Plett and Roy supervised the experiments

All authors contributed to the discussion of the results

By signing this statement of contributions, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis:

Petra Marschner

Signature: Date: 25/06/2014

Mark Tester

Signature: Date: 25/06/2014

Darren Plett

Signature: Date: 25/06/2014

Stuart Roy

Signature: Date: 25/06/2014

Evaluating a commercially relevant transgenic barley cultivar (cv. WI4330) with salt stress-inducible (*ZmRab17*) or constitutive (*ZmUbi1*) expression of *AVP1* in saline conditions

Running title: the type of promoter controlling *AVP1* expression is important

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Keywords: H^+-PPase, GUS activity, promoters, salinity, *CaMV 35S*, stress-inducibility

Abstract

Transgenic barley with constitutive *CaMV 35S* expression of *AVP1*, a gene encoding an *Arabidopsis* vacuolar H^+-pyrophosphatase, had improved shoot growth and grain yield in saline conditions. A commercially relevant transgenic barley cultivar with well-regulated expression of *AVP1* and without a promoter of viral origin is needed for this transgenic technology to be applicable for grain growers. In this study, we investigate whether the expression of *AVP1* in a commercially relevant Australian barley breeding line (cv. WI4330) using a stress-inducible promoter (*ZmRab17*) or a constitutive promoter with a plant-based origin (*ZmUbi1*) is beneficial. We show that the *ZmRab17* promoter is salt stress-inducible in barley root stelar cells at 200 mM NaCl, with basal transgene expression in non-saline conditions. Furthermore, we find that transgenic T_2 *ZmRab17*:*AVP1* and *ZmUbi1*:*AVP1* barley (cv. WI4330) had no difference in shoot or root biomass compared to null segregants in hydroponic conditions at 0 and 200 mM NaCl. However, the shoot and root biomass of two independent T_1 *35S*:*AVP1* barley (cv. WI4330) lines was greater than null segregants and *ZmRab17*:*AVP1* barley (cv. WI4430) in hydroponic conditions at 200 mM NaCl. This indicates that the type of promoter driving transgene expression is an important factor influencing the phenotype of transgenic plants and that the constitutive *CaMV 35S* promoter is likely to be contributing, atleast in part, to the improved plant growth of transgenic *AVP1* barley in saline conditions. Overall, this study has identified promising *35S*:*AVP1* barley (cv. WI4330) lines that could benefit Australian grain growers with saline soils in the future.

Introduction

The constitutive cauliflower mosaic virus (*CaMV*) *35S* driven expression of an *Arabidopsis* vacuolar H^+-pyrophosphatase gene (*AVP1*) in barley appears to be a promising option to increase grain yield in a saline field (Schilling et al., 2014). However, for this technology to be applicable to Australian grain producers with saline soils, a commercially relevant transgenic *AVP1* barley cultivar is needed. Ideally, this transgenic *AVP1* barley cultivar would be well-adapted to Australian growing conditions and high yielding in non-saline and saline conditions. It would also satisfy Australian government transgenic crop deregulation requirements (FSANZ, 2007) and, to increase the likelihood of consumer acceptance, should comprise minimal perceived adverse aspects of transgenic technology, such as uncontrolled transgene expression or a promoter of viral origin (Ho et al., 1999; Potenza et al., 2004). The development of a high yielding commercially relevant *AVP1* barley cultivar with well-regulated control of *AVP1* expression and without a promoter of viral origin is therefore needed.

Stress-inducible promoters help to refine transgene expression in transgenic plants (Potenza et al., 2004). A stress-inducible promoter allows temporal control of transgene expression by initiating transcription of a transgene only when a particular stress, such as drought or salinity, is present (Potenza et al., 2004). Fine-tuning transgene expression can conserve cellular energy (Potenza et al., 2004) and reduce the chance of detrimental growth phenotypes if the transgene to be expressed is important in developmental processes (Morran et al., 2011). Accordingly, the stress-inducible expression of a transgene improved transgenic plant growth in both non-stressed and stressed conditions compared to constitutive transgene expression (Kasuga et al., 1999; Kovalchuk et al., 2013; Morran et al., 2011; Su and Wu, 2004; Waterer et al., 2010). A salt stress-inducible promoter, which activates a transgene only when salinity is present, could therefore be advantageous in developing a commercially relevant high yielding transgenic salt tolerant crop (Roy et al., 2014; Tester and Bacic, 2005).

The stress-inducible maize (*Zea mays*) *ZmRab17* promoter may be useful for developing transgenic barley with salt stress-inducible *AVP1* expression. The *ZmRab17* promoter was inducible in the shoot and root tissue of maize by drought stress and applied abscisic acid (ABA) (Busk et al., 1997; Vilardell et al., 1991). Furthermore, in transgenic wheat and barley, the expression of a transcription factor (*TaDREB2* and *TaDREB3*) driven by the *ZmRab17* promoter was drought stress-inducible (Morran et al., 2011). As salinity causes an osmotic stress similar to drought stress, it is possible that the *ZmRab17* promoter may also be salt stress-inducible. Under high NaCl (> 250 mM), mRNA levels of the *ZmRab17* gene increased in leaf and root tissue of maize (Busk et al., 1997). Preliminary testing of the salt stress inducibility of the *ZmRab17* promoter after a saline solution (200 mM NaCl) was applied to T_1 *ZmRab17*:*uidA* barley seedlings on Petri dishes has been conducted (Schilling, 2010). However, further work is needed to test the salt stress inducibility of T_2 *ZmRab17*:*uidA* barley in more controlled growth conditions, such as hydroponics, where it can be ensured plants have sufficient nutrients and water at all times (Schilling, 2010). Thus, characterisation of the *ZmRab17* promoter in barley under non-saline and saline conditions is required to test if this promoter is salt stress-inducible and to identify in what tissue(s) and cell type(s) this stress-inducible promoter drives transgene expression.

To date, the constitutive *CaMV 35S* (Gaxiola et al., 2001; Li et al., 2010; Paez-Valencia et al., 2013; Pasapula et al., 2011; Qin et al., 2013; Schilling et al., 2014; Zhao et al., 2006) or maize *Ubiquitin* (*ZmUbi1*) (Kim et al., 2013) promoters have been solely used to drive the expression of *AVP1* in transgenic plants to improve salinity tolerance. However, in dryland agriculture, fluctuating levels of soil electrical conductivity often occur within a field causing variable crop growth (Richards, 1983; Richards et al., 1987). Given that AVP1 is a H^+-pumping pyrophosphatase which utilises a high-energy phosphoanhydride bond from the hydrolysis of cytosolic pyrophosphate (PP_i) to orthophosphate (P_i) (Maeshima, 2000), the salt stress-inducible expression of *AVP1* may help to conserve cellular energy in transgenic *AVP1* barley, particularly in areas of a field where no salinity is present. A transgenic barley variety with well-regulated

salt stress-inducible expression of *AVP1* may also be better perceived by consumers compared to constitutive driven *AVP1* expression (Potenza et al., 2004). Conversely, it is probable that the stress-inducible expression of *AVP1* may not be advantageous, as the expression of this transgene can improve plant growth in non-saline conditions (Schilling et al., 2014) and may be required from an early growth stage and prior to the onset of salinity stress to enable increased seedling vigour, and thus improved plant growth under saline conditions (Chapter 6). It is therefore important to evaluate the growth of transgenic barley with salt stress-inducible expression of *AVP1* to determine whether this provides any advantage or disadvantage over constitutive *CaMV 35S* driven expression of *AVP1*. Preliminary testing of T_1 *ZmRab17*:*AVP1* barley (cv. WI4330) in soil with a 75 mM NaCl treatment was previously completed (Schilling, 2010). However, results for shoot biomass and leaf ion contents were inconclusive, due to the low NaCl treatment and the detection of *AVP1* expression in non-stressed conditions (Schilling, 2010). Thus, further testing of the T_2 *ZmRab17*:*AVP1* barley at higher concentrations of salinity and in a more controlled set-up, such as hydroponic conditions, is needed

Alternatively, the maize *ZmUbi1* promoter could be a useful plant-derived constitutive promoter to control *AVP1* expression in transgenic barley. There is concern that the use of viral DNA in transgenic plants, such as the *CaMV 35S* promoter, may cause non-specific host recombination issues, such as viruses arising from the incorporation of the *CaMV 35S* promoter into dormant, endogenous viruses in transgenic plants (Ho et al., 1999). Although there is limited scientific evidence to support this perceived concern (Hull, 2000), it has been suggested that the expression of a transgene using a promoter sourced from a plant origin rather than a viral origin could help to reduce these safety concerns (Potenza et al., 2004). The *ZmUbi1* promoter is thought to drive constitutive and high transgene expression in most plant tissues (Christensen and Quail, 1996). This plant derived promoter could therefore be a useful alternative to the viral derived *CaMV 35S* promoter, particularly for transgene expression in monocotyledonous plants (Christensen and Quail, 1996). Transgenic rice with *ZmUbi1* driven expression of *AVP1* had improved

plant growth in greenhouse conditions under salinity compared to wild-type (Kim et al., 2013). Preliminary testing of T_1 *ZmUbi1*:*AVP1* barley in soil with a 75 mM NaCl treatment was previously completed (Schilling, 2010). However, due to the low NaCl treatment, biomass and leaf ion contents results were inconclusive and thus further testing of the T_2 *ZmUbi1*:*AVP1* barley at higher concentrations of salinity is needed to determine whether the constitutive *ZmUbi1* driven expression of *AVP1* alters the growth or ion contents of transgenic barley.

Additionally, for this AVP1 technology to be applicable to Australian grain growers with saline soils, the development of a high yielding commercially relevant transgenic *AVP1* barley cultivar adapted to Australian growing conditions is required. Previously, transgenic barley expressing *AVP1* were generated using the barley cultivar Golden Promise (Schilling et al., 2014). Golden Promise, a gamma-ray induced mutant barley cultivar developed from the cultivar Maythorpe (Forster et al., 1994), was selected due to an established *Agrobacterium*-mediated transformation protocol for this cultivar (ACPFG Transformation Group, Adelaide, Australia). However, Golden Promise is not well-adapted to Australian conditions and is not suitable for commercial use by Australian grain growers. Recent advances in cereal transformation efficiency have facilitated the transformation of a commercially relevant barley breeding line, WI4330, with the *AVP1* transgene (ACPFG Transformation Group, Adelaide, Australia). This advanced Waite Institute barley breeding line, WI4330, is a high yielding barley cultivar adapted to South Australian growing conditions and suitable for Australian grain growers (Jason Eglinton, personal communication, University of Adelaide). However, it is yet to be established if the expression of *AVP1* can improve the growth of this commercially relevant WI4330 barley cultivar in saline conditions.

The aim of this study was to characterise the stress-inducible *ZmRab17* promoter under salinity stress in barley and to evaluate the growth of a commercially relevant transgenic barley (cv. WI4330) with

constitutive (*ZmUbi1*) and stress-inducible (*ZmRab17*) expression of *AVP1* in non-saline and saline conditions.

Experimental Design

Evaluation of stress-inducible *ZmRab17* promoter in barley in saline conditions

Seeds of wild-type (cv. Golden Promise) and two independent transformation events of T_2 *ZmRab17:uidA* barley (cv. Golden Promise) (provided by Nataliya Kovalchuk, ACPFG, Australia) were germinated for 5 d on moist filter paper in 145 mm diameter Petri dishes. Seedlings were transplanted to a small hydroponics set-up on individual sections of 8 mm mesh held inside a container filled with 2 L of nutrient solution. The nutrient solution consisted of reverse osmosis (RO) water with the following nutrients (in mM): 0.2 NH_4NO_3, 5.0 KNO_3, 2.0 $Ca(NO_3)_2.4H_2O$, 2.0 $MgSO_4.7H_2O$, 0.1 KH_2PO_4, 0.5 $Na_2Si_3O_7$, 0.05 NaFe(III)EDTA, 0.05 H_3BO_3, 0.005 $MnCl_2.4H_2O$, 0.01 $ZnSO_4.7H_2O$, 0.005 $CuSO_4.5H_2O$ and 0.0001 $Na_2MoO_4.2H_2O$. An aerator stone attached to a Precision SR 7500 aerator (Aqua One, Sydney, Australia) was used to provide continuous aeration to each hydroponic container. The nutrient solution was changed every 5 d to ensure all nutrients were adequately supplied. Following 10 d after seed imbibition, salinity treatments of 0 and 200 mM NaCl with supplementary $CaCl_2$ were supplied to the nutrient solution. Following 24 h of salinity treatment, a section of root and 1st leaf of each plant were sampled for ß-glucuronidase (GUS) staining and a section of root tissue was collected for genotyping each plant for the presence of the *ZmRab17* promoter and *uidA* gene using specific primers and a control gene *HvVRT* (data not shown).

Histochemical GUS analysis of salt treated *ZmRab17:uidA* barley tissue

Collected root and leaf samples of wild-type and T_2 *ZmRab17:uidA* barley (control and salt treated) were vacuum infiltrated for 20 min at -20 kPa in small Petri dishes (5 cm diameter × 2 cm depth) containing 15 mL of GUS solution (50 mM sodium phosphate at pH 7, 10 mM EDTA, 0.1 % Triton X-100, 2 mM

potassium ferrocyanide, 2 mM potassium ferricyanide, 0.5 mg/mL X-Gluconide (5-bromo-4-chloro-3-indoxyl-beta-D-glucuronic acid, cyclohexlammonium salt) (Cat# G1281C1, Gold Biotechnology, St Louis, MO, USA) and 25 mg/mL chloramphenicol). Samples were incubated in the dark (Petri dishes wrapped in aluminium foil) in an oven (Contherm Scientific Ltd, Wellington, New Zealand) at 37 °C for 8 h. After incubation, the root and shoot tissue was rinsed in a series of 2 h ethanol dilutions of 20 %, 35 %, 50 % and 70 % to remove leaf chlorophyll colouring. The extent of GUS staining was visualised and digital images recorded using a Leica MZ FLIII stereo microscope, equipped with a Leica DC 300F camera and Leica IM50 image manager software (Leica Microsystems Ltd, Heerbrugg, Switzerland).

Evaluation of *ZmRab17:AVP1* and *ZmUbi1:AVP1* barley (cv. WI4330) lines in salinity

Seeds of wild-type (cv.WI4330) and four independent transformation lines of T_2 *ZmRab17:AVP1* and *ZmUbi1:AVP1* selected for a low *AVP1* copy number (1-2 copies, ACPFG Transformation Group) were germinated for 4 d in Petri dishes on moist filter paper. Uniform size seedlings were transplanted to a fully-supported hydroponics set-up (Genc et al., 2007). Briefly, each hydroponic trolley contained 42 PVC tubes (4 cm diameter × 28 cm depth) containing black polyethylene pellets (~2 mm diameter) and positioned into two individual trays connected to an 80 L tank containing nutrient solution. A uniform size seedling was randomly transplanted to each tube. Hydroponic plants were supplied with nutrient solution in a volume of 80 L with cycling every 30 mins between nutrient solution and free drainage within the tanks. The nutrient solution consisted of reverse osmosis (RO) water with the following nutrients (in mM): 0.2 NH_4NO_3, 5.0 KNO_3, 2.0 $Ca(NO_3)_2.4H_2O$, 2.0 $MgSO_4.7H_2O$, 0.1 KH_2PO_4, 0.5 $Na_2Si_3O_7$, 0.05 NaFe(III)EDTA, 0.05 H_3BO_3, 0.005 $MnCl_2.4H_2O$, 0.01 $ZnSO_4.7H_2O$, 0.005 $CuSO_4.5H_2O$ and 0.0001 $Na_2MoO_4.2H_2O$. The nutrient solution was replaced every 10 d to ensure all nutrients were maintained at a sufficient level for the duration of experiments. Following 10 d after transplanting, as the 3rd leaf blade began to emerge, salinity treatments were imposed in the nutrient solution with increments of 25 mM NaCl (116.88 g NaCl with 3.8 g $CaCl_2$) twice a day until 0, 200 and 300 mM NaCl treatments were established.

Following 21 d of salinity treatment, the 3rd leaf blade was sampled for ion analysis and the youngest fully-emerged leaf blade was collected for genotyping and gene expression analysis. Roots were rinsed in 10 mM $CaCl_2$ and blotted dry prior to oven drying. SPAD meter readings were measured at the centre of the 3rd leaf blade using a hand-held SPAD 502 meter (Minolta, Osaka, Japan) and a ruler was used to measure the length of the longest root for each plant.

Determination of leaf and root Na^+, K^+ & Cl^- concentrations

The 3rd leaf blade and roots were oven dried for 3 d at 70 °C and digested in 1 % nitric acid (v/v) at 95 °C for 5 h in a 54-well HotBlock (Environmental Express, Mount Pleasant, SC, USA). The Na^+ and K^+ concentrations of digested leaf and roots were determined using a flame photometer (Model 420, Sherwood Scientific, Cambridge, UK) following the protocol of Shavrukov et al., (2010). The concentration of Cl^- in digested leaf and roots was determined using a chloride analyser (Model 926, Sherwood Scientific, Cambridge, UK). Briefly, 1 mL of the same 1% nitric acid (v/v) digest solution (described above) was added to a solution containing combined acid buffer (0.006 % nitric acid (v/v), 90 % water and 10 % acetic acid) with gelatine (1:4 ratio) and titrated using a conditioned chloride analyser with a silver anode.

Semi-quantitative RT-PCR analysis of *AVP1* expression

Root tissue was ground to a fine powder and total RNA was extracted (Chomczynski, 1993). Extracted RNA was treated with DNase-free (Ambion, Madison, WI, USA) to remove DNA contamination. DNase-treated RNA (1 µL) was used to synthesize cDNA with a Superscript III RT kit (Invitrogen). Semi-quantitative *AVP1* expression in each plant was determined using reverse transcription PCR (RT-PCR) amplification of 1 µL of cDNA template with *AVP1*-specific forward primer: 5' – GCA GCT CTT AAG ATG GTT GAA – 3' and reverse primer 5' – AGA GGT GTG AGC ATG ACA AGG – 3'. The PCR conditions used to amplify a fragment of the *AVP1* transcript (expected band size of 164 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 reactions contained 1× Platinum® *Taq* PCR buffer, 2 mM $MgCl_2$, 200 µM of each dNTPs, 0.5 U of Platinum® Taq DNA polymerase (Invitrogen) and 10 µM of each primer. Gel electrophoresis with a 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 check PCR products and record gel images. To account for differences in initial concentrations of cDNA, the amount of gene expression was determined by normalising the level of PCR product as gel band intensity (minus background intensity) obtained using *HvGAP* relative to that of *AVP1* using GIMP 2.6.11 GNU Image Manipulation Program (www.gimp.org).

Statistical analysis

Data was 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 within lines compared to null segregants at a probability level of $P \leq 0.05$.

Results

The activity and localisation of the *ZmRab17* promoter at 0 and 200 mM NaCl was evaluated using histochemical *uidA* (GUS) analysis of two independent transgenic T_2 *ZmRab17:uidA* barley (cv. Golden Promise) lines (*ZmRab17*:*uidA*-1 and *ZmRab17*:*uidA*-2). No blue colour (indicative of GUS activity) was detected in leaf and root tissue of both *ZmRab17:uidA* barley lines at 0 mM NaCl (Figure 1a, b & S1). Additionally, no GUS activity was present in the leaf tissue of *ZmRab17:uidA* barley lines following 24 h at 200 mM NaCl (Figure 1c & S1 c). However, GUS activity was present in root tissue following 24 h at

200 mM NaCl (Figure 1d & S1 d). Specifically, GUS activity in salt-treated transgenic barley containing the *uidA* gene was only visualised in mature roots regions within stelar cells, with predominant staining occurring at lateral root tip junctions (Figure S1).

Semi-quantitative RT-PCR of root tissue from *ZmRab17:AVP1* and *ZmUbi:AVP1* barley (cv. WI4330) plants at 0 and 200 mM NaCl indicates that the transgenic barley is expressing *AVP1* (Figure 2a & b). Notably, the *ZmRab17:AVP1* barley requires a higher number of PCR cycles (30 to 32 cycles) for *AVP1* expression to be detected compared to the *ZmUbi1:AVP1* barley, which shows *AVP1* expression from a lower number of PCR cycles (28 cycles) (Figure 2a). However, the relative *AVP1* expression in the roots of *ZmRab17:AVP1* barley at 0 mM NaCl at 32 cycles is similar to that observed in roots of *ZmRab17:AVP1* barley at 200 mM NaCl (Figure 2b). The *ZmUbi:AVP1* barley also had greater expression of *AVP1* than *ZmRab17:AVP1* at both 0 and 200 mM NaCl (Figure 2b).

The shoot biomass of wild-type (cv. WI4330), null segregants and four independent lines of *ZmRab17:AVP1* and *ZmUbi1:AVP1* transgenic barley were reduced at 200 mM (37 to 58 %) and 300 mM NaCl (63 to 74 %) compared to 0 mM NaCl (Figure 3a & b). Additionally, the root biomass of all plants was reduced at 200 mM (16 to 42%) and 300 mM NaCl (54 to 71%) (Figure 3c). However, all transgenic *AVP1* barley lines had no difference in shoot and root biomass compared to wild-type and null segregants at 0, 200 and 300 mM NaCl with one exception, transgenic line *ZmUbi1-AVP1*-4, which had a consistently smaller shoot and root biomass compared to null segregants (*Nulls ZmUbi1*) within treatments (Figure 3b & c). Additionally, root length of all plants was reduced at 200 mM (28 to 44 %) and 300 mM NaCl (33 to 44%) compared to 0 mM NaCl (Figure 4). All plants had a higher 3rd leaf blade SPAD reading at 200 mM NaCl (4 to 17%) and similar SPAD values at 300 mM NaCl compared to 0 mM NaCl (Figure 5).

As expected, the sodium (Na^+) concentration (µM) in the leaf and roots of all plants increased at 200 and 300 mM NaCl stress compared to plants at 0 mM NaCl (Figure 6). Several transgenic *AVP1* barley lines had a significantly greater root Na^+ concentration at 200 mM NaCl (*ZmRab17-AVP1*-2, *ZmRab17-AVP1*-3, *ZmUbi1:AVP1-1*, *ZmUbi1:AVP1-2* & *ZmUbi:AVP1-4*) and 300 mM NaCl (*ZmUbi1:AVP1-2*, *ZmUbi1:AVP1-3* & *ZmUbi:AVP1-4*) compared to respective null segregants (Figure 6b). Additionally, the potassium (K^+) concentration (µM) of all plants was lower in leaf tissue at 200 mM (12 to 30%) and 300 mM (46 to 59%) NaCl and in root tissue at 200 mM (62 to 74%) and 300 mM (69 to 83%) NaCl compared to plants at 0 mM NaCl (Figure 7). There was no difference in leaf or root K^+ concentration between lines at 200 mM or 300 mM NaCl, except one line, *ZmRab17-AVP1*-4, which a lower leaf K^+ concentration compared to null segregants (Figure 7a). However, at 0 mM NaCl, several *ZmRab17:AVP1* barley lines had greater K^+ concentrations in leaf (*ZmRab17-AVP1*-2 & *ZmRab17-AVP1*-3) and roots (*ZmRab17-AVP1*-1, *ZmRab17-AVP1*-2 & *ZmRab17-AVP1*-4) compared to *ZmRab17* null segregants (Figure 7b).

Discussion

Previously, the maize *ZmRab17* promoter was inducible by abscisic acid (ABA) in mature maize embryos (Vilardell et al., 1990), drought and ABA stress in the shoot and roots of maize (Busk et al., 1997) and by drought in leaf tissue of transgenic wheat and barley (Morran et al., 2011). Maize seedlings at high NaCl stress (> 250 mM) also had greater *ZmRab17* mRNA levels with a larger response in leaves compared to roots (Busk et al., 1997). However, in non-stressed conditions basal expression of a transgene controlled by the *ZmRab17* promoter in transgenic wheat and barley leaf tissue was detected (Morran et al., 2011). In this study, an increase in *AVP1* expression was detected in the roots of transgenic *ZmRab17:AVP1* barley at 200 mM NaCl compared to 0 mM NaCl (Figure 1 & 2b) and high basal expression of *AVP1* in at 0 mM NaCl was also detected in the *ZmRab17:AVP1* barley roots and leaf tissue (Figure 2b & S3). Nonetheless, it is possible that there are transcription factors in maize which alter the inducibility of the *ZmRab17* promoter compared to wheat or barley.

In this study, GUS activity only in root tissue, particularly in root stelar cells within mature roots and at lateral root tip junctions, was detected in *ZmRab17*:*uidA* barley seedlings after 24 h at 200 mM NaCl (Figure 1d & S1e). This suggests *ZmRab17* driven transgene expression may be specific to the root stele. This could potentially explain why Busk et al. (1997) needed to over-expose a northern blot to measure maize root *ZmRab17* mRNA levels under high salinity and why in this study a high PCR cycle number was needed to detect *AVP1* expression in the roots of *ZmRab17*:*AVP1* barley (Figure 2a). It is probable that sampling the whole root diluted the overall level of *ZmRab17* mRNA and that a higher level of expression and/or inducible expression may be present if root stelar cells are specifically tested. In contrast to the semi-quantitative PCR expression analysis of *ZmRab17*:*AVP1* barley, no GUS activity was detected in leaf tissue of salt stressed *ZmRab17*:*uidA* barley (Figure 1c) and no basal expression of GUS was detected in *ZmRab17*:*uidA* barley roots at 0 mM NaCl (Figure 1b). This absence of GUS could be due to the shorter duration of salinity stress or younger plant age. It is also probable that the GUS staining was insufficient to visualise GUS present in leaf tissue, with GUS staining tending to be more effective where there is a greater number of small cells per unit area, such as in the phloem, or that the sensitivity of the semi-quantitative PCR was greater than GUS staining (Jefferson et al., 1987; Terada and Shimamoto, 1990). Nonetheless, an increase in GUS activity by the *ZmRab17* promoter at 200 mM NaCl compared to 0 mM NaCl suggests this promoter is salt stress-inducible in barley roots with basal expression in non-saline conditions (Figure 1d). This promoter could therefore be useful for developing transgenic cereal crops with salt stress-inducible expression of salinity tolerance genes.

In this study, a commercially relevant barley cultivar (cv. WI4330) expressing *AVP1* via a stress-inducible (*ZmRab17*) or plant-derived constitutive (*ZmUbi1*) promoter had a similar shoot and root biomass compared to wild-type and null segregants at 0 mM, 200 and 300 mM NaCl (Figure 3). There was also no difference in root length or leaf SPAD values between the transgenic *ZmRab17*:*AVP1* and *ZmUbi1*:*AVP1* barley, wild-type and null segregants (Figures 4, 5, 6 & 7). However, several of the

transgenic *ZmRab17:AVP1* and *ZmUbi1:AVP1* barley lines did have greater root Na^+ concentrations at 200 mM and 300 mM NaCl compared to null segregants, suggesting AVP1 may be facilitating increased sequestration of Na^+ into vacuoles. These findings support previous preliminary results testing the same transgenic *AVP1* barley lines in a pot experiment with non-saline and saline conditions (Schilling, 2010). However, the lack of a larger shoot biomass in the *ZmRab17*:*AVP1* and *ZmUbi1*:*AVP1* barley (cv. WI4330) is in contrary to the larger shoot biomass of T_4 *35S*:*AVP1* barley (cv. Golden Promise) compared to wild-type previously observed under salinity (Schilling et al., 2014).

Differences between promoters (*ZmRab17*, *ZmUbi1* and *35S*) may explain the absence of a larger shoot biomass phenotype in the transgenic *ZmRab17:AVP1* and *ZmUbi1:AVP1* barley (cv. WI4330) compared to the *35S*:*AVP1* barley (cv. Golden Promise). A preliminary screen of T_1 *35S*:*AVP1* barley (cv. WI4330) barley showed that two independent lines (*35S-AVP1*-1 and *35S-AVP1*-5) had significantly greater shoot and root biomass compared to null segregants at 200 mM NaCl (Figure S2). Although not all the *35S*:*AVP1* (cv. WI4330) lines had a significant increase in shoot and root growth (Figure S2), these findings for the T_1 plants suggest that the benefits of *AVP1* expression is not exclusive to a set of experimental conditions or to the barley variety Golden Promise. It also suggests that the *35S*:*AVP1* barley (cv. WI4330) can outperform *ZmRab17*:*AVP1*-2 barley (cv. WI4330) in saline conditions (Figure S2). This suggests that the *CaMV 35S* driven expression of *AVP1* in barley may be more beneficial than the expression of *AVP1* using either the *ZmRab17* or *ZmUbi1* promoters. With the exception of one study (Kim et al., 2013), all other previous studies showing improved growth of transgenic *AVP1* plants in saline conditions have involved the constitutive expression of *AVP1* using the *CaMV 35S* promoter (Gaxiola et al., 2001; Li et al., 2010; Pasapula et al., 2011; Qin et al., 2013; Schilling et al., 2014; Zhao et al., 2006).

Potentially, the *CaMV 35S* promoter has a more suitable level of *AVP1* expression or cell specificity compared to the *ZmRab17* or *ZmUbi1* promoters. The *CaMV 35S* promoter reportedly expresses a

transgene at high levels and in most cell types throughout the lifecycle of a transgenic plant (Battraw and Hall, 1990; Odell et al., 1985; Terada and Shimamoto, 1990). Histochemical GUS analysis of *CaMV 35S* driven expression in rice shows a high level of GUS activity in the embryo of imbibed seeds (Terada and Shimamoto, 1990). The presence of *CaMV 35S* expression in a germinating embryo could be important, with analysis of *35S:AVP1* barley seedlings (cv. Golden Promise) (refer to Chapter 6) suggesting AVP1 is required before the onset of salinity stress and during barley germination to increase seedling vigour. The location or high level of *CaMV 35S* driven *AVP1* expression could be contributing to the improved shoot growth of transgenic *35S:AVP1* barley in saline conditions.

In contrast, the *ZmRab17* and *ZmUbi1* promoters may have an insufficient level of expression or cell specificity for *AVP1* expression in barley. For example, the *ZmRab17* promoter is salt stress-inducible, appears to be specific to mature root stelar cells and has a lower level of *AVP1* expression compared to the *CaMV 35S* promoter (Figure 1d & S3). In transgenic *Arabidopsis*, *ZmRab17* promoter activity was also low after 8 to 10 d of germination (Vilardell et al., 1994). This promoter could therefore be reducing *AVP1* expression in the transgenic barley during a critical time-point for regulating seedling vigour. Additionally, the maize *ZmUbi1* promoter is thought to express a transgene constitutively, yet the level of *ZmUbi1* driven gene expression has been shown to decline across the development of a rice plant (Cornejo et al., 1993). There is one report of increased growth of transgenic rice with *ZmUbi1* driven expression of *AVP1* under saline conditions (Kim et al., 2013). However, a separate study reports that transgenic rice (*Oryza sativa*) expressing *AVP1* via the *ZmUbi1* promoter have a larger shoot biomass under drought stress but no increase in growth under salinity (Lee et al., 2012). A similar lack of a larger shoot biomass phenotype in a transgenic Indonesian rice variety expressing *AVP1* via the *ZmUbi1* promoter in saline conditions has also been observed (A. Hairmansis ACPFG, unpublished). Potentially, *AVP1* expression driven by either the *ZmRab17* or *ZmUbi1* promoters is either activated too late prior to the onset of stress, at an insufficient level, in the incorrect cell-type or developmental stage or a

combination of these factors, and thus, in this study does not facilitate improved shoot biomass of transgenic *AVP1* barley.

Although a precise explanation for the lack of a larger shoot biomass phenotype in barley with *ZmRab17* or *ZmUbi1* driven *AVP1* expression is unknown, it does indicate that the type of promoter controlling *AVP1* expression is important. It also highlights the need for well characterised promoters to control transgenes of interest and that stress-inducible expression of a transgene may only be beneficial for certain transgenes. Further research using well characterised promoters to activate *AVP1* expression at specific expression levels or at specific developmental stages, such as within a germinating barley embryo or seedling, could provide further insight into the role of AVP1. This study also suggests that the *CaMV 35S* promoter is likely to be contributing to the larger shoot biomass phenotype of transgenic *AVP1* barley in saline conditions. Furthermore, this study supports the concept that *35S:AVP1* can improve the shoot biomass of barley (Schilling et al., 2014).

A mechanism for the improved shoot growth of transgenic *35S:AVP1* barley in saline conditions is yet to be fully elucidated (Schilling et al., 2014). No increase in Na^+ or K^+ concentrations were measured in the 4th leaf tissue of *35S:AVP1* barley (cv. Golden Promise) in saline conditions compared to null segregants (Schilling et al., 2014). However, root tissue was not analysed due to plant growth in pot and field conditions involving root growth in soil (Schilling et al., 2014). In this study, the T_1 *35S:AVP1* barley (cv. WI4330) also had no increase in 4th leaf or root Na^+ or K^+ concentrations at 0 and 200 mM NaCl Figure S4 & S5). However, the *35S:AVP1* barley (cv. WI4330) lines (*35*S-*AVP1*-1, *35*S-*AVP1*-3, *35*S-*AVP1*-4 & *35*S-*AVP1*-6) had significantly higher root Cl^- concentrations than null segregants in hydroponic conditions at 200 mM NaCl (Figure S4). This suggests that AVP1 may be altering Cl^- transport in transgenic barley under salinity stress, which is consistent with increased root Cl^- concentrations measured in transgenic *AVP1* bentgrass compared to wild-type under salinity (Li et al., 2010) and a proposed role of AVP1 in

facilitating anion transport (Dale Sanders, personal communication). Furthermore, in this study, the transgenic *35S*:*AVP1* barley (cv. WI4330) lines (*35S-AVP1*-1 & *35S-AVP1*-5) had a greater root biomass compared to null segregants at 200 mM NaCl (Figure S2c). This is also consistent with a larger root biomass potentially improving water uptake of transgenic *AVP1* plants under salinity (Bao et al., 2009; Gaxiola et al., 2012; Li et al., 2010; Pasapula et al., 2011).

Conclusions

This study showed that the *ZmRab17* promoter is salt stress-inducible in barley root stelar cells at 200 mM NaCl, with basal gene expression in non-saline conditions. It also showed that stress-inducible (*ZmRab17*) and constitutive (*ZmUbi1*) expression of *AVP1* does not alter the shoot or root biomass of commercially relevant WI4330 barley at 0, 200 or 300 mM NaCl. A precise explanation for why transgenic *ZmRab17*:*AVP1* and *ZmUbi1*:*AVP1* barley (cv. WI4330) had no increase in plant growth under salinity stress is unknown. However, two independent T_1 *35S*:*AVP1* barley (cv. WI4330) lines had a larger shoot and root biomass than null segregants and *ZmRab17*:*AVP1* barley (cv. WI4330) at 200 mM NaCl. This suggests that the constitutive *CaMV 35S* promoter is likely to be contributing, at least in part, to the improved plant growth of transgenic *35S*:*AVP1* barley in saline conditions. Overall, this study was unable to identify commercially relevant transgenic barley (cv. WI4330) lines with improved growth in saline conditions with either stress-inducible or constitutive expression of *AVP1* using plant-derived promoters. Nonetheless, promising *35S*:*AVP1* barley (cv. WI4330) lines with larger shoot and root biomass in saline conditions have been identified in this study, which could be a valuable resource for Australian grain growers in the future.

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Figure Legends

Figure 1 Representative images of T_2 *ZmRab17*:*uidA* barley (cv. Golden Promise) showing histochemical GUS staining of leaf and root tissue after 24 h at (a & b) 0 mM or (c & d) 200 mM NaCl. Blue colouring indicates the presence of GUS activity. Digital images were recorded using a Leica MZ FLIII stereo microscope equipped with a Leica DC 300F camera and Leica IM50 image manager software.

Figure 2 (a) Individual gel images of showing reverse-transcription PCR (RT-PCR) band intensity for three replicates of T_2 *ZmRab17*:*AVP1* and *ZmUbi1*:*AVP1* barley (cv. WI4330) root tissues at 0 and 200 mM NaCl for 28, 30 and 32 PCR cycles using *AVP1* and *HvGAP* specific primers (internal control) (b) Relative *AVP1* expression levels at 32 PCR cycles (compared to *HvGAP* at 28 PCR cycles) in root tissue of three independent *ZmRab17*:*AVP1* (1, 2 & 3) and *ZmUbi1*:*AVP1* (1, 2 & 3) barley (cv WI4330) plants at 0 mM (light grey) and 200 mM (dark grey) NaCl. Values are presented as the mean ± s.e.m (n = 4).

Figure 3 (a) An image of the fully-supported 80 L hydroponics set-up showing the growth of wild-type (cv. WI4330) and T_2 *ZmRab17*:*AVP1* and *ZmUbi1*:*AVP1* barley at 0, 200 and 300 mM NaCl from left to right. (b) Shoot and (c) root biomass (g DW plant^{-1}) of wild-type, null segregants from *ZmRab17:AVP1* transformations, four independently transformed T_2 *ZmRab17*:*AVP1* lines (1, 2, 3 & 4), null segregants from *ZmUbi1:AVP1* transformations and four independently transformed T_2 *ZmUbi1*:*AVP1* barley lines (1, 2, 3 & 4) at 0 (light grey), 200 (grey) and 300 (dark grey) mM NaCl. Values are presented as the mean ± s.e.m with 0 mM (n = 5-7), 200 mM (n = 3-7) and 300 mM (n = 5-7) and an asterisks (*) indicating a significant difference compared to respective Nulls (*ZmRab17*) or Nulls (*ZmUbi1*) within treatments (one-way ANOVA, LSD, $P \leq 0.05$).

Figure 4 Average root length (cm plant^{-1}) of wild-type, null segregants from *ZmRab17:AVP1* transformations, four independently transformed T_2 *ZmRab17*:*AVP1* lines (1, 2, 3 & 4), null segregants

from *ZmUbi1:AVP1* transformations and four independently transformed T_2 *ZmUbi1*:*AVP1* barley lines (1, 2, 3 & 4) at 0 (light grey), 200 (grey) and 300 (dark grey) mM NaCl. Values are presented as the mean ± s.e.m with 0 mM (*n* = 5-7), 200 mM (*n* = 3-7) and 300 mM (*n* = 5-7).

Figure 5 Average SPAD reading (unit) at middle of 3rd leaf blade for wild-type (cv. WI4330), null segregants from *ZmRab17:AVP1* transformation, four independently transformed T_2 *ZmRab17:AVP1* lines (1, 2, 3 & 4), null segregants from *ZmUbi1:AVP1* transformations and four independently transformed T_2 *ZmUbi1*:*AVP1* barley lines (1, 2, 3 & 4) at 0 (light grey), 200 (grey) and 300 (dark grey) mM NaCl. Values are presented as the mean ± s.e.m with 0 mM (*n* = 5-7), 200 mM (*n* = 3-7) and 300 mM (*n* = 5-7).

Figure 6 (a) Leaf and (b) root Na^+ concentrations (mM) of wild-type, null segregants from *ZmRab17:AVP1* transformations, T_2 *ZmRab17*:*AVP1* (1, 2, 3 & 4), null segregants from *ZmUbi1:AVP1* transformations and T_2 *ZmUbi1*:*AVP1* (1, 2, 3 & 4) barley (cv. WI4330) at 0 (light grey), 200 (grey) and 300 (dark grey) mM NaCl. Values are presented as the mean ± s.e.m with 0 mM (*n* = 5-7), 200 mM (*n* = 3-7) and 300 mM (*n* = 5-7) NaCl and an asterisks (*) indicating a significant difference compared to respective Nulls (*ZmRab17*) or Nulls (*ZmUbi1*) within treatments (one-way ANOVA, LSD, $P \leq 0.05$).

Figure 7 (a) Leaf and (b) root K^+ concentrations (mM) of wild-type (cv. WI4330), null segregants from *ZmRab17:AVP1* transformation, four independently transformed T_2 *ZmRab17*:*AVP1* lines (1, 2, 3 & 4), null segregants from *ZmUbi1:AVP1* transformation and four independently transformed T_2 *ZmUbi1:AVP1* barley lines (1, 2, 3 & 4) at 0 (light grey), 200 (grey) and 300 (dark grey) mM NaCl. Values are presented as the mean ± s.e.m with 0 mM (*n* = 5-7), 200 mM (*n* = 3-7) and 300 mM (*n* = 5-7) NaCl with an asterisks (*) indicating a significant difference compared to respective Nulls (*ZmRab17*) or Nulls (*ZmUbi1*) within treatments (one-way ANOVA, LSD, $P \leq 0.05$).

Figures

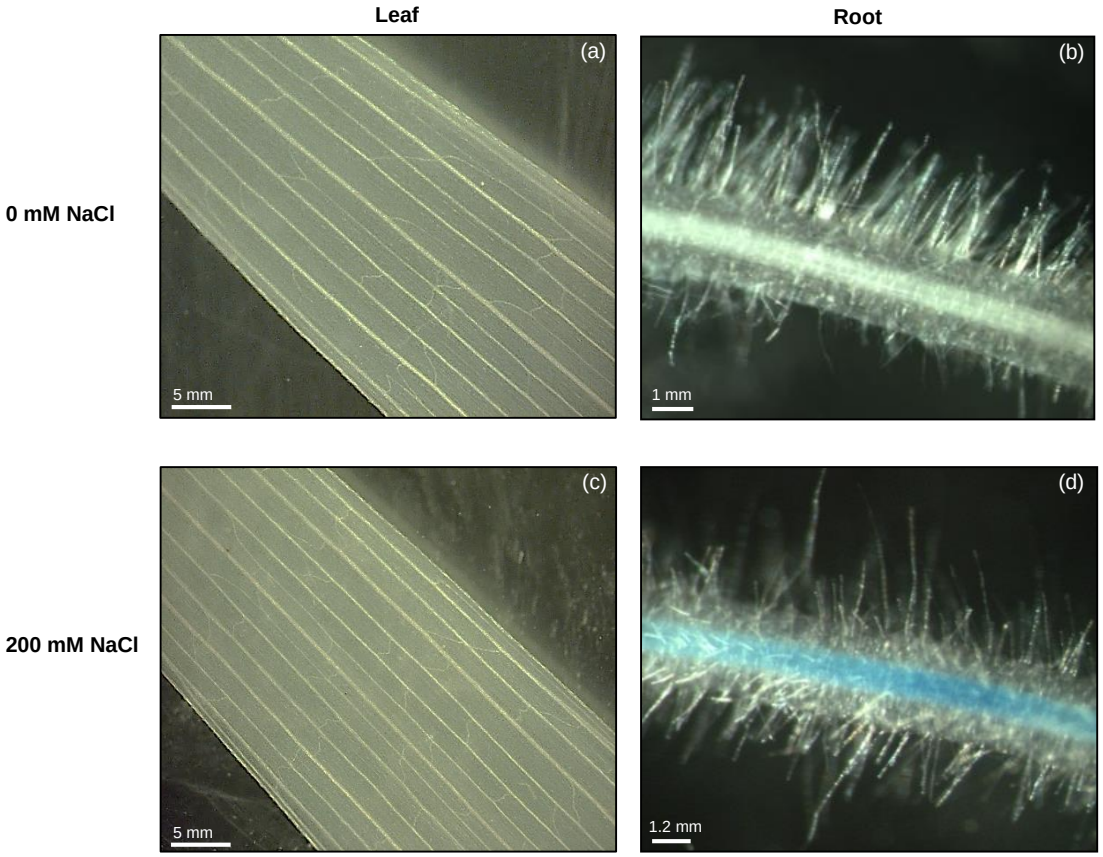
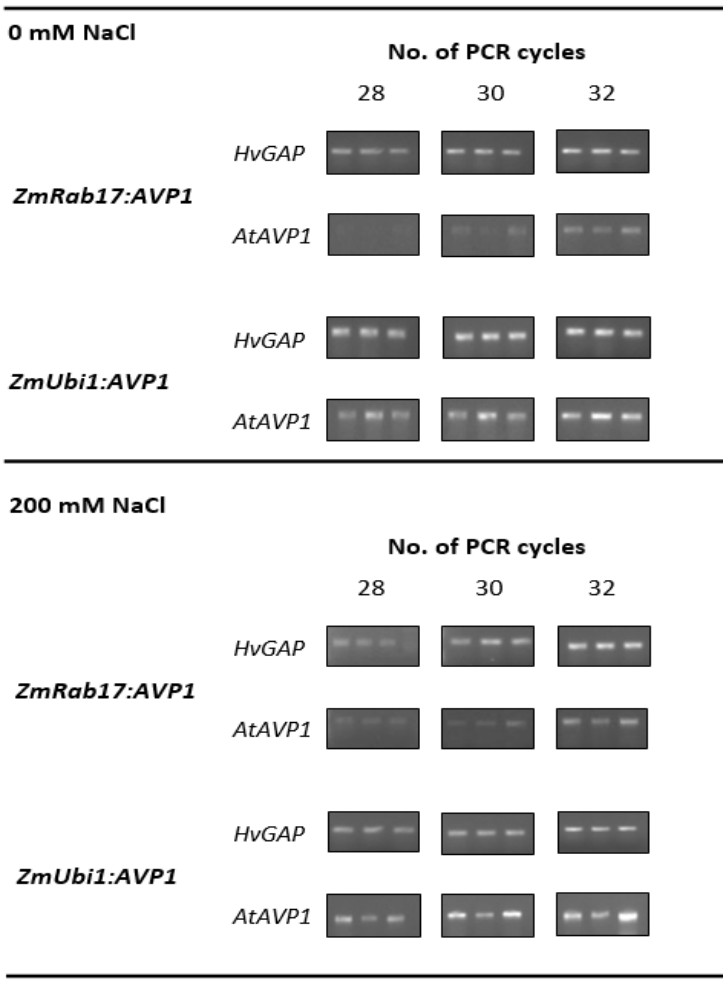


Figure 1

(a)


0 mM NaCl
No. of PCR cycles
28
30
32
HvGAP
ZmRab17:AVP1
AtAVP1
HvGAP
ZmUbi1:AVP1
AtAVP1
200 mM NaCl
No. of PCR cycles
28
30
32
HvGAP
ZmRab17:AVP1
AtAVP1
HvGAP
ZmUbi1:AVP1
AtAVP1

(b)

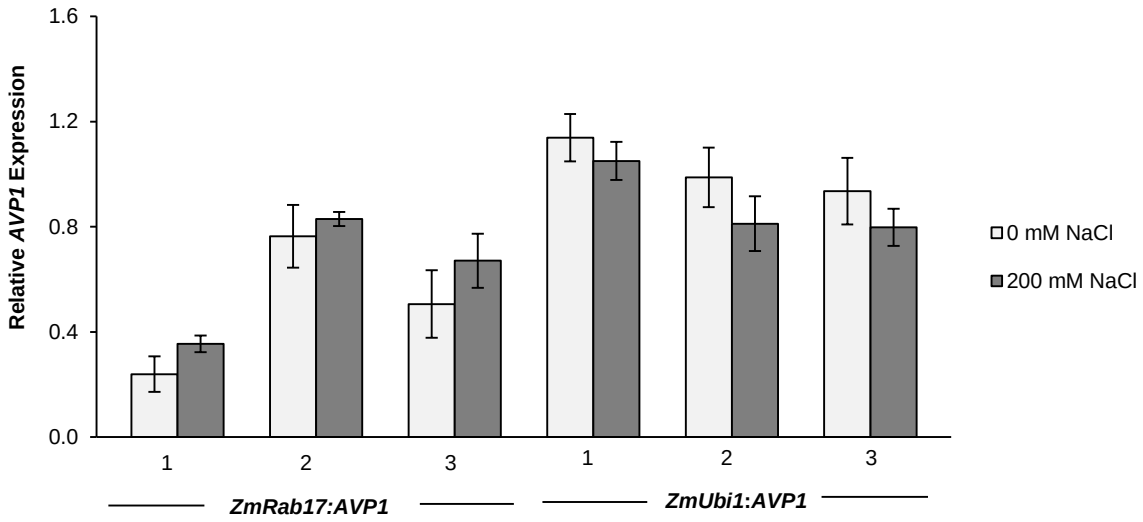
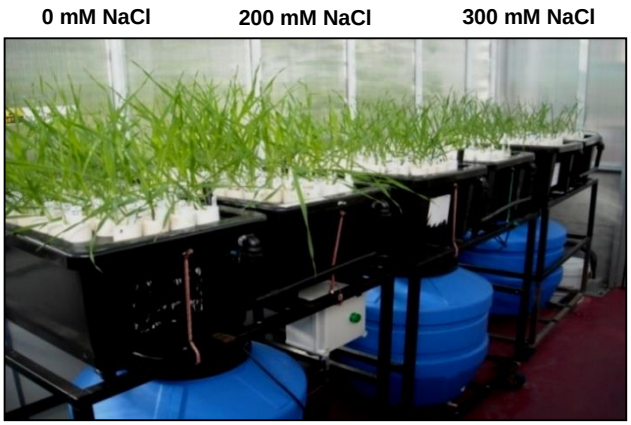
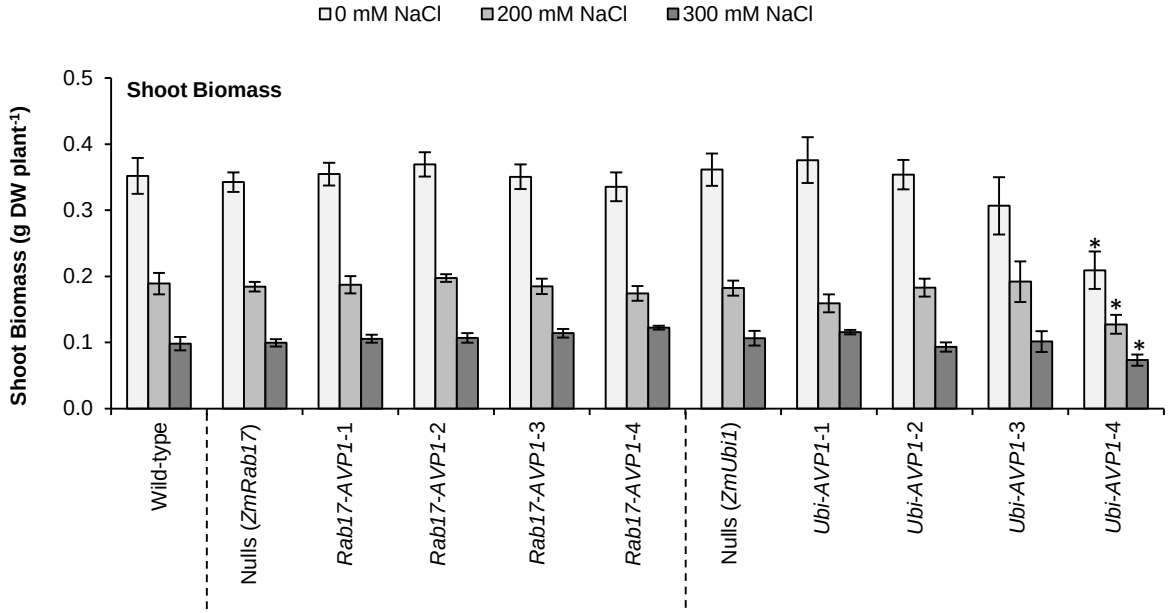

Relative AVP1 Expression
1.6
1.2
0.8
0.4
0.0
1
2
3
1
2
3
ZmRab17:AVP1
ZmUbi1:AVP1
0 mM NaCl
200 mM NaCl

Figure 2

(a)


0 mM NaCl
200 mM NaCl
300 mM NaCl

(b)


0 mM NaCl
200 mM NaCl
300 mM NaCl
Shoot Biomass
Shoot Biomass (g DW plant-1)
0.5
0.4
0.3
0.2
0.1
0.0
Wild-type
Nulls (ZmRab17)
Rab17-AVP1-1
Rab17-AVP1-2
Rab17-AVP1-3
Rab17-AVP1-4
Nulls (ZmUbi1)
Ubi-AVP1-1
Ubi-AVP1-2
Ubi-AVP1-3
Ubi-AVP1-4
*
*
*

(c)

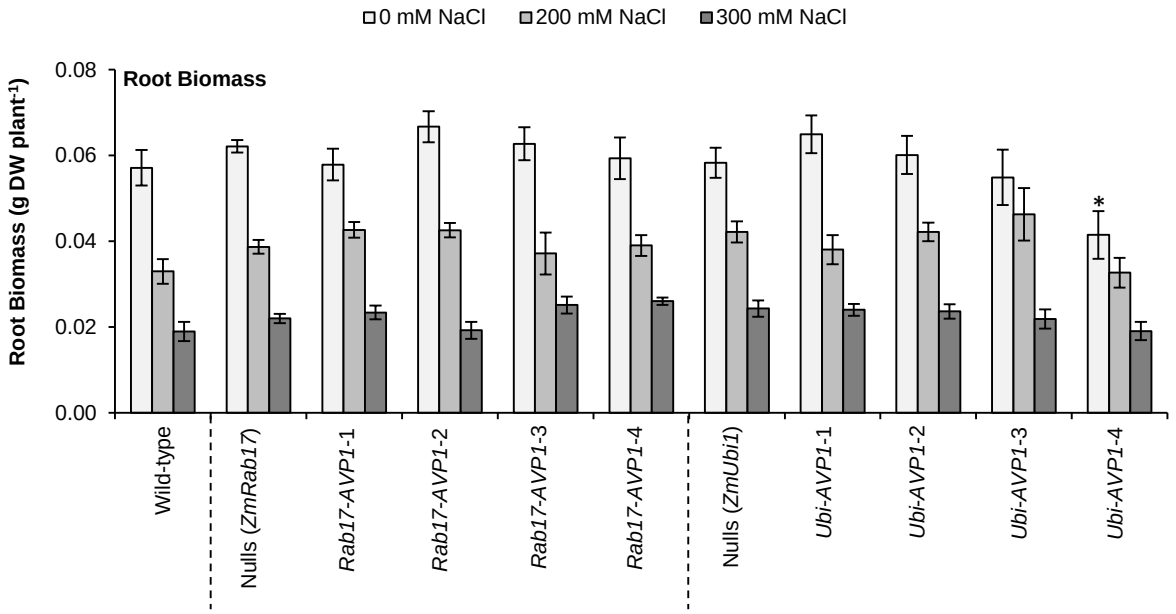

0 mM NaCl
200 mM NaCl
300 mM NaCl
Root Biomass
Root Biomass (g DW plant-1)
0.08
0.06
0.04
0.02
0.00
Wild-type
Nulls (ZmRab17)
Rab17-AVP1-1
Rab17-AVP1-2
Rab17-AVP1-3
Rab17-AVP1-4
Nulls (ZmUbi1)
Ubi-AVP1-1
Ubi-AVP1-2
Ubi-AVP1-3
Ubi-AVP1-4
*

Figure 3

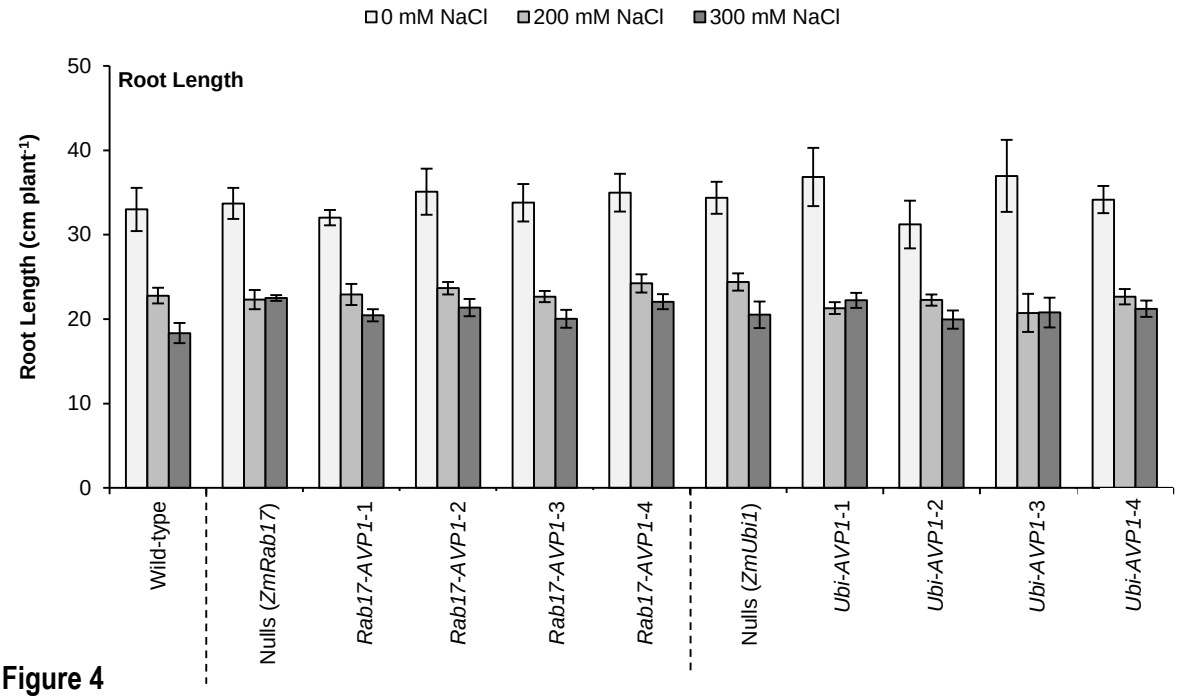

0 mM NaCl
200 mM NaCl
300 mM NaCl
Root Length
Root Length (cm plant-1)
50
40
30
20
10
0
Wild-type
Nulls (ZmRab17)
Rab17-AVP1-1
Rab17-AVP1-2
Rab17-AVP1-3
Rab17-AVP1-4
Nulls (ZmUbi1)
Ubi-AVP1-1
Ubi-AVP1-2
Ubi-AVP1-3
Ubi-AVP1-4

Figure 4

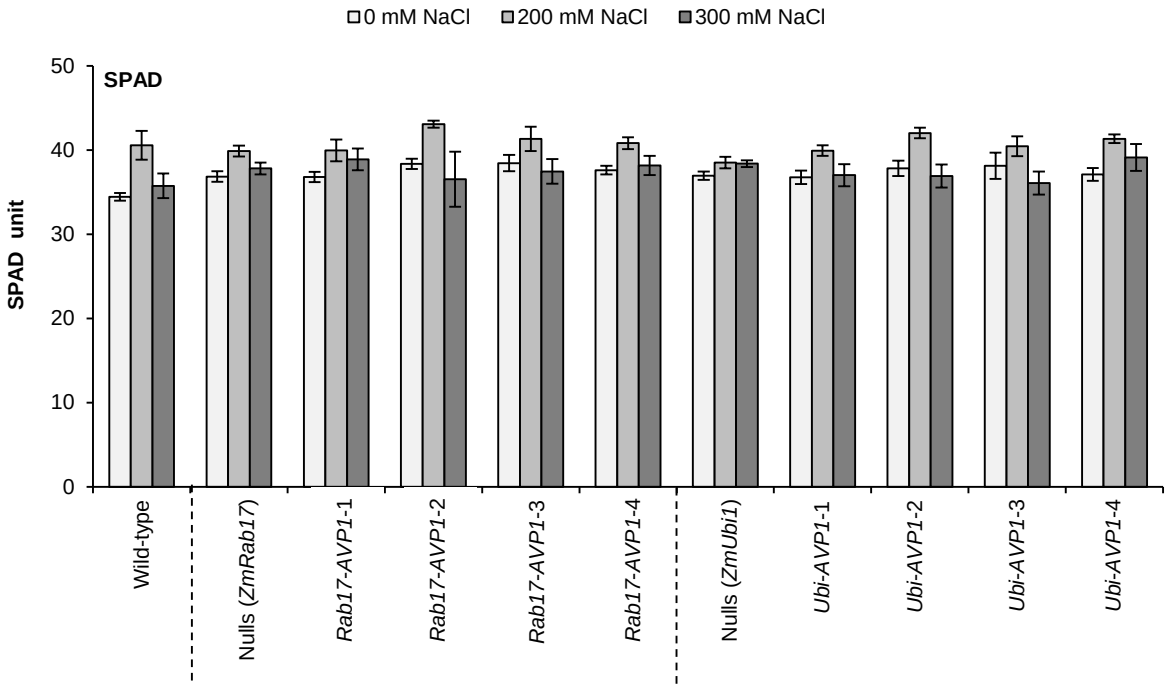

0 mM NaCl
200 mM NaCl
300 mM NaCl
SPAD
SPAD unit
50
40
30
20
10
0
Wild-type
Nulls (ZmRab17)
Rab17-AVP1-1
Rab17-AVP1-2
Rab17-AVP1-3
Rab17-AVP1-4
Nulls (ZmUbi1)
Ubi-AVP1-1
Ubi-AVP1-2
Ubi-AVP1-3
Ubi-AVP1-4

Figure 5

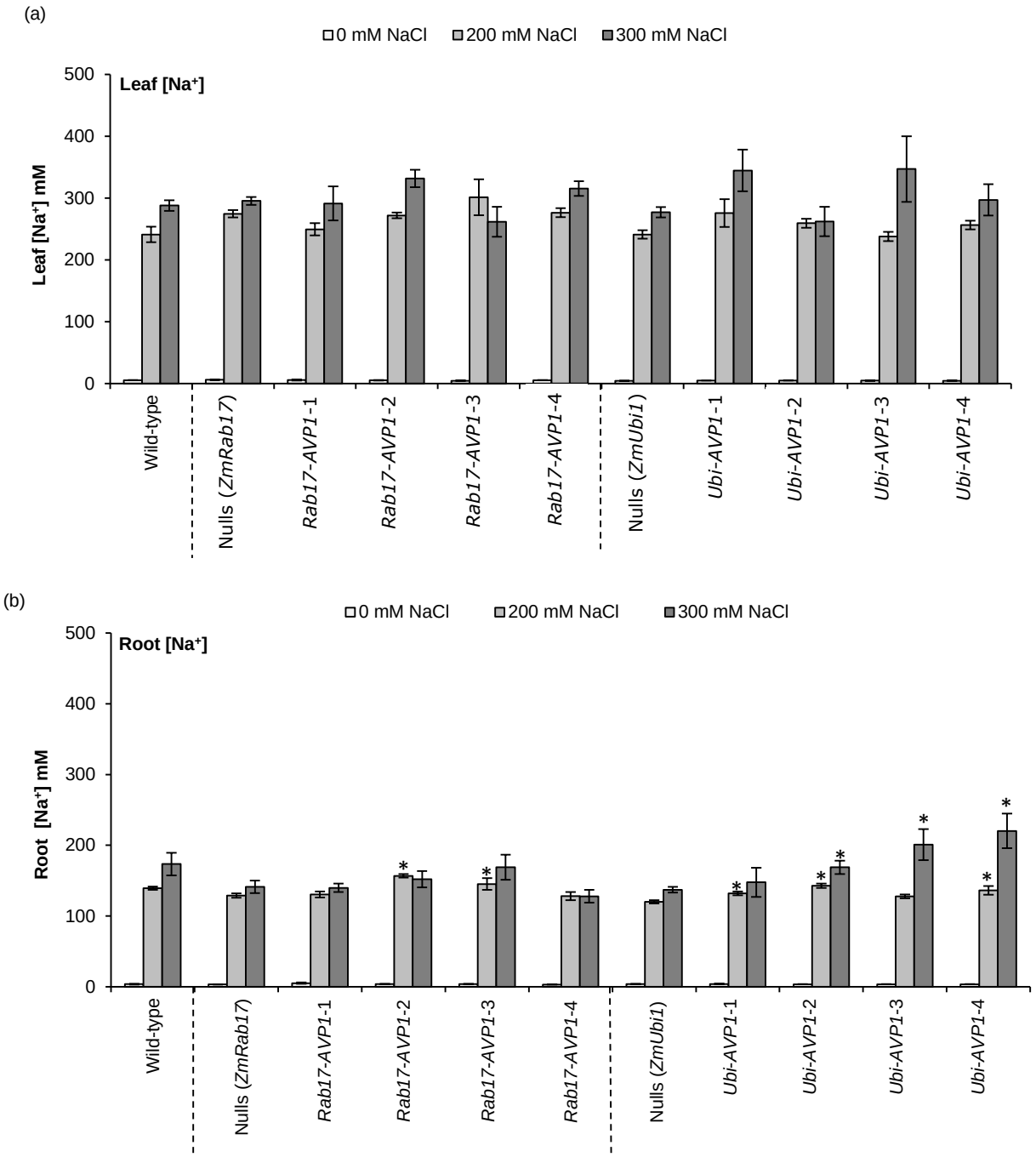

(a)
0 mM NaCl
200 mM NaCl
300 mM NaCl
Leaf [Na+]
Leaf [Na+] mM
500
400
300
200
100
0
Wild-type
Nulls (ZmRab17)
Rab17-AVP1-1
Rab17-AVP1-2
Rab17-AVP1-3
Rab17-AVP1-4
Nulls (ZmUbi1)
Ubi-AVP1-1
Ubi-AVP1-2
Ubi-AVP1-3
Ubi-AVP1-4
(b)
0 mM NaCl
200 mM NaCl
300 mM NaCl
Root [Na+]
Root [Na+] mM
500
400
300
200
100
0
Wild-type
Nulls (ZmRab17)
Rab17-AVP1-1
Rab17-AVP1-2
Rab17-AVP1-3
Rab17-AVP1-4
Nulls (ZmUbi1)
Ubi-AVP1-1
Ubi-AVP1-2
Ubi-AVP1-3
Ubi-AVP1-4

Figure 6

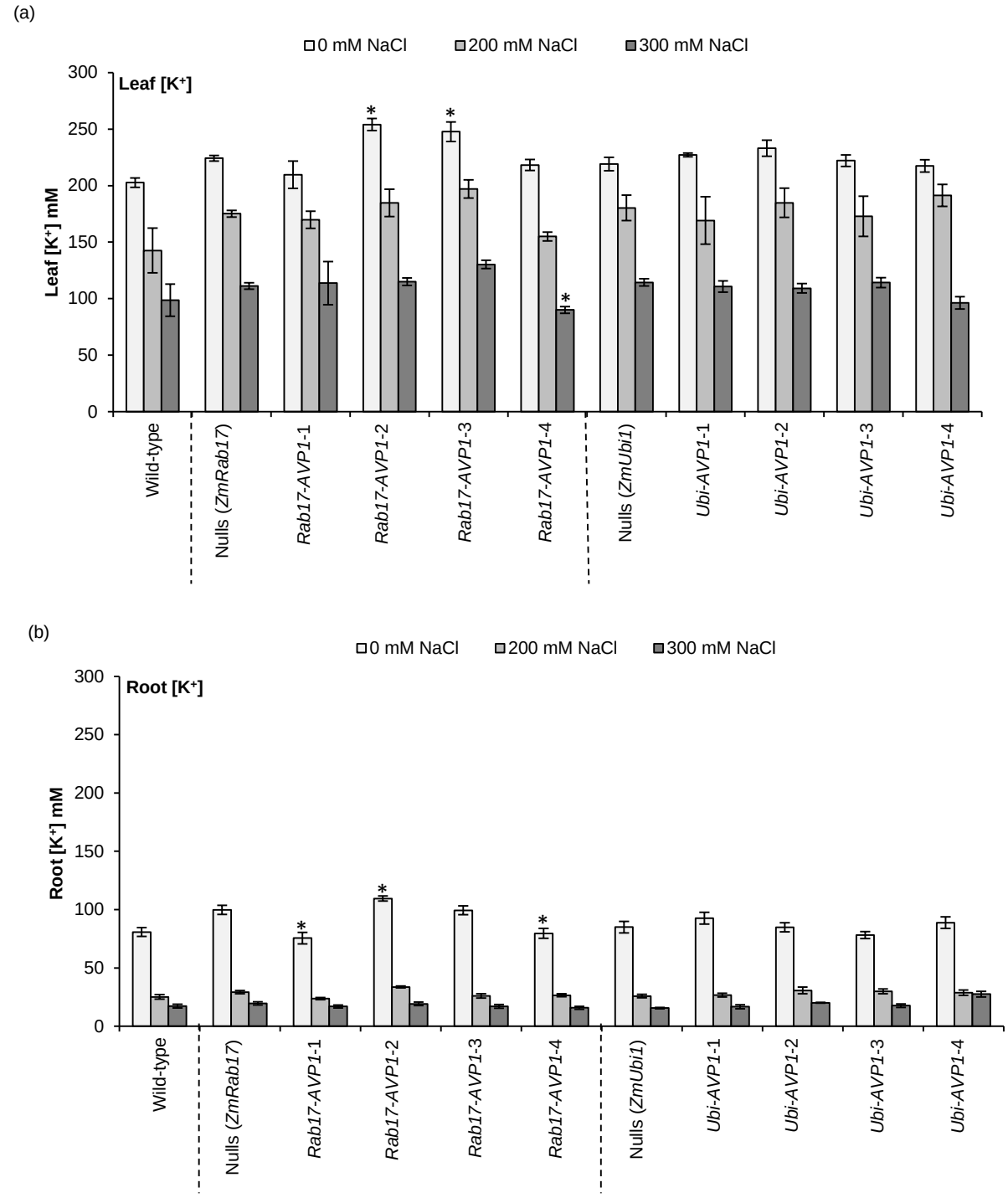

(a)
0 mM NaCl
200 mM NaCl
300 mM NaCl
Leaf [K+]
Leaf [K+] mM
300
250
200
150
100
50
0
Wild-type
Nulls (ZmRab17)
Rab17-AVP1-1
Rab17-AVP1-2
Rab17-AVP1-3
Rab17-AVP1-4
Nulls (ZmUbi1)
Ubi-AVP1-1
Ubi-AVP1-2
Ubi-AVP1-3
Ubi-AVP1-4
(b)
0 mM NaCl
200 mM NaCl
300 mM NaCl
Root [K+]
Root [K+] mM
300
250
200
150
100
50
0
Wild-type
Nulls (ZmRab17)
Rab17-AVP1-1
Rab17-AVP1-2
Rab17-AVP1-3
Rab17-AVP1-4
Nulls (ZmUbi1)
Ubi-AVP1-1
Ubi-AVP1-2
Ubi-AVP1-3
Ubi-AVP1-4

Figure 7

Supplementary Figures

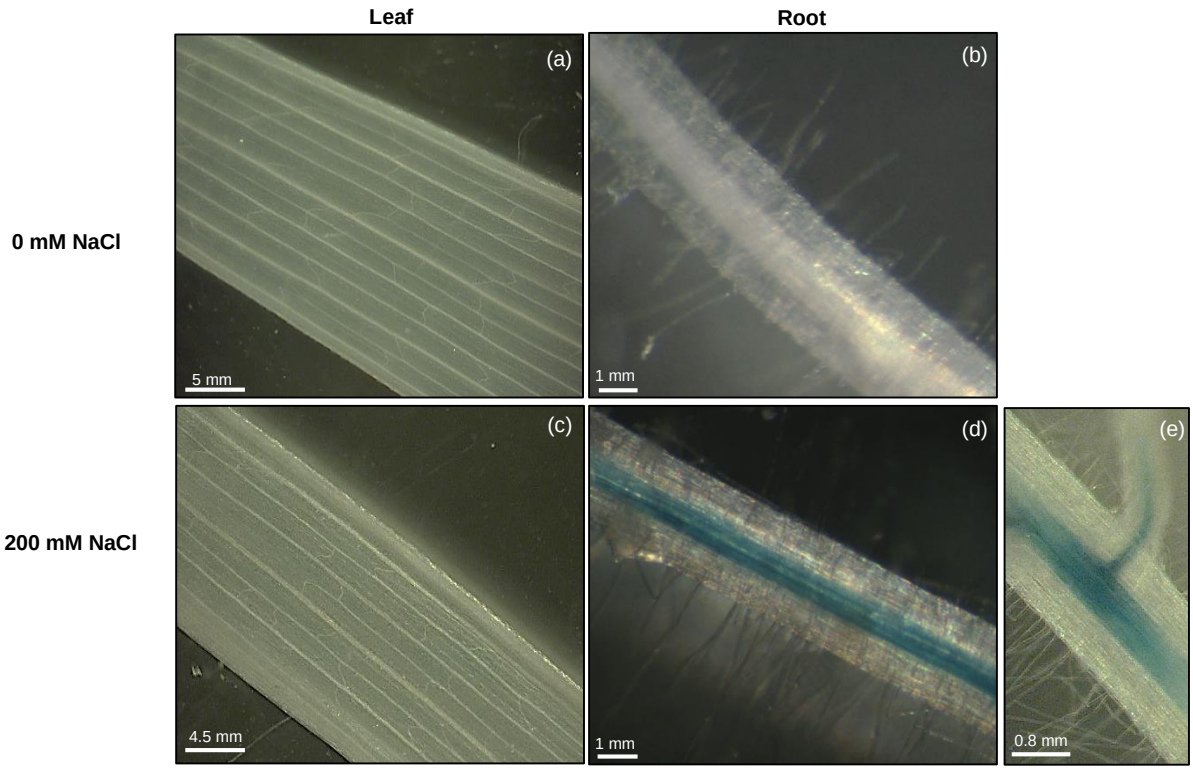


Figure S1 Representative images of second independent T_2 *ZmRab17*:*uidA* barley (cv. Golden Promise) event showing histochemical GUS staining (blue colouring) after 24 h at (a & b) 0 mM or (c & d) 200 mM NaCl in leaf and root tissue respectively (e) GUS activity at the lateral root tip junction at 200 mM NaCl. Digital images were recorded using a Leica MZ FLIII stereo microscope equipped with a Leica DC 300F camera and Leica IM50 image manager software.

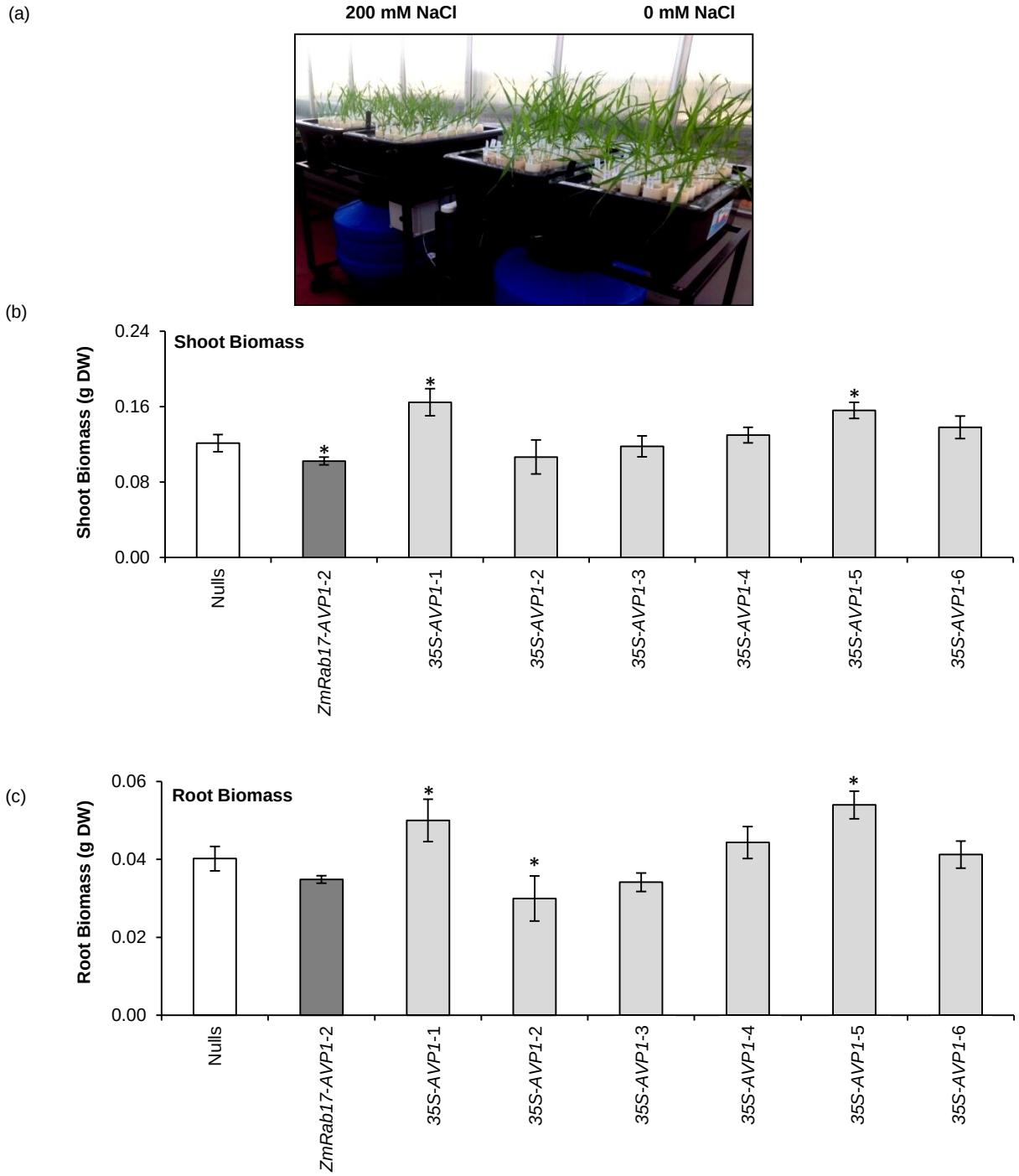


Figure S2 (a) An image of null segregants, T_2 *ZmRab17:AVP1-2* and six independent transformation events of T_1 *35S:AVP1* (1, 2, 3, 4, 5 & 6) barley (cv. WI4330) in hydroponics after 21 d at 200 and 0 mM NaCl treatments (left to right) (b) Shoot and (c) root biomass (g DW plant^{-1}) of null segregants (white), T_2 *ZmRab17:AVP1-2* (dark grey) and T_1 *35S:AVP1* lines (1, 2, 3, 4, 5 & 6) (light grey) at 200 mM NaCl treatment. Values are presented as the mean ± s.e.m (n = 4-9) with an asterisks (*) indicating a significant difference to null segregants (one-way ANOVA, LSD, $P \leq 0.05$).

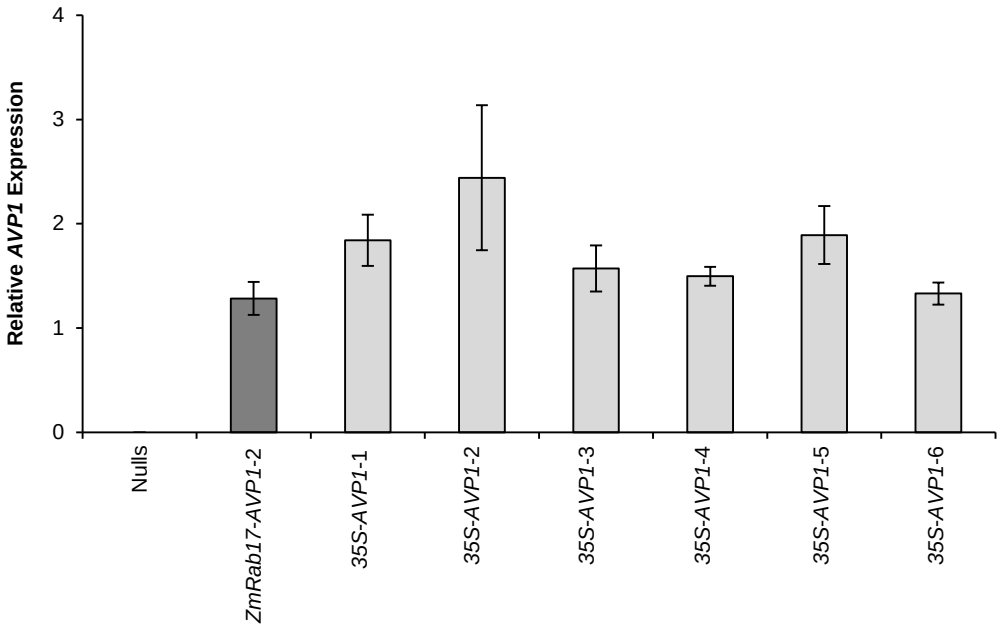


Figure S3 Relative *AVP1* expression (compared to *HvGAP*) in leaf tissue of null segregants (Nulls), T_2 *ZmRab17*:*AVP1*-2 and T_1 *35S*:*AVP1* lines (1, 2, 3, 4, 5 & 6) barley (cv. WI4330) in non-saline conditions (0 mM NaCl) using semi-quantitative RT-PCR with *AVP1* and *HvGAP* specific primers (internal control). Values are presented as the mean ± s.e.m (*n* = 3). This confirms expression of *AVP1* in T_2 *ZmRab17*:*AVP1*-2 and all six transgenic T_1 *35S*:*AVP1* barley lines and the absence of *AVP1* expression in null segregants.

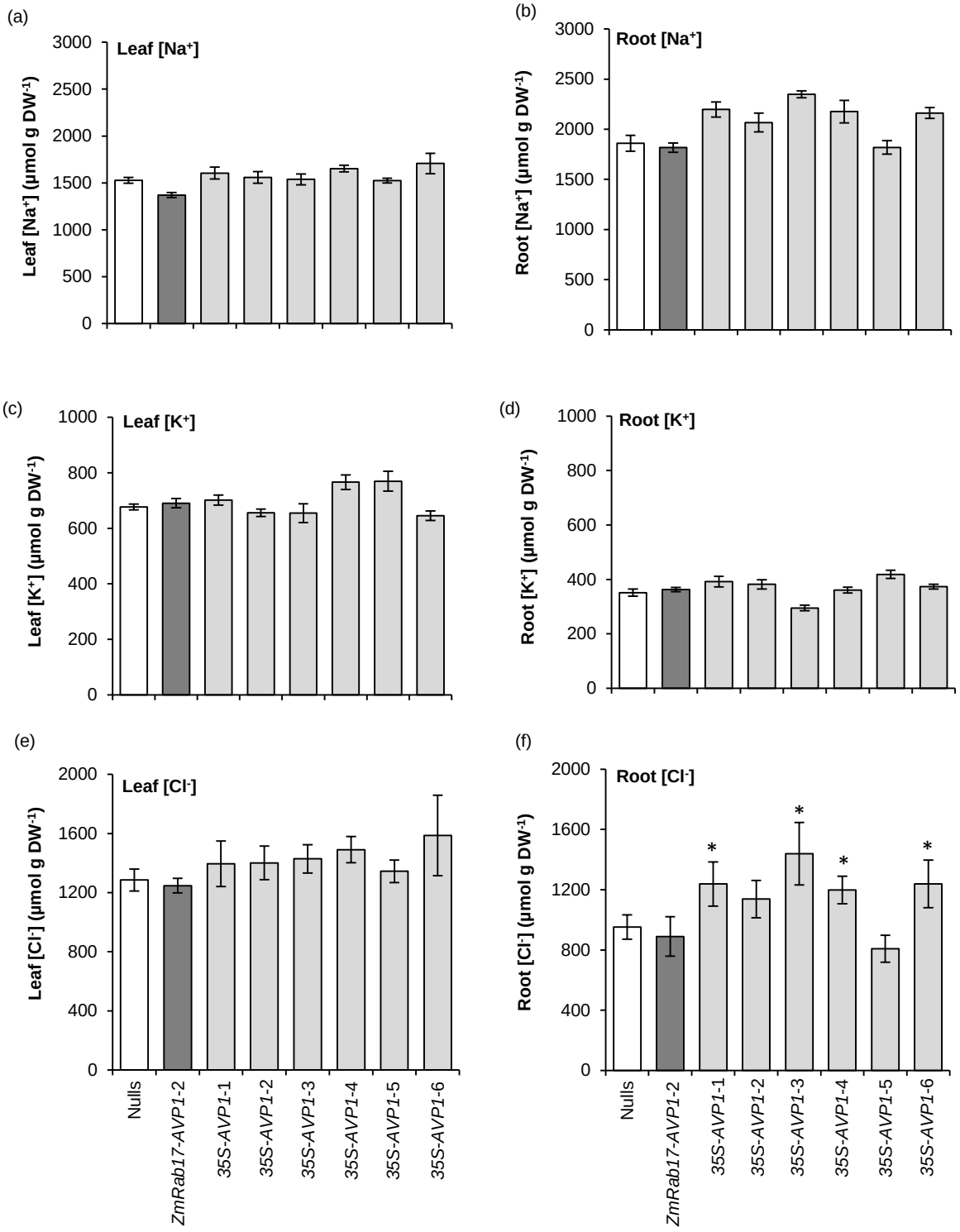


Figure S4 (a & b) Na^+, (c & d) K^+ and (e & f) Cl^- concentrations (µmol g DW^{-1}) in 3rd leaf and roots respectively of null segregants (white), T_2 *ZmRab17*:*AVP1*-2 (dark grey) and T_1 *35S*:*AVP1* barley lines (1, 2, 3, 4, 5 & 6) (grey) (cv. WI4330) after 21 d at 200 mM NaCl. Values are presented as the mean ± s.e.m. (*n* = 4-9) with an asterisks (*) indicating a significant difference to nulls (one-way ANOVA, LSD, $P \leq 0.05$).

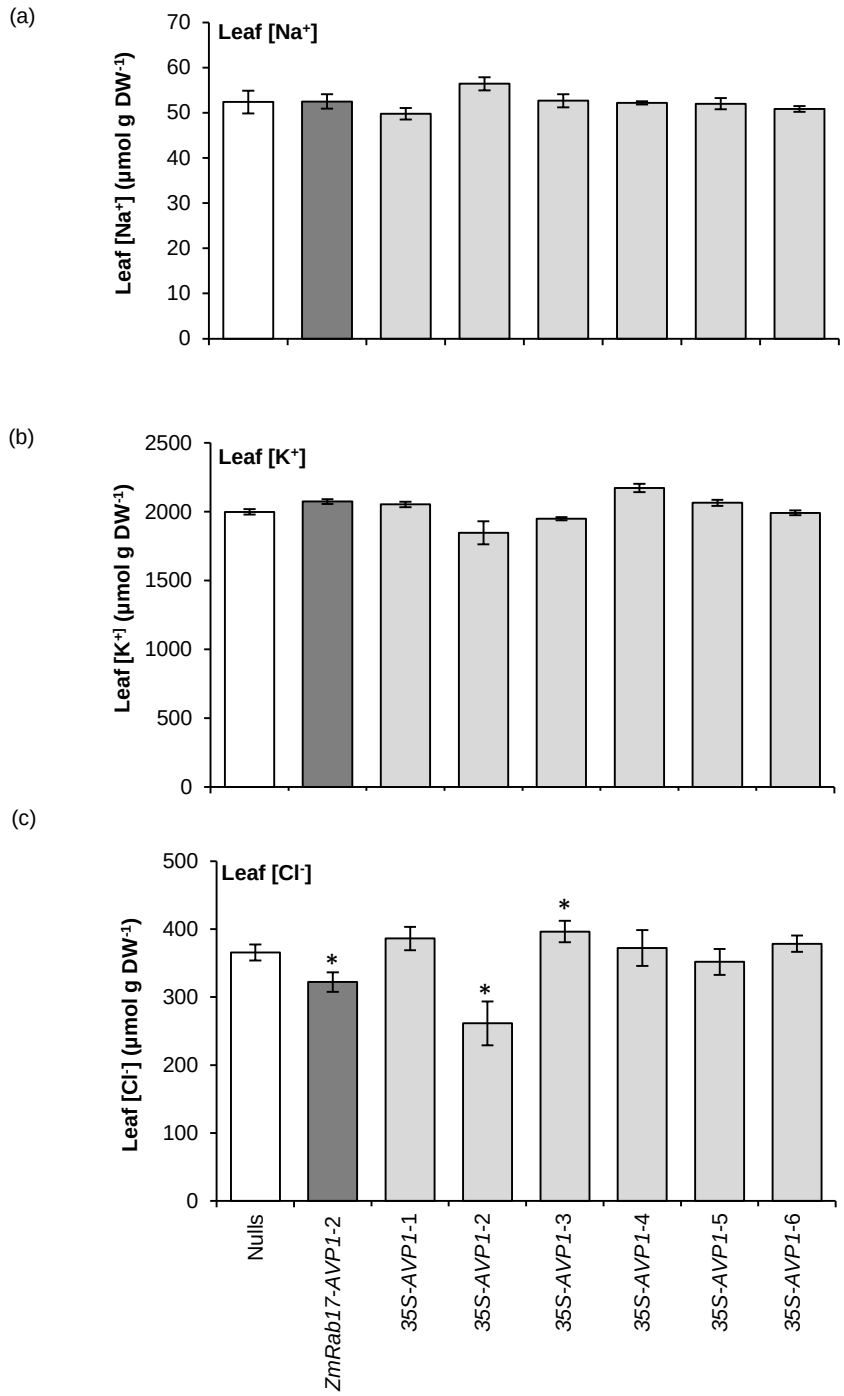


Figure S5 (a) Na^+, (b) K^+ and (c) Cl^- concentrations (µmol g DW^{-1}) in the 3rd leaf blade of null segregants (white), T_2 *ZmRab17:AVP1-2* (dark grey) and T_1 *35S:AVP1* barley lines (1, 2, 3, 4, 5 & 6) (light grey) at 0 mM NaCl. Values are presented as the mean ± s.e.m (n = 4-9) with an asterisks (*) indicating a significant difference to null segregants (one-way ANOVA, LSD, $P \leq 0.05$).

Additional Information

To increase the quantity of seed for future large scale field trials, the *ZmRab17*:*AVP1* and *ZmUbi1*:*AVP1* (cv. WI4330) barley was hand sown and harvested at a non-saline field site near O'Halloran Hill, South Australia (Glenthorne farm, The University of Adelaide) in 2011 (Figure A1). However, due to the lack of improved salinity tolerance in these lines compared to null segregants in the greenhouse they were not tested at the saline field site in Kunjin, Western Australia.

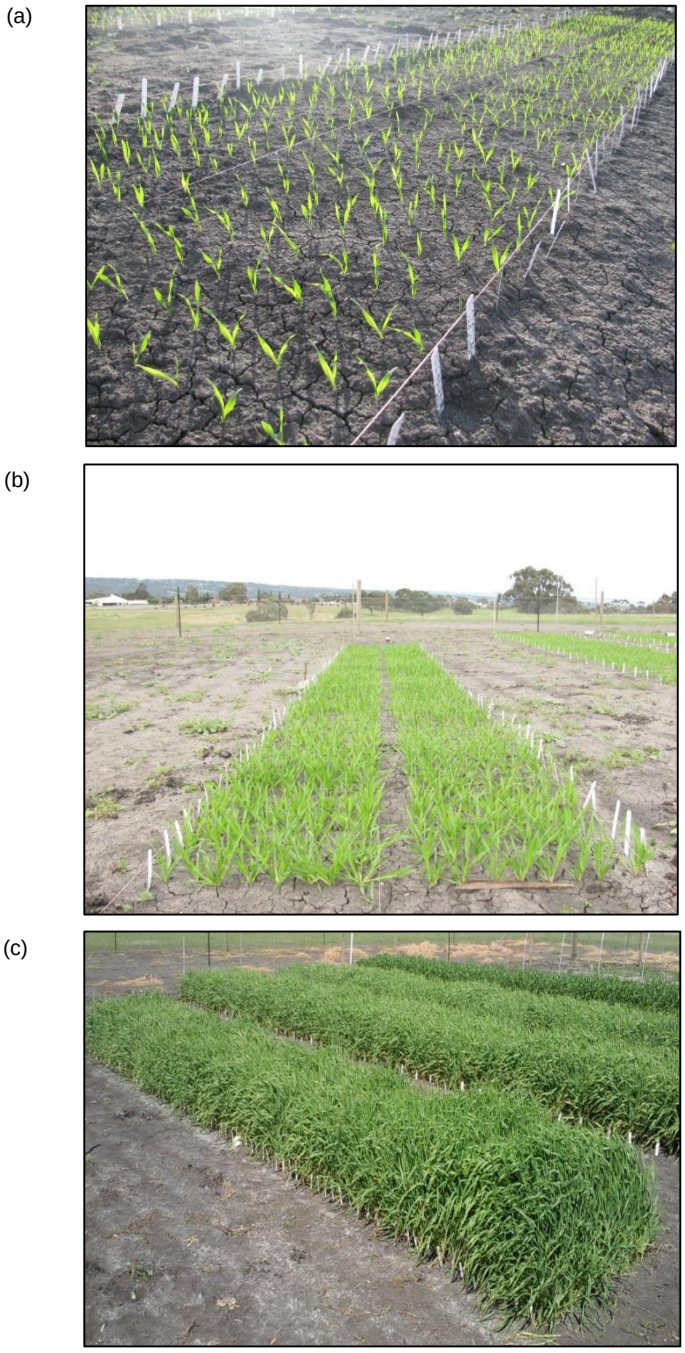


Figure A1 Images of plots with mixed rows of wild-type and T_3 *ZmRab17:AVP1 and ZmUbi1:AVP1* barley (cv. WI4330) hand sown at a non-saline GM field site near O'Halloran Hill, Adelaide, South Australia (Glenthorne Farm, The University of Adelaide) in June 2011. Images taken in (a) July, (b) August and (c) September 2011.

Chapter 8

General discussion

Review of thesis aims

Previous work established that transgenic barley (cv. Golden Promise) with the constitutive *CaMV 35S* expression of *AVP1*, a gene encoding the type I *Arabidopsis* vacuolar H^+-pyrophosphatase (H^+-PPase), had significantly larger projected shoot area in non-saline and saline soil compared to null segregants in a greenhouse-based experiment (Schilling, 2010). However, the growth and grain yield of the transgenic *AVP1* barley was yet to be evaluated in a saline field. It was also yet to be explored whether the larger shoot biomass of transgenic *AVP1* barley in both non-saline and saline conditions arose from changes in tissue solute accumulation, plant water use, plant nutrition, carbohydrate metabolism, heterotrophic growth or a combination of these traits. In addition, for this *AVP1* technology to be applicable for barley grain growers, a commercially relevant transgenic *AVP1* barley cultivar with well-regulated control of *AVP1* expression was needed.

The aims of this PhD project were:

1. To evaluate the shoot biomass and grain yield of wild-type and transgenic *AVP1* barley in a field with low and high salinity **(Chapter 2)**

2. To investigate the abiotic stress tolerance and potential factors contributing to the larger shoot biomass of transgenic *AVP1* barley by evaluating:
    a. the growth, rhizosphere acidification and P uptake of transgenic *AVP1* barley at low and sufficient P supply **(Chapter 3)**
    b. the growth and nitrate (NO_3^-) uptake capacity of transgenic *AVP1* barley at low and sufficient NO_3^- supply **(Chapter 4)**
    c. the growth, tissue ion contents and water use of transgenic *AVP1* barley under salinity, low water availability and a combination of the two stresses **(Chapter 5)**
    d. the seedling vigour and carbohydrate metabolism of transgenic *AVP1* barley **(Chapter 6)**

3. To characterise the salt stress inducibility of the *ZmRab17* promoter and to evaluate the growth of commercially relevant barley (cv. WI4330) with *AVP1* expression via the stress-inducible promoter (*ZmRab17*) or the plant-derived constitutive promoter (*ZmUbi1*) in non-saline and saline conditions **(Chapter 7)**

Summary of the main findings

In Chapter 2, the shoot biomass and grain yield traits of transgenic *AVP1* barley was evaluated in a field with low and high salinity near Kunjin, Western Australia. The transgenic *AVP1* barley had a larger shoot biomass with no change in leaf Na^+ accumulation compared to wild-type in both the low and high salinity field (Schilling et al., 2014). In addition, the transgenic *AVP1* barley had increased grain yield per plant in the high salinity field. An increase in the number of grains per plant appeared likely to be contributing to this increased grain yield. To our knowledge this is the first time that such effects of *AVP1* expression in transgenic plants have been measured in a field with salinity. These findings suggests that transgenic *AVP1* barley is a promising option to help increase the grain yield of cereal crops in a saline field.

In both Chapter 3 and 4, the growth of transgenic *AVP1* barley was evaluated at low and sufficient P or NO_3^- supply to determine whether the transgenic *AVP1* barley had altered nutrient use. At low P supply, the transgenic *AVP1* barley had a larger shoot biomass, greater root P uptake and increased rhizosphere acidification compared to wild-type. No significant difference in shoot or root biomass of transgenic *AVP1* barley compared to null segregants was observed at sufficient P supply. In the low NO_3^- treatment, transgenic *AVP1* barley (*35S-AVP1*-2 and *35S-AVP1*-3) had a larger shoot biomass but no significant difference in root biomass or NO_3^- uptake compared to null segregants. In the sufficient NO_3^- treatment, one transgenic *AVP1* barley line (*35S-AVP1*-2) had a larger shoot and root biomass, higher shoot and root N contents and greater low-affinity NO_3^- uptake capacity compared to null segregants. These findings suggest that transgenic *AVP1* barley has improved shoot growth at low P and low NO_3^- supply and that

enhanced nutrient uptake, potentially through increased rhizosphere acidification, could be contributing to the improved growth of transgenic *AVP1* barley compared to null segregants.

In Chapter 5, the effect of salinity (osmotic only), low water availability (matric only) and a combination of the two stresses (osmotic and matric) on growth and ion uptake of transgenic *AVP1* barley was assessed. Confirming previous findings, with salinity only the transgenic *AVP1* barley had a larger shoot biomass with no difference in leaf Na^+, K^+ and Cl^- concentrations compared to null segregants. Two sibling transgenic *AVP1* barley lines (*35S-AVP1*-1a & *35S-AVP1*-1b) also had a larger shoot biomass under low water availability compared to null segregants. Cumulative plant water use was similar in the transgenic *AVP1* barley and null segregants. Furthermore, two transgenic *AVP1* barley lines *(35S-AVP1*-1a and *35S-AVP1*-2) had a larger shoot and root biomass compared to null segregants in the treatment with combined salinity and low water availability. These findings suggest that transgenic *AVP1* barley is a promising option to improve crop growth under concurrent stresses.

In Chapter 6, a paper roll method was optimised to evaluate the seedling vigour of transgenic *AVP1* barley at 0 and 100 mM NaCl in a non-destructive manner. Eleven days after seed imbibition at 0 mM NaCl, transgenic *AVP1* barley had a larger shoot and root biomass and total plant biomass compared to null segregants. This larger plant biomass was detectable from 6 days after seed imbibition with the transgenic *AVP1* barley having a faster growth rate between 0 to 5 days after seed imbibition compared to null segregants. However, at 150 mM NaCl, the total plant biomass of transgenic *AVP1* barley at 11 d after seed imbibition was similar to null segregants. At 0 mM NaCl, transgenic *AVP1* barley and null segregants did not differ in metabolites involved with sucrose metabolism. However, compared to wild-type, the leaf metabolomic analysis indicates that transgenic *AVP1* barley had significantly lower galactose and significantly higher ascorbic acid and dehydroascorbic acid concentrations. Overall, these findings suggests that transgenic *AVP1* barley had improved seedling vigour and enhanced ascorbic acid

synthesis compared to null segregants. It is also suggests that both these traits could be contributing to the larger plant biomass of transgenic *AVP1* barley compared to null segregants. However, further work is needed to investigate this hypothesis.

In Chapter 7, the salt stress inducibility of the *ZmRab17* promoter was characterised and the growth of a commercially relevant cultivar (cv. WI4430) expressing *AVP1* using via the *ZmRab17* or constitutive *ZmUbi1* promoter was evaluated in hydroponics conditions with 0, 200 and 300 mM NaCl. The findings suggest that the *ZmRab17* promoter is salt-stress inducible in barley root stelar cells but that there is also basal transgene expression at 0 mM NaCl. Both shoot and root biomass of *ZmRab17*:*AVP1* and *ZmUbi1*:*AVP1* barley (cv. WI4330) did not differ from wild-type and null segregants at 0, 200 and 300 mM NaCl. However, results of T_1 *35S*:*AVP1* barley (cv. WI4330) showed that two transgenic barley lines had a larger shoot and root biomass and higher root chloride (Cl^-) concentrations compared to null segregants at 200 mM NaCl. This suggests that the larger shoot biomass phenotype of transgenic *AVP1* barley is not limited to the Golden Promise cultivar and that the *CaMV 35S* promoter could be important for increasing the shoot biomass of transgenic plants.

Implications of thesis findings

This project showed that transgenic barley with the constitutive *CaMV 35S* expression of *AVP1* had a larger shoot biomass than plants without this gene under various abiotic stresses, including salinity, drought, low P supply, low NO_3^- supply and combined salinity and low water availability. Furthermore, it showed that transgenic *AVP1* barley had increased grain yield per plant compared to wild-type in a field with high salinity. It has previously been suggested that altering the expression of one specific gene would be unlikely to increase crop abiotic stress tolerance, due to the multiple tolerance mechanisms involved (Roy et al., 2011; Shabala, 2013). However, in this case, *AVP1* appears to be one such gene that can improve the growth of transgenic plants under various individual abiotic stresses and even concurrent

abiotic stresses. The use of transgenic barley expressing *AVP1* thus appears to be a promising option to increase growth and grain yield of cereal crops exposed to abiotic stress.

A common phenotype of transgenic plants expressing *AVP1* or the gain of function allele *AVP1D* is a larger shoot biomass, and occasionally a larger root biomass, compared to plants without this gene under abiotic stress (Bao et al., 2009; Gaxiola et al., 2001; Gonzalez et al., 2010; Li et al., 2005; Li et al., 2010; Paez-Valencia et al., 2011; Paez-Valencia et al., 2013; Park et al., 2005; Pasapula et al., 2011; Qin et al., 2013; Schilling et al., 2014; Yang et al., 2007; Yang et al., 2014). Transgenic plants expressing *AVP1* or *AVP1D* also have a larger shoot biomass in non-stress conditions (Li et al., 2005; Li et al., 2010; Schilling et al., 2014; Vercruyssen et al., 2011; Yang et al., 2007). The larger shoot and root biomass of transgenic *AVP1* plants under salinity stress was attributed to increased vacuolar Na^+ sequestration (Gaxiola et al., 2001; Li et al., 2010; Pasapula et al., 2011) and at low water availability was attributed to a larger root biomass enabling increased water uptake (Bao et al., 2009; Park et al., 2005; Pasapula et al., 2011). Whilst the larger shoot biomass of transgenic *AVP1* plants at low P and NO_3^- supply was ascribed to a larger root biomass and greater rhizosphere acidification increasing nutrient uptake (Paez-Valencia et al., 2013; Yang et al., 2007; Yang et al., 2014).

However, the findings of this project (Chapters 2 to 6) with those from previous studies (Bao et al., 2009; Ferjani et al., 2011; Gaxiola et al., 2001; Gaxiola et al., 2012; Gonzalez et al., 2010; Li et al., 2005; Li et al., 2010; Paez-Valencia et al., 2013; Park et al., 2005; Pasapula et al., 2011; Qin et al., 2013; Schilling et al., 2014; Vercruyssen et al., 2011; Yang et al., 2007; Yang et al., 2014) suggest that a combination of traits is contributing to the larger biomass of transgenic barley expressing *AVP1* compared to null segregants under abiotic stress. It is likely that both enhanced vacuolar acidification (Gaxiola et al., 2001) and reduced cytoplasmic PP_i levels (Ferjani et al., 2011) are responsible for various traits, such as increased nutrient and water uptake, increased ascorbic acid synthesis, enhanced carbohydrate

metabolism and increased seedling vigour, and thus the larger growth of transgenic *AVP1* barley (summarised in Figure 1). Some traits, such as increased auxin fluxes, increased sucrose phloem loading, enhanced gluconeogenesis and increased ascorbic acid synthesis (Chapter 6), increase the shoot and/or root growth of transgenic *AVP1* plants compared to plants without this gene (Chapter 2, 3, 4 and 5) (Figure 1). Thus, compared to plants without this gene transgenic *AVP1* plants appear to tolerate abiotic stress conditions because they are larger in size. Whilst other traits, such as increased vacuolar ion sequestration, increased nutrient uptake (Chapter 3 and 4), increased water uptake (Chapter 5), increased photosynthesis and enhanced seedling vigour (Chapter 6), would be advantageous to transgenic *AVP1* plants under abiotic stress enabling increased shoot (Chapter 2, 3, 4 and 5) and root biomass and increased grain or fruit yield (Chapter 2) compared to plants without this gene (Figure 1). Overall, it is difficult to distinguish between cause and effect of these traits; some or all of these traits are likely to be contributing to the increased growth of transgenic barley expressing *AVP1* at different stages throughout their lifecycle (Figure 1).

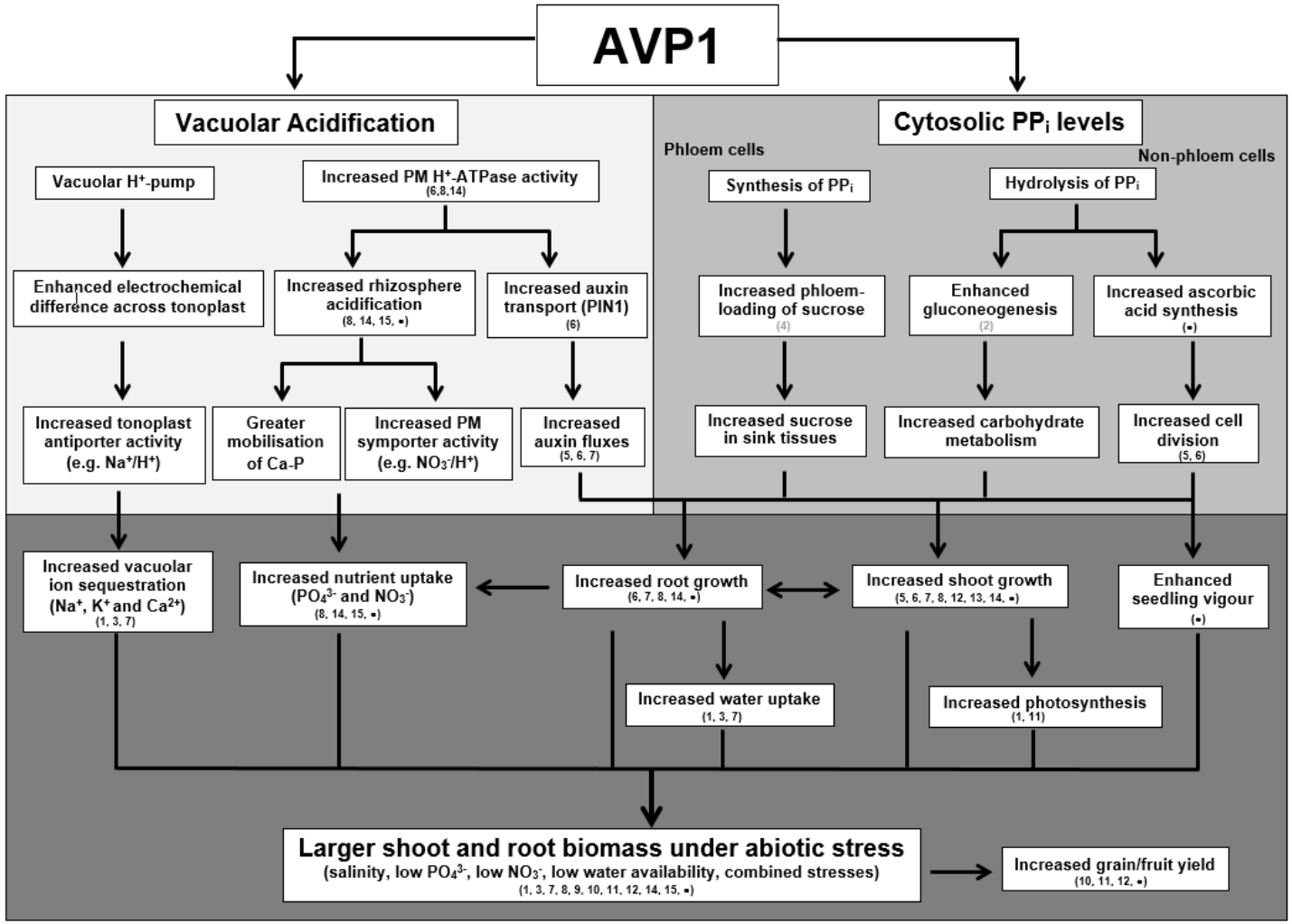


Figure 1 A flowchart outlining various traits observed (black number) or hypothesised (grey number) to be contributing to the larger growth of transgenic plants expressing the *Arabidopsis* vacuolar H^+-PPase gene (*AVP1*) under abiotic stress based on previous studies 1. Bao et al., 2009; 2. Ferjani et al., 2011; 3. Gaxiola et al., 2001; 4. Gaxiola et al., 2012; 5; Gonzalez et al., 2010; 6. Li et al., 2005; 7. Li et al., 2010; 8. Paez-Valenica et al., 2013; 9. Park et al., 2005; 10. Pasapula et al., 2011; 11. Qin et al., 2013; 12. Schilling et al., 2014; 13. Vercruyssen et al., (2011); 14. Yang et al., 2007; 15. Yang et al., 2014 and (●) the results in this project (Chapters 2 to 6). AVP1 has two main mechanisms (1) vacuolar acidification (light-grey box) and (2) regulation of cytoplasmic inorganic pyrophosphate (PP_i) concentrations within specific cell types (non-phloem vs phloem) (grey box). Both mechanisms underpin various traits (dark-grey box) that contribute to the greater shoot and root biomass of transgenic plants expressing *AVP1* throughout the plant lifecycle. Abbreviations: Ca^{2+}: calcium, Ca-P: calcium phosphates, H^+: proton, K^+: potassium, Na^+: sodium, PIN1: Pinformed 1 auxin efflux facilitator, PP_i: inorganic pyrophosphate. Solid lines with arrows indicate a putative link between traits and the respective direction of flow.

Previously, transgenic *AVP1* barley also had a greater shoot biomass than null segregants in non-saline conditions (Schilling, 2010) and other studies have reported a larger shoot biomass of transgenic plants expressing *AVP1* in non-saline conditions compared to plants without this gene (Gonzalez et al., 2010; Li et al., 2005; Li et al., 2010; Paez-Valencia et al., 2013; Vercruyssen et al., 2011; Yang et al., 2007). However, in this project, this was not always the case in non-stressed conditions. Compared to null segregants, transgenic *AVP1* barley had a greater shoot biomass in non-stressed conditions in the nitrate (Chapter 4) and seedling vigour (Chapter 6) experiments. However, the shoot biomass of transgenic *AVP1* barley did not differ to null segregants or wild-type in non-stressed conditions in the phosphorus (Chapter 3) or soil water potential (Chapter 5) experiments. Given that transgenic *AVP1* barley has improved seedling vigour (Chapter 6), unintentional variations in nutrient or water availability in non-stressed conditions between experiments in these chapters may explain this inconsistency. Seedling vigour may or may not increase growth of transgenic *AVP1* barley in non-stressed conditions depending on whether the non-stressed conditions are indeed non-limiting in all aspects. If nutrients or water are limiting, transgenic *AVP1* barley could be larger due to enhanced seedling vigour allowing better uptake of nutrients or water. Whilst, if the conditions were non-limiting, the seedling vigour should to an extent still increase the growth of transgenic *AVP1* barley compared to null segregants in non-stressed conditions (as seen at the seedling stage, Chapter 6). However, it is possible that this growth advantage in transgenic *AVP1* barley may plateau, if there are no limitations enabling the enhanced seedling vigour to be an advantage to the transgenic *AVP1* barley, which would allow the null segregants to reach a similar size by the biomass sampling time-point (generally 3 weeks after treatment). Overall, this project highlights the importance of ensuring all experimental conditions, such as nutrient and water availability, are well controlled in non-stressed conditions especially when phenotyping transgenic plants which can tolerate multiple abiotic stresses.

In this project, the shoot and root biomass of *ZmRab17:AVP1* barley (cv. WI4330) did not differ from wild-type or null segregants in saline conditions. Whilst *35S*:*AVP1* barley (cv. WI4330) had a greater shoot and root biomass compared to null segregants at 200 mM NaCl (Chapter 7). In addition, plant biomass of *35S*:*AVP1* barley (cv. Golden Promise) did not differ from null segregants at 100 mM NaCl applied from seed imbibition (Chapter 6). Given the proposed role of AVP1 in facilitating seedling vigour (Chapter 6) (Ferjani et al., 2011), these finding collectively suggest that an initial non-stress period just after seed imbibition may be needed for transgenic *AVP1* barley growth to be enhanced via increased seedling vigour, increased photosynthesis, increased nutrient and water uptake and enhanced sucrose transport to sink tissues. If AVP1 is activated too late, such as when activated by the salt-stress inducible promoter *ZmRab17* (Chapter 7) or if salt stress occurs from the start of seed imbibition (Chapter 6), it is likely that the larger growth of transgenic *AVP1* barley may not occur due to insufficient time for these beneficial traits to be established. Thus, it may be necessary for transgenic *AVP1* plants to be larger prior to the onset of the stress (i.e. to have the traits within the light-grey and grey shaded boxes of Figure 1 prior to the onset of a stress) for these transgenic plants to have increased shoot growth under abiotic stress.

In addition, the lack of a larger shoot biomass phenotype in the *ZmRab17:AVP1* and *ZmUbi1*:*AVP1* barley compared to the *35S*:*AVP1* barley (cv. WI4330) (Chapter 7), the GUS activity in *ZmRab17*:*uidA* barley root stelar cells only and the low level of *AVP1* expression in the *ZmRab17:AVP1* and *ZmUbi1:AVP1* barley suggest that not only the timing but also potentially the level and cell specificity of *AVP1* expression may influence the phenotype of transgenic *AVP1* plants. Considering this finding and that the transgene is only half the story with the promoter also contributing to the phenotype of transgenic plants (Roy et al., 2014), it is evident that the choice of promoter used to control the expression of a transgene, such as *AVP1*, is important. This project suggests that when phenotyping transgenic plants, a transgene should be tested under the control of several different promoters with various tissue specificity, stress inducibility

and expression levels to determine the extent of effects and the optimal promoter to use for controlling transgene expression.

Future research

GM field trials of transgenic *AVP1* barley in Australia

This project established some of the first saline GM field trials of transgenic barley in Australia (OGTR, 2014) (Chapter 2). However, there were several factors that limited the results of these field trials, particularly for the field trials in 2012 and 2013. Salinity was extremely variable across the field site with $EC_{1:5}$ (soil:water) values within plots (1.2 m × 2 m) varying by > 1000 µS cm^{-1} and likewise between individual plots within the high salinity area with $EC_{1:5}$ values ranging from 79 ± 7 µS cm^{-1} to 1592 ± 18 µS cm^{-1} (Figure 2). The amount and timing of rainfall was also variable with both low and high rainfall periods causing drought and waterlogging throughout the growing season which limited plant establishment and growth (Figure 3). For example, the total average rainfall in Corrigin, WA (near the field site at Kunjin, WA) for the months of June and July is usually about 50 mm. However, after sowing in June 2013 only 0.3 mm of rainfall occurred (low water availability) until July when a total of 71 mm of rainfall occurred in 4 days (waterlogging) (BOM, 2014). Thus, although a sowing density of 160 plants m^{-2} was desired, variation in planting density occurred, particularly in the high salinity field area where sections of plots had no plant growth (Figure 2). Given that the final grain yield measurements from each plot is dependent on the number of plants in each plot (i.e. a higher plot grain yield due to a greater number of plants rather than an increase in grain yield per plant), this variation in plant density made interpretation of plot grain yield results difficult. For this reason, only plant biomass and yield measurements from individual plants randomly sampled from plots were used. To help minimise the variability in plot plant densities, replicates were blocked in the field trial design based on an EM map of the field site and soil $EC_{1:5}$ values were measured for each plot. However, future work will need to further address the variation in plant density between field trial plots. The number of field trial plot replicates for each line should be

increased and the field site could be cultivated to help increase the uniformity of salinity. Furthermore, raised beds to help reduce the occurrence of waterlogging (Bakker et al., 2010; Bakker et al., 2005; Holland et al., 2007) and irrigation to help reduce the impact of dry conditions could be used in future trials.

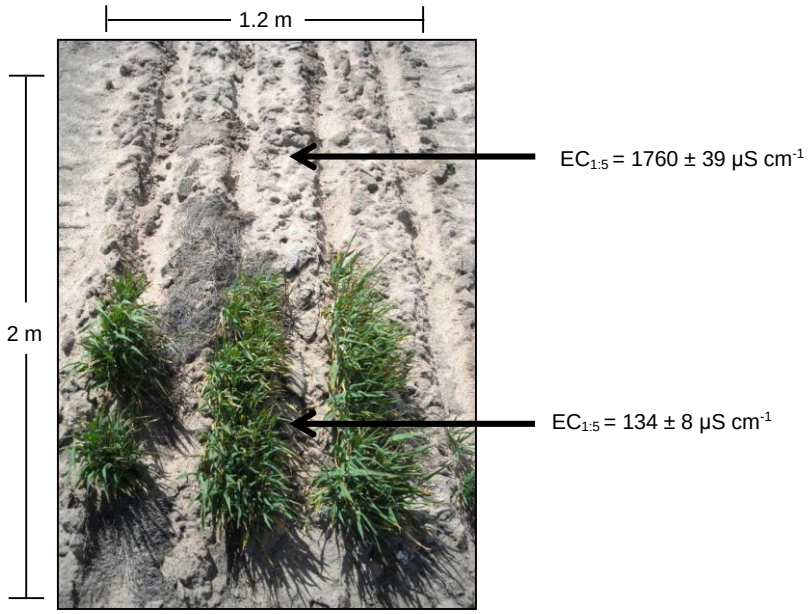


Figure 2 Large variation in salinity influences plant density in field trial plots. An image of a field trial plot (2 m length x 1.2 m wide) in the high salinity area at Kunjin, Western Australia in 2012 with the electrical conductivity (EC) of a 1:5 (soil:water) extract (µS cm^{-1}) of soil sampled to <10 cm deep from areas of plots with plant and without plant establishment.

Figure 3 An image of the saline GM field trial plots at Kunjin, Western Australia in July (2013) showing the occurrence of waterlogging after a rainfall event of 71 mm in 4 days (BOM, 2014).

In this project, all field grown plants were sampled by hand for plant biomass measurements and thus biomass results were limited to the number of plants that were sampled per plot (n = 6) and to one time-point in the growing season at Z37 (Zadoks et al., 1974). High-throughput field phenomics tools, such as the Phenomobile® developed at the High Resolution Plant Phenomics Centre (HRPPC) of the Australian Plant Phenomics Facility (http://www.plantphenomics.org.au/services/phenomobile/) or aerial drones fitted with high resolution cameras (Huang et al., 2013), could be used in the future to assist with large scale phenotyping of transgenic plants in the field through time. Digital images of each plot would allow plant growth to be monitored from seedling establishment to plant maturity and infrared thermal-imaging could also be used to monitor leaf canopy temperatures (Prashar et al., 2013). The extent of plant establishment in each plot could also be quantified, allowing differences in plant density between plots to be incorporated into the statistical analysis of individual plot grain yields.

In the greenhouse, transgenic *35S:AVP1* barley (cv. Golden Promise) had increased shoot biomass at low P supply (Chapter 3), low NO_3^- supply (Chapter 4), low water availability and combined salinity and low water availability (Chapter 5). Given that field trials of transgenic plants are needed to validate greenhouse-based findings of improved abiotic stress tolerance (Nelissen et al., 2014; Roy et al., 2014), future work should investigate whether transgenic *AVP1* barley has improved grain yield compared to null segregants in the field at different rates of P and N fertilisers and at low water availability (i.e. with and without a rainout shelter). Furthermore, this project identified promising lines of commercially relevant *35S:AVP1* barley (cv. WI4330) with improved shoot and root growth in hydroponic conditions at 200 mM NaCl (Chapter 7). Seed of these lines (T_3 generation) should also be tested in future saline GM field trials to investigate whether these commercially relevant lines have improved shoot growth and grain yield in a field with salinity. In addition, future work should measure grain quality traits, such as grain screening levels and protein contents, to investigate effects of AVP1 on barley grain quality under non-stress and abiotic stress conditions.

Ideally, testing of transgenic *AVP1* barley lines across multiple growing seasons and at multiple field sites is needed in the future to characterise this transgenic barley to a greater extent. Alternate field sites with less severe salinity and waterlogging would also be more desirable for testing the transgenic plants expressing *AVP1* in the future. However, current Australian government regulations for GM plant material limit the extent to which transgenic plants can be tested in field trials with the Office of the Gene Technology Regulator (OGTR) licence conditions restricting the location of GM field trial sites, the size of GM field trials and the transport and storage of transgenic plant material (http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/DIR102). The inspection of a 250 m buffer zone surrounding a GM field trial site for volunteer species (closely related plant species to barley) required every 2 weeks when transgenic plants are flowering is also a time-consuming process. In addition, one of the main limitations to conducting extensive GM field trials is the high cost involved, including the need to monitor GM field sites for up to 2 years postharvest. Nonetheless, considering the importance of testing transgenic plants in the field, effort should be made to ensure that this translational research continues in the future (Nelissen et al., 2014). The recent establishment of the New Genes for New Environments (NGNE) facilities at Merredin and Katanning in Western Australia (https://agric.wa.gov.au/n/1423) for testing transgenic plants in the field is ideal and, in the long term, the establishment of such facilities across different soil types and climatic conditions across Australia would be advantageous.

Use of AVP1 technology to improve the abiotic stress tolerance of cereal crops

In this project, transgenic *AVP1* barley (cv. Golden Promise) had a larger shoot biomass and increased grain yield in saline conditions (Chapter 2). This suggests that the AVP1 technology is useful for grain growers and can improve the abiotic stress tolerance of cereal crops. Future work should backcross *AVP1* into commercially relevant Australian barley cultivars, such as Fathom™, Compass™, Commander™, Fleet™, Hindmarsh™ or Schooner™, to improve the abiotic stress tolerance of these cultivars. In the long

term, attempts should also be made to deregulate and commercialise the transgenic *35S:AVP1* barley seed for grain growers. Future work should aim to establish a commercialisation process for the delivery of GM barley seed to Australian grain growers to help ensure growers benefit from this material. Furthermore, the findings of this project suggest that it could be worthwhile transferring the AVP1 technology into wheat, one of the most important cereal crops in the world (FAO, 2013), to help improve the grain yield of this crop in abiotic stress conditions. Transgenic wheat expressing *AVP1* has recently been generated at the ACPFG and characterisation of this transgenic *AVP1* wheat under various abiotic stresses, such as drought, salinity and low nutrient availability, should be conducted in the future.

Use of HVP1 or HVP10 to improve the abiotic stress tolerance of barley

This project also suggests that further research on the barley homologues of *AVP1*, including *HVP1* and *HVP10* (Fukuda et al., 2004; Shavrukov et al., 2010), could be beneficial. It is likely that public acceptance of transgenic barley over-expressing *HVP1* or *HVP10* (cisgenics) would be greater than transgenic barley expressing *AVP1* given this involves the over-expression of a barley gene in barley rather than an *Arabidopsis* gene in barley. Transgenic barley over-expressing either *HVP1* (M. Krishnan unpublished) or *HVP10* (J. Bovill unpublished) have been generated at the Australian Centre for Plant Functional Genomics (ACPFG). Future work should continue to investigate the abiotic stress tolerance of these transgenic barley lines in both greenhouse and field experiments.

Future work should also aim to develop a non-GM barley variety with increased H^+-PPase activity. An attempt should be made to identify allelic diversity in *HVP1* or *HVP10*, for an allele that leads to greater protein activity. Marker assisted selection (MAS), using molecular markers designed to superior alleles of *HVP1* and *HVP10,* could also be used to generate a non-GM barley variety with increased H^+-PPase activity. Furthermore, other non-GM approaches, such as Transcription Activator-Like Effector Nucleases (TALEN) (Li et al., 2012) or Clustered Regularly Interspersed Short Palindromic Repeats

(CRISPR)/CRISPR-associated (Cas) (Feng et al., 2014) could be used to either (a) modify the native promoter of *HVP1* and/or *HVP10* to increase protein abundance in barley or to (b) modify the genes themselves to enhance protein activity.

The vacuole, apoplastic and rhizosphere acidification of transgenic *AVP1* barley

It has been proposed that AVP1 helps to establish an electrochemical potential difference for H^+ across the tonoplast, which facilitates vacuolar Na^+/H^+ antiporter activity and thereby increases Na^+ sequestration into vacuoles (Duan et al., 2007; Gaxiola et al., 2001; Kim et al., 1994; Zhen et al., 1997). Previously, transgenic plants expressing *AVP1* had increased shoot Na^+ accumulation compared to plants without this gene (Bao et al., 2009; Gaxiola et al., 2001; Li et al., 2010). However, in this study, leaf Na^+ concentrations did not differ between transgenic *AVP1* barley and plants without this gene (Chapters 2). It is possible that the same Na^+ concentration is present in the transgenic *AVP1* barley and null segregants, but that the transgenic *AVP1* barley has a higher amount of Na^+ in vacuoles rather than the cytoplasm. However, future work is needed to determine whether the transgenic *AVP1* barley has increased vacuolar acidification and vacuolar Na^+ sequestration compared to null segregants. The activity of vacuolar Na^+/H^+ antiporters, such as *HvNHX1, HvNHX2, HvNHX3 or HvNHX4* (Ershov et al., 2007; Fukuda et al., 2004; Roslyakova et al., 2009; Vasekina et al., 2005), and vacuolar H^+-PPase activity could be measured in the transgenic *AVP1* barley and null segregants. Furthermore, the Na^+ concentration in the cytoplasm, which is smaller than the vacuole and thus more likely to show a greater proportional change than the vacuole, should be measured in transgenic *AVP1* barley and null segregants. However, reliable methodology for such cytoplasmic Na^+ measurements are currently not available and, thus alternatively, methods to determine vacuolar Na^+ concentrations using a fluorescent Na^+ indicator, such as Sodium Green (Amorino and Fox, 1995; Duan et al., 2007) or cryo-scanning electron microscopy (SEM) X-Ray microanalysis (James et al., 2006) may be more useful. In addition, measurements of vacuolar pH using a pH-sensitive dye (Duan et al., 2007) or pH measurements of plant sap exuded from

the roots (Yu et al., 1999) of transgenic *AVP1* barley and null segregants is needed to evaluate the level of vacuolar acidification.

A previous study suggests that transgenic *Arabidopsis* over-expressing *AVP1* has lower apoplastic pH levels than wild-type (Li et al., 2005), which could be important for the movement of ions and compounds, such as Na^+, NO_3^-, PO_4^{3-} or dehydroascorbic acid across the plasma membrane (Rautenkranz et al., 1994). Future work should measure apoplastic pH in transgenic *AVP1* barley and null segregants using a method such as a confocal laser scanning microscopy with pH sensitive and pH in-sensitive fluorescent dyes (Fan and Neumann, 2004; Yu et al., 2001).

In this project, bromocresol purple staining showed that transgenic *AVP1* barley had increased rhizosphere acidification compared to wild-type (Chapter 3). Attempts were made to quantify this pH change by measuring the pH of rhizosphere soil collected from the transgenic *AVP1* barley roots (Chapter 3). However, future work could use micro-electrode ion flux (MIFE™) measurements to quantify H^+ release in specific root zones (Shabala et al., 2013). In addition, it has been previously suggested that greater rhizosphere acidification in transgenic *AVP1* plants is due to the up-regulation of the plasma membrane (PM) H^+-ATPase (Paez-Valencia et al., 2013; Yang et al., 2007). However, it is yet to be tested whether the rhizosphere acidification observed in the transgenic *AVP1* barley in this project is due to the up-regulation of the PM H^+-ATPase or the presence of AVP1 in the PM as observed in *Arabidopsis* over-expressing *AVP1* (Li et al., 2005). Future work should measure the expression and activity of PM H^+-ATPase in transgenic *AVP1* barley and null segregants and determine the localisation of the AVP1 protein in the transgenic *AVP1* barley using either immunogold labelling or immunohistocehmical labelling with an antibody raised against the AVP1 protein (Paez-Valencia et al., 2011; Pasapula et al., 2011; Sarafian et al., 1992). Furthermore, the enhanced rhizosphere acidification observed in transgenic *AVP1* barley (Chapter 3) suggests that this transgenic barley may be advantageous for crop growth in alkaline soils

and, likewise, potentially disadvantageous in acidic soils. Future work should evaluate the growth and grain yield of transgenic *AVP1* barley and null segregants in both alkaline and acidic soils.

How does AVP1 influence cytosolic PP_i levels in transgenic *AVP1* barley?

It was recently suggested that the main function of AVP1 is the hydrolysis of cytosolic pyrophosphate (PP_i), which at high concentrations is an inhibitor of gluconeogenesis (Ferjani et al., 2011). In plants, PP_i is a by-product of various metabolic reactions including the synthesis of amino acids, DNA and RNA, sucrose, starch and fatty acids (Maeshima, 2000). A decrease in cytoplasmic PP_i levels could therefore potentially increase many of these processes in transgenic plants expressing *AVP1*. More research is needed to determine to what extent, if any, each of these processes are altered in the transgenic *AVP1* plants. Future work should also measure H^+-PPase activity and the level of PP_i in the transgenic *AVP1* barley and null segregants. Given that AVP1 mediates PP_i hydrolysis on the cytoplasmic side of the tonoplast, measurements of cytosolic PP_i are needed. However, currently this is not feasible due to a lack of accurate methodology to specifically measure cytosolic PP_i concentrations. Until a reliable method is established, PP_i measurements in the whole leaf and root could be used (Edwards et al., 1984; Smyth and Black, 1984) and should be measured in transgenic *AVP1* barley and null segregants through time to help distinguish between the effects of PP_i synthesis versus hydrolysis.

Does transgenic *AVP1* barley have altered sucrose phloem-loading or starch mobilisation?

It was also recently hypothesised that in phloem sieve element companion cells AVP1 may be localised on the PM helping to regulate sucrose phloem-loading (Gaxiola et al., 2012; Paez-Valencia et al., 2011). It was suggested that AVP1 increases PP_i synthesis in phloem companion cells, and thus sucrose respiration and ATP supply, enabling the PM H^+-ATPase to mediate sucrose phloem-loading (Gaxiola et al., 2012). There is evidence that AVP1 is localised on the PM of sieve element companion cells (Paez-Valencia et al., 2011) and that it is thermodynamically feasible *in vitro* for H^+-PPases to synthesise PP_i

(Baltscheffsky H et al., 1966; Davies et al., 1997; Rocha Façanha and de Meis, 1998; Seufferheld et al., 2004). However, to determine whether transgenic *AVP1* barley has increased sucrose phloem transport, the concentration of sucrose in the phloem sap of transgenic barley expressing *AVP1* and null segregants should be measured. Leaf isotope feeding experiments using labelled $^{14}CO_2$ or $^{13}CO_2$ and mass spectrometry (Kölling et al., 2013) could also be used to measure carbon export from leaf (source) to root (sink) tissue, as well as the extent of carbon partitioning into these tissues, in transgenic *AVP1* barley and null segregants. The findings of this project also suggest that transgenic *AVP1* barley seedlings may have increased sucrose loading from the scutellum vascular parenchyma (source) into phloem sieve-element cells, and thus increased sucrose transport to the embryo and subsequently the developing cotyledon and roots (sinks) (Chapter 6). To investigate this concept further, future work should measure sucrose concentrations in the scutellum, embryo and developing cotyledon and roots of transgenic *AVP1* barley and null segregants through time following seed imbibition. Furthermore, considering plants mobilise starch to sucrose during the night (Geiger and Servaites, 1994; Geiger et al., 2000) and that an increase in density of starch granules was qualitatively observed from transgenic *AVP1* barley seedlings compared to wild-type (Chapter 6), it is possible that increased starch levels could benefit the growth of transgenic *AVP1* barley during extended dark periods. The amount of starch in transgenic *AVP1* barley should be quantified and the growth of transgenic *AVP1* barley and null segregants should be tested under varied day lengths to further investigate this observation.

Does AVP1 have a role in ascorbic acid synthesis in transgenic *AVP1* barley?

In this project, transgenic barley expressing *AVP1* had significantly lower galactose and significantly higher ascorbic acid and dehydroascorbic acid compared to null segregants at 11 d after seed imbibition (Chapter 5). This suggests that ascorbic acid synthesis is increased in transgenic *AVP1* barley compared to null segregants, which may be contributing the larger biomass of transgenic *AVP1* barley. Potentially, given that PP_i is produced as a by-product of ascorbic acid synthesis and is an inhibitor of metabolic

processes at high concentrations, the hydrolysis of PP_i in transgenic *AVP1* barley could facilitate ascorbic acid synthesis. However, the metabolomics findings in this project were based on fold-change values and future work should measure the concentration of ascorbic acid and dehydroascorbic acid in the leaf tissue (Hewitt and Dickes, 1961) of transgenic *AVP1* barley compared to null segregants. In addition, the ascorbic acid concentration should be measured in mutant *Arabidopsis* plants defective in the *AVP1* gene, such as the *fugu5* mutants (Ferjani et al., 2011) or the *avp1* mutants (Li et al., 2005). Potentially, ascorbic acid concentrations could be lower in *fugu5* or *avp1* mutants compared to wild-type and the supply of ascorbic acid to these mutants may recover their growth. Future work could evaluate the expression of genes such as L-galactose dehydrogenase (*GalDH*) (GenBank DQ456874), or the activity of enzymes, such as GDP-D-mannose pyrophosphorylase (EC 2.7.7.13), which are involved in the Smirnoff-Wheeler ascorbic acid synthesis pathway (Lisko et al., 2013; Wheeler et al., 1998), to confirm if this pathway is up-regulated in transgenic *AVP1* barley compared to nulls segregants (Chapter 6). Given that ascorbic acid is known to influence cell division (Liso et al., 1988; Pignocchi and Foyer, 2003), an attempt was made in this project to measure the number and size of between vein (bv) adaxial epidermal cells in transgenic *AVP1* barley and null segregants (Chapter 6). However, no significant difference in number or size of bv adaxial epidermal cells was observed. Future work should measure the number and size of other cell types, such as mesophyll cells, in transgenic *AVP1* barley and null segregants. Cell division in the transgenic *AVP1* barley could also be evaluated by measuring the number of cells in the metaphase using colchicine solution to determine cell doubling time (CDT) (Evans et al., 1957; Harrison et al., 1998).

Use of a systems biology approach to investigate the larger biomass of transgenic *AVP1* barley

In this study, metabolomics analysis was used to evaluate differences in leaf and root metabolites between transgenic *AVP1* barley and null segregants at 11 d after seed imbibition. However, this metabolomics analysis only provides a snapshot of metabolites at one time-point. Future work should evaluate the metabolic profile of transgenic *AVP1* barley and null segregants across their lifecycle,

particularly prior to and following 6 d after seed imbibition when changes in seedling vigour were observed (Chapter 6). A more targeted metabolomics approach could also be used by evaluating metabolite changes in specific regions of shoots and roots of transgenic *AVP1* barley. In addition, AVP1 appears to regulate a number of different and complex traits involving plant growth (Figure 1), therefore future research should use a systems biology approach (Cramer et al., 2011; Kitano, 2002) to further investigate the effects of AVP1 in transgenic *AVP1* barley, other transgenic plants expressing *AVP1* and mutant plants defective in the *AVP1* gene. The use of omics technologies, such as metabolomics, transcriptomics and proteomics, would help to provide a more comprehensive insight into the many apparent functions arising from this one gene.

Concluding remarks

In this project, transgenic barley with constitutive *CaMV 35S* expression of *AVP1* had a larger shoot biomass and, importantly, higher grain yield per plant compared to wild-type in a field with high salinity. The *35S:AVP1* barley also had increased shoot growth under various other abiotic stresses including low P supply, low NO_3^- supply, low water availability and combined salinity and low water availability. It is likely that a combination of traits, such as increased nutrient use, enhanced water use, altered carbohydrate metabolism, improved seedling vigour and increased ascorbic acid synthesis, are contributing to the larger shoot biomass of *35S:AVP1* barley compared to plants without this gene. The findings of this project also suggest that the *ZmRab17* promoter is salt stress inducible in root stelar cells with some basal transgene expression and that the type of promoter used to control the expression of *AVP1* in transgenic barley is important. Overall, this project suggests that *35S:AVP1* barley is a promising option for increasing cereal crop productivity under abiotic stress.

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Chapter 9

Appendix

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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

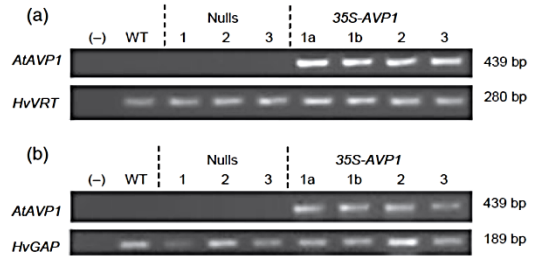


Figure 1 Molecular characterization of wild-type, null segregants and transgenic barley expressing *AVP1*. (a) Genotyping for the presence or absence of *AVP1* using polymerase chain reaction (PCR) with *AVP1*-specific primers and *HvVRT2*-specific primers (internal control) (b) Expression analysis of *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

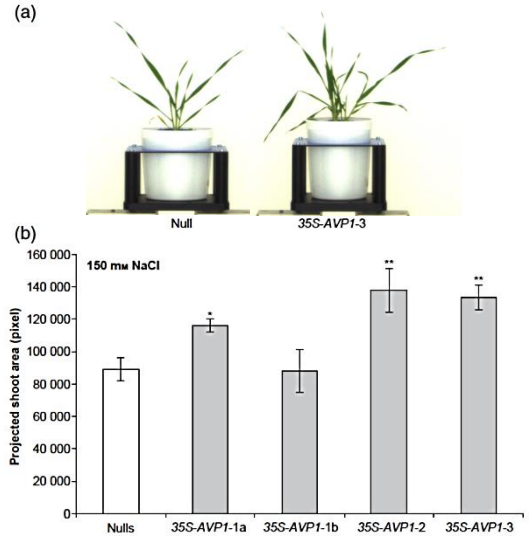


Figure 2 Projected shoot area of salt-treated transgenic barley expressing *AVP1* and null segregants in the greenhouse. (a) High-resolution visible light (RGB) side-view image of a representative null segregant (cv. Golden Promise) and transgenic barley line expressing *AVP1* (*35S-AVP1*-3) 47 days 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).

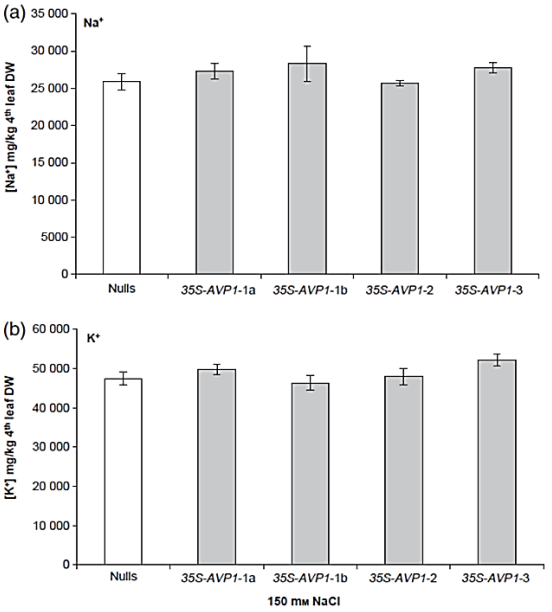


Figure 3 Leaf Na^+ and K^+ concentrations of transgenic barley expressing *AVP1* and null segregants in saline soil. (a) Na^+ and (b) K^+ concentrations (mg/kg DW) of the 4th leaf blade of null segregants (cv. Golden Promise) (white bars) and transgenic barley expressing *AVP1* (*35S-AVP1*-1a, *35S-AVP1*-1b, *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

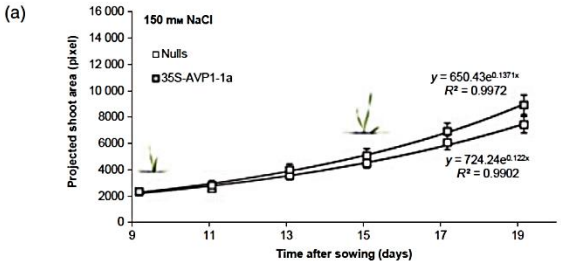


Figure 4 Nondestructive plant imaging of salt-treated transgenic barley expressing *AVP1* and null segregants. The projected shoot area (pixel) of null segregants (white squares) and *35S-AVP1*-1a line (grey squares) between (a) 9–19 days and (b) 28–47 days after sowing in soil with 150 mM NaCl. Representative 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

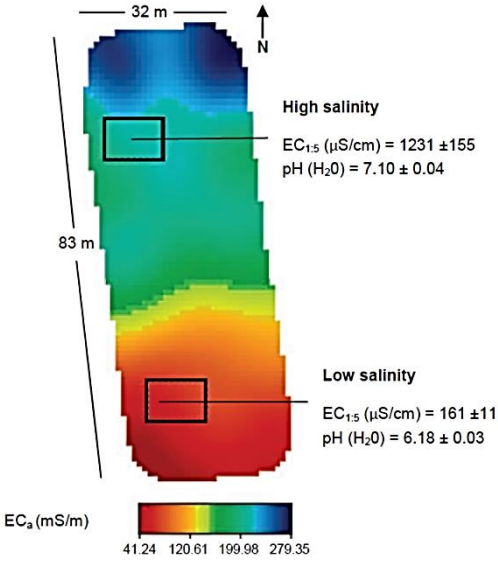
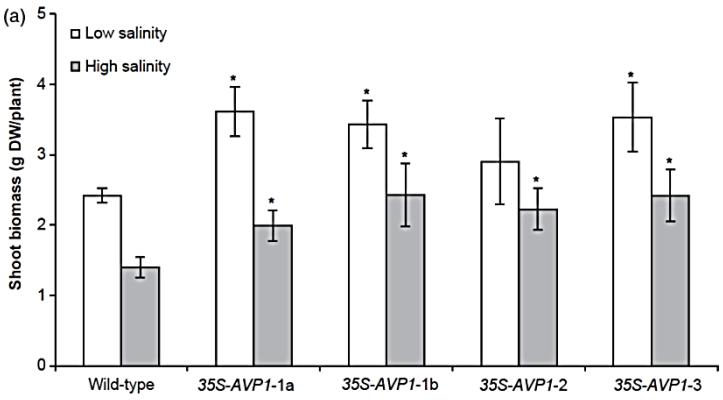


Figure 5 Soil characterization of a saline field trial site. An electromagnetic (EM) map showing the apparent electrical conductivity (EC_a) of a saline field trial site (83 m length × 32 m width, N = north) with red indicating low EC_a (41 mS/m) and blue high EC_a (199 mS/m). Black rectangles indicate 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



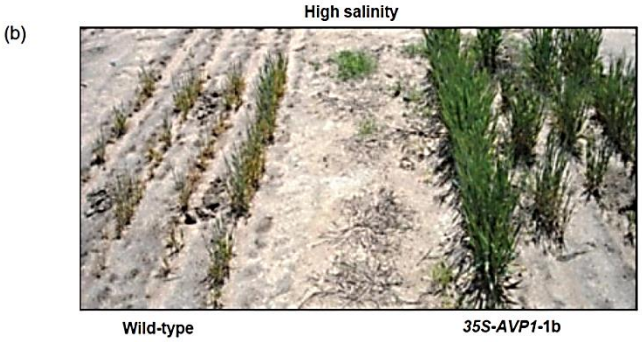


Figure 6 Shoot biomass of wild-type and transgenic barley expressing *AVP1* in a saline field trial (a) Shoot biomass (g DW/plant) of wild-type (cv. Golden Promise) and four transgenic barley lines expressing *AVP1* (*35S-AVP1*-1a, *35S-AVP1*-1b, *35S-AVP1*-2 & *35S-AVP1*-3) after 12 weeks growth in 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.