Confirmation of the barley vacuolar pyrophosphatase *HvHVP10* as a candidate gene for salinity tolerance

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LIST OF ABBREVIATIONS

%	percent
~	approximately
×	times
°C	degrees Celsius
3′	three prime of nucleic acid sequence
35S	promoter of cauliflower mosaic virus 35S
5′	five prime of nucleic acid sequence
ABA	abscisic acid
ACPFG	Australian Centre for Plant Functional Genomics
AGRF	Australian Genome Research Facility
ANOVA	analysis of variance
AMBP	Aminomethylene bisphosphonate
Arabidopsis	*Arabidopsis thaliana*
At	*Arabidopsis thaliana*
AtAVP	Arabidopsis vacuolar pyrophosphatase
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BLAST	basic local alignment search tool
bp	base pairs of nucleic acids
BSA	bovine serum albumin
Ca^{2+}	calcium ion
$CaCl_2$	calcium chloride
CaMV	cauliflower mosaic virus
Cat. No	Catalogue number
CBL	calcineurin like-B protein
cDNA	complimentary deoxyribonucleic acid
CIPK	calcineurin like-B interacting protein kinase
Cl^-	chloride ion
cm	centimetre(s)
Col-0	Arabidopsis ecotype Columbia-0
CSIRO	Commonwealth Scientific and Industrial Research Organisation
d	day(s)
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dH_20	deionised water
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DREB	Dehydration Response Element Binding
dS	deciSiemens

ECe	electrical conductivity
EDTA	ethylenediaminetetraacetic acid
g	gram(s)
g	Gravity
gDNA	genomic deoxyribonucleic acid
GFP	green fluorescent protein
GUS	β-glucuronidase protein
h	hour(s)
H^+	hydrogen ion
H^+V-PPase	H^+ translocating vacuolar pyrophosphatase
H_20	Water
HCl	hydrochloric acid
HvHVP10	Barley vacuolar pyrophosphatase
K^+	potassium ion
kb	kilo base pairs of nucleic acid
KCl	potassium chloride
kg	kilogram(s)
KOH	potassium hydroxide
L	litre(s)
LB	left border of T-DNA sequence
LB media	Luria Bertani media
M	molar
mg	milligram(s)
Mg^{2+}	magnesium ion
$MgCl_2$	magnesium chloride
min	minute(s)
mL	millilitre(s)
mm	millimetre(s)
mM	millimolar
mol	mole
mRNA	messenger ribonucleic acid
MS	Murashige and Skoog media
n	sample size
N_2	nitrogen
NA	not available
Na^+	sodium ion
NaCl	sodium chloride
NaOH	sodium hydroxide
NCBI	National Centre for Biotechnology Information
ng	nanogram(s)
NHX	Na+/H+ exchanger
NO^{3-}	nitrate

Nos	bacterial nopaline synthase terminator sequence
OD	optical density
Os	*Oryza sativa*
OsOVP	rice vacuolar pyrophosphatase
PCR	polymerase chain reaction
Pi	phosphate
PPase	pyrophosphatase
PPi	pyrophosphate
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
QTL	quantitative trait loci
RB	right border of T-DNA sequence
RIL	recombinant inbred line
RNA	ribonucleic acid
RO	reverse osmosis
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
Sec	second(s)
SOS	salt overly sensitive
T_0	primary transformant containing T-DNA insert
T_1	progeny of T_0 plant
T_2	progeny of T_1 plant
TAE	tris-acetate-EDTA
T-DNA	transfer deoxyribonucleic acid
TE	transposable element
Tm	melting temperature of primers
U	units
UAS	upstream activation sequence
Ubi	promoter of maize Ubiquitin-1
UidA	*β*-glucuronidase gene
UV	ultraviolet
V	voltage
V-PPase	vacuolar pyrophosphatase
v/v	volume per volume
w/v	weight per volume
WT	wild type
X-Gluc	5-bromo-4-chloro-3-indoyl-glucuronide
µL	microlitre(s)
µm	micrometre
µM	micromolar
µmol	micromole(s)

ABSTRACT

Salinity is one of the most significant environmental issues affecting agricultural production in Australia and around the world. One main salinity tolerance mechanism among plants is the exclusion of sodium ions from the aerial parts of the plants. Conventional breeding techniques and genetic engineering approaches have been employed in the effort to develop varieties with lower levels of shoot sodium accumulation under saline conditions. A gene identification approach in the salt laboratory at the Australian Centre for Plant Functional Genomics (ACPFG) identified a major quantitative trait locus (QTL) for sodium exclusion (*HvNax3*) in the barley mapping population Barque-73 (*Hordeum vulgare*; a high yielding South Australian cultivar) × CPI-71284-48-48, (a wild barley; *Hordeum spontaneum*). Syntenic mapping revealed a number of candidate genes underlying the *HvNax3* QTL, the most promising of which encodes a vacuolar pyrophosphatase proton pump (V-PPase, *HVP10* gene in barley) responsible for establishing an electrochemical gradient across the tonoplast that drives the secondary transport of ions such as sodium into the vacuole. The compartmentalisation of sodium in the vacuole reduces the toxic effect of high sodium concentration in the cytoplasm, whilst at the same time facilitating water uptake into the plant. Orthologues of this gene have been shown to confer salinity and drought tolerance in a variety of plant species.

In order to confirm that V-PPase is the gene responsible for the *HvNax3* phenotype, analysis of the accumulation of sodium ions and *HvHVP1*0 gene expression in root and shoot tissue was conducted over 10 days of salinity stress. Results of this work suggested that the accumulation of sodium in the roots of lines carrying the CPI-71284-48 *HvNax3* allele may be attributed to a peak in *HvHVP10* expression at day 3 of salinity stress. This finding is further supported by analysis of pyrophosphatase activity within root tonoplast enriched vesicles, results of which suggest that pyrophosphatase activity was higher in the line with the CPI-71284-48 *HvNax3* allele in response to salinity stress. Sequence analysis of the *HvHVP10* CDS, gene and promoter region suggests that this difference in expression and enzyme activity may be due to the presence of a DREB transcription binding site just upstream of the *HvHVP10* start codon in CPI-71824-48. Future analysis is required to validate this finding and warrants further investigation, possibly by employing the newly emerging CRISPR technology.

A significant component of this research project was the development of *HvHVP10* overexpression (OEX) lines. The salinity tolerance of these lines was characterised in the T_1

and T_2 generation using a supported hydroponics system and destructive analysis. Results from this study indicated that is some cases the constitutive OEX of *HvHVP10* lead to increased biomass of transgenic plants under both salinity stress and control conditions. Several transgenic lines also displayed altered patterns of sodium accumulation in the root and shoot tissue under 150 mM and 200 mM NaCl. Further analyses of these transgenic lines at later generations is required to confirm this phenotype and warrants future investigation.

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CERTIFICATION OF DISSERTATION

I certify that 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 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. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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ENDORSEMENT

_____ _____

Signature of Principal Supervisor Date

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1 Background and Literature Review

1.1 Barley

Barley is a member of the Triticeae group within the family Graminaceae, and thus shares a common ancestry with rice, rye, wheat and oats (Gramene, 2010). The genus *Hordeum* comprises 32 species and contains both annual and perennial species, all with 7 chromosomes. Cultivated barley, *Hordeum vulgare*, has a genome size of 4900 Mb, is a diploid and is the only *Hordeum* species to have undergone domestication approximately 10,000 years ago in the Fertile Crescent, from its wild progenitor *H. spontaneum*. This species continues to grow wild in the Middle East, along the coast of the Dead Sea and in areas of South Africa and Asia in both natural and disturbed habitats (Badr *et al.*, 2000).

Barley is the second most significant cereal crop in Australia, accounting for 25 % of total cereal production (Barley Australia, 2010) and generates approximately 2 billion Australian dollars in revenue per year (Australian Bureau of Statistics, 2010). In Australia, barley occupies approximately 5 million hectares of cultivated land and is grown in the states of Western Australia, South Australia, Queensland, Victoria and to a small extent in Tasmania (Barley Australia, 2010). Australia is the world's third largest exporter of barley and produces high quality 2-row spring-type feed and malt barley. The Australian malting selection rate is the highest of the world's exporting nations, with around 35-40% of our national crop selected as malt for the Brewery Industry (Australian Bureau of Statistics, 2010).

Barley is grown over a broader environmental range than any other cereal and much of the world's barley is produced in regions with climates unfavourable for the production of other major cereals (Langridge and Barr, 2003). Whilst barley is a hardy crop, adapted to a wide range of environmental conditions, many abiotic factors continue to threaten barley production, including salinity (Rengasamy, 2002), drought (Nevo and Chen, 2010) and nutrient deficiency (Yang *et al.*, 2007).

1.2 Salinity as a limitation on crop production

Salinity is one of the most important abiotic factors influencing the productivity of agricultural systems around the world, with approximately 190 million hectares of land affected by salinity worldwide (FAO, 2008). In Australia in 2001 an estimated 2.5 million hectares of land were affected by dryland salinity, and it is predicted that this area may increase to 17 million hectares by 2050 (Commonwealth of Australia, 2010). Soil salinisation

is caused by excess accumulation of salts, predominantly sodium chloride (NaCl), magnesium (Mg^{2+}), calcium sulphates ($CaSO_4$) and bicarbonates (HCO_3^-) at the soil surface. Soils are referred to as saline when their electrical conductivity (EC) is equal to or greater than 4.0 dS/m (approximately 40 mM NaCl). Primary salinity is a result of the naturally occurring processes associated with rock mineral weathering, whereas secondary salinity (referred to as dryland or irrigated salinity when occurring in rain-fed or irrigated environments, respectively) is a direct result of human activities such as land clearing, over-irrigation and urbanisation (Nevo and Chen, 2010). These activities result in groundwater rising to the surface which mobilises stored salts in the soil profile bringing them to the root zone of plants. Although over-irrigation and urbanisation contribute to dryland and irrigated land salinity, the main cause of rising groundwater in Australia is the clearing of deep-rooted, perennial native vegetation and its replacement with shallow-rooted, annual crop and pasture species (Clarke *et al.*, 2002).

When salt accumulates at the soil surface, plants respond in two phases. The osmotic phase results from the accumulation of salts in the soil solution (to a threshold of 40mM NaCl), thereby lowering the external water potential and reducing cell turgor, causing an immediate decrease in plant growth. Plants experience the osmotic phase of salinity stress for as long as they are growing in media (substrate) containing salt. The second phase, referred to as the ionic phase, occurs only when salts taken up by plants in the transpiration stream accumulate to toxic levels in leaves, resulting in leaf necrosis and a further decline in growth (Munns and Tester, 2008, Roy *et al.*, 2014).

Salt stress affects many physiological and biochemical process within plants and significantly reduces yield. The deleterious effect of salt on a plant is a consequence of both reduced soil water potential and water deficit, responsible for osmotic stress, and the toxic effect of excess ions on critical biochemical processes within cells respectively. In cereals such as barley it is the sodium ion (Na^+) which is primarily responsible for ionic toxicity (Hasegawa *et al.*, 2000). However, the accumulation of Cl^- has been shown in some cases to have a greater or additive effect on the effect of Na^+ stress (Tavakkoli *et al.*, 2010). Na^+ specific damage in the cytoplasm of plant cells inhibits the activity of a range of enzymes and transcription factors involved in cellular metabolic processes (Tester and Davenport, 2003). Na^+ toxicity is mainly due to competition with K^+ for enzyme binding sites as many enzymatic reactions are activated by potassium, including those involved in protein synthesis; thus, plants require a high K^+/Na^+ ratio for normal metabolic functioning. In addition, high Na^+ levels also affect

the plants ability to absorb essential nutrients, such as calcium and may result in nutrient deficiency (Tester and Davenport, 2003, Roy *et al.*, 2014). Leaves are more susceptible to sodium toxicity than roots and recover growth less effectively than roots following salt exposure (Munns and Tester, 2008). As Na^+ is the primary cause of salt toxicity and ion-specific damage in plants, for the purpose of this review Na^+ will be used interchangeably with the word 'salt'.

1.3 Mechanisms of salinity tolerance

Plants have evolved various tolerance mechanisms and three components of salinity tolerance have been identified: 1) osmotic stress tolerance; 2) Na^+ or Cl^- (chloride) exclusion from the shoot; and 3) shoot ionic tissue tolerance (Munns and Tester 2008, Tilbrook and Roy 2014). Osmotic stress tolerance allows the plant to maintain growth whilst under osmotic stress, through adjustment mechanisms which may involve long distance signalling within the plant (Silva and Geros, 2009). Na^+ exclusion reduces sodium accumulation in the shoots by restricting the transport of sodium at the root zone (Munns and Tester 2008, Tilbrook and Roy 2014). Tissue tolerance is related to the plant's ability to accumulate Na^+ and chloride (Cl^-) ions in leaf tissue by compartmentalisation in the cell vacuole or in the apoplastic space, away from the cytoplasm where it does the most damage (Blumwald *et al.*, 2000).

Barley has been identified as the most salt tolerant cereal (Munns and Tester, 2008; Figure 1-1). The superior salt tolerance of barley has been associated with increased sodium accumulation in the vacuole of roots (Fukuda *et al.*, 2004), thereby restricting Na^+ transfer to the shoot. The ability of a barley plant to maintain a high K^+/Na^+ cytosolic ratio has also been suggested to play a key role in its salinity tolerance (Garbarino and Dupont, 1988). This has been confirmed by work by Leonova *et al.* (2005), who compared the concentration of Na^+ and K^+ in shoots and roots of cultivars differing in their salinity tolerance. The concentration of Na^+ in the shoots was significantly higher in the salt sensitive phenotype, whereas a reciprocal pattern was observed in the roots in the salt tolerant cultivar. K^+ levels were also increased in the shoots in the salt tolerant phenotype, helping to maintain a high K^+/Na^+ ratio (Leonova *et al.*, 2005). This is further supported by recent work by Ligaba & Katsuhara (2010) who examined the physiological and molecular responses of barley cultivars to salt stress. Salt treatment was associated with increased xylem sap osmolarity, enhanced accumulation of sodium ions in the shoot and reduced plant growth in the salt sensitive

cultivar. These results indicate that the ability to restrict sodium entry to the shoots and accumulate Na^+ in the vacuoles of roots is a key salinity tolerance mechanism in barley.

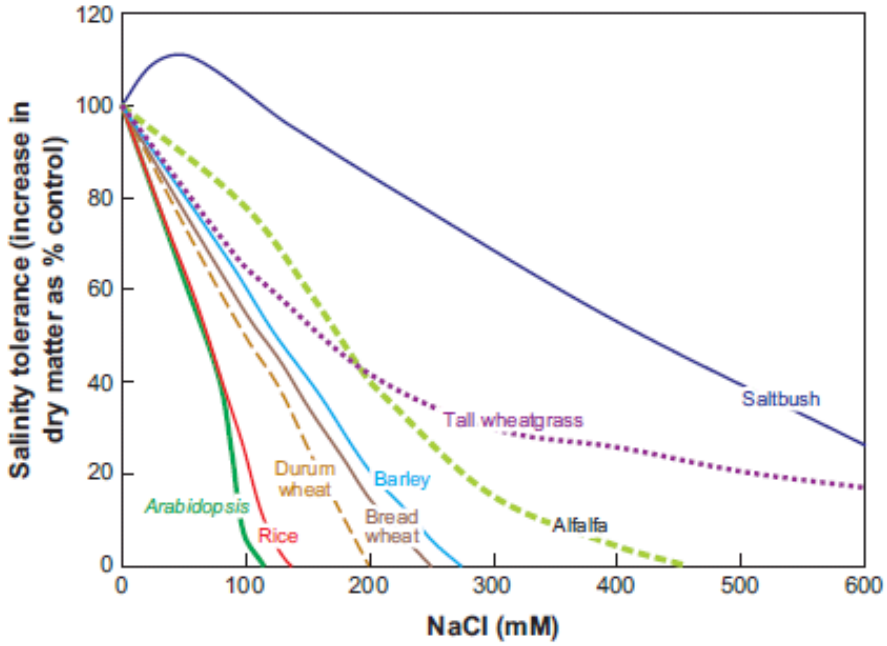

Figure 1-1 Diversity in the salt tolerance of various plant species (Munns & Tester, 2008)

Although *H. vulgare* is regarded as a salt tolerant monocot compared with bread wheat and other cultivated Triticeae, barley cultivars still experience a 60 % decline in biomass at 150 mM NaCl (Garthwaite *et al.* 2005, Tilbrook and Roy 2014). Numerous reports have suggested that wild barley species offer a source of favourable alleles for salinity tolerance breeding and germplasm enhancement to increase genetic diversity in breeding populations (Mano and Takeda, 1998, Nevo and Chen, 2010). A study by Garthwaite *et al.* (2005), identified a number of wild *Hordeum* species, including *H. spontaneum*, that were more salt tolerant than domesticated barley. In this report they found that *H. vulgare* exhibited up to 6 fold more dead leaf material than wild *Hordeum* species and growth rates of the wild barley species were on average 30 % higher than domesticated barley in 450 mM NaCl (Garthwaite *et al.*, 2005). The increased salinity tolerance in the wild species was attributed to more efficient Na^+ and Cl^- exclusion from the shoots and better maintenance of leaf K^+ concentration.

1.4 Sodium exclusion QTL (*HvNax3*) in barley

Previous work in the Tester laboratory at ACPFG identified a Quantitative Trait Locus (QTL) for sodium exclusion in barley, in an F_1-derived Doubled Haploid (DH) population (Shavrukov *et al.*, 2010). The mapping population was produced from a cross of a South Australian adapted cultivar (Barque-73) with a *H. spontaneum* accession (CPI-71284-48). The Na^+ exclusion QTL (*HvNax3*) was detected on the short arm of chromosome 7H (Figure 1-2) and was deemed highly significant with a likelihood ratio statistic (LRS) of 45.6. The *HvNax3* QTL accounted for approximately 51% of the phenotypic variation explained, where the CPI-71284-48 allele reduced shoot Na^+ accumulation by 10-25% in plants grown in 150 mM NaCl. *HvNax3* was validated in an advanced backcross (BC_2F_2) population (AB-QTL). Fine mapping of the DH population and AB-QTL lines with CAPs markers (Cleaved Amplified Polymorphic Sequences) reduced the *HvNax3* interval (Shavrukov *et al.*, 2010).

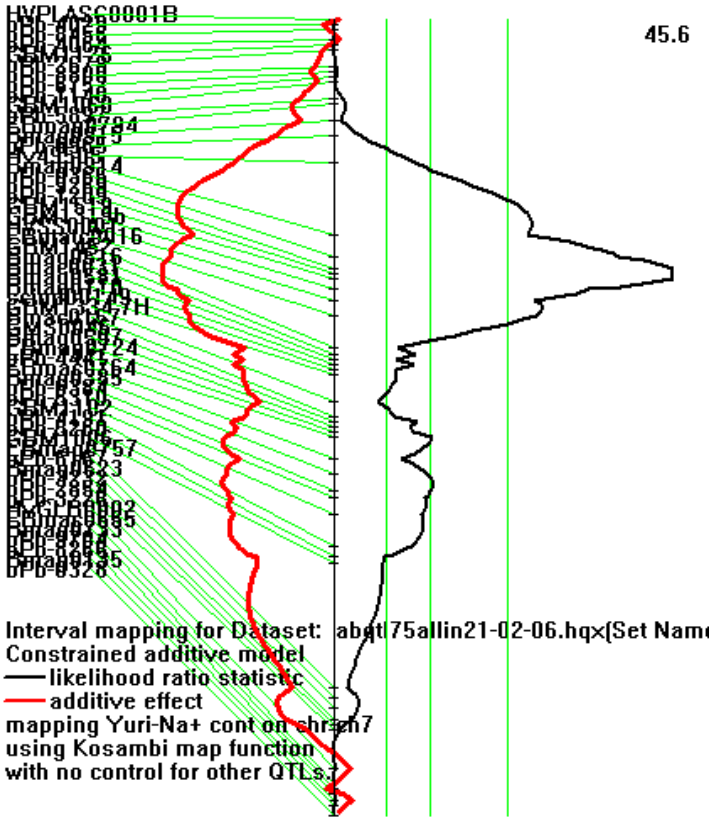

Figure 1-2 Chromosome 7H *HvNax3* QTL for sodium exclusion mapped in the F_1-derived DH population (LRS = 45.6). Green vertical lines represent levels of significance (from left to right: suggestive (P≤0.1), significant (P≤0.05) and highly significant (P≤0.01) (Shavrukov *et al.*, 2010).

Synteny and comparative mapping with the sequenced rice and *Brachypodium* genomes revealed a good co-linearity between the three genomes in the *HvNax3* interval (Figure 1-3). A conserved gene order and content was observed, thereby providing a good prediction of gene content and function in barley. A repetitive region in the fragment of the rice chromosome was identified and removed, narrowing the *HvNax3* interval from 30 to 16 genes. Sixteen genes were found to correspond to the HvNax3 interval (Table 1-1), several of which encode for proteins that have been associated with salinity tolerance in a variety of plant species: namely; H^+- pyrophosphatase (vacuolar proton pump), R-SNARE (R-VAMP17 in *Arabidopsis* which mediates vesicle fusion with the tonoplast), ANTH (facilitates clathrin protein assembly in clathrin-dependent vesicle trafficking) and DELLA (group of proteins involved in the regulation of plant structure and development). The strongest candidate for the *HvNax3* QTL is a gene which encodes for a vacuolar proton pump, H^+-pyrophosphatase (V-PPase, gene *HVP10* in barley). V-PPase is one of two proton pumps in the vacuole of plants, responsible for establishing an electrochemical gradient across the tonoplast (Hasegawa *et al.*, 2000). This electrochemical gradient provides the energy required for antiporters to transport ions against their concentration gradient across the tonoplast into the vacuole (Silva and Geros, 2009).

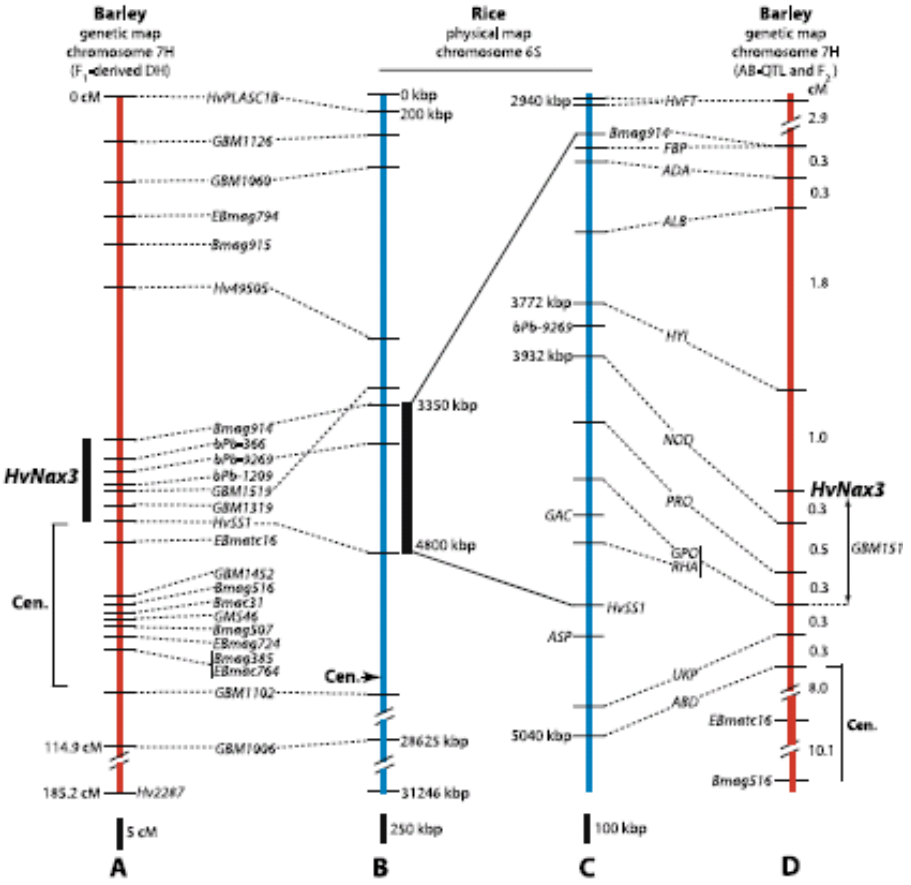

Figure 1-3 Barley and rice comparative maps A. Barley genetic map made from the F_1-derived DH population including the *HvNax3* QTL interval. B. Corresponding interval on rice chromosome 6. C. Expanded view of rice interval. D. Barley composite genetic map using AB-QTL. The centromere location in the barley map is inferred by links to wheat deletion mapping data (Shavrukov *et al.*, 2010).

Table 1-1 Genes in the *HvNax3* intervals of rice and *Brachypodium* (Shavrukov *et al.* 2010).

Rice or *Brachypodium* ortholog[a] (barley marker)	Homology/inferred function
LOC_Os06g07770 (HYI)	Contains DCD domain. DCD proteins are a plant-specific group of proteins and presently have no ascribed function (Tenhaken et al. 2005)
LOC_Os06g07780	R-SNARE. Facilitate membrane fusion. Most similar to the three R-VAMP71 subfamily members of *Arabidopsis* (http://www.tc.umn.edu/~sande099/atsnare.htm), which mediate vesicle fusion with the tonoplast (Uemura et al. 2004; Carter et al. 2004). Vesicle trafficking
LOC_Os06g07800	Hypothetical protein
LOC_Os06g07820	BSD-domain-containing protein (Doerks et al. 2002). BSD1 (At1g10720) is among the most similar proteins in *Arabidopsis* and has been shown to have transcription-factor-like properties (Park et al. 2009). Unknown biological function
LOC_Os06g07830	AP180 N-terminal homology (ANTH)-domain-containing protein. Most similar to ANTH proteins of *Arabidopsis* (Holstein and Oliviusson 2005). Facilitate clathrin assembly in clathrin-dependent vesicle trafficking
LOC_Os06g07840	Hypothetical protein
LOC_Os06g07860, LOC_Os06g07960	Cystathionine beta-lyase. Most similar to a CBL protein in *Arabidopsis* (At5g57050) required for methionine biosynthesis (Levin et al. 2000)
Bradi1g47900	GRAS family transcription factor. Shows most similarity to the five members of the DELLA subgroup in *Arabidopsis* (Bolle 2004) involved in gibberellins signaling (Cheng et al. 2004; Achard et al. 2006). Regulation of plant stature and development
LOC_Os06g07869, LOC_Os06g07969	Ubiquinol-cytochrome C reductase complex ubiquinone-binding protein. This complex is a component of the mitochondrial respiratory chain
LOC_Os06g07878, LOC_Os06g07978	26S proteasome α-3 subunit. Corresponds to PAC1 in *Arabidopsis* (At3G22110; Fu et al. 1999). Ubiquitin-mediated protein degradation
LOC_Os06g07887, LOC_Os06g07987	CAZy glycosyltransferase family 47, subgroup C2 (Li et al. 2004; http://www.cazy.org/), which includes the *Arabidopsis* XGD1 xylogalacturonan xylosyltransferase (At5g332290; Jensen et al. 2008). Polysaccharide metabolism
LOC_Os06g07905, LOC_Os06g08005	CAZy glycosyltransferase family 31 (http://www.cazy.org/). Most similar to members of subgroup A-III in *Arabidopsis*, which include members with β-(1,3)-GalT activity (Qu et al. 2008). Polysaccharide metabolism
LOC_Os06g07896, LOC_Os06g07996	S15 type ribosomal protein. Protein translation
LOC_Os06g07914, LOC_Os06g07923, LOC_Os06g07932, LOC_Os06g07941, LOC_Os06g08014, LOC_Os06g08023, LOC_Os06g08032, LOC_Os06g08041, LOC_Os06g08060	2-oxoglutarate-Fe (II) oxygenase. Oxidoreductases of this type have a range of substrates. One of the closest matches in *Arabidopsis* is the LDOX leucoanthocyanidin dioxygenase (At4G22880), which contributes to the synthesis of flavonol glycosides (Stracke et al. 2009)
LOC_Os06g08080 (HVP10)	Pyrophosphate-energized vacuolar membrane proton pump, H^+-pyrophosphatase. Most similar to AVP1 (At1G15690) in *Arabidopsis*, responsible for establishing a difference in electrochemical potential for H^+ across the tonoplast. Corresponds to HVP10 in barley (Fukuda et al. 2004)
LOC_Os06g08090	Beta-glucuronidase (GUS). Most homologous to the three CAZy glycoside hydrolase family 79 members of *Arabidopsis* (Woo et al. 2007; http://www.cazy.org/), at least one of which has been shown to have GUS activity (Eudes et al. 2008). Polysaccharide metabolism
LOC_Os06g08100	Hypothetical protein
LOC_Os06g08110 (NOD)	Similar to nodulins and the major facilitator superfamily (MFS) of proteins. MFS proteins are transporters for various solutes (Pao et al. 1998)

1.4.1 Vacuolar proton pumps

Physiological studies of plant responses to salt stress indicate that Na^+ accumulation in root vacuoles or salt sequestration in leaf cell vacuoles are critical determinants of salinity tolerance (Blumwald, 2000, Hasegawa *et al.*, 2000, Inan *et al.*, 2004, Munns and Tester, 2008, Møller *et al.*, 2009, Tilbrook and Roy, 2014). The intracellular compartmentalisation of sodium allows the plant to partition toxic ions away from the cytoplasm through energy dependent transport into the vacuole (Hasegawa *et al.*, 2000).

As alluded to above, the active transport of compounds across the tonoplast is driven by two vacuolar H^+-translocating proton pumps - V-ATPase and V-PPase (Figure 1-4). The vacuolar V-ATPase consists of 8 subunits and is an enzyme common to all eukaryotes. It requires the energy derived from ATP hydroloysis to transport protons across the tonoplast. The vacuolar V-PPase is an enzyme ubiquitous to plants and present in a few phototrophic bacteria, however, it is absent in mammals and fungi (Maeshima 2000, Schilling *et al*. 2017). It uses the energy derived from the hydrolysis of pyrophosphate (PPi) to transport protons across the tonoplast. Barley is known to possess at least three V-PPases (*HVP1*, *HVP3*, and *HVP10*), which are expressed in a variety of tissues under different conditions. Sequence similarity between the V-PPase located underneath the *HvNax3* and the known barley V-PPases suggest that this gene corresponds to *HVP10*.

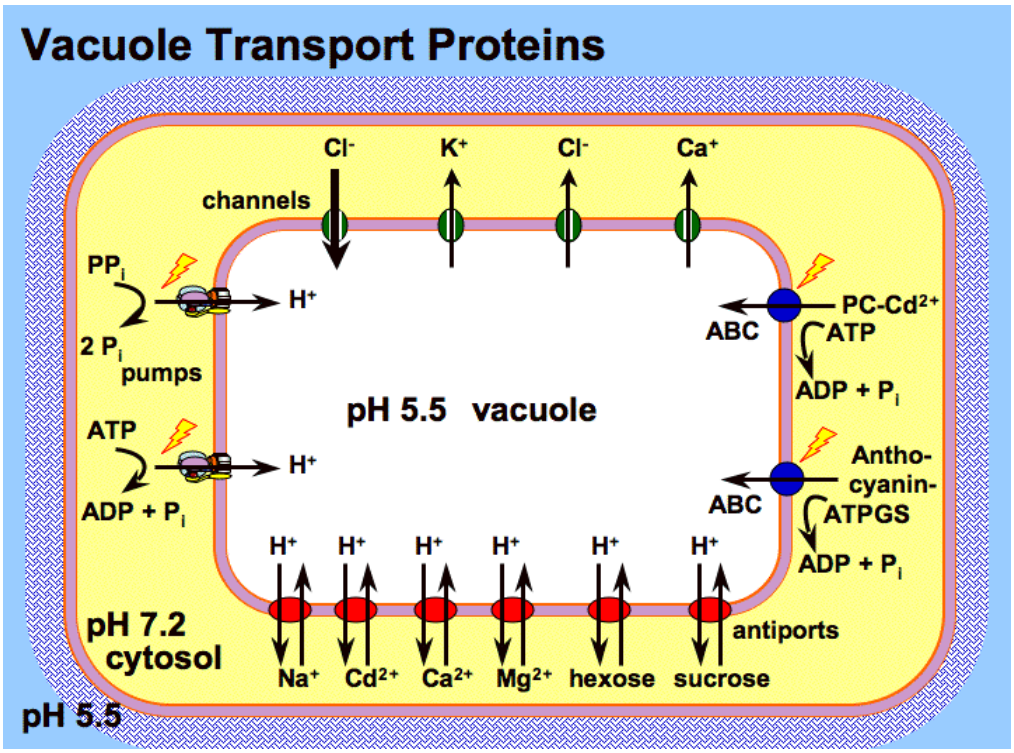

Figure 1-4 Diagram of a plant cell identifying locations of transport proteins located at the tonoplast required for cell homeostasis. Both V-PPases and V-ATPases pump protons (H^+) into the vacuole, using the energy released from the hydrolysis of either PPi or ATP, respectively. Red circles represent antiport co-transporters which require the energy from proton pumps to couple the transport of ions and sugars into the vacuole against their electrochemical gradient to the movement of H^+ down its concentration gradient into the cytosol. Green circles represent channels which transport ions passively across the tonoplast. Blue circles represent ATP-Binding Cassettes (ABC proteins) which transport cadmium and anthocyanin into the vacuole using the energy derived from ATP hydrolysis (Koning, 1994).

1.4.2 Vacuolar H^+-Pyrophosphatase (V-PPase)

In plants V-PPases are highly hydrophobic, single polypeptides of 75-81 kD with 14-16 trans-membrane domains (Maeshima and Yoshida, 1989). Three highly conserved segments have been identified within the primary structure, the first of which is the catalytic domain for substrate hydrolysis (CS1), while the other two conserved segments (CS2 and CS3) are critical for enzymatic functioning (Figure 1-5). The amino acid sequences of V-PPases are highly conserved among higher plants exhibiting a 86-91 % identity, the least conserved region can be found in the first 60 residues of the N-terminal domain, upstream of the substrate binding site (Maeshima, 2000). In plants V-PPase are potassium (K^+) dependent and are moderately sensitive to inhibition by Ca^{2+} (Belogurov and Lahti, 2001), and require Mg^{2+} as a co-factor for catalytic activity (Gaxiola *et al.*, 2007).

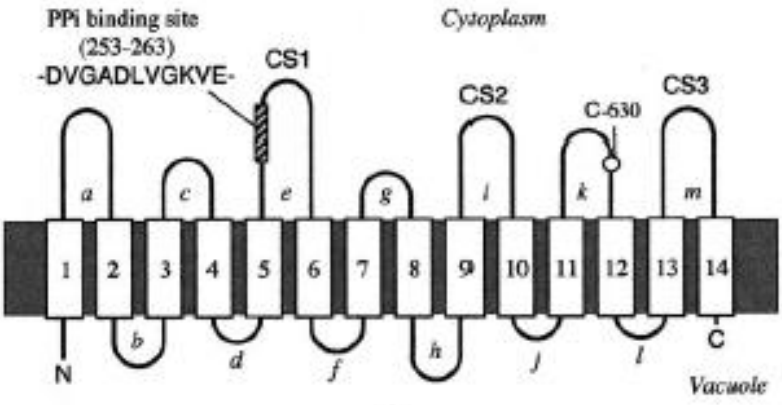

Figure 1-5 Topological model of V-PPase from mung bean. Fourteen trans-membrane domains were predicted from the TMpred program. Three conserved segments are marked (CS1, CS2 and CS3) (Maeshima and Yoshida, 1989).

The V-PPase is the enzyme responsible for the hydrolysis of pyrophosphate, a secondary metabolite of cellular processes such as DNA, RNA, sucrose and cellulose synthesis, and is also formed during the conversion of pyruvate to phosphoenolpyruvate by pyruvate phosphate dikinase (Martinoia *et al.*, 2006). V-PPases utilise inorganic pyrophosphate as a substrate to generate the proton electrochemical gradient across the vacuolar membrane, acidifying vacuoles in plant cells (Figure 1-6; Maeshima, 2000). This electrochemical gradient provides proton motive force for the secondary active transport of ions across the tonoplast by the action of antiporters (Martinoia *et al.*, 2006), responsible for transporting sodium (Na^+/H^+ antiporter), calcium (Ca^{2+}/H^+ antiporter), cadmium (Cd^{2+}/H^+ antiporter), and magnesium

(Mg^{2+}/H^+ antiporter) into the vacuole (Figure 4). In plants vacuolar Na^+/H^+ antiporters (such as *AtNHX1* from Arabidopsis), use the electrochemical gradient generated by V-PPase to move H^+ down their electrochemical potential into the cytoplasm and Na^+ against its electrochemical potential into the vacuole (Duan *et al.*, 2007). The compartmentalisation of Na^+ in the vacuole reduces the toxic effect of high salt concentrations in the cytoplasm, whilst at the same time facilitating water uptake in conditions of low external osmotic potential (Gaxiola *et al.*, 2007).

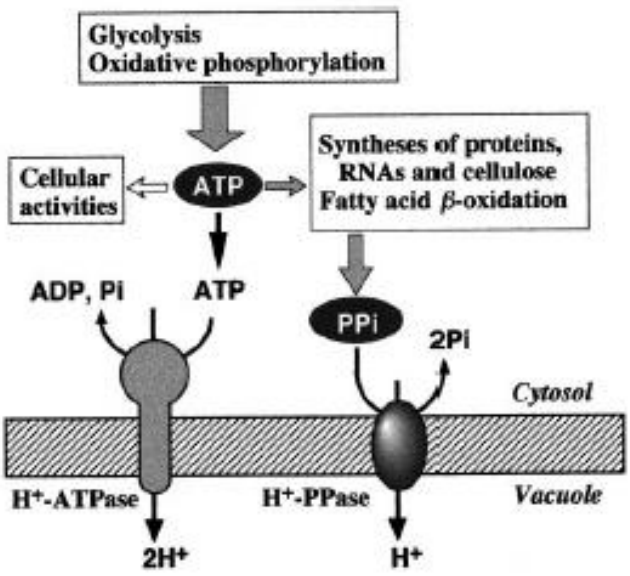

Figure 1-6 Pyrophosphate (PPi) production and vacuolar proton pumps H^+-PPase and H^+-ATPase. PPi is supplied as a by-product of biosyntheses of macromolecules such as RNAs, proteins, and cellulose and L-oxidation of fatty acids. It is used by H^+-PPase as an energy source to pump H^+ ions into the vacuole. Conversely H^+-ATPase requires the energy derived from ATP hydrolysis to pump protons into the vacuole (Maeshima and Yoshida, 1989).

1.4.2.1 Barley V-PPase isoforms

Comparison of the cDNA sequence identity of two barley V-PPase genes (*HVP1* and *HVP10*) indicated that they share 79.8 % identity in their open reading frame (ORF) and 86.2 % identity in their amino acid sequence; however they differ significantly in their 5' and 3' untranslated regions (UTR), with only 53.3 % and 47.7 % identity respectively (Fukuda *et al.*, 2004, Wang *et al.*, 2009). At the time the Fukuda *et al.* (2004) paper was published the third V-PPase isoform of barley (*HVP3*) had not yet been identified. More recently, the consensus peptide sequences of three wheat V-PPase paralogs (*TaVP1, TaVP2* and *TaVp3*) were aligned with 23 V-PPases from barley, maize, rice, sorghum, tobacco and Arabidopsis (Figure 1-7). It was found that the V-PPase genes form three distinctive phylogenetic groups, with each group containing a member from wheat and barley, and two members from rice, sorghum and maize. This multiple alignment revealed that *HVP10* was very similar to the wheat V-PPase paralog *TaVP1* and belonged to the same group as rice *OVP2* and *OVP3*; maize *ZmVP2* and *Zm*VP4; and *SbVP2 and SbVP4* (Wang *et al.*, 2009). The highly characterised Arabidopsis V-PPase gene, *AVP1*, can be found in the same group as *HVP1* and *OVP1*.

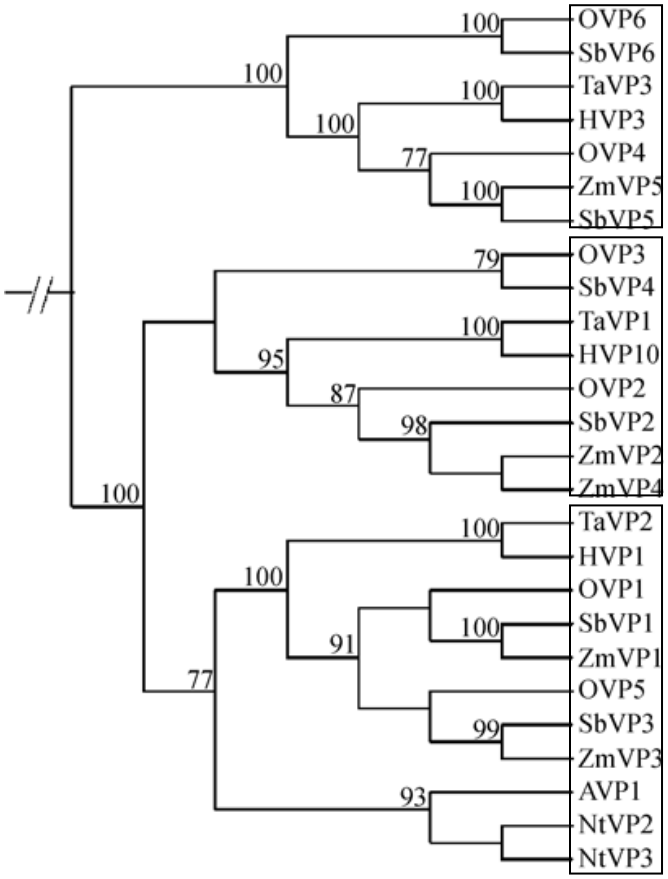

Figure 1-7 Phylogenic tree of 26 V-PPase genes from different species (Wang *et al.*, 2009). V-PPase peptide sequences from different species were aligned using the program MacVector 9.0, using default parameters.

1.4.2.2 V-PPase and its response to salinity stress

Many studies have demonstrated that V-PPase activity and gene expression is up-regulated in response to various abiotic stresses, particularly salinity stress, in a number of plant species (Fukuda *et al.*, 2004, Liang *et al.*, 2005, Fukuda and Tanaka, 2006, Jha *et al.*, 2010, Schilling *et al.*, 2017). Fukuda *et al.*(2004) demonstrated that the proton-translocating activity of V-PPase in tonoplast vesicles from barley roots increased within one hour of salt application (100 mM NaCl) and that the activity was higher than that of the control. In this study the expression level of two barley V-PPase isoforms, *HVP1* and *HVP10*, and the barley Na^+/H^+ antiporter (*HvNHX1*) were examined in response to ionic and osmotic stresses. Northern blot analysis of total RNAs revealed that the transcript of *HVP10* was more abundant in roots,

whereas that of *HVP1* was more abundant in shoots, indicating that their individual activity may vary between different plant tissues. Ionic stress (200 mM NaCl) significantly increased the expression levels of both *HVP1* and *HVP10* in barley; however the isoforms were differentially expressed in root tissue over time. *HVP1* expression increased 4 fold in the roots by 5 hours, and then decreased after 24 hours of treatment, whereas the *HVP10* transcript increased 1.5 fold in roots after 24 hours. Increases in both *HVP1* and *HvNHX1*, but not *HVP10* mRNA transcripts were observed in barley roots after exposure to 400 mM mannitol for 5 hours to simulate osmotic stress (Fukuda *et al.*, 2004). Despite the differences detected at the root zone, no differences in the mRNA levels of barley V-PPases were detected in the shoots under salinity stress at 5 and 24 hours (Fukuda *et al.*, 2004). Due to the sequence identity of the barley isoforms in their coding sequence, this result suggests that differences in their 5' and 3' UTR caused the different and individual regulation of these genes in response to salinity stress (Maeshima, 2000, Fukuda *et al.*, 2004). The then undiscovered third V-PPase isoform of barley could not be investigated, and further work is warranted to characterise this V-PPase isoform.

Wang *et al.* (2009) demonstrated that the wheat paralog to *HVP10*, *TaVP1*, had higher activity in root tissue and was also inducible by salinity stress. In this study Wang *et al.* (2009) demonstrated that wheat V-PPase gene paralogs were also spatially differentially regulated in response to stress; *TaVP1* was expressed in roots and induced by salinity stress, *TaVP2* was mainly expressed in shoot tissue and down regulated in leaves under dehydration, and *TaVP3* was only detected in developing seeds and was not inducible by stress (Wang *et al.*, 2009). The increased activity of V-PPase in response to salt stress has also been demonstrated in dicot species, including potato (Queiros *et al.*, 2009), sunflower (Ballesteros *et al.*, 1997) and carrot (Colombo and Cerana, 1993).

Increased V-PPase activity and sodium accumulation in the vacuole has also been detected in halophytic species under conditions of high NaCl. The marine succulent *Salicornia bigelovii* exhibits optimal growth in highly saline environments of between 100 and 400 mM NaCl (Parks *et al.*, 2002). Its proposed mechanism of salinity tolerance is the accumulation of Na^+ in the vacuole of shoots, protecting salt-sensitive enzymes in the cytoplasm, whilst at the same time providing the osmotic driving force for the uptake of water in highly saline waters. Increased hydrolysis of pyrophosphate and proton translocation was correlated with increased V-PPase protein accumulation in *S. bigelovii* grown in 200 mM NaCl (Parks *et al.*, 2002).

Several reports have indicated that V-PPase activity decreases in some species in response to salt stress. In some of these reports V-PPase activity was inhibited by NaCl treatment and V-ATPase activity was significantly increased, indicating it was the predominant vacuolar proton pump in some species under saline conditions (Wang *et al.*, 2001, Silva *et al.*, 2010). These inconsistencies in results may reflect species differences (Maeshima, 2000), or individual experimental conditions, including exposure time and the concentration of the salt solution. As previously discussed, there is significant temporal variation in pump activity in response to salinity stress, indicating that the length of salt exposure is an important component and should be considered when designing an experiment. The concentration of the salt treatment is also an important factor when examining salinity tolerance mechanisms, and should be applied in increment applications to ensure the experiment accurately reflects the ability of a plant to withstand salinity stress, not osmotic stock. Another consideration is the reproductive stage of the plant. The V-PPase pump is thought to be the major proton pump in young, rapidly dividing tissue (Maeshima and Yoshida, 1989, Façanha and de Meis, 1998), or in ATP limiting conditions such as anoxia and chilling (Carystinos *et al.*, 1995).

The relative contribution of the two vacuolar proton pumps in response to various abiotic stresses remains to be determined. The role of the two pumps was elucidated in a study by Kreb *et al.* in 2010, who examined Arabidopsis mutants lacking one or both V-ATPase isoforms at the tonoplast. Analysis of the double mutant indicated that there was no residual V-ATPase activity and that the electrochemical gradient across the tonoplast was due to the sole action of V-PPase. The double mutant remained viable, indicating that V-PPase is sufficient for embryo and seedling development; however the loss of ATPase activity resulted in reduced nitrogen storage and increased nitrogen assimilation in the mutant, resulting in significant growth inhibition. Surprisingly V-ATPase was not limiting for sodium accumulation, as no differences were detected between the mutants and wild type plants in their ability to sequester excess sodium ions into the vacuole under salt stress at 50 and 100 mM NaCl.

1.4.2.3 Overexpression studies of V-PPases

Genetic manipulation of V-PPases and Na^+/H^+ antiporters in plants have been identified as an important avenue for crop improvement (Maeshima, 2000, Gaxiola *et al.*, 2007). The overexpression of the vacuolar proton pump should enhance the proton gradient across the tonoplast driving the Na^+/H^+ antiporter, thereby increasing the ability of the plant to withstand

salinity and drought stress. Gaxiola *et al.* (2001) showed that the overexpression of the Arabidopsis *AVP1* increased the salinity and drought tolerance of transgenic Arabidopsis. Transgenic plants were more tolerant to high concentrations of NaCl (250 mM) and accumulated more Na^+ and K^+ in their leaf tissue compared to wild type plants. Arabidopsis plants overexpressing *AVP1* were also more tolerant to water deprivation than isogenic wild type strains, maintaining higher relative water content in their leaf tissue under drought stress (Gaxiola *et al.*, 2001). More recently, Park *et al.* (2017) demonstrated with a gene editing approach that increasing the native expression of the *AVP1* gene in Arabidopsis resulted in greater plant biomass and improved drought tolerance. The enhanced tolerance of transgenic plants to salinity and drought is explained by an enhanced uptake of Na^+ ions into the vacuole, driven by the greater H^+ electrochemical gradient across the tonoplast. The elevated solute content in the vacuole allowed transgenic plants to uptake water to maintain cell turgor under conditions of low soil water potential. The increased drought tolerance of Arabidopsis plants overexpressing *AVP1* has also been demonstrated in other studies (Park *et al.*, 2005, Ibrahim *et al.*, 2009). Transgenic Arabidopsis plants overexpressing the V-PPase gene from the succulent halophyte *Suaeda salsa* have also displayed increased salinity and drought tolerance (Guo *et al.*, 2006). These authors have proposed that the overexpression of vacuolar H^+ pyrophosphatase from Arabidopsis (*AVP*) and homologues from other species could provide a means of increasing the salinity and drought tolerance of agriculturally important crops (Brini *et al.*, 2007, Duan *et al.*, 2007, Bao *et al.*, 2009).

The overexpression of *AVP* gene has also been associated with increased root development, which may also contribute to the enhanced salinity and drought tolerance of transgenic plants. Li *et al.* (2005) showed that the overexpression of *AVP* resulted in increased vegetative growth and enhanced auxin mediated root development, resulting in higher water absorption and water retention capacity of transgenic plants. Conversely null knockout avp1-1 mutants displayed disrupted shoot and root formation and reduced auxin transport. *AVP* also contributed to the regulation of apoplastic pH homeostasis (Li *et al.*, 2005). Under control conditions there was no significant difference in apoplastic, cytoplasmic and vacuolar pH in wildtype and *AVP1*-constitutive overexpressing transgenic plants; however the apoplastic pH of *AVP1* overexpressing plants was significantly more acidic under salt stress (Li *et al.*, 2005). Other studies have shown that the overexpression of V-PPase also leads to increased tolerance to other abiotic factors, including nutrient deficiency. Yang *et al.* (2007) demonstrated that Arabidopsis, tomato and rice plants overexpressing *AVP* were also more

tolerant to low phosphorus conditions due to enhanced rhizosphere acidification, root proliferation and organic acid extrusion from roots, implying that the overexpression of V-PPase confers tolerance to a wide range of abiotic stresses.

Recently, a review of the multiple roles of AVP1 in abiotic stress tolerance has been discussed, based on current literature relating to the constitutive expression of this protein, and analysis of loss of function mutants (Schilling *et al.*, 2017). It is widely accepted that the increased biomass of plants overexpressing the gene under abiotic stress and non-stressed conditions is due to increased vacuolar ion sequestration, improved auxin transport, and enhanced heterotrophic growth; however more recently it has also been implicated in the regulation of cytosolic PPi levels and regulation of transport of sucrose from source and sink tissues (**Error! Reference source not found.**). In 2011, Ferjani *et al.* demonstrated that Arabidopsis loss of function *fugu5* mutants, defective in AVP1, failed to maintain heterotrophic growth after germination and were shown to contain 2.5 fold higher PPi and 50% less sucrose than wild type. The phenotype of wild type plants could be effectively restored by exogenous supply of sucrose to the growth media or removal of the cytosolic pyrophosphate via heterologous expression of the inorganic pyrophosphatase1 gene (IPP1) from budding yeast (*Saccharomyces cervisiae*). These results provide evidence that high levels of cytosolic PPi inhibits gluconeogenesis and that hydrolysis of cytosolic PPi is a major metabolic function of AVP1 at the early stages of postembryonic growth in plants.

There is also mounting evidence that *AVP1* is localised to the plasma membrane of phloem companion cells in Arabidopsis and can function as a PPi-synthase (Paez-Valencia *et al*. 2011, Regmi *et al*. 2016). Unfortunately no in planta studies have demonstrated the role of H-PPase in PPi synthesis and it may be that the localisation of V-PPase on the plasma membrane may be attributed to remnants of the tonoplast adhering to the cell surface during sieve-element formation. These reports do provide some evidence that V-PPase may have multiple roles and interact in different tissue and cell types throughout the life cycle of the plant. For a full review of the literature see Shilling *et al.* (2017).

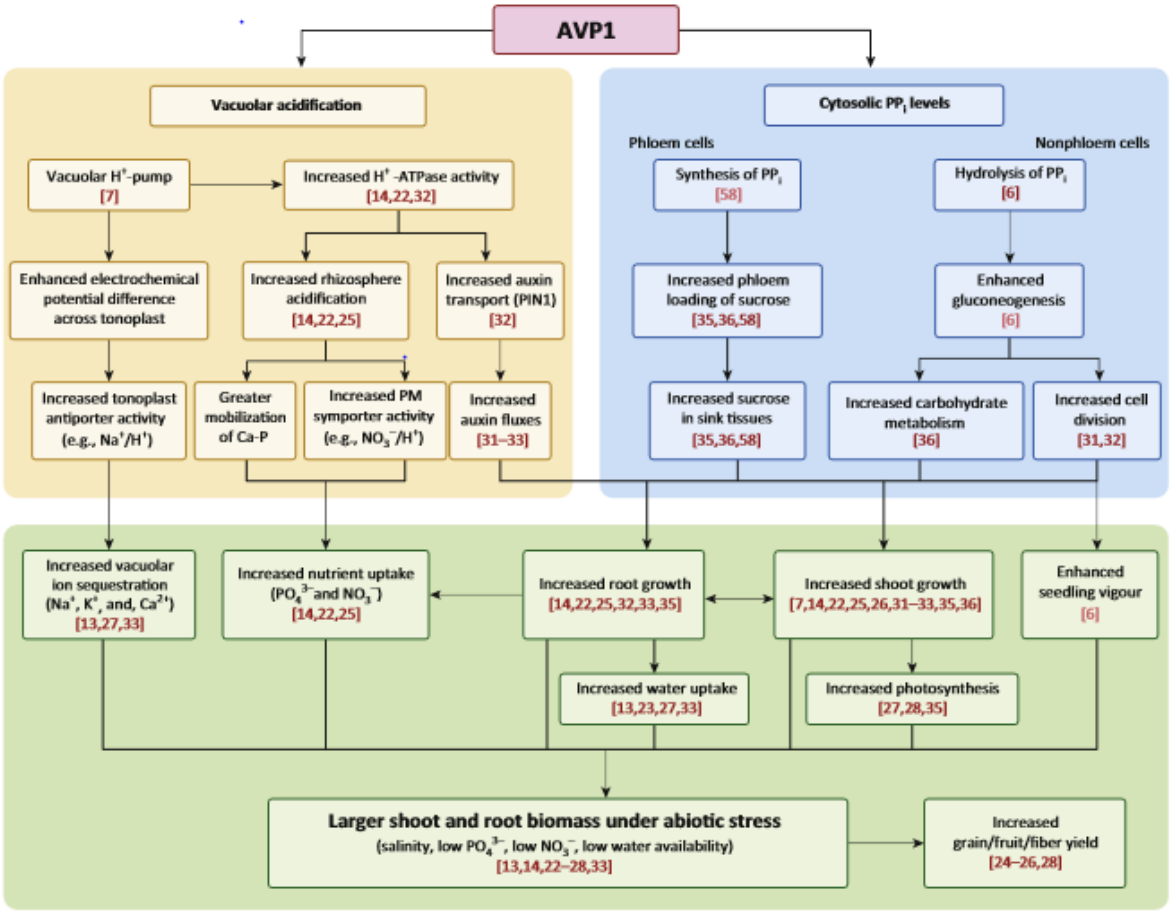

Figure 1-8 Multiple roles of AVP1 as described in Shilling *et al.* (2017). AVP1 has two main functions: 1) vacuolar acidification and 2) regulation of cytoplasmic inorganic pyrophosphate (PPi) concentrations within specific cell types. Both mechanisms contribute to the larger growth of transgenic plants expressing the Arabidopsis gene *AVP1*. Solid lines with arrows indicate a putative link between traits and the respective direction of flow.

The increased salinity and drought tolerance of transgenic plants overexpressing orthologous V-PPase genes from other species has been demonstrated in a number of important agricultural crops. The increased salinity and drought tolerance of transgenic plants overexpressing V-PPase from the halophyte *Thellungiella halophila* has been reported in cotton (Lv *et al.*, 2008, Lv *et al.*, 2009), maize (Li *et al.*, 2008), and tobacco (Gao *et al.*, 2006). *T. halophila*, which is endemic to China, has been shown to survive and produce viable seed in the presence of 500 mM NaCl (Inan *et al.*, 2004). Transgenic cotton plants constitutively overexpressing V-PPase from *T. halophila* were shown to have between 22-75% greater V-PPase activity compared with wild type plants under control conditions (Lv *et al.*, 2008). The hydrolytic activity of the V-PPase increased in both control and transgenic plants under osmotic stress, however, it was significantly greater in transgenic plants (Lv *et al.*, 2008). The transgenic plants demonstrated greater accumulation of Na^+, K^+, Ca^{2+}, Cl^- in their vacuoles, and exhibited improved shoot and root growth as well as sustained photosynthetic performance under saline conditions (Lv *et al.*, 2008). Under drought conditions, plants overexpressing V-PPase from *T. halophila* were characterised by higher chlorophyll content, improved photosynthesis, higher relative water content in leaves and less cell membrane damage following exposure to drought stress, compared to control plants (Lv *et al.*, 2009). These reports demonstrate that genetic manipulation of V-PPase genes results in the enhanced performance of agriculturally important crops to salinity and drought stress conditions.

Other studies have focused on the overexpression of Na^+/H^+ antiporters to increase the salinity and drought tolerance of plants. Apse *et al.* (1999) showed that the overexpression of a vacuolar Na^+/H^+ antiporter (*AtNHX1*) from Arabidopsis allowed transgenic Arabidopsis plants to grow in solutions containing 200 mM NaCl, a concentration at which Arabidopsis growth is usually inhibited. A study by Xue *et al.* (2004) demonstrated that transgenic wheat overexpressing *AtNHX1* also showed a significant improvement in germination rates biomass production, and grain weight under saline conditions. Transgenic plants exhibited a reduced level of sodium in the leaves and enhanced ration of K^+/Na^+ under salt stress, indicating that overexpression of the Na^+/H^+ antiporter also leads to greater sodium accumulation in root vacuoles.

A more recent study indicated that the OEX of *NHX1* in barley on its own did not improve performance under salinity stress (Aden et al 2015). It was hypothesised that this was due to

plants not having enough of a proton gradient and that this could be overcome by the duel co-expression of *NHX1* and *AVP1*.

1.4.3 Other candidate genes within the HvNax3 *QTL*

Although the evidence present in the literature regarding the likely role of V-PPase in conferring salinity tolerance in barley, a number of other candidate genes have been identified within the *HvNax3* interval which may encode for proteins involved in mechanisms of salinity tolerance. One of the gene products belongs to the super-family of proteins SNARE, which facilitates membrane fusion (Jahn *et al.*, 2003). This group shows the closest similarity to the R-VAMP71 subgroup in Arabidopsis responsible for vesicle fusion with the tonoplast. Site directed mutagenesis and silencing of VAMP-71 was shown to increase the salinity tolerance of Arabidopsis (Leshem *et al.*, 2006). In this study, exposure to high salt concentrations resulted in the increased production of hydrogen peroxide (H_2O_2) in endosomes, which fused with the tonoplast in wild type plants. Suppression of this gene inhibited fusion of H_2O_2 containing vesicles with the tonoplast and reduced the release of calcium from the vacuole. It was suggested that fusion inhibition of these vesicles with the tonoplast preserved tonoplast function, allowing mutant plants to maintain vacuolar pH and tolerate salt stress. In a recent study the reduced expression of the VAMP-71 also reduced the drought tolerance of transgenic plants by suppression of abscisic acid-dependent stomatal closure (Leshem *et al.*, 2006). These results suggest that the regulation of these genes may represent part of the natural salinity and drought tolerance mechanism in plants.

A *Brachypodium* orthologue of a gene present in the *HvNax3* interval shares closest similarity to five DELLA transcription factors in Arabidopsis. DELLA is a family of nuclear growth repressing proteins involved in the integrated response of plants to adverse environmental conditions, and whose degradation is stimulated by gibberellin (Achard *et al.*, 2006). Archard *et al.* (2006) demonstrated that DELLA factors contribute to salinity induced growth inhibition under saline conditions; however the relationship of these factors to sodium accumulation in the shoot was not reported.

Another candidate gene in the *HvNax3* interval belongs to the group of proteins required for clathrin-mediated vesicle budding, termed ANTH proteins. A related ANTH protein was detected in an *Agrobacterium tumefaciens* functional screen of genes involved in salt tolerance in the mangrove plant *Bruguiera gymnorhiza*. In this study, an mRNA for vacuolar proton-inorganic pyrophosphatase pump was also identified to confer salt tolerance to *A.*

tumefaciens bacteria. A direct association of this ANTH gene with salinity tolerance in plants is yet to be determined.

While these other genes in the *HVNax3* interval have been shown to have a putative association with mechanisms of salinity tolerance, as demonstrated by the literature, barley V-PPase (HVP) represents the strongest candidate for the sodium exclusion phenotype in the Barque-73/CPI-71284-48 population. This project seeks to confirm that *HVP10* is responsible for the *HvNax3* phenotype, and to characterise the role of this gene in salinity tolerance in barley. It is envisioned that the generation of transgenic barley plants with modified *HVP* expression will assist in the development of genetic stocks adapted to abiotic stresses, as well as increase our understanding of the molecular basis of salinity tolerance in this agriculturally important crop.

1.5 Research Questions

The specific research questions that will be addressed by this project are as follows:

1) Is the *HVP10* V-PPase gene responsible for the different sodium exclusion phenotypes observed in Barque-73 and CPI-71284-48

2) Is V-PPase activity up-regulated in the roots and/or in shoots of barley in response to salt stress?

3) Does overexpression of the *HVP10* V-PPase gene result in enhanced salinity and drought tolerance of barley?

4) Is *HVHVP10* localised only to the tonoplast and in what cell type is *HVP10* V-PPase expressed?

1.6 Aims/Objectives of the project

This project will address the following aims and objectives:

1) Confirm that V-PPase is responsible for *HvNax3* sodium exclusion QTL;

2) Characterise V-PPase activity *in planta* in parents CPI-71284-48, Barque-73 and double-haploid lines (DH) from the cross in response to salt stress;

3) Clone and sequence the V-PPase promoter and gene from CPI-71284-48 and Barque-73;

4) Generate transgenic barley plants with constitutive and stress inducible expression of V-PPase; and

5) Develop GFP and GUS constructs to determine tissue distribution and sub-cellular localisation of V-PPase.

1.7 Significance/Contribution to the discipline

Given the significant negative impact salinity has on agricultural production, the identification and introgression of tolerance genes into elite cereal germplasm has been identified as an important area of scientific research by the Australian Grains Industry. This research will provide long-term benefits to the grains industry by assisting in the development of genetic stocks which are adapted to salinity stress, thereby allowing the maintenance of stable crop yields in increasingly saline environments. This research will also increase our understanding of the molecular and physiological mechanisms of salinity tolerance in barley. It is hoped that this technology can then be transferred to other agriculturally important crop species, such as wheat.

2 Confirmation of the barley V-PPase *HvHVP10* as a candidate gene for the *HvNax3* sodium exclusion QTL in barley

2.1 Introduction

A major quantitative trait locus (QTL) for sodium exclusion (*HvNax3*) has previously been identified in a barley DH mapping population Barque-73 (*Hordeum vulgare*) x CPI-71284-48, (*H. spontaneum*) (Shavrukov *et al.*, 2010). The phenotypic difference in 3rd leaf sodium accumulation observed in this population explained 51% of the phenotypic variation and was validated in an AB-QTL (BC_2F_2) population. Synteny mapping with rice and *Brachypodium* genomes identified 16 classes of proteins within the *HvNax3* interval which may be responsible for the observed difference in sodium accumulation in this population (Shavrukov *et al.* 2010).

During the course of this PhD, fine mapping of the *HvNax3* region conducted by a visiting scholar, further reduced the QTL interval to 4 families of proteins (Shavrukov *et al.*, 2013); Appendix 8.1). The most promising candidate gene for the *HvNax3* locus is one which encodes for a vacuolar pyrophosphatase proton pump (V-PPase), *HvHVP10* in barley. This protein contributes to generating the electrical potential difference across the tonoplast (inside the vacuole is positive compared to the cytoplasm). The membrane potential alters the electrochemical gradient for some ions such as sodium and H^+ which can drive the secondary-active transport of sodium into the vacuole via antiporters. Storage of sodium in the vacuole has been shown to be an important mechanism of salinity tolerance in plants (Munns and Tester, 2008).

To date, three V-PPase isoforms have been identified in barley; *HvHVP10* (Tanaka *et al.*, 1993), identified as the gene most likely responsible for the *HvNax3* QTL; and *HvHVP1* (Fukuda *et al.*, 2004), both of which are located on chromosome 7H (Shavrukov, 2014), and HVP3 (Wang *et al*. 2009). Investigation of these barley V-PPase isoforms has demonstrated that they differ in both their spatial and temporal expression, where *HvHVP10* is mainly expressed in the root and *HvHVP1* is mainly expressed in the shoot (Fukuda *et al.*, 2004). It is hypothesised that lines carrying the CPI-71284-48 allele for *HvNax3* have increased expression of *HvHVP10* in the root in response to salinity stress. The increased activity of *HvHVP10* at the tonoplast leads to better compartmentalisation of sodium ions in the vacuoles of root cell. This prevents the sodium ions from reaching the aerial parts of the plant and

disrupting important metabolic processes such as photosynthesis, whilst at the same time facilitating water uptake into the plant. It has also been suggested that increased HVP10 activity during the seedling stage may enhance seedling vigour (Fukuda *et al.*, 2004, Schilling *et al.*, 2014, Schilling *et al.*, 2017), perhaps through better sucrose transport (Gaxiola *et al.*, 2016, Regmi *et al.*, 2016), resulting in larger plants.

The aims of this chapter are to confirm that the barley V-PPase gene *HvHVP10* is responsible for the *HvNax3* sodium exclusion QTL in the barley population Barque-73 x CPI-71284-48 and is broken into 2 sections; 1) Examination of the phenotypic effects of the *HvNax3* QTL *in planta* in CPI-71284-48, Barque-73 and two AB-QTL lines (18D/014 carries the CPI-71284-48 *HvNax3* allele and 18D/011 carries the Barque allele); and 2) Transcriptional profiling of barley V-PPases *HvHVP1* and *HvHVP10*.

The specific hypotheses are;

1. CPI-71284-48 and the CPI-71284-48 AB-QTL line 18D/014 (line carrying the CPI-71284-48 *HvNax3* allele) will retain more sodium in the root and less in the shoot over time (10 days) when compared with Barque-73 and the Barque-73 AB-QTL line 18D/011 (line carrying the Barque-73 *HvNax3* allele) in response to the imposed salinity stress of 150mM NaCl.
2. CPI-71284-48 and the CPI-71284-48 AB-QTL line (18D/014) will have greater *HvHVP10* expression in the root when compared with Barque-73 and the Barque-73 AB-QTL line (18D/011) in response to the imposed salinity stress.

2.2 Methods

2.2.1 Examination of HvNax3 QTL in planta in CPI-71284-48, Barque-73 and AB-QTL lines

2.2.1.1 Plant material

The barley genotypes CPI-71284-48, Barque-73 (parents of the DH population) and the AB-QTL lines, 18D/011 (Barque-73 *HvNax3* allele) and 18D/014 (CPI-71284-48 *HvNax3* allele) were used to investigate the accumulation of ions in the roots and shoots over time under 150 mM NaCl. This material was also generated for expression analysis of *HvHVP10* and *HvHVP1* in root and shoot tissue over 10 days of salt stress (150mM NaCl) and under control conditions (no added NaCl).

2.2.1.2 Salt hydroponics screen

The experiment consisted of a completely randomised block design with 4 replicates and was conducted in SARDI (South Australian Research and Development Institute) glasshouses at the University of Adelaide, Waite Campus. Seeds were sterised in petri dishes by exposure to UV light for 5 minutes, then germinated on moist filter paper at room temperature (21°C) for 4 days. Uniformly germinated seedlings were transplanted to tubes filled with polycarbonate fragments and grown in a supported hydroponics system (Figure 2-1) under salt (150 mM NaCl) and control treatments (no added NaCl). Plants were grown to the third leaf stage in a modified Hoglands growth solution (0.2 mM NH_4NO_3, 5.0 mM KNO_3, 2.0 mM $Ca(NO_3)_2$ $4H_2O$, 2.0 mM $MgSO_4$ $7H_2O$, 0.1 mM KH_2PO_4, 0.5 mM $Na_2Si_3O_7$, 0.05 mM NaFe(III)EDTA, 50 µM H_3BO_3, 5.0 µM $MnCl_2$ $4H_2O$, 10 µM $ZnSO_4$ $7H_2O$, 0.5 µM $CuSO_4$ $7H_2O$, 0.1 µM Na_2MoO_4 $2H_2O$) before NaCl was applied to the solution in 25 mM increments with additional calcium chloride (2 mM) morning and night until the target concentration was reached. Plants were grown for 10 days post initial salt application and harvested for root and shoot material over the course of the experiment.

Figure 2-1 Images of the hydroponics system used for the QPCR and phenotyping experiments described in this chapter. (A) At salt application (third leaf emergence) (B) Front view of control tanks at third leaf emergence.

2.2.1.3 Plant tissue sampling

Root and shoot material was harvested at the same time during the day, approximately 2:00pm in the afternoon (due to the potential diurnal nature of V-PPase gene expression (Lerch *et al.* 1995) at 0, 1, 2, 3, 5, 7 and 10 days post initial salt treatment. When sampling earlier time points plants were pooled for each replicate to obtain enough material for analysis (0 days = 4 plants, 1 and 2 days = 3 plants, 3 and 5 days = 2 plants, 7 and 10 days = 1 plant). Plants were gently removed from the pots and the roots were rinsed with 10 mM $CaCl_2$ to remove residual NaCl and nutrient solution from the root surface. The roots were then blotted dry on paper towel to remove excess moisture. Shoot tissue was separated from the root mass, then the root and shoot samples were weighed immediately to determine the fresh weight. The roots were separated into 3 equal portions and 1 portion of the root material and the third leaf were harvested at each time point for gene expression analysis of *HvHVP10* and *HvHVP1*. Plant material was placed into 10 mL tubes, immediately snap frozen in liquid nitrogen and stored at -80°C.

2.2.1.4 Measurement of ion concentration in roots and shoots

Root tissue and the 2nd leaf were sampled at each time point to determine the accumulation of sodium in the tissues over the course of the experiment. The fourth leaf at day 10 was also

harvested to determine final sodium and potassium concentrations at the completion of the experiment.

Root and shoot material for ion concentration analysis was dried at 68°C for 2 days and the dry weight recorded. Plant material was digested in 20 mL of 1% HNO_3 at 85°C for 4 hours in a Teflon hot block. Na^+ and K^+ concentrations were determined using a flame photometer (Sherwood, UK model 420) and expressed as concentration (mM) in the plant sap (tissue water basis).

2.2.2 Transcriptional profiling of barley V-PPases HvHVP1 and HvHVP10

2.2.2.1 RNA extraction

Total RNA was extracted from the third leaf and total roots of plants using the TRIZOL® based extraction method (Chomczynski and Sacchi, 2006). TRIZOL® reagent was replaced with TRIZOL-like reagent composed of 38% (v/v) phenol pH 4.3, 12% (w/v) guanidine thiocyanate, 7% (w/v) ammonium, 3 M sodium acetate pH 5.0, and 5% (v/v) glycerol. Briefly, approximately 100 mg of frozen ground leaf tissue was placed into a 2 mL Eppendorf tube and plant material was lysed with 1 mL of TRIZOL-like reagent. Samples were mixed on an orbital rotor for 5 minutes at room temperature. Then 200 µL of chloroform was added to each sample and vortexed for 10 s. Samples were incubated for 3 minutes at room temperature then centrifuged at 12,000 *g* for 15 minutes at 4°C. The upper aqueous phase was transferred to a clean 1.5 mL Eppendorf tube and 500 µL of isopropanol was added to precipitate the RNA. Samples were incubated at room temperature for 10 minutes, and then centrifuged at 12,000 *g* for 10 minutes and the supernatant removed. The RNA pellet was washed with 1 mL of 75% (v/v) ethanol, samples mixed and then centrifuged at 7,500 *g* for 5 minutes at 4°C. Ethanol was removed with a pipette and the RNA pellet was air dried in a fume hood for 10 minutes to remove residual traces of ethanol. The RNA was resuspended in 25 µL of RNase-free water. DNA contamination was removed from each RNA sample using a DNA Free kit following the manufacturer's instructions. Briefly, 2.5 µL of 10 × DNase I buffer and 2 Units of DNase were added to each RNA sample, and then samples were gently mixed and incubated at 37°C for 30 minutes. 5 µL of DNase inactivation agent was added to each sample and samples were mixed. Samples were then incubated at room temperature for 2 minutes and centrifuged at 10,000 *g* for 1 minute. The aqueous phase was transferred to a clean Eppendorf tube and stored at $^{-}$80°C. RNA concentration was measured using a Nano-drop and RNA integrity was checked using gel electrophoresis (Figure 2-2) on a 1% TAE agarose gel.

2.2.2.2 Complementary DNA (cDNA) synthesis

First strand cDNA was synthesised using SuperScript™ III Reverse Transcriptase following the manufacturer's instructions. cDNA synthesis was performed in 20 µL reactions composed of 1 µL of oligo (dT)18 (50µM), 1µL of dNTP mix (10mM each of dATP, dGTP, dCTP, dTTP), 1 µg of total RNA and sterile milli-q water to a final volume of 13 µL. The reaction was incubated for 5 minutes at 65°C then placed immediately on ice for 1 minute. The samples were briefly centrifuged then 4 µL of 5×First –Strand Buffer, 1 µL of 0.1M DTT, 1 µL of RNaseOut™ Recombinant RNase Inhibitor and 1µL of SuperScript™III RT (50 Units) were added to each sample and contents mixed by pipetting up and down. The reaction was incubated at 50°C for 60 minutes then heated at 70°C for 15 minutes to terminate the reaction.

All cDNA samples were checked via PCR using primers targeting the internal control gene *HvGAPDH* (forward 5′-GTGAGGCTGGTGCTGATTACG-3′, reverse 5′TGGTGCAGCTAGCATTTGACA-3′) in 15 µL reactions composed of 1 µL of cDNA (10ng/µL), 1.5 µL of dNTPs (10 µM), 0.3 µL of forward and reverse primers, 0.3 µL of $MgCl_2$ (10 mM), 0.1 µL of Platnium Taq (Invitrogen) and 11.2 µL of sterile H_2O. PCR cycling conditions consisted of an initial heat activation step at 95°C for 2 minutes, followed by 26 cycles of denaturation at 94°C for 10 seconds, annealing at 58°C for 10 seconds and extension at 68°C for 30 seconds. A final extension step was carried out at 68°C for 2 minutes. All PCR products were visualised on a 1% agarose gel (for an example see Figure 2-2) and missing or degraded samples were re-synthised. All cDNA samples were then submitted to the ACPFG qPCR laboratory for mRNA expression analysis of *HvHVP10* and *HvHVP1*.

2.2.2.3 Agarose gel electrophoresis

All PCR products were visualised on Agarose gels prepared using 1×TAE buffer (40 mM Tris-Acetate, 1 mM EDTA and 1 % or 0.8% (w/v) agarose. DNA was stained with 0.5 $\mu g \cdot mL^{-1}$ ethidium bromide or 0.05 $\mu l \cdot mL^{-1}$ SYBR-safe. DNA marker ladders HyperLadder™ 1kb or HyperLadder™ 100 bp were used to determined DNA size and quantity. Electrophoresis was run at 90-100 volts for 40 minutes to separate DNA fragments. DNA fragments were visualised using a GeneFlash gel documentation system (Syngene, Cambridge, UK).

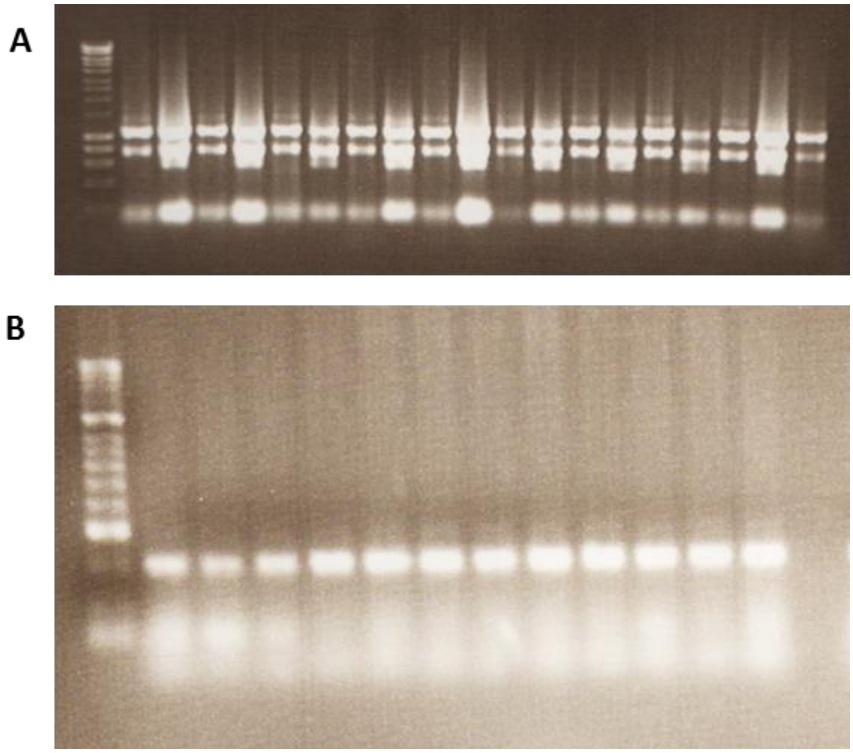

Figure 2-2 An example of a gel electrophoresis integrity check of root and shoot control and salt RNA samples. (A) expression analysis of root and shoot control and salt cDNA samples using qPCR of the internal control gene *HvGAPDH* (B). 4µL of RNA and 1ul of cresol red loading dye was loaded onto a 1.0 % agarose gel in TAE buffer with a 1 kB RNA ladder. The integrity of cDNA samples was checked via PCR using *HvGAPDH* primers.

2.2.2.4 qRT-PCR

qRT-PCR of *HvHVP10* and *HvHVP1* was performed using gene-specific primers and was conducted by Yuan Li at the ACPFG. A total of six control genes were used in this experiment to normalise *HvHVP1* and *HvHVP10* transcript copy number in root and shoot cDNA samples; *HSP70 Hv*, *Cyclophilin Hv*, *GAPDH Hv*, *rt 1312*, *rt1313* and *rt1314*. Root cDNA transcript copy numbers were normalised using the control genes *HSP70 Hv*, *Cyclophilin Hv*, *rt1312* and *rt1314*. Shoot cDNA trranscript copy numbers were normalised using the control genes *GAPDH*, *rt1312*, *rt1313* and *rt1314*. Primers used for qRT-PCR of barley genes are shown in Table 2-1. Salt-treated tissues which showed significant variability between the different control genes were omitted from the final analysis of *HvHVP10* and *HvHVP1* expression in response to salt stress. The root and shoot expression data from the different time points was compared to the expression of the same gene just prior to salt application, to examine the response of the relevant gene to salt stress over time.

Table 2-1 Primers used for QRT-PCR analysis of barley V-PPases *HvHVP10* and *HvHVP1*

Gene	Sequence (5´-3´)	Designed by/Reference
HSP70 Hv	**F**-CGACCAGGGCAACCGCACCAC **R**-ACGGTGTTGATGGGGTTCATG	(Burton *et al.*, 2008)
Cyclophilin Hv	**F**-CCTGTCGTGTCGTCGGTCTAAA **R**-ACGCAGATCCAGCAGCCTAAAG	(Burton *et al.*, 2008)
GAPDH Hv	**F**-GTGAGGCTGGTGCTGATTACG **R**-TGGTGCAGCTAGCATTTGAGAC	(Burton *et al.*, 2008)
rt1312 Hv	**F**-GTGAGGATGGAACTTGAATAGC **R**-TGCCACACAGTAAATATAAGAGC	Stephen Fletcher, ACPFG
rt1313 Hv	**F**-GAGCAAGTATCATCCAGGCAACC **R**-ACATCACAGACCAGCAGCATTG	Stephen Fletcher, ACPFG
rt1314 Hv	**F**-AGATACTACGCAAGCAGAAG **R**-ACAATCAAATAAATAGGGAGTGG	Stephen Fletcher, ACPFG
HvHVP10	**F**-GGTCTGTGGGCTGGTCTGATTATTG **R**-GCTGACGTAGATGCTGACAGCAATAG	Yuri Shavrukov, ACPFG
HvHVP1	**F**-AAAGAGCCTGGGCCCGAAAGGC **R**-TCTTGAAGAGGATTCCTCCATAG	Yuri Shavrukov, ACPFG

2.3 Results

2.3.1 Examination of HvNax3 QTL in planta in CPI-71284-48, Barque-73 and AB-QTL lines

2.3.1.1 CPI-71284-48 grew more slowly under salt and control conditions

The barley accessions CPI-71284-48 (*H. spontaneum*), Barque-73 (*H. vulgare*) and the AB-QTL lines 18D/014 (CPI-71284-48 *HvNax3* allele), and 18D/011 (Barque-73 *HvNax3* allele) were grown in supported hydroponics under 150 mM NaCl and control conditions (0 mM). A significant reduction in growth was observed when the plants were exposed to salt stress. However, the different species were found to have quite different growth habits over the course of the experiment, where CPI-71284-48 grew more slowly under both salt and control conditions, but had wider, larger leaves. Barque-73 and both AB-QTL lines all displayed a similar growth pattern, consisting of a faster growth rate and thinner longer leaves. The fresh weight of each genotype under salt treatment is therefore expressed as a percentage of their control at each time point (salt/control ×100). CPI-71284-48 maintained a greater relative fresh weight of shoots at 1, 2 and 3 days post salt stress (

Figure 2-3) and maintained greater relative root fresh weight at day 1 and 2 post salt stress. CPI-71284-48 also maintained a greater shoot to root ratio at day 3 of salt stress when compared with the other genotypes. Conversely, Barque-73 maintained greater root and shoot fresh weight at 5 and 7 days post salt stress, but had lower relative shoot to root ratios at 5, 7 and 10 days. The AB-QTL line 18D/014 maintained higher root and shoot FW at day 7 compared with 18D/011; however they both displayed similar FW by day 10 of salt stress.

2.3.1.2 CPI-71284-48 accumulated more sodium and less potassium in the leaf

The second leaf and total root was sampled from each genotype at each time point to investigate the accumulation of ions in the shoot and root over the 10 days of imposed salinity stress. In this experiment CPI-71284-48 maintained lower sodium and higher potassium concentration in the roots from day 2 of salt stress and accumulated higher shoot sodium in the second leaf at 7 and 10 days post salt stress, although it had significantly less shoot sodium at 5 days when compared with the other genotypes (

Figure 2-4). This is a different phenotype to the *HvNax3* QTL, where CPI-71284-48 and lines carrying the CPI-71284-48 *HvNax3* allele had significantly reduced sodium accumulation in the third leaf after 10 days of salt stress (Shavrukov *et al.*, 2010)..

The fourth leaf was also sampled to determine sodium and potassium concentrations in this leaf at the completion of the experiment (

Figure 2-3). CPI-71284-48 had significantly more sodium and less potassium in the fourth leaf under salt stress compared with the other genotypes, supporting the observation of higher sodium accumulation in the second leaf. These results could suggest that CPI-71284-48 is better at keeping Na^+ ions from young tissue by keeping it in older tissue.

2.3.1.3 The AB-QTL line 18D/014 displays the HvNax3 phenotype

Despite these differences observed in the parental genotypes and the inconsistency between the parents of the DH population, the AB-QTL line 18D/014 carrying the CPI-71284-48 *HvNax3* allele tended to maintain higher root sodium at day 5, 7 and 10, but similar shoot sodium to Barque-73 throughout the imposed salinity stress. The AB-QTL line 18D/014 also tended to have less sodium and more potassium in the fourth leaf at 10 days as expected, when compared with the AB-QTL line 18D/011 and retained greater sodium in the roots from day 3 of salinity stress (

Figure 2-4). This phenotype is consistent with the hypothesis that lines carrying the *HvNax3* allele from CPI-71284-48 contain more sodium in the roots and less in the shoots under periods of salinity stress, possibly via compartmentalisation of sodium in the root vacuole.

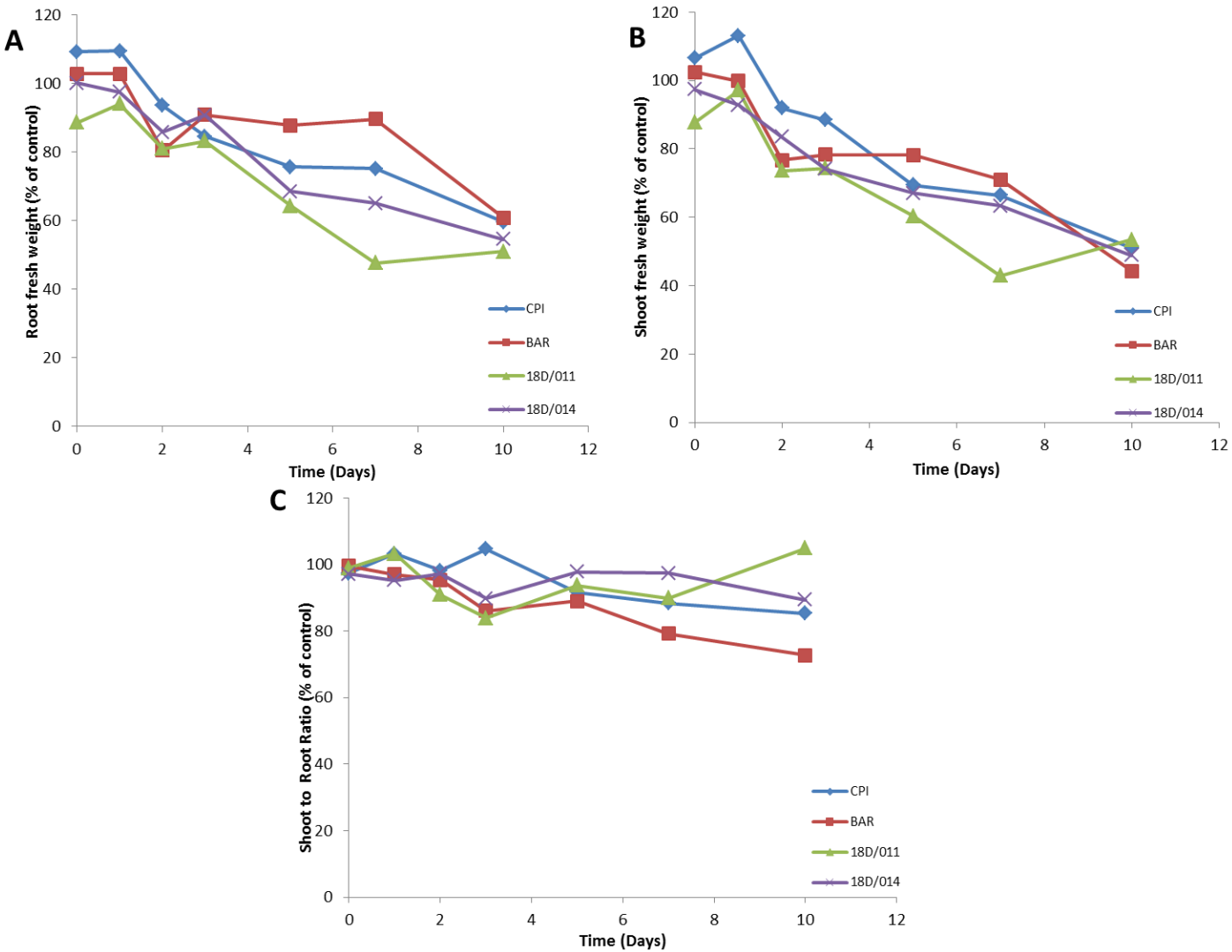
A
Root fresh weight (% of control)
Time (Days)
CPI
BAR
18D/011
18D/014
B
Shoot fresh weight (% of control)
Time (Days)
CPI
BAR
18D/011
18D/014
C
Shoot to Root Ratio (% of control)
Time (Days)
CPI
BAR
18D/011
18D/014

Figure 2-3 (A) Root fresh weight, (B) shoot fresh weight and (C) shoot to root ratio of barley genotypes CPI-71284-48, Barque-73, and AB-QTL lines, 18D/011 (Barque-73 allele) and 18D/014 (CPI-71284-48 allele) grown in supported hydroponics under 150 mM NaCl. Data are presented as a %, relative to control treatment (no NaCl). NaCl was applied to growth solutions at third leaf emergence twice daily in 25 mM increments with additional calcium (2 mM) for 3 days. Plants were grown for 10 days and root and the second leaf from each plant were harvested at 0, 1, 2, 3, 5, 7 and 10 days.

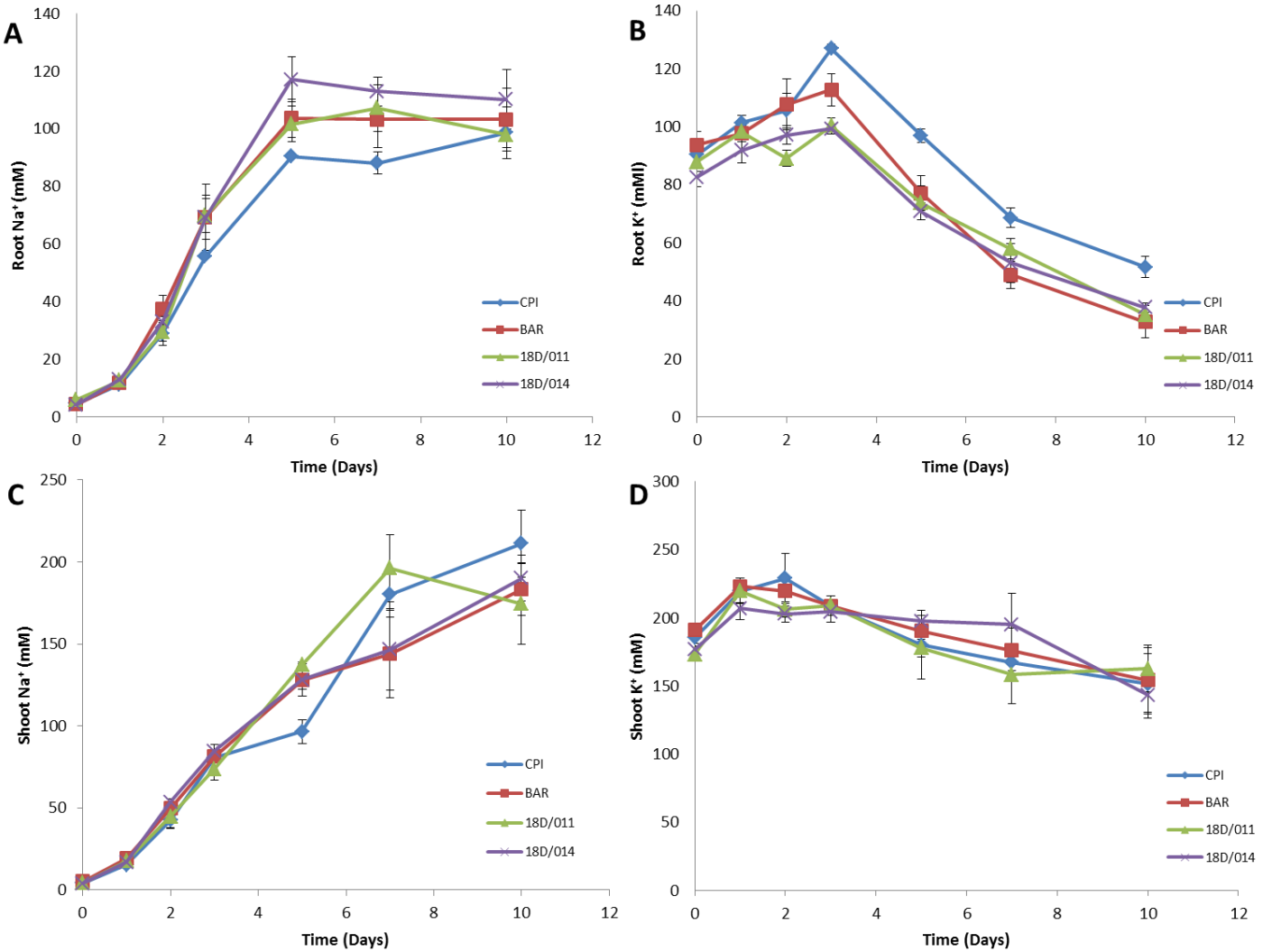
A
Root Na⁺ (mM)
Time (Days)
B
Root K⁺ (mMl)
Time (Days)
C
Shoot Na⁺ (mM)
Time (Days)
D
Shoot K⁺ (mM)
Time (Days)
CPI
BAR
18D/011
18D/014

Figure 2-4 (A) Root Na^+, (B) root K^+, (C) shoot Na^+ and (D) shoot K^+ of barley genotypes CPI-71284-48, Barque-73, and AB-QTL lines, 18D/011 (Barque-73 allele) and 18D/014 (CPI-71284-48 allele) grown in supported hydroponics under 150 mM NaCl. NaCl was applied to growth solution at third leaf emergence twice a day in 25 mM increments with additional calcium (2mM) for 3 days. Plants were grown for 10 days and root and the second leaf from each plant were harvested at 0, 1, 2, 3, 5, 7 and 10 days. Values are the means ± SE.

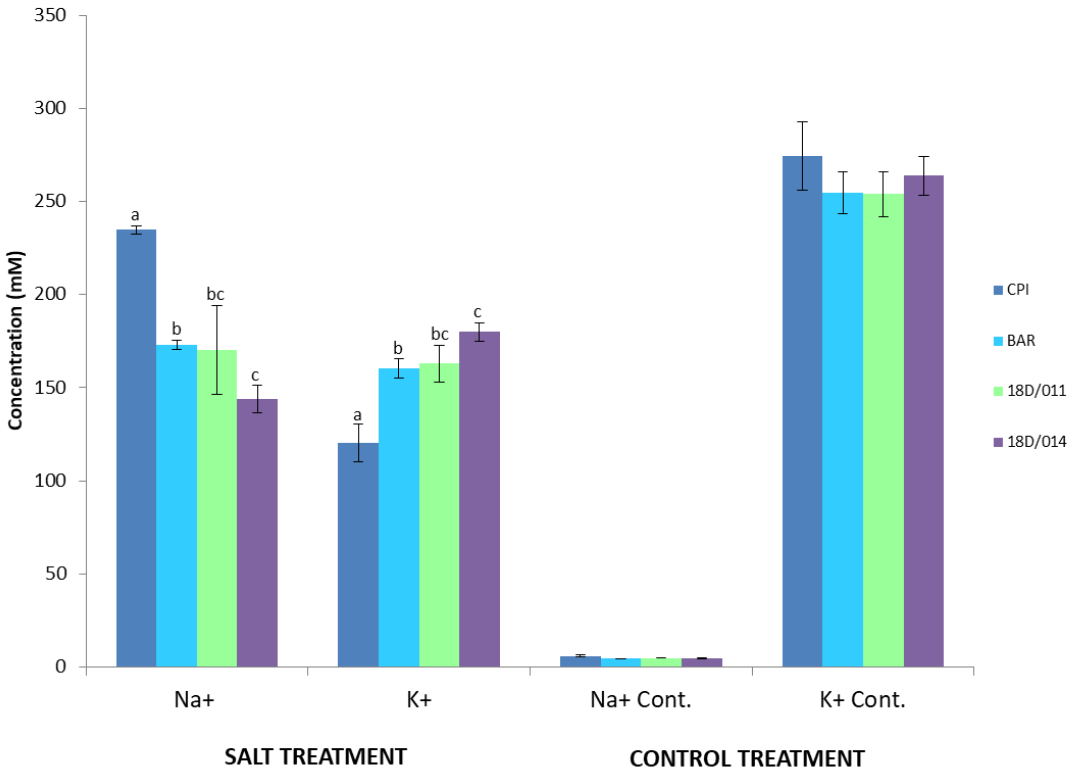

Figure 2-5 Sodium (Na^+) and potassium (K^+) concentrations in the fourth leaf of barley genotypes CPI-71284-48, Barque-73, and AB-QTL lines, 18D/011 (Barque-73 allele) and 18D/014 (CPI-71284-48 allele) after growth in supported hydroponics and exposure to 150mM (Salt treatment) or 0mM NaCl (Control treatment0; Na^+ Cont.; K^+ Cont.). Plants were subjected to NaCl for 10 days. Values are the means ± SEM.

2.3.1.4 Variation in sodium accumulation in the 2nd, 3rd and 4th leaf in CPI-71284-48

Due to the inconsistency between the *HvNax3* phenotype observed in the CPI-71284-48 × Barque-73 DH population (lines carrying the CPI-71284-48 *HvNax3* allele had reduced sodium accumulation in the third leaf at day 10 of salt stress at 150 mM NaCl) (Shavrukov *et al.*, 2010) and the sodium accumulation observed in CPI-71284-48 in this experiment (increased sodium accumulation in the second (

Figure 2-4) and fourth leaf (Figure 2-5) at 10 days salt stress at 150mM NaCl, a second experiment was conducted comparing the growth rate (Figure 2-6) as well as the sodium and potassium concentrations in the second, third and fourth leaves of Yuri Shavrukov's material CPI-71284-48 (Y-CPI), Barque-73 (Y-BARQUE-73) and the AB-QTL lines 18D/014 and 18D/011 at day 10 of 150mM NaCl stress (Figure 2-7). An additional independent seed source for CPI-71284-48 (S-CPI) and Barque-73 (S-BARQUE-73) was obtained from Stewart

Coventry of the Barley Breeding Programme at the University of Adelaide's Waite Campus, to determine if seed quality or genetic variation was a contributing factor. Plants were all growth staged (according to leaf number) throughout the course of the experiment and fresh weights were measured at the completion of the experiment, prior to harvesting the leaves for flame analysis. Barque-73 and the AB-QTL lines 18D/014 and 18D/011 all had similar development rate under salt stress (Zadok's Growth Stage 14 at day 10), whereas CPI-71284-48 was slower to produce more leaves (Zadok's Growth Stage 13.5 at day 10) and had a lower fresh weight at day 10.

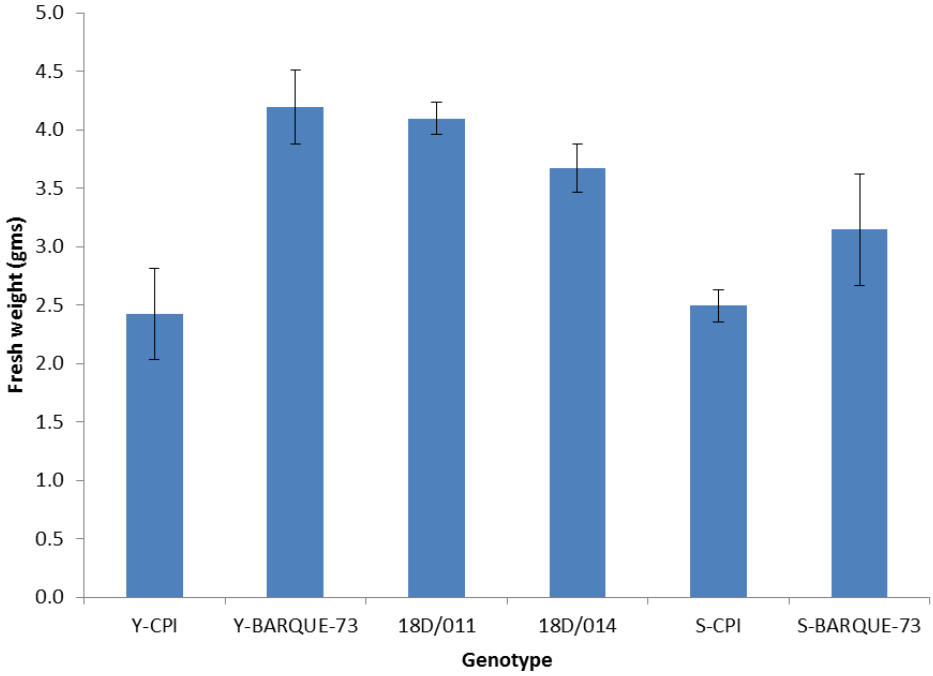

Figure 2-6 Fresh weight (g) of genotypes CPI-71284-48 (Y-CPI and S-CPI), Barque-73 (Y-BARQUE-73 and S-BARQUE-73), and AB-QTL lines, 18D/011 (Barque-73 allele) and 18D/014 (CPI-71284-48 allele) grown in supported hydroponics under 150 mM NaCl. Y refers to seeds obtained by Yuri Shavrukov (ACPFG) and S refers to seed obtained from Stewart Coventry (University of Adelaide). NaCl was applied to growth solution at third leaf emergence twice a day in 25 mM increments with additional calcium (2 mM) for 3 days. Plants were grown for 10 days after the initial application of salt. Values are the means ± SE of 10 replicates.

The accumulation of sodium in the second, third and fourth leaves was also variable in CPI-71284-48 in this experiment, where higher sodium concentrations were detected in the second leaf compared with the second leaf of Barque-73 and both the AB-QTL lines 18D/014 and

18D/011 (Figure 2-7). This result however, was not reflected in the third or fourth leaf, which displayed significantly lower sodium concentrations and higher potassium concentrations. There was also variation between the seed sources, where S-Barque-73 displayed similar sodium concentrations in the second and third leaves, but significantly higher sodium in the fourth leaf than CPI-71284-48 from both sources (Figure 2-7). Nonetheless, these results cumulatively show that CPI-71284-48 is displaying the *HvNax3* phenotype in this experiment.

2.3.1.5 The AB-QTL line 18D/014 maintains lower shoot Na^+: K^+

The AB-QTL line 18D/014 (CPI-71284-48 *HvNax3* allele) only displayed a slight reduction in shoot sodium in the second, third and fourth leaves, when compared with Barque-73 and 18D/011. However, this line maintained better sodium to potassium ratio in all of the leaves tested (Figure 2-8). Despite the inconsistencies with these results and those obtained from the original QRT-PCR experiment, the cDNA samples generated from this experiment were analysed for QRT-PCR of *HvHVP10* and *HvHVP1* mRNA expression.

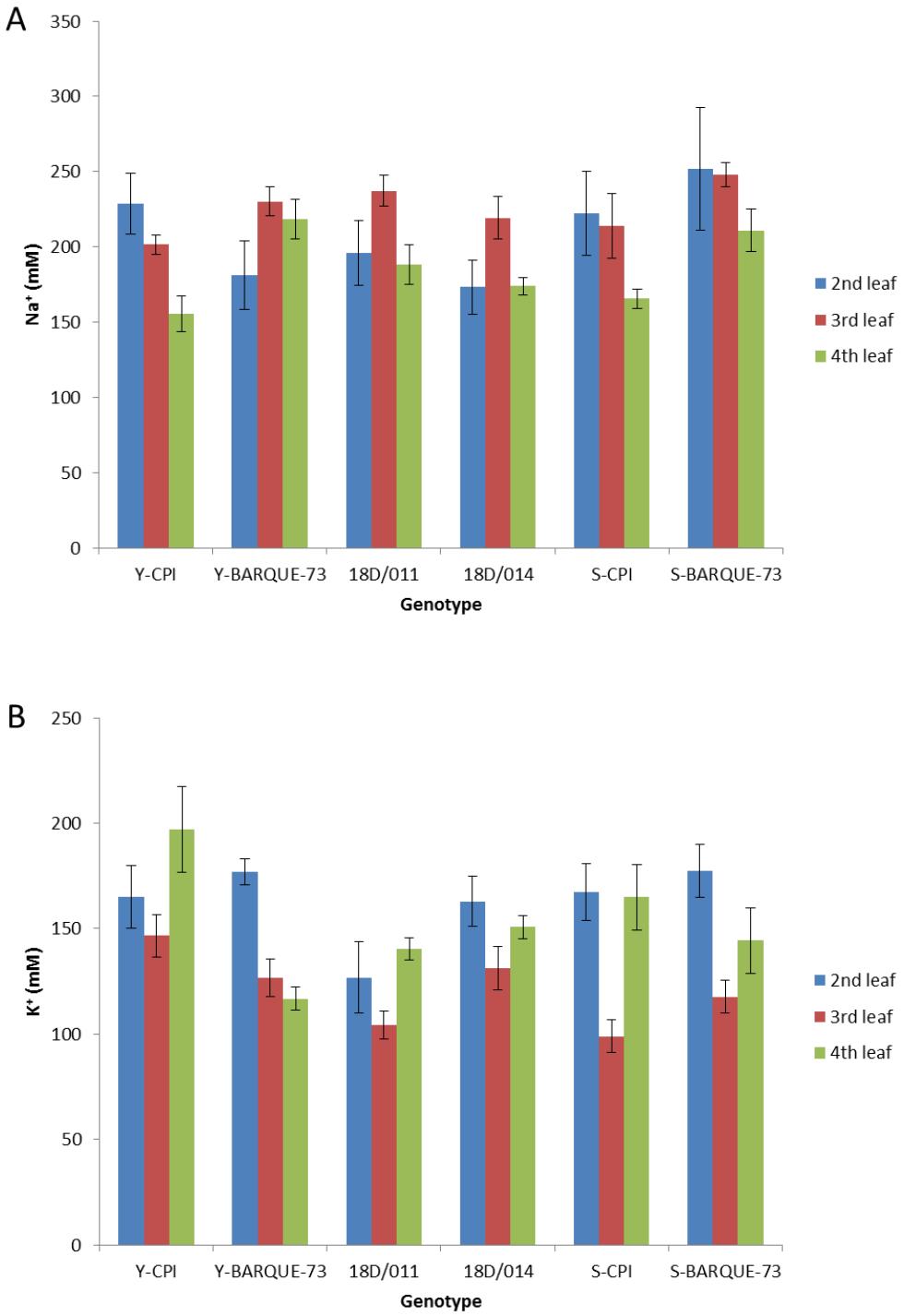

Figure 2-7 Sodium (Na^+) and potassium (K^+) concentrations of genotypes CPI-71284-48, Barque-73, and AB-QTL lines, 18D/011 (Barque-73 allele) and 18D/014 (CPI-71284-48 allele) grown in supported hydroponics under salt stress for ten days. Values are the means ± SE of 10 replicates.

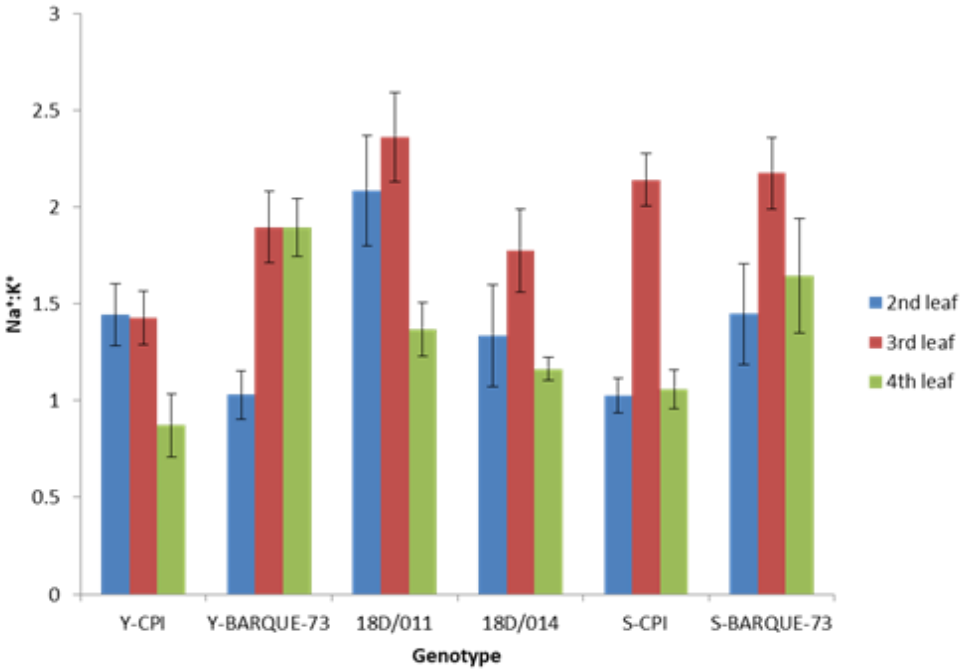

Figure 2-8 Na^+:K^+ of genotypes CPI-71284-48, Barque-73, and AB-QTL lines, 18D/011 (Barque-73 allele) and 18D/014 (CPI-71284-48 allele) grown in supported hydroponics under salt stress for ten days. Values are the means ± SE of 10 replicates.

2.3.2 Transcriptional profiling of barley V-PPases HvHVP1 and HvHVP10

2.3.2.1 HvHVP10 expression occurs mainly in the root of salt stressed barley

qRT-PCR of the barley V-PPases *HvHVP10* and *HvHVP1* was performed on cDNA isolated from salt stressed root and shoot material from the barley genotypes CPI-71284-48, Barque-73 and AB-QTL lines 18D/014 (CPI-71284-48 *HvNax3* allele) and 18D/011 (Barque-73 *HvNax3* allele). The mRNA expression profile of *HvHVP10* and *HvHVP1* in the roots and shoots over time in plants grown at 150 mM NaCl indicates that *HvHVP10* is mainly expressed in the roots and *HvHVP1* expression occurs mainly in the shoot of salt stressed barley (

).

When comparing the mRNA expression profile of *HvHVP10* in the roots of the AB-QTL lines in response to salinity stress, *HvHVP10* mRNA expression peaked in the root of 18D/014 to over 600,000 copies at day 1 of salt application, then the line 18D/011 seems to display a peak in *HvHVP10* expression at day 3 of salinity stress, indicating a delayed response

compared with 18D/014. However a lot of variability was observed in transcript abundance at this time point in both of the AB-QTL lines, considerably confounding the validity of this result.

HvHVP10 transcript levels in CPI-71284-48 peaked in the root at day 1 of salinity stress; to similar mRNA levels of that observed for the AB-QTL line 18D/014. This was followed by a second peak in *HvHVP10* expression in the roots of CPI-71284-48 at day 3 post salt application, to approximately 1,200,000 copies. This was 4 times greater than the levels observed for Barque-73. The mRNA expression profile of *HvHVP1* yielded a dissimilar pattern of transcript abundance, with expression mainly being detected in the shoot. The AB-QTL line 18D/014 displayed a high level of *HvHVP1* expression in the shoot at day zero, prior to initial salt application, followed by a second peak at day 7, which was 2 times that of the AB-QTL line 18D/011. The AB-QTL line 18D/011, carrying the Barque-73 *HvNax3* allele, displayed a very different pattern of *HvHVP1* expression, where higher levels of transcript abundance were detected in the shoot at day 3 of salt application, with a second peak being detected at day 7 of salt stress, similar to but much lower than AB-QTL line 18D/014.

Transcriptional profiling of *HvHVP1* in CPI-71284-48 and Barque-73 shoot mRNA indicated that CPI-71284-48 had peaked *HvHVP1* expression at day 0, 1 and 3, however this dropped to similar levels to those of Barque-73 by day 3 and further declined in both genotypes by day 5 and 7 of salt stress.

It also should be noted that the transcript levels of *HvHVP1* in the shoot were considerably lower compared with the levels of *HvHVP10* mRNA transcript in the root, however different control genes were used to normalise transcript abundance therefore we are unable to directly compare transcript copy number between the tissues.

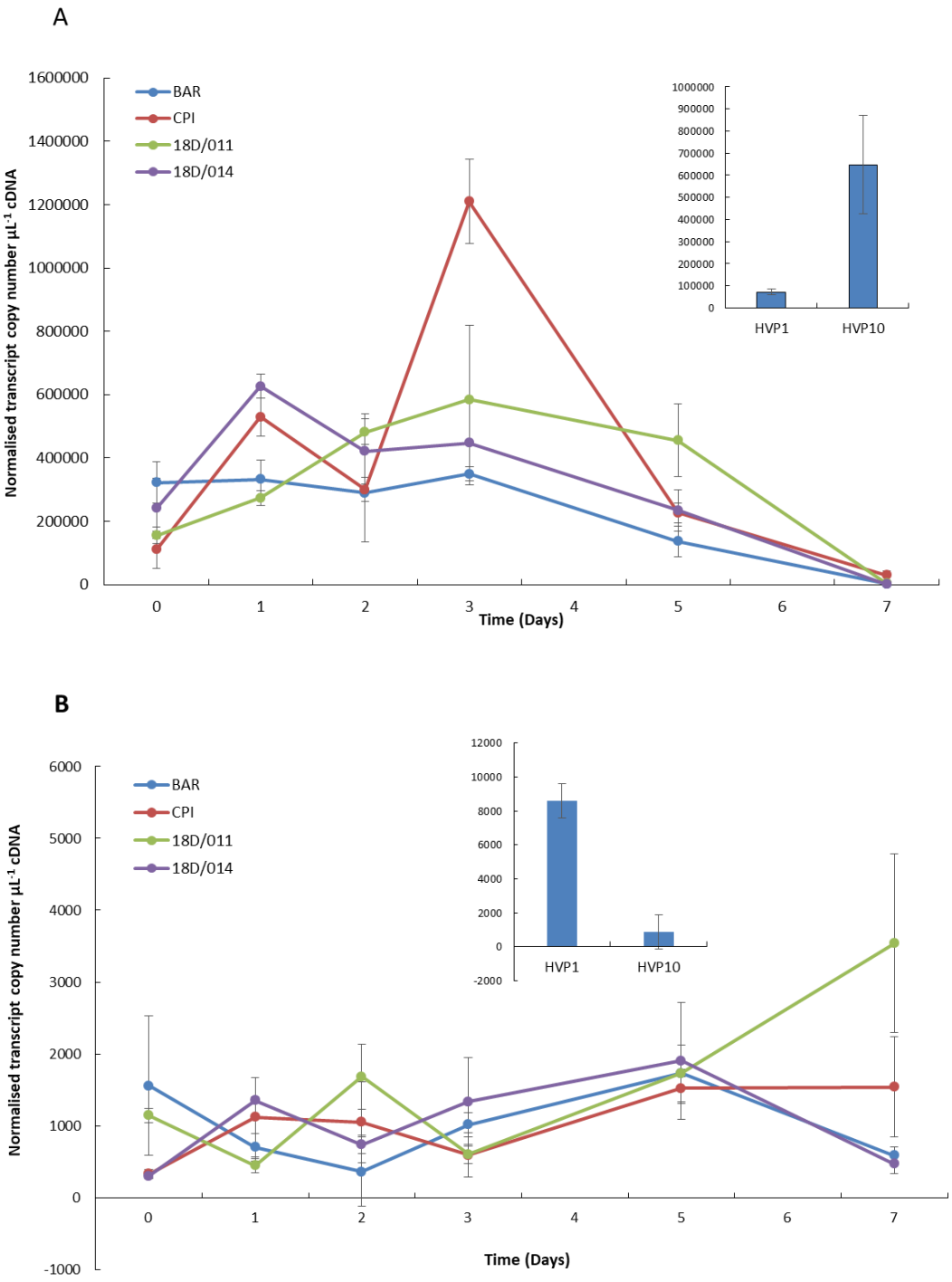

Figure 2-9 Expression profiles of *HvHVP10* in root (A) and shoot (B) of Barque-73 (BAR), CPI-71284-48 (CPI) and AB-QTL lines 18D/014 (CPI-71284-48 *HvNax3* allele) and 18D/011 (Barque-73 *HvNax3* allele). Insert shows average relative expression of *HvHVP10* to *HvHVP1* of all cultivars at day 3 of salt stress. Mean of 4 replicates ± SEM. cDNA was synthesized from plants grown in supported hydroponics under 150 mM NaCl. NaCl was applied to growth solution at third leaf emergence twice a day in 25 mM increments with additional calcium (2 mM) for 3 days. Plants were grown for 10 days and root and the third leaf from each plant were harvested at 0, 1, 2, 3, 5, 7 and 10 days.

2.4 Discussion

2.4.1 Examination of HvNax3 QTL in Planta in CPI-71284-48, Barque-73 and AB-QTL lines

2.4.1.1 CPI-71284-48 has a different growth habit

Barque-73 (*H. vulgare*), CPI-71284-48 (*H. spontaneum*) and AB-QTL (BC_2F_2) lines 18D/014 and 18D/011 were transferred to increasing salt stress from the third leaf emergence for a period of 21 days. CPI-71284-48 developed more slowly under both salt and control conditions and produced wider, larger leaves. This result has also been observed in pot-grown plants in the glasshouse (Figure 2-10) and has been reflected under field conditions (J. Eglinton, pers. comm.). The different growth form of progenitor accessions is well documented (Chapin *et al.*, 1989, Kernich *et al.*, 1995, Feuillet *et al.*, 2008). Kernich *et al.* (1995) examined the variation in developmental patterns of wild and cultivated barley and demonstrated that the durations of the leaf initiation and spikelet initiation phases were longer in *H. spontaneum* accessions when compared with Australian spring barley cultivars Bandulla and Schooner. Chapin (1989) has specifically shown that the rates of leaf elongation of both desert and coastal *H. spontaneum* accessions are significantly lower than in *H. vulgare* cultivars and this growth form is characteristic of its adaptation to harsh environments. The *H. vulgare* cultivars also had lower root to shoot ratios than progenitor accessions in the early stages of plant development, which is consistent with the observations reported here.

Figure 2-10 Image of wild barley accession CPI-71284-48 (*Hordeum vulgare* ssp. *spontaneum*) and Australian malting barley cv. Clipper (*Hordeum vulgare* L.) showing different growth habits. Image provided curtesy of Professor Jason Eglinton (University of Adelaide)

2.4.1.2 CPI-71284-48 accumulated more sodium in the shoot and less in the root

In order to gain a better understanding of the *HvNax3* QTL, the concentrations of sodium and potassium ions in root and shoot tissue were examined in the barley genotypes CPI-71284-48, Barque-73 and the AB-QTL lines 18D/014 (CPI-71284-48 allele) and 18D/011 (Barque-73 allele) over 10 days of salt stress at 150 mM NaCl. In this experiment CPI-71284-48 accumulated less sodium in the root and more in the shoot from day 3 of the imposed salinity stress, as indicated by total root and second leaf ion concentrations (

Figure 2-4). This result was unexpected and is an opposite phenotype to the *HvNax3* QTL identified in the Barque-73 × CPI-71284-48 DH population, determined from third leaf sodium concentrations. The second leaf was chosen for the time course series, as the newest emerged leaf (third leaf) was sampled for QRT-PCR of *HvHVP10* and *HvHVP1*, and this may account for the discrepancy in these results. The fourth leaf also had higher sodium concentrations in this experiment (Figure 2-5); however it was noted at the time of sampling that the fourth leaf in CPI-71284-48 was not fully expanded, which may significantly confound these results. A repeat experiment examining the relative concentrations of sodium in the second, third and fourth leaf of each genotype revealed that there was also variation between second leaf, and third and fourth leaf sodium accumulation in CPI-71284-48, where higher sodium concentrations were detected in the second leaf compared with the second leaf of the other barley genotypes (Figure 2-7). This result however, was not reflected in the third or fourth leaf, which displayed significantly lower sodium concentrations and higher potassium concentrations, consistent with the *HvNax3* phenotype. This observation could indicate that CPI-71284-48 restricts sodium accumulation in the younger leaves by keeping it in older leaves. It also indicates that the examination of second leaf sodium concentration is not reflective of what is occurring in the other leaves, which may explain, in part, the variation between the results observed here and the lower sodium concentration in shoots of lines carrying the CPI-71284-48 *HvNax3* allele. It may also indicate that plants load Na^+ into the leaf sheath and then the blade during leaf development. These hypothesises could be tested in future experiments.

This experiment tested the hypothesis that the *HvNax3* phenotype of CPI-71284-48 was due to the retention of more sodium ions in the root via increased vacuolar sequestration of sodium, being driven by increased root *HvHVP10* expression. This would then restrict the accumulation of sodium in the shoot, which would otherwise disrupt photosynthesis and other important metabolic processes. Therefore the finding here that sodium and potassium concentration in the root tissue of CPI-71284-48 were reduced from day 3 of salt stress and increased in the shoot was unexpected the contrasts with previous reports (Shavrukov *et al.*, 2010). One of the most likely reasons for the unexpected root leaf sodium phenotype in the CPI-71284-48 parent may be attributed the different rate of development between this species and the other genotypes used in the experiment, or indeed multiple haplotypes within CPI-71284-48. It is highly probable that many genes within the *H. spontaneum* genome contribute to its increased salinity and drought tolerance. These could include other genes that restrict

the entry of sodium into the root; possibly via non-selective cation channels. Indeed, the second peak located on the long arm of 7H in the original QTL mapping study of the CPI-71284-48 and Barque-73 DH population (Shavrukov *et al.*, 2010) has since been identified as the pyrophosphatase homologue *HvHVP1*(Shavrukov, 2014).

2.4.1.3 The AB-QTL line 18D/014 displays the HvNax3 phenotype

The AB-QTL 18D/014 carrying the *HvNax3* allele from CPI-71284-48 retained greater sodium in the roots from day 3 of salinity stress (

Figure 2-4) and had less sodium and more potassium in the 2nd leaf. This line also had less sodium and more potassium in the 4th leaf at day 10 days (Figure 2-5), when compared with the AB-QTL line 18D/011. In the repeat experiment 18D/014 maintained a lower sodium to potassium ratio in the second, third and fourth leaf (Figure 2-8), compared with Barque-73 and 18D/011. This pattern of sodium accumulation was observed over multiple experiments and is consistent with the *HvNax3* phenotype, where this line retains more sodium in the roots and accumulates less sodium in the shoots under salt stress, possibly via increased root vacuolar sequestration of sodium.

2.4.2 Transcriptional profiling of barley V-PPases HvHVP1 and HvHVP10

2.4.2.1 HvHVP10 is mainly expressed in the root of salt stressed barley

Expression analysis of *HvHVP10* and *HvHVP1* in the root and shoot of barley genotypes Barque-73, wild barley CPI-71284-48, and the AB-QTL lines 18D/014 and 18D/011 during exposeure to salt indicate that *HvHVP10* expression occurs mainly within the root and *HvHVP1* expression occurs mainly within shoot tissue (

). This result is consistent with other reports of barleyV-PPase expression. Fukuda (2004) showed that the expression patterns of *HvHVP1* and *HvHVP10* differed significantly, where *HvHVP1* was mainly expressed in the shoot and *HvHVP10* had higher transcript abundance in the roots of the barley cv. Kashima (Fukuda *et al.*, 2004). Furthermore, Fukuda *et al.* (2004) demonstrated that salt and osmotic stress stimulated transcript levels of *HvHVP1* and *HvHVP10* in the roots but not in the shoots of barley. Differential tissue-specific expression of V-PPase isoforms has also been examined in tobacco, where the V-PPase cDNA clone *TVP31* transcripts were more abundant in roots than in other tissues (Lerchl *et al.*, 1995). Here it was further reported that the accumulation of the specific transcripts were also differentially regulated during leaf development, where it was higher in young actively

dividing tissue (sink leaves) and significantly lower in older (source) leaves. This result has also been reported in mung bean, where RNA analysis indicated the transcript abundance was highest in the elongating region of the hypocotyl but extremely low in the mature region. The decline in transcript level was specifically correlated with decreased pyrophosphatase activity and enzyme protein content (Nakanishi and Maeshima, 1998).

Very low levels of *HvHVP10* expression were observed in the third leaf of all four barley genotypes, and expression was not responsive to salt stress (

). These results are somewhat contradictory to Shavrukov *et al.* (2013), who reported high mRNA levels of *HvHVP10* in the shoot tissue of CPI-71284-48 at day 3 of salinity stress; although at this time point *HvHVP10* expression in the roots was still 10-fold higher than the levels observed in the shoots. This discrepancy may be attributed to variation between experimental procedures used in the two experiments, particularly sampling time. It has been demonstrated that V-PPase isoforms from tobacco display diurnal expression within the leaves, with minimum transcript accumulation occurring at high noon and a 10-fold increase in V-PPase transcript abundance at midnight (Lerchl *et al.*, 1995). During the current investigation, plant tissue sampling occurred at the same time during the day at each time point (2:00pm), to eliminate variation caused by the potential diurnal nature of *HvHVP10* gene expression. It is not reported whether the sampling time was consistent across the duration of the experiment in the study conducted by Shavrukov *et al.* (2013), which account for this discrepancy in results.

2.4.2.2 HvHVP10 expression peaks at day 3 of salt stress in roots of CPI-71284-48

qRT-PCR of root and shoot tissue from Barque-73 and CPI-71284-48 and AB-QTL lines indicated that CPI-71284-48 and 18D/014 (CPI-71284-48 *HvNax3* allele) had increased *HvHVP10* expression in the root at one day of salt stress (

). Fukuda *et al.* (2004) has also resported similar increases in *HvHVP10* expression in barley after 24 h of treatment with NaCl. In CPI-71284-48 this was followed by a second peak in *HvHVP10* expression at day three of salinity stress; at this time point, *HvHVP10* expression in CPI-71284-48 roots was 3-fold higher than in Barque-73. Roots of the AB-QTL lines also tended to show a peak in *HvHVP10* expression at day three of salt stress, although there were no significant differences between 18D/014 and 18D/011 at this time point.

A peak in *HvHVP10* expression in the roots of CPI-71284-48 at day three of salt stress at 150mM NaCl was also reported by Shavrukov *et al.* (2013). A similar increase in *HvHVP10* expression was reported for Barque-73, although at lower levels. Although there are some discrepancies between the current study and those obtained by Shavrukov *et al.* (2013), they collectively suggest that *HvHVP10* expression peaks in the root at day 3 of salinity stress. Therefore it is possible that increases in root *HvHVP10* expression, though unconfirmed in this study due to large variability of the data at day three, may account for the lower shoot sodium concentration in lines carrying the CPI-71284-48 *HvNax3* allele. Another possibility is that plants carrying the CPI-71284-48 *HvNax3* allele have early seedling vigour, resulting in larger plants which have a dilution effect on the Na^+ ion concentrations in the leaf tissue. Despite the mechanism by which *HvHVP10* expression results in lower sodium concentrations in the shoot, *HvHVP10* remains a candidate controlling the salinity tolerance QTL on chromosome 7H in the Barque-73/CPI-71284-48 DH population.

2.4.2.3 HvHVP1 expression mainly occurs in the shoots

HvHVP1 in the shoot tissue of salt-stressed barley displayed a very different pattern of transcript abundance compared with the transcription profile of *HvHVP10*. *HvHVP1* expression was much higher in the shoots compared to *HvHVP10* expression and peaked in the shoots of 18D/011 at day 7 of salinity stress. This result however, was not reflected in the other genotypes, where HvHVP1 transcript copy number tended to peak at day 5 then decreased in the shoot tissue by day 7. Our results are consistent with Shavrukov *et al.* (2013), who also reported a peak in *HvHVP1* in the roots of Barque-73 at day 5. They also reported a peak in *HvHVP1* expression in the shoot of CPI-71284-48 and Barque-73 on day 5 of salt stress, indicating that *HvHVP1* had delayed response to salt compared to *HvHVP10* expression. It was proposed *HvHVP10* and *HvHVP1* had a complementary expression profile in response to salinity stress. This inconsistency in our results may be due to differential expression during leaf development. In our experiments, the youngest growing tissue was collected for cDNA synthesis, which may have influenced our results. RNA analysis of V-PPase isoforms in tobacco has also shown that the accumulation of specific transcripts is differentially regulated during leaf development, where generally V-PPase is highest in young actively growing tissue (Lerchl *et al.*, 1995). This may explain the comparatively high *HvHVP1* activity observed in the CPI-71284-48 on days 0-2 of salt stress.

Interestingly, Fukuda *et al.* (2004) reported that *HvHVP1* transcript abundance increased 4 fold and 2 fold after 5 hrs and 24 hrs, respectively in roots, treated with 200 mM NaCl. This

was significantly greater than observations in the current study. This inconsistency in results could be attributed to differences in salt application. In the Fukuda *et al.* (2004) study, NaCl application was performed in a single step, possibly causing osmotic shock to the plants, which may cause a different response to salt when compared with our experiments, where salt and additional calcium were applied to the growth solution incrementally.

HvHVP10 provides a strong candidate gene for the salinity tolerance observed in the *Hordeum spontaneum* accession CPI-71284-48. We will investigate this hypothesis further by conducting physiological experiments to examine its activity *in planta* and then use a biotech approach to determine if by modifying the expression levels of *HvHVP10* we can affect the plant's salt tolerance phenotype.

2.5 Summary

In this chapter it is proposed that the vacuolar pyrophosphatase gene *HvHVP10* in barley is responsible for the sodium exclusion *HvNax3* QTL identified in the Barque-73 x CPI-71284-48 DH population. The AB-QTL line carrying the *HvNax3* allele from CPI-71284-48, 18D/014, had higher sodium concentrations in the roots and lower concentrations in the shoots compared with the AB-QTL line 18D/011 (Barque-73 *HvNax3* allele) over the period of imposed salinity stress. Expression analysis of *HvHVP10* and *HvHVP1* in the roots and shoots over the 10 day period indicated that *HvHVP10* was mainly expressed in the root and *HvHVP1* was mainly expressed in the shoots of salt stressed barley. *HvHVP10* transcript abundance peaked in the root at day 3 of salinity stress in CPI-71284-48, but no differences were detected between the AB-QTL lines with and without the *HvNax3* allele. Large variation in expression at this time point could have prevented significant differences being detected if they were present. Based on these results, day three of salinity stress has been selected for analysis of pyrophosphatase activity in planta in the AB-QTL lines.

3 Analysis of V-PPase activity *in planta* in AB-QTL lines in response to salinity stress

3.1 Introduction

Many studies have demonstrated that the OEX of V-PPase results in enhanced growth and Na^+ sequestration in transgenic plants, however early evidence from the literature suggests that the protein itself is inhibited by increasing Na^+ concentration. The inhibitory effect of sodium ions on the PPi-dependent proton pumping activity of plant cells has been demonstrated in many experiments (Wang *et al.*, 1986, Rea and Sanders, 1987, Nakamura *et al.*, 1992). Nakamura *et al.* (1992) showed that H^+-PPase of the tonoplast from mung bean roots was severely inhibited with increasing Na^+ concentration; however vesicles from the plasma-lemma showed a rapid increase in ATPase activity in response to salt stress. It was suggested that high NaCl stress increased the intracellular concentration of Na^+ ions of root vacuoles, which inhibited the tonoplast H^+-PPase and stimulated the activity of the plasma-lemma H^+-ATPase. Kabala and Klobus (2001) demonstrated that the addition of NaCl to the assay medium caused a great decrease in PP-ase activity in tonoplast vesicles from cucumber (*Cucumis sativus* L.) roots. This result was shown to be specifically caused by the addition of sodium and not chloride ions to the reaction medium, where KCl increased PP-ase activity five-fold, however using NaCl instead of KCl caused a significant decrease in PP-ase activity. In that study vacuolar ATPase activity was elevated by 79% in the presence of NaCl, again suggesting that V-ATPase activity is stimulated by exposure to NaCl. In a further study Kabala and Klobus (2008) examined the time dependent effect of 50mM NaCl on the activities of both ATPase and V-PPase in cucumber and found that PP-ase activity was inhibited at all times tested over an eight day period, whereas V-ATPase increased after 24 hours of exposure then decreased at 4 and 8 days. It was demonstrated that changes in enzyme activity were not due to the salt action on the expression of encoding genes, as transcript levels of the genes were similar from both salt stressed and untreated cucumber roots. The authors suggested that alterations of proton pump activities under salinity are due to post-translational alterations induced by NaCl.

Others have reported increased V-PPase activity under salt stress which has led to the hypothesis that under normal environmental conditions the vacuolar ATPase is mainly responsible for energising the tonoplast, however under stress conditions when the level of

ATPase in the cell drops, PPase becomes the dominant pump in establishing an electrochemical potential gradient across the tonoplast (Carystinos *et al.*, 1995, Darley *et al.*, 1995, Nakanishi and Maeshima, 1998). Although the regulation of both V-PPase and ATPase activities by salt is well reported in the literature, there is yet no clear pattern of activation and deactivation of both pumps in response to salinity stress and the actual role of V-PPase in response to NaCl remain unclear.

In the previous chapter it was proposed that the sodium exclusion *HvNax3* QTL is due to the compartmentalisation of sodium ion within the vacuoles of the root tissue due to the increased expression of *HvHVP10* in lines carrying the CPI-71284-48 *HvNax3* allele. QRT-PCR results (Chapter 2) indicated that V-PPase expression peaks in the roots of barley at day three of salinity stress and thus this was the selected target for the analysis of enzyme kinetics of vacuolar pyrophosphatase within the roots of the AB-QTL lines, to determine if differences in the pyrophosphatase activity of root tonoplast enriched vesicles (TEV) exists in barley plants with and without the CPI-71284-48 *HvNax3* allele. The AB-QTL lines were selected for analysis as they only differ for the region of the *HvNax3* QTL, thus eliminating the confounding effects caused by growth rate variation or the presence of other genes within the *H. Spontenaum* genome which may influence vacuolar pyrophosphatase activity.

The specific hypothesis for this chapter is that roots from plants carrying the CPI-71284-48 *HvNax3* allele (18D/014) will have greater V-PPase activity than plants with the Barque-73 *HvNax3* allele (18D/011) in response to salinity stress.

3.2 Methods

3.2.1 Plant Material

The barley cultivar Finniss was used to develop the protocols for growth conditions, isolation of tonoplast enriched vesicles from roots and an enzymatic assay to investigate the hydrolytic activities of barley vacuolar H^+-Pyrophosphatase (V-PPase). The AB-QTL barley lines 18D/014 (CPI-71284-48 *HvNax*3 allele) and 18D/011 (Barque-73 *HvNax3* allele) were then screened for V-PPase activity under salt stress (150 mM NaCl) and control conditions (0 mM). Day 3 of salt stress at 150 mM NaCl was selected as the appropriate target for examination of pyrophosphatase activity based on QRT-PCR results.

3.2.2 Barley growth conditions

All experiments were carried out in PC2 laboratories at CSIRO, Black Mountain, ACT, Australia. Approximately 200 seeds of each genotype were germinated in petri dishes on moist filter paper and kept in the dark for 2 to 3 days. Germinated seedlings were then transferred to two areated 5 L lunch box hydroponic systems (Figure 3-1) containing half strength modified Hoagland's Nutrient Solution (6.5 mM KNO_3, 4.0 mM $Ca(NO_3)_2$ $4H_2O$, 100 μM $NH_4H_2PO_4$, 2.0 mM $MgSO_4$ $7H_2O$, 4.6 μM H_3BO_3, 0.5 μM $MnCl_2$ $4H_2O$, 0.2 μM $ZnSO_4$ $7H_2O$, 0.1 μM $(NH_4)_6Mo_7O_{24}$ 4H2O, 0.2 μM $CuSO_4$ $5H_2O$, 45 μM $FeCl_3$). Plants were grown at 25°C with natural daylength for 6 days and the growth solution was changed daily to maintain health of the roots and prevent the build up of organic matter in the growth solution. Salt and additional calcium chloride (2 mM) was added to one of the tanks in 25 mM increments twice a day from day 6. On day eight (day three of salt stress), root tissue from salt stressed and control plants was excised with a scalpel and immediately placed in ice cold growth solution for isolation of tonoplast enriched vesicles.

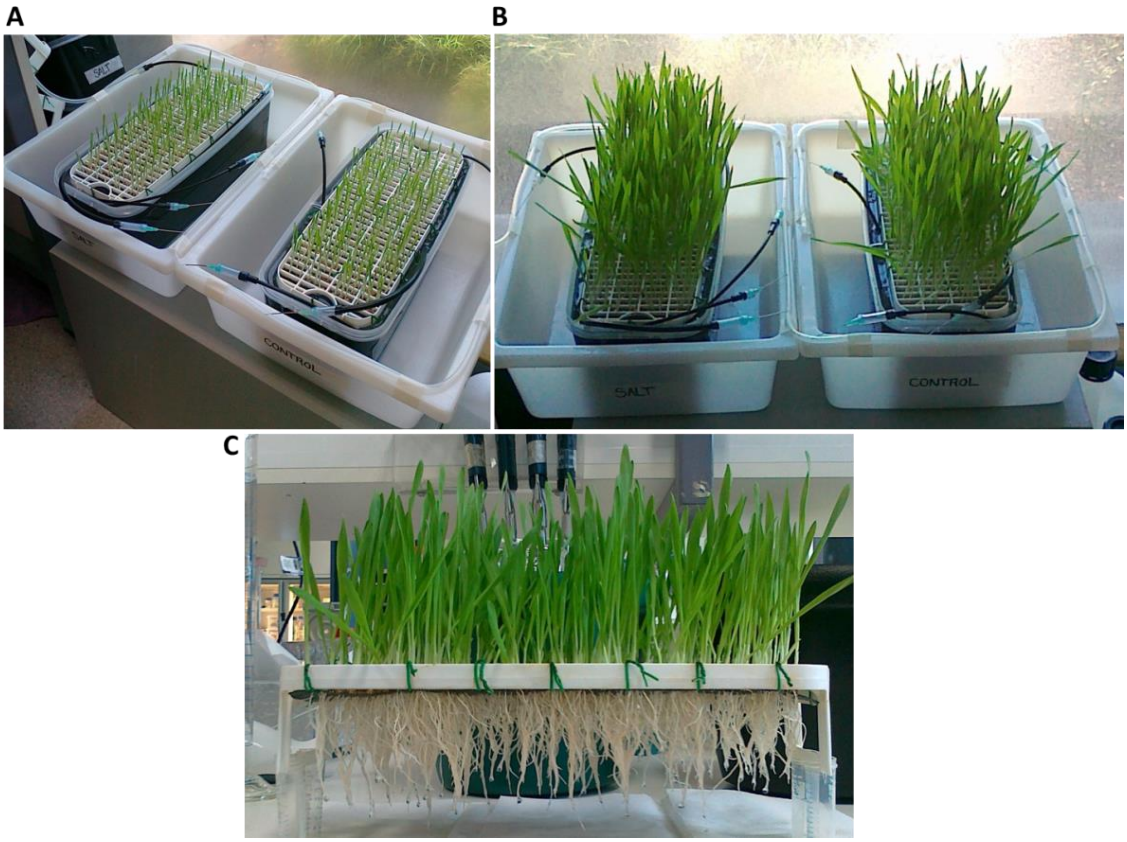

Figure 3-1 Images of 5L hydroponics system used to grow roots for tonoplast enriched vesicle isolation. (A) 3 days post-transplanting; (B) prior to salt application at 6 days post-transplanting; and (C) root growth prior to salt application at 6 days post-transplanting. All images are of cultivar Finniss.

3.2.3 Isolation of Tonoplast Enriched Vesicles (TEV)

Isolation of tonoplast enriched vesicles from barley roots was performed according to the protocol of Maeshima and Yoshida (1989), with some modifications. All procedures were carried out at 4°C and plant tissues maintained on ice. Fresh excised root tissue was removed from the growth solution and blotted dry on paper towel. The root tissue was immediately added to ice cold homogenising medium (1:1 volume), consisting of 0.25 M sorbitol, 50 mM Tris/acetate (pH 7.5), 1 mM EGTA, 1% (w/v) PVP (Polyvinylpyrrolidone), 2 mM DTT (Dithiothreitol), and 20 µM APMSF (p-amidinophenyl-methanesulfonyl-fluoride hydrochloride), which was added to the homogenising medium directly before the experiment. Root tissue was homogenised using a Kambrook Essentails KSB7 hand blender with attached chopper bowl. The homogenate was filtered through 2 layes of teflon cloth and then centrifuged twice at 7000 rpm for 10 minutes in pre-chilled 50 mL Pyrex centrifuge

tubes in a Beckman Aventi® J25 Centrifuge. The supernatant was transferred to Beckman Coulter Thinwall, Ultra-Clear™ centrifuge tubes (13.5 mL), then centrifuged at 34,317 *g* in a Beckman SW 41Ti ultracentrifuge for 30 minutes. The supernatant was removed and the precipitate (crude membrane fraction) was gently resuspended in 1 mL of pre-chilled 0.5 M Sucrose, 20 mM Tris/acetate, 1 mM EGTA, 2 mM EDTA and 2 mM DTT. Crude membrane fraction suspensions were then pooled into a single ultacentrifuge tube, adjusting the total volume to 6.5 mL. The crude membrane fraction was gently overlaid with 6.7 mL of pre-chilled 0.25 M sorbital, 20 mM Tris/acetate, 1 mM EGTA, 2 mM EDTA and 2 mM DTT, then centrifuged for 50 minutes at 34,317 *g*. The interface between the two solutions (tonoplast enriched vesicles) was extracted from the ultra clear centrifuge tube using a 0.5 mL syringe, resuspended in 13 mL 0.25 M sorbital/tris solution and centrifuged for 30 minutes at 34,317 *g*. The supernatant was removed gently with a pipette and discarded. The resulting white pellet was resuspended in 120 µl of 0.25 M sorbitol/tris solution then split between two Eppendorf microcentrifuge tubes, for either the enzyme assay (100 µL) or protein determination (20 µL). The samples were stored at -80°C. The amount of protein from each sample was quantified using the Bradford method of protein determination and BSA as a standard.

3.2.4 Malachite green assay for measuring V-PPase hydrolytic activity

The malachite green colorimetric assay was used to measure the liberation of free inorganic phosphate (Pi) from pyrophosphate (PPi) of isolated tonoplast enriched vesicles, according to the method of van Veldhoven and Mannaerts (1987). This method is based on the ionic association of malachite green with phosphomolybdate under acidic conditions and has been shown to accurately measure phosphorus in the micromolar range.

Enzyme kinetics were examined using the substrate sodium pyrophosphate in a series of dilutions ranging from 0.05 µM to 1200 µM. Magnesium chloride is a known co-factor of pyrophosphatases (Maeshima and Yoshida, 1989). Therefore assays were also performed with 0, 0.5, 1 2 and 3 mM $MgSO_4$. In addition, 30 µM aminomethylene-bisphosphonate (AMBP), a know competitive inhibitor of both soluble and membrane bound inorganic pyrophosphatases (Gordon-Weeks *et al.*, 1999) was also tested with 1.0 mM $MgSO_4$.

All enzyme assay procedures were carried out in 96-well flat bottomed microtitre plates using calibrated multi-channel pipettes. Samples were removed from the -80°C freezer and briefly

thawed on ice. five µl of sample (containing approximately 0.5 µg of TEV) was resuspended in 150 µL of ice cold sterile H_2O in a microtitre plate on ice. Reactions were started by the addition of 150 µL of sample to 150 µL of 2 X reaction buffer. The standard reaction mixture contained 0.3 mM sodium pyrophosphate, 50 mM KCl, 1 mM sodium molybdate, 0.03% Triton X-100, 30 mM Tris/MES pH 7.3 and 1 mM $MgSO_4$ pre warmed to 30°C on a heating block. The last columm of the microtitre plate was left blank for the generation of a phosphorus standard curve. Immediately 90 µL of the assay medium was taken and added to an equal volume of ice cold 10% Trichloroacetic acid (TCA) and gently mixed via pipetting to terminate the enzymatic reaction (T0 = blank) and left on ice. This procedure was carried out again after 30 minutes of incubation at 30°C (T30), then again after 60 minutes (T60). TCA-treated samples were centrifuged in an Eppendorf centrifuge at 6,000 *g* for 10 minutes. To each plate a standard curve containing a series of dilutions of a Pi standard (10 ug/mL) in standard reaction buffer to 90 µL and 90 µL of 10% TCA was added to the final column of wells. Then 40 uL of Reagent 1, containg 14.2 mM ammonium molybdate tetrahydrate in 3.1 M sulfuric acid, was added to each well and plates were incubate at room tempertaure (RT) for 10 minutes. 40 µL of Reagent 2, containing 3.5 g/L polyvinyl alcohol in 0.35 g/L Malachite green carbinol HCl was added to each well and plates were incubated at RT for 20 minutes. Absorbance was measured at 640 nm with a spectrophotometer (plate reader) and liberated P was determined by the difference in Pi between T30 or T60 and T0 (Pi release). Specific activity was expressed as nmol Pi/min/µg of protein.

3.3 Results

3.3.1 Analysis of V-PPase activity in planta in AB-QTL lines in response to salinity stress

The malachite green colourimetric assay was first conducted on isolated root tonoplast enriched vesicles (TEV) from salt stressed and control grown barley (cv. Finniss), using a standard reaction assay medium according to that of Stewart (2005). The experiment consisted of four biological replicates under both salt (100mM) and controlled conditions. The colourimetric assay followed the kinetic properties of enzymes, where there was a linear relationship between change in absorbance and time for up to 60 min (Figure 3-2).

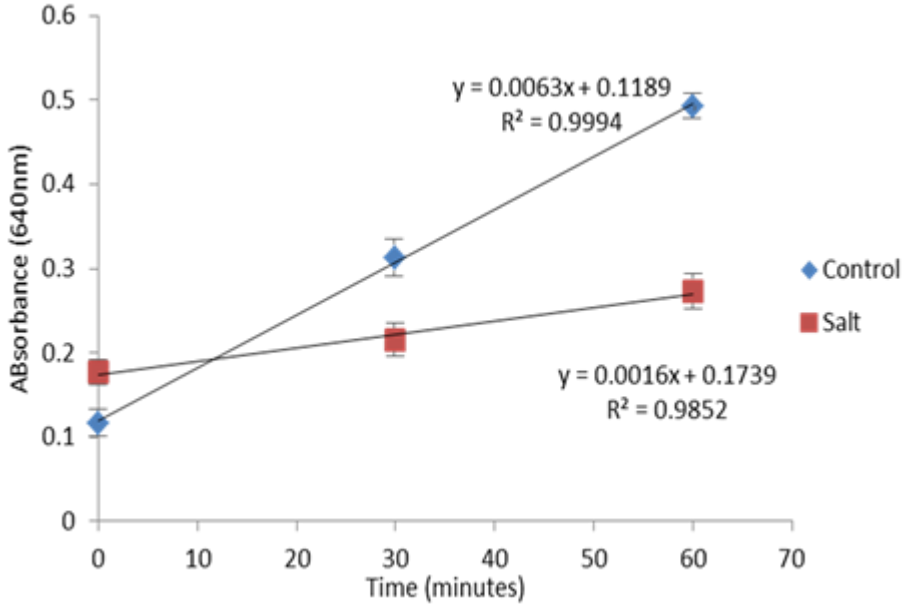

Figure 3-2 Malachite green assay on vacuolar preps isolated from Finniss roots, showing a linear relationship between absorbance and time. Mean of four biological replicates.

3.3.2 V-PPase activity follows classic Michaelis-Menten Kinetics

TEVs isolated from the AB-QTL lines 18D/014 and 18D/011 grown under salt and control conditions were examined for vacuolar pyrophosphatase hydrolytic activity. A total of 3 biological replicates for each genotype were prepared for analysis. However, due to particularly low TEV yields one of the biological replicates was eliminated from the analysis. Therefore only two biological replicates × 2 technical replicates were used in the enzyme assay. Additionally, due to low yields a longer incubation time was employed, thus the time points taken were T0 (0 minutes), T30 (30 mins) and T210 (210 mins). The later time point

was selected for enzyme analysis and was shown to be linearly associated with change in absorbance (data not shown).

The PPi substrate dependency of barley V-PPase displayed classic Michaelis-Menten kinetics, where the initial rate of reaction (velocity) was linearly dependent on substrate concentration, and then the rate reached maximal activity (Vmax) and did not change as the substrate concentration increased (Figure 3-3). Results also indicate that the line 18D-014 carrying the *HvNax3* allele has less of a reduction in V-PPase activity when exposed to salt than the 18D/011 line. Barley root vacuolar pyrophosphatase reached maximal catalytic activity (Vmax) between 200 and 300 µM sodium pyrophosphate. This result is consistent with reports of V-PPase activity from other plant species, including mung bean (*Vigna radiata*) and red beet (*Beta vulgaris*) (Rea and Poole, 1985, Maeshima and Yoshida, 1989).

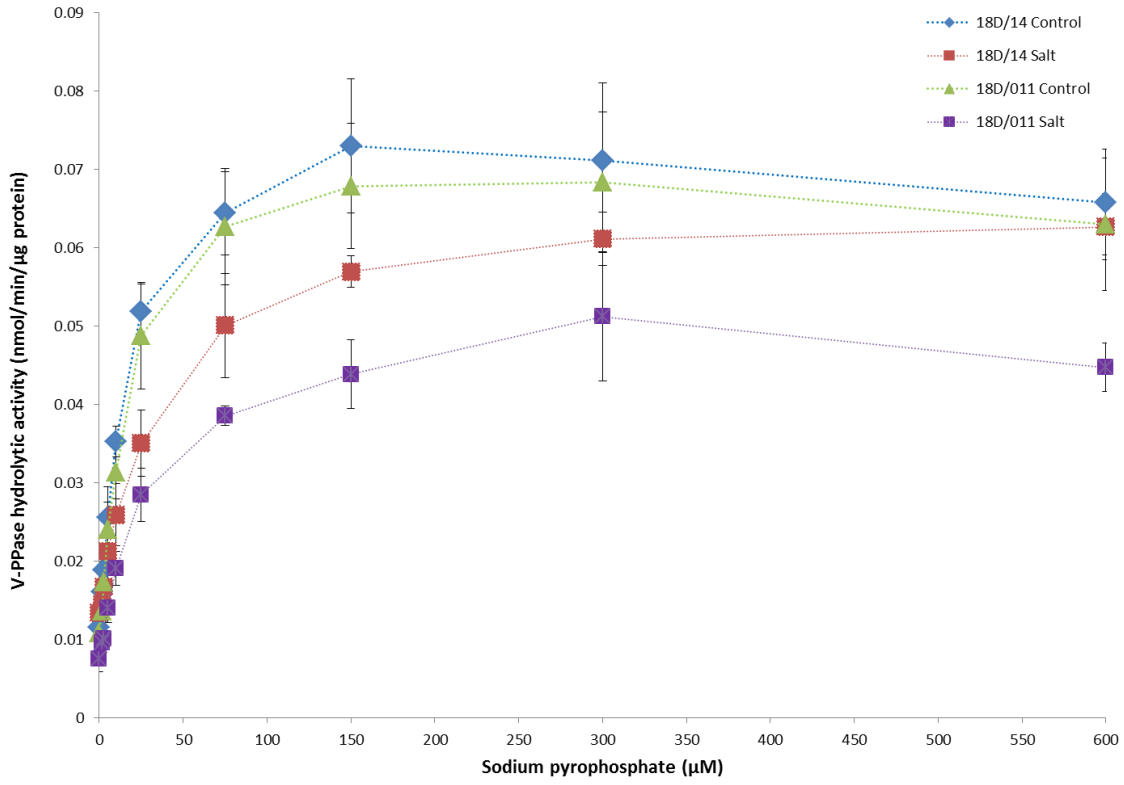

Figure 3-3 Michaelis-Menten curve for barley vacuolar pyrophosphatase (velocity vs. substrate concentration). The sodium pyrophosphate series was fixed at 1 mM $MgSO_4$. PPi hydrolytic activity was measured as the liberation of Pi from PPi (calculated using T210) of root TEV isolated from AB-QTL lines 18D/014 and 18D/011 grown under salt and control conditions. Values are the means ± SEM (n=2 biologicl replicates and 4 technical replicates).

3.3.3 Inhibition of vacuolar pyrophosphatase activity by salt

A Lineweaver-Burk (double reciprocal) plot (Figure 3-4) was used to calculate the apparent Vmax and Km values of root vacuolar pyrophosphatase hydrolytic activity for the AB-QTL lines under salt and control conditions. The plot shows that TEV isolated from the roots of control grown plants maintained a good linearity between velocity and substrate concentration (r^2 >0.96), however TEV isolated from salt stressed plants had a slight departure from linearity with an r^2 of 0.92, possibly indicating that salt is affecting the colorimetric assay is some way.

The calculated Vmax and Km values (Table 3-1) indicate that salt is significantly inhibiting root V-PPase activity in both the AB-QTL lines. Root TEV isolated from control grown plants of the AB-QTL line 18D/014 had a Vmax of 0.062 nmol/min/µg of protein and the AB-QTL line 18D/011 had a similar Vmax of 0.06 nmol/min/µg of protein. However, the AB-QTL lines had a slightly different Km under controlled conditions of 5.4 and 6.2 µM, respectively. Salt stress was shown to significantly reduce the Vmax, and to a lesser extent the Km of pyrophosphatase activity, in both the AB-QTL lines. However, the AB-QTL line carrying the *HvNax3* allele from CPI-71284-48 retained a higher Vmax under salt stress (0.046 nmol/min/µg of protein) when compared with the AB-QTL line 18D/011 carrying the Barque-73 *HvNax3* allele (0.036 nmol/min/µg of protein). These results suggest that salt is acting as a non-competitive inhibitor of V-PPase activity, but is having less of an inhibitory effect on enzyme activity in the AB-QTL line carrying the *H. spontaneum* derived *HvNax3* allele (18D/014).

It is hypothesised that differences in the amino acid sequence of the V-PPase protein in CPI-71284-48 and Barque-73 is responsible for the differences in V-PPase expression and activity, possibly through alterations to the PPi binding site. It also could be that the proteins which regulate the activity of the PP-ase are different in *H. spontaneum* and *H. vulgare*.

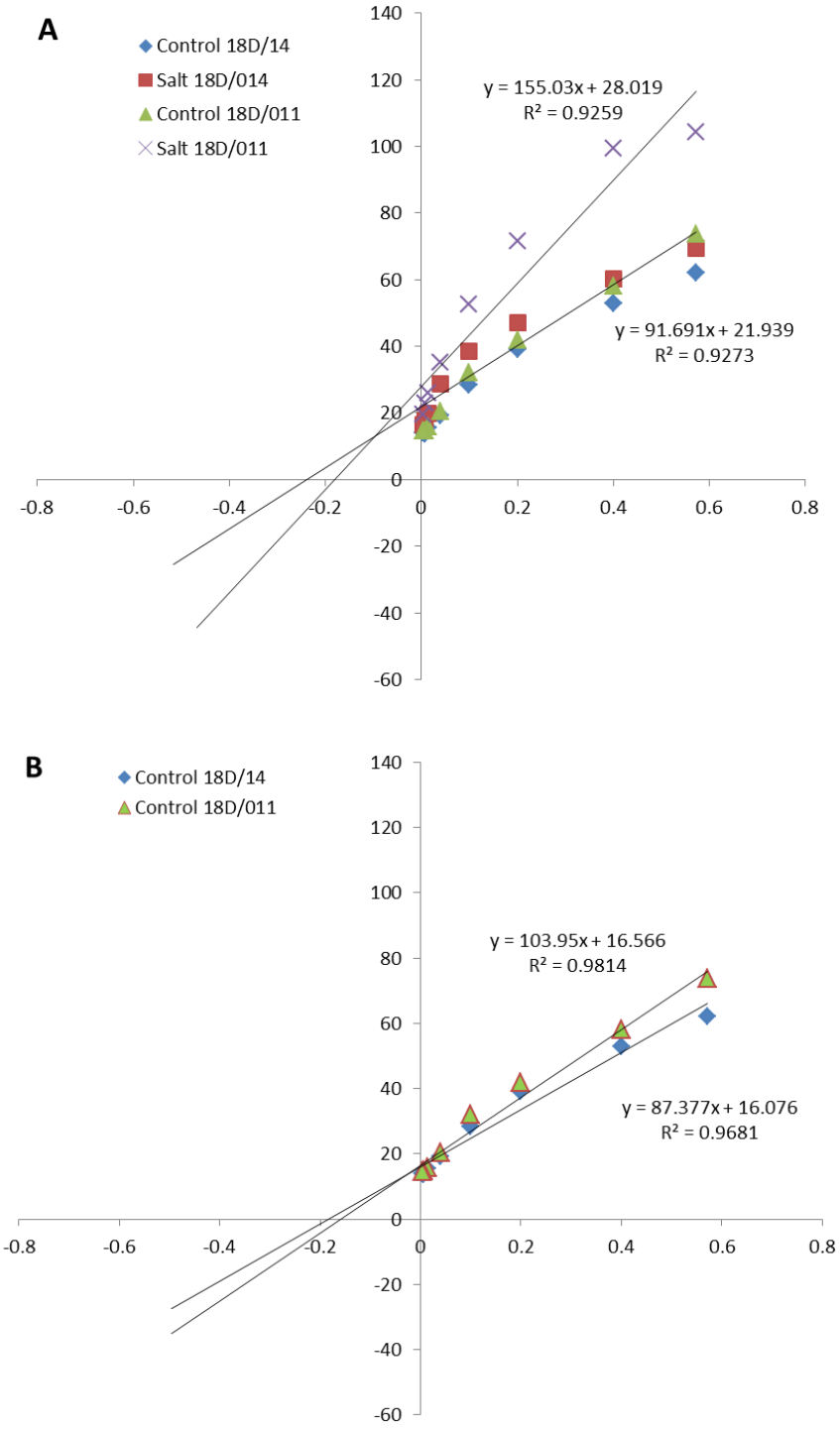

Figure 3-4 Lineweaver-Burk (Double reciprocal) plot for pyrophosphatase activity of TEV isolated from the roots of (A) salt stressed and (B) control grown barley.

Table 3-1 Vmax and Km of pyrophosphatase activity measured on TEV isolated from AB-QTL lines.

	Vmax (nmol/min/µg of protein)		Km (µM)	
AB-QTL Line	Control	Salt	Control	Salt
18D/014	0.062	0.046	5.4	4.1
18D/011	0.060	0.036	6.2	5.5

3.3.4 Barley V-PPase has an absolute requirement for $MgSO_4$

The malachite green colourimetric assay was also performed on root TEV using an $MgSO_4$ series, a known co-factor of pyrophosphatases (Maeshima, 2000), with a fixed sodium pyrophosphate concentration of 300µM. Unfortunately TEV from both the AB-QTL lines under salt stress had to be combined due to a limited number of assay replicates. Barley root V-PPase had an absolute requirement for $MgSO_4$, where activities were undetectable in the absence of magnesium (Figure 3-5). TEV maintained the highest V-PPase catalytic activity between 2 and 3 mM $MgSO_4$, a result supported by reports of other V-PPases. V-PPase activity of TEV isolated from salt stressed barley roots only maintained half the activity of those isolated from control grown plants, again supporting the observation that salt is having an inhibitory effect on enzyme activity. The inhibitor AMBP (amino-methylene-bisphosphonate), in the presence of 1.0 mM $MgSO_4$, was also shown to significantly inhibit V-PPase hydrolytic activity to levels slightly higher than those observed at 0 mM $MgSO_4$ (Figure 3-5).

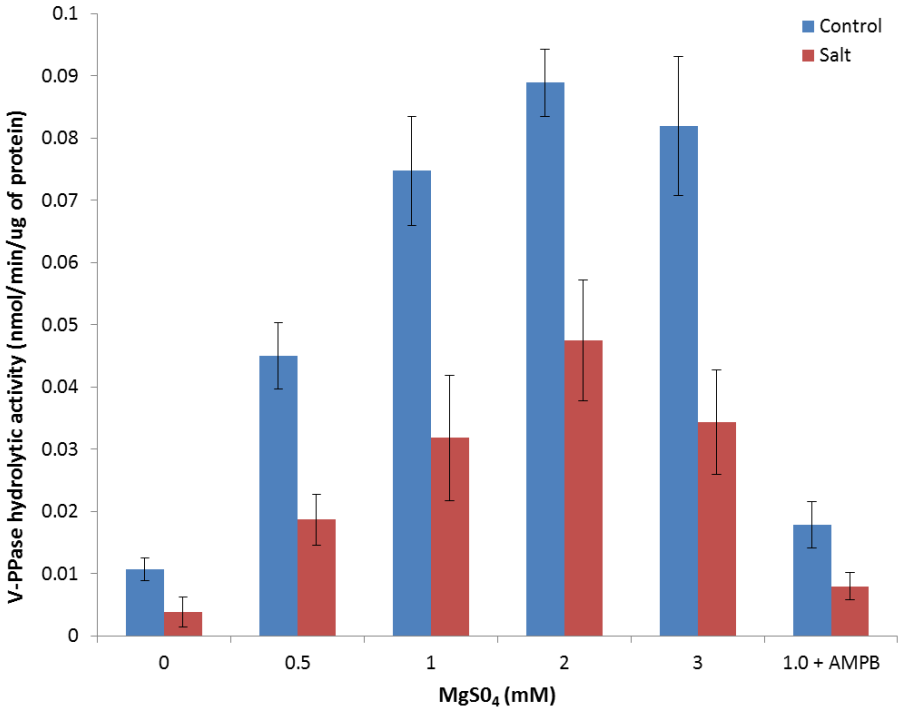

Figure 3-5 V-PPase activity under different $MgSO_4$ concentrations using a fixed substrate of 30 µM sodium pyrophosphate. Values are the means ± SEM.

3.4 Discussion

3.4.1 V-PPase activity follows classic Michaelis-Menten kinetics.

Based on the results obtained from the expression analysis of *HvHVP10* in the roots of salt stressed barley, day 3 of salt stress at 150 mM was the selected target for analysis of root vacuolar pyrophosphatase activity in the AB-QTL lines 18D/014 and 18D/011. The AB-QTL lines were selected for analysis of pyrophosphatase activity, as they are genetically similar and only differ for the region of the *HvNax3* QTL. Tonoplast enriched vesicles (TEV) isolated from the roots of the AB-QTL lines grown under control and salt treatment were used to measure pyrophosphatase activity The relationship between enzyme activity and substrate concentration followed classic Michaelis-Menten kinetics (Figure 3-3). The maximal velocity of the pyrophosphatase enzyme was reached between 200-300 µM PPi in the presence of 1 mM $MgCl_2$, which is consistent with reported V-PPase hydrolytic activities from other species (Maeshima, 2000). It should be noted that TEV isolated from salt stressed roots displayed a slight departure from linearity in the Double Reciprocal Plot (Figure 3-4), as indicated by the lower r^2 value of 0.92 compared to the control of 0.996. This suggests that salt may be interfering with the colorimetric assay in some way, possibly due to increased contamination during vacuole membrane preparation. It was noted during experiments that TEV isolated from salt stressed barley roots were more opaque than those isolated from control grown roots and this may result in variation between spectrophotometric readings. This may be attributed to increased contamination of the vacuolar preparations of salt stressed plants.

The AB-QTL lines were selected for analysis of V-PPase activity as they are only differ for the region of the *HvNax3* QTL, thus eliminating the potential confounding effects of other genes within the *H. spontaneum* genome, which may also contribute to pyrophosphatase activity. It should be noted however, that the malachite green colourimetric assay measures Pi release generated by all H^+-PPases located on the vacuolar membrane, thus it may also measure *HvHVP1* and other pyrophosphatases in barley. Furthermore it should also be noted that the vacuolar membrane isolation procedure employed during this research may result in cross contamination with other cellular organelles or soluble PPases.

3.4.2 Inhibition of V-PPase activity by salt

Salt stress was shown to decrease the Vmax of pyrophosphatase activity in roots of all of the barley genotypes (Figure 3-3), and to a lesser extent the Km of the enzyme (Table 3-1). The apparent Vmax of root V-PPase activity for the AB-QTL line 18D/014 were 0.062 nmol/min/µg protein under control conditions and 0.046 nmol/min/µg of protein under salt stress; and a Km of 5.4 and 4.1 µM respectively (Table 3-1). The AB-QTL line carrying the Barque-73 *HvNax3* allele (18D/011) had a similar Vmax of 0.060 nmol/min/µg of protein under control conditions and a reduced Vmax of 0.36 under salt stress. This line had a slightly higher Km of 6.6 and 5.5 under control and salt respectively. The decrease in Vmax suggests that salt is acting as a non-competitive inhibitor of root vacuolar pyrophosphatase activity. Interestingly, barley V-PPase activity was inhibited in salt stressed roots to a lesser extent in the line carrying the *HvNax3* allele from CPI-71284-48 (18D/014), as indicated by its higher Vmax value. This is an interesting observation, indicating that this line can better maintain pyrophosphatase activity within its roots under periods of salt stress. This may account for the higher root sodium and lower shoot sodium accumulation observed in this line, consistent with the *HvNax3* phenotype. It would have been interesting to conduct similar investigations of V-PPase enzyme activity on TEV isolated from CPI-71284-48 and Barque-73; to see if they reflect a similar pattern of activity under salt and control conditions as their respective AB-QTL line. A similar result would provide further evidence that *HvHVP10* is the gene responsible for the sodium exclusion *HvNax3* QTL identified in the Barque-73 x CPI-71284-48 DH population. However, the different developmental rates identified in the parental genotypes in chapter 2 may significantly confound the results, given that pyrophosphatases are typically highly expressed in young growing tissue (Lerchl *et al.*, 1995).

The Vmax and Km values reported here are consistent with those reported for other V-PPases; however specific H^+-PPase activities vary significantly between plant species, tissues and assay conditions (for full review of literature see (Maeshima, 2000). Typical Vmax values have been reported between 0.02 – 1.1 µmol/min/mg protein for seedling hypocotyl of mung bean (*Vigna radiate*, cv. Wilczek; (Maeshima and Yoshida, 1989)), storage tissue of red beet (*Beta vulgaris*; (Sarafian and Poole, 1989, Kim *et al.*, 1994)), oat roots (*Avena sativa* L.; (Leigh *et al.*, 1992) and Arabidopsis leaf tissue (Krebs *et al.*, 2010).

The effect of salt stress on V-PPase activity has been extensively studied in a various plant species. Reports show inhibition of V-PPase under salt stress in mung bean (Matsumoto and Chung, 1988, Nakamura *et al.*, 1992), and in wheat roots (Wang *et al.*, 2001). Other reports indicate salt treatment increases V-PPase hydrolytic activity. Fukuda *et al.* (2004) reported an increase in V-PPase activity in tonoplast vesicles from salt-stressed barley. This supports previous research by Matsumoto and Chang (1988) who recorded an increase in proton-transport activity in tonoplast vesicles isolated from salt stressed barley plants, indicating it was an adaptive response to NaCl stress

An increase in V-PPase activity has similarly been reported in salt-treated suspension cells of wild carrot (*Daucus carota*) (Colombo and Cerana, 1993), in salt adapted potato callus (*Solanum tuberosum* L.) (Queiros *et al.*, 2009) and in sunflower roots (*Helianthus annuus*) (Ballesteros *et al.*, 1997). Queiros *et al.* (2009) specifically demonstrated that tonoplast enriched vesicles isolated from 150 mM NaCl-tolerant calli lines were higher than the PPi dependent H^+ transport of tonoplast vesicles from control cells. Furthermore, they showed that changes in V-PPase activity were correlated with protein amount. Discrepancies in these results has been attributed to differences in experimental conditions (Ballesteros *et al.*, 1997) and are dependent on the plant species being tested (Wang *et al.*, 2001).

3.4.3 V-PPase has an absolute requirement for $MgSO_4$

Mg^{2+} is a known co-factor of pyrophosphatase (Leigh *et al.*, 1992). The substrate for vacuolar pyrophosphatase is a pyrophosphate magnesium complex (Rea and Poole, 1985) which acts to stabilise and activate the enzyme. The pyrophosphatase activity of TEV isolated from salt stressed and control grown barley was also analysed using a $MgSO_4$ series using a fixed sodium pyrophosphate concentration of 0.3 mM. Our results support this conclusion since the V-PPase enzyme from root TEV did not hydrolyse PPi in the absence of Mg^{2+} regardless of previous treatments (Figure 3-5). The low levels of activity detected at 0 mM $MgSO_4$ was considered background caused by within-plate and between plate-variations as no statistical differences were found between these levels and the negative control (no TEV).

Our results suggest that an optimal $MgSO_4$ concentration for barley V-PPase is between 1 - 2 mM, a concentration commonly used in the solubilisation and purification of the enzyme (Nakanishi and Maeshima, 1998). Higher concentrations of $MgSO_4$ were shown to inhibit enzyme activity, a result which has been reported previously (Queiros *et al.*, 2009). It has

been suggested that High Mg^{2+} concentrations may form precipitates with PPi, which may impair kinetic analysis.

The $MgSO_4$ series measurements consistently showed lower pyrophosphatase activity of TEV isolated from salt stressed barley roots compared with those isolated from control-grown plants, again indicating that salt had an inhibitory effect on root vacuolar pyrophosphatase activity. Unfortunately, the V-PPase activities of the individual AB-QTL lines could not be analysed using the $MgSO_4$ dilution series due to a limited number of replicates. It would have been interesting to examine if the AB-QTL line carrying *HvNax3* allele from CPI-71284-48 similarly displayed increased hydrolytic activity in this experiment under salt stress compared with the AB-QTL line carrying the Barque-73 *HvNax3* allele.

3.4.4 V-PPase activity is inhibited by AMPB

This research indicated that amino-methylene-bisphosphonate (AMPB) significantly inhibited the V-PPase hydrolytic activity of TEV isolated from salt stressed and control grown barley roots, in the presence of 1.0 mM $MgSO_4$ (Figure 3-5). AMBP is a known potent competitive inhibitor to both soluble pyrophosphatases (Zhen *et al.*, 1994) and membrane-bound pyrophosphatases from higher plant vacuoles (Gordon-Weeks *et al.*, 1999). It is theorised that this compound competes with pyrophosphate for the catalytic binding site on the enzyme, and due to the presence P-C-P structure, is more stable than the pyrophosphate substrate (Gordon-Weeks *et al.*, 1999). The inhibitor AMBP is used extensively in therapeutic drugs to treat chronic human disease caused by parasitic protozoa (Rodrigues *et al.*, 1999, Szabo and Oldfield, 2001), due to its resistance to enzymatic hydrolysis and chemical breakdown. In addition, it has little to no effect on ATPase and is therefore highly specific (Gordon-Weeks *et al.*, 1999, Rodrigues *et al.*, 1999). Collectively, these results do suggest that our observations reflect true vacuolar pyrophosphatase activity of tonoplast vesicles from barley roots.

3.5 Summary

In this chapter the vacuolar pyrophosphatase activity of the roots of the AB-QTL lines was examined in response to salinity stress, to determine if differences in the sodium exclusion *HvNax3* phenotype may be attributed to differences in hydrolytic V-PPase activity. Salt was shown to significantly decrease the pyrophosphatase activity of tonoplast enriched vesicles isolated from root tissue of the AB-QTL lines. Furthermore, differences in enzyme activity were observed between the AB-QTL lines under salt, where the AB-QTL line carrying the CPI-71284-48 *HvNax3* allele (18D/014) retained a slightly higher Vmax under salt stress. These results do collectively support the hypothesis that *HvHVP10* is the gene responsible for the sodium exclusion QTL, *HvNax3* in the barley Barque-73 x CPI-71284-48 DH population. It is the recommendation of this chapter that further testing, using a greater number of biological replicates, be undertaken to confirm this hypothesis. To determine if differences in the sequences of the gene and the coding sequence of the mRNA may account for the differences in V-PPase activity and sodium exclusion phenotype observed in the line carrying the CPI-71284-48 *HvNax3* allele, both versions of *HvHVP10* (including cDNA, gDNA and the promoter) were sequenced from CPI-71824 and Barque-73.

4 Molecular characterisation of the barley V-PPase *HvHVP10* in CPI-71284-48 and Barque-73

4.1 Introduction

In the previous chapters it was proposed that the H^+ translocating pyrophosphatase protein in barley, *HvHVP10* (Tanaka *et al.*, 1993), is responsible for the sodium exclusion QTL *HvNax3* identified in the Barque-73 (*H. vulgare*) x CPI-71284-48-48 (*H. spontaneum*) doubled haploid (DH) population; based on synteny mapping with rice and Brachypodium (Shavrukov *et al.*, 2010) gene expression analysis (Shavrukov *et al.*, 2013) and differences in protein activity (Chapter 3). However, the precise nature of the allelic difference between *HvHVP10* from Barque-73 and CPI-71284-48 remains unknown. This chapter is focused on the molecular characterisation of *HvHVP10* in Barque-73 and CPI-71284-48 and is comprised of two sections: Part 1. The genetic characterisation of *HvHVP10* in CPI-71284-48 and Barque-73; and Part 2. *HvHVP10* membrane localisation and cell-type specific expression profiling of *HvHVP10* in CPI-71284-48 and Barque-73.

The specific aims of Part 1 of this chapter are to: 1) isolate and clone the *HvHVP10* CDS, full-length gene and promoter regions from CPI-71284-48 and Barque-73, generating pCR8/GW/TOPO TA Gateway® entry_vectors for use in downstream applications; 2) sequence and compare the CDS, full-length gene and promoter sequences in CPI-71284-48 and Barque-73 to identify potential regions of polymorphism which may explain the *HvHax3* QTL; and 3) determine if there are differences in *HvHVP10* gene copy number between the two genotypes.

The specific localisation of H^+-PPase to the vacuole has been demonstrated for many plant species (for literature review see (Rea and Poole, 1993)) and is considered to be a bona fide tonoplast marker (Maeshima and Yoshida, 1989). Later studies using a combination of immune-localisation and proteomic approaches have demonstrated a dual localisation of H^+-PPase at the vacuole and plasma membrane (Langhans *et al.*, 2001). The specific aim of Part 2 of this chapter is to examine the membrane localisation of barley *HvHVP10* using a transient expression system; and to determine the cell-type specific expression of *HvHVP10* in CPI-71284-48 and Barque-73.

It should be noted that the laboratory work of this chapter was conducted at the same time as the Shavrukov et al 2013 publication, thus a lot of this work is included in the above mentioned publication.

4.2 Materials and Methods

4.2.1 Isolation of HvHVP10 CDS, full-length gene and promoter region from CPI-71284-48 and Barque -73

4.2.1.1 Plant Material

Barque-73 (*H. vulgare*) and CPI-71284-48 (*H. spontaneum*) root RNA was kindly provided by Dr Yuri Shavrukov (ACPFG, University of Adelaide). RNA was extracted from plants that had been grown hydroponically and salt stressed at 3rd leaf emergence (approx. 17 days post germination). NaCl was applied to the growth solution (Modified Hoagland's Nutrient Solution, section 2.2.1.2), in increments of 25 mM twice daily (morning and afternoon) for three consecutive days to reach a final concentration of 150 mM NaCl. At each addition of NaCl, supplementary $CaCl_2$ (2 mM) was also added to the nutrient solution.

4.2.1.2 Complementary DNA (cDNA) synthesis

At 0, 1, 2, 3, 5 and 10 days after initial salt application shoots and roots were harvested for RNA isolation and gene expression analysis. Tissue samples were immediately frozen in liquid nitrogen and stored at -80° C. RNA was isolated using Trizol-like reagent composed of 38% (v/v) phenol pH 4.3, 12% (w/v) guanidine thiocyanate, 7% (w/v) ammonium thiocyanate, 3 M sodium acetate pH 5.0, and 5% (v/v) glycerol. cDNA synthesis was performed on RNA isolated from roots of CPI-71284-48 and Barque-73 exposed to salt for three days. First strand cDNA was synthesised using SuperScript™ III reverse transcriptase, following manufacturer's instructions (Invitrogen, USA). cDNA synthesis was performed in 20 µL reactions comprised of 1 µL of oligo (dT)18 (50µM), 1µL of dNTP mix (10mM each dATP, dGTP, dCTP, dTTP), 1 µg of total RNA and sterile milli-q water to a final volume of 13 µL. The reaction was incubated for 5 minutes at 65°C then placed immediately on ice for 1 minute. The samples were briefly centrifuged then 4 µL of 5x First –Strand Buffer, 1 µL of 0.1M DTT, 1 µL of RNaseOut™ Recombinant RNase Inhibitor (ThermoFisher Scientific, USA) and 1 µL of SuperScript™III RT (Invitrogen) were added to each sample and contents mixed by pipetting up and down. The reaction was incubated at 50°C for 60 minutes then heated at 70°C for 15 minutes to terminate the reaction.

4.2.1.3 Genomic DNA extraction

Barque-73 and CPI-71284-48 seeds were germinated in petri dishes on moist tissue for 7 days and approximately 100 mg of leaf tissue was harvested into 10 mL tubes and snap frozen in liquid nitrogen. Frozen plant material was ground using ball bearings and an Eppendorf vortex, before being transferred to a 2 mL microcentrifuge tube for DNA extraction. DNA was extracted using the phenol/chloroform/iso-amyl alcohol method. Briefly, 600 µL of extraction buffer (1% sarkosyl, 100 mM Tris-HCL, 100 mM NaCl, 10 mM EDTA and 2% PVPP) was added to frozen leaf tissue and tubes were gently mixed by hand. An equal volume of phenol/chloroform/iso-amyl alcohol (25:24:1) was added and tubes were mixed on an orbital mixer for 10 mins. Tubes were centrifuged for 10 mins in a microcentrifuge (11,000 *g*). The upper phase was transferred to a fresh 1.5 mL microcentrifuge tube and 60 µL of 3 M sodium acetate (pH4 .8) and 600 µL of isopropanol was added, then samples were mixed via gentle in version. The DNA was allowed to precipitate from the solution at room temperature for 30 to 60 minutes. Tubes were again centrifuged and the supernatant discarded. The pellet was air dried in a fume cupboard for 10 minutes and then resuspended in 40 µL of R40 (40ng/ml RNase A (Qiagen, Germany) in 1× TE buffer).

4.2.1.4 PCR amplification of HvHVP10 CDS from CPI-71284-48 and Barque-73 cDNA

The coding sequence of *HvHVP10* was amplified from CPI-71284-48 and Barque-73 cDNA using PCR primers based on the annotated *HvHVP10* sequence (D13472.2 NCBI). The primer pair PYR-20-for and PYR-20-rev (Table 4-1) were used to amplify *HvHVP10* from the gene's start codon (ATG) to the stop codon (TAG) at end of the gene, corresponding to an open reading frame of 2289 bp. The coding sequence minus the stop codon (2286 bp) was also amplified via PCR using the primer pair PYR20-F and HVP10_no_stop-R, for use in transient expression systems.

Three independent PCR reactions were carried out for each construct and genotype, using Platinum *Taq* DNA Polymerase High Fidelity (Hi-Fi) (ThermoFisher, Scientific), which contains proofreading 3-5 exonuclease activity to remove sequence errors introduced during PCR amplification (excise mismatched nucleotides in the 3′-5′direction). The 20 µl reaction mix composed of 1.0 µL of cDNA (10ng/µL) , 2 µL of Hi Fi buffer, 2 µL of dNTPs (2 mM) 1.0 µL of forward primer (10 µM), 1.0 µL of reverse primer (10 µM), 1.0 µL of DMSO, 0.8 µL $MgSO_4$ (10 mM), 0.2 µL Hi-Fi Taq and 11.00 µL of H_2O. The PCR conditions were

initial denaturation at 94°C for 3 minutes; 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 2.5 minutes extension at 68°C.

4.2.1.5 PCR amplification of HvHVP10 from gDNA using FastStart Hi-fidelity PCR system

FastStart High fidelity PCR system, dNTPack (Roche) was used to amplify the full-length *HvHVP10* gene from CPI-71284-48 and Barque-73 genomic DNA using the primer pair PYR20-F and PYR20-R, according to the manufacturer's instructions. The PCR reaction was comprised of 1.5 µL Fast Start Buffer, 0.3 µL of nucleotide mix, 0.6 µL of PYR-20-for, 0.6 µL of PYR-20-rev, 0.6 µL of DMSO, 0.45 µL of gDNA, 0.15 µL of *Taq* and 10.8 µL of H_2O. Thermal B conditions were used to amplify the gene, involving 2 amplification steps. (94°C for 2 mins – denaturation; 10 cycles for step 1 amplification; 94°C for 30 secs, 58°C for 30 secs, 68°C for 5 minutes; 31 cycles for step 2 amplification; 94°C for 30 secs, 58°C for 30 secs and 68°C 5 minutes + 20 secs each cycle; 68°C for 7 mins for final extension.

Table 4-1 Primers used for the isolation, sequencing and cloning of *HvHVP10* CDS, full-length gene and promoter regions in CPI-71284-48 (*H. spontaneum*) and Barque-73 (*H. vulgare*).

Primer Name	Orientation	Length	Sequence 5´to 3´ (length bp)	Purpose	Size of Fragment
PYR20-F	Forward	22	ATGGCGATCCTCGGGGAGCTCG	isolation and cloning of CDS and full-length gene	
PYR20-R	Reverse	24	CTAGATGTACTTGAACAGCAGACC	isolation and cloning of CDS and full-length gene	2289
GW1	Forward	25	GTTGCAACAAATTGATGAGCAATGC	Sequencing CDS and full-length gene	NA
GW2	Reverse	25	GTTGCAACAAATTGATGAGCAATTA	Sequencing CDS and full-length gene	NA
PYR-1	Forward	25	CTCTTTAGCACTGCATCTTTCTTGC	Sequencing full-length gene	NA
PYR-2	Reverse	25	CTGGAAGAGCCAACCAGCTGATGAC	Sequencing full-length gene	NA
PYR-3	Forward	25	GTCATCAGCTGGTTGGCTCTTCCAG	Sequencing CDS and full-length gene	NA
PYR-4	Reverse	25	TCCAACACTCTTCATGGTCATGGCA	Sequencing CDS and full-length gene	NA
PYR-7	Reverse	24	GATGGCTCCAAGCAAGAAAGATGC	Sequencing CDS and full-length gene	NA
HVP10_no_stop-R	Reverse	24	GATGTACTTGAACAGCAGACCTCC	Cloning	2286
pPYR-9	Forward	24	TCATGCCTTGAAGAATGTTGTTGC	Promoter isolation and cloning	2349
pPYR-10	Forward	24	GCCAAAACCTCACCACGGAACCCT	Promoter isolation	1829

pPYR-11	Forward	24	GCCTGCTTAGGGCATGTACAATGG	Promoter isolation	1206
G-PYR-9	Reverse	22	TCGATGAGGTAGTCGCCGTAGC	Promoter isolation	
HVP10-Prom-Rev	Reverse	18	GGCTGTCTGGCTGGCAGG	Promoter cloning	2256

PCR amplification of 2.2 kb *HvHVP10* promoter region from CPI-71284-48 and Barque -73 gDNA

4.2.1.6 Identification of Morex BAC Clone containing HvHVP10

A barley (cv. Morex) BAC library with 3.7 × genome-coverage was screened for BAC clones containing the *HvHVP10* gene (Shavrukov *et al.* 2013). The BAC library, constructed by Amplicon Express (http://ampliconexpress.com/), was screened via PCR using two primer pairs: PYR-31F + PYR-32R (455 bp product) and PYR-33F + PYR-34R (567 bp product). A single positive clone, 0262H05, was identified and was kindly provided by Nils Stein (IPK, Gatersleben, Germany).

4.2.1.7 BAC plasmid miniprep

The BAC clone DNA was purified using a QIAGEN Large-Construct Kit according to the manufacturer's protocol. Briefly, the BAC clone was bulked up in a starter culture of 5 ml of selective LB medium for 8 hrs at 37°C with shaking. One mL of the starter culture was diluted into 500 mL of selective LB medium and grown overnight at 37°C with shaking. The cells were harvested by centrifugation at 6000 *g* at 4°C for 15 minutes in a Beckman® JA-10 rotor and the bacterial pellet completely resuspended in 20 mL of Buffer P1 by gently pipetting up and down. 20 mL of Buffer P2 was added and the contents were mixed by gentle inversion then contents were incubated at RT for 5 min. 20 mL of chilled Buffer P3 was added and cells were immediately mixed by gentle inversion, and then incubated on ice for 10 min. The sample was centrifuged at 20,000 *g* for 30 min at 4°C in a Beckman JA-17 rotor and the lysate was filtered through pre-moistened filter paper. Thirty six ml of RT isopropanol was added to the cleared lysate and the contents mixed and centrifuged at 15,000 *g* in a Beckman JS-13 rotor. The DNA pellet was washed with 70% (v/v) RT ethanol and centrifuged at 15, 000 *g* for 15 minute. The supernatant was gently removed with a pipette and the pellet air dried for 3 min. The DNA pellet was dissolved in 9.5 mL of Buffer XE by gentle mixing to avoid shearing the large BAC DNA. 200 µl of ATP-Dependent Exonuclease and 300 µL of ATP solution were added to the dissolved DNA to remove genomic DNA and nicked BAC DNA, leaving only supercoiled DNA for further purification. The sample was incubated in a water bath at 37°C for 60 min. The BAC DNA was applied to an equilibrated QIAGEN-tip 500 and 10 mL of Buffer QS was added to the tip and allowed to enter the resin by gravity flow. The tip was washed twice with 30 ml of Buffer QC. DNA was eluted from

the QIAGEN-tip with 15 mL of pre-warmed (65°C) Buffer QF. DNA was precipitated from the solution with 10.5 mL of RT isopropanol, gently mixed and centrifuged at 15000 *g* for 30 min at 4°C in a Beckman JS-13 rotor and the supernatant gently removed with a pipette. The DNA pellet was washed with 5 mL of RT ethanol (70% v/v), centrifuged at 15000 *g* for 15 minutes and the supernatant removed. The DNA pellet was allowed to air-dry for 5-10 minutes then was redissolved in 10 mM Tris-HCl (pH 8.5) overnight at RT. Yield and DNA quality was determined via gel electrophoresis.

4.2.1.8 BAC Clone sequencing

BAC sequencing was performed at the Australian Genome Research Facility (Brisbane, Australia) using a 454 GSFLX analyser. Sequence contigs were assembled using the Newbler software (Roche Diagnostics) and used in blast searches of NCBI databases to identify genes.

4.2.1.9 Primer design based on Morex HvHVP10 sequence

A contig (contig 2), containing a full copy of *HvHVP10*, shared 100% sequence identity with the annotated *HvHVP10* coding sequence from *Hordeum vulgare* mRNA (NCBI Accession No. D13472.2). The 5´ sequence of Morex *HvHVP10* (approximately -2200 bp upstream of start codon) was used to design a series of 5´ primers which were paired with a gene-specific reverse primer approximately 60 bp downstream of the start codon to isolate the promoters of *HvHVP10* in CPI-71284-48 and Barque-73 genotypes (Figure 4-1). Primers used to amplify the promoter region are listed within Table 4-1. PCR reactions were performed using Hi-Fi platinum *Taq* following the protocol as described previously.

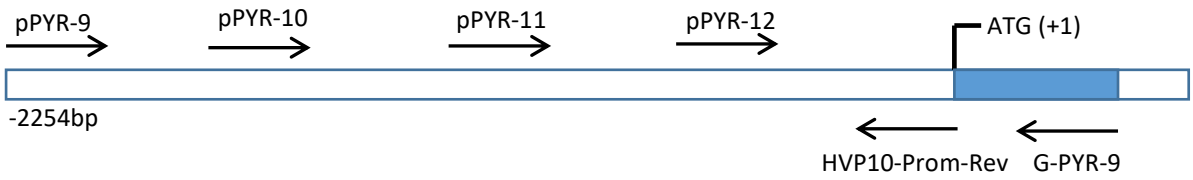

Figure 4-1 Schematic diagram of primers designed to isolate ~2200 of *HvHVP10* in CPI-71284-48 and Barque-73 based on BAC clone sequence (cv. Morex).

4.2.1.10 DNA extraction from agarose gels

Amplified DNA fragments from CPI-71284-48 and Barque-73 cDNA and gDNA were excised from agarose gels and purified using a Macherey Nagel (Germany) NucleoSpin Gel and PCR Clean-up Kit as per the manufacturer's instructions. Briefly, the gel slice containing the PCR product was dissolved in NTI solution for 10 minutes at 50°C. The sample was loaded into a clean-up column placed in a collection tube and centrifuged for 30 seconds at 11,000 *g*. The silica membrane was washed with 700 µL of NT3 Buffer and centrifuged for 30 seconds. This step was repeated to minimise salt carry-over. The silica membrane was dried by centrifugation for 1 minute at 11,000 *g* then 30 µL of pre-warmed (50°C) NE Buffer was added to the column and incubated at RT for 1 minute. Purified DNA was eluted into a fresh microfuge tube by centrifugation for 11,000 *g* for 1 minute.

4.2.2 Generation of pCR8/GW/TOPO TA Gateway® entry vectors carrying HvHVP10 CDS, full-length gene and promoter regions from CPI-71284-48 and Barque -73

Gateway® cloning was used to generate entry vectors carrying the *HvHVP10* CDS, full-length gene and promoter regions from CPI-71284-48 and Barque-73 for use in plant expression systems. The advantage of this technology is that PCR product can be directly cloned into the pCR8 vector to generate entry clones, eliminating the need for restriction enzyme digestion and ligation. A further advantage is the ease of sub-cloning the gene of interest into a wide range of destination vectors through the LR reaction (Xu and Li, 2008).

Amplified PCR *HvHVP10* fragments from CPI-71284-48 and Barque-73 cDNA (CDS) and gDNA (full-length gene and promoter) were cloned into pCR8/GW/TOPO® TA Gateway® entry vector (Invitrogen) according to the manufacturer's instructions. In brief, 4 µl of PCR product, 1 µL salt solution and 1 µL of PCR8 vector were incubated on ice for 30 minutes. Four µl of the cloning reaction was added to 50 µL of competent One Shot TOP10 *E. coli* cells, and the mixture incubated on ice for 30 minutes. The cells were heat shocked at 42°C for 30 seconds to allow entry of the plasmid, then the reaction was immediately transferred to ice for 5 minutes. 250 µL of liquid Luria Betani (LB) media (tryptone 10 g/L, yeast extract 5 $g \cdot L^{-1}$, NaCl 5 $g \cdot L^{-1}$, pH7.5) was added and cells were incubated at 37°C with shaking for 45 minutes. 50 µL of bacterial culture were then plated out on LB plates containing the antibiotic spectinomycin (50 $\mu g \cdot mL^{-1}$) for positive selection and plates were incubated at 37°C overnight. Positive clones resistant to the antibiotic were selected and bulked up in LB liquid

media with the antibiotic at 37°C overnight. At least 5 clones from each PCR reaction were bulked up for sequencing analysis.

4.2.2.1 Isolation of plasmid DNA

Plasmid DNA was isolated using the Bioline (UK) Plasmid Mini Kit according to the manufacturer's instructions. Briefly, 2 mL of bacterial culture was transferred to a 2 mL microfuge tube and centrifuged for 1 minute at 16,100 *g* to pellet the cells. The supernatant was removed, and then cells were resuspended in 250 µL of resuspension buffer and an equal volume of Lysis buffer to lyse the cells. The reaction was neutralised and the precipitated DNA was separated from cellular components via centrifugation at 16,100 *g* for 10 minutes. The upper phase containing the plasmid DNA was collected and transferred to a spin column placed in a 2 mL collection tube and centrifuged for 1 minute at 10,000 *g* to bind DNA to the column membrane. Bound plasmid DNA was washed with 500 µL of Wash Buffer AP, centrifuged and then 700 µL of wash buffer BP was added and again the sample was centrifuged at 10,000 *g* for I minute. An additional centrifugation step of 2 minutes was carried out to remove residual ethanol. 30 µL of elution buffer was added to the column and the sample was incubated at room temperature for 1 minute. Plasmid DNA was eluted from the column membrane via centrifugation at 10,000 *g* for 1 minute.

4.2.2.2 "Quick and Dirty" plasmid preparation of full-length gene clones

Due to the low transformation efficiency of the full-length *HvHVP10* gene into pCR8/GW/TOPO® entry vectors (Figure 4-3), a "Quick and Dirty" plasmid preparation for restriction enzyme analysis was employed, following the protocol of (Birnboim and Doly, 1979). Briefly, *E.coli*, containing the full gene in PCR, were grown overnight in 2.5 mL of LB plus spectinomycin antibiotic at 37°C with shaking. The bacteria was transferred to a 2 mL microfuge tube and centrifuged in a bench top centrifuge max speed (13, 000 *g*) for 1 minute. The supernatant was discarded. Cells were resuspend in 100 µL of solution I (5 mM Glucose, 25 mM Tris-HCl and 10 mM EDTA at pH 8.0). 200 µL of solution II (0.2 M NaOH and 1% SDS) and 150 µL of solution III (3 M potassium acetate (pH 4.8) was added to the cells and the tube was mixed by gentle inversion 6-8 times. Cells were centrifuged for 10-15 minutes at 10,000 *g* and the supernatant transferred to a fresh tube. 750 µL of isopropanol was added and the tubes were inverted a number of times and DNA allowed to precipitate at RT for 1 hour. DNA was collected at the bottom of the tube via centrifugation at 10,000 *g* for 10-15 minutes

and the supernatant removed. One (1) ml of ethanol (70%) was added and the DNA was briefly vortexed then centrifuged for 10 minutes at 10,000 *g*. The supernatant was completely removed with a pipette and the DNA pellet was air dried on a heat block (45°C) for a 3-5 minutes. The DNA was resuspend in 30 µL of H20 containing RNase (Roche).

4.2.2.3 Restriction enzyme digestion

Amplicons cloned into the pCR8/GW/TOPO TA Gateway system can orientate in the entry vector in either direction. Therefore, the orientation of the gene must be confirmed using restriction enzyme analysis. The integration and orientation of plasmid DNA was confirmed using restriction digestion with the restriction enzyme *Blp*I (New England Biolabs, USA) for CDS and full-length gene entry clones and *Bam*HI and *Eco*RV for *HvHVP10* promoter entry clones. The 20 µL digestion reaction consisted of 2.5 µL of 1x NEBuffer 4, 3 µL of plasmid DNA, 0.1 µL restriction enzyme and 4.4 µl of sterile H_20. Digestion reactions were incubated at 37°C for at least 4 hours. The DNA products of restriction digestion were separated by agarose gel electrophoresis and positive clones carrying *HvHVP10* CDS in the correct orientation were selected for sequencing analysis.

4.2.2.4 PCR clean-up of full-length gene pCR8/GW/TOPO® clones isolated using 'Quick and Dirty' Protocol

pCR8/GW/TOPO® clones carrying the full-length gene in the correct orientation that were isolated using the Quick and Dirty protocol underwent PCR clean-up prior to Sanger sequencing, using the Isolate II PCR and Gel Kit (Bioline) according to the manufacturer's instructions. Briefly, one volume of sample was mixed with 2 × volume of Binding Buffer CB and the sample was loaded into a column placed in a 2 mL collection tube. The column was centrifuged for 30 seconds at 11,000 *g* and the flow-through discarded. The silica membrane was washed with 700 µL of Wash Buffer CW and centrifuged for 30 seconds at 11,000 *g*. This step was repeated to minimise salt carry-over which may affect downstream applications. The silica membrane was dried by centrifugation for 1 minute at 11,000 *g* to remove residual ethanol. 30 µL of pre-warmed Elution Buffer C was added to the silica membrane and the column was incubated at RT for 1 minute. The DNA was eluted into a fresh microcentrifuge tube via centrifugation for 1 minute at 11,000 *g*.

4.2.2.5 Big Dye sequencing reaction

Plasmids carrying the *HvHVP10* CDS, full-length gene and promoter regions from CPI-71284-48 and Barque-73 in the correct orientation were selected for sequencing analysis. Purified plasmid DNA was labelled and prepared for sequencing using the BigDye®Terminator (BDT) v3.1 Cycle Sequencing Kit (ThermoFisher Scientific). Reactions were performed in a 10 µL mix consisting of 0.3 µL of plasmid DNA, 0.32 µL of sequencing primer (10 µM of either PYR20for, PYR20rev, PYR-3 or PYR-4), 1.0 µL BDT v3.1, 3.5 µL BDT Buffer and 4.88 µL H_20. Thermal cycling conditions consisted of one cycle at 96°C for 1 min; 35 cycles at 96°C for 10 secs, 50°C for 5 secs and 60°C for 4 minutes. The sequencing reaction was then allowed to cool to room temperature and transferred to a 1.5 mL Eppendorf tube. 75 µL of 2.0 mM $MgSO_4$ was added to the reaction mixture, samples were briefly vortexed, and then incubated at room temperature for 15 minutes. Tubes were centrifuged for 15 minutes at 15,000 *g*, the supernatant removed and 75 µL of 70% ethanol was added to each sample. The tubes were centrifuged for 10 minutes and the supernatant was removed by pipette or decanting. Tubes were wrapped in foil and allowed to air dry in a fume cupboard for 10 minutes and then were submitted to the Australian Genome Research Facility (Waite Campus, Urrbrae, South Australia) for capillary separation using an AB3730x/sequencing platform (Applied Biosystems, USA). DNA sequencing analysis and alignments were performed using Vector NTI Advance™ 11.0 (Invitrogen, Mulgrave, Vic, Australia).

4.2.3 Southern Blot Analysis of HvHVP10 copy number in CPI-71284-48 and Barque-73

Southern blot hybridisation was performed, following the protocol described by Sambrook *et al.* (1989) to determine if CPI-71284-48 and Barque contained variation in *HvHVP10* copy number within their genome. A number of other barley cultivars were also included for comparison (Morex, WI4330, Barque-73, CPI-71284-48, recombinant line 1 (18D/011) recombinant line 2 (18D/014), Chinese Spring (Wheat), Betzes (Barley) and Chinese Spring + Betzes 7H chromosome addition line). Briefly, gDNA from each accession were digested using the restriction enzymes *Hind*III, *Xba*I, *Bam*HI and *Eco*RV (New England Biolabs). Restriction enzyme digestions were performed in 20 µL reactions consisting of 4 µL of gDNA, 1 x reaction buffer and 5 Units of restriction enzyme. Reactions were incubated at 37°C for 4-5 hours then heat inactivated at 65°C for 20 minutes. DNA fragments were separated via electrophoresis on UltraPure™ Agarose (TermoFisher Scientific) gels at a

constant voltage of 35 V overnight, then transferred to a Hybond N+ nylon membrane using 0.4 M NaOH as the transfer buffer. Membranes had been pre-incubated at 37°C for approximately 24 hours in pre-hybridisation solution consisting of 10 × SSC (3 M NaCl, 0.3M tris-sodium citrate), Denhardt's III solution (TermoFisher Scientific), and salmon sperm DNA (Sigma-Aldric). A PCR amplification product corresponding to cDNA fragment was used to probe for *HvHVP10*, using the primers PYR-9 and PYR 12 (corresponding to a 220 bp region approximately in the middle of the coding sequence) (Table 4-2). The PCR product was radio-labelled with [α-32P] dCTP using Klenow's fragment and a standard protocol. For hybridisation with the labelled probe, membranes were incubated at 65°C for 16 hours in a hybridisation solution containing HSB buffer, Denhardt's III solution, Dextran sulphate and salmon sperm DNA. Membranes were then washed at 65°C for 20 minutes in 3 solutions (2×SSC, 1×SSCans 0.5× SSC) of decreasing salt concentration. Washed membranes were covered with plastic cling wrap and exposed to film (Fuji HR-T), in appropriate cassettes with intensifying screens fitted at -80°C for 4-6 days. The film was developed using a CP1000 automatic film processor (AGFA, Belgium). Assistance with probe labelling, hybridisation and autoradiography was provided by Ms. Margaret Pallotta at the ACPFG.

Table 4-2 Primers for southern analysis of *HvHVP10* copy number in barley genotypes

Forward Primer	Sequence	Reverse Primer	Sequence	Target
PYR-9	GGTCTGTGGGCTGGTCTGATTATTG	PYR-12	GCTGACGTAGATGCTGACAGCAATAG	Middle of gene
HVP103-F	CGTCGCTCAACATCCTCATCAAGC	HVP103-R	CGAGGGAGGACATCTGGTCTCTCT	3'UTR
HVP105-F	GAATCCCCTCGCAAATCACACCGG	HVP105-R	ACGAAACCCTAACGACGGACCGGA	5'UTR

4.2.4 Bioinformatics

The *HvHVP10* protein and cDNA sequence (Accession No. D13472.2) was obtained from the National Centre for Biotechnology information (NCBI) website (http://www.ncbi.nlm.nih.gov). The Basic Local Alignment Search Tool (BLAST) version 2.2.23 was used to find regions of local similarlity between DNA sequences. Spidey, an mRNA-to-genomic alignment tool available on the NCBI website was used to align *HvHVP10* CDS with the full-length gene in order to identify exon/intron boudaries in the *HvHVP10* gene sequence. Vector NTI version 11.0 (Invitrogen) was used for a number of purposes including the alignment of DNA sequences, primer design and analysis, restriction enzyme analysis of DNA sequences and vector design using *in silico* Gateway® cloning reactions. Primers were designed using Primer3 online software (http://bioinfo.ut.ee/primer3-0.4.0/).

4.2.5 Promoter Motif Analysis

HvHVP10 promoter motif analysis was performed using standard variables of MatInspector (https://www.genomatix.de/matinspector.html.), a software tool used to detect transcription factor binding site motifs in DNA sequences.

4.2.6 HvHVP10 membrane localisation

4.2.6.1 Generation of C and N terminal YFP fusion protein expression vectors

The *HvHVP1*0 CDS with and without the stop codon from pCR8/GW-TOPO TA Gateway® entry vectors was recombined into a Gatewaty-enabled destination vectors for N and C terminal reporter fusion constructs, *YFP:HvHVP10* and *HvHVP10:YFP* respectively. Recombination reactions were performed using Gateway LR Clonase II enzyme mix (Invitrogen), according to the protocol provided by the manufacturer. Briefly, in a 1.5 mL microcentrifuge tube 1-7µL of purified TOPO® entry clone was added to 1 µL of destination vector and TE Buffer (pH 8.0) to a final volume of 8 µL. 1 µl of LR Clonase II enzyme mix was added and the reaction was incubated at 25°C for 1 hour. The reaction was terminated with the addition of 1 µL of Proteinase K solution (Invitrogen) and samples were incubated for 10 minutes at 37°C. A 4 µL sub sample was used for transformation into *Escherichia coli* competent cells using the heat shock method. Plasmid DNA was isolated from 6 colonies of each transformation and checked via restriction enzyme digestion and sequencing to confirm integration of the target sequence into the plasmid in the correct orientation.

4.2.6.2 Transient expression of HvHVP10:YFP in onion epidermal cells

Transient expression of the *HvHVP10*:YFP and YFP:*HvHVP10* fusion proteins in leek (*Allium ampeloprasum*) epidemal cells was performed by particle bombardment technique using a Bio-Rad Biolistic Particle Delivery System Model PDS-1000/He (Bio-Rad), as decribed in Delhaize *et al.* (2007). Briefly, gold particles with a 0.6 µm diameter (Bio-Rad) were coated with the desired plasmid (0.5 µg) using 0.1 M spermidine. DNA-coated gold particles were centruifuged at 7000 *g* for 2 minutes, washed with ethanol (100%), recentrifiged and then resuspended in 50µL of ethanol (100%). Sections of leek leaves were excised approximately 5 to 10 cm from the base of a mature plant and sliced into 1.0 cm^2 squares. Several squares were rested on moist tissue at the centre of 100 × 15 mm Petri dishes with the curved side of the leaf facing upward. Petri dishes holding the leek tissue were placed approximately 8 cm from the rupture disk containg the DNA-coated gold particles. Pressure in the delivery chamber was reduced to an 88 kPa vaccuum and leek tissue was bombarded with gold particles driven by 6000 kPa helium. Confocal images were taken using a Leica-TCS SP2 system (Leica, Germany) and processed using Adobe PHOTOSHOP® software (Adope, USA).

4.2.6.3 Agrobacterium tumefaciens-mediated transient expression of HvHVP10:YFP in Nicotiana benthamiana

Due to issues with the agrobacterium strain AGL-1 and conferred resistance to ampicillin in our laboratory (Hellens *et al.*, 2000), YFP fusion plasmids were engineered (using restriction enzymes *Xmal* and *HindIII*) to replace the ampicillin selectable marker with spectinomycin selectable marker from cloning vector pPLEX-502 (Figure 8-6).

4.2.6.4 Preparation of chemically-competent A. tumefaciens strain AGL-1 cells

Laboratory stocks of *A. tumefaciens* strain AGL-1 cells were multiplied according to the method described by Hõfgen and Wilmitzer (1988). *Argrobacterium* was streaked on an LB plate with rifampicin (20µL/mL) and grown for 2 days at 28 °C. A single colony was transferred to 5 mL of liquid LB with rifampicin and incubated for 2 days at 28 °C with shaking. The 5 mL culture was then added to 200 mL of YEP media (Bacto peptone 10g L^{-1}; yeast axtract 10 g L^{-1}; NaCl 5 g L^{-1}; pH 7.0) and incubated at 28 °C with shaking until culture reached OD600 0.5-1.0 (approx. 5 h). The culture was split into 4 × 50 mL falcon tubes and tubes were centrifuged at 3000 g for 20 mins at 4 °C in an Eppendorf 581R centrifuge. The supernatant was removed and cells were washed with 2.5 mL of 1 × TE Buffer (pH 8.0), then centrifuged at 3000 *g* for 10 mins at 4 °C. The supernatant was again removed and cells were resuspended in 5 mL of YEP media. Cells were separated into 250 µL aliquots in prechilled 1.5 mL Eppendorf tubes, snap frozen in liquid nitrogen and stored at -80 °C.

4.2.6.5 Transformation of YFP fusion protein expression vectors into chemically competent A. tumefaciens strain AGL-1

Binary expression vectors pPLEX502 harbouring HVP10_no_stop:YFP fusion were transformed into *A. tumefaciens* strain AGL1 using the freeze-thaw method based on that described by (Höfgen and Willmitzer, 1988). Briefly, 1 µL of expression vector was added to 100 µL of chemically competent AGL-1 cells thawed on ice. The cells were gently mixed and incubated on ice for 5 minutes, followed by snap freezing in liquid nitrogen for 5 minutes and incubation in a 37°C water bath for a further 5 minutes. 1 mL of LB broth was added to the cells then cells were shaken at 28°C for 1-4 hours. Cells were centrifuged then plated onto agar containing rifampicin (20 mg/L) and spectinomycin (50 mg/L). Single colonies were bulkup up overnight in 100 mL of LB broth containg rifampicin and spectinomycin.

4.2.6.6 Agro-infiltration of Nicotiana benthamiana

A. tumefaciens strain AGL-1 harbouring YFP:*HvHVP10* and p19 (kindly provided by Dr Craig C. Wood (CSIRO) was grown overnight at 28 °C in LB broth supplemented with the appropriate antibiotic and rifampicilin. Turbid cultures were supplemented with 100 µM acetosyringone and grown for a further 2 hours. Cultures were centrifuged (4000 *g* for 5 min at RT) and gently resuspended in infiltration buffer (5 mM MES, 5 mM $MgSO_4$, pH 5.7, 100 µM acetosyringone) to an optical density ~2.0. A final combination of cultures was prepared so that each *Agrobacterium* construct equalled OD 600 nm. The final mixture of *Agrobacterium* cells was infiltrated by the gentle squeezing of cultures from a 1 mL syringe barrel into the underside of fully-expanded leaves of 5-6 week-old *N. benthamiana* plants. Plants were housed in a 24°C plant growth room with overhead lighting using 9:15 h light:dark cycle, where the light intensity was 400–500 µEinsteins m^{-2} s^{-1} at the leaf surface. Typically only two leaves per plant (each ~12 cm in size) were used for infiltrations with non-ideal leaves (too old or too young) removed from the plant 1 day prior to the infiltration. Infiltrated areas of leaves, commonly 3 to 4 cm were circled by a permanent marker. YFP was visualised 2 days post infiltration.

4.2.1 Cell-type specific expression profiling of HvHVP10 in CPI-71284-48 and Barque-73

4.2.1.1 Construction of expression vectors for barley transformation

To investigate the cell-type specific expression profile of *HvHVP10* in CPI-71284-48 and Barque-73 genotypes, the promoter from each genotype was cloned into expression vectors containing either GFP or GUS and transformed into the barley accession WI4330 (University of Adelaide Barley Breeders, Adelaide, Australia). The *HvHVP10* promoter regions cloned from CPI-71284-48 and Barque-73 from pCR8/GW/TOPO TA Gateway® entry vectors were recombined into the Gateway® enabled destination vectors pMDC107 (GFP) and pMDC162 (GUS) (Curtis and Grossniklaus, 2003) was performed using Gateway® LR Clonase II enzyme mix accoding to manufactures instructions. Briefly, in a 1.5 mL microfuge tube 4 µL of purified TOPO® entry clone was added to 1 µL of destination vector and TE Buffer (pH 8.0) to a final volume of 8 µL. 1 µL of LR Clonase II enzyme mix was added and the reaction was incubated at 25°C for 1 hour. The reaction was terminated with the addition of 1 µL of Proteinase K solution and samples were incubated for 10 minutes at 37°C.

4.2.1.2 A. tumefaciens-mediated barley transformation

Plant expression vectors carrying the *HvHVP10* promoter from CPI-71284-48 and Barque-73 fused to GFP and GUS reporter constructs were transformed into the barley accession WI4330 (University of Adelaide Barley Breeders) via *A. tumefaciens*-mediated transformation, followed by the regeneration of barley plantlets in soil (Singh *et al.*, 1997, Jacobs *et al.*, 2007). Transformation of barley with plant expression vectors was conducted by the barley transformation team at ACPFG.

4.2.1.3 Analysis of Promoter:GFP Reporter Constructs

12 seeds of 6 T_1 plants were germinated on moist filter paper then grown for 10 days in supported hydroponics under control and salt stressed conditions for 3 days as previuosly described in section 3.2.2. Whole root tissues were analysed for GFP fluorecense using a using a Leica TCS SP2 confocal microscope with a GFP filter set. Images were processed using Leica TCS SP2 software and Adope PHOTOSHOP® software.

4.3 Results

4.3.1 Identifying the genetic basis for the HvNax3 QTL in CPI-71284-48 x Barque-73 DH population

4.3.1.1 PCR amplification of HvHVP10 CDS, full-length gene and 2.2 kb promoter region from CPI-71284-48 and Barque 73

The *HvHVP10* CDS was PCR-amplified from both CPI-71284-48 and Barque-73 root cDNA (Figure 4-2; A). The PCR amplified only a single product of approximately 2.3 kb in length, which was consistent with the annotated *HVP10* sequence of 2289bp (Accession No. D13472.2. NCBI Database). No PCR product was visualised in the negative control.

The full-length *HvHVP10* gene and 2.2 kb of the promoter region was isolated from CPI-71284-48 and Barque-73 gDNA. The full-length gene was amplified using the Fast start Hi-fidelity PCR system and produced a strong band of approximately 4.5 kb in length in both genotypes (Figure 4-2; B). Other bands were detected in the gel due to non-specific annealing of the primers. The promoter region was isolated from both genotypes using primers based on the sequence for cv. Morex (Appendix 8.5). Morex was included as a positive control in the PCR reaction. Three different primer pairs amplifying different lengths of the promoter region produced the expected size fragments in each of the reactions, ranging in size from 2.3 kb to 1.2 kb (Figure 4-2; C). Other bands were detected in the gels arising from non-specific annealing of primers.

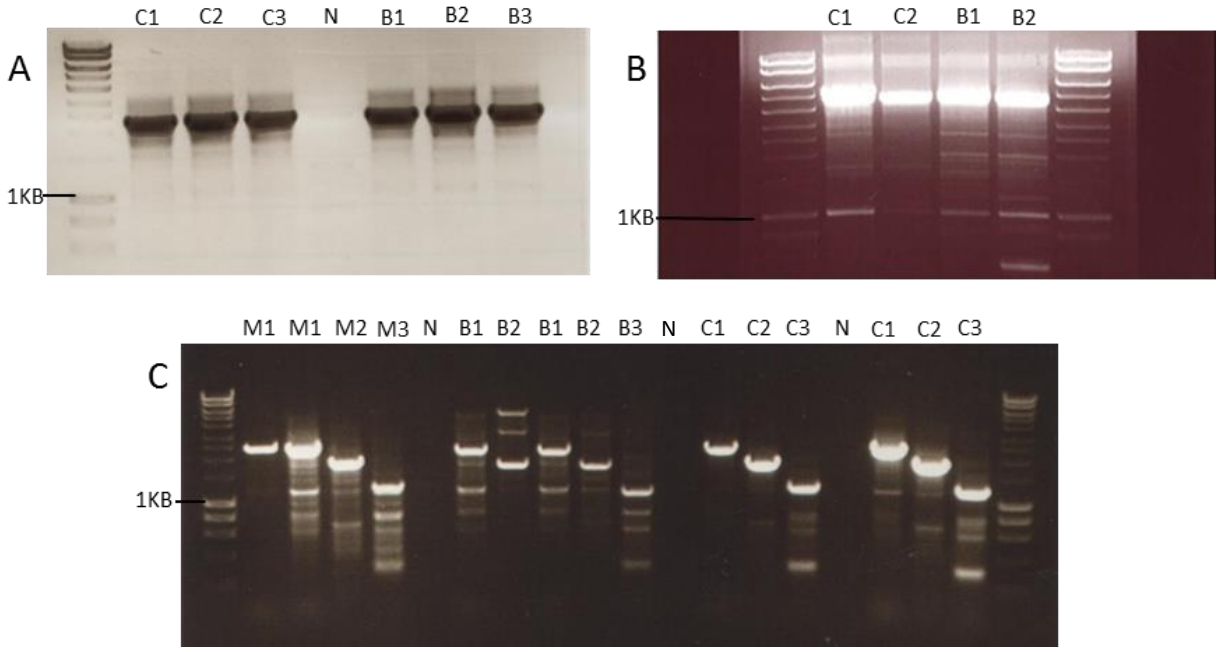

Figure 4-2 PCR amplification of *HvHVP10*. (A) CDS (B) full-length gene and (C) promoter regions from CPI-71284-48 (C#) and Barque -73 DNA (B#). Template cDNA was synthesized from RNA isolated from root tissue of CPI-71824-48 (C1-3) and Barque-73 (B1-3) plants grown hydroponically until 3rd leaf emergence and salt stressed for 3 days. gDNA for *HvHVP10* full-length gene and promoter isolations was extracted using phenol-chloroform extraction from the first leaf of germinated CPI-71824-48 and Barque-73 seedlings. 1KB: DNA Hyperladder 1 (Bioline), M1-3: Morex (positive control), N: negative control.

4.3.1.2 Generation of pCR8/GW/TOPO TA Gateway® entry vectors carrying HvHVP10 CDS, full-length gene and promoter regions from CPI-71284-48 and Barque -73

The PCR products from CPI-71284-48 and Barque-73 cDNA (*HvHVP10* CDS) and gDNA (*HvHVP10* full-length gene and promoter) were cloned into pCR8/GW/TOPO TA Gateway® entry vectors. The construction of pCR8/GW/TOPO TA Gateway® entry vectors was undertaken for a number of purposes, including sequence analysis of the *HvHVP10* CDS, full-length gene and promoter region from CPI-71284-48 and Barque-73; as well as the generation of entry clones for use in expression systems and other downstream applications, such as the generation of *HvHVP10* OEX lines outlined in the following chapter (Section 5.3.1). The integration and orientation of the *HvHVP10* CDS, full-length gene and promoter regions from CPI-71284-48 and Barque-73 into the pCR8/GW/TOPO TA Gateway® entry vectors was

confirmed via restriction enzyme digestion. The integration of the *HvHVP10* CDS and full-length gene into gateway entry clones was confirmed with the restriction enzyme *Blp*I. Insertion of the *HvHVP10* CDS in the correct orientation produced digested fragments of 3365 and 1741 bp (Figure 4-3; A) and the correct orientation of the *HvHVP10* full-length gene produced digested products of 5055 bp and 2092bp (Figure 4-3; B). The restriction enzymes *Bam*HI and *Eco*RV were used for entry clones carrying the *HvHVP10* promoter region, where correct orientation of the insert produced 2 digested fragments of 4728 bp and 476 bp (Figure 4-3; C).

At least 3 clones from 3 independent PCR reactions were sequenced to obtain a consensus *HvHVP10* CDS, full-length gene and promoter sequence for each genotype and to identify errors which may have been introduced during the cloning process. Plasmids carrying the *HvHVP10* CDS or full-length gene from CPI-71284-48 and Barque-73 were sequenced using the primers PYR-20-F, PYR-3, PYR-4 PYR-20-R (as described in Table 5-2), with additional primers PYR-1 and PYR-7 for full-length gene clones. Promoter clones were sequenced using the primers PYR-9, PYR-10, PYR-11 and HVP10-prom-rev.

Once a plasmid carrying the correct *HvHVP10* CDS was identified, the *HvHVP10* CDS minus the stop codon was amplified from the plasmid and cloned into pCR8/GW/TOPO TA Gateway® entry vectors for use in transient expression systems. The correct CDS from Barque-73 was used for downstream applications as the CDS from CPI-71284-48 contained a SNP introduced during the cloning process.

The integration of the full-length *HvHVP10* gene into pCR8 entry vectors displayed very low transformation efficiency, possibly due to the size of the product being transformed. A total of 17 clones carrying the Barque-73 full-length gene and 16 clones carrying the CPI-71284-48 full-length gene were successfully cloned into pCR8/GW/TOPO TA Gateway® entry vectors in the correct orientation. A consensus sequence was obtained for the *HvHVP10* full-length gene from both genotypes. However, all clones contained SNPs introduced during PCR amplification or the cloning process; therefore they could not be used for downstream applications.

Approximately 2200 bp of the promoter region of *HvHVP10* from CPI-71284-48 and Barque-73 were cloned into pCR8/GW/TOPO TA Gateway® entry vectors. The primer pair PYR-9

and HVP10-prom-rev produced different sized PCR products in CPI-71824-48 and Barque-73 (2256 bp and 2154 bp respectively). A consensus sequence was obtained for each promoter and plasmids carrying the correct promoter sequence were identified for Gateway cloning into expression vectors carrying GUS and GFP reporter genes.

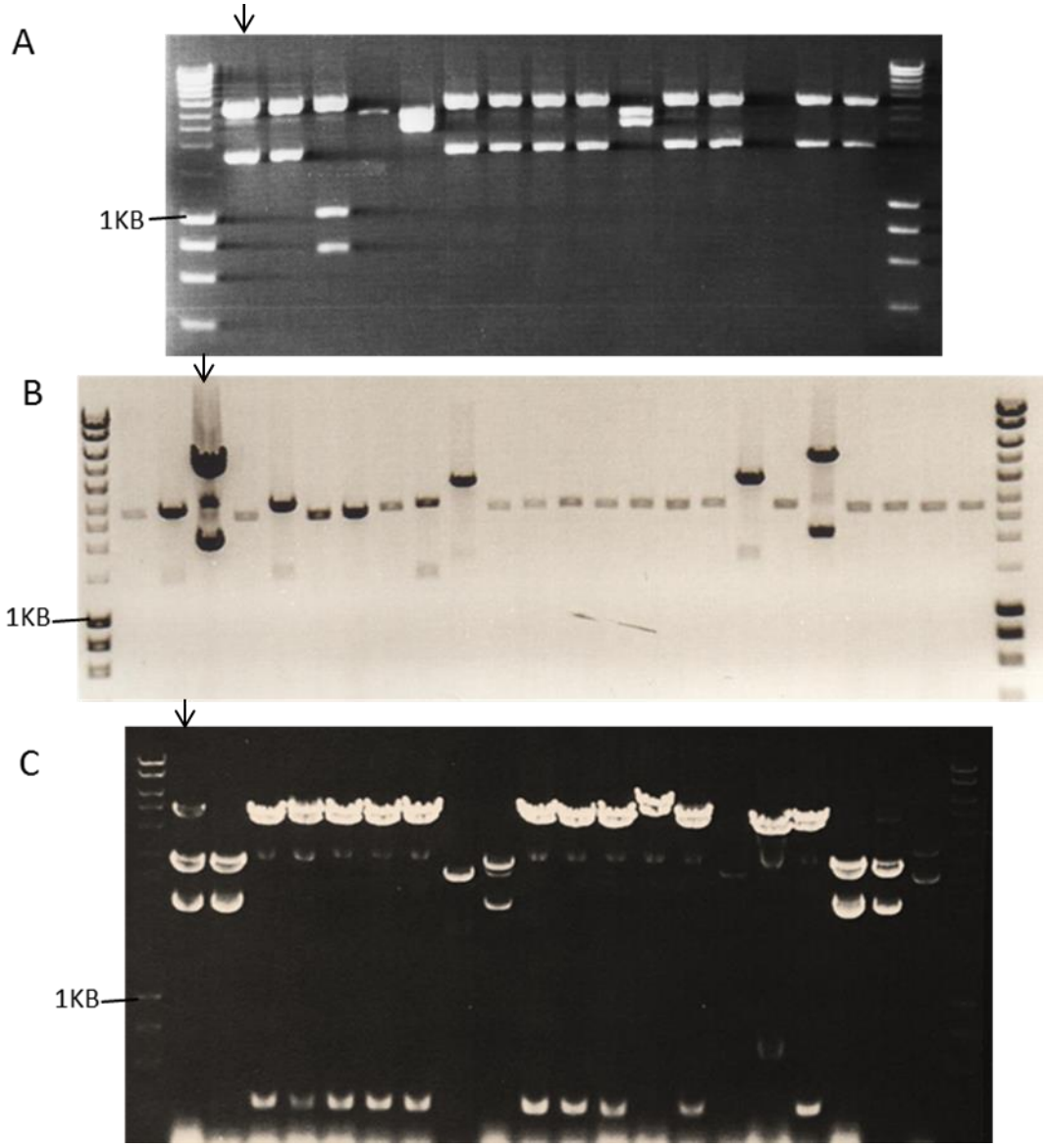

Figure 4-3 Restriction enzyme digestions of pCR8 entry clones carrying the *HvHVP10* A) CDS, B) full-length gene and C) promoter regions from CPI-71284-48 and Barque-73. A. lanes 1-8 is CDS from CP-71284I and lanes 9-15 is CDS from Barque-73. B. lanes 1-12 is FLG (Full Length Gene) from CPI-71284-48 and lanes 13-24 is FLG from Barque-73. C. lanes 1-10 is promoter region from CPI-71824-48 and lanes 11-20 is promoter region from Barque-73. Plasmid DNA was digested with *Blp*I for the CDS and full-length gene and *Bam*HI and *Eco*RV for promoter constructs. Arrows indicate clones of the correct size and orientation on each gel.

4.3.1.3 Sequence analysis of HvHVP10 CDS indicates the HvHVP10 Protein is highly conserved

The coding sequence of *HvHVP10* isolated from CPI-71284-48 and Barque cDNA consisted of an ORF of 2289 bp (Appendix 8.3), corresponding to 762 amino acids (Figure 4-4) with 14 predicted transmembrane helices (Figure 4-5) (TMHMM Server v. 2; http://www.cbs.dtu.dk/services/TMHMM/). Sequence analysis comparing the *HvHVP10* CDS from CPI-71824-48 and Barque-73 identified 7 SNPs between the 2 genotypes, however no SNPs altered the amino acid sequence, indicating that allelic variation in the *HvHVP10* CDS is not responsible for the sodium exclusion QTL (*HvNax3*).

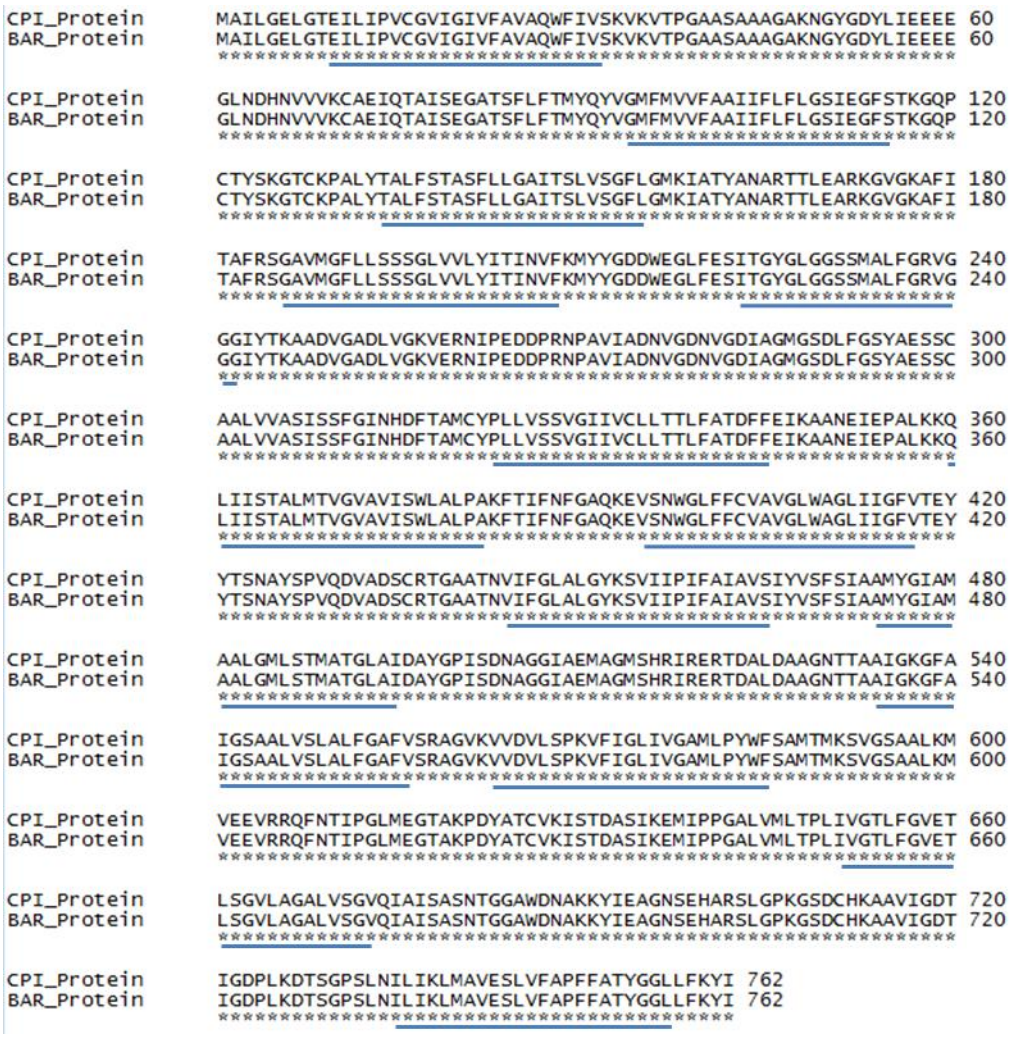

Figure 4-4 Multiple sequence alignment of protein sequences of *HvHVP10* from CPI-71284-48 (CPI_Protein) and Barque 73 (BAR_Protein), showing identical amino acid sequences. Alignment was constructed using ClustalW (NCBI website). Blue lines indicate transmembrane helices predicted using TMHMM Server v. 2.0.

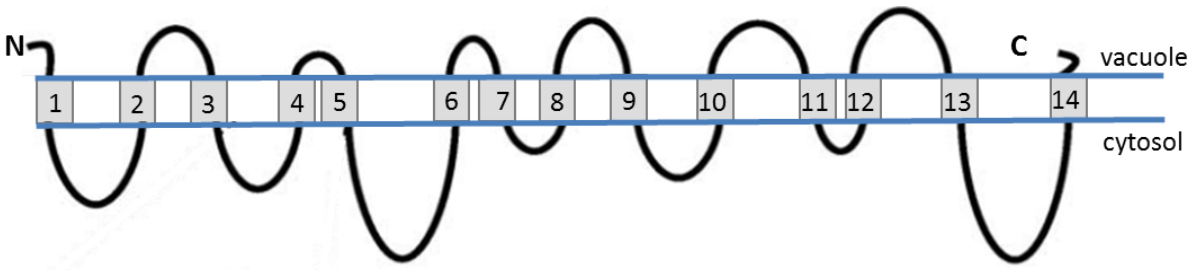

Figure 4-5 Topological model of *HvHVP10* containing 14 transmembrane domains predicted using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Adapted from Van *et al.* (2005).

4.3.1.4 Identification of other V-PPase proteins in Barley

The *HvHVP10* protein sequence from CPI-71824-48 and Barque-73 was blasted against the draft barley genome sequence (The International Barley Genome Sequencing Consortium, 2012), using the Barley Blast Server (http://webblast.ipk-gatersleben.de/barley/), to identify protein sequences homologous to *HvHVP10* in barley. As expected, contigs corresponding to the previously isolated barley cDNA clones for HVP10 (GenBank Accession No. D13472.2) and HVP1 (GenBank Accession No. AK360389) were identified. The blast search identified three additional predicted barley vacuolar pyrophosphatase sequences (

Table 4-3), denoted HVP3 (GenBank Accession No. AK362588.1), HVP4 (GenBank Accession No. AK375042.1) and HVP5 (GenBank Accession No. AK363930.1). The protein sequences of barley V-PPases range in length between 762 amino acids for HVP10 to 799 amino acids for HVP5 . Proteins for HVP10 and HVP1 (771 amino acids) shared the highest homology, with 86% sequence identity. By contrast, HVP10 shared only 38% homology with HVP5. Both HVP10 and HVP1 are located on chromosome 7H (long arm and short arm, respectively) and contain 14 or 15 transmembrane domains. HVP3 and HVP4 were shown to both be located on chromosome 1H, with 13 predicted transmembrane helices. These proteins share 75% and 74% homology with HVP10, respectively. HVP5 is located on chromosome 6H and is predicted to have 14 transmembrane domains.

Table 4-3 Vacuolar pyrophosphatase sequences in barley identified using IPK Barley Blast Server (http://webblast.ipk-gatersleben.de/barley/) (e-value threshold <0.01)

V-PPase	GenBank Accession no.	Morex contig	Amino Acid Length	Chromosome	TMH	Sequence identity with HVP10
HVP1	AK360389	137615	771	7HL	15	86%
HVP10	D13472.2	2547568	762	7HS	14	NA
HVP3	AK362588.1	2552365	775	1H	13	75%
HVP4	AK375042.1	60645	763	1H	13	74%
HVP5	AK363930.1	46291	799	6H	14	38%

TMH - Transmembrane domains prediction for V-PPases in Barley using TMHMM Server v 2.0

4.3.1.5 Sequence alignment of the vacuolar pyrophosphatase proteins in barley confirms the presence of highly conserved sequences

Multiple sequence alignment of the barley vacuolar pyrophosphatases HVP1 and HVP10 with the three putative barley V-PPase proteins using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) revealed that the first 60 residues at the N-terminus of the protein were the least conserved (Figure 4-6). Multiple sequence alignment also confirmed the presence of highly conserved motifs common to all V-PPase proteins (Maeshima, 2000, Nakanishi *et al.*, 2001, Fukuda *et al.*, 2004, Zancani *et al.*, 2007). The first conserved sequence (CS1) is located in cytosolic loop (CL3; Figure 4-6) and contains the motif (DVGADLVGKVE), the putative PPi binding site (Maeshima, 2000). The second conserved sequence (CS2) is also located in a hydrophilic loop (CL5). The third segment (CS3) is located in the carboxyl-terminal part of the protein (CL7) and contains 12 charged residues, similar to mung bean V-PPase (Maeshima, 2000)

Other common conserved sequences within the barley V-PPase were also identified (Figure 4-7). *HvHVP1* (HVP1), *HvHVP10* (HVP10), *HvHVP3* (HVP3: AK362588.1) and *HvHVP4* (HVP4: AK375042.1) all shared the conserved sequence EYYTS, however for *HvHVP5* (HVP5: AK363930.1), this sequence is replaced with KYYTD (Drozdowicz and Rea, 2001). The conserved sequence DX3DX3D (Nakanishi *et al.*, 2001, Zancani *et al.*, 2007) was located on CL5 and CL7 at the C-terminus of the protein. The putative 14-3-3 protein ligand-binding sequence RQFNTIP (Venter *et al.*, 2006) was also detected in HVP1 and HVP10. This sequence differed by 1 amino acid in HVP3 (RQF*TIP), 2 amino acids in HVP4 and 3 amino

acids in HVP5. The conserved sequence GDT(I/V)GDPXKDTXGP (Zancani *et al.*, 2007) was very highly conserved in HVP1, 10, 3 and 4, however it differs by 2 amino acids in HVP5.

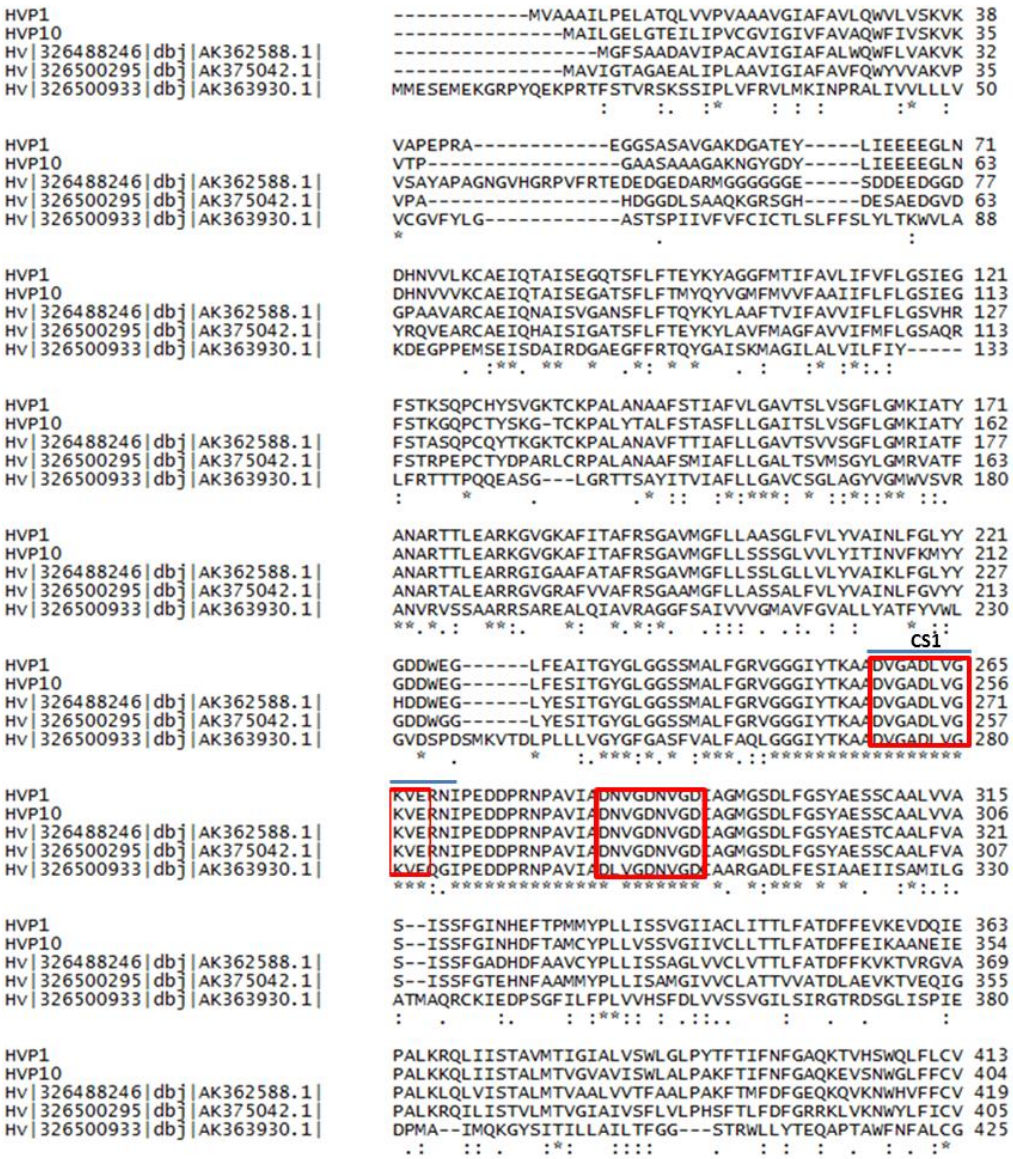

Figure 4-6 Amino acid alignment of vacuolar pyrophosphatase proteins in barley. The blue underlined region shows conserved sequence (CS1) proposed as the putative catalytic site of V-PPase (DVGADLVGKVE) (Maeshima, 2000). The other conserved sequence DXVGDNVGD, an acidic motif involved enzyme function (Nakanishi *et al.*, 2001)is also highlighted. AK362588.1, AK375042.1 and AK363930.1 are HVP3, HVP4 and HVP5 respectively.

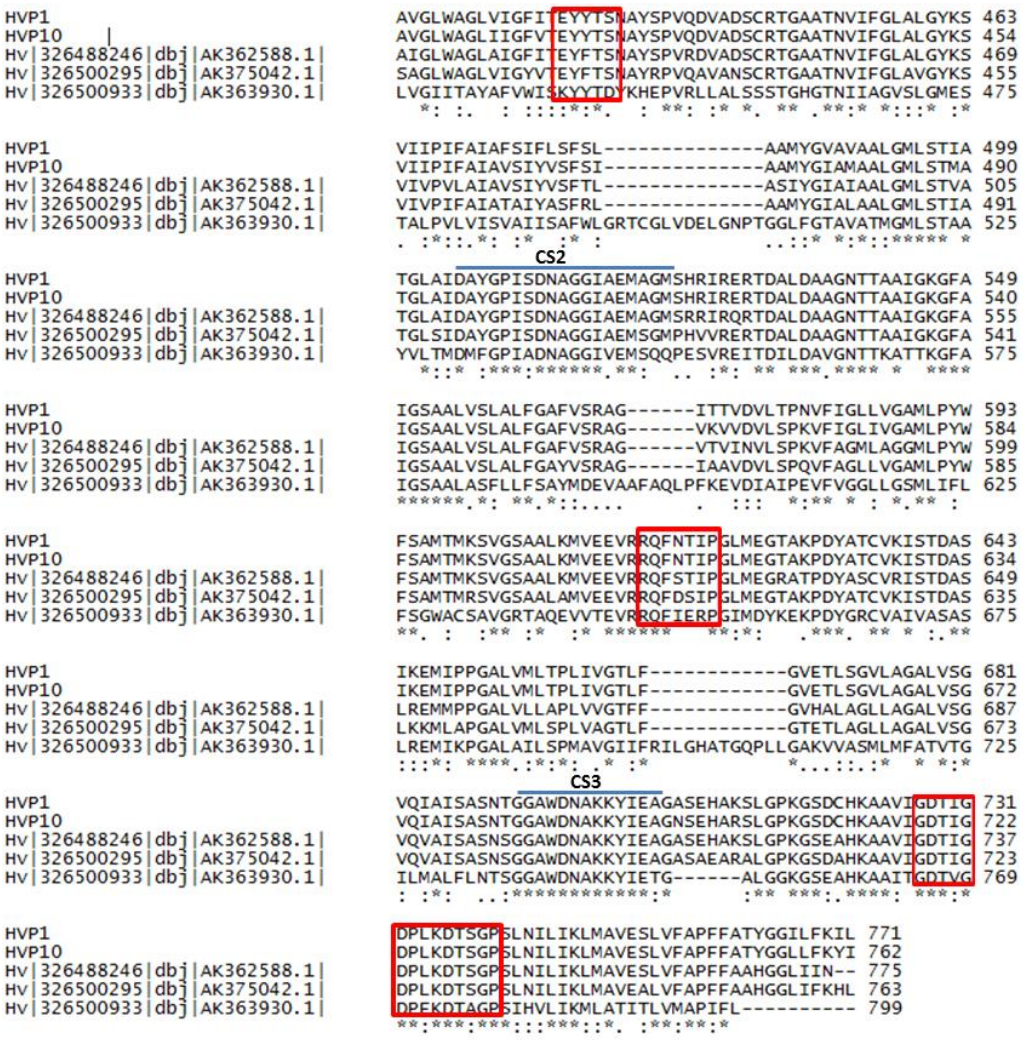

Figure 4-7 Amino acid alignment of vacuolar pyrophosphatase proteins in barley. The blue underlined regions show conserved sequence CS2 and CS3 (Maeshima, 2000). The highlighted region EYYTS replaced in HVP5 by KYYTD in K^+-insensitive V-PPases (Drozdowicz and Rea, 2001). The other conserved sequences RQNTIP (Venter *et al.*, 2006)and GDTIGDPLKDTSGP (Zancani *et al.*, 2007) are also highlighted. AK362588.1, AK375042.1 and AK363930.1 are HVP3, HVP4 and HVP5 respectively.

4.3.1.6 Phylogenetic analysis of vacuolar H^+-pyrophosphatases from barley and other plant species

The V-PPase amino acid sequences from barley and other monocot and dicot species were compiled and examined for phylogenetic relationships between V-PPase members. Identification of homologues was based primarily on sequence similarity between the five Barley V-PPases and the predicted amino acid sequences of V-PPases from other species. Rice (*Oryza sativa* L.), Brachypodium (*Brachypodium distachyon*) and sorghum (*Sorghum bicolor*) protein sequences were downloaded from public databases, including Phytozome

(https://phytozome.jgi.doe.gov/), NCBI (http://www.ncbi.nlm.nih.gov/) and Ensemble Plants Brachypodium distachyon (v1.0) (https://plants.ensembl.org/Brachypodium_distachyon/Info/Index)

Phylogenetic analysis revealed that the V-PPase protein sequences clustered into two distinct groups (Figure 4-8), as previously described (Drozdowicz and Rea, 2001). In addition, each V-PPase member from barley clustered with a single member from Brachypodium, rice and sorghum. Phylogenetic analysis showed that *HvHVP10* formed a clade with *Bradi1g47767.1* (a putative molecule located on chromosome 1 of Brachypodium. It has a protein length of 762 amino acids and shares 95% sequence identity with *HvHVP10*), *Sobic.010G060600* (located on Chromosome 10 of *Sorghum bicolor*. It has a protein length of 763 amino acids and shares 93% sequence identity with *HvHVP10*) and rice *LOC Oso6g08080.1* (located on Chromosome 6. It has a protein length of 767 amino acids and shares 92% sequence identity with *HvHVP10*.

Protein alignment of *HvHVP10* (using Clustal Omega) with the closest Brachypodium, rice and sorghum orthologues indicated the V-PPase protein is the least conserved within the first 60 residues of the N terminal domain of the protein. This is consistent with the results of Fukuda *et al.* (2004), who also reported that *HvHVP10* and *HvHVP1* shared the least sequence identity within the first 60 amino acids of the protein.

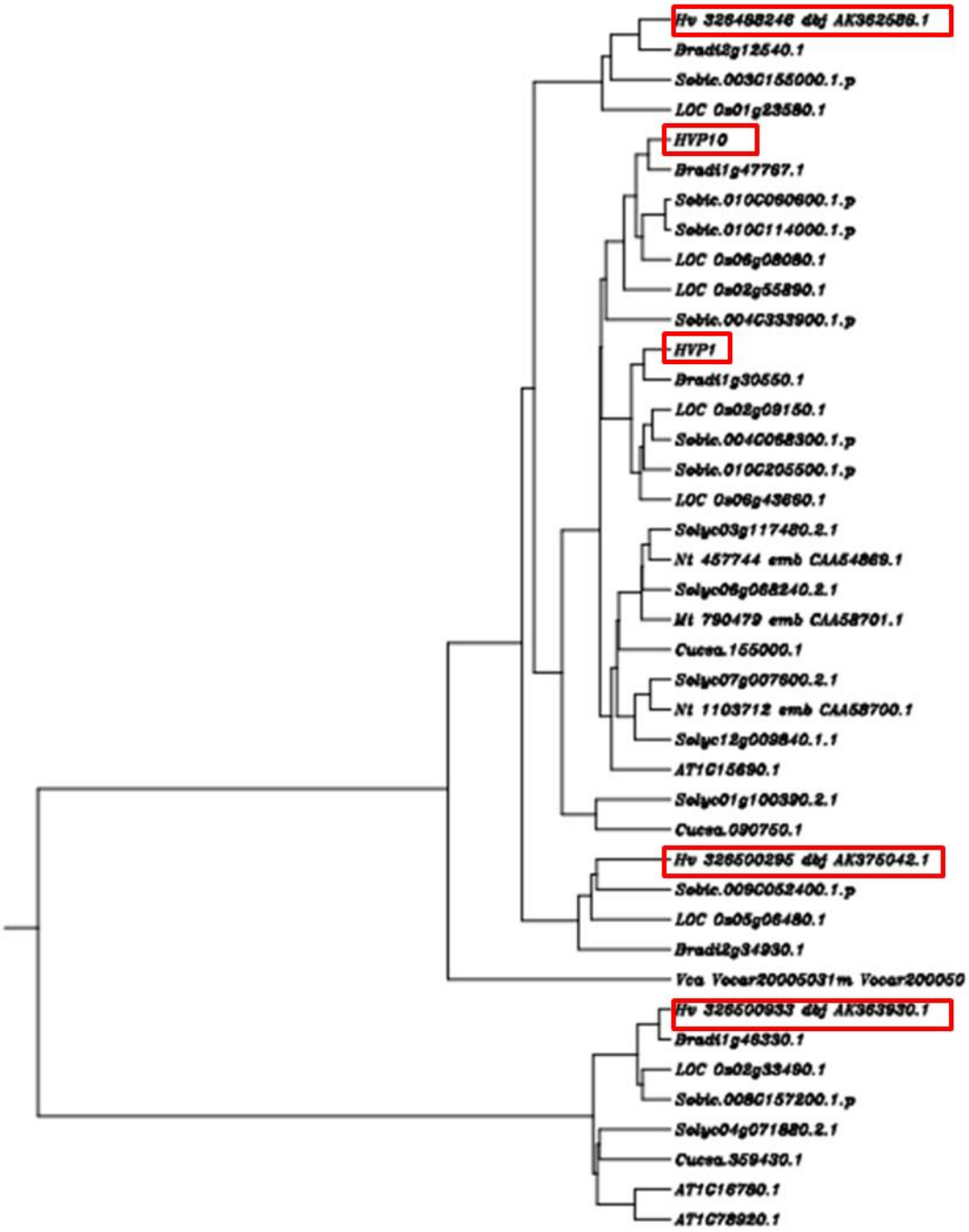

Figure 4-8 Unrooted phylogenetic tree of V-PPase protein sequences in selected monocot and dicot species. (Hv = *Hordeum vulgare*; Bradi = *Brachypodium distachyon*; Sobic = *Sorghum bicolor*; LOC = *Oryza sativa*; Solyc = *Solanum lycopersicum*; Nt = *Nicotiana tabacum;* Cucsa = *Cucumis sativus*; AT = *Arabidopsis thaliana*). Phylogenetic tree created using Genome workbench – NCBI (https://www.ncbi.nlm.nih.gov/tools/gbench/). The red

boxes highlight the five HVPs. AK362588.1, AK375042.1 and AK363930.1 are HVP3, HVP4 and HVP5 respectively.

4.3.1.7 Sequence analysis of HvHVP10 full-length gene in CPI-71284-48 and Barque-73

The full-length *HvHVP10* gene from CPI-71284-48 and Barque-73 was determined via sequencing of multiple pCR8/TOPO/GW1 Gateway® entry clones. In order to identify exon/intron boundaries within the *HvHVP10* full-length gene, an mRNA (CDS) to genomic alignment was performed using SPIDEY (http://www.ncbi.nlm.nih.gov/spidey/). SPIDEY is an NCBI database that predicts intron/exon boundaries using a BLAST alignment engine for splice site selection based on plant-based algorithms. The full-length *HvHVP10* gene from CPI-71284-48 and Barque-73 (Figure 4-9) consists of 8 exons and 7 introns (Table 4-4). The co-ordinates of the exon-intron boundaries varied slightly between the two genotypes.

Table 4-4 mRNA to genomic alignment of *HvHVP10* CDS and full-length gene from CPI-71824-48 and Barque-73 (predicted using SPIDEY)

Exon	Genomic coordinates CPI-71284-48	Genomic coordinates Barque-73	mRNA coordinates
1	1-241	1-241	1-241
2	1635-2206	1640-2211	242-813
3	2340-2719	2345-2725	813-1193
4	2799-2885	2804-2890	1194-1280
5	2971-3301	2975-3305	1281-1611
6	3499-3909	3503-3913	1612-2022
7	3986-4045	3997-4056	2023-2082
8	4154-4360	4162-4368	2083-2289

Figure 4-9 *HvHVP10* gene structure from CPI-71284-48 (predicted using SPIDEY) with in/dels highlighted for Barque-73 (alignment conducted using ClustalW2)

Sequencing of a BAC clone from cv. Morex allowed comparison of the full-length *HvHVP10* gene from CPI-71284-48 and Barque-73 with the Morex *HvHVP10* gene sequence (Appendix 8.4). The *HvHVP10* gene was 4360 bp in length for both Morex and CPI-71284-48, whereas for Barque-73 the *HvHVP10* gene was 4367 bp.

Multiple sequence alignment of *HvHVP10* gene sequence from CPI-71824-48, Barque-73 and Morex confirmed that the *HvHVP10* gene is highly conserved within the exons. In total there were eight SNPs between the 3 genotypes, none of which altered the amino acid sequence: two SNPs exon 1, two SNPs exon 2, one SNP exon 3, one SNP in exon 6, two SNPs exon 8. Exons, 4, 5 and 7 were identical between CPI-71284-48, Barque-73 and Morex.

Despite conservation of the gene in the coding regions, a number of insertions, deletions and SNPs were observed within the non-coding regions. Intron 1 is the longest and least conserved between the genotypes with 14 SNPs. Barque-73 also contained a number of in/dels not present in CPI-71284-48, namely a 3 bp insertion in intron 1 (423bp) and an insertion of 7 bp in the 6th intron (3929bp) (Figure 4-9). Barque also had a deletion of 3 bp in the 7th intron (4142bp). Other SNPs were detected within the non-coding regions with two SNPs in intron 2, four SNPs in intron 3 and one SNP in intron 4.

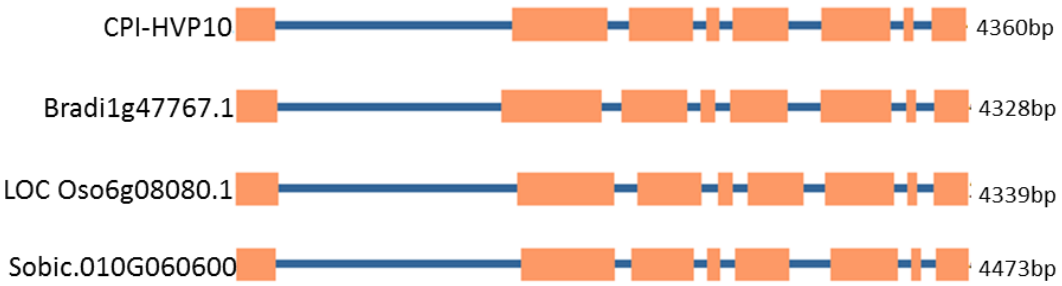

Figure 4-10 Gene structure of *HvHVP10* from CPI-71824-48 and orthologous V-PPase genes from Brachypodium (Bradi1g47767.1), rice (LOC Oso6g08080.1) and *Sorghum bicolor* (Sobic.010G060600). Exon/intron boundaries were predicted using SPIDEY (http://www.ncbi.nlm.nih.gov/spidey/). Drawn to scale.

The genetic structure of the *HvHVP10* full-length gene form CPI-71284-48 was compared with V-PPases orthologues from other species (Figure 4-10). The V-PPase CDS and full-length gene sequences for the closest Brachypodium, rice and *Sorghum bicolor* orthologues were obtained from NCBI and Phytozome databases and analysed for exon/intron boundaries using SPIDEY (http://www.ncbi.nlm.nih.go v/spidey/) *HvHVP10*, and its closest orthologue from Brachypodium, *Bradi1g47767.1*, and rice, *LOC Oso6g08080.1* all had gene sequences of similar lengths, of between 4330 and 4360 bp (Brachypodium = 4328 bp, and rice = 4339 bp). The *Sorghum bicolor* homologous gene *(Sobic.010G060600*) was approximately 100bp longer (4473b bp). All mRNA to genomic alignments of V-PPase sequences produced single splice variants. Comparison of the homologous genes with *HvHVP10* revealed that gene structure is highly conserved between species, with all V-PPase members comprising eight exons and seven introns. Intron 1 and Intron 5 were the most variable in length between the V-PPase orthologues.

Multiple sequence alignment of the V-PPase gene sequences was conducted using MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/). The full-length *HvHVP10* gene from CPI-71284-48 shared 84% sequence identity with the Brachypodium orthologue (*Bradi1g47767.1*), 79% sequence identity with the rice orthologue (*Oso6g08080.1*) and 76% sequence identity with the *Sorghum bicolor* homologous gene *(Sobic.010G060600*). Sequence alignment of the full-length V-PPase genes also revealed that the genes were the least conserved at the start of the gene (N-terminus). A large insertion in intron 5 was also detected in the *Sorghum bicolor*

homologous gene (*Sobic.010G060600*) which was not present in the other V-PPase orthologues.

4.3.1.8 HvHVP10 gene copy number variation between CPI-71284-48 and Barque-73

Southern blot hybridisation was used to determine endogenous *HvHVP10* gene copy number within CPI-71284-48, Barque-73 and advanced backcross AB-QTL (BC_2F_2) lines 18D/014 (CPI-71284-48 allele) and 18D/011 (Barque-73 allele). Other gDNA samples from a range of genetic backgrounds were also included in the Southern screen, including the barley genotypes Morex, and WI4330, Chinese Spring wheat (*Triticum aestivum* L.), Betzes barley (*H. vulgare* L.), and a Chinese Spring-Betzes disomic chromosome addition line containing chromosome 7H from barley (cv. Betzes) (Figure 4-10). Genomic DNA was digested with the restriction enzymes *Hind*III and *Xba*I and the PCR-amplified product of the primer pair PYR-9 and PYR-12 (Table 4-2) was used for the hybridisation probe. The target sequence flanked by this primer pair corresponds to a region of approximately 272 bp in the middle of the gene, from exon 4 (2824 bp) to exon 5 (3096 bp) and spanning the 4th intron. Multiple alignment of this sequence with the other barley V-PPase identified in this study indicates that this probe potentially hybridises to the other V-PPase orthologues (sequence identity between 34 and 84%), which significantly confounds our results. Unfortunately, these sequences were not available at the time of Southern Blot analysis and the probe was believed to be specific to *HvHVP10*.

The presence of multiple hybridisation bands in both the *Hind*III and *Xba*I restriction digestions (Figure 4-11), however does suggest that all of the barley genotypes screened may contain multiple copies of the *HvHVP10* gene. Furthermore, Southern blot analyses indicate that there are potential differences in *HvHVP10* gene copy number between CPI-71284-48 and Barque-73, where CPI-71284-48 appears to have less copies of *HvHVP10* than Barque-73. A similar pattern of hybridisation was observed between Barque-73 and its AB-QTL line 18D/011 whereas and the AB-QTL line carrying the CPI-71284-48 allele (18D/014) contains similar bands to CPI-71284-48 and Barque-73, confirming the probe is targeting the area of the *HvHax3* QTL on chromosome 7H. Nothing was detected in wheat cultivar Chinese Spring, as expected.

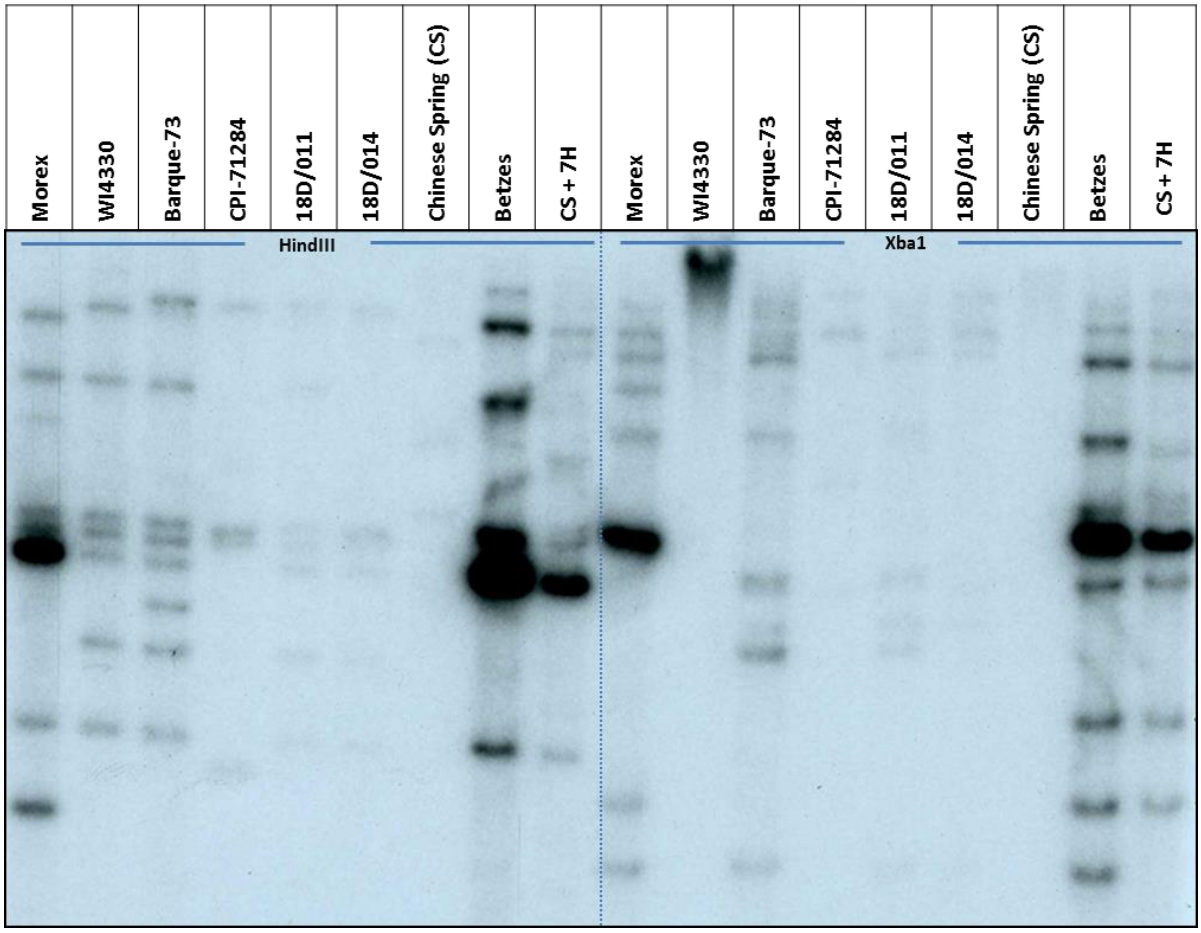

Figure 4-11 Southern blot analysis of selected barley genotypes and wheat cultivar Chinese spring and Chinese Spring addition line containing chromosome 7H from barley (cv. Betzes). The restriction enzyme *Hind*III was used to digest genomic DNA on the left of dotted line The restriction enzyme *Xba*I was used to digest genomic DNA on the right of the dotted line. The PCR-amplified product of the *HvHVP10* fragment using primers PYR-9 + PYR-12 was radiolabelled with [α-^{32}P]dCTP and was used as a probe.

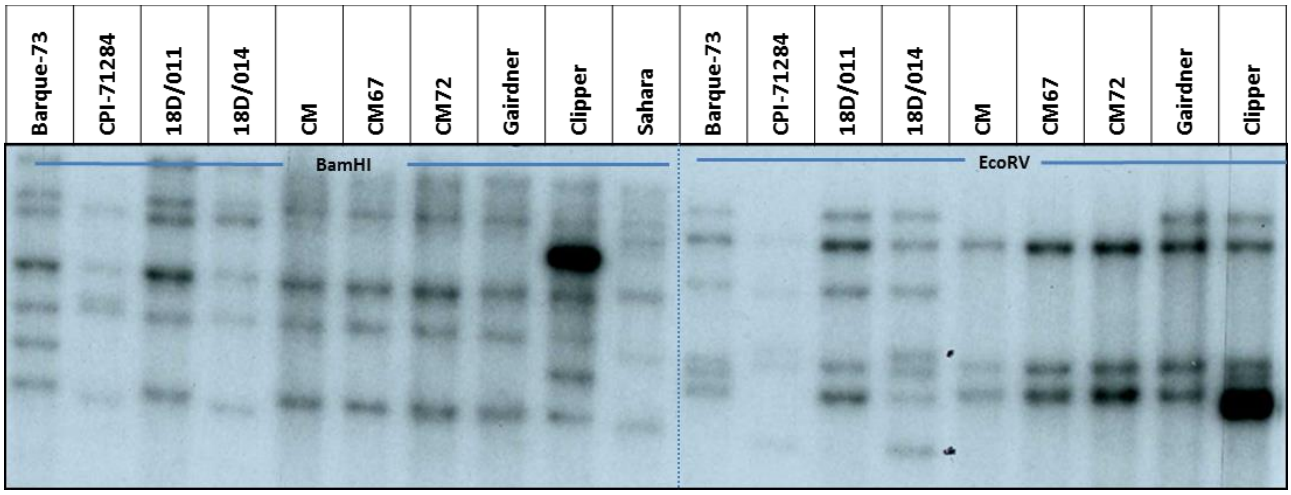

Figure 4-12 Southern blot analysis of selected barley genotypes. The restriction enzyme *BamHI* was used to digest genomic DNA in lanes 1-10 and the restriction enzyme *EcoRV* was used to digest genomic DNA in lanes 11-19. The PCR-amplified product of the *HvHVP10* fragment using primers PYR-9 + PYR-12 was radiolabelled with [α-^{32}P]dCTP was used as a probe. Black dots on 18D/014 represent CPI-71284-48 derived *HvHVP10* fragments.

CPI-71284-48, Barque-73 and advanced Backcross AB-QTL lines 18D/014 (CPI-71284-48 allele) and 18D/011 (Barque-73 allele) and a range of selected barley genotypes were also screened via southern blot using the restriction enzymes *BamHI* and *EcoRV* (Figure 4-12). Barley genotypes CM, CM67, CM72 (North Africa) and Gardiner (Australia) were also screened and all have similar patterns of hybridisation. Again, the AB-QTL lines displayed a similar pattern of hybridisation to their parental line (18D/014 (CPI-71284-48 allele) and 18D/011 (Barque-73 allele). The presence of multiple hybridisation bands, and therefore multiple copies of *HvHVP10* gene, confirm the previous results. These results also suggest that there are fewer copies of *HvHVP10* in CPI-71284-48 than in Barque-73.

4.3.1.9 HvHVP10 promoter regions in CPI-71284-48 and Barque-73 differ significantly

The *HvHVP10* promoter region (≈2200 bp) from CPI-71284-48 and Barque-73 was determined via sequencing of multiple pCR8/TOPO/GW1 Gateway® entry clones carrying amplified promoter products. Sequencing of these pCR8/TOPO/GW1 Gateway® entry clones revealed that the *HvHVP10* promoter regions of CPI-71284-48 and Barque-73 differed significantly. The most significant differences between the *HvHVP10* promoter regions were

due to the presence of 2 large insertions in Barque-73, which are not present in CPI-71284-48. The presence of these insertions in Barque-73 resulted in a larger PCR product of 2256 bp, compared to that of CPI-71284-48 of 2154 bp, despite the fact that the same primer pair was used on gDNA from both genotypes (Figure 4-2). Sequencing of multiple entry clones identified the first insertion of 63bp at 1390bp and a second insertion of 31 bp (at -1794 bp) in Barque-73 (relative to the translation start site) (Figure 4-13). Further sequencing revealed that the *HvHVP10* promoter region was very highly conserved between the two genotypes at -2200 to -1700 bp, however, closer to start of gene is less conserved due to the presence of a SNP at position -8 in Barque-73, which alters the nucleotide sequence from an A in CPI-71284-48 to a T. The alignment of the promoter *HvHVP10* regions from CPI-71284-48 and Barque-73 is contained within (Appendix 8.5).

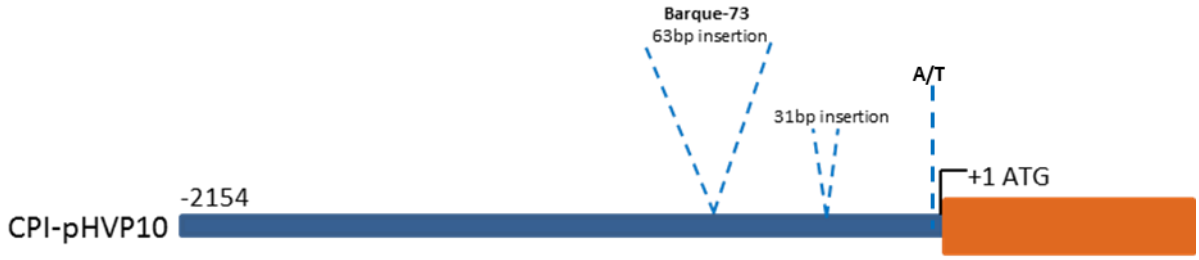

Figure 4-13 Schematic diagram of -2154 bp of the *HvHVP10* promoter region in CPI-71284-48 showing the position of 2 large insertions present in Barque-73 *HvHVP10* promoter region.

4.3.1.10 Promoter Motif analysis

The promoter regions from CPI-71284-48 and Barque-73 were compared using Matinspector Release professional 8.0.5 on Genomatix Software Suite website (https://www.genomatix.de/solutions/genomatix-software-suite.html.). Matinspector is a software tool that utilises a large library of matrix descriptions for transcription factor binding sites to locate matches in DNA sequences. Similar and/or functionally related transcription factor binding sites are grouped into matrix families and the software allows the user to analyse sequences using predefined libraries. Two libraries were used to compare CPI-71284-48 and Barque-73 promoter sequences;

A. Matrix Family Library Version 8.4 (Selected groups = General Core Promoter Elements (core/matrix sim = optimised for plants (0.75/Optimised)

B. Plant IUPAC Library Version 7.0 (based on PLACE Release 30.0) (Selected groups = PLACE) (search parameters = max.0% mismatches)

Matinspector analysis gave a graphical representation of results (

). The main differences in the transcription factor binding sites identified between the two genotypes were due to the two large insertions present in Barque-73, as expected and slight differences at the start of the promoter region just downstream of ATG start site of *HvHVP10*. Both Libraries gave similar results in terms of the identification of transcription factor binding sites, suggesting there is a lot of cross-over between libraries; however the 2nd Library gave more information regarding the transcription factor binding site core sequence. The first library, General Core Promoter Elements (optimised for plants), was selected for further analysis to directly compare general transcription factor binding sites in CPI-71284-48 and Barque-73 promoter regions.

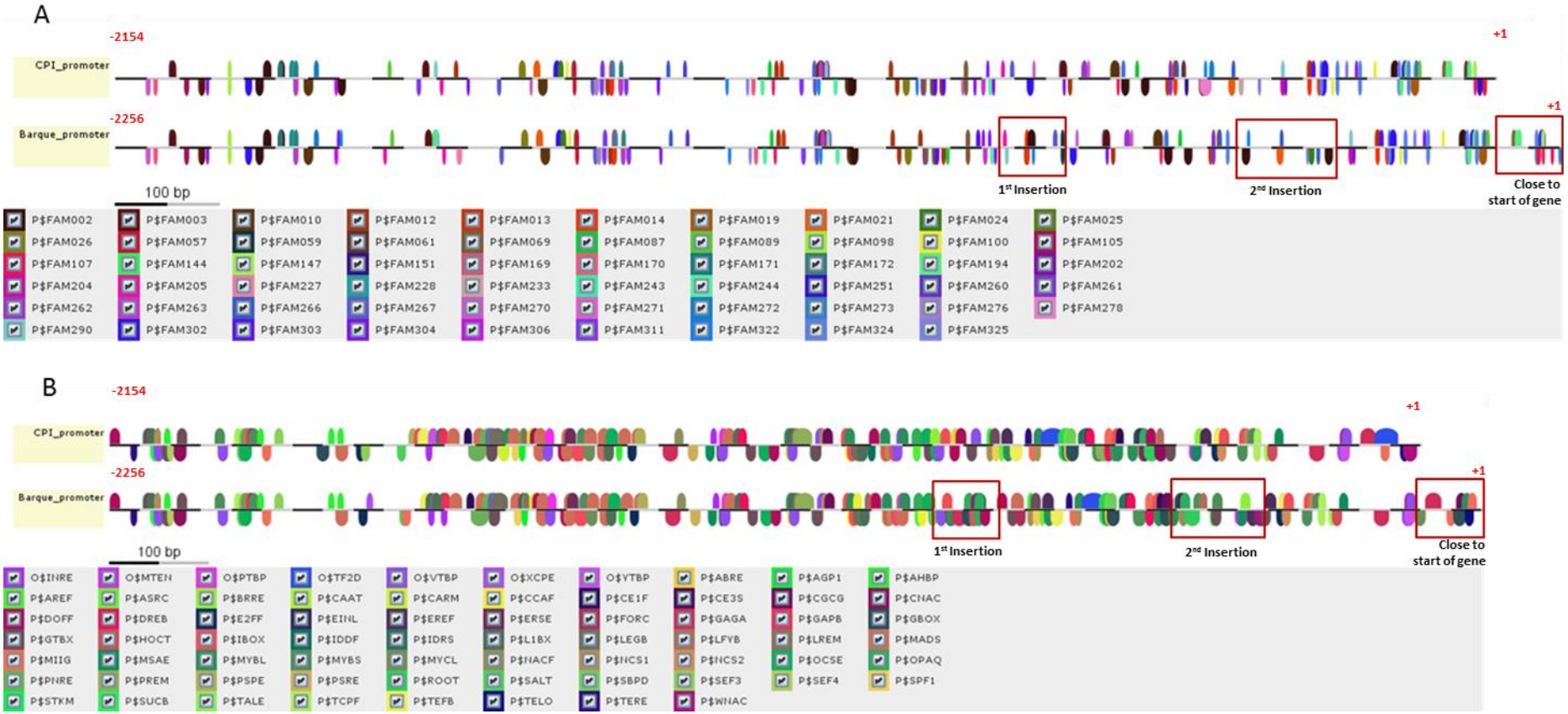

Figure 4-14 Motif analysis of *HvHVP10* promoter regions from CPI-71284-48 and Barque-73 using Matinspector (release professional 8.0.5 https://www.genomatix.de/matinspector.html.; Date accessed: March 2011). A General core promoter elements optimised for plants. B Plant cis-acting regulatory DNA elements (PLACE). Highlighted regions correspond to the two large insertions present in Barque-73 and variation between CPI-71284-48 and Barque-73 close to the start (+1) of the *HvHVP10* gene

Table 4-5 Transcription factor binding sites in *HvHVP10* promoter regions from CPI-71284-48 (-2154) and Barque-73 (-2256) (General core promoter elements (optimised for plants) were identified using Matinspector Release professional 8.0.5 (https://www.genomatix.de/matinspector.html). (red letters=ci value >60, capital letters indicate core sequence)

Genotype	Position	strand	Sequence #	Core promoter element	Core similarity	Matrix similarity	Sequence	Reference
Barque-73	1st insert	+	-853 to -837	Opaque-2 like transcriptional activators	1	0.947	tcctCCACctcatcatt	(Schmidt *et al.* 1992)
		-	-845 to -829	L1 box motif for L1 layer-specific expression	1	0.863	gtaggaTAAAtgatgag	(Abe *et al.* 2001)
		-	-844 to -828	Plant I-Box site	1	0.957	cgtagGATAaatgatga	(Giuliano *et al.* 1988)
		+	-841 to -825	MYB proteins with single DNA binding repeat	1	0.949	tcatttATCCtacgtga	(Lu *et al.*, 2002)
		+	-837 to -821	ABA response elements	1	0.91	ttatcctACGTgacact	(Gomez-Porras *et al.* 2007)
		+	-836 to -820	Opaque-2-like transcriptional activators	1	0.987	tatcctACGTgacactt	(Schmidt *et al.* 1992)
		-	-827 to -801	DNA binding with one finger (DOF)	1	0.987	ctatcttaAAAGtgtca	(Noguero *et al.* 2013).
Barque-73	2nd insert	-	-390 to -370	Ethylene response element factors	1	0.876	aTCGAgagaaaccacggct	(Fujimoto *et al.* 2000)
CPI-71284-48	Close to start of gene	-	-27 to -13	Dehydration responsive element binding factor	1	0.914	gcaggCCAGacgaaac	(Yamaguchi-Shinozaki and Shinozaki, 1994)

A number of sequences were identified giving a significant hit for general core promoter elements within the 2 inserts in Barque-73, with a core similarity of 1 and Matrix similarity >0.85. The first insert of 63 bp contained 7 significant hits; 2 Opaque-2-like transcriptional activators; a L1 box motif for L1 layer specific expression; a Plant I-box site; a MYB protein with single DNA binding repeat; an ABA response elements and a DNA binding site with one zinc finger. The 2nd insert of 31 bp only identified a single transcription factor binding site, namely an ethylene response element factor. In addition a Dehydration Response Element Binding Factor (DREB) was identified in the CPI-71284-48 *HvHVP10* promoter just upstream of the *HvHVP10* translation start site which was absent in the Barque-73 *HvHVP10* promoter transcription factor analysis. This identification of the DREB transcription factor binding site in CPI-71284-48 was also confirmed by PLACE analysis.

4.3.2 HvHVP10 membrane localisation and cell-type specific expression profiling of HvHVP10 in CPI-71284-48 and Barque-73

4.3.2.1 HvHVP10 membrane localisation in onion epidermal cells

In order to determine whether the membrane localisation of the barley *HvHVP10* protein to the tonoplast, the *HvHVP10* ORF sequence with and without the stop codon was cloned into expression vectors to encode both N (YFP:HVP10) and C terminal (HVP10_no_stop:YFP) YFP fusion proteins. The expression vectors were transformed into leek (*A. ampeloprasum*) epidermal cells by micro-projectile bombardment to assess subcellular membrane localisation of *HvHVP10* by YFP fluorescence. YFP fluorescence from the HVP10_no_stop:YFP fusion was observed on epidermal cells 1 day post inoculation, however the pattern of fluorescence was not clear or restricted to a single membrane (Figure 4-15). This result could be indicative that *HvHVP10* is not restricted to the tonoplast, or a result of incorrect YFP trafficking within the cell. Shorter inoculation times were attempted in an effort to reduce the build-up of YFP in the cell, but this did not alter the pattern of fluorescence (data not shown). No fluorescence was observed in N-terminal YFP fusion proteins.

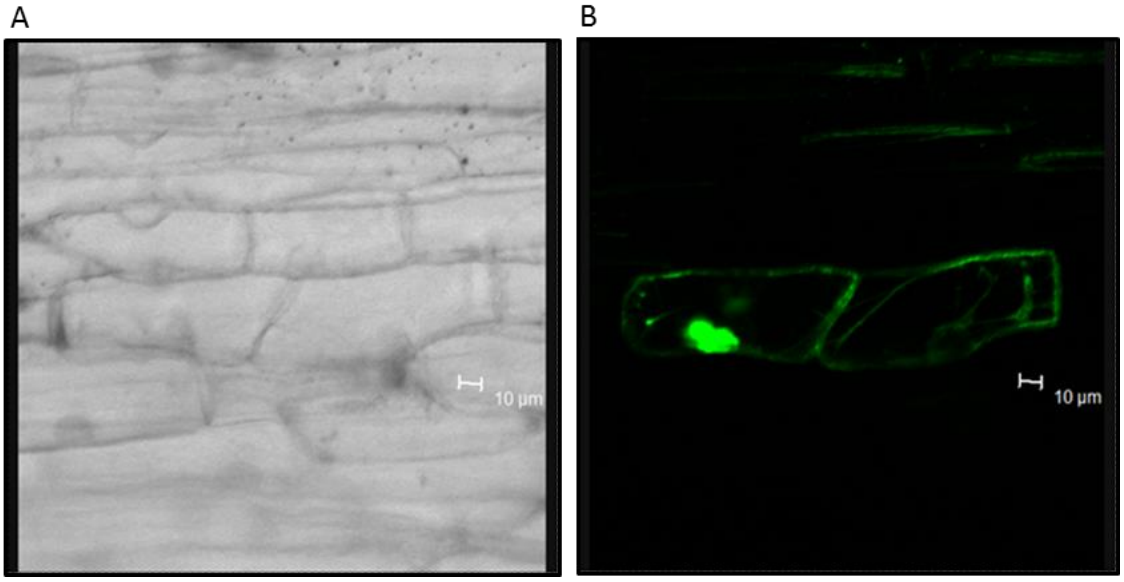

Figure 4-15 Subcelluler localisation of flourescence in leek (*A. ampeloprasum* var. porrum) epidemal cells transiently expressing a fusion of YFP with the C-terminus of *HvHVP10* (HVP10_no_stop :YFP). YFP fluorescence was visualised by confocal microscopy with excitation and emission wavelength of 514 nm and 525-610 nm, respectively. (A) Bright-field image and (B) False colour image showing YFP fluorescence.

4.3.2.2 Agrobacterium-mediated transient expression of HvHVP10:YFP in Nicotiana benthamiana

The *HvHVP10*::YFP reporter fusion was also transiently expressed in *Nicotiana benthamiana*. The *Agrobacterium* strain used for transformation (AGL1) had acquired resistance to ampicillin in our laboratory, so an alternative vector carrying the spectinomycin selectible marker, pPLEX502, was modified via restriction enzyme digestion and ligation to produce a new expression vector carrying *HvHVP10*_no_stop:YFP and the spectinomycin resistance gene (pPLEX HVP10-YFP) (Figure 8-6).

The binary expression vector pPLEX502 harbouring HVP10_no_stop:YFP fusion was infiltrated into *N. benthamiana*. Agroinfiltration was performed on 5-6 week old plants and analysed for YFP flourescence two days post-infiltration using a Leica TCS SP2 system. Transient expression of *HvHVP10* in tobacco similarly did not show localisation of the *HvHVP10* fusion protein to a specific membrane or organelle (data not shown) .

4.3.2.3 Cell-type specific expression profiling of HvHVP10 in CPI-71284-48 and Barque-73

To investigate the cell-type specific expression profile of *HvHVP10* in CPI-71284-48 and Barque-73, the promoter from each genotype was cloned into expression vectors to drive the expression of GFP (Figure 8-7) and GUS (Figure 8-8) in a barley (line WI4330) background.

T_1 plants harbouring the promoter from each genotype were grown under supported hydroponics for 10 days and whole root tissue was analysed for GFP fluorescence. No GFP signal could be visualised under a con-focal microscope, despite several attempts at different stages of plant development. T_1 seed was only obtained shortly before the end of the PhD candidature, so further detailed analysis of the promoter GFP and GUS reporter constructs could not be undertaken. Preliminary screening of whole root tissue from these plants by a Bachelor of Science Honours student at the University of Adelaide indicated that GUS activity was low, and could not be readily detected, however GUS expression could be detected in these lines by semi-q RT-PCR (Menadue, 2014, unpublished data). This indicates that the promoter functions enough to produce GUS transcript.

4.4 Discussion

4.4.1 Identifying the genetic basis for the HvNax3 QTL in CPI-71284-48 x Barque-73 DH population

Wild populations are often adapted to extreme environments and offer an important source of stress tolerance alleles for cereal breeding programmes (Korff *et al.*, 2006, Sutton *et al.*, 2007, Wu *et al.*, 2011). Many of these studies often begin with the identification of a QTL, or region of the genome conferring the expression of a phenotypic trait in a mapping population derived from a direct cross (Tanksley and McCouch, 1997, Xiao *et al.*, 1998, Blair *et al.*, 2006, Xiong *et al.*, 2006). Further functional characterisation of these regions of the genome, usually involving a combination of both quantitative analysis of gene transcripts and protein or metabolite abundance and activity (transcriptomic, proteomic, or metabolomic approaches), allow the identification of the gene and the nucleotide polymorphisms altering the functions of those genes (Alonso-Blanco *et al.*, 2009, Hayes *et al.*, 2015, Hove *et al.*, 2015). In order to identify the genetic basis for phenotypic variation in 3rd leaf sodium concentration (*HvNax3* QTL) in the Barque-73 (*H. vulgare*) x CPI-71284-48 (*H. spontaneum*) DH and AB-QTL

populations, the *HvHVP10* CDS, full-length gene and -2200 of the promoter regions were isolated, sequenced, and compared; to identify potential nucleotide polymorphisms in *HvHVP10* which may account for the different sodium exclusion phenotypes observed in these genetically related but distinct species.

It should be noted however, that a better strategy for sequencing *HvHVP10* may have been to sequence directly from cDNA and genomic DNA, prior to cloning, thus eliminating Taq-derived sequence errors introduced during the cloning process. Employing Next Generation Sequencing (NGS) technologies such as exon capture and Illumina HiSeq, with high coverage of the genome, would have also overcome this problem, however these technologies are expensive.

4.4.1.1 The HvHVP10 CDS is highly conserved

Sequence analysis of the *HvHVP10* CDS from CPI-71284-48 and Barque-73 revealed that the *HvHVP10* CDS from both genotypes consisted of an ORF of 2289 bp, translating to 762 amino acids. The two proteins were identical, and shared 100% sequence identity with the annotated *HvHVP10* sequence (D13472.2; NCBI database http://www.ncbi.nlm.nih.gov/). The CDS was very highly conserved between the genotypes, with 7 synonymous SNPs in the CDS between CPI-71284-48 and Barque-73. Allelic variation in the protein sequence is not responsible for the sodium exclusion phenotype observed in lines carrying the *HvNax3* allele from *H. spontaneum* accession CPI-71284-48.

The *HvHVP10* CDS sequence was analysed using TMHMM Server v. 2 (http://www.cbs.dtu.dk/services/TMHMM/) to predict the number of transmembrane domains in the protein sequence. Results indicated that *HvHVP10* contains 14 transmembrane domains, with both the C and N termini exposed to the vacuole. This result is consistent with reports by Maeshima (2000) who showed that the V-PPase from mung bean is a 14-16 span intrinsic tonoplast protein which consists of a single polypeptide of 75-82 kDa (**Error! Reference source not found.**). Topological prediction of *AtAVP1* has also revealed a similar protein structure to *HvHVP10* (Drozdowicz and Rea, 2001).

4.4.1.2 Identification of other barley pyrophosphatase members is confirmed by the presence of highly conserved motifs

In this study an additional three putative V-PPase gene sequences were identified in barley from BLAST searches of the draft barley genome sequence (http://webblast.ipk-gatersleben.de/barley/). This is consistent with rice (*Oryza sativa*), which also has five V-PPase members, identified by TBLASTN searches of the sequenced rice genome (Choura and RebaÏ, 2005). Additionally, Lerchl *et al.* (1995) reported four paralogues in tobacco (*Nicotiana tabacum*). Sequence alignment of these putative V-PPase barley sequences with *HvHVP10* and *HvHVP1* confirmed the presence of highly conserved motifs in the protein sequence, common to all V-PPase (Maeshima, 2000, Zancani *et al.*, 2007). The V-PPase family is reported to have a number of highly conserved segments within the gene, referred to as CS1, CS2, CS3, and have been proposed as sites for substrate binding and catalytic activity (Maeshima, 2000). The first conserved sequence (CS1) is located in cytosolic loop (CL3) and contains the motif (DVGADLVGKVE). This sequence has been proposed as the catalytic domain involved in substrate hydrolysis (Maeshima, 2000, Drozdowicz and Rea, 2001), and has been confirmed to be exposed to the cytosol (Takasu *et al.*, 1997). The sequence is conserved in all V-PPases sequenced to date (Zancani *et al.*, 2007) is common to both soluble PPases and H^+-PPases (Rea *et al.*, 1992). The second conserved sequence (CS2) is located in a hydrophilic loop (CL5(i)). The third segment (CS3) is located in the carboxyl-terminal part of the protein (CL7(m)) and contains 12 charged residues (Nakanishi *et al.*, 1999). Site directed mutagenesis and chemical modification studies of these conserved sequences have confirmed these regions to be directly involved in enzymatic and proton-translocating reactions of V-PPase (Zhen *et al.*, 1997, Zancani *et al.*, 2007, Lin *et al.*, 2012) .

Multiple sequence alignment of *HvHVP10* CDS with the other putative barley V-PPases sequences also confirmed the presence of other highly conserved motifs common to all V-PPase (Figure 4-7). The conserved sequences with the acidic motifs DX3DX3D and GDTIGDPLKDTSGP were also present, located on CL5 and at the C-terminus to transmembrane domain 14 respecively. Both of these conserved sequences have been subject to site directed mutagenesis and in each case resulted in loss of hydrolytic function and transport activities, suggesting involvement in substrate binding (Nakanishi *et al.*, 2001, Van *et al.*, 2005, Zancani *et al.*, 2007). In addition, HVP1, HVP10, HVP3 (AK362588.1) and HVP4 (AK375042.1) all shared the conserved sequence EYYTS, however for HVP5

(AK363930.1), this sequence is replaced with KYYTD (Figure 4-7) which has been shown to be involved in K^+ sensitivity (Drozdowicz and Rea, 2001). The conserved sequence RQFNTIP, a putative 14-3-3 protein ligand binding sequence (Venter *et al.*, 2006) was also identified in the Barley V-PPase sequences.

4.4.1.3 Phylogenetic analysis of vacuolar H^+-pyrophosphatases from barley and other plant species

All characterised V-H^+-PPases comprise two structurally and functionally distinct types: Type 1, which is K^+-sensitive and characterised by *AtAVP1*; and Type 2 which are K^+-insensitive (but Ca^2+ hypersensitive) and characterised by *AtAVP2* (Maeshima, 2000, Drozdowicz and Rea, 2001). Phylogenetic analysis indicated that V-PPase proteins in barley (

Table 4-3) also clustered into two functionally distinct groups, where *HvHVP1* (HVP1), *HvHVP10* (HVP10), *HvHVP3* (HVP3: AK362588.1) and *HvHVP4* (HVP4: AK375042.1) clustered into one group and *HvHVP5* (HVP5: AK363930.1) was contained within the other group (Figure 4-8). In addition, *HvHVP1*, *HvHVP10*, *HvHVP3* and *HvHVP4* all contained the sequence EYYTS (Figure 4-6), which is the motif conserved in most other Type 1 V-PPases (Drozdowicz and Rea, 2001, Fukuda *et al.*, 2004), however *HvHVP5* contains the sequence KYYTD (Figure 4-7), indicating it is a Type 2 V-PPase and K^+-insensitive (Drozdowicz and Rea, 2001), a result which is reinforced by its clustering with *AtAVP2* (Figure 4-8). Future work could include detailed analysis of the other HVPs indentified in this study to determine in which tissue they are expressed.

Each V-PPase member from barley clustered with a single Brachypodium (*Brachypodium distachyon*: Bradi_), rice (*Oryza sativa*: LOC Oso_) and sorghum (*Sorghum bicolor*: Sorbic._) V-PPase gene (Figure 4-8). Phylogenetic analysis showed that *HvHVP10* formed a clade with: *Bradi1g47767.1* (a putative molecule located on chromosome 1 of *B. distachyon* genome, consisting of 2289 bp within the CDS and translating to 762 amino acids. Sequence alignment indicated that this orthologue shared 95% sequence identity with *HvHVP10)*; *Sobic.010G060600* (a putative molecule located on chromosome 10 of the *S. bicolor* genome, with a CDS consisting of 2292 bp and translating to 763 amino acids. It was found to share 93% sequence identity with *HvHVP10*); and *LOC Oso6g08080.1* (located on chromosome 6 of *O. sativa*, consisting of 2304 bp and translating to 767 amino acids. It was found to share 92% sequence identity with *HvHVP10*).

Brini *et al.* (2007) showed that the HVP10 protein sequence shared the highest homology with the wheat (*Triticum aestivum*) V-PPase orthologue *TVP1* (Accession no. AY296911), with 100% sequence identity in the protein sequence, followed by the rice orthologue OVP2 (Accession no. AB012766), with 98% sequence identity at the protein level (Liu *et al.*, 2009). This is consistent with our results. Protein alignment of OVP2 (Protein id. BAA31524.1) with rice *LOC Oso6g08080.1* indicates that they share an identical protein sequence, and are therefore presumed to be the same gene. Similarly, the *Sorghum bicolor* orthologue shares the same protein sequence as SVP1, respectively, and are presumed to be the same gene.

4.4.1.4 The HvHVP10 gene sequence differs significantly within the non-coding regions

Sequence analysis of the full-length gene *HvHVP10* gene sequence from CPI-71284-48 and Barque-73, and subsequent alignment with Morex *HvHVP10* gene sequence, revealed that the *HvHVP10* gene differed significantly between the two genotypes within the non-coding regions of the gene. In particular Barque-73 contained numerous SNPs, insertions and a deletion not present in the *HvHVP10* gene sequence from CPI-71284-48. Intron 1 is the longest and least conserved with 14 SNPs identified between the genotypes. Barque-73 also contained a number of insertions not present in CPI-71284-48, namely a 3 bp insertion in intron 1 (423bp) and an insertion of 7bp in the 6th intron (3929bp) (Figure 4-9). Barque also had a deletion of 3bp in the 7th intron (4142bp). Other SNPs were detected within the non-coding regions with 2 SNPs in intron 2, 4 SNPs in intron 3 and 1 SNP in intron 4. Any of these differences may affect gene regulatory elements, which may account for the difference in V-PPase expression and different sodium exclusion phenotype observed in CPI-71284-48 and Barque-73.

In order to identify if the full-length *HvHVP10* gene is responsible for the *HvNax3* QTL and different sodium exclusion phenotypes observed in the CPI-71284-48 x Barque-73 DH population, 17 clones carrying the Barque-73 full-length gene and 16 clones carrying the CPI-71284-48 full-length gene were cloned into pCR8/GW/TOPO TA Gateway® entry vectors in the correct orientation, for the generation of gene-reporter constructs. Sequencing of multiple entry vectors revealed that, despite numerous attempts to clone the gene, we were unable to obtain the full-length gene from CPI-71284-48 and Barque-73 without SNPs being introduced during the cloning process. This is probably due to the length of the product being cloned. Therefore, these entry vectors could not be used for Gateway® cloning into plant expression vectors. Although not possible within the time frame for my PhD candidature, one solution to overcoming the sequencing errors would be to cut and paste, by restriction and ligation, from multiple entry plasmids to create the correct gene sequence.

The full-length *HvHVP10* gene sequences were obtained for CPI-71284-48 and Barque-73. Unfortunately there are currently no full length gene sequences available for the other V-PPase members in barley for genetic comparison. Thus, the gene sequences for the closest rice (*Oryza sativa*), Brachypodium (*Brachypodium distachyon*) and sorghum (*Sorghum bicolor*) orthologues were also downloaded from available databases, including NCBI,

PHYTOZOME and Brachypodium distachyon (v1.0) (http://plants.ensembl.org/Brachypodium_distachyon/) and examined for genomic structure using Spidey (mRNA to genomic alignment). Comparison of full-length genes from rice Brachypodium and sorghum revealed they all consisted of a single splice variant composed of 8 exons and 7 introns (Figure 4-10), therefore the homologous genes display a similar gene structure to *HvHVP10*. Interestingly, the *Sorghum bicolor* orthologue had longer gene due to large insertion in intron five.

4.4.1.5 Potential HvHVP10 copy number variation in CP-71284 and Barque-73

Copy number variations (CNV) resulting from replication of large regions of the genome, usually larger than 1kb, have been identified as a key contributor to intra-species genetic variation in plants, along with single nucleotide polymorphisms and short insertions and deletions (Zmienko *et al.,* 2014). This type of natural mutation occurs on all chromosomes, usually in hotspots in the telomeric region of the chromosome and can result in having too few or too many copies of the gene (Muñoz-Amatriaín *et al.*, 2013). In plants only a few traits have been associated with gene duplication. For instance, a tandem duplication of the *Bot1* (Boron tolerance) gene was found to confer boron toxicity tolerance in the barley Algerian landrace Sahara 3771, as compared with the boron-intolerant Australian malting cultivar Clipper, which contained only a single copy of the gene (Sutton *et al.*, 2007, Hayes *et al.*, 2015). CNV at the *MATE1* transporter gene in maize has also been associated with increased aluminium tolerance (Maron *et al.*, 2013) and variation in *CBF* genes clustered at the frost-tolerance locus FR-2 contributed to cold tolerance in winter barley genotypes 'Igri' and 'Franka' (Knox *et al.*, 2010) and in wheat (Vagujfalvi *et al.*, 2005).

HvHVP10 copy number in CPI-71284-48, Barque-73, AB-QTL lines (18D/011 and 18D/014) and other selected genotypes was examined via Southern blot analysis. The *HvHVP10* copy number in the wheat-barley disomic 7H chromosome addition line (Islam *et al.*, 1981) was also investigated, since the location of the *HvNax3* QTL is on 7HS (Shavrukov *et al.*, 2010). These lines are a useful genetic resource for a range of applications (Bilgic *et al.*, 2007). A probe was produced targeting the middle of the gene from exon 4 (2824b) to exon 5 (3096bp) and spanning the 4th intron. Unfortunately, this region also shared sequence identity with the other barley V-PPase members identified in this study, indicating it may not be specific to *HvHVP10*.

The presence of multiple hybridisation bands however, does suggest the presence of multiple copies of the *HvHVP10* gene in both CPI-71284-48 and Barque-73. In addition, Southern results indicate that there is potential variation in *HvHVP10* copy number between CPI-71284-48 and Barque-73, where Barque-73 has more copies of the *HvHVP10* gene. This result is consistent across multiple restriction digests (Figure 4-11 and Figure 4-12). The exact number of *HvHVP10* copies that exist in these genotypes is not clear. It should be noted that a similar pattern of hybridisation was observed between the AB-QTL lines with the *HvNax3* allele from either Barque-73 or CPI-71284-48 and the parental line, indicating that the right locus is being targeted. Examination of the hybridisation pattern of the 7H chromosome addition line suggests that 5 copies of the gene are located on chromosome 7H of barley cultivar Betzes. This is inconsistent with results reported from Fukuda *et al.* (2004), who identified only a single copy of HVP10 in barley using a probe targeting the 3'-UTR of HVP10. This could be explained by the fact that the probe they used was specific to HVP10. It should also be noted that this analysis was conducted on the Japanese barley cultivar Kashima (Kitakanto 3*Musashinomugi), which may also account for the discrepancy in results. Unfortunately, despite several attempts at Southern blot analysis with probes specific to the 3'-UTR and 5'-UTR of *HvHVP10*, a clearer result could not be obtained.

The Southern results do suggest that Barque-73 contains more copies of the *HvHVP10* gene than the progenitor accession CPI-71284-48. It is possible that having more than one copy of the *HvHVP10* gene in Barque-73 has a negative (deleterious) effect on *HvHVP10* gene expression in this genotype. Interestingly, Munoz-Amatriain *et al.* (2013) found that barley breeding and domestication has resulted in a depletion of CNV diversity, where higher levels of CNV diversity are present in wild accessions relative to cultivated barley, a result supported by the *Bot1* example (Sutton *et al.*, 2007). A different result was observed in wheat, where gene duplications were predominant in a cultivated wheat accession and more gene deletions than duplications were identified in wild wheat (Saintenac *et al.*, 2011). It may be that some genes have lost CNV diversity through domestication and narrowing of the gene pool, however, for other genes this may have resulted in increased CNV diversity, possibly due to their position on the chromosome and whether it is in a region of increased homologous recombination.

4.4.1.6 Differences in the HvHVP10 promoter region in CPI-71284-48 and Barque-73

Wallace *et al.* (2014) showed that while variation in gene sequence is important, a large portion of functional variation in maize results from differences in gene copy number and gene regulation rather than modifications to the protein coding sequence. Hayes *et al.* (2015) investigated the diversity in boron toxicity of Australian barley genotypes via sequencing and expression analysis of the barley boron tolerance genes *HvBot1* and *HvNIP2;1*. Results of this work demonstrated that the coding sequence of *HvNIP2;1* (a transport protein belonging to the NIP family of aquaporins on chromosome 6H of barley) was highly conserved across barley germplasm, and differences in boron tolerance were associated with differences in the 5ÚTR of this gene. The increased boron tolerance of landrace Sahara was proposed to be caused by a SNP in the 5'UTR of *HvNIP2;1*, creating a small open reading frame that interferes with *HvNIP2;1* translation. Also in barley, a CACTA-like transposon insertion 5kb upstream of the ORF of the aluminium tolerance gene *HcAACT1* enhances and alters the tissue localisation of *HcAACT1* expression (Fujii *et al.*, 2012). Liu *et al.* (2013) showed that polymorphisms in the *ZmDREB2.7* promoter, but not the protein coding region itself, were associated with different levels of drought tolerance among maize varieties.

Sun *et al.* (2010), examined the promoter region of the V-PPases from both *Thellungiella* (*TsVp1*) and Arabidopsis (*AVP1*) which are involved in the salt stress response. To determine which tissue the promoters are active in, under controlled and stress conditions the GUS reporter gene was placed under the control of each of the promoters and transformed into Arabidopsis plants. Analysis of the transgenic plants with the *TsVP1* promoter revealed that GUS had activity in all tissues, except in the seed, and activity was strongly induced in both the roots (particularly the root tips) and the shoots when plants were exposed to salt stress. This induction was not seen in transgenic Arabidopsis plants containing the *AVP1* promoter. Using a series of 5' deletion mutants of the *TsVP1* promoter, an 856bp region (-2200 bp upstream from the nucleotide position +1) was found to contain enhancer elements that increased gene expression levels and a 130bp region was finally identified as the key sequence for the salt stress response in *Thellungiella*. These results indicate that while these genes share high sequence identity in their coding sequence, differences in the promoter region are responsible for their individual regulation and varied expression under salt stress.

In order to identify if significant differences exist in the *HvHVP10* promoter regions between CPI-71284-48 and Barque-73, -2200 of the promoter regions was isolated, cloned into multiple PCR8/GW/TOPO entry vectors and sequenced. Sequence analysis of entry vectors indicated that two large insertions of 63 and 31 bp were present in the Barque-73 *HvHVP10* promoter region. The *HvHVP10* promoter region from both genotypes was analysed using Matinspector and compared for transcription binding sites.

The main regions of variation in transcription binding sites between CPI-71284-48 and Barque-73 were in the locations of the 2 inserts in Barque -73, and close to the *HvHVP10* translation start site in CPI-71284-48. The first insert of 63 bp contained a number of transcription binding sites which had a core similarity of 1 and a high matrix similarity >0.94. These transcription binding sites included; an Opaque-2-like transcriptional activator (Opaque-2 is a regulatory locus in Maize that controls the expression of Zein proteins (Schmidt *et al.*, 1992); an L1 box motif for L1 layer specific expression (*cis*-acting regulatory element involved in cotyledon development and epidermal cell differentiation (Abe *et al.*, 2001); a Plant I-box site (a conserved sequence upstream of light-regulated genes (Giuliano *et al.*, 1988); a site for MYB proteins (Lu *et al.*, 2002) found in the promoter of the dehydration responsive gene rd22 in Arabidopsis (Abe *et al.*, 1997); an ABA response element (a hormone involved in regulation of stomatal closure seed and bud dormancy and physiological response to cold, drought and salinity stress (Gómez-Porras *et al.*, 2007) also present in early response to dehydration (ERD) in Arabidopsis (Kariola *et al.*, 2006); and a DNA binding sitewith one zinc finger (a family of transcription factors involved in response to light and seed maturation and germination), where factor binding may activate or repress transcription (Noguero *et al.*, 2013). The 2nd insert of 31 bp in the Barque-73 *HvHVP10* promoter region contained only a single transcription binding site, namely an ethylene response element factor, which have been shown to function as activators of GCC box dependent transcription (Fujimoto *et al.*, 2000). Many of these transcription binding sites are associated with the expression of stress response genes and these insertions may disrupt gene regulatory elements involved in transcription of the *HvHVP10* gene in Barque-73.

In addition, potential variation in transcription factor binding sites was observed close to the *HvHVP10* translation start site (+1) in CPI-71284-48 and Barque-73 (Figure 4-14). Matinspector analysis identified a dehydration response element binding factor (DREB) close

to start of the *HvHVP10* gene in CPI-71284-48; however the analysis did not detect this in Barque-73. This is believed to be due to a SNP at position -8 in Barque-73, from an A to a T (Barque-73 (A/GCCTGAC), CPI-71284-48 (A/GCCAGAC). The identification of a dehydration response element in CPI-71284-48 was confirmed by PLACE analysis using Matinspector. DRE/CRT (Dehydration Response Element- C-repeat) acting elements have been identified in the promoters of stress-inducible genes in various plant species (Liu *et al.*, 2000, Ito *et al.*, 2006, Dietz *et al.*, 2010, Rae *et al.*, 2011, Mizoi *et al.*, 2012). In Arabidopsis, the dehydration-responsive element (DRE) with the core sequence A/GCCGAC has been identified as an ABA-independent cis-acting element important for the regulation of gene expression in response a range of abiotic stresses, including, salinity, drought and cold stress (Yamaguchi-Shinozaki and Shinozaki, 1994).

Studies have demonstrated that DREB1 (induced by cold) and DREB2 (induced by dehydration and high salinity) (Liu *et al.*, 1998) have different DNA-binding specificities (Sakuma *et al.*, 2006) and preferentially bind to different sequences (Peng *et al.*, 2013). The most preferred binding sequence of barley HvDRF1 is TT/ACCGCCTT (Xue and Loveridge, 2004). In addition PgDREB2 was shown to be phosphorylated and could not bind to DRE/CRT (Agarwal *et al.*, 2007), indicating that different species have different target sequences.

It should be noted that the software program Matinspector on the Genomatix website is only a predictive tool that utilises a large library of matrix descriptions for transcription binding sites to locate probable matches in DNA sequences. The real significance of these variations between CPI-71284-48 and Barque-73 *HvHVP10* promoter regions can only be confirmed with detailed analysis of different promoter-reporter constructs or employing emerging genome editing technologies such as CRISPR-Cas9 (Arora and Narula 2017) to modify specific nucleotides within the sequence.

4.4.2 HvHVP10 membrane localisation and cell-type specific expression profiling of HvHVP10 in CPI-71284-48 and Barque-73

4.4.2.1 HvHVP10 may not just be localised to the tonoplast?

The transient expression of GFP and derivatives in intact protoplasts and plant tissue is a useful tool for determining the subcellular membrane localisation of proteins and the elucidation of protein function (Kokkirala *et al.*, 2010). A number of studies have examined

the localisation of V-PPase from a range of plant species, including monocots (Mitsuda *et al.*, 2001) and dicots (Regmi *et al.*, 2016), as well as bacteria (Ramos *et al.*, 2003) and parasites (Drozdowicz *et al.*, 2003). Plant V-PPases are mostly associated with the tonoplast, where they co-exist with V-ATPase proteins (Rea and Poole, 1993), but they are also reported to be located at the golgi cisternae (Mitsuda *et al.*, 2001) and at the plasma membrane (Maeshima and Yoshida, 1989). Immuno-localisation of Type 1 H^+-PPase AVP1 with a protein-specific antibody in Arabidopsis cross sections indicated that the AVP1 protein is localised to the plasma membrane of sieve element-companion cells (Paez-Valencia *et al.*, 2011). These reports suggest that V-PPase proteins are active on various membranes within the cell.

HvHVP10::YFP and YFP::*HvHVP10* fusions were produced to verify the localisation of *HvHVP10* to the tonoplast in a transient expression system. YFP fluorescence from the *HvHVP10*::YFP fusion was observed 1 day post inoculation in onion epidermal cells; however the pattern of fluorescence did not appear to be restricted to a single membrane (Figure 4-15). YFP florescence was observed throughout the cell, which suggests that *HvHVP10* is not just localised to the tonoplast, but also to the plasma membrane, nucleus and also the cytoplasm. The transient expression of *HvHVP10* using agro-benth infiltration revealed a similar pattern of YFP flourescence, despite several attempts using different inoculation times and Agrobacterium concentrations.

These findings suggest that *HvHVP10* is not localised to a single membrane, although the possibility that there was a problem with YFP trafficking in the cell cannot be ruled out. The presence of YFP may hinder proper localisation encoded by a transit sequence on the attached protein. Furthermore, YFP fusion may cause conformational change in the attached protein, causing a false active result (Hanson and Köhler, 2001). The use of an *HvHVP10* specific antibody in immune-localisation studies would overcome the problems associated with YFP interfering with trafficking within the cell. It would also provide conclusive evidence as to whether *HvHVP10* is targeted specifically to the tonoplast in barley.

4.4.2.2 Cell-type specific expression profiling of HvHVP10 in CPI-71284-48 and Barque-73

Studies have shown that V-PPase isoforms are stress-inducible and have tissue-specific roles. The rice V-PPase orthologue OVP1 has been shown to be induced by cold stress (Zhang *et al.*, 2011), anoxia and chilling (Carystinos *et al.*, 1995) and OVP2 (*Os06g0178900*) is

induced by chilling and 100mM NaCl (Sakakibara *et al.* unpublished results). Chromosomal mapping by RFLP techniques demonstrated that OVP1 and OVP2 are isoforms encoded by different genes both located on chromosome 6 (homologous to barley chromosome 7H). Other studies have also demonstrated that the expression of the two proteins is regulated differently (Sakakibara *et al.*, 1996). Northern analysis indicated that OVP2 has higher expression in undifferentiated calli and lower expression in the roots and shoots of intact rice plants, compared with OVP1.

CPI-71284-48 and Barque-73 *HvHVP10* promoter-GFP and GUS reporter lines were created for cell-type specific expression profiling in barley. GFP reporter lines were grown in supported hydroponics under 150 mM NaCl and whole root sections of 10 day old plants were examined for GFP fluorescence. No GFP fluorescence was observed in whole root tissues, despite several attempts at different stages of plant development. An ACPFG Honours student examined T_1 GUS-reporter constructs and was able to confirm that the WI4330 barley did have the *uidA* (β-glucuronidase) gene, which was absent in wild type barley. Semi-quantitative RT-PCR analysis of these GUS-reporter lines indicated that the *uidA* gene was being expressed in 17 day old hydroponically grown *pHvHVP10* transgenic lines (grown in 300 mM NaCl for 3 days), however it was low compared to the control gene used (*HvVRT2*). Despite observing *uidA* expression, GUS activity was not observed in the root or the shoot of *pHvHVP10* WI4330 plants grown in 300 mM NaCl for 3 days, however the rice positive control (*pPRP1-11* in Nipponbare rice) did exhibit GUS activity, suggesting there was no issue with the GUS assay itself.

These results collectively suggest that the *HvHVP10* promoter may be a stress-inducible promoter and require a specific set of growth conditions at a specific stage of plant development to be expressed. It could be that *HvHVP10* is only expressed in a specific cell type in the meristematic region of the root, which may not be observed in whole root sections. The use of techniques like *in situ* PCR (Athman *et al.*, 2014) or laser assisted micro dissection (Roy *et al.*, 2012) may allow the determination of the specific cell type in which the gene is being expressed.

Examination of the expression data for *OVP2* (available on the Rice Expression Profile Database (http://ricexpro.dna.affrc.go.jp/) (Figure 4-16), the closest rice orthologue to *HvHVP10*, indicates that this gene is mainly expressed in the leaf sheath and root at the

vegetative stage. This may provide some information for further investigation of promoter reporter constructs in terms of plant development stage, but suggests that root tissue remains a likely target. Further detailed analysis of GUS reporter constructs under different abiotic stresses, involving microscopic examination of root cross sections of cells at different zones of maturation is required to confirm the cell type specific expression of *HvHVP10* in barley.

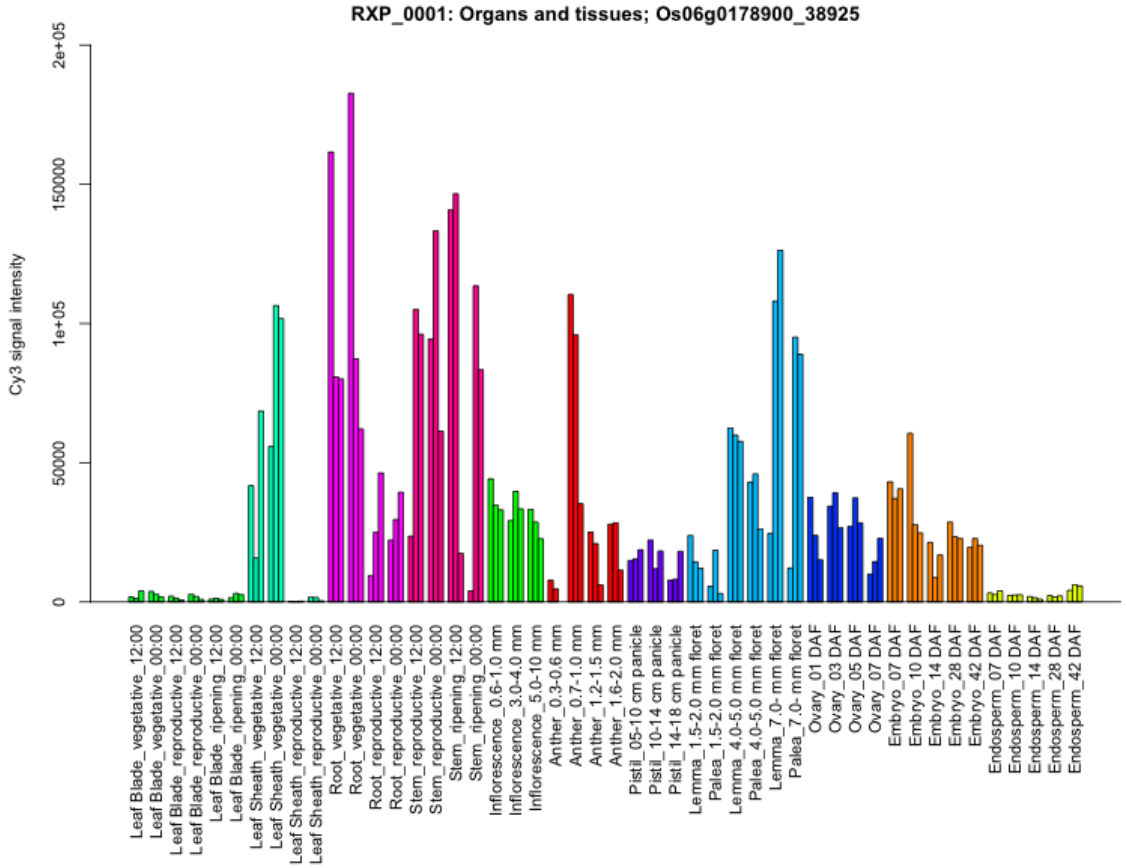

Figure 4-16 Spatio-temporal OVP2 (Os06g0178900) gene expression of various tissues /organs throughout entire growth in the field. Data obtained from Rice Expression Profile Database (raw data) (http://ricexpro.dna.affrc.go.jp/)

4.5 Summary

In this chapter we examined if nucleotide polymorphisms exist in the CDS, full-length gene and promoter region of *HvHVP10*, which may account for the different sodium exclusion phenotype (*HvNax3* QTL) and *HvHVP10* gene expression observed in CPI-71284-48 and Barque-73 under salinity stress (section 2.3.2). The *HvHVP10* CDS was highly conserved

between the genotypes, indicating that allelic variation in the *HvHVP10* protein sequence is not responsible for the observed difference in 3rd leaf sodium concentration in these genetically diverse but related species, when exposed to 150mM NaCl. In this study we also identified an additional three putative V-PPase members in barley, based on the *HvHVP10* protein sequence, which contained a number of highly conserved motifs common to all V-PPase. We also identified differences in the *HvHVP10* full-length gene and promoter region in CPI-71284-48 and Barque-73; however the actual significance of these sequence variations could not be determined due to time constraints. It is the recommendation of this chapter that detailed microscopic analysis of promoter reporter constructs is undertaken under a range of abiotic stresses, including salt, drought and cold stress, in order to determine if the sodium exclusion (*HvNax3*) phenotype is due to variations in the *HvHVP10* promoter in CPI-71284-48 and Barque-73, and whether this allelic variation also confers resistance to other abiotic stress.

5 Constitutive and stress inducible expression of *HvHVP10* for improving salinity tolerance of barley

5.1 Introduction

Genetic resources from wild relatives offer an important source of stress tolerance genes for use it current breeding programs. However this approach can often be time consuming and it is often challenging to isolate the target gene without associated deleterious genes. Genetic engineering approaches offer an alternative approach to conventional breeding methods to improve stress tolerance of plants and can also be used as "proof of concept" studies to show that the candidate gene identified from QTL mapping studies indeed has an effect on the desired phenotype.

Advances in genetic engineering and molecular biology have led to improvements in plant salt tolerance by altering the expression of genes that encode different sodium transporters. Many studies have demonstrated that the modification of these genes can lead to increased salinity tolerance of a range of plant species, either by increasing Na^+ compartmentalisation in the vacuole (tissue tolerance) or by reducing Na^+ accumulation in the shoot (Na^+ exclusion) (Adem *et al.*, 2014). Many of these reports involving the modification of Na^+ transport use constitutive promoters to express candidate genes that encode for various ion transporters (Apse *et al.*, 1999), and/or proton pumps (Gaxiola *et al.*, 2001), and vacuolar and plasma membrane antiporters (Shi *et al.*, 2003). Two of the most common constitutive promoters used in these expression studies are the cauliflower mosaic virus *35S* promoter and the maize *Ubiquitin-1* promoter. Despite the fact that both these promoters are constitutive, and are therefore expressed throughout the plant, the level of expression and tissue specificity have been shown to vary greatly between the promoters and the plant species in which these genes are expressed (Gallo-Meagher and Irvine, 1993, Holtorf *et al.*, 1995, Li *et al.*, 1997, Bassie *et al.*, 2000).

Other studies have demonstrated that the constitutive expression of some genes can have a detrimental effect on normal plant growth and development (Kasuga *et al.*, 1999). The OEX (Over Expression) of cDNA encoding *DREB1a* (a protein which specifically interacts with dehydration response element and induces expression of stress tolerance genes), when driven by the strong constitutive *35S* (CaMV) cauliflower mosaic virus promoter, activated the expression of many stress tolerance genes and resulted in severe growth retardation under

normal growing conditions. A similar effect has been observed in rice (Ito *et al.*, 2006), *Arabidopsis* (Kasuga *et al.*, 1999) and tobacco (Kasuga *et al.*, 2004, Gutha and Reddy, 2008). However the expression of *DREB1a* under the control of the stress inducible *rd29A* promoter resulted in minimal growth effects under normal conditions and greater tolerance to a range of abiotic stresses, including drought, salt loading and freezing (Kasuga *et al.*, 1999, Kasuga *et al.*, 2004).

Barley typically has a high salt tolerance (>250 mM NaCl) (Munns *et al.*, 2006) and represents a valuable experimental model for a number of small grain cereals, including wheat. This agronomically important crop also has reliable transformation technologies for functional gene analysis, making it a suitable candidate for transgenic studies. Currently *A. tumefaciens*-based transformation in barley is predominantly in the Golden Promise cultivar, due to its greater transformation efficiency than other barley genotypes (Goedeke *et al.*, 2007). Golden Promise is a non-commercial barley cultivar in Australia and is not high yielding or suited to Australian growing conditions. Little research has been conducted on commercially relevant Australian barley cultivars. In this study, the high yielding elite Australian barley breeding line WI4330 was transformed with the cDNA of the vacuolar H^+-pumping pyrophosphatase (V-PPase) *HvHVP10* from barley (Barque-73) under the control of the constitutive expression promoter cauliflower mosaic virus *35S* or maize *Ubiquitin-1*. WI4330 was also transformed with *HvHVP10* under the control of the stress inducible promoter *Rab17* to determine if the stress inducible expression of *HvHVP10* also results in enhanced salinity tolerance.

It was hypothesised that the constitutive and stress inducible expression of *HvHVP10* will increase the salinity tolerance of the cultivar WI4330 above its current level of tolerance. To test this hypothesis, the study in this chapter aimed to;

1. Develop transgenic commercially relevant barley expressing *HvHVP10* under the constitutive expression of *35S* and *Ubiquitin-1* promoters.
2. Develop transgenic commercially relevant barley expressing *HvHVP10* under the stress inducible expression of *Rab17* promoter.
3. Characterise the genotype and assess the salinity tolerance of the T_1 and T_2 transgenic lines.

4. Produce new genetic material for future experiments that can be used to confirm that enhances expression of *HvHVP10* enhances that salt tolerance of barley.

5.2 Methods

5.2.1 Plant materials

The barley cultivar Barque-73 was used as the genomic source to amplify the *HvHVP10* open reading frame (2289bp) for use in plant expression vectors (4.2.1.4). RNA for cDNA synthesis was extracted from salt stressed roots of Barque-73 and was kindly supplied by Yuri Shavrokov (ACPFG). The barley line WI4330, an advanced breeding line with a similar pedigree to the cultivar Flagship (University of Adelaide Barley Breeders, Adelaide, Australia) was used as the genetic background for Agrobacterium-mediated transformation of barley with constitutive and stress inducible expression of *HvHVP10*.

5.2.2 Generation of plant expression vectors using Gateway® cloning

Gateway cloning was used to generate plant expression vectors with constitutive and stress inducible expression of *HvHVP10*. The complete open reading frame of *HvHVP10* (2289bp) was amplified from Barque-73 cDNA and ligated into a *pCR®8/GW/TOPO®* vector (Invitrogen, USA) to create Gateway® enabled entry vectors, as described in Chapter 4. LR Clonase II (Invitrogen), was used to transfer *HvHVP10* from *pCR®8/GW/TOPO®* vectors into destination vectors containing either the cauliflower mosaic virus (*35S*) or maize Ubiquitin-1 (*Ubi*) promoter for constitutive expression of the transgene. *HvHVP10* was also transferred into a destination vector containing a stress inducible *Rab17* promoter. LR recombination reactions were performed according to the methods described in section 4.2.1.1.

5.2.2.1 Plasmid DNA transformation into chemically competent Escherichia coli cells

Transformation into chemically competent *Escherichia coli* cells was performed via the heat shock method. Briefly, 4 µL of LR reaction was added to 50 µL of competent One Shot® TOP10 *E. coli* cells (Invitrogen, USA) and cells were incubated on ice for 30 minutes. The cells were incubated at 42°C for 30 seconds to allow entry of the plasmid, then the reaction was immediately transferred to ice for 5 minutes. 250 µL of liquid Luria Betani (LB) media (tryptone 10 $g \cdot L^{-1}$, yeast extract 5 $g \cdot L^{-1}$, NaCl 5 $g \cdot L^{-1}$, pH7.5) was added and cells were incubated at 37°C with shaking for 45 minutes. 50 µL of bacterial culture were then plated out on LB plates containing the appropriate antibiotic (50 µM either Spectomycin, Hygromycin etc) for positive selection and plates were incubated at 37°C overnight. Positive clones

resistant to the antibiotic were selected and bulked up in 5 mL of LB liquid media with the correct antibiotic at 37°C overnight.

5.2.2.2 Isolation of plasmid DNA

Plasmid DNA was isolated using the Bioline Plasmid Mini Kit (Bioline, UK) according to the protocol provided by the manufacturer. Briefly, 2 mL of bacterial culture was transferred to a 2 mL microfuge tube and centrifuged for 1 minute at 16,100 *g* to pellet the cells. The supernatant was removed, and the cells were resuspended in 250 µL of resuspension buffer and an equal volume of Lysis buffer to lyse the cells. The reaction was neutralised with buffer and the precipitated DNA was separated from cellular components via centrifugation at 16,100 *g* for 10 minutes. The upper phase containing the plasmid DNA was collected and transferred to a spin column placed in a 2 mL collection tube and centrifuged for 1 minute at 10,000 *g* to bind DNA to the column membrane. Bound plasmid DNA was washed with 500 µL of Wash Buffer AP, centrifuged and then 700 µL of wash buffer BP was added and again the sample was centrifuged at 10,000 *g* for 1 minute. An additional centrifugation step of 2 minutes was carried out to remove residual ethanol. To elute plasmid DNA from the column membrane, 30 µL of elution buffer was added to the column and the sample was incubated at room temperature for 1 minute. The sample was then centrifuged at 10,000 *g* for 1 minute to elute plasmid DNA into a fresh labelled 1.5 mL microfuge tube.

5.2.2.3 Restriction digestion

The integration and orientation of plasmid DNA was confirmed using restriction digestion with the restriction enzyme *Blp*1 (New England Biolabs, USA). The 20 µL digestion reaction was composed of 2.5 µL of 1× NE Buffer 4, 3 µL of plasmid DNA, 0.1 µL of restriction enzyme *Blp*1 and 14.4 µL of sterile H_2O. Reactions were incubated at 37°C for 5 hours then heat inactivated at 65°C for 20 minutes.

5.2.2.4 Gel electrophoresis

DNA products of restriction digestion were separated by agarose gel electrophoresis. The gels were prepared using 1×TAE buffer (40 mM Tris-Acetate, 1 mM EDTA) and 1 % (w/v) agarose. DNA was stained with 0.5 $\mu g/mL^{-1}$ ethidium bromide or 0.05 $\mu L \cdot mL^{-1}$ SYBR-safe (ThermoFisher Scientific, USA). DNA marker ladders HyperLadder™ 1kb or HyperLadder™ 100bp were used to determined DNA size and estimate quantity. Electrophoresis was run at 90-100 volts to separate DNA fragments.

5.2.2.5 DNA sequencing

Purified plasmid DNA was labelled and prepared for sequencing using the BigDye®Terminator (BDT) v3.1 Cycle Sequencing Kit (ThermoFisher Scientific, USA). Reactions were performed in a 10 µL mix consisting of 0.3 µL of plasmid DNA, 0.32 µL of sequencing primer (10 µM), 1.0 µl BDT v3.1, 3.5 µL BDT Buffer and 4.88 µL H_2O. Thermal cycling conditions consisted of one cycle at 96°C for 1 min; 35 cycles at 96°C for 10 secs, 50°C for 5 secs and 60°C for 4 minutes. The sequencing reaction was then allowed to cool to room temperature and transferred to a 1.5 mL microfuge tube. 75 µL of 2.0 mM $MgSO_4$ was added to the reaction mixture to precipitate the DNA, samples were briefly vortexed, and then incubated at room temperature for 15 minutes. Tubes were centrifuged for 15 minutes at 15,000 *g*, the supernatant removed and 75 µL of 70% ethanol was added to each sample. The tubes were centrifuged for 10 minutes and the supernatant was removed by pipette or decanting. Tubes were allowed to air dry in a fume cupboard for 10 minutes and then were submitted to the Australian Genome Research Facility (Waite Campus, Urrbrae, South Australia) for capillary separation using an AB3730×/sequencing platform (Applied Biosystems, USA). DNA sequencing analysis and alignments were performed using Vector NTI Advance™ 11.0.

5.2.3 Generation of transgenic plants with constitutive and stress inducible expression of HvHVP10

5.2.3.1 Agrobacterium mediated barley transformation

The barley line WI4330 (University of Adelaide Barley Breeders) was used as the genetic background for the generation of transgenic barley with constitutive and stress inducible expression of *HVP10*. Plant expression vectors carrying the *HVP10* CDS were transformed into WI4330 via *Agrobacterium tumefaciens*-mediated transformation, followed by the regeneration of barley plantlets in soil (Singh *et al.*, 1997, Jacobs *et al.*, 2007). The transformation of barley with plant expression vectors was conducted by the Barley Transformation Team at ACPFG.

5.2.3.2 Molecular confirmation of the transgene via Southern Analysis

Southern blot hybridisation was performed to confirm presence of the transgene in the T_1 generation and to determine the number of T-DNA inserts in *35S* and *Ubi* transgenic barley plants, following the protocol described by (Sambrook *et al.*, 1989). T1 seeds were segregating into null segregants and those that contain the transgene, therefore 16 seeds from

each independent transformation event were germinated and the leaf tissue pooled to prevent mistaking nulls for negative transformants. High quality gDNA was extracted from each line using phenol chloroform extraction (section 4.2.1.3) and digested using the restriction enzyme appropriate to the construct. The *35S:HvHVP10* OEX lines were digested with the enzyme *BamHI* (New England Biolabs) and labelled with a 2×35S probe. The *35S:HvHVP10* construct contains 2 copies of the 2×35S sequence and the single *BamHI* restriction site is located between the 2×35S promoter and the HVP10 CDS. Therefore a single copy of the transgene would produce 2 hybridisation bands, with a minimum band of 5130bp being detected for each full transgene insertion. The *Ubi:HvHVP10* OEX lines were digested with the restriction enzyme *HindIII* and labelled with 2×35S probe. As there is only one copy of 2 x 35S promoter sequence located downstream of the NOS terminator sequence, only a single hybridisation band (minimum 6663bp) would be detected for each full insert.

Restriction enzyme digestions were performed in 20 µL reactions consisting of 4 µL of gDNA, 1×supplied manufactures reaction buffer and 5 units of the appropriate restriction enzyme (New England Biolabs). Reactions were incubated at 37°C for 4-5 hours then heat inactivated at 65°C for 20 minutes. DNA fragments were separated via electrophoresis on UltraPure™ Agarose gels at a constant voltage of 35 V overnight, then transferred to a Hybond N+ nylon membrane using 0.4 M NaOH as the transfer buffer. Membranes had been pre-incubated at 37°C for approximately 24 hours in pre-hybridisation solution consisting of 10 × SSC (3 M NaCl, 0.3 M tris-sodium citrate), Denhardt's III solution, and salmon sperm DNA. The PCR product of the 2×35S product was radio labelled with [α-32P]dCTP using Klenow's fragment and a standard protocol. For hybridisation with the labelled probe membranes were incubated at 65°C for 16 hours in a hybridisation solution containing HSB buffer, Denhardt's III solution, Dentran sulphate and salmon sperm DNA. Membranes were then washed at 65°C for 20 minutes in 3 solutions of decreasing salt concentration. Washed membranes were covered with plastic film and exposed to film (Fuji HR-T), in appropriate cassettes with intensifying screens fitted at -80°C for 4-6 days. The film was developed using a CP1000 automatic film processor (AGFA, Belgium). Assistance with probe labelling, hybridisation and Autoradiography were provided by Ms. Margaret Pallotta at ACPFG.

5.2.4 Salinity tolerance screening

5.2.4.1 Barley growth conditions

Salinity tolerance screening of transgenic barley lines was conducted on the T1 generation in the greenhouse of The Plant Accelerator® at the University of Adelaide, Waite Campus and the T_2 generation in the glasshouse at CSIRO Black Mountain Laboratories in Canberra, ACT. The specific conditions and lines tested in each experiment are summarised in Table 5-1. The performance of the transgenic lines was compared to the null segregants, which had lost the transgene through segregation and/or untransformed WI4330 as controls.

Table 5-1. Summary of hydroponics experiments screening T_1 and T_2 *HvHVP10* OEX lines for salinity tolerance

Experiment	Construct	Lines tested	Date	Location	Conditions
T_1 salinity tolerance screen	*35S:HvHVP10*	WI4330 (WT) WI465-1 WI465-2 WI465-3 WI465-5 WI465-6	May/June 2012	Plant Accelerator®, University of Adelaide, SA	12-22°C natural day length (photoperiod 16 hours)
	Ubi:HvHVP10	WI4330 (WT) WI467-1 WI467-2 WI467-3 WI467-4 WI467-7	May/June 2012		
T_2 salinity tolerance screen	*35S:HvHVP10*	WI4330 (WT) WI465-1 WI465-3 WI465-7	April/May 2013	CSIRO Black Mountain Laboratories, ACT	12-28°C with natural daylength (photoperiod 12 hours)
	Ubi:HvHVP10	WI4330 (WT) WI467-1 WI467-2 WI467-4 WI467-7	June/July 2013		

5.2.4.2 T_1 screen for salinity tolerance at Plant Accelerator®, University of Adelaide, Waite Campus

T_1 seeds of uniform size of 5 lines of the *35S:HvHVP10* and 5 lines of the *Ubi:HvHVP10* construct were surface sterilised under UV light for five minutes and then germinated on moist Whatman™ filter paper (90 mm) in plastic petri dishes (145 mm diameter). The dishes were sealed in plastic bags to prevent evaporation and seeds were left to germinate at 21°C for 5 days under natural daylight. Uniformly germinated seedlings were transferred into tubes filled with polycarbonate fragments and grown in supported hydroponics systems employing a completely randomised block design as described in Results Chapter 2 (section 2.2.1.2), with the following amendments. Plants were grown to the third leaf stage (day 10 in hydroponics) and then salt (25 mM NaCl) and additional calcium chloride (3 mM) was incrementally applied twice daily to growth solution until a final concentration of 250 mM NaCl was reached. Nutrient solution was renewed every 10 days. Plants were grown for 21 days after initial salt application and symptoms of salt stress were monitored and recorded as per section 2.2.2.2.

5.2.4.3 T_2 screen for salinity tolerance at CSIRO Black Mountain Laboratories, Canberra (ACT)

Transgenic T_2 seeds of *35S:HvHVP10* and *Ubi:HvHVP10* lines were surface sterilised under UV light for 5 minutes and placed on moist paper towel in petri dishes (90mm) and left in the dark at 4°C for 2 days to assist in uniform germination. Seeds were removed from the cold room and allowed to germinate at room temperature for 3 to 4 days. Individual lines were screened for germination efficiency and the 3 best lines from the *35S:HvHVP10* construct and 4 best germinating lines from the *Ubi:HvHVP10* construct were selected for subsequent salinity screening. WI4330 seedlings were also selected in this manner to ensure unbiased seedling selection for tolerance screening. Germinated seedlings were transferred to 6.5 × 15.8 cm pots filled with quartz gravel and placed in 50 L hydroponic tanks and with a flood/drain cycle of 3 minutes and 20 minutes. Seedlings were initially watered with tap water, then half strength Modified Hoagland's Nutrient Solution (6.5 mM KNO_3, 4.0 mM $Ca(NO_3)_2$ $4H_2O$, 100 µM $NH_4H_2PO_4$, 2.0 mM $MgSO_4$ $7H_2O$, 4.6 µM H_3BO_3, 0.5 µM $MnCl_2$ $4H_2O$, 0.2 µM $ZnSO_4$ $7H_2O$, 0.1 µM $(NH_4)_6Mo_7O_{24}$ $4H_2O$, 0.2 µM $CuSO_4$ $5H_2O$, 45 µM $FeCl_3$), was added after 2 days. After a further 4 days of growth the concentration of nutrient solution was increased to full strength Modified Hoagland's Nutrient Solution. Pots were later

reordered employing a completely randomised block design, with treatments of 0 mM, 150 mM and 200 mM NaCl. Salt stress initiated when the 4th leaf was fully emerged (approximately 17 days post germination). Salt stress was applied incrementally (25 mM NaCl with additional 2 mM $CaCl_2$) in the morning and evening until the desired concentration was reached. The nutrient solution was renewed every 7 days. Plants were grown for 21 days after initial salt application and symptoms of salt stress were monitored and recorded (Figure 5-1).

Figure 5-1 Salinity tolerance hydroponics screen of T_2 transgenic WI4330 lines carrying the *UBI:HvHVP10* insert. Photographs show the growth stages of the plant at: A, three days after transplant; B, salt application (4th leaf emergence); C, D and E, Harvest (21 days post salt stress). Experiment was conducted in a PC2 Glasshouse at CSIRO Black Mountain Laboratories, ACT. Seedlings were transplanted into hydroponic tanks 5 days post germination and grown until the fourth leaf was fully emerged (approximately 17 days post germination). Salt stress was applied in 25 mM increments with additional calcium (2mM CaCl) until the desired salt concentration was reached (150 mM and 200 mM NaCl). Plants were harvested for root and shoot biomass measurements 21 days after salt stress was initiated.

5.2.4.4 Measurements of stomatal conductance

Four days after the final salt application the fourth leaf of transgenic plants carrying the *35S:HvHVP10* insert were measured with a Steady State Diffusion Poromoter (model SC-1) (Decagon). Leaf porosity measurements of each plant were conducted randomly between the hours of 10:00am and 2:00pm over a 2 day period. Measurements from the middle of the leaf were taken gently to prevent the leaf tissue from being damaged.

5.2.4.5 Measurement of biomass

Destructive sampling was performed to determine final plant biomass. Plants were removed from the pots and then their roots were rinsed with 10 mM $CaCl_2$ to remove residual NaCl and other nutrients. The roots were blotted dry on paper towels to remove excess moisture. The shoot tissue was separated from the root mass with scissors and samples were weighed immediately to determine fresh weight. Dry weights were measured after samples were dried in an oven at 65°C overnight.

5.2.4.6 Measurement of ion concentration in roots and shoots

Tissue Na^+ and K^+ concentrations were measured in the 4^{th} leaf and total roots and calculated based on tissue water content as previously described in section 2.2.1.4.

5.2.5 Molecular characterisation of transgenic barley plants

5.2.5.1 Genomic DNA extraction

To test for the presence or absence of the T-DNA insert, genomic DNA was isolated from T_1 and T_2 barley plants. High quality gDNA for Southern Blot analysis was extracted from leaves of T_1 plants using the phenol chloroform extraction protocol as previously described in section 4.2.1.3. For routine PCR gDNA was extracted from leaf tissue using a high throughput freeze-dried extraction method. Briefly; a 4 cm section of leaf tissue was harvested from each plant and placed in each well of a deep-well microtiter plate and samples were freeze-dried overnight. A sterile ball bearing was then added to each well and samples were ground in a Qiagen shaker at a frequency of 23 vibrations per second. Pre-warmed extraction buffer (375 µL) containing 0.1 M Tris-HCl (pH 8.0), 0.05 M EDTA (pH 8.0) and 1.25 % w/v SDS was added to each well and plates were incubated at 65°C for 1 hour. Plates were cooled in the fridge for 30 minutes and 187 µL of ammonium acetate was added to each well. Plates were inverted and placed in the fridge for 30 minutes, then centrifuged for 30 minutes at 4000 *g*. The supernatant (340 µl) was recovered from each well and transferred to

a fresh microtitre plate containing 220 µL of 100% (v/v) isopropanol. DNA was allowed to precipitate for 5 minutes at room temperature. Plates were centrifuged at 4000 *g* for 30 minutes and 320 µL of the supernatant was removed and added to a new deep-well microtiter plate. To each well 70% (v/v) ethanol was added and the plate was centrifuged for 30 minutes at 4000 *g*. DNA was resuspended in 225 µL of dH_2O.

5.2.5.2 RNA extraction and Complementary DNA (cDNA) synthesis

Total RNA was extracted from the newest emerged leaves of T_1 plants using the TRIZOL® based extraction method (Chomczynski and Sacchi 1987) as previously described in section 2.2.2.1. First strand cDNA was synthesised using SuperScript™ III reverse transcriptase as previously described in section 2.2.2.2

5.2.5.3 Primer Design

Primers were designed for sequencing, genotyping and expression analysis to confirm the presence and level of expression of the transgene in plant expression vectors and transgenic barley plants. Primers targeting the *HvHVP10* ORF were designed using the computer software Vector NTI Advance™ 11.00 (Invitrogen, Mulgrave, Vic, Australia). Primers targeting the Nos terminator sequence in the transformation vector were kindly provided by Dr Nick Collins (ACPFG).

5.2.5.4 Polymerase Chain R*eaction (PCR)*

The presence or absence of the transgene from individual lines was determined via PCR using the transgene specific primers PYR-25 (5´-CTTGGTCCCAAGGGTTCAGACTGC-3´) and NosT-nick (5´-CATCGCAAGACCGGCAACAGGATTC-3´) (Table 5-2) which would not amplify a product from the native *HvHVP10* gene. The gDNA samples that were extracted using the high-throughput microtitre plate method was first screened with *VRT* primers (for- 5´-CCGAATGTACTGCCGTCATCACAG-3´, rev- 5´ TGGCAGAGGAAAATATGCGCTTGA-3´) to confirm DNA integrity prior to genotyping. HotStar*Taq*® DNA Polymerase was used for PCR reactions following manufactures instructions. PCR was performed in 10 µL reactions composed of 5 µL of 2×HotStar*Taq* Master Mix, 0.2 µM PYR-25 and 0.2 µM NosT-nick, 2 µL of template DNA (50-100 ng) and sterile dH_2O water.

PCRs were carried out in a Bio-Rad C-1000 thermal cycler. PCRs were started with an initial heat activation step of 15 mins at 95°C, followed by 30 cycles of: denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72° for 2 minutes and 30 seconds. A final extension step was carried out at 72°C for 10 minutes. DNA products of PCR were separated by agarose gel electrophoresis as previously described in section 2.2.2.3.

Table 5-2 Primers used for molecular characterisation of transgenic WI4330 lines carrying the 35S:*HvHVP10* and *Ubi:HvHVP10* insert

Primer Name	Orientation	Length	Sequence (5´-3´)	Purpose	Size
PYR20-F	Forward	22	ATGGCGATCCTCGGGGAGCTCG	Amplifying HVP10 CDS, Sequencing	2289
PYR20-R	Reverse	24	CTAGATGTACTTGAACAGCAGACC	Amplifying HVP10 CDS, Sequencing	
GW1	Forward	25	GTTGCAACAAATTGATGAGCAATGC	Sequencing	N/A
GW2	Reverse	24	GTTGCAACAAATTGATGAGCAATTA	Sequencing	N/A
PYR-3	Forward	25	GTCATCAGCTGGTTGGCTCTTCCAG	Sequencing	N/A
PYR-4	Reverse	24	CTACCGAGGTTCGTTCTTTCTACG	Sequencing	N/A
PYR-25	Forward	24	CTTGGTCCCAAGGGTTCAGACTGC	Expression analysis, Genotyping	346
NOS-R3	Reverse			Expression analysis	305
NosT-Nick	Reverse	25	CATCGCAAGACCGGCAACAGGATTC	Genotyping	
VRT-for	Forward	24	CCGAATGTACTGCCGTCATCACAG	Genotyping	280
VRT-rev	Reverse	24	TGGCAGAGGAAAATATGCGCTTGA	Genotyping	
HvGAP	Forward	21	GTGAGGCTGGTGCTGATTACG	Expression analysis	189
HvGAP	Reverse	21	TGGTGCAGCTAGCATTTGACA	Expression analysis	

5.2.5.5 Reverse Transcription (RT)-PCR

RT-PCR was performed to determine the expression of the transgene barley lines using primers specific to the *HVP10* transgene and not the native *HvHVP10*. The primer pair PYR-25 5′-CTTGGTCCCAAGGGTTCAGACTGC-3′and NOS-R3 amplifies a region that contains vector specific backbone, thus making it very specific. Primers to the endogenous HvGAP control gene (forward 5′-GTGAGGCTGGTGCTGATTACG-3′, reverse 5′-TGGTGCAGCTAGCATTTGACA-3′ (Table 5-2) were used as positive controls. The RT PCR was set up in a similar manner to PCR using Platinum®*Taq* DNA polymerase and cDNA as the template.

5.2.6 Data Analysis

Data analyses, including means, SE and independent 2-tailed t-tests, were performed using Microsoft Excel (Microsoft, Redmond, WA, USA). Mean comparisons were performed using the mixed linear model function with spatial analysis in GenStat 15th Edition (VSN International, Hemel Hemstead, UK).

The relative biomass of transgenic plants carrying the *35S:HvHVP10* or the *Ubi:HvHVP10* insert under 150 mM and 200 mM were compared to the same lines under control conditions. SE was calculated for these measurements using the following formula described in Payton *et al.* (2003) based on overlapping confidence limits;

$$SE = RB\ [(SE_x/x)^2+(SE_y/y)^2]^{1/2}$$

Where:), SE = SE of the relative biomass, RB = relative biomass (treatment biomass/control biomass × 100), SE_x = treatment SE, x = treatment mean, SE_y = control SE and y = control mean.

5.3 Results

5.3.1 Generation of plant expression vectors

5.3.1.1 35S:HvHVP10 construct

The binary expression system *35S:HvHVP10* (12413bp) was developed in this study to express *HvHVP10* under the control of the cauliflower mosaic virus *35S* promoter (CaMV35S). The Gateway® destination vector *pMDC32* containing the *35S* promoter was kindly provided by Ms Jodie Kretschmer and Dr Andrew Jacobs (ACPFG). The resultant expression vector (12413bp) is shown in Figure 5-2

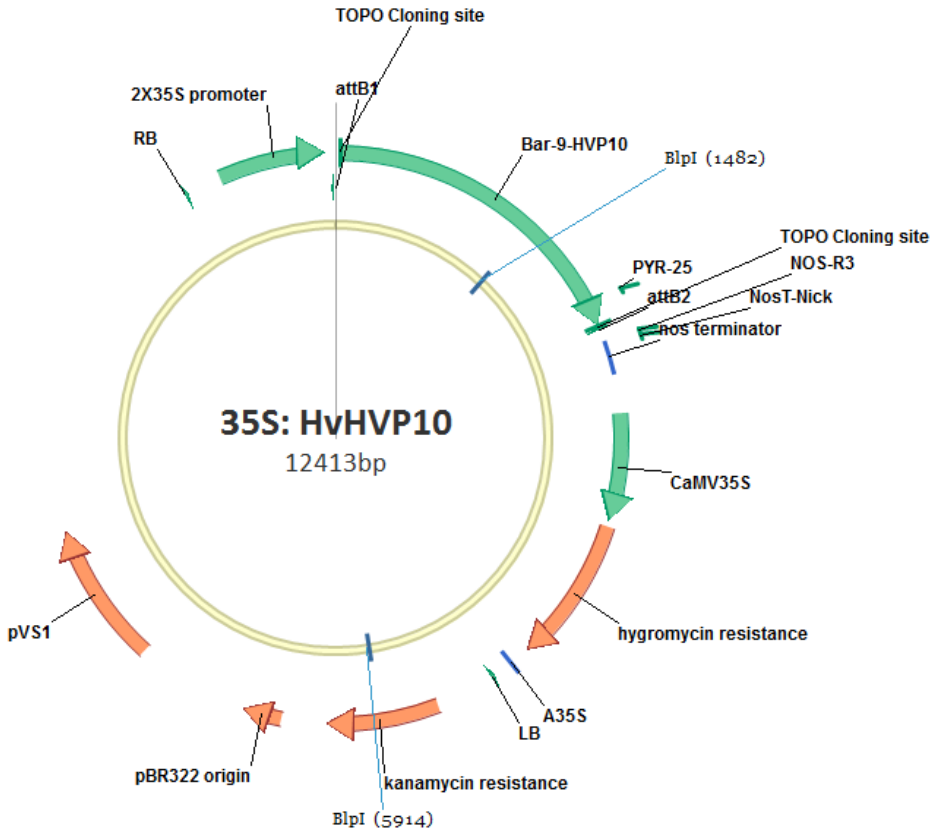

Figure 5-2 Map of the plant expression vector *35S:HvHVP10* containing *HvHVP10* CDS under the control of the cauliflower mosaic virus *35S* promoter (CaMV35S). The vector backbone contains the following components: kanamycin resistance gene, the origin of replication for *E. coli* (*pBR322*), and the minimal replicon (*pVS1*) for stable maintenance in *E. coli*. The T-DNA cassette contains the right border sequence (RB), dual cauliflower mosaic virus 35S promoter (2×35S), the gateway recombination sequences (*attB1 and attB2*), the Barque-73 *HVP10* coding sequence (Bar-9-HVP10), the nopaline synthase (*Nos*) terminator, cauliflower mosaic virus 35S promoter (CaMV35S), hygromycin resistance gene, cauliflower mosaic virus 35S terminator (A35S) and left border sequence (LB). The location of the NosT-Nick and PYR25 primers are indicated.

5.3.1.2 Ubi:HvHVP10 construct

The binary expression system *Ubi:HvHVP10* was developed in this study to express *HvHVP10* under the control of the maize *Ubiquitin-1* promoter. The Gateway compatible destination vector pTOOL37-pUBI, modified from pMDC32, was kindly provided by Ms Jodie Kretschmer and Dr Andrew Jacobs (ACPFG). The resultant expression vector (13164bp) is shown in Figure 5-3

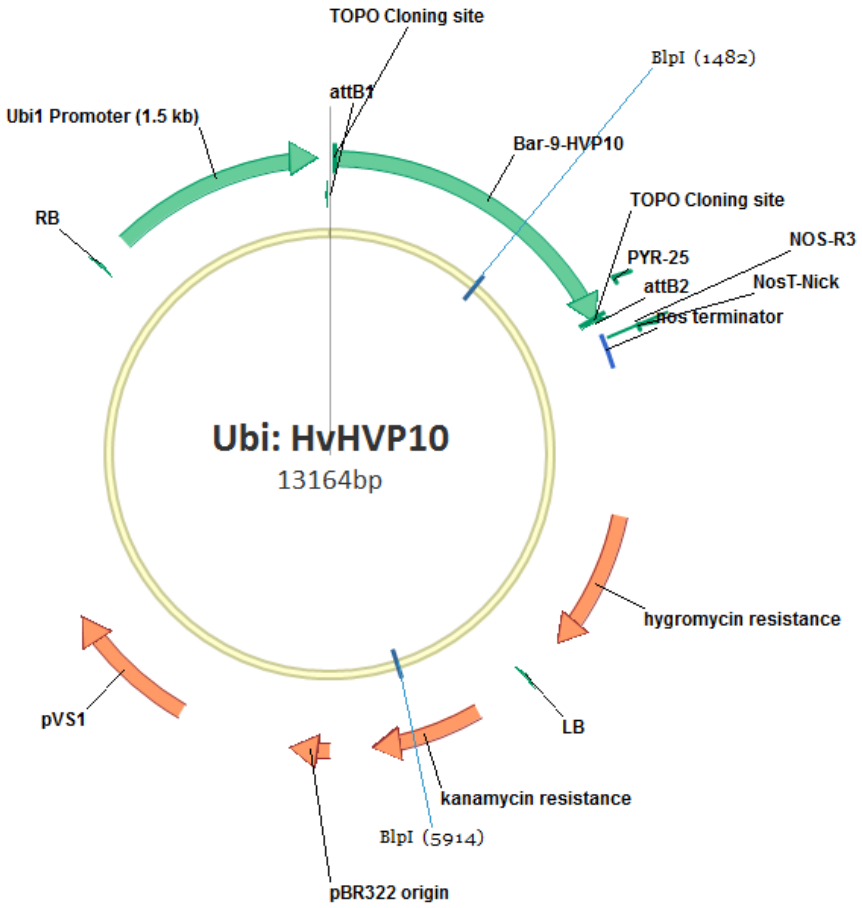

Figure 5-3 Map of the plant expression vector *Ubi:HvHVP10* containing *HvHVP10* CDS under the control of the *Ubiquitin-1* promoter (Ubi 1). The vector backbone contains the following components: kanamycin resistance gene, the origin of replication for *E. coli* (*pBR322*), and the minimal replicon (*pVS1*) for stable maintenance in *E. coli*. The T-DNA cassette contains the right border sequence (RB), the maize *Ubiquitin-1* promoter (Ubi 1), the gateway recombination sequences (*attB1 and attB2*), the Barque-73 *HvHVP10* coding sequence (Bar-9-HVP10), the nopaline synthase (*Nos*) terminator, the hygromycin resistance gene and left border sequence (LB). The location of the NosT-Nick and PYR25 primers are indicated.

5.3.1.3 Rab17:HvHVP10 construct

The binary expression system *Rab17:HvHVP10* was developed in this study to express *HvHVP10* under the control of the stress inducible *Rab17* promoter. The Gateway compatible *Rab17* destination vector, modified from pMDC32, was provided by Dr Sergiy Lopato (ACPFG). The resultant expression vector (12264bp) is shown in Figure 5-4

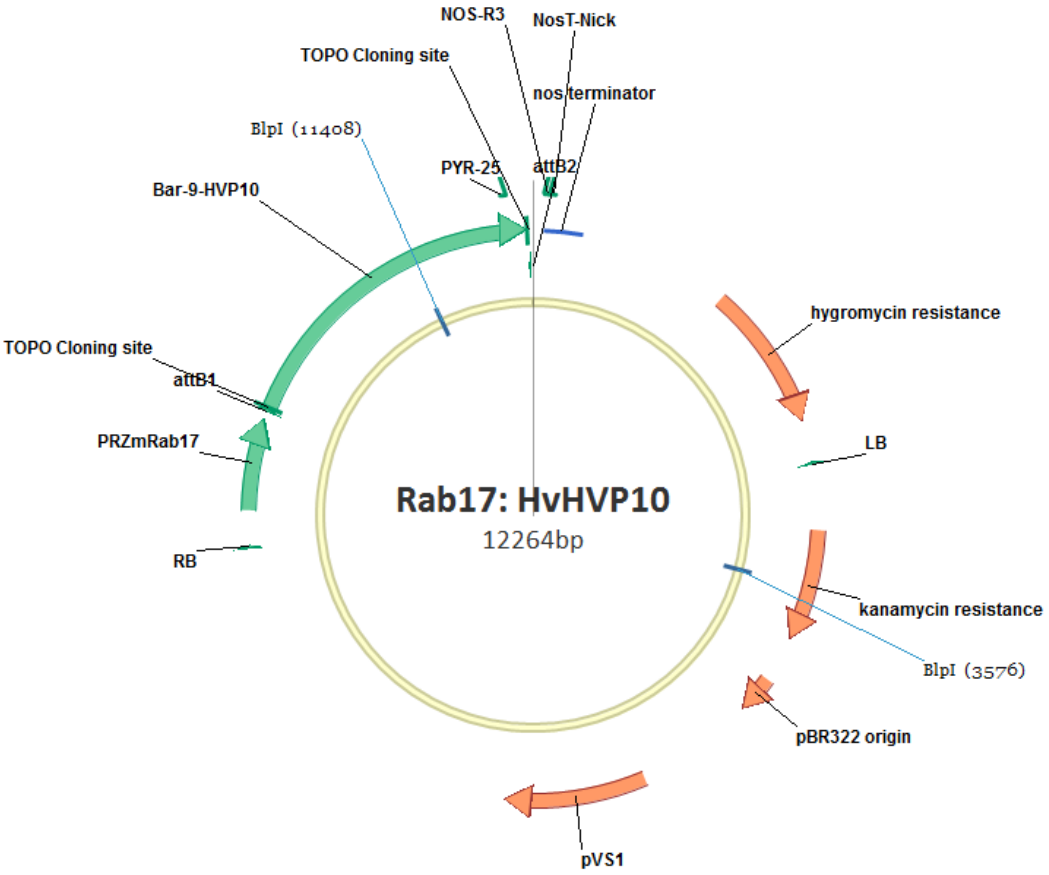

Figure 5-4 Map of the plant expression vector *Rab17:HvHVP10* containing *HvHVP10* CDS under the control of the stress inducible *Rab17* promoter (*PRZmRab17*). The vector backbone contains the following components: kanamycin resistance gene, the origin of replication for *E. coli* (pBR322), and the minimal replicon (pVS1) for stable maintenance in *E. coli*. The T-DNA cassette contains the right border sequence (RB), the *PRZmRab17* promoter, the gateway recombination sequences (attB1 and attB2), the Barque *HvHVP10* coding sequence, the nopaline synthase (Nos) terminator, the hygromycin resistance gene and left border sequence (LB). The location of the NosT-Nick and PYR25 primers are indicated.

5.3.2 Generation of transgenic barley with constitutively and stress inducible expression of HvHVP10

Six transgenic WI4330 barley lines constitutively expressing *HvHVP10* under the control of the cauliflower mosaic virus *35S* promoter and nine transgenic WI4330 lines constitutively expressing *HvHVP10* driven by the maize *Ubiquitin-1* promoter were generated by *A. tumefaciens*-mediated transformation into barley cultivar WI4330. Details of the constitutive expression lines generated in this study are summarised in Table 5-3.

Table 5-3 Summary of the result of *A.tumefaciens*-mediated transformation of WI4330 lines with constitutive expression of *HvHVP10* driven by the cauliflower mosaic virus *35S* promoter and the maize *Ubiquitin-1* promoter (Copy number is based on results of Q-PCR and Southern blot hybridisation)

Construct	Line Identification #	Copy Number (Q-PCR)	Copy Number (Southern blot)
HVP10:35S	WI465-1	1-2	1
	WI465-2	1	1
	WI465-3	1-2	1 (Possible truncated construct)
	WI465-5	1-2	1
	WI465-6	1-2	1
	WI465-7	1-2	1
HVP10:Ubi	WI467-1	3-4	3
	WI467-2	2-3	2
	WI467-3	1	1
	WI467-4	1	1
	WI467-5	2-3	2
	WI467-7	-	3
	WI467-13	-	1
	WI467-14	-	1
	WI467-16	-	1

22 WI4330 lines with *HvHVP10* driven by the *Rab17* promoter (WI466-#) were also generated in this study to determine if stress inducible expression of *HvHVP10* would also improve the salinity tolerance of WI4330 above its normal background tolerance. Unfortunately, subsequent analysis of the *Rab17* promoter by other researchers at ACPFG indicated that this promoter was also expressed under control conditions (Schilling *et al.*, 2014). Therefore due to the time constraints of the PhD it was decided to concentrate

experimental effort on the characterisation of the transgenic lines with constitutive expression of *HvHVP10*.

5.3.3 Molecular characterisation of transgenic barley WI4330 constitutively expressing HvHVP10

The integration of the transgene was confirmed using Southern-blot analysis and PCR in both the T_1 and T_2 generations. The number of T-DNA insertions in the transgenic lines constitutively expressing *HvHVP10* was analysed using Southern Blot analysis on the T_1 generation (Figure 5-5; Table 5-3). All 35S lines had a single insertion of the transgene as demonstrated by the presence of 2 bands, with the exception of WI465-3 which has a single band, perhaps suggesting the T-DNA insert had been truncated and has lost one of the *35S* promoters. There is variation in the number of T-DNA inserts in the *Ubi:HvHVP10* lines, with WI467-1 and WI467-7 having 3 copies, WI467-2 and WI467-5 having 2 copies, and WI467-3, WI467-4, WI467-13, WI467-14 and WI467-16 having a single copy of the transgene (Figure 5-6). The integration of the transgene in the T_1 and T_2 generations were also confirmed by PCR using transgene specific primers targeting the *HvHVP10* CDS and the *Nos* terminator sequence to prevent amplification of the endogenous barley *HvHVP10* gene (Figure 5-7).

RT-PCR analysis was performed to determine whether the transgene was expressed in five lines from each construct in the T_1 generation. Total RNA was extracted from leaf tissue of control and salt stressed plants and used to synthesise cDNA on which PCR was performed. Primers used in the PCR amplified a region from the middle of the transgene to the *Nos* terminator, to differentiate from endogenous *HvHVP10* gene expression. The analysis confirmed the expression of *HvHVP10* under the control of *35S* (Figure 5-8) and *Ubiquitin-1* (Figure 5-9) promoters and, as expected, no expression was detected in un-transformed WI4330.

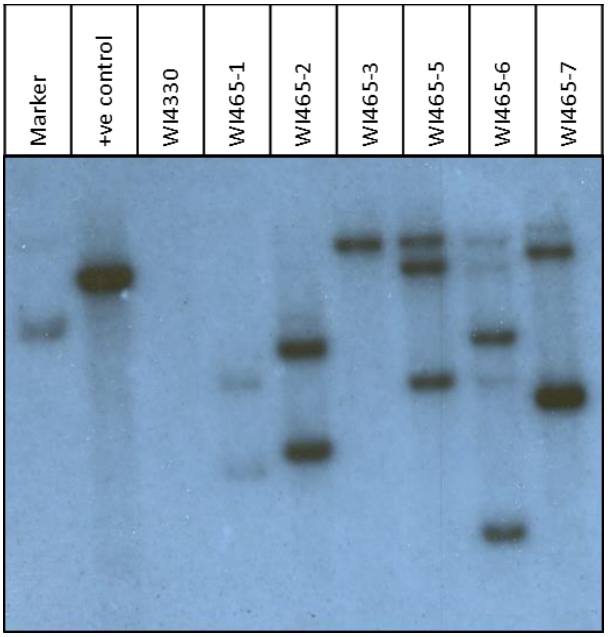

Figure 5-5 Southern blot analysis of pooled T_1 plants of transgenic WI4330 constitutively expressing *HvHVP10* CDS driven by *35S* promoter. Sixteen (16) T_1 plants were pooled for analysis. The restriction enzyme *BamHI* was used to digest gDNA of the transgenic plants and gDNA from wild type WI4330 was included as a control. PCR-amplified product of the 2×35S marker was radiolabelled with [α-^{32}P]dCTP and used as a probe. Marker λ*Hind*III.

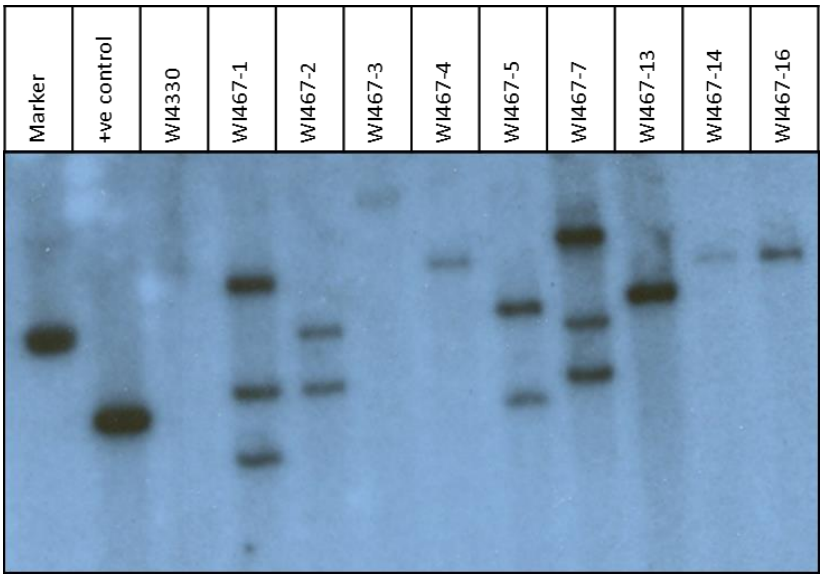

Figure 5-6 Southern blot analysis of pooled T_1 plants of transgenic WI4330 constitutively expressing *HvHVP10* CDS driven by *UBI* promoter. 16 T_1 plants were pooled for analysis Restriction enzyme *Hind*III was used digest gDNA of the transgenic plants and gDNA from wild type WI4330 was included as a control. PCR-amplified product of the 2×35S marker was radiolabelled with [α-^{32}P]dCTP and used as a probe.

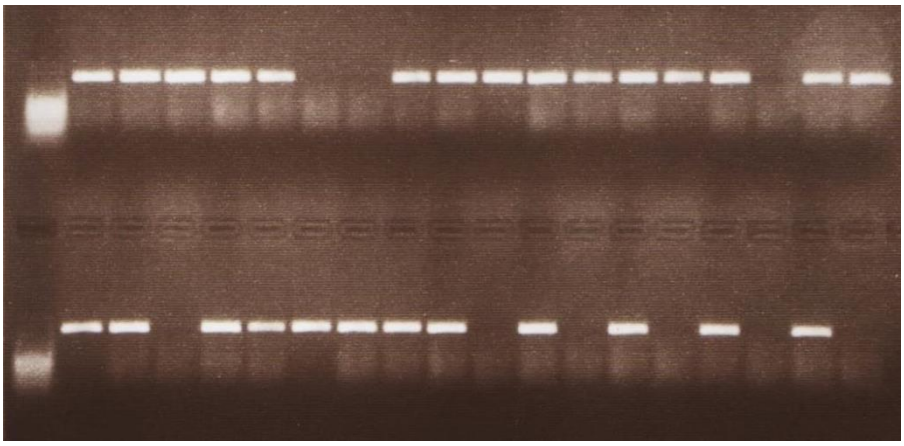

Figure 5-7 Example of molecular analysis of T_1 and T_2 transgenic WI4330 carrying *35S:HVP10* and *UBI:HVP10* inserts where each one line shows the presence/absence of the amplicon (black wells indicate nulls). The presence of *HvHVP10* in transgenic WI4330 was confirmed by PCR using gDNA as the template and transgene specific primers PYR-25 and NosT-Nick. Untransformed WI4330 was included as a negative control. gDNA was extracted from leaf tissue of barley plants grown under 0 mM, 150 mM and 200 mM NaCl using the high throughput microtitre plate method. Primers targeting the barley vernalisation gene VRT were used as an internal control confirming the presence of gDNA (data not shown).

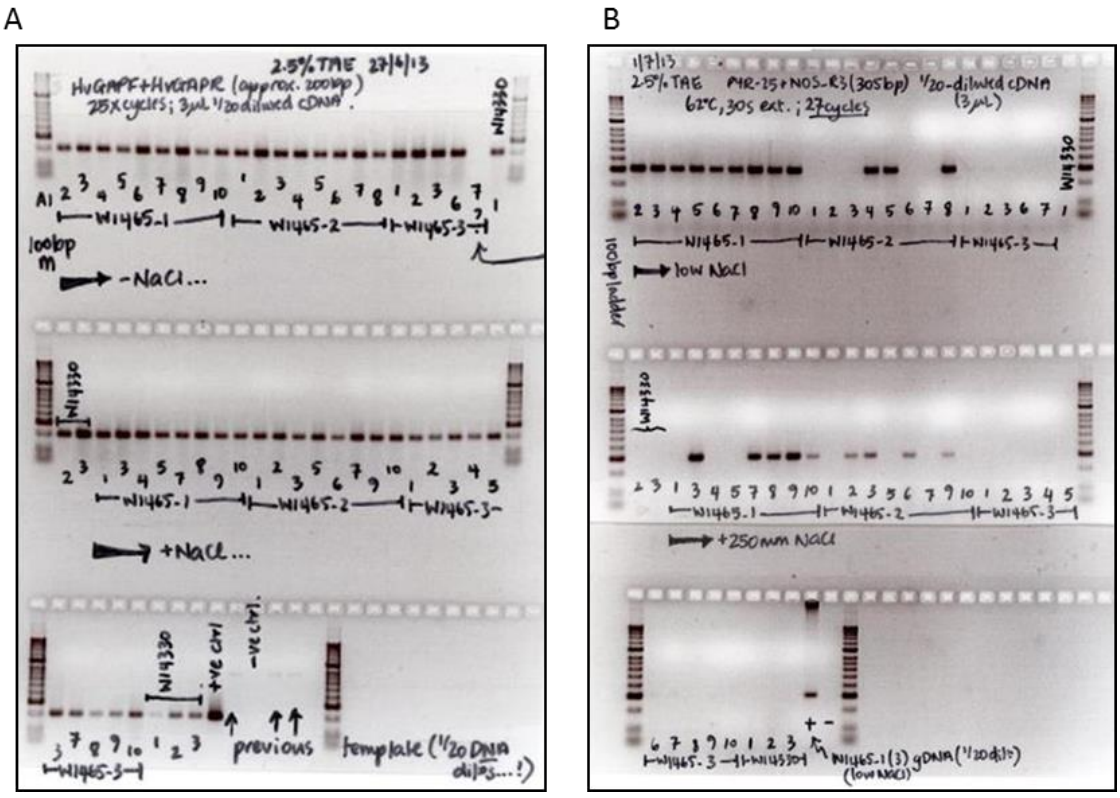

Figure 5-8 Analysis using semi-qPCR of internal control gene *HvGAP* (A) and *HvHVP10* (B) in the shoot of wild type WI4330 and T_1 transgenic WI4330 constitutively expressing *HvHVP10* under the control of the *35S* promoter. The cDNA was synthesised from total RNA extracted from leaf tissue of barley plants grown under 0 mM and 250 mM NaCl. The barley plants were grown hydroponically with salt treatment imposed in 25 mM increments over 5 days. Leaf samples were taken 21 days after salt treatment was initiated.

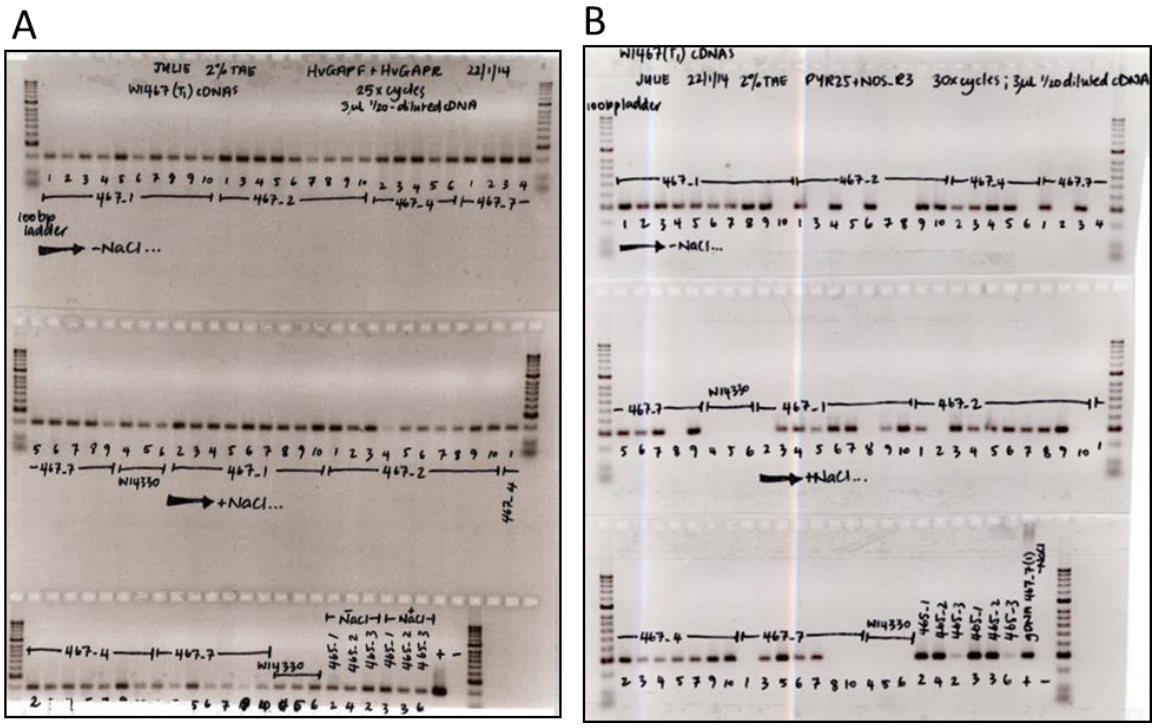

Figure 5-9 Analysis using semi-qPCR of internal control gene HvGAP (A) and HVP10 (B) in the shoot of wild type WI4330 and T_1 transgenic WI4330 constitutively expressing HVP10 under the control of *Ubiquitin-1* promoter. The cDNA was synthesised from total RNA extracted from leaf tissue of barley plants grown under 0 mM and 25 0mM NaCl. The barley plants were grown hydroponically with salt treatment imposed in 25 mM increments over 5 days. Leaf samples were taken 21 days after salt treatment was initiated.

Lines constitutively expressing *HvHVP10* were selected for salinity tolerance screening based on the quality and germination efficiency of seed from independent transformation events in the T1 and T2 generation. Ideally a minimum of 3 independent transformation events, each with on a single T-DNA insert would have been selected for phenotypic evaluation, however limited seed number and seed quality, resulted in the best germinating lines being selected for screening.

5.3.4 Salinity tolerance of transgenic barley constitutively expressing HvHVP10

5.3.4.1 Transgenic barley WI4330 lines carrying the 35S:HvHVP10 insert have variation in root and shoot biomass under salt stress

Five T_1 lines constitutively expressing *HvHVP10* under control of the cauliflower mosaic virus *35S* promoter were screened for salinity tolerance in a supported hydroponics system at the University of Adelaide, Waite Campus. At harvest the barley plants showed an obvious

reduction in plant size compared to the plants grown under control conditions. Leaf senescence, a symptom of ionic stress was also observed on the salt stressed plants.

Biomass measurements were analysed to determine the effect of salinity stress on the overall yield of transgenic plants, nulls lines and untransformed WI4330 upon completion of the experiment. The number of biological replicates for each line varied due to segregation in the T_1 generation. Therefore the nulls for each construct were combined, as there were not enough nulls for each independent transformation event to carry out appropriate statistical analyses.

Three *35S:HvHVP10* OEX lines displayed some variation in root and shoot biomass in the T_1 generation (Figure 5-10). In particular Line WI445-6 had significantly greater shoot FW and root FW and DW compared to the null segregates grown under 250 mM NaCl. WI465-2 had similar phenotype with significantly greater root DW and shoot FW compared with the null lines grown under 250 mM NaCl. Other lines showed no significant difference in root and shoot biomass. The null lines performed more poorly than the wild type, indicating that the plants were still recovering from the transformation process.

It should be noted that in all of the experiments involving the analysis of transgenic lines carrying the 35S:*HvHVP10* insert and the Ubi:*HvHVP10* insert, un-transformed WI4330 was included as a control. Un-transformed WI4330 consistently performed better that both the nulls in all the experiments, despite the fact that they are the same species, indicating that the null lines and lines carrying the transgene are suffering from transgenerational effect due to the transformation process. Therefore only the nulls will be compared to the transgenic lines as they share the same experimental history.

5.3.4.2 No variation in shoot and root Na status among transgenic plants carrying the 35S:HvHVP10 insert

The Na^+ and K^+ concentrations in the roots and shoots of transgenic WI4330 barley constitutively expressing *HvHVP10* under the 35S promoter was determined from the 4th leaf and total root at 21 days after salt stress in the screen for salinity tolerance.

Salt treatment increased Na^+ concentrations and decreased K^+ concentrations in roots and shoots of transgenic lines transformed with *HvHVP10* in the T_1 salinity tolerance screen (Figure 5-12) as expected. However, no clear differences in root or shoot Na^+ and K^+

concentrations between transgenic barley carrying the *35S:HvHVP10* insert and the null lines after salt stress were observed.

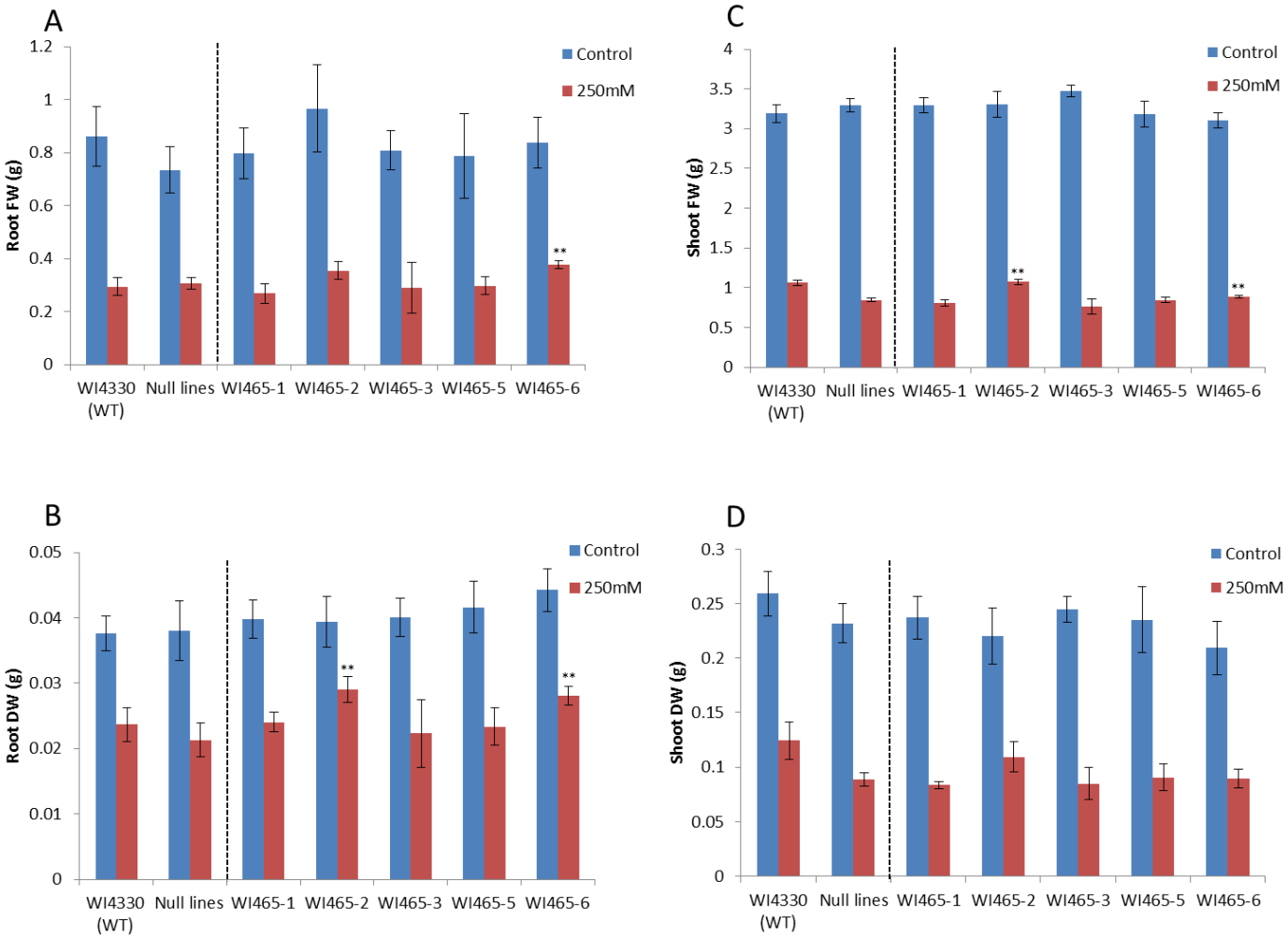
A
Root FW (g)
1.2
1
0.8
0.6
0.4
0.2
0
Control
250mM
**
WI4330 (WT)
Null lines
WI465-1
WI465-2
WI465-3
WI465-5
WI465-6
C
Shoot FW (g)
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
250mM
**
**
WI4330 (WT)
Null lines
WI465-1
WI465-2
WI465-3
WI465-5
WI465-6
B
Root DW (g)
0.05
0.04
0.03
0.02
0.01
0
Control
250mM
**
**
WI4330 (WT)
Null lines
WI465-1
WI465-2
WI465-3
WI465-5
WI465-6
D
Shoot DW (g)
0.3
0.25
0.2
0.15
0.1
0.05
0
Control
250mM
WI4330 (WT)
Null lines
WI465-1
WI465-2
WI465-3
WI465-5
WI465-6

Figure 5-10 (A) Root fresh weight, (B) root dry weight, (C) shoot fresh weight and (D) shoot dry weight of *35S:HvHVP10* T_1 lines grown in hydroponics under 0 mM and 250 mM NaCl. Salt stress was imposed in 2 5mM increments over 4 days starting when the fourth leaf was starting to emerge. Barley plants were harvested 3 weeks after initial salt application. The vertical dashed line separates wild type and nulls from plants containing the transgene. Nulls were derived from segregation of transgenic barley lines in the T_1 generation. Values are the means ± SEM with asterisks (* or **) indicating a significant difference from the null (2-tailed t-test assuming equal variances) of $P \leq 0.05$ or ≤ 0.01. The number of biological replicates differs for each line where; WI4330 = 9, 8; Null lines = 10, 15; WI465-1 = 10, 5; WI465-2 = 3, 4; WI465-3 = 4, 3; WI465-5 = 5, 4; WI465-6 = 7, 5.

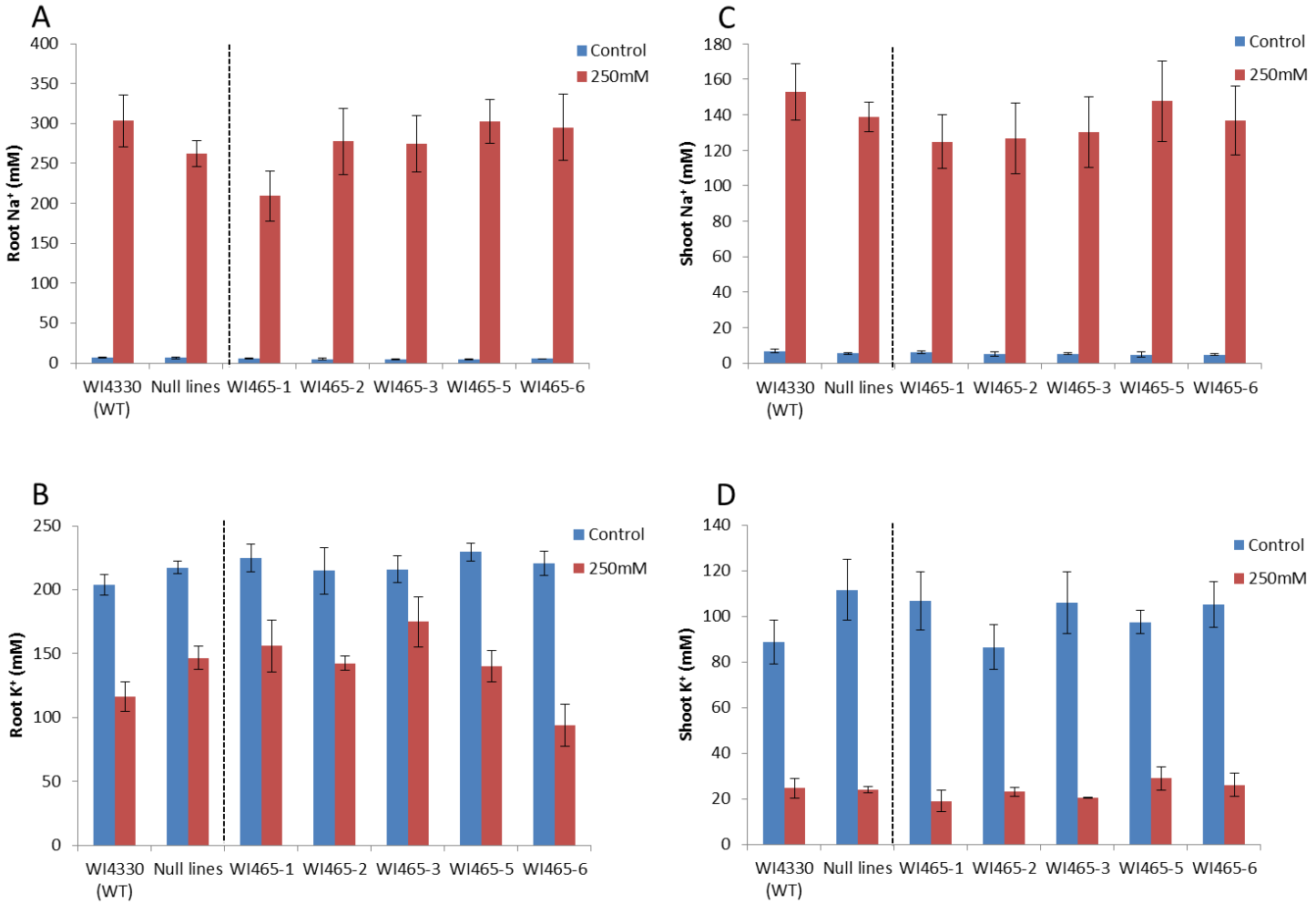
A
Root Na+ (mM)
400
350
300
250
200
150
100
50
0
Control
250mM
WI4330 (WT)
Null lines
WI465-1
WI465-2
WI465-3
WI465-5
WI465-6
C
Shoot Na+ (mM)
180
160
140
120
100
80
60
40
20
0
Control
250mM
WI4330 (WT)
Null lines
WI465-1
WI465-2
WI465-3
WI465-5
WI465-6
B
Root K+ (mM)
250
200
150
100
50
0
Control
250mM
WI4330 (WT)
Null lines
WI465-1
WI465-2
WI465-3
WI465-5
WI465-6
D
Shoot K+ (mM)
140
120
100
80
60
40
20
0
Control
250mM
WI4330 (WT)
Null lines
WI465-1
WI465-2
WI465-3
WI465-5
WI465-6

Figure 5-11 (A) Root Na^+, (B) root K^+, (C) shoot Na^+ and (D) shoot K^+ of *35S:HvHVP10* T_1 lines grown in hydroponics under 0 mM and 250 mM NaCl. Salt stress was imposed in 25 mM increments over 4 days starting when the fourth leaf was starting to emerge. Barley plants were harvested 3 weeks after initial salt application. The vertical dashed line separates wild type and nulls from plants containing the transgene. Nulls were derived from segregation of transgenic barley lines in the T_1 generation. Values are the means ± SEM. The number of biological replicates differs for each line where; WI4330 = 9, 8; Null lines = 10, 15; WI465-1 = 10, 5; WI465-2 = 3, 4; WI465-3 = 4, 3; WI465-5 = 5, 4; WI465-6 = 7, 5. Differences between lines were not statistically significant ($P \leq 0.05$).

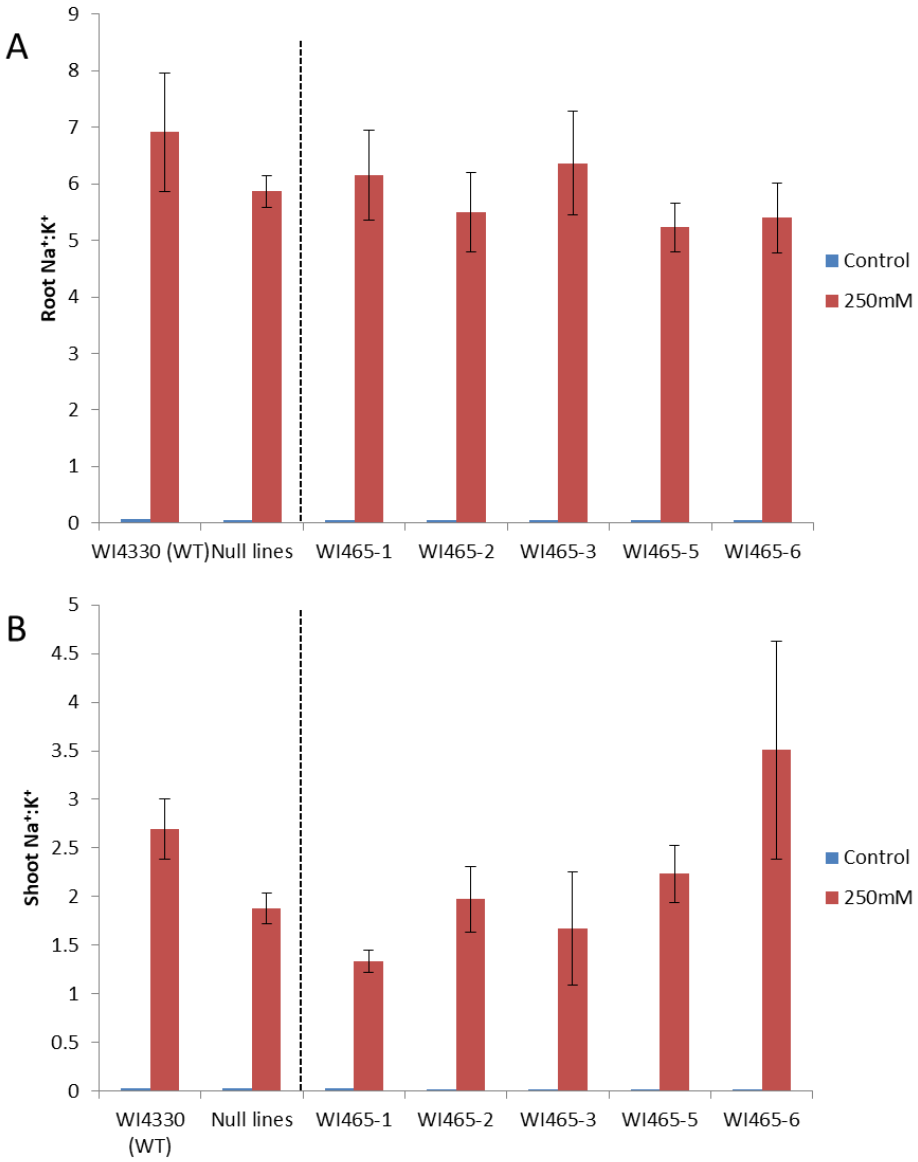

Figure 5-12 (A) Root Na^+ to root K^+ ratio and (B) shoot Na^+ to K^+ ratio of *35S:HvHVP10* T_1 lines grown in hydroponics under 0 mM and 250 mM NaCl. Salt stress was imposed in 25m M increments over 4 days starting when the fourth leaf was starting to emerge. Barley plants were harvested 3 weeks after initial salt application. The vertical dashed line separates wild type and nulls from plants containing the transgene. Nulls were derived from segregation of transgenic barley lines at the T_1 generation. Ratio-based data did not allow the calculation of standard error. The number of biological replicates differs for each line where; WI4330 = 9, 8; Null lines = 10, 15; WI465-1 = 10, 5; WI465-2 = 3, 4; WI465-3 = 4, 3; WI465-5 = 5, 4; WI465-6 = 7, 5. Differences between lines were not statistically significant ($P \leq 0.05$).

5.3.4.3 Transgenic barley WI4330 lines carrying the 35S:HvHVP10 insert have variation in root and shoot biomass in the T_2 salt tolerance screen

Three T2 lines (WI465-1, WI465-3 and WI465-7) carrying *HvHVP10* under the constitutive *35S* promoter were screened for salinity tolerance under two salt treatments of 150 mM NaCl and 200 mM NaCl for 21 days. A significant difference in the biomass of transgenic plants and their null segregates was observed when plants were subject to salinity stress (Figure 5-13). WI465-1 had significantly greater root FW and shoot FW and DW at 200 mM NaCl. WI465-7 had significantly greater root and shoot FW and DW at both 150 mM and 200 mM NaCl than their respective nulls. Interestingly WI465-3, having a truncated copy of the gene, displayed no difference in biomass between transgenic plants and nulls, although it had a biomass similar to that of those carrying the transgene. Unfortunately, due to seed availability the number of biological replicates for WI465-3 was limited and only 3 nulls of line WI465-3 were screened for salinity tolerance under control conditions.

To assess the salinity tolerance of the transgenic barley plants, the ratio of biomass in salt stress and controlled conditions was calculated for each line in the T_2 generation for both salt treatments (Table 5-4). In the T_2 generation two transgenic lines containing the full transgene insert were significantly more salt tolerant than their respective nulls. The line WI465-3 retained 21% more root DW compared with its respective null at 150 mM NaCl (59% and 38% respectively). WI465-7 was even more salt tolerant, retaining 12 % more root FW at 150 mM (56% compared to 44%) and 8% more shoot FW at 150 mM (39% compared to 31%) and 7% under 200 mM NaCl (31% to 24%). This line also retained 22% more root DW and 24% more root DW under 150 mM and 200 mM NaCl respectively, and up to 12% more shoot DW under both salt treatments. In contrast the transgenic line WI465-1 often performed equal to or worse than its null line under both salt treatments, however this result was not statically significant.

Table 5-4 Relative biomass (%) of T_2 transgenic WI4330 lines carrying *35S:HvHVP10* and nulls grown under 150 mM and 200 mM NaCl compared to the same lines grown under 0 mM NaCl Total biomass under 150 mM and 200mM NaCl ÷ total biomass under 0 mM NaCl (%) ± SEM.

		Root FW		Root DW		Shoot FW		Shoot DW	
Genotype		150 mM NaCl	200 mM NaCl	150 mM NaCl	200 mM NaCl	150 mM NaCl	200 mM NaCl	150 mM NaCl	200 mM NaCl
WI4330	WT	36(±5.3)	33(±5.5)	39(±4.51)	39(±6.83)	28(±3.49)	24(±3.4)	44(±3.6)	39(±4.7)
WI465-1	null	54(±10.8)	39(±5.9)	58(±14.7)	45(±10.0)	39(±5.8)	29(±2.9)	59(±8.3)	48(±4.2)
	gene	55(±7.9)	39(±5.6)	58(±8.7)	44(±7.05)	37(±3.4)	28(±2.8)	53(±5.1)	44(±4.5)
WI465-3	null	42(±7.1)	34(±7.0)	38(±6.1)	41(±12.1)	36(±7.3)	27(±5.9)	43(±5.8)	36(±5.6)
	gene	50(±7.6)	37(±4.7)	59(±8.2)**	47(±7.06)	36(±3.73)	26(±2.1)	54(±6.1)	42(±4.1)
WI465-7	null	43(±5.1)	38(±7.7)	46(±5.3)	44(±7.17)	31(±3.0)	24(±2.8)	45(±4.9)	37(±4.9)
	gene	56(±6.0)*	43(±4.4)	68(±6.7)**	68(±8.92)**	39(±2.8)*	31(±2.2)*	57(±4.7)*	48(±3.5)*

** = significant at P≤0.01; * = significant at P≤0.05

5.3.4.4 Transgenic barley WI4330 lines carrying the 35S:HvHVP10 insert have variation in shoot and root Na status in the T_2 salinity tolerance screen

A mixed linear model with spatial analysis using REML variance component analysis in GenStat (v.6.0) was performed to eliminate confounding experimental edge effects and to determine if significant differences exist in the response variables tested between genotypes and those containing the transgene under salinity stress (Table 5-5). Each line had enough nulls for statistical analysis, thus allowing direct examination of the effect of the transgene and salt treatment on biomass measurements and Na^+ and K^+ concentrations. The presence of the transgene was found to have a slight effect, though not significant, on shoot Na^+ (where the presence of the transgene increases shoot sodium in line WI465-7, and to a lesser extent in line WI465-3 (

Figure 5-14). Interestingly, those lines carrying the transgene were shown to maintain lower root Na^+: K^+ under salt stress (Figure 5-15), possibly suggesting that these lines are actively transporting Na^+ away from the roots.

Table 5-5 Mixed linear model with spatial analysis (REML variance component analysis) of T_2 transgenic WI4330 lines carrying *35S:HvHVP10* and nulls grown under 0 mM, 150 mM and 200 mM NaCl

Source of variation	F-test									
	Root FW (g)	Root DW (g)	Shoot FW (g)	Shoot DW (g)	Root Na^+ (mM)	Root K^+ (mM)	Shoot Na^+ (mM)	Shoot K^+ (mM)	Root Na^+/K^+ (mM)	Shoot Na^+/K^+ (mM)
Genotype	ns	*	ns	**	**	ns	*	*	**	**
Transgene	**	**	**	*	ns	*	**	ns	ns	ns
Treatment	**	**	**	**	**	*	**	**	**	**
Genotype x Transgene	ns	ns	ns	*	**	ns	ns	ns	ns	ns
Genotype x Treatment	ns	ns	**	*	ns	ns	ns	ns	**	**
Transgene x Treatment	ns	ns	ns	ns	ns	ns	*	ns	**	ns
Genotype x Transgene x Treatment	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

** = significant at P≤0.01; * = significant at P≤0.05; ns = not significant

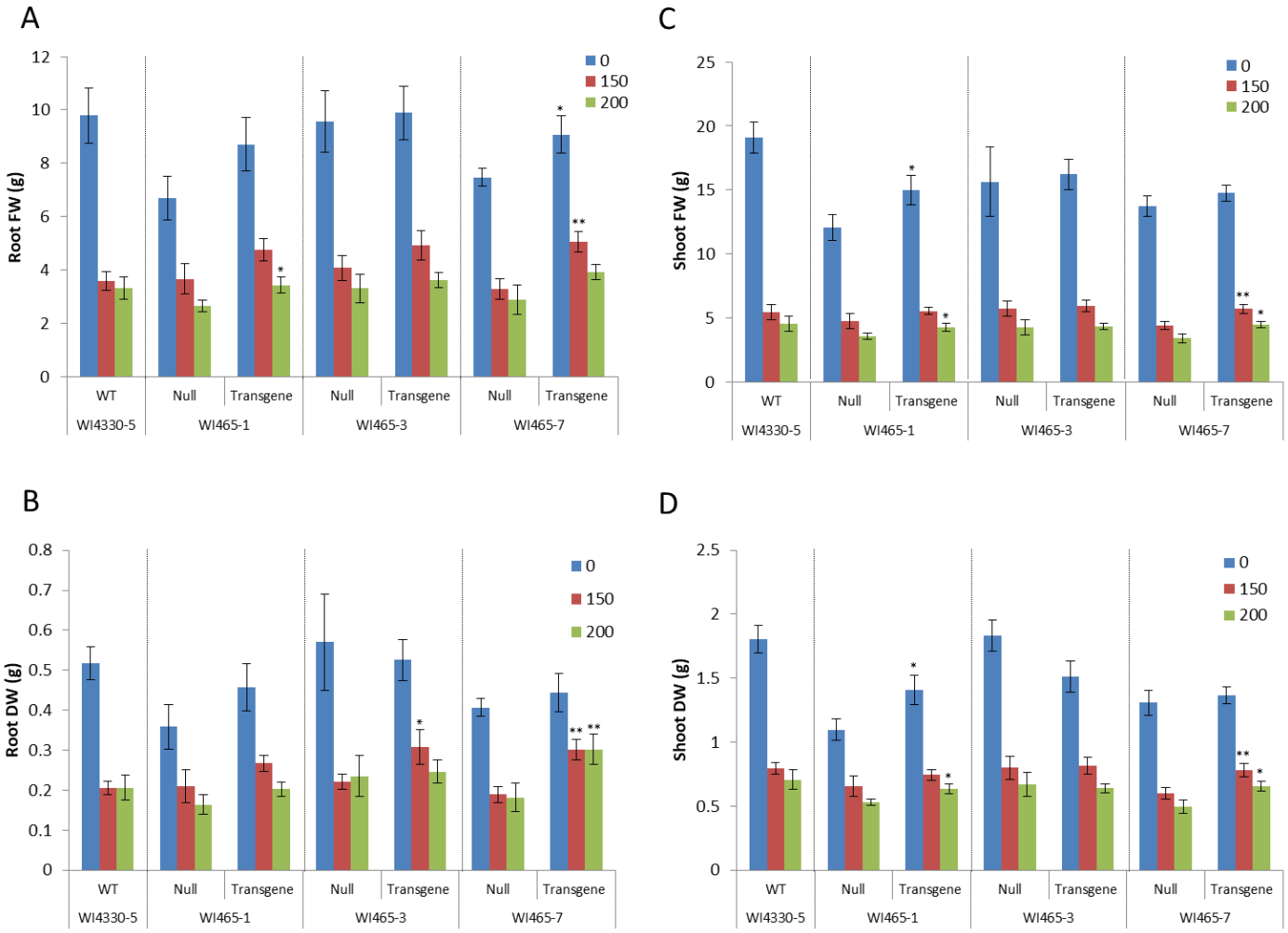
A
Root FW (g)
0
150
200
WT
Null
Transgene
WI4330-5
WI465-1
WI465-3
WI465-7
C
Shoot FW (g)
B
Root DW (g)
D
Shoot DW (g)

Figure 5-13 (A) Root fresh weight, (B) root dry weight, (C) shoot fresh weight and (D) shoot dry weight of *35S:HvHVP10* T_2 lines grown in hydroponics under 0 mM, 150 mM and 200 mM NaCl. Salt stress was initiated when the fourth leaf was starting to emerge and was imposed in 25 mM increments applied daily morning and night until the desired concentration was reached. Barley plants were harvested 3 weeks after initial salt application. Lines are separated into those containing the transgene and their respective nulls. Values are the means ± SEM with asterisks (* or**) indicating a significant difference (2-tailed t-test assuming equal variances) of $P \leq 0.05$ or ≤ 0.01. The number of biological replicates differs for each line where; WI4330-5 (WT) = 4, 5, 5; WI465-1 null = 9, 7, 8; transgene = 11, 12, 11; WI465-3 null = 3, 7, 5; transgene = 10, 8, 10; WI465-7 null = 7, 5, 4; transgene = 11, 13, 13.

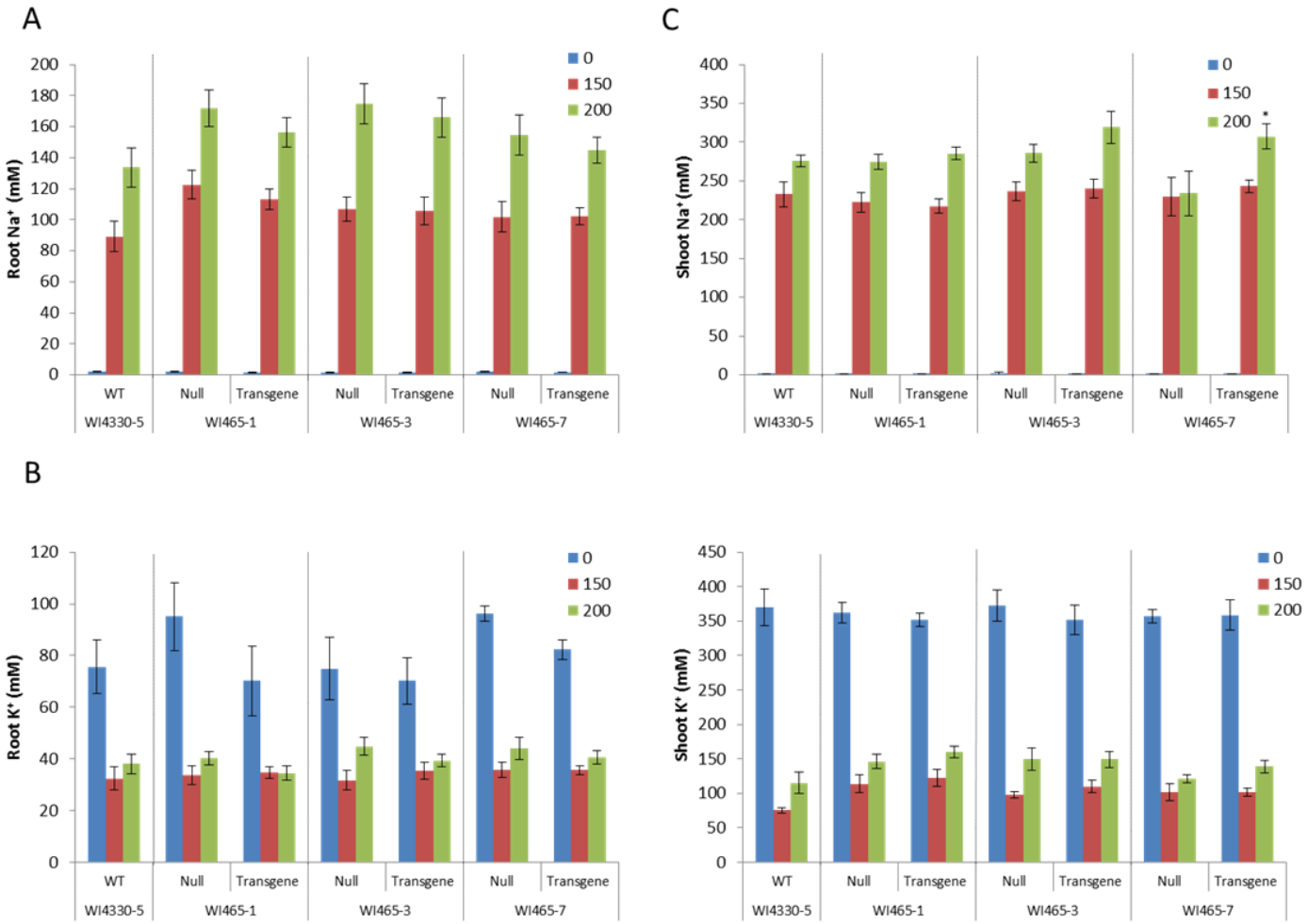
A
C
B
Root Na+ (mM)
Shoot Na+ (mM)
Root K+ (mM)
Shoot K+ (mM)
0
150
200
*
WT
Null
Transgene
WI4330-5
WI465-1
WI465-3
WI465-7

Figure 5-14 (A) Root Na^+, (B) root K^+, (C) shoot Na^+ and (D) shoot K^+ of *35S:HvHVP10* T_2 lines grown in hydroponics under 0 mM, 150 mM and 200 mM NaCl. Salt stress was initiated when the fourth leaf was starting to emerge and was imposed in 25 mM increments applied daily morning and night until the desired concentration was reached. Barley plants were harvested 3 weeks after initial salt application. Lines are separated into those containing the transgene and their respective nulls. Values are the means ± SEM with asterisks (* or**) indicating a significant difference (2-tailed t-test assuming equal variances) of $P \leq 0.05$ or ≤ 0.01. The number of biological replicates differs for each line where; WI4330-5 (WT) = 4, 5, 5; WI465-1 null = 9, 7, 8; transgene = 11, 12, 11; WI465-3 null = 3, 7, 5; transgene = 10, 8, 10; WI465-7 null = 7, 5, 4; transgene = 11, 13, 13.

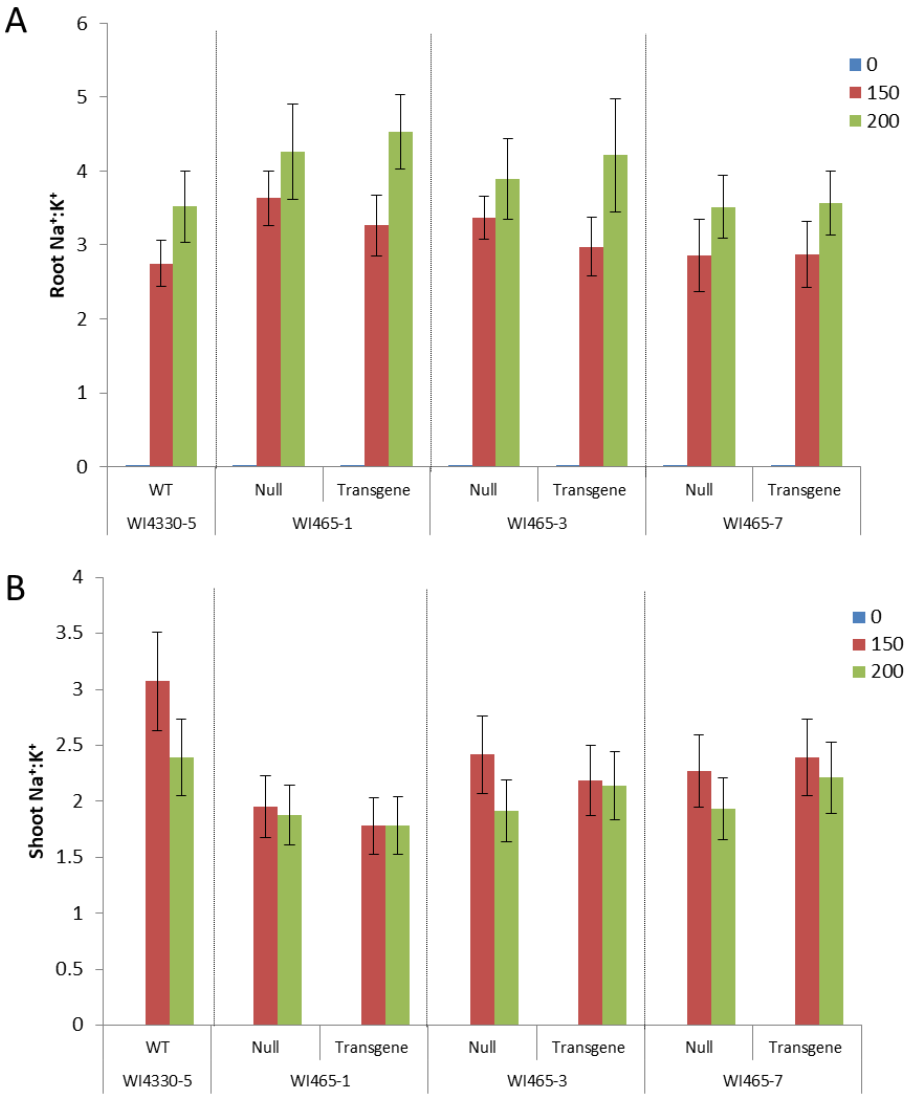

Figure 5-15 (A) Root Na^+ to root K^+ ratio and (B) shoot Na^+ to K^+ ratio of *35S:HvHVP10* T_1 lines grown in hydroponics under 0 mM, 150 mM and 200 mM NaCl. Salt stress was initiated when the fourth leaf was starting to emerge and was imposed in 25 mM increments applied daily morning and night until the desired concentration was reached. Barley plants were harvested 3 weeks after initial salt application. Values are the means ± SEM. The number of biological replicates differs for each line where; WI4330-5 (WT) = 4, 5, 5; WI465-1 null = 9, 7, 8; transgene = 11, 12, 11; WI465-3 null = 3, 7, 5; transgene = 10, 8, 10; WI465-7 null = 7, 5, 4; transgene = 11, 13, 13.

5.3.4.5 Salt treatment lowers leaf stomatal conductance of T_2 35S:HvHVP10 OEX lines

T_2 *35S:HvHVP10* lines were screened with a steady state diffusion porometer (model SC-1) in order to determine if there were variations between lines in their ability to adjust their stomatal conductance in response to salinity stress. The *35S* OEX lines were evaluated for leaf porosity 4 days after salt application was completed, in order to give the plants time to adjust to the imposed salinity stress (150 mM or 200 mM NaCl). Measurements were taken in between 10:00 am and 2:00 pm during the day over a two day period. Salt treatment was shown to significantly decrease the stomatal conductance of the plants (Figure 5-16), however there was no clear phenotypic difference observed between the lines carrying the transgene and the null segregates.

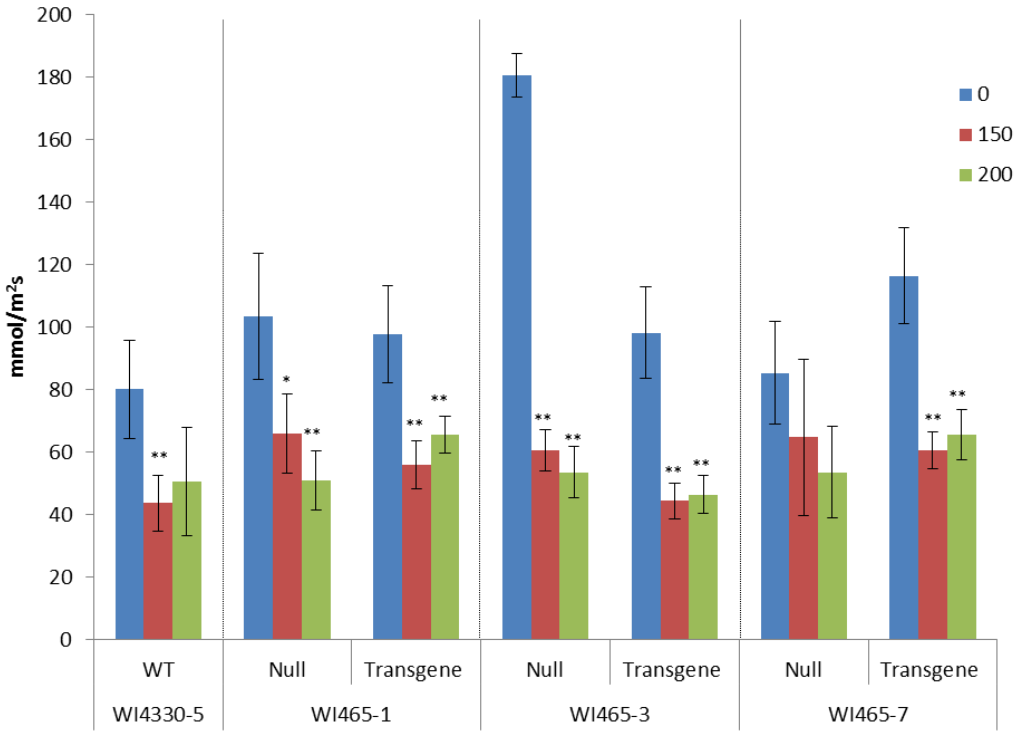

Figure 5-16 Leaf porometer measurements (model SC-1) of T_2 *35S:HvHVP10* OEX lines grown in hydroponics under 0 mM, 150 mM and 200 mM NaCl. Salt stress was initiated when the fourth leaf was starting to emerge and was imposed in 25 mM increments applied daily morning and night until the desired concentration was reached. The *35S* OEX lines were evaluated for leaf porosity 4 days after salt application was completed. Measurements were taken in between 10:00am and 2:00 during the day over a two day period. Barley plants were then harvested 3 weeks after initial salt application. Values are the means ± SEM with asterisks (* or**) indicating a significant difference (2-tailed t-test assuming equal variances) of $P \leq 0.05$ or ≤ 0.011. The number of biological replicates differs for each line where; WI4330-5 (WT) = 4, 5, 5; WI465-1 null = 9, 7, 8; transgene = 11, 12, 11; WI465-3 null = 3, 7, 5; transgene = 10, 8, 10; WI465-7 null = 7, 5, 4; transgene = 11, 13, 13.

5.3.4.6 Transgenic barley WI4330 lines carrying the Ubi:HvHVP10 insert have variation in root and shoot biomass

Five T_1 lines constitutively expressing *HvHVP10* under the control of the *Ubiquitin-1* promoter were also screened for salinity tolerance in supported hydroponics at the Plant Accelerator®, at the University of Adelaide Waite Campus. This experiment was conducted at the same time and in a similar manner as the 35S experiment. After 21 days plants showed significant signs of salinity stress, with reduced growth and increased leaf senescence compared with control grown plants.

The number of biological replicates varied for each line due to segregation in the T_1 generation. Some lines tested had 3 copies of the transgene and no null segregants (Table 5-3); therefore the nulls from each independent transformation event were combined to enable appropriate statistical analysis.

Some variation in root and shoot biomass among transgenic plants carrying the *Ubi:HvHVP10* insert was observed in the T_1 generation (Figure 5-17). In particular line WI467-7, carrying 3 copies of the transgene, had significantly greater root FW and shoot FW compared with null lines under control conditions (0 mM NaCl), however this phenotype was not observed in response to salinity stress. Under salinity stress (250 mM NaCl) the transgenic line WI467-2 had significantly greater root DW and Shoot FW and DW and line WI467-3 had significantly greater root DW.

5.3.4.7 Transgenic lines carrying the Ubi:HvHVP10 insert have less root sodium and higher shoot sodium

Ion concentration analysis suggests that lines containing *HvHVP10* under the control of the *Ubiquitin-1* promoter displayed some variation in root and shoot Na^+ and K^+ concentrations after salt stress, compared with the null lines. Transgenic lines WI467-1 and WI467-2 had significantly less root Na^+ (106 mM and 107 mM) compared with the null segregates at 250 mM NaCl (142 mM) (Figure 5-18).

5.3.4.8 Transgenic lines carrying the Ubi:HvHVP10 insert have greater root Na^+:K^+ ratio

Variation in the root and shoot Na^+: K^+ ratio of transgenic lines carrying the *Ubi:HvHVP10* insert was observed in response to salinity stress. In the T_1 screen the transgenic lines WI467-1 and WI467-2 had lower root Na^+: K^+ and higher shoot Na^+: K^+ compared with the null segregates under 250 mM NaCl (Figure 5-19).

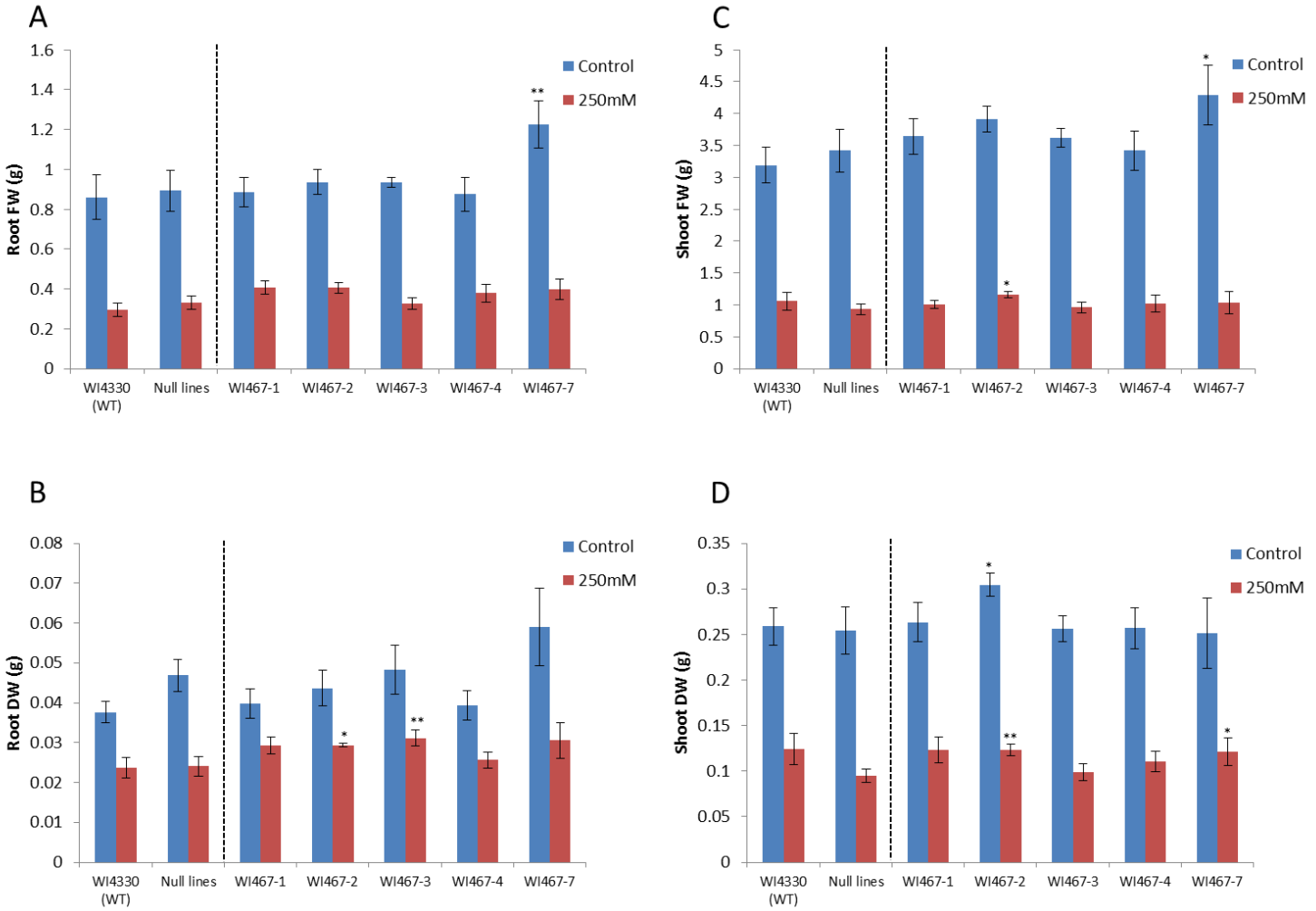
A
Root FW (g)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
250mM
**
WI4330 (WT)
Null lines
WI467-1
WI467-2
WI467-3
WI467-4
WI467-7
C
Shoot FW (g)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
250mM
*
*
WI4330 (WT)
Null lines
WI467-1
WI467-2
WI467-3
WI467-4
WI467-7
B
Root DW (g)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Control
250mM
*
**
WI4330 (WT)
Null lines
WI467-1
WI467-2
WI467-3
WI467-4
WI467-7
D
Shoot DW (g)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Control
250mM
*
**
*
WI4330 (WT)
Null lines
WI467-1
WI467-2
WI467-3
WI467-4
WI467-7

Figure 5-17 (A) Root fresh weight, (B) root dry weight, (C) shoot fresh weight and (D) shoot dry weight of *Ubi:HvHVP10* T_1 lines grown in hydroponics under 0 mM and 250 mM NaCl. Salt stress was imposed in 25 mM increments over 4 days starting when the fourth leaf was starting to emerge. Barley plants were harvested 3 weeks after initial salt application. The vertical dashed line separates wild type and nulls from plants containing the transgene. Nulls were derived from segregation of transgenic barley lines at the T_1 generation. Values are the means ± SEM with asterisks (* or**) indicating a significant difference (2-tailed t-test assuming equal variances) of $P \leq 0.05$ or ≤ 0.01. The number of biological replicates differs for each line where; WI4330 (WT) = 9, 8; Null lines = 11, 12; WI467-1 = 9, 7; WI467-2 = 5, 8; WI467-3 = 3, 5; WI467-4 = 5, 6; WI467-7 = 4, 4.

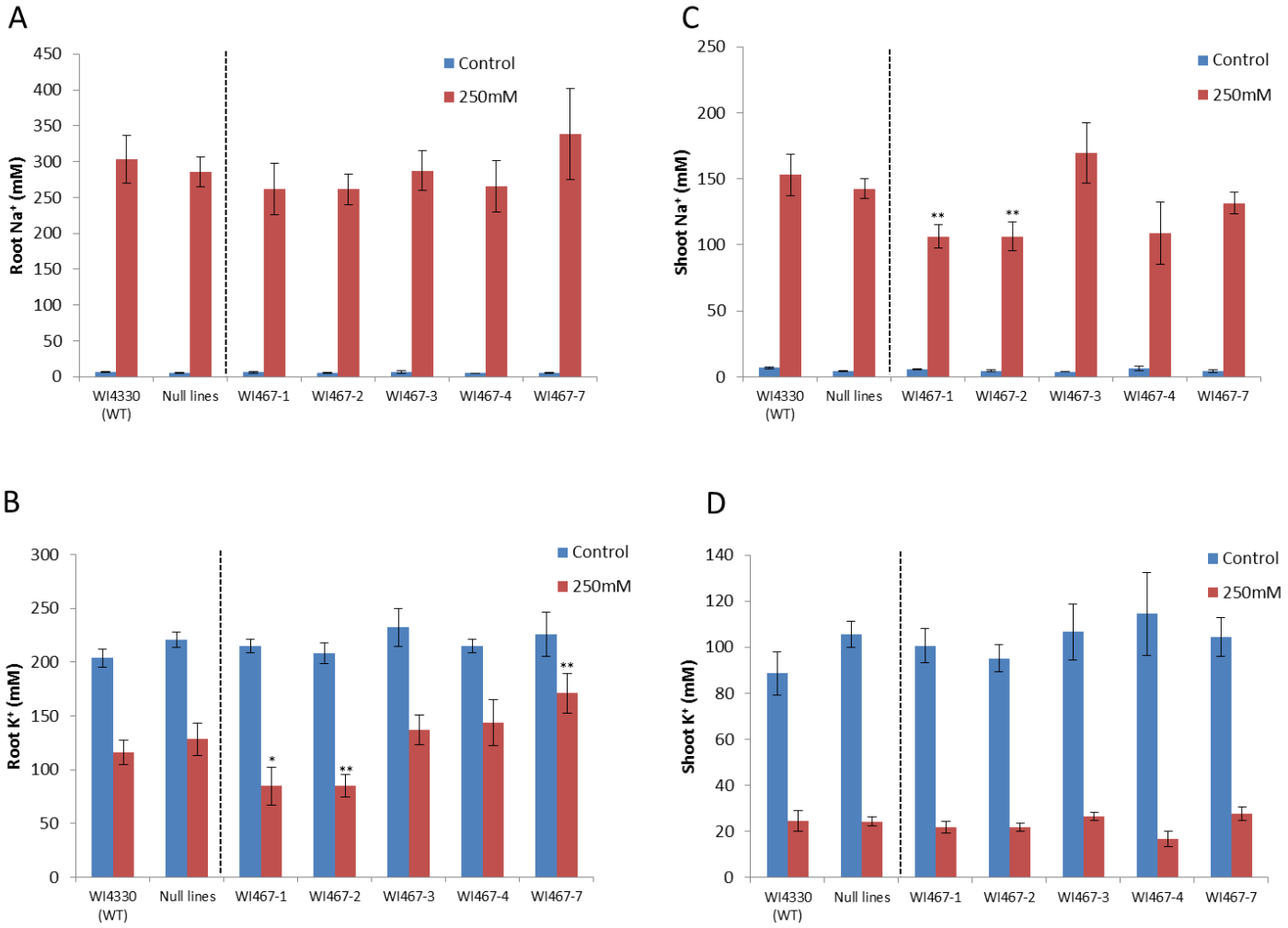
A
Root Na⁺ (mM)
Control
250mM
WI4330 (WT)
Null lines
WI467-1
WI467-2
WI467-3
WI467-4
WI467-7
B
Root K⁺ (mM)
C
Shoot Na⁺ (mM)
D
Shoot K⁺ (mM)

Figure 5-18 (A) Root Na^+, (B) root K^+, (C) shoot Na^+ and (D) shoot K^+ of *Ubi:HvHVP10* T_1 lines grown in hydroponics under 0 mM and 250 mM NaCl. Salt stress was imposed in 25 mM increments over 4 days starting when the fourth leaf was starting to emerge. Barley plants were harvested 3 weeks after initial salt application. The vertical dashed line separates wild type and nulls from plants containing the transgene. Nulls were derived from segregation of transgenic barley lines at the T_1 generation. Values are the means ± SEM with asterisks (* or**) indicating a significant difference (2-tailed t-test assuming equal variances) of $P \leq 0.05$ or ≤ 0.01. The number of biological replicates differs for each line where; WI4330 (WT) = 9, 8; Null lines = 11, 12; WI467-1 = 9, 7; WI467-2 = 5, 8; WI467-3 = 3, 5; WI467-4 = 5, 6; WI467-7 = 4, 4.

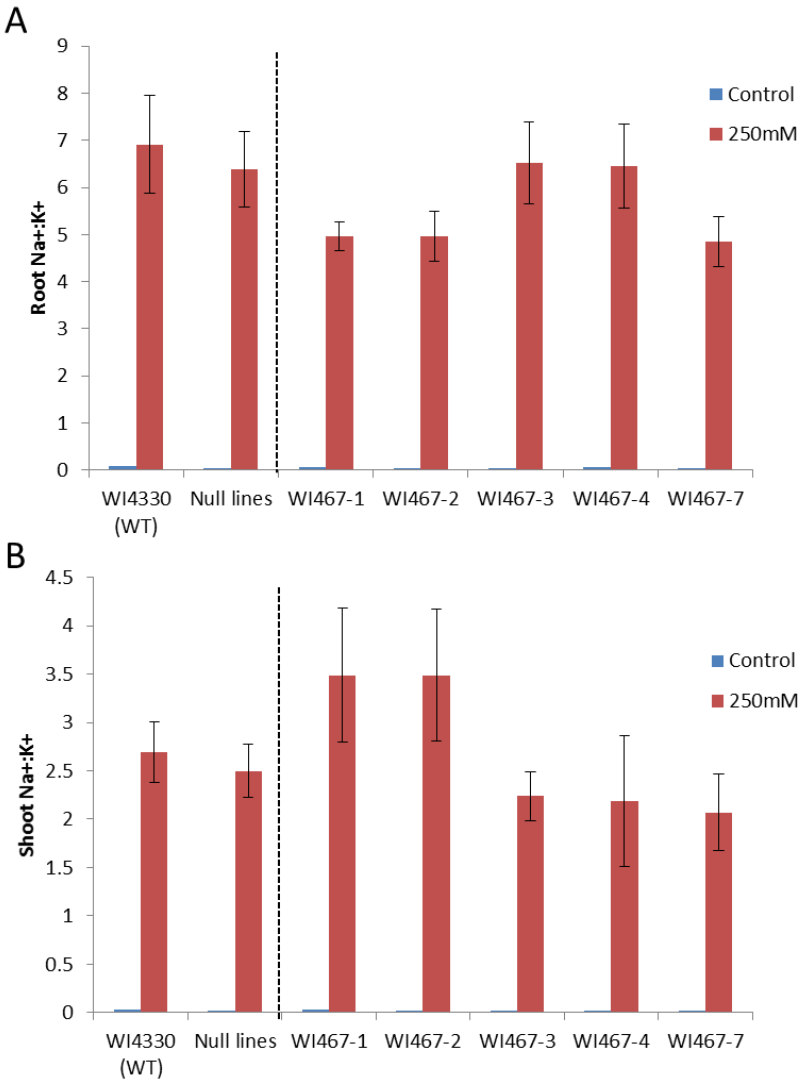

Figure 5-19 (A) Root Na^+ to root K^+ ratio and (B) shoot Na^+ to K^+ ratio of *Ubi:HvHVP10* T_1 lines grown in hydroponics under 0 mM and 250 mM NaCl. Salt stress was imposed in 25 mM increments over 4 days starting when the fourth leaf was starting to emerge. Barley plants were harvested 3 weeks after initial salt application. The vertical dashed line separates wild type and nulls from plants containing the transgene. Nulls were derived from segregation of transgenic barley lines at the T_1 generation. Values are the means ± SEM .The number of biological replicates differs for each line where; WI4330 (WT) = 9, 8; Null lines = 11, 12; WI467-1 = 9, 7; WI467-2 = 5, 8; WI467-3 = 3, 5; WI467-4 = 5, 6; WI467-7 = 4, 4.

5.3.4.9 Transgenic barley WI4330 lines carrying the Ubi:HvHVP10 insert have some variation in root and shoot biomass in the T_2 salt tolerance screen

Four lines and untransformed WI4330 were screened for salinity tolerance in the T_2 hydroponics screen at CSIRO Black Mountain Laboratories in Canberra. The number of biological replicates again varied due to segregation in the T_2 generation and null lines were combined to allow for statistical comparison between null lines and those carrying the transgene. Lines were selected based on germination efficiency and 3 levels of salt were imposed (0 mM, 150 mM and 200 mM NaCl). Plants were destructively harvested for root and shoot biomass measurements 21 days post initial salt application. Variation in biomass measurements were observed between those plants carrying the transgene and the nulls (Figure 5-20, Figure 5-21). Null lines performed worse compared to untransformed WI330 under both control and 150 mM NaCl, indicating that the plants may still be recovering from the transformation process. In general, transgenic *UBi: HvHVP10* performed better than the null lines in terms of biomass measurements under both salt and control treatments. In particular, the transgenic line WI467-7 consistently displayed significantly greater shoot FW and shoot DW, root FW and DW compared with the null lines under both the control and salt treatments. The other transgenic lines were larger than the null segregants under 0 mM NaCl, but not significantly so.

The ratio of biomass of the transgenic lines carrying the *Ubi:HvHVP10* insert under salt and control conditions was calculated to assess the salinity tolerance of these lines (Table 5-6). Transgenic WI4330 lines carrying the *Ubi:HvHVP10* insert were not shown to be significantly more salt tolerant than the null lines. The transgenic line WI467-7, whilst maintaining significantly greater biomass under salt stress, was not shown to be more salt tolerant than the null lines when compared relative to its control.

Table 5-6 Relative biomass (%) of T_2 transgenic WI4330 lines carrying *Ubi:HvHVP10* and nulls grown under 150 mM and 200 mM NaCl compared to the same lines grown under 0 mM NaCl (Total biomass under 150 mM and 200 mM NaCl ÷ total biomass under 0 mM NaCl (%) ± SE.

	Root FW		**Root DW**		**Shoot FW**		**Shoot DW**	
	150 mM	200 mM	150 mM	200 mM	150 mM	200 mM	150 mM	200 mM
WI4330(WT)	51 (±4.01)	34 (±6.5)	61 (±4.57)	45 (±8.81)	37 (±2.68)	23 (±5.43)	51 (±3.76)	38 (±6.07)
Null Lines	53 (±5.11)	47 (±9.3)	62 (±6.84)	46 (±11.35)	36 (±2.74)	29 (±7.36)	56 (±4.35)	41 (±8.69)
WI467-1	53 (±5.57)	38 (±9.6)	61 (±6.93)	42 (±9.51)	31 (±2.11)	23 (±8.34)	48 (±3.57)	37 (±8.62)
WI467-2	58 (±5.61)	42 (±8.25)	61 (±8.00)	51(±14.99)	40 (±2.55)	29 (±7.36)	59 (±4.04)	48 (±7.27)
WI467-4	51 (±6.10)	44 (±13.03)	56 (±6.96)	49 (±16.51)	36 (±2.90)	32 (±11.2)	52 (±4.53)	48 (±10.52)
WI467-7	48 (±5.07)	43 (±8.37)	56 (±6.38)	46 (±9.32)	34 (±2.39)	27 (±4.86)	50 (±3.51)	43 (±5.53)

A Mixed linear model with spatial analysis was performed on the T_2 salinity tolerance screen of WI4330 lines carrying the *Ubi:HvHVP10* insert and null lines to determine if significant differences exist between the genotypes in their response to salt stress (

Table 5-7). It was found that there was a significant genotype effect for all measured response variables. As expected, treatment effects were detected for all shoot variables, but interestingly not for root biomass measurements. Also no significant genotype × treatment effects was observed for root FW and DW measurements. This is very surprising and possibly highlights the fact that there was overall only very small variation in root biomass measurements between the 3 treatments (Figure 5-20). There was a significant interaction between the genotype and salt treatment in terms of shoot FW (Figure 5-21), root Na^+, shoot Na^+, Shoot Na^+: K^+, and Root to shoot Na^+ (

Table 5-7).

Table 5-7 Mixed linear model with spatial analysis (REML variance component analysis) on T_2 transgenic WI4330 carrying *Ubi:HvHVP10* and nulls grown under 150 mM and 200 mM NaCl.

Source of variation	F-test										
	Root FW (g)	Root DW (g)	Shoot FW (g)	Shoot DW (g)	Root Na^+ (mM)	Root K^+ (mM)	Shoot Na^+ (mM)	Shoot K^+ (mM)	Root Na+/K^+ (mM)	Shoot Na^+/K^+ (mM)	Root: Shoot Na^+
Genotype	**	**	**	**	**	**	**	**	**	**	*
Treatment	ns	Ns	**	*	**	**	**	**	**	**	**
Genotype x treatment	ns	Ns	*	ns	**	ns	*	ns	ns	**	**

** = significant at $P \leq 0.01$; *= significant at $P \leq 0.05$; ns = not significant

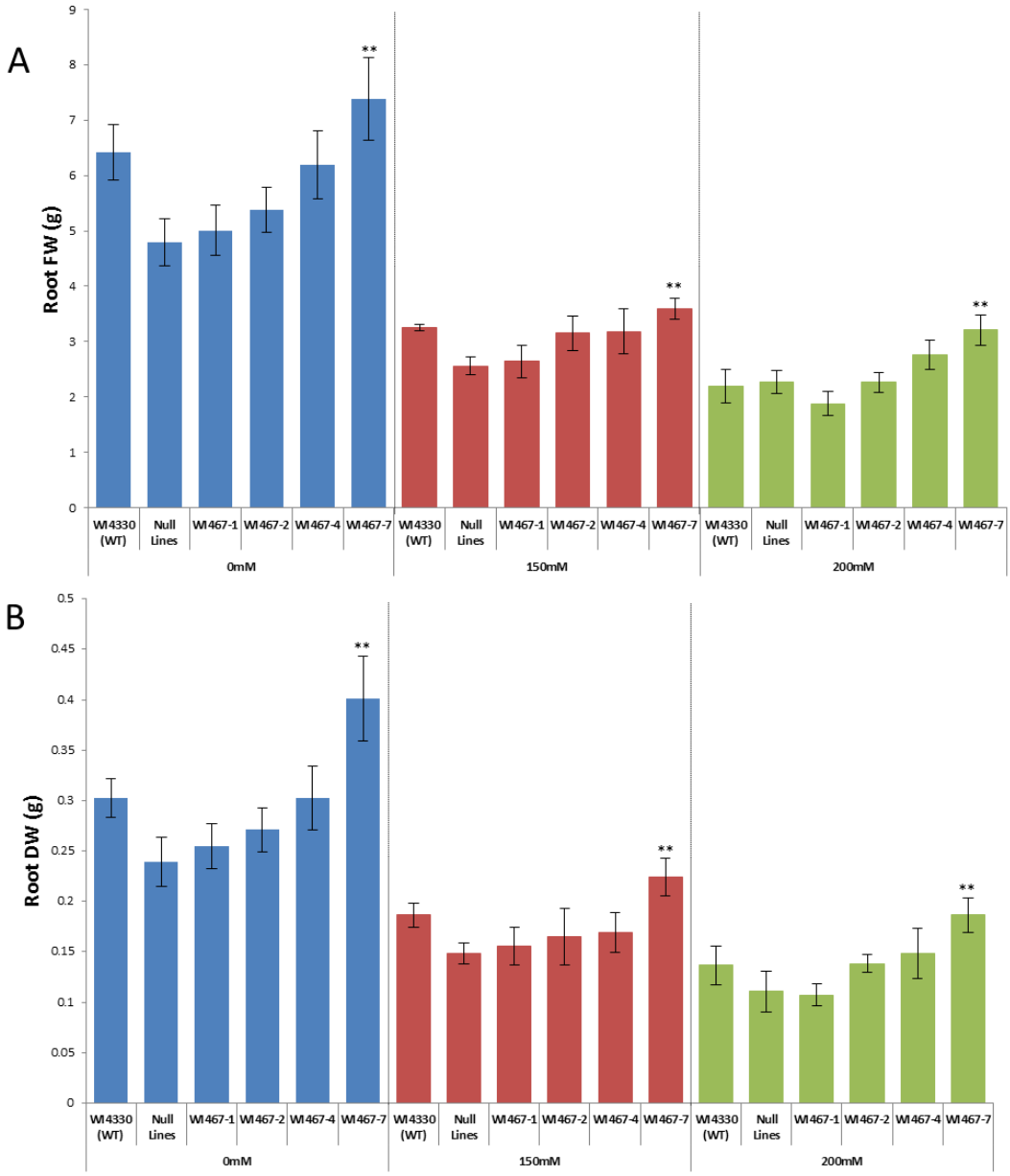

Figure 5-20 (A) Root fresh weight, and (B) root dry weight, of *Ubi:HvHVP10* T_2 lines grown in hydroponics under 0 mM, 150 mM and 200 mM NaCl. Salt stress was initiated when the fourth leaf was starting to emerge and was imposed in 25 mM increments applied daily morning and night until the desired concentration was reached. Barley plants were harvested 3 weeks after initial salt application. Nulls were derived from segregation of transgenic barley lines at the T_2 generation. Values are the means ± SEM with asterisks (* or**) indicating a significant difference (2-tailed t-test assuming equal variances) of $P \leq 0.05$ or ≤ 0.01. The number of biological replicates differs for each line where; WI4330 (WT) = 9, 4, 6; Null lines = 14, 15, 13; WI467-1 = 19, 18, 18; WI467-2 = 12, 14, 13; WI467-4 = 8, 7, 9; WI467-7 = 16, 16, 15.

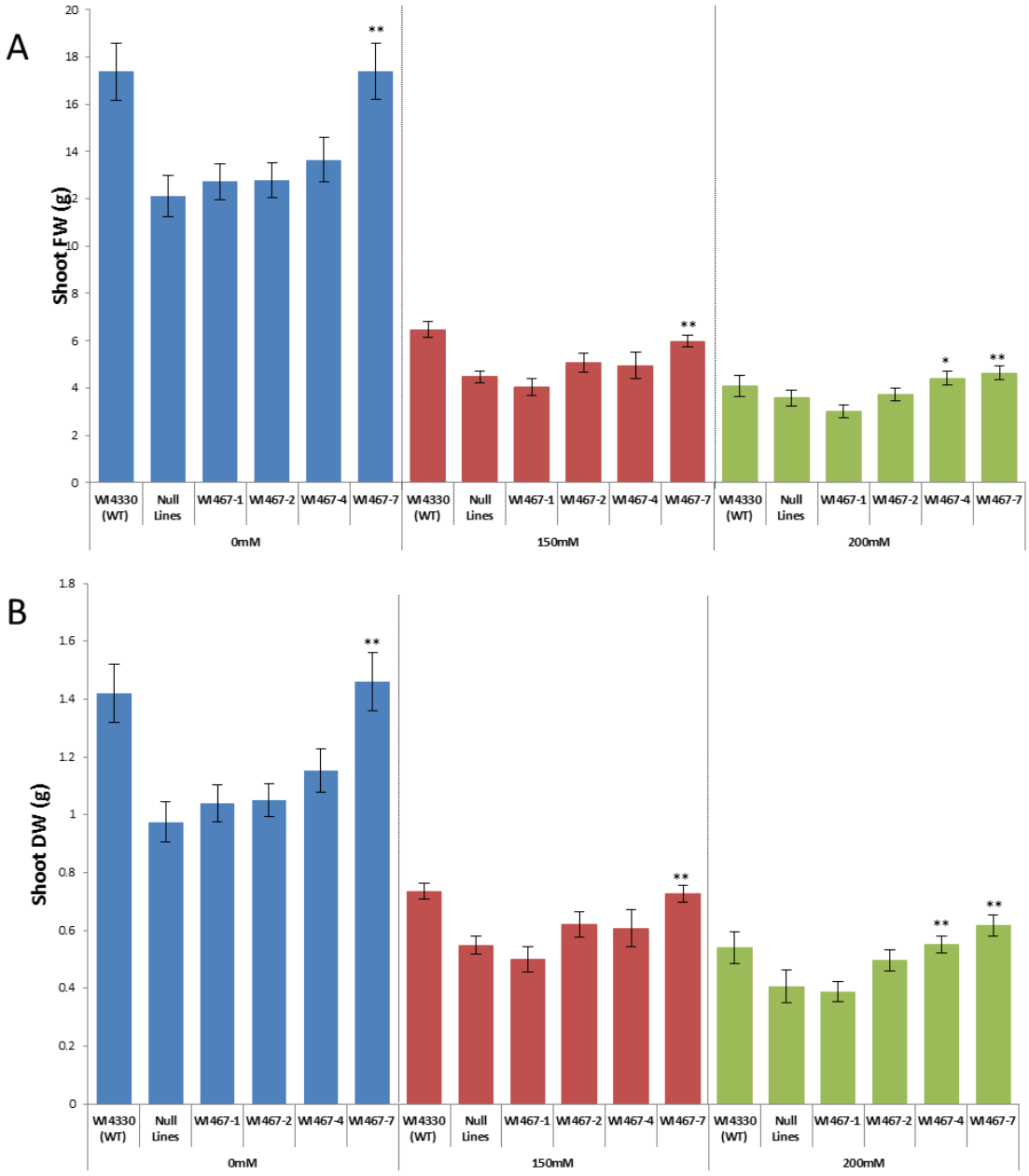

Figure 5-21 (A) Shoot fresh weight and (B) shoot dry weight of *Ubi:HvHVP10* T_2 lines grown in hydroponics under 0 mM, 150 mM and 200 mM NaCl. Salt stress was initiated when the fourth leaf was starting to emerge and was imposed in 25 mM increments applied daily morning and night until the desired concentration was reached. Barley plants were harvested 3 weeks after initial salt application. Nulls were derived from segregation of transgenic barley lines at the T_2 generation. Values are the means ± SEM with asterisks (* or**) indicating a significant difference (2-tailed t-test assuming equal variances) of $P \leq 0.05$ or ≤ 0.01. The number of biological replicates differs for each line where; WI4330 (WT) = 9, 4, 6; Null lines = 14, 15, 13; WI467-1 = 19, 18, 18; WI467-2 = 12, 14, 13; WI467-4 = 8, 7, 9; WI467-7 = 16, 16, 15.

5.3.4.10 Transgenic lines carrying the Ubi:HvHVP10 insert have higher shoot sodium in the T_2 screen

Limited variation was detected in root Na^+ and K^+ concentrations of transgenic Ubi:*HvHVP10* lines in response to salinity stress (Figure 5-22), with only line WI467-7 displaying significantly less Na^+ in its roots and shoots under salinity stress. In contrast, significant variation in shoot Na^+ and K^+ concentrations was observed (Figure 5-23). The transgenic WI4330 lines WI467-2, WI467-4 and WI467-7 had significantly more Na^+ in their shoot at 150 mM NaCl (246 mM, 243 mM and 245 mM) compared to the nulls (223 mM) (Figure 5-23). The transgenic line WI467-7 also had significantly more shoot Na^+ (297 mM) at 200 mM NaCl compared with the null lines (274 mM), however this line also had significantly less shoot K^+ at 150 mM (61 mM) and 200 mM (80 mM) NaCl. In contrast, the transgenic line WI467-4 had significantly more K^+ in the shoots at both 150 mM (94 mM) and 200 mM (111 mM) NaCl compared with the null segregates (71 mM and 94 mM).

In the T_2 salinity tolerance screen all transgenic lines had greater root Na^+: K^+ at 150 mM NaCl. WI467-7 had higher shoot Na^+: K^+ at both 150 mM and 200 mM NaCl respectively (Figure 5-24), whereas transgenic line WI465-4 displayed lower Na^+: K^+ in the shoot under both salt treatments.

Root: shoot Na^+ ratio was also calculated in order to determine if the transgenic lines maintained a different ratio than the null lines, possibly indicating Na^+ partitioning within specific tissue. The transgenic lines tended to maintain a lower root: shoot Na^+ ratio compared with the null segregates (Figure 5-25), indicating that the lines carrying the transgene maintain a higher sodium concentration in their shoots relative to their roots.

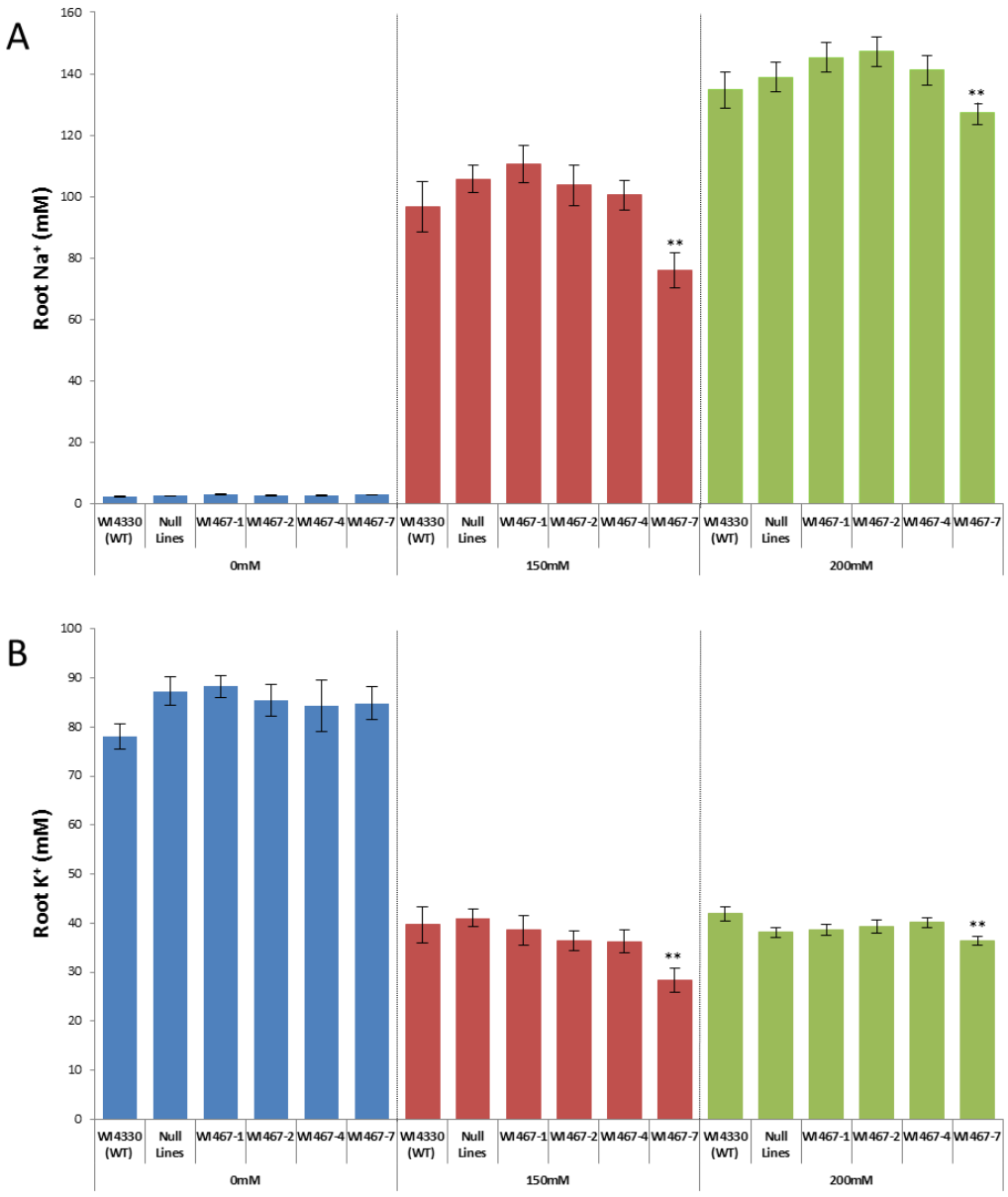

Figure 5-22 (A) Root Na^+ and (B) root K^+ of *Ubi:HvHVP10* T_2 lines grown in hydroponics under 0 mM, 150 mM and 200 mM NaCl. The vertical dashed lines separate salt treatments. Salt stress was initiated when the fourth leaf was starting to emerge and was imposed in 25 mM increments applied daily morning and night until the desired concentration was reached. Barley plants were harvested 21 d after initial salt application. Total roots were used for the measurement of ion concentration. Nulls were derived from segregation of transgenic barley lines at the T_2 generation. Values are the means ± SEM with asterisks (* or**) indicating a significant difference (2-tailed t-test assuming equal variances) of $P \leq 0.05$ or ≤ 0.01. The number of biological replicates differs for each line where; WI4330 (WT) = 9, 4, 6; Null lines = 14, 15, 13; WI467-1 = 19, 18, 18; WI467-2 = 12, 14, 13; WI467-4 = 8, 7, 9; WI467-7 = 16, 16, 15 for 0mM, 150mM and 200mM NaCl respectively.

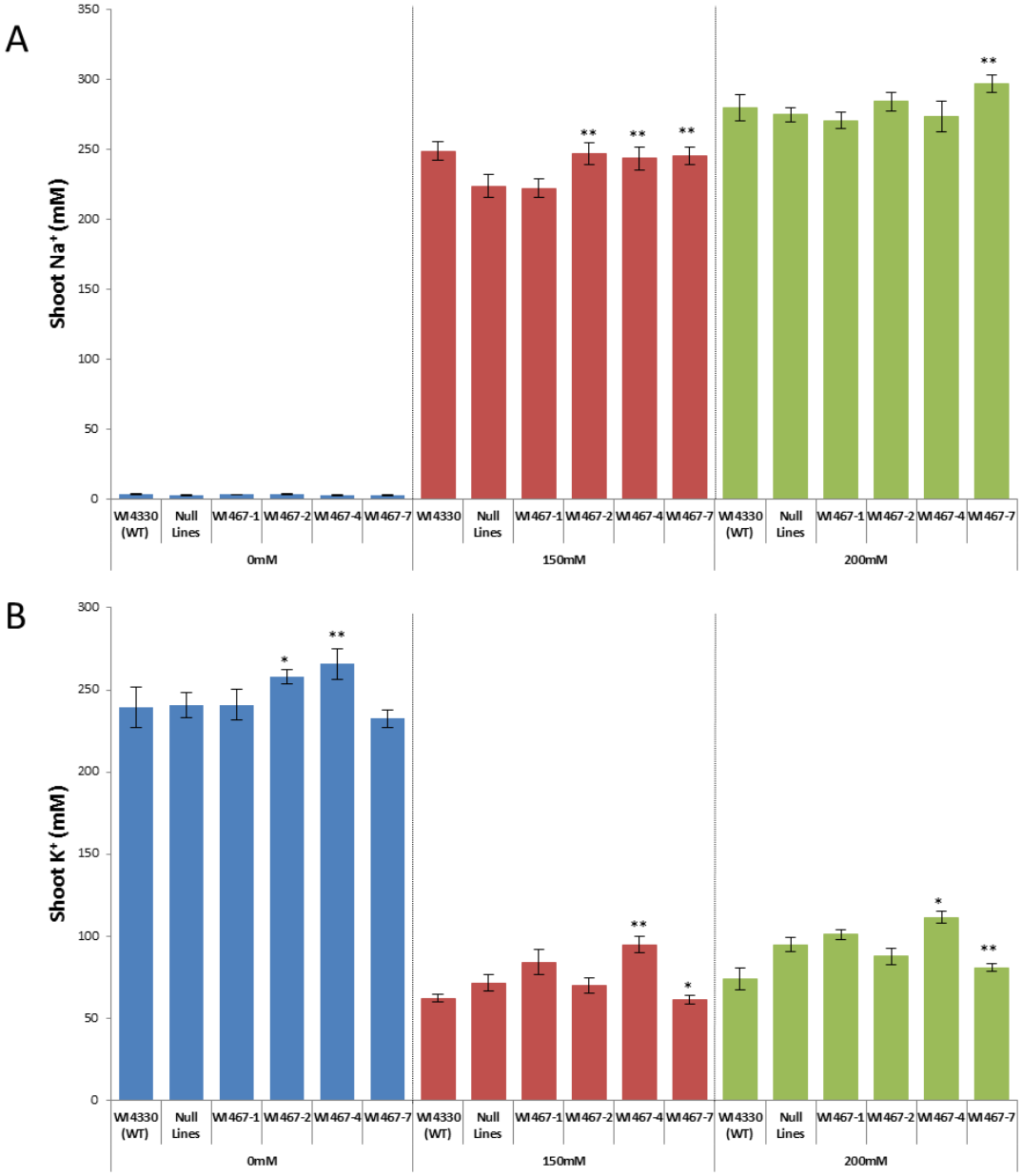

Figure 5-23 (A) shoot Na^+ and (B) shoot K^+ of *Ubi:HvHVP10* T_2 lines grown in hydroponics under 0 mM, 150 mM and 200 mM NaCl. Salt stress was initiated when the fourth leaf was starting to emerge and was imposed in 25 mM increments applied daily morning and night until the desired concentration was reached. Barley plants were harvested 3 weeks after initial salt application. Nulls were derived from segregation of transgenic barley lines at the T_1 generation. Values are the means ± SEM with asterisks (* or**) indicating a significant difference (2-tailed t-test assuming equal variances) of $P \leq 0.05$ or ≤ 0.01. The number of biological replicates differs for each line where; WI4330 (WT) = 9, 4, 6; Null lines = 14, 15, 13; WI467-1 = 19, 18, 18; WI467-2 = 12, 14, 13; WI467-4 = 8, 7, 9; WI467-7 = 16, 16, 15.

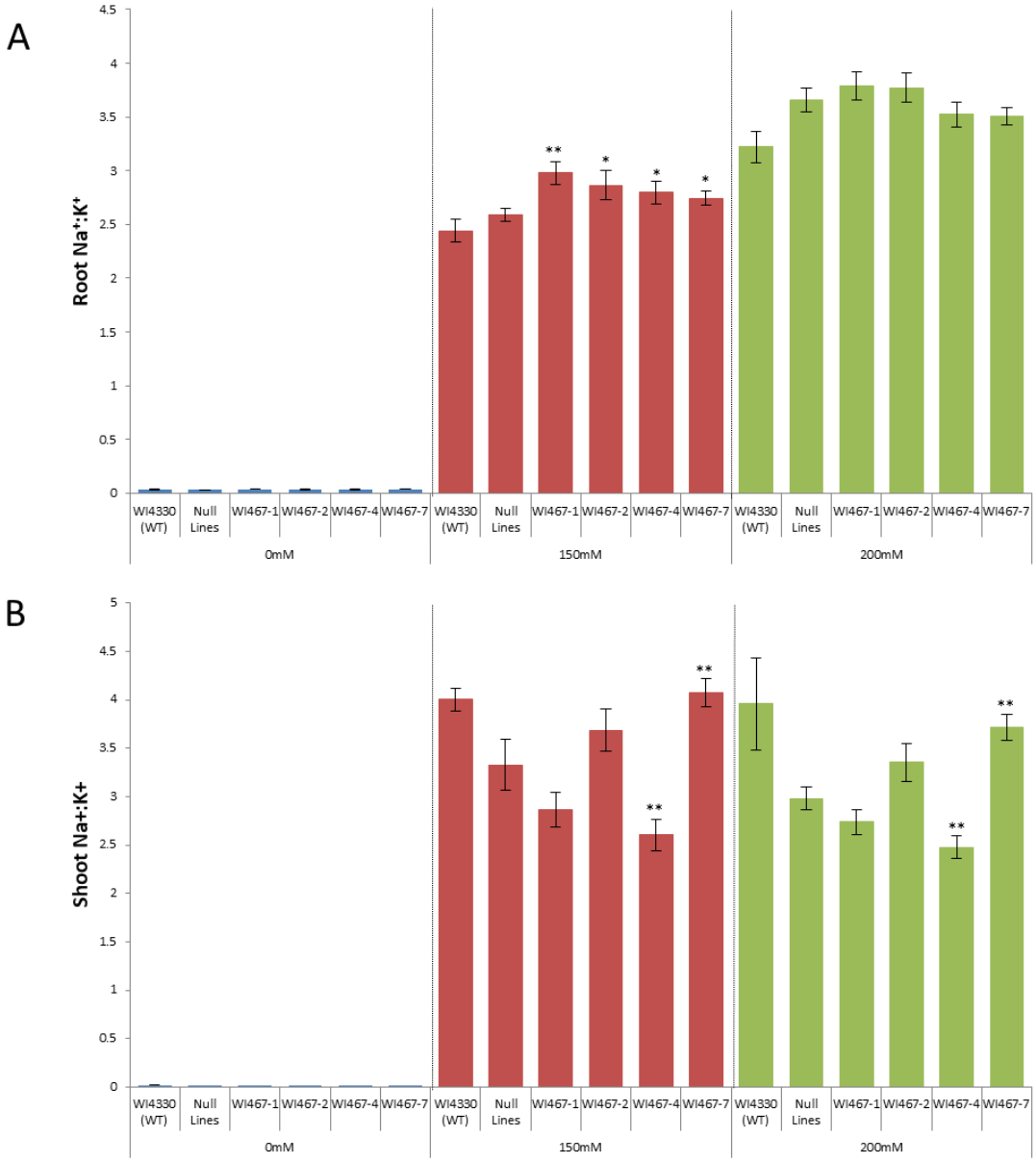

Figure 5-24 (A) Root Na^+ to root K^+ ratio and (B) shoot Na^+ to K^+ ratio of *Ubi:HvHVP10* T_2 lines grown in hydroponics under 0 mM, 150 mM and 200 mM NaCl. Salt stress was initiated when the fourth leaf was starting to emerge and was imposed in 25 mM increments applied daily morning and night until the desired concentration was reached. Barley plants were harvested 3 weeks after initial salt application. Nulls were derived from segregation of transgenic barley lines at the T_2 generation. Values are the means ± SEM with asterisks (* or**) indicating a significant difference (2-tailed t-test assuming equal variances) of $P \leq 0.05$ or ≤ 0.01. The number of biological replicates differs for each line where; WI4330 (WT) = 9, 4, 6; Null lines = 14, 15, 13; WI467-1 = 19, 18, 18; WI467-2 = 12, 14, 13; WI467-4 = 8, 7, 9; WI467-7 = 16, 16, 15.

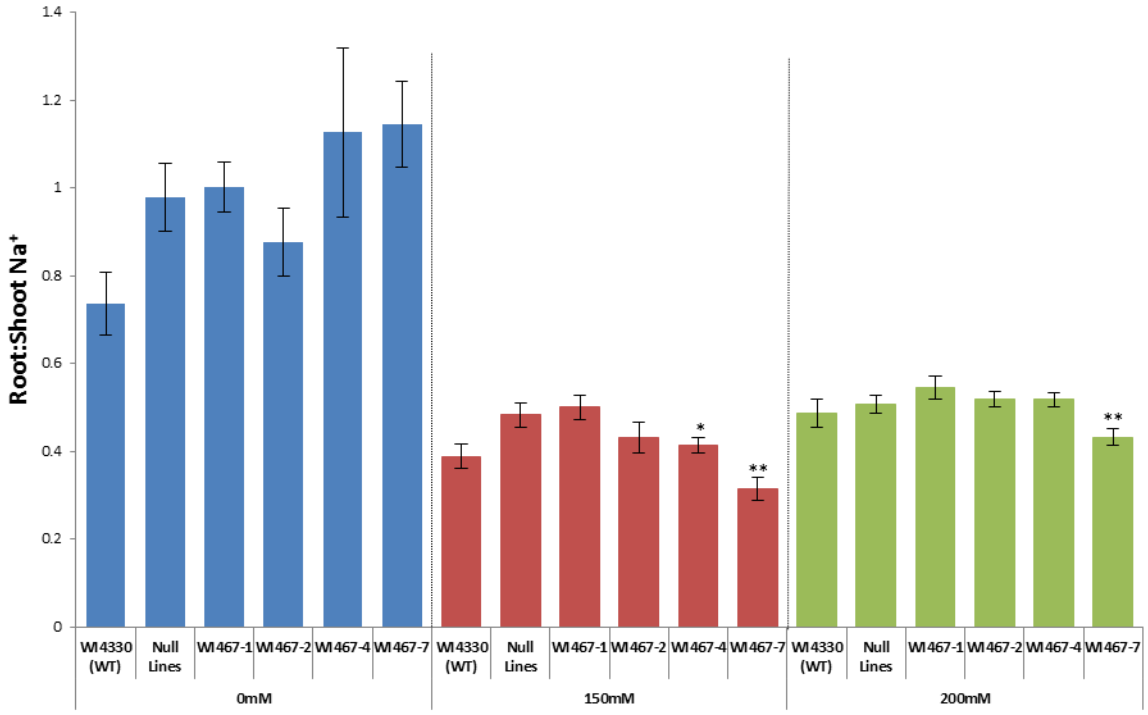

Figure 5-25 Root to shoot Na^+ of *Ubi:HvHVP10* T_2 lines grown in hydroponics under 0 mM, 150 mM and 200 mM NaCl. Salt stress was initiated when the fourth leaf was starting to emerge and was imposed in 25 mM increments applied daily morning and night until the desired concentration was reached. Barley plants were harvested 3 weeks after initial salt application. Nulls were derived from segregation of transgenic barley lines at the T_2 generation. Values are the means ± SEM with asterisks (* or**) indicating a significant difference (2-tailed t-test assuming equal variances) of $P \leq 0.05$ or ≤ 0.01. The number of biological replicates differs for each line where; WI4330 (WT) = 9, 4, 6; Null lines = 14, 15, 13; WI467-1 = 19, 18, 18; WI467-2 = 12, 14, 13; WI467-4 = 8, 7, 9; WI467-7 = 16, 16, 15.

5.4 Discussion

5.4.1 Constitutive expression of HvHVP10 tended to increase the root and shoot biomass of transgenic barley

The overexpression of the *Arabidopsi*s vacuolar H^+-PPase (*AtAVP1*) and it's homologues have been shown to improve the salinity tolerance of a range of plant species including *Arabidopsis* (Gaxiola *et al.*, 2001), tobacco (Gao *et al.*, 2006), alfalfa (Bao *et al.*, 2009), corn (Li *et al.*, 2008), cotton (Pasapula *et al.*, 2011), creeping bentgrass (Li *et al.*, 2010), rice (Liu *et al.*, 2010, Kim *et al.*, 2014), peanut (Banjara *et al.*, 2012), and barley (Schilling *et al.*, 2014). Initially, the improved salinity tolerance was presumably due to a higher proton electrochemical gradient within these plants, which facilitates enhanced sequestering of ions into the vacuole via the Na^+/H^+ antiporter (Gaxiola *et al.*, 2001). This both alleviates the toxic effect of high cytosolic sodium concentration and facilitates water uptake into the plant (Gaxiola *et al.*, 2001, Duan *et al.*, 2007). Other studies suggest that the increased salinity tolerance of plants OEX V-PPases is due primarily to the increased biomass of the transgenic plants under both control and stress conditions (Schilling *et al.*, 2014).

In this study the barley V-PPase *HvHVP10* was constitutively expressed in the barley (*Hordeum vulgare*) cultivar WI4330 under the control of the maize *Ubiquitin-1* promoter and the cauliflower mosaic virus *35S* promoter to determine if the overexpression of *HVP10* could increase the salinity tolerance of WI4330 above its current level of tolerance. To our knowledge, this is the first report to date of the overexpression of a barley V-PPase within a barley genetic background. Transgenic WI4330 plants constitutively expressing *HvHVP10* were evaluated for salinity tolerance in supported hydroponics at a range of salt concentrations at both the T_1 and T_2 generation.

A number of transgenic plants carrying the *35S:HvHVP10* insert were found to have improved root and shoot growth under salinity stress compared to their null segregates in the T_2 salinity tolerance screen. When comparing the ratio of biomass under salt and control conditions two transgenic *35S:HvHVP10* OEX lines, WI465-3 and WI465-7, were more salt tolerant than their respective nulls, retaining between 21% and 24% more root DW and up to 12% more shoot DW under salinity stress. The increased biomass of these lines under salt stress was not reflected in T_1 experiment; however this is common in transgenic studies, as the transgenic T_1 generation contain hemizygous plants which are still segregating and lack transgene stability (Vain *et al.*, 2002). In addition the plants are often suffering from

epigenetic effects from having been through the transformation process, as demonstrated in this study. Here we found that wildtype consistently performed better than both the nulls and the transgenic lines in all experiments, indicating that the null and transgenic lines were still suffering from a transgenerational effect. This result highlights the fact that in transgenic studies it is important to compare transgenic lines to the null lines, rather than the wildtype, providing further evidence that peer-reviewed journals should use nulls to compare transgene effects rather than the un-transformed genotype.

A significant increase in biomass was also observed in a single transgenic line carrying the *Ubi:HvHVP10* insert under salinity stress, however unlike the *35S:HvHVP10* OEX lines this phenotype was also reflected under non-stressed conditions. This may be due to the expression level of the transgene as directed by the promoter. The line WI467-7, containing 3 copies of the transgene had significantly greater root and shoot FW compared with the null lines in the T_1 screen (Figure 5-17) and in the T_2 screen, where WI467-7 had significantly greater root biomass (Figure 5-20) and shoot biomass (Figure 5-21) under both salt treatments as well as control conditions. Our results demonstrate that this line consistently maintains greater biomass under all experimental conditions, however when comparing its biomass under salt stress relative to the control it was not found to be salt tolerant than the null lines. The transgenic lines WI467-2 and WI467-4 also displayed greater root and shoot biomass in the T_1 and T_2 salinity tolerance screen. Whilst these lines do not appear to be more salt tolerant, further experimental investigation of the *Ubi:HvHVP10* lines is warranted in the T_3 generation to determine the underling mechanism for the increase in biomass of these lines, as these lines could be interesting to breeders for enhancing yield.

The increase in biomass of the plants constitutively expressing V-PPases under salt stress is consistent with other studies involving the over expression of *AtAVP1* and its homologues. Gaxiola *et al.* (2001) demonstrated that transgenic Arabidopsis plants OEX *35S:AtAVP1* were more resistant to high concentrations of NaCl than isogenic wild type strains. This phenotype was also observed under control conditions. Similarly, OEX of H-PPase from *Thellungiella halophia* enhanced the salinity tolerance of cotton and also resulted in significant increase in root biomass under non-stressed conditions (Lv *et al.*, 2008).

This increase in biomass resulting from *AtAVP1* OEX has also been reported in barley in the glasshouse and under saline field conditions (Schilling *et al.*, 2014). In the glasshouse

transgenic *35S:AtAVP1* barley (cv. Golden Promise) had increased projected shoot area compared with null lines in a pot experiment using non-destructive phenotyping to quantify the growth of transgenic plants over time. The increased biomass of transgenic barley plants was also observed under non-saline conditions. This result was also reflected under saline field conditions where transgenic plants showed an increase in shoot biomass and greater grain yield per plant compared to wild-type plants (Schilling *et al.*, 2014). Homozygous *35S:HvHVP10* and *Ubi:HvHVP10* lines generated in this study should also be screened in a saline field site, such as the ACPFG field trial site at Corrigin, WA to determine if the phenotype is also expressed under field condition, similar to transgenic barley expressing *AtAVP1*.

The increase in biomass of transgenic plants overexpressing *AtAVP1* under non-stressed conditions, as seen with barley (Schilling *et al.*, 2014) and Arabidopsis (Gaxiola *et al.*, 2001, Gaxiola *et al.*, 2012) appears to be dependent on the construct and the species being transformed. The transgenic line WI467-7 carrying the *Ubi:HvHVP10* insert was shown to be consistently significantly larger under control conditions, however transgenic plants OEX *HvHVP10* under the control of the 35S promoter did not display increased biomass under non-stressed conditions. This is consistent with other reports. Pasapula *et al.* (2011) showed that the OEX of *AtAVP1* in cotton resulted in transgenic plants exhibiting greater growth than wildtype plants grown under hydroponic conditions in the presence of 200 mM NaCl, however there were no difference between wild type Coker 312 and transgenic lines grown under control conditions.

It has been well documented that the overexpression of *AtAVP1* leads to increased biomass of transgenic plants. However, the underlying molecular mechanism of how *AtAVP1* overexpression leads to larger plants is only now becoming clearer (Schilling *et al.*, 2017). Transgenic Arabidopsis plants over-expressing *AtAVP1* produced larger leaf area due to an increase in cell numbers (Gonzalez *et al.*, 2010). The OEX of *AtAVP1* in *Arabidopsis* has also been shown to play an important role in organ development through the facilitation of auxin fluxes, where enlarged root and shoot systems regulated by *AtAVP1* were caused by the upregulated expression of auxin distributers and auxin transport associated genes, including *P-adenosine triphosphatase* and *pinformed 1 auxin efflux facilitator* (Li *et al.*, 2005). A similar result was observed in transgenic tobacco expressing the wheat vacuolar H^+-

pyrophosphatase gene (*TaVP*), where plants had enlarged root and shoot systems and displayed elevated expressions of auxin-transporter associated genes, including *NtPIN1*, *NtPIN1b*, *NtPIN3* and *NtPIN3b* (Li *et al.*, 2014). The mechanism of how V-PPase regulates the transcription of auxin-transport associated genes remains to be elucidated.

Schilling *et al.* (2014) hypothesised that the increased biomass of *AtAVP1* OEX lines was associated with improvements in seedling vigour. Indeed, it has been shown that V-PPase plays an important role in plant development during the early post-germinative stage, where it has been shown that removal of PPi from the cytosol is necessary to increase gluconeogenesis and optimise postembryonic heterotrophic growth (Ferjani *et al.*, 2011). This suggests that by OEX of the V-PPase gene, plant cells may be better able to hydrolyse cytosolic pyrophosphate PPi, thereby optimising the plant's metabolic function during the early stages of plant growth and development. It would be interesting to conduct germination assays on the lines generated in this study to establish if the up-regulation of *HvHVP10* leads to increased post-germinative growth of transgenic plants. A PhD student from Melbourne University is working with the lines generated in this project and is currently performing germination assays and metabolomic analysis to examine if the OEX of V-PPase is increasing the germination rate and accumulation of metabolites of transgenic plants.

The improvement in biomass as a result of V-PPase OEX has also been suggested to be a result of improvements in sucrose transport (Gaxiola *et al.*, 2012, Khadilkar *et al.*, 2015). Swart (2005) has also suggested that V-PPase may have a duel role in sucrose accumulation; through contributing to the disposal of cytosolic PPi (which would otherwise inhibit sucrose synthesis), but also through use of the energy derived from the hydrolysis of PPi to increase the sucrose sink pool by activation of the proton motive force across the vacuolar membrane. This drives the secondary transport of sucrose from the cytosol into the vacuole.

The reported multiple roles of vacuolar pyrophosphatase have led to the hypothesis that the protein has different functions in different tissue (Gaxiola *et al.*, 2012). Therefore, it is necessary to express the gene in specific cell types to examine the various functions of the gene in different tissue types.

5.4.2 Transgenic WI4330 carrying the Ubi:HvHVP10 insert tended to contain more Na^+ concentrations in their shoots

Sodium sequestration in vacuoles is considered one of the main salinity tolerance mechanisms among many plant species (Munns and Tester, 2008, Gaxiola *et al.*, 2012), including many glycophytic plants (Munns *et al.*, 2006) and those adapted to saline environments (Lv *et al.*, 2008). Many OEX studies have demonstrated that the constitutive expression of type 1 vacuolar H^+-PPase from *Arabidopsis* (*AtAVP1*) and from the halophilic species *Thellungiella halophila* (*TsVP*) have increased the salinity tolerance of a range of species, which has been linked to an enhanced electrochemical gradient across the vacuolar membrane and improved sodium compartmentalisation in the vacuole (Gaxiola *et al.*, 2001, Guo *et al.*, 2006, Duan *et al.*, 2007 , Lv *et al.*, 2008). This results in the maintenance of osmotic potential and increased water retention in the plant under saline conditions (Apse *et al.*, 1999, Duan *et al.*, 2007).

In this study salt treatment increased Na^+ concentrations and decreased K^+ concentrations in roots and shoots of transgenic lines transformed with *HvHVP10*, as expected (Chen *et al.*, 2005, Munns and Tester, 2008). However the accumulation of tissue Na^+ varied significantly between the lines under the control of *35S* and *Ubiquitin-1* promoters in both hydroponics experiments, indicating that these constitutive promoters invoke different responses to salinity stress and result in altered mechanisms of sodium transport and accumulation within the plant.

There were no clear differences in root or shoot Na^+ and K^+ concentrations between transgenic barley carrying the *35S:HvHVP10* insert and the null lines after salt stress in the T_1 (Figure 5-11) or T_2 screen (

Figure 5-14). WI465-7 was screened in the T_2 generation and did display significantly higher shoot sodium (307 mM) compared with its respective null (233 mM) at 200 mM NaCl, however no other transgenic lines carrying the *35S:HvHVP10* insert displayed this phenotype. This result is consistent with the analysis of *AtAVP1* OEX in transgenic barley (cv. Golden Promise), driven by the cauliflower mosaic virus *35S* promoter (Schilling *et al.,* 2014).This work demonstrated that the constitutive OEX of *AtAVP1* in barley did not result in increased sodium accumulation in the shoot under saline conditions in the glasshouse or in the field, suggesting that sequestration alone was not responsible for the increase in biomass observed in these lines.

Lines containing *HvHVP10* under the control of the *Ubiquitin-1* promoter however, displayed significant variation in root and shoot Na^+ and K^+ concentrations after salt stress, compared with the null lines in both the T_1 and T_2 screen. In the T_1 generation WI467-1 and WI467-2 had significantly less root Na^+ compared with the null segregates at 250 mM NaCl (Figure 5-18). In the T_2 screen, the transgenic line WI467-7 also had significantly less root Na^+ and root K^+ compared with null lines, under 150 mM and 200 mM NaCl respectively (Figure 5-22). This result could be attributed to the large root biomass and increased water retention in this plant having a "dilution effect" on Na^+ and K^+ concentrations (Asch *et al.*, 1999, Kaya *et al.*, 2002). The decreased root sodium concentration in this line may also indicate that these plants are actively transporting sodium from the root to the shoot, via the transpiration stream, referred to as xylem loading (Munns *et al.,* 2006). Recently, an investigation into the salinity tolerance of barley revealed that the active transport of sodium from the root to the shoot was a characteristic of the tissue tolerant phenotype observed in the salt tolerant barley cultivar Numar (Adem *et al.,* 2014).

There was also significant variation in shoot Na^+ and K^+ concentrations of transgenic *Ubi:HvHVP10* lines after salinity stress. In the T_2 screen the transgenic WI4330 lines WI467-2, WI467-4 and WI467-7 had significantly more Na^+ in their shoot at 150 mM NaCl compared to the nulls (Figure 5-23). The transgenic line WI467-7 also had significantly more shoot Na^+ at 200 mM NaCl compared with the null segregates, however this line also had significantly less shoot K^+ at 150 mM and 200 mM NaCl. In contrast, the transgenic line WI467-4 had significantly more K^+ in the shoots at both 150 mM and 200 mM NaCl respectively. These results suggest that the transgenic lines carrying the *Ubi:HvHVP10* insert

can maintain higher shoot sodium concentrations and use the accumulation of Na^+ in the shoot to lower the osmotic potential in the leaf to drive water uptake into the plant. This physiological response to salinity stress has previously been identified as a key component contributing to the overall salinity tolerance of barley (Munns *et al.*, 2006, Shabala *et al.*, 2010, Adem *et al.*, 2014).

The high shoot sodium and potassium concentrations observed in some of these transgenic plants, along with their ability to maintain growth under saline conditions, presumably through sodium sequestration, is consistent with other studies involving the OEX of V-PPases; although many of these studies report a similar increase in root sodium concentrations. Plants expressing *AtAVP1* displayed increased Na^+ concentration in the shoot and the root under salt stress (Gaxiola *et al.*, 2001, Bao *et al.*, 2009, Li *et al.*, 2010), as well as decreased vacuole membrane leakage and increased net photosynthesis (Bao *et al.*, 2009, Pasapula *et al.*, 2011). Gaxiola *et al.* (2001) demonstrated that isolated vacuolar membrane vesicles from transgenic *Arabidopsis* constitutively expressing *AtAVP1* had enhanced cation uptake capability, and as a result plants were shown to have greater solute accumulation and increased water retention under saline conditions. The OEX of H^+-PPase from *Thellungiella halophia* (*TaVP*) in cotton similarly resulted in transgenic plants having a greater accumulation of Na^+, K^+ Ca^{2+}, Cl^- and osmotic solutes, such as soluble sugars, in the root and leaf tissue, resulting in increased water retention compared with wild-type plants (Lv *et al.*, 2008, Lv *et al.*, 2009). These plants were also shown to have lower membrane ion leakage and malondialdehyde (MDA) levels, suggesting that the Na^+ was compartmentalised in the vacuole instead of the cytoplasm, thus reducing its toxic effect (Lv *et al.*, 2008). Similarly, the OEX of *TVP1* in *Arabidopsis* resulted in increased Na^+ and K^+ content in shoot tissue in plants exposed to 200 mM NaCl. Furthermore, the increased salinity tolerance observed in these lines was positively correlated with increased vacuolar solute accumulation in *Arabidopsis* cells (Brini *et al.*, 2007).

It should be noted that plants with high shoot Na^+ also need to accumulate compatible solutes within the leaf tissue to maintain cell membrane integrity and osmotic potential. It could be hypothesised that increased *HvHVP10* expression and activity is helping to move sugars from source to sink tissues, allowing the plant to synthesise more compatible solutes, thereby allowing the plant to accumulate more Na+ in the vacuole.

5.4.3 *Transgenic WI4330 carrying the Ubi:HvHVP10 insert tended to maintain a higher Na^+: K^+ in their roots*

In the T_2 salinity tolerance screen transgenic plants carrying the *Ubi:HvHVP10* insert displayed a higher Na^+: K^+ in their root tissue compared to the null segregants under 150 mM NaCl (Figure 5-24), despite the fact that some lines maintained significantly lower root Na^+ concentration. This result was not expected. It is widely accepted that the ability of plants to maintain a low root Na^+: K^+ is critical component of plant salinity tolerance (Munns and Tester, 2008), although direct measurements of this ratio have been few (Kronzucker *et al.*, 2006). Other reports have demonstrated that low cytosolic Na^+: K^+ ratio in the root is not central to the plants ability to tolerate salinity stress. Kronzucker *et al.* (2006) used non-invasive short lived radiotracers $^{42}K^+$ and $^{22}N^+$ in intact barley seedlings to evaluate uni-directional plasma membrane fluxes and cytosolic concentrations of K^+ and Na^+ in root tissue. This work demonstrated that the Na^+: K^+ ratio in the cytosol of root cells was a poor predictor of barleys response to salinity stress.

There was variation in shoot Na^+: K^+ under both salt treatments (150 mM and 200 mM NaCl), however no clear trend was observed in the transgenic lines carrying the *Ubi:HvHVP10* insert. The transgenic lines WI467-1 and WI467-4 had lower Na^+: K^+ in the shoots and under both 150 mM and 200 mM NaCl, whereas transgenic lines WI467-2 and WI467-7 had higher Na^+: K^+ in the shoots. In order to evaluate the relative accumulation of Na^+ in root tissue compared to shoot tissue the ratio of root to shoot sodium concentration was calculated. Most lines displayed a lower root to shoot sodium ratio, indicating that lines are accumulate more sodium in the shoot relative to the root. This result again supports the notion that transgenic plants carrying the *Ubi:HvHVP10* insert are using Na^+ in the shoots as an osmoticum, to lower the osmotic potential in the leaf, thereby driving water uptake into plant under periods of salinity stress.

5.4.4 *Plants with HvHVP10 have no variation in stomatal conductance*

Gas exchange has been identified as a selectable physiological trait that can be used by breeding programs to measure the photosynthetic capacity and yield potential of agricultural crops (Munns *et al.*, 2010, Rahnama *et al.*, 2010). It has also been used under glasshouse conditions to investigate the relative contribution of ionic, osmotic and oxidative stress components towards the salinity tolerance of barley (Adem *et al.*, 2014) and the acclimation to salinity in pea (*Pisum sativum*) (Pandolfi *et al.*, 2012). Transgenic plants carrying the

35S:HvHVP10 insert were screened with a steady state diffusion porometer (model SC-1) in the T_2 salinity tolerance screen, in order to determine if there were variations between lines in their ability to adjust their leaf stomatal conductance in response to salinity stress. The *35S:HvHVP10* OEX lines were evaluated for leaf porosity 4 days after salt application was completed, in order to give the plants time to adjust to the imposed salinity stress.

Salt treatment was shown to significantly decrease the stomatal conductance of the plants (Figure 5-16), however there was no significant difference observed between the lines carrying the transgene and the null segregates. It is noted however, that there was no difference in the stomatal conductance between the two salt treatments (150 mM and 250 mM NaCl), indicating that NaCl concentration was not a limiting factor in determining the overall rate of transpiration. Given the accumulation of Na^+ in the shoots of transgenic barley plants carrying the *Ubi:HvHVP10* insert, it would have been interesting to determine if these transgenic plants could maintain photosynthetic efficiently and water uptake under saline conditions, as demonstrated from other V-PPase OEX studies (Gaxiola *et al.*, 2001). Unfortunately, weather conditions prevented leaf porometer measurements being taken 4 days after salinity stress was imposed in this experiment. It is recommended that these studies be carried out on homozygous lines in the T3 or later generations, to determine if the OEX of *HvHVP10* resulted in increased osmotic tolerance.

5.4.5 Constitutive expression of HvHVP10 may increase tolerance to other abiotic stresses

Here we show the constitutive expression of the barley vacuolar V-PPase *HvHVP10* in transgenic WI4330 increased the root and shoot biomass of transgenic plants under salinity stress. The increase in biomass of transgenic plants, particularly the root system, allows the plants to be more resistant to other abiotic stresses, including water deficit and mineral deficiencies. The establishment of an extensive root system allows the plant to absorb water and nutrients from a greater surface area within the soil profile, thereby reducing the stress on the plant posed by limited water and nutrient availability. Many studies have demonstrated that transgenic plants with enlarged root systems resulting from up-regulation of *AtAVP1* and its homologues also display increased drought tolerance, where transgenic plants are able to maintain greater photosynthetic activity, higher stomatal conductance and transpiration rates under water deficit conditions (Gaxiola *et al.*, 2001, Park *et al.*, 2005, Guo *et al.*, 2006, Brini

et al., 2007, Li *et al.*, 2008, Bao *et al.*, 2009, Pasapula *et al.*, 2011). In addition, the overexpression of *AtAVP1* in Arabidopsis has also revealed that phosphorus deficiency induces the increased expression of *AtAVP1* and subsequent increased expression of the type 1 plasma membrane adenosine triphosphatase (P-ATPase), resulting in increased rhizosphere acidification and root proliferation and increased phosphorus use efficiency of transgenic plants (Yang *et al.*, 2007). A similar result was observed under nitrate NO_3^- limiting conditions in romaine lettuce transformed with *AtAVP1* (Paez-Valencia *et al.*, 2013) and tobacco transformed with the wheat V-PPase *TaVP* (Li *et al.*, 2014), a close homologue of *HvHVP10* (Liu *et al.*, 2011). Others have reported improvements in cold tolerance in rice constitutively expressing *OsOVP1*, a rice V-PPase, however interestingly these lines did not display increased salinity tolerance (Zhang *et al.*, 2011). Collectively, these reports support the notion that the OEX of V-PPases results in enhanced tolerance to a range of abiotic stresses. Therefore, it is important to test the *HvHVP10* OEX lines generated in this study under a range of growth limiting conditions to determine if the OEX of *HvHVP10* in WI4330 increases its tolerance to other abiotic factors. Indeed, preliminary screening of *Ubi:HvHVP10* lines with Bromophenol blue (3',3'',5',5''-tetrabromophenolsulfonphthalein) pH agar indicated that these lines may also display increased acidification of the rhizosphere under phosphorus deficient conditions, which warrants further detailed investigation. Ideally, these studies would be performed on multiple homozygous lines in the T3 or later generations.

5.5 Summary

Our results suggest that the constitutive overexpression of *HvHVP10* in transgenic barley (cv. WI4330) increased the root and shoot biomass of transgenic plants under salinity stress. Furthermore, transgenic plants carrying the *35S:HvHVP10* insert were found to be more salt tolerant than their null segregates under a range of salt concentrations. Transgenic WI4330 plants of both constructs displayed an increase in root and shoot biomass; however they had different phenotypes of sodium accumulation in root and shoot tissue in response to salinity stress. Transgenic WI4330 carrying the *Ubi:HvHVP10* insert contained significantly more Na^+ in the shoots under 150 mM NaCl, indicating that these transgenic plants may be using the accumulation of Na^+ in the shoot to lower the osmotic potential in the leaf to drive water uptake into the plant. No significant difference in Na^+ or K^+ concentrations was observed in the root or shoot of transgenic plants constitutively expressing *HvHVP10* under the control of the *35S* promoter, indicating that sodium sequestration was not responsible for the increase in

biomass observed in these plants. It is the recommendation of this chapter that further analysis be carried out on the transgenic lines generated in this study in the T3 generation in the glasshouse under a range of different stresses and under saline field conditions at the ACPFG field site in Corrigin, WA.

6 General discussion and future directions

6.1 Review of thesis aims

Salinity is a major abiotic constraint to agricultural production worldwide. Saline soils impose on plants both an osmotic stress, due to decreased soil water potential and an ionic stress, due to the accumulation of Na^+ ions in the shoots. These stresses affect all of the metabolic processes occurring within the plant and result in an immediate reduction in plant growth and decreased overall final yield (Tester and Davenport, 2003, Munns *et al.*, 2006, Munns and Tester, 2008, Roy *et al.*, 2014). Plants utilise various strategies at both the cellular and tissue level to tolerate the osmotic and ionic stress caused by high soil salt concentration. Plants can minimise the ionic effect of high shoot Na^+ by excluding Na^+ from cells, compartmentalising Na^+ within the vacuole, or reducing the amount of Na^+ entering the transpiration stream, bringing water and nutrients from the roots to the shoots (Tester and Davenport, 2003, Apse and Blumwald, 2007, Munns and Tester, 2008). These processes involve an array of different sodium transporters and signalling elements located within different regions of the plant, all of which contribute to the overall maintenance of Na^+ homeostasis under saline conditions (Tester and Davenport, 2003, Apse and Blumwald, 2007).

Here we report on the characterization of the H^+ translocating pyrophosphatase (V-PPase) *HvHVP10* gene as a candidate gene for salinity tolerance in barley. The *HvHVP10* gene was proposed to be the gene responsible for the sodium exclusion *HvNax3* QTL, identified in a DH population of a cross between between wild barley (*Hordeum spontaneum*; CPI-71284-48) and locally adapted domesticated barley (*H. vulgare*; Barque-73) (Shavrukov *et al.*, 2010, Shavrukov *et al.*, 2013) (section 2.4.1.3). The V-PPase protein is responsible for establishing an electrochemical gradient across the tonoplast which drives the secondary transport of sodium into the vacuole via the sodium antiporter. The compartmentalisation of sodium in the vacuole is an important salinity tolerance mechanism and the OEX of orthologues of this gene has been shown to increase the salinity tolerance of a variety of plant species (Gaxiola *et al.*, 2001, Gao *et al.*, 2006, Guo *et al.*, 2006, Brini *et al.*, 2007, Duan *et al.*, 2007, Li *et al.*, 2008, Lv *et al.*, 2008, Yue *et al.*, 2008, Bao *et al.*, 2009, Liu *et al.*, 2011). The specific aims of this project were to: 1) test that *HvHVP10* is responsible for

HvNax3 sodium exclusion QTL; 2) characterise *HvHVP10* in planta in CPI-71284-48, Barque-73 and advanced backcross (AB QTL) lines in response to salt stress; 3) clone and sequence the *HvHVP10* CDS, gene and promoter from CPI-71284-48 and Barque-73; 4) generate transgenic barley plants with constitutive expression of V-PPase; and 5) develop GFP and GUS reporter constructs to determine tissue distribution and sub-cellular localisation of the V-PPase protein.

6.2 Confirmation of *HvHVP10* as a candidate gene for the sodium exclusion *HvNax3* QTL

The first aim of this research was to confirm that the gene *HvHVP10* in barley is responsible for the sodium exclusion *HvNax3* phenotype observed in the Barque-73 x CPI-71284-48 DH population. The general hypothesis is that increased sodium accumulation in the roots in the line carrying the *HvNax3* allele of CPI-71284-48 and less accumulation in the shoot under salt stress is caused by increased *HvHVP10* mRNA expression and or pyrophosphatase activity, leading to increased sequestration of sodium ions in the root vacuole. This in turn leads to increased water uptake into the plant under periods of osmotic stress.

In Chapter 2 the relative concentrations of sodium and potassium ions were examined in the roots and shoots of CPI-71284-48, Barque-73 and the AB-QTL lines 18D/014 (CPI-71284-48 *HvNax3* allele) and 18D/011 (Barque-73 *HvNax3* allele) over 10 days of salinity stress. Results of this experiment indicated that 18D/014 accumulated more sodium ions in the roots and less in the shoots, thus maintaining better sodium to potassium ratio in the shoot, which is consistent with the *HvNax3* phenotype (Figure 2-8). On further examination, the mRNA expression profile of *HvHVP10* in CPI-71284-48 was shown to have 4 fold higher expression in the roots at day 3 of salinity stress (Figure 2-9), with the AB-QTL line carrying the *HvNax3* allele from CPI-71284-48 (18D/014) appearing to maintain a greater V-PPase activity in root vacuoles isolated from salt stressed plants than the AB-QTL line with the Barque-73 allele (18D/011) (Figure 3-3). These results support the hypothesis that increased root accumulation of sodium improves the salinity tolerance of barley and may be due to a difference in *HvHVP10* expression and or activity derived from the *H.spontaneum HvNax3* allele. Despite these promising trends, often the differences were not statically significant and more replicates would be required to demonstrate if different V-PPase alleles have a significant effect on the plant's phenotype. Further evidence may be gained by examining the pyrophosphatase activity in the recurrent backcross parent Barque-73, to determine if it

maintains similar V-PPase activity in response to salinity stress as the AB-QTL line 18D/011 (Barque-73 *HvNax3* allele).

6.3 Molecular characterisation of *HvHVP10* in CPI-71284-48 and Barque-73

The second aim of this project was to identify polymorphisms in the CDS, gene or promoter which may account for the different phenotypes in the lines carrying the CPI-71284-48 *HvNax3* allele and in the lines carrying the Barque-73 *HvNax3* allele. Sequence analysis revealed that the *HvHVP1*0 CDS was very highly conserved, with no allelic differences in the amino acid sequence being detected. Variation in the gene sequence was identified, due to the presence of a number of insertions in the Barque-73 *HvHVP10* gene. However the true significance of these genetic differences remains to be elucidated. Significant differences were observed in the promoter region of the *HvHVP10* genes between CPI-71284-48 and Barque-73, particularly in transcription factor binding sites, including the presence of a DREB binding site, and these are hypothesised to be responsible for the differences in gene expression observed between the two alleles.

Further bioinformatic analysis of *HVP10* CDS (IPK barley blast server) identified two other putative V-PPase orthologues in barley, in addition to the previously described *HVP1* (Fukuda *et al.* 2004), HVP10 (Tanaka *et al.*, 1993)and *HVP3* (Wang *et al.*, 2009), bringing the total number of *HVP*s to five. Alignment and sequence analysis of the five barley V-PPase sequences (Figure 4-6) revealed the presence of several highly conserved motifs, common to all V-PPases, specifically involved in pyrophosphate binding (Maeshima and Yoshida, 1989, Nakanishi *et al.*, 2001) and enzyme activation (Zancani *et al.*, 2007). *HVP1* and *HVP10* belonged to the Type I, or potassium sensitive, PPase group while *HVP3* and *HVP5* contained a motif associated with the type II class, potassium insensitive, V-PPases. Further investigation of the barley V-PPase orthologues identified in this study is required to determine their specific function and tissue specificity.

6.4 Constitutive expression of *HvHVP10* for improving the salinity tolerance of barley

A significant component of this research project was the generation and characterisation of transgenic barley plants overexpressing the *HvHVP10* CDS under the control of the cauliflower mosaic virus *35S* promoter and maize *Ubiquitin-1* promoter, in order to investigate of role of *HvHVP10* in conferring increased salinity tolerance of barley. The performance of the transgenic lines depended on the promoter used to control the gene.

35S:HvHVP10 expressing barley displayed significantly greater root fresh weight and dry weight under control and salt stress (150 mM and 200 mM NaCl), compared with plants lacking the transgene. Specific lines accumulated up to 25% more shoot dry weight (Table 5-4) than the null segregates under both 150 mM and 200 mM NaCl. Despite the increase in biomass, no significant differences were observed in root and shoot sodium and potassium concentrations. Some *Ubi-1*:*HVP10* barley lines displayed significantly greater root fresh weight (17%; Table 5-6) under control and salt stress at 150 mM NaCl, when compared with wild type and null lines. Significant differences were also detected in root sodium concentrations, with transgenic lines having statisically less sodium contained within the roots at both 150 mM and 200 mM NaCl and significantly more sodium and potassium in their shoots, thus maintaining beter sodium to potassium ratios.

The different patterns of sodium accumulation in the tissues of the transgenic plants indicate constitutive promoters can invoke different responses to salinity stress. There may be post-translational or transcriptional modifications which affect the expression of the gene or the activity of the protein, or these promoters are not truly constitutively expressed in all cell types, and subtle differences in expression level and or localisation are responsible for the different phenotypes observed in the transgenic plants.

6.5 Further insight in to the role of vacuolar PPases

6.5.1 V-PPase and increased sucrose accumulation

Literature suggests that V-PPase may also play a crucial role in sucrose metabolism. Enhancement of sucrose metabolism in salt stressed plants could enhance a plant's ability to synthesis compatible solutes and/or provide more ATP for growth. V-PPases have been proposed to have a duel role in sugar metabolism, 1) the proton motive force created by the enzyme causes enhanced sucrose translocation and 2) enhanced sucrose synthesis via PPi removal (Swart, 2005, Gaxiola *et al.*, 2016, Regmi *et al.*, 2016, Schilling *et al.*, 2017).

New roles of V-PPases have been hypothesised due to its apparent localisation on the plasma membrane of phloem companion cells in leaf tissue. It has been hypothesised that the upregulation of plasma membrane bound V-PPase, which act as a PPi synthases, enhances sucrose fluxes from source to sink tissues by improving phloem loading capacity (Fuglsang *et*

al., 2011). It has been suggested that the PPi produced by that process can be used to enhance the conversion of sucrose to Fru-1, 6-BP, which is used in the glycolysis pathway, thus enhancing ATP production (Khadilkar *et al.*, 2015). Plasma membrane bound ATPases would use this energy source to pump protons out of the cell to the apoplast, thereby setting up the conditons for sucrose/H^+ symporters to move more sucrose from mesophyll cells in to the phloem (Paez-Valencia *et al.*, 2011, Khadilkar *et al.*, 2015). Khadilkar *et al.* (2015) demonstrated by knocking down *AVP1* gene expression, specifically in phloem tissue, using targeted amiRNA of *AVP1,* resulted in a disruption in sucrose transport. The precise role of V-PPases in sucrose transport and metabolism however remains to be elucidated. The extra energy resulting from this enhanced growth could be used to maintain growth under salinity and/or in the production of compatible solutes which enable a plant to store more toxic ions in the vacuole. Interestingly, comparison of the accumulation of compatible solutes in Barque-73 and CPI-71284-48 in response to osmotic stress indicates that CPI-71284-48 has significantly increased levels of the free sugars sucrose, glucose and fructose (Table 6-1) (Jason Eglington (2012) pers. comm).

Table 6-1 Accumulation of compatible solutes (mM) in response to osmotic stress in Barque-73 (*Hordeum vulgare*) and CPI-71284-48 (*H. spontaneum*) (Jason Eglington (2012) pers.comm.)

	Barque-73		**CPI-71284-48**	
	Control	Stress	Control	Stress
Sucrose	275	223	239	665
Glucose	302	383	78	586
Fructose	179	511	239	1337
Glycinebetaine	24	114	24	105
Proline	0	238	0	271
Total	**780**	**1505**	**580**	**2964**

Osmotic stress applied as PEG 1000

Both CPI-71284-48 and Barque-73 also displayed increased levels of the amino acids proline and glycinebetaine in response to osmotic stress. Increased levels of proline has been shown to be induced in both halophytes and glycophytes by growing them under non-saline conditions then subjecting the plants to increasing levels of salinity stress (Ashraf and Foolad, 2007, Hoque *et al.*, 2007, Huang *et al.*, 2013). The observation that proline production is an

adaptive response to salinity is reinforced by findings that inland populations of the halophyte Armeria have proline concentrations of 1.4 µmol/g fr wt, whereas coastal populations reach concentrations of 26 µmol/g fr wt (Stewart and Lee, 1974). Glycine-betaine also contributes to cytoplasmic osmotic adjustment and may also protect cellular enzymes against ion toxicity and assist in the maintenance of membrane integrity (Greenway and Munns, 1980). Glycine-betaine has been shown to accumulate specifically in the youngest expanding leaves of *Hordeum marinum* (sea barley grass) when grown at 200 mM NaCl and was positively correlated with increased salinity tolerance (Islam *et al.*, 2007). Despite these findings, the exact mechanism of how increased proline and glycine betaine accumulation contributes to salinity tolerance remains controversial. It is argued that it is not an adaptive response but a direct product of osmotic stress (Ashraf and Foolad, 2007).

Based upon the above observations, it would be interesting to examine if either the tolerant AB-QTL line (18D/14) or over-expressing *HVP10* lines also had increased levels of sucrose accumulation and transport and proline and glycine betaine concentrations in response to salinity stress, further supporting their role in conferring increased tolerance to abiotic stress. If this is the case, the question could be asked whether this is due to the CPI-71284-48 HvNax3 allele having more sugars to synthesize compatible solutes or more energy due to increased vacuolar pyrophospahtase activity.

Two independent T_2 *35S: HvHVP10* overexpression lines were examined by an ACPFG student based in Melbourne who employed a metabolomics approach to examine the accumulation of solutes in these transgenic lines under control conditions. Results indicated that both lines had metabolites levels 1.3 times higher in the shoot than the null lines, in particular the amino acids proline and glycine betaine were approximately 1.5 times higher in the overexpression lines compared with the nulls. It is recommended that future experiments be undertaken on these lines, examining the accumulation of compatible solutes under salinity stress.

Further experimental comparison of the lines generated in this study is recommended to determine if the differences observed in the T_2 generation is also displayed over multiple generations. Future investigation into *HvHVP10* may also involve the use of a promoter that is constitutively expressed at the germination stage to examine whether early seedling vigour contributes to the increased salinity tolerance of transgenic plants.

6.5.2 Why do plants have multiple vacuolar PPases and are they used to enhance tolerance to other abiotic stresses?

The question should be raised as to why plants possess so many V-PPases. Barley has 5 V-PPases (section 4.3.1.4), rice has 6 OVPs (Liu *et al.*, 2009), and wheat has 3 published V-PPases (Wang *et al.*, 2009). Why are so many needed? Are they specialising for different stresses? Are they specialising for different functions? These questions should be investigated to elucidate the specific role and function of the various plant PPases and would provide useful insight into their exact role in stress tolerance.

Further investigation is also warranted examining variation in the expression V-PPase genes to identify other beneficial alleles which may be incorporated into elite barley germplasm. This may involve screening a range of diverse barley germplasm and landrace accessions for alleleic variation in the expression of the barley V-PPase genes, possibly by employing emerging high-throughput technologies such as RNA-seq, to determine which alleles have the highest expression and to determine if this is correlated with improved growth rate under stress. This would not only provide the scope for new allele discovery, but also could be used for functional analysis studies. These genes can then be introduced into crop plants and elite breeding germplasm using conventional breeding techniques.

The use of GM technology to modify the various barley V-PPases would also provide useful insight into their roles in conferring tolerance to other abiotic stresses. Many studies have demonstrated a link between V-PPase overexpression and increased tolerance to mineral deficiencies (Yang *et al.*, 2007, Krebs *et al.*, 2010, Paez-Valencia *et al.*, 2013, Schilling *et al.*, 2017). It is the recommendation of this research project that the transgenic material generated in this study be examined under multiple stresses; including water deficit, anoxia and mineral deficiencies, to examine if *HvHVP10* also plays a role in conferring increased tolerance to other stresses. Ideally this would be performed on lines from the T3 or later generations.

6.5.3 Transgenic barley had improved performance but GM barley is not going to be grown soon.

The transgenic barley generated in chapter 5 had clear growth improvements and salinity tolerance over null transformants type plants. However, the cost of de-regulating a GM plant is extremely expensive and it is unlikely that any GM barley plant will go through this process, as they are not currently going to be readily acceptable by many consumers. So while the transgenic barley generated by this project has been effective proof of concept, other technologies are required to manipulate the expression of these genes in a non GM way, to enhance yield. New genome editing technologies are emerging as an attractive alternative to genetic modification. These techniques can make targeted changes to a plant's DNA, either substituting a nucleotide for another, deleting regions of DNA or inserting new DNA into the organism. This technology has specifically been shown to be an extremely useful tool for genome editing in both plant (Feng *et al.*, 2013, Mao *et al.*, 2013) and animal based applications (Hwang *et al.*, 2013). In the USA, the USDA has declared that it will not regulate genome edited plants in the same way it regulates GM plants. In Australia the OGTR has recommended that any edit made to a plant, which in theory could be done using current breeding practices (the introduction of a beneficial allele from a landrace by years of backcrossing, or the mutation of a plant's DNA by chemical or radiological approaches) should not be considered GM. As such, genome editing technologies, like TALENs and CRISPR-Cas9 are become attractive options in generating a non-GM plant with a phenotype similar to transgenic plants.

In this study, it is hypothesised that a difference in a DREB transcriptional factor binding site, may explain the difference between the tolerance of CPI-71284-48 and Barque-73. The use of CRISPR-Cas9 technology (Jinek *et al.*, 2012) to specifically modify the DREB sequence by mutating it, deleting it or inserting more in the promoter of *HVP10*, would result in the generation of plants which have potentially different levels of *HVP10* expression. The use of CRISPR-Cas9 technology to specifically modify the promoter region of *HvHVP10* would provide "proof of concept" and would overcome some of the limitations associated with GMOs and the introduction of foreign genes into plant genomes for crop improvement. In the case where the CRISPR-Cas9 DNA-free genome editing technology, which introduces Cas9 ribonucleoproteins that are delivered into plant cells as RNA molecules, is currently not regulated in the same way as plasmid based cloning systems (Arora and Narula, 2017). Thus,

it could be used to edit the DREB factor in the *HvHVP10* promoter region of elite barley germplasm and then it could be selected for using conventional breeding techniques (Khatodia *et al.*, 2016).

6.6 Limitations to current study

It should be noted that one of the major limitations to this research project was the lack of biological replicates for the pyrophosphatase assay. This was caused, in part, due to the delay in receiving the qPCR results to determine the appropriate target for the colometric assay. This time delay, combined with poor laboratory growing conditions over the winter period, lead to lower tonoplast enriched vesicle yields and the removal of a single biological replicate from the analysis. This limited significant statistical inference of the results, despite the fact that the AB-QTL line 18D-014 carrying the CPI-71284-48 *HvNax3* allele may display increased affinity to pyrophosphate protein binding and/or pyrophosphatase activity under salinity stress. Future research, involving more biological replicates is required to determine if the CPI-71284-48 *HvNax-3* allele has a protein with a higher affinity under salt stress and warrants further investigation.

The second major limitation to the current research was the lack of phenotypic evaluation of transgenic plants at later generations, or even field evaluation, when lines are homozygous for the transgene and no longer segregating. This is caused by the length of time required to develop the construct and perform the agrobacterium-mediated transformation into barley. Upon reflection, this limitation could have been eliminated if the transgenic material had been developed prior to the commencement of this research project. This would have allowed screening of transgenic plants for salinity tolerance at the T_3 or later generations and would increase the possibility of the publication of this research material in high impact scientific journals.

6.7 Future directions

The transgenic *35S:HvHVP10* and *UBI: HvHVP10* lines that performed well in the T_2 salinity tolerance screen were genotyped and T3 lines bulked up for further screening. It is recommended that this transgenic plant material be further characterised in both the laboratory and in the field. It is expected that the *35S* and *Ubi* over-expression lines will be bulked up during the coming year at the OTGR approved site at Glenthorne in Adelaide, in preparation for field screening to be carried out the following year at the University of Adelaide OTGR

approved salt site in Western Australia. It is proposed that an honours student will also continue further characterisation of the transgenic and reporter lines in the laboratory, including examination of promoter-GUS reporter constructs. Future research opportunities include the characterisation of the OEX transgenic barley lines expressing this vacuolar pyrophosphatase gene to establish whether pyrophosphatase activity is correlated with phenotype and to understand how activity of these enzymes improves tolerance to other abiotic stresses, including water deficit and mineral nutrient deficiencies.

Other research opportunities include:

1) Employ current gene editing technologies to manipulate the promoter of the HVP genes to enhance their native expression in non-GM systems.

2) Determine whether enhanced *HVP* expression can increase the tolerance to other abiotic stresses

3) Examine allelic variation in *HvHVP10* alleles across a wide range of germplasm to identify the best allele to use in barley breeding programs.

4) Examine the role of *HvHVP10* in sodium sequestration and sugar transport and accumulation in more detail, to determine the underlying mechanism for increased biomass in transgenic plants.

5) Determine which cells *HvHVP10* is being expressed in and at what stage of plant development and determine if expression changes over the life cycle of the plant.

6.8 Summary

Wild genetic resources adapted to a wide range of environmental conditions can provide tremendous potential for adaptive genetic diversity against abiotic and biotic stresses. It is clear from this research, and other research examining plant V-PPases, that *HvHVP10* from CPI-71284-48 *(H. spontaneum*) may play a significant role in the maintenance of growth of barley under saline conditions; through either increased sodium sequestration into the root vacuole via enhanced protein affinity in pryrophosphate binding and/or pyrophospahatase activity, or by promoting metabolic processes such as gluconeogenesis and sucrose

accumulation. Furthermore, the potential for the different barley V-PPases to be involved in different processes within the plant at different stages of plant development requires detailed investigation, and may lead to the identification of favourable alleles against a variety of abiotic stresses that can be used for breeding purposes and future crop improvement. This research has contributed to the growing knowledge of barley V-PPases, and more specifically the role of *HvHVP10* in conferring salinity tolerance, but also advances the understanding of the molecular mechanisms underlying salinity tolerance in an agronomically important crop species.

6.9 Conclusion

The exclusion of sodium ions from the shoots of both glycophytes and halophytes is considered to be an important salinity tolerance mechanism among plants. The aim of this research project was to confirm that the barley V-PPase *HvHVP10* is a candidate gene for salinity tolerance in barley, due to increased compartmentalisation of sodium ions in root vacuoles. The sodium exclusion QTL *HvNax3*, derived from *H.spontaneum* accession CPI-71284-48 has been shown to be involved in increased V-PPase activity and expression within the roots, providing evidence that *HvHVP10* is a strong candidate gene for *HvNax3*. However more experimental examination and further genetic characterisation is required to confirm this hypothesis. A significant component of this research was the generation of transgenic barley constitutively expressing V-PPase *HvHVP10*. While the effect of *HvHVP10* overexpression in barley requires further assessment, the transgenic lines generated in this study have displayed promising phenotypes related to increased biomass or alterations in tissue ion concentrations. Results of this research also increase our current understanding of the molecular and physiological mechanisms involved in the salinity tolerance of an agronomical important crop species; and specifically the role the barley V-PPase *HvHVP1*0 plays in altering sodium transport within the plant.

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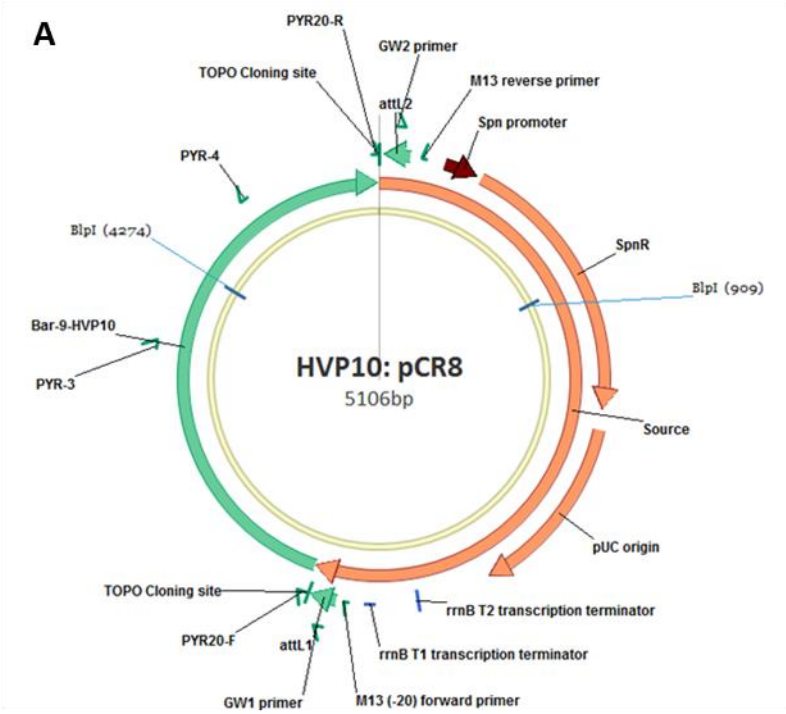
8

Appendices

8.1 Publication

Shavrukov, Y., Bovill, J., Afzal, I., Hayes, J.E., Roy, S.J., Tester, M. and Collins, N.C. (2013) HVP10 encoding V-PPase is a prime candidate for the barley HvNax3 sodium exclusion gene: evidence from fine mapping and expression analysis. *Planta*, **237**, 1111-1122.

8.2 Vector Diagrams



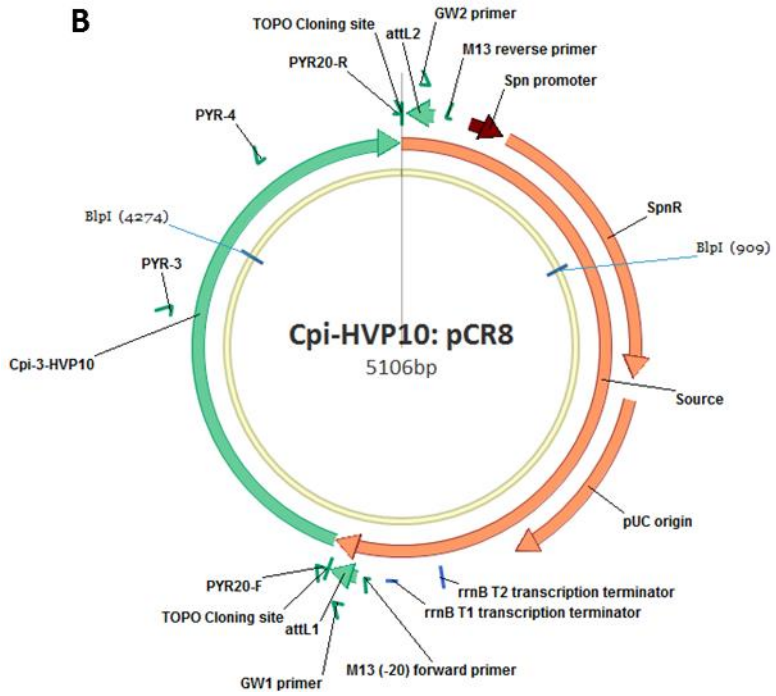


Figure 8-1 Vector diagrams of pCR8/GW/TOPO Gateway® entry vectors containing *HvHVP10* CDS from Barque-73 (A; Bar-9-HVP10) and CPI-71284-48 (B; Cpi-3-HVP10) cloned into the vector at the TOPO® cloning site between the gateway recombination sites (attL1 and attL2). The vector contains spectinomycin promoter (Spn promoter); spectinomycin resistance gene (SpnR); pUC origin of replication (pUC origin); rrn T1 and rrn T_2 transcription terminator sites; M13 forward and M13 reverse primer binding sites and GW1 and GW2 primer binding sites. Vector diagrams include restriction sites for the restriction enzyme *Blp*I and primer (PYR20-F; PYR-3; PYR-4; PYR20-R) binding sites used to confirm insertion and orientation of *HvHVP10* CDS.

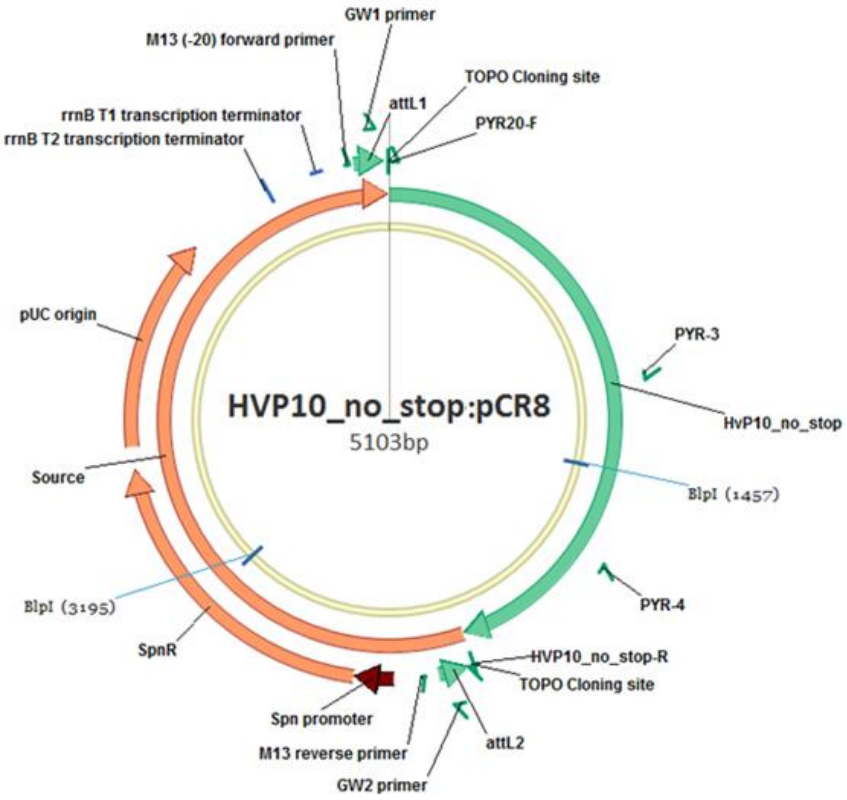


Figure 8-2 Vector diagram of pCR8/GW/TOPO Gateway® entry vectors containing *HvHVP10* CDS from Barque-73 without the stop codon (HvP10_no_stop) cloned into the vector at the TOPO® cloning site between the gateway recombination sites (attL1 and attL2). The vector contains spectinomycin promoter (Spn promoter); spectinomycin resistance gene (SpnR); pUC origin of replication (pUC origin); rrn T1 and rrn T2 transcription terminator sites; M13 forward and M13 reverse primer binding sites and GW1 and GW2 primer binding sites. Vector diagrams include restriction sites for restriction enzyme *Blp*I and primer (PYR20-F; PYR-3; PYR-4; PYR20-R HVP10-no-stop) binding sites used to confirm insertion and orientation of *HvHVP10* CDS.

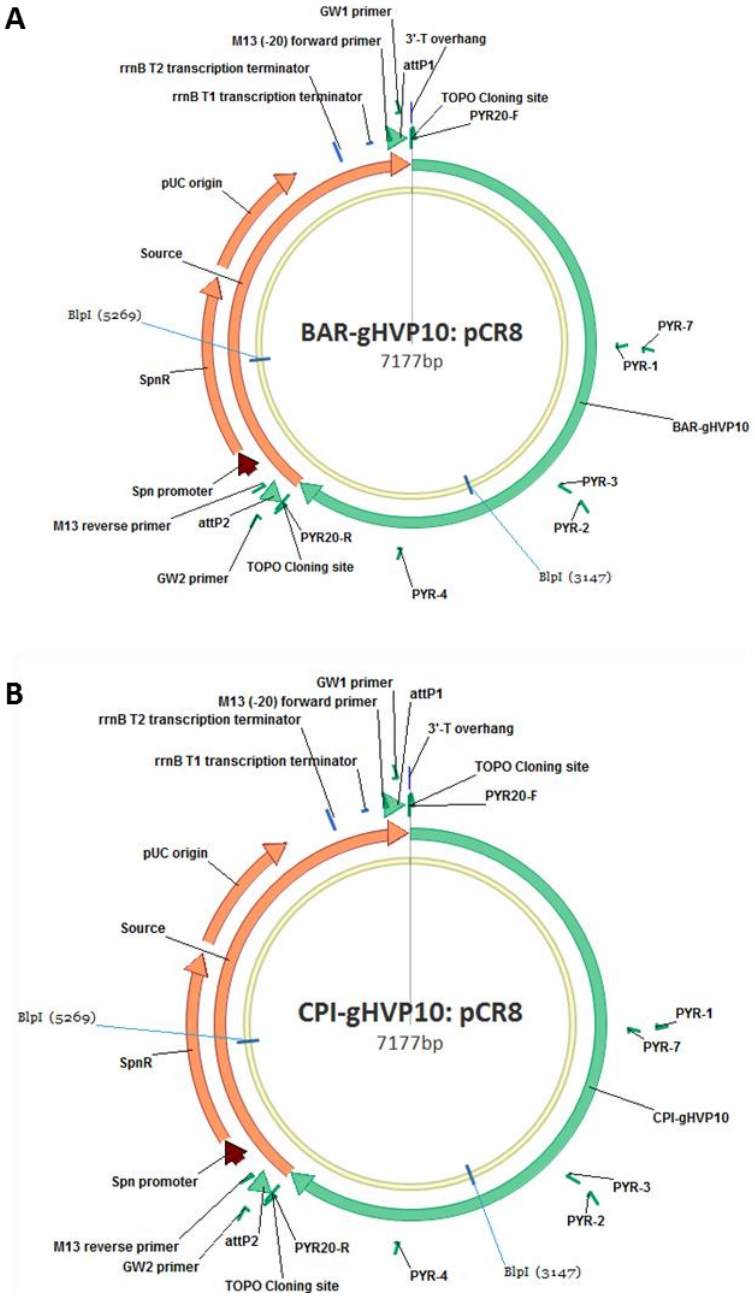


Figure 8-3 Vector diagrams of pCR8/GW/TOPO® Gateway entry vectors containing the *HvHVP10* full-length gene from Barque-73 (A; Bar-gHVP10) and CPI-71284-48 (B; CPI-gHVP10) cloned into the vector at the TOPO® cloning site between the gateway recombination sites (attL1 and attL2). The vector contains spectinomycin promoter (Spn promoter); spectinomycin resistance gene (SpnR); pUC origin of replication (pUC origin); rrn T1 and rrn T2 transcription terminator sites; M13 forward and M13 reverse primer binding sites and GW1 and GW2 primer binding sites. Vector diagrams include restriction sites for the restriction enzyme *Blp*I and primer (PYR20-F; PYR-1; PYR-2; PYR-3; PYR-4; PYR-7 and PYR20-R) binding sites used to confirm insertion and orientation of *HvHVP10* full-length gene.

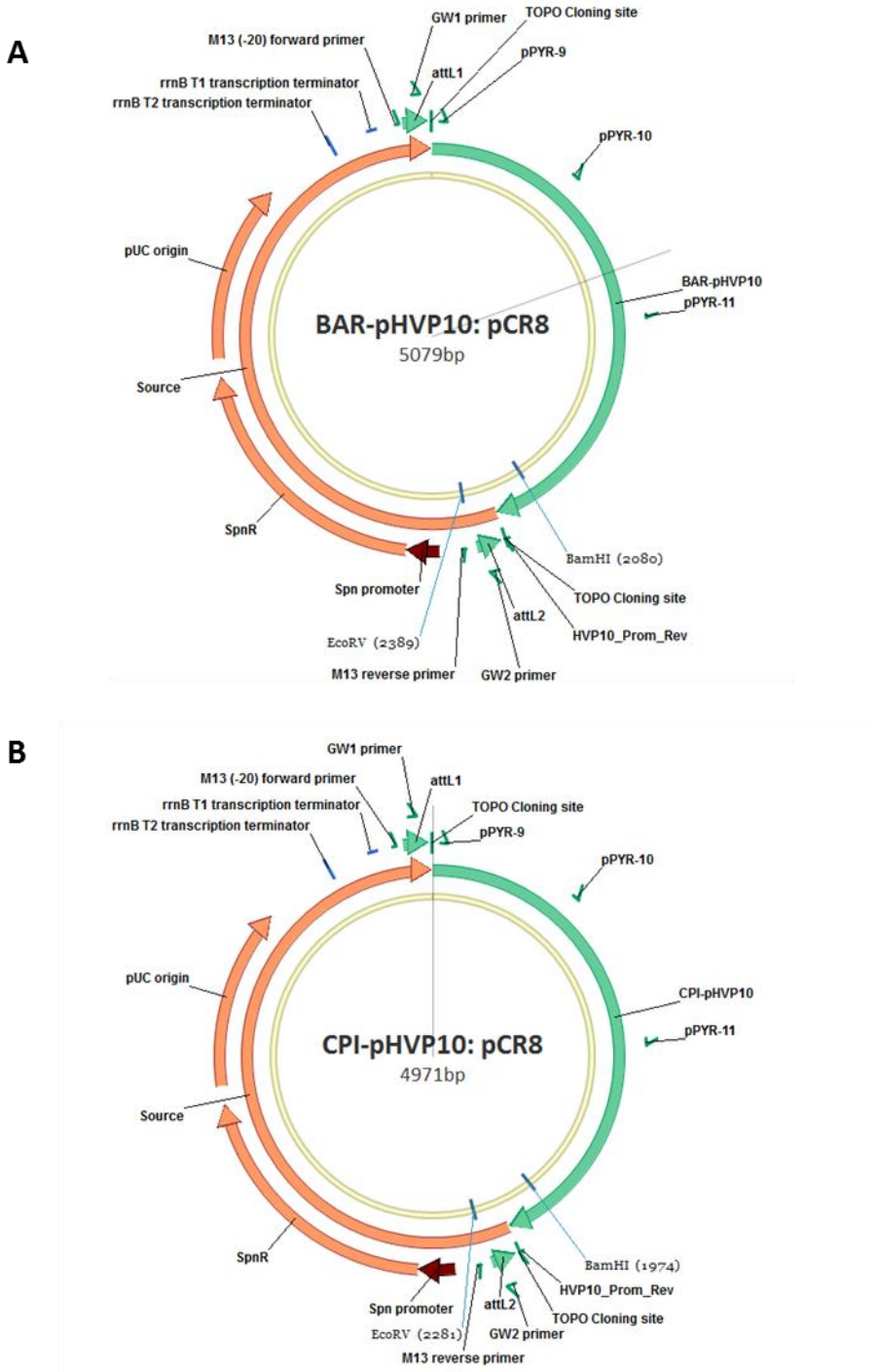


Figure 8-4 Vector diagrams of pCR8/GW/TOPO® Gateway entry vectors containing *HvHVP10* promoter regions from Barque-73 (A; BAR-pHVP10) CPI-71284-48 (B; CPI-pHVP10) cloned into the vector at the TOPO® cloning site between the gateway recombination sites (attL1 and attL2). The vector contains spectinomycin promoter (Spn promoter); spectinomycin resistance gene (SpnR); pUC origin of replication (pUC origin); rrn T1 and rrn T2 transcription terminator sites; M13 forward and M13 reverse primer binding sites and GW1 and GW2 primer binding sites. Vector diagrams include restriction sites for the restriction enzymes *Bam*HI and *Eco*RV and primer (pPYR-9, pPYR-10, pPYR-11) binding sites used to confirm insertion and orientation of *HvHVP10* promoter.

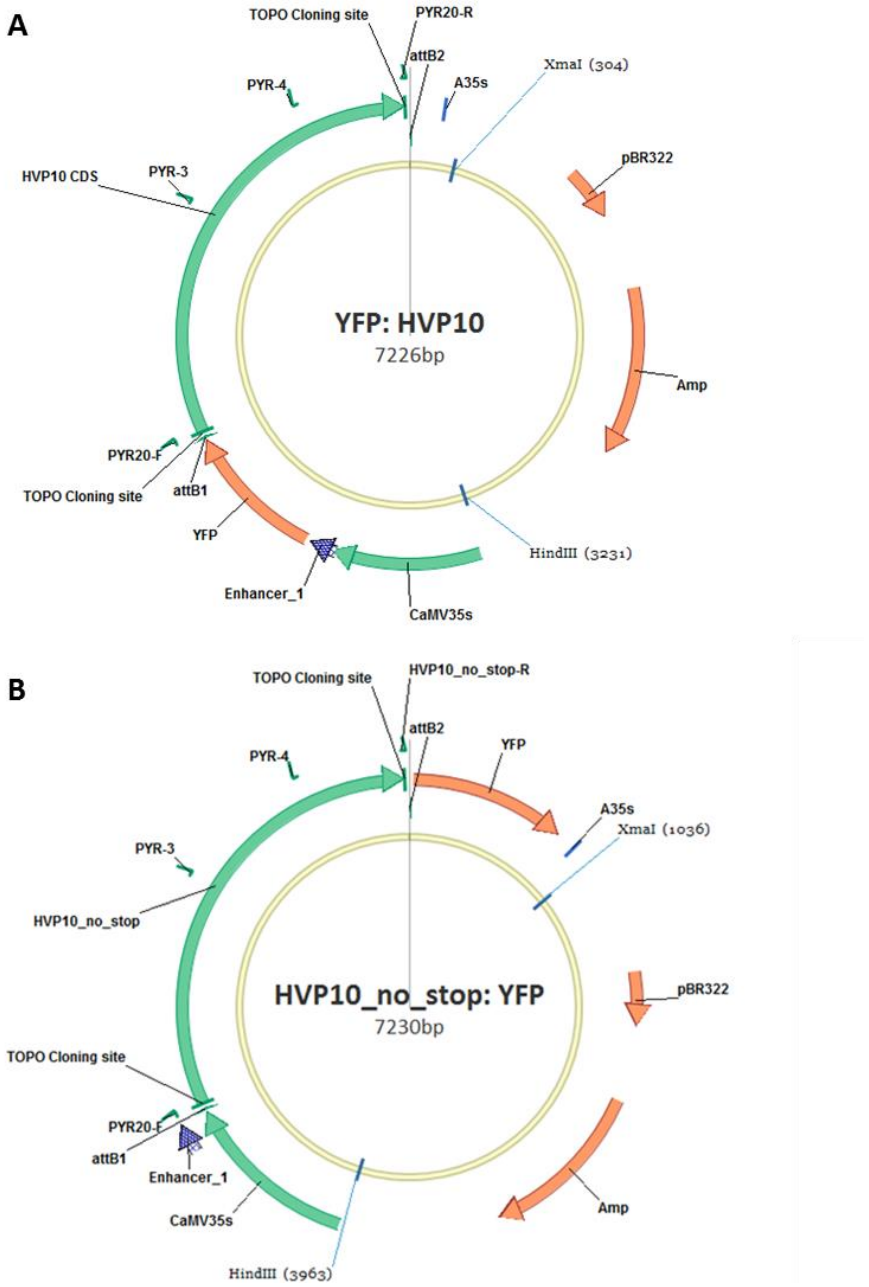


Figure 8-5 Vector diagrams of N (A) and C terminal (B) YFP fusion vectors carrying the *HvHVP10* CDS with the stop codon (HVP10) and without the stop codon (HVP10_no_stop). The *HvHVP10* CDS with and without the stop codon was inserted between the recombination sites (*attB1* and *attB2*). The vector backbone contains the following components: the origin of replication for *E. coli* (pBR322); ampicillin resistance gene (Amp); cauliflower mosaic virus 35S promoter (CaMV35S); Enhancer_1, the enhancer for transient expression of YFP; The gene encoding YFP (YFP); and the cauliflower mosaic virus 35S terminator sequence (A35S). The position of primers used to sequence expression vectors (PYR20-F, PYR20-R, PYR-3, PYR-4 and HVP10_no_stop-R) and the restriction sites for enzymes *Hind*III and *Xma*I are indicated.

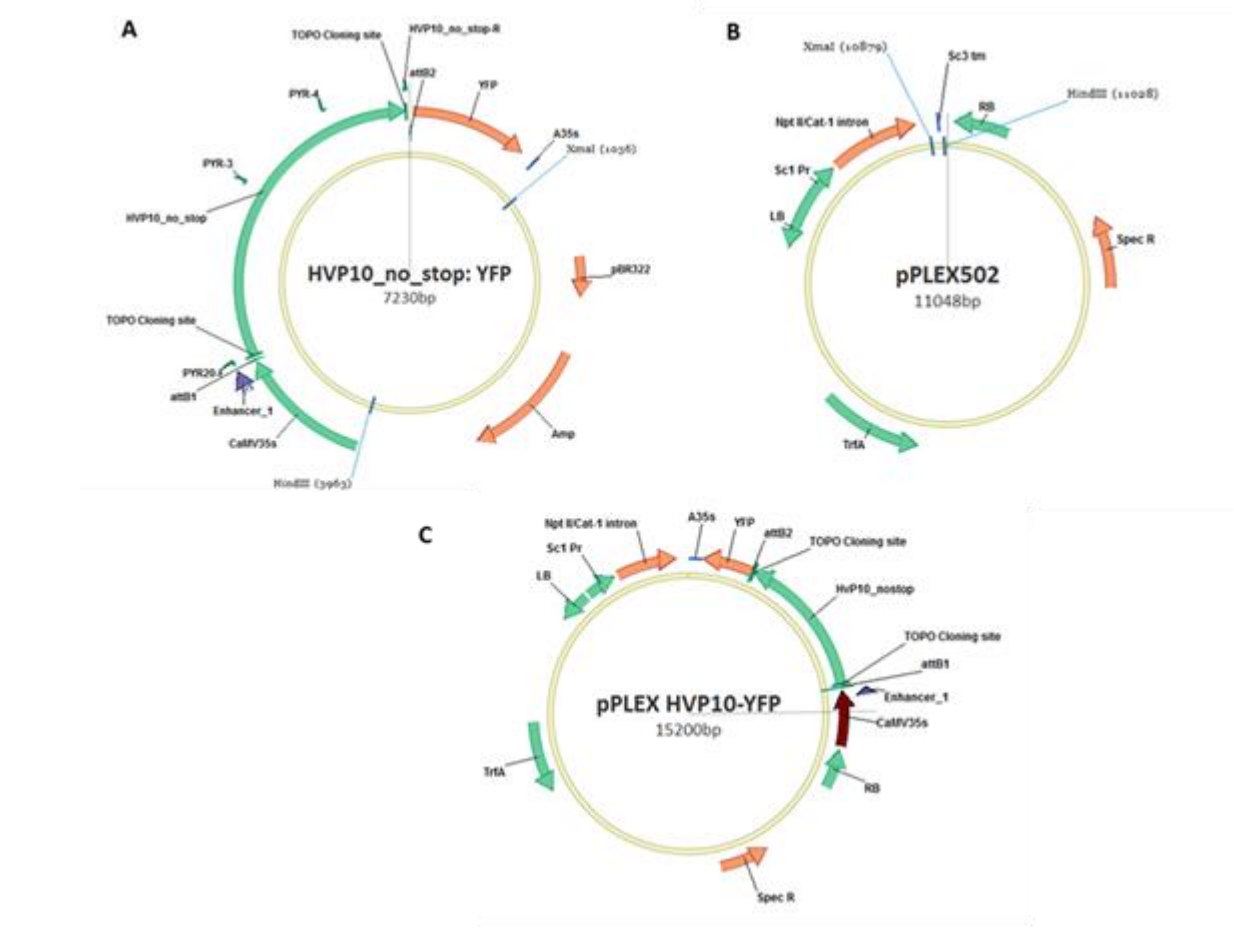


Figure 8-6 Vector diagrams of the HVP10_no_stop :YFP expression vector carrying ampicillin resistance gene (A), the entry vector pPLEX502 carrying spectinomycin resistance gene (B) and the resultant expression vector pPLEX HVP10-YFP (C) carrying HVP10_no_stop :YFP expression vector with spectinomycin resistance produced via restriction enzyme digestion and ligation. The vector backbone contains the following components: the origin of replication for E. coli (pBR322); ampicillin resistance gene (Amp); cauliflower mosaic virus 35S promoter (CaMV35S); Enhancer_1, the enhancer for transient expression of YFP; The gene encoding YFP (YFP); and the cauliflower mosaic virus 35S terminator sequence (A35S). The position of the restriction sites for enzymes HindIII and XmaI are included in the vector map.

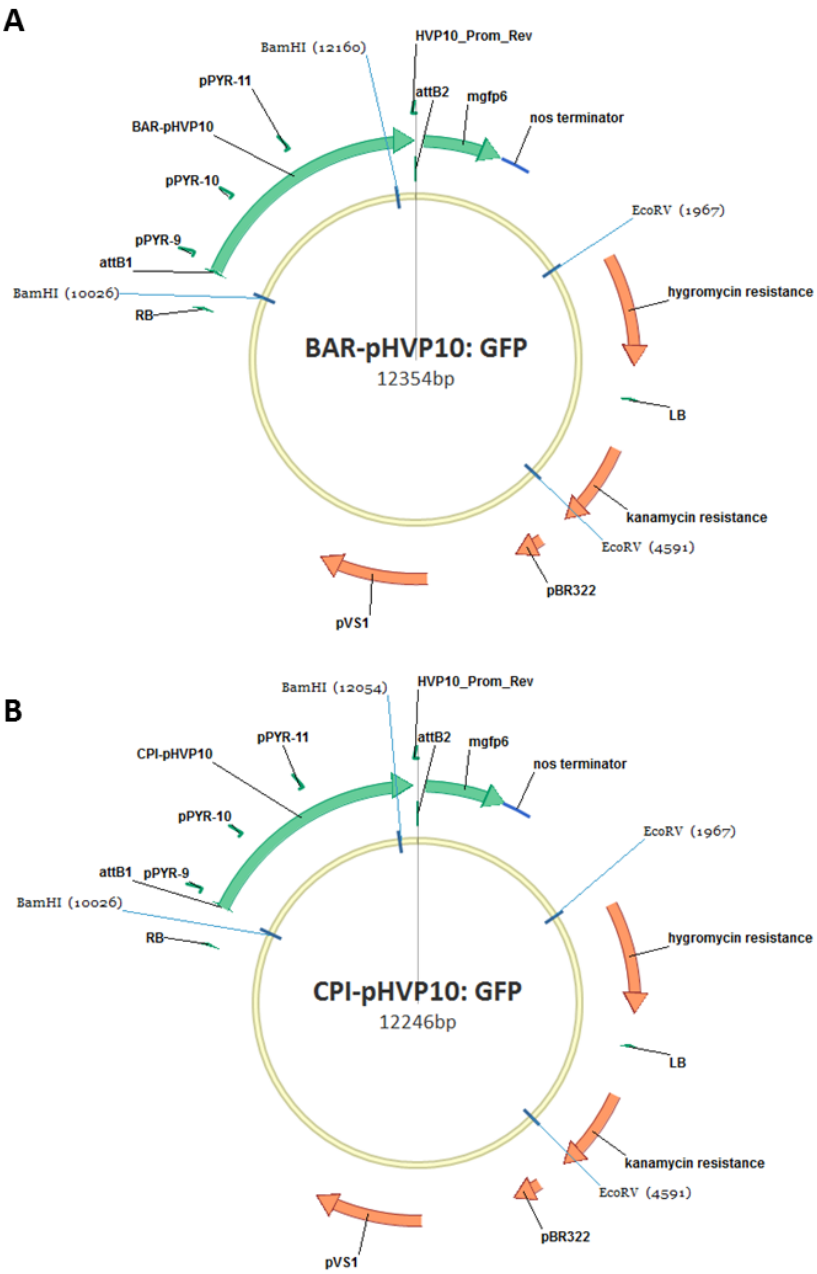


Figure 8-7 Map of the destination vectors carrying the HVP10 promoter region from Barque-73 (A)and CPI-71284-48 (B) fused to the green fluorescent protein gene (*mgfp6*). The vector backbone contains the following components: kanamycin resistance gene, the origin of replication for E. coli (pBR322), and the minimal replicon (pVS1) for stable maintenance in E. coli. The T-DNA cassette contains the right border sequence (RB), dual cauliflower mosaic virus 35S promoter (2X35S), the gateway recombination sequences (attB1 and attB2), the Barque HVP10 coding sequence, the nopaline synthase (Nos) terminator, cauliflower mosaic virus 35S promoter (CaMV35S), hygromycin resistance gene, cauliflower mosaic virus 35S terminator (A35S) and left border sequence (LB).

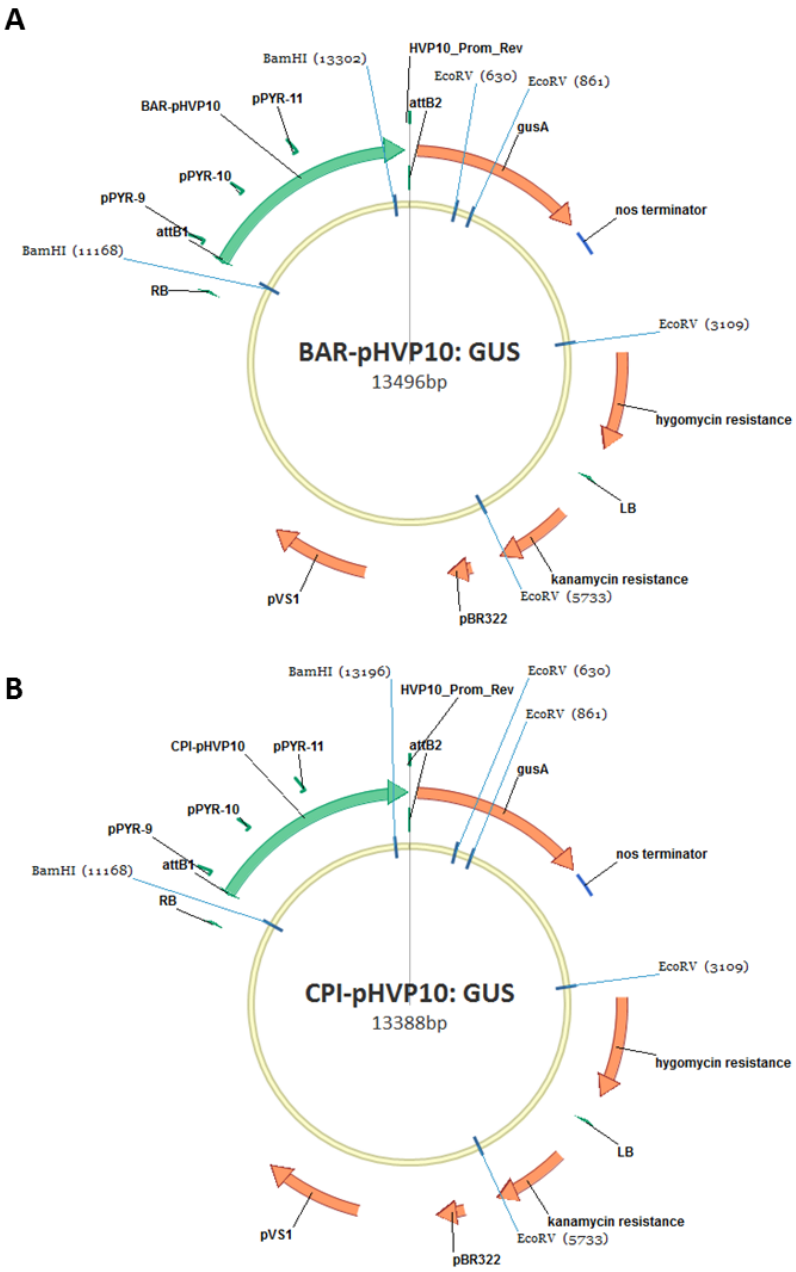


Figure 8-8 Map of the destination vectors carrying the HVP10 promoter region from Barque-73 (A)and CPI-71284-48 (B) fused to the β-glucuronidase gene (*UidA*). The vector backbone contains the following components: kanamycin resistance gene, the origin of replication for E. coli (pBR322), and the minimal replicon (pVS1) for stable maintenance in E. coli. The T-DNA cassette contains the right border sequence (RB), dual cauliflower mosaic virus 35S promoter (2X35S), the gateway recombination sequences (attB1 and attB2), the Barque HVP10 coding sequence, the nopaline synthase (Nos) terminator, cauliflower mosaic virus 35S promoter (CaMV35S), hygromycin resistance gene, cauliflower mosaic virus 35S terminator (A35S) and left border sequence (LB).

8.3 The *HvHVP10* ORF sequence for CPI-71284-48 and Barque-73 showing the position of SNPs in the *HvHVP10* CDS (Highlighted in red). Alignments were performed using CLUSTALW.

```
CPI_CDS         GCTGCTCTTGTTGTTGCTTCCATCTCATCTTTTGGAATCAACCATGATTTCACTGCGATG 960
BAR_CDS         GCTGCTCTTGTTGTTGCTTCCATCTCATCTTTTGGAATCAACCATGATTTCACTGCGATG 960
                ************************************************************

CPI_CDS         TGCTACCCACTGCTCGTGAGCTCTGTAGGCATCATTGTTTGCTTGCTCACCACACTCTTT 1020
BAR_CDS         TGCTACCCACTGCTCGTGAGCTCTGTAGGCATCATTGTTTGCTTGCTCACCACACTCTTT 1020
                ************************************************************

CPI_CDS         GCAACTGATTTCTTTGAGATTAAGGCTGCAAACGAAATTGAACCTGCTCTGAAGAAGCAG 1080
BAR_CDS         GCAACTGATTTCTTTGAGATTAAGGCTGCAAACGAAATTGAACCTGCTCTGAAGAAGCAG 1080
                ************************************************************

CPI_CDS         CTCATCATCTCCACTGCTCTAATGACTGTTGGTGTTGCGGTCATCAGCTGGTTGGCTCTT 1140
BAR_CDS         CTCATCATCTCCACTGCTCTAATGACTGTTGGTGTTGCGGTCATCAGCTGGTTGGCTCTT 1140
                ************************************************************

CPI_CDS         CCAGCTAAGTTCACCATCTTCAACTTCGGTGCTCAGAAGGAAGTGTCCAACTGGGGCCTG 1200
BAR_CDS         CCAGCTAAGTTCACCATCTTCAACTTCGGTGCTCAGAAGGAAGTGTCCAACTGGGGCCTG 1200
                ************************************************************

CPI_CDS         TTCTTCTGCGTGGCAGTTGGTCTGTGGGCTGGTCTGATTATTGGATTTGTGACTGAATAC 1260
BAR_CDS         TTCTTCTGCGTGGCAGTTGGTCTGTGGGCTGGTCTGATTATTGGATTTGTGACTGAATAC 1260
                ************************************************************

CPI_CDS         TACACTAGCAACGCCTACAGCCCTGTGCAAGATGTTGCCGATTCCTGCAGAACTGGTGCT 1320
BAR_CDS         TACACTAGCAACGCCTACAGCCCTGTGCAAGATGTTGCCGATTCCTGCAGAACTGGTGCT 1320
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CPI_CDS         GCCACCAACGTCATCTTCGGTCTTGCTCTGGGGTACAAGTCTGTTATCATCCCAATTTTC 1380
BAR_CDS         GCCACCAACGTCATCTTCGGTCTTGCTCTGGGGTACAAGTCTGTTATCATCCCAATTTTC 1380
                ************************************************************

CPI_CDS         GCTATTGCTGTCAGCATCTACGTCAGCTTCTCCATTGCTGCAATGTACGGCATTGCAATG 1440
BAR_CDS         GCTATTGCTGTCAGCATCTACGTCAGCTTCTCCATTGCTGCAATGTACGGCATTGCAATG 1440
                ************************************************************

CPI_CDS         GCTGCTCTTGGCATGCTAAGCACAATGGCAACTGGTCTTGCCATCGATGCTTATGGTCCC 1500
BAR_CDS         GCTGCTCTTGGCATGCTAAGCACAATGGCAACTGGTCTTGCCATCGATGCTTATGGTCCC 1500
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CPI_CDS         ATTAGTGACAATGCTGGTGGAATTGCTGAGATGGCTGGCATGAGCCACAGAATCCGTGAG 1560
BAR_CDS         ATTAGTGACAATGCTGGTGGAATTGCTGAGATGGCTGGCATGAGCCACAGAATCCGTGAG 1560
                ************************************************************

CPI_CDS         AGGACTGATGCTCTTGATGCTGCTGGCAACACAACCGCTGCTATTGGGAAGGGTTTCGCC 1620
BAR_CDS         AGGACTGATGCTCTTGATGCTGCTGGCAACACAACCGCTGCTATTGGGAAGGGTTTCGCC 1620
                ************************************************************

CPI_CDS         ATTGGATCAGCTGCTCTCGTGTCCCTGGCACTTTTCGGTGCCTTTGTCAGCAGAGCTGGT 1680
BAR_CDS         ATTGGATCAGCTGCTCTCGTGTCCCTGGCACTTTTCGGTGCCTTTGTCAGCAGAGCTGGT 1680
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CPI_CDS         GTGAAGGTCGTTGATGTCCTATCTCCCAAGGTGTTCATTGGTCTGATTGTCGGAGCCATG 1740
BAR_CDS         GTGAAGGTCGTTGATGTCCTATCTCCCAAGGTGTTCATTGGTCTGATTGTCGGAGCCATG 1740
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CPI_CDS         CTTCCGTACTGGTTCTCTGCCATGACCATGAAGAGTGTTGGAAGTGCTGCCCTCAAGATG 1800
BAR_CDS         CTTCCGTACTGGTTCTCTGCCATGACCATGAAGAGTGTTGGAAGTGCTGCCCTCAAGATG 1800
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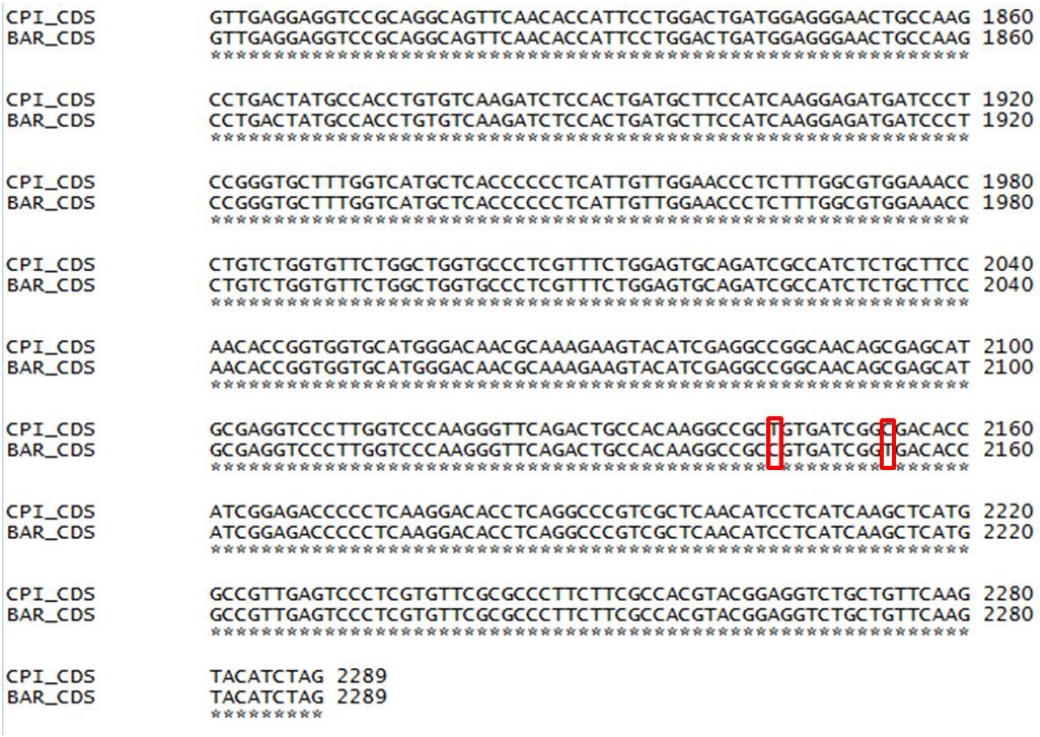
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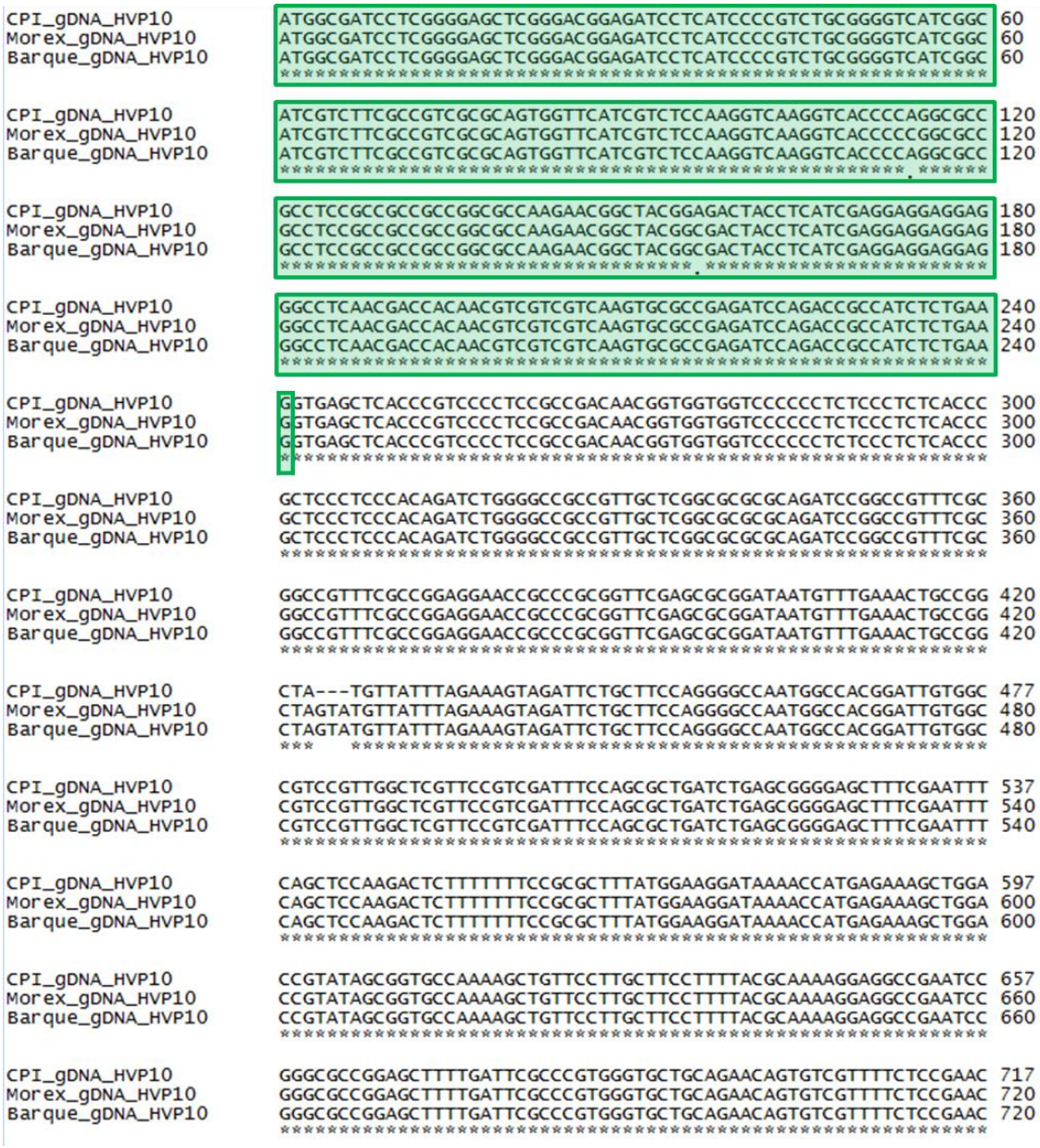
8.4 ClustalW alignment of the full-length HvHVP10 gene showing the position of exons (highlighted in green) and non-coding introns



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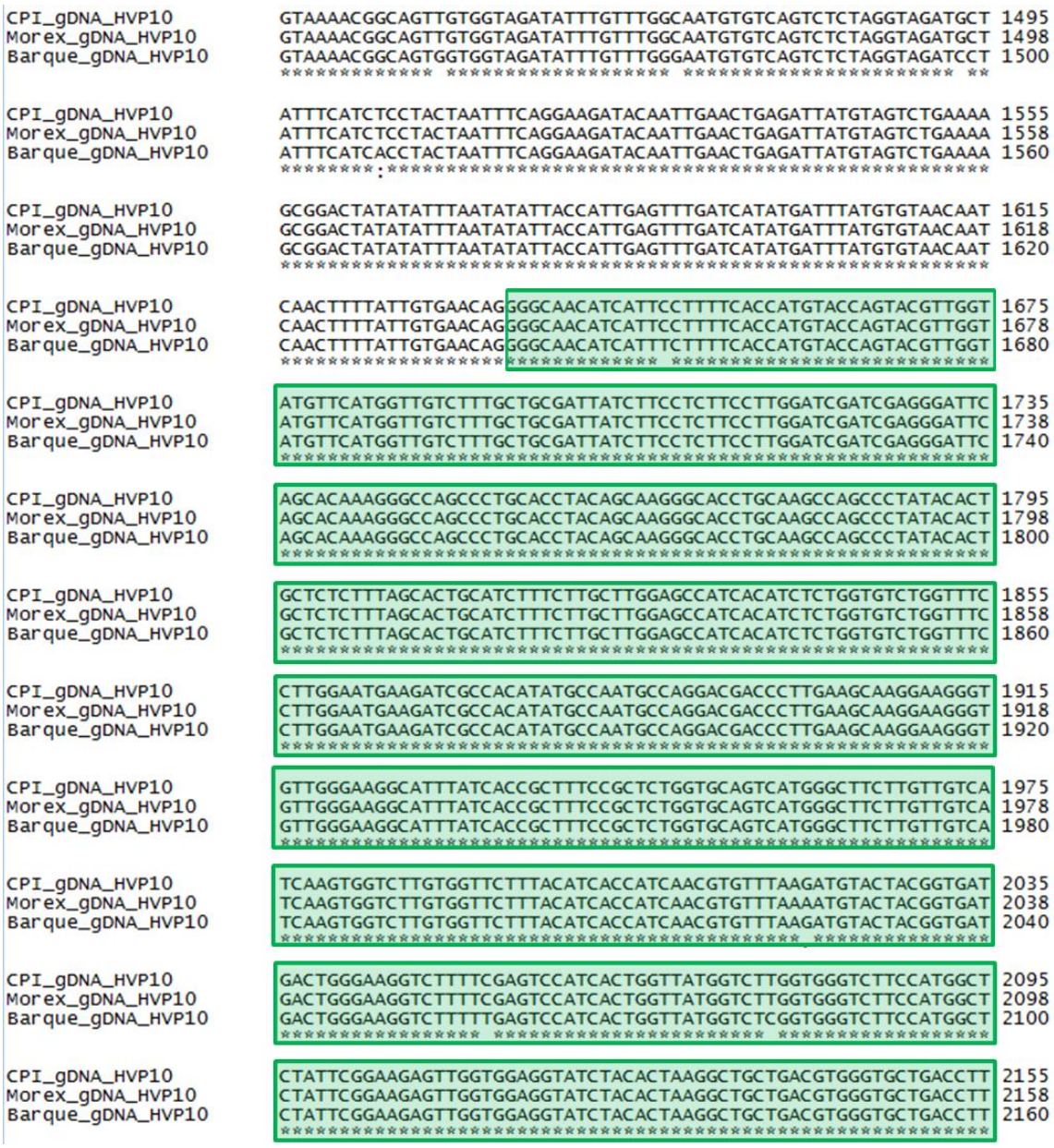
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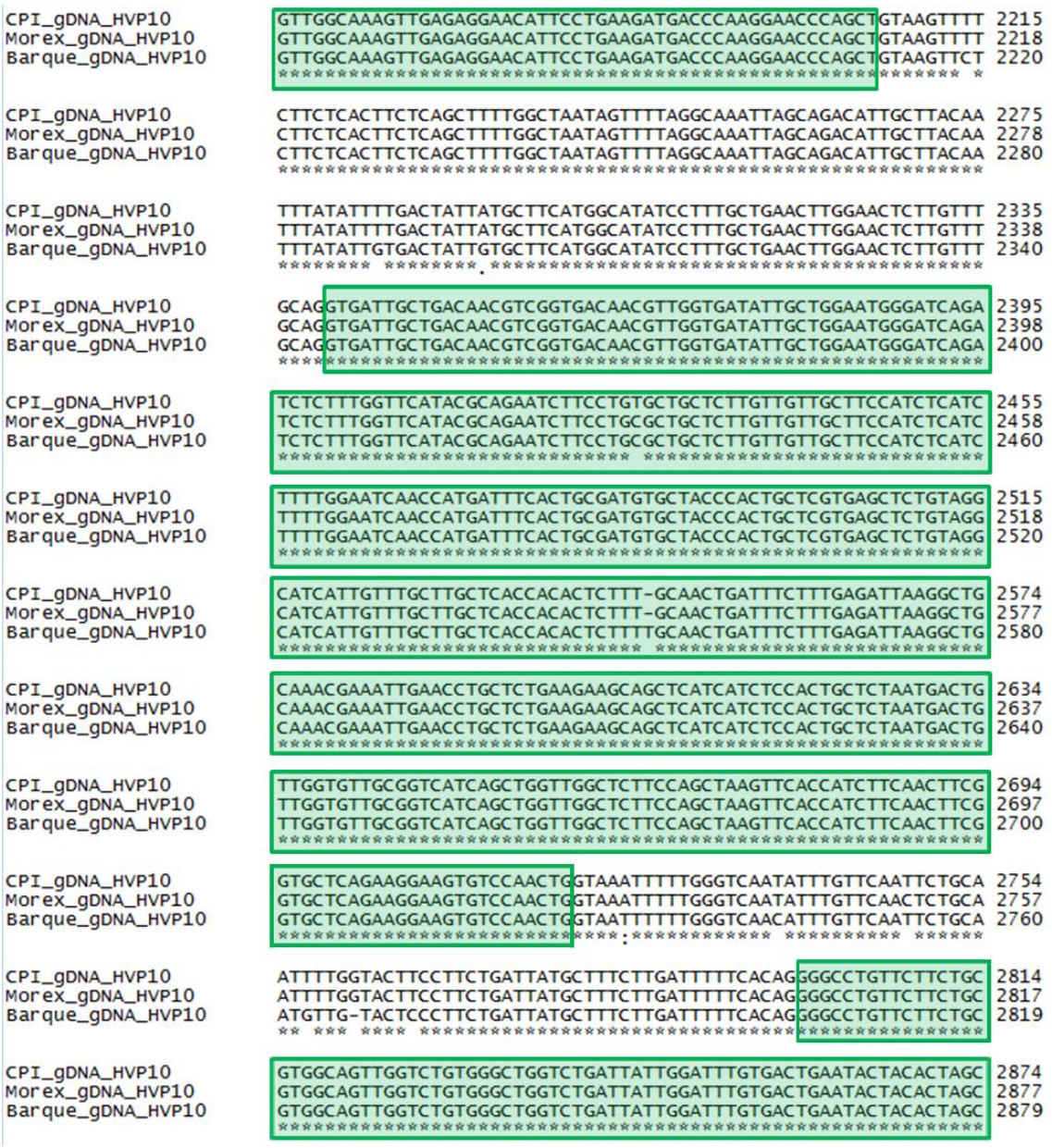
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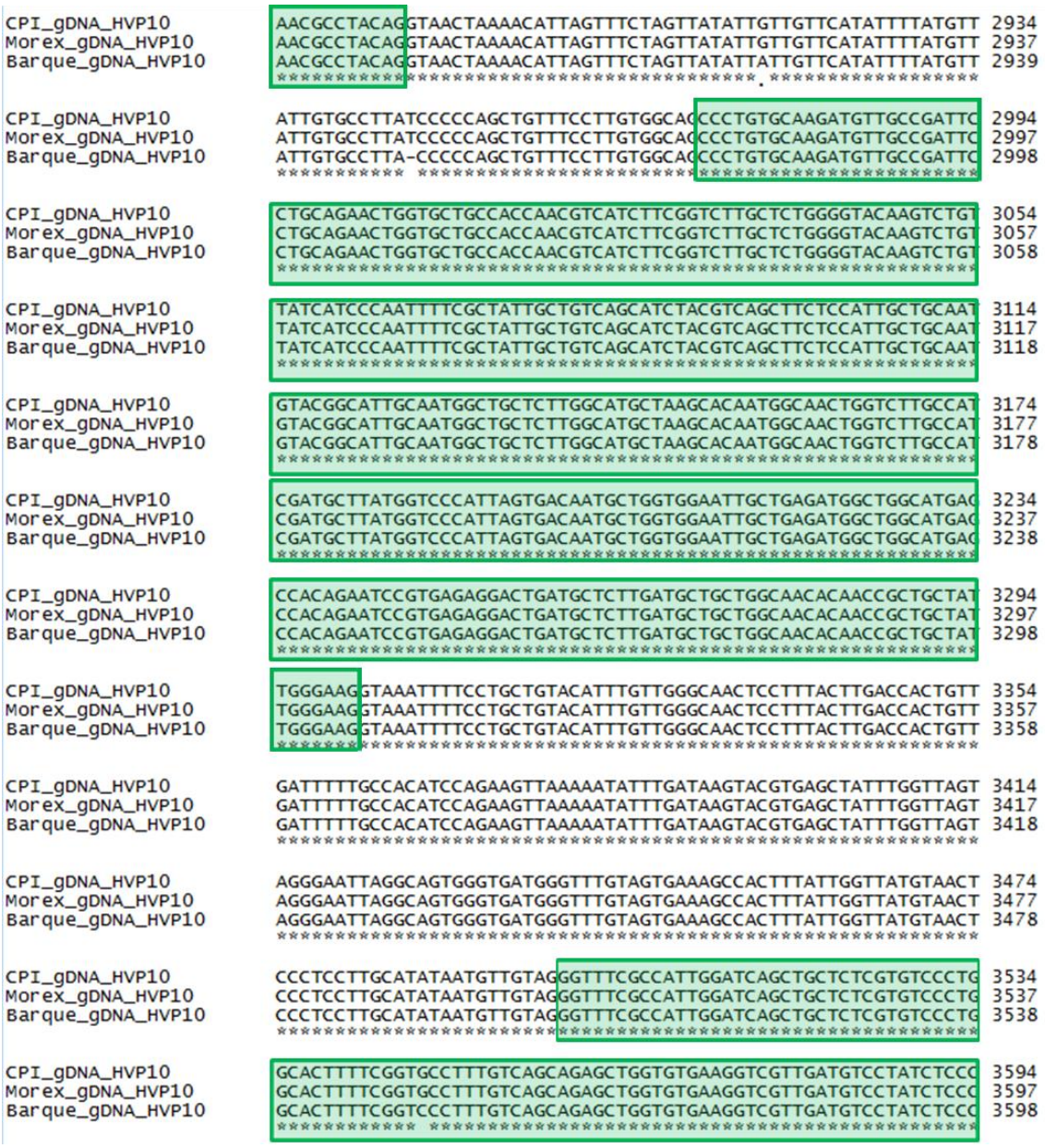
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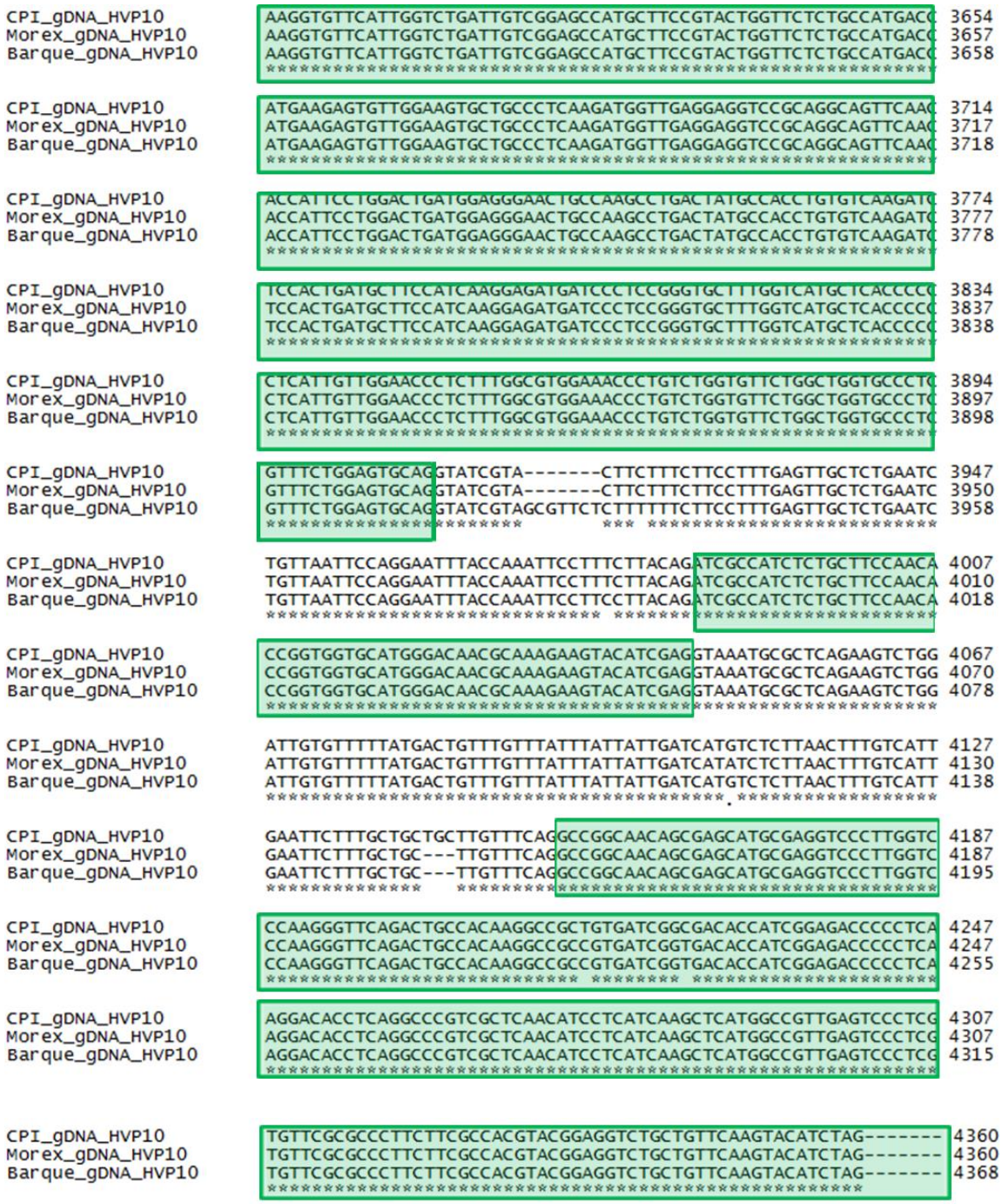
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CCGGTGGTGCATGGGACAACGCAAAGAAGTACATCGAGGTAAATGCGCTCAGAAGTCTGG 4067
CCGGTGGTGCATGGGACAACGCAAAGAAGTACATCGAGGTAAATGCGCTCAGAAGTCTGG 4070
CCGGTGGTGCATGGGACAACGCAAAGAAGTACATCGAGGTAAATGCGCTCAGAAGTCTGG 4078
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ATTGTGTTTTTATGACTGTTTGTTTATTTATTATTGATCATATCTCTTAACTTTGTCATT 4130
ATTGTGTTTTTATGACTGTTTGTTTATTTATTATTGATCATGTCTCTTAACTTTGTCATT 4138
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CCAAGGGTTCAGACTGCCACAAGGCCGCTGTGATCGGCGACACCATCGGAGACCCCCTCA 4247
CCAAGGGTTCAGACTGCCACAAGGCCGCCGTGATCGGTGACACCATCGGAGACCCCCTCA 4247
CCAAGGGTTCAGACTGCCACAAGGCCGCCGTGATCGGTGACACCATCGGAGACCCCCTCA 4255
AGGACACCTCAGGCCCGTCGCTCAACATCCTCATCAAGCTCATGGCCGTTGAGTCCCTCG 4307
AGGACACCTCAGGCCCGTCGCTCAACATCCTCATCAAGCTCATGGCCGTTGAGTCCCTCG 4307
AGGACACCTCAGGCCCGTCGCTCAACATCCTCATCAAGCTCATGGCCGTTGAGTCCCTCG 4315
TGTTCGCGCCCTTCTTCGCCACGTACGGAGGTCTGCTGTTCAAGTACATCTAG------- 4360
TGTTCGCGCCCTTCTTCGCCACGTACGGAGGTCTGCTGTTCAAGTACATCTAG------- 4360
TGTTCGCGCCCTTCTTCGCCACGTACGGAGGTCTGCTGTTCAAGTACATCTAG------- 4368

8.5 HvHVP10 Promoter alignment from CPI-71284-48, Barque-73 and Morex. Alignments were performed using CLUSTALW.

```
cpi_promoter          TTTGTACAAAAAGCAGGCTCCGAAT-----TCGCCCTTTCATGCCTTGAAGAATGTTGTT 55
barque_promoter       TTTGTACAAAAAGCAGGCTCCGAAT-----TCGCCCTTTCATGCCTTGAAGAATGTTGTT 55
morex_promoter        TATCTTCAAAATGCATGGTCATATAGTTTTTTAAATATTCATGCCTTGAAGAATGTTGTT 60
                      *:* *:*****:*** * **. *::     * ... :**********************

cpi_promoter          GCACTTCTGTTAATAAATAAAAATGATTTAAACCAAAATTTAATAAAAATACAAGTTTTC 115
barque_promoter       GCACTTCTGTTAATAAATAAAAATGATTTAAACCAAAATTTAATAAAAATACAAGTTTTC 115
morex_promoter        GCACTTCTGTTAATAAATAAAAATGATTTAAACCAAAATTTAATAAAAATACAAGTTTTC 120
                      ************************************************************

cpi_promoter          ATGGAAGCATAAGGCCTTGTTTGGTGTAAGAGGTTTTGGGAGGTTCAAAAAGAAATACCC 175
barque_promoter       ATGGAAGCATAAGGCCTTGTTTGGTGTAAGAGGTTTTGGGAGGTTCAAAAAGAAATACCC 175
morex_promoter        ATGGAAGCATAAGGCCTTGTTTGGTGGAAGAGGTTTTGGGAGGTTCAGAAAGAAATACCC 180
                      *************************** ********************.************

cpi_promoter          CCATATGCCCTAAAATACCCAAAAATCCCCATCAGCCCATTTCGTACGCACGTAATGAAC 235
barque_promoter       CCATATGCCCTAAAATACCCAAAAATCCCCATCAGCCCATTTCGTACGCACGTAATGAAC 235
morex_promoter        CCATATGCCCTAGAATACCCAAAAATCCCTATCAGCCCATTTCGTACGCACGGAATGAAC 240
                      ************.**************** ********************* ********

cpi_promoter          ATGCCACTGCCATGATCACCACCAACCCCTCCCAAGTCCCAACCCATGTGTTACAGAAAA 295
barque_promoter       ATGCCACTGCCATGATCACCACCAACCCCTCCCAAGTCCCAACCCATGTGTTACAGAAAA 295
morex_promoter        ATGCCACTCCCATGATCACCACCAACCCCTCCCAAGTCCCAACCCATGTGTTACAGAAAA 300
                      ******** ************************************************

cpi_promoter          CTCCAGGAGTCACTCGTGGAATGGGATGAAACAAGGGCGCTCGTTGTGCTGCTCCCGTCA 355
barque_promoter       CTCCAGGAGTCACTCGTGGAATGGGATGAAACAAGGGCGCTCGTTGTGCTGCTCTTGTCA 355
morex_promoter        CTCCAGGAGTCACTCGTGGAATGGGATGAAACAAGGGCGCTCGTTGTGCTGCTCCCGTCA 360
                      ************************************************  ****

cpi_promoter          TAGGAGGTTTAATCGAACAAAACTTATTTTGGGGACTGGGAGGGGGGGGGG----CTAGC 411
barque_promoter       TAGGAGGTTTAATCGAACAAAACTTATTTTGGGGACTGGGAGGGGGGGGGGGGGCTAGC 415
morex_promoter        TAGGAGGTTTAATCGAACAAAACTTATTTTGGGGACTGGGAGGGGG-GGGG----CTAGC 415
                      ********************************************** ****    *****

cpi_promoter          CGGAAAATGGGTGGAGCGGGTCATCAAATCCCCTCTGATCCCCACCCCTTGGGGCTGCAG 471
barque_promoter       CGGAAAAGGGGGGGAGCGGGTCATCAAATCCCCTCTGATCCCCACCCCTTGGGGCTGCAA 475
morex_promoter        CGGGAAATGGGTGGAGCGGGTCATCAAATCCCCTCTGATCCCCACCCCTTGGGGCTGCAG 475
                      ***.*** *** ************************************************.

cpi_promoter          AAACTCACCACCAAACAAGGTGTGAAGAAAAAAAGAAAAGGAAAATATAGGAAATGAGAT 531
barque_promoter       AAACTCCCCACCAAACAAGGTGTGAAAAAAAAAAAAAAAAGGAAAATATAGGAAATGAAAT 535
morex_promoter        AAACTCACCACCAAACAAGGTGTGAAGAAGAAAAGAAAAGGAAAATATAGGAAAGGAGAT 535
                      ******.******************.**.****.****************** **.**

cpi_promoter          AAATATTGAAATAAATGAAGAGAAAGGTAAAAAAAATAGCCATGCCAAAACCTCACCACCG 591
barque_promoter       AAATATTGAAATAAATGAAAAGAAAGGTAAAAAAAATAGCCATGCCAAAACCTCACCACCG 595
morex_promoter        AAATATTGAAATAAATGAAGAGAAAGGTAAAAAAAATAGCCATGCCAAAACCTCACCACGG 595
                      ******************.****************************************** *

cpi_promoter          AACCCTTTATTCCACATAATGAGCATGTAAAACATTTAAGCATGCACACTTAACATGTTG 651
barque_promoter       AACCCTTTATTCCACATAATGAGCATGTAAAACATTTAAGCATGCACACTTAACATGTTC 655
morex_promoter        AACCCTTTATTCCACATAATGAGCATGTAAAACATTTTAGCATGCACACTTAACATGTTG 655
                      ****************************************:********************

cpi_promoter          GAGGTTAACTCAAAATAGTCATGAAGATCTAAGGGGCCTCTAACACAAAAGCCAATTATG 711
barque_promoter       GAGGTTAACTCAAAATAGTCATGGAGATCTAAGGGGCCTCTAACACAAAAGCCAATTATG 715
morex_promoter        GAGGTTAATTCAAAATAGTCATGGAAATCTAAGGGGCCTCTAACACAAAAGCCAATTATG 715
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cpi_promoter          GAGGTTAACTCAAAATAGTCATGAAGATCTAAGGGGCCTCTAACACAAAAGCCAATTATG 711
barque_promoter       GAGGTTAACTCAAAATAGTCATGGAGATCTAAGGGGCCTCTAACACAAAAGCCAATTATG 715
morex_promoter        GAGGTTAATTCAAAATAGTCATGGAAATCTAAGGGGCCTCTAACACAAAAGCCAATTATG 715
                      ******** **************.*.************************************

cpi_promoter          GAGACGTCTATATACACGGAGAGGTGATGGCTTTAGCCATTTTTGGAATTCCTCGTTATC 771
barque_promoter       GAGACGTCTATATACACGGAGAGGTGATGGCTTTAGCCATTTTTGGAATTCCTCGTTATC 775
morex_promoter        GAGACGTCTATATACACGGAGAGGTGATGGCTTTAGCCATTTTTAGAATTCCTCGTTATC 775
                      ********************************************.***************

cpi_promoter          TTATCCATGCCTCCCATGTGTCGAAATTCGCCCTTCTAAATATGGAAATTAGTGCTCACG 831
barque_promoter       TTATCCATGCCTCCCATGTGTCGAAATTCGCCCTTCTAAATATGGAAATTAGTGCTCACG 835
morex_promoter        TTATCCATGCCTCCCATGTGTCGAAATTCGCCTTTCTAAATATGGAAATTAGTGCTCACG 835
                      ********************************* ****************************

cpi_promoter          GCAATCCTTCCCTAAGAGGTTATTCGGGAAACCAGAAGAAGAAAACTTGCAATACAACTC 891
barque_promoter       GCAATCCTTCCCTAAGAGGTTATTCGGGAAACCAGAAGAAGAAAACTTGCAATACAACTC 895
morex_promoter        GCAATCCTTCCCTAAGAGGTTATTCGGAAAATCAGAAGAAGAAAACTTGCAATACAACTC 895
                      ****************************.*** ****************************

cpi_promoter          TAGTTGTACAACCGTGCAGTTTTGCATCGACTCTCTCTGCTACGACATACACGCCTCTCA 951
barque_promoter       TAGTTGTACAACCGTGCAGTTTTGCATCGACTCTCTCTGCTACGACATACACGCCTCTCA 955
morex_promoter        TAGTTGTACAACCGTACAGTTTTGCATCGACTCTCTCTGCTACGACATACACGCCTCTCG 955
                      ***************.*********************************************.

cpi_promoter          TGACAATTTCTACGATCAAATACCTCCCTAGCCCGTTTCATTTGTTAGGTCCATTTAAAT 1011
barque_promoter       TGACAATTTCTACGATCAAATACCTCCCTAGCCCGTTTCATTTGTTAGGTCCATTTAAAT 1015
morex_promoter        TGATAATTTCTACGATCAAATACCTCCCTAGCCCGTTTCATTTGTTAGGTCCATTTAAAT 1015
                      *** ************************************************************

cpi_promoter          AAAAAAATAGAACGTCGATAAATTCCCGACCTTCGGATTTTAAGCATGGCAAATTGAAAG 1071
barque_promoter       -AAAAAATAGAACGTCGATAAATTCCCGACCTTCGGATTTTAAGCATGGCAAATTGAAAG 1074
morex_promoter        -AAAAAATAGAACGTCGATAAATTCCCGACCTTCGGATTTTAAGCATGGCAAATTGAAAG 1074
                       ************************************************************

cpi_promoter          TCTTTTTTTACATCAACTTTAATTGACACGGGGTGAAAAATTTAGTTGTTAAATACGACA 1131
barque_promoter       TCTTTTTTTACATCAACTTTAATTGACACGAGGTGAAAAATTTAGTTGTTAAATACGACA 1134
morex_promoter        ACTTTTTTTACATCAACTTTAATTGACACGAGGTGAAAGATTTAGTTGTTAGATACGACA 1134
                      :*********************************.*******.************.********

cpi_promoter          ACCTTGCAATCTGGCGTCAACTAAAACTGCCATAAAAACGATCGGGTTACCTGCTTAGGG 1191
barque_promoter       ACCTTGCAATCTGGCGTCAACTAAAACTGCCATAAAAATGATCGGGTTACCTGCTTAGGG 1194
morex_promoter        ACCTTGCAATCTGGCGTCAACTAAAACTACCATAAAAACGATCGGGTTGCCTGCTTAGGG 1194
                      ********************************.********* *********.***********

cpi_promoter          CATGTACAATGGTTGATAAGATAGTTTTATCTTAAACCTTGCATGTAATTTAGAGATGAC 1251
barque_promoter       CATGTACAATGGTTGATAAGATAGTTTTATCTTAAATCTTGCATGTAATCTAGAGATGAT 1254
morex_promoter        CATGTACAATGGTTGATAAGATAGTTTTATCTTAAATCTTGCATGTAATTTAGAGATGAC 1254
                      ************************************ ************ **********

cpi_promoter          AAAAAAGAGTCTCTACAATGGGTTATCTTTTAGCCTTATCTTTTATAATTAGTTATTCCT 1311
barque_promoter       AAAAAAGAGTCTCTACAATGGGTTATCTTTTAGCCTTATCTTCTATAATTAGTTATTCCT 1314
morex_promoter        AAAAAAGAGTCTCCACAATGGGTTATCTTTTAGCCTTATCTTCTATAATTAGTTATTTCT 1314
                      ************* ***************************** ************** **

cpi_promoter          AAAAACATGTTGAGACATATTGTGCTAAGAGATCACCTCTCGTGTTCTCTTAAATAAGAG 1371
barque_promoter       AAAAACATGGTGAGACATATTTTGCTAAAAGATCACCTCTTGTGTTCTCTTAAATAAGAG 1374
morex_promoter        AAAAACACGGTGAGACATATTGTGCTAAGAGATCACCTCTTGTGTTCTCTTAAATAAGAG 1374
                      ******* * ************ *******.************ ******************
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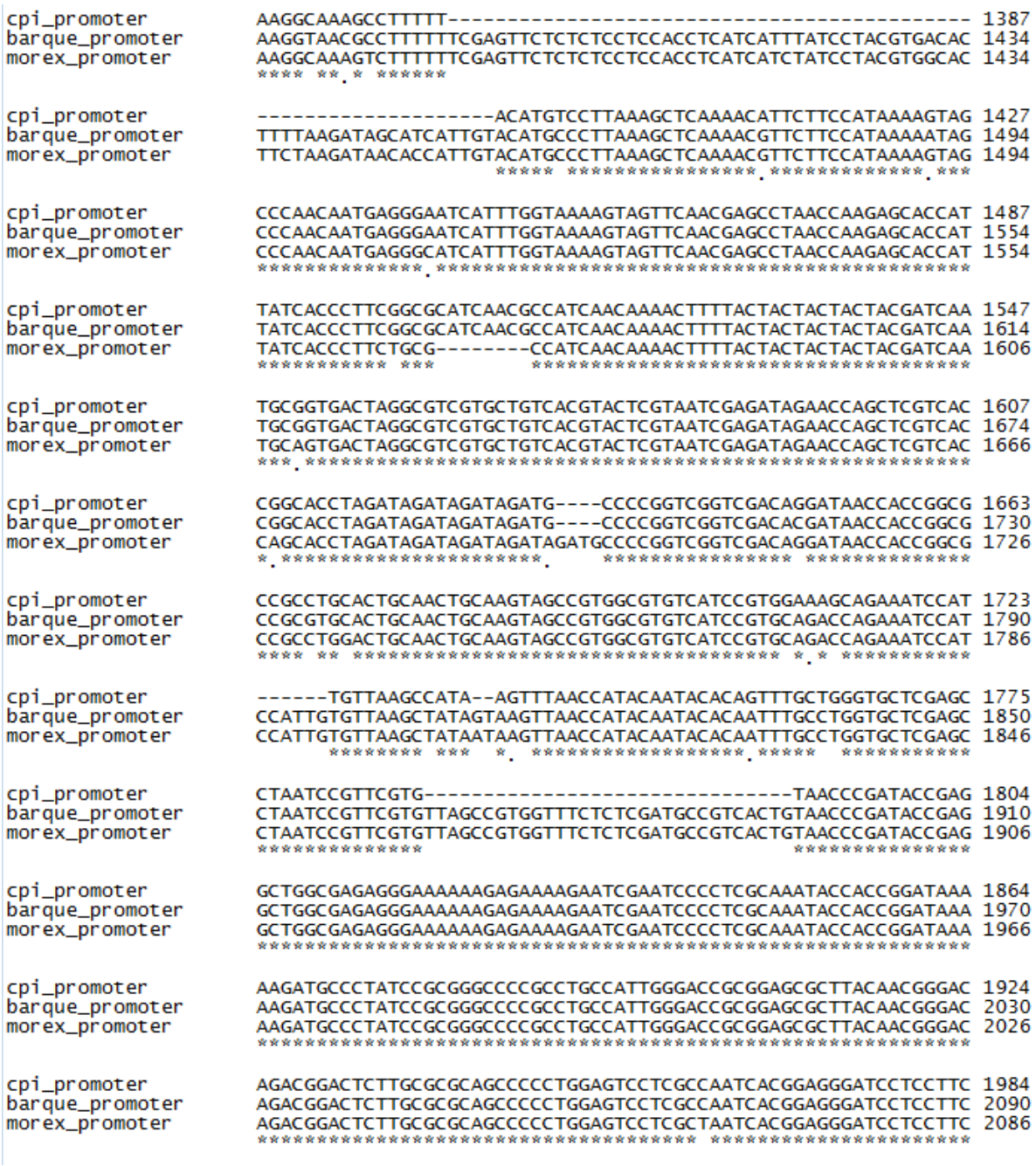
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cpi_promoter ----------ACATGTCCTTAAAGCTCAAAACATTCTTCCATAAAAGTAG 1427
barque_promoter TTTTAAGATAGCATCATTGTACATGCCCTTAAAGCTCAAAACGTTCTTCCATAAAAATAG 1494
morex_promoter TTCTAAGATAACACCATTGTACATGCCCTTAAAGCTCAAAACGTTCTTCCATAAAAGTAG 1494
cpi_promoter CCCAACAATGAGGGAATCATTTGGTAAAAGTAGTTCAACGAGCCTAACCAAGAGCACCAT 1487
barque_promoter CCCAACAATGAGGGAATCATTTGGTAAAAGTAGTTCAACGAGCCTAACCAAGAGCACCAT 1554
morex_promoter CCCAACAATGAGGGCATCATTTGGTAAAAGTAGTTCAACGAGCCTAACCAAGAGCACCAT 1554
cpi_promoter TATCACCCTTCGGCGCATCAACGCCATCAACAAAACTTTTACTACTACTACTACGATCAA 1547
barque_promoter TATCACCCTTCGGCGCATCAACGCCATCAACAAAACTTTTACTACTACTACTACGATCAA 1614
morex_promoter TATCACCCTTCTGCG--------CCATCAACAAAACTTTTACTACTACTACTACGATCAA 1606
cpi_promoter TGCGGTGACTAGGCGTCGTGCTGTCACGTACTCGTAATCGAGATAGAACCAGCTCGTCAC 1607
barque_promoter TGCGGTGACTAGGCGTCGTGCTGTCACGTACTCGTAATCGAGATAGAACCAGCTCGTCAC 1674
morex_promoter TGCAGTGACTAGGCGTCGTGCTGTCACGTACTCGTAATCGAGATAGAACCAGCTCGTCAC 1666
cpi_promoter CGGCACCTAGATAGATAGATAGATG----CCCCGGTCGGTCGACAGGATAACCACCGGCG 1663
barque_promoter CGGCACCTAGATAGATAGATAGATG----CCCCGGTCGGTCGACACGATAACCACCGGCG 1730
morex_promoter CAGCACCTAGATAGATAGATAGATAGATGCCCCGGTCGGTCGACAGGATAACCACCGGCG 1726
cpi_promoter CCGCCTGCACTGCAACTGCAAGTAGCCGTGGCGTGTCATCCGTGGAAAGCAGAAATCCAT 1723
barque_promoter CCGCGTGCACTGCAACTGCAAGTAGCCGTGGCGTGTCATCCGTGCAGACCAGAAATCCAT 1790
morex_promoter CCGCCTGGACTGCAACTGCAAGTAGCCGTGGCGTGTCATCCGTGCAGACCAGAAATCCAT 1786
cpi_promoter ------TGTTAAGCCATA--AGTTTAACCATACAATACACAGTTTGCTGGGTGCTCGAGC 1775
barque_promoter CCATTGTGTTAAGCTATAGTAAGTTAACCATACAATACACAATTTGCCTGGTGCTCGAGC 1850
morex_promoter CCATTGTGTTAAGCTATAATAAGTTAACCATACAATACACAATTTGCCTGGTGCTCGAGC 1846
cpi_promoter CTAATCCGTTCGTG------------------------------TAACCCGATACCGAG 1804
barque_promoter CTAATCCGTTCGTGTTAGCCGTGGTTTCTCTCGATGCCGTCACTGTAACCCGATACCGAG 1910
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cpi_promoter GCTGGCGAGAGGGAAAAAAGAGAAAAGAATCGAATCCCCTCGCAAATACCACCGGATAAA 1864
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cpi_promoter AAGATGCCCTATCCGCGGGCCCCGCCTGCCATTGGGACCGCGGAGCGCTTACAACGGGAC 1924
barque_promoter AAGATGCCCTATCCGCGGGCCCCGCCTGCCATTGGGACCGCGGAGCGCTTACAACGGGAC 2030
morex_promoter AAGATGCCCTATCCGCGGGCCCCGCCTGCCATTGGGACCGCGGAGCGCTTACAACGGGAC 2026
cpi_promoter AGACGGACTCTTGCGCGCAGCCCCCTGGAGTCCTCGCCAATCACGGAGGGATCCTCCTTC 1984
barque_promoter AGACGGACTCTTGCGCGCAGCCCCCTGGAGTCCTCGCCAATCACGGAGGGATCCTCCTTC 2090
morex_promoter AGACGGACTCTTGCGCGCAGCCCCCTGGAGTCCTCGCTAATCACGGAGGGATCCTCCTTC 2086

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cpi_promoter          TCTCTCCTCGCCACCGCGGACGGGCCGTCCGCGGCCGCTATATAAAGCGGGAGGCATCAC 2044
barque_promoter       TCTCTCCTCGCCACCGCGGACGGGCCGTCCGCGGCCGCTATATAAAGCGGGAGGCAGCAC 2150
morex_promoter        TCTCTCCTCGCCACCGCGGACGGGCCGTCCGCGGCCGCTATATAAAGCGGGAGGCATCAC 2146
                      ************************************************************ ***

cpi_promoter          GAGCTCCGGGACACAGAGAG--CGAGAGACCCGTCCGTCCCCCCTCATCCCACCCCGCCC 2102
barque_promoter       GAGCTCCGGGACACAGAGAGAGCGAGAGACCCGTCCGTCCCCCCTCATCCCACCCCGCCC 2210
morex_promoter        GAGCTCCGGGACACACAGAGAGCGAGAGACCCGTCCGTCCCC---CATCCCACCCCGCCC 2203
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cpi_promoter          GCCGCCGTCCGGTCCGTCGTTAGGGTTTCGTCGGCCTGCCAGCCAGACAGCC 2154
barque_promoter       GCCGCCGTCCGGTCCGTCGTTAGGGTTTCGTCGGCCTGCCAGCCTGACAGCC 2262
morex_promoter        GCCGCCGTCCGGTCCGTCGTTAGGGTTTCGTCGGCCTGCCAGCCAGACAGCC 2255
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