Regulation of candidate genes in black point formation in barley

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Declaration

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K. Ryan Walker

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Abstract

Black point of barley refers to discolouration of the embryo end of the grain. Downgrading of malting barley to feed grade due to black point results in significant economic loss to the Australian barley industry. Given that black point normally occurs in regions of Australia that experience high humidity during grain fill, humidity most probably contributes to the severity of black point in susceptible varieties. Previous studies have excluded fungal infection as a cause but enzymatic browning reaction has been recently hypothesised as responsible for black point. More specifically, a role for peroxidases has been proposed.

The first major focus of this study was to confirm under what environmental conditions black point formation was likely to occur and whether there was genetic variation contributing to the phenotype. The occurrence of high humidity and low temperatures was associated with the formation of black point in susceptible varieties, with early maturing varieties being more susceptible to black point. These environmental conditions probably create a moist environment during grain development in which the developing grain cannot dry out, enabling stress or wounding to the embryo that subsequently results in black point formation. Analysis combining two South Australian sites (Hatherleigh and Port Wakefield, SA) identified QTL for black point formation on chromosomes 2H (*QBpt.AlSl-2H*) and 3H *QBpt.AlSl-3H*) at positions 83.4 cM and 102.6 cM respectively. Additive by environment effects were substantial at both QTL. Linkage of the QTL on chromosome 2H with the *earliness per se* (*eps2*) locus and the observation that early maturing varieties were usually more susceptible to black point established a probable association between earliness and black point susceptibility. When an early maturing (susceptible) variety was

planted later so that it matured at the same time as a later maturing (tolerant) variety there was no significant difference in black point scores.

The second focus of this study was to characterise a number of candidate genes more than likely linked to black point by investigating expression levels during grain fill and subsequently mapping the genomic regions responsible for those changes in expression. Candidate genes chosen were *Quinone Reductase* (*HvQR*), *Phenylalanine Ammonia Lyase* (*HvPAL*), *Barley Peroxidase 1* (*HvBP1*), *stress-related Peroxidase* (*HvPrx7*) and *Lipoxygenase A* (*HvLoxA*). Differential expression as detected using northern analysis, between susceptible and tolerant varieties, was only observed for *HvBP1, HvPrx7* and *HvQR*. Quantitative PCR (qPCR) confirmed that *HvBP1* and *HvPrx7* expression was up to two times higher in black point susceptible varieties during all stages of grain development, while *HvQR* expression was significantly higher in the hard dough and mature stages of grain fill in susceptible varieties. Increased expression for *HvBP1* and *HvPrx7* (approximately two-fold) was also apparent in the tolerant variety Alexis between symptomatic and asymptomatic grains. The qPCR data was then used as a quantitative trait, to score the expression of these candidate genes in an Alexis/Sloop double haploid (DH) mapping population. Areas of the genome potentially involved in the regulation of these candidates (expression QTL or eQTL) were mapped on chromosomes 2H (for *HvPrx7 and HvBP1)* and 5H (for *HvQR and HvBP1)*. The eQTL for *HvPrx7* and *HvQR* were located in the same regions as the corresponding genes, suggesting their expression is regulated via *cis*-acting factors. In contrast, while *HvBP1* is located on 3H, eQTL were located on 2H and 5H suggesting *trans*-acting factors were involved. The use of comparative mapping studies between barley and rice identified a number of transcription factor genes within these eQTL.

The final component of this study was to investigate how *HvBP1* and *HvPrx7* expression might be affected by examining their promoters and potential interactors with those promoters. Promoter regions for the susceptible variety Sloop and tolerant variety Alexis were isolated, compared and analysed for known motifs. Particular emphasis was placed on those elements that were associated with embryo and endosperm specific expression or responses to environmental stresses. Several regions containing single nucleotide polymorphisms (SNPs) between the promoters from the tolerant and susceptible varieties were identified. A 160 bp region for *HvBP1* and 380 bp region for *HvPrx7* were used in Yeast One Hybrid (Y1H) screening to identify potential regulatory proteins. In particular, a potential bZIP-containing factor which interacted with the promoter of *HvPrx7* was further characterised. Interaction was confirmed by a gel shift assay and gene expression by northern analysis showed expression at the milk, soft dough and hard dough stages of grain development. Increased expression was apparent in the susceptible variety Sloop.

The eQTL, Y1H and environmental studies have furthered our understanding of genes that could be involved in the regulation of black point formation under conditions of low temperature and high humidity. This information will contribute to assessing the roles these genes play in black point formation under certain environmental conditions, and more broadly, will assist in improving breeding for resistant barley varieties.

Abbreviations

%	Percent
°C	Degrees Celsius
µL	Microlitre
3-AT	3-amino-1,2,4-triazole
A	Alexis
ABA	Abscisic acid
AFLP	Amplified Fragment Length Polymorphism
ANOVA	one-way analysis of variance
AP2	Homeodomain, Apetala 2
AP2/ERF	Apetala 2/ethylene responsive factor
BAC	Bacterial artificial chromosomes
bHLH	Basic helix-loop-helix
BOM	Bureau of Meteorology
bp	Base pairs
BP1	Barley Peroxidase 1
BSA	Bovine Serum Albumin
bZIP	Basic-leucine zipper
cM	Centimorgans
CT	Cycle threshold
denso	Plant stature locus
DH	Doubled haploid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E2F	Elongation factor 2
ea	Actual vapour pressure
EDTA	Ethylenediamine tetra acetic acid
EFA	Elongation factor A
eps2	Earliness per se locus
eQTL	Expression QTL
es	Saturation vapour pressure
EST	Expressed sequence tag
F	Forward
g	Gravitational force
GA	Gibberellic acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBSSI	Granule Bound Starch Synthase I
h	Hour
H	Hatherleigh
H_2O_2	Hydrogen peroxide
HD-ZIP	Homeodomain leucine zipper
HPLC	High Pressure Liquid Chromatography
HSP	Heat shock protein
Hv	*Hordeum Vulgare*
IPTG	Isopropyl-beta-D-thiogalactopyranoside
KDa	Kilodaltons
L	Ladder
LB	Luria Bertani
LEA	Late embryogenesis abundant

LOX	Lipoxygenase
LRR	Leucine rich repeats
LRS	Likelihood ratio statistic
LSD	Least significant difference
m	Metre
M	Molar
mg	Milligrams
Min	Minute
mL	Millilitre
mm	Millimetre
mM	Millimolar
MW	Molecular weight
MYB	Myeloblastosis
MYC	Myelocytomatosis
nm	Nanomolar
O/L	Overlap
O_2^-/ HO_2^-	Superoxide/Perhydroxyl radical
OH	Hydroxyl radical
ORF	Open reading frame
PAC	P1-derived artificial chromosome
PAL	Phenylalanine ammonia-lyase
PCR	Polymerase chain reaction
pI	Isoelectric point
POX	Peroxidase
Ppd-H1	Photoperiod response gene
PPO	Polyphenol oxidase
pQTL	Protein QTL
Prx7	Peroxidase 7
PVVP	Polyvinylpyrrolidone
PW	Port Wakefield
qPCR	Quantitative real time polymerase chain reaction
QR	Quinone Reductase
QTL	Quantitative trait loci
R	Reverse
RFLP	Restriction fragment length polymorphism
RH	Relative humidity
RIL	Recombinant inbred line
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
S	Sloop
SD	Synthetic Defined Medium
SDS	Sodiumdodecylsulfate
sdw1	Denso locus
sec	Seconds
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
T	Temperature
TAE	Tris-acetate-EDTA
Tm	Temperature
Tris-Cl	Trizma hydrochloride
V	Volts

VPD	Vapour pressure deficit
X-∞-GAL	bromo-chloro-indolyl-galactopyranoside
X-gal	Bromo-4-chloro-3-indolyl β-D-galactopyranoside
Y1H	Yeast one-hybrid
YPD	Yeast Peptone Dextrose
μm	Micrometre

Chapter One. Literature Review

1.1 An introduction to black point

Black point of barley (*Hordeum vulgare* L.) is characterised by brown-black discolouration at the embryo end of the grain. In barley, black point is confined to the lemma and palea (or husk), which remains adhered tightly to the caryopsis (outer seed coat) (Figure 1.1).

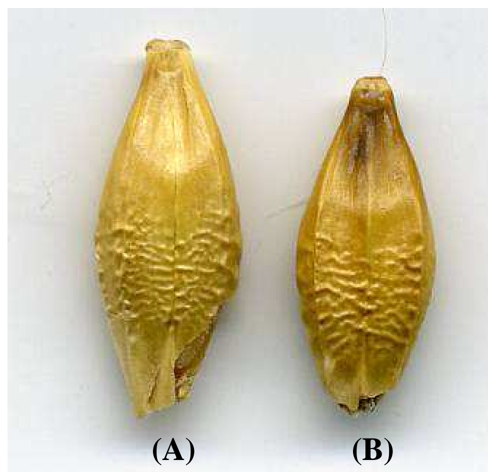

Figure 1.1 Black point symptoms. A healthy barley grain (A) compared with a barley grain showing black pointed symptoms (B).

1.2 Significance of black point

Black point is a serious but intermittent problem in Australia, occurring most severely in Queensland but also occurring in Western Australia, South Australia, Victoria and northern New South Wales. Barley is the second most widely grown crop in Australia, with only wheat occupying a greater area. Australia produced 7.804 million tonnes of barley over an area of 4.523 million hectares on average over the last five years (The Australian Bureau of Agricultural and Resource Economics and Sciences ABARE 2011). Barley production is important to the Australian economy with the annual gross value of Australian barley

estimated to be worth $1.974 billion in the 2010/11 season [Grains Research and Development Corporation (GRDC 2010)].

Barley production can be divided into two main categories: Feed grain, which is a preferred grain for many feed lots and stockfeed manufacturers and malt barley, which is used in the production of beer and food products. While Australian barley production only occupies 3% of the world barley production, the Australian malting barley trade accounts for 30% of the world malting barley trade with the major competitors being Canada and the European Union (Department of Primary Industries and Fisheries 2005). Approximately 40% of Australia's barley production is of malting quality. Black point is a problem facing Australian barley producers, causing a downgrading of malting quality to feed grade at recieval. Downgrading of malting quality barley due to black point has been estimated to reach economic losses of 10 million dollars per year (Peter Sidley, *personal communication*, Australian Barley Board Grain Ltd). With such a large proportion of Australia's barley production aiming for malting quality, investigating the genetic basis of black point is important. The identification of candidate genes and their incorporation into breeding programs, will allow these genes to be targeted and resistance to black point achieved. This would ensure maximum export of Australia's malting barley and minimum economic losses due to black point. Therefore, an understanding of the mechanism of black point formation and how that might be manipulated is also important.

1.3 Proposed causes of black point

The literature on black point is unclear and often contradictory, with suggested causes of black point formation including fungal infection (Waldron 1934; Machacek and Greaney 1938; Southwell *et al.* 1980; Rees *et al.* 1984), environmental conditions (Waldron 1934;

Rees *et al.* 1984; Conner *et al.* 1992; Fernandez *et al.* 1994) and potential biochemical changes (Whitaker and Chang 1996; Walker and Ferrar 1998). The discoloration associated with black point occurs in both wheat and barley. The following section deals with each of the proposed causes and the validity of evidence that has been published to date.

1.3.1 Fungal infection

Research on black point initially focused on the assumption that the discolouration associated with black point was the result of a saprophytic infection (Waldron 1934; Machacek and Greaney 1938; Southwell *et al.* 1980) by fungi including *Alternaria infectoria* (Perelló *et al.* 2008), *Bipolaris sorokiniana* (Kumar *et al.* 2002), *Fusarium proliferatum* (Conner *et al.* 1992; Desjardins *et al.* 2007) and most often *Alternaria alternata* (Southwell *et al.* 1980; Rees *et al.* 1984; Conner and Davidson 1988; Conner and Kuzyk 1988; Cromey and Mulholland 1988; Ellis *et al.* 1996). Black point symptoms were often described in relation to the mycelial density in the tissues affected (Rees *et al.* 1984). However, many other fungi have also been associated with grain discolouration, including *Bipolaris, Epicoccum, Fusarium, Cladosporium, Stemphylium* and *Chaetomium* spp.(Machacek and Greaney 1938; Rees *et al.* 1984; Conner and Kuzyk 1988). Intriguingly, Hyde and Galleymore (1951) found that the tip of the wheat grain had far more fungal mycelium than the base (embryo end) where black point is observed. In contrast, Bhowmink (1969) and Cromey and Mulholland (1988) reported that the symptoms of black point in wheat were due to a dense mycelial mat at the embryo end of the grain. In many cases the fungus deemed responsible was also observed in healthy grain or inoculation of the grain did not consistently induce symptoms (Conner and Kuzyk 1988;

Maloy and Specht 1988; Conner *et al.* 1996; Ellis *et al.* 1996; Williamson 1997a; Williamson 1997b; Desjardins *et al.* 2007) suggesting fungi is not responsible for black point formation.

Although early reports suggested that a fungus may have been involved in the discolouration process, no evidence of any direct association between black point and fungal infection has been provided (Jacobs and Rabie 1987; Basson *et al.* 1990; Ellis *et al.* 1996). Direct association between the presence of fungi and black point formation has also been discounted by Williamson (1997a) after observing a similar infection process for *A. alternata* in both susceptible and tolerant varieties. These results have since been replicated by Hadaway (2002) and Hudec (2007) who found *Alternaria* spp. in both healthy and black pointed grain.

1.3.2 Environmental conditions

Adverse environmental conditions appear to be associated with black point symptoms (Waldron 1934; Rees *et al.* 1984; Conner *et al.* 1992; Fernandez *et al.* 1994). Waldron (1934) observed that high temperatures and low moisture conditions were associated with severe symptoms, whereas Rees (1984) reported that moist conditions during grain filling and ripening increased the incidence of symptoms. However, the higher average minimum temperature, higher rainfall and slightly higher relative humidity at a coastal site in Bundaberg, Queensland were shown to increase black point symptoms (Tah *et al.* 2010). Prolonged ripening due to cold and frosts also appears to increase the likelihood of black point formation (Fernandez *et al.* 1994). Specifically increased temperature and moisture

between grain development stages of milk and dough appear to be associated with an increase in the incidence of black point in wheat (Moschini *et al.* 2006).

The influence of irrigation and precipitation on the incidence of black point in spring wheat has been investigated, with the authors concluding that the incidence of black point was strongly influenced by the amount of overhead irrigation applied during the milk and mealy-dough stages (Conner 1987). Symptom severity has been reported to be largely dependent on seasonal conditions and is most serious under irrigation (Madariaga and Mellado 1988; Maloy and Specht 1988), also when frequent rainfalls and heavy dews occur during kernel development (Southwell *et al.* 1980). Rainfall and the timing of the rainfall may therefore be an influential factor in black point formation. Interestingly intermittent precipitation during grain development increased symptoms in comparison to once off heavy rainfall events (Petr and Capouchova 2001).

Black point appears to be a consistent problem when barley is grown outside of its natural Mediterranean environment, where the grain usually ripens and dries rapidly in an almost moisture-free atmosphere. Under conditions of high humidity where ripening and drying is prolonged, black point occurs at higher levels with distinct differences between susceptible and resistant genotypes (Sulman *et al.* 2001a). Given this evidence and the observation that black point tends to occur more readily in regions where the environment is humid at grain fill (such as Queensland and northern New South Wales), humid conditions seem to play an important role in the formation of black point. Humid conditions during grain fill may trigger biochemical changes in the cell that subsequently induce black point formation. Although research indicates that black point may be linked to a combination of temperature, humidity and rainfall, further research is required to identify exactly what

environmental conditions are contributing to black point and if the timing of these conditions contributes to severity.

1.3.3 Biochemical changes (enzymatic browning)

The induction of biochemical changes within the grain is likely to result in the formation of black point symptoms, which can be linked to enzymatic browning. Enzymatic browning is a characteristic reaction of plant tissues subjected to stressful conditions or wounding, which involves the oxidation of phenolic compounds by polyphenol oxidase (PPO) and peroxidases (POX) and the transformation of the oxidation products to brown or black pigments, such as melanins (Whitaker and Chang 1996; Walker and Ferrar 1998) and quinines (Tomás-Barberán and Espín 2001).

Williamson (1997a) discovered a relationship between black point susceptibility in wheat and the presence of peroxidase isozymes. Peroxidases and the phenols considered necessary for the development of black point symptoms are also components of the barley grain (Cochrane 1994b). Endogenous hydrogen peroxide (H_2O_2) which is essential for peroxidation, has also been shown to be produced by barley germ aleurone cells. The quinones formed as a result of phenolase activity are highly reactive and give rise to insoluble polymers by self-polymerisation or by condensation reactions with compounds such as proteins and amino acids (Barz and Koster 1981), resulting in the discolouration associated with black point. Although these enzymes and substrates are believed to be involved in black point, the mechanism by which they may combine to create symptoms is not clear.

Stress conditions or disruptions, such as barley pre-germination, might bring the germ aleurone peroxidases to react with phenols under certain environmental conditions during grain filling and ripening (Cochrane 1994a). Any disruption of the immature caryopsis under certain environmental conditions may also bring these enzymes and substrates together, giving rise to extensive melanisation (Cochrane 1994b). Williamson (1997a) has also concluded that the symptoms shown in the formation of black point in wheat is likely to be an oxidized phenol resulting from the biochemical disruption of the ripening process brought about by stressful conditions. This would support previous discussions that high humidity at grain fill is linked to black point formation, bringing together the substrates discussed in the oxidation of phenols to quinones and hence black point formation. Such an interaction of substrates may well be occurring during black point formation in barley grain from susceptible varieties, thus suggesting a genotype x environment effect.

1.3.3.1 Enzymes and substrates involved in biochemical changes

The following section discusses the role of substrates involved in the oxidation of phenols by peroxidase and polyphenol oxidase in the formation of black point and redox status during abiotic stress. Figure 1.2 outlines the proposed model for enzymatic browning and the subsequent formation of black point.

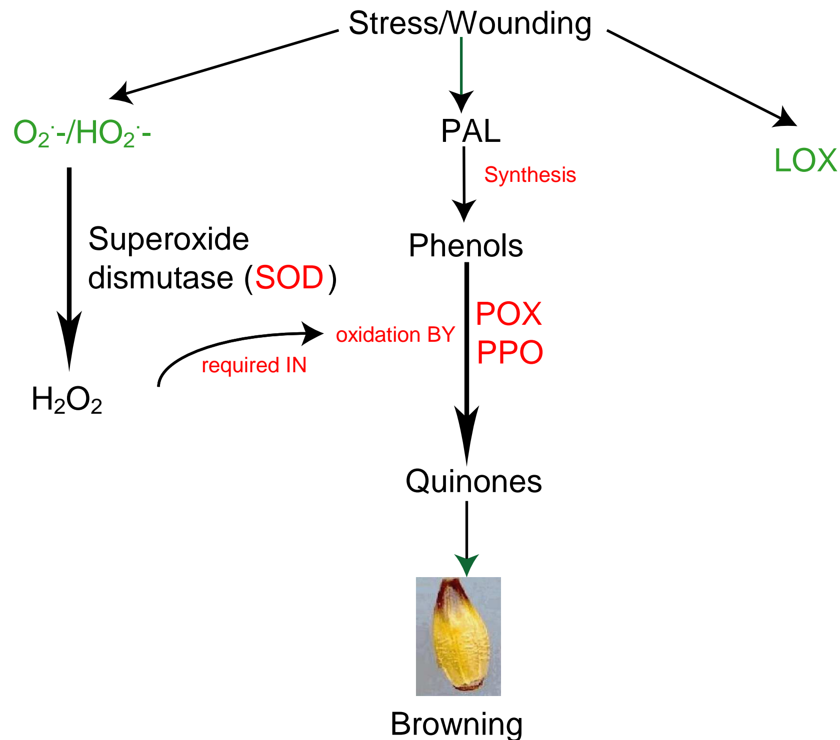

Figure 1.2 A model illustrating the characteristic reaction of plant tissues subjected to stressful conditions or wounding. This typically involves the oxidation of phenolic compounds by polyphenol oxidase (PPO) and peroxidases (POX) and the transformation of the oxidation products to brown or black pigments (Quinones). Production of lipoxygenase (LOX) and superoxide (O_2^{-}/ $HO_2^{\cdot -}$) are also characteristic of plants subjected to stress or wounding. O_2^{-}/ $HO_2^{\cdot -}$ is dismutated in the cell to hydrogen peroxide (H_2O_2) by Superoxide Dismutase (SOD). Hydrogen peroxide is used as a substrate in the oxidation of phenols by PPO and POX (Droillard *et al.* 1987).

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1.3.3.1.1 Reactive oxygen species and their removal

Reactive oxygen species (ROS) are products of many biological processes occurring in different sub-cellular locations, especially in the oxygen-evolving functions of plant chloroplasts and the mitochondrial electron transport system (Bowler *et al.* 1994). ROS include superoxide and its protonated form perhydroxyl radical (O_2^{-}/ $HO_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$). Plants have well developed defence systems

against ROS, involving both limiting the formation of ROS as well as instituting its removal.

The abundant O_2^-/HO_2^- is formed by univalent electron transfer to O_2 and can contribute to the synthesis of the particularly damaging $OH^·$, so that control of this ROS is essential (Halliwell and Gutteridge 1989). $OH^·$ can cause DNA mutation, protein denaturation and lipid peroxidation (Liu *et al.* 1999). The dismutation of O_2^-/HO_2^- results in the formation of H_2O_2. Peroxidases and catalase are oxygen scavengers (Droillard *et al.* 1987) and catalyse the reaction that degrades H_2O_2 to water. The fact that H_2O_2 is essential in the oxidation of phenolic compounds by peroxidase, suggests that H_2O_2 could be a rate-limiting factor in the formation of black point.

Within a cell, superoxide dismutase (SOD) constitutes the first line of defence against ROS. SOD catalyses the dismutation of O_2^-/HO_2^- to H_2O_2. Without catalysis by SOD this reaction is relatively slow, but with catalysis by SOD it proceeds at an extremely rapid rate (Bielski *et al.* 1985). The role of SOD is to remove O_2^-/HO_2^- before it reacts with H_2O_2 to form the reactive species OH-·.

Experiments conducted by (Hadaway 2002) found the majority of barley varieties analysed showed an increase in SOD activity in black pointed barley grains compared to healthy barley grains. Increased levels of SOD could indicate an increase in the level of H_2O_2 through the dismutation of O_2^-/HO_2^-, required in the oxidation of phenols to quinones and hence black point formation. However no one has reported measuring ROS in black pointed grain.

1.3.3.1.2 Lipoxygenase (LOX)

Lipoxygenase is an iron-containing protein which catalyses a direct reaction of polyunsaturated fatty acids with oxygen to give 13- and 9- hydroperoxides. LOXs are normally present in the seeds of plants where they are involved in mobilisation of storage lipids during germination (Feussner *et al.* 2001). LOXs have been shown to play important roles in seed germination and seedling growth and development (Terp *et al.* 2006), defence against wounding or pathogens and during senescence (Siedow 1991). LOX gene expression is regulated by different forms of stress, such as wounding, water deficiency or pathogen attack (Porta and Rocha-Sosa 2002). A typical response of a stressed plant is the production of H_2O_2, which in turn results in an increase of LOX activity (Porta and Rocha-Sosa 2002).

In the case of black point, stress or wounding of cells may occur due to the high humidity at grain fill resulting in an oxidation reaction of phenols to quinones which requires H_2O_2. The plant stress response of an increase in H_2O_2 has been shown to be associated with an increase in LOX activity, suggesting LOX could be associated with black point formation through the plant's response to stress/wounding.

1.3.3.1.3 Phenols

Phenylalanine ammonialyase (PAL) is a wound-induced enzyme that initiates an increase in the concentration of phenolic compounds, ferulic acid and p-coumaric acid from phenylalanine (Michalowitz *et al.* 2001). In rice PAL has been shown to be expressed in response to different stress stimuli (Sarma and Sharma 1999). PAL gene expression in

wheat has been shown to be up-regulated in response to cold stress (Gaudet *et al.* 2003), however whether the stress of humidity previously associated with black point formation (Tah *et al.* 2010) affects the regulation of PAL remains to be determined.

Phenols are natural components of healthy grain (Cochrane 1994b) and the release of phenols from damaged plant tissue is well documented and is a likely outcome when pericarp cells are crushed during grain filling and ripening. A study by Michalowitz (2001) found there was up to a 60-fold increase in ferulic and p-coumaric acid in the husks of black point-affected barley grain compared to healthy grain. These results suggest that PAL activity increases with black point formation and could also be rate limiting. In contrast, the tissue covering the embryo in black pointed wheat had reduced levels of ferulic and p-coumaric acid (Michalowitz *et al.* 2001). This indicates that other phenols may be involved in black point formation in wheat.

1.3.3.1.4 Peroxidases

Peroxidase is a heme-containing enzyme usually associated with wound-healing processes such as lignification. Peroxidase performs single-electron oxidation of phenolic compounds in the presence of H_2O_2 (Dunford 1991). Germ aleurone peroxidases appear to be involved in the germination process of barley and they react with phenols during germination (Cochrane 1994a; Cochrane 1994b). Sulman (2001b) have suggested that the level of peroxidase in mature barley kernels of all varieties analysed was sufficient to cause black point and differences in substrate or H_2O_2 may be the factor that distinguishes between resistance and susceptibility, with H_2O_2 required in the oxidation of phenols to quinones and black point formation.

Hadaway (2003) found that the activity of peroxidase enzymes increased during grain development. Additionally, peroxidases with a higher isoelectric point have only been found in susceptible varieties to date (Hadaway *et al.* 2003). Mak (2006) investigated differentially expressed proteins in black point affected and black point free grains. Enzymes involved in phenolic compound metabolism and peroxidases were found to be differentially expressed between germ and endosperm bran fractions, with the percentage of 'stress' proteins greatest in the black pointed samples (Mak *et al.* 2006). Similarly using a proteomics approach March (2007) identified *HvBP1* as present in black pointed grain and not healthy grain of the susceptible variety, Sloop. Peroxidases are therefore likely to be involved in black point formation in barley and further understanding of the environmental factors triggering black point formation, peroxidase gene expression and regulation will contribute to our understanding of black point.

1.3.3.1.5 Polyphenol oxidases

Polyphenol oxidase (PPO), a copper containing metalloprotein, catalyses the oxidation of phenolics to quinones which make brown pigments in wounded tissue (Kim *et al.* 2001). Browning in fruit and vegetables, such as lettuce and potato, is initiated by the enzymatic oxidation of phenolic compounds by PPOs (Martinez and Whitaker 1995). Monophenol mono-oxygenase (tyrosinase), diphenol oxidase (catechol oxidase), and laccase which are common PPOs oxidise mono-phenols (o-diphenols and p-diphenols) using molecular oxygen. The oxidation of these phenols results in the formation of the highly reactive quinones, and possibly black point formation. PPO was not examined in this study as it

was examined by Hadaway and Able (*unpublished data*) and no differences were identified in PPO suggesting there may be a different mechanism involved.

1.4 Candidate genes in black point formation

Applying what knowledge there is about the biochemical events that may occur during black point formation (section 1.2.3), we can speculate that the candidate genes involved in these processes may include SOD, POX, PAL and LOX.

1.4.1 POX genes

The literature has clearly indicated that the formation of black point is associated with the oxidation of phenols by peroxidases (Williamson 1997a; Williamson 1997b; Hadaway 2002), most likely those with a basic isoelectric point (Hadaway 2002; Hadaway *et al.* 2003).

There are a number of peroxidase genes that have been cloned and sequenced that are found in grain and germinating tissue. These include *BP1* (Rasmussen *et al.* 1991), *BP2*, *BP2A* (Theilade and Rasmussen 1992), *Prx7* (Kristensen *et al.* 1999), and *Prx8* (Thordal-Christensen *et al.* 1992). *BP1* has been characterised and found to be highly tissue-specific, occurring maximally in the endosperm 15 days after flowering (Rasmussen *et al.* 1991). *BP1* was identified as being differentially expressed between barley varieties differing in black point susceptibility (March 2003). Expression of *BP1* was observed for one developmental stage longer in susceptible varieties, remaining expressed until soft dough in susceptible varieties and only until late milk in tolerant varieties. BP1 was also

identified as more abundant in black pointed grains (March *et al.* 2007) than healthy grains further supporting its potential as a candidate gene in black point formation.

Prx7 has also been identified as being differentially expressed between barley varieties (March 2003). *Prx7* was expressed at a consistently high level in black point susceptible varieties towards the end of grain development. *Prx7* and *Prx8* have also been shown to be upregulated in emerging coleoptile tissue when inoculated with powdery mildew fungus or by wounding of epidermal cells (Kristensen *et al.* 1999). *Prx7* is localised in the vacuoles, while *Prx8* is localised in the cell walls of mesophyll cells, presumably to crosslink phenolic compounds to inhibit fungal penetration of the cell wall (Kristensen *et al.* 1997). The high expression of peroxidases towards the end of grain development is consistent with a role in the oxidation of phenols and hence black point formation.

Plant development and environmental changes, including biotic stress, are often followed by dramatic changes in peroxidase activity and in the number of isoenzymes present in specific tissues (Kristensen *et al.* 1999). This could indicate that the differential expression of peroxidase genes observed is due to environmental factors, such as humidity, that are known to be associated with black point.

1.4.2 LOX genes

The occurrence of LOX enzymes in cereal grain has been well documented, with barley containing two distinct isozymes, LOX 1 and LOX 2 (Doderer *et al.* 1992). LOX 2 is present in the early stages of grain development, whereas LOX 1 accumulates during the later stages of grain development (Schmitt and Van Mechelen 1997).

LOX gene expression is regulated by different forms of stress, such as wounding, water deficiency or pathogen attack (Porta and Rocha-Sosa 2002). The literature has indicated that black point is likely to be associated with a form of stress or wounding, indicating that LOX could be involved in black point formation. With LOX 1 accumulating later in grain development, when black point forms, there could be a correlation between black point formation and LOX-1 accumulation.

Porta and Rocha-Sosa (2002) have reported an increase in LOX activity in association with the production of hydrogen peroxide in response to stress or wounding. With hydrogen peroxide required in the oxidation of phenols to quinones and hence black point formation, this could indicate that LOX is involved in the formation of black point through the oxidation reaction.

1.4.3 SOD genes

SOD catalyses the dismutation reaction that results in the formation of H_2O_2 from O_2^- /$HO_2^.$ Barley germ aleurone cells are able to produce endogenous H_2O_2 (Cochrane 1994a) required for peroxidation to take place. Four classes of SOD have been identified, containing either a dinuclear Cu/Zn or mononuclear Fe, Mn or Ni cofactor (Whitaker and Chang 1996). Typically, MnSOD is mitochondrial, FeSOD is plastidic, mitochondrial, or peroxisomal; and CuZnSOD can be plastidic, cytosolic or peroxisomal (Bowler *et al.* 1994).

Hadaway (2002) observed that an increase in SOD activity within the barley grain may be associated with black point formation. Initial findings by March (2003) were inconclusive with respect to whether SOD gene expression is associated with the development of black

point. In susceptible varieties, there were, higher levels of expression of FeSOD transcripts during later stages of grain development but MnSOD was expressed at high levels in all varieties during grain development, and CnZnSOD expression was down regulated towards the end of grain development.

1.5 Identification of candidate genes for black point tolerance

The candidate genes discussed above may be involved in black point formation based on the assumption that the gene expression differs between susceptible and tolerant varieties (especially during grain fill). These candidate genes are also regulated by environmental factors often associated with black point formation. The following section deals with identifying regions of the genome that control or contribute to black point formation.

1.5.1 Mapping studies and proposed QTL for black point

Quantitative trait loci (QTL) have been identified for black point in barley using a doubled haploid (DH) mapping population. Severity of black point can be measured by visual assessment of a sample of grains and scored as a trait as described by Hadaway (2002). A preliminary study using the DH populations of Arapiles x Franklin and Sloop x Alexis identified QTL associated with black point tolerance on chromosome 2H (Hadaway 2002).

Black point has also been investigated in wheat using DH mapping populations derived from Sunco x Tasman and Cascades x AUS1408, resulting in the detection of QTL on chromosomes 2B and 2D respectively (Williamson 2002). This group of chromosomes is largely homologous with chromosome 2H in barley (Devos *et al.* 1993). More recently QTL for black point tolerance have been further mapped in the Sunco x Tasman and

Cascades x AUS1408 populations, identifying QTL on chromosomes 1D, 2B, 3D, 4A, 5A, 7A and 2A, 2D, 7A respectively (Lehmensiek *et al.* 2004).

Similarly in barley the genetic regions associated with black point tolerance in the F2 population, Valier/Binalong, was investigated. QTL contributed by the tolerant variety Valier, were detected on 2HS, 2HC, 3HL, 4HL and QTL contributed by the susceptible variety Binalong were detected on 5HL (Tah *et al.* 2010). QTL in seven barley populations controlling kernel discolouration in barley has also been investigated using brightness, redness and yellowness to identify QTL on 2H, 3H, 4H, 5H and 7H (Li *et al.* 2003).

Comparative-mapping techniques have been employed with barley (chromosome 2H) and rice (chromosome 7), to identify candidate genes in the chromosome region underlying the black point QTL on 2H in barley (March *et al.* 2008). Bacterial artificial chromosomes (BACs) and phage artificial chromosomes (PACs) of rice sequence information were aligned to give a consensus sequence that was searched against barley expressed sequence tags (ESTs) to specify candidate genes. A number of candidate genes thought to be associated with black point were identified, including genes encoding POX, LOX, PAL and a quinone reductase (QR) (March *et al.* 2008), confirming potential involvement of our candidate genes in black point formation.

1.5.2 Other candidate gene identification techniques

Genetic data sets and associated mapping populations provide a powerful resource for the cloning and analysis of genes controlling grain development and the properties of mature grain (Milligan *et al.* 2005). A number of techniques have been employed to identify

candidate genes involved in a given pathway or trait. Genetic loci have been discussed (section 1.5.1) where the quantitative trait (black point) has been mapped to associated markers and QTL identified but only March *et al* has identified candidate genes using a comparative mapping technique (March *et al.* 2008). However, the genes can only be associated with the proposed model for black point formation (Figure 1.2) and a role for them in black point has not been proven.

Bioinformatics-based approaches such as that used by March (2008) are frequently used for subdividing genes within QTL intervals into alternate groups of highly probable candidates. This has been successfully done in Poplus plants studying cell wall traits resulting in a manageable set of genes with known and putative cell wall biosynthesis function (Ranjan *et al.* 2010). *Arabidopsis thaliana*, like barley, as a model organism for seed plants, is a suitable target for QTL studies due to the availability of highly developed molecular and genetic tools, and the extensive knowledge accumulated on the metabolite profile (Brotman *et al.* 2011). Similar to mapping QTL, levels of transcript and protein abundance have been mapped to identify genomic loci controlling the observed variation in mRNA and protein levels, generating expression QTL (eQTL) and protein QTL (pQTL) (Schadt *et al.* 2003; Keurentjes *et al.* 2007; Wentzell *et al.* 2007; Fu *et al.* 2009). The eQTL approach in barley has yielded information that led to the identification of strong candidate genes underlying phenotypic QTL for resistance to leaf rust in barley and on the general pathogen response pathway hence facilitating a systems appraisal of this host-pathogen interaction (Chen *et al.* 2010). Similarly Potokina (2008) successfully undertook genome-wide analyses of transcript abundance by eQTL mapping in barley. Generally eQTL studies in the literature have used microarray techniques. Microarrays and macroarrays offer a technique for screening the expression profile of very large numbers of

genes simultaneously with both types of arrays used to study grain development in cereals (Milligan *et al.* 2005).

Proteomics has also been used to identify candidate genes for a number of plant processes. Using proteomics, barley peroxidase 1 (BP1) was found to be more abundant in black pointed grain (March *et al.* 2007) than healthy grain, supporting a potential role for peroxidases in black point formation. Similary peroxidases were found to be differentially expressed between germ and endosperm bran fractions, with the percentage of 'stress' proteins greatest in the black pointed samples (Mak *et al.* 2006).

Candidate genes for black point formation may not only contribute directly but also include candidates that prevent germination and wounding. Black pointed grain has been shown to have started germination and to have increased alpha-amylase levels (Hadaway and Able, *unpublished data*). Further evidence for the link with black point and the germination pathway was presented by March (2007), identifying an late embryogenesis abundant (LEA) protein in healthy grain but not black pointed grain, suggesting that grains have entered the germination process where LEA is usually degraded.

A clear genotype x environment interaction also occurs with humid conditions at grain fill being associated with the formation of black point (Sulman *et al.* 2001a; Moschini *et al.* 2006; Tah *et al.* 2010). The question therefore arises as to whether the regulation of gene and protein expression is affected by the environmental conditions proposed to favour black point formation.

1.6 Identifying regulatory factors contributing to black point

Physiological knowledge of black point as well as comparative mapping techniques of a known putative QTL on chromosome 2H has identified a number of candidate genes in black point formation. Differential gene expression determines the development of a plant. Each gene exhibits a specific temporal and spatial expression pattern and level, resulting in each tissue expressing a unique set of proteins (Deplancke *et al.* 2004). Although differential expression can be regulated at different steps, including protein synthesis and protein and mRNA degradation, it is widely appreciated that developmental gene expression patterns are predominantly established at the level of transcription regulation (Lee and Young 2000). Specifically, differential gene expression is controlled by regulatory transcription factors that bind to *cis*-regulatory DNA elements, often located on or near a gene's promoter (Deplancke *et al.* 2004). These regulatory DNA-binding proteins function as *trans*-acting activators of transcription, stimulating RNA polymerase catalysed transcription, or in some instances heterodimers or larger complexes that are formed by two or more different proteins that bind to the *cis*-acting element before a gene can be transcribed (Zhu *et al.* 2003). Thus the regulation of differentially expressed genes in black point formation could be a single transcription factor or involve a complex series of events.

Black point has been strongly linked with environmental stress and a possible wounding mechanism (Figure 1.2). Transcriptional control of the expression of stress responsive genes is a crucial part of the plant's response to stress (Singh *et al.* 2002). Transcription factors interact with *cis*-elements in the promoter regions of various abiotic stress related genes and thus up-regulate the expression of many secondary responsive genes resulting in abiotic stresses tolerance (Agarwal and Jha 2010). A number of *cis*-elements and corresponding transcription factors in *Arabidopsis thaliana* have been identified that are

important for regulating the plants response to stress including: AP2/ERF (apetala 2/ethylene responsive factor), basic leucine zipper, HD-ZIP (homeodomain leucine zipper), MYC (myelocytomatosis), MYB (myeloblastosis), WRKY and different classes of zinc finger domains (Shinozaki and Yamaguchi-Shinozaki 2000; Pastori and Foyer 2002). MYB proteins have been linked to plant responses to ultra-violet light, wounding, anaerobic stress and pathogens (Rushton and Somssich 1998). ERF genes have been shown to be regulated by cold, drought, pathogen infection and wounding (Singh et al. 2002). WRKY family members have shown enhanced expression and DNA binding activity following induction by a range of pathogens, defence signals and wounding (Eulgem et al. 1999). If wounding and stress is involved in black point formation, these transcription factors may therefore play a role.

Understanding the transcription factors involved in the regulation of genes that affect the outcome of black point formation will be important in our knowledge of the trait. Similar to mapping QTL, the literature has identified the ability to use levels of transcript abundance to identify genomic loci controlling the observed variation in mRNA (eQTL). This would allow the identification of candidates in the regulation of black point formation as completed for the trait itself by March and colleagues (2008). Potokina *et al* (2008) successfully used Affymatrix microarray to study genome wide gene expression and identify eQTLs in barley. Furthermore eQTL that regulate gene activity can be correlated with QTLs identified for traditional phenotypic traits to provide additional clues to the genetic basis of quantitative genetic variation (Schadt *et al.* 2003; Hubner *et al.* 2005).

A powerful method, Yeast one-hybrid (Y1H) has been used to identify-protein DNA interactions (Bartel and Fields 1995; Zhu *et al.* 2001). This technique allows the

investigation of regulatory regions of the candidate genes and the identification of proteins (usually transcription factors) involved in the gene's regulation. The method has successfully identified transcription factors involved in a gene's regulation in wheat (Shen *et al.* 2003; Lopato *et al.* 2006), rice (Zhu *et al.* 2003), barley (Müller *et al.* 2000) and parsley (Cormack *et al.* 2002). Understanding how the candidate genes involved in black point formation are regulated through genomic regions involved in the gene's regulation (eQTL) and candidate genes (Y1H) could be an effective approach for understanding the trait and environmental stress responses involved. Furthermore genetic modification of the identified transcription factors may be a tool in enhancing the tolerance of barley varieties to black point.

1.7 Research justification

Black point has been proposed to be of a biochemical nature and that more specifically an enzymatic browning reaction causes the discolouration. This reaction is characteristic of plants subjected to stress or wounding. The wounding triggers a reaction in which an interaction between peroxidases and phenols may lead to the discoloration observed. A number of genes have been identified as candidates for black point formation based on a model for enzymatic browning (Figure 1.2). The detection and mapping of a QTL for black point has also allowed the identification of candidate genes through comparative mapping between barley and rice (March *et al.* 2008).
Research described herein therefore aimed to:

1. Determine the environmental conditions that induce black point by simulating the environmental conditions thought to induce black point within controlled conditions, incorporating high humidity. Environmental data from field sites over a period

of 4 years aimed to confirm the required environmental conditions while black point scoring was used for determining areas of the genome contributing to black point formation via quantitative trait loci (QTL) mapping. The potential role in timing of grain fill on black point formation was also analysed through assessment of the effect of planting date on the incidence of black point. Chapter 2 describes this research while the majority of this chapter was published in 2008 in the Australian Journal of Agricultural Research (Walker *et al.* 2008)

2. Determined the expression of a number of candidate genes in susceptible and tolerant cultivars during grain fill (Chapter 3). Peroxidase gene expression has been shown to be expressed for longer in susceptible varieties during grain fill (March 2003), while *QR, LOX* and *PAL* have also been shown to be potential candidates. Research aimed to characterise *HvBP1*, *HvPrx7*, *HvPAL*, *HvQR* and *HvLox1* gene expression during grain development in cultivars of varying susceptibilities to black point. Differential gene expression between susceptible and tolerant cultivars may allow for a potential breeding target in the future. When differential expression was established, gene expression was further examined within healthy and black pointed grains.

3. Determine areas of the genome contributing to differential expression of candidate genes for black point formation by combining QTL mapping and fine mapping with gene expression data (Chapter 4). This research therefore aimed to identify eQTLs or areas of the genome contributing to gene expression for genes found to be differentially expressed. Candidates were also mapped to a chromosomal location in the barley genome to enable identification of whether eQTLs were *cis*- or *trans*-acting. If *trans*-regulatory mechanisms were identified, comparative mapping studies between barley, wheat and rice allowed the identification of candidate regulatory factors (such as transcription factors) potentially involved in the genes' regulation.

4. Determine potential regulatory mechanisms for candidates identified as differentially expressed between tolerant and susceptible varieties (Chapter 5). This component aimed to firstly determine if susceptibility is correlated with differences in regulatory elements by analysing the promoter regions of candidate genes in the susceptible variety Sloop and tolerant variety Alexis. Secondly, the research aimed to identify transcription factors that might regulate gene expression by using Y1H screening. Although the regulatory networks of the candidate genes identified have not been explored to date, an understanding of how these genes are regulated will be a major step in increasing our knowledge of the mechanisms involved, allowing for the breeding of tolerant barley varieties. Knowledge of the role of transcription factor genes in black point formation also provides a valuable tool for the manipulation of plants. Tolerant varieties are needed in order to reduce the losses for growers, which in turn would ultimately lead to an increased market share for Australia’s malting barley industry.

Chapter Two. The association of environmental conditions with black point formation and the identification of QTL

2.1 Introduction

Given that no direct association between black point and fungal infection in barley (Jacobs and Rabie 1987; Basson *et al.* 1990; Ellis *et al.* 1996) or wheat (Williamson 1997a) has been found, the involvement of fungi in black point formation has generally been discounted. However, there has been a suggestion that black point results from the induction of enzymatic browning during exposure to unfavourable environmental conditions during grain fill (Williamson 1997b). Peroxidases from the germ aleurone have been shown to react with phenols when cellular disruption occurs (Cochrane 1994a). Any disruption of the immature caryopsis may also bring these enzymes and substrates together, giving rise to extensive melanisation (Cochrane 1994b). Environmental conditions at grain fill may therefore be linked to the associated enzymatic browning process and black point formation through disruption of cells at the embryo end of the grain. The accurate establishment of which environmental conditions can be considered unfavourable is therefore critical to ensure understanding of black point formation.

In Australia, the incidence of black point in wheat and barley crops is variable and seems to depend largely on seasonal conditions, although these conditions have not been clearly established. Prolonged ripening due to cold and frosts has been reported to increase the likelihood of black point formation in durum wheat (Fernandez *et al.* 1994). An early study associated high temperatures and low moisture conditions with severe symptoms in common wheat (Waldron 1934), but other evidence (Rees *et al.* 1984) indicated that the occurrence of moist and humid conditions during grain filling and ripening increases the

intensity and frequency of black point in common wheat. Limited evidence that high humidity contributes to black point formation (Sulman *et al.* 2001a; Hudec 2007) and kernel discolouration in barley (Li *et al.* 2003) has also been provided.

Even though there is some evidence that varieties of differing maturities vary in their susceptibility to black point formation, there have been limited genetic studies on black point in barley. Recent research has detected quantitative trait loci (QTL) affecting black point in two populations of wheat, a Sunco x Tasman-derived population and a Cascades x AUS1408-derived population (Lehmensiek *et al.* 2004). In barley, QTL have been reported for kernel discolouration (de la Penna *et al.* 1999; Li *et al.* 2003) but not specifically for black point. Given the current confusion over correctly categorising and separating the two discolourations as two distinct categories, whether these QTL affect black point is not known. Mapping of QTL that affect black point formation in barley will permit comparison with genomic regions that have been reported to contribute to kernel discolouration and genomic regions identified in wheat. However, given that the environmental conditions that contribute to black point are not well understood, there is a need to identify the environmental conditions to be able to replicate black point *in vitro*. This would allow more comprehensive genetic studies to be undertaken.

The research presented in this chapter (and in Walker *et al.* 2008, Appendix 4), therefore, aimed to simulate the environmental conditions thought to induce black point within controlled conditions, incorporating high humidity. The conditions responsible for black point formation at 2 South Australian field sites over 5 years were investigated and the areas of the genome contributing to black point formation determined via quantitative trait loci (QTL) mapping of black point scores. Furthermore investigating the potential role of

timing of grain fill on black point formation was investigated through assessment of the effect of planting date on the incidence of black point.

2.2 Materials and methods

2.2.1 Simulation of humid conditions for black point formation

To examine whether black point can be induced under humid conditions, barley (*Hordeum vulgare* L.) plants were grown within a glasshouse where either a humid environment (70 to 80% relative humidity) or a non-humid environment (40% relative humidity) was established during the grain fill period. The susceptible varieties Sloop and Keel were grown as well as the tolerant variety Alexis, with five replicates of each variety planted for use in each environment. The experiment was repeated twice in each controlled environment. Plants were grown in a University of California soil mix (Baker 1957), in a glasshouse under natural light at the Waite Campus of the University of Adelaide (Adelaide, South Australia, latitude 34°56"S, longitude 138°36"E). An average glasshouse temperature of 22°C ± 3°C was maintained. Plants were hand-watered every second day until anthesis, between Zadoks' stage 60 (beginning of anthesis) and 65 (mid-way through anthesis) [Zadoks' scores determined as per (Zadoks *et al.* 1974)]. Plants were then separated into humid (Figure 2.1) and non-humid (or standard glasshouse) growing conditions. Humid conditions were maintained by enclosing the plants with plastic sheeting and the use of overhead misters (Figure 2.1). Misters were turned on for 15 min at 4 h intervals until towards the end of grain development (Zadoks stage 91) when the interval time was increased to 8 h to allow grain to dry and mature. Relative humidity was monitored using a thermo-hygrometer clock (Digitor, model # 241/Y 5189).

Figure 2.1 Simulation of humid conditions during grain fill in the glasshouse. Plants were grown in humid conditions (70-80%) after anthesis (Zadoks' stage 60) by enclosing plants with plastic sheeting and addition of overhead misters which were run for 15 min at 4 h intervals until maturity (stage 91) (Zadoks *et al.* 1974).

Because issues with fungal infection and poor grain fill occurred in the humid conditions created in the glasshouse, a growth chamber was also used to simulate humid conditions. Plants were either grown in a Bigfoot growth chamber (Bigfoot Model # GC-20, Econair Ecological Chambers Inc., Winnipeg, MB Canada) for their entire lifecycle or grown in the glasshouse environment until anthesis and then placed into the chamber at anthesis. The chamber conditions involved a cycle of 13 h light and 11 h dark at 28°C and a relative humidity of 80%. These conditions were set due to the limited space availability within the Bigfoot chamber. The same varieties and five replicates for each variety (n=5) were used in the chamber experiment as for the glasshouse.

2.2.2 Plant material for field trials to study black point formation

Environmental conditions necessary for black point formation and the mapping of QTL were studied in the field. The varieties Alexis and Sloop, as well as Arapiles, Barque, Baudin, Fitzroy, Franklin, Gairdner, Golden Promise, Keel, Mundah, Schooner, Sloop SA, Sloop Vic and VB9935 were grown at Port Wakefield (138°8" E, 34°11" S; near Adelaide) in 1999/2000 [provided by South Australian Research and Development Institute (SARDI) stage 4 trials, courtesy of Rob Wheeler, SARDI] and Hatherleigh (140°16" E, 37°29" S; in south-eastern South Australia) in 2001/2002, 2004/2005, 2005/2006 and 2006/2007 (Figure 2.2).

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Figure 2.2 Sites in South Australia where barley was grown to assess for black point formation. Port Wakefield (Trial site 1) and Hatherleigh (Trial site 2) are marked in yellow. Sites, from which, weather data was available from the Australian Bureau of Meteorology (Price, Robe and Mount Gambier) are marked in red. Image generated using Google Earth (version 4.3).

Based on previous experience (Trent Potter, SARDI, *personal communication*), the conditions at Hatherleigh were expected to favour black point formation as trials were planted slightly later than normal in order to increase the probability that there would be high humidity during the grain filling period. Field experiments were planted in serpentine, with the experimental design completely randomised in 1.25 by 4.5 m, five-row plots. A plot was considered to be one replicate. In the 2004/2005 and 2005/2006 growing seasons five replicates (or plots) of each cultivar were planted except for Sloop and Alexis for which 10 plots of each were planted. In the 1999/2000, 2001/2002 and 2006/2007 growing seasons, cultivars were duplicated (two plots). Differences in black point susceptibility between the parental varieties Sloop and Alexis in the 2001/2002 season (where Sloop is susceptible and Alexis is tolerant) gave the basis for evaluating 92 doubled haploid (DH) lines derived from a cross between Alexis and Sloop (Barr *et al.* 2003) in field experiments at Port Wakefield in 1999/2000 and Hatherleigh in 2004/2005. The full DH Alexis/Sloop mapping population was grown in the 2004/2005 (in triplicate) and 1999/2000 season (in duplicate) to use for QTL analysis of black point scores. The full Alexis/Sloop population was also planted in duplicate during the 2006/2007 season.

2.2.3 Phenotyping black point and maturity of field-grown material

Observations (the extent of discolouration) were recorded and photographed to provide a definition for black point and examine variation in symptom severity. Black point (observed as distinct discolouration at the embryo end of the grain) was examined in five samples of 100 grains for each plot in the field trials.

Because the environmental conditions during grain fill are associated with black point

formation, how the grain stages vary between varieties was determined. Grain stages in the 2006/2007 season were therefore recorded in each plot according to Zadoks' growth stage, by measuring maturity at time intervals over grain fill from stages 71 (medium milk), 85 (soft dough), 87 (hard dough) and 95 (onset of maturity) (Zadoks *et al.* 1974) (Figure 2.3). Meteorological data were then analysed during these developmental stages, which corresponded with the months of November and December for each growing season at Hatherleigh. Sampling times in the 2005/2006 season at each of the Zadoks' stages indicated that the maturity times were similar between years. The Zadoks' score for the varieties grown at Port Wakefield was recorded at one time-point (30th October, 1999) (data kindly provided by Mr Stewart Coventry, The University of Adelaide).

Figure 2.3 Zadoks' growth stages for barley. Barley grain representative of medium milk (71), soft dough (85), hard dough (87) and maturity (95) are shown (Zadoks *et al.* 1974).

2.2.4 Weather observations at field trial sites

A Tinytag data logger (Hastings, Port Macquarie, New South Wales) was used on site at Hatherleigh in 2004/2005 and 2005/2006 to record temperature and humidity readings at one hour intervals. In 2001/2002 and 2006/2007 no data logger was available on site but data were available from regional weather stations operated by the Australian Bureau of Meteorology (www.bom.gov.au) of which the two nearest locations to Hatherleigh are Mount Gambier (140°46" E, 37°49" S) and Robe (139°76" E, 37°16" S) (Figure 2.2). The 2004/2005 data from the data logger were compared with meteorological data (maximum

and minimum temperature and humidity as well as 9 am and 3 pm temperature and humidity in the 2004/2005 season and maximum and minimum temperature and humidity in 2005/2006) from Mount Gambier airport (aero station) and Robe weather stations (Appendix 1, Figure A1.1-A1.6). Temperature and humidity patterns at Hatherleigh were similar to those at Mount Gambier (Appendix 1, Table A1.1). Mount Gambier meteorological data were therefore chosen for analysis for 2001/2002 and 2006/2007. These data included minimum and maximum air temperatures measured in a shaded enclosure at a height of approximately 1.2 m above the ground, average relative humidity (%) readings of synoptic observations taken at 3 h intervals from 12 am and precipitation as mm of precipitation to 9 am daily. No on-site observations were available in 1999/2000 at the Port Wakefield site and therefore data was obtained from the nearest BOM weather station, which was Price (138°0" E 34°29" S) (Figure 2.2).

To further examine the weather data, they were analysed by counting the number of days on which: (1) the maximum was above 20°C; (2) the minimum was above 10°C; (3) the maximum humidity was above 90%; and (4) the minimum relative humidity was above 50%. The total extent to which the temperature or relative humidity differed from the nominal values for each of these categories was also calculated by summing the total degrees above 20°C for the maximum temperature; the total degrees above 10°C for the minimum temperature; the total percentage above 90% for the maximum relative humidity; and the total percentage above 50% for the minimum relative humidity.

Using temperature (*T*) and relative humidity (*RH*) measured daily at 9 am (when humidty was at its maximum) during grain fill; saturation vapour pressure (e_s), actual vapour

pressure (e_a) and vapour pressure deficit (*VPD*) were also calculated as per equations 1 to 3 (Wang *et al.* 2004).

$$e_s = 0.6108 \exp\left(\frac{17.27T}{T+237.3}\right) \quad \text{kPa} \quad (1)$$

$$e_a = (RH/100) \times e_s \quad \text{kPa} \quad (2)$$

$$VPD = e_s - e_a \quad \text{kPa} \quad (3)$$

2.2.5 QTL mapping

QTL for black point tolerance were either generated using Windows QTL Cartographer 2.5 and QTLNetwork 2.0. Composite interval mapping of black point scores for the two sites, Port Wakefield (1999/2000) and Hatherleigh (2005/2006) were treated individually using Windows QTL Cartographer 2.5 (Basten *et al.* 2005) with significance threshold values set at a genome-wide significance level of 0.05 using 500 permutations. The marker map used was an updated version of those previously reported for the Alexis/Sloop DH mapping population (Barr *et al.* 2003; Willsmore *et al.* 2006). All available marker information was collated, the map order was reconstructed using RECORD (Van Os *et al.* 2005) and refined through comparisons with the map order obtained from a larger recombinant inbred line (RIL) population (kindly provided by Greg Lott, SARDI). The percentage of phenotypic variation explained and allele contribution by each QTL was also estimated.

Because Windows QTL Cartographer did not allow the combined analysis of both years in the two environments, QTL analysis was also conducted by mixed linear composite interval mapping (Yang *et al.* 2007) using the software QTLNetwork 2.0 (Yang *et al.*

2008), which was the better package to analyse the combined data (in this case). Significance thresholds corresponding to experiment-wise significance levels of 0.05 were set using 10,000 permutations. The additive main effects of QTL were treated as fixed and the environmental effects and additive-environmental interaction effects were treated as random. QTL effects were estimated using a Bayesian method via 20,000-cycle Gibbs sampling. For each QTL, heritability was estimated for both additive and additive-by-environment effects.

2.2.6 The effect of planting date on black point formation

To determine whether maturity affects black point formation, the early maturing and black point susceptible variety Sloop and the later maturing variety Alexis were planted at different times in the 2005/2006 and 2006/2007 seasons at the Hatherleigh site. Plots were hand sown in single rows, as 1.25 by 4.5 m plots. In the 2005/2006 season individual plots of Sloop were planted two weeks and one month following the original planting date. Thus allowing varieties to mature at comparable times. An individual plot of Alexis was planted 2 weeks prior to the original planting date, and individual plots of Sloop were planted either 2 weeks after or 1 month after the original planting date. Grain was harvested, hand threshed and scored for black point as per section 2.2.3.

2.2.7 Statistical analysis

Data for black point scores were analysed with Genstat (8th Edn, Release 8.2, 2005, Lawes Agricultural Trust, VSN International Ltd., Hemel Hempstead, UK) using one-way analysis of variance (ANOVA) for each year's data and two-way ANOVA to compare

cultivars across years. One-way ANOVA was also used to compare data from plots with altered planting dates. Data for *VPD* were analysed using one-way ANOVA to compare values across years and sites. The least significant difference (LSD) at P = 0.05 was used to test for significant differences between means.

Correlations were calculated between the Hatherleigh trial site and nearby weather stations at Mount Gambier and Robe for temperature and humidity measurements at 9am and 3pm, as well as maximum/minimum temperature and humidity in the 2004/2005 season. Due to the lack of availability of 9 am/3 pm data in the 2005/2006 season, correlations were made only for maximum and minimum temperature and humidity.

2.3 Results

2.3.1 Defining black point symptoms

For grain grown in the field, black point was visualised as brown-black discolouration confined to the lemma and palea at the embryo end of the grain (Figure 2.4). Black point was rarely observed in grain before the hard dough stage of development (Zadoks' stage 87, Figure 2.3). Symptom severity varied with respect to the intensity of discolouration and the extent to which the grain was covered by the discolouration (Figure 2.4). Black point was recorded when the discolouration was equal to or greater than 1mm. Black point formation appeared to occur randomly throughout the head and was not isolated to a particular region of the head (e.g. top or bottom) (data not shown).

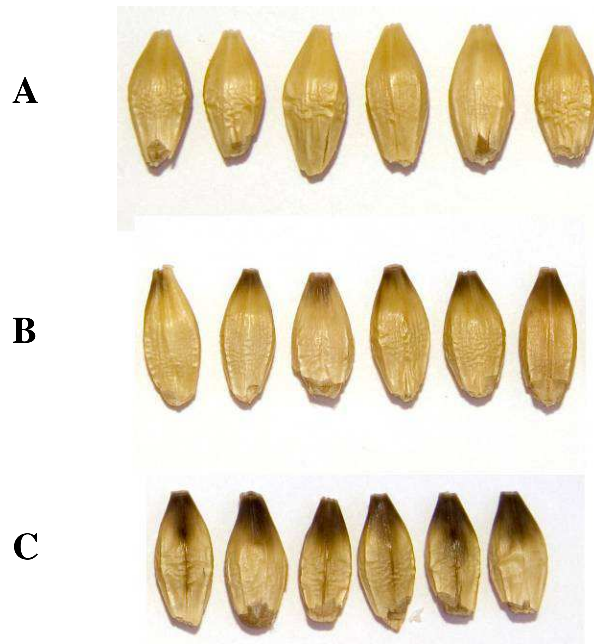

Figure 2.4 Grain displaying varying levels of black point symptoms from no symptoms (A) to moderate (B) and severe symptoms (C). The varying colour and the degree to which the grain can be affected are shown.

2.3.2 Simulating black point in humid conditions

When barley was placed in humid conditions within the glasshouse or in a growth chamber during grain fill, neither environment was suitable for the healthy growth of barley nor was black point induced. Barley plants grown within the humid environment simulated in the glass house displayed symptoms of a black mould after anthesis and grain fill was affected, with grain not forming properly (data not shown). Similarly, when barley was placed in a growth chamber for its entire lifecycle, the conditions did not sustain healthy growth with the majority of plants not heading and for those that did, grain fill did not occur. When plants were added to the growth chamber upon anthesis, black point formation was also not induced.

2.3.3 Black point has a genotypic basis

At the Hatherleigh site, black point symptoms on the variety Keel consistently exceeded the 10% threshold with significantly higher levels in 2001/2002 compared to those observed in 2004/2005 and 2005/2006 (Table 2.1). Other varieties usually considered susceptible to black point (including Barque, Schooner, Sloop, Sloop SA and Sloop Vic) also exceeded the 10% threshold in the 2001/2002 season as did Sloop Vic and VB9935 in the 2005/2006 season. Some varieties including Gairdner, Franklin, Mundah, Golden Promise, Arapiles, Baudin and Alexis consistently showed tolerance (with scores below 10%) across years and sites (Table 2.1). In two environments (Port Wakefield in 1999/2000 and Hatherleigh in 2004/2005), Alexis had significantly more black point than Sloop but where black point levels were generally higher (such as Hatherleigh in 2001/2002 and 2005/2006), the opposite was true. Under the extreme weather conditions associated with drought in 2006/2007 there were very few symptoms, which provided a basis for comparing weather data to years in which extreme symptoms were observed (such as 2001/2002) (Figures 2.5 to 2.9).

Table 2.1 The incidence of black point (%) in barley varieties grown at Port Wakefield (PW) in 1999/2000 and Hatherleigh (H) in 2001/2002, 2004/2005, 2005/2006 and 2006/2007. Where black point scores exceed the industry standards of 10%, varieties are considered susceptible (indicated in bold). Within columns, means followed by the same letter in superscript are not significantly different (P>0.05, n=10 for 1999/2000, 2001/2002 and 2006/2007, n=25 for 2004/2005 and 2005/2006 except for Sloop and Alexis in those years where n=50). * denotes an early to mid-maturing variety.

	Field trial location (year)				
Parent	**PW (1999/2000)**	**H (2001/2002)**	**H (2004/2005)**	**H (2005/2006)**	**H (2006/2007)**
Alexis	7.2^{cd}	4.3^{ab}	6.3^{g}	7.0^{e}	0.1^{ab}
Arapiles		8.0^{ab}	1.6^{b}	4.8^{d}	0.1^{ab}
Barque	3.3^{ab}	**23.3^{d}**	2.4^{c}	7.5^{ef}	0.0^{a}
Baudin *		6.2^{ab}	4.1^{e}	6.6^{e}	0.4^{bc}
Fitzroy				9.4^{gh}	0.0^{a}
Franklin	5.3^{bc}	3.8^{a}	0.6^{a}	1.7^{a}	0.0^{a}
Gairdner	2.2^{a}	8.8^{b}	1.0^{ab}	1.5^{a}	0.0^{a}
Golden Promise			1.8^{b}	4.7^{cd}	0.1^{ab}
Keel*	9.0^{d}	**37.8^{f}**	**19.1^{i}**	**22.6^{j}**	0.7^{cd}
Mundah		7.8^{ab}	1.8^{b}	2.8^{b}	1.0^{d}
Schooner*	1.7^{a}	**22.3^{cd}**	2.7^{cd}	3.5^{bc}	0.0^{a}
Sloop*	2.7^{a}	**17.8^{c}**	3.3^{de}	8.7^{fg}	0.1^{ab}
Sloop SA*		**28.5^{e}**	5.4^{fg}	9.2^{g}	0.1^{ab}
Sloop Vic*		**24.2^{de}**	5.3^{f}	**10.0^{h}**	0.4^{bc}
VB9935			6.6^{h}	**14.0^{i}**	0.0^{a}
LSD	**2.0**	**4.8**	**0.9**	**1.2**	**0.3**

2.3.4 Environmental conditions associated with black point

Because attempts to simulate the humidity (which was thought to be a contributing factor in black point formation) in the glasshouse or growth chamber (section 2.3.2) did not sustain the healthy growth of barley plants, the environmental conditions associated with black point formation at two field sites were determined. On-site weather data was not available at Port Wakefield in 1999/2000 and Hatherleigh in the years 2001/2002 and 2006/2007. Data from the Price weather station was considered representative for Port Wakefield due to its close proximity and the presence of no other weather stations in the

immediate vicinity. Mount Gambier was found to be the most appropriate weather station, representative of the Hatherleigh site (Appendix 1, Figures A1.1 to A1.6) as per section 2.2.4. Maximum and minimum temperatures and humidity were chosen for further analysis because the 9 am and 3 pm data did not truly reflect the extremes of temperature and humidity observed (Figures A1.1, A1.3, A1.5). General day-to-day maximum and minimum trends for the weather station and Mount Gambier followed one another (Figures A1.1, A1.3 and A1.5). However, the maximum humidity data provided for Mount Gambier were lower probably because the Mount Gambier readings were taken at 6 am rather than at sunrise when maximum humidity is normally is at its highest (BOM, *personal communication*). Thus, the maximum humidity may not be accurate. Indeed there was no significant correlation between the maximum humidity at the weather station and those at either site regardless of season (Table A1.1). Nevertheless, correlations generally supported the visual assessment of graphs for both temperature and humidity in the 2004/2005 and 2005/2006 seasons. For the 2004/2005 season, temperature and humidity at 9 am and 3 pm were strongly correlated for the Mount Gambier aero station with the Hatherleigh weather station ($r = >0.8$ for humidity and $r =0.9$ for temperature as per Table A1.1) as were maximum and minimum temperatures ($r = 0.96$ and $r = 0.87$ respectively; Table A1.1).

In the 2005/2006 season, strong correlations with the Hatherleigh weather station for maximum temperature were observed for both the Mount Gambier and Robe sites ($r = 0.95$ and $r = 0.91$ respectively, Table A1.1). Strong correlations for minimum humidity were also evident with the Mount Gambier station ($r = 0.92$, Table A1.1). Mount Gambier data were therefore used for analysis as they were more closely correlated to the on-site conditions.

At Hatherleigh in 2001/2002 when black point symptoms were most severe (Table 2.1), the minimum humidity was generally high during grain fill (Figure 2.5) while both maximum and minimum temperatures were generally low (Figure 2.5). When black point was less severe in the 2004/2005 (Figure 2.6) and the 2005/2006 (Figure 2.7) seasons, the maximum humidity during grain fill was as high as in 2001/2002 but the minimum humidity was generally lower, with higher temperatures. During the 2006/2007 season (Figure 2.8), when few symptoms were observed, temperatures were higher and humidity was generally lower than other years. Further, limited rain fall events occurred during the grain fill period in 2006/2007 (Figure 2.8). In the years when black point was apparent, there were rainfall events of differing magnitudes during the grain fill period. In the 2004/2005 season significant rainfall events were observed consistently through the 60 day grain filling period (Nov to Dec 2004), with late rainfall observed at days 54 to 58 (Figure 2.6). In contrast during the 2005/2006 season rainfall events were observed early in the grain filling period from days 3 to 9, with small events on days 33, 38 and 43. The next significant rainfall event was not until days 46 and 47 (Figure 2.7).

Varieties grown at the Hatherleigh site (2001/2002) were grouped into categories based on black point susceptibility in a problematic year, with less than 5% black point (Figure 2.9 A), less than 10% black point (Figure 2.9 B) and greater than 15% black point (Figure 2.9 C). A clear segregation in maturity can be made between parental groups. The early maturing varieties emerge as susceptible to black point (Figure 2.9 C) and the later maturing varieties (Figure 2.9 A) tolerant. The early rainfall events appear associated with the milk to soft dough stages of grain development in susceptible varieties (Figure 2.9 C). Rainfall events occurred later, when the susceptible varieties had passed the hard dough

stage (Figure 2.9 C) and the tolerant varieties ranged from late milk to soft dough (Figure 2.9 A). Late rainfall was also observed in 2001/2002 on days 41 and 42 (Figure 2.5) which is more than likely when susceptible varieties would be progressing past the hard dough stage of grain fill (Figure 2.9 C). A significant rainfall event (>25mm) was also observed at the Port Wakefield site on day 43 (Figure 2.10), but the grain fill stage could not be estimated due to limited maturity information. Even so there is a clear difference in the maturity of parents (Figure 2.10 C), thus suggesting a clear difference in grain fill timing. In addition, differences in maturity between varieties appeared similar between sites (Figure 2.9 and 2.10 compared), with the only exception being Barque. Barque is normally susceptible to black point in appropriate conditions and this was reflected by its grouping with early maturers at Hatherleigh. However, at Port Wakefield Barque was of similar maturity to Alexis and Franklin and also had similar levels of black point (Table 2.1, Figure 2.9 C).

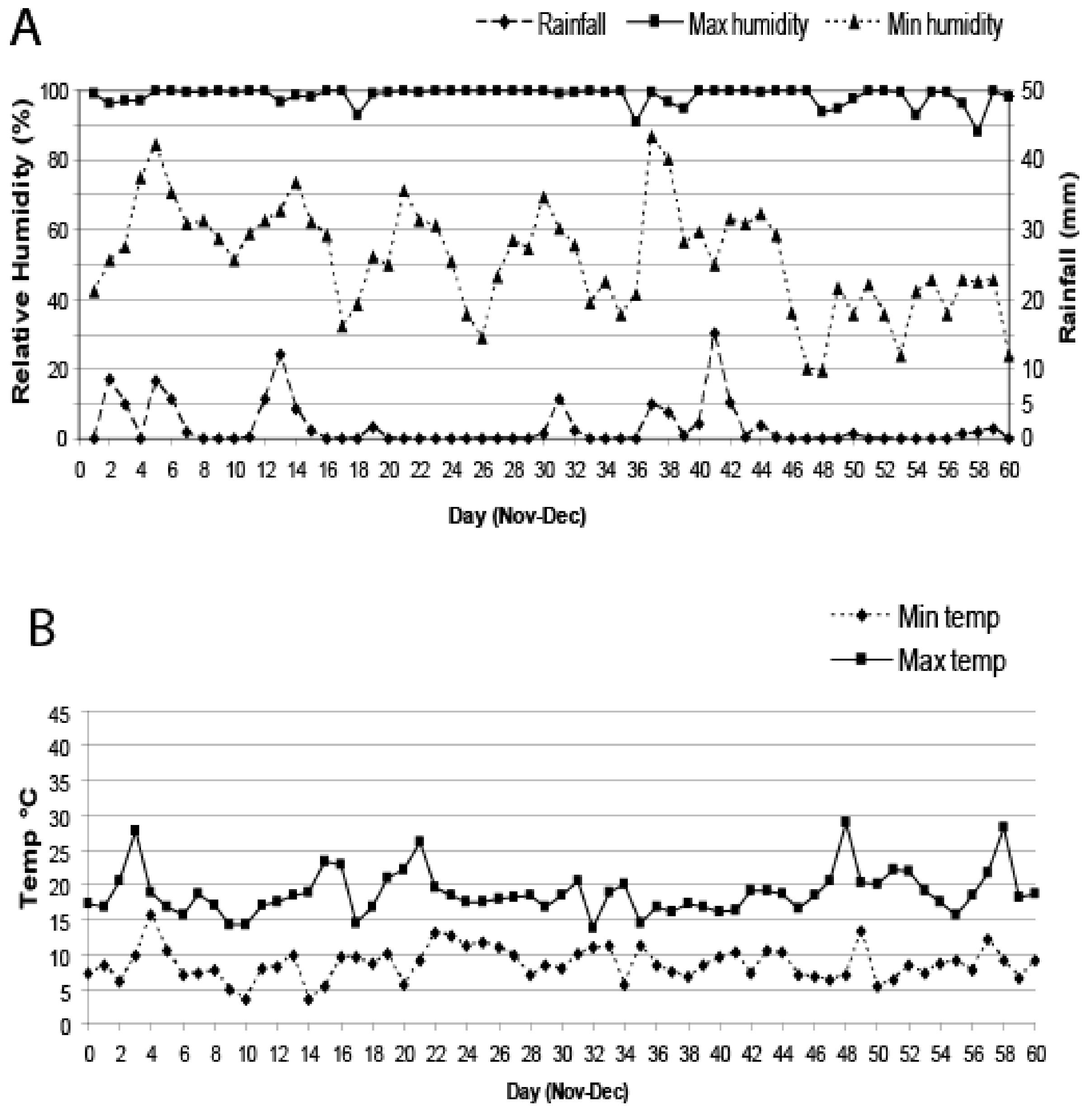

Figure 2.5 Weather conditions during grain fill representative of Hatherleigh in the 2001/2002 season. Daily rainfall; maximum and minimum relative humidity (A); and maximum and minimum temperatures (B) in November and December for 2001. Data were collected from Mount Gambier aero station (BOM).

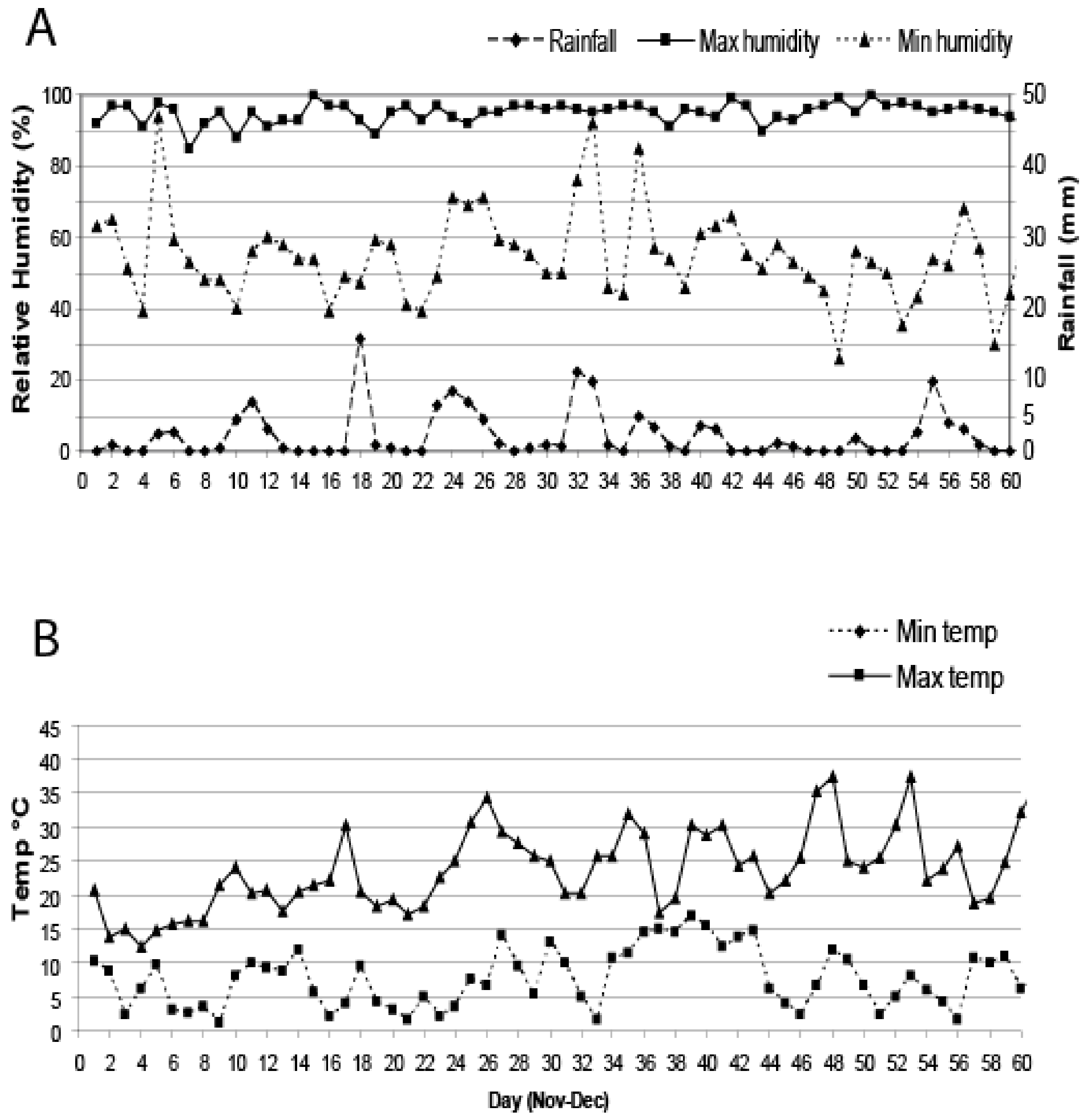

Figure 2.6 Weather conditions during grain fill at Hatherleigh in the 2004/2005 season. Daily rainfall; maximum and minimum relative humidity (A); and maximum and minimum temperatures (B) in November and December for 2004. Data were collected from Hatherleigh (on-site weather station).

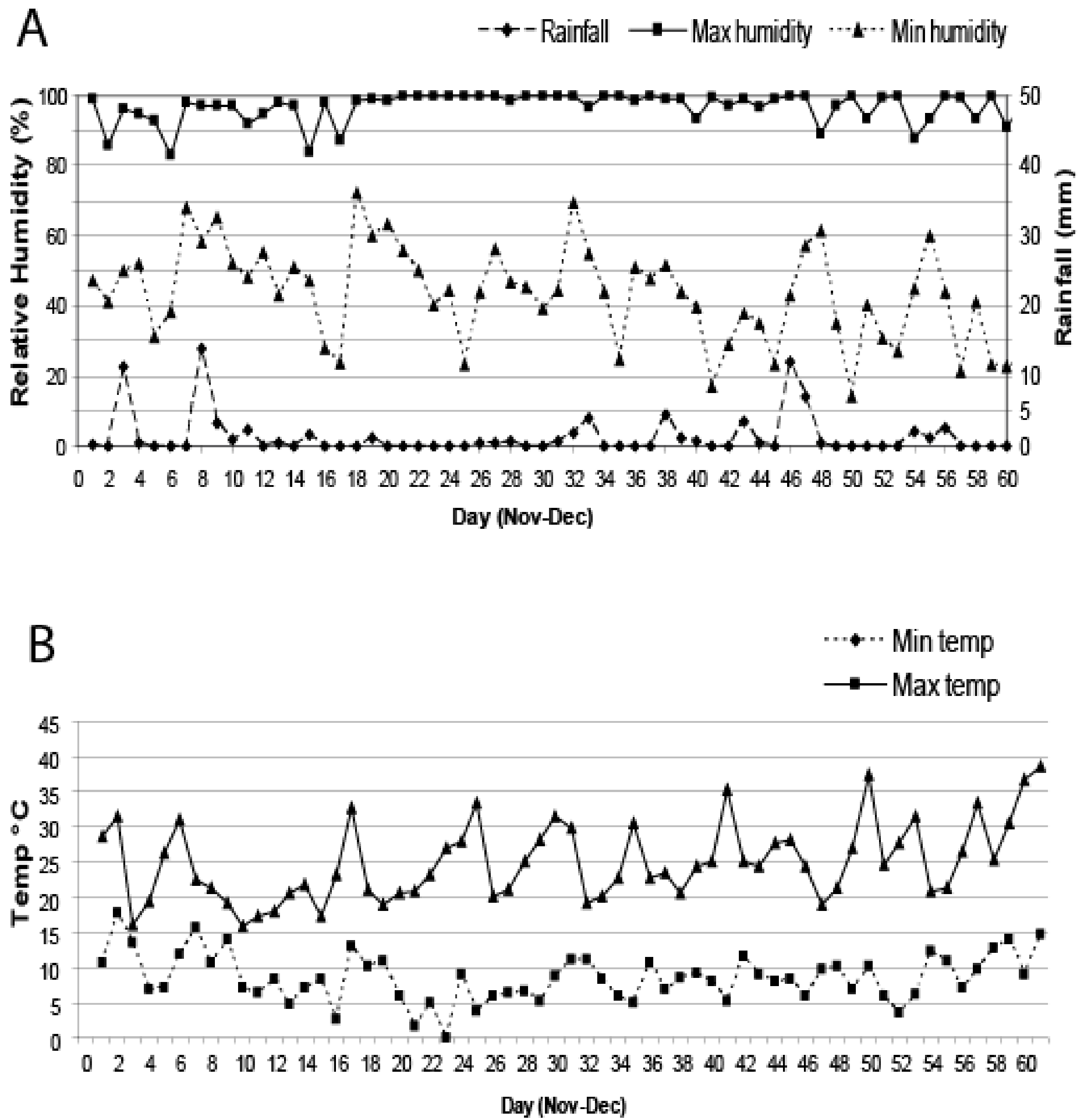

Figure 2.7 Weather conditions during grain fill at Hatherleigh in the 2005/2006 season. Daily rainfall; maximum and minimum relative humidity (A); and maximum and minimum temperatures (B) in November and December for 2005. Data were collected from Hatherleigh (on-site weather station).

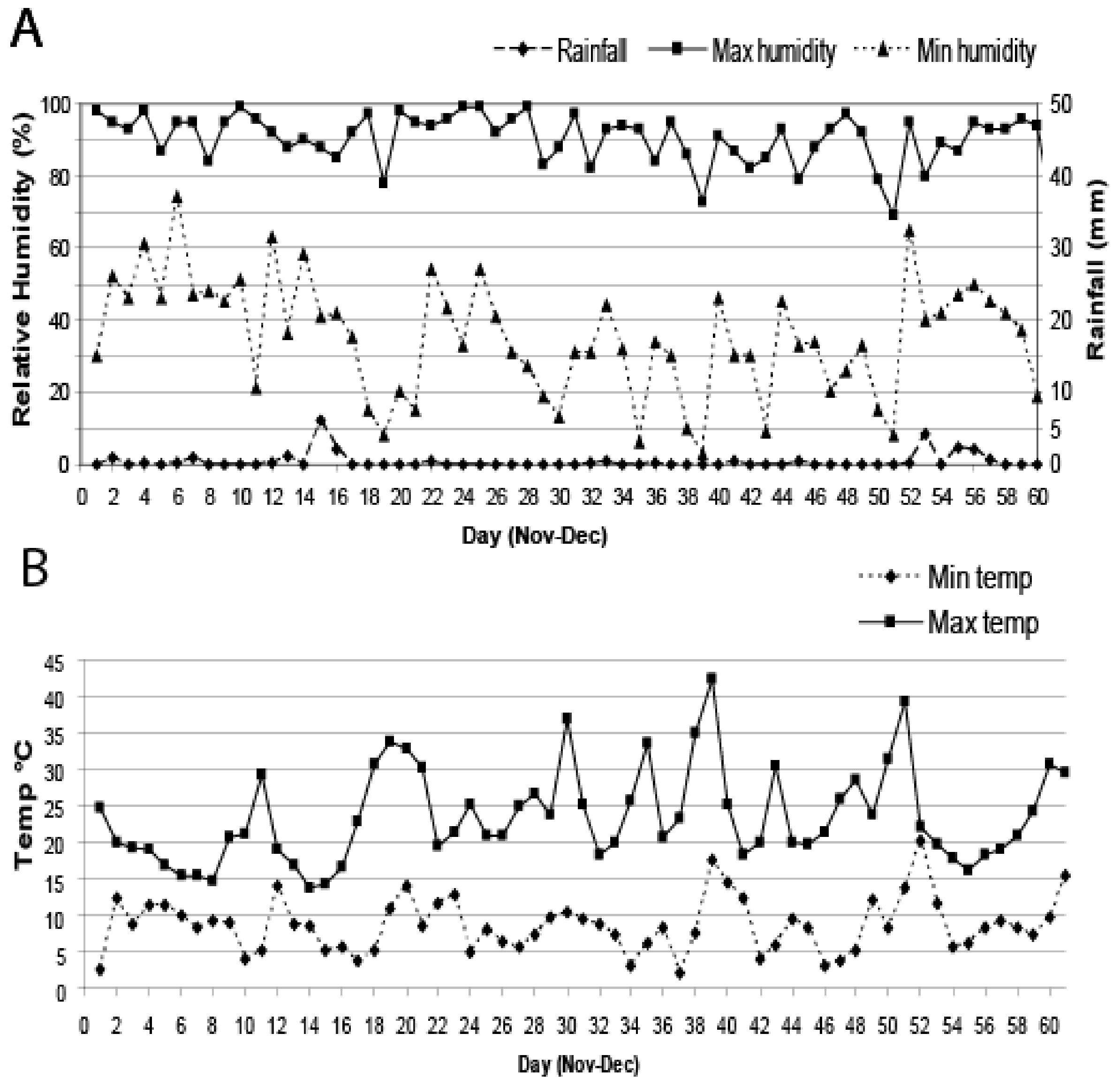

Figure 2.8 Weather conditions during grain fill representative of Hatherleigh in the 2006/2007 season. Daily rainfall; maximum and minimum relative humidity (A); and maximum and minimum temperatures (B) in November and December for 2006. Data were collected from Mount Gambier aero station (BOM).

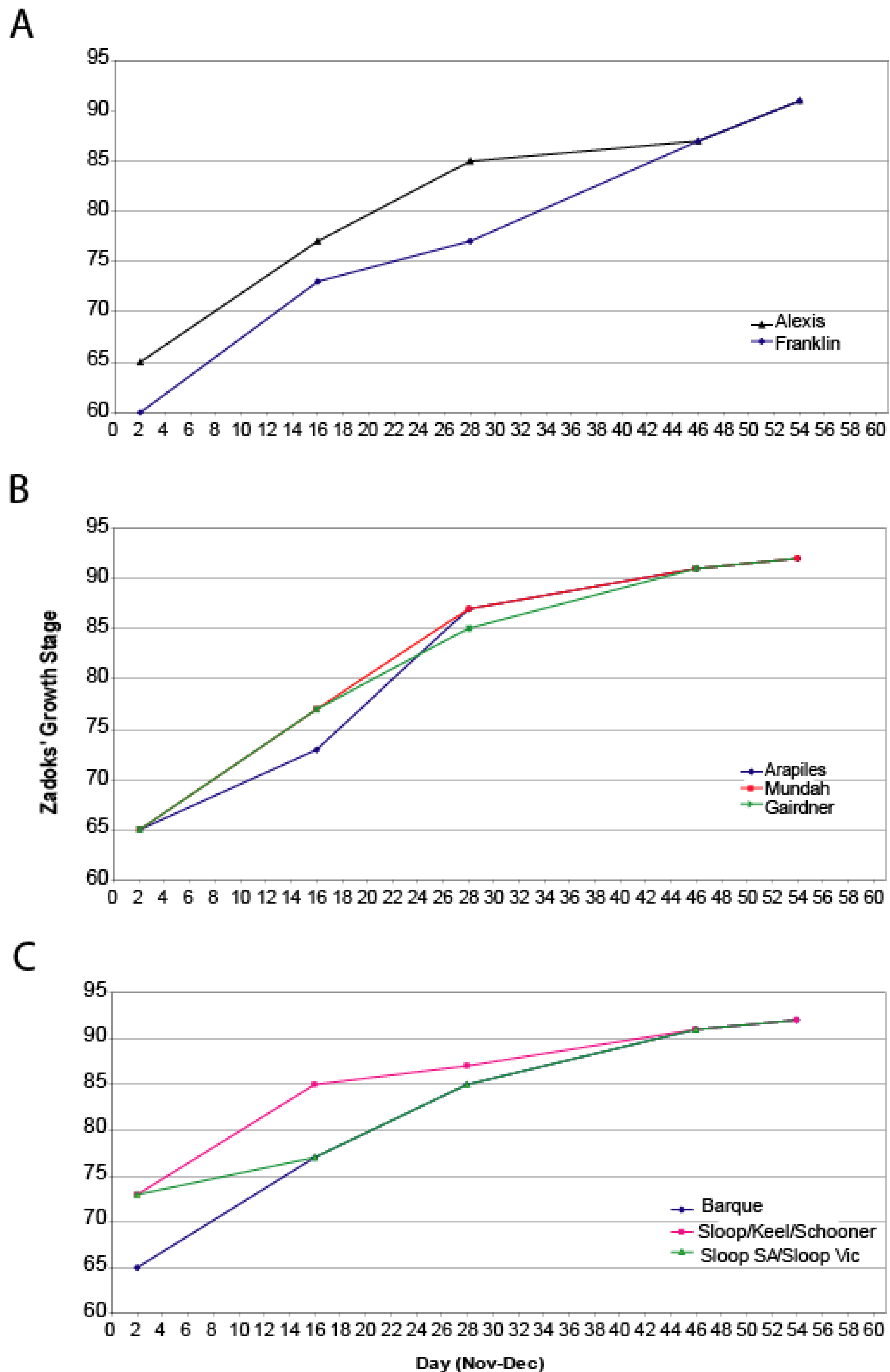

Figure 2.9 Association of maturity with black point susceptibility. Zadoks' growth stages were recorded on the 2nd, 16th and 28th of November and the 12th and 20th of December 2006 (days 2, 16, 28, 46 and 54). Each parent was categorised into normally <5% black point (A) (Alexis, Franklin), <10% black point (B) (Arapiles, Baudin, Gairdner, Mundah) and >15% black point (C) (Barque, Keel, Schooner, Sloop, Sloop SA, Sloop Vic). Categories were formed based on a problematic year (2001/2002) (Table 2.1).

Observations of the Port Wakefield data, showed the maximum humidity was lower than that measured at Hatherleigh in all years (Figure 2.10), except in 2006/2007 (Figure 2.8) where humidity was also mostly below 80% at Hatherleigh. Temperatures were similar to that of the 2004/2005 (Figure 2.6) and 2005/2006 (Figure 2.7) seasons at Hatherleigh. In the 2001/2002 season where significant black point scores were observed at Hatherleigh, the maximum temperature did not exceed 30ºC, indicating that the lower temperatures combined with the high humidity (not observed at Port Wakefield) were associated with black point formation.

To further analyse weather data across years at the Hatherleigh site, each aspect was categorised based on a set of arbitrary values (Chapter 2.2.4). For the grain fill periods of the 2001/2002, 2004/2005 and 2005/2006 seasons (Table 2.2) which were determined as per section 2.2.3, the maximum relative humidity was above 90% for a similar number of days. In 2001/2002, the total percent humidity above 90% for the grain fill period was less than that of 2004/2005 (Table 2.2) while there was a greater number of days with a humidity above 50% in the 2001/2002 season (when incidence of black point was highest) compared to other years (Table 2.2). Both maximum and minimum humidity were considerably lower in 2006/2007 (Table 2.2). The number of days on which the minimum temperature was above 10°C varied little between years. However, the number of days on which the maximum was above 20°C and the extent to which the maximum was greater than 20°C, reduced with black point symptoms. In 2001/2002, when symptoms were severe, the temperature exceeded 20°C on only 17 days compared to 40 days in 2006/2007, when only minimal symptoms were observed (Table 2.2). This categorisation was not analysed at the Port Wakefield site because with only one maturity point recorded during

the grain fill period (section 2.2.3), an accurate representation of the grain fill period could not be made.

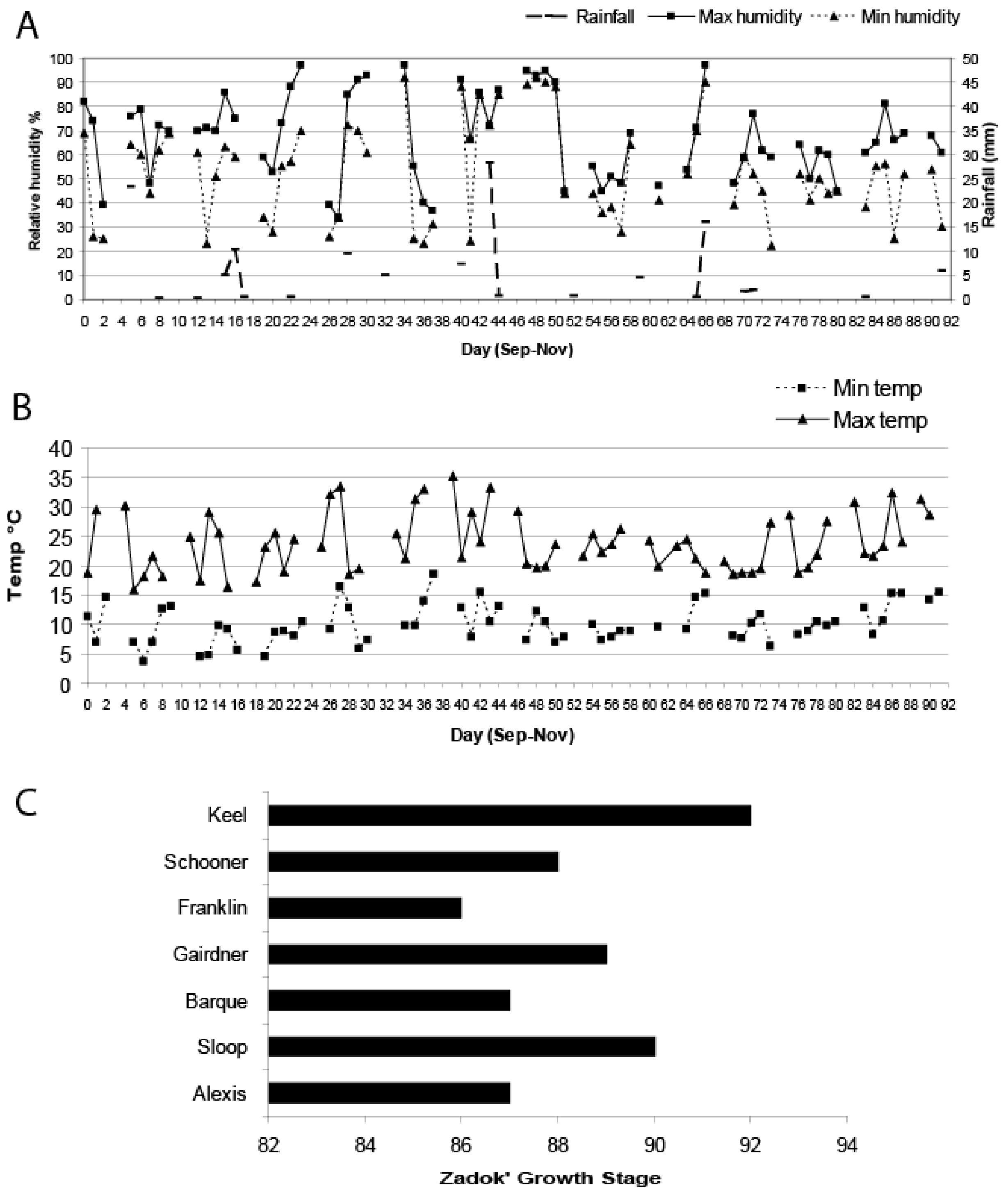

Figure 2.10 Weather conditions during the months of September to November at Port Wakefield in the 1999 season. Daily rainfall; maximum and minimum relative humidity (A); and maximum and minimum temperatures (B) for the months of September, October and November (Days 0-91), 1999. Data was collected from the Price weather station (BOM). Zadoks' scores of parents were recorded on the 30th of October 1999 (C).

Table 2.2 Numbers of days during grain fill (in November and December) with maximum temperature above 20°C, minimum temperature above 10°C, maximum relative humidity above 90% and minimum relative humidity above 50% in the 2001/2002, 2004/2005, 2005/2006 and 2006/2007 seasons. Data were collected from Mount Gambier for 2001 and 2006 and from Hatherleigh for 2004 and 2005. Numbers in parentheses indicate the cumulative number of degrees or percent humidity above these threshold values. Data was derived from data presented in Figures 2.6 (A and B) to 2.9 (A and B).

YEAR	**Days with maximum temperatures above 20°C** (total °C >20)	**Days with minimum temperature above 10°C** (total °C >10)	**Days with a maximum relative humidity above 90%** (total % > 90)	**Days with a minimum relative humidity above 50%** (total % > 50)
2001	**17** (48.3)	**17** (25.2)	**58** (311)	**41** (440)
2004	**45** (283.8)	**18** (44.8)	**60** (521.3)	**33** (421.1)
2005	**51** (326.4)	**20** (43.7)	**55** (440.1)	**20** (163)
2006	**40** (267)	**17** (52.7)	**38** (187)	**10** (82)

Because there appeared to be a relationship between black point formation and the occurrence of low temperatures with high humidity, the *VPD* was also determined. Vapour pressure deficit is a representation of the difference (deficit) between the amount of moisture in the air and the amount of moisture the air can hold when saturated (Prenger and Ling 2000). *VPD* was significantly greater in years in which black point formation was minimal (Figure 2.11).

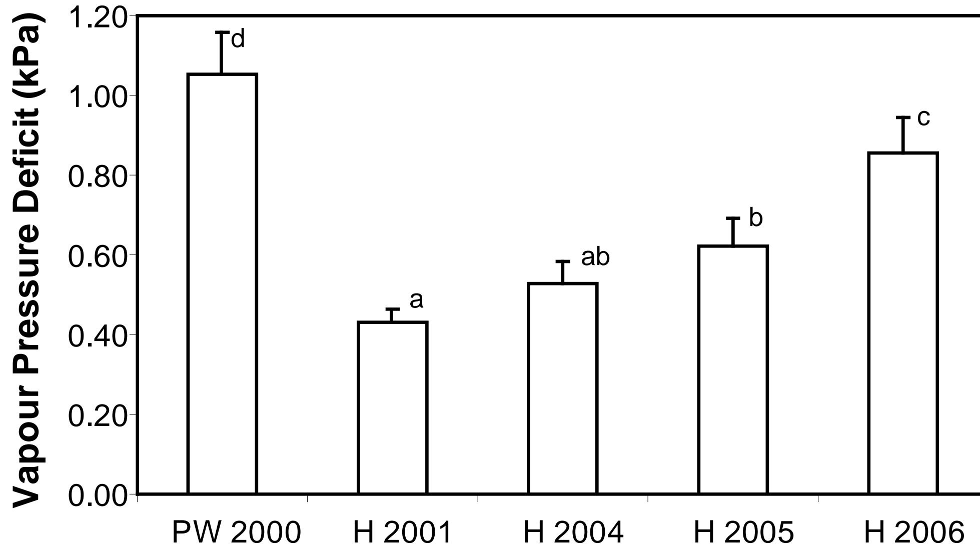

Figure 2.11 Mean vapour pressure deficit (kPa) for November and December at the Port Wakefield (P) and Hatherleigh (H) sites. Bars with similar letters are not significantly different. LSD=0.186 at P<0.001.

2.3.5 QTL identification

The mean black point scores of lines within the DH Alexis/Sloop mapping population grown at Port Wakefield in 1999/2000 and at Hatherleigh in 2004/2005 were distributed as shown in Figure 2.12. In both of these environments, neither of the parents and very few of the lines had black point scores exceeding the 10% threshold.

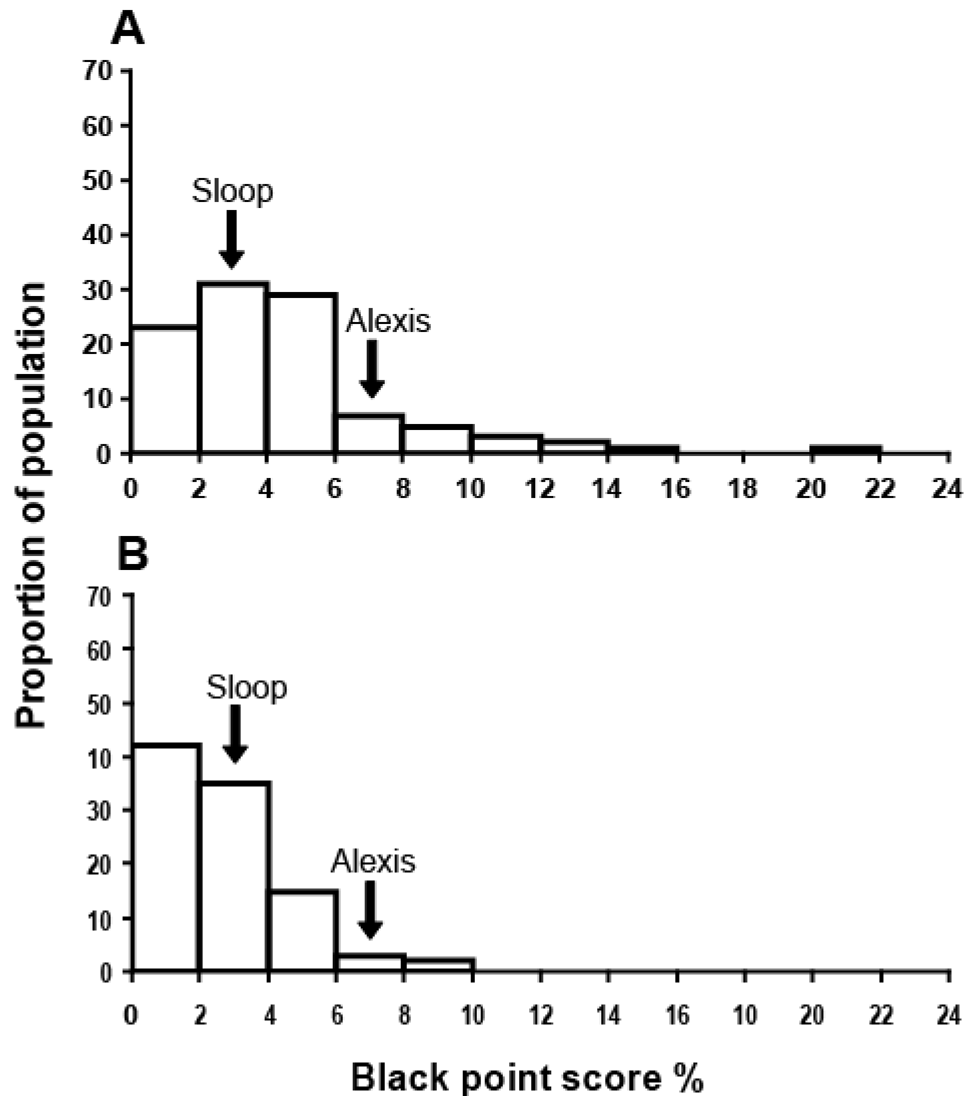

Figure 2.12 Frequency distribution of black point scores (represented as a %) in the Alexis/Sloop DH mapping population at Port Wakefield 1999/2000 (A) and Hatherleigh 2004/2005 (B). Tick marks indicate limits of the frequency classes. Black point scores for the parents, Alexis and Sloop, are indicated.

Using black point data from the Port Wakefield site (at which Sloop had less black point than Alexis), QTL were detected on chromosomes 1H, 2H and 3H (Figure 2.13). Alexis contributed to the QTL on chromosome 1H, which explained 14% of the phenotypic variance while the Sloop allele contributed to the QTL on chromosomes 2H and 3H explaining 16% and 11% of the phenotypic variance observed, respectively. The *earliness per se* locus (*eps2*) (Laurie *et al.* 1995) and the closely linked microsatellite marker EBmac684 both fall under the QTL on 2H for Port Wakefield in 2000 (Figure 2.13). The

QTL on chromosome 3H was also detected at Hatherleigh explaining 17% of the phenotypic variance, but the allelic effects were reversed, with the allele contributed by Alexis. Two other QTL were identified for the Hatherleigh site (on chromosomes 2H and 5H) (Figure 2.13). The Alexis allele contributed to these QTL on 2H and 5H explaining 15% and 14% of the phenotypic variance, respectively. The *denso* locus (*sdw1*) (Barua *et al.* 1993; Laurie *et al.* 1993) and the closely-linked abg4 marker (Hellewell *et al.* 2000) also appear to be closely linked to the black point QTL identified on chromosome 3H at the Port Wakefield and Hatherleigh sites.

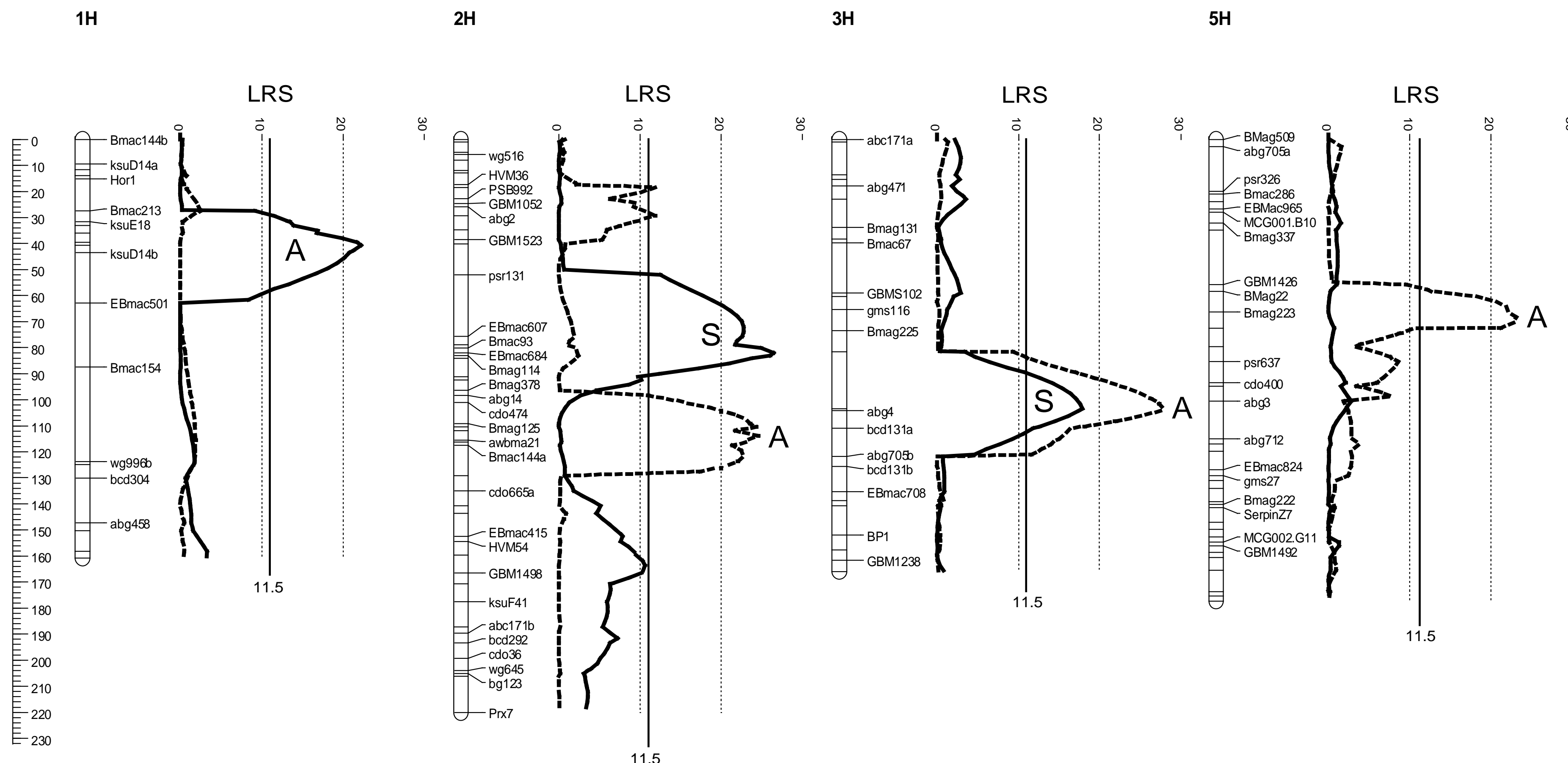

Figure 2.13 Likelihood ratio test statistics from composite interval mapping of black point incidence in the Alexis/Sloop DH population at Port Wakefield, SA in 1999/2000 (bold line) and Hatherleigh, SA in 2004/2005 (dashed line) showing QTL detected on chromosomes 1H, 2H, 3H and 5H. Distances within chromosomes are displayed in centimorgans (cM). At each QTL peak, the allele contributing to tolerance is identified as coming from Sloop(S) or Alexis (A). Significance thresholds set by permutation (LRS = 11.5 for both environments) are shown by solid vertical lines. For clarity, AFLP markers have been removed. The marker map is an updated version of those previously reported for the Alexis/Sloop DH mapping population (Barr *et al.* 2003; Willsmore *et al.* 2006).

QTL network allowed for a combined analysis to include the two environments and differing years. Two QTL were detected for black point, one on chromosome 2H (*QBpt.AlSl-2H*) and one on chromosome 3H (*QBpt.AlSl-3H*) (Figure 2.14), resulting in QTL in the same positions on chromosomes 2H and 3H as the individual site analysis using QTL cartographer. Additive by environment effects were important at both QTL. No QTL epistasis was detected. At *QBpt.AlSl-2H*, the additive by environment effect accounted for 10.6% of the phenotypic variance while the additive main effect accounted for only 2.4% of this variance (Table 2.3). At *QBpt.AlSl-3H*, the additive by environment interaction effect accounted for 7.8% of the phenotypic variance while there was no significant additive main effect (Table 2.3). At Port Wakefield in 1999/2000, the Sloop alleles at both QTL contributed to tolerance. At Hatherleigh in 2004/2005, the allelic effects were reversed so that the Alexis alleles contributed towards tolerance (Table 2.3) in a manner similar to the allele contributions in the individual site analysis. The position of *QBpt.AlSl-2H* corresponds closely with that of an *earliness per se* locus (*eps2*) (Laurie *et al.* 1995) and the closely linked microsatellite marker EBmac684 while the position of *QBpt.AlSl-3H* corresponds closely with the *denso* locus (*sdw1*) (Barua *et al.* 1993; Laurie *et al.* 1993) and the closely-linked abg4 marker (Hellewell *et al.* 2000). The QTL identified on chromosome 3H was therefore consistent using each method of analysis. Although the QTL detected on chromosome 2H for the individual analysis at Port Wakefield was in the equivalent position for the combined analysis, the second QTL detected at the Hatherleigh site no longer exists. The effect of the combined analysis resulted in the support interval being reduced from approximately 50 cM (Figure 2.13) to 5 cM (Table 2.3).

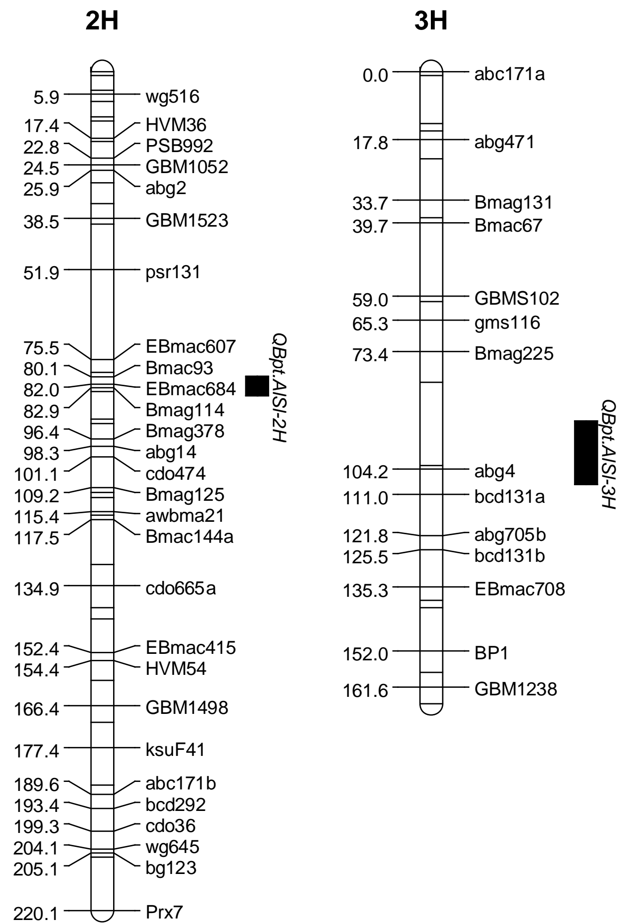

Figure 2.14 Linkage maps of chromosomes 2H and 3H showing the positions of QTL detected using QTL Network as affecting the incidence of black point in a DH population of barley derived from a cross between Alexis and Sloop (Barr *et al.* 2003; Willsmore *et al.* 2006). For clarity, AFLP markers are not labelled in this diagram.

Table 2.3 QTL detected as affecting tolerance to black point in a doubled haploid population of barley derived from a cross between Alexis and Sloop. Positive effects indicate that the Sloop allele contributed towards the tolerance, while negative effects indicate that the Alexis allele contributed towards tolerance. * indicates effects that are significant at an experiment-wise significance level of 0.05.

	Quantitative trait locus	
	QBpt.AlSl-2H	***QBpt.AlSl-3H***
Chromosome	2H	3H
Position	83.4 cM	102.6 cM
Support interval	80.5 to 85.5 cM	91.6 to 108.2 cM
Additive main effect (*a*)	0.45*	0.22
Additive by environment interaction effects:		
Port Wakefield 1999/2000 (ae_1)	0.90*	0.75*
Hatherleigh 2004/2005 (ae_2)	-0.90*	-0.79*
QTL heritabilities:		
Additive ($h^2(a)$)	0.024	0.004
Additive by environment ($h^2(ae)$)	0.106	0.078

2.3.6 The effect of planting date on black point formation

Because the timing of environmental conditions as it relates to grain maturity appears to influence black point formation (Figure 2.9), the effect of planting date was examined. In the 2006 season (Figure 2.15A), when Sloop was planted one month later than normal sowing date, the black point score was significantly lower than Sloop planted at the normal sowing date and Sloop planted two weeks after the original sowing. The black point scores for Sloop planted one month later were not significantly different to the Alexis control

planted at the normal sowing date. The 2007 season resulted in very low black point scores that were not statistically different (Figure 2.15).

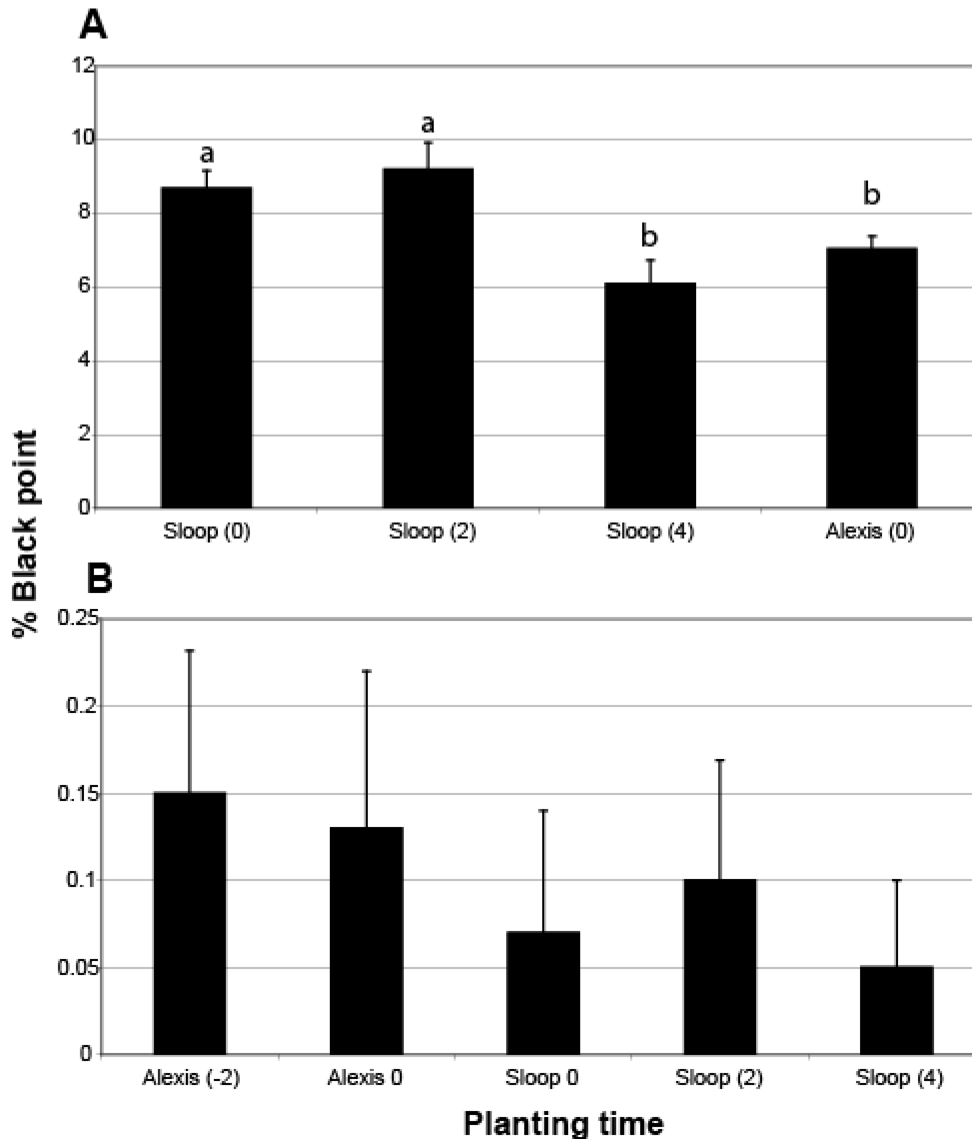

Figure 2.15 The effect of planting date on black point formation. Value in brackets after cultivar denotes the number of weeks different to the usual planting date. In the 2005/2006 season (A), Sloop was planted at the original planting date (0), two weeks (2) or four weeks (4) after the original planting date. In the 2006/2007 season (B), Alexis was also planted two weeks before the original planting date (-2). Means ± SE are represented for (A) n=50 for Alexis/Sloop (0) and n=20 for Sloop (2) and Sloop (4) and (B) n=15 for Alexis/Sloop (0) and n=20 for the remaining treatments. In the 2005/2006 season, bars with similar letters are not significantly different. LSD=1.271 at P<0.001. Scores in the 2006/2007 season were not significantly different.

2.4 Discussion

The incidence of black point in Australian barley crops varies among seasons and seems to depend upon seasonal conditions. Black point occurs most severely in Queensland but is also found in Western Australia, South Australia, Victoria and northern New South Wales. Although anecdotal evidence has suggested that high humidity plays a role in black point formation, there have been limited studies in Australian conditions confirming this role (Sulman *et al.* 2001a). This study therefore aimed to simulate the humid conditions thought to induce the disorder and establish the role of the environment and genotype in black point formation. In particular, the symptoms of black point of barley have now been clearly defined; the environmental influences in South Australian conditions established; QTL for black point tolerance identified and a potential role for maturity considered.

Black point has at times been considered synonymous with 'kernel discolouration' (de la Penna *et al.* 1999) or as a type of kernel discolouration (Li *et al.* 2003). Here, we have clearly defined black point of barley and consider kernel discolouration and black point to be two distinct phenomena. Black point describes darkening at the embryo end of the grain while kernel discolouration (also referred to as weather staining) involves a caramelisation or darkening of the whole grain, with the extreme form of greyish hue or distinctive spots appearing on the grain as visible mould formation (Li *et al.* 2003).

Composite interval mapping of the Alexis/Sloop DH mapping population has identified QTL for black point tolerance on chromosomes 1H, 2H and 3H for the Port Wakefield site and chromosomes 2H, 3H and 5H for Hatherleigh. However, there was a difference between sites for genetic contribution by the parents to the QTL even though neither parent exhibited what is regarded as susceptibility at either site (Table 2.1). The normally

susceptible cultivar (Sloop) was shown to contribute to each QTL at the Port Wakefield site explaining 11 to 16% of the variance observed, except in the case of the QTL on chromosome 1H at which the usually tolerant variety Alexis contributed, explaining 14% of variance observed. The tolerant variety (Alexis) contributed to all QTL identified at the Hatherleigh site in 2004/2005, explaining between 14 to 17% of the variance observed. Given that both parents exhibited few symptoms (both below the 10% threshold) for susceptibility under the Port Wakefield conditions but were clearly differentiated at the Hatherleigh site in 2001/2002, the results from Hatherleigh showing Alexis contributing alleles for black point tolerance are likely to be more relevant to seasons in which black point is problematic and tolerant varieties are required.

Analysis to incorporate both environments resulted in refinement to two QTL, on chromosome 2H (*QBpt.AlSl-2H*) and on chromosome 3H (*QBpt.AlSl-3H*). Additive by environment effects were important at both QTL. At *QBpt.AlSl-2H*, the additive by environment effect accounted for 10.6% of the phenotypic variance while the additive main effect accounted for only 2.4% of this variance (Table 2.3). At *QBpt.AlSl-3H*, the additive by environment interaction effect accounted for 7.8% of the phenotypic variance while there was no significant additive main effect (Table 2.3). These results are a strong indication that the environment has a significant influence on black point formation as was expected. At Port Wakefield in 1999/2000, the Sloop alleles at both QTL contributed to tolerance. At Hatherleigh in 2004/2005, the allelic effects were reversed so that the Alexis alleles contributed towards tolerance (Table 2.3), indicating that the Alexis allele contributed the tolerance in the problematic environment.

Black point in wheat has been reported to be affected by QTL on chromosomes 1D, 2B, 3D, 4A, 5A and 7A in a Sunco x Tasman-derived population and on 2A, 2D and 7A in a Cascades x AUS1408-derived population with each QTL explaining up to 18% of the observed phenotypic variance (Lehmensiek *et al.* 2004). Given the high levels of synteny and sequence similarity between chromosome 2H in barley and chromosome 2B in wheat (Dubcovsky *et al.* 1996) and the identification of QTL in the same regions, it is plausible to suggest that the underlying genes for barley and wheat black point formation are similar (if not the same).

QTL from combined analysis detected in this study correspond in position with QTL that have previously been reported for kernel discolouration in the same population. Using measures of grain brightness to assess tolerance to kernel discolouration, Li *et al.* (2003) also detected QTL on chromosomes 2H and 3H near the markers EBmac684 and abg4 respectively (Li *et al.* 2003). Alexis contributed the alleles for tolerance in both of these chromosome regions in both studies. Further similarities between black point and other forms of kernel discolouration can be seen by considering the environmental conditions under which they tend to occur. A greater incidence of kernel discolouration has been associated with high relative humidity late in the grain filling stage and a high incidence of rainy days until harvest (Young 1997), while an allele for grain brightness was also associated with the late heading date (Li *et al.* 2003). This is similar to the observation that the late maturing variety Alexis is tolerant to black point. Although the black point symptoms that were assessed for this study are clearly distinct from the kernel discolouration symptoms that have been assessed elsewhere, the two conditions may share common biochemical pathways, may be affected by some of the same genes and seem to be favoured by similar environmental conditions. In particular, with QTL in similar

positions for black point and kernel discolouration, the underlying genes contributing to these two traits may be the same. However, given that black point is confined to the embryo end of the grain whereas kernel discolouration is indiscriminate, the mechanism by which black point and kernel discolouration are expressed (to display their symptoms) are evidently different.

Developmental loci in barley include a photoperiod response gene (*Ppd-H1*) (Laurie *et al.* 1994), an *earliness per se* locus (*eps2*) (Laurie *et al.* 1995) and a plant stature locus (*denso*) (Barua *et al.* 1993; Laurie *et al.* 1993), all of which segregate in the Alexis/Sloop DH population (Coventry *et al.* 2003). The discovery of QTL contributing to black point tolerance in the same regions supports a link with maturity and suggests a potential connection with plant stature, with Sloop displaying a tall early flowering phenotype compared with the semi-dwarf, later flowering Alexis. The QTL identified on 3H aligns with the marker abg4 which is closely linked to the plant stature locus, *denso*. Even though semi-dwarf varieties would probably maintain a humid micro-climate, the semi-dwarf Alexis is considered not susceptible, suggesting that the timing of flowering and/or grain development may be more important for black point formation. The QTL identified on 2H falls in the position of the *eps2* locus, suggesting a role in maturity. This finding supports the results obtained, that susceptible varieties were found to mature earlier (Figure 2.9). Further evidence of the importance of maturity was provided by the observation that when planting dates were altered so that the tolerant cultivar Alexis and the susceptible cultivar Sloop matured at the same time, they had similar black point scores. This data alone therefore suggests that there is a strong genotype by environment interaction and that the timing of environmental effects during grain fill is likely to be important in inducing symptoms.

Comparison of weather data during the years that field trials were grown has established the likely conditions required for black point formation. Previous reports have indicated that the intensity and frequency of black point in wheat (Rees *et al.* 1984) and barley (Sulman *et al.* 2001a) increases with the occurrence of moist and humid conditions during grain fill and ripening. Kernel discolouration has also been associated with high relative humidity late in the grain filling stage and with a high incidence of rainy days prior to harvest (Young 1997; Hudec 2007). A high incidence of black point was observed in 2001/2002 at the Hatherleigh site under high humidity, an increased *VPD* and relatively low temperatures. Fewer symptoms were observed in other environments where even though there was a similar maximum humidity, the minimum and maximum temperatures were greater; this was again supported by a decrease in *VPD* values correlating with lower black point scores. The combination of low temperature and high humidity may be important therefore in favouring the formation of black point.

Rain events during the grain fill period may also play a role (particularly towards the end). Early rainfall events (Days 0 to 14) occurred in the 2001/2002 season when black point was severe. It is likely that this was when the susceptible cultivars were entering the milk to soft dough stages of grain development (Figure 2.9C), suggesting that rainfall during the early stages of grain fill could be a contributing factor. Indeed, it has been shown that in spring wheat, black point was significantly increased when irrigation was applied during the milk or mid-dough stages (Conner 1987). The milk and dough stages occurred earlier in Sloop than in Alexis (Figure 2.9C) such that the later rainfall events could also be associated with soft dough stage and hard dough stage. These rainfall events could also be associated with black point formation (Figure 2.5, day 41*)* in a year where black point was

prevalent. This confirms the earlier discussion that the environmental conditions at a specific time in development could be crucial to black point formation.

Further evidence that the temperature and not just humidity plays a role was provided by the controlled environment experiments. Due to issues with the availability of growth chamber space, plants were grown in parallel with rice plants which are commonly grown at high temperatures. Although the humidity was high to theoretically suit the formation of black point, the conditions (temperature) did not allow healthy growth of the plants. Similarly when plants were contained within plastic in the glass house to try and mimic a humid environment, the area was very small and there was not sufficient air flow. This resulted in mould formation within the chamber and on the plants themselves. Although a field screening method using a high humidity tunnel with overhead and ground irrigation has been previously described, issues with maintenance of temperature still remain (Sulman *et al.* 2001a). Further research is needed to confirm that the low temperature and high humidity association can be recreated in a simulated environment satisfactorily. The establishment of high humidity and associated low temperatures in a growth chamber and the simulation of rainfall events throughout the grain fill period would allow us to determine if a rainfall event at a specific stage of development is also contributing to black point severity.

In conclusion, genotypic and environmental factors have been found to contribute to the severity of black point. QTL for black point tolerance on chromosomes 2H and 3H have been identified. The association of QTL on chromosome 2H with the *eps2* locus has provided apparent evidence for the impact of maturity, further supported through the establishment of contributing environmental conditions, and demonstration that early

maturing varieties are more susceptible to black point. High humidity associated with low temperatures (or a low *VPD*) appears to induce black point symptoms in susceptible varieties, possibly as a result of stress or wounding to the embryo through the creation of a moist environment in which the grain cannot dry out. Disruption or wounding in the barley grain during grain filling and ripening has been proposed to allow the release of peroxidases from the germ aleurone such that they react with phenols (Cochrane 1994a; Cochrane 1994b). Given that black point is confined to the embryo end of the grain, it is likely that wounding and subsequent oxidation of phenols by peroxidases is also confined to that region of the grain. Although general environmental trends have been established, further research consisting of more detailed maturity studies is required to precisely determine if a specific event (such as rainfall) is triggering symptom development at a specific developmental stage. The conditions identified will allow a starting point for simulation experiments so that barley may be easily screened during breeding for black point and the physiological basis for black point studied. Studies of the impact of maturity in barley black point formation and comparisons with kernel discolouration and the disorder in wheat could also occur. A genetic basis for black point formation has been identified, yet the environmental effects contribute extensively to severity. Understanding what regulates the expression of black point formation will therefore be important in understanding how the environment impacts upon it.

Chapter Three. Characterising expression of candidate genes in black point formation

3.1 Introduction

The research presented in the previous chapter has established that high humidity and low temperatures during the grain-fill period contributed to black point formation in susceptible varieties of barley. Given that black point is probably a form of enzymatic browning; a number of genes could be involved in black point formation including those that encode for polyphenol oxidase and peroxidases proposed to have a role in browning; phenylalanine ammonia-lyases which are expressed in response to different stress stimuli as observed in rice (Sarma and Sharma 1999); quinone oxidoreductase which has been proposed by March *et al* (2008) to act as a defence mechanism in response to wounding within the grain; and lipoxygenases where gene expression is regulated by different forms of stress, such as wounding, water deficiency or pathogen attack (Porta and Rocha-Sosa 2002). Polyphenol oxidase activity in mature grains is barely detectable with no differences between tolerant and susceptible varieties or between black pointed and healthy grain (Hadaway, *unpublished data*). On this basis polyphenol oxidase was not investigated as a candidate gene in the research presented here.

Given that peroxidases with a higher isoelectric point have only been found in varieties susceptible to black point (Hadaway *et al.* 2003), peroxidases are likely to have a role in black point formation. The peroxidase genes Barley Peroxidase 1 (*HvBP1*; Accession: M73234) (Rasmussen *et al.* 1991) and Peroxidase 7 (*HvPrx7*; Accession: AJ003141) (Kristensen *et al.* 1999) have both been cloned and sequenced in barley and found to be

expressed within the grain. *HvBP1* has been characterised to be highly tissue-specific, occurring maximally in the endosperm 15 days after flowering (Rasmussen *et al.* 1991). However, its expression in different barley varieties during grain development has not been previously examined. Using a proteomics approach, *HvBP1* was also identified as present in black pointed grain and not healthy grain of the susceptible cultivar, Sloop (March *et al.* 2007). Preliminary analysis has suggested that *HvPrx7* and *HvBP1* are expressed for longer in susceptible varieties (March 2003). *HvBP1* and *HvPrx7* would therefore be ideal candidates for a role in black point formation.

Genetic mapping studies within this project (Chapter 2, Figure 2.14) have identified a putative QTL for barley black point on chromosome 2HS supporting previous studies identifying QTL in the same location for black point and kernel discolouration (de la Penna *et al.* 1999; Hadaway 2002; Li *et al.* 2003). March *et al.* (2008) have used *in silico* comparative mapping between barley and rice to identify candidate genes with proposed roles in enzymatic browning from this region, including a phenylalanine ammonia lyase (*HvPAL*, Accession: AB367438.1) and a quinone reductase (*HvQR*, Accession: AJ474981). Because these genes fall within the *QBpt.AlSl-2H* QTL, characterisation of their expression will provide insight with regards to their proposed roles in black point formation.

Lox genes, and in particular, *HvLox1*, is an ideal candidate in black point formation because Lox gene expression is regulated by different forms of stress, such as wounding (Porta and Rocha-Sosa 2002) and more specifically, *HvLox1* has been shown to accumulate in the later stages of grain development (Schmitt and Van Mechelen 1997) when black point symptoms also typically occur. An increase in Lox1 activity in

association with the production of hydrogen peroxide in response to stress/wounding (Porta and Rocha-Sosa 2002) also provides a direct link with the enzymatic browning model (Figure 1.2).

The aims of the research presented in this chapter therefore were to characterise *HvBP1*, *HvPrx7*, *HvPAL*, *HvQR* and *HvLox1* gene expression during grain development in cultivars of varying susceptibilities to black point. Differential gene expression between susceptible and tolerant cultivars may allow for a potential breeding target in the future. If differential expression was established, gene expression was further examined within healthy and black pointed grains.

3.2 Materials and methods

3.2.1 Plant material and sampling

Plant material available from the field experiments described in section 2.2.2 was used to characterise gene expression. The tolerant varieties Alexis, Arapiles, Baudin, Franklin, Gairdner, Mundah and susceptible varieties Sloop, Barque, Fitzroy, Golden Promise, Keel, Schooner, Sloop SA, Sloop Vic and VB9935 (Chapter 2 and Walker *et al.* (2008)) were grown in field experiments at Hatherleigh (140°16" E, 37°29" S; in south-eastern South Australia; Figure 2.2) in the 2005/2006 season. Grain was sampled randomly from three plots for Sloop and Alexis and from two plots for other varieties when plants were at Zadoks' growth stages 75 (medium milk), 85 (soft dough), 87 (hard dough) and stage 95 (maturity) (as per Figure 2.3 and section 2.2.3) (Zadoks *et al.* 1974). At each stage up to 12 individual heads were removed from the main stem using scissors into a 50 mL Falcon

tube, snap frozen using liquid nitrogen in the field and then packed into dry ice for transportation to Adelaide. Samples were stored at -80°C until required. Leaf tissue was also sampled from plant material grown in the glass house (section 2.2.1), samples were snap frozen using liquid nitrogen and stored at -80°C until required.

3.2.2 Establishing differential gene expression using northern analysis

3.2.2.1 RNA isolation and gel electrophoresis

TRIzol® (Invitrogen) was used for all RNA isolation. The method used was adapted from the protocol supplied by the manufacturer. Whole barley grains were ground in liquid nitrogen using the IKA® A11 basic analytical mill (IKA Works, Pataling Jaya Selangor, Malaysia). Leaf tissue was snap frozen in a 10 mL microcentrifuge tube containing 2 mm ball bearings and vortexed until ground to a fine powder. Ground tissue (approximately 8-10 heads for grain and 10-12 leaves for leaf tissue) was transferred to a 10 mL tube (pre-chilled in liquid nitrogen) to which 5 mL of TRIzol® was added, vortexed for 1 min following incubation at room temperature for 5 min and centrifuged at 4000 *g* for 45 min at 4°C. The supernatant was removed to a fresh 10 mL tube to which 1 mL of chloroform was added. The tube was shaken vigorously for 15 sec and then incubated at room temperature for 10 min before being centrifuged again at 4000 *g* for 45 min at 4°C. The clear supernatant was transferred to two microfuge tubes (≈1 mL per tube) making sure not to take any of the interphase. RNA was precipitated by the addition of 450 µL of isopropanol and 450 µL of 1.2 M NaCl to each tube. Tubes were shaken to mix, and incubated at room temperature for 10 min before centrifuging at 12000 *g* for 10 min at 4°C. The supernatant was removed and the remaining pellet washed in 2 mL of 75%

ethanol and then centrifuged at 7000 *g* for 5 min at room temperature. The supernatant was carefully removed using a pipette and the pellet air dried for 5 min before being resuspended in 50 µL of 100mM Trizma hydrochloride (Tris-Cl), 10 mM Ethylenediamine tetra acetic acid (EDTA, pH 8.0) (TE) buffer. To pellet any insoluble material such as polysaccharides, tubes were incubated at 65°C for 15 min and then centrifuged at 12000 *g* at 4°C for 5 min. The supernatant containing RNA was transferred to a 1.5 mL microfuge tube. RNA concentration and purity was determined by measuring the absorbance of a 1/100 dilution at the wavelengths of 260 nm and 280 nm on a UV/VIS SP8001 spectrophotometer (Metertech). RNA concentration was calculated using the following formula:

Concentration (µg/µL) = Absorbance at 260 nm x dilution factor / 25

Purity was determined by dividing the absorbance at 260 nm by that at 280 nm and samples were only used if above 1.8. RNA quality was also assessed by agarose gel electrophoresis. Agarose gels (1.5% w/v) were prepared by boiling 250 mL agarose (Promega, Madison, WI, USA) in 1x 2 M Tris-acetate, 50 mM EDTA, pH 8.0 (TAE) buffer, allowed to cool and 1.5 µL of ethidium bromide added for staining purposes. This was mixed and immediately poured into the gel mould and left to set for 30 min for horizontal gel electrophoresis using horizontal gel tanks (EasyCast Electrophoresis Systems, OWL Scientific Inc., Cambridge, UK) in 1 X TAE buffer. Gels were electrophoresed at 100 V for 45 min before visualisation and photographed under a short wavelength UV transilluminator (BioDoc-*It*™ Imaging System). RNA was stored at -80°C until required.

3.2.2.2 Probe preparation for northern blotting

The full length cDNA sequences of peroxidase genes *HvBP1* (Accession: M73234), *HvPrx7* (Accession: AJ003141) and *HvLox1* (Accession: L35931) were obtained from the National Centre for Biotechnology Information NCBI database (www.ncbi.nlm.nih.gov/ version 2.2.10 of BlastN, October 2004). Similarity in sequences between peroxidases that have been cloned, sequenced and found in grain or germinating tissue (*HvBP1:* M73234, *HvBP2:* Z23131, *HvBP2A:* M83671, *HvPrx7:* AJ003141 and *HvPrx8:* X62438) and other identified *Lox* genes (*HvLox1:* L35931 and *HvLox2:* L37358) were established through alignment using Vector NTI 9.0 software (InforMax, USA). Primers were designed to non-conserved regions specific to each of the candidates identified in Table 3.1 to amplify cDNA probes for northern blotting. Probes for candidate genes *HvPAL* (Hv.t49) and *HvQR* (Hv.t38) identified through comparative mapping studies were kindly provided by Tim March (March *et al.* 2008).

Table 3.1 Primers designed to amplify probes for candidate genes *HvBP1, HvPrx7* and *HvLox1*. Forward (F) and reverse (R) primers and the melting temperature (Tm°C) used for amplification are indicated. Probe sizes are indicated in base pairs (bp).

Candidate	Primer	Tm (°C)	Size (bp)
BP1	**F** 5′ CACACACAAAGGAGAGAGGAGATGGCTCG 3′	55	195
	R 5′ CAGTCGTGGAAGTGGAGTCGAAGGAGG 3′		
Prx7	**F** 5′ AACCAGGGCGCTTTCTTCGAGCAGTT 3′	58	368
	R 5′ TGGCTAGACATCACACTTCCACGATTCAAAG 3′		
Lox1	**F** 5′ GGCGGCGACTCCCTGCTTAA 3′	58	444
	R 5′ CCTTGCTCTTGGCCGTGGTAAG 3′		

Probes were amplified from cDNA (from leaf tissue) using high fidelity DNA polymerase and Elongase® Enzyme mix (Invitrogen) as per the manufacturer's instructions. cDNA synthesis was performed using SuperScript III (Invitrogen), according to the manufacturer's instructions. Total RNA (1 µg) extracted from leaf material as per section 3.2.2.1 was used in a final reaction volume of 25 µL for the first strand cDNA synthesis. PCR of the product of this reaction using the primers designed to *HvBP1, HvPrx7* and *HvLox1* resulted in a probe length of 195 bp, 368 bp and 444 bp respectively (Table 3.1).

The PCR mixture contained Buffer A (4 µL), Buffer B (6 µL) (1.6mM Mg^{2+}), dNTPs (10mM, 1 µL), the forward and reverse primer combinations identified in Table 3.1 (10mM, 1 µL), cDNA (1 µL), Elongase® enzyme mix (1 µL) and nanopure water (36 µL). The cycling conditions were 94°C for 2 min; then 35 cycles of 94°C for 30 sec, *x*°C (Tm as per Table 3.1) and 1 min at 68°C; followed by 68°C for 5 min. Bromophenol blue loading dye (6 X) (Promega, Madison, WI, USA) was then added to the PCR samples to a final concentration of 1 X and the PCR products separated by horizontal gel electrophoresis (1.5% agarose, w/v) as per section 3.2.2.1 to ensure the products were of the correct size. After approximately 30 min, PCR products were visualised and photographed under UV light (BioDoc-*It*™ Imaging System).

Products of the expected size were excised from the agarose gel using a scalpel blade with the aid of UV light. The fragments were purified using the Wizard®SV Gel and PCR Clean-Up System (Promega) as per manufacturer's instructions.

Purified PCR products were ligated into the pDrive cloning vector according to the manufacturer's instructions (Qiagen, Hilden, Germany). Reactions consisted of 2 X buffer

(5 µL), PCR product (4 µL) and pDrive (1 µL) to a total volume of 10 µL and were incubated at 4°C overnight to maximise ligation efficiency.

The *Escherichia coli* strain DH5-α was used for all bacterial transformation experiments using heat shock methods, in which competent cells were prepared as per the manufacturer's instructions (Promega). Ligated products (10 µL) were added to 50 µL of competent cells, incubated on ice for 15 min, heat shocked at 42°C for 30 sec followed by incubation on ice for 2 min. Transformed cells (100 µL) were plated onto Luria Bertani (LB) + ampicillin (100 µg μL^{-1}) + isopropyl-beta-D-thiogalactopyranoside (IPTG, 100 µL of 100 µM) + 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal, 40 µL of 20 mg mL^{-1}) selection plates. Plates were incubated for 16 to 24 h at 37°C, then at 4°C for 30 min to enhance the blue colour development of colonies not containing inserts.

Colony PCR was used to ensure only colonies with the correct size insert were chosen for subsequent sequencing and probe preparation. A sterile 100 µL pipette tip was used to remove the desired colony (white). A reference plate (incubated at 37°C) was created by touching the same tip on a LB/ampicillin/X-gal plate and into a subsequent Go-Taq® PCR mixture (12 µL) (Promega) containing Go-Taq® (6.25 µL); T7 primer (5' TAATACGACTCACTATAGGG 3'; 10 µM, 1.25 µL); SP6 primer (5' ATTTAGGTGACACTATAGAA 3'; 10 µM, 1.25 µL) and sterile nanopure water (3.25 µL). Cycling conditions comprised of 94°C for 2 min (1 cycle), 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min (35 cycles) and finally 72°C for 10 min.

A 100 µL pipette tip was used to inoculate a 5 mL culture (LB broth containing 100 µg mL^{-1} ampicillin) from the reference plate prepared, using positive colonies identified

through colony PCR. Tubes were incubated at 37°C overnight (16 h) with shaking (150 rpm). Cultures were centrifuged at 5000 *g* for 10 min followed by DNA plasmid mini-preparations using Wizard® Plus SV Minipreps DNA purification system (Promega) according to the manufacturer's instructions. DNA was eluted in 30 µL of nanopure water for sequencing.

Transformed PCR products were sequenced by The Australian Genome Research Facility (AGRF) using BigDye™ chemistry (Applied Biosystems, Foster City, CA, USA). Preparation of purified DNA samples was achieved as per the manufacturer's instructions (AGRF, http://www.agrf.org.au). Reactions containing 500 ng of template, 1 µL of forward (T7) or reverse (SP6) primer (6.4 µM) and sterile nanopure water to a total volume of 13 µL were prepared in a 1.5 mL microfuge tube before sending to AGRF for sequencing. Vector NTI Advance™ 10 (Invitrogen) was used for sequence analysis, using the Contig Express element of the software to align and assemble sequencing reactions, ensuring the correct sequences were amplified.

3.2.2.3 Northern blot

RNA from each of the four grain developmental stages of the varieties described in Section 3.2.1 was used for northern analysis. Gel electrophoresis was undertaken as per section 3.2.2.1, using denaturing agarose gels. Denaturing agarose gels were prepared by boiling 1.8 g of agarose (Promega, Madison, WI, USA) in 127.5 mL nanopure water and 15 mL of 10 X MOPS buffer [0.2 M MOPS (pH 7.0), 20 mM sodium acetate, 10 mM EDTA (pH 8.0)]. Once cooled to ~60°C, 7.5 mL of 37% formaldehyde and 4 µL of 10 mg/µL

ethidium bromide were added. This was mixed and immediately poured into the gel mould and left to set for 30 min for horizontal gel electrophoresis as per section 3.2.2.1.

RNA samples were prepared by adding 2 µL of 10 X MOPS buffer, 3.5 µL formaldehyde, and 10 µL formamide to 10 µg of total RNA. Samples were heated at 65°C for 15 min and loading dye (6 X) (Promega, Madison, WI) was added to a final concentration of 1 X. Electrophoresis was carried out in 1 X MOPS buffer at 60 V for 30 min, then 100 V for a further 90 min. Ribosomal RNA bands were visualised under UV light (BioDoc-*It*™ Imaging System).

RNA transfer was performed using downward capillary transfer (Sambrook and Russell 2001). Total RNA was transferred to Hybond-N^+ membrane (Amersham Biosciences, Australia) overnight and cross-linked using a GS GENE LINKER™ (BIO-RAD, Richmond, CA, USA). Membranes were pre-hybridised in 5 mL of hybridisation buffer for 4 h at 68°C (in a hybridisation bottle) in a hybridisation oven with rotation.

Probes (prepared as per section 3.2.2.2) were radioactively labelled with P^{32} (GE Healthcare) using Ready-To-Go DNA Labelling Beads (GE Healthcare) as per the manufacturer's instructions. Labelled probes were denatured for 2 min at 95°C and added to 20 mL of hybridisation buffer [0.5 M sodium phosphate (pH 7.4), 7% (w/v) sodiumdodecylysulfate (SDS), and 1 mM EDTA (pH 7.0)], which was subsequently added to the labelling beads (GE Healthcare).

The P^{32} labelled probe was denatured for 5 min at 95°C and placed on ice for a further 2 min before being added directly to the hybridisation bottle containing the membrane and hybridisation buffer. Hybridisation was overnight at 68°C.

The following day, membranes were washed with 2 X SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 0.1% SDS (w/v); 1X SSC, 0.1% SDS; and 0.5 X SSC, 0.1% SDS at 68°C for 20 min each. Membranes were subsequently exposed to Hyperfilm™ MP autoradiography film (Amersham Pharmacia Biotech, Buckinghamshire, England) in an autoradiograph cassette containing an intensifying screen at -80°C for three days. Films were developed using a CP1000 developer (AGFA-Geveart Group, Mortsel, Belgium).

3.2.3 Confirming differential gene expression using quantitative real time PCR (qPCR)

qPCR was used to further characterise the differential expression of *HvBP1, HvPrx7* and *HvQR* observed between the black point susceptible cultivar Sloop and tolerant cultivar Alexis using northern analysis.

3.2.3.1 qPCR Probe design

Probes for qPCR were designed from unique regions (3′) of the candidate genes *HvBP1, HvPrx7* and *HvQR* with the aim of obtaining products between 150 and 300 bp. Primer 3 (www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) was used for primer design and NetPrimer (www.premierbiosft.com/netprimer/netprlaunch/netprlaunch.html) to test primer quality through prediction of primer dimers and hairpin loops. Primers (Table 3.2)

designed to *HvBP1, HvPrx7 and HvQR* resulted in a probe length of 246 bp, 307 bp and 240 bp respectively (Table 3.2).

Table 3.2 qPCR primers designed to amplify probes for candidate genes *HvBP1, HvPrx7* and *HvQR*. Forward (F) and reverse (R) primers and the melting temperature (Tm°C) used for amplification are indicated.

Candidate	Primer	Tm (°C)	Size (bp)
BP1	**F** 5′ CCCACCATAAGCCCCACCTT 3′	55	246
	R 5′ ATGAGGGTCCGCACCAGC 3′		
Prx7	**F** 5′ CGTGCCCACCCTCATCATCTCCTCCTT 3′	55	307
	R 5′ GCCCTGGTCCGACTTGAACA 3′		
QR	**F** 5′ GAAGGGCGACTATGTCTTTGTGT 3′	55	240
	R 5′ CCCACGTTCTCGAAGTAGATGT 3′		

Probes were amplified as per section 3.2.2.2, purified using High Pressure Liquid Chromatography (HPLC) as detailed by Burton *et al.* (2004) and sequences confirmed as per section 3.2.2.2.

3.2.3.2 RNA isolation and cDNA synthesis

RNA from grain at each of the four stages of grain fill from Sloop and Alexis was extracted using TRIzol® (Invitrogen) as per section 3.2.2.1. The exception was that tissue was added to a 2 mL tube to equal roughly 0.2 to 0.5 cc of volume and 1 mL of TRIzol® reagent used. cDNA synthesis reactions were performed as per section 3.2.2.2. cDNA (1 µL) was firstly checked for quality using Go-Taq® PCR mixture (Section 3.2.2.2) containing *HvGAPDH* primers (Forward-5’ACAAGCTTGACAAAGTTGTCGTTCAGAG-3’, Reverse-

5'TGTCTGTGGTGTCAACGAGAAGGAATAC-3'). *HvGAPDH* is considered to be a housekeeping gene on the basis of its high level and stable transcriptional activity in a range of barley tissues (Burton *et al.* 2004). Reactions were performed as per section 3.2.2.1 using 1 µg total RNA as per manufacturer's instructions. The resultant cDNA was only used for qPCR if a distinct single product was observed at the correct size on an agarose gel.

RNA was also extracted from black pointed and healthy grain (from mature grain of both Sloop and Alexis) using unpublished methods provided by Dr Andrew Milligan (Australian Centre for Plant Functional Genomics, ACPFG). Before extraction, enough tissue was added to a 2 mL tube to equal roughly 0.2 to 0.5 cc of volume. A volume of 0.5 mL of extraction buffer [50mM Tris (pH 9.0), 200 mM NaCl, 1% Sarcosyl, 20mM EDTA, 5mM dithiothreitol (DTT) made freshly before use] was added and vortexed until homogenous. Following the addition of 0.5 mL of phenol/chloroform/isoamyl alcohol (49:49:2 v/v), samples were vortexed until thoroughly suspended followed by centrifugation for 5 min at 14000 rpm at 4ºC. The aqueous upper phase was removed (0.5 mL) to a fresh 2 mL tube and TRIzol® methods continued as outlined in section 3.2.2.1. Synthesis of cDNA for use in qPCR was undertaken as outlined in section 3.2.2.2.

3.2.3.3 qPCR

A dilution series of the probe covering seven orders of magnitude from a 10^9 copies/µL stock solution was created as detailed by Burton *et al.* (2004). Three replicates of each of the seven standard concentrations were included in the qPCR together with a minimum of three no template controls. qPCRs were assembled by a liquid handling robot (CAS-1200

robot; Corbett Life Sciences, New South Wales, Australia). Three replicate PCRs for each of the cDNA samples were included in every run. cDNA solution (2 µL of a 1 in 20 dilution), the diluted standard or water was used in a reaction containing 5 µL of IQ SYBR Green PCR reagent (Bio-Rad Laboratories, California, USA), 1.2 µL each of the forward and reverse primers at 4 µM, 0.3 µL of 10 X SYBR Green in water and 0.3 µL of sterile nanopure water. Reactions were performed in a RG 3000 Rotor-Gene Real Time Thermal Cycler (Corbett Life Sciences) as follows: 3 min at 95°C followed by 45 cycles of 1 sec at 95°C, 1 sec at 55°C, 30 sec at 72°C and 15 sec at the optimal acquisition temperature (83°C). A melt curve was obtained from the product at the end of the amplification by heating from 70°C to 99°C. Using the Rotor-Gene V6 software (Corbett Life Sciences) the optimal cycle threshold (CT) was determined from the dilution series, with the raw expression data derived. The mean expression level and standard deviation of each set of three replicates for each cDNA was calculated.

Normalisation of the raw data was performed using the strategy of Burton *et al.* (2004). Five control genes were assessed [barley glyceraldehyde-3-phosphate dehydrogenase (*HvGAPDH*), barley elongation factor A (*HvEFA*), barley heat shock protein 70 (*HvHSP70*), barley tubulin (*HvTubulin*) and barley cyclophilin (*HvCycl*)]. The three best control genes (*HvGAPDH, HvCycl, HvHSP70*) from this set were selected, with normalisation factors calculated using the geNorm program (Vandesompele *et al.* 2002). A measure of consistency was obtained by examining the M value (Vandesompele *et al.* 2002), where a high M value indicates that a control gene has a very disparate expression with respect to other control genes. The raw expression values for *HvBP1, HvPrx7* and *HvQR* in the cDNA sample were divided by the normalisation factor for that cDNA to produce the normalised expression data.

3.2.4 Statistical analysis

qPCR gene expression data were analysed with Genstat (10th Edn, Release 10.1, 2007, Lawes Agricultural Trust, VSN International Ltd., Hemel Hempstead, UK) using a two-sided T-test (unpaired) at a confidence interval of 95%. A probability of $P \leq 0.05$ was used to test for significant difference of means between the two cultivars, Sloop and Alexis. This form of statistical analysis was deemed appropriate due to variation in sampling times between cultivars. One-way analysis of variance (ANOVA) was used to compare qPCR gene expression data at the mature stage of grain development in black pointed and healthy grain. The least significant difference (LSD) at $P = 0.05$ was used to test for significant differences between means.

3.3 Results

3.3.1 Differential gene expression established using northern analysis

Gene expression was investigated in a number of varieties displaying varying levels of susceptibility to black point (as described in Table 2.1, Chapter 2). Varieties susceptible to black point, commonly showed higher *HvBP1* expression during the early stages of grain fill, in particular, milk and soft dough (Figure 3.1) except for Sloop SA. Although *HvBP1* is highly expressed during the milk stage of grain fill for Keel (which is most susceptible; Table 2.1), expression was not detected during the soft dough stage of development. However, this was more than likely because of the poor RNA loadings in the corresponding lanes (Figure 3.1). Mundah, Franklin and Alexis, varieties which show

tolerance to black point even in environmental conditions considered suitable for its formation (refer to section 2.3.4), had low *HvBP1* expression levels (Figure 3.1). However Golden Promise and Gairdner have high expression only at the milk stage of development.

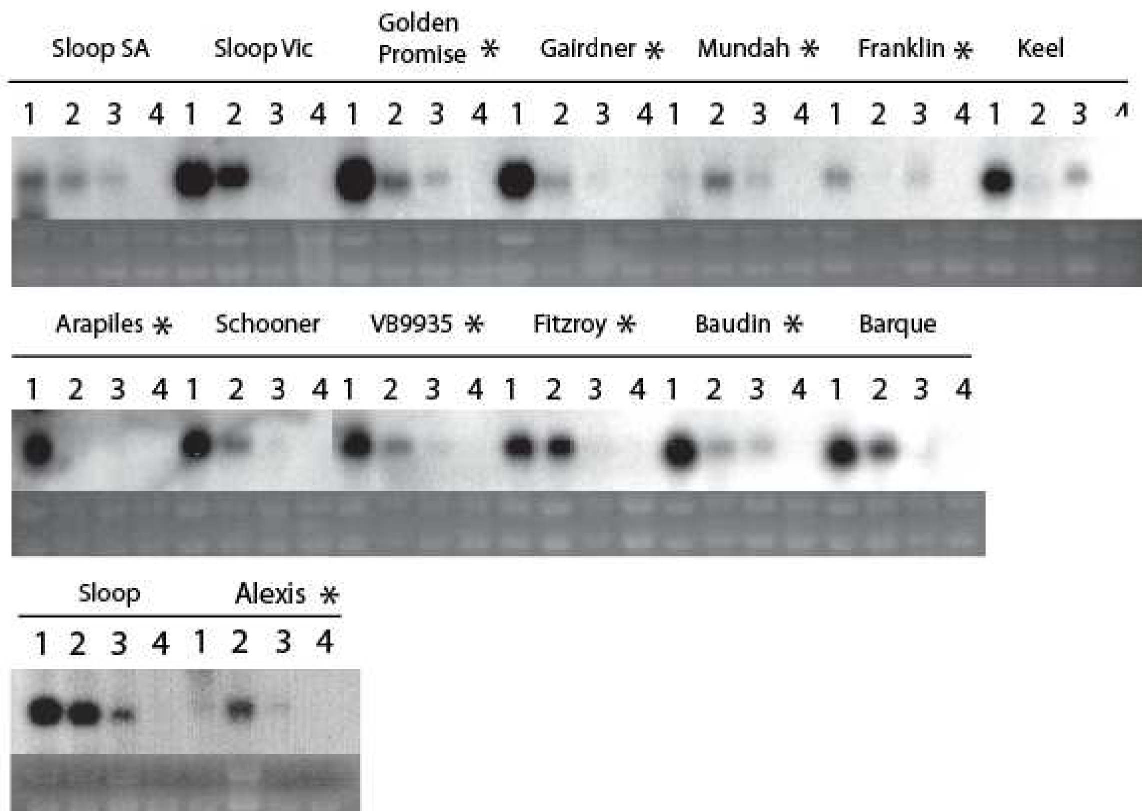

Figure 3.1 Northern blot analysis of *HvBP1*. Gene expression across developmental stages: 1=Milk, 2=Soft Dough, 3=Hard Dough and 4=Maturity (Zadoks *et al.* 1974) of varieties showing a range of susceptibilities to black point. The lower panel represents ethidium bromide stained ribosomal RNA (rRNA) bands as a control. * after the variety name represents tolerant varieties (Walker *et al.,* 2008, chapter 2). Representative blot (n=2).

Because *HvPrx7* expression was not apparent using northern analysis (even with three biological replicates, data not shown), northern analysis was repeated for Sloop and Alexis (Figure 3.2), increasing the concentration of RNA (2X). *HvPrx7* expression was higher in

all stages of grain development in the susceptible variety Sloop in comparison to the tolerant variety Alexis (Figure 3.2).

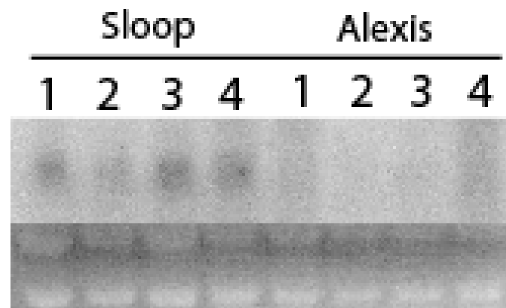

Figure 3.2 Northern blot analysis of *HvPrx7*. Sloop and Alexis gene expression across developmental stages: 1=Milk, 2=Soft Dough, 3=Hard Dough and 4=Maturity (Zadoks *et al.* 1974). The lower panel represents ethidium bromide stained ribosomal RNA (rRNA) bands as a loading control. This is a representative blot (n=2).

HvPAL was expressed at low levels in all varieties at various stages (Figure 3.3). However, even though higher expression seemed particularly evident in Keel and Baudin at hard dough and in Sloop and Franklin at milk (Figure 3.3), not all susceptible varieties displayed higher *HvPAL* expression. Expression at the milk, soft dough and mature stages was observed in Sloop and Barque, with decreased expression apparent at the hard dough stage (Figure 3.3) in comparison to other susceptible varieties. Both Sloop and Barque were found to be susceptible in the optimal environmental conditions for black point formation (Chapter 2, Table 2.1). A similar expression profile was apparent in Fitzroy, however black point data was not recorded for this variety in the 2002 season when the environmental conditions were found to favour black point formation (Chapter 2). Fitzroy was found to be tolerant in the 2005-2006 season (Chapter 2 and Walker *et al.* (2008)).

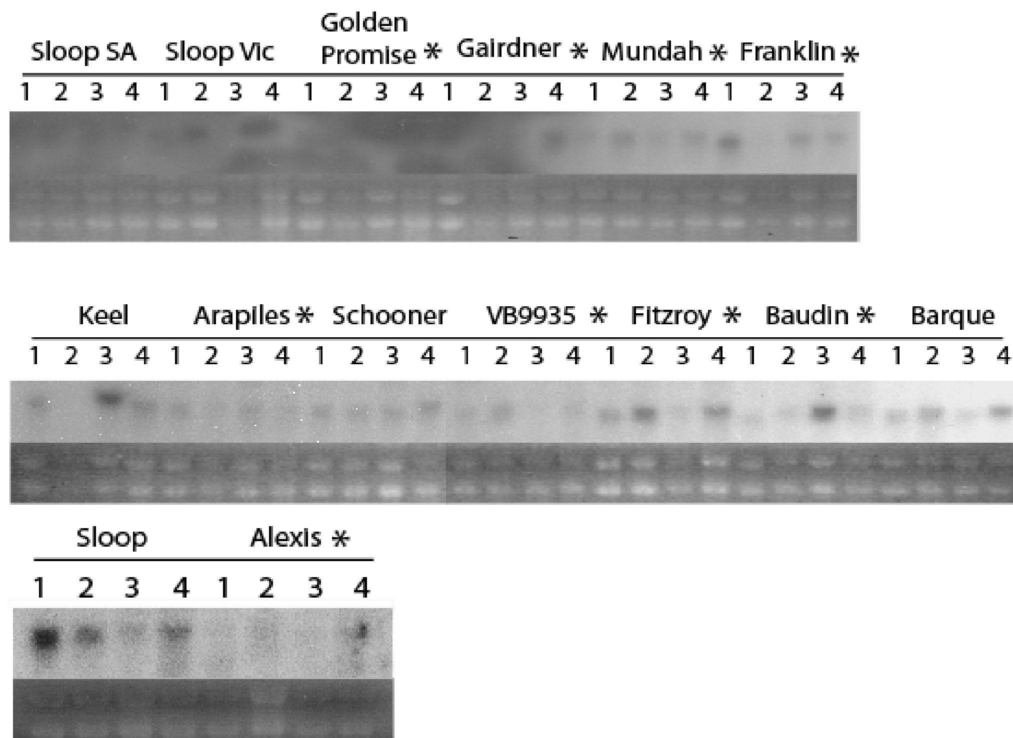

Figure 3.3 Northern blot analysis of *HvPAL*. Gene expression across developmental stages: 1=Milk, 2=Soft Dough, 3=Hard Dough and 4=Maturity (Zadoks *et al.* 1974) of varieties showing a range of susceptibilities to black point. The lower panel represents ethidium bromide stained ribosomal RNA (rRNA) bands as a loading control. * after the variety name represents tolerant varieties (Walker *et al.,* 2008, chapter 2). Representative blot (n=2).

Northern analysis of *HvQR* indicated a general increase in expression throughout the later stages of grain development regardless of variety (Figure 3.4). However, *HvQR* expression was lower in the tolerant cultivars Mundah and Franklin. The reduced levels of expression in the normally susceptible cultivar Sloop Vic can be explained by depleted loadings of RNA, similarly for Golden Promise where no expression was observed in the mature sample.

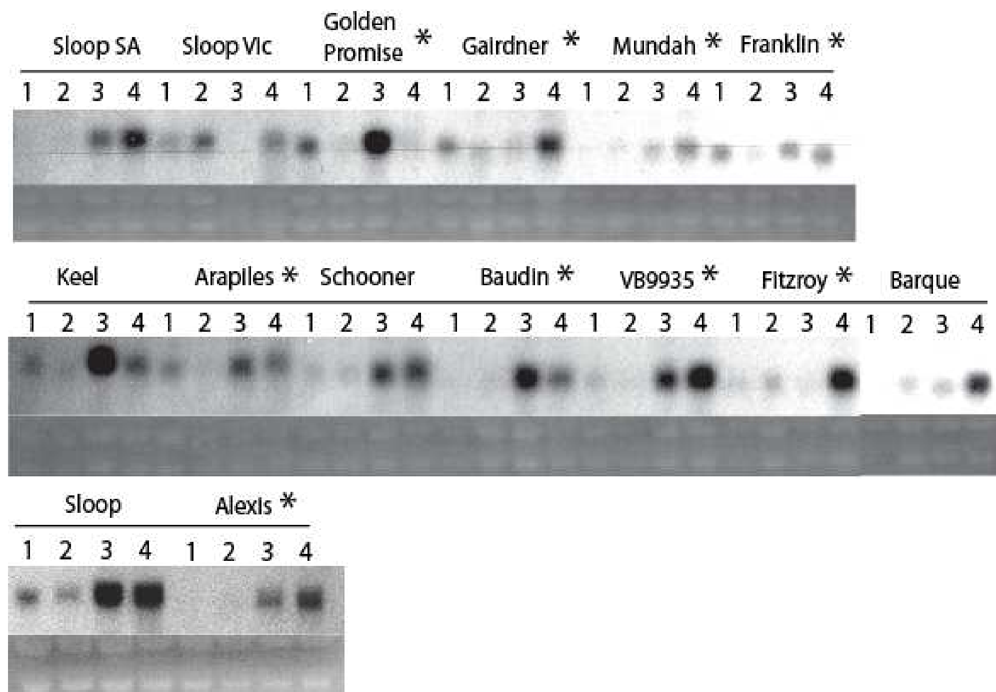

Figure 3.4 Northern blot analysis of *HvQR*. Gene expression across developmental stages: 1=Milk, 2=Soft Dough, 3=Hard Dough and 4=Maturity (Zadoks *et al.* 1974) of varieties showing a range of susceptibilities to black point. The lower panel represents ethidium bromide stained ribosomal RNA (rRNA) bands as a loading control. * after the variety name represents tolerant varieties (Walker *et al.,* 2008, chapter 2). Representative blot (n=2).

Northern analysis of *HvLox1* suggests there are no obvious or consistent signs of differential expression between susceptible and tolerant cultivars (Figure 3.5). Expression was greatest in the cultivars Keel, Baudin, Sloop and Golden Promise at the hard dough stage of grain development. An increase in expression was also observed in the soft dough and mature samples from varieties Fitzroy and Barque (Figure 3.5).

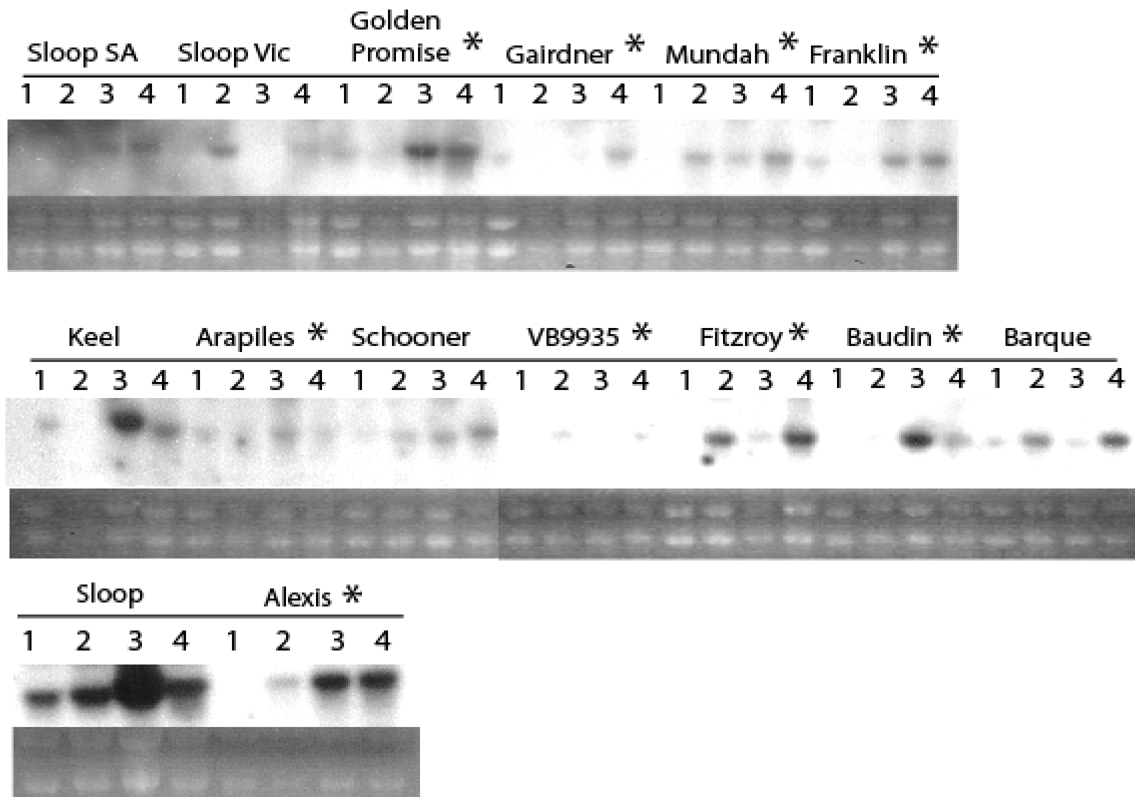

Figure 3.5 Northern blot analysis of *HvLox1*. Gene expression across developmental stages: 1=Milk, 2=Soft Dough, 3=Hard Dough and 4=Maturity (Zadoks *et al.* 1974) of varieties showing a range of susceptibilities to black point. The lower panel represents ethidium bromide stained ribosomal RNA (rRNA) bands. * after the variety name represents tolerant varieties (Walker *et al.,* 2008, chapter 2). Representative blot (n=2).

3.3.2 Confirmation of differential gene expression using qPCR

Given the differential expression observed between the susceptible variety Sloop and tolerant variety Alexis for candidate genes *HvBP1* and *HvQR*, gene expression was further characterised using qPCR. Although low expression was observed in the candidate *HvPrx7* using Northern analysis, increased expression was evident in Sloop (Figure 3.2). Because previous research also showed increased *HvPrx7* levels in susceptible varieties (March 2003) and the proposed role of peroxidase in black point formation (Section 1.3.3.1.4), *HvPrx7* was also included for further characterisation.

Differential expression of *HvBP1*, *HvPrx7* and *HvQR* was observed between stages of grain development and between the black point susceptible cultivar Sloop and the tolerant cultivar Alexis (Figures 3.6 to 3.8). Generally, higher expression for all three genes was observed in the black-point susceptible variety Sloop (Figures 3.6 to 3.8), consistent with the northern analysis (Figures 3.1, 3.2 and 3.4). Although northern analysis suggested *HvBP1* expression was elevated in the milk and soft dough stages in both varieties (Figure 3.1), the qPCR data suggested that there was no significant difference in expression between Sloop and Alexis at the milk and soft dough stages of grain development. Significantly lower *HvBP1* expression was observed in hard dough and mature samples from the tolerant variety Alexis when compared with the susceptible variety Sloop (Figure 3.6).

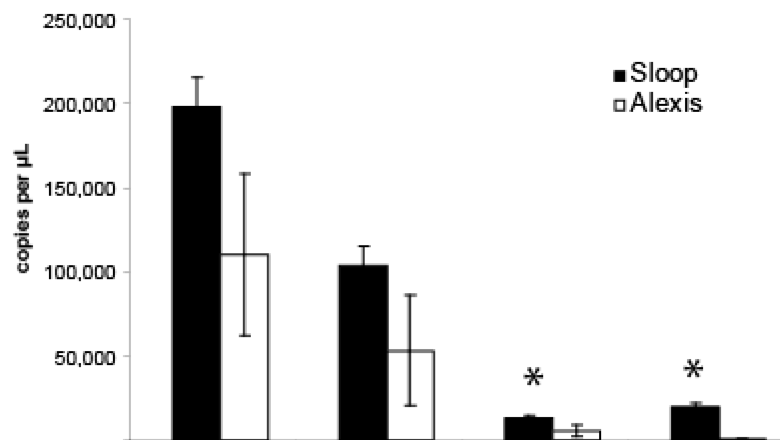

Figure 3.6 Normalised expression levels (level of mRNA presented as number of copies of cDNA per µL of candidate gene *HvBP1* obtained from qPCR (n = 3) in barley varieties Sloop (susceptible and solid bars) and Alexis (tolerant and empty bars) grown in field experiments at Hatherleigh (as per Section 3.2.1). Mean expression (bars represent standard error) for each stage of grain fill is shown (milk, soft dough, hard dough and mature – refer to (Zadoks *et al.* 1974)).* Denotes a probability of $P \leq 0.05$ (Section 3.2.4).

HvPrx7 expression (Figure 3.7) was significantly greater in Sloop than in Alexis for the milk, hard dough and mature stages. Expression at the mature stage of development for Sloop was greater than any other stage (Figure 3.7), with expression increasing with grain maturity. For Alexis, *HvPrx7* expression was greatest at the soft dough stage of development.

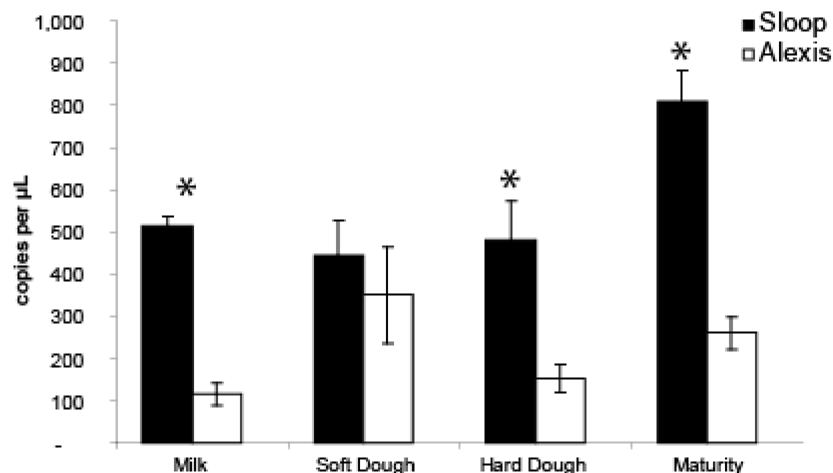

Figure 3.7 Normalised expression levels (level of mRNA presented as number of copies of cDNA per µL) of candidate gene *HvPrx7* obtained from qPCR (n = 3) in barley varieties Sloop (susceptible and solid bars) and Alexis (tolerant and empty bars) grown in field experiments at Hatherleigh (as per Section 3.2.1). Mean expression (bars represent standard error) for each stage of grain fill is shown (milk, soft dough, hard dough and mature – refer to (Zadoks *et al.* 1974)).* Denotes a probability of $P \leq 0.05$ (Section 3.2.4).

Gene expression was shown to be greater with increasing maturity in both varieties in the candidate *HvQR* (Figure 3.8). Greater expression is observed in the hard dough and mature stages when comparing Sloop and Alexis (Figure 3.8). No significant difference in gene expression was observed in the early, milk and soft dough stages of grain development for both Sloop and Alexis (Figure 3.8).

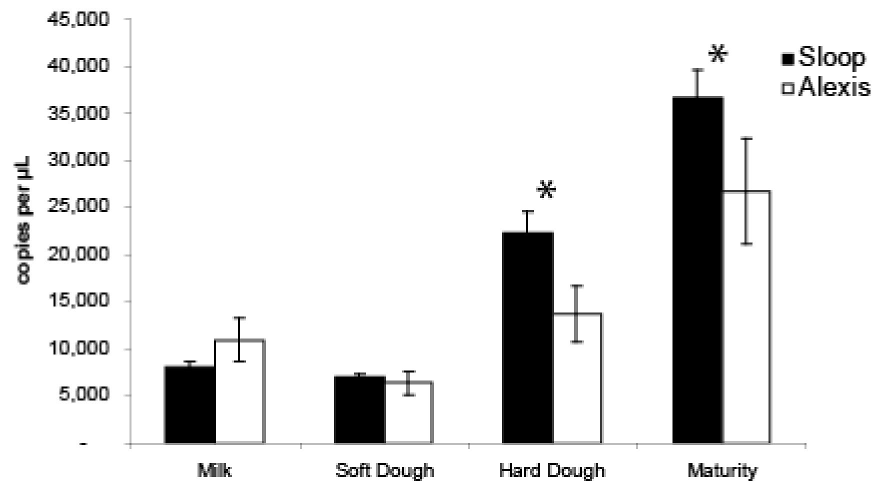

Figure 3.8 Normalised expression levels (level of mRNA presented as number of copies of cDNA per µL) of candidate gene *HvQR* obtained from qPCR (n = 3) in barley varieties Sloop (susceptible and solid bars) and Alexis (tolerant and empty bars) grown in field experiments at Hatherleigh (as per Section 3.2.1). Mean expression (bars represent standard error) for each stage of grain fill is shown (milk, soft dough, hard dough and mature – refer to (Zadoks *et al.* 1974).* Denotes a probability of $P \leq 0.05$ (Section 3.2.4).

Candidate genes *HvBP1, HvPrx7* and *HvQR* were further characterised through investigation of gene expression by qPCR in black pointed and healthy grain. *HvBP1* and *HvPrx7* expression in black pointed and healthy grain from Sloop was not significantly different (Figure 3.9A, B) but was significantly greater in black pointed grain from Alexis (Figure 3.9A, B). Expression of *HvQR* showed no difference between black pointed and healthy grain in the tolerant cultivar Alexis, though greater expression was observed in the healthy Sloop sample (Figure 3.9C).

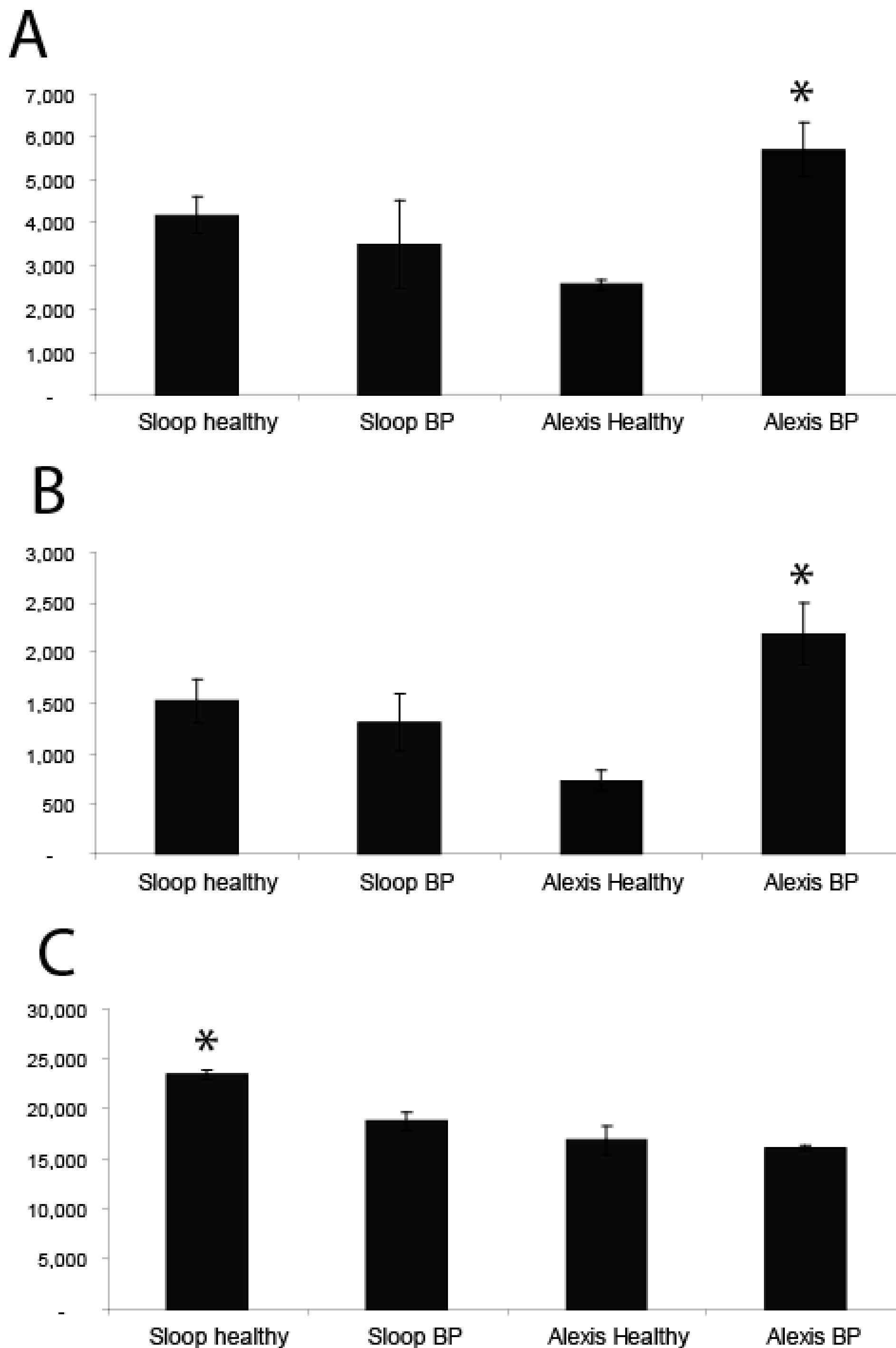

Figure 3.9 Normalised expression levels in black pointed and healthy grain (level of mRNA presented as number of copies per µL) of candidate gene *HvBP1* (A), *HvPrx7* (B) and *HvQR* (C) obtained from qPCR (n = 3, standard deviation of each set of three replicates for each cDNA was calculated) in barley varieties Sloop (susceptible and solid bars) and Alexis (tolerant and empty bars) grown in field experiments at Hatherleigh (Section 3.2.1). Expression was established in mature grain. *Denotes a probability of $P \leq 0.05$ (Section 3.2.4).

3.4 Discussion

There has been some suggestion that black point results from the induction of enzymatic browning during exposure to unfavourable environmental conditions during grain fill (Cochrane 1994b; Williamson 1997b; Sulman *et al.* 2001a; Walker *et al.* 2008). The research presented in the previous chapter indicated that high humidity and low temperatures during the grain fill period contributed significantly to black point severity in susceptible varieties. These environmental conditions could potentially induce expression of genes that encode for enzymes that contribute to the enzymatic browning process (reviewed in section 1.4). The research presented in this chapter therefore aimed to characterise some of the candidate genes likely to be induced under the appropriate environmental conditions that lead to black point.

Differential gene expression was observed for the peroxidases, *HvBP1* and *HvPrx7*, as well as a quinone reductase, *HvQR,* which was identified within the QTL for black point on 2H (*QBpt.AlSl-2H)* (March *et al.* 2008). No differential expression was observed for *HvPAL* and *HvLox1*. Whether differential expression observed in candidate genes *HvBP1, HvPrx7* and *HvQR* was in any way correlated with black point was also established by examining gene expression in black pointed and healthy mature grain. Because black point cannot be visualised in the early stages of grain fill, correlations between black point and gene expression could not be made for grain from other developmental stages. However, while *HvBP1* and *HvPrx7* expression in black pointed and healthy grain from the susceptible cultivar Sloop was not significantly different, significantly greater expression was apparent in black pointed grain from the tolerant cultivar Alexis. An increased level of peroxidase gene expression associated with the susceptible cultivar was not apparent, suggesting that

differences in susceptibility may be correlated with the protein level only. Expression of *HvQR* showed no difference between black pointed and healthy grain in the tolerant cultivar Alexis, even though greater expression was observed in the healthy Sloop sample. Quinone reductase enzymes remove quinone (Harborne 1979) and have been proposed to protect grain from black point formation if induced as a defence mechanism in response to wounding within the grain (March *et al.* 2008). If quionone reductase was contributing to tolerance we would therefore expect higher levels in grain from the tolerant variety. However, the greater levels of *HvQR* expression in the healthy grain from Sloop might suggest a role in the reduction of quinones and hence black point symptoms. Whether wounding played a role in that induction or not remains to be clarified.

High humidity associated with low temperatures (or a low *VPD*) appears to induce black point symptoms in susceptible varieties (Section 2.3.4), possibly as a result of stress or wounding to the embryo through the creation of a moist environment in which the grain cannot dry out. Candidate genes *HvLox1* and *HvPAL* were chosen for their potential roles in response to wounding (Sarma and Sharma 1999; Porta and Rocha-Sosa 2002). PAL is a wound-induced enzyme that initiates an increase in the concentration of phenolic compounds, ferulic acid and *p*-coumaric acid from phenylalanine (Michalowitz *et al.* 2001) and in rice, *PAL* has been shown to be expressed in response to different stress stimuli (Sarma and Sharma 1999). *Lox1* was included as a candidate gene as its expression has been shown to be regulated by different forms of stress, such as wounding (Porta and Rocha-Sosa 2002) and more specifically *HvLox1* has been shown to accumulate in the later stages of grain development (Schmitt and Van Mechelen 1997) when black point symptoms also typically occur. When examined by northern analysis *HvLox1* and *HvPAL* were found to not be differentially expressed between black point susceptible and tolerant

cultivars, suggesting that in this case differential expression cannot be targeted for breeding purposes. The presence or absence of black point during the early stages of grain fill was not able to be confirmed because black point is not evident until maturity. The wounding to the grain proposed to occur during black point formation probably did not occur and might therefore explain the lack of differential gene expression. Generally low levels of the stress-inducible *HvPAL* support this argument. However, why *HvLOX1* is induced later in grain development (regardless of cultivar) requires further investigation.

Given the proposed role of the enzymatic browning reaction and the identification of higher isoelectric points for peroxidases in varieties susceptible to black point (Hadaway *et al.* 2003), peroxidases are ideal candidates in black point formation. Peroxidase gene expression was greater across all stages of grain development in the susceptible cultivar Sloop, suggesting that increased peroxidase expression at an undetermined stage may be a contributing factor in black point formation. Prior research using northern analysis had indicated that peroxidase genes *HvBP1* and *HvPrx7* may be expressed for longer during grain fill in susceptible varieties (March 2003). Northern analysis and qPCR performed during this research confirmed that *HvBP1* and *HvPrx7* expression was greatest in susceptible varieties during the earlier stages of grain fill (Figure 3.2, Figure 3.6) with gene expression increased for longer during grain fill in susceptible cultivars suggesting a role in black point formation.

The increased levels of expression in the later stages of grain development in the susceptible variety Sloop correspond with the timing of black point symptoms where we would expect increased oxidation of phenolic compounds and increased levels of peroxidase (see section 1.3.3.1). Sulman *et al.* (2001b) and Hadaway *et al.* (2003) found no

correlation between total peroxidase activity and the levels of black point in susceptible and tolerant cultivars. Mature barley kernels could therefore contain sufficient peroxidase in all varieties to cause black point but differences in substrate (Sulman *et al.* 2001b) or types of peroxidase (Hadaway *et al.* 2003) may be the distinguishing factor between susceptibility and tolerance. Provided the increased gene expression at the later stages of grain fill observed in susceptible varieties is translated to increased protein activity, a link between susceptibility and *HvPrx7* and *HvBP1* could be concluded. Indeed *HvBP1* proteins have been found to be greater in black pointed grain and not healthy grain from the susceptible cultivar Sloop (March *et al.* 2007) but both tolerant and susceptible varieties need to be examined to confirm this link. The susceptibility observed in Sloop may therefore be due to the increased peroxidase levels especially at later stages of grain fill. However, the levels of peroxidase enzyme extracted from barley grain exhibiting black point symptoms has been previously shown to be lower than that of healthy grain (Sulman *et al.* 2001b) suggesting that the type and amount of individual peroxidases may be more important. In this study, no significant difference in gene expression was observed between healthy and black pointed grain from Sloop but in the tolerant Alexis there was a two-fold increase in expression in black pointed grain suggesting that peroxidase protein levels would be greater in black-pointed tolerant grain. While these results contrast those of Sulman *et al.* (2001b) who found higher peroxidase activity in healthy grain, that study only measured total peroxidase activity using other varieties. At a protein level, three isoforms of *HvBP1* were identified as present in black pointed grain and not healthy grain of the susceptible cultivar Sloop (March *et al.* 2007) suggesting post-translational modification occurs. Combined with the results presented here, this also suggests that there are low levels of BP1 protein in the black-pointed tolerant grain because the protein is not produced from the transcript or is degraded. Future experiments should therefore focus on

establishing protein levels of *HvBP1* in the tolerant cultivar Alexis and *HvPrx7* protein levels in both cultivars.

HvQR gene expression increased with maturity and showed elevated levels in the susceptible cultivar Sloop, which may be associated with the increased levels of quinone proposed with the enzymatic browning model (Chapter 1, Figure 1.2). Comparative mapping studies of the putative QTL identified on chromosome 2HS (*QBpt.AlSl-2H*, Figure 2.14) identified an EST with sequence similarity to a quinone reductase (NAD(P)H-QR) (March *et al.* 2008). NAD(P)H-QR is a typical flavoprotein which has shown catalytic activity with short-chain acceptor quinones (Trost *et al.* 1995). Unlike other flavoproteins catalysing a one electron reduction of quinones NAD(P)H-QR is a soluble protein producing fully reduced quinols without semiquinone intermediates, therefore reducing the build up of reactive oxygen species from semiquinone autooxidation (Trost *et al.* 1995) and protecting plant cells from oxidative damage (Sparla *et al.* 1999). The proposed stress or wounding of plant tissue in black point formation may result in the oxidation of phenolic compounds to quinones by enzymes such as peroxidases and polyphenol oxidases (Walker and Ferrar 1998). Quinones are highly reactive compounds proposed to be involved in cross linking cell walls to provide a physical barrier for protection (Lynn and Chang 1990). In order to regulate the levels of quinones, plants are able to produce quinone reductase enzymes, resulting in the reduction of quinones into hydroquinones that can be removed from the quinone redox cycle by conjugation (Harborne 1979). Given that quinones are likely to contribute to the browning observed during black point formation, *HvQR* could have a potential role in a tolerance mechanism where the enzymatic browning process is disrupted through removal of quinone reactivity. One would therefore expect that *HvQR* would be at higher levels in tolerant grains during grain fill. However, increased

HvQR expression was observed at the hard dough and mature stages of grain development in the susceptible cultivar Sloop (Figure 3.8) suggesting that HvQR may also have been greater. The genes that encode quinone reductase in plants and fungi have been previously shown to be up-regulated by quinones (Cohen *et al.* 2004), supporting the observation that quinones (and therefore black point) are likely to form during the later stages of grain fill (under adverse environmental conditions). The observation that higher *HvQR* expression was observed in the healthy grains of the susceptible variety Sloop, suggests that gene expression may have been induced in response to quinone formation leading to its removal through *HvQR*. However, even though low levels of *HvQR* were present in the tolerant Alexis, no significant difference in *HvQR* expression was observed between healthy and black pointed grains. Future experiments need to characterise protein expression of *HvQR* in susceptible and tolerant cultivars.

In conclusion, differential expression between susceptible and tolerant cultivars has been established during different stages of grain fill for the candidate genes *HvBP1*, *HvPrx7* and *HvQR* using both northern and qPCR analysis. An increased level of gene expression in susceptible varieties confirms a possible role in black point formation. Further investigation into how the candidate genes identified are regulated will allow us to further understand the differential expression observed and their possible roles in black point formation.

Chapter Four. Establishing areas of the genome contributing to *HvQR*, *HvPrx7* and *HvBP1* gene expression

4.1 Introduction

Extensive variation in gene expression has been shown in all organisms studied to date (Oleksiak *et al.* 2002; Gilad *et al.* 2006; Genissel *et al.* 2008). Sequence polymorphisms that produce altered (or absent) proteins and qualitative and quantitative differences in gene expression that generate varying amounts of protein in a cell or tissue result in phenotypic differences among individuals (Druka *et al.* 2010). Transcript expression levels, when assessed in an experimental or mapping population, can be considered as quantitative traits and used to map quantitative trait loci (QTL) for gene expression (Jansen and Nap 2001; Doerge 2002; Schadt *et al.* 2003). Schadt *et al.* (2003) used a genome wide genetic analysis of gene expression in maize, mice and humans to identify differential expression. Using this data as a quantitative trait and standard statistical tools allowed identification of the genetic regions contributing to variation in gene expression (or eQTLs). Mapping expression profiles in yeast (Brem *et al.* 2002) and Eucalyptus (Kirst *et al.* 2004) has also demonstrated the utility of this method in understanding complex traits. Expression QTL (eQTL) mapping studies are therefore a powerful tool in the identification of genetic variants contributing to gene regulation.

eQTLs are categorised as *cis*- or *trans*-acting; where *cis*-eQTLs represent a polymorphism physically located near the gene itself or within the promoter and *trans*-eQTLs represent a polymorphism at a location in the genome other than the actual position of the gene whose transcript is being measured, or a polymorphism at the physical position of a regulatory

factor elsewhere in the genome (Hansen *et al.* 2008). Regions controlling seed development in wheat have been investigated, identifying both *cis*- and *trans*-acting eQTLs (Jordan *et al.* 2007). Similarly, gene expression QTL analysis of 16000 genes in barley identified 23738 eQTLs affecting expression of 12987 genes, regulated by both *cis*- and *trans*- effects (Potokina *et al.* 2008).

The differential expression of *HvPrx7*, *HvBP1* and *HvQR* between black point susceptible and tolerant cultivars observed previously (see Chapter 3) implies different regulatory effects between cultivars. Combining QTL mapping and fine mapping with gene expression data would allow areas of the genome contributing to that differential gene expression of *HvPrx7*, *HvBP1* and *HvQR* to be identified. The research presented in this chapter therefore identified eQTLs or areas of the genome contributing to gene expression for *HvBP1, HvPrx7* and *HvQR*, thus providing preliminary insight into their regulation. *HvBP1, HvPrx7* and *HvQR* were also mapped to a chromosomal location in the barley genome to enable identification of whether eQTLs were *cis*- or *trans*-acting. If *trans*-regulatory mechanisms were identified, comparative mapping studies between barley, wheat and rice allowed the identification of candidate regulatory factors (such as transcription factors) potentially involved in the genes' regulation.

4.2 Materials and methods

4.2.1 Plant material and sampling

Plant material from field experiments described in section 2.2.2 was used in the identification of eQTL. Doubled haploid (DH) lines (92) derived from a cross between

Alexis and Sloop (Barr *et al.* 2003) were planted in field experiments at Hatherleigh in 2004 and 2005 as per section 2.2.2.

Grain was sampled as per section 3.2.1, with sampling from two separate plots (two biological replicates) at Zadoks' growth stages 75 (medium milk), 85 (soft dough), 87 (hard dough) and stage 95 (maturity) (Figure 2.3) (Zadoks *et al.* 1974) from each of the 92 lines of the Alexis/Sloop DH population. Grain samples were used for DNA and RNA isolation for open reading frame (*ORF*) characterisation, genome localisation and gene expression studies respectively.

4.2.2 *ORF* characterisation and genome localisation

Prior to performing gene expression analysis across DH populations, the *ORF* of *HvBP1* and *HvPrx7* was sequenced for Sloop and Alexis while chromosomal location was also identified. This was not required for *HvQR* as it was an EST previously identified through comparative mapping studies as residing within the black point QTL identified on 2H (Chapter 2 and March *et al.* 2008).

4.2.2.1 RNA and DNA isolation

RNA was extracted as per section 3.2.2.1 and used in cDNA amplification for Sloop and Alexis as per section 3.2.3.2. Genomic DNA was isolated from young leaf tissue of Sloop and Alexis barley plants (approximately 1 month old, grown in a controlled growth room at a constant 18°C with a 12 h light/ 12 h dark regime). Tissue was ground (approximately 100 mg) to a fine powder using liquid nitrogen in a pre-cooled sterilised mortar and pestle

before addition to 0.5 mL DNA extraction buffer (1% sarcosyl, 100 mM NaCl, 10 mM EDTA, 100 mM Tris-HCl, 1% polyvinylpyrrolidone PVVP; pH 8.0). This was then vortexed at low speed until thawed. Phenol/chloroform/isoamyl alcohol (25:24:1 v/v) (1 mL) was then added to the samples, mixed using an orbital mixer for 15 min and centrifuged at 6000 rpm for 15 min. The upper aqueous layer was transferred to a fresh tube to which 90 µL of sodium acetate (pH 5.2) and 900 µL of isopropanol were added and DNA was allowed to precipitate at room temperature for 5 min on an orbital mixer. Following centrifugation at 13000 rpm for 10 min the supernatant was discarded and 1 mL of 70% ethanol carefully added to the tube to wash the DNA pellet. The tube was slowly and gently agitated for 2 min followed by removal of the ethanol and the pellet air dried for 20 min. DNA was re-suspended in 50 µL of R40 (40 µg per mL RNase A in 1 x TE), placed at 4°C overnight and stored at -20°C until required. Quantification of the DNA was performed by measuring absorbance at 260 nm using a UV/VIS SP8001 spectrophotometer (Metertech) as per section 3.2.2.1.

4.2.2.2 Sequence variation within the *ORF*

Sequence variation between the parents of the Alexis x Sloop DH mapping population was determined through sequencing the full length *ORF* of *HvBP1* (Accession: M73234) and *HvPrx7* (Accession: AJ003141) cDNA. Primers were designed to the *ORF* of each gene with resultant primer combinations as per Table 4.1.

Table 4.1 Primers designed to amplify the *ORF* for candidate genes *HvBP1* and *HvPrx7*. Forward (F) and reverse (R) primers, expected product size and the melting temperature (Tm°C) used for amplification are indicated.

Candidate	Primer	Tm (°C)	Size (bp)
HvBP1	**F** 5′ ATGGCTCGTGTTCCTCTGCTAGCA 3′	59	1079
	R 5′ TAGCCAATGCTTCCTGCGGCTTCGT 3′		
HvPrx7	**F** 5′ ATGGCGTCCAGAGCAGCAGCGGCCATC 3′	65	1025
	R 5′ TCACATGTCAGCGGCGATCCCCTCGTC 3′		

The PCR mixture contained: Buffer A (4 µL); Buffer B (6 µL); (1.6 mM Mg^{2+}); dNTPs (10 mM, 1 µL); Forward primer (10 µM, 1 µL); Reverse primer (10 µM, 1 µL), cDNA (1 µL Alexis/Sloop), Elongase® enzyme mix (1 µL) and nanopure water (36 µL). The cycling conditions were 94°C for 2 min; then 35 cycles of 94°C for 30 sec, x°C (Tm°C indicated above) and 1.5 min at 68°C; followed by 68°C for 5 min. PCR products were separated by gel electrophoresis (1.5% agarose, w/v) and visualised using ethidium bromide, cloned and sequenced as per section 3.2.2.2. PCR conditions above were repeated using genomic DNA (isolated as per Section 4.2.2.1) to identify the presence/absence of introns.

4.2.2.3 Mapping of candidates to the barley genome

4.2.2.3.1 PCR of barley:wheat addition lines

Barley:wheat addition lines, where each addition line contains the full complement of wheat chromosomes and a single homologous chromosome pair from barley (Islam *et al.* 1981) were screened to determine the chromosomal location of candidate genes *HvBP1*

and *HvPrx7*. Genomic DNA was extracted as described in section 4.2.2.1, from each of the seven lines, Betzes (as a barley positive control) and Chinese Spring (as a wheat positive control). DNA was screened by PCR using oligonucleotide combinations for each of the candidates, (Table 4.1). PCR reaction mixture (20 µL) contained 10X PCR Buffer (2 µL); $MgCl_2$ (50 mM, 0.8 µL): dNTPs (10 mM, 1 µL): forward primer (10 mM, 1 µL): reverse primer (10mM 1 µL), *Taq* polymerase (1.25 U, Invitrogen), template (1 µL) and sterile distilled water (11.75 µL). Cycling conditions were previously outlined in section 3.2.2.2. PCR products were separated by gel electrophoresis (1.5% agarose, w/v) and visualised using ethidium bromide. Bands of the correct size (Table 3.1) were cloned and sequenced as per section 3.2.2.2 as confirmation.

4.2.2.3.2 Fine mapping of *HvPrx7* and *HvBP1*

After chromosomal location of candidate genes (Section 4.2.2.3.1), fine mapping was undertaken with the aim of placing the genes on the Alexis x Sloop DH map. No sequence variation between the *ORFs* of Alexis and Sloop for *HvBP1* was identified making it difficult to easily place on the map. However, Restriction Fragment Length Polymorphism (RFLP) analysis of *HvBP1* was previously reported by March *et al.* (2007) in the Alexis x Sloop DH mapping population, placing the candidate gene on chromosome 3H.

A single nucleotide polymorphism (SNP) (at bp 463) identified in the *ORF* of *HvPrx7* between Sloop and Alexis allowed the use of Amplified Fragment Length Polymorphism (AFLP) to map the gene to the barley genome. Go-Taq® PCR mixture (25 µL) (Promega) was used to obtain a PCR product of 696 bp, containing Go-Taq® (12.5 µL); Forward primer (5' ACCTGGAGCGCATCGTGGAGTTCC 3'; 10 µM, 2.5 µL); Reverse primer (5'

AGGCCCTGGTCCGACTTGAACAG 3'; 10 µM, 2.5 µL) and sterile distilled water (3.25 µL). Cycling conditions comprised of 94°C for 2 min (1 cycle), 94°C for 30 sec, 58°C for 30 sec, 72°C for 1 min (35 cycles) and finally 72°C for 10 min. PCR products were digested using Hyp81 (New England Biolabs), by adding Hyp81 enzyme (1 µL) and sterile distilled water (18 µL) to the PCR mixture (10 µL) and 10 X buffer (2 µL) and incubating at 37°C for 1 h. Digestion patterns were analysed through separation by gel electrophoresis (1.5% agarose, w/v) and visualisation with ethidium bromide (as per section 3.2.2.1.4). Sloop and Alexis parents were digested as controls and polymorphisms scored A (Alexis) and B (Sloop) across Alexis x Sloop DH mapping population lines. The *HvPrx7* marker was placed on an updated version of the Alexis/Sloop DH map (as per section 2.2.5, kindly provided by Greg Lott, SARDI) using Map Manager (Manly *et al.* 2001). The position of *HvQR* was determined by March and colleagues as 2H by RFLP mapping in the Alexis x Sloop DH mapping population (March *et al.* 2008). This location was therefore used for all experimentation.

4.2.3 Gene expression in the Alexis x Sloop DH population

Gene expression in the Alexis x Sloop DH population was viewed as a quantitative trait because we can measure differences in gene transcript levels using qPCR. This allowed the subsequent identification of eQTL across the barley genome for *HvBP1*, *HvPrx7* and *HvQR*.

4.2.3.1 RNA isolation and cDNA synthesis

An adapted TRIzol® (Invitrogen) method was used for RNA isolation as per section 3.2.2.1. cDNA was synthesised from RNA extracted from the hard dough stage of grain development as per section 3.2.3. The hard dough stage was used as this stage was found to be differentially expressed between the Alexis and Sloop parents for all three genes analysed (Figures 3.6-3.8).

Whole barley grains from 92 Alexis x Sloop DH mapping population lines were ground in liquid nitrogen using the IKA® A11 basic analytical mill (IKA Works, Pataling Jaya Selangor, Malaysia) as per section 3.2.2.1. Ground tissue (approximately 150 mg) was transferred to a 2 mL tube containing 1 mL of TRIzol® and RNA extracted as per the manufacturers' instructions. RNA was assessed by gel electrophoresis as per section 3.2.2.1 following subsequent cDNA amplification.

A single cDNA synthesis reaction was performed for each of 92 Alexis x Sloop DH lines for the first biological replicate (replicate 1) for *HvBP1*, *HvPrx7* and *HvQR*. For a second biological replicate (Replicate 2, using grain from an alternate plot), single cDNA synthesis reactions were also undertaken. However, due to some of the lines in replicate 2 having considerably lower expression levels than replicate 1 or no measurable expression, cDNA synthesis was repeated for a subset of 72 lines from replicate 2 to confirm that observation. Reactions were performed as per section 3.2.3.2 using 1 µg total RNA as per manufacturer's instructions. Preceding qPCR, cDNA was checked for quality using Go-Taq® PCR containing *HvGAPDH* primers (Forward- 5'

ACAAGCTTGACAAAGTTGTCGTTCAGAG -3’, Reverse- 5’ TGTCTGTGGTGTCAACGAGAAGGAATAC -3’).

4.2.3.2 Detection of gene expression (qPCR)

Expression data was obtained for the 92 DH Alexis x Sloop mapping population lines. qPCR methods were undertaken as per section 3.2.3.3. Normalisation of the raw data was performed using the control gene *HvGAPDH* as per Burton *et al.* (2004). Due to the large size of the data set, technical PCR replicates were limited to two for each biological replicate.

A trouble shooting step was also included using *HvGAPDH* to ensure reliability of data used to identify eQTL. In a small subset of lines gene expression was studied at the milk stage (milk 73, refer to Zadoks *et al*. 1974), where expression in candidates was evident. Three biological replicates and three technical replicates were undertaken.

4.2.4. eQTL analysis

Composite interval mapping of expression data obtained (from section 4.2.3.2) was completed using Windows QTL Cartographer 2.5 (Basten *et al.* 2005) with significance threshold values set at a genome-wide significance level of 0.05 using 1000 permutations. Genome-wide significance levels were re-calculated using Map Manager QTX (Manly *et al.* 2001), calculating the probability for the likelihood ratio statistic (LRS) in 2 cM steps for 1000 permutations using an additive regression model. QTL analysis was only completed on biological replicate 1 due to the low expression values obtained in the second

replicate. The marker map used was an updated version of those previously reported for the Alexis/Sloop DH mapping population (Barr *et al.* 2003; Willsmore *et al.* 2006). All available marker information was collated, the map order was reconstructed using RECORD (Van Os *et al.* 2005) and refined through comparisons with the map order obtained from a larger recombinant inbred line (RIL) population as per section 2.2.5 (kindly provided by Greg Lott, SARDI).

4.2.5 Comparative mapping studies

Comparative mapping studies between barley, wheat and rice, were conducted with the aim of identifying candidate genes residing within the eQTL identified (see Section 4.2.4). Only those eQTL which exceeded the highly significant LRS threshold (>0.05), calculated using Map Manager QTX (Manly *et al.* 2001), were explored using comparative mapping. eQTLs identified in replicate one of the qPCR were targeted, due to the inability to detect expression in replicate two.

Markers which flanked the eQTL in the Alexis/Sloop DH population were used as a starting point to identify putative syntenous regions in rice. Due to a lack of sequence information for markers on the Alexis/Sloop map, a variety of barley maps were aligned by the identification of common markers between maps. Wheat was used to bridge the gap between barley and rice, as even after aligning numerous barley maps, there was still a lack of sequence data for many of the barley markers. A Basic Alignment Search Tool (BLAST) nucleotide (BLASTn) analysis of barley markers with sequence data against bin-mapped wheat ESTs, allowed the identification of corresponding bins in wheat. Wheat ESTs from these corresponding bins were identified using the Wheat Binmap viewer from

the GrainGenes database (http://wheat.pw.usda.gov/GG2/index.shtml, 10/08/2008). BLASTn analysis of wheat ESTs was then performed against all rice bacterial artificial chromosome (BAC) and P1-dervied artificial chromosome (PAC) sequences in Genbank, using the Institute for Genomic Research database (TIGR; http://rice.plantpathology.msu.edu, accessed 10/08/2008). The chromosomal location of the BAC/PAC with the highest *e*-value was used to align the wheat EST sequences with rice chromosomes. Brief information of the gene models within the identified syntenous regions was then downloaded from TIGR and searched for genes whose annotation suggested a role in the regulation of transcription.

4.3 Results

4.3.1 Identifying and characterising areas of the genome regulating *HvBP1* expression

4.3.1.1 Sequence variation within the *ORF*

Sequencing of the *ORF* of *HvBP1* showed no sequence variation between the parents of the Alexis/Sloop DH population. The full length *ORF* (1079 bp) has been previously sequenced in the parental variety Bomi (*HvPrx5* Accession; M73234, Rasmussen *et al* 1991), where they report using the *PcR7* probe to study gene expression. The *PcR7* probe aligns to the last 543 bp of the 3' end of the Bomi sequence, however, 4 bp appear to be different in the PcR7 probe (Figure 4.1). The cDNA sequence obtained for the parents Sloop and Alexis is identical to that previously reported for Bomi (Figure 4.1). Further characterisation of the *HvBP1 ORF* involved sequencing reactions using genomic DNA as template, revealing the presence of no introns (Figure 4.1). The genomic sequence of Sloop

is an identical match to the cDNA sequences for both Alexis and Sloop (Figure 4.1). However, genomic sequencing for Alexis *HvBP1* differed from the cDNA *HvBP1* sequence with seven single nucleotide polymorphisms (SNPs) identified (Figure 4.1). To check for sequencing errors three replications were undertaken, ensuring a correct result. The gDNA for Alexis *HvBP1* appears to be identical with the *PcR7* sequence identified by Rasmussen and colleagues (Figure 4.1) between 536 and 1079 bp.

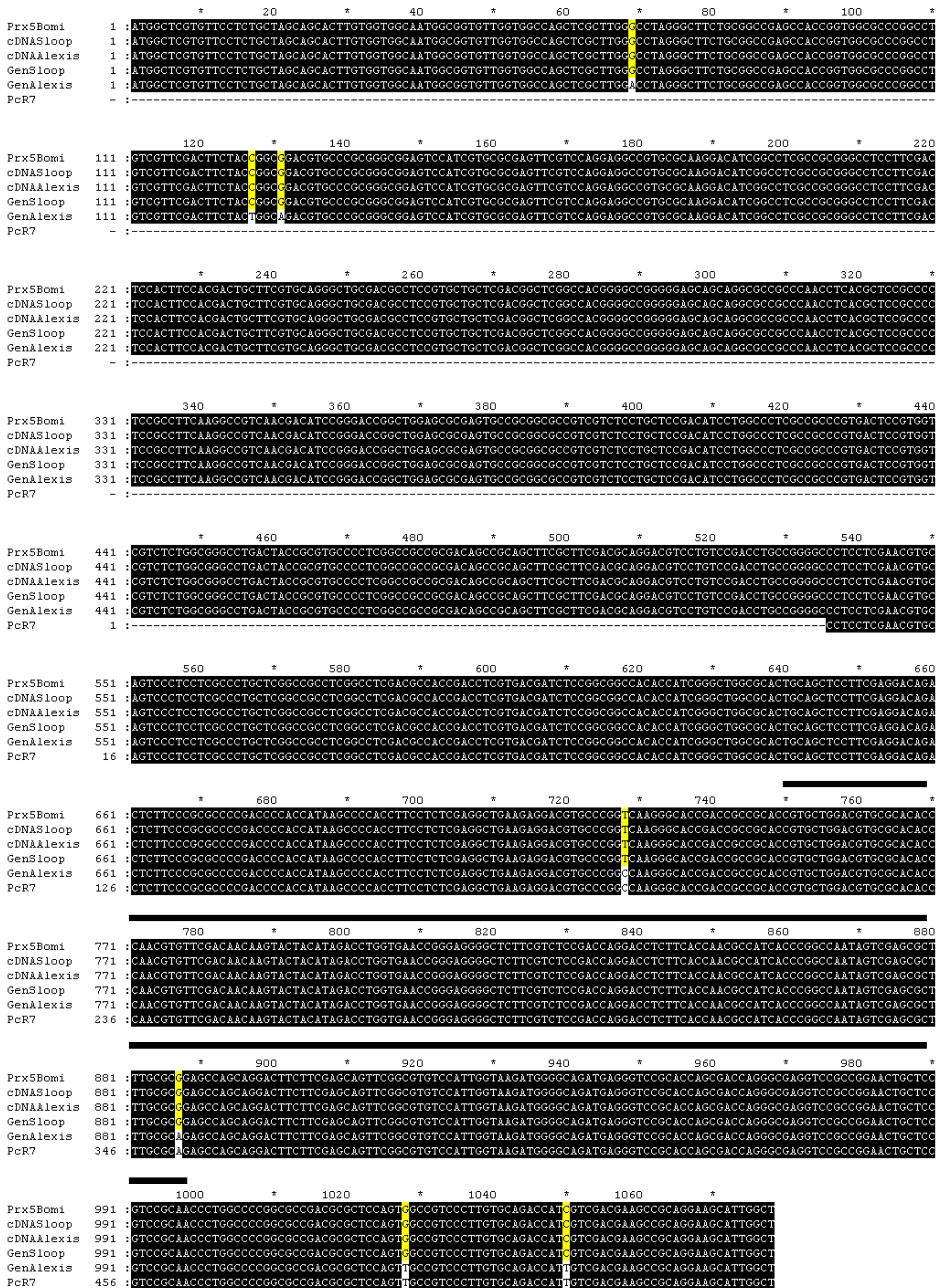

Figure 4.1 Alignment of sequences for *HvBP1*. The *ORF* of *HvBP1* was sequenced from cDNA and genomic DNA (Gen) of Sloop and Alexis (n=3). Previously reported sequences for *HvBP1* (*HvPrx5* Bomi and *PcR7*) are also shown (Rasmussen *et al.* 1991). The sequence used for the qPCR probe is shown as a line above the sequence.

4.3.1.2 Chromosomal location of *HvBP1*

Using PCR of barley:wheat addition lines, *HvBP1* was localised to chromosome 7H (Figure 4.2).

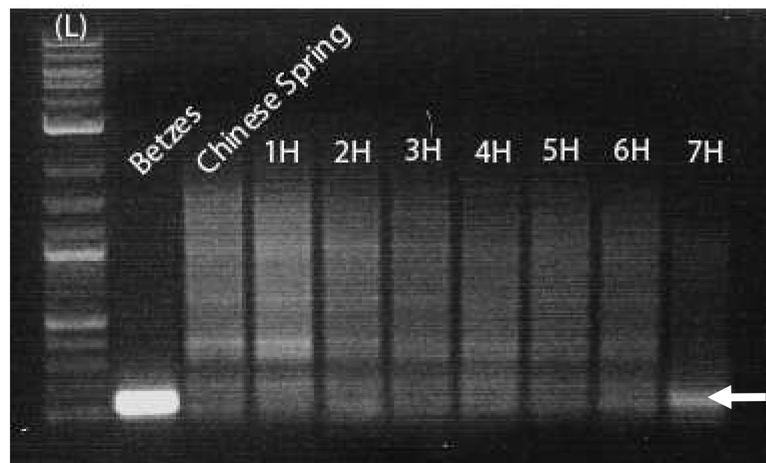

Figure 4.2 Chromosomal localisation of *HvBP1* using PCR of barley:wheat addition lines. Lines included barley control (Betzes), wheat control (Chinese Spring) and wheat addition lines containing one of each of the barley chromosomes (1H-7H). (L)=1kb plus ladder. The arrow represents the amplification of the PCR product for *HvBP1* in the 7H addition line.

The absence of SNPs within the *ORF* did not allow the fine mapping of *HvBP1* using Amplified Fragment Length Polymorphism analysis. Previous studies using RFLP techniques have mapped *HvBP1* to chromosome 3H (March *et al.* 2007). Rasmusson and colleagues (1991) using the *PcR7* probe also reported the location of *HvBP1* to be on chromosome 3H.

4.3.1.3 Expression data across the Alexis/Sloop mapping population

Variation in gene expression was observed across the Alexis/Sloop DH population for biological replicate 1 (Figure 4.3A). The variation observed indicates that gene expression

can be used in further experiments as a quantitative trait to map gene expression, with the aim of identifying eQTL. When gene expression was studied in a second biological replicate (an alternate plot at Hatherleigh) gene expression was not detected in more than half of the lines (Figure 4.3B), suggesting technical problems with the qPCR. To address this, the qPCR was repeated (a repeat of replicate 2) for the 72 lines where little or no expression was apparent. cDNA quality was firstly examined through assessment of *HvGAPDH* and results compared to the first replicate (Figure 4.4). When no expression was observed for replicate 2 (Figure 4.3B), *HvGAPDH* levels were low or absent in comparison to the first replicate (Figure 4.4), suggesting that the results obtained were due to cDNA quality. *HvGAPDH* levels were substantially increased in the repeat of replicate 2 (Figure 4.4, 72 lines represented by a line under the A x S number). Comparable and higher *HvGAPDH* levels were observed in comparison to the first replicate where all 92 lines of the population displayed expression.

After normalisation against *HvGAPDH,* little or no expression was detected for *HvBP1* in a large number of lines for the repeat of replicate 2 (data not shown). Expression appears to be higher than that of the first replicate in a large number of the lines, where higher candidate gene (*HvBP1*) expression was observed. Where no expression was present in replicate 2, *HvGAPDH* expression appeared sound. Establishment of gene expression in the second biological replicate revealed that 38 of the 92 lines displayed no gene expression for *HvBP1.* A scatter plot also indicated no relationship between replicate 1 and 2 (Figure 4.5). Results would therefore suggest we are observing considerable biological variation between plots.

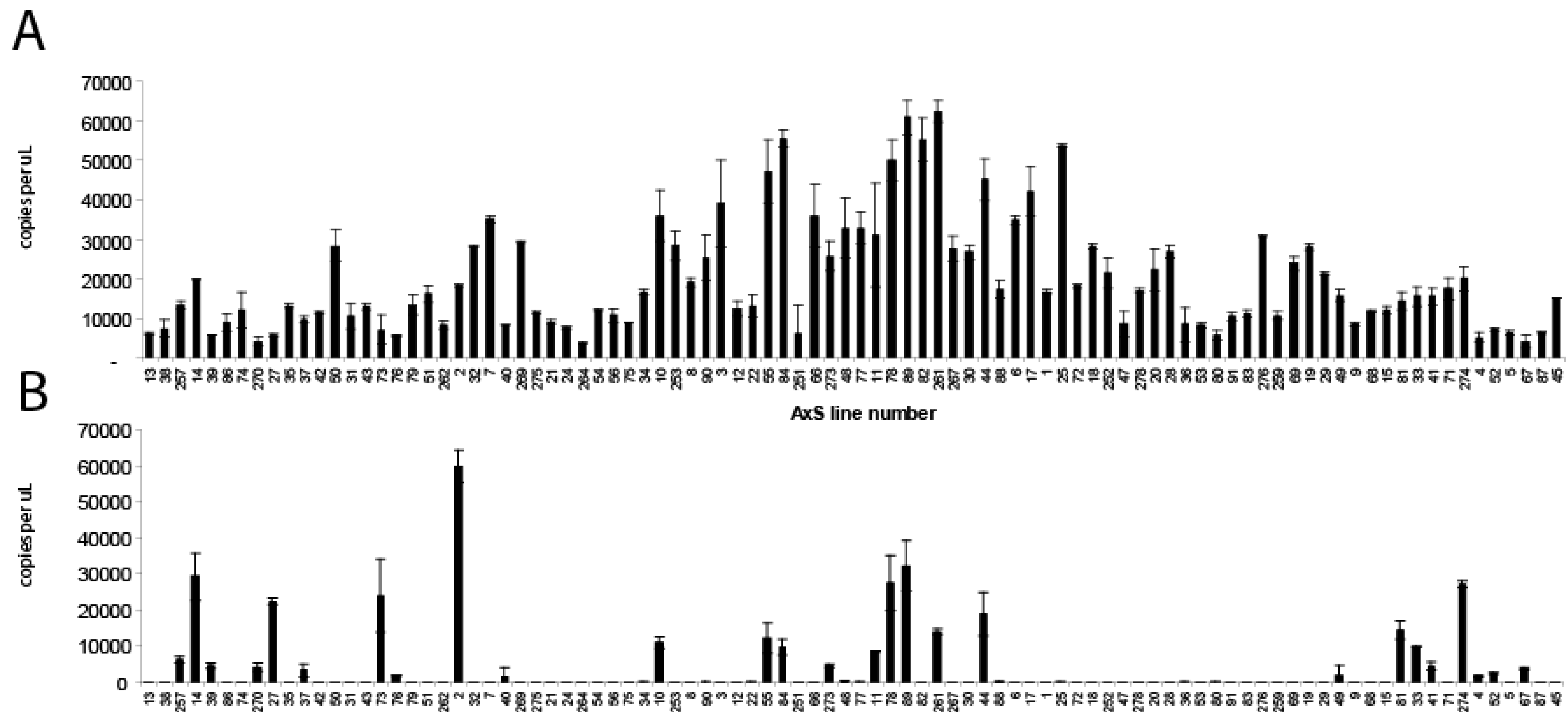

Figure 4.3 Normalised expression levels (level of mRNA presented as number of copies per µL) of candidate gene *HvBP1* obtained from qPCR across the Alexis x Sloop (A x S) DH mapping population. Normalisation of the raw data was performed using the control gene *HvGAPDH* as per (Burton et al., 2004). Two biological replicates (separate plots) are presented, in A and B respectively (mean ± standard deviation of 2 technical replicates for each biological replicate are shown). Expression was observed at the hard dough (77) stage of grain development (refer to Zadoks *et al*. 1974, see section 3.2.1).

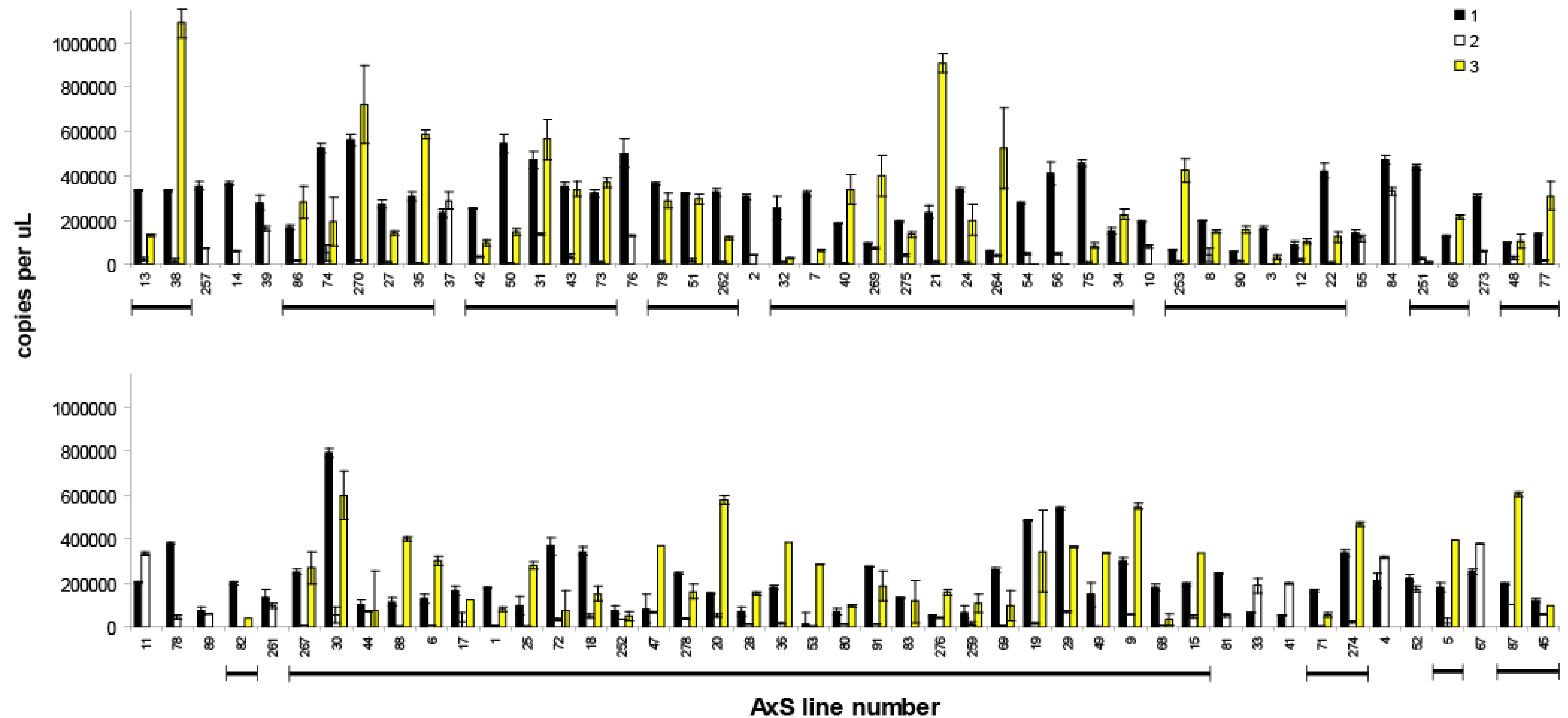

Figure 4.4 *HvGAPDH* expression levels (level of mRNA presented as number of copies per µL) for cDNA obtained from qPCR across the Alexis x Sloop DH mapping population [n = 2 (Replicate 1 and 2 combined), n=3 (Repeat of Replicate 2), standard deviation for each cDNA was calculated]. Replicate 1 (solid black bar) is representative of a biological replicate while Replicate 2 (white bar) and the repeat (yellow bar) are representative of a second biological replicate. The repeat of Replicate 2 (shown by a line under the AxS number) was a subset of lines created from replicate 2 where little or

Figure 4.4 cont.

no candidate gene expression was observed (Figure 4.3). Expression was observed at the hard dough (77) stage of grain development (refer to Zadoks *et al*., 1974).

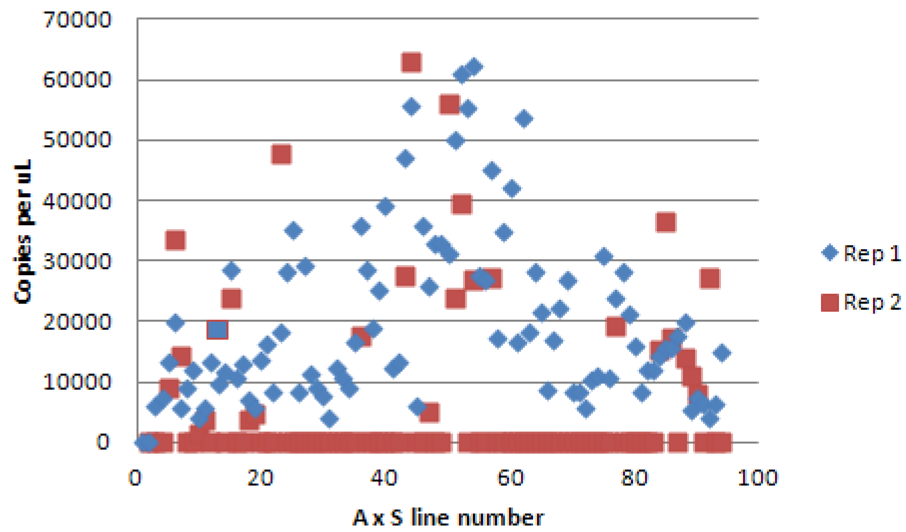

Figure 4.5 Comparison of qPCR data for *HvBP1* in biological replicate 1 and 2. Data from figures 4.3 and 4.4 have been displayed in a scatterplot for comparison.

A further trouble shooting step was therefore completed to ensure reliability and decide which data should be used to identify eQTL. Gene expression was studied at the milk stage (73, refer to Zadoks *et al*. 1974) of grain development for *HvBP1* where previous higher expression levels were observed (refer to section 3.3.2, Figure 4.6). Candidate genes *HvQR* and *HvPrx7* were also included in this experiment. The small subset of lines displayed high *HvGAPDH* levels (Figure 4.6A) and elevated levels of expression for *HvBP1* (Figure 4.6B) compared to the hard dough stage (Figure 4.4) as expected, suggesting data from replicate 1 was reliable for eQTL analysis. *HvQR* (Figure 4.6B) and *HvPrx7* (Figure 4.6C) were also expressed.

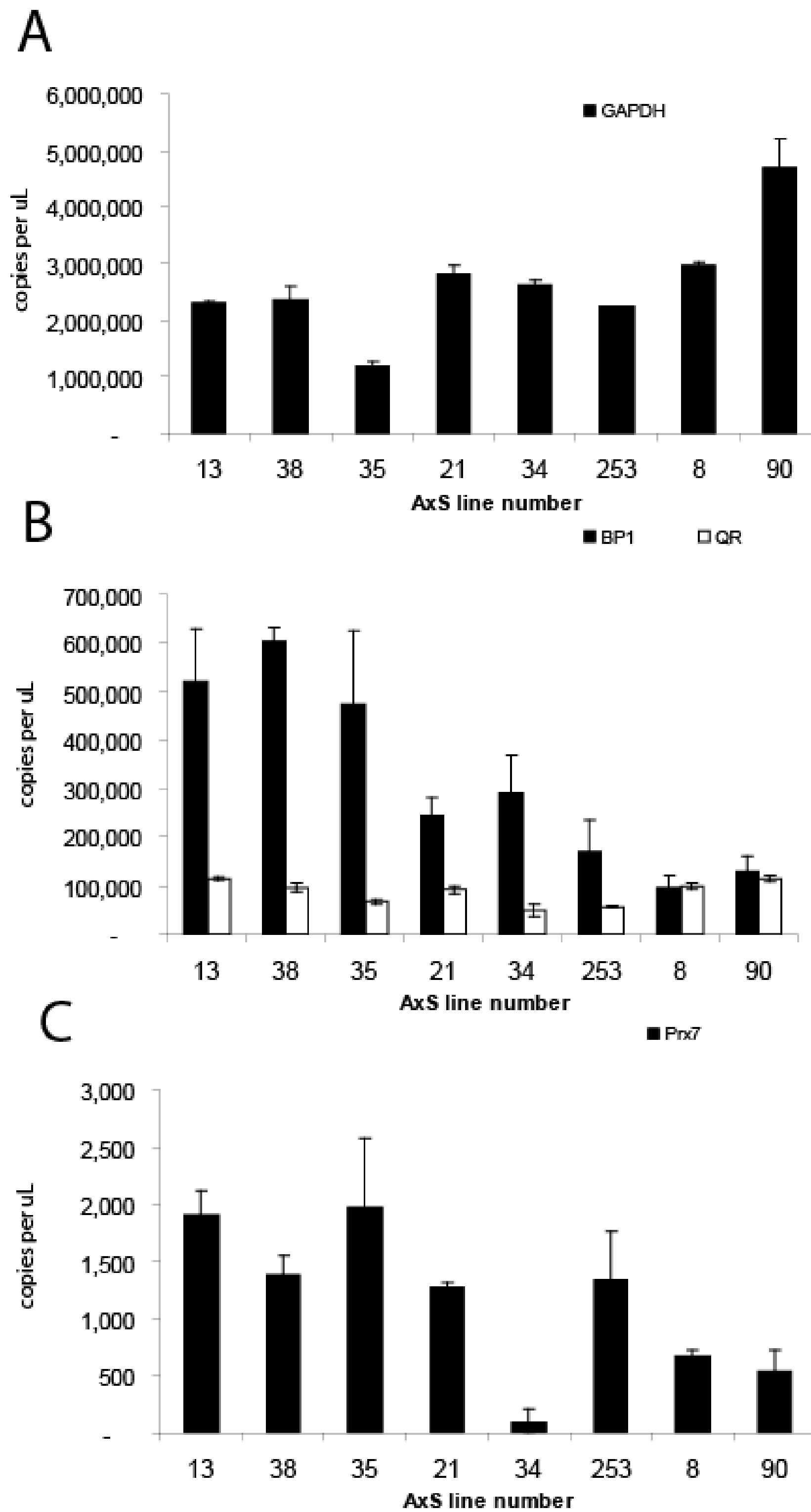

Figure 4.6 *HvGAPDH* expression levels for cDNA (A) and normalised expression levels of candidate gene *HvBP1, HvQR* (B) and *HvPrx7* (C) (level of mRNA presented as number of copies per µL) obtained from qPCR across a subset of lines from Alexis x Sloop DH mapping population (mean ± standard deviation of 3 technical replicates). The milk stage of grain development was examined (milk 73, refer to Zadoks *et al*. 1974).

4.3.1.4 Identification of *trans*-acting eQTL for *HvBP1*

Due to the large variation present between biological replicates, eQTL analysis was undertaken on replicate 1. Gene expression was distributed across the Alexis/Sloop DH population as shown for biological replicate 1 (Figure 4.3 A) and biological replicate 2 (Figure 4.3B and 4.5). Using gene expression data from replicate 1 (where gene expression was apparent across all lines), eQTL were detected on chromosomes 1H, 2H and 5H (Figure 4.7). The Alexis allele contributed to the QTL on all chromosomes, explaining 5.6%, 13% and 21% of the phenotypic variance for chromosomes 1H, 2H and 5H, respectively. Calculation of genome-wide significance levels indicated that only the QTL on chromosomes 2H and 5H are considered highly significant (>0.05 LRS). No significant QTL were detected for replicate 2 and its repeat (data not shown). The *HvBP1* gene was mapped to chromosome 7H (Figure 4.2) indicating that for *HvBP1* a *trans*-regulatory mechanism (polymorphism elsewhere in the genome) is observed with loci on chromosomes 2H and 5H affecting the expression of the *HvBP1* gene on 7H.

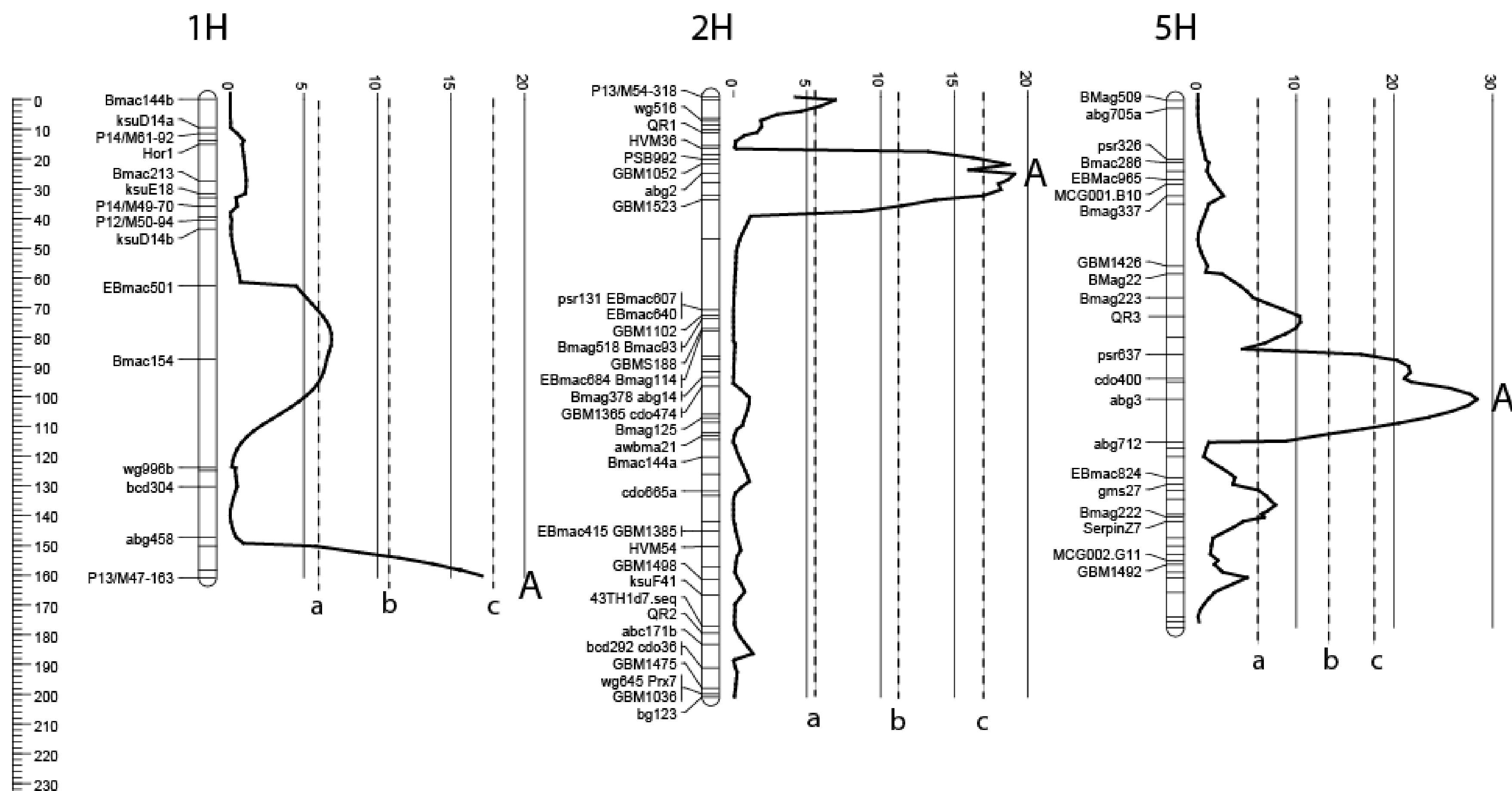

Figure 4.7 Likelihood ratio test statistics from composite interval mapping of *HvBP1* gene expression (Replicate 1) (eQTLs) in the Alexis /Sloop DH population grown at Hatherleigh, SA in 2004/2005 showing QTL detected on chromosomes 1H, 2H and 5H. Distances within chromosomes are displayed in centimorgans (cM). At each QTL peak, the allele contributing is identified as coming from Sloop(S) or Alexis (A). Genome-wide significance levels were re-calculated using Map Manager QTX (Manly *et al.* 2001) with a = suggestive, b = significant and c = highly significant. The marker map is an updated version of those previously reported for the Alexis/Sloop DH mapping population (Barr *et al*. 2003; Willsmore *et al*. 2006).

4.3.1.5 Comparative mapping studies of the *trans*-acting eQTL for *HvBP1*

Comparative mapping studies between barley, wheat and rice, were conducted with the aim of identifying candidate genes residing within the *trans*-eQTL identified. eQTL on chromosome 2H and 5H for *HvBP1* were chosen for comparative mapping studies because these eQTL were found to be highly significant.

Markers which flanked the eQTL in the Alexis/Sloop doubled haploid population were used as a starting point to identify putative syntenous regions in rice. Due to a lack of sequence information for markers on the Alexis/Sloop map, a variety of barley maps were aligned by the identification of common markers between maps (Figure 4.8 and Figure 4.9). Flanking markers of the eQTL identified on chromosome 2H of the Alexis/Sloop DH map (Barr *et al*. 2003; Willsmore *et al*. 2006) were identified as HVM36 and GBM1523 (Figure 4.8). Flanking markers of the eQTL identified on chromosome 5H in the Alexis/Sloop DH map (Barr *et al*. 2003; Willsmore *et al*. 2006) were identified as psr637 and abg712 (Figure 4.9). BLASTn analysis of barley markers on chromosome 2H of barley (Figure 4.8) against bin-mapped wheat ESTs, identified hits to bins on the short arm of chromosomes 2A, 2B and 2D. The entire short arm of wheat chromosome 2 was used for further analysis, resultant of hits to all bins (http://wheat.pw.usda.gov/GG2/index.shtml). A total of 810 wheat ESTs from these corresponding bins were identified using the Wheat Binmap viewer from the GrainGenes database (http://wheat.pw.usda.gov/GG2/index.shtml). BLASTn analysis of these 810 wheat ESTs was performed against all rice BAC and PAC sequences in Genbank, with a noticeable trend in hits to rice chromosome 4 and 7 as expected (Figure 4.10). A total of 102 wheat ESTs (12.6%) aligned to rice chromosome 4 and 308 (38%) to rice chromosome 7.

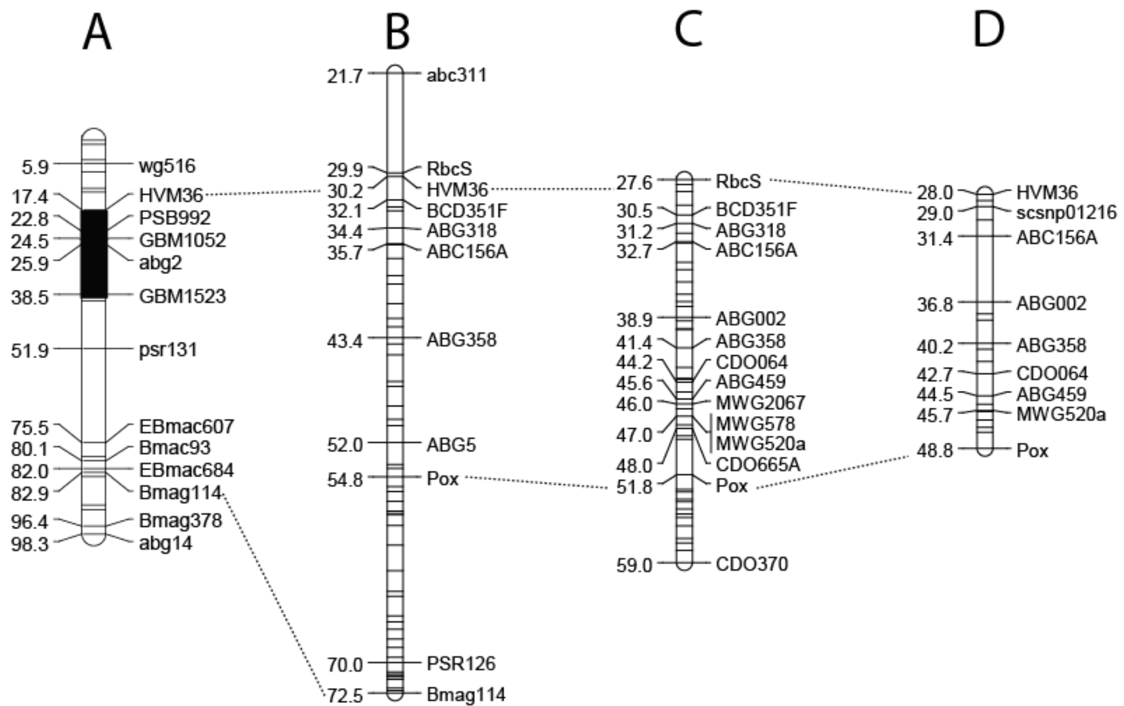

Figure 4.8 Barley chromosome 2H maps aligned to show common markers within the eQTL identified. Flanking markers of the eQTL identified in the Alexis/Sloop DH map (A) (Barr *et al*. 2003; Willsmore *et al*. 2006) were identified as HVM36 and GBM1523; Identification of further marker sequence information through alignment with maps; (B) DArT/SSR/RFLP/STS consensus map (Wenzl *et al.* 2006); (C) Barley BinMap 2005 (http://barleygenomics.wsu.edu/); (D) Barley Consensus 2005, SNP map (Rostoks *et al.* 2005).

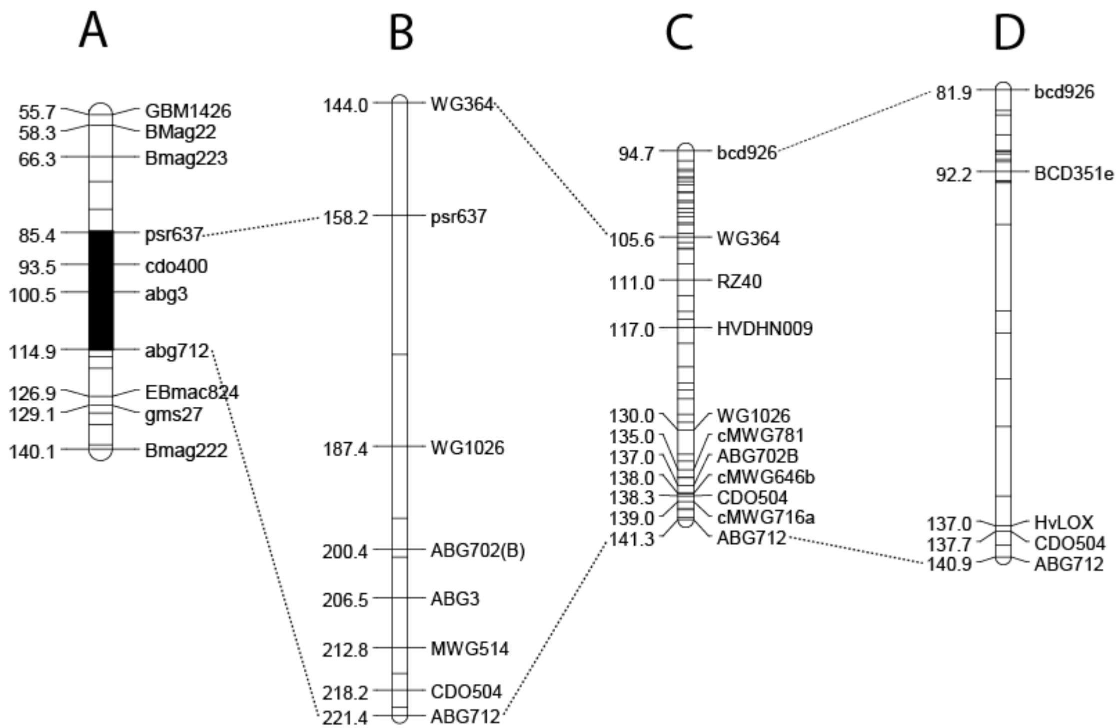

Figure 4.9 Barley chromosome 5H maps aligned to show common markers within the eQTL identified. Flanking markers of the eQTL identified in the Alexis/Sloop DH map (A) (Barr *et al.* 2003; Willsmore *et al.* 2006) were identified as psr637 and abg712; Identification of further marker sequence information through alignment with maps; (B) Barley G x H (Galleon x Haruna) (http://greengenes.cit.cornell.edu/WaiteQTL/GxH.html); (C) Barley BinMap 2005 (http://barleygenomics.wsu.edu/); (D) Barley Consensus 2005, SNP map (Rostoks *et al.* 2005).

BLASTn analysis of barley markers on chromosome 5H of barley (Figure 4.11) against bin-mapped wheat ESTs, identified hits to bins on the long arm of chromosomes 5A, 5B and 5D. A total of 743 wheat ESTs from these corresponding bins (Figure 4.11) were identified using the Wheat Binmap viewer from the GrainGenes database (http://wheat.pw.usda.gov/GG2/index.shtml). The bin 12-0.35-0.57 on chromosome 5AL displayed 23 hits (47%) to rice chromosome 9. There was a noticeable trend of hits to rice chromosome 3 (326 or 44%) for the remaining bins identified as identified (Figure 4.11).

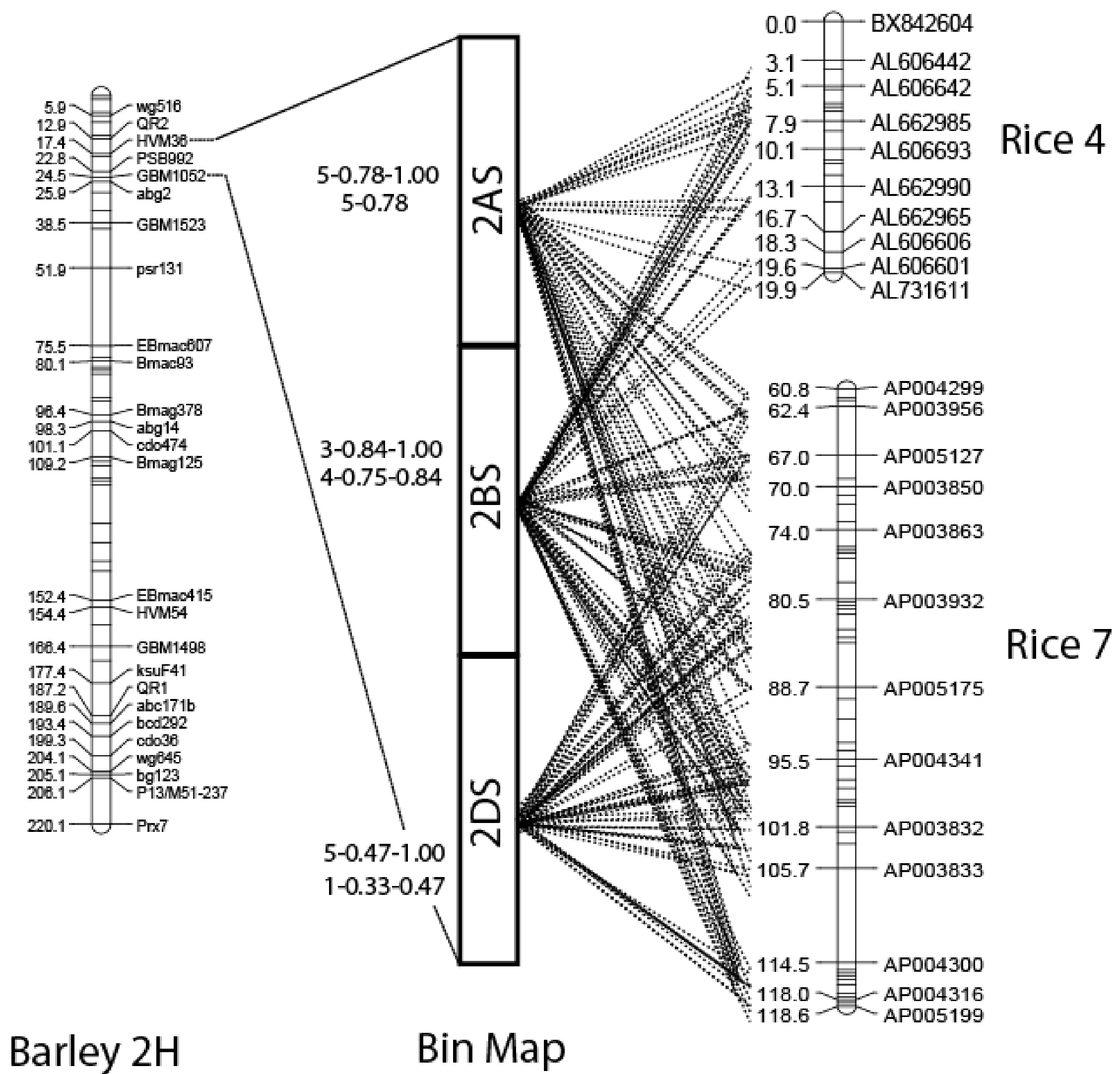

Figure 4.10 Alignment of the eQTL on barley chromosome 2H and rice chromosome 4 and 7. Marker sequence data from Barley 2H [Alexis x Sloop DH map (Barr *et al.* 2003; Willsmore *et al.* 2006)] resulted in hits to wheat bin map, chromosomes 2AS, 2BS and 2DS. The corresponding bin names are represented to the left of the bin map. Syntenous regions are linked to rice chromosome 4 (0-19.9 cM) and 7 (60.8-118.6 cM).

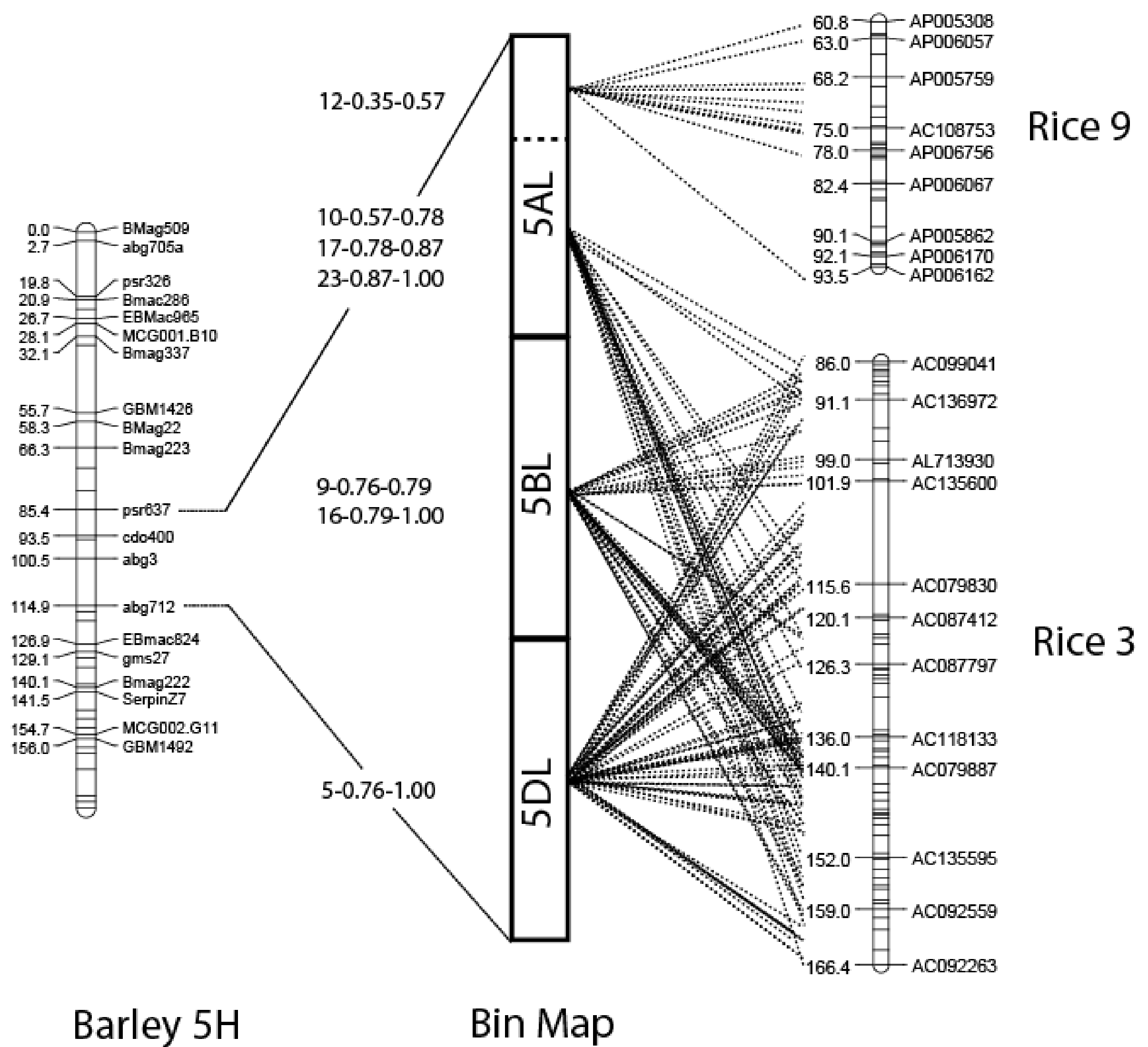

Figure 4.11 Alignment of the eQTL on barley chromosome 5H and rice chromosome 9 and 3. Marker sequence data from Barley 5H [Alexis x Sloop DH map (Barr *et al*. 2003; Willsmore *et al*. 2006)] resulted in hits to wheat bin map, chromosomes 5AL, 5BL and 5DL. The corresponding bin names are represented to the left of the Bin map. Syntenous regions are linked to rice chromosome 9 (60.8-93.5 cM) and 3 (86-166.4 cM).

Regions of Rice Chromosome 4 and 7, which were found to be syntenous to the eQTL on barley 2H (Figure 4.10) revealed a total of 19 candidates for chromosome 4 (Table 4.2) and 76 candidates for chromosome 7 (Table 4.3) through comparative mapping between barley, wheat and rice. Genes were chosen as candidates based on their proposed role in transcription or whether they had DNA binding domains or domains previously ascribed to transcription factors. The full list of candidates identified through comparative mapping can be found in Appendix 2. Regions of Rice Chromosome 9 and 3, which were found to be syntenous to the eQTL on barley 5H (Figure 4.11) revealed a total of 60 candidates for chromosome 9 (Table 4.4) and 95 candidates for chromosome 3 (Table 4.5) through comparative mapping.

Table 4.2 Proposed candidate genes within the identified syntenous region (Rice 4) for the *HvBP1* eQTL identified on barley chromosome 2H. Candidates identified through comparative mapping between barley, wheat and Rice (Figure 4.10). Locus represents the gene number and accession, the rice BAC number in which the gene resides (TIGR; http://rice.plantpathology.msu.edu). Candidate represents brief information of the gene. models, with annotations suggesting a proposed role in the regulation of transcription.

Locus	Accession	Candidate
LOC_Os04g02000	AL606642	Zinc-finger, RanBP-type, containing protein, expressed
LOC_Os04g08060	AL606654	Zinc finger, C2H2 type family protein, expressed
LOC_Os04g08290	AL662959	Zinc finger, C2H2 type family protein
LOC_Os04g08600	AL663013	Zinc finger, C2H2 type family protein
LOC_Os04g17200	AL662989	GRF zinc finger family protein
LOC_Os04g16970	AL606611	Zinc finger, C3HC4 type family protein, expressed
LOC_Os04g10890	AL663018	Zinc knuckle family protein
LOC_Os04g16270	AL662961	Zinc knuckle family protein
LOC_Os04g09560	AL731589	DNA binding protein-like, putative
LOC_Os04g10260	AL662934	DNA binding protein, putative
LOC_Os04g10610	AL731620	SWIM zinc finger family protein
LOC_Os04g11830	AL662965	TCP-domain protein, putative, expressed
LOC_Os04g12460	AL606449	Leucine Rich Repeat family protein, expressed
LOC_Os04g08390	AL662959	Leucine Rich Repeat family protein, expressed
LOC_Os04g15650	AL662993	Leucine Rich Repeat family protein, expressed
LOC_Os04g02520	AL606992	Leucine Rich Repeat family protein
LOC_Os04g08370	AL662959	Leucine Rich Repeat family protein
LOC_Os04g14990	AL731592	BURP domain-containing protein, putative
LOC_Os04g19684	AL731611	Methyl-CpG binding domain containing protein, expressed

Table 4.3 Proposed candidate genes within the identified syntenous regions (Rice 7) for eQTL identified on barley chromosome 2H. Candidates identified through comparative mapping between barley, wheat and Rice (Figure 4.10). Locus represents the gene number and accession, the rice BAC number in which the gene resides (TIGR; http://rice.plantpathology.msu.edu). Candidate represents brief information of the gene models, with annotations suggesting a proposed role in the regulation of transcription.

Locus	Accession	Candidate
LOC_Os07g31470	AP004259	MYB transcription factor, putative, expressed
LOC_Os07g37210	AP005195	MYB transcription factor, putative, expressed
LOC_Os07g44090	AP004334	Myb-related protein Hv33, putative, expressed
LOC_Os07g43420	AP004009	Myb, DNA-binding, putative, expressed
LOC_Os07g31500	AP004259	leucine-rich repeat receptor protein kinase EXS precursor, putative, expressed
LOC_Os07g35110	AP003863	Leucine Rich Repeat family protein
LOC_Os07g31720	AP005177	ZAC, putative, expressed
LOC_Os07g32170	AP005186	SBP domain containing protein, expressed
LOC_Os07g32350	AP005127	WD-repeat protein 74, putative, expressed
LOC_Os07g32420	AP003815	DNA binding protein, putative, expressed
LOC_Os07g37800	AP003705	DNA binding protein, putative, expressed
LOC_Os07g38170	AP003981	DNA binding protein, putative, expressed
LOC_Os07g39320	AP004276	DNA binding protein, putative, expressed
LOC_Os07g41640	AP005193	DNA binding protein, putative, expressed
LOC_Os07g42750	AP004309	DNA binding protein, putative, expressed
LOC_Os07g44950	AP003765	DNA binding protein, putative, expressed
LOC_Os07g49290	AP004333	DNA binding protein, putative, expressed
LOC_Os07g39940	AP003985	DNA binding protein, putative
LOC_Os07g48200	AP005243	B3 DNA binding domain containing protein, expressed
LOC_Os07g33720	AP003930	NB-ARC domain containing protein
LOC_Os07g33730	AP003930	NB-ARC domain containing protein, expressed
LOC_Os07g34880	AP006753	Homeobox domain containing protein
LOC_Os07g35870	AP005156	bHLH transcription factor, putative, expressed
LOC_Os07g36390	AP004401	CRP1, putative, expressed
LOC_Os07g36820	AP004261	Uncharacterized Cys-rich domain, putative, expressed
LOC_Os07g37650	AP005296	ARF GAP-like zinc finger-containing protein ZIGA3, putative, expressed
LOC_Os07g37920	AP003932	NAM-like protein, putative, expressed
LOC_Os07g38240	AP003981	AN1-type zinc finger protein 2B, putative, expressed
LOC_Os07g38750	AP003845	AP2 domain containing protein
LOC_Os07g39110	AP004182	AP2/EREBP transcription factor BABY BOOM, putative, expressed
LOC_Os07g38440	AP005908	Regulatory protein, DeoR, putative, expressed
LOC_Os07g39310	AP004276	Zinc finger, C2H2 type family protein, expressed
LOC_Os07g40780	AP003915	Zinc finger, C2H2 type family protein, expressed
LOC_Os07g39960	AP005149	Zinc finger, C2H2 type family protein
LOC_Os07g40950	AP003840	Zinc finger, C2H2 type family protein
LOC_Os07g39970	AP005149	Zinc finger protein PIF1, putative, expressed
LOC_Os07g40300	AP003846	Zinc finger protein 7, putative, expressed
LOC_Os07g40080	AP003750	Zinc-finger protein 1, putative, expressed
LOC_Os07g42610	AP004988	Ring-H2 zinc finger protein, putative, expressed
LOC_Os07g48680	AP003818	RING-H2 finger protein ATL4L, putative, expressed
LOC_Os07g42640	AP004309	FYVE zinc finger family protein, expressed
LOC_Os07g45180	AP005455	SWIM zinc finger family protein, expressed
LOC_Os07g43400	AP004009	SWIM zinc finger family protein, expressed

Table 4.3 cont.

LOC_Os07g45250	AP005455	SWIM zinc finger family protein
LOC_Os07g47010	AP003825	SWIM zinc finger family protein
LOC_Os07g47360	AP004570	CW-type Zinc Finger family protein, expressed
LOC_Os07g39430	AP004185	mTERF family protein, expressed
LOC_Os07g39480	AP003747	OsWRKY78 - Superfamily of rice TFs having WRKY and zinc finger domains, expressed
LOC_Os07g48260	AP005243	OsWRKY78 - Superfamily of rice TFs having WRKY and zinc finger domains, expressed
LOC_Os07g40570	AP004275	WRKY transcription factor 3, putative, expressed
LOC_Os07g48450	AP005167	NAC domain-containing protein 18, putative, expressed
LOC_Os07g48550	AP005167	NAC domain-containing protein 21/22, putative, expressed
LOC_Os07g39800	AP005437	transcription repressor HOTR, putative, expressed
LOC_Os07g39810	AP005437	triacylglycerol lipase, putative, expressed
LOC_Os07g39820	AP005437	SHR, putative, expressed
LOC_Os07g40020	AP005149	GRAS family transcription factor containing protein, expressed
LOC_Os07g40130	AP003750	transcriptional regulatory protein algP, putative
LOC_Os07g44200	AP003749	transcription regulator, putative, expressed
LOC_Os07g41580	AP005193	nuclear transcription factor Y subunit B-3, putative, expressed
LOC_Os07g41720	AP006458	nuclear transcription factor Y subunit A-3, putative, expressed
LOC_Os07g40580	AP004275	Eukaryotic translation initiation factor 5A, putative, expressed
LOC_Os07g41340	AP005175	B12D protein, expressed
LOC_Os07g41350	AP005175	B12D protein, expressed
LOC_Os07g41370	AP005175	MADS-box transcription factor 18, putative, expressed
LOC_Os07g41560	AP005193	STF-1, putative
LOC_Os07g42370	AP005198	pnFL-2, putative, expressed
LOC_Os07g42800	AP004309	AT hook-containing MAR binding 1-like protein, putative, expressed
LOC_Os07g44030	AP004339	TKI1, putative
LOC_Os07g44690	AP005292	AT-HSFB4, putative, expressed
LOC_Os07g45350	AP003822	ZCF61, putative, expressed
LOC_Os07g47110	AP004274	Phosphoric diester hydrolase/ transcription factor, putative, expressed
LOC_Os07g47790	AP006268	ERF-like protein, putative, expressed
LOC_Os07g48180	AP005243	transcription factor RF2b, putative, expressed
LOC_Os07g48820	AP003813	transcription factor HBP-1b, putative, expressed
LOC_Os07g48870	AP003813	typical P-type R2R3 Myb protein, putative, expressed
LOC_Os07g49380	AP005199	PWWP domain containing protein, expressed

Table 4.4 Proposed candidate genes within the identified syntenous region (Rice 9) for the *HvBP1* eQTL identified on barley chromosome 5H. Candidates identified through comparative mapping between barley, wheat and Rice (Figure 4.10). Locus represents the gene number and accession, the rice BAC number in which the gene resides (TIGR; http://rice.plantpathology.msu.edu). Candidate represents brief information of the gene models, with annotations suggesting a proposed role in the regulation of transcription.

Locus	Accession	Candidate
LOC_Os09g27650	AP005308	zinc finger, C2H2 type family protein, expressed
LOC_Os09g39660	AP005546	zinc finger, C2H2 type family protein, expressed
LOC_Os09g28110	AP005393	RING zinc finger protein-related, putative, expressed
LOC_Os09g32730	AC108753	zinc finger-like protein, putative, expressed
LOC_Os09g29130	AP005676	ZF-HD protein dimerisation region containing protein, expressed
LOC_Os09g29310	AP005399	RING/C3HC4/PHD zinc finger-like protein, putative, expressed
LOC_Os09g29370	AP005399	RING-H2 finger protein ATL5F, putative, expressed
LOC_Os09g37050	AP006149	RING-H2 finger protein ATL2B, putative, expressed
LOC_Os09g38110	AC137596	RING-H2 finger protein ATL2A, putative, expressed
LOC_Os09g36500	AP006067	RING-H2 finger protein ATL2A, putative, expressed
LOC_Os09g33670	AC137594	zinc finger, C3HC4 type family protein, expressed
LOC_Os09g33740	AC137594	zinc finger, ZZ type family protein, expressed
LOC_Os09g33550	AC137595	zinc finger protein CONSTANS-LIKE 15, putative, expressed
LOC_Os09g38400	AC137592	zinc finger protein hangover, putative, expressed
LOC_Os09g38610	AC137592	zinc finger protein 2, putative, expressed
LOC_Os09g38790	AP005396	zinc finger protein 207, putative, expressed
LOC_Os09g34980	AP006859	zinc knuckle family protein, expressed
LOC_Os09g27730	AP005559	protein HVA22, putative, expressed
LOC_Os09g28200	AP005655	AT-HSFB4, putative, expressed
LOC_Os09g28210	AP005655	DNA binding protein, putative, expressed
LOC_Os09g29360	AP005399	DNA binding protein, putative, expressed
LOC_Os09g31390	AC108758	DNA binding protein, putative, expressed
LOC_Os09g31470	AC108762	DNA binding protein, putative, expressed
LOC_Os09g37760	AP005679	DNA binding protein, putative, expressed
LOC_Os09g29830	AP006169	DNA binding protein, putative, expressed
LOC_Os09g28900	AP005755	DNA binding protein, putative
LOC_Os09g28310	AP005655	bZIP transcription factor, putative, expressed
LOC_Os09g36760	AP006174	bZIP-like protein, putative, expressed
LOC_Os09g36910	AP006149	bZIP transcription factor family protein, expressed
LOC_Os09g29820	AP006169	BZIP family transcription factor, putative, expressed
LOC_Os09g28440	AP005891	AP2 domain containing protein, expressed
LOC_Os09g28890	AP005755	AHM1, putative, expressed
LOC_Os09g29460	AP005574	homeobox-leucine zipper protein ATHB-6, putative, expressed
LOC_Os09g35910	AP005681	homeodomain-leucine zipper transcription factor TaHDZipI-1, putative, expressed
LOC_Os09g29550	AP005555	dof zinc finger protein, putative

Table 4.4 cont.

LOC_Os09g29960	AP005759	dof zinc finger protein MNB1A, putative, expressed
LOC_Os09g29930	AP006169	transcription factor BIM2, putative, expressed
LOC_Os09g30310	AP005633	nuclear transcription factor Y subunit C-2, putative, expressed
LOC_Os09g30320	AP005633	BURP domain containing protein, expressed
LOC_Os09g30400	AP005392	OsWRKY80 - Superfamily of rice TFs having WRKY and zinc finger domains, expressed
LOC_Os09g31200	AC108756	multiple stress-responsive zinc-finger protein ISAP1, putative, expressed
LOC_Os09g31300	AC108758	helix-loop-helix DNA-binding domain containing protein, expressed
LOC_Os09g31454	AC108762	myb-like DNA-binding domain containing protein, expressed
LOC_Os09g36730	AP006174	myb-related protein Hv1, putative, expressed
LOC_Os09g32010	AC099403	ternary complex factor MIP1, putative, expressed
LOC_Os09g32260	AC099404	ANAC079/ANAC080, putative, expressed
LOC_Os09g32510	AC108763	BHLH transcription factor, putative, expressed
LOC_Os09g33580	AC137595	bHLH transcription factor GBOF-1, putative, expressed
LOC_Os09g32948	AC108759	MADS-box transcription factor 8, putative, expressed
LOC_Os09g33490	AC137595	NAC domain-containing protein 18, putative, expressed
LOC_Os09g38010	AC137596	NAC domain-containing protein 78, putative, expressed
LOC_Os09g38000	AC137596	ANAC086, putative
LOC_Os09g33590	AC137595	retrotransposon protein, putative, LINE subclass
LOC_Os09g34060	AP006756	transcription factor RF2a, putative, expressed
LOC_Os09g34330	AP007254	transcription factor AtMYC2, putative
LOC_Os09g35700	AP005864	YY1 protein precursor, putative, expressed
LOC_Os09g35760	AP005864	OCL3 protein, putative, expressed
LOC_Os09g36160	AP005567	SHI, putative, expressed
LOC_Os09g37250	AP006548	ARID/BRIGHT DNA binding domain containing protein
LOC_Os09g37910	AP005742	HMG1/2-like protein, putative, expressed

Table 4.5 Proposed candidate genes within the identified syntenous regions (Rice 3) for the *HvBP1* eQTL identified on barley chromosome 5H. Candidates identified through comparative mapping between barley, wheat and Rice (Figure 4.10). Locus represents the gene number and accession, the rice BAC number in which the gene resides (TIGR; http://rice.plantpathology.msu.edu). Candidate represents brief information of the gene models, with annotations suggesting a proposed role in the regulation of transcription.

Locus	Accession	Candidate
LOC_Os03g31880	AC133861	SHR, putative, expressed
LOC_Os03g32220	AC147803	zinc-finger protein 1, putative, expressed
LOC_Os03g41110	AC133860	zinc-finger protein 1, putative, expressed
LOC_Os03g41390	AC135500	zinc-finger protein 1, putative, expressed
LOC_Os03g55540	AC090713	zinc-finger protein 1, putative, expressed
LOC_Os03g62230	AC104487	zinc-finger protein 1, putative, expressed
LOC_Os03g39040	AC135502	zinc knuckle family protein, expressed
LOC_Os03g39880	AC120537	zinc knuckle family protein, expressed
LOC_Os03g45730	AC135600	zinc knuckle family protein, expressed
LOC_Os03g40710	AC109601	zinc finger, C2H2 type family protein
LOC_Os03g49132	AC097368	zinc finger, C2H2 type family protein
LOC_Os03g60540	AC104433	zinc finger, C2H2 type family protein
LOC_Os03g41640	AC136972	GRF zinc finger family protein
LOC_Os03g44600	AL731878	GRF zinc finger family protein
LOC_Os03g57260	AC133340	GRF zinc finger family protein, expressed
LOC_Os03g43840	AC128646	zinc finger protein LSD2, putative, expressed
LOC_Os03g52740	AC118133	SWIM zinc finger family protein
LOC_Os03g57410	AC084296	RING-H2 finger protein ATL5D, putative, expressed
LOC_Os03g57890	AC090871	zinc finger A20 and AN1 domains-containing protein, putative, expressed
LOC_Os03g57920	AC090871	zinc finger A20 domain-containing protein 2, putative, expressed
LOC_Os03g59540	AC135595	RING zinc finger protein, putative, expressed
LOC_Os03g59760	AC137507	RING finger protein 126, putative, expressed
LOC_Os03g60570	AC104433	zinc finger DNA-binding protein, putative, expressed
LOC_Os03g32270	AC106887	sigma factor sigB regulation protein rsbQ, putative, expressed
LOC_Os03g32590	AC097367	transcription initiation factor, putative, expressed
LOC_Os03g33012	AC105743	WRKY transcription factor 4, putative, expressed
LOC_Os03g53050	AC096855	WRKY transcription factor 21, putative, expressed
LOC_Os03g63810	AC120506	WRKY transcription factor 14, putative, expressed
LOC_Os03g45450	AC133859	OsWRKY60 - Superfamily of rice TFs having WRKY and zinc finger domains, expressed
LOC_Os03g55080	AC079887	OsWRKY3 - Superfamily of rice TFs having WRKY and zinc finger domains, expressed
LOC_Os03g55164	AC079887	OsWRKY4 - Superfamily of rice TFs having WRKY and zinc finger domains, expressed
LOC_Os03g58420	AC093713	OsWRKY6 - Superfamily of rice TFs having WRKY and zinc finger domains
LOC_Os03g37670	AC093312	DNA binding protein, putative
LOC_Os03g46790	AC146718	DNA binding protein, putative
LOC_Os03g62100	AC104487	DNA binding protein, putative
LOC_Os03g46860	AC116369	DNA binding protein, putative, expressed
LOC_Os03g53630	AC087852	DNA binding protein, putative, expressed
LOC_Os03g55590	AC099043	DNA binding protein, putative, expressed
LOC_Os03g56090	AC133450	DNA binding protein, putative, expressed
LOC_Os03g58530	AC104321	DNA binding protein, putative, expressed
LOC_Os03g59670	AC137507	DNA binding protein, putative, expressed
LOC_Os03g60120	AC139172	DNA binding protein, putative, expressed

Table 4.5 cont.

LOC_Os03g63710	AC120506	DNA binding protein, putative, expressed
LOC_Os03g38990	AC133003	DNA-binding protein SMUBP-2, putative, expressed
LOC_Os03g59460	AC135595	DNA-binding protein EMBP-1, putative
LOC_Os03g38210	AC147962	myb-like DNA-binding domain containing protein, expressed
LOC_Os03g55760	AC099043	myb-like DNA-binding domain, SHAQKYF class family protein, expressed
LOC_Os03g51110	AC147426	MYB52, putative, expressed
LOC_Os03g38610	AC133333	MADS-box transcription factor PHERES2, putative
LOC_Os03g54160	AC092556	MADS-box transcription factor 14, putative, expressed
LOC_Os03g54170	AC092556	MADS-box transcription factor 34, putative, expressed
LOC_Os03g38870	AC133003	dof domain, zinc finger family protein, expressed
LOC_Os03g42200	AC107206	dof domain, zinc finger family protein, expressed
LOC_Os03g55610	AC099043	dof domain, zinc finger family protein, expressed
LOC_Os03g39432	AC137921	helix-loop-helix DNA-binding domain containing protein, expressed
LOC_Os03g53020	AC096855	helix-loop-helix DNA-binding domain containing protein, expressed
LOC_Os03g55220	AC084282	helix-loop-helix DNA-binding domain containing protein, expressed
LOC_Os03g55550	AC090713	helix-loop-helix DNA-binding domain containing protein, expressed
LOC_Os03g40080	AC109602	GRAS family transcription factor containing protein, expressed
LOC_Os03g40440	AC092778	B12D protein, expressed
LOC_Os03g42230	AC107206	B3 DNA binding domain containing protein, expressed
LOC_Os03g42370	AC097280	B3 DNA binding domain containing protein
LOC_Os03g42250	AC107206	B3 DNA binding domain containing protein
LOC_Os03g42630	AC092780	GRAB2 protein, putative, expressed
LOC_Os03g43390	AC145780	Leucine Rich Repeat family protein, expressed
LOC_Os03g43650	AC120505	leucine-rich repeat receptor protein kinase EXS precursor, putative, expressed
LOC_Os03g43930	AC147427	class III HD-Zip protein 4, putative, expressed
LOC_Os03g44900	AC145381	CCR4-NOT transcription complex subunit 3, putative, expressed
LOC_Os03g44944	AC138001	CCR4-NOT transcription complex subunit 3, putative
LOC_Os03g52594	AC118133	CCR4-NOT transcription complex subunit 2, putative, expressed
LOC_Os03g45410	AC133859	TATA-binding protein 2, putative, expressed
LOC_Os03g47140	AC090683	atGRF2, putative, expressed
LOC_Os03g47200	AC079830	ocs element-binding factor 1, putative
LOC_Os03g47740	AC079736	BEL1-related homeotic protein 30, putative, expressed
LOC_Os03g47780	AC079736	WD-repeat protein pop3, putative, expressed
LOC_Os03g47970	AC087851	GATA transcription factor 25, putative, expressed
LOC_Os03g48450	AC097277	DELLA protein RGL1, putative, expressed
LOC_Os03g49990	AC087797	DELLA protein SLR1, putative, expressed
LOC_Os03g51330	AC146936	DELLA protein SLR1, putative, expressed
LOC_Os03g48970	AC123974	nuclear transcription factor Y subunit A-1, putative, expressed
LOC_Os03g50310	AC087181	CCT motif family protein, expressed
LOC_Os03g51690	AC145380	homeobox protein OSH1, putative, expressed
LOC_Os03g51910	AC135956	BHLH transcription factor, putative, expressed
LOC_Os03g52320	AC103550	GIF2, putative, expressed
LOC_Os03g56050	AC133450	ANT-like protein, putative, expressed
LOC_Os03g60260	AC133007	ANT1, putative, expressed
LOC_Os03g56580	AC091494	NAC domain-containing protein 42, putative, expressed
LOC_Os03g56970	AC084320	ATARP7, putative, expressed
LOC_Os03g57149	AC133340	mTERF-like protein, putative, expressed
LOC_Os03g57190	AC133340	TCP family transcription factor containing protein, expressed
LOC_Os03g62470	AC096856	ATNAC3, putative
LOC_Os03g63270	AC092559	regulatory protein, putative, expressed

Table 4.5 cont.

LOC_Os03g63400	AC096688	transcription factor BTF3, putative, expressed
LOC_Os03g63920	AC128647	KAP-2, putative, expressed
LOC_Os03g64300	AC092263	transcriptional corepressor LEUNIG, putative, expressed

4.3.2 Identifying and characterising areas of the genome regulating *HvPrx7* expression

4.3.2.1 Sequence variation within the *ORF*

Sequencing of the *ORF* of *HvPrx7* identified sequence variation between the parents of the Alexis/Sloop DH population. The full length *ORF* has been previously sequenced in a P-02 line (Kristensen *et al.* 1999), and matches that of the Alexis sequence identified (Figure 4.12). However, Sloop varied at positions 18, 321, 463, 504 and 853. The cDNA and gDNA were found to match, indicating that there were no introns present for *HvPrx7*. The translated nucleotides reveal that amino acids at residue 155 and 285 are different as a result of the SNPs present in the *ORF* (Figure 4.13). At residue 155 Sloop has a threonine while Alexis has an alanine. Sloop has an asparagine at residue 285, while Alexis has a histidine.

```
                         *        20         *        40         *        60         *        80
cDNAAlexis    1 :atggcgtccagagcagcagcggccatcgccgtcctggccttggtctgcgccgccgtccactcgtcggagggccagctgtc
cDNASloop     1 :atggcgtccagagcagcggcggccatcgccgtcctggccttggtctgcgccgccgtccactcgtcggagggccagctgtc
Prx7P-02      1 :atggcgtccagagcagcagcggccatcgccgtcctggccttggtctgcgccgccgtccactcgtcggagggccagctgtc
GenSloop      1 :atggcgtccagagcagcggcggccatcgccgtcctggccttggtctgcgccgccgtccactcgtcggagggccagctgtc
GenAlexis     1 :atggcgtccagagcagcagcggccatcgccgtcctggccttggtctgcgccgccgtccactcgtcggagggccagctgtc

                         *       100         *       120         *       140         *       160
cDNAAlexis   81 :accaaacttccacgccgccacgtgcccggacctggagcgcatcgtggagttccacgtcgccgagacgttccggcgcgacg
cDNASloop    81 :accaaacttccacgccgccacgtgcccggacctggagcgcatcgtggagttccacgtcgccgagacgttccggcgcgacg
Prx7P-02     81 :accaaacttccacgccgccacgtgcccggacctggagcgcatcgtggagttccacgtcgccgagacgttccggcgcgacg
GenSloop     81 :accaaacttccacgccgccacgtgcccggacctggagcgcatcgtggagttccacgtcgccgagacgttccggcgcgacg
GenAlexis    81 :accaaacttccacgccgccacgtgcccggacctggagcgcatcgtggagttccacgtcgccgagacgttccggcgcgacg

                         *       180         *       200         *       220         *       240
cDNAAlexis  161 :tgggcgtggcgccggcgctcatccgcatcctcttccacgactgcttcccgcagggctgcgacgcctccgtgctgctcaag
cDNASloop   161 :tgggcgtggcgccggcgctcatccgcatcctcttccacgactgcttcccgcagggctgcgacgcctccgtgctgctcaag
Prx7P-02    161 :tgggcgtggcgccggcgctcatccgcatcctcttccacgactgcttcccgcagggctgcgacgcctccgtgctgctcaag
GenSloop    161 :tgggcgtggcgccggcgctcatccgcatcctcttccacgactgcttcccgcagggctgcgacgcctccgtgctgctcaag
GenAlexis   161 :tgggcgtggcgccggcgctcatccgcatcctcttccacgactgcttcccgcagggctgcgacgcctccgtgctgctcaag

                         *       260         *       280         *       300         *       320
cDNAAlexis  241 :ggcgccggcagcgagctcaacgagatccccaaccagacgctccgccccgtggcgctcgacctcatcgagcgcatccgcgc
cDNASloop   241 :ggcgccggcagcgagctcaacgagatccccaaccagacgctccgccccgtggcgctcgacctcatcgagcgcatccgcgc
Prx7P-02    241 :ggcgccggcagcgagctcaacgagatccccaaccagacgctccgccccgtggcgctcgacctcatcgagcgcatccgcgc
GenSloop    241 :ggcgccggcagcgagctcaacgagatccccaaccagacgctccgccccgtggcgctcgacctcatcgagcgcatccgcgc
GenAlexis   241 :ggcgccggcagcgagctcaacgagatccccaaccagacgctccgccccgtggcgctcgacctcatcgagcgcatccgcgc

                         *       340         *       360         *       380         *       400
cDNAAlexis  321 :tgccgtgcaccgcgcatgcgggcccaccgtctcctgcgccgacatcaccgtgctcgccacccgcgactccctcgtcaagg
cDNASloop   321 :cgccgtgcaccgcgcatgcgggcccaccgtctcctgcgccgacatcaccgtgctcgccacccgcgactccctcgtcaagg
Prx7P-02    321 :tgccgtgcaccgcgcatgcgggcccaccgtctcctgcgccgacatcaccgtgctcgccacccgcgactccctcgtcaagg
GenSloop    321 :cgccgtgcaccgcgcatgcgggcccaccgtctcctgcgccgacatcaccgtgctcgccacccgcgactccctcgtcaagg
GenAlexis   321 :tgccgtgcaccgcgcatgcgggcccaccgtctcctgcgccgacatcaccgtgctcgccacccgcgactccctcgtcaagg

                         *       420         *       440         *       460         *       480
cDNAAlexis  401 :ccggcggccccagcttcgacgtcgccctcggccgccgcgacgggctcgccccggcgtcgtccgcactcgtcggcctcctg
cDNASloop   401 :ccggcggccccagcttcgacgtcgccctcggccgccgcgacgggctcgccccggcgtcgtccacactcgtcggcctcctg
Prx7P-02    401 :ccggcggccccagcttcgacgtcgccctcggccgccgcgacgggctcgccccggcgtcgtccgcactcgtcggcctcctg
GenSloop    401 :ccggcggccccagcttcgacgtcgccctcggccgccgcgacgggctcgccccggcgtcgtccacactcgtcggcctcctg
GenAlexis   401 :ccggcggccccagcttcgacgtcgccctcggccgccgcgacgggctcgccccggcgtcgtccgcactcgtcggcctcctg

                         *       500         *       520         *       540         *       560
cDNAAlexis  481 :ccggcgcccttcttcgacgtgcccaccctcatctcctccttcgccaaccggagcctcgacgtcgccgacctcgtgtccct
cDNASloop   481 :ccggcgcccttcttcgacgtgccgaccctcatctcctccttcgccaaccggagcctcgacgtcgccgacctcgtgtccct
Prx7P-02    481 :ccggcgcccttcttcgacgtgcccaccctcatctcctccttcgccaaccggagcctcgacgtcgccgacctcgtgtccct
GenSloop    481 :ccggcgcccttcttcgacgtgccgaccctcatctcctccttcgccaaccggagcctcgacgtcgccgacctcgtgtccct
GenAlexis   481 :ccggcgcccttcttcgacgtgcccaccctcatctcctccttcgccaaccggagcctcgacgtcgccgacctcgtgtccct

                         *       580         *       600         *       620         *       640
cDNAAlexis  561 :ctccggcgcccacaccttcggcgtcgcccactgcccggccttcgaggaccggttcaagccggtgttcgacaccaacccgg
cDNASloop   561 :ctccggcgcccacaccttcggcgtcgcccactgcccggccttcgaggaccggttcaagccggtgttcgacaccaacccgg
Prx7P-02    561 :ctccggcgcccacaccttcggcgtcgcccactgcccggccttcgaggaccggttcaagccggtgttcgacaccaacccgg
GenSloop    561 :ctccggcgcccacaccttcggcgtcgcccactgcccggccttcgaggaccggttcaagccggtgttcgacaccaacccgg
GenAlexis   561 :ctccggcgcccacaccttcggcgtcgcccactgcccggccttcgaggaccggttcaagccggtgttcgacaccaacccgg

                         *       660         *       680         *       700         *       720
cDNAAlexis  641 :ccatcgacggcaagttcgccacggcgctgaggaacaagtgcgccggggacaaccccgccggcacgctgacccagaacctc
cDNASloop   641 :ccatcgacggcaagttcgccacggcgctgaggaacaagtgcgccggggacaaccccgccggcacgctgacccagaacctc
Prx7P-02    641 :ccatcgacggcaagttcgccacggcgctgaggaacaagtgcgccggggacaaccccgccggcacgctgacccagaacctc
GenSloop    641 :ccatcgacggcaagttcgccacggcgctgaggaacaagtgcgccggggacaaccccgccggcacgctgacccagaacctc
GenAlexis   641 :ccatcgacggcaagttcgccacggcgctgaggaacaagtgcgccggggacaaccccgccggcacgctgacccagaacctc

                         *       740         *       760         *       780         *       800
cDNAAlexis  721 :gacgtgcgcacgccggacgtgttcgacaacaagtactacttcgacctgatcgcgaggcaggggctgttcaagtcggacca
cDNASloop   721 :gacgtgcgcacgccggacgtgttcgacaacaagtactacttcgacctgatcgcgaggcaggggctgttcaagtcggacca
Prx7P-02    721 :gacgtgcgcacgccggacgtgttcgacaacaagtactacttcgacctgatcgcgaggcaggggctgttcaagtcggacca
GenSloop    721 :gacgtgcgcacgccggacgtgttcgacaacaagtactacttcgacctgatcgcgaggcaggggctgttcaagtcggacca
GenAlexis   721 :gacgtgcgcacgccggacgtgttcgacaacaagtactacttcgacctgatcgcgaggcaggggctgttcaagtcggacca

                         *       820         *       840         *       860         *       880
cDNAAlexis  801 :gggcctgatcgaccacccgaccaccaagcgcatggccacacgcttctccctccaccagggcgctttcttcgagcagttcg
cDNASloop   801 :gggcctgatcgaccacccgaccaccaagcgcatggccacacgcttctccctcaaccagggcgctttcttcgagcagttcg
Prx7P-02    801 :gggcctgatcgaccacccgaccaccaagcgcatggccacacgcttctccctccaccagggcgctttcttcgagcagttcg
GenSloop    801 :gggcctgatcgaccacccgaccaccaagcgcatggccacacgcttctccctcaaccagggcgctttcttcgagcagttcg
GenAlexis   801 :gggcctgatcgaccacccgaccaccaagcgcatggccacacgcttctccctccaccagggcgctttcttcgagcagttcg

                         *       900         *       920         *       940         *       960
cDNAAlexis  881 :cgaggtccatgaccaagatgagcaacatggacattctcaccggcaccaagggcgagatccggaacaactgcgccgtcccc
cDNASloop   881 :cgaggtccatgaccaagatgagcaacatggacattctcaccggcaccaagggcgagatccggaacaactgcgccgtcccc
Prx7P-02    881 :cgaggtccatgaccaagatgagcaacatggacattctcaccggcaccaagggcgagatccggaacaactgcgccgtcccc
GenSloop    881 :cgaggtccatgaccaagatgagcaacatggacattctcaccggcaccaagggcgagatccggaacaactgcgccgtcccc
GenAlexis   881 :cgaggtccatgaccaagatgagcaacatggacattctcaccggcaccaagggcgagatccggaacaactgcgccgtcccc

                         *       980         *      1000         *      1020         *      1040
cDNAAlexis  961 :aacaggcgtgtccaggacatcgagaccgccgtcgccggcgacgaggggatcgccgctgacatgtg---------------
cDNASloop   961 :aacaggcgtgtccaggacatcgagaccgccgtcgccggcgacgaggggatcgccgctgacatgtg---------------
Prx7P-02    961 :aacaggcgtgtccaggacatcgagaccgccgtcgccggcgacgaggggatcgccgctgacatgtg---------------
GenSloop    961 :aacaggcgtgtccaggacatcgagaccgccgtcgccggcgacgaggggatcgccgctgacatgtga--------------
GenAlexis   961 :aacaggcgtgtccaggacatcgagaccgccgtcgccggcgacgaggggatcgccgctgacatgtg---------------
```

Figure 4.12 ORF sequence summary for *HvPrx7*. ORF of *Prx7* was sequenced from cDNA and genomic DNA (Gen) of Sloop and Alexis (n=3). Previously reported sequences for *HvPrx7* (*Prx7 P-02*) are shown (Kristensen *et al* 1999).

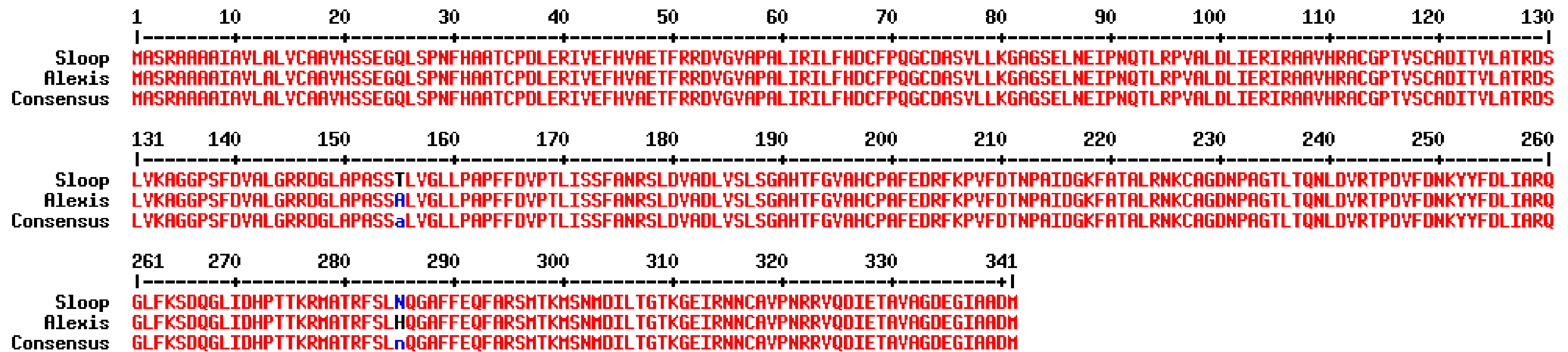

Figure 4.13 Alignment of the amino acid sequences for *HvPrx7* from Alexis and Sloop. The ORF sequence presented in Figure 4.12 was translated using Multalin (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html).

4.3.2.2 Chromosomal location of *HvPrx7*

Chromosomal localisation of *Prx7* to chromosome 2H was shown using barley;wheat addition line PCR (Figure 4.14). The SNP at 463 bp identified in the ORF of *HvPrx7* between Sloop and Alexis (Figure 4.12) allowed the use of Amplified Fragment Length Polymorphism to map the gene to the long arm of chromosome 2H (Figure 4.15) confirming the barley:wheat addition line PCR.

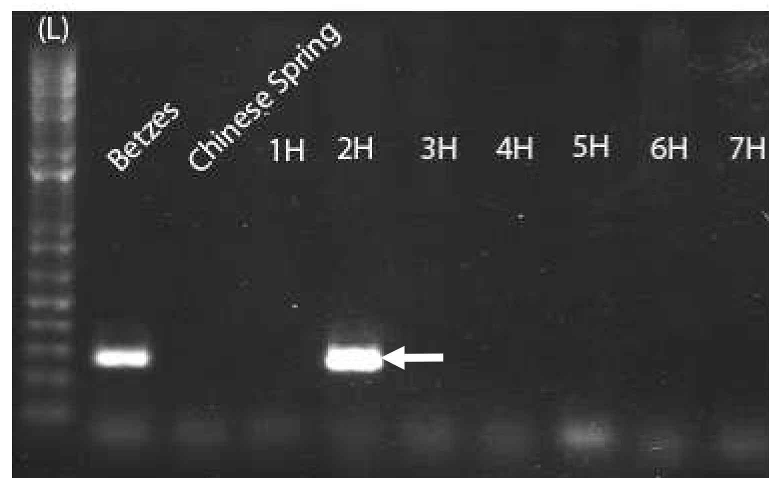

Figure 4.14 Chromosomal localisation of *HvPrx7* by PCR. Lines including barley control (Betzes), wheat control (Chinese Spring) and each of the barley:wheat addition lines containing an extra barley chromosome (1H-7H) were used. (L)=1Kb plus ladder. The arrow represents the amplification of the PCR product in the 2H addition line.

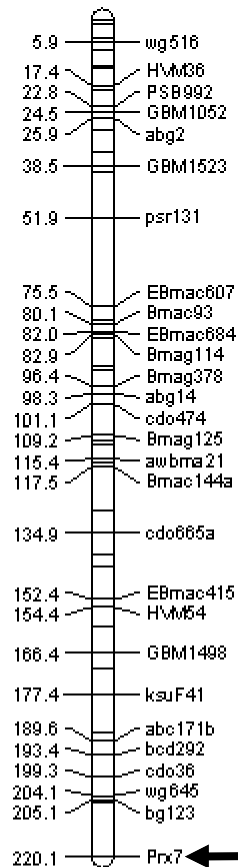

Figure 4.15 Location of *HvPrx7* on chromosome 2H as determined using Amplified Fragment Length Polymorphism (AFLP). The arrow represents the map location of *HvPrx7* on the long arm of chromosome 2H.

4.3.2.3 Expression of *HvPrx7* across Alexis/Sloop mapping population lines

Variation in gene expression was observed across the Alexis/Sloop DH population for biological replicate 1 (Figure 4.16A). In the second biological replicate, gene expression was not detected in a large number of lines (Figure 4.16B), consistent with technical problems observed for the qPCR of *HvBP1*. Trouble shooting experiments were carried out as previously outlined (4.3.1.3). Identical results were obtained, such that when no expression was observed for replicate 2 (Figure 4.16B), *HvGAPDH* levels were low or

absent in comparison to the first replicate (Figure 4.4), suggesting that the results obtained were due to cDNA quality. Experiments to assess any problems with the probe indicated it was of good quality, showing similar levels of *HvPrx7* expression to previous experiments at the milk stage of development (Figure 4.6). Similar to the observation for *HvBP1* a scatterplot indicated no relationship between replicate 1 and replicate 2 (Figure 4.17) while the repeat of replicate 2 also had little or no expression (data not shown).

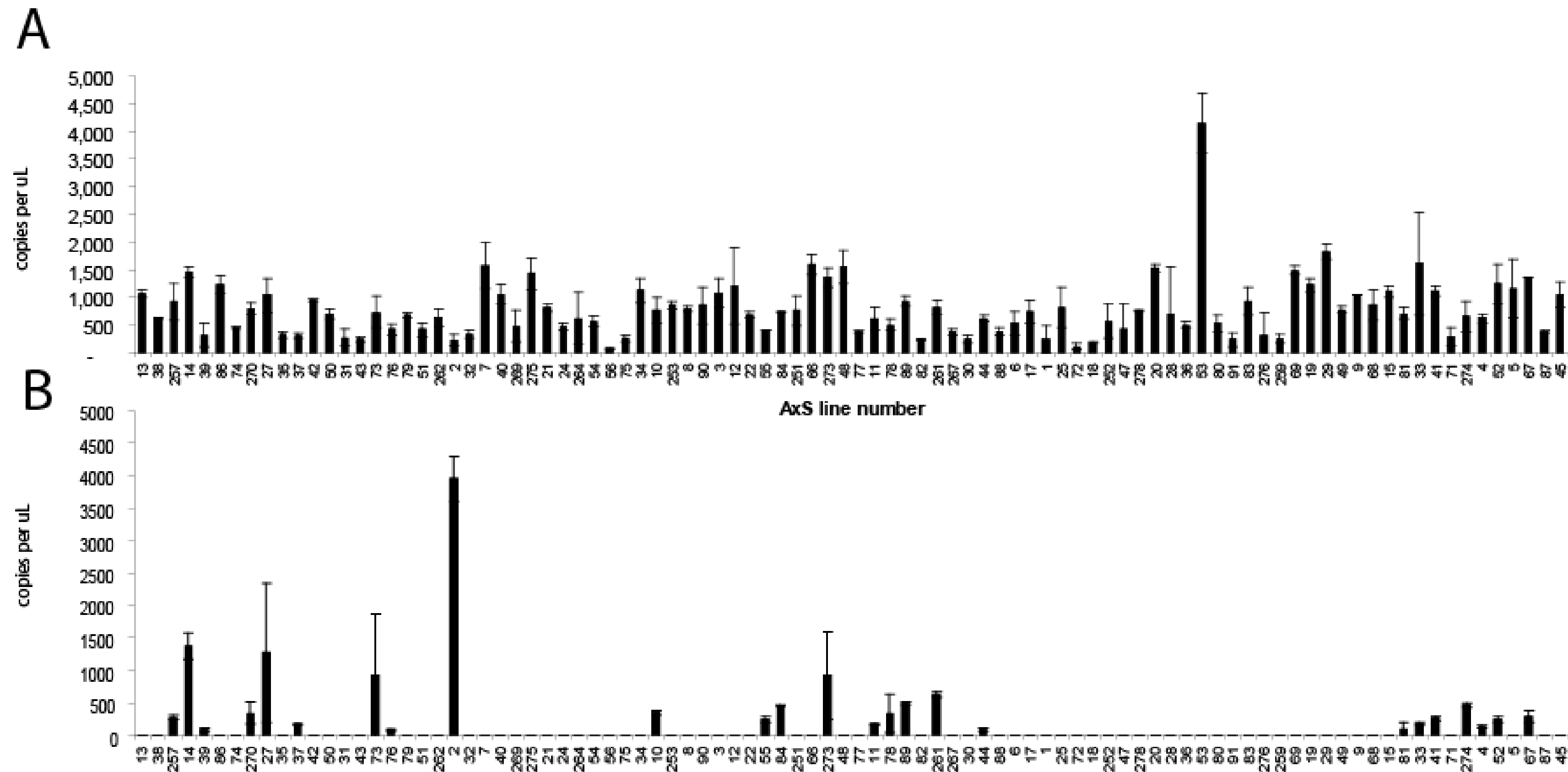

Figure 4.16 Normalised expression levels (level of mRNA presented as number of copies per µL) of candidate gene *Prx7* obtained from qPCR (n = 2, standard deviation of each set replicates for each cDNA was calculated) across the Alexis/Sloop (A x S) DH mapping population. Normalisation of the raw data was performed using the control gene *HvGAPDH* as per Burton *et al.* (2004).Two biological replicates (separate plots) are presented, A and B. Expression was observed at the hard dough (77) stage of grain development (refer to Zadoks *et al.* 1974).

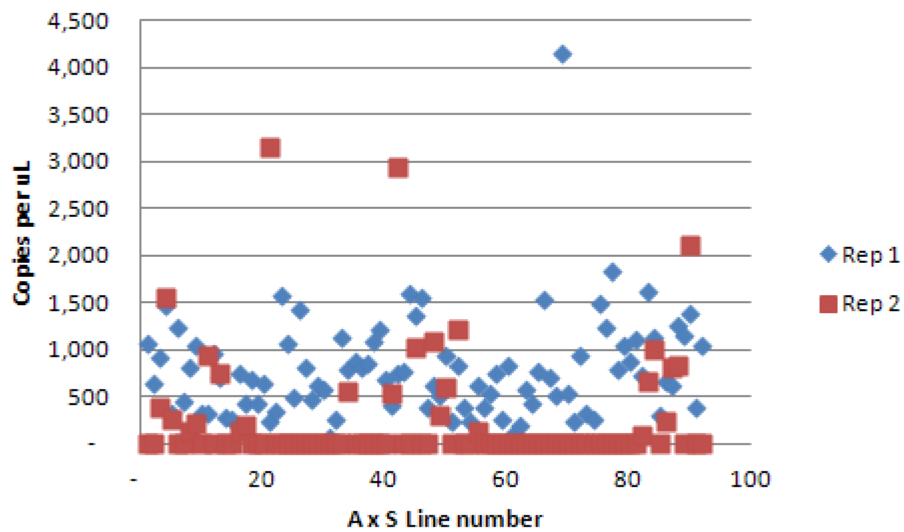

Figure 4.17 Comparison of qPCR data for *HvPrx7* in biological replicate 1 and 2. Data from figures 4.3 and 4.4 have been displayed in a scatterplot for comparison.

4.3.2.4 Identification of a *cis*-acting eQTL for *HvPrx7*

As for *HvBP1* (section 4.3.1.3), large variation was present between biological replicates, such that eQTL analysis was undertaken using replicate 1. Using gene expression data from replicate 1, QTL were detected on chromosomes 1H, 2H, 3H, 4H and 6H (Figure 4.18). However, the QTL on chromosome 2H was the only QTL found to be highly significant when genome-wide significance levels were calculated using Map Manager QTX (Manly *et al.* 2001) (Figure 4.18). The Alexis allele contributed to the QTL on chromosomes 1H, 3H and 6H, explaining 4%, 8% and 5% of the phenotypic variance respectively. The Sloop allele contributed to the QTL on chromosomes 2H and 4H, explaining 25% and 9% of the phenotypic variance respectively. QTLs on chromosomes 1H, 3H and 4H were found to be significant while QTL represented on 2H were highly significant and QTL on 6H were only suggestive (Figure 4.18).

The 2H eQTL was located in the same position as the *HvPrx7* gene (mapped using barley:wheat addition line PCR and AFLP mapping, Section 4.3.2.1). Given that *cis*-acting QTLs are defined as messages whose levels are linked to markers within 10 kb of their own gene (Brem *et al.* 2008) and that at least one of its eQTL mapped within a distance of ±5 cM (Potokina *et al.* 2008), we can conclude that *HvPrx7* is under a model of *cis*-regulation.

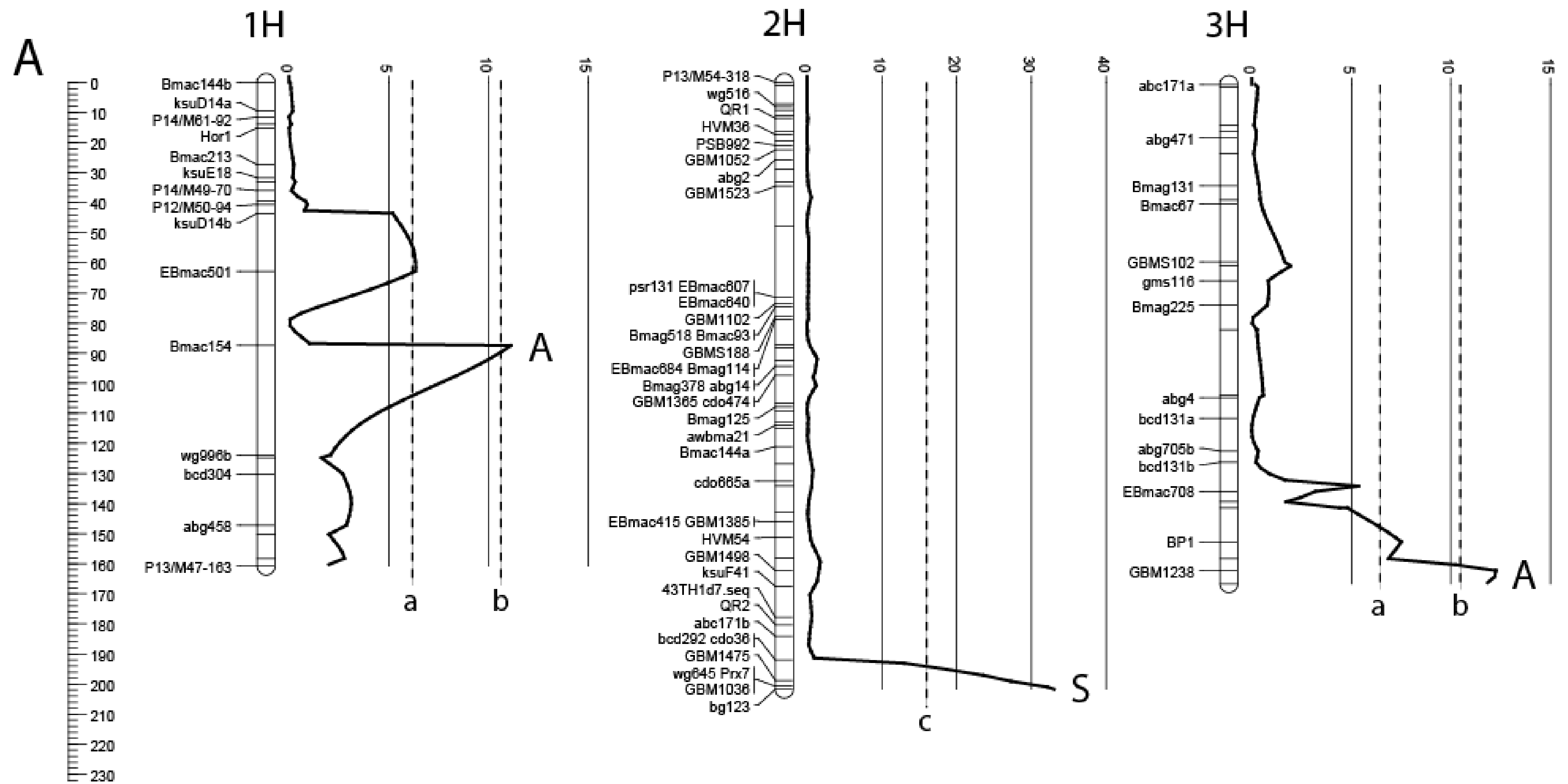

Figure 4.18 cont.

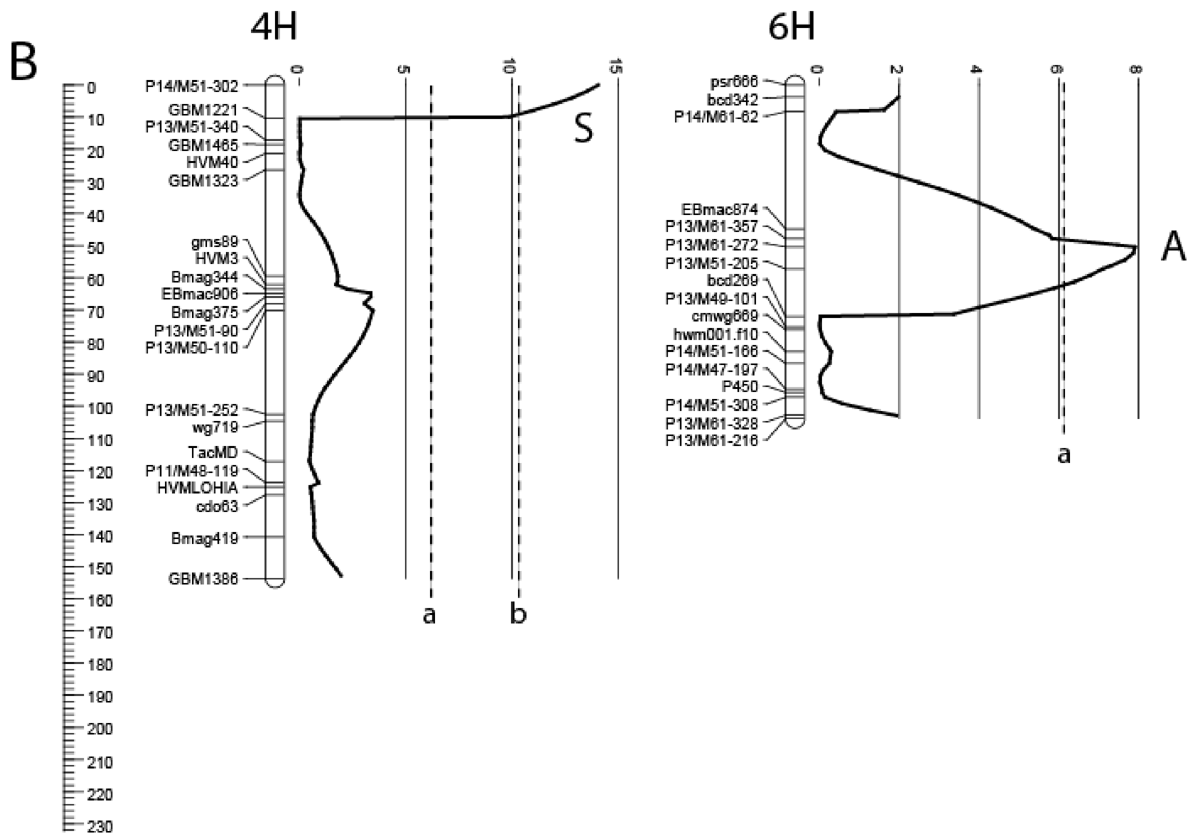

Figure 4.18 Likelihood ratio test statistics from composite interval mapping of *Prx7* gene expression (Replicate 1) (eQTLs) in the Alexis x Sloop DH population grown at Hatherleigh, SA in 2004/2005 showing QTL detected on chromosomes 1H, 2H, 3H (A), 4H and 6H (B). Distances within chromosomes are displayed in centimorgans (cM). At each QTL peak, the allele contributing is identified as coming from Sloop(S) or Alexis (A). Genome-wide significance levels were re-calculated using Map Manager QTX (Manly *et al.* 2001) with a = suggestive, b = significant and c = highly significant. The marker map is an updated version of those previously reported for the Alexis/Sloop DH mapping population (Barr *et al*. 2003; Willsmore *et al*. 2006).

4.3.2.5 Comparative mapping studies for eQTL of *HvPrx7*

Cis-regulation of the Prx7 gene suggests that we are observing a polymorphism physically located near or in the gene itself, or have identified a promoter polymorphism. Indeed, polymorphisms were identified within the gene (Figure 4.12 and Figure 4.13). For this reason, the eQTL identified on 2H using gene expression data from replicate 1 (Section 4.3.2.4) was not further investigated through comparative mapping studies. The other

eQTL also were not further investigated as their likelihood ratio statistic (LRS) threshold was not considered highly significant.

4.3.3 Identifying and characterising areas of the genome regulating *HvQR* expression

4.3.3.1 Expression data across Alexis/Sloop mapping population lines for *QR*

Variation in gene expression was observed across the Alexis x Sloop DH population for biological replicate 1 (Figure 4.19A). As for *HvBP1* and *HvPrx7*, gene expression was not detected in a large number of lines for the second biological replicate (Figure 4.19B) or a repeat of the second replicate (data not shown). Similarly a scatter plot revealed no relationship between replicates (Figure 4.20). Trouble shooting experiments were carried out as outlined in section 4.3.1.3. Results replicated those for *HvBP1* and *HvPrx7* such that cDNA quality was confirmed in a repeat of replicate 2 and using the milk stage of development (Figure 4.6).

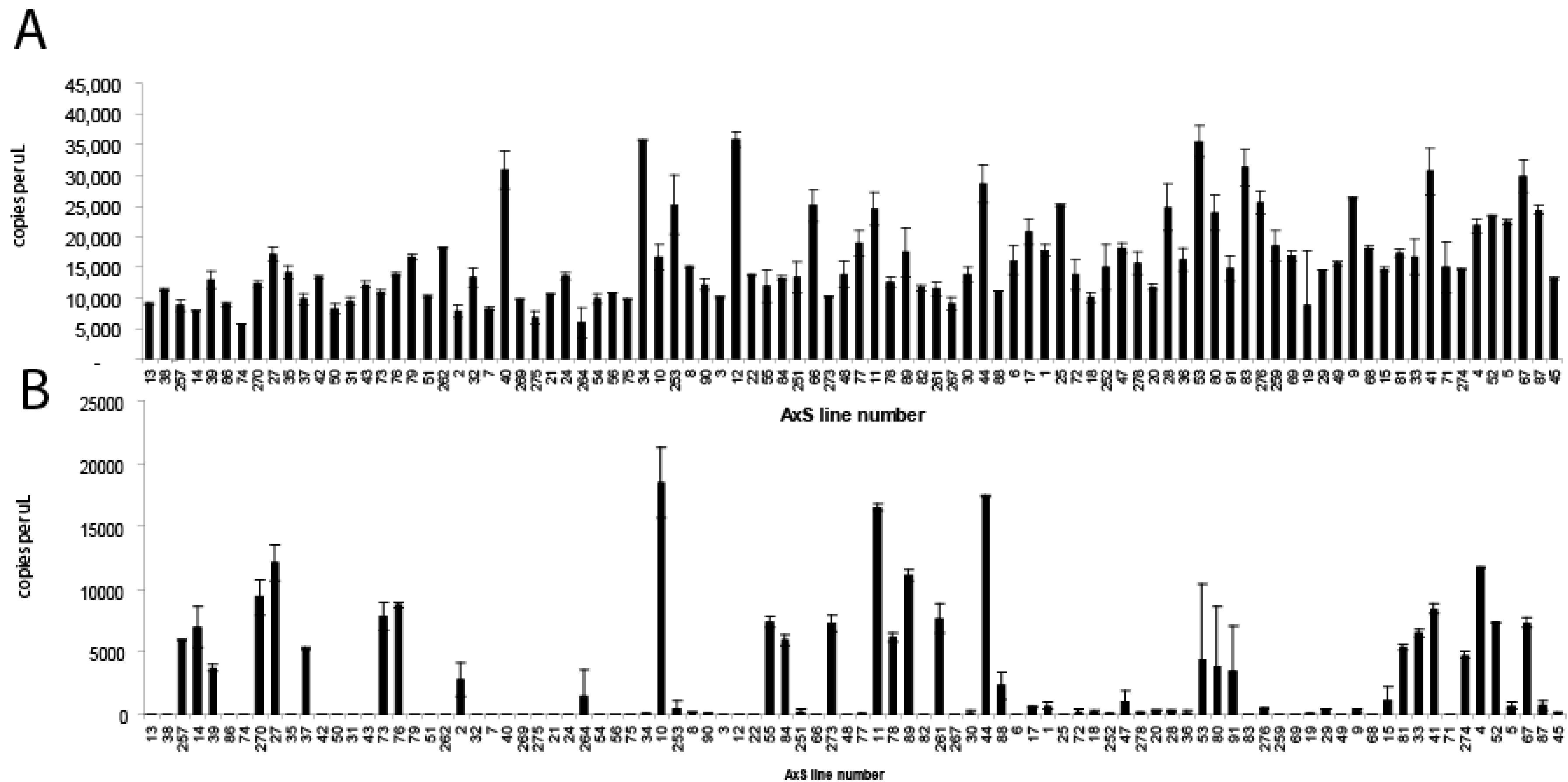

Figure 4.19 Normalised expression levels (level of mRNA presented as number of copies per µL) of candidate gene *QR* obtained from qPCR (n = 2, standard deviation of each set replicates for each cDNA was calculated) across the Alexis/Sloop (A x S) DH mapping population. Normalisation of the raw data was performed using the control gene *HvGAPDH* as per Burton *et al.* (2004).Two biological replicates (separate plots) are presented, A and B. Expression was observed at the hard dough (77) stage of grain development (refer to Zadoks *et al*. 1974).

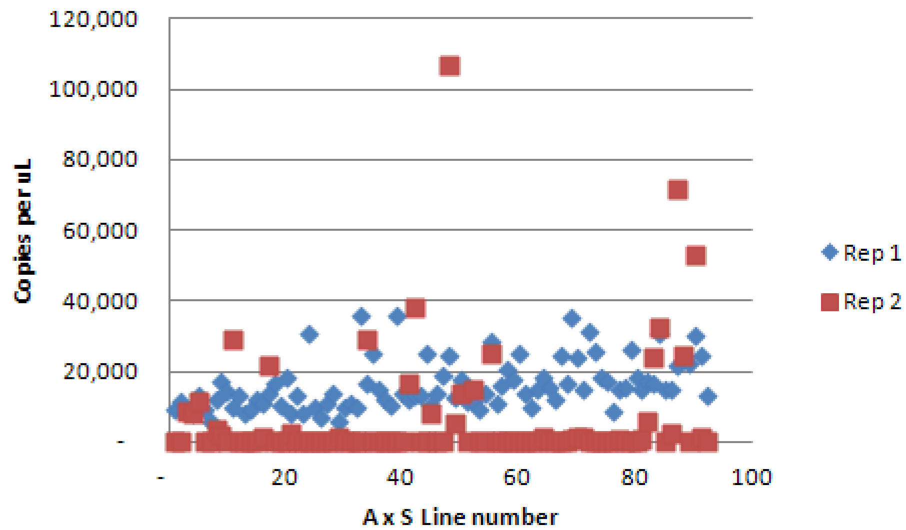

Figure 4.20 Comparison of qPCR data for *HvQR* in biological replicate 1 and 2. Data from figures 4.3 and 4.4 have been displayed in a scatterplot for comparison.

4.3.3.3 Identification of a *cis*-acting eQTL for *HvQR*

As for *HvBP1* (section 4.3.1.3) and *HvPrx7* (section 4.3.2.3), eQTL analysis was undertaken on replicate 1. Using gene expression data from replicate 1, QTL were detected on chromosomes 1H, 2H, 5H, 6H and 7H (Figure 4.20). However, the QTL on chromosome 5H was the only QTL found to be highly significant. This QTL is a *cis*-eQTL given its position is the same as the *HvQR* gene reported by March and colleagues (March *et al.* 2008). The Alexis allele contributed to the QTL on chromosomes 1H and 2H, explaining 4% of the phenotypic variance for both QTL which were only suggestive (Figure 4.20). The Sloop allele contributed to the QTL on chromosomes 5H, 6H and 7H, explaining 33%, 8% and 6% of the phenotypic variance respectively (Figure 4.21). QTL on chromosome 5H were found to be highly significant while the 6H QTL was significant and the 7H QTL suggestive.

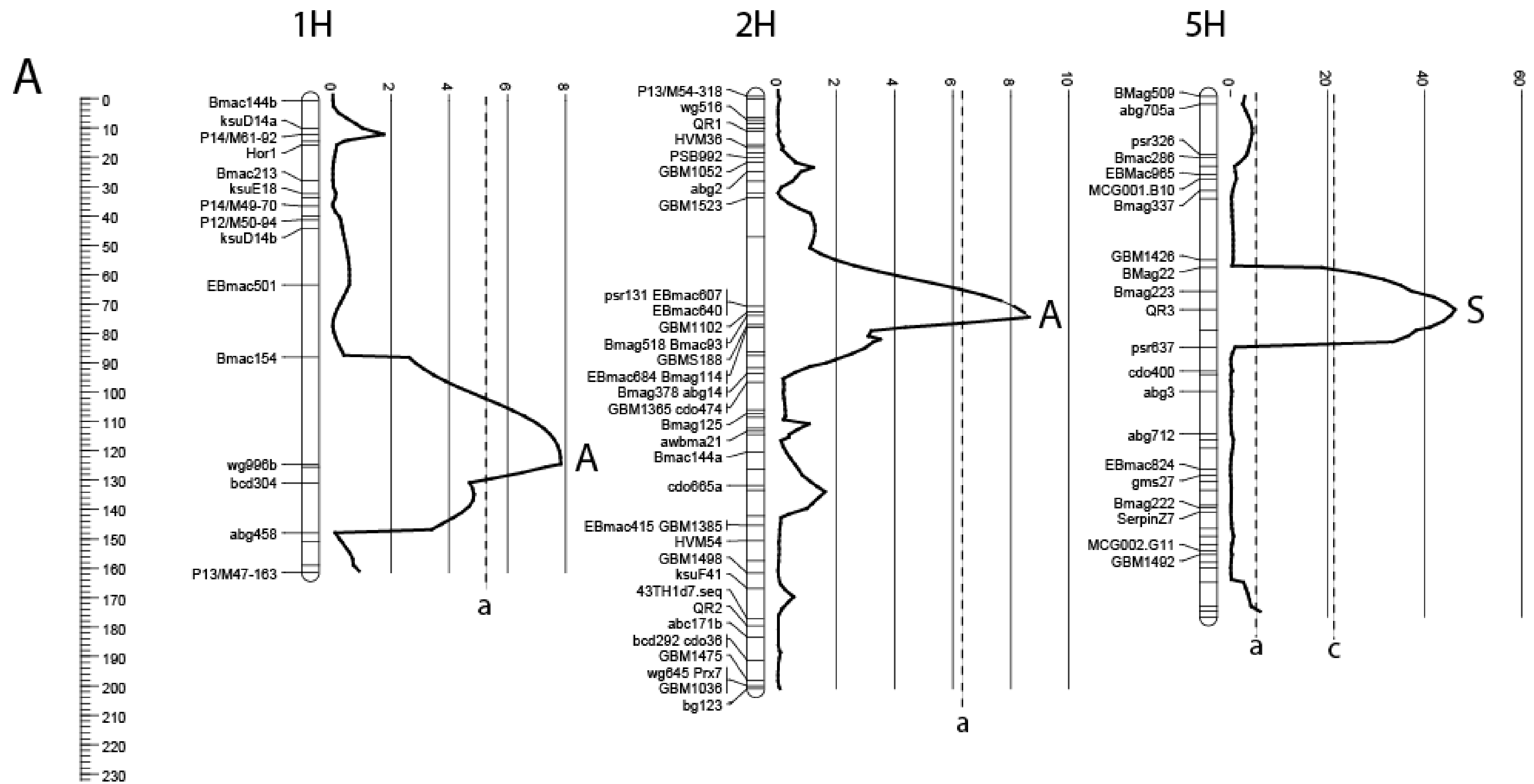

Figure 4.21 cont.

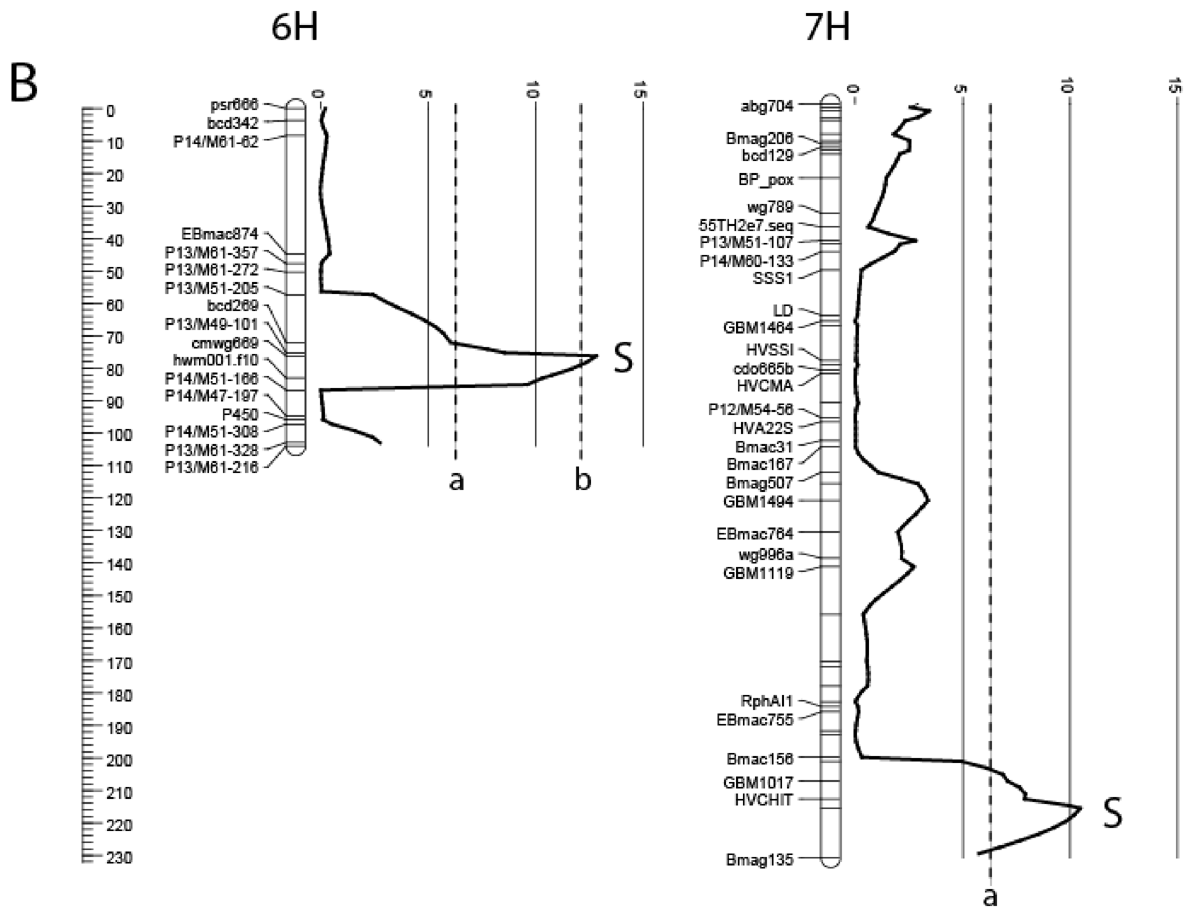

Figure 4.21 Likelihood ratio test statistics from composite interval mapping of *HvQR* gene expression (B) (Replicate 1) (eQTLs) in the Alexis x Sloop DH population grown at Hatherleigh, SA in 2004/2005 showing QTL detected on chromosomes 1H, 2H, 5H, 6H and 7H. Distances within chromosomes are displayed in centimorgans (cM). At each QTL peak, the allele contributing is identified as coming from Sloop (S) or Alexis (A). Genome-wide significance levels were re-calculated using Map Manager QTX (Manly *et al.* 2001) with a = suggestive, b = significant and c = highly significant. The marker map is an updated version of those previously reported for the Alexis/Sloop DH mapping population (Barr *et al*. 2003; Willsmore *et al*. 2006).

4.3.3.3 Comparative mapping studies for *HvQR*

Cis-regulation of the *QR* gene suggests that we are observing a polymorphism physically located near the gene itself, or have identified a promoter polymorphism. For this reason the QTL identified using gene expression data from replicate 1 (Section 4.3.2.2) was not further investigated through comparative mapping studies.

4.4 Discussion

QTL for black point formation were successfully identified on chromosomes 2H and 3H in previous research (Chapter 2 and Walker *et al.* 2008). Further experiments suggested that *HvBP1, HvPrx7* and *HvQR* (Chapter 3) may be candidates in black point formation given their differential expression between tolerant and susceptible cultivars. eQTL mapping has been successfully used previously to allow the identification of candidate genes involved in seed development in wheat (Jordan *et al.* 2007). The research presented in this chapter therefore aimed to identify eQTL for these genes. Using this approach, *trans*-acting candidate genes involved in the regulation of *HvBP1* were identified and *HvPrx7* and *HvQR* were shown to be most likely *cis*-regulated.

The 'genetical genomics' (Jansen and Nap 2001) or 'expression genetics' (Varshney *et al.* 2006) used in this study is a powerful tool for explaining differential gene expression such as that seen for *HvBP1*, *HvPrx7* and *HvQR* in black point susceptible and tolerant barley varieties. Rather than use a whole genome approach like many previous studies in wheat and barley (Jordan *et al.* 2007; Potokina *et al.* 2008), this research focused specifically on the genes differentially expressed between black point susceptible varieties and black point tolerant varieties (identified in chapters 2 and 3). This was achieved through the use of qPCR across the Alexis/Sloop DH population. Due to the logistics involved with undertaking qPCR on each DH line individually and material constraints, replication was limited. Although significant variation was observed between biological replicates for all candidate genes (Figures 4.3, 4.16 and 4.19), trouble shooting experiments (Figure 4.6) revealed that cDNA and probes used for qPCR were of good quality. Because of this variation in data between lines and a lack of relationship between replicates (Figures 4.5,

4.17 and 4.20), QTL analysis was undertaken using replicate 1. Further biological replication in future experiments would therefore allow investigation of the variation in gene expression between plots and validate the eQTLs described herein. Another limiting factor of the experiment could be the small population size (110 DH lines) where a larger population size should allow for more accurate results (Jordan *et al.* 2007). However, previous studies with microarrays have used comparable population sizes and/or replication [such as one replicate of 160 recombinant inbred lines (RIL) lines or two independent replicates per RIL in Arabidopsis, 139 lines with one single replicate in barley and 41 DH lines in wheat (Jordan *et al.* 2007; Keurentjes *et al.* 2007; West *et al.* 2007; Potokina *et al.* 2008)].

As mentioned earlier (section 4.1), eQTLs are categorised as *cis*- or *trans*-acting; where *cis*-eQTLs represent a polymorphism physically located near or within the gene itself or within the promoter and *trans*-eQTLs represent a polymorphism at a location in the genome other than the actual position of the gene whose transcript is being measured, or a polymorphism at the physical position of a regulatory factor elsewhere in the genome (Hansen *et al.* 2008). A study by Potokina *et al.* (2008) analysing eQTL of 16000 barley genes identified 23738 significant eQTLs with genome wide significance ($P \leq 0.05$). A large proportion of the transcripts were regulated by both *cis*- and *trans*- effects, however more than half of the quantitatively controlled transcripts were primarily regulated by *cis*-eQTLs in the Steptoe x Morex population. Although *HvPrx7* and *HvQR* appear to be *cis*-regulated, *HvBP1* seems to be *trans*-regulated. A transcription factor elsewhere in the genome is therefore likely to be regulating *HvBP1* gene expression. In contrast, *HvPrx7* and *HvQR* are likely to contain a SNP within the promoter regions affecting chromatin structure or transcription factor binding sites and hence the expression of the gene (Wittkopp *et al.*

2004). However there were also SNPs in the *HvPrx7 ORF* which also affected the amino acid sequence (Figure 4.13). While this change in amino acids may affect the function of the protein itself, whether these are responsible for changes in gene expression requires investigation. Sequence variation within the ORF or 3’ untranslated region may however also have a downstream effect on mRNA stability (Wittkopp *et al.* 2004). Amino acid changes within the coding sequence that affect the activity of the gene product, or codon usage changes that affect the level of protein, may lead to a change in gene expression either directly through auto-regulation of the gene by its protein product or indirectly through a pathway of intermediates (Ronald *et al.* 2005). The Sloop allele (from the susceptible parent) was found to contribute to both *cis*-eQTLs for *HvPrx7* and *HvQR*, suggesting a SNP within the gene or promoter may be responsible for differential expression. Further analysis of the effect of any SNPs on mRNA stability, binding of transcription factors to the promoter or protein activity is therefore necessary.

The full length *ORF* of *HvBP1* has been previously sequenced in the parental variety Bomi and the PcR7 probe used to study gene expression (Rasmussen *et al.* 1991). March and colleagues (March *et al.* 2008) have mapped *HvBP1* to chromosome 3H. Using barley:wheat addition lines, this research indicated that *HvBP1* resides on chromosome 7H (Figure 4.2). Sequencing revealed that the PcR7 probe (Rasmussen *et al.* 1991) and the genomic sequence for *HvBP1* from Alexis are the same but different to Prx5 Bomi and gDNA of Sloop. The Alexis cDNA sequence.aligned with that of the Sloop genomic and cDNA sequences (Figure 4.1). This suggests that there are two similar copies of the gene on chromosomes 3H and 7H. Furthermore this could make the two copies of the gene indistinguishable in qPCR experiments. With primers designed to the 3´ end of the HvBP1 sequence (Section 3.2.3.1) including 3 SNPs (Figure 4.1) experiments could be amplifying

either of the peroxidase genes. Future experiments could design primers that use any of the SNPs to differentiate the 7H and 3H versions of the gene. Highly significant eQTLs for *HvBP1* were identified on 2H and 5H. Because *HvBP1* was mapped to a different location (7H or 3H), a *trans*-regulatory mechanism is suggested. The Alexis allele (tolerant) was found to contribute to both *trans*-acting eQTLs for *HvBP1*, suggesting that a transcription factor may be affecting gene regulation and contributing to tolerance through inhibition of *HvBP1* gene expression at later stages of maturity.

Candidates in transcriptional repression at both *HvBP1* eQTL were identified through comparative mapping studies (Table 4.2-4.5, Table 4.6). Of particular interest are genes encoding for a Hordeum repressor of transcription (HRT) protein and a Short internodes (SHI)-like protein identified on rice chromosome 7, aligning with chromosome 2H in barley. Both proteins have been shown to repress expression of genes usually responsive to gibberellic acid (GA) including α -amylase (Raventós *et al.* 1998; Fridborg *et al.* 2001). A key response to GA in a mature cereal grain is to initiate germination and allow the production of α-amylase, synthesised in the aleurone cells during germination for breakdown and mobilisation of the starch in the endosperm of seed (Fridborg *et al.* 2001). SHI has been shown to specifically block the activity of a high-isoelectric point α -amylase promoter following GA treatment (Fridborg *et al.* 2001). Black pointed grain has also been shown to have started germination and to have increased alpha-amylase levels (Hadaway and Able, *unpublished data*). In addition, late embryogenesis abundant (LEA) proteins present in healthy grains but not black pointed grains (March *et al.* 2007) have been shown to degrade when a grain enters the process of germination (Hsing *et al.* 1998) suggesting a potential link between germination state of a grain and black point. SHI and HRT may

therefore play a role in repressing expression of genes associated with germination (and by association black point) including *HvBP1*.

Other candidates that may be associated with germination but that have also been associated with stress include T-complex protein (TCP), *B12* and *BURP* domain genes. TCP genes have been implicated in the control of seed germination in Arabidopsis (Tatematsu *et al.* 2008) and early seed development or abiotic stress in rice (Sharma *et al.* 2010). *B12* transcripts in barley have been reported in the aleurone layer and the embryo of developing seed, disappearing at seed maturity and reappearing in the germinating embryo (Aalenf *et al.* 1994). The basic B12 protein has also been suggested to play a fundamental role in the vegetative tissues of sweet potato under unfavourable environmental conditions leading to leaf senescence (Huang *et al.* 2001), implying a role in response to environmental stress. SCB1, a seed coat BURP-domain protein which is detected within the seed coat during the early stages of soybean seed development, has been proposed to be involved in the formation of the seed coat by governing the differentiation of the seed coat parenchyma cells (Batchelor *et al.* 2002). However, the majority of the genes containing BURP domains have been suggested to be crucial for responses to stress. *BnBDC1*, a shoot specific gene in oil seed rape has been shown to be up-regulated by salt and down–regulated by salicylic acid (Yu *et al.* 2004) while rice BURP family members (*OsBURP)* have been shown to be induced by drought cold, salt and abscisic acid (ABA) (Ding *et al.* 2009). Their presence in the eQTL for *HvBP1* may therefore suggest an ability to up-regulate *HvBP1* under the unfavourable conditions that lead to black point (low temperature, high humidity as shown in chapter 2). However, to date, the molecular function of the BURP domain is still unknown (Xu *et al.* 2010).

The RD22 class of BURP proteins have been shown to be drought responsive and mediated by abscisic acid (ABA) signalling in Arabidopsis (Yamaguchi-Shinozaki and Shinozaki 1993). Dehydration triggers the production of ABA (Abe *et al.* 1997) and ABA then activates the gene expression of *AtMYB2*, which in turn induces the expression of the *RD22* gene transcription factors (Abe *et al.* 1997). Given that environmental stress has been identified as playing a key role in the formation of black point and the possible up-regulation of *HvBP1*, we could be observing a pathway regulated by ABA and subsequently a BURP domain protein that regulates *HvBP1* expression. Interestingly, ABA also controls germination and seed development (Kim 2007) allowing post-germination growth only in favourable conditions (Lopez-Molina *et al.* 2001). Previous findings have indicated that the stage of grain maturity is important in determination of black point formation and *HvBP1* expression (Chapter 2, Chapter 3) while black pointed grain may have germinated (Able and Hadaway, *unpublished data*) suggesting that ABA-regulated transcription factors and proteins may play an important role. ABA prevents germination and could therefore act as part of a tolerance mechanism for black point. This argument is further supported by the observation that Alexis (tolerant allele) has been identified to contribute to the two eQTL identified for *HvBP1*.

Other candidates within the eQTL include those that have also previously been shown to have a role in ABA signalling or responses to ABA (Table 4.6) such as the basic helix-loop-helix (bHLH), basic-leucine zipper (bZIP), B3 DNA binding domain and Arabidopsis Transcription Factor (ARF). bHLH transcription factors are up-regulated by ABA to induce ABA-mediated gene expression of the BURP domain proteins *RD29A* and *RD22* (Kim and Kim 2006). *bZIP* EMBP-1 have been implicated in ABA-induced gene expression in wheat (Guiltinan *et al.* 1990) and in maize during embryo development

(Vasil *et al.* 1995). Given that the B3 proteins and ARF families also have involvement in ABA responses (Romanel *et al.* 2009) and ABA controls germination, this group of transcription factors can be considered candidates. However the response of *HvBP1* to ABA is yet to be determined.

Zinc fingers are one of the most common motifs implicated in regulation through their interaction with the *cis*-elements of target genes (Takatsuji 1999) especially those involved in stress tolerance (Table 4.3). Msn2p and Msn4p, members of the C2H2 family of zinc fingers, have been shown to be key regulators of stress responsive gene expression in *Saccharomyces cerevisiae* (Görner *et al.* 1998), to be involved in the putative repression activity of defence and stress responses by Arabidopsis and to have key roles in different developmental pathways (Ciftci-Yilmaz and Mittler 2008). GIS, another member of the zinc finger family, plays a role in trichome initiation downstream of the gibberellin (GA)-signaling pathway during inflorescence development (Gan *et al.* 2007). Given that the ratio of GA and ABA controls germination (Kent Bradford 2007), zinc finger family proteins may therefore play a role in black point formation. MYB transcription factors (Table 4.6) have been shown to play important roles in response to gibberellic acid (GA) (Gubler *et al.* 2002) and stress signals (Chen *et al.* 2005). MYB proteins appear to control secondary metabolism and in particular, phenylpropanoid metabolism (Martin and Paz-Ares 1997). Phenylpropanoids, derived from trans-cinnamic acid, are formed by the deamination of L-Phenylalanine by PAL and are responsible for the production of anthocyanins, aurones and phlobaphenes (Solecka 1997). Given the potential involvement of PAL, the synthesis of phenols and the role of peroxidases in the oxidation to quinones (Figure 1.2), the MYB transcription factors may be responsible for the up-regulation of *HvBP1* during black point formation. This conclusion is supported by previous results, showing on up-regulation of

HvBP1 (Chapter 3) in the susceptible cultivar Sloop. A MYB transcription factor gene, HvGAMYB has been isolated from a barley aleurone cDNA library and the gene product has been shown to be upregulated by α-amylase and to respond to GA (Gubler *et al.* 1995; Gubler *et al.* 2002), indicating a link with germination as discussed earlier.

Wounding of plant tissue results in the oxidation of phenolic compounds to quinones by enzymes such as peroxidases and polyphenol oxidases (Whitaker and Chang 1996). Black point is more than likely due to wounding within the embryo allowing peroxidases and phenolic substrates to mix (Cochrane 1994b), initiating enzymatic browning and hence black point formation. Proteins that contain leucine rich repeats (LRR) have been proposed to play a role in the regulation of responses to wounding (Table 4.6, Shanmugam 2005)| and plant pathogens (Shanmugam 2005). Black point is the result of an enzymatic browning reaction and is therefore more likely to be a result of an abiotic stress such as low temperatures and high humidity as identified in Chapter 2. LRR-proteins have been shown to accumulate in soybean after wounding (Favaron *et al.* 1994) and to increase in response to wounding in apple collected at varying maturity stages, indicating a role in stress response and fruit development (Conway *et al.* 1998).

Table 4.6 Proposed candidate genes and function within the identified syntenous regions (Rice 4 and 7) and (Rice 9 and 3) for eQTL identified on barley chromosome 2H and 5H. Candidates identified through comparative mapping between barley, wheat and rice (Figures 4.11 and 4.12). Candidate genes highlighted in grey are further discussed based on potential roles in abiotic stresses, seed development or germination and therefore potentially black point

Candidate gene (Family)	Function	References
Zinc Finger C2H2	Key cellular processes including transcriptional regulation, development, pathogen defence, and stress responses	(Ciftci-Yilmaz and Mittler 2008)
Dof zinc Finger	Key transcription factor for light regulation	(Yanagisawa and Sheen 1998)
(GRF/C3HC4/SWIM)	Transcription activators in growth and development	(Choi *et al.* 2004)
	Regulatory role in stem elongation	(Kim *et al.* 2003)
	Regulation of cell expansion in leaf and cotyledon tissues	(Zhang *et al.* 2007; Ciftci-Yilmaz and Mittler 2008)
	Stress Tolerance	(Wang *et al.* 2007)
	Disease resistance	(Ciftci-Yilmaz and Mittler 2008)
Zinc Finger (ISAP1)	Confers cold, dehydration, and salt tolerance in transgenic tobacco	(Mukhopadhyay *et al.* 2004)
Zinc Finger (LSD2)		
Zinc Finger A20/AN1	Stress response	(Vij and Tyagi 2008)
TCP Domain	Abiotic stress	(Sharma e*t al.* 2010)
	Growth and Development	(Kosugi and Ohashi 2002)
	Positive regulators of gene expression during cell proliferation	(Kosugi and Ohashi 2002)
	Negative regulators of cell proliferation	(Gaudin *et al.* 2000)
	Control of cell elongation	(Palatnik *et al.* 2003; Schommer *et al.* 2008)
		(Koyama *et al.* 2007; Broholm *et al.* 2008)
		(Costa *et al.* 2005; Hervé *et al.* 2009)
	Male and female gametophyte development	(Pagnussat *et al.* 2005; Takeda *et al.* 2006)
	Embryogenesis	(Ruuska *et al.* 2002)
	Embryo growth	(Tatematsu *et al.* 2008)
	Jasmonic acid synthesis and leaf senescence	(Schommer *et al.* 2008)
	Photomorphogenesis	(López-Juez *et al.* 2008)
Leucine Rich Repeat	Induced by infection and stress related signals	(Shanmugam 2005)
	Regulated by wounding and pathogen Infection	(Shanmugam 2005)

Candidate gene (Family)	Function	References
	G-Protein mediated signalling Vesicular transport	(Jensen *et al.* 2000) (Jensen *et al.* 2000)
BURP Domain	Plant Development Response and adaption to stresses (drought, salt, cold, and abscisic acid treatment)	(Ding *et al.* 2009)
Methyl-CpG	Controlling chromatin structure mediated by CpG methylation	(Grafi *et al.*)
MYB	Secondary Metabolism Regulating cellular morphogenesis Responses to hormone and stress signals Circadian rhythm,and dorsoventrality	(Martin and Paz-Ares 1997; Chen *et al.* 2005) (Martin and Paz-Ares 1997; Chen *et al.* 2005) (Chen *et al.* 2005) (Riechmann *et al.* 2000)
ZAC	G-protein mediated signaling Vesicular transport	(Jensen *et al.* 2000) (Jensen *et al.* 2000)
SBP	Leaf and glume development Local regulator of GA-mediated signalling Growth and flower development	(Moreno *et al.* 1997; Wang *et al.* 2005) (Zhang *et al.* 2007) (Yang *et al.* 2008)
WD-repeat	Signal transduction, RNA processing Cytoskeletal dynamics Chromatin modification Cell division Apoptosis Light signaling and vision, Cell motility Flowering and floral development Meristem organization	(Neer *et al.* 1994; van Nocker and Ludwig 2003) (Neer *et al.* 1994; van Nocker and Ludwig 2003) (Neer *et al.* 1994; van Nocker and Ludwig 2003) (Neer *et al.* 1994; van Nocker and Ludwig 2003) (Chantha *et al.* 2006) (Chantha *et al.* 2006) (Chantha *et al.* 2006) (Chantha *et al.* 2006) (Chantha *et al.* 2006) (Chantha *et al.* 2006)

Candidate gene (Family)	Function	References
NB-ARC	Regulator of cell death Regulate R (Resistance) proteins	(van der Biezen and Jones 1998) (van Ooijen *et al.* 2008)
bHLH	ABA-mediated signal transduction Anthocyanin biosynthesis Phytochrome signaling Fruit dehiscence Carpel and epidermal development, Stress response Transcription of structural anthocyanin gene (AP1)	(Kim and Kim 2006) (John 2003; Toledo-Ortiz *et al.* 2003) (Duek and Fankhauser 2005) (John 2003; Toledo-Ortiz *et al.* 2003) (John 2003; Toledo-Ortiz *et al.* 2003) (John 2003; Toledo-Ortiz *et al.* 2003) (Spelt *et al.* 2000)
BIM2 AtMYC2	*Member of the bHLH family* *bHLH related protein*	(Yu *et al.* 2005) (Abe *et al.* 2003)
CRP1	Mitochondrial gene expression	(Fisk *et al.* 1999)
ARF GAP	Vesicle budding (Acts catalytically to recruit COPI components)	(Rein *et al.* 2002)
NAC Domain	Central role in senescence Nutrient remobilization to the developing grain Grain protein content variation Pattern formation and organ seperation	(Ostersetzer and Adam 1997; Nakabayashi *et al.* 1999) (Waters *et al.* 2009; Jamar *et al.* 2010) (Jamar *et al.* 2010) (Riechmann and Ratcliffe 2000)
NAM	Member of NAC family	
AN1	Encodes a bHLH protein	(Spelt *et al.* 2000)
AP2/EREBP Transcription Factor	Signal transduction pathways of biotic and environmental stress responses Cambial tissue development Key developmental regulators in reproductive and vegetative organs Hormonal regulation	(Riechmann and Meyerowitz 1998) (van der Graaff *et al.* 2000) (Riechmann and Meyerowitz 1998) (Feng *et al.* 2005)

Candidate gene (Family)	Function	References
	ABA response and ethylene response	(Riechmann and Ratcliffe 2000)
DeoR Regulatory Protein	Transcriptional regulation, Ligand interactions	(Anantharaman and Aravind 2006)
C2H2	Flower development, flowering time, seed development, and root nodule development	(Riechmann and Ratcliffe 2000)
mTERF	Regulation of transcription of the mitochondrial genome	(Linder *et al.* 2005)
	Localized in mitochondria, transcription termination, also transcription initiation and the control of mtDNA replication	(Roberti *et al.* 2003)
WRKY Transcription Factor	Regulation of plant defense response pathways	(Eulgem *et al.* 1999; Ülker and Somssich 2004) (Zhang and Wang 2005)
	Responses to the abiotic stresses of wounding	(Hara *et al.* 2000; Cheong *et al.* 2002)
	Response to combination of drought and heat	(Rizhsky *et al.* 2002)
	Response to cold	(Huang and Duman 2002; Pnueli *et al.* 2002)
	Regulatory roles;	
	Morphogenesis of trichomes	(Johnson *et al.* 2002)
	Embryos	(Alexandrova and Conger 2002)
	Senescence	(Chen *et al.* 2002; Robatzek and Somssich 2002)
	Dormancy	(Pnueli *et al.* 2002)
	Plant growth	(Chen *et al.* 2002)
	Metabolic pathways	(Rushton *et al.* 1995; Willmott *et al.* 1998; Johnson *et al.* 2002; Sun *et al.* 2003)
HOTR	Transcription Repressor	(Mutisya *et al.* 2006)
Triacylglycerol lipase	Anabolic and catabolic processes in yeast and plants	
	Membrane repair	(Rajakumari *et al.* 2009)
SHR	Acts both as a signal from the stele and as an activator of endodermal cell fate, SCR-mediated cell division	(Nakajima *et al.* 2001)

Candidate gene (Family)	Function	References
GRAS	root and shoot development	(Bolle 2004)
	Gibberellic acid (GA) signalling	(Bolle 2004)
	Phytochrome A signal transduction	(Bolle 2004)
	Nodule morphogenesis in legumes	(Bolle 2004)
		(Kaló *et al.* 2005; Heckmann *et al.* 2006)
algP	Regulating Mucoidy in *Pseudomonas aeruginosa*	(Konyecsni and Deretic 1990)
Nuclear Transcription Factor Y	Coordinate plant responses to drought tolerance	(Nelson *et al.* 2007)
B12D	Protein known to be accumulated in plants during embryo development, seed maturation, and leaf senescence	(Aalenf *et al.* 1994; Huang *et al.* 2001)
MADS-box	Developmental processes (seed and fruit development)	(Becker and Theißen 2003)
	Floral homeotic functions	(Becker and Theißen 2003)
	Flowering time genes	(Michaels and Amasino 1999; Sheldon *et al.* 1999; Hartmann *et al.* 2000; Lee *et al.* 2000; Sheldon *et al.* 2000)
STF-1	Light and hormone signalling	(Song *et al.* 2008)
pnFL-2	Associated with photoperiodic events	(Kim *et al.* 2003)
MAR	Important in plants at higher levels of gene regulation	
	Chromosomal organization	(Morisawa *et al.* 2000)
AT-HSFB4	Genes responsive to both heat stress and a large number of chemical stressors	(Schöffl *et al.* 1998; Baniwal *et al.* 2004)
ERF (AP2 family)	Ethylene response factor (ERF)-type transcription factor	(Jung *et al.* 2007)
	Response to biotic and abiotic stresses in plants	(Jung *et al.* 2007)
	Pathogen attack and high salinity	(Jung *et al.* 2007)
	Essential *cis*-acting element in;	
	Ethylene, methyl jasmonate and salicylic acid responsive genes	(Ohme-Takagi and Shinshi 1995; Park *et al.* 2001; Lee *et al.* 2004; Yi *et al.* 2004; Jung *et al.* 2007)
	Several cold, high salt and drought-inducible genes	

Candidate gene (Family)	**Function**	**References**
RF2a/RF2 b Transcription Factor	bZIP transcription activator. RF2a/2b is involved in transcriptional regulation of the rice tungro bacilliform virus promoter.	(Dai *et al.* 2004)
HBP-1b Transcription Factor	Leucine Zipper Transactivator in the cell cycle-dependant transcription of wheat histone genes	(Tabata 1991)
PWWP	Cell growth and differentiation Protein- protein interactions	(Stec *et al.* 2000)
AT-HSFB4	Genes responsive to heat and chemical stresses	(Nover 1991; Morimoto 1998)
bZIP	Regulate diverse biological processes such as pathogen defence, light and stress signalling, seed maturation and flower development.	(Jakoby *et al.* 2002)
bZIP EMBP-1	Implicated in ABA induced gene expression in wheat Interacts with VIVIPAROUS1, a maize regulatory protein involved in the Response to ABA during maize embryo development	(Guiltinan *et al.* 1990) (Vasil *et al.* 1995)
Homeodomain Leucine Zipper	Water Stress Responsive in an ABA dependant signalling pathway	(Lee and Chun 1998; Söderman *et al.* 1999)
ATHB-6	Up regulated by ABA during drought stress Target of ABI1 (Protein phosphatase), displays a reduced sensitivity towards ABA during seed germination and stomatal closure in Arabidopsis	(Söderman *et al.* 1999) (Himmelbach *et al.* 2002)
SHI	Suppressor of GA responses	(Fridborg *et al.* 2001)
ARID/BRIGHT domain	Regulate cell proliferation, development, and differentiation	(Wang *et al.* 2007)
sigB	Provide for the interaction of multisubunit RNA polymerase (PEP) with Promoter	(Lysenko 2007)

Candidate gene (Family)	Function	References
SMUBP-2	DNA binding protein	
B3 DNA binding domain	Proteins with the B3 domain are involved in a number of processes: Transcriptional activation: FUSCA3 (FUS3), LEAFY COTYLEDON2 (LEC2) and ABSCISIC ACID INSENSITIVE3 (ABI3) Transcriptional repression: HIGH-LEVEL EXPRESSION OF SUGAR-INDUCIBLE GENE 2 (HSI), HSI L1 and HSIL2 or VP1/ ABI3-LIKE (VAL) from the ABI3 and HSI/VAL families are all shown to be involved in seed development and maturation. RAV genes: growth, development and flowering time ARF family: regulates a range of responses to auxin and have additional systems of regulation The B3 proteins functionally characterized from the ABI3, HSI, RAV and ARF families have shown that they are mainly involved in hormone, signaling pathways such as those for auxin, abscisic acid, brassinosteroid and gibberellins.	(Tsukagoshi *et al.* 2005) (Suzuki *et al.* 2007) (Baumlein H *et al.* 1994) (Stone *et al.* 2001) (Hu *et al.* 2004) (Sessions *et al.* 1997) (Mallory *et al.* 2005) (Romanel *et al.* 2009)
TATA	Physical interaction between OsTBP2 (TATA binding protein 2) and RF2a, a rice bZIP transcription factor	(Zhu *et al.* 2002)
atGRF2	Play a role in the regulation of cell expansion in leaf and cotyledon tissues	(Kim *et al.* 2003)
ocs element	A promoter element transferred to the host plant nucleus by certain DNA viruses.	(Ellis *et al.* 1993)
GATA transcription factor	Implicated in light-dependent and nitrate-dependent control of transcription (Zinc Finger)	(Reyes *et al.* 2004)
DELLA protein	DELLA proteins have an important role in integrating multiple environmental and hormonal signals to coordinate plant growth and development Transcriptional regulation of the DELLA genes also has a role in controlling	(Sun and Gubler 2004) (Oh *et al.* 2007)

Candidate gene (Family)	Function	References
	GA responsiveness and subsequent plant growth and development	(Zentella *et al.* 2007)
	In barley, the DELLA protein, SLN1 acts to repress the expression of a transcription factor, GAMYB which is directly responsible for inducing α-amylase expression	(Gubler *et al.* 2002)
OSH1	Overexpression of *OSH1* causes a reduction of the level of GA_1 by suppressing GA 20-oxidase expression	(Kusaba *et al.* 1998)
ATARP7	May be involved in the modulation of chromatin structure and transcriptional regulation mainly in interphase cells	(Kandasamy *et al.* 2003)
KAP-2	Binds to the H-box (CCTACC) element in the bean CHS15 chalcone synthase promoter	
	Stimulates transcription from a promoter harboring the H-box cis element	(Lindsay *et al.* 2002)
LEUNIG	Key regulator of the Arabidopsis floral homeotic gene AGAMOUS	(Conner and Liu 2000)

ERF proteins are a sub family of the AP2/EREBP (Table 4.6) transcription factor family, unique to plants. ERF genes are regulated by cold, drought, pathogen infection, wounding or treatment with ethylene, SA or jasmonic acid (Singh *et al.* 2002). ERF proteins can act as both transcriptional activators and repressors (Fujimoto *et al.* 2000). WRKY transcription factors have shown enhanced DNA binding and/or expression following induction by pathogens, defence signals and wounding (Eulgem *et al.* 2000). Given the link LRRs, ERF proteins and WRKY have with wounding a link with peroxidases is plausible.

The eQTL identified can be correlated with those QTL for traditional phenotypic traits (or in this case black point) so as to provide additional information about the genetic basis of quantitative genetic variation (Schadt *et al.* 2003; Kirst *et al.* 2004; Bystrykh *et al.* 2005; Hubner *et al.* 2005). The eQTL identified did not align with QTL identified for black point formation [Chapter 2 and Walker *et al.* (2008)]. The eQTL identified on 7H for *HvPrx7* is in a similar position to a spot blotch QTL (Steffenson *et al.* 1996), suggesting that *HvPrx7* may be involved in a regulatory pathway contributing to spot blotch. The *HvBP1* eQTL observed on chromosome 2H is in the same region as a photoperiod response gene (Coventry *et al.* 2003) which affects flowering time and the duration of grain filling. Previous results (Chapter 2) indicated that the stage of grain maturity when grain was exposed to conditions thought to induce black point played a role in black point formation. Indeed, the QTL identified for black point formation [Chapter 2 and Walker *et al.* (2008)] was also linked to the earliness *per se* locus (Laurie *et al.* 1995). The presence of the 2H eQTL may therefore reflect differences in gene expression due to developmental differences (grain maturity and flowering time) associated with the photoperiod response.

This is further supported by the fact that differences in *HvBP1* expression are observed at different grain fill stages (Chapter 3).

In conclusion, a number of processes that could involve or regulate expression of the peroxidase gene *HvBP1* have been suggested. The stress response of plants is regulated by multiple signalling pathways (Jane 2001; Knight and Knight 2001). A combination of the identified proteins or domains are therefore likely to be regulating the expression of peroxidase genes. eQTL for candidate genes have been identified using expression data across the Sloop/Alexis population. *Cis*-eQTLs (identified for *HvPrx7* and *HvQR*) represent a polymorphism physically located near the gene itself, or identification of a promoter polymorphism. *Trans*-eQTLs are the result of a polymorphism at a location in the genome other than the actual position of the gene whose transcript is being measured, or a polymorphism at the physical position of a regulatory factor elsewhere in the genome (Hansen *et al.* 2008). Little is known about the architecture of gene regulation or about the genetic basis for variation in gene expression levels (Gilad *et al.* 2008). Mutations in putative regulatory regions have been associated with >100 human phenotypes (Gilad et al. 2008), therefore investigation of the promoter regions and analysis of interacting factors will allow us to further understand the regulation of the identified candidate genes and black point formation. Specifically the promoter regions of peroxidase genes *HvBP1* and *HvPrx7* will allow us to investigate the regulatory interactions with peroxidase genes and black point formation.

Chapter Five. Promoter analysis for *HvBP1* and *HvPrx7* and identification of a potential regulator of *HvPrx7* expression

5.1 Introduction

Peroxidase genes are probably involved in the formation of black point through their role in enzymatic browning (as per section 1.3, Figure 1.2). A greater level of gene expression in susceptible varieties (Chapter 3) compared to tolerant varieties further supports a role. Studies described in the previous chapter further analysed the differential expression observed, using expression data to map eQTL in the Alexis/Sloop DH population. A *cis*-acting QTL for *HvPrx7* expression was identified suggesting that the main difference between susceptible and tolerant cultivars might be polymorphisms physically located in or near the gene itself, or within the promoter leading to differential expression. On the other hand, the *trans*-acting eQTL detected for *HvBP1* suggests that a polymorphism at the physical position of a regulatory factor elsewhere in the genome might lead to differential expression between black point susceptible and tolerant varieties.

The major mechanism of differential gene expression is transcriptional regulation (Lee and Young 2000) whereby gene expression is controlled by whether transcription factors bind to DNA *cis*-elements located in a gene's promoter or not (Lopato *et al.* 2006). Single nucleotide polymorphisms (SNPs) are the most common form of genetic variation in organisms, occurring at a frequency of one in every 1000 bp in humans (Brookes 1999) and one in every 170 bp in rice (Yu *et al.* 2002). SNPs can be located in the promoter or coding regions of plants and many traits in plants are attributed to SNPs and their

variations (Bryan *et al.* 2000; Edwards 2007). A SNP identified within transcription factor binding sites between a susceptible and tolerant variety could have downstream effects on gene regulation. For example, in rice, a SNP within the Granule Bound Starch Synthase I (*GBSSI*) promoter regulates expression of *GBSSI* and affects its function, resulting in a deformed loop on the outer layer (surface) altering the 3D shape, structure and function of the protein, possibly owing to a change in the substrate binding site (Kharabian 2010). Genetic variations that alter the amino acid sequence of proteins are relatively easy to identify, however sequence variations that affect the regulation of genes are more difficult to pinpoint due to the large amount of non-functional polymorphisms in the vicinity of a gene (Andersen *et al.* 2008).

Yeast-One hybrid (Y1H) technology is a powerful method to identify protein-DNA interactions and has successfully identified transcription factors involved in gene regulation in barley (Müller *et al.* 2000; Ogo *et al.* 2007). Similarly, several transcription factors from the homeodomain, Apetala 2 (AP2) domain and elongation factor 2 (E2F) families have been identified and isolated in wheat (Lopato *et al*. 2006). MYC transcription factors have also been identified in rice (Zhu *et al.* 2003), WRKY transcription factors in parsley (Cormack *et al.* 2002) and a drought responsive element (DRE) transcription factor in wheat (Shen *et al.* 2003) using Y1H technology. Y1H technology therefore presents an opportunity to identify transcription factors that bind to the promoters of *HvBP1* and *HvPrx7*.

The research presented in this chapter aimed to determine if susceptibility was correlated with differences in regulatory elements by analysing the promoter regions of candidate

genes *HvBP1* and *HvPrx7* in the susceptible variety Sloop and tolerant variety Alexis. The second aim was to identify transcription factors that might regulate gene expression by using Y1H screening.

5.2 Materials and methods

5.2.1 – *HvBP1* and *HvPrx7* promoter isolation and *in silico* characterisation

Gene promoters were isolated using a genome walking approach. Information from the full length gene sequence (*HvBP1* and *HvPrx7*) identified in chapter 4 allowed promoter isolation and *in silico* characterisation of likely transcription factor binding sites. *In silico* characterisation also allowed the identification of SNPs between a susceptible and tolerant variety which may affect the function of those transcription factor binding sites.

5.2.1.1 – Genome walking library construction

A genome walking library was constructed using genomic DNA (gDNA) isolated from *Hordeum vulgare* (cv. Sloop) as described in Section 4.2.2.1. The technique was adapted from the protocol outlined in the Clontech Universal GenomeWalker Kit (Clontech, U.S.A., Scientifix, Australia). Isolated gDNA was digested using nine blunt end cutting enzymes: *Dra*I; *Eco*RV; *Pvu*II; *Stu*I; and *Sca*I as per the manufacturer's instructions (Clontech). *Nru*I; *Hinc*II; *Nae*I and *Msc*I were also included as per (Boden *et al.* 2009). The digested DNA was then purified using phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1). The DNA was precipitated by the addition of 0.1

volume 3 M sodium acetate (pH 4.8), 20 µg of glycogen (Ambion, Victoria, Australia) and 2 volumes of ice-cold 95% ethanol. The samples were then centrifuged at 13000 *g* for 10 min at 4°C. The DNA pellet was washed with ice-cold 80% ethanol, before being air-dried and then resuspended in 20 µL of TE buffer (pH 7.5).

The GenomeWalker Adapter was ligated onto the digested gDNA by combining gDNA (4 µL), the GenomeWalker Adapter (25 µM, 1.9 µL), 10 X ligation buffer (1.6 µL) and T4 DNA ligase (3 U) in a reaction volume of 16 µL. Tubes were incubated overnight at 16°C and then at 70°C the following day for 5 min to cease the reaction. TE buffer (1 X; pH 7.4; 72 µL) was then added to each reaction tube and the libraries were stored at -20°C.

5.2.1.2 – Genome walking

Genome walking consisted of three successive walks (two PCR rounds for each walk) for the *HvBP1* promoter and three walks for the *HvPrx7* promoter. The third round of genome walking for *HvPrx7* used the same primer pair as the second walk. The isolated *HvBP1* promoter was 2416 bp while the *HvPrx7* promoter isolated was 1569bp. A schematic diagram of the genome walking is represented in Figure 5.1, including primer combinations to confirm promoter specificity (Table 5.1).

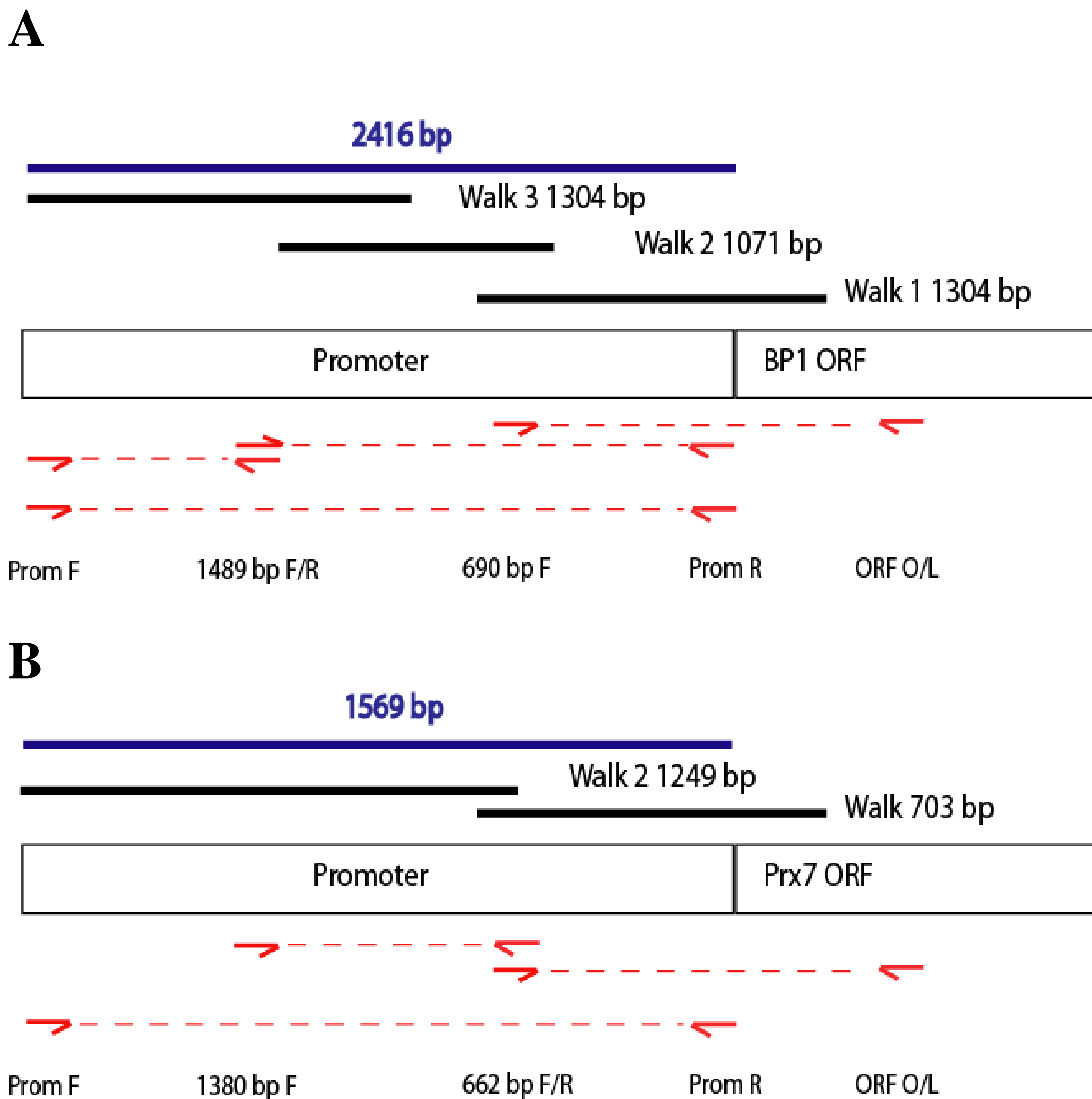

Figure 5.1 Schematic summary of genome walking and specificity PCR for *HvBP1* (A) and *HvPrx7* (B). For *HvBP1* (A) three genome walks resulted in fragments of 1304 bp, 1071 bp and 1304 bp respectively with 2416 bp of the promoter isolated (represented by blue line). For *HvPrx7* (B) two genome walks resulted in fragments of 703 bp and 1249 bp respectively and a total of 1569 bp (represented by blue line) of the promoter. Primers were designed to confirm specificity to the open reading frame (ORF). Arrows represent primer and direction of amplification, while the red dotted lines represent the amplified fragments. F = forward primer, R = reverse primer, ORF O/L = primer designed to overlap into the ORF of the candidate gene to confirm specificity (as per Table 5.1).

Each walk or amplification of promoter fragments was performed by two successive rounds of PCR, a primary PCR followed by a secondary (or nested) PCR, using Elongase

components (Invitrogen). Primary PCR solutions contained Elongase buffer A (4 µL), Elongase buffer B (6 µL), dNTPs (10 mM, 1 µL), Elongase enzyme mix (1 U, 1 µL) and 1 µL of the respective genome walking library. In addition (and in each case), the forward primer for each primary PCR was Adapter primer 1 (AP1) (5' GTAATACGACTCACTATAGGGC 3') (10 µM, 1 µL), with reverse primers (primary) used that were specific to *HvBP1* or *HvPrx7* (10 µM, 1 µL) (Table 5.1). The reaction volume was made up to 50 µL with nanopure water. Thermal cycle conditions for the primary PCRs were as follows: denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 68°C for 3 min, and a final extension at 68°C for 10 min. Products from the primary PCRs were then diluted two-fold for use in the secondary PCR.

The secondary PCR was a repeat of the primary PCR, with primers being the only difference, using the secondary (S) primers (Table 5.1) and 3 µL of the respective diluted (2-fold) primary PCR product as the template. In addition, the forward primer for each reaction was Adapter primer 2 (AP2) (5' ACTATAGGGCACGCGTGGT 3') (10 µM, 1 µL), with secondary (nested) gene specific primers used as the reverse primers (10 µM, 1 µL) (Table 5.1). The thermal cycling conditions were identical to those used for the primary PCR. Secondary PCR products were electrophoresed, visualised and then excised for each walk (as shown in Figure 5.2 and Figure 5.3). Excised fragments were purified, ligated into the pDrive cloning vector and subsequently transformed into *E. coli* as outlined in section 3.2.2.2. Products were sequenced and chromatogram files containing the sequence data uploaded into the ContigExpress program (Invitrogen, Vector NTI Advance 10, Australia) for analysis as per section 3.2.2.2. Unnecessary sequence information including plasmid DNA sequence or adapter sequence was removed and a contig of files

with expected overlapping sequence information from each walk compiled (section 3.2.2.2).

Table 5.1 Gene specific primers used during each genome walking experiment to isolate the *HvBP1* (A) and *HvPrx7* (B) promoters. Three walks were undertaken for *HvBP1* (A) and two walks for *HvPrx7* (B). For each walk two PCR reactions were performed, combining the adaptor primers (AP1/AP2) with gene specific primary PCR primers (P) for the first PCR reaction and secondary PCR primers (S) for the second nested reaction.

A

Walk Number	**Primer**
1	**P** 5′ ACCAACACCGCCATTGCCACCACAA 3′
	S 5′ ACAAGTGCTGCTAGCAGAGGAACACGA 3′
2	**P** 5’ CTAGTTACTCATACTCCCTCCGTCATGAT 3′
	S 5′ CCTAGCTGGTTATTGATTGGCTGTGAAATG 3′
3	**P** 5′ 3′ TGCTGTCTCTGATAGGGATATGTATCTA
	S 5′ 3′ TAGTCCTGACCTACATGTCCTACCTAT

B

Walk number	**Primer**
1	**P** 5′ CACACACAAAGGAGAGAGGAGATGGCTCG 3′
	S 5′ CAGTCGTGGAAGTGGAGTCGAAGGAGG 3′
2	**P** 5′ GTACCCGCAAATTCGTGTCTCTTATTCTAAC 3′
	S 5′ ACACCACAGTGACGGGCATGTTGGACA 3′

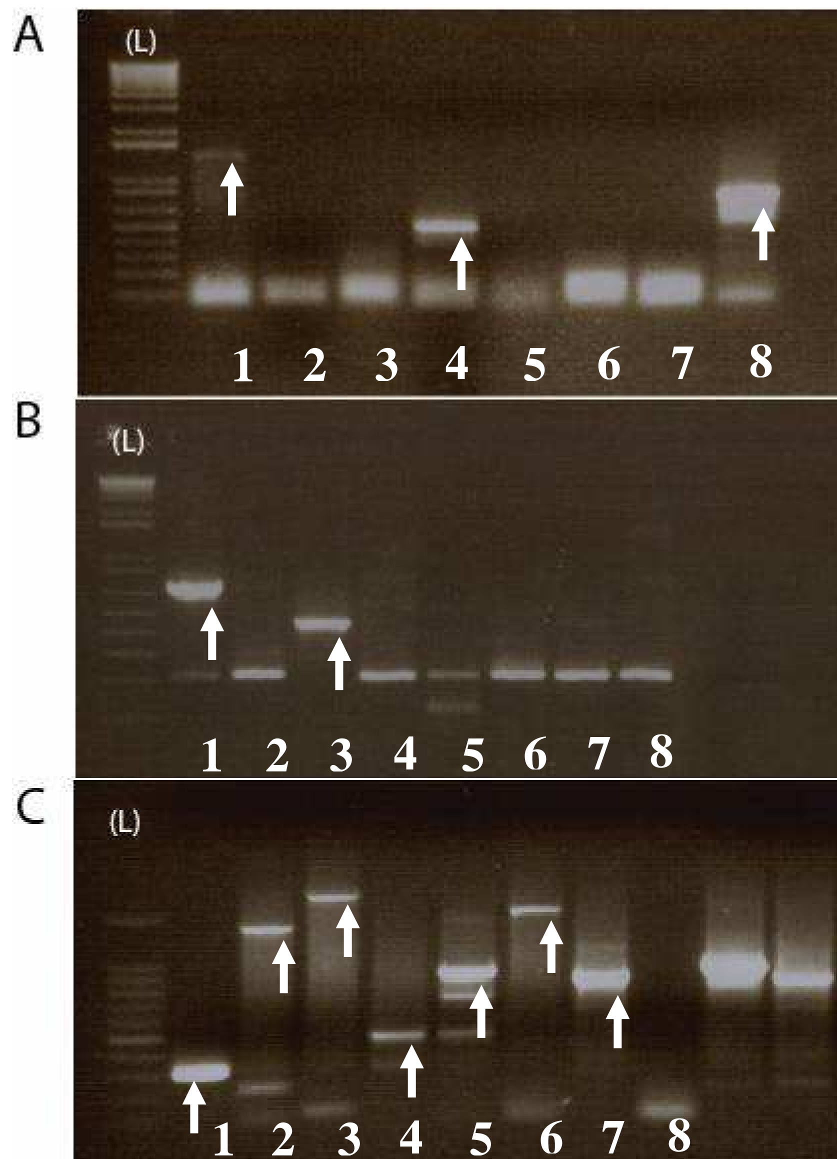

Figure 5.2 Genome walking to isolate the *HvBP1* promoter region. Digested cDNA libraries are represented in lanes: (1) *Dra*I (2) *Pvu*II (3) *Eco*RV (4) *Stu*I (5) *Sca*I (6) *Hinc*II (7) *Nae*I and (8) *Msc*I. The arrowhead represents fragments that were successfully cloned and sequenced. The first round of genome walking (A) resulted in amplification from the *Dra*I, *Stu*I and *Msc*I libraries. The second round (B) resulted in amplification from the *Dra*I and *Eco*RV libraries. The third round (C) resulted in amplification from the *Dra*I, *Pvu*II, *Eco*RV, *Stu*I, *Sca*I, *Hinc*II and *Nae*I libraries. (L)=1Kb plus ladder.

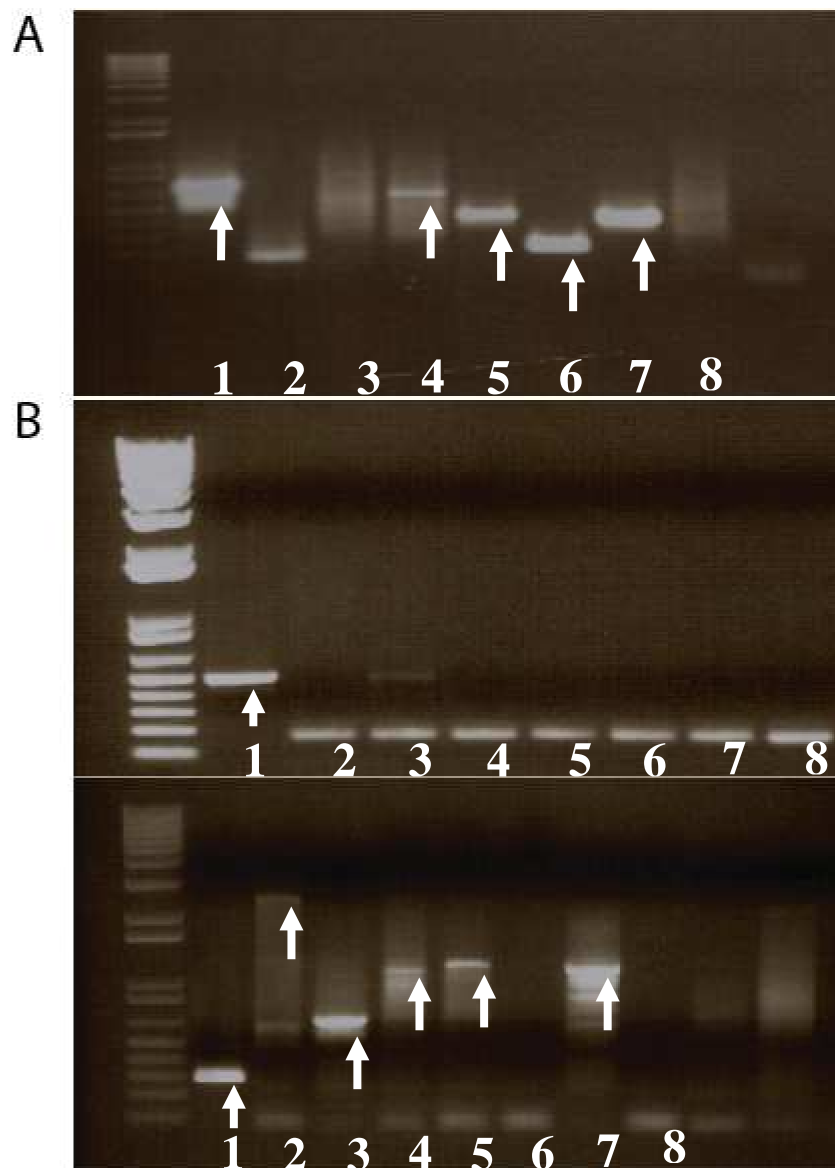

Figure 5.3 Genome walking to isolate the *HvPrx7* promoter region. Digested cDNA libraries are represented in lanes: (1) *Dra*I (2) *Pvu*II (3) *Eco*RV (4) *Stu*I (5) *Sca*I (6) *Hinc*II (7) *Nae*I and (8) *Msc*I. The arrowhead represents fragments that were successfully cloned and sequenced. The first round of genome walking (A) resulted in amplification from the *Dra*I, *Pvu*II, *Stu*I, *Sca*I, *Hinc*II and *Nae*I libraries. The second round (B) was repeated twice resulting in amplification from the *Dra*I library (top panel) and secondly the *Dra*I, *Pvu*II, *Eco*RV, *Stu*I, *Sca*I and *Nae*I libraries (bottom panel). (L)=1Kb plus ladder.

5.2.1.4 Confirmation of promoter specificity

Following sequencing and contig alignment of the promoter fragments (as discussed in the previous section), PCR was performed to confirm the sequences of the products isolated through genome walking as specific to the *HvBP1* and *HvPrx7* promoters. Primers were designed to the *HvBP1* and *HvPrx7* isolated promoters (Figure 5.1, Table 5.2). Reverse primers that were complementary to the *ORF* of *HvBP1* and *HvPrx7* were designed to confirm specificity to the *ORF* (Table 5.2). PCR solutions contained Elongase buffer A (4 µL), Elongase buffer B (6 µL), dNTPs (10 mM, 1 µL), Elongase enzyme mix (1 U, 1 µL), forward primer (10 µM, 1µL), reverse primer (10 µM, 1 µL), Sloop DNA (50 ng/ µL, 1 µL) and nanopure water (up to 50 µL). PCR cycling parameters were: denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 30 sec at the respective annealing temperatures for each primer combination (Table 5.2), 72°C for 2 min and 30 sec, with a final extension step at 72°C for 10 min. PCR products were separated by gel electrophoresis using 1.5% (w/v) agarose gels and visualised by ethidium bromide staining. The fragments were purified, ligated and subsequently transformed into *E. coli* and products sequenced as outlined in section 3.2.2.2. Sequence data was analysed as described in section 5.2.

Table 5.2 List of primers used to confirm that the obtained promoter sequence was specific to *HvBP1/HvPrx7*. The distance of the primer from the ORF is represented (690 BP F (forward primer) represents 690 bp into the promoter from the ATG, 1489 R (reverse primer), 1480 bp into the promoter). Primers were designed to overlap into the ORF (ORF O/L) and also isolate the full length promoter (Prom F/R). Tm (°C) represents melting temperature of primer reaction. Expected product size (bp) is represented.

Promoter	Primer	Tm (°C)	Expected Size (bp)
HvBP1			
690 bp F	**F** 5′ AGATCCATTGCATTTACTCCTAACAGCTAA 3′	55	928
ORF O/L	**R** 5′ AGCAGTCGTGGAAGTGGAGTCGAA 3′		
1489 bp F	**F** 5′ GCAGAGTCCTAGCTACGACAAGCT 3′	59	1489
Prom R	**F** 5′ CTCCTCTCTCCTTTGTGTGTGACC 3′		
Prom F	**F** 5′ CGCGAGCCCAGCATGTTGGGATTA 3′	57	936
1489 R	**F** 5′ GGACTCTGCCCTCTCCTTTCGTA 3′		
HvPrx7			
662 bp F	**F** 5′ TCACBACAAATACAATGAAAGGTCAAGT 3′	54	946
ORF O/L	**R** 5′ CGGAGCGTCTGGTTGGGGATCT 3′		
1380 bp F	**F** 5′ GAAGGAGCGGCGACGATAGAAGAG 3′	52	745
662 bp R	**R** 5′ AAGTGGTACAGATTGCTAGACAGACTC 3′		

5.2.1.5 Amplification of the full length promoter

DNA was isolated as per section 4.2.2.1 but for both varieties (Sloop and Alexis). Consensus sequences for the *HvBP1* and *HvPrx7* promoters in both Sloop and Alexis were generated through multiple rounds of cloning and sequence PCR analysis. Primer 3 (www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) was used for primer design to the sequence originally isolated from Sloop using genome walking and NetPrimer (www.premierbiosft.com/netprimer/netprlaunch/netprlaunch.html) to test primer quality through prediction of primer dimers and hairpin loops. Primer combinations are represented in Table 5.3 and PCR conditions were as per section 5.2.1.2. Three biological replications of the full length were undertaken to ensure sequence quality. Two cloning/sequencing reactions were undertaken as per section 3.2.2.2.

Table 5.3– List of primers used to obtain the full length promoter sequence for *HvBP1/HvPrx7* in parental varieties Sloop and Alexis. F represents forward primers, R represents reverse primers. Tm (°C) represents melting temperature of primer reaction. Expected product size (bp) is represented.

Promoter	Primer	Tm (°C)	Expected Size (bp)
HvBP1	**F** 5′ CGCGAGCCCAGCATGTTGGGATTA 3′	57	2416
	R 5′ CTCCTCTCTCCTTTGTGTGTGACC 3′		
HvPrx7	**F** 5′ CAAATAGGCGAAAAGCGGACACATGTCAAT 3′	58	1569
	R 5′ TGCTGAAGCTGAGCTTCTTCTTGCACCT 3′		

5.2.1.6 *In silico* promoter analysis

Sequence data from successive PCRs were assembled using ContigExpress software (Invitrogen, Vector NTI Advance 10). Promoter sequences were uploaded into the Vector NTI 10 software (Invitrogen, Vector NTI Advance 10) and SNPs between Sloop and Alexis identified through alignments.

The *HvBP1* and *HvPrx7* promoter consensus sequences were analysed using the PLACE database (Plant Cis-Acting Regulatory DNA Elements, http://www.dna.affrc.go.jp/PLACE/; *accessed 12/06/10*) to identify *cis*-elements within the sequence (Higo *et al.* 1999). Alexis and Sloop sequences were analysed to identify the presence or absence of regulatory elements. Regions were chosen for Y1H analysis based on the presence of SNPs between the sequence from Sloop (susceptible) and the sequence from Alexis (tolerant) and based on function in relation to peroxidase genes or potential role(s) in black point formation.

5.2.2 Y1H Analysis

Y1H analysis was undertaken as per the Matchmaker™ Library Construction and Screening Kits User manual (Clontech) with modifications as per Lopato *et al.* (2006). A flow diagram outlining the methods for the Y1H screen and analysis of positive clones is shown in Figure 5.4.

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held by the University of Adelaide Library.

Figure 5.4 Flow diagram outlining methods for Y1H screening and analysis of positive clones. Adapted from Lopato *et al.* (2006).

5.2.2.1 – Construction and screening Y1H library

5.2.2.1.1 – cDNA library construction

cDNA libraries for Y1H were constructed for the varieties Sloop (susceptible) and Alexis (tolerant) using RNA pooled from each of the developmental stages milk, soft dough, hard dough and maturity (as per section 3.2.1)

5.2.2.1.1.1 – RNA extraction and cDNA synthesis

RNA was extracted as per section 3.2.2.1, but a DNA-free protocol was undertaken to remove contaminating DNA. A 50 µL reaction was set up to contain 0.1 volume 10 X DNase I buffer and 1 µL of DNase plus the RNA sample. The sample was incubated at 37°C for 30 min, resuspended in 0.1 volume of DNase inactivation reagent (Clontech) and incubated at room temperature for 2 min before centrifugation at 10000 *g* for 1.5 min. The supernatant containing RNA was transferred to a fresh tube before storage at -80°C until required. RNA was quantified to check quality as per section 3.2.2.1.

First strand cDNA synthesis was performed using 2 µg of pooled RNA (500 ng from each of the developmental stages: milk, soft dough, hard dough, maturity) and an oligo(d)T primer. Two µL of RNA (2 µg) was combined with 1 µL of CDS III primer (MATCHMAKER Library construction and screening Kit, Clontech), incubated at 72°C for 2 min followed by cooling on ice for 2 min. Tubes were spun briefly and the following added to the reaction tube: 2 µL 5 X first strand buffer, 1 µL DTT (20 mM), 1 µL dNTP

(10 mM) and 1 µL of MMLV reverse transcriptase (Clontech). After incubation at 42°C for 10 min 1 µL of SMART III Oligonucleotide (MATCHMAKER Library construction and screening kit, Clontech) was added and incubated at 42°C in a hot lid thermal cycler. First strand synthesis was terminated by placing tubes at 75°C for 10 min. Tubes were cooled to room temperature, 1 µL (2 U) of RNase H added and incubated at 37°C for 20 min. First strand cDNA products were stored at -20°C until required.

ds cDNA was amplified by long distance PCR (LD-PCR). Two x 100 µL PCR reactions were set up, containing: 2 µL first-strand cDNA, 70 µL deionised water, 10 µL 10 X advantage 2 PCR buffer (Clontech), 2 µL 50 X dNTP mix, 2 µL 5´ PCR primer, 2 µL 3´ PCR primer, 10 µL of 10 X GC-Melt solution and 50 X advantage 2 polymerase mix. Tubes were mixed gently. PCR cycling parameters were denaturation at 95°C for 30 sec, followed by 22 cycles of 95°C for 10 sec, 68°C for 6 min, with a final extension step at 68°C for 5 min. ds cDNA was purified with a CHROMA SPIN™ TE-1000 column as per the Matchmaker™ Library Construction and Screening Kits User manual (Clontech).

5.2.2.1.1.2 – cDNA library transfer to yeast

5.2.2.1.1.2.1 – Preparation of competent yeast cells

Yeast competent cells were prepared using the LiAc method as per the Yeast Protocols Handbook (PT3024-1 Clontech™). AH109 yeast stock was streaked on a Yeast Peptone Dextrose Adenine YPDA agar plate and incubated at 30°C for approximately 3 days, or until colonies appeared. One colony was inoculated into 3 mL of YPDA medium in a sterile 15 mL centrifuge tube and incubated at 30°C for 8 h. Fifty µL of the culture was

transferred to a 250 mL conical flask containing 50 mL of YPDA. The culture was further incubated at 250 rpm for 16-20 h until the OD_{600} sample reached 0.15 to 0.3, taking approximately 8 h. Cells were centrifuged at 700 x *g* for 5 min at room temperature, the supernatant was discarded and the cell pellet resuspended in 100 mL YPDA. Samples were incubated at 30°C for 3 to 5 h until OD_{600} reached 0.4 to 0.5. Cells were centrifuged at 700 *g* for 5 min at room temperature and the cell pellet resuspended in 60 mL dH_20. Cells were further centrifuged at 700 x *g* for 5 min at room temperature and the cell pellet resuspended in 3 mL of 1.1 X TE/ lithium acetate (LiAc). The resuspension was split between 2 x 1.5 mL centrifuge tubes, centrifuged at high speed for 15 sec and the pellet resuspended in 600 µL of 1.1 X TE/LiAc. Tubes were snap frozen in liquid nitrogen and stored at -80°C until required.

cDNA (section 5.2.2.1.1.1) was transformed into competent yeast cells. In a sterile 15 mL tube the following was combined: 10 µL of cDNA, 6 µL of pGADT7-Rec (0.5 µg) and 20 µL of herring testes carrier DNA (10 mg/mL) (denatured by heating to 100°C for 5 min, chilling on ice and repeating the process a second time). Competent cells (600 µL) were added followed by gentle mixing. polyethylene glycol (PEG/LiAc) solution (2.5 mL) was then added and mixed by gentle vortexing followed by incubation at 30°C for 45 min, mixing cells every 15 min. DMSO (160 µL) was then added, mixed and incubated in a 42°C water bath for 20 min (with further mixing after 10 min). Centrifugation at 700 x *g* for 5 min was undertaken and pellet resuspended in 3 mL of YPD Plus liquid medium. Tubes were incubated at 30°C for 90 min, transferred to a 50 mL sterile centrifuge tube and centrifuged at 700 *g* for 5 min. The supernatant was discarded and the pellet

resuspended in 30 mL of NaCl solution (0.9 %). Two hundred µL was spread on SD/-Leu plates, incubated upside down at 30°C until colonies appeared. Transformants were harvested by firstly chilling plates at 4°C for 3 to 4 hours. Five mL of freezing medium (YPDA containing 25% glycerol and 25 µg kanamycin) was added to each plate, using a sterile glass rod to gently swirl and dislodge cells into liquid. Liquids were combined in a sterile flask, mixed well, incubated at 30°C for 30 min with rotation (220 rpm) and checked using a haemocytometer to calculate cell density to ensure the library contained an adequate number of cells for screening. Cell density was calculated for yeast containing the Sloop and Alexis cDNA libraries, resulting in 8.75 x 10^8cells/mL and 1.15 x 10^9cells/mL respectively. Cell density exceeded the recommended threshold of 2.7 x 10^7cells/mL. Aliquots (1.5 mL) were snap frozen in liquid nitrogen and stored at -80°C until further use. Library titer was also tested by spreading 100 µL of a 1:100, 1:1000 and 1:10000 dilutions on 100 mm SD/-Leu plates. Plates were incubated at 30°C until colonies appeared. Colonies were counted and number of colonies in the library calculated using the following formula: colonies X dilution factor / volume plated (mL). This allowed the calculation of mating efficiency and indication of successful screens (found to be greater than 2%).

5.2.2.1 Construction of yeast reporter strains

5.2.2.1.1 Construction of the pINT-1-HIS 3 reporter plasmid

Promoter regions were chosen for Y1H analysis based on the presence of SNPs between the sequence from Sloop (susceptible) and the sequence from Alexis (tolerant) (resulting in the presence/absence of binding domains) and based on function in relation to peroxidase function and potential role(s) in black point formation. Selected promoter sequences for *HvBP1* and *HvPrx7* containing the *cis*-target elements were cloned into the pINT1-HI3NB vector (kindly provided by Dr. PBF Ouwerkerk, Institute of Molecular Plant Sciences, Leiden University, Netherlands). Target sequences from Sloop and Alexis were cloned into the pINT1-HI3NB, resulting in two reporter plasmids for *HvBP1* and two reporter plasmids for *HvPrx7*.

Primers were designed to amplify the *HvBP1* and *HvPrx7* promoter fragments and include the restriction sites *Not*I and *Spe*I allowing 2 extra base pair overhangs for enzyme binding specificity (Table 5.4). The two unique restriction sites were used for single step directional cloning of the DNA fragments into the binary vector. For each construct combination (*HvBP1* Sloop-pINT1-HI3NB, Alexis-pINT1-HI3NB, *HvPrx7* Sloop-pINT1-HI3NB, Alexis- pINT1-HI3NB) 0.1 µg of vector and *HvBP1* or *HvPrx7* promoter region were digested at 37°C for 2 h in: Buffer 2 (10 X), *Not*I and *Spe*I, 10 X Bovine Serum Albumin (BSA) and deionised water up to 20 µL. PCR clean up was performed as per the manufacturer's instructions [Wizard®SV Gel and PCR Clean-Up System (Promega)]. Ligation of the promoter fragments into the pINT1-HI3NB were performed by the addition

of 5 µL of digested plasmid, 1 µL of digested PCR product, 1.2 µL of 10 X buffer (Clontech) and 0.8 µL of T4 DNA ligase, incubating at room temperature for 4 h. Vectors were transformed and the presence of *HvBP1* or *HvPrx7* promoter fragments confirmed through sequencing as per section 3.2.2.2. Transformed PCR products were sequenced as per section 3.2.2.2.

Table 5.4 Primer combinations containing the unique *Not* I (in red) or *Spe* I (in blue) for *HvBP1* and *HvPrx7* promoter sequence cloning into the pINT1-HI3NB vector. Primer combinations were used to amplify promoter fragments in the varieties Sloop and Alexis. Tm (°C) represents melting temperature of primer reaction. Expected product size (bp) is represented.

Primer	**Tm (°C)(*Size*)**
HvBP1	
F (*Not* I) 5′ AT**GCGGCCGC**CTCTGTTGGTGTTA 3′	55 *(196 bp)*
R (*Spe* I) 5′ GG**ACTAGT**CAAGTGTCTGATGTCAAGTAGTTCCAA 3′	
HvPrx7	
F (*Not* I) 5′ AT**GCGGCCGC**AATTTTTCACACAAATACAATGAA 3′	50 *(393 bp)*
R (*Spe* I) 5′ GC**ACTAGT**GAGAGAGAGAGAGACTAATTACA 3′	

5.2.2.1.2 Transformation of pHIS3-pINT1 reporter to yeast

pINT1-HI3NB vector containing the sequenced *HvBP1/HvPrx7* (from Sloop or Alexis) promoter fragments were transformed into yeast as per section 5.2.2.1.1.2.1, with Y187 competent yeast cells used instead of AH109. Cells were spread on YPDA-G418 plates and incubated at 30°C for 3 days. Colonies were picked and re-streaked on YPDA-G418 plates for a further 3 days. After incubation plates were stored at 4°C and re-streaked after 2 months. Overnight cultures were mixed with glycerol to 25% final concentration and stored as 1 mL aliquots at -80°C until further use.

5.2.2.1.3 Determining 3-AT concentration

3-amino-1,2,4-triazole (3-AT) is a competitive inhibitor of the yeast *HIS3* protein, able to inhibit low levels of *HIS3* expressed in a leaky manner and hence suppress background growth on SD medium lacking histidine [Matchmaker™ Library Construction and Screening Kits User manual (Clontech)] controlling selection gene dependency. G418 resistant colonies (section 5.2.3.2.2) were plated on a concentration series of 0, 5, 10, 25 and 50 mM 3-AT and plates incubated at 30°C for 7 days counting colonies to determine the optimal concentration that is required to reduce growth.

5.2.2.2 Overnight yeast mating (cDNA library screen)

Overnight yeast mating of the cDNA library (section 5.2.2.1.1) and the pINT-1-HIS 3 reporter plasmid (containing the promoter sequence) allows the identification of transcription factors binding to sequences produced by the library with only positive colonies being able to survive on SD medium lacking histidine.

5.2.2.2.1 Overnight yeast mating

Fifty mL of culture for each reporter strain was grown overnight (section 5.2.2.1.2). A 1.5 mL aliquot of the reporter was combined with 50 mL 1 x YPDA (plus 50 µL of G418). Flasks were incubated at 30°C with shaking overnight. Cells were harvested by centrifugation at 2000 rpm for 5 min. Cells were resuspended in 50 mL of 2 x YPDA plus 25 µg/mL of kanamycin, mixed with 1.5 mL aliquot of cDNA library in the AH109 strain and incubated in a 2 L conical flask at 30°C overnight with slow (30 to 50 rpm) rotation. After 22 h, the yeast cells were harvested by centrifugation at 1000 x *g* for 10 min, washed in 1 X TE (plus 25 µg/mL kanamycin), resuspended in 5 mL of the same buffer and spread (200 µL) on SD/–His –Leu selective plates containing the optimal level of 5 mM 3-AT (as determined in section 5.2.2.1.3). Transformation efficiency was calculated by spreading samples on SD/–Leu plates at 1 in 10, 1 in 100, 1 in 1000 and 1 in 10000 dilutions. Plates were incubated at 30°C until colonies appeared (up to 2 weeks).

Positive colonies (His+) colonies were restreaked on SD/–His –Leu selective plates containing 5 mM 3-AT. Use of the ∞-galactosidase reporter gene (*MEL1*) allowed the

identification of false positives directly on the plates using X-∞-GAL (25 µg/mL), allowing the selection of blue colonies as false positives. Two hundred µL of X-∞-GAL (25 µg/mL) was spread on the SD/–His –Leu selective plates, allowed to dry followed by re-streaking of positive colonies. Plates were incubated at 30°C for 3 to 5 days.

5.2.2.2.2 Assessment of positive colonies

PCR reactions were performed directly on the His + colonies. The Y-DER DNA extraction reagent kit (Pierce) was used to extract DNA from positive colonies as per the manufacturer's instructions. PCR reactions on the positive colonies used Platinum Taq™. PCR solutions contained Failsafe Buffer G (12.5 µL), Platinum Taq (0.5 µL), ADLD forward primer (5' CTATTCGATGATGAAGATACCCCACCAAACCC 3') (10 µM, 1µL), ADLD reverse primer (5' AGTGAACTTGCGGGGTTTTTCAGTATCTACGAT 3') (10 µM, 1 µL), DNA (1 µL) and nanopure water (up to 25 µL). PCR cycling parameters were: denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 30 sec at 68°C, 68°C for 3 min with a final extension step at 68°C for 3 min. PCR products were separated by gel electrophoresis using 1.5 % (w/v) agarose gels and visualised by ethidium bromide staining.

PCR fragments were further digested with *Hae*III to determine if there were any conserved banding patterns, reducing the number of potential sequencing reactions. Reactions contained 10 X Buffer 2 (2 µL), HaeIII (0.5 µL) and nanopure water (up to 10 µL). Tubes were incubated at 37°C for 4 h, followed by incubation at 65°C for 20 min. Digestion products were separated by gel electrophoresis using 1.5% (w/v) agarose gels and

visualised by ethidium bromide staining as per section 3.2.2.2. Candidate fragments which displayed differentiating banding patterns after digestion were ligated into the pDrive cloning vector and subsequently transformed into *E. coli* and transformed PCR products sequenced as outlined in section 3.2.2.2.

5.2.2.2.3 Plasmid isolation from yeast

Plasmids identified as putative positives were isolated from yeast for further analysis. Overnight cultures (10 mL) of the His+ colonies were grown in CM –Leu medium in 50 mL tubes at 30°C with shaking. Cells were harvested by centrifugation at 2400 x *g*, resuspended in 200 µL of 0.9 M sorbitol/50 mM EDTA containing 4 mg/mL lyticase. The resuspended cells were transferred to a 1.5 mL microcentrifuge tube and incubated at 30°C for 1 h. Tubes were centrifuged at 3000 rpm for 5 min and a standard alkaline lysis miniprep procedure performed as per section 3.2.2.2. Fragments were ligated into the pDrive cloning vector according to the manufacturer's instructions (Qiagen, Hilden, Germany) and subsequently transformed into *E. coli* and sequenced as outlined in section 3.2.2.2.

5.2.2.2.4 Verification of positive interactions and specificity

Protein DNA interactions were confirmed by re-transformation of the reporter and control strains. Yeast strains were co-transformed with the reporter plasmid (pINT-1HIS3) and the library plasmid containing the identified sequence. Primers were designed with the appropriate restriction sites for ligation into the library vector pGADT7 (Table 5.5). The

library vector containing the identified sequences was then co-transformed with the original bait vector (section 5.2.2.1.1).

Table 5.5 Primer combinations containing the unique restriction sites (in red) for ligation of the candidate YIH sequences for *HvBP1* and *HvPrx7* into the library plasmid vector (pGADT7). Fragment represents positive interactions with the *HvBP1* promoter region (Sloop and Aleixis) and *HvPrx7* promoter region (Sloop and Alexis). Tm (°C) represents melting temperature of primer reaction. RE sites represents the unique restriction sites included in primer design with an extra 2 bp overhang.

Fragment	Primer	Tm (°C)	RE Sites
***HvBP1* Sloop**			
3	**F** 5′ ATGAATTCCGCCCGCGCAAGCCCTT 3′ **R** 5′ GCATCGATACAAACAGATCCACATTAGCT 3′	59	*EcoRI/ClaI*
4	**F** 5′ ATGAATTCCGGCCGCCCGCCTTACAT 3′ **R** 5′ CTGGATCCATATATAAATCAGGTCCATGAT 3′	57	*EcoRI/BamHI*
5	**F** 5′ ATGAATTCGGGGACTGCCCAAGGCTACTG 3′ **R** 5′ ATGGATCCACGTAACAGAGACCCTTTTTTGA 3′	60	*EcoRI/BamHI*
9	**F** 5′ ATGAATTCGGGGGCGCAAGTGAAATACCA 3′ **R** 5′ ATGGATCCGCCCCGCTTCCGACCCACG 3′	61	*EcoRI/BamHI*
11	**F** 5′ ATGAATTCGGAAAGGCAAAAATTCTGATGTTGTT 3′ **R** 5′ ATGGATCCGTACATCAATTATATATTTTTTAAACT 3′	55	*EcoRI/BamHI*
***HvBP1* Alexis**			
2	**F** 5′ ATGAATTCGGGGGCTGGCCGAAACAGT 3′ **R** 5′ ATGGATCCATATAGATACAACCAGCTCTAAAAG 3′	59	*EcoRI/BamHI*
8	**F** 5′ ATGAATTCGGGACAGCAACAAGTCGGACA 3′ **R** 5′ ATGGATCCGGATAAAACTTTATTTATATTTTATTCCAG 3′	58	*EcoRI/BamHI*
9	**F** 5′ ATCCCGGGCCAAGCTCTAATACGACTCCCTAT 3′ **R** 5′ CGATCGATATATAGCATATAGATACAACCAGCTCT 3′	60	*XmaI/ClaI*
***HvPrx7* Sloop**			
1	**F** 5′ ATGAATTCGGGCTCCGCAAGCGTGC 3′ **R** 5′ GCATCGATATAGCTAACATATAGTAGAACCAAC 3′	59	*EcoRI/ClaI*
4	**F** 5′ ATGAATTCGCCGCCCGCGCAAGCC 3′ **R** 5′ ATCTCGAGTCACTAACCAACACCGTTAATCC 3′	62	*EcoRI/XhoI*
13	**F** 5′ ACGAATTCAAACAATTTCAGATTAATGATATTCAATCC 3′ **R** 5′ ATGGATCCGGGCAGGAAACCATGATCATC 3′	58	*EcoRI/BamHI*
***HvPrx7* Alexis**			
1	**F** 5′ ATGAATTCGGGGAAGCAGCAGAAGAAGAAAAG 3′ **R** 5′ ATGGATCCGTAAGATAAGAATTTTCTTTTGGCCT 3′	60	*EcoRI/BamHI*
11	**F** 5′ ATGAATTCGGGGGAGAGCCGAAAGAGATCT 3′ **R** 5′ ATGGATCCGAATTTGACATCAACGTCATTCTGG 3′	62	*EcoRI/BamHI*

PCR reactions were undertaken for each of the primers in Table 5.5 and the products ligated into the pGADT7 library vector as per section 5.2.2.1.1. Co-transformations were performed with the library vector containing the target sequences and the bait used for Y1H screening (section 5.2.2.1.1). A transformation was performed with the empty library vector as a control as per section 5.2.2.1.1 One hundred and fifty µL was plated onto SD/-His-Leu plates and incubated at 30°C for 3 to 7 days. Positive colonies were analysed as per section 5.2.2.2.2.

5.2.2.4 Further characterisation of positive clones

Positive clones were further analysed by firstly isolating the full length sequence and then identifying gene expression during grain fill and the chromosomal location of the gene. Binding specificity was confirmed through a gel shift assay. One positive clone was identified (*HvPrx7* 11, section 5.2.2.2.4) and further investigated.

5.2.2.4.1 Isolation of the full length sequence of positive clones

The sequence of the positive clone containing an interacting partner for the *HvPrx7* promoter was identified by using the Basic Local Alignment Search Tool (tBLASTx and tBLASTn; http://blast.ncbi.nlm.nih.gov/Blast.cgi, NCBI-GenBank Flat File Release 172.0, August 2008). Potential candidates were identified and primers for isolation designed using the *in silico* (full length) sequence identified in rice as a basic-leucine zipper (bZIP) transcription factor domain containing protein (NP_001058100) (Table 5.6). tBLASTx and

tBLASTn were searched and results updated in July 2011. PCR was performed to isolate the full length sequence and confirmation of sequence performed as described earlier (Section 5.2.1.4).

Table 5.6 Primers used to obtain the full length *bZIP* for *HvPrx7*.
Tm (°C) represents melting temperature of primer reaction. Expected product size (bp) is represented.

Primer	Tm (°C)	Expected Size (bp)
F 5′ ATGGACGCCGACCTCGACCTG 3′	57	909
R 5′ GAAACTTGCGAATAAGCTGTCACTAGTCTA 3′		

5.2.2.4.2 Northern analysis and chromosomal location

Northern analysis was performed as per section 3.2.2. Using RNA from the varieties Sloop and Alexis during grain fill. Primers for the full length *HvbZIP* sequence (Table 5.6) were used to prepare the probe for northern analysis and for the PCR of barley:wheat addition lines to establish chromosomal location (as per section 4.2.2.3.1).

5.2.2.4.3 Confirmation of interaction between protein and promoter for *HvPrx7*

5.2.2.4.3.1 Protein expression vector preparation

The ORF of the positive interacting partner was amplified in PCR reactions as per section 5.2.2.4.3. The amplified PCR reaction mixture was used in a ligation reaction with the pCR8®/GW/TOPO® vector and subsequently transformed into competent cells. Colony PCR and confirmation by sequencing was performed as per section 5.2.2.2.2. ORFs

(Alexis) were transferred into the pDEST17 vector for protein expression. Gateway® recombination technology was used to transfer the coding regions of the genes from the pCR®8/GW/TOPO® vector to the pDEST17® protein expression vector; with 1µL of ORF region:pCR®8/GW/TOPO® used in the recombination reaction as per the manufacturer's protocol (Gateway® LR ClonaseTM II Enzyme Mix product, Invitrogen). Transformation of OneShot® TOP10 competent *E. coli* cells with 2 µL of the recombination reaction was conducted as per the manufacturer's protocol (Gateway® LR ClonaseTM II Enzyme Mix product, Invitrogen). Transformed cells were subsequently plated onto LB agar containing ampicillin (100 µg/mL as a selective agent). Confirmation of the recombinant vectors was conducted via PCR and sequence analysis. Colony PCR reactions were performed as per section 3.2.2.2 using the forward and reverse primer combinations identified in Table 5.6 (10mM, 1 µL). PCR products were visualised as per section 3.2.2.2. Confirmation of the ORFs being in-frame in the pDEST17® vector was conducted via sequence analysis (Chapter 3, section 3.2.2.2).

5.2.2.4.3.2 Heterologous protein expression

The *HvbZip*ORF:pDEST17® protein expression vectors were transformed into BL21-AI protein expression optimised cells. Four overnight starter cultures of the BL21-AI protein expression cells were commenced by inoculating 200 µL of cells into 8 mL of LB/Carbenicillin (50 µg/ mL) at 37°C with agitation. The following day, four 200 mL LB/Carbenicillin (50 µg/mL) cultures were each inoculated with a 8 mL starter culture, and were subsequently incubated at 37°C with agitation until an OD_{600} measurement of 0.4 was reached. Upon recording an OD_{600} measurement of 0.4, L-(+)-arabinose was added to 0.4%

w/v to two of the cultures for induced samples; with D-glucose added to 0.5% w/v to the other two cultures for repressed samples. Cultures were then incubated at either 23°C or 37°C for 5 hours with agitation, followed by cell collection at 3000 x *g* for 15 min at 4°C for cell pelleting. The resulting supernatant was discarded and the cell pellets were snap-frozen using liquid nitrogen and stored at -80°C until protein extraction.

5.2.2.4.3.3 Protein extraction and DNA binding assay

For protein isolation, 1 L of both induced and non-induced cell cultures were removed from storage at -80°C and resuspended in 30 mL of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 5mM imidazole, 1% Triton-X, pH 8), and mixed vigorously. To the resuspended cells, lysozyme (1 mg/mL), RNase A (10 µg/mL) and DNase I (166 µg/µL) was added, gently shaken to mix, and incubated on ice for 30 min. The suspension mixture was then snap-frozen in liquid nitrogen, thawed and vortexed for 30 sec; with this process repeated three times in total. Cells were then sonicated six times for 10 sec each time, with resting on ice for 30 sec between each sonication. The homogenised mixture was then centrifuged at 10000 x *g* for 20 min at 4°C with a 100 µL aliquot taken for gel analysis (cell lysate).

The resulting supernatant was transferred to a new 50 mL tube for selective ammonium sulphate precipitation, where 0.24 mg/mL ammonium sulphate was added and shaken vigorously to mix. The mixture was centrifuged at 10000 x *g* for 20 min at 4°C, with supernatant being transferred to a new 50 mL tube. Ammonium sulphate (0.13 mg/mL) was added to the suspension, shaken vigorously to mix and centrifuged at 10000 x *g* for 20

min at 4°C with supernatant being discarded. To the cell lysate pellet, 10 mL of binding buffer (20 mM NaH_2PO_4, 500 mM NaCl, 20 mM imidazole, pH 7.4) was added and the pellet resuspended. An aliquot of 100 µL was loaded for gel analysis.

At 4°C the remaining sample was loaded very slowly into a previously equilibrated HisTrapTMHP 5 mL column (GE Healthcare Life Sciences, UK), with a 100 µL aliquot of flow through (flow through) collected for gel analysis. The column was washed with 100 mL of binding buffer, with 100 µL aliquots taken at 2 mL (wash 2), 50 mL (wash 50) and 100 mL (wash 100) for gel analysis. The protein was then eluted from the column with 3 X 5 mL elution buffer (20 mM NaH_2PO_4, 500 mM NaCl, 250 mM imidazole, pH 7.4), with 100 µL aliquots of the resulting elution fractions taken for gel analysis (Elution 1, 2 and 3). Protein purification samples [9 µL of collected aliquots with 3 µL of NuPAGE® LDS Sample Buffer (4x) (Invitrogen)] were heated at 70°C for 10 min before being loaded into 15-well NuPAGE® Novex® 4-12% Bis-Tris mini gels. BIO-RAD Precision Plus Dual Colour Protein Ladder (10 µL) was also loaded onto the gels, which were electrophoresed according to the manufacturer's instructions. After electrophoresis, the protein gels were removed from the plastic casing and placed into 50 mL of fixing solution (15 mL ethanol, 5 mL acetic acid, 30 mL sterile deionised water) for 1 h. After incubation, the fixing solution was then replaced with staining solution [50% methanol, 7% acetic acid, 0.125 w/v Brilliant Blue G (Sigma)] and left at room temperature overnight with gentle agitation. The protein gels were then destained using coomassie destain (50% methanol, 10% acetic acid) at room temperature. The protein gel was then scanned using an Epson Perfection 4180 Photo Scanner.

Protein-DNA interactions were assessed using a DNA binding assay. DNA from the PCR products used for Y1H screening (promoter regions) was used in the binding assay. The concentration of the annealed DNA fragment was determined on a 1% Agarose gel. Thirty μM of DNA was mixed with a concentration series (0.2-10 μM) of extracted protein in 30 μL of 20 mM Tris-HCL buffer (pH 7.4), 100 mM NaCl, 100% glycerol and 1 mM $MgCl_2$. The reaction mixture was incubated at 37°C for 10 min. Products were resolved at 4°C in 1 % agarose using gel electrophoresis in 1 x TAE buffer, run at 9 V for 1.5 h and visualised by ethidium bromide staining.

5.3 Results

5.3.1 Genome Walking

Full length promoter sequences for *HvBP1* were aligned to identify SNPs between Sloop and Alexis (Figure 5.5). Four SNPs were identified within the 2416 bp promoter at 308, 618, 1508 and 1712 bp upstream of the ATG start site. For *HvPrx7* (Figure 5.6), four SNPs were identified within the 2720 bp isolated at 244, 639, 972 and 1092 upstream of the ATG start site.

```
S
                         *        20         *        40         *        60         *        80
BP1 Sloop  : GAGGAGAGAGGAAACACACACTGGCCCGACGATGCCGAAAGACAGGTGCGACGACTAGTCCTGGAGTTAAACATCCTTTC : 2335
BP1 Alexis : GAGGAGAGAGGAAACACACACTGGCCCGACGATGCCGAAAGACAGGTGCGACGACTAGTCCTGGAGTTAAACATCCTTTC : 2335
             GAGGAGAGAGGAAACACACACTGGCCCGACGATGCCGAAAGACAGGTGCGACGACTAGTCCTGGAGTTAAACATCCTTTC

                         *       100         *       120         *       140         *       160
BP1 Sloop  : TAAGTACTCACCGGCACATCGTGTCGTGAACTAATGAGCAGCGAAGTTGAATGTTTTCCTAGCTGTTCACAGACTACAGT : 2255
BP1 Alexis : TAAGTACTCACCGGCACATCGTGTCGTGAACTAATGAGCAGCGAAGTTGAATGTTTTCCTAGCTGTTCACAGACTACAGT : 2255
             TAAGTACTCACCGGCACATCGTGTCGTGAACTAATGAGCAGCGAAGTTGAATGTTTTCCTAGCTGTTCACAGACTACAGT

                         *       180         *       200         *       220         *       240
BP1 Sloop  : TCATCAAGGTTGTTTGATTTCACTCCGATCCATATCGATCCGCGGTCTCAGCTCCGAATTGGCGTACAGATTCCTAACCC : 2175
BP1 Alexis : TCATCAAGGTTGTTTGATTTCACTCCGATCCATATCGATCCGCGGTCTCAGCTCCGAATTGGCGTACAGATTCCTAACCC : 2175
             TCATCAAGGTTGTTTGATTTCACTCCGATCCATATCGATCCGCGGTCTCAGCTCCGAATTGGCGTACAGATTCCTAACCC

                         *       260         *       280         *       300         *       320
BP1 Sloop  : TATCCACTGTACCGTAACGGTTCCGCGGTTAGAACGGTACAGAAATCCAACGAATGATATGTGTGCTTCCAGTAGTGCGG : 2095
BP1 Alexis : TATCCACTGTACCGTAACGGTTCCGCGGTTAGAACGGTACAGAAATCCAACGAATGATATGTGTGCTCCCAGTAGTGCGG : 2095
             TATCCACTGTACCGTAACGGTTCCGCGGTTAGAACGGTACAGAAATCCAACGAATGATATGTGTGCT CCAGTAGTGCGG

                         *       340         *       360         *       380         *       400
BP1 Sloop  : TATCTCATTGTGGTTGTCTCCGCGACAGTATGTACTAAAACTCACTTTTGGTACGCGATGCGTGCCGTGGAGGTAGATAG : 2015
BP1 Alexis : TATCTCATTGTGGTTGTCTCCGCGACAGTATGTACTAAAACTCACTTTTGGTACGCGATGCGTGCCGTGGAGGTAGATAG : 2015
             TATCTCATTGTGGTTGTCTCCGCGACAGTATGTACTAAAACTCACTTTTGGTACGCGATGCGTGCCGTGGAGGTAGATAG

                         *       420         *       440         *       460         *       480
BP1 Sloop  : CTGCTTTAACGAATCCGATAGGTGTTACTTTCATTGTGTCTACAGTAGATTCTTTTTTGTTTTTATTACATCGTCGATTA : 1935
BP1 Alexis : CTGCTTTAACGAATCCGATAGGTGTTACTTTCATTGTGTCTACAGTAGATTCTTTTTTGTTTTTATTACATCGTCGATTA : 1935
             CTGCTTTAACGAATCCGATAGGTGTTACTTTCATTGTGTCTACAGTAGATTCTTTTTTGTTTTTATTACATCGTCGATTA

                         *       500         *       520         *       540         *       560
BP1 Sloop  : ATTACTCCTTACAATGTTTAACTTATTGTATTGATCAATGAGTATGAGGGAGGCAGTACTAAATCTTCTGCATGTACATT : 1855
BP1 Alexis : ATTACTCCTTACAATGTTTAACTTATTGTATTGATCAATGAGTATGAGGGAGGCAGTACTAAATCTTCTGCATGTACATT : 1855
             ATTACTCCTTACAATGTTTAACTTATTGTATTGATCAATGAGTATGAGGGAGGCAGTACTAAATCTTCTGCATGTACATT

                         *       580         *       600         *       620         *       640
BP1 Sloop  : TAAAAGATCTTGGATCGACCAATAACTAACCGACACTTTACTCAACTTTTTATCGTGAGTGTGATGAGTACGTATATATT : 1775
BP1 Alexis : TAAAAGATCTTGGATCGACCAATAACTAACCGACACTTTACTCAACTTTTTATCGTGGGTGTGATGAGTACGTATATATT : 1775
             TAAAAGATCTTGGATCGACCAATAACTAACCGACACTTTACTCAACTTTTTATCGTG GTGTGATGAGTACGTATATATT

                         *       660         *       680         *       700         *       720
BP1 Sloop  : CATCGTGTTGCTTTATGATTAATCGACAATCCTCATTTACGTTACCTAGAATTTGGAACAGATAACACCTTTACGTGCAT : 1695
BP1 Alexis : CATCGTGTTGCTTTATGATTAATCGACAATCCTCATTTACGTTACCTAGAATTTGGAACAGATAACACCTTTACGTGCAT : 1695
             CATCGTGTTGCTTTATGATTAATCGACAATCCTCATTTACGTTACCTAGAATTTGGAACAGATAACACCTTTACGTGCAT

                         *       740         *       760         *       780         *       800
BP1 Sloop  : TTAAATTAGTACGGAAGATTTGGCACTGTTTCCATTATGATATTCATTCTAGTGTCTATTTTCTGTTCTTCTCAGATATA : 1615
BP1 Alexis : TTAAATTAGTACGGAAGATTTGGCACTGTTTCCATTATGATATTCATTCTAGTGTCTATTTTCTGTTCTTCTCAGATATA : 1615
             TTAAATTAGTACGGAAGATTTGGCACTGTTTCCATTATGATATTCATTCTAGTGTCTATTTTCTGTTCTTCTCAGATATA

                         *       820         *       840         *       860         *       880
BP1 Sloop  : CGATTATTTACTTTTGAAGATGAGGTAGAATAATAAATGAGTTTATTATTGAATCTAATCGTCTATTGTTGAATCTGTCT : 1535
BP1 Alexis : CGATTATTTACTTTTGAAGATGAGGTAGAATAATAAATGAGTTTATTATTGAATCTAATCGTCTATTGTTGAATCTGTCT : 1535
             CGATTATTTACTTTTGAAGATGAGGTAGAATAATAAATGAGTTTATTATTGAATCTAATCGTCTATTGTTGAATCTGTCT

                         *       900         *       920         *       940         *       960
BP1 Sloop  : ACAATGATAAATGATACGTATATTATTGAATTTGATCATCGTATACGTATCCCTCTTAAACACTGAGACCCCACGACAGA : 1455
BP1 Alexis : ACAATGATAAATGATACGTATATTATTGAATTTGATCATCGTATACGTATCCCTCTTAAACACTGAGACCCCACGACAGA : 1455
             ACAATGATAAATGATACGTATATTATTGAATTTGATCATCGTATACGTATCCCTCTTAAACACTGAGACCCCACGACAGA

                         *       980         *      1000         *      1020         *      1040
BP1 Sloop  : GACTATCCCTATACATAGATCCTATCGCGGTATCAGGACTGGATGTACAGGATGGATACCCGTGGAGTAATTTCTGAACT : 1375
BP1 Alexis : GACTATCCCTATACATAGATCCTATCGCGGTATCAGGACTGGATGTACAGGATGGATACCCGTGGAGTAATTTCTGAACT : 1375
             GACTATCCCTATACATAGATCCTATCGCGGTATCAGGACTGGATGTACAGGATGGATACCCGTGGAGTAATTTCTGAACT

                         *      1060         *      1080         *      1100         *      1120
BP1 Sloop  : TCTGATGTTCCTCCCGAGGCTGATATATCAATATCGCCAACTCAGTGAGCCATTCGTTAGTGAGCTTCTTAAACGGGGGA : 1295
BP1 Alexis : TCTGATGTTCCTCCCGAGGCTGATATATCAATATCGCCAACTCAGTGAGCCATTCGTTAGTGAGCTTCTTAAACGGGGGA : 1295
             TCTGATGTTCCTCCCGAGGCTGATATATCAATATCGCCAACTCAGTGAGCCATTCGTTAGTGAGCTTCTTAAACGGGGGA

                         *      1140         *      1160         *      1180         *      1200
BP1 Sloop  : AGTGAGCTGTTAGTTGAATGTGAGCCTTCTTGGTCTTTGGTCAGCTGCGTCTTCTGGTCTTTGGTGAGGTCCCTCCGGCT : 1215
BP1 Alexis : AGTGAGCTGTTAGTTGAATGTGAGCCTTCTTGGTCTTTGGTCAGCTGCGTCTTCTGGTCTTTGGTGAGGTCCCTCCGGCT : 1215
             AGTGAGCTGTTAGTTGAATGTGAGCCTTCTTGGTCTTTGGTCAGCTGCGTCTTCTGGTCTTTGGTGAGGTCCCTCCGGCT
```

Figure 5.5 cont.

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                        *        20         *        40         *        60         *        80
BP1 Sloop  : GAGGAGAGAGGAAACACACACTGGCCCGACGATGCCGAAAGACAGGTGCGACGACTAGTCCTGGAGTTAAACATCCTTTC : 2335
BP1 Alexis : GAGGAGAGAGGAAACACACACTGGCCCGACGATGCCGAAAGACAGGTGCGACGACTAGTCCTGGAGTTAAACATCCTTTC : 2335
             GAGGAGAGAGGAAACACACACTGGCCCGACGATGCCGAAAGACAGGTGCGACGACTAGTCCTGGAGTTAAACATCCTTTC

                        *       100         *       120         *       140         *       160
BP1 Sloop  : TAAGTACTCACCGGCACATCGTGTCGTGAACTAATGAGCAGCGAAGTTGAATGTTTTCCTAGCTGTTCACAGACTACAGT : 2255
BP1 Alexis : TAAGTACTCACCGGCACATCGTGTCGTGAACTAATGAGCAGCGAAGTTGAATGTTTTCCTAGCTGTTCACAGACTACAGT : 2255
             TAAGTACTCACCGGCACATCGTGTCGTGAACTAATGAGCAGCGAAGTTGAATGTTTTCCTAGCTGTTCACAGACTACAGT

                        *       180         *       200         *       220         *       240
BP1 Sloop  : TCATCAAGGTTGTTTGATTTCACTCCGATCCATATCGATCCGCGGTCTCAGCTCCGAATTGGCGTACAGATTCCTAACCC : 2175
BP1 Alexis : TCATCAAGGTTGTTTGATTTCACTCCGATCCATATCGATCCGCGGTCTCAGCTCCGAATTGGCGTACAGATTCCTAACCC : 2175
             TCATCAAGGTTGTTTGATTTCACTCCGATCCATATCGATCCGCGGTCTCAGCTCCGAATTGGCGTACAGATTCCTAACCC

                        *       260         *       280         *       300         *       320
BP1 Sloop  : TATCCACTGTACCGTAACGGTTCCGCGGTTAGAACGGTACAGAAATCCAACGAATGATATGTGTGCTTCCAGTAGTGCGG : 2095
BP1 Alexis : TATCCACTGTACCGTAACGGTTCCGCGGTTAGAACGGTACAGAAATCCAACGAATGATATGTGTGCTCCCAGTAGTGCGG : 2095
             TATCCACTGTACCGTAACGGTTCCGCGGTTAGAACGGTACAGAAATCCAACGAATGATATGTGTGCT CCAGTAGTGCGG

                        *       340         *       360         *       380         *       400
BP1 Sloop  : TATCTCATTGTGGTTGTCTCCGCGACAGTATGTACTAAAACTCACTTTTGGTACGCGATGCGTGCCGTGGAGGTAGATAG : 2015
BP1 Alexis : TATCTCATTGTGGTTGTCTCCGCGACAGTATGTACTAAAACTCACTTTTGGTACGCGATGCGTGCCGTGGAGGTAGATAG : 2015
             TATCTCATTGTGGTTGTCTCCGCGACAGTATGTACTAAAACTCACTTTTGGTACGCGATGCGTGCCGTGGAGGTAGATAG

                        *       420         *       440         *       460         *       480
BP1 Sloop  : CTGCTTTAACGAATCCGATAGGTGTTACTTTCATTGTGTCTACAGTAGATTCTTTTTTGTTTTTATTACATCGTCGATTA : 1935
BP1 Alexis : CTGCTTTAACGAATCCGATAGGTGTTACTTTCATTGTGTCTACAGTAGATTCTTTTTTGTTTTTATTACATCGTCGATTA : 1935
             CTGCTTTAACGAATCCGATAGGTGTTACTTTCATTGTGTCTACAGTAGATTCTTTTTTGTTTTTATTACATCGTCGATTA

                        *       500         *       520         *       540         *       560
BP1 Sloop  : ATTACTCCTTACAATGTTTAACTTATTGTATTGATCAATGAGTATGAGGGAGGCAGTACTAAATCTTCTGCATGTACATT : 1855
BP1 Alexis : ATTACTCCTTACAATGTTTAACTTATTGTATTGATCAATGAGTATGAGGGAGGCAGTACTAAATCTTCTGCATGTACATT : 1855
             ATTACTCCTTACAATGTTTAACTTATTGTATTGATCAATGAGTATGAGGGAGGCAGTACTAAATCTTCTGCATGTACATT

                        *       580         *       600         *       620         *       640
BP1 Sloop  : TAAAAGATCTTGGATCGACCAATAACTAACCGACACTTTACTCAACTTTTTATCGTGAGTGTGATGAGTACGTATATATT : 1775
BP1 Alexis : TAAAAGATCTTGGATCGACCAATAACTAACCGACACTTTACTCAACTTTTTATCGTGGGTGTGATGAGTACGTATATATT : 1775
             TAAAAGATCTTGGATCGACCAATAACTAACCGACACTTTACTCAACTTTTTATCGTG GTGTGATGAGTACGTATATATT

                        *       660         *       680         *       700         *       720
BP1 Sloop  : CATCGTGTTGCTTTATGATTAATCGACAATCCTCATTTACGTTACCTAGAATTTGGAACAGATAACACCTTTACGTGCAT : 1695
BP1 Alexis : CATCGTGTTGCTTTATGATTAATCGACAATCCTCATTTACGTTACCTAGAATTTGGAACAGATAACACCTTTACGTGCAT : 1695
             CATCGTGTTGCTTTATGATTAATCGACAATCCTCATTTACGTTACCTAGAATTTGGAACAGATAACACCTTTACGTGCAT

                        *       740         *       760         *       780         *       800
BP1 Sloop  : TTAAATTAGTACGGAAGATTTGGCACTGTTTCCATTATGATATTCATTCTAGTGTCTATTTTCTGTTCTTCTCAGATATA : 1615
BP1 Alexis : TTAAATTAGTACGGAAGATTTGGCACTGTTTCCATTATGATATTCATTCTAGTGTCTATTTTCTGTTCTTCTCAGATATA : 1615
             TTAAATTAGTACGGAAGATTTGGCACTGTTTCCATTATGATATTCATTCTAGTGTCTATTTTCTGTTCTTCTCAGATATA

                        *       820         *       840         *       860         *       880
BP1 Sloop  : CGATTATTTACTTTTGAAGATGAGGTAGAATAATAAATGAGTTTATTATTGAATCTAATCGTCTATTGTTGAATCTGTCT : 1535
BP1 Alexis : CGATTATTTACTTTTGAAGATGAGGTAGAATAATAAATGAGTTTATTATTGAATCTAATCGTCTATTGTTGAATCTGTCT : 1535
             CGATTATTTACTTTTGAAGATGAGGTAGAATAATAAATGAGTTTATTATTGAATCTAATCGTCTATTGTTGAATCTGTCT

                        *       900         *       920         *       940         *       960
BP1 Sloop  : ACAATGATAAATGATACGTATATTATTGAATTTGATCATCGTATACGTATCCCTCTTAAACACTGAGACCCCACGACAGA : 1455
BP1 Alexis : ACAATGATAAATGATACGTATATTATTGAATTTGATCATCGTATACGTATCCCTCTTAAACACTGAGACCCCACGACAGA : 1455
             ACAATGATAAATGATACGTATATTATTGAATTTGATCATCGTATACGTATCCCTCTTAAACACTGAGACCCCACGACAGA

                        *       980         *      1000         *      1020         *      1040
BP1 Sloop  : GACTATCCCTATACATAGATCCTATCGCGGTATCAGGACTGGATGTACAGGATGGATACCCGTGGAGTAATTTCTGAACT : 1375
BP1 Alexis : GACTATCCCTATACATAGATCCTATCGCGGTATCAGGACTGGATGTACAGGATGGATACCCGTGGAGTAATTTCTGAACT : 1375
             GACTATCCCTATACATAGATCCTATCGCGGTATCAGGACTGGATGTACAGGATGGATACCCGTGGAGTAATTTCTGAACT

                        *      1060         *      1080         *      1100         *      1120
BP1 Sloop  : TCTGATGTTCCTCCCGAGGCTGATATATCAATATCGCCAACTCAGTGAGCCATTCGTTAGTGAGCTTCTTAAACGGGGGA : 1295
BP1 Alexis : TCTGATGTTCCTCCCGAGGCTGATATATCAATATCGCCAACTCAGTGAGCCATTCGTTAGTGAGCTTCTTAAACGGGGGA : 1295
             TCTGATGTTCCTCCCGAGGCTGATATATCAATATCGCCAACTCAGTGAGCCATTCGTTAGTGAGCTTCTTAAACGGGGGA

                        *      1140         *      1160         *      1180         *      1200
BP1 Sloop  : AGTGAGCTGTTAGTTGAATGTGAGCCTTCTTGGTCTTTGGTCAGCTGCGTCTTCTGGTCTTTGGTGAGGTCCCTCCGGCT : 1215
BP1 Alexis : AGTGAGCTGTTAGTTGAATGTGAGCCTTCTTGGTCTTTGGTCAGCTGCGTCTTCTGGTCTTTGGTGAGGTCCCTCCGGCT : 1215
             AGTGAGCTGTTAGTTGAATGTGAGCCTTCTTGGTCTTTGGTCAGCTGCGTCTTCTGGTCTTTGGTGAGGTCCCTCCGGCT
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Figure 5.5 cont.

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                          *        1220         *        1240         *        1260         *        1280
BP1 Sloop  : GTCTGCTCGCAAGCCGACTAGCAGTATTCATTGTAATTAATACATGCAATAGTCATTACATGCGGAAATTTGAGGTGATT : 1135
BP1 Alexis : GTCTGCTCGCAAGCCGACTAGCAGTATTCATTGTAATTAATACATGCAATAGTCATTACATGCGGAAATTTGAGGTGATT : 1135
             GTCTGCTCGCAAGCCGACTAGCAGTATTCATTGTAATTAATACATGCAATAGTCATTACATGCGGAAATTTGAGGTGATT

                          *        1300         *        1320         *        1340         *        1360
BP1 Sloop  : AGCTTGGGAACCAACGCACCATAACTACTCCCCTTCGCGTGAGATATATTCAGTGGGGAGGGGAGACCGTGTTCCCAAAC : 1055
BP1 Alexis : AGCTTGGGAACCAACGCACCATAACTACTCCCCTTCGCGTGAGATATATTCAGTGGGGAGGGGAGACCGTGTTCCCAAAC : 1055
             AGCTTGGGAACCAACGCACCATAACTACTCCCCTTCGCGTGAGATATATTCAGTGGGGAGGGGAGACCGTGTTCCCAAAC

                          *        1380         *        1400         *        1420         *        1440
BP1 Sloop  : GTGGGGGACATTGTGTGTATAAGGTGAGTGTGTTTCGAGATCTCGTGACTCTGCATCCCAACAATGGAAGTGTTCTCCCC :  975
BP1 Alexis : GTGGGGGACATTGTGTGTATAAGGTGAGTGTGTTTCGAGATCTCGTGACTCTGCATCCCAACAATGGAAGTGTTCTCCCC :  975
             GTGGGGGACATTGTGTGTATAAGGTGAGTGTGTTTCGAGATCTCGTGACTCTGCATCCCAACAATGGAAGTGTTCTCCCC

                          *        1460         *        1480         *        1500         *        1520
BP1 Sloop  : GGACTTGAGTATGTAGCCCACATGTTCGAACAGCATCGATCCTGAGACGGGAGAGGAAAGCATGGGGTGTGGAGATGACA :  895
BP1 Alexis : GGACTTGAGTATGTAGCCCACATGTTCGAACAGCATCGATCCTGAGACGGGAGAGGAAAGCATGGGGCGTGGAGATGACA :  895
             GGACTTGAGTATGTAGCCCACATGTTCGAACAGCATCGATCCTGAGACGGGAGAGGAAAGCATGGGG GTGGAGATGACA

                          *        1540         *        1560         *        1580         *        1600
BP1 Sloop  : GTCCTAGTAAGGGTTCTATCAACCGCGGGTGGCACCCCGTCCGCAGTTTCGCTGATTGCCGTTCAAACGTTAAAAGTAGA :  815
BP1 Alexis : GTCCTAGTAAGGGTTCTATCAACCGCGGGTGGCACCCCGTCCGCAGTTTCGCTGATTGCCGTTCAAACGTTAAAAGTAGA :  815
             GTCCTAGTAAGGGTTCTATCAACCGCGGGTGGCACCCCGTCCGCAGTTTCGCTGATTGCCGTTCAAACGTTAAAAGTAGA

                          *        1620         *        1640         *        1660         *        1680
BP1 Sloop  : AGTAGTACAGAAGGTCACCGCTAAACAAGAAGCCGGTGCTCTAGCCAGAGCCACACGAGCAGAAGCAGTGGCTGCTAAGC :  735
BP1 Alexis : AGTAGTACAGAAGGTCACCGCTAAACAAGAAGCCGGTGCTCTAGCCAGAGCCACACGAGCAGAAGCAGTGGCTGCTAAGC :  735
             AGTAGTACAGAAGGTCACCGCTAAACAAGAAGCCGGTGCTCTAGCCAGAGCCACACGAGCAGAAGCAGTGGCTGCTAAGC

                          *        1700         *        1720         *        1740         *        1760
BP1 Sloop  : CGTACCGAAGTCCTCCGCGGAGATCTGTAACTCCGTGAGGGCTAGGCGCTCCGTTGCGTAATGGCACGTTCGGGGACGCC :  655
BP1 Alexis : CGTACCGAAGTCCTCCGCGGAGATCTGTAACCCCGTGAGGGCTAGGCGCTCCGTTGCGTAATGGCACGTTCGGGGACGCC :  655
             CGTACCGAAGTCCTCCGCGGAGATCTGTAAC CCGTGAGGGCTAGGCGCTCCGTTGCGTAATGGCACGTTCGGGGACGCC

                          *        1780         *        1800         *        1820         *        1840
BP1 Sloop  : ACATGAAGAAGCTGTTGGCAGCTGGGGCTACAGCTGACGAGAGGGACTACAAGCAGTGGTGTTCGCTAGGCCAGCGAGTG :  575
BP1 Alexis : ACATGAAGAAGCTGTTGGCAGCTGGGGCTACAGCTGACGAGAGGGACTACAAGCAGTGGTGTTCGCTAGGCCAGCGAGTG :  575
             ACATGAAGAAGCTGTTGGCAGCTGGGGCTACAGCTGACGAGAGGGACTACAAGCAGTGGTGTTCGCTAGGCCAGCGAGTG

                          *        1860         *        1880         *        1900         *        1920
BP1 Sloop  : CCGAAGTCACGACCCACTCAGTGCGTCGCCCGACAGTTCGCAGAAGGGACGTGCAACACCGGTAGCTCGCGTGACTTAGT :  495
BP1 Alexis : CCGAAGTCACGACCCACTCAGTGCGTCGCCCGACAGTTCGCAGAAGGGACGTGCAACACCGGTAGCTCGCGTGACTTAGT :  495
             CCGAAGTCACGACCCACTCAGTGCGTCGCCCGACAGTTCGCAGAAGGGACGTGCAACACCGGTAGCTCGCGTGACTTAGT

                          *        1940         *        1960         *        1980         *        2000
BP1 Sloop  : GGAATGCTGTGCAGGTTGAGCCGTAGGCTAGCCAGACTACCAGGAAGGCTCATGTTTACGACGTTGGTACACTACTTCCA :  415
BP1 Alexis : GGAATGCTGTGCAGGTTGAGCCGTAGGCTAGCCAGACTACCAGGAAGGCTCATGTTTACGACGTTGGTACACTACTTCCA :  415
             GGAATGCTGTGCAGGTTGAGCCGTAGGCTAGCCAGACTACCAGGAAGGCTCATGTTTACGACGTTGGTACACTACTTCCA

                          *        2020         *        2040         *        2060         *        2080
BP1 Sloop  : GAAGTACCGTTCGAGTGTCCTTTCGGGCGGACCTAAGCCGGCACCGCTCGTGTCCGACCTCCTGGTAGCTCACTAGTGCA :  335
BP1 Alexis : GAAGTACCGTTCGAGTGTCCTTTCGGGCGGACCTAAGCCGGCACCGCTCGTGTCCGACCTCCTGGTAGCTCACTAGTGCA :  335
             GAAGTACCGTTCGAGTGTCCTTTCGGGCGGACCTAAGCCGGCACCGCTCGTGTCCGACCTCCTGGTAGCTCACTAGTGCA

                          *        2100         *        2120         *        2140         *        2160
BP1 Sloop  : GGCGGTCGCGGGAGGTTGAAGGCCAGTCGCAGTAGCTGTTGAAAAGGGCTTACTTAGAGTCTTGTTACAACTCCATAAAG :  255
BP1 Alexis : GGCGGTCGCGGGAGGTTGAAGGCCAGTCGCAGTAGCTGTTGAAAAGGGCTTACTTAGAGTCTTGTTACAACTCCATAAAG :  255
             GGCGGTCGCGGGAGGTTGAAGGCCAGTCGCAGTAGCTGTTGAAAAGGGCTTACTTAGAGTCTTGTTACAACTCCATAAAG

                          *        2180         *        2200         *        2220         *        2240
BP1 Sloop  : CGTGTGGACAAGACTTACAAGGTACAGCCTGGAACGTCTAGTAGCGGGCTGATCGGTTGGTGGATCCGATGGGGTGCGTG :  175
BP1 Alexis : CGTGTGGACAAGACTTACAAGGTACAGCCTGGAACGTCTAGTAGCGGGCTGATCGGTTGGTGGATCCGATGGGGTGCGTG :  175
             CGTGTGGACAAGACTTACAAGGTACAGCCTGGAACGTCTAGTAGCGGGCTGATCGGTTGGTGGATCCGATGGGGTGCGTG

                          *        2260         *        2280         *        2300         *        2320
BP1 Sloop  : TGCAATGCGGAAGCATGCCTGAGTCGGGGCACGTGCGTCGATGCGTGTGGACTTTAGTCACACGTGTGTGCGTGATCTCA :   95
BP1 Alexis : TGCAATGCGGAAGCATGCCTGAGTCGGGGCACGTGCGTCGATGCGTGTGGACTTTAGTCACACGTGTGTGCGTGATCTCA :   95
             TGCAATGCGGAAGCATGCCTGAGTCGGGGCACGTGCGTCGATGCGTGTGGACTTTAGTCACACGTGTGTGCGTGATCTCA

                          *        2340         *        2360         *        2380         *        2400
BP1 Sloop  : GGGCAGTGCCGAGCCTGGGTTGTGCGGGTCGCGCGCGTATGCGAATGCACTGGCCCGGAGACGGCACCGAATTAGGGTTG :   15
BP1 Alexis : GGGCAGTGCCGAGCCTGGGTTGTGCGGGTCGCGCGCGTATGCGAATGCACTGGCCCGGAGACGGCACCGAATTAGGGTTG :   15
             GGGCAGTGCCGAGCCTGGGTTGTGCGGGTCGCGCGCGTATGCGAATGCACTGGCCCGGAGACGGCACCGAATTAGGGTTG

                          *
BP1 Sloop  : TACGACCCGAGCGC :    1
BP1 Alexis : TACGACCCGAGCGC :    1
             TACGACCCGAGCGC
```

Figure 5.5 Alignment of promoter sequences for *HvBP1*. Promoter of *HvBP1* was sequenced from genomic DNA of Sloop and Alexis (n=3). Four SNPs were identified at 308, 618, 1508 and 1712 bp (highlighted yellow) upstream of the start site. S represents start site.

S

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                         *        20         *        40         *        60         *        80
Prx7 Sloop : ACGACTTCGACTCGAAGAAGAACGTGGACCTGGATCGATCCATCGATGAATCCTGACTCCCTCCCAGCACACCGAACGTG : 1490
Prx7 Alexi : ACGACTTCGACTCGAAGAAGAACGTGGACCTGGATCGATCCATCGATGAATCCTGACTCCCTCCCAGCACACCGAACGTG : 1490
             ACGACTTCGACTCGAAGAAGAACGTGGACCTGGATCGATCCATCGATGAATCCTGACTCCCTCCCAGCACACCGAACGTG

                         *       100         *       120         *       140         *       160
Prx7 Sloop : TTCTATGGGTGATGTGGAACAATAAATATGTAGCTCGACCCAACGAGCTAGACTAGGGTAGTACACTTCAATTTCAGCAT : 1410
Prx7 Alexi : TTCTATGGGTGATGTGGAACAATAAATATGTAGCTCGACCCAACGAGCTAGACTAGGGTAGTACACTTCAATTTCAGCAT : 1410
             TTCTATGGGTGATGTGGAACAATAAATATGTAGCTCGACCCAACGAGCTAGACTAGGGTAGTACACTTCAATTTCAGCAT

                         *       180         *       200         *       220         *       240
Prx7 Sloop : CTCCAATTGATCTCTTCAACTATCTGCTGCTAATCGATGGAGGTAGAGGTACGACCCGTGGGTGGGTGCGTCGATCGTGC : 1330
Prx7 Alexi : CTCCAATTGATCTCTTCAACTATCTGCTGCTAATCGATGGAGGTAGAGGTACGACCCGTGGGTGGGTGCGTCGATCGTGC : 1330
             CTCCAATTGATCTCTTCAACTATCTGCTGCTAATCGATGGAGGTAGAGGTACGACCCGTGGGTGGGTGCGTCGATCGTGC

                         *       260         *       280         *       300         *       320
Prx7 Sloop : TACACTAGGTGTTCTCTCTTCTGATCAGCGTCTCTCTCTCTCTCTCTGATTAATGTTTAATTTCTCCTTGTACGTTGT : 1250
Prx7 Alexi : TACGCTAGGTGTTCTCTCTTCTGATCAGCGTCTCTCTCTCTCTCTCTGATTAATGTTTAATTTCTCCTTGTACGTTGT : 1250
             TAC CTAGGTGTTCTCTCTTCTGATCAGCGTCTCTCTCTCTCTCTCTGATTAATGTTTAATTTCTCCTTGTACGTTGT

                         *       340         *       360         *       380         *       400
Prx7 Sloop : ACCCGTTCAACGTACCTGTTTCGACCATTCCTTTTAGTTGACCGGCCGATCCCATGATCCCATGGGCGTTTAAGCACAGA : 1170
Prx7 Alexi : ACCCGTTCAACGTACCTGTTTCGACCATTCCTTTTAGTTGACCGGCCGATCCCATGATCCCATGGGCGTTTAAGCACAGA : 1170
             ACCCGTTCAACGTACCTGTTTCGACCATTCCTTTTAGTTGACCGGCCGATCCCATGATCCCATGGGCGTTTAAGCACAGA

                         *       420         *       440         *       460         *       480
Prx7 Sloop : GAATAAGATTGAAAGGAGGACCAAAGGTGCACTTGAAAGTGGATTGTGGTGTCACTGCCCGTACAACCTGTATGATGAGG : 1090
Prx7 Alexi : GAATAAGATTGAAAGGAGGACCAAAGGTGCACTTGAAAGTGGATTGTGGTGTCACTGCCCGTACAACCTGTATGATGAGG : 1090
             GAATAAGATTGAAAGGAGGACCAAAGGTGCACTTGAAAGTGGATTGTGGTGTCACTGCCCGTACAACCTGTATGATGAGG

                         *       500         *       520         *       540         *       560
Prx7 Sloop : ATCATATATGAGGATGTACGTTTAATAAATACGTTCCTTTTTTCACATCTAACGCCTGCCCTTGAAAACTTTGGATCTAC : 1010
Prx7 Alexi : ATCATATATGAGGATGTACGTTTAATAAATACGTTCCTTTTTTCACATCTAACGCCTGCCCTTGAAAACTTTGGATCTAC : 1010
             ATCATATATGAGGATGTACGTTTAATAAATACGTTCCTTTTTTCACATCTAACGCCTGCCCTTGAAAACTTTGGATCTAC

                         *       580         *       600         *       620         *       640
Prx7 Sloop : ATATATGTAAGACATACTTTTTTTGTAAATATTAAGTTGTTTTTTAGTTTTTTTAACTTTTACAAAAAGTTTATTTGAGC :  930
Prx7 Alexi : ATATATGTAAGACATACTTTTTTTGTAAATATTAAGTTGTTTTTTAGTTTTTTTAACTTTTACAAAAAGTTTATTTGAAC :  930
             ATATATGTAAGACATACTTTTTTTGTAAATATTAAGTTGTTTTTTAGTTTTTTTAACTTTTACAAAAAGTTTATTTGA C

                         *       660         *       680         *       700         *       720
Prx7 Sloop : TGGAAAGTAACATAAACACACTTTTTAAAAATGTTTTTATTTTAATATGAAATTGAAGAAAAAATTTTTTATTTTTTTTAG :  850
Prx7 Alexi : TGGAAAGTAACATAAACACACTTTTTAAAAATGTTTTTATTTTAATATGAAATTGAAGAAAAAATTTTTTATTTTTTTTAG :  850
             TGGAAAGTAACATAAACACACTTTTTAAAAATGTTTTTATTTTAATATGAAATTGAAGAAAAAATTTTTTATTTTTTTTAG

                         *       740         *       760         *       780         *       800
Prx7 Sloop : TAATTTTAACATACTTATTACTGGATATTATCGATTATTTAAACAGAAAAGAGAGTCTTCAGTTACAACAAAAACAACTT :  770
Prx7 Alexi : TAATTTTAACATACTTATTACTGGATATTATCGATTATTTAAACAGAAAAGAGAGTCTTCAGTTACAACAAAAACAACTT :  770
             TAATTTTAACATACTTATTACTGGATATTATCGATTATTTAAACAGAAAAGAGAGTCTTCAGTTACAACAAAAACAACTT

                         *       820         *       840         *       860         *       880
Prx7 Sloop : TTAAATATATGAACACGTTTTGTTTTAGTTCAAACTATATTTTTATAAAGGTTTGATAAAATTGAAGAATTAATTAATAA :  690
Prx7 Alexi : TTAAATATATGAACACGTTTTGTTTTAGTTCAAACTATATTTTTATAAAGGTTTGATAAAATTGAAGAATTAATTAATAA :  690
             TTAAATATATGAACACGTTTTGTTTTAGTTCAAACTATATTTTTATAAAGGTTTGATAAAATTGAAGAATTAATTAATAA

                         *       900         *       920         *       940         *       960
Prx7 Sloop : TTTTAAAATTATATATATATACAACGTAGATGTTGGTCTTTGGTTTCATATAAAAGGATATCTAACGATCTGTCTAAGGTAC :  610
Prx7 Alexi : TTTTAAAATTATATATATATACAACGTAGATGTTGGTCTTTGGTTTCATATAAAAGGATATCTAACGATCTGTCTAAGGTAC :  610
             TTTTAAAATTATATATATATACAACGTAGATGTTGGTCTTTGGTTTCATATAAAAGGATATCTAACGATCTGTCTAAGGTAC

                         *       980         *      1000         *      1020         *      1040
Prx7 Sloop : TTAGCACATCCACCAACGATTAAACCATCCCGTATTAAATGCCTAGTTCGTTCCCGTAGGTCAACCTAGATGAATTGCAT :  530
Prx7 Alexi : TTAGCACATCCGCCAACGATTAAACCATCCCGTATTAAATGCCTAGTTCGTTCCCGTAGGTCAACCTAGATGAATTGCAT :  530
             TTAGCACATCC CCAACGATTAAACCATCCCGTATTAAATGCCTAGTTCGTTCCCGTAGGTCAACCTAGATGAATTGCAT
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Figure 5.6 cont.

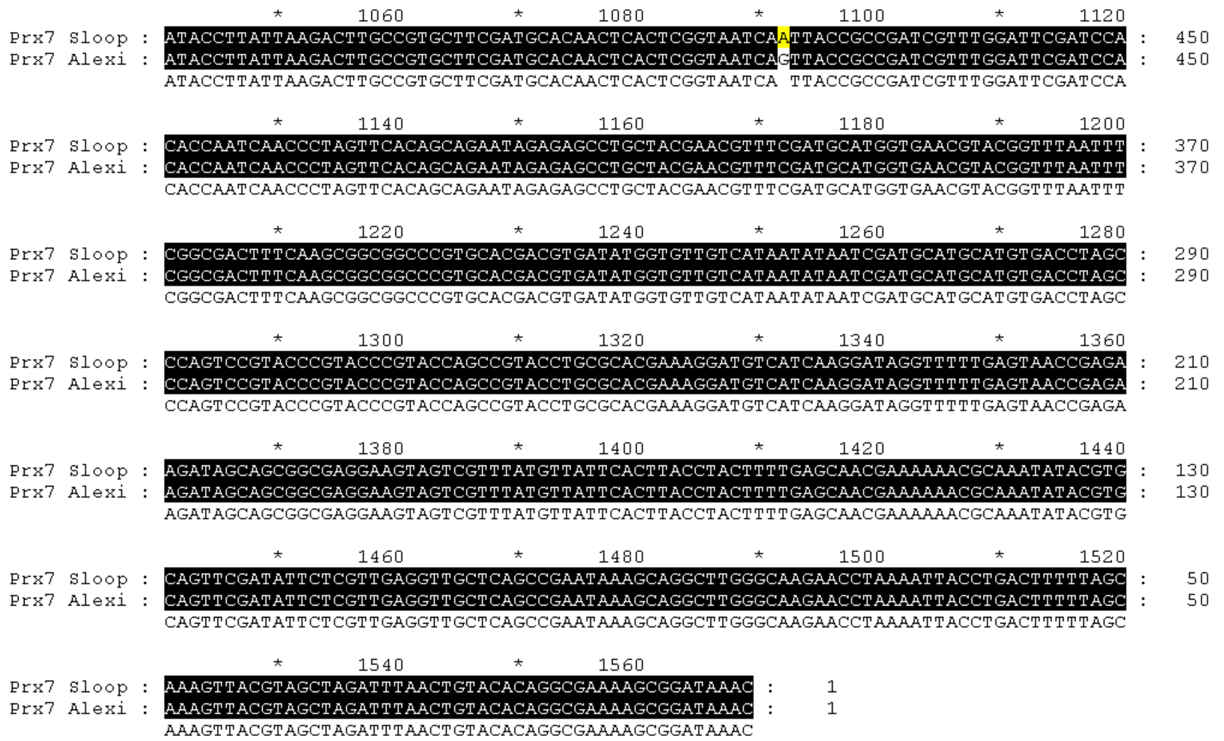


Figure 5.6 Alignment of promoter sequences for *HvPrx7*. Promoter of *HvPrx7* was sequenced from genomic DNA of Sloop and Alexis (n=3). Four SNPs were identified at 244, 639, 972 and 1092 bp (highlighted in yellow) upstream of the start site. S represents start site.

5.3.2 – *In silico* promoter analysis

In silico promoter elements identified many regulatory elements potentially involved in peroxidase gene regulation (Table 5.7). *In-silico* analysis of the promoter regions was undertaken using PLACE (Plant Cis-Acting Regulatory DNA Elements, http://www.dna.affrc.go.jp/PLACE/) database. The full list of regulatory DNA elements identified for *HvBP1* and *HvPrx7* is shown in Appendix 3.

Table 5.7 Summary of *cis*-elements within the promoter regions of *HvBP1* (A) and *HvPrx7* (B). Positions of the element (and motif name) are given in the direct strand (+) as well as the complementary strand (-). Green highlighted elements were included in the chosen sequence for Y1H screening. Nucleotide abbreviations; V=A, C or G; B= C, G or T; R= A or G; W= A or T; Y= C or T; H = A, C or T; D= A, G or T; S= G or C; N= A, G, C or T. Elements in red are present only in Alexis. Full details are in Appendix 3

A

***HvBP1* Promoter**

Motif Name	Location and Strand	SIGNAL SEQUENCE
-300ELEMENT	1803 (-)	TGHAAARK
AACACOREOSGLUB1	2239 (-)	AACAAAC
ABRELATERD1	171 (+)	ACGTG
ABREOSRAB21	331 (-)	ACGTSSSC
ARE1	2161 (-)	RGTGACNNNGC
ARFAT	1454 (-)	TGTCTC
CANBNNAPA	71 (-)	CNAACAC
CAREOSREP1	1809 (+), 475 (-)	CAACTC
CATATGGMSAUR	1489 (+), 1489 (-)	CATATG
CBFHV	619 (+), 631 (+), 1248 (+), 297 (-), 619 (-), 631 (-), 741 (-), 1214 (-), 1248 (-), 2012 (-), 2270 (-)	RYCGAC
CEREGLUBOX2PSLEGA	1598 (-)	TGAAAACT
CGACGOSAMY3	633 (+), 2293 (+), 740 (-), 1247 (-), 2011 (-)	CGACG
CRTDREHVCBF2	619 (+), 631 (+), 1248 (+), 619 (-), 631 (-), 1248 (-)	GTCGAC
DOFCOREZM	291 (+), 916 (+), 1147 (+), 2130 (+), 2375 (+), 2401 (+), 391 (-), 845 (-), 1019 (-), 1381 (-), 1632 (-), 1983 (-), 2234 (-), 2277 (-), 2335 (-)	AAAG
DPBFCOREDCDC3	953 (+), 2264 (+), 104 (-), 123 (-), 246 (-), 1270 (-), 2109 (-), **2107 (-)**	ACACNNG
GADOWNAT	482 (+)	ACGTGTC
GARE2OSREP1	1165 (+)	TAACGTA
GCN4OSGLUB1	553 (-), 1329 (-)	TGAGTCA
LTRECOREATCOR15	226 (+), 204 (-), 741 (-), 1214 (-), 1353 (-)	CCGAC
MYB2CONSENSUSAT	835 (-)	YAACKG
MYBCORE	636 (+), 835 (+), 2077 (+), 2192 (+), 1334 (-), 1745 (-)	CNGTTR
MYCATRD22	423 (+)	CACATG
MYCCONSENSUSAT	123 (+), 246 (+), 423 (+), 436 (+), 1489 (+), 1533 (+), 1972 (+), 2265 (+), 2304 (+), 123 (-), 246 (-), 423 (-), 436 (-), 1489 (-), 1533 (-), 1972 (-), 2265 (-), 2304 (-)	CANNTG

Table 5.7 cont.

HvBP1* Promoter *cont.		
Motif Name	**Location and strand**	**SIGNAL SEQUENCE**
POLASIG1	1606 (-)	AATAAA
PYRIMIDINEBOXOSRAMY1A	2276 (+)	CCTTTT
QARBNEXTA	170 (+)	AACGTGT
RYREPEATBNNAPA	1782 (-)	CATGCA
TATCCACHVAL21	1991 (-)	TATCCAC
WBOXATNPR1	537 (+), 847 (+), 2254 (+)	TTGAC
WRKY71OS	88 (+), 302 (+), 308 (+), 538 (+), 553 (+), 598 (+), 746 (+), 848 (+), 893 (+), 1081 (+), 1190 (+), 1329 (+), 1878 (+), 1969 (+), 2066 (+), 2102 (+), 2255 (+), 43 (-), 1414 (-), 1470 (-), 1667 (-), 2166 (-), 2392 (-),	TGAC

B

***HvPrx7* Promoter**		
Motif Name	**Location and Strand**	**SIGNAL SEQUENCE**
-300ELEMENT	52 (-), 903 (-)	TGHAAARK
ABRELATERD1	128 (+), 342 (+), 1139 (+), 341 (-), 496 (-), 1138 (-)	ACGTG
ABREOSRAB21	342 (+)	ACGTSSSC
ARFAT	1169 (-)	TGTCTC
CANBNNAPA	1122 (-)	CNAACAC
CAREOSREP1	109 (-)	CAACTC
CBFHV	97 (+) 261 (+)	RYCGAC
CEREGLUBOX2PSLEGA	156 (-)	TGAAAACT
CGACGOSAMY3	200 (+) 1380 (-)	CGACG
DOFCOREZM	12 (+), 147 (+), 246 (+), 801 (+), 869 (+), 878 (+), 924 (+), 1023 (+), 1156 (+), 359 (-), 369 (-), 398 (-), 645 (-), 1046 (-), 1228 (-), 1264(-), 1415 (-)	AAAG
DPBFCOREDCDC3	19 (+), 428 (+), 294 (+)	ACACNNG
GARE2OSREP1	530 (-)	TAACGTA
LTRECOREATCOR15	98 (+), 262 (+)	CCGAC
MYB2CONSENSUSAT	545 (+), 1208 (-)	YAACKG
MYBCORE	322 (+), 1208 (+), 545 (-), **593 (-)**	CNGTTR
MYCATRD22	20 (+), 1424 (+)	CACATG
MYCCONSENSUSAT	20 (+), 341 (+), 384 (+), 429 (+), 545 (+), 1138 (+), 1208 (+), 1424 (+), 1541 (+), 20 (-), 341 (-), 384 (-), 429 (-), 545 (-), 1138 (-), 1208 (-), 1424 (-), 1541 (-)	CANNTG
POLASIG1	810 (-), 857 (-), 888 (-), 934 (-)	AATAAA

Table 5.7 cont.

WBOXATNPR1	125 (+), 786 (+), 25 (+), **476 (+), 928 (-)**	TTGAC
WRKY71OS	126 (+), 285 (+), 787 (+), 25 (-), 827 (-), 1113 (-), **477 (+), 928 (-)**	TGAC
ELRECOREPCRP1	**927 (-)**	TTGACC
AGMOTIFNTMYB2	**540 (+)**	AGATCCAA
CACGTMOTIF	**341(+), 1138 (+), 341 (-), 1138 (-)**	CACGTG
GAREAT	**1467 (+)**	TAACAAR
PALBOXACP	**1030 (+)**	CCGTCC
POLASIG2	**370 (-), 1265 (-)**	AATTAAA
PYRIMIDINEBOXHVEPB1	**1047 (+)**	TTTTTTCC

Elements were further investigated based on any relationship to the peroxidase pathway, response to environmental stress, wounding or links to germination. Figure 5.7 summarises the *HvBP1* target sequence for Y1H screening, SNPs and *cis*-element binding sites. The sequence was from 144 to 340 bp into the promoter, with a screening sequence of 196 bp and 1 SNP between Sloop and Alexis at 308 bp. PLACE database analysis indicated that the SNP resulted in an extra *cis*-element in the tolerant variety Alexis, DBFCOREDCDC3 (Table 5.7). This signal site has been shown to interact with a novel bZIP transcription factor that is ABA responsive and embryo-specific (Kim *et al.* 1997). Elements linked to endosperm gene expression, ABA or GA signalling and WRKY DNA binding proteins were also present in the Y1H region used for screening (Table 5.7).

Figure 5.8 summarises the *HvPrx7* target sequence for Y1H screening, SNPs and *cis*-element binding sites. The Y1H fragment was designed from 275 to 668 bp into the promoter, with a screening sequence of 393 bp and 1 SNP between Sloop and Alexis at 639 bp (Figure 5.6). PLACE database analysis indicated that the SNP resulted in an extra *cis*-element in the tolerant variety Alexis, WBOXATNPR1 (Table 5.6). This element has

been suggested to have a putative function in response to environmental stress (Chen *et al.* 2002), specifically by salicylic acid (SA) induced WRKY DNA binding proteins. The SNP resulting in the extra WBOXATNPR1 was the focus of the Y1H screen but the promoter region used for screening was expanded to 393 bp to include elements specific to gene expression in the endosperm or germinating embryos; and in response to ABA or GA and WRKY DNA binding proteins (Table 5.7).

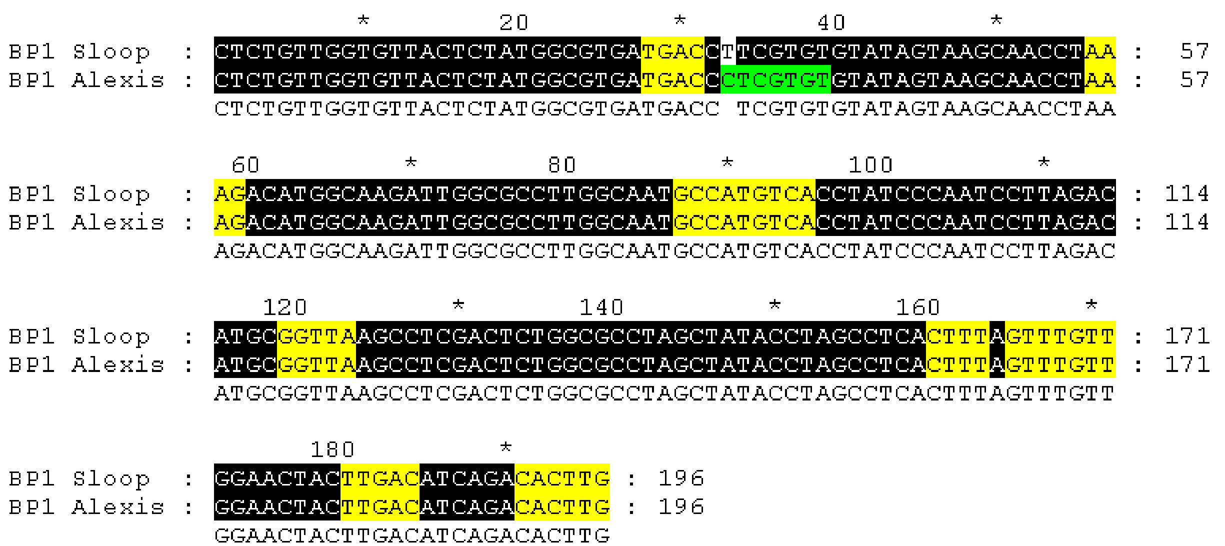


Figure 5.7 *HvBP1* promoter region targeted for Y1H screening. Promoter region from *HvBP1* used in yeast one hybrid screening. Common *cis*-elements are highlighted in yellow, *cis*-elements highlighted in green are specific to the tolerant cultivar Alexis. SNP (308 bp into the *HvBP1* promoter) remains white.

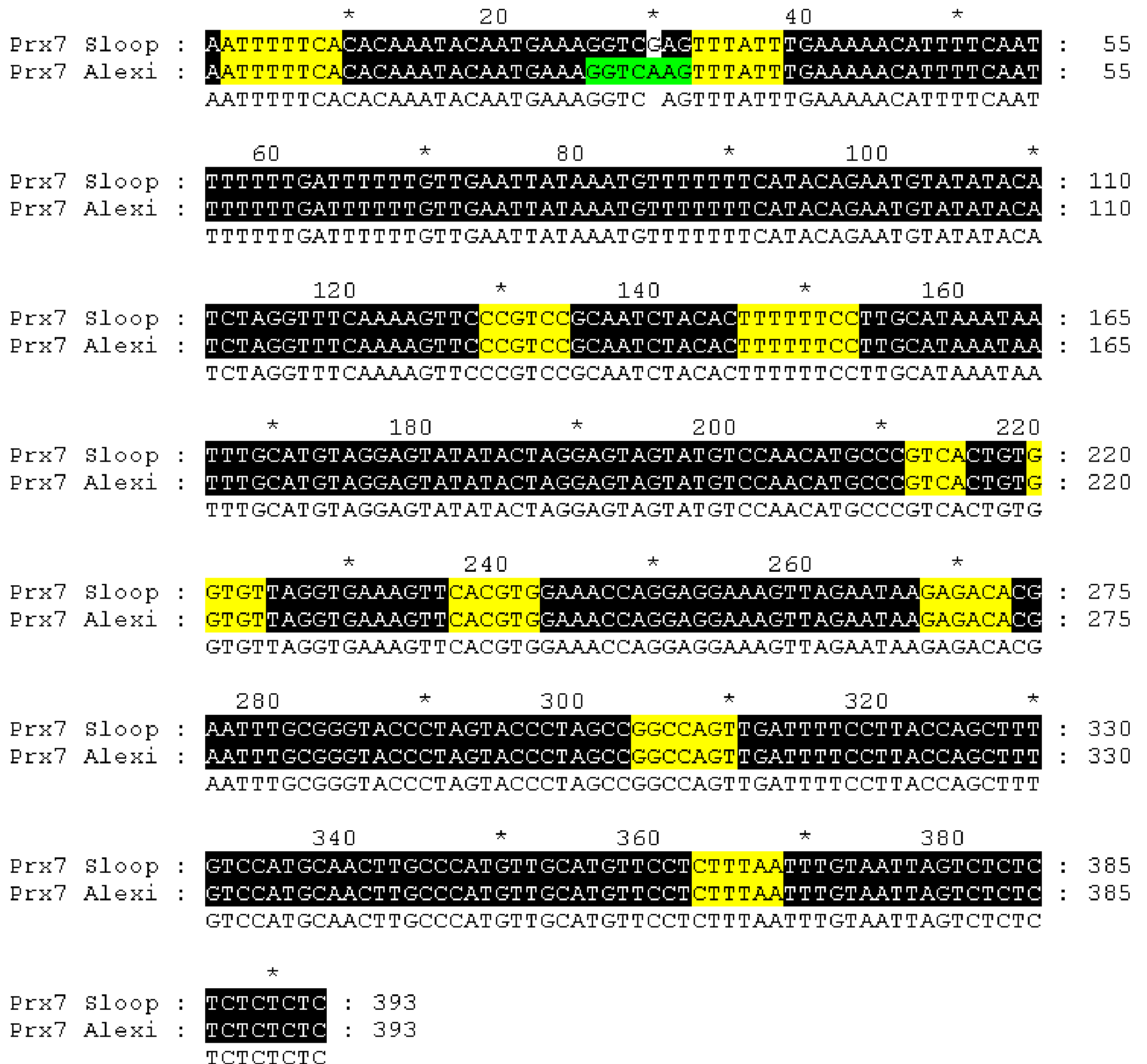


Figure 5.8 *HvPrx7* promoter region targeted for Y1H screening. Promoter region from *HvPrx7* used in yeast one hybrid screening. Common *cis*-elements are highlighted in yellow, *cis*-elements highlighted in green are specific to the tolerant cultivar Alexis. SNP (639 bp into the *HvPrx7* promoter) remains white.

5.3.3 – Yeast one hybrid screening

Initial screening of the Y1H library for the *HvBP1* bait sequence resulted in 112 positive colonies for Alexis and 136 positive colonies for Sloop. Initial screening for the *HvPrx7* bait sequence resulted in greater than 500 positive colonies. All of the positive colonies for *HvBP1* or *HvPrx7* were re-streaked on plates utilising the α--galactosidase reporter gene (*MEL1*) to allow the identification of false positives directly on the plates using X-α-GAL. The colonies that turned blue were excluded as false positives. Positive interactions (white colonies) were restriction digested to remove conserved banding patterns. This resulted was 16 positive interactions for *HvBP1* Sloop, 17 positive interactions for *HvBP1* Alexis, 12 positive interactions for *HvPrx7* Sloop and 13 positive interactions for *HvPrx7* Alexis. Sequencing identified five unique clones for *HvBP1* Sloop and three for *HvBP1* Alexis, similarly identifying three unique clones for *HvPrx7* Sloop and two for *HvPrx7* Alexis (Table 5.8).

In silico sequence searches for clones identified as interactors with the *HvPrx7* promoter suggested a potential bZIP domain containing protein was isolated (Table 5.8). Co-transformation of the library vector containing the candidate sequence and the original bait sequence showed one positive interaction. Transformation and plating on SD/-His-Leu plates revealed a positive interaction for the *HvPrx7* Alexis 11 clone. Co-transformation was repeated using Sloop as the bait, also confirming binding suggesting the SNP identified between Sloop and Alexis is not contributing to the presence or absence of a

transcription factor binding and differential gene expression. Co- transformation resulted in no positive interactions for *HvBP1*.

Table 5.8 BlastX and Blast N analysis from positively identified sequences for Y1H screening. *HvBP1* or *HvPrx7* Sloop and Alexis Y1H screen clone number is represented. Accession number, BLAST N and BLAST X results and *e* value is represented. The clone highlighted by grey shading was the only positive confirmed by co-transforming the library vector containing the identified sequence and the original bait sequence.

	Accession	Blast N	e value	Accession	Blast X	e value
***HvBP1* Sloop**						
3		**No Results**		EAW80031	isoform CRA_b	0.23
4	AY692477.1	*Triticum* alpha-expansion EXPA3	$8.00E^{-35}$	AAS48878.1	expansion EXPA (*Triticum*)	3.00E-14
5		**No Results**		ABB90545.1	Lipid transfer protein (*Triticum*)	4.7
				NP_181959.1	Xylogen-like protein (*Arabidopsis)*	1.90E-01
				EAZ39035.1	Hypothetical protein OsJ_022518 (*Oryza*)	5.00E-05
9	AK248318.1	*Hordeum* clone: FLbaf52b15	$1.00E^{-25}$	BAB33421.1	Putative senescence-associated protein	3.00E-96
				T02955	Probable cytochrome P450 monooxygenase	5.00E-76
11	AK252409.1	*Hordeum* clone: FLbaf152a06	$4.00E^{-146}$	NP_563825.1	GPI-anchor transamidase (*Aradidopsis*)	4.00E-14
***HvBP1* Alexis**						
2	X16276.1	Barley mRNA for alpha-amylase/subtilisin	8.00E-25	P07596	Alpha-amylase/subtilisin inhibitor (BASI)	1.00E-110
8	X01777.1	Barley mRNA for B3-hordein	0			
	DQ148297.1	*Hordeum* clone Hn6 B hordein gene	5.00E-157	P06471	B3-hordein	4.00E-38
9	X16276.1	Barley mRNA for alpha-amylase/subtilisin	5.00E-27	CAM57979.2	NAC transcription factor (*Hordeum*)	1.00E-13

Table 5.8 cont.

		(BASI)				
	AM500855.1	*Hordeum* mRNA NAC transcription factor (Nac 1)	2.00E-16			

	Accession	Blast N	e value	Accession	Blast X	e value
***HvPrx7* Sloop**						
1		Cytosolic heat shock protein 90 (*Hordeum*)	100%	AAP87284	Cytosolic heat shock protein 90 (*Hordeum*)	2.00E-42
4		**No Results**			PREDICTED:similar to SAM and SH3 domain	
13		*Hordeum Mla* locus	8.00E-81		Dipeptide ABC transporter, permease protein	
	EF067844	*Hordeum vrs1* locus, and *Hox1* gene	1.00E-79		DppC (*Aeropyrum*)	
***HvPrx7* Alexis**						
1		**No Results**		AAP87284	Cytosolic heat shock protein 90 (*Hordeum*)	2.00E-42
				P36183	Endoplasmic homolog precursor (*Hordeum*)	1.00E-06
11		**No Results**		NP_001058100	Os06g0622700 (*Oryza*) Basic-leucine zipper (bZIP) transcription factor domain containing protein	2.00E-05

5.3.4 Further characterisation of positive clones

One positive interaction (418 bp) was identified with the *HvPrx7* promoter region isolated, shown to bind in both parents, Sloop and Alexis. Using the Basic Local Alignment Search Tool (tBLASTx and tBLASTn; http://blast.ncbi.nlm.nih.gov/Blast.cgi, NCBI-GenBank Flat File Release 172.0, August 2008) the clone was further analysed. The *in silico* sequence identified in rice allowed sequencing in barley (*HvbZIP)* and further characterisation through northern analysis, chromosomal location and confirmation of binding specificity by a gel shift assay.

5.3.4.1 *In silico* sequence search and identification of full length sequence

The positive interaction identified for *HvPrx7* resulted in a sequence of 418 bp (Figure 5.9). A BLASTx search indicated no results. A BLASTn search identified a rice candidate Os06g062270, a basic-leucine zipper (bZIP) transcription factor domain containing protein. Primer design to the rice sequence (Table 5.9) resulted in successful amplification of 909 bp in barley, *HvbZIP* (Figure 5.10).

5’GGGGGAGAGCCGAAAGAGATCTCGCAATGGTAGCCGGAAAGCCAAGCAGTGATCA
ACCAGAGACCTTGGAGCTTCTACTCCATGGAAGACGCTGGAGGGGCACAAGGGAGAG
GATCAAGCTAGATATTCTGCCGTTGCGTGCAGCTGCTGCTTGCTAGACTAGTGACAGC
TTATTCGCAAGTTTCCAGTATGTAGTGTAGTTATGTGTGTTCTCTTGCTGCAACCGTGG
ATTTATCCATGAGTACCTTTCTTCTCTCTCCGTCCCCTCTTGTTTTATGATCTTCTAATC
AGATGCTAGTTTTGAAATCTGGCATTCCGTGTTACTTTATGTCTCTGGCGTAAGTTCGG
GCACCCTCTGGGTTTATGTAACTATGTGAATCCTGTTTTGCCAATGCCAGAATGACGTT
GATGTCAAATT 3’

Figure 5.9 *HvPrx7* prey sequence that was found to bind the selected region of the *HvPrx7* promoter.

Table 5.9 Nucleotide blast of the full length barley sequence (Figure 5.10) (A) nucleotide Blast results of *Hordeum* predicted protein identified (AK369957.1) (B) Blast X results of *Hordeum* predicted protein identified (AK369957.1). Accession number and e value are represented.

A

Accession	Nucleotide Blast	e value
AK369957.1	*Hordeum vulgare* subsp. *vulgare* mRNA for predicted protein	0
AK369957.1	*Hordeum vulgare* subsp. *vulgare* mRNA for predicted protein	0
AK365505.1	*Hordeum vulgare* subsp. *vulgare* mRNA for predicted protein	0

B

Accession	Blast X	
BAJ99768.1	Predicted protein [*Hordeum* vulgare subsp. Vulgare]	2.00E-69
BAJ96708.1	Predicted protein [*Hordeum* vulgare subsp. Vulgare]	4.00E-68
ACR36817.1	unknown [Zea mays]	2.00E-40
XP002437297.1	Hypothetical protein SORBIDRAFT_10g024430 [*Sorghum* bicolour]	3.00E-38
EEC80996.1	Hypothetical protein OsI_23742 [*Oryza sativa* Indica Group]	7.00E-38
NP001058100.1	Os06g0622700 bZIP transcription factor-like [*Oryza sativa* Japonica Group	7.00E-38
NP001147256.1	LOC100280864 bZIP transcription factor protein [*Zea mays*]	4.00E-35

```
                  *        20         *        40         *        60         *
1.TXT : ATGGACGCCGACCTCGACCTGGACGCCCTCCTCGCCACCTTCGCCGCCGATTCCGCAGTCTCCGAGCTCC :  70
2.TXT : atggacgccgacctcgacctggacgccctcctcgccaccttcgccgccgattccgcagtctccgagctcc :  70
3.TXT : ATGGATGTAGAGTTCTTCGCCGACCTCGACCTCGACGCGCTCCTCGCCTCCTTCTCCTCCTCCGCCGCCG :  70
        ATGGAcGccGAccTCgaCctgGACgcCctCCTCGcCaCctTCgcCGCCgatTcCgCagtCTCCGagctCc

                80         *       100         *       120         *       140
1.TXT : TCGCCCCGCCTCCGCATCTCGATGCGGAGGCGGGGTCGCCGGAGTCGGTGACCTCCCGGTCCAGCCCCGC : 140
2.TXT : tcgccccgcctccgcatctcgatgcggaggcggggtcgccggagtcggtgacctcccggtccagccccgc : 140
3.TXT : CCGCCGGCTCCGGCGTCTCGGGCCTCTTCGCCCCGTCACCGCCGCACGATGCGGAGGCGGGGTCGCCGGA : 140
        tCGCCccgcCtccgcatctcGatgcggagGCgggGTCgCCGgaGtcgGtgaCctcccgGtccagcCCcGc

                  *       160         *       180         *       200         *
1.TXT : CGGCGAGGAGGCGCTGTCGGAGATCGAGAGGTTTCTGATGCAGGAGGAGGAGGCGGCGGGGGTGGAGCCG : 210
2.TXT : cggcgaggaggcgctgtcggagatcgagaggtttctgatgcaggaggaggaggcggcgggggtggagccg : 210
3.TXT : GTCGGTGAGCTCCCGGCGGCCCAGCCCTTCCCGGGAGGCGGCGCTGTCGGAGATCGAGAGGTTCCTGATG : 210
        cggcGaGgaggCgCtGtcGgagAtCgagaggtttctGatGcaGgaGgaGGAGgcgGcGgGGgTggaGccG

               220         *       240         *       260         *       280
1.TXT : GTGGACGGGATCAGCGTGGATGAGTTCATCGACGCCCTGTTCGACGGTGCGGAGGAGGGGGGCGAGAAGG : 280
2.TXT : gtggacgggatcagcgtggatgagttcatcgacgccctgttcgacggtgcggaggaggggggcgagaagg : 280
3.TXT : GAGGAGGGCCCCGCGGCGGAGGAGGGGGTCGGCGCGGAGGATTTCTTCGACGCGCTCCTCGTCGACGGCG : 280
        GtGGAcGGgatCagcGtGGAtGAGttcaTCGaCGCcctGttcgaCggtGcgGaGgaGgggGgCGAgaagG

                  *       300         *       320         *       340         *
1.TXT : GGAACGGGAGTGAGGCTGAGGCTGGGGGCAGCACTGATGGGGACTCTAGGAGGGGGGATGAAGAGGGGGT : 350
2.TXT : ggaacgggagtgaggctgaggctgggggcagcactgatggggactctaggaggggggatgaagagggggt : 350
3.TXT : GGGAGCAGCGAGGAGGAAGAAGAGGGGAAGGCGAGTGAGGCGGGGGAAGCACGGATGGGGATTCCGGGAA : 350
        GGaAcGgGagtGAGGctGAgGctGGGggcaGcAcTGAtGgGGactctAGgAgGGggGatGAagagGGGgt

               360         *       380         *       400         *       420
1.TXT : GGAGGTGGTGACGCCGGAGACGGAGGTTGATGGCGATGATCCCATCAGCAAAAAGAAGAGGAGGCAAATG : 420
2.TXT : ggaggtggtgacgccggagacggaggttgatggcgatgatcccatcagcaaaaagaagaggaggcaaatg : 420
3.TXT : GGAGAATGAGGTGGCTACCCCGGACGCGGAGAAGGAGGATGTGGAGGCGGAGGTGGATGGCGATGATCCC : 420
        GGAGgtgGtGacGcCggagaCGGAgGttGAtggcGAtGATcccatcagcaAaaaGaAgaGgaggcAaatg

                  *       440         *       460         *       480         *
1.TXT : AGGAATAGGGATTCTGCCATGAAGTCGAGGGAGAGGAAAAAGTCATATGTTAAGGACTTGGAGACGAAGA : 490
2.TXT : aggaatagggattctgccatgaagtcgagggagaggaaaaagtcatatgttaaggacttggagacgaaga : 490
3.TXT : ATGAGCAAGAAGAAGAGGAGGCAGATGAGAAATAGGGATTCTGCCATGAAATCGAGGGAGAGGAAGAAGA : 490
        AgGAatAgGgAttctgccAtGaAGtcGAGggAgAGGaAaaagtCatatgttaaGgacttGgaGAcGAAGA

               500         *       520         *       540         *       560
1.TXT : GCAAGTATCTCGAGGCGGAGTGTCGCCGCCTCAGCTACGCACTTCAGTGCTGCGCAGCTGAGAACATGGC : 560
2.TXT : gcaagtatctcgaggcggagtgtcgccgcctcagctacgcacttcagtgctgcgcagctgagaacatggc : 560
3.TXT : TGTATGTTAAGGACCTAGAGACTAAGAGCAAGTATCTAGAGGCCGAGTGTCGTCGCCTCAGCTACGCGCT : 560
        gcaAgtaTctcGAggcgGAGtgTcgccGCctcagctacGcacttcAGTGctGcgcagctgagaACatGgc

                  *       580         *       600         *       620         *
1.TXT : ACTGCGCCAGAACATGTTGAAGGATAGGCCTATTGGTGCTCACACAGTCATGCAGGAGTCTGCCGTACTT : 630
2.TXT : actgcgccagaacatgttgaaggataggcctattggtgctcacacagtcatgcaggagtctgccgtactt : 630
3.TXT : TCAGTGCTGCGCAGCTGAGAACATGGCGCTGCGCCAGAGCTTGTTGAAGGATAGGCCTGTCGGTGCCGCC : 630
        aCtGcGCcagaacatgttGAAggatagGCctattggtgctcacacagtcatgcaGgagtctGccGtactt

               640         *       660         *       680         *       700
1.TXT : ATGGAAACCCTGCCGTTGGTTTCCCTGCTTTGTCTAGTGAGCATCGTGTGCCTATTCCTAACGCCCGGTC : 700
2.TXT : atggaaaccctgccgttggtttccctgctttgtctagtgagcatcgtgtgcctattcctaacgcccggtc : 700
3.TXT : ACAGCCATGCAGGAGTCTGCCGTACTCACGGAAACCCTGCCGCTGGTTTCCCTGCTTTGGCTCGTGAGCA : 700
        AtgGaaAccCtGccGTtgGtttccCTgctttgtctagTGagcaTcGTgTgCCTatTcctaacgcccgGtc

                  *       720         *       740         *       760         *
1.TXT : TACCCAACCGAAGTCTGGTGGCTCCAAGGAGAGCCGAAAGAGATCTCGCAATGGTAGCCGGAAAGCCAAG : 770
2.TXT : tacccaaccgaagtctggtggctccaaggagagccgaaagagatctcgcaatggtagccggaaagccaag : 770
3.TXT : TCGTGTGCCTACTCCCGGTGCCCGGTCTACCCAACCGAAACCCGGTGGCTCGAAGCAGCGCCGGAAGAGA : 770
        TacccaaCCgAagtCtGGTGgCtccaaggagagcCgAAagagatcTcGCaatggtagcCGgaaagccAag

               780         *       800         *       820         *       840
1.TXT : CAGTGATCAACCAGAGACCTTGGAGCTTCTACTCCATGGAAGACGCTGGAGGGGCACAAGGGAGAGGATC : 840
2.TXT : cagtgatcaaccagagaccttggagcttctactccatggaagacgctggaggggcacaagggagaggatc : 840
3.TXT : TCTCGCGACGGTAACCGGAAAGAAGACAAGCAGTGAACAACAGCTAGAAGAAACATTACTACTCCATGGG : 840
        cagtGatcaaccAgagaccttGgAGcttctactccAtggAagaCgctggaggggcacAagggagaggatc

                  *       860         *       880         *       900         *
1.TXT : AAGCTAGATATTCTGCCGTTGCGTGCAGCTGCTGCTTGCTAGACTAGTGACAGCTTATTCGCAAGTTTC- : 909
2.TXT : aagctagatattctgccgttgcgtgcagctgctgcttgctag---------------------------- : 882
3.TXT : AGGCGTTGCAAGGGCTCGAGGGCGAGGATCAAGCTAGATACCGGACCGTTCCGTTTAGCAGCAGCTGCTT : 910
        AaGCtagatAttctgcCGttGcgtgcagctgctgcttgctag        c g tta   gca  t
```

Figure 5.10 Full length uncharacterised *HvbZIP* transcription factor in barley (1) alignment with *Hordeum* predicted protein (2) and rice bZIP transcription factor domain containing protein (3) (Os06g062270) (Table 5.9, full length nucleotide blast of barley sequence).

The identification of rice candidate Os06g062270, a basic-leucine zipper (bZIP) transcription factor domain containing protein allowed successful amplification in barley (Figure 5.10). A recent BLASTn has revealed 100 % identity to a *Hordeum* predicted protein (Table 5.9, Figure 5.10), with the identification of conserved domains indicating similarity with a bZIP transcription factor. An BLASTx of the *Hordeum* clone resulted in hits not only to the original rice candidate Os06g062270, a basic-leucine zipper (bZIP) transcription factor domain containing protein but other bZIP transcription factor proteins (Table 5.9).

5.3.4.2 Northern analysis and chromosomal location

HvbZIP expression was apparent in the early stages of grain fill, observing expression in the milk, soft dough and hard dough stages of maturity (Figure 5.11). Expression appears greater in the susceptible cultivar Sloop through the soft dough and hard dough stages of grain development (Figure 5.11). No expression was observed at maturity in Sloop or Alexis. Chromosomal location using barley:wheat addition lines resulted in amplification on all chromosomes (data not shown). The original rice candidate, Os06g062270, mapped to chromosome 6 (Yu *et al.* 2005).

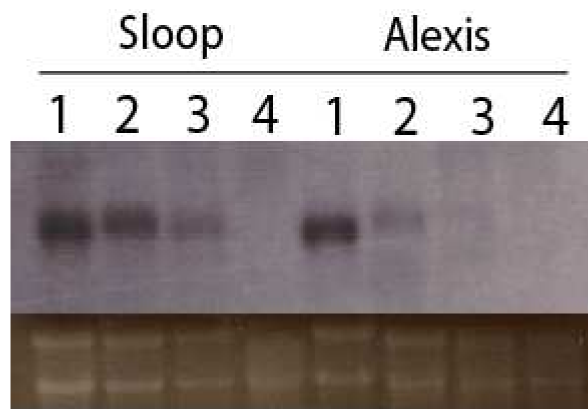


Figure 5.11 Northern blot analysis of *HvPrx7 (bZip)*. Sloop and Alexis gene expression across developmental stages: 1=Milk, 2=Soft Dough, 3=Hard Dough and 4=Maturity (Zadoks' *et al.* 1974). The lower panel represents ethidium bromide stained ribosomal RNA (rRNA) bands as a loading control. This is a representative blot (n=2).

5.3.4.3 Protein expression

The theoretical MW and p*I* values of bZIP were determined to be 72.3 kDa and 5.07 respectively. Heterologous expression of the HvbZIP protein resulted in bands present in the induced sample at approximately 30, 23 and 20 kDa (Figure 5.12). A more prevalent band was present in the induced sample closer to the predicted MW of 72 kDa (Figure 5.12), suggesting some degradation of HvbZIP during heterologous expression.

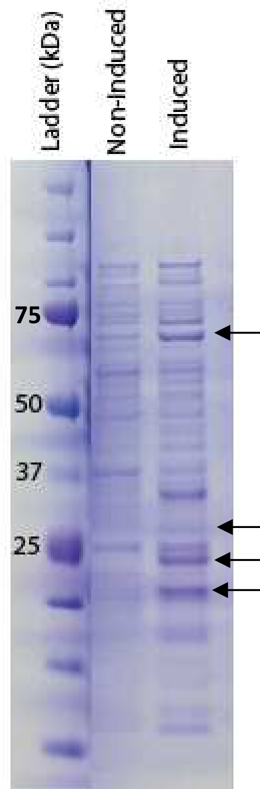


Figure 5.12 *bZIP* (uncharacterised barley protein) protein expression in induced and non-induced cell cultures. A strong product band is apparent at approximately 30, 23 and 20 kDa in the induced culture (represented by arrow). A stronger band is present in the induced sample closer to the predicted size of 72 kDa. BIO-RAD Precision Plus Dual Colour Protein Ladder used, not all sizes shown. To determine the theoretical MW and p*I* values the compute p*I*/MW tool was used (http://www.expasy.org/tools/pi_tool.html).

5.3.4.4 Gel shift assay

A DNA binding assay was performed with increasing concentrations of the recombinant protein (section 5.2.2.4.3.3). As protein concentration increased using the identified promoter region, the size of the band increased, indicating binding of the protein with the promoter sequence (Figure 5.13).

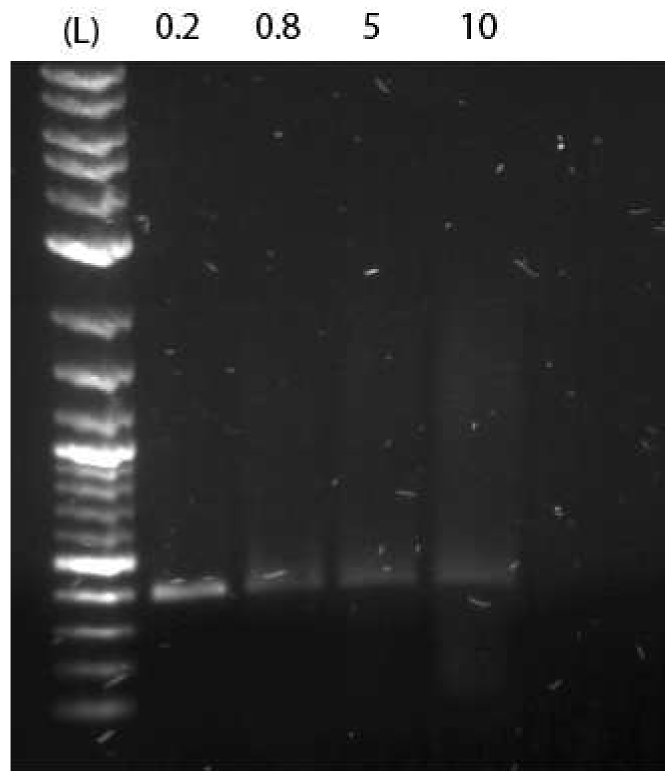


Figure 5.13 DNA Binding Assay. *HvPrx7* Promoter bait (ssDNA) (section 5.2.2.1.1) was incubated with uncharacterised bZIP (section 5.2.2.4.3.3) induced protein. Increasing amounts of protein were incubated ranging from 0.2-10 μM (represented at top of figure). (L)=1Kb plus ladder.

5.4 Discussion

Peroxidases were previously established as candidate genes for black point susceptibility given their differential expression between susceptible and tolerant varieties. Two peroxidase genes, *HvBP1* and *HvPrx7*, were identified as candidates. eQTL were then identified for these two genes using expression data across the Sloop/Alexis population (Chapter 4). Given the identified areas and candidates contributing to gene regulation this research aimed to determine if susceptibility is correlated with differences in regulatory elements by analysing the promoter regions of candidate genes in the susceptible variety Sloop and tolerant variety Alexis. Secondly, the research presented in this chapter aimed to

identify transcription factors that might regulate gene expression of *HvBP1* and *HvPrx7* by using Y1H screening. Isolation of the promoter regions of the peroxidase genes *HvBP1* and *HvPrx7* allowed the identification of a number of the regulatory regions possibly controlling peroxidase gene expression and therefore black point formation (Table 5.7). However, in the regions of the promoter analysed, SNPs appeared to have no effect on gene regulation between susceptible and tolerant varieties. The only interactor found was a bZIP for the *HvPrx7* promoter which bound regardless of which variety the promoter was isolated from.

For each of the peroxidase promoters identified, 2416 bp were isolated for *HvBP1* and 1569 bp for *HvPrx7*. Four SNPs were identified for each promoter between Sloop (susceptible) and Alexis (tolerant) varieties. SNPs are the main source of DNA variation in most plant and animal genomes (Garcés-Claver *et al.* 2007). There is a good understanding of how mutations in coding regions affect the amino acid composition of proteins and in some cases how these lead to differences in phenotype, but the effect of variation at the DNA level on transcript abundance remains elusive (Gilad *et al.* 2008). Identifing regulatory regions in the genome and predicting how polymorphisms in regulatory regions affect gene expression levels temporally or spatially has been shown to be difficult (Wray 2007). *In-silico* analysis of the promoter regions for *HvBP1* and *HvPrx7* identified a large number of transcription factor binding domains. SNPs within the transcription factor binding sites of the promoters of these genes in barley varieties that differ in black point susceptibility may therefore be responsible for differences not only observed in gene expression but black point too. Elements in the Y1H screening sequences were further

investigated based on any relationship to the peroxidase pathway, response to environmental stress, wounding or links to germination (Table 5.10).

Table 5.10 Elements and Function included in promoter regions chosen for Y1H screening. Element and Function are represented for the selected promoter regions of *HvBP1* and *HvPrx7*. Highlighted elements indicate an extra element in the tolerant variety Alexis.

Element	Function
HvBP1	
-300ELEMENT	Differential gene expression in the developing barley endosperm
AACACOREOSGLUB1	Endosperm-specific gene expression
ARE1	Antioxidant response element of NAD(P)H:quinone reductase genes
CBFHV	Binding site of barley (H.v.) CBF1, and also of barley CBF2,dehydration-responsive element (DRE) binding proteins (DREBs)
DOFCOREZM	Core site required for binding of Dof proteins in maize PBF is an endosperm specific Dof protein
DPBFCOREDCDC3	A novel class of bZIP transcription factors, interact with ABA-responsive and embryo-specification elements
MYBCORE	Involved in regulation of genes that are responsive to water stress in Arabidopsis
MYCCONSENSUSAT	Function as transcriptional activators in abscisic acid signalling.
PYRIMIDINEBOXOSRAMY1A	Found in the promoter of barley alpha-amylase (Amy2/32b) gene which is induced in the aleurone layers in response to GA
WBOXATNPR1	Recognized specifically by salicylic acid (SA)-induced WRKY DNA binding proteins. Response to environmental stress.
WRKY71OS	A transcriptional repressor of the gibberellin signalling pathway
HvPrx7	
-300ELEMENT	*As above*
ABRELATERD1	Transcriptional regulation of ABI3- and ABA-responsive genes including RD29B and RD29A in seeds, germinating embryos, and seedlings of Arabidopsis.
ARFAT	RF (auxin response factor) binding site found in the promoters of primary/early auxin response genes of Arabidopsis thaliana
CANBNNAPA	Embryo- and endosperm-specific transcription of napin (storage protein) gene, napA; seed specificity; activator and repressor
DOFCOREZM	*As above*
MYCCONSENSUSAT	*As above*
MYBCORE	*As above*
POLASIG1	Poly A signal found in legA gene of pea, rice alpha-amylase
WBOXATNPR1	*As above*
WRKY71OS	*As above*
PYRIMIDINEBOXHVEPB1	Required for GA induction

Promoter Y1H screening sequences of 196 bp for *HvBP1* and of 393 bp for *HvPrx7* were chosen, including SNPs which lead to different elements being present and therefore potentially the presence of different transcription factors or repressors. Typically Y1H screening uses tandem repeats of the binding domain, targeting a specific transcription factor. However, in this study a greater length was chosen to screen, including other elements in the vicinity with any relationship to the peroxidase pathway, response to environmental stress, wounding or links to germination. Other studies in rice (Zhu *et al.* 2003) and barley (Müller *et al.* 2000; Ogo *et al.* 2007) have successfully identified regulatory factors using longer bait sequences. This screen focused on the effect of the SNP in the promoter regions, resulting in an extra bZIP (embryo specific) element in *HvBP1* (DPBFCOREDCDC3) and an extra WRKY element in *HvPrx7* (WBOXATNPR1), with known links to environmental stress (Table 5.10). However these interactions were not identified suggesting that the identified SNPs are having no effect on the presence or absence of a transcription factor or repressor. Black pointed grain has been shown to have started germination and to have increased alpha-amylase levels (Hadaway and Able, *unpublished data*). As a result elements associated with germination (or GA/ABA) or with links to germination were included in the screen (Table 5.10). WRKY binding sites were also identified for *HvBP1*, given the have shown enhanced DNA binding and/or expression following induction by pathogens, defence signals and wounding (Eulgem *et al.* 2000). A MYB element was also identified for *HvBP1* promoter, because *HvGAMYB* has been isolated from a barley aleurone cDNA library and the gene product has been shown to be upregulated by α-amylase and to respond to GA (Gubler *et al.* 1995; Gubler *et al.* 1997).

Screening for *HvPrx7* identified a positive interaction with an uncharacterised bZIP transcription factor in barley (*HvbZIP).* The interaction was confirmed by co-transformation in Sloop and Alexis, further confirming binding specificity by a gel shift assay (Figure 5.13). The gel shift assay revealed information about the protein bound, however we could still be observing multiple complexes and this assay does not allow localisation of the binding site.

In Chapter 3 *HvPrx7* expression was significantly greater in Sloop than in Alexis for the milk, hard dough and mature stages. Expression at the mature stage of development for Sloop was greater than any other stage (Figure 3.7), with expression increasing with grain maturity. Expression of the proposed bZIP transcription factor was evident in the early stages of grain development (milk, soft dough and hard dough stages of maturity) (Figure 5.11) in both Sloop and Alexis. However expression of bZIP was higher in the susceptible variety Sloop and expression does not appear correlated with *HvPrx7* expression as determined by northern analysis. bZIP expression should therefore be investigated by qPCR in future research to make correlations with *HvPrx7* and confirm the northern analysis.

The bZIP transcription factor family is one of the largest families in plants, having diverse roles in plant stress responses and hormone transduction (Uno *et al.* 2000; Jakoby *et al.* 2002; Rodriguez-Uribe and O'Connell 2006). For example the bZIP transcription, OsABF2 in rice, regulates expression of abiotic stress-responsive genes through an ABA dependant pathway (Hossain *et al.* 2010); and HvBL22 (from barley) activates seed storage protein genes (Oñate *et al.* 1999). The super family identified was further confirmed by

chromosomal location, resulting in amplification on all chromosomes (Section 5.4.3.2). Given these results we cannot be certain that we are not detecting other copies of the gene through northern analysis (Figure 5.12).The literature indicates that the gene resides on rice chromosome 6 (Yu *et al.* 2005), which is syntenous with barley chromosome 7.

There is only limited information regarding the mechanisms by which plants regulate specific expression of peroxidase genes (Yoshida *et al.* 2003). To activate downstream gene expression, the bZIP transcription factors interact with ABA-responsive elements (ABREs). Indeed, the element was present in the promoter of *HvPrx7* for both Sloop and Alexis and we observed binding in both varieties. Given that ABA is known to be associated with the control of germination (Kim 2007) and black pointed grain has been shown to have started germination and to have increased alpha-amylase levels (Hadaway and Able, *unpublished data*), then the bZIP transcription factor and ABA may be important in controlling black point formation. However, this remains to be confirmed as does whether bZIP regulates expression of *HvPrx7*. ABA is more likely to play a role in the tolerant variety Alexis where grain is unlikely to have started germination and therefore ABA would be probably at higher levels. bZIP may therefore bind to the ABA responsive element to repress expression of *HvPrx7* in the presence of ABA. TaABF1, a seed specific bZIP transcription factor involved in ABA signal transduction of developing wheat has been proposed to play a role in the regulation of seed dormancy and ABA sensitivity in wheat (Rikiishi *et al.* 2010). *TaABF1* has been proposed to influence pre harvest sprouting as resistance to pre-harvest sprouting requires a high level of seed dormancy (Gubler *et al.* 1997).

Similarly in Arabidopsis the *abi5* mutant, (bZIP) has shown decreased sensitivity to ABA inhibition of seed germination and an altered ABA-regulated gene expression, indicating a link between ABA signal transduction and seed specific gene expression (Finkelstein and Lynch 2000).

Due to multiple complexes often involved with gene regulation, an interacting partner with HvbZIP may also be contributing to *HvPrx7* expression. *TRAB1* and *HvABI5* in rice and barley (*AtABI5* homologs) have been shown to physically interact with their corresponding *AtABI3* homologs, *OsVP1* and *HvVP1*, and regulate seed maturation and dormancy by activating ABA-responsive genes (Hobo *et al.* 1999; Nakamura *et al.* 2001; Casaretto and Ho 2003). Although HvbZIP appears to interact with the promoter of *HvPrx7* this remains to be confirmed as does whether differences in expression are due to multiple complexes and contribute to black point.

No interacting partners were identified for *HvBP1*. Expanding the promoter region and investigation into other regulatory elements is therefore required. Screening areas of the *HvBP1* promoter containing the same domains as in the *HvPrx7* promoter could confirm the involvement of bZIP transcription factors in the regulation of expression of other peroxidase genes.

There is a possible link with germination and the regulation of *HvPrx7*, however this more than likely involves other interacting partners. Confirmation of gene expression in the later stages of grain development in the tolerant cultivar Alexis would confirm a link with the regulation of the peroxidase gene *HvPrx7* by the proposed bZIP transcription factor and

ABA. Research in this chapter has successfully isolated the promoters *HvBP1* and *HvPrx7* and identified a large number of regulatory elements. Yeast-one hybrid screening has indicated that HvbZIP may be part of a large complex of events regulating *HvPrx7* and contributing to black point formation.

Chapter Six. General Discussion

6.1 Introduction

The results of this study have established the contributing weather conditions and characterised several candidate genes which might contribute to black point. Where weather conditions are appropriate black point can be a serious but intermittent problem, downgrading malt barley, used for beer and food production, to feed grade. The causes of black point have often been contradictory, with suggested causes including fungal infection (Rees *et al*. 1984, Waldron 1934; Machecek and Greany 1938; Southwell *et al*. 1980), environmental conditions (Waldron 1934; Rees *et al.* 1984; Conner *et al.* 1992; Fernandez *et al.* 1994) and potential biochemical changes (Walker 1998; Whitaker and Chang 1996). Fungal infection has been excluded (Conner and Kuzyk 1988; Maloy and Specht 1988; Conner *et al.* 1996; Ellis *et al.* 1996; Williamson 1997b; Desjardins *et al.* 2007), and the trait is likely to result from the enzymatic oxidation of phenolic compounds to quinones and the transformation of those oxidation products to brown or black pigments during low temperature and high humidity. However the major problem faced is consistent replication of the environmental conditions deemed necessary to induce symptoms, resulting in large variations in phenotypic screening between years.

Previous studies have identified QTL associated with tolerance to discolouration of the embryo end of the grain on chromosome 2H (Hadaway 2002). Similarly, using measures of grain brightness, redness and yellowness to assess tolerance to kernel discoloration, QTLs have been detected on chromosomes 2H, 3H, 4H, 5H and 7H (Li *et al,* 2003). In this study the symptoms of black point and kernel discolouration were clearly differentiated (Chapter 2, Walker *et al.* (2008)). March and colleagues have identified several candidate

genes underlying the QTL for black point susceptibility on chromosome 2H, narrowing the QTL size to 10 cM (March *et al*. 2008). This study also identified QTL on 1H, 2H, 3H and 5H by screening for black point formation in a mapping population across a number of sites and years. Association of weather conditions across years with black point formation also occurred. Genes were then targeted based on the candidates identified by QTL studies as well as the observation that peroxidases are likely to be involved (Williamson 2002; Hadaway *et al.* 2003; March 2003). Differential gene expression of these candidates between susceptible and tolerant varieties was then characterised. Identifying candidate genes in black point formation may allow breeding programs to screen for tolerant varieties.

This study investigated alternative methods to identify areas of the genome and or mechanisms that had an effect on the candidate genes involved in black point formation. Given the environmental triggers identified, regulation of the candidate genes may be influential in black point formation. Candidates were therefore further studied by identifying areas of the genome (eQTL) and genes contributing to their regulation by comparative mapping. The promoter regions of peroxidase genes *HvBP1* and *HvPrx7* were analysed and SNPs identified in susceptible and tolerant cultivars; however these had no effect on binding of regulatory factors. Although no link can be made directly with black point, bZIP transcription factors were identified as a candidate regulating *HvPrx7* gene expression. *HvPrx7* gene expression therefore appears to be part of a complex series of regulatory events. Through comparative mapping studies a number of candidate genes potentially regulating *HvBP1* gene expression were also identified. Y1H studies utilising other regions of the promoter will allow confirmation of regulatory factors. Understanding what regulates these genes may provide the link between differential peroxidase gene

expression and the environment which leads to black point formation. Future research with the simulation of the necessary environmental conditions will allow focus on the candidates identified in the QTL regions proposed to regulate *HvBP1*. Furthermore studying *bZIP* gene expression in induced and non-induced environments or the use of *bZIP* mutants could determine if there is an effect on black point formation.

6.2 Simulating environmental conditions to induce symptoms

Generally moist humid conditions during the grain fill period has been previously shown to lead to black point formation (Southwell *et al.* 1980; Rees *et al.* 1984; Conner 1987; Moschini *et al.* 2006; Tah *et al.* 2010). This study identified that the occurrence of low vapour pressure deficit (high humidity and low temperature) is associated with the formation of black point in susceptible varieties. These environmental conditions probably create a moist environment during grain development so that the developing grain cannot dry out. Stress, wounding or pre-germination of the embryo caused by this environment might then lead to black point formation. We now have the ability to simulate the high humidity and associated low temperatures to allow more accurate phenotypic screening and analysis of material in studying the expression of candidate genes in susceptible grains.

Experiments altering planting dates to account for maturity differences between susceptible and tolerant varieties indicated that the timing of these environmental triggers is important for severity of the trait, with the earlier maturing variety Sloop being most susceptible. Simulation of the conditions identified will now allow a more comprehensive study to identify the stage of grain fill where the greatest impact is observed. This will allow farmers to plan their crop planting especially having later maturing varieties sown earlier

to avoid the necessary environmental conditions. Likewise, use of the appropriate conditions in a greenhouse environment will allow the incorporation of quicker and more detailed screens into breeding programs. Further research may involve developing modelling software to enable early detection.

6.3 Candidate genes and areas of the genome contributing to black point

In plant genetics, the most common way to identify candidates is to look for map co-segregation between candidates and loci affecting the trait. The 2H black point QTL has been confirmed across a large number of populations and sites. Environmental effects have a profound influence on the expression of quantitative traits. Replication across different sites and a number of years has allowed further investigation into the environmental influences and confirmation of the 2H black point QTL. To further define this region of the genome and more accurately identify candidates through comparative mapping, fine mapping and increasing the density of markers is required. Marker saturation would allow differentiation and a more refined comparative mapping study to narrow and investigate candidates.

Recent sequencing of the Brachypodium genome will allow a more detailed analysis of the candidate genes involved in black point formation from barley. A novel approach that incorporated chromosome sorting, next-generation sequencing, array hybridisation, and systematic exploitation of conserved synteny with model grasses assigned ~86% of the estimated ~32000 barley (*Hordeum vulgare*) genes to individual chromosome arms (Mayer *et al.* 2011). As a result of this study we now have the ability to simulate the environmental

conditions in growth chambers and utilise the Brachypodium genome to investigate and confirm candidates.

Peroxidase genes *HvBP1* and *HvPrx7* as well as *HvQR* were found to be differentially expressed between tolerant and susceptible varieties during grain fill, implicating a role in black point formation. The parental varieties Sloop and Alexis differ in maturity and in plant stature, with Sloop displaying a tall, early flowering phenotype compared with the semi-dwarf, later flowering Alexis. They are known to differ at three developmental loci: a photoperiod response gene (Ppd-H1) (Laurie *et al.* 1994), an earliness per se locus (eps2) (Laurie *et al.* 1995), and a plant stature locus (*sdw1*) (Barua *et al.* 1993; Laurie *et al.* 1993; Coventry *et al.* 2003). Simulation of the identified environmental influences would allow a more detailed study on the effect of maturity on black point formation and expression of candidate genes at different maturities.

6.4 Regulation of peroxidase genes

Peroxidase genes have been confirmed to be differentially expressed between tolerant and susceptible varieties (Chapter 3). Hadaway *et al.* (2003) found that the activity of peroxidase enzymes increased during grain development. Additionally, peroxidases with a higher isoelectric point have only been found in susceptible varieties to date (Hadaway *et al.* 2003) while HvBP1 is more abundant in black pointed grains (March *et al.* 2007). Peroxidases therefore appear to play an important role in black point formation. Peroxidases are part of a large gene family (Hiraga *et al.* 2001) and individual peroxidase types may have several copies within the genome. Indeed, this study appears to have identified two copies of the *HvBP1* gene in expression studies. Differential gene

expression of the peroxidases *HvBP1* and *HvPrx7* between susceptible and tolerant cultivars was confirmed by northern analysis and qPCR. Futhermore *HvQR*, a candidate identified through comparative mapping studies by March and colleagues (2008) was confirmed to be differentially expressed.

This study only looked at a specific set of candidates identified through comparative mapping and previous knowledge of peroxidase involvement. Given that we can now simulate the environmental conditions necessary for black point formation, future research should investigate all genes that are differentially expressed during black point formation rather than the targeted approach used in this study. Genome-wide expression profiling through microarray technology offers the opportunity to screen the entire genome and regions identified through QTL studies. This can be accomplished using a closed format hybridization technology such as cDNA microarrays (Schena *et al.* 1995) or an oligonucleotide GeneChip (Lockhart *et al.* 1996). The ~8,000 gene array used by Hazen *et al.* (2003) and Chen and Chen (2002) was used to profile Arabidopsis transcriptional response to wounding stress (Cheong *et al.* 2002). Applying this technology to plants grown in the simulated environmental conditions would provide a more detailed and comprehensive analysis of the genes involved.

Differentially expressed genes elsewhere in the genome might share pathways with genes in the QTL region and reflect downstream effects of the QTL (or regulation). Consequently, this study focused on identifying areas of the genome contributing to regulation and utilised Y1H technology. *Cis*-eQTLs (identified for *HvPrx7* and *HvQR*) represent a polymorphism physically located near the gene itself, or identification of a promoter polymorphism. *Trans*-eQTLs identified for *HvBP1* (Chapter 4) are the result of a

polymorphism at a location in the genome other than the actual position of the gene whose transcript is being measured, or a polymorphism at the physical position of a regulatory factor elsewhere in the genome (Hansen *et al.* 2008). Comparative mapping between barley, wheat and rice identified potential candidates for regulation, thereby providing a data set of genes to be further investigated through expression studies. A number of transcription factors involved in stress responses were identified, including DRE-related binding factors, leucine zipper DNA-binding proteins, putative zinc finger proteins, MYB proteins, bZIP/HD-ZIPs, and AP2/EREBP (Seki *et al.* 2001; Chen *et al.* 2002).

SNPs were identified within the *ORF* of peroxidase genes *HvBP1* and *HvPrx7* between susceptible and tolerant varieties, resulting in an alteration in the amino acid sequence of the encoded protein and therefore affecting protein function directly/indirectly or interactions in a multi-protein complex by increasing/decreasing the activity (Uzun *et al.* 2007). As observed by March and colleagues, *HvBP1* was identified as present in black pointed grain and not healthy grain of the susceptible variety. SNPs within the *ORF* could therefore be a contributing factor in protein synthesis and the symptoms observed. However, whether *HvBP1* is present in the black pointed grains of the tolerant variety would need to be investigated to confirm this hypothesis.

Mutant and over-expression transgenic plants are also very useful in revealing gene interactions within complex transcriptional pathways (Hazen *et al.* 2003). To further evaluate the effects of candidate genes in black point formation and to assign functions, it would be useful to have a gene ‘knock-out’ system. An example of this is the approach used to manipulate the mechanistic end-point of stress tolerance such as over expression of superoxide dismutase in order to detoxify oxygen radicals produced under stress

(McKersie *et al.* 1996; Roxas *et al.* 1997). Transgenic plants designed to synthesize high levels of osmoprotectants show elevated levels of stress tolerance, but often suffer from deleterious pleiotropic effects such as dwarfing (Tarczynski *et al.* 1993; Romero *et al.* 1997). Gene knockout studies in the environmental conditions known to induce black point (low temperature/high humidity) would allow a direct link to black point formation to be concluded. Furthermore gene knockouts of the transcription factors identified through comparative mapping studies and the bZIP identified by Y1H would lead to a greater understanding of the regulatory pathways involved.

6.5 Conclusions

This study has successfully identified the environmental conditions that can be simulated to induce symptoms (Walker *et al.* 2008), solving a problem faced by researchers in this field. Candidate genes have been identified after confirming the black point QTL on chromosome 2H. Furthermore, candidates have been identified in the regulation of peroxidase genes. Black point probably occurs due to an environmental trigger involving low vapour pressure deficit, high humidity and low temperatures, resulting in a reaction involving germination in symptomatic grain.

A likely model is that phenolic compounds are oxidised by peroxidases and transformed to quinones resulting in black point formation. Candidate peroxidase genes are differentially expressed between susceptible and tolerant varieties implicating a role in response to stress and enzymatic browning. The observation that higher *HvQR* expression was observed in

the healthy grains of the susceptible variety Sloop, suggests that gene expression may have been induced in response to quinone formation leading to its removal through *HvQR*. Furthermore a number of stress related transcription factors have been identified in regulating *HvBP1* gene expression and a bZIP transcription factor is likely to be part of a complex series of events regulating *HvPrx7* gene expression.

Black point research has advanced to a point where the necessary environmental conditions can be induced, thus allowing larger genomic scans and investigation into current candidate genes to be undertaken. Understanding such candidates and the regulatory role they play will enable modelling scenarios to be included into breeding programs of the future to breed for tolerant varieties.

References

Aalenf, R. B., H.-G. Opsahl-Ferstad, C. Linnestad and O.-A. Olsen (1994). "Transcripts encoding an oleosin and a dormancy-related protein are present in both the aleurone layer and the embryo of developing barley (*Hordeum vulgare L.*) seeds." *The Plant Journal* **5**(3): 385-396.

Abe, H., T. Urao, T. Ito, M. Seki, K. Shinozaki and K. Yamaguchi-Shinozaki (2003). "Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) Function as Transcriptional Activators in Abscisic Acid Signaling." *The Plant Cell Online* **15**(1): 63-78.

Abe, H., K. Yamaguchi-Shinozaki, T. Urao, T. Iwasaki, D. Hosokawa and K. Shinozaki (1997). "Role of Arabidopsis MYC and MYB homologs in drought- and abscisic acid-regulated gene expression." *Plant Cell* **9**: 1859 - 1868.

Agarwal, P. and B. Jha (2010). "Transcription factors in plants and ABA dependent and independent abiotic stress signalling." *Biologia Plantarum* **54**(2): 201-212.

Alexandrova, K. and B. Conger (2002). "Isolation of two somatic embryogenesis-related genes from orchardgrass (*Dactylis glomerata*)." *Plant Sci* **162**: 301 - 307.

Anantharaman, V. and L. Aravind (2006). "Diversification of Catalytic Activities and Ligand Interactions in the Protein Fold Shared by the Sugar Isomerases, eIF2B, DeoR Transcription Factors, Acyl-CoA Transferases and Methenyltetrahydrofolate Synthetase." *Journal of Molecular Biology* **356**(3): 823-842.

Andersen, M. C., P. G. Engström, S. Lithwick, D. Arenillas, P. Eriksson, B. Lenhard, W. W. Wasserman and J. Odeberg (2008). "*In Silico* Detection of Sequence Variations Modifying Transcriptional Regulation." *PLoS Comput Biol* **4**(1): e5.

Baker, K. F. (1957). The U.C. system for producing healthy container-grown plants. , University of California**:** 1957. 1332 p..

Baniwal, S., K. Bharti, K. Chan, M. Fauth, A. Ganguli, S. Kotak, S. Mishra, L. Nover, M. Port, K.-D. Scharf, J. Tripp, C. Weber, D. Zielinski and P. von Koskull-Doring (2004). "Heat stress response in plants: a complex game with chaperones and more than twenty heat stress transcription factors." *Journal of Biosciences* **29**(4): 471-487.

Barr, A. R., S. P. Jefferies, S. Broughton, K. J. Chalmers, J. M. Kretschmer, W. J. R. Boyd, H. M. Collins, S. Roumeliotis, S. J. Logue, S. J. Coventry, D. B. Moody, B. J. Read, D. Poulsen, R. C. M. Lance, G. J. Platz, R. F. Park, J. F. Panozzo, A. Karakousis, P. Lim, A. P. Verbyla and P. J. Eckermann (2003). "Mapping and QTL analysis of the barley population Alexis x Sloop." *Australian Journal of Agricultural Research* **54**(12): 1117-1123.

Bartel, P. L. and S. Fields (1995). "Analysing protein-protein interactions using 2-hybrid system." *Oncogene Techniques* **254**: 241-263.

Barua, U. M., K. J. Chalmers, W. T. B. Thomas, C. A. Hackett, V. Lea, P. Jack, B. P. Forster, R. Waugh and W. Powell (1993). "Molecular mapping of genes determining height, time to heading, and growth habit in barley (*Hordeum vulgare*)." *Genome* **36**: 1080-1087.

Barz, W. and J. Koster (1981). *The Biochemistry of Plants*. New York.

Basson, A. B. K., O. T. de Villiers and C. J. Rabie (1990). "Effect of black ends on quality characteristics of Clipper barley and malt." *American Society of Brewing Chemists* **48**: 8-13.

Basten, C., B. S. Weir and Z.-B. Zeng (2005). QTL Cartographer. Department of Statistics, North Carolina State University, Raleigh, NC.

Batchelor, A., K. Boutilier, S. Miller, J. Hattori, L. Bowman, M. Hu, S. Lantin, D. Johnson and B. Miki (2002). "SCB1, a BURP-domain protein gene, from developing soybean seed coats." *Planta* **215**: 523 - 532.

Baumlein H, S. Misera, H. Luerssen, K. Kolle and C. Horstmann (1994). The FUS3 gene of *Arabidopsis thaliana* is a regulator of gene expression during late embryogenesis. Oxford, ROYAUME-UNI, Blackwell.

Becker, A. and G. Theißen (2003). "The major clades of MADS-box genes and their role in the development and evolution of flowering plants." *Molecular Phylogenetics and Evolution* **29**(3): 464-489.

Bhowmink, T. P. (1969). "Alternaria seed infection of wheat." *Plant Disease Reporter* **53**: 77-78.

Bielski, B. H. J., D. E. Cabelli, R. L. Arudi and A. B. Ross (1985). "Reactivity of HO_2/O_2- radicals in aqueous solution." *Journal of Physical and Chemical Reference Data* **14**: 1041-1100.

Boden, S. A., P. Langridge, G. Spangenberg and J. A. Able (2009). "TaASY1 promotes homologous chromosome interactions and is affected by deletion of Ph1." *The Plant Journal* **57**(3): 487-497.

Bolle, C. (2004). "The role of GRAS proteins in plant signal transduction and development." *Planta* **218**(5): 683-692.

Bowler, C., W. V. Camp, M. V. Montagu and D. Inze (1994). "Superoxide dismutase in plants." *Critical Reviews in Plant Science* **13**(3): 199-218.

Brem, R. B., G. Yvert, R. Clinton and L. Kruglyak (2002). "Genetic Dissection of Transcriptional Regulation in Budding Yeast." *Science* **296**(5568): 752-755.

Broholm, S. K., S. Tähtiharju, R. A. E. Laitinen, V. A. Albert, T. H. Teeri and P. Elomaa (2008). "A TCP domain transcription factor controls flower type specification along the radial axis of the Gerbera (Asteraceae) inflorescence." *Proceedings of the National Academy of Sciences* **105**(26): 9117-9122.

Brookes, A. J. (1999). "The essence of SNPs." *Gene* **234**(2): 177-186.

Brotman, Y., D. Riewe, J. Lisec, R. C. Meyer, L. Willmitzer and T. Altmann (2011). "Identification of enzymatic and regulatory genes of plant metabolism through QTL analysis in Arabidopsis." *Journal of Plant Physiology* **168**(12): 1387-1394.

Bryan, G. T., K.-S. Wu, L. Farrall, Y. Jia, H. P. Hershey, S. A. McAdams, K. N. Faulk, G. K. Donaldson, R. Tarchini and B. Valent (2000). "A Single Amino Acid Difference Distinguishes Resistant and Susceptible Alleles of the Rice Blast Resistance Gene Pi-ta." *The Plant Cell Online* **12**(11): 2033-2046.

Burton, R. A., N. J. Shirley, B. J. King, A. J. Harvey and G. B. Fincher (2004). "The CesA Gene Family of Barley. Quantitative Analysis of Transcripts Reveals Two Groups of Co-Expressed Genes." *Plant Physiology* **134**(1): 224-236.

Bystrykh, L., E. Weersing, B. Dontje, S. Sutton, M. T. Pletcher, T. Wiltshire, A. I. Su, E. Vellenga, J. Wang, K. F. Manly, L. Lu, E. J. Chesler, R. Alberts, R. C. Jansen, R. W. Williams, M. P. Cooke and G. d. Haan (2005). "Uncovering regulatory pathways that affect hematopoietic stem cell function using 'genetical genomics'." *Nature* **37**(3).

Casaretto, J. and T.-h. D. Ho (2003). "The Transcription Factors HvABI5 and HvVP1 Are Required for the Abscisic Acid Induction of Gene Expression in Barley Aleurone Cells." *The Plant Cell Online* **15**(1): 271-284.

Chantha, S.-C., B. Emerald and D. Matton (2006). "Characterization of the plant Notchless homolog, a WD repeat protein involved in seed development." *Plant Molecular Biology* **62**(6): 897-912.

Chen, C. and Z. Chen (2002). "Potentiation of developmentally regulated plant defense response by AtWRKY18, a pathogen-induced Arabidopsis transcription factor." *Plant Physiol* **129**: 706 - 716.

Chen, L., D. Segal, N. Hukriede, A. Podtelejnikov, D. Bayarsaihan, J. Kennison, V. Ogryzko, I. Dawid and H. Westphal (2002). "Ssdp proteins interact with the LIM-domain-binding protein Ldb1 to regulate development." *Proc Natl Acad Sci U S A* **99**: 14320 - 14325.

Chen, R., Z. Ni, X. Nie, Y. Qin, G. Dong and Q. Sun (2005). "Isolation and characterization of genes encoding Myb transcription factor in wheat (*Triticum aestivem L.*)." *Plant Science* **169**(6): 1146-1154.

Chen, W., N. Provart, J. Glazebrook, F. Katagiri, H. Chang, T. Eulgem, F. Mauch, S. Luan, G. Zou, S. Whitham, P. Budworth, Y. Tao, Z. Xie, X. Chen, S. Lam, J. Kreps, J. Harper, A. Si-Ammour, B. Mauch-Mani, M. Heinlein, K. Kobayashi, T. Hohn, J. Dangl, X. Wang and T. Zhu (2002). "Expression profile matrix of Arabidopsis transcription factor genes suggests their putative functions in response to environmental stresses." *Plant Cell* **14**: 559 - 574.

Chen, X., C. A. Hackett, R. E. Niks, P. E. Hedley, C. Booth, A. Druka, T. C. Marcel, A. Vels, M. Bayer, I. Milne, J. Morris, L. Ramsay, D. Marshall, L. Cardle and R. Waugh (2010). "An eQTL Analysis of Partial Resistance to *Puccinia hordei in Barley*." *PLoS ONE* **5**(1): e8598.

Cheong, Y., H. Chang, R. Gupta, X. Wang, T. Zhu and S. Luan (2002). "Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses in Arabidopsis." *Plant Physiology* **129**: 661 - 677.

Choi, D., J. H. Kim and H. Kende (2004). "Whole Genome Analysis of the OsGRF Gene Family Encoding Plant-Specific Putative Transcription Activators in Rice (Oryza sativa L.)." *Plant and Cell Physiology* **45**(7): 897-904.

Ciftci-Yilmaz, S. and R. Mittler (2008). "The zinc finger network of plants." *Cellular and Molecular Life Sciences* **65**(7): 1150-1160.

Cochrane, M. P. (1994a). "Observations on the germ aleurone of barley phenol oxidase and peroxidase activity." *Annals of Botany* **73**: 121-128.

Cochrane, M. P. (1994b). "Observations of the germ aleurone of barley. Morphology and histology." *Annals of Botany* **73**: 113-119.

Cohen, R., M. R. Suzuki and K. E. Hammel (2004). "Differential Stress-Induced Regulation of Two Quinone Reductases in the Brown Rot Basidiomycete *Gloeophyllum trabeum*." *Applied and Environmental Microbiology* **70**(1): 324-331.

Conner, J. and Z. Liu (2000). "LEUNIG, a putative transcriptional corepressor that regulates AGAMOUS expression during flower development." *Proceedings of the National Academy of Sciences* **97**(23): 12902-12907.

Conner, R. L. (1987). "Influence of irrigation timing on black point incidence in soft white spring wheat." *Canadian Journal of Plant Pathology* **9**: 301-306.

Conner, R. L., J. M. Carefoot, J. B. Bole and G. C. Kozub (1992). "The effect of nitrogen fertilizer and irrigation on black point incidence in soft white spring wheat." *Plant and Soil* **140**: 41-47.

Conner, R. L. and J. G. N. Davidson (1988). "Resistance in wheat to black point caused by *Alternaria-Alternata* and *Cochliobolus-Sativus*." *Canadian Journal of Plant Science* **68**(2): 351-359.

Conner, R. L., S. F. Hwang and R. R. Stevens (1996). "*Fusarium proliferatum*: a new causal agent of black point in wheat." *Canadian Journal of Plant Pathology* **18**: 419-423.

Conner, R. L. and A. D. Kuzyk (1988). "Effectiveness of fungicides in controlling stripe rust, leaf spot and black point in soft white spring wheat." *Canadian Journal of Plant Pathology* **10**: 321-326.

Conway, W. S., C. Yao, G. S. Ross and C. E. Sams (1998). "Apple polygalacturonase-inhibiting protein cDNA cloning and transcription." *ICPP* **98**: 5.4.11.

Cormack, R., T. Eulgem, P. Rushton, P. Kochner, K. Hahlbrock and I. Somssich (2002). "Leucine zipper-containing WRKY proteins widen the spectrum of immediate early elicitor-induced WRKY transcription factors in parsley." *Biochim Biophys Acta* **1576**: 92 - 100.

Cormack, R. S., T. Eulgem, P. J. Rushton, P. Köchner, K. Hahlbrock and I. E. Somssich (2002). "Leucine zipper-containing WRKY proteins widen the spectrum of immediate early elicitor-induced WRKY transcription factors in parsley." *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression* **1576**(1-2): 92-100.

Costa, M. M. R., S. Fox, A. I. Hanna, C. Baxter and E. Coen (2005). "Evolution of regulatory interactions controlling floral asymmetry." *Development* **132**(22): 5093-5101.

Coventry, S. J., A. R. Barr, J. K. Eglinton and G. K. McDonald (2003). "The determinants and genome locations influencing grain weight and size in barley (*Hordeum vulgare* L.)." *Australian Journal of Agricultural Research* **54**(12): 1103-1115.

Cromey, M. G. and R. I. Mulholland (1988). "Blackpoint of wheat: fungal associations, cultivar susceptibility, and effects on grain weight and germination." *New Zealand Journal of Agricultural Research* **31**: 51-55.

Dai, S., Z. Zhang, S. Chen and R. N. Beachy (2004). "RF2b, a rice bZIP transcription activator, interacts with RF2a and is involved in symptom development of rice tungro disease." *Proceedings of the National Academy of Sciences of the United States of America* **101**(2): 687-692.

de la Penna, R. C., K. P. Smith, F. Capettini, G. J. Muehlbauer, M. Gallo-Meagher, R. Dill-Macky, D. A. Sommers and D. C. Rasmusson (1999). "Quantitative trait loci associated with resistance to Fusarium head blight and kernal discoloration in barley." *Theor Appl Genet* **99**: 561-569.

Department of Primary Industries and Fisheries. (2005). 2008, from http://www.daff.gov.au/.

Deplancke, B., D. Dupuy, M. Vidal and A. J. M. Walhout (2004). "A Gateway-Compatible Yeast One-Hybrid System." *Genome Research* **14**(10b): 2093-2101.

Desjardins, A. E., M. Busman, R. H. Proctor and R. Stessman (2007). "Wheat kernel black point and fumonisin contamination by *Fusarium proliferatum*." *Food Additives and Contaminants* **24**(10): 1131-1137.

Devos, K. M., T. Millan and M. D. Gale (1993). "Comparative RFLP maps of the homoeologous group-2 chromosomes of wheat, rye and barley." *Theoretical and Applied Genetics* **85**(6): 784-792.

Ding, X.-P., H. Xin, X. Kabin and X. Lizhong (2009). "Genome-wide identification of BURP domain-containing genes in rice reveals a gene family with diverse structures and responses to abiotic stresses." *Planta* **230**: 149 - 163.

Ding, X., X. Hou, K. Xie and L. Xiong (2009). "Genome-wide identification of BURP domain-containing genes in rice reveals a gene family with diverse structures and responses to abiotic stresses." *Planta* **230**(1): 149-163.

Doderer, A., I. Kokkelink, S. van der Veen, B. E. Valk, A. Schram and A. C. Douma (1992). "Purification and characterization of two lipoxygenase isoenzymes from

germinating barley." *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology* **1120**(1): 97-104.

Doerge, R. W. (2002). "Mapping and analysis of quantitative trait loci in experimental populations." *Nature Review Genetics* **3**(1): 43-52.

Droillard, M. J., A. Paulin and J. C. Massot (1987). "Free radical production, catalase and superoxide dismutase activities and membrane integrity during senescence of petals of cut carnations (*Dianthus caryophyllus*)." *Physiologia Plantarum* **71**(2): 197-202.

Druka, A., E. Potokina, Z. Luo, N. Jiang, X. Chen, M. Kearsey and R. Waugh (2010). "Expression quantitative trait loci analysis in plants." *Plant Biotechnology Journal* **8**(1): 10-27.

Dubcovsky, J., M. C. Luo, G. Y. Zhong, R. Bransteitter, A. Desai, A. Kilian, A. Kleinhofs and J. Dvorak (1996). "Genetic Map of Diploid Wheat, Triticum monococcum L., and Its Comparison With Maps of Hordeum vulgare L." *Genetics* **143**(2): 983-999.

Duek, P. D. and C. Fankhauser (2005). "bHLH class transcription factors take centre stage in phytochrome signalling." *Trends in Plant Science* **10**(2): 51-54.

Dunford, H. B. (1991). *Peroxidase in Biology and Chemistry*. Boca Raton

Edwards, D., Forster,J.W.,Cogan,N.O.I.,Batley,J.,Chagne, D (2007). Single nucleotide polymorphism discovery, in:Association Mapping in Plants, Springer, NewYork.

Ellis, J. G., J. G. Tokuhisa, D. J. Llewellyn, D. Bouchez, K. Singh, E. S. Dennis and W. J. Peacock (1993). "Does the ocs-element occur as a functional component of the promoters of plant genes?" *The Plant Journal* **4**(3): 433-443.

Ellis, S. A., M. J. Gooding and A. J. Thompson (1996). "Factors influencing the relative susceptibility of wheat cultivars (*Triticum aestivum* L.) to blackpoint." *Crop Protection* **15**: 69-76.

Eulgem, T., P. Rushton, S. Robatzek and I. Somssich (2000). "The WRKY superfamily of plant transcription factors." *Trends Plant Sci* **5**: 199 - 206.

Eulgem, T., P. Rushton, E. Schmelzer, K. Hahlbrock and I. Somssich (1999). "Early nuclear events in plant defence signalling: rapid gene activation by WRKY transcription factors." *EMBO Journal* **18**: 4689 - 4699.

Eulgem, T., P. J. Rushton, S. Robatzek and I. E. Somssich (2000). "The WRKY superfamily of plant transcription factors." *Trends Plant Sci* **5**(5): 199-206.

Eulgem, T., P. J. Rushton, E. Schmelzer, K. Hahlbrock and I. E. Somssich (1999). "Early nuclear events in plant defence signalling: rapid gene activation by WRKY transcription factors." *EMBO J* **18**(17): 4689-4699.

Favaron, F., R. D'Ovidio, E. Porceddu and P. Alghisi (1994). "Purification and molecular characterization of a soybean polygalacturonase-inhibiting protein." *Planta* **195**(1): 80-87.

Feng, J.-X., D. Liu, Y. Pan, W. Gong, L.-G. Ma, J.-C. Luo, X. Deng and Y.-X. Zhu (2005). "An Annotation Update via cDNA Sequence Analysis and Comprehensive Profiling of Developmental, Hormonal or Environmental Responsiveness of the Arabidopsis; AP2/EREBP Transcription Factor Gene Family." *Plant Molecular Biology* **59**(6): 853-868.

Fernandez, M. R., R. M. Clarke, R. M. DePauw, R. B. Irvine and R. E. Knox (1994). "Black point and red smudge in irrigated durum wheat in southern Saskatchewan in 1990-1992." *Canadian Journal of Plant Pathology* **16**: 221-227.

Fernandez, M. R., R. M. Clarke, R. M. DePauw, R. B. Irvine and R. E. Knox (1994). "Black point and red smudge in irrigated durum wheat in southern Saskatchewan in 1990-1992." *Canadian Journal of Plant Pathology* **16**: 221-227.

Feussner, I., H. Kühn and C. Wasternack (2001). "Lipoxygenase-dependent degradation of storage lipids." *Trends in Plant Science* **6**(6): 268-273.

Finkelstein, R. R. and T. J. Lynch (2000). "The Arabidopsis Abscisic Acid Response Gene ABI5 Encodes a Basic Leucine Zipper Transcription Factor." *The Plant Cell Online* **12**(4): 599-610.

Fisk, D. G., M. B. Walker and A. Barkan (1999). "Molecular cloning of the maize gene crp1 reveals similarity between regulators of mitochondrial and chloroplast gene expression." *EMBO J* **18**(9): 2621-2630.

Fridborg, I., S. Kuusk, M. Robertson and E. Sundberg (2001). "The Arabidopsis Protein SHI Represses Gibberellin Responses in Arabidopsis and Barley." *Plant Physiology* **127**(3): 937-948.

Fu, J., J. J. B. Keurentjes, H. Bouwmeester, T. America, F. W. A. Verstappen, J. L. Ward, M. H. Beale, R. C. H. de Vos, M. Dijkstra, R. A. Scheltema, F. Johannes, M. Koornneef, D. Vreugdenhil, R. Breitling and R. C. Jansen (2009). "System-wide molecular evidence for phenotypic buffering in Arabidopsis." *Nat Genet* **41**(2): 166-167.

Fujimoto, S. Y., M. Ohta, A. Usui, H. Shinshi and M. Ohme-Takagi (2000). "Arabidopsis Ethylene-Responsive Element Binding Factors Act as Transcriptional Activators or Repressors of GCC Box–Mediated Gene Expression." *The Plant Cell Online* **12**(3): 393-404.

Gan, Y., C. Liu, H. Yu and P. Broun (2007). "Integration of cytokinin and gibberellin signalling by Arabidopsis transcription factors GIS, ZFP8 and GIS2 in the regulation of epidermal cell fate." *Development* **134**(11): 2073-2081.

Garcés-Claver, A., S. Fellman, R. Gil-Ortega, M. Jahn and M. Arnedo-Andrés (2007). "Identification, validation and survey of a single nucleotide polymorphism (SNP) associated with pungency in *Capsicum*." *Theoretical and Applied Genetics* **115**(7): 907-916.

Gaudet, D. A., A. Laroche, M. Frick, R. Huel and B. Puchalski (2003). "Plant development affects the cold-induced expression of plant defence-related transcripts in winter wheat." *Physiological and Molecular Plant Pathology* **62**(3): 175-184.

Gaudin, V., P. A. Lunness, P. R. Fobert, M. Towers, C. Riou-Khamlichi, J. A. H. Murray, E. Coen and J. H. Doonan (2000). "The Expression of D-Cyclin Genes Defines Distinct Developmental Zones in Snapdragon Apical Meristems and Is Locally Regulated by the Cycloidea Gene." *Plant Physiology* **122**(4): 1137-1148.

Genissel, A., L. M. McIntyre, M. L. Wayne and S. V. Nuzhdin (2008). "Cis and trans regulatory effects contribute to natural variation in transcriptome of Drosophila melanogaster." *Molecular Biology and Evolution* **25**(1): 101-110.

Gilad, Y., A. Oshlack and S. A. Rifkin (2006). "Natural selection on gene expression." *Trends in Genetics* **22**(8): 456-461.

Gilad, Y., S. A. Rifkin and J. K. Pritchard (2008). "Revealing the architecture of gene regulation: the promise of eQTL studies." *Trends in Genetics* **24**(8): 408-415.

Görner, W., E. Durchschlag, M. T. Martinez-Pastor, F. Estruch, G. Ammerer, B. Hamilton, H. Ruis and C. Schüller (1998). "Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity." *Genes & Development* **12**(4): 586-597.

Grafi, G., A. Zemach and L. Pitto "Methyl-CpG-binding domain (MBD) proteins in plants." *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression* **1769**(5-6): 287-294.

GRDC. (2010). "Australian Grains Focus 2011-12." from www.grdc.com.au.

Gubler, F., P. M. Chandler, R. G. White, D. J. Llewellyn and J. V. Jacobsen (2002). "Gibberellin Signaling in Barley Aleurone Cells. Control of SLN1 and GAMYB Expression." *Plant Physiology* **129**(1): 191-200.

Gubler, F., R. Kalla, J. K. Roberts and J. V. Jacobsen (1995). "Gibberellin-Regulated Expression of a myb Gene in Barley Aleurone Cells: Evidence for Myb Transactivation of a High-pl [alpha]-Amylase Gene Promoter." *The Plant Cell Online* **7**(11): 1879-1891.

Gubler, F., R. J. Watts, R. Kalla, P. Matthews, M. Keys and J. V. Jacobsen (1997). "Cloning of a Rice cDNA Encoding a Transcription Factor Homologous to Barley GAMyb." *Plant and Cell Physiology* **38**(3): 362-365.

Guiltinan, M., W. Marcotte and R. Quatrano (1990). "A plant leucine zipper protein that recognizes an abscisic acid response element." *Science* **250**(4978): 267-271.

Hadaway, T. K. (2002). The genetic and biochemical basis for black point in barley. Hons Thesis. Department of Plant Science. Adelaide, South Australia, University of Adelaide**:** 71.

Hadaway, T. K., T. J. March and A. J. Able (2003). The involvement of peroxidases in the formation of black point in barley. Proceedings of the 11th Australian Barley Technical Symposium, Adelaide.

Halliwell, B. and J. M. C. Gutteridge (1989). Free radicals in biology and medicine. Oxford, Clarendon Press.

Hansen, B. G., B. A. Halkier and D. J. Kliebenstein (2008). "Identifying the molecular basis of QTLs: eQTLs add a new dimension." *Trends in Plant Science* **13**(2): 72-77.

Hara, K., M. Yagi, T. Kusano and H. Sano (2000). "Rapid systemic accumulation of transcripts encoding a tobacco WRKY transcription factor upon wounding." *Mol Gen Genet* **263**: 30 - 37.

Harborne, J. B. (1979). "Variation and functional significance of phenolic conjugation in plants." *Biochemistry of plant phenolics*: 457-474.

Hartmann, U., S. Höhmann, K. Nettesheim, E. Wisman, H. Saedler and P. Huijser (2000). "Molecular cloning of SVP: a negative regulator of the floral transition in Arabidopsis." *The Plant Journal* **21**(4): 351-360.

Hazen, S. P., Y. Wu and J. A. Kreps (2003). "Gene expression profiling of plant responses to abiotic stress." *Functional & Integrative Genomics* **3**(3): 105-111.

Heckmann, A. B., F. Lombardo, H. Miwa, J. A. Perry, S. Bunnewell, M. Parniske, T. L. Wang and J. A. Downie (2006). "Lotus japonicus Nodulation Requires Two GRAS Domain Regulators, One of Which Is Functionally Conserved in a Non-Legume." *Plant Physiology* **142**(4): 1739-1750.

Hellewell, K. B., D. C. Rasmusson and M. Gallo-Meagher (2000). "Enhancing yield of semi-dwarf barley " *Crop Science* **40**: 352-358.

Hervé, C., P. Dabos, C. Bardet, A. Jauneau, M. C. Auriac, A. Ramboer, F. Lacout and D. Tremousaygue (2009). "In Vivo Interference with AtTCP20 Function Induces Severe Plant Growth Alterations and Deregulates the Expression of Many Genes Important for Development." *Plant Physiology* **149**(3): 1462-1477.

Higo, K., Y. Ugawa, M. Iwamoto and T. Korenaga (1999). "Plant cis-acting regulatory DNA elements (PLACE) database: 1999." *Nucleic Acids Res* **27**: 297 - 300.

Higo, K., Y. Ugawa, M. Iwamoto and T. Korenaga (1999). "Plant cis-acting regulatory DNA elements (PLACE) database: 1999." *Nucleic Acids Research* **27**(1): 297-300.

Himmelbach, A., T. Hoffmann, M. Leube, B. Hohener and E. Grill (2002). "Homeodomain protein ATHB6 is a target of the protein phosphatase ABI1 and regulates hormone responses in Arabidopsis." *EMBO J* **21**(12): 3029-3038.

Hiraga, S., K. Sasaki, H. Ito, Y. Ohashi and H. Matsui (2001). "A Large Family of Class III Plant Peroxidases." *Plant and Cell Physiology* **42**(5): 462-468.

Hobo, T., Y. Kowyama and T. Hattori (1999). "A bZIP factor, TRAB1, interacts with VP1 and mediates abscisic acid-induced transcription." *Proceedings of the National Academy of Sciences* **96**(26): 15348-15353.

Hossain, M. A., J.-I. Cho, M. Han, C.-H. Ahn, J.-S. Jeon, G. An and P. B. Park (2010). "The ABRE-binding bZIP transcription factor OsABF2 is a positive regulator of abiotic stress and ABA signaling in rice." *Journal of Plant Physiology* **167**(17): 1512-1520.

Hsing, Y.-i. C., C.-h. Tsou, T.-f. Hsu, Z.-y. Chen, K.-l. Hsieh, J.-s. Hsieh and T.-y. Chow (1998). "Tissue- and stage-specific expression of a soybean (*Glycine max L.*) seed-maturation, biotinylated protein." *Plant Molecular Biology* **38**(3): 481-490.

Hu, Y. X., Y. H. Wang, X. F. Liu and J. Y. Li (2004). "Arabidopsis RAV1 is down-regulated by brassinosteroid and may act as a negative regulator during plant development." *Cell Research* **14**(1): 8-15.

Huang, T. and J. Duman (2002). "Cloning and characterization of a thermal hysteresis (antifreeze) protein with DNA-binding activity from winter bittersweet nightshade, Solanum dulcamara." *Plant Mol Biol* **48**: 339 - 350.

Huang, Y.-J., K.-Y. To, M.-N. Yap, W.-J. Chiang, D.-F. Suen and S.-C. G. Chen (2001). "Cloning and characterization of leaf senescence up-regulated genes in sweet potato." *Physiologia Plantarum* **113**(3): 384-391.

Hubner, N., C. A. Wallace, H. Zimdahl, E. Petretto, H. Schulz, F. Maciver, M. Mueller, O. Hummel, J. Monti, V. Zidek, A. Musilova, V. Kren, H. Causton, L. Game, G. Born, S. Schmidt, A. Muller, S. A. Cook, T. W. Kurtz, J. Whittaker, M. Pravenec and T. J. Aitman (2005). "Integrated transcriptional profiling and linkage analysis for identification of genes underlying disease." *Nature Genetics* **37**(3): 243-253.

Hudec, K. (2007). "Pathogenicity of fungi associated with wheat and barley seedling emergence and fungicide efficacy of seed treatment." *Biologia* **62**(3): 287-291.

Hyde, M. B. and H. B. Galleymore (1951). "The subepidermal fungi of cereal grains. 2. The nature, identity and origin of the mycelium in wheat." *Annals of Applied Biology* **38**(348-347).

Islam, A., K. W. Shepherd and D. H. B. Sparrow (1981). "Isolation and characterization of euplasmic wheat-barley chromosome addition lines." *Heredity* **46**(2): 161-174.

Jacobs, B. and C. J. Rabie (1987). "The correlation between mycelial presence and black point in barley." *Phytophylactica* **19**: 77-81.

Jakoby, M., B. Weisshaar, W. Dröge-Laser, J. Vicente-Carbajosa, J. Tiedemann, T. Kroj and F. Parcy (2002). "bZIP transcription factors in Arabidopsis." *Trends in Plant Science* **7**(3): 106-111.

Jamar, C., F. Loffet, P. Frettinger, L. Ramsay, M.-L. Fauconnier and P. du Jardin (2010). "NAM-1gene polymorphism and grain protein content in Hordeum." *Journal of Plant Physiology* **167**(6): 497-501.

Jane, G. (2001). "Genes controlling expression of defense responses in Arabidopsis — 2001 status." *Current Opinion in Plant Biology* **4**(4): 301-308.

Jansen, R. C. and J.-P. Nap (2001). "Genetical genomics: the added value from segregation." *Trends in Genetics* **17**(7): 388-391.

Jensen, R. B., K. Lykke-Andersen, G. I. Frandsen, H. B. Nielsen, J. Haseloff, H. M. Jespersen, J. Mundy and K. Skriver (2000). "Promiscuous and specific phospholipid binding by domains in ZAC, a membrane-associated Arabidopsis protein with an ARF GAP zinc finger and a C2 domain." *Plant Molecular Biology* **44**(6): 799-814.

John, S. (2003). "Cell-fate specification in the epidermis: a common patterning mechanism in the root and shoot." *Current Opinion in Plant Biology* **6**(1): 74-78.

Johnson, C., B. Kolevski and D. Smyth (2002). "TRANSPARENT TESTA GLABRA2, a trichome and seed coat development gene of Arabidopsis, encodes a WRKY transcription factor." *Plant Cell* **14**: 1359 - 1375.

Jordan, M. C., D. J. Somers and T. W. Banks (2007). "Identifying regions of the wheat genome controlling seed development by mapping expression quantitative trait loci." *Plant Biotechnology Journal* **5**(3): 442-453.

Jung, J., S. Won, S. Suh, H. Kim, R. Wing, Y. Jeong, I. Hwang and M. Kim (2007). "The barley ERF-type transcription factor HvRAF confers enhanced pathogen resistance and salt tolerance in *Arabidopsis*." *Planta* **225**(3): 575-588.

Kaló, P., C. Gleason, A. Edwards, J. Marsh, R. M. Mitra, S. Hirsch, J. Jakab, S. Sims, S. R. Long, J. Rogers, G. B. Kiss, J. A. Downie and G. E. D. Oldroyd (2005). "Nodulation Signaling in Legumes Requires NSP2, a Member of the GRAS Family of Transcriptional Regulators." *Science* **308**(5729): 1786-1789.

Kandasamy, M. K., E. C. McKinney and R. B. Meagher (2003). "Cell cycle-dependent association of Arabidopsis actin-related proteins AtARP4 and AtARP7 with the nucleus." *The Plant Journal* **33**(5): 939-948.

Kent Bradford, H. N. (2007). *Seed Development, Dormancy and Germination*, Blackwell Publishing.

Keurentjes, J. J. B., J. Fu, I. R. Terpstra, J. M. Garcia, G. van den Ackerveken, L. B. Snoek, A. J. M. Peeters, D. Vreugdenhil, M. Koornneef and R. C. Jansen (2007). "Regulatory network construction in Arabidopsis by using genome-wide gene expression quantitative trait loci." *Proceedings of the National Academy of Sciences* **104**(5): 1708-1713.

Kharabian, A. (2010). "An efficient computational method for screening functional SNPs in plants." *Journal of Theoretical Biology* **265**(1): 55-62.

Kim, J. and H.-Y. Kim (2006). "Molecular characterization of a bHLH transcription factor involved in Arabidopsis abscisic acid-mediated response." *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression* **1759**(3-4): 191-194.

Kim, J. H., D. Choi and H. Kende (2003). "The AtGRF family of putative transcription factors is involved in leaf and cotyledon growth in Arabidopsis." *The Plant Journal* **36**(1): 94-104.

Kim, J. Y., Y. S. Seo, J. E. Kim, S.-K. Sung, K. J. Song, G. An and W. T. Kim (2001). "Two polyphenol oxidases are differentially expressed during vegetative and reproductive development and in response to wounding in the Fuji apple." *Plant Science* **161**(6): 1145-1152.

Kim, S. (2007). "Recent advances in ABA signaling." *Journal of Plant Biology* **50**(2): 117-121.

Kim, S. Y., H.-J. Chung and T. L. Thomas (1997). "Isolation of a novel class of bZIP transcription factors that interact with ABA-responsive and embryo-specification elements in the Dc3 promoter using a modified yeast one-hybrid system." *The Plant Journal* **11**(6): 1237-1251.

Kirst, M., A. A. Myburg, J. P. G. De León, M. E. Kirst, J. Scott and R. Sederoff (2004). "Coordinated Genetic Regulation of Growth and Lignin Revealed by Quantitative Trait Locus Analysis of cDNA Microarray Data in an Interspecific Backcross of Eucalyptus." *Plant Physiology* **135**(4): 2368-2378.

Knight, H. and M. R. Knight (2001). "Abiotic stress signalling pathways: Specificity and cross-talk." *Trends in Plant Science* **6**(6): 262-267.

Konyecsni, W. M. and V. Deretic (1990). "DNA sequence and expression analysis of algP and algQ, components of the multigene system transcriptionally regulating

mucoidy in *Pseudomonas aeruginosa*: algP contains multiple direct repeats." *J. Bacteriol.* **172**(5): 2511-2520.
Kosugi, S. and Y. Ohashi (2002). "DNA binding and dimerization specificity and potential targets for the TCP protein family." *The Plant Journal* **30**(3): 337-348.
Koyama, T., M. Furutani, M. Tasaka and M. Ohme-Takagi (2007). "TCP Transcription Factors Control the Morphology of Shoot Lateral Organs via Negative Regulation of the Expression of Boundary-Specific Genes in Arabidopsis." *The Plant Cell Online* **19**(2): 473-484.
Kristensen, B. K., H. Bloch and S. K. Rasmussen (1999). "Barley coleoptile peroxidases. Purification, molecular cloning, and induction by pathogens." *Plant Physiology* **120**: 501-512.
Kristensen, B. K., J. Brandt, K. Bojsen, H. Thordal-Christensen, K. B. Kerby, D. B. Collinge, J. D. Mikkelsen and S. K. Rasmussen (1997). "Expression of a defence-related intercellular barley peroxidase in transgenic tobacco." *Plant Science* **122**(2): 173-182.
Kumar, J., P. Schäfer, R. Hückelhoven, G. Langen, H. Baltruschat, E. Stein, S. Nagarajan and K.-H. Kogel (2002). "*Bipolaris sorokiniana*, a cereal pathogen of global concern: cytological and molecular approaches towards better control‡." *Molecular Plant Pathology* **3**(4): 185-195.
Kusaba, S., M. Fukumoto, C. Honda, I. Yamaguchi, T. Sakamoto and Y. Kano-Murakami (1998). "Decreased GA1 Content Caused by the Overexpression ofOSH1 Is Accompanied by Suppression of GA 20-Oxidase Gene Expression." *Plant Physiology* **117**(4): 1179-1184.
Laurie, D. A., J. H. Pratchett, J. H. Bezant and J. W. Snape (1995). "RFLP mapping of five major genes and eight quantitative trait loci controlling flowering time in a winter x spring barley (*Hordeum vulgare* L.) cross." *Genome* **38**: 575-585.
Laurie, D. A., N. Pratchett, J. H. Bezant and J. W. Snape (1994). "Genetic analysis of a photoperiod response gene on the short arm of chromosome 2 (2H) of *Hordeum vulgare* (barley)." *Heredity* **vol. 72** 619-627.
Laurie, D. A., N. Pratchett, K. M. Devos, I. J. Leitch and M. D. Gale (1993). "The distribution of RFLP markers on chromosome 2(2H) of barley in relation to the physical and genetic location of 5S rDNA." *Theoretical and Applied Genetics* **87**: 177-183.
Lee, H., S.-S. Suh, E. Park, E. Cho, J. H. Ahn, S.-G. Kim, J. S. Lee, Y. M. Kwon and I. Lee (2000). "The AGAMOUS-LIKE 20 MADS domain protein integrates floral inductive pathways in Arabidopsis." *Genes & Development* **14**(18): 2366-2376.
Lee, J.-H., J.-P. Hong, S.-K. Oh, S. Lee, D. Choi and W. Kim (2004). "The ethylene-responsive factor like protein 1 (CaERFLP1) of hot pepper (*Capsicum annuum L.*) interacts in vitro with both GCC and DRE/CRT sequences with different binding affinities: Possible biological roles of CaERFLP1 in response to pathogen infection and high salinity conditions in transgenic tobacco plants." *Plant Molecular Biology* **55**(1): 61-81.
Lee, T. I. and R. A. Young (2000). "Transcription of Eukaryotic Protein-coding genes." *Annual Review of Genetics* **34**(1): 77-137.
Lee, Y.-H. and J.-Y. Chun (1998). "A new homeodomain-leucine zipper gene from Arabidopsis thaliana induced by water stress and abscisic acid treatment." *Plant Molecular Biology* **37**(2): 377-384.
Lehmensiek, A., A. W. Campbell, P. M. Williamson, M. Michalowitz, M. W. Sutherland and G. Daggard (2004). "QTLs for black point resistance in wheat and the

identification of potential markers for use in breeding programs." *Plant Breeding* **123**(5): 410-416.

Li, C. D., R. C. M. Lance, H. M. Collins, A. Tarr, S. Roumeliotis, S. Harasymow, M. Cakir, G. P. Fox, C. R. Grime, S. Broughton, K. J. Young, H. Raman, A. R. Barr, D. B. Moody and B. J. Read (2003). "Quantitative trait loci controlling kernel discoloration in barley (Hordeum vulgare L.)." *Australian Journal of Agricultural Research* **54**: 1251-1259.

Linder, T., C. Park, J. Asin-Cayuela, M. Pellegrini, N.-G. Larsson, M. Falkenberg, T. Samuelsson and C. Gustafsson (2005). "A family of putative transcription termination factors shared amongst metazoans and plants." *Current Genetics* **48**(4): 265-269.

Lindsay, W. P., F. M. McAlister, Q. Zhu, X.-Z. He, W. Dröge-Laser, S. Hedrick, P. Doerner, C. Lamb and R. A. Dixon (2002). "KAP-2, a protein that binds to the H-box in a bean chalcone synthase promoter, is a novel plant transcription factor with sequence identity to the large subunit of human Ku autoantigen." *Plant Molecular Biology* **49**(5): 503-514.

Liu, L., K.-E. L. Eriksson and J. F. D. Dean (1999). "Localization of hydrogen peroxide production in Zinnia elegans L. stems." *Phytochemistry* **52**(4): 545-554.

Lockhart, D. J., H. Dong, M. C. Byrne, M. T. Follettie, M. V. Gallo, M. S. Chee, M. Mittmann, C. Wang, M. Kobayashi, H. Norton and E. L. Brown (1996). "Expression monitoring by hybridization to high-density oligonucleotide arrays." *Nat Biotech* **14**(13): 1675-1680.

Lopato, S., N. Bazanova, S. Morran, A. Milligan, N. Shirley and P. Langridge (2006). "Isolation of plant transcription factors using a modified yeast one-hybrid system." *Plant Methods* **2**(1): 3.

López-Juez, E., E. Dillon, Z. Magyar, S. Khan, S. Hazeldine, S. M. de Jager, J. A. H. Murray, G. T. S. Beemster, L. Bögre and H. Shanahan (2008). "Distinct Light-Initiated Gene Expression and Cell Cycle Programs in the Shoot Apex and Cotyledons of Arabidopsis." *The Plant Cell Online* **20**(4): 947-968.

Lopez-Molina, L., S. Mongrand and N.-H. Chua (2001). "A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in Arabidopsis." *Proceedings of the National Academy of Sciences* **98**(8): 4782-4787.

Lynn, D. G. and M. Chang (1990). "Phenolic Signals in Cohabitation: Implications for Plant Development." *Annual Review of Plant Physiology and Plant Molecular Biology* **41**(1): 497-526.

Lysenko, E. (2007). "Plant sigma factors and their role in plastid transcription." *Plant Cell Reports* **26**(7): 845-859.

Machacek, J. E. and F. J. Greaney (1938). "The black point or kernal smudge disease of cereals." *Canadian Journal of Research* **16**: 84-113.

Madariaga, B. R. and Z. M. Mellado (1988). "Study on black point disease on spring cultivars sown in the south-central part of Chile." *Agricultura Técnica* **48**: 42-45.

Mak, Y., R. D. Willows, T. H. Roberts, C. W. Wrigley, P. J. Sharp and L. E. S. Copeland (2006). "Black Point is associated with reduced levels of stress, disease- and defence-related proteins in wheat grain." *Molecular Plant Pathology* **7**(3): 177-189.

Mallory, A. C., D. P. Bartel and B. Bartel (2005). "MicroRNA-Directed Regulation of Arabidopsis AUXIN RESPONSE FACTOR17 Is Essential for Proper Development and Modulates Expression of Early Auxin Response Genes." *The Plant Cell Online* **17**(5): 1360-1375.

Maloy, O. C. and K. L. Specht (1988). "Black point of irrigated wheat in central Washington." *Journal of Plant Diseases and Protection* **72**: 1031-1033.
Manly, K. F., J. R. H. Cudmore and J. M. Meer (2001). "Map Manager QTX, cross-platform software for genetic mapping." *Mammalian Genome* **12**(12): 930-932.
March, T. J. (2003). Black Point-Related Gene Expression during Barley Grain Development. Hons Thesis. Department of Plant and Pest Science. South Australia., University of Adelaide**:** 69.
March, T. J., J. A. Able, K. Willsmore, C. J. Schultz and A. J. Able (2008). "Comparative mapping of a QTL controlling black point formation in barley." *Functional Plant Biology* **35**(5): 427-437.
March, T. J., J. A. Jason, C. J. Schultz and A. J. Able (2007). "A novel late embryogenesis abundant protein and peroxidase associated with black point in barley grains." *Proteomics* **7**(20): 3800-3808.
Martin, C. and J. Paz-Ares (1997). "MYB transcription factors in plants." *Trends in Genetics* **13**(2): 67-73.
Martinez, M. V. and J. R. Whitaker (1995). "The biochemistry and control of enzymatic browning." *Trends in Food Science & Technology* **6**(6): 195-200.
Mayer, K. F. X., M. Martis, P. E. Hedley, H. Šimková, H. Liu, J. A. Morris, B. Steuernagel, S. Taudien, S. Roessner, H. Gundlach, M. Kubaláková, P. Suchánková, F. Murat, M. Felder, T. Nussbaumer, A. Graner, J. Salse, T. Endo, H. Sakai, T. Tanaka, T. Itoh, K. Sato, M. Platzer, T. Matsumoto, U. Scholz, J. Doležel, R. Waugh and N. Stein (2011). "Unlocking the Barley Genome by Chromosomal and Comparative Genomics." *The Plant Cell Online* **23**(4): 1249-1263.
McKersie, B. D., S. R. Bowley, E. Harjanto and O. Leprince (1996). "Water-Deficit Tolerance and Field Performance of Transgenic Alfalfa Overexpressing Superoxide Dismutase." *Plant Physiology* **111**(4): 1177-1181.
Michaels, S. D. and R. M. Amasino (1999). "FLOWERING LOCUS C Encodes a Novel MADS Domain Protein That Acts as a Repressor of Flowering." *The Plant Cell Online* **11**(5): 949-956.
Michalowitz, M., M. Sulman, G. Fox, K. Spann and P. M. Williamson (2001). Unravelling the biochemical basis of black point formation in wheat and barley grains. APPS Conference, Cairns, QLD.
Milligan, A., S. Lopato and P. Langridge (2005). Functional Genomics of Seed Development in Cereals. Cereal Genomics. P. Gupta and R. Varshney, Springer Netherlands**:** 447-481.
Moreno, M. A., L. C. Harper, R. W. Krueger, S. L. Dellaporta and M. Freeling (1997). "Liguleless1 encodes a nuclear-localized protein required for induction of ligules and auricles during maize leaf organogenesis." *Genes & Development* **11**(5): 616-628.
Morimoto, R. I. (1998). "Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators." *Genes & Development* **12**(24): 3788-3796.
Morisawa, G., A. Han-yama, I. Moda, A. Tamai, M. Iwabuchi and T. Meshi (2000). "AHM1, a Novel Type of Nuclear Matrix–Localized, MAR Binding Protein with a Single AT Hook and a J Domain–Homologous Region." *The Plant Cell Online* **12**(10): 1903-1916.
Moschini, R., M. Sisterna and M. Carmona (2006). "Modelling of wheat black point incidence based on meterological variables in the southern Argentinean Pampas region." *Australian Journal of Agricultural Research* **57**: 1151-1156.

Mukhopadhyay, A., S. Vij and A. K. Tyagi (2004). "Overexpression of a zinc-finger protein gene from rice confers tolerance to cold, dehydration, and salt stress in transgenic tobacco." *Proceedings of the National Academy of Sciences of the United States of America* **101**(16): 6309-6314.

Müller, K., C. Pozzi, J. Müller, F. Salamini and W. Rohde (2000). "Molecular analysis of homeotic genes involved in barley development." *Pflügers Archiv European Journal of Physiology* **439**(7): r014-r015.

Mutisya, J., C. Sun, S. Palmqvist, Y. Baguma, B. Odhiambo and C. Jansson (2006). "Transcriptional regulation of the sbeIIb genes in sorghum (Sorghum bicolor) and barley (*Hordeum vulgare*): Importance of the barley sbeIIb second intron." *Journal of Plant Physiology* **163**(7): 770-780.

Nakabayashi, K., M. Ito, T. Kiyosue, K. Shinozaki and A. Watanabe (1999). "Identification of clp Genes Expressed in Senescing Arabidopsis Leaves." *Plant and Cell Physiology* **40**(5): 504-514.

Nakajima, K., G. Sena, T. Nawy and P. N. Benfey (2001). "Intercellular movement of the putative transcription factor SHR in root patterning." *Nature* **413**(6853): 307-311.

Nakamura, S., T. J. Lynch and R. R. Finkelstein (2001). "Physical interactions between ABA response loci of Arabidopsis." *The Plant Journal* **26**(6): 627-635.

Neer, E., C. Schmidt, R. Nambudripad and T. Smith (1994). "The ancient regulatory-protein family of WD-repeat proteins." *Nature* **371**: 297 - 300.

Nelson, D. E., P. P. Repetti, T. R. Adams, R. A. Creelman, J. Wu, D. C. Warner, D. C. Anstrom, R. J. Bensen, P. P. Castiglioni, M. G. Donnarummo, B. S. Hinchey, R. W. Kumimoto, D. R. Maszle, R. D. Canales, K. A. Krolikowski, S. B. Dotson, N. Gutterson, O. J. Ratcliffe and J. E. Heard (2007). "Plant nuclear factor Y (NF-Y) B subunits confer drought tolerance and lead to improved corn yields on water-limited acres." *Proceedings of the National Academy of Sciences* **104**(42): 16450-16455.

Nover, L. (1991). Heat Shock Response. Boca Raton, Florida CRC Press.

Ogo, Y., R. Nakanishi Itai, H. Nakanishi, T. Kobayashi, M. Takahashi, S. Mori and N. K. Nishizawa (2007). "The rice bHLH protein OsIRO2 is an essential regulator of the genes involved in Fe uptake under Fe-deficient conditions." *The Plant Journal* **51**(3): 366-377.

Oh, E., S. Yamaguchi, J. Hu, J. Yusuke, B. Jung, I. Paik, H.-S. Lee, T.-p. Sun, Y. Kamiya and G. Choi (2007). "PIL5, a Phytochrome-Interacting bHLH Protein, Regulates Gibberellin Responsiveness by Binding Directly to the GAI and RGA Promoters in Arabidopsis Seeds." *The Plant Cell Online* **19**(4): 1192-1208.

Ohme-Takagi, M. and H. Shinshi (1995). "Ethylene-Inducible DNA Binding Proteins That Interact with an Ethylene-Responsive Element." *The Plant Cell Online* **7**(2): 173-182.

Oleksiak, M. F., G. A. Churchill and D. L. Crawford (2002). "Variation in gene expression within and among natural populations." *Nature Genetics* **32**(2): 261-266.

Oñate, L., J. Vicente-Carbajosa, P. Lara, I. Díaz and P. Carbonero (1999). "Barley BLZ2, a Seed-specific bZIP Protein That Interacts with BLZ1 in Vivo and Activates Transcription from the GCN4-like motif of B-hordein Promoters in Barley Endosperm." *Journal of Biological Chemistry* **274**(14): 9175-9182.

Ostersetzer, O. and Z. Adam (1997). "Light-Stimulated Degradation of an Unassembled Rieske FeS Protein by a Thylakoid-Bound Protease: The Possible Role of the FtsH Protease." *The Plant Cell Online* **9**(6): 957-965.

Pagnussat, G. C., H.-J. Yu, Q. A. Ngo, S. Rajani, S. Mayalagu, C. S. Johnson, A. Capron, L.-F. Xie, D. Ye and V. Sundaresan (2005). "Genetic and molecular identification

of genes required for female gametophyte development and function in Arabidopsis." *Development* **132**(3): 603-614.
Palatnik, J., E. Allen, X. Wu, C. Schommer, R. Schwab, J. Carrington and D. Weigel (2003). "Control of leaf morphogenesis by microRNAs." *Nature* **425**(6955): 257-263.
Park, J. M., C.-J. Park, S.-B. Lee, B.-K. Ham, R. Shin and K.-H. Paek (2001). "Overexpression of the Tobacco Tsi1 Gene Encoding an EREBP/AP2–Type Transcription Factor Enhances Resistance against Pathogen Attack and Osmotic Stress in Tobacco." *The Plant Cell Online* **13**(5): 1035-1046.
Pastori, G. M. and C. H. Foyer (2002). "Common Components, Networks, and Pathways of Cross-Tolerance to Stress. The Central Role of "Redox" and Abscisic Acid-Mediated Controls." *Plant Physiology* **129**(2): 460-468.
Perelló, A., M. Moreno and M. Sisterna (2008). "*Alternaria infectoria* species-group associated with black point of wheat in Argentina." *Plant Pathology* **57**(2): 379-379.
Petr, J. and I. Capouchova (2001). "Cause of the occurance of malting barley kernal discolouration." *Monatsschrift fur Brauwissenschaft* **54**: 104-113.
Pnueli, L., E. Hallak-Herr, M. Rozenberg, M. Cohen, P. Goloubinoff, A. Kaplan and R. Mittler (2002). "Molecular and biochemical mechanisms associated with dormancy and drought tolerance in the desert legume *Retama raetam*." *The Plant Journal* **31**: 319 - 330.
Porta, H. and M. Rocha-Sosa (2002). "Plant Lipoxygenases. Physiological and Molecular Features." *Plant Physiology* **130**(1): 15-21.
Potokina, E., A. Druka, Z. Luo, R. Wise, R. Waugh and M. Kearsey (2008). "Gene expression quantitative trait locus analysis of 16 000 barley genes reveals a complex pattern of genome-wide transcriptional regulation." *The Plant Journal* **53**(1): 90-101.
Prenger, J. J. and P. P. Ling (2000). Greenhouse Condensation Control. Fact Sheet (Series) AEX-800. Columbus, OH, Ohio State University Extension.
Rajakumari, S., R. Rajasekharan and G. Daum (2009). "Triacylglycerol lipases of yeast and plants: more than just hydrolases." *Chemistry and Physics of Lipids* **160**(0): S7.
Ranjan, P., T. Yin, X. Zhang, U. Kalluri, X. Yang, S. Jawdy and G. Tuskan (2010). "Bioinformatics-Based Identification of Candidate Genes from QTLs Associated with Cell Wall Traits in *Populus*." *BioEnergy Research* **3**(2): 172-182.
Rasmussen, S. K., K. G. Welinder and J. Hejgaard (1991). "cDNA cloning, characterization and expression of an endosperm-specific barley peroxidase." *Plant Molecular Biology* **16**: 317-327.
Raventós, D., K. Skriver, M. Schlein, K. Karnahl, S. W. Rogers, J. C. Rogers and J. Mundy (1998). "HRT, a Novel Zinc Finger, Transcriptional Repressor from Barley." *Journal of Biological Chemistry* **273**(36): 23313-23320.
Rees, R. G., D. J. Martin and D. P. Law (1984). "Black point in bread wheat: effects on quality and germination, and fungal associations." *Australian Journal of Experimental Agriculture* **24**(127): 601-605.
Rees, R. G., D. J. Martin and D. P. Law (1984). "Black point in bread wheat: effects on quality and germination, and fungal associations." *Australian Journal of Experimental Agriculture and Animal Husbandry* **24**: 601-605.
Rein, U., U. Andag, R. Duden, H. D. Schmitt and A. Spang (2002). "ARF-GAP–mediated interaction between the ER-Golgi v-SNAREs and the COPI coat." *The Journal of Cell Biology* **157**(3): 395-404.

Reyes, J. C., M. I. Muro-Pastor and F. J. Florencio (2004). "The GATA Family of Transcription Factors in Arabidopsis and Rice." *Plant Physiology* **134**(4): 1718-1732.

Riechmann, J., J. Heard, G. Martin, L. Reuber, C. Jiang, J. Keddie, L. Adam, O. Pineda, O. Ratcliffe, R. Samaha, R. Creelman, M. Pilgrim, P. Broun, J. Zhang, D. Ghandehari, B. Sherman and G. Yu (2000). "Arabidopsis transcription factors: genome-wide comparative analysis among Eukaryotes." *Science* **290**: 2105 - 2110.

Riechmann, J. L. and E. M. Meyerowitz (1998). THE AP2/EREBP FAMILY OF PLANT TRANSCRIPTION FACTORS. Berlin, ALLEMAGNE, de Gruyter.

Riechmann, J. L. and O. J. Ratcliffe (2000). "A genomic perspective on plant transcription factors." *Current Opinion in Plant Biology* **3**(5): 423-434.

Rikiishi, K., T. Matsuura and M. Maekawa (2010). "TaABF1, ABA response element binding factor 1, is related to seed dormancy and ABA sensitivity in wheat (*Triticum aestivum L.*) seeds." *Journal of Cereal Science* **52**(2): 236-238.

Rizhsky, L., H. Liang and R. Mittler (2002). "The combined effect of drought stress and heat shock on gene expression in tobacco." *Plant Physiol* **130**: 1143 - 1151.

Robatzek, S. and I. Somssich (2002). "Targets of AtWRKY6 regulation during plant senescence and pathogen defense." *Genes Dev* **16**: 1139 - 1149.

Roberti, M., P. L. Polosa, F. Bruni, C. Musicco, M. N. Gadaleta and P. Cantatore (2003). "DmTTF, a novel mitochondrial transcription termination factor that recognises two sequences of Drosophila melanogaster mitochondrial DNA." *Nucleic Acids Research* **31**(6): 1597-1604.

Rodriguez-Uribe, L. and M. A. O'Connell (2006). "A root-specific bZIP transcription factor is responsive to water deficit stress in tepary bean (Phaseolus acutifolius) and common bean (P. vulgaris)." *Journal of Experimental Botany* **57**(6): 1391-1398.

Romanel, E. A. C., C. G. Schrago, R. M. Couñago, C. A. M. Russo and M. Alves-Ferreira (2009). "Evolution of the B3 DNA Binding Superfamily: New Insights into REM Family Gene Diversification." *PLoS ONE* **4**(6): e5791.

Romero, C., J. Bellés, J. Vayá, R. Serrano and F. Culiáñez-Macià (1997). "Expression of the yeast <i>trehalose-6-phosphate synthase</i> gene in transgenic tobacco plants: pleiotropic phenotypes include drought tolerance." *Planta* **201**(3): 293-297.

Ronald, J., J. M. Akey, J. Whittle, E. N. Smith, G. Yvert and L. Kruglyak (2005). "Simultaneous genotyping, gene-expression measurement, and detection of allele-specific expression with oligonucleotide arrays." *Genome Research* **15**(2): 284-291.

Rostoks, N., S. Mudie, L. Cardle, J. Russell, L. Ramsay, A. Booth, J. Svensson, S. Wanamaker, H. Walia, E. Rodriguez, P. Hedley, H. Liu, J. Morris, T. Close, D. Marshall and R. Waugh (2005). "Genome-wide SNP discovery and linkage analysis in barley based on genes responsive to abiotic stress." *Molecular Genetics and Genomics* **274**(5): 515-527.

Roxas, V. P., R. K. Smith, E. R. Allen and R. D. Allen (1997). "Overexpression of glutathione S-transferase/glutathioneperoxidase enhances the growth of transgenic tobacco seedlings during stress." *Nat Biotech* **15**(10): 988-991.

Rushton, P., H. Macdonald, A. Huttly, C. Lazarus and R. Hooley (1995). "Members of a new family of DNA-binding proteins bind to a conserved cis-element in the promoters of alpha-Amy2 genes." *Plant Molecular Biology* **29**: 691 - 702.

Rushton, P. J. and I. E. Somssich (1998). "Transcriptional control of plant genes responsive to pathogens." *Current Opinion in Plant Biology* **1**(4): 311-315.

Ruuska, S. A., T. Girke, C. Benning and J. B. Ohlrogge (2002). "Contrapuntal Networks of Gene Expression during Arabidopsis Seed Filling." *The Plant Cell Online* **14**(6): 1191-1206.

Sambrook, J. and D. W. Russell (2001). *Molecular cloning: a laboratory manual*, Cold Spring Harbour Laboratory Press.

Sarma, A. D. and R. Sharma (1999). "Purification and characterization of uv-b induced phenylalanine ammonia-lyase from rice seedlings." *Phytochemistry* **50**(5): 729-737.

Schadt, E. E., S. A. Monks, T. A. Drake, A. J. Lusis, N. Che, V. Colinayo, T. G. Ruff, S. B. Milligan, J. R. Lamb, G. Cavet, P. S. Linsley, M. Mao, R. B. Stoughton and S. H. Friend (2003). "Genetics of gene expression surveyed in maize, mouse and man." *Nature* **422**(6929): 297-302.

Schena, M., D. Shalon, R. W. Davis and P. O. Brown (1995). "Quantitative Monitoring of Gene Expression Patterns with a Complementary DNA Microarray." *Science* **270**(5235): 467-470.

Schmitt, N. F. and J. R. Van Mechelen (1997). "Expression of lipoxygenase isoenzymes in developing barley grains." *Plant Science* **128**(2): 141-150.

Schöffl, F., R. Prändl and A. Reindl (1998). "Regulation of the Heat-Shock Response." *Plant Physiology* **117**(4): 1135-1141.

Schommer, C., J. F. Palatnik, P. Aggarwal, A. Chételat, P. Cubas, E. E. Farmer, U. Nath and D. Weigel (2008). "Control of Jasmonate Biosynthesis and Senescence by miR319 Targets." *PLoS Biol* **6**(9): e230.

Seki, M., M. Narusaka, H. Abe, M. Kasuga, K. Yamaguchi-Shinozaki, P. Carninci, Y. Hayashizaki and K. Shinozaki (2001). "Monitoring the Expression Pattern of 1300 Arabidopsis Genes under Drought and Cold Stresses by Using a Full-Length cDNA Microarray." *The Plant Cell Online* **13**(1): 61-72.

Sessions, A., J. L. Nemhauser, A. McColl, J. L. Roe, K. A. Feldmann and P. C. Zambryski (1997). "ETTIN patterns the Arabidopsis floral meristem and reproductive organs." *Development* **124**(22): 4481-4491.

Shanmugam, V. (2005). "Role of extracytoplasmic leucine rich repeat proteins in plant defence mechanisms." *Microbiological Research* **160**(1): 83-94.

Sharma, R., M. Kapoor, A. Tyagi and S. Kapoor (2010). "Comparative transcript profiling of TCP family genes provide insight into gene functions and diversifications in rice and *Arabodopsis*." *Journal of Plant Molecular Biology and Biotechnology* **1**(1): 24-38.

Sheldon, C. C., J. E. Burn, P. P. Perez, J. Metzger, J. A. Edwards, W. J. Peacock and E. S. Dennis (1999). "The FLF MADS Box Gene: A Repressor of Flowering in Arabidopsis Regulated by Vernalization and Methylation." *The Plant Cell Online* **11**(3): 445-458.

Sheldon, C. C., D. T. Rouse, E. J. Finnegan, W. J. Peacock and E. S. Dennis (2000). "The molecular basis of vernalization: The central role of FLOWERING LOCUS C (FLC)." *Proceedings of the National Academy of Sciences* **97**(7): 3753-3758.

Shen, Zhang, He, Liu and Chen (2003). "An EREBP/AP2-type protein in *Triticum aestivum* was a DRE-binding transcription factor induced by cold, dehydration and ABA stress." *Theoretical and Applied Genetics* **106**(5): 923-930.

Shen, Y. G. S., W. K. Z. Zhang, S. J. H. He, J. S. Z. Zhang, Q. L. Liu and S. Y. C. Chen (2003). "An EREBP/AP2-type protein in <SMALL>Triticum aestivum</SMALL> was a DRE-binding transcription factor induced by cold, dehydration and ABA stress." *TAG Theoretical and Applied Genetics* **106**(5): 923-930.

Shinozaki, K. and K. Yamaguchi-Shinozaki (2000). "Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways." *Current Opinion in Plant Biology* **3**(3): 217-223.

Siedow, J. N. (1991). "Plant Lipoxygenase: Structure and Function." *Annual Review of Plant Physiology and Plant Molecular Biology* **42**(1): 145-188.

Singh, K. B., R. C. Foley and L. Oñate-Sánchez (2002). "Transcription factors in plant defense and stress responses." *Current Opinion in Plant Biology* **5**(5): 430-436.

Söderman, E., M. Hjellström, J. Fahleson and P. Engström (1999). "The HD-Zip gene ATHB6 in Arabidopsis is expressed in developing leaves, roots and carpels and up-regulated by water deficit conditions." *Plant Molecular Biology* **40**(6): 1073-1083.

Solecka, D. (1997). "Role of phenylpropanoid compounds in plant responses to different stress factors." *Acta Physiologiae Plantarum* **19**(3): 257-268.

Song, Y. H., C. M. Yoo, A. P. Hong, S. H. Kim, H. J. Jeong, S. Y. Shin, H. J. Kim, D.-J. Yun, C. O. Lim, J. D. Bahk, S. Y. Lee, R. T. Nagao, J. L. Key and J. C. Hong (2008). "DNA-Binding Study Identifies C-Box and Hybrid C/G-Box or C/A-Box Motifs as High-Affinity Binding Sites for STF1 and LONG HYPOCOTYL5 Proteins." *Plant Physiology* **146**(4): 1862-1877.

Southwell, R. J., J. F. Brown and P. T. Wong (1980). "Effect of inoculum density, stage of plant growth and dew period on the incidence of black point caused by *Alternaria alternata* in durum wheat." *Annals of Applied Biology* **96**: 29-35.

Sparla, F., G. Tedeschi, P. Pupillo and P. Trost (1999). "Cloning and heterologous expression of NAD(P)H:quinone reductase of Arabidopsis thaliana, a functional homologue of animal DT-diaphorase." *FEBS Letters* **463**(3): 382-386.

Spelt, C., F. Quattrocchio, J. N. M. Mol and R. Koes (2000). "Anthocyanin1 of Petunia Encodes a Basic Helix-Loop-Helix Protein That Directly Activates Transcription of Structural Anthocyanin Genes." *The Plant Cell Online* **12**(9): 1619-1632.

Stec, I., S. B. Nagl, G.-J. B. van Ommen and J. T. den Dunnen (2000). "The PWWP domain: a potential protein–protein interaction domain in nuclear proteins influencing differentiation?" *FEBS Letters* **473**(1): 1-5.

Steffenson, B., P. Hayes and A. Kleinhofs (1996). "Genetics of seedling and adult plant resistance to net blotch (*Pyrenophora teres*) and spot blotch (*Cochliobolus sativus*) in barley." *Theoretical and Applied Genetics* **92**(5): 552-558.

Stone, S. L., L. W. Kwong, K. M. Yee, J. Pelletier, L. Lepiniec, R. L. Fischer, R. B. Goldberg and J. J. Harada (2001). "LEAFY COTYLEDON2 encodes a B3 domain transcription factor that induces embryo development." *Proceedings of the National Academy of Sciences* **98**(20): 11806-11811.

Sulman, M., G. Fox, K. Bell, K. Young and A. Inkerman (2001a). Field screening using a controlled environment for black point resistance in barley. Proceedings of the 10th Australian Barley Technical Symposium, Canberra.

Sulman, M., G. Fox, A. Osman, A. Inkerman, P. Williamson and M. Michalowitz (2001b). Relationship between total peroxidase activity and susceptibility to black point in mature grain of some barley cultivars. Proceedings of the 10th Australian Barley Technical Symposium, Canberra.

Sun, C., S. Palmqvist, H. Olsson, M. Boren, S. Ahlandsberg and C. Jansson (2003). "A novel WRKY transcription factor, SUSIBA2, participates in sugar signaling in barley by binding to the sugar-responsive elements of the iso1 promoter." *Plant Cell* **15**: 2076 - 2092.

Sun, T. and F. Gubler (2004). "Molecular Mechanism of Gibberellin Signalling in Plants." *Annual Review of Plant Biology* **55**: 197-223.

Suzuki, M., H. H.-Y. Wang and D. R. McCarty (2007). "Repression of the LEAFY COTYLEDON 1/B3 Regulatory Network in Plant Embryo Development by VP1/ABSCISIC ACID INSENSITIVE 3-LIKE B3 Genes." *Plant Physiology* **143**(2): 902-911.

Tabata, T. (1991). "Leucine-zipper type transcription factors of wheat." *Journal of Plant Research* **104**(2): 171-181.

Tah, P. R., A. Lehmensiek, G. P. Fox, E. Mace, M. Sulman, G. Bloustein and G. E. Daggard (2010). "Identification of genetic regions associated with black point in barley." *Field Crops Research* **115**(1): 61-66.

Takatsuji, H. (1999). "Zinc-finger proteins: the classical zinc finger emerges in contemporary plant science." *Plant Molecular Biology* **39**(6): 1073-1078.

Takeda, T., K. Amano, M.-a. Ohto, K. Nakamura, S. Sato, T. Kato, S. Tabata and C. Ueguchi (2006). "RNA Interference of the *Arabidopsis* Putative Transcription Factor TCP16Gene Results in Abortion of Early Pollen Development." *Plant Molecular Biology* **61**(1): 165-177.

Tarczynski, M. C., R. G. Jensen and H. J. Bohnert (1993). "Stress Protection of Transgenic Tobacco by Production of the Osmolyte Mannitol." *Science* **259**(5094): 508-510.

Tatematsu, K., K. Nakabayashi, Y. Kamiya and E. Nambara (2008). "Transcription factor AtTCP14 regulates embryonic growth potential during seed germination in *Arabidopsis thaliana*." *The Plant Journal* **53**(1): 42-52.

Terp, N., C. Göbel, A. Brandt and I. Feussner (2006). "Lipoxygenases during *Brassica napus* seed germination." *Phytochemistry* **67**(18): 2030-2040.

The Australian Bureau of Agricultural and Resource Economics and Sciences ABARE. (2011). Retrieved 7th July 2011 from http://www.abare.gov.au/publications_html/crops/crops_10/crops_10.html.

Theilade, B. and S. K. Rasmussen (1992). "Structure and chromosomal localization of the gene encoding barley seed peroxidase BP2A." *Gene* **118**: 261-266.

Thordal-Christensen, H., J. Brandt, B. Cho, S. Rasmussen, P. Gregersen, V. Smedegaard-Petersen and C. DB (1992). "cDNA cloning and characterization of two barley peroxidase transcripts induced differentially by the powdery mildew fungus *Erysiphe graminis*." *Physiological and Molecular Plant Pathology* **40**: 395-409.

Toledo-Ortiz, G., E. Huq and P. H. Quail (2003). "The Arabidopsis Basic/Helix-Loop-Helix Transcription Factor Family." *The Plant Cell Online* **15**(8): 1749-1770.

Tomás-Barberán, F. A. and J. C. Espín (2001). "Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables." *Journal of the Science of Food and Agriculture* **81**(9): 853-876.

Trost, P., P. Bonora, S. Scagliarini and P. Pupillo (1995). "Purification and Properties of NAD(P)H:(quinone-acceptor) Oxidoreductase of Sugarbeet Cells." *European Journal of Biochemistry* **234**(2): 452-458.

Tsukagoshi, H., T. Saijo, D. Shibata, A. Morikami and K. Nakamura (2005). "Analysis of a Sugar Response Mutant of Arabidopsis Identified a Novel B3 Domain Protein That Functions as an Active Transcriptional Repressor." *Plant Physiology* **138**(2): 675-685.

Ülker, B. and I. E. Somssich (2004). "WRKY transcription factors: from DNA binding towards biological function." *Current Opinion in Plant Biology* **7**(5): 491-498.

Uno, Y., T. Furihata, H. Abe, R. Yoshida, K. Shinozaki and K. Yamaguchi-Shinozaki (2000). "Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions." *Proceedings of the National Academy of Sciences* **97**(21): 11632-11637.

Uzun, A., C. M. Leslin, A. Abyzov and V. Ilyin (2007). "Structure SNP (StSNP): a web server for mapping and modeling nsSNPs on protein structures with linkage to metabolic pathways." *Nucleic Acids Research* **35**(suppl 2): W384-W392.

van der Biezen, E. A. and J. D. G. Jones (1998). "The NB-ARC domain: a novel signalling motif shared by plant resistance gene products and regulators of cell death in animals." *Current Biology* **8**(7): R226-R228.

van der Graaff, E., A. D. Dulk-Ras, P. J. Hooykaas and B. Keller (2000). "Activation tagging of the LEAFY PETIOLE gene affects leaf petiole development in Arabidopsis thaliana." *Development* **127**(22): 4971-4980.

van Nocker, S. and P. Ludwig (2003). "The WD-repeat protein superfamily in Arabidopsis: conservation and divergence in structure and function." *BMC Genomics* **4**(1): 50.

van Ooijen, G., G. Mayr, M. M. A. Kasiem, M. Albrecht, B. J. C. Cornelissen and F. L. W. Takken (2008). "Structure–function analysis of the NB-ARC domain of plant disease resistance proteins." *Journal of Experimental Botany* **59**(6): 1383-1397.

Van Os, H., P. Stam, R. Visser and H. Van Eck (2005). "RECORD: a novel method for ordering loci on a genetic linkage map." *TAG Theoretical and Applied Genetics* **112**(1): 30-40.

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe and F. Speleman (2002). "Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes." *Genome Biology* **3**(7): research0034.0031 - research0034.0011.

Varshney, R. K., D. A. Hoisington and A. K. Tyagi (2006). "Advances in cereal genomics and applications in crop breeding." *Trends in Biotechnology* **24**(11): 490-499.

Vasil, V., W. R. Marcotte Jr, L. Rosenkrans, S. M. Cocciolone, I. K. Vasil, R. S. Quatrano and D. R. McCarty (1995). "Overlap of Viviparous1 (VP1) and Abscisic Acid Response Elements in the Em Promoter: G-Box Elements Are Sufficient but Not Necessary for VP1 Transactivation." *The Plant Cell Online* **7**(9): 1511-1518.

Vij, S. and A. Tyagi (2008). "A20/AN1 zinc-finger domain-containing proteins in plants and animals represent common elements in stress response." *Functional & Integrative Genomics* **8**(3): 301-307.

Waldron, L. R. (1934). "Increase of kernal weight in common wheat due to black point disease." *Journal of Agricultural Research* **48**: 1017-1024.

Walker, J. R. L. and P. H. Ferrar (1998). Diphenol oxidases, enzyme catalyzed browning and plant disease resistance. *Biotechnology and Genetic Engineering Reviews*. M. P. Tombs. Andovar, England, Intercept Ltd. **15:** 457-498.

Walker, K. R., J. A. Able, D. E. Mather and A. J. Able (2008). "Black point formation in barley: environmental influences and quantitative trait loci." *Australian Journal of Agricultural Research* **59**(11): 1021-1029.

Wang, C.-H., L.-H. Su and C.-H. Sun (2007). "A Novel ARID/Bright-like Protein Involved in Transcriptional Activation of Cyst Wall Protein 1 Gene in Giardia lamblia." *Journal of Biological Chemistry* **282**(12): 8905-8914.

Wang, E., C. J. Smith, W. J. Bond and K. Verburg (2004). "Estimations of vapour pressure deficit and crop water demand in APSIM and their implications for prediction of crop yield, water use, and deep drainage." *Australian Journal of Agricultural Research* **55**(12): 1227-1240.

Wang, H., T. Nussbaum-Wagler, B. Li, Q. Zhao, Y. Vigouroux, M. Faller, K. Bomblies, L. Lukens and J. F. Doebley (2005). "The origin of the naked grains of maize." *Nature* **436**(7051): 714-719.

Waters, B. M., C. Uauy, J. Dubcovsky and M. A. Grusak (2009). "Wheat (Triticum aestivum) NAM proteins regulate the translocation of iron, zinc, and nitrogen compounds from vegetative tissues to grain." *Journal of Experimental Botany* **60**(15): 4263-4274.

Wentzell, A. M., H. C. Rowe, B. G. Hansen, C. Ticconi, B. A. Halkier and D. J. Kliebenstein (2007). "Linking Metabolic QTLs with Network and eQTLs Controlling Biosynthetic Pathways." *PLoS Genet* **3**(9): e162.

Wenzl, P., H. Li, J. Carling, M. Zhou, H. Raman, E. Paul, P. Hearnden, C. Maier, L. Xia, V. Caig, J. Ovesna, M. Cakir, D. Poulsen, J. Wang, R. Raman, K. Smith, G. Muehlbauer, K. Chalmers, A. Kleinhofs, E. Huttner and A. Kilian (2006). "A high-density consensus map of barley linking DArT markers to SSR, RFLP and STS loci and agricultural traits." *BMC Genomics* **7**(1): 206.

West, M. A. L., K. Kim, D. J. Kliebenstein, H. van Leeuwen, R. W. Michelmore, R. W. Doerge and D. A. St. Clair (2007). "Global eQTL Mapping Reveals the Complex Genetic Architecture of Transcript-Level Variation in Arabidopsis." *Genetics* **175**(3): 1441-1450.

Whitaker, J. R. and Y. L. Chang (1996). Enzymatic browning and its prevention. Washington DC, American Chemical Society.

Williamson, P. (2002). Black point: role of peroxidase isozymes, development of screening tests and improved germplasm, Final Report., GRDC Winter Cereal Improvement Project no. CWQ4c.

Williamson, P. M. (1997a). "Black point of wheat: in vitro production of symptoms, enzymes invoIoved, and association with *Alternaria alternata*." *Australian Journal of Agricultural Research* **48**: 13-19.

Williamson, P. M. (1997b). Black point in wheat and its relevance to barley. Proceedings of the 8th Barley Technical Symposium Gold Coast, Australia.

Willmott, R., P. Rushton, R. Hooley and C. Lazarus (1998). "DNase1 footprints suggest the involvement of at least three types of transcription factors in the regulation of alpha- Amy2/A by gibberellin." *Plant Molecular Biology* **38**: 817 - 825.

Willsmore, K. L., P. Eckermann, R. K. Varshney, A. Graner, P. Langridge, M. Pallotta, J. Cheong and K. J. Williams (2006). "New eSSR and gSSR markers added to Australian barley maps." *Australian Journal of Agricultural Research* **57**(9): 953-959.

Wittkopp, P. J., B. K. Haerum and A. G. Clark (2004). "Evolutionary changes in cis and trans gene regulation." *Nature* **430**(6995): 85-88.

Wray, G. A. (2007). "The evolutionary significance of cis-regulatory mutations." *Nat Rev Genet* **8**(3): 206-216.

www.bom.gov.au. "Bureau of Meteorology " Retrieved Feb 2007.

Xu, H., Y. Li, Y. Yan, K. Wang, Y. Gao and Y. Hu (2010). "Genome-scale identification of Soybean BURP domain-containing genes and their expression under stress treatments." *BMC Plant Biology* **10**(1): 197.

Yamaguchi-Shinozaki, K. and K. Shinozaki (1993). "The plant hormone abscisic acid mediates the drought-induced expression but not the seed-specific expression of rd22, a gene responsive to dehydration stress in Arabidopsis thaliana." *Mol Gen Genet* **238**: 17 - 25.

Yanagisawa, S. and J. Sheen (1998). "Involvement of Maize Dof Zinc Finger Proteins in Tissue-Specific and Light-Regulated Gene Expression." *The Plant Cell Online* **10**(1): 75-90.

Yang, J., C. Hu, H. Hu, R. Yu, Z. Xia, X. Ye and J. Zhu (2008). "QTLNetwork: mapping and visualizing genetic architecture of complex traits in experimental populations." *Bioinformatics* **24**(5): 721-723.

Yang, J., J. Zhu and R. W. Williams (2007). "Mapping the genetic architecture of complex traits in experimental populations." *Bioinformatics* **23**(12): 1527-1536.

Yi, S. Y., J.-H. Kim, Y.-H. Joung, S. Lee, W.-T. Kim, S. H. Yu and D. Choi (2004). "The Pepper Transcription Factor CaPF1 Confers Pathogen and Freezing Tolerance in Arabidopsis." *Plant Physiology* **136**(1): 2862-2874.

Yoshida, K. Y., P. K. Kaothien, T. M. Matsui, A. K. Kawaoka and A. S. Shinmyo (2003). "Molecular biology and application of plant peroxidase genes." *Applied Microbiology and Biotechnology* **60**(6): 665-670.

Young, K. J. (1997). Weather staining of barley in Western Australia Proceedings of the 8th Barley Technical Symposium, Gold Coast, ABT Ltd.

Yu, J., S. Hu, J. Wang, G. K.-S. Wong, S. Li, B. Liu, Y. Deng, L. Dai, Y. Zhou, X. Zhang, M. Cao, J. Liu, J. Sun, J. Tang, Y. Chen, X. Huang, W. Lin, C. Ye, W. Tong, L. Cong, J. Geng, Y. Han, L. Li, W. Li, G. Hu, X. Huang, W. Li, J. Li, Z. Liu, L. Li, J. Liu, Q. Qi, J. Liu, L. Li, T. Li, X. Wang, H. Lu, T. Wu, M. Zhu, P. Ni, H. Han, W. Dong, X. Ren, X. Feng, P. Cui, X. Li, H. Wang, X. Xu, W. Zhai, Z. Xu, J. Zhang, S. He, J. Zhang, J. Xu, K. Zhang, X. Zheng, J. Dong, W. Zeng, L. Tao, J. Ye, J. Tan, X. Ren, X. Chen, J. He, D. Liu, W. Tian, C. Tian, H. Xia, Q. Bao, G. Li, H. Gao, T. Cao, J. Wang, W. Zhao, P. Li, W. Chen, X. Wang, Y. Zhang, J. Hu, J. Wang, S. Liu, J. Yang, G. Zhang, Y. Xiong, Z. Li, L. Mao, C. Zhou, Z. Zhu, R. Chen, B. Hao, W. Zheng, S. Chen, W. Guo, G. Li, S. Liu, M. Tao, J. Wang, L. Zhu, L. Yuan and H. Yang (2002). "A Draft Sequence of the Rice Genome (*Oryza sativa L. ssp. indica*)." *Science* **296**(5565): 79-92.

Yu, J., J. Wang, W. Lin, S. Li, H. Li, J. Zhou, P. Ni, W. Dong, S. Hu, C. Zeng, J. Zhang, Y. Zhang, R. Li, Z. Xu, S. Li, X. Li, H. Zheng, L. Cong, L. Lin, J. Yin, J. Geng, G. Li, J. Shi, J. Liu, H. Lv, J. Li, J. Wang, Y. Deng, L. Ran, X. Shi, X. Wang, Q. Wu, C. Li, X. Ren, J. Wang, X. Wang, D. Li, D. Liu, X. Zhang, Z. Ji, W. Zhao, Y. Sun, Z. Zhang, J. Bao, Y. Han, L. Dong, J. Ji, P. Chen, S. Wu, J. Liu, Y. Xiao, D. Bu, J. Tan, L. Yang, C. Ye, J. Zhang, J. Xu, Y. Zhou, Y. Yu, B. Zhang, S. Zhuang, H. Wei, B. Liu, M. Lei, H. Yu, Y. Li, H. Xu, S. Wei, X. He, L. Fang, Z. Zhang, Y. Zhang, X. Huang, Z. Su, W. Tong, J. Li, Z. Tong, S. Li, J. Ye, L. Wang, L. Fang, T. Lei, C. Chen, H. Chen, Z. Xu, H. Li, H. Huang, F. Zhang, H. Xu, N. Li, C. Zhao, S. Li, L. Dong, Y. Huang, L. Li, Y. Xi, Q. Qi, W. Li, B. Zhang, W. Hu, Y. Zhang, X. Tian, Y. Jiao, X. Liang, J. Jin, L. Gao, W. Zheng, B. Hao, S. Liu, W. Wang, L. Yuan, M. Cao, J. McDermott, R. Samudrala, J. Wang, G. K.-S. Wong and H. Yang (2005). "The Genomes of *Oryza sativa*: A History of Duplications." *PLoS Biol* **3**(2): e38.

Yu, S., L. Zhang, K. Zuo, Z. Li and K. Tang (2004). "Isolation and characterization of a BURP domain-containing gene BnBDC1 from *Brassica napus* involved in abiotic and biotic stress." *Physiol Plant* **122**: 210 - 218.

Zadoks, J. C., T. T. Chang and C. F. Konzak (1974). "A decimal code for the growth of stage of cereals." *Weed Research* **14**: 415-421.

Zadoks, J. C., T. T. Chang and C. F. Konzak (1974). "A decimal code for the growth stages of cereals." *Weed Research* **14**(6): 415-421.

Zentella, R., Z.-L. Zhang, M. Park, S. G. Thomas, A. Endo, K. Murase, C. M. Fleet, Y. Jikumaru, E. Nambara, Y. Kamiya and T.-p. Sun (2007). "Global Analysis of DELLA Direct Targets in Early Gibberellin Signaling in Arabidopsis." *The Plant Cell Online* **19**(10): 3037-3057.

Zhang, Y., S. Schwarz, H. Saedler and P. Huijser (2007). "SPL8, a local regulator in a subset of gibberellin-mediated developmental processes in Arabidopsis." *Plant Molecular Biology* **63**(3): 429-439.
Zhang, Y. and L. Wang (2005). "The WRKY transcription factor superfamily: its origin in eukaryotes and expansion in plants." *BMC Evolutionary Biology* **5**(1): 1.
Zhu, Q., M. I. Ordiz, T. Dabi, R. N. Beachy and C. Lamb (2002). "Rice TATA Binding Protein Interacts Functionally with Transcription Factor IIB and the RF2a bZIP Transcriptional Activator in an Enhanced Plant *in Vitro* Transcription System." *The Plant Cell Online* **14**(4): 795-803.
Zhu, Y., X.-L. Cai, Z.-Y. Wang and M.-M. Hong (2003). "An Interaction between a MYC Protein and an EREBP Protein Is Involved in Transcriptional Regulation of the Rice Wx Gene." *Journal of Biological Chemistry* **278**(48): 47803-47811.
Zhu, Y. Y., E. M. Machleder, A. Chenchik, R. Li and P. D. Siebert (2001). "Reverse transcriptase template switching: A SMART^{TM} approach for full length cDNA library construction." *Biotechniques* **30**: 892-897.

Appendix

1. Comparisons on weather data from Hatherleigh, Mount Gambier and Robe (Chapter 2).

2. Candidate genes within the eQTL for *HvBP1* (Chapter 4)

3. Full list of regulatory DNA elements identified for *HvBP1* and *HvPrx7* (Chapter 5) using PLACE database.

4. Publications from the research presented in this thesis

Appendix 1. Representative weather data for Hatherleigh in years where the on site weather station was absent, through correlations with the Mount Gambier aero and Robe weather stations.

Table A1.1 Correlations between the Hatherleigh trial site, Mount Gambier Aero and Robe weather stations. 9 am and 3 pm temperature and humidity as well as maximum and minimum humidity and temperature correlations are shown for the 04/05 season. Maximum and minimum humidity and temperature are shown for the 05/06 season. * represents where data not available.

	04/05 Season (correlation value)				05/06 Season (correlation value)			
HUMIDITY	**9am**	**3pm**	**Maximum**	**Minimum**	**9am**	**3pm**	**Maximum**	**Minimum**
Mount Gambier	0.81	0.82	0.55	0.78	*	*	0.38	0.92
Robe	0.72	0.46	0.11	0.49	*	*	-0.05	0.52
TEMPERATURE								
Mount Gambier	0.92	0.95	0.96	0.87	*	*	0.95	0.64
Robe	0.9	0.89	0.92	0.8	*	*	0.91	0.65

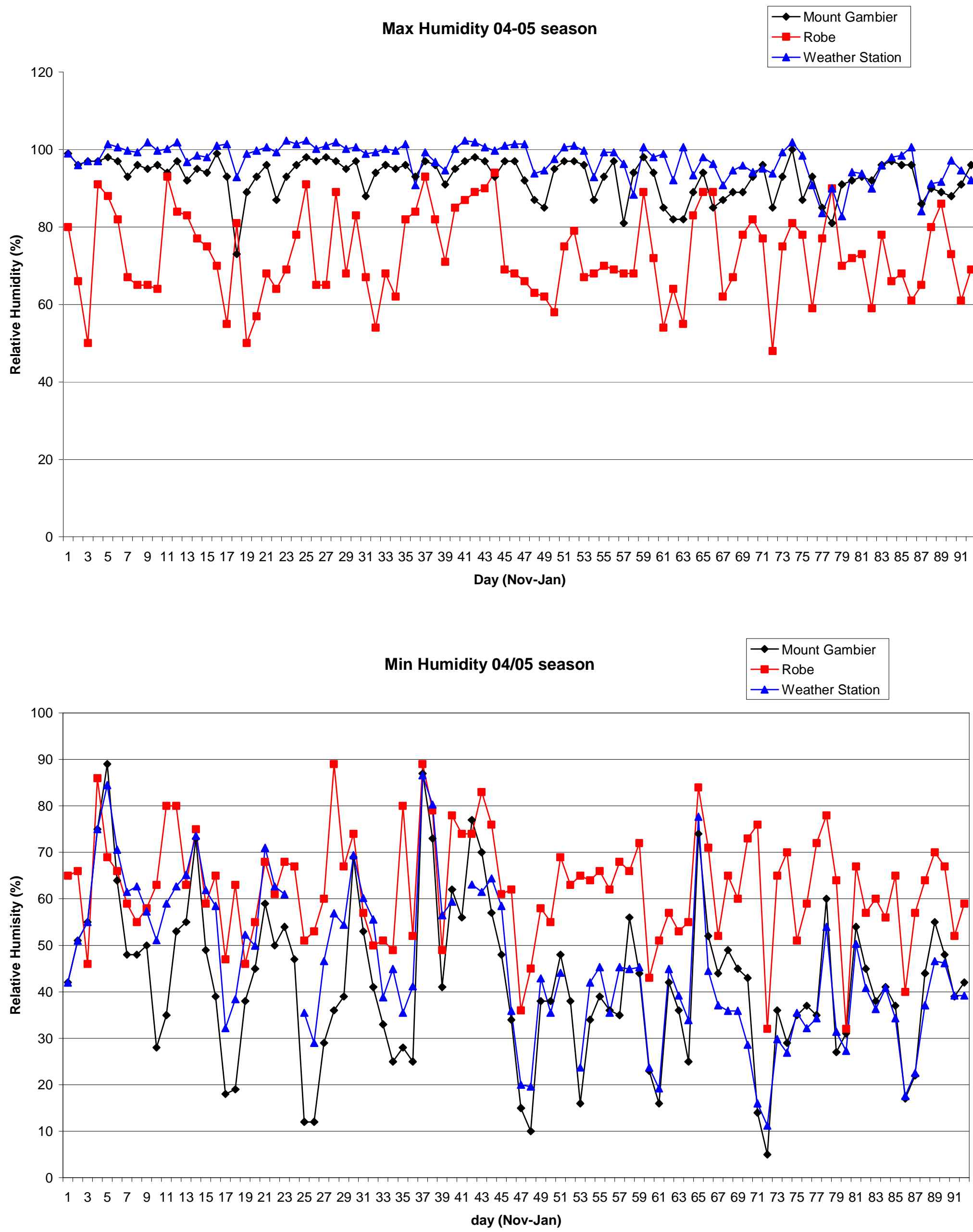
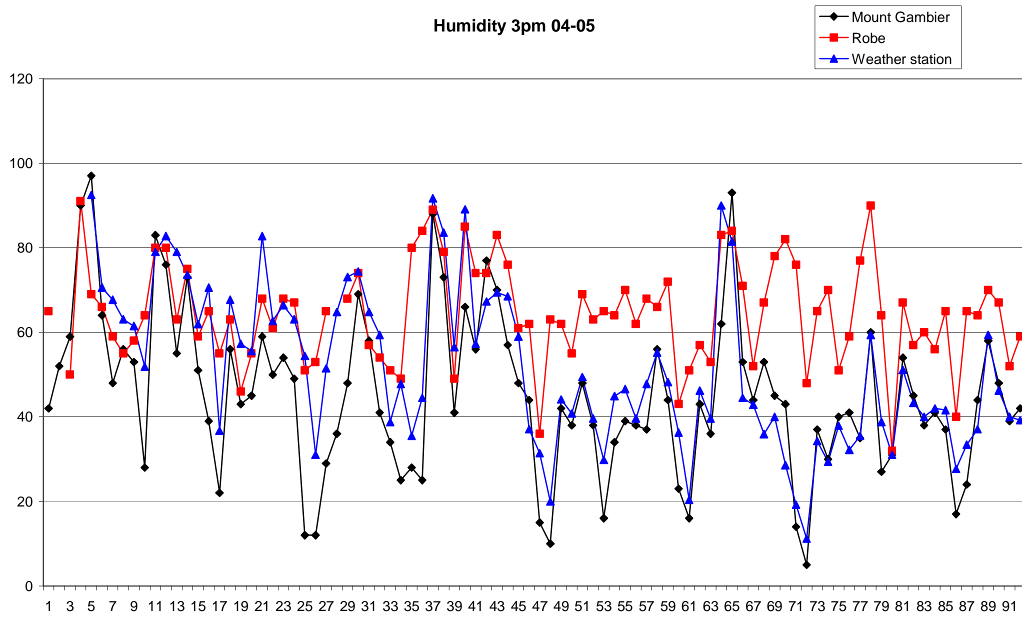


Figure A1.1 Comparison of maximum (A) and minimum humidity (B) for the Mount Gambier aero and Robe weather stations (2004/2005 season). The months of November through to January are represented. Mount Gambier follows the trend of the on site station.



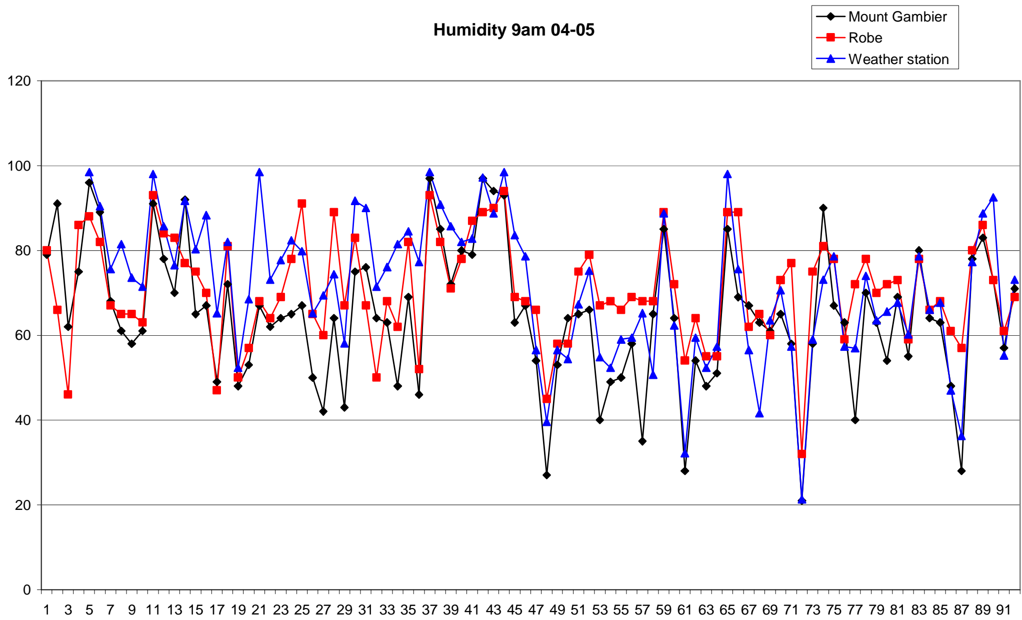


Figure A1.2 Comparison of 3 pm (A) and 9 am (B) humidity for the Mount Gambier aero and Robe weather stations (2004/2005 season). The months of November through to January are represented. Mount Gambier follows the trend of the on site station, though daily extremes reached are not represented when compared to A1.1.

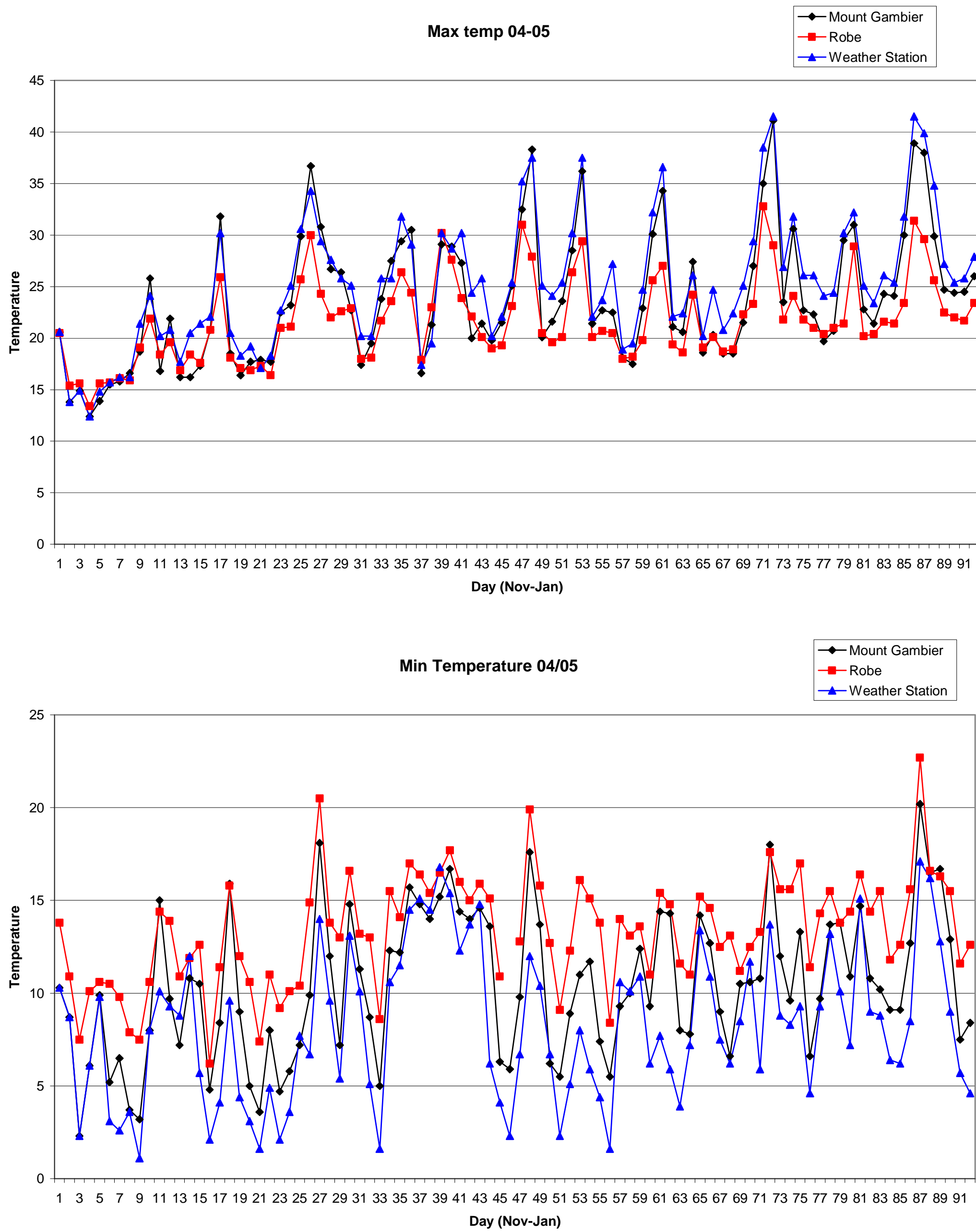


Figure A1.3 Comparison of maximum (A) and minimum temperature (B) for the Mount Gambier aero and Robe weather stations (2004/2005 season). The months of November through to January are represented. Mount Gambier follows the trend of the on site station and to some extent with the lower temperatures observed at the trial site (Hatherleigh).

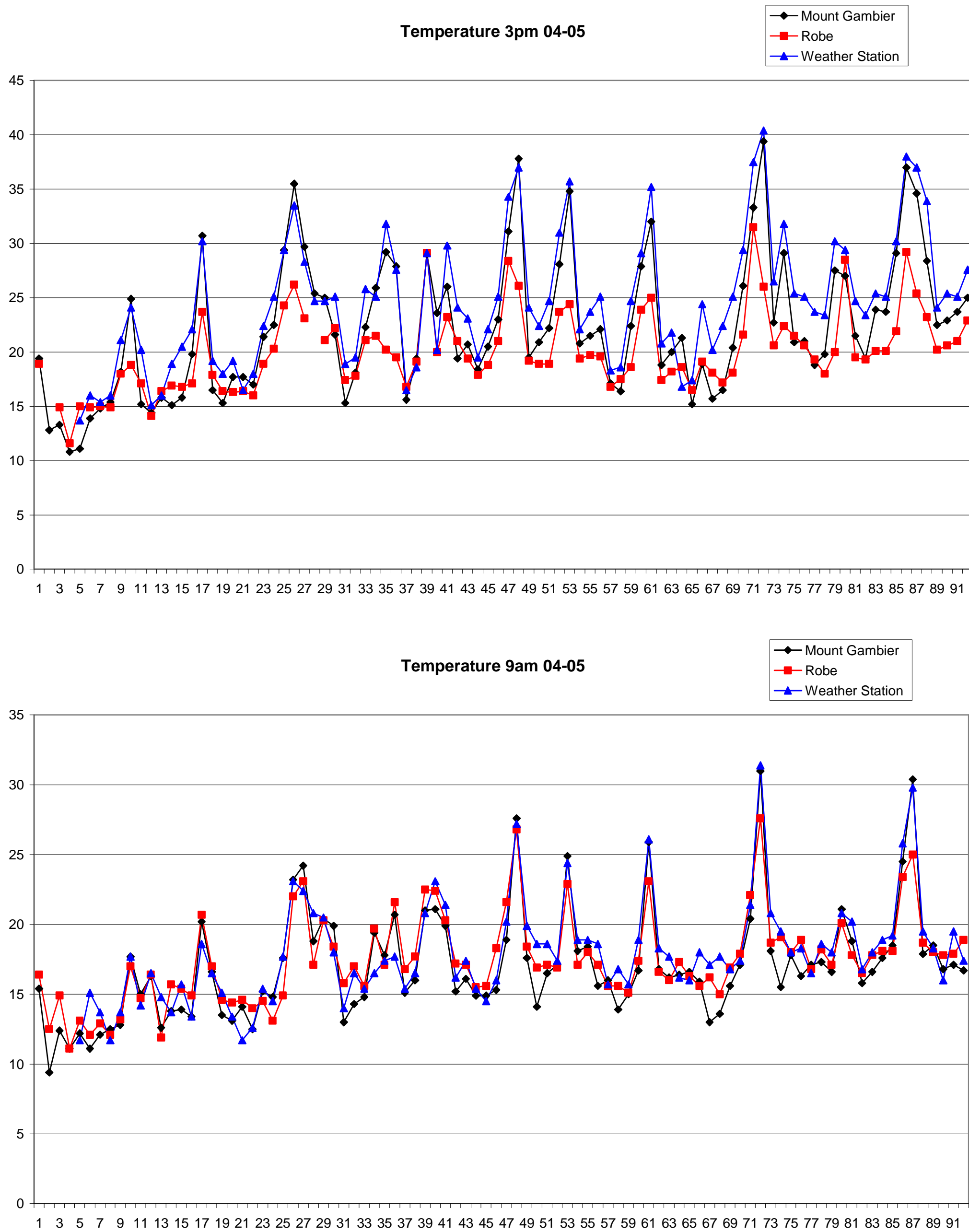


Figure A1.4 Comparison of 3 pm (A) and 9 am (B) temperature for the Mount Gambier aero and Robe weather stations (2004/2005 season). The months of November through to January are represented. Mount Gambier and Robe follow trends of the on site station, though daily extremes reached are not represented when compared to A1.3.

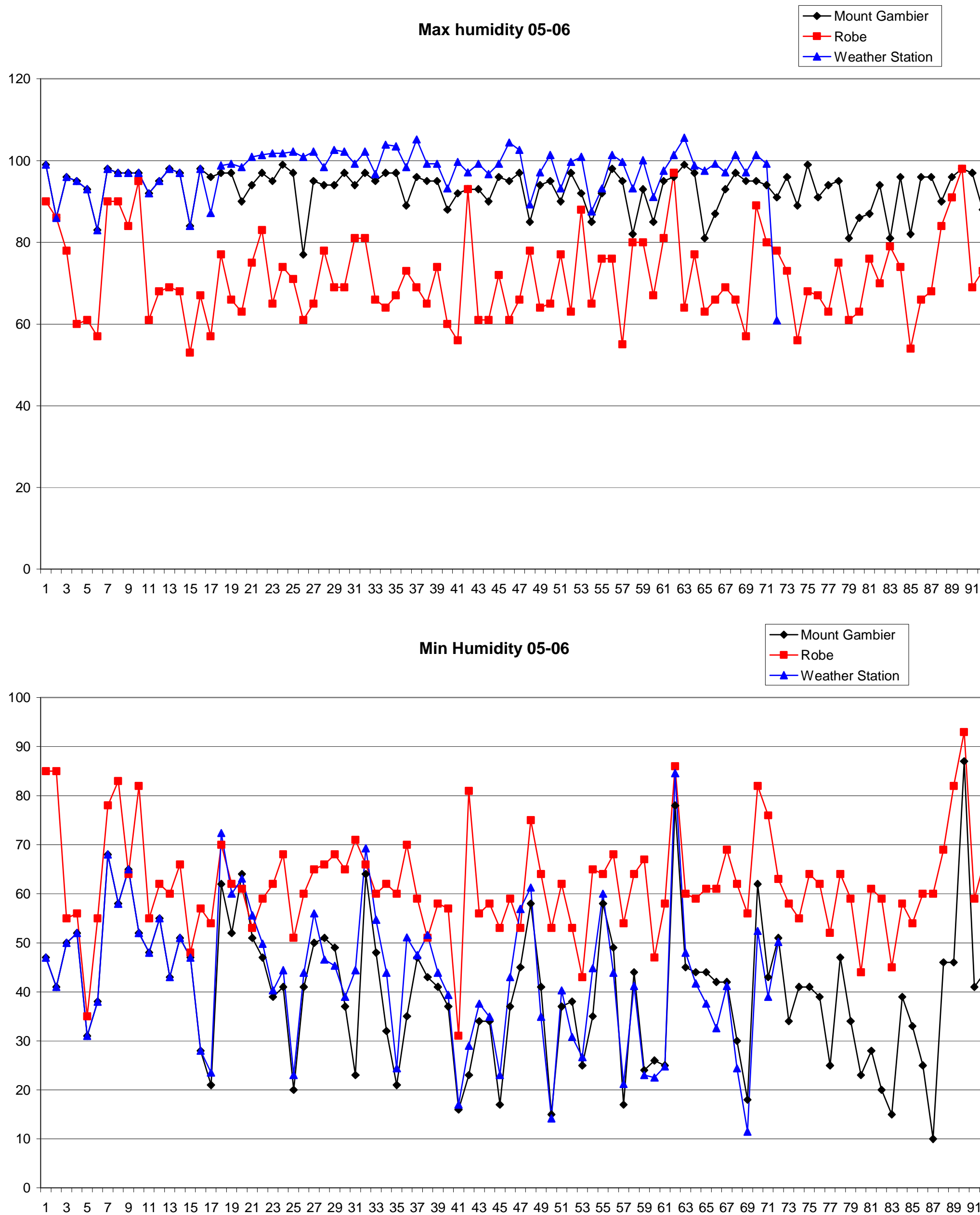


Figure A1.5 Comparison of maximum (A) and minimum humidity (B) for the Mount Gambier aero and Robe weather stations (2005/2006 season). The months of November through to January are represented. Mount Gambier follows the trend of the on site station.

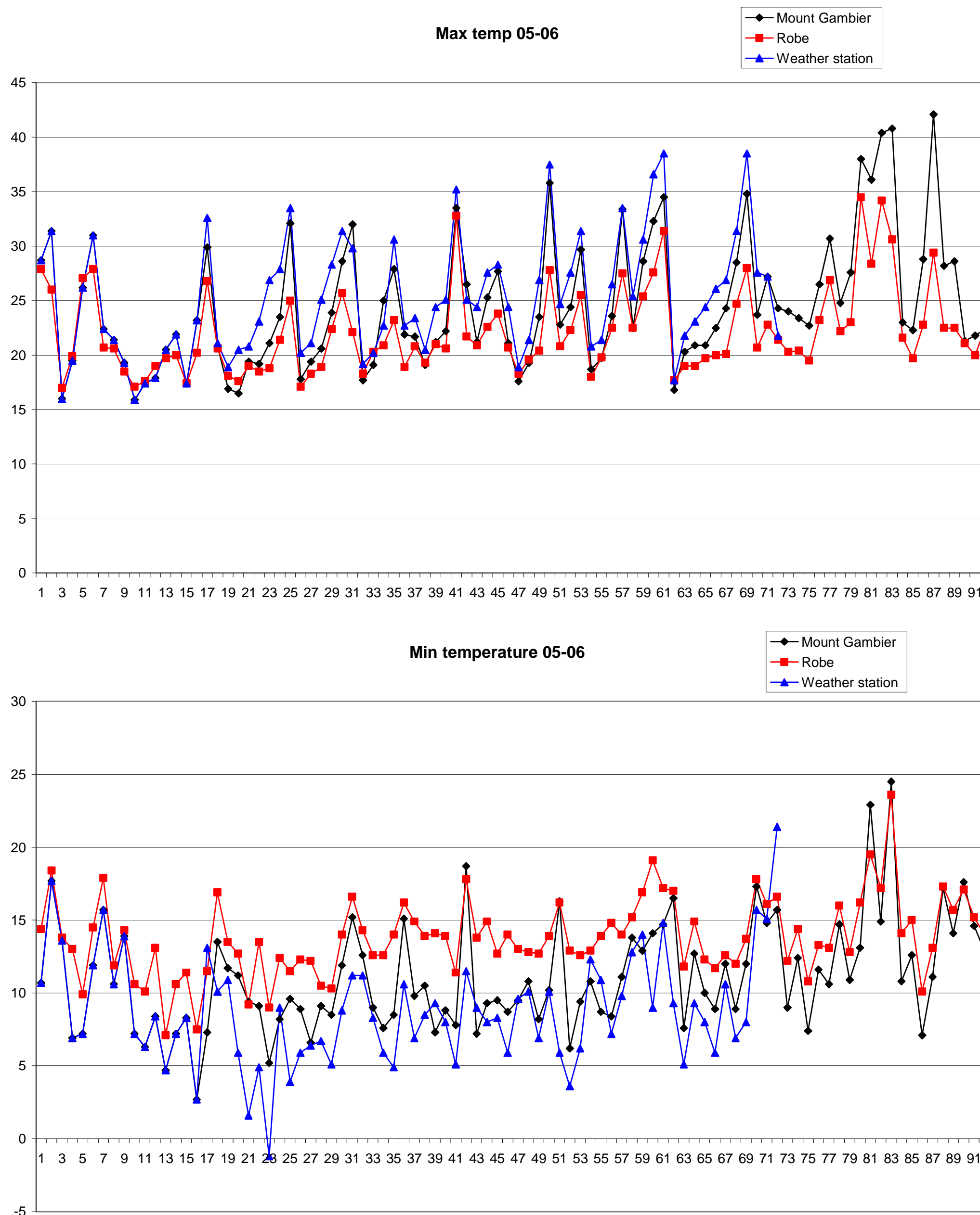


Figure A1.6 Comparison of maximum (A) and minimum temperature (B) for the Mount Gambier aero and Robe weather stations (2005/2006 season). The months of November through to January are represented. Mount Gambier follows the trend of the on site station and to some extent with the lower temperatures observed at the trial site (Hatherleigh).

Appendix 2. Candidate genes for the eQTL for *HvBP1* (Chapter 4) in the regions of Rice Chromosomes syntenous to barley identified through comparative mapping. Regions of Rice Chromosome 4 and 7, which were found to be syntenous to barley 2H (Figure 4.11). Regions of Rice Chromosome 9 and 3, which were found to be syntenous to barley 5H (Figure 4.12).

Please refer to attached file:in the CD on the back cover of the thesis.

Candidate genes in the regions of Rice Chromosomes syntenous to barley identified through comparative mapping.xls

Appendix 3. Regulatory DNA elements identified for *HvBP1* and *HvPrx7* promoter regions (Chapter 5). Analayis was undertaken using PLACE database analysis (Higo *et al.* 1999)

Please refer to attached files:in the CD on the back cover of the thesis.

1. *BP1 Place database analysis*
2. *Prx7 Place database analysis*

Appendix 4.

Peer-reviewed publications

K. Ryan Walker, Jason A. Able, Diane E. Mather, and Amanda J. Able.
Black point formation in barley: environmental influences and quantitative trait loci
Australian Journal of Agricultural Research, 2008, **59**, 1021–1029

Conference Proceedings

Walker, K.R., Able, J.A., Mather, E.D., Able A.J (2008)
Investigating the expression and regulation of two peroxidase genes in barley
10th International Barley Genetics Symposium, Bibliotheca Alexandrina, Alexandria, Egypt.

Ryan Walker, Jason A. Able, Diane E. Mather, Amanda J. Able (2007)
Differential gene expression associated with Black Point formation in barley
13th Australian Barley Technical Symposium, Freemantle, Western Australia.