Regulation of candidate genes in black point formation in barley

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Declaration

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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