Genetic Control of Hydrolytic Enzymes in Germinated Barley (*Hordeum vulgare* L.)

By

**Cheng-dao Li (M.Ag.)**
Zejiang Agricultural University, China

A thesis submitted for the Degree of Doctor of Philosophy in the Faculty of Agricultural and Natural Resource Sciences at the University of Adelaide

Department of Plant Science
Waite Agricultural Research Institute
University of Adelaide

November 1997
# Table of Content

Acknowledgement i

Declaration ii

Papers published from this thesis iii

List of abbreviations iv

Summary vi

## Chapter 1 Introduction and literature review

1.1 Introduction 1

1.2 Literature review 3

1.2.1 The factors which influence malt quality 3

1.2.1.1 The cell wall and its degradation 3

1.2.1.1.1 Endo 1,3-1,4-β-glucanase 4

1.2.1.1.2 Endo 1,3-β-glucanase 6

1.2.1.1.3 Other hydrolytic enzymes degrading cell walls 7

1.2.1.2 Degradation of starch granules 8

1.2.1.2.1 Alpha-amylases 9

1.2.1.2.2 Beta-amylases 14

1.2.1.2.3 Limit dextrinase 17

1.1.1.2.4 Alpha-glucosidase 18

1.2.1.3 Degradation of the protein 21

1.2.1.4 GA induced hydrolytic enzyme expression in germinated barley 22

1.2.2 Mapping genes and quantitative trait loci (QTL) using genetic markers 25

1.2.2.1 Development of genetic markers 25

1.2.2.1.1 From morphological markers to RFLP markers 25

1.2.2.1.2 Development of PCR markers 27

1.2.2.1.2.1 RAPD markers 29

1.2.2.1.2.2 Microsatellite DNA markers 30

1.2.2.1.3 Development of AFLP markers 31
### 1.2.2.2 Gene mapping in barley

### 1.2.2.3 Mapping quantitative trait loci (QTL) using molecular markers

### 1.2.2.4 QTL mapping in barley

### 1.3 Aims of the present project

---

#### Chapter 2 Materials and methods

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Materials</td>
<td>43</td>
</tr>
<tr>
<td>2.2 Methods</td>
<td>44</td>
</tr>
<tr>
<td>2.2.1 DNA isolation and Southern blot analysis</td>
<td>44</td>
</tr>
<tr>
<td>2.2.1.1 Small scale DNA isolation</td>
<td>44</td>
</tr>
<tr>
<td>2.2.1.2 Large scale DNA isolation</td>
<td>45</td>
</tr>
<tr>
<td>2.2.1.3 Digestion of genomic DNA with restriction enzymes</td>
<td>45</td>
</tr>
<tr>
<td>2.2.1.4 Agarose gel electrophoresis</td>
<td>46</td>
</tr>
<tr>
<td>2.2.1.5 Transfer of DNA onto nylon membranes</td>
<td>46</td>
</tr>
<tr>
<td>2.2.1.6 Probe preparation and labeling</td>
<td>46</td>
</tr>
<tr>
<td>2.2.1.7 Hybridisation and autoradiography</td>
<td>47</td>
</tr>
<tr>
<td>2.2.1.8 Removal of radioactive probe from membranes</td>
<td>48</td>
</tr>
<tr>
<td>2.2.2 PCR amplification of specific genes from the barley genome</td>
<td>48</td>
</tr>
<tr>
<td>2.2.3 Gene cloning</td>
<td>49</td>
</tr>
<tr>
<td>2.2.3.1 RNA isolation from developing seeds</td>
<td>49</td>
</tr>
<tr>
<td>2.2.3.2 Reverse transcriptase PCR (RT-PCR)</td>
<td>50</td>
</tr>
<tr>
<td>2.2.3.3 Ligation of DNA-sequences into plasmid T-vector</td>
<td>50</td>
</tr>
<tr>
<td>2.2.3.4 Transformation of ligated PCR:pGEM-T vector</td>
<td>51</td>
</tr>
<tr>
<td>2.2.3.5 Isolation of recombinant plasmids</td>
<td>51</td>
</tr>
<tr>
<td>2.2.3.6 DNA sequencing</td>
<td>52</td>
</tr>
<tr>
<td>2.2.4 Preparation of green malt</td>
<td>52</td>
</tr>
<tr>
<td>2.2.5 Isoelectric focusing electrophoresis and Western blotting analysis</td>
<td>52</td>
</tr>
<tr>
<td>2.2.6 Assay for activity of the enzymes</td>
<td>53</td>
</tr>
<tr>
<td>2.2.6.1 Assay for alpha-amylase activity</td>
<td>53</td>
</tr>
</tbody>
</table>
2.2.6.2 Assay for beta-amylose activity
2.2.6.3 Assay for beta-glucanase activity
2.2.6.4 Assay for the activity of limit dextrinase
2.2.7 Gene mapping and QTL analysis
2.2.8 Reverse bulked segregant-analysis
2.2.10 Seed dormancy

Chapter 3 Sequence variation, isoenzyme type and activity QTL of beta-amylose in barley (Hordeum vulgare L.)

3.1 Introduction
3.2 Results
3.2.1 Isolating beta-amylose cDNA
3.2.2 Mapping of beta-amylose genes with RFLP
3.2.3 Mapping of isoenzyme polymorphisms
3.2.4 Mapping of QTLs controlling the enzyme activity in germinated barley to the enzyme activity in barley grain
3.2.5 Reverse bulked segregant-analysis of the contribution of the beta-amylose locus
3.3 Discussion
3.3.1 Chromosomal location of beta-amylose genes
3.3.2 Genes controlling beta-amylose enzyme activity
3.3.3 Sequence variation and isoenzyme type

Chapter 4 Molecular mapping of structural loci for hydrolytic enzymes of germinating barley

4.1 Introduction
4.2 Results
4.2.1 Mapping structural loci of hydrolytic enzymes degrading cell walls
4.2.1.1 RFLP and PCR mapping of 1,3-1,4-β-glucanase genes
4.2.1.2 Molecular mapping of the 1,3-\(\beta\)-glucanase gene family
4.2.1.3 Chromosomal location of \(\beta\)-glucosidase
4.2.1.4 RFLP mapping of the 1,4-\(\beta\)-xylanase genes
4.2.2 Mapping structural loci of hydrolytic enzymes degrading starch
4.2.2.1 RFLP and isoenzyme mapping of alpha-amylase genes
4.2.2.2 RFLP mapping of limit dextrinase gene
4.2.2.3 Chromosomal location of alpha-glucosidase gene
4.3 Discussion
4.3.1 Gene locations of 1,3-1,4-\(\beta\)-glucanases
4.3.2 Gene locations of 1,3-\(\beta\)-glucanases
4.3.3 Relationship of 1,3-\(\beta\)-glucanase and 1,3-1,4-\(\beta\)-glucanase
4.3.4 Gene location and function of 1,4-\(\beta\)-xylanase
4.3.5 Relationship of the RFLP and IEF bands of alpha-amylase
4.3.6 Gene location of limit dextrinase
4.3.7 Summary

Chapter 5 Molecular mapping of QTLs for the enzyme activity of beta-glucanase, alpha-amylase and limit dextrinase

5.1 Introduction
5.2 Results
5.2.1 QTLs controlling the enzyme activity of 1,3-1,4-\(\beta\)-glucanase
5.2.1.1 Mapping QTLs controlling the enzyme activity
5.2.1.2 Comparison of the QTLs in different mapping populations
5.2.2 QTLs controlling the enzyme activity of alpha-amylase
5.2.2.1 Mapping QTLs controlling alpha-amylase activity
5.2.2.2 Comparison of the QTLs for alpha-amylase in different mapping populations
5.2.3 QTLs controlling the enzyme activity of limit dextrinase
5.2.3.1 Mapping QTLs controlling the LD activity
5.2.3.2 Comparison of QTL locations for limit dextrinase in two mapping populations 91
5.2.3.3 Marker-assisted selection for the limit dextrinase activity 92
5.2.4 Mapping QTLs controlling seed dormancy, kernel weight and kernel shape 93
5.3 Discussion 94
5.3.1 Relationship between structural genes and enzyme activity 94
5.3.2 Genes controlling the activity of LD 95
5.3.3 Interaction of hydrolytic enzymes for degrading starch 97
5.3.4 Heritability and QTL mapping 98
5.3.5 Common QTLs controlling multiple traits 98

Chapter 6 Molecular mapping of GA$_3$-regulated genes and GA-responsive loci 100
6.1 Introduction 100
6.2 Results 101
6.2.1 Chromosomal locations of the GA$_3$ induced genes 101
6.2.2 Mapping the GA$_3$-induced genes 102
6.2.3 Reverse bulked-analysis to identify a GA-responsive locus 103
6.2.4 Selecting the enzyme activity using RFLP marker ABC468 104
6.2 Discussion 105

Chapter 7 General discussion 109
7.1 Gene locations and functions of hydrolytic enzymes 109
7.2 Regulatory loci for the enzyme activity 110
7.3 Common QTLs controlling the activity of several hydrolytic enzymes 110
7.4 Selecting the enzyme activity by molecular markers 111
7.5 Isolating the genes underlying favourable QTL 112

Bibliography 114
Summary

Hydrolytic enzymes are not only physiologically important for seed germination, but also commercially important for the malting and brewing industry. The aims of the present study include (1) mapping the structural genes of hydrolytic enzymes and determining the contribution of each gene to the activity of the enzyme and (2) identifying regulatory loci (QTL) controlling the activity of the enzymes.

Using RFLP, STS-PCR and isoenzyme techniques, three doubled haploid populations and the wheat-barley addition lines, genes encoding most of the important hydrolytic enzymes for degrading barley endosperm cell walls and starch were mapped. The seven genes encoding endo 1,3-β-glucanase are all located on the long arm of chromosome 3H, with the genes encoding isoenzymes G1, GII, GIII, GIV, GV and GVII clustered in a region of less than 20 cM. The region is flanked by the RFLP marker MWWG2099 on the proximal side and the Barley Yellow Mosaic Virus (BYMV) resistance gene ym9 at the distal end. The gene encoding isoenzyme VI lies approximately 50 cM outside this cluster, towards the centromere. All of the 1,3-β-glucanase genes, except GIV, are represented by single copies on the barley genome. A single gene for endo 1,3-1,4-β-glucanase isoenzyme EI was mapped on the long arm of chromosome 1H with both RFLP and STS-PCR markers. The gene is closely flanked by the RFLP markers PSR121 and AWBMA34; each only 0.3 cM distant. However, no polymorphism could be detected for the isoenzyme EII on chromosome 7H. The β-glucosidase gene was located on chromosome 2H and its surrounding regions are highly conserved. In contrast, the genes encoding 1,4-β-xylanase are highly polymorphic. The three xylanase genes are closely linked on the long arm of chromosome 5H, flanked by the RFLP markers CDO506 on the proximal side and PSR370 at the distal end.

Several RFLP and major isoenzyme bands of α-amylase were mapped to the long arm of chromosome 6H. The single genes encoding limit dextrinase and α-glucosidase were located chromosomes 7H and 2H, respectively.
There are three β-amylase genes in the barley genome. One is located on chromosome 2H, close to the centromere, and two are on chromosome 4H, close to the telomere. The four major isoenzyme bands of β-amylase were mapped on chromosome 4H and a series of minor bands on chromosome 6H. The four major isoenzyme bands and the two β-amylase genes cosegregated on chromosome 4H.

Particular emphasis was placed on analysing the effect of β-amylase activity since this is the key enzyme in determining diastatic power. Two cDNAs, encoding the Sd1 and Sd2 isoenzymes of β-amylase, were isolated. One amino acid difference (Met-527 to Ile-527) in the C-terminus of β-amylase could result in the partial loss of binding ability with the enzyme inhibitor (s), and alter the pI and banding-pattern of β-amylase.

QTL and reverse bulked-segregant analysis showed that the β-amylase locus (*Bmy1*) on chromosome 4H controlled the total and free activity of this enzyme and the free/bound enzyme ratio in barley grain. The activity of free enzyme in green malt is also controlled by a locus in this region. The effect of this locus on the enzyme activity was consistently detected and accounted for 12% to 20% of the total variation for β-amylase activity in the three mapping populations. This locus also explained nearly 100% of the variation in the ratio of free/bound enzyme in the “Chebec X Harrington” population. However, the β-amylase locus (*5my2*) on chromosome 2H had no detectable effect on enzyme activity.

The structural locus (*Amy1*) of α-amylase on chromosome 6H contributes 5.3% and 12.9% of the variation of the enzyme activity in the “Haruna Nijo X Gillean” and “Chebec X Harrington” populations, respectively, but no effect in the “Clipper X Sahara377” population. No significant effect on enzyme activity was found at the chromosome 7H locus (*Amy2*).

One QTL for 1,3-1,4-β-glucanase activity was detected on chromosome 7H, which could be related to the EI structural locus (*GhB1*), but no significant contribution to the enzyme activity was found from the EI locus on chromosome 1H. Interestingly, the region of the 1,3-β-glucanase gene cluster contributed to the enzyme activity of 1,3-
1,4-β-glucanase, which suggests that 1,3-β-glucanases may join 1,3-1,4-β-glucanase to degrade 1,3-1,4-β-glucan during seed germination. The limit dextrinase locus (Ld) on chromosome 7H only controlled the total enzyme activity but not the free enzyme activity in green malt.

In addition to the structural loci, regulatory loci were also identified as controlling the activity of 1,3-1,4-β-glucanase, α-amylase, β-amylase and limit dextrinase. One, two and five regulatory loci were found for the activity of 1,3-1,4-β-glucanase in the three mapping populations, respectively, with one QTL on chromosome 2H explaining 21.5% of the total variation of the enzyme activity. Five to six QTLs were identified controlling the activity of α-amylase in the three populations with the QTLs on chromosomes 2H and 5H making the most significant contributions. One QTL linked to the bordein gene family on chromosome 1H was consistently implicated in the control α-amylase activity in all three mapping populations. The QTLs for the activity of limit dextrinase showed similar chromosomal distribution as for α-amylase, but less variation could be explained by the given QTLs. Three, four and six regulatory QTLs were found for the activity of β-amylase in the three mapping populations. In addition, QTLs for seed dormancy, kernel weight and kernel shape were mapped.

Common QTLs were identified as controlling the activity of several hydrolytic enzymes. The common QTLs on chromosome 5H were consistent with the major QTLs for seed dormancy. A QTL on chromosome 2H additionally controlled plant height and photoperiod response. Reverse bulked-segregant analysis demonstrated that the common QTL on chromosome 2H is related to GA-responsiveness. The two GA-induced genes, EshA and Esh2A, mapped on chromosomes 2H and 5H could be the candidate genes. Using the RFLP marker ABC468 linked with the common QTL on chromosome 2H as reference, the activity of α-amylase, β-glucanase and limit dextrinase could be increased by 27.7%, 31.1% and 15.2%, respectively, by selecting for the favourable allele at this locus. The locus was predicted to account for 29.5%, 31.4% and 23.4% of the total variation of activity of α-amylase, β-glucanase and limit dextrinase, respectively.
The major finding of this work has been the demonstration of common loci influencing the activity of several key hydrolytic enzymes involved in the mobilisation of seed reserves. This result will allow a more controlled selection of diastatic power and extract levels that has previously been possible. It is clear from the results obtained that major gains can be achieved through the selection of only a few desired alleles.