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 wish to sincerely thank my supervisors Dr. Peter Langridge and Dr. Reg Lance. Their stimulating ideas, wisdom, guidance, supervision, encouragement, patient and critical review of the manuscript were invaluable for me to complete my study.

I am grateful to Professor GB Fincher for his encouragement and advice for mapping the genes encoding 1,3-beta-glucanase, 1,4-beta-xylanase and limit dextrinase, and to Dr. Lesley MacLeod for her supervision during the early stages of my study.

Many thanks go to all the staff and students, past and now, in Dr. Langridge's Laboratory for their help and friendship, especially to Mr. Angelo Karakousis for his help on techniques and computer; to Ms Jan Nield, Ms Jodie Kretschmer, Ms Sue Manning, Dr. Xinmin Li and Dr. Ute Bauman for their excellent advice and technical support.

I am indebted to Dr. AKMR Islam, Dr. Sue Logue, Dr. Peilin Xu, Dr. Xiaoqi Zhang, Dr. J Jacobsen and Dr. Evan Evans for their precious plant materials, DNA clones, antibodies or protein.

I express my thanks to Dr. Evan Evans, Mr. Jason Eglinton and Ms Cathy Gibson for the techniques of enzyme analysis.

I appreciate the help from the staff of barley group for preparing plant materials, especially Dr. Andrew Barr, Miss Sophie Roumeliotis and Ms Amanda Box.

Many thanks go to Ms Margret Cargill, Ms Mandy Pace, Mr. Peter Cassidy, Mr. Barry Felberg and Ms Ruth Ellickson for their help and advice.

I acknowledge the Australian government for providing an Aus-Aid scholarship and the Chinese government to allow me pursuing my study.

Finally, I thank my parents, wife and son for their love, patience and encouragement.

Declaration

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

I give consent to this copy of my thesis, when deposited in the University of Adelaide Library, being available for loan and photocopying

Cheng-dao Li

November 1997

Papers published from this thesis

1. **Li CD**, Langridge P, Lance RCM, Xu P, Fincher GB (1996) Seven members of the 1,3-β-glucanase gene family in barley (*Hordeum vulgare*) are clustered on the long arm of chromosome 3 (3HL). Theor Appl Genet 92:791-796

2. Banik M, **Li CD**, Langridge P, Fincher GB (1997) Structure, hormonal regulation and chromosomal location of gene encoding barley 1,4-β-xylan endohydrolases. Mol Gen Genet, 253: 599-608

3. **Li CD**, Langridge P, Lance RCM, Fincher GB (1996) Molecular mapping the cloned genes and the QTLs related to the hydrolytic enzyme activity. In 6th International Barley Genetics Symposium, Saskatoon, Canada

4. **Li CD**, Lance RCM, Langridge P, Fincher GB (1996) Mapping the QTLs controlling aspects of malting quality. In I.T.M.I. Public Workshop, Sydney, Australia. p10

5. Langridge P, A Karakousis, J Kretschmer, S Manning, R Boyd, **C Li**, S Logue, R Lance and AARDI Chebec X Harrington RFLP & QTL analysis. http://greengenes.cit.cornell.edu/waiteQTL/CXH.html

6. Langridge P, A Karakousis, J Kretschmer, S Manning, **C Li**, S Logue, R Lance Clipper X Sahara 3371 RFLP & QTL analysis.
http://greengenes.cit.cornell.edu/waiteQTL/CXS.html

7. Langridge P, A Karakousis, J Kretschmer, S Manning, **C Li** Galleon x Haruna Nijo RFLP & QTL analysis. http://greengenes.cit.cornell.edu/waiteQTL/GXH.html

List of abbreviations

aa	amino acid
A-PCR	arbitrarily primed PCR
AFLP	amplified fragment length polymorphism
BASI	bifunctional α-amylase subtilisin inhibitor
BSA	bulked segregant-analysis
BC	backcross population
bp	base pair
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
Ci	curie
cM	centiMorgan
cm	centimeter
DAF	DNA amplification fingerprinting
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosin triphosphate
DH	doubled haploid population;
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DP	diastatic power
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EBC	European Brewery Convention
EDTA	ethleneiaminetetraacetic acid
EMBL	European Molecular Biology Laboratory
g	gram
GA	gibberellic acid
HRP	horse radish peroxidase
IEF	isoelectric focusing
IPTG	isopropyl-1-thio-β-D-galactoside
kD	kiloDalton
LD	limit dextrinase
M	molar
mA	milliampere
µg	microgram/s
ml	milliliter/s
µl	microliter/s
ML	maximium likelihood
mM	millimolar
mRNA	messenger ribonucleic acid
ng	nanogram
nm	nanometer
°C	degrees centrigrade
OD	optical density
oligo(dT)	oligodeoxythymidylic acid
PAGE	polyacrylamide gel eletrophoresis
PCR	Polymerase Chain Reaction
PIPES	piperazine-N,N'-bis-2-ethane-sulfonic acid
PVP	polyvinyl polypyrrolidone
QTL	Quantitative Trait Loci
RAMP	random amplification of microsatellite DNAs
RAPD	random amplified polymorphic DNA
rDNA	ribosomal deoxyribonucleic acid

Reg	regression analysis
RFLD	restriction fragment length difference
RFLP	restriction fragment length polymorphism
RI	recombinant inbred lines
RNA	ribonucleic acid
RNase A	ribonuclease
rpm	revolution per minute
RT-PCR	reverse transcriptase PCR
Sarkosyl	N-lauroylsarcosine
Sd	starch degrading enzyme
SDS	sodium dodecyl sulfate
SSC	sodium chloride/sodium citrate (buffer)
SSR	simple sequence repeats
STS	sequence tagged site
TAE	Tris/acetate (buffer)
TBE	Tris/boric acid (buffer)
Taq	Thermus aquatacus DNA polymerase
TE	Tris/EDTA (buffer)
UV	ultraviolet
V	volt
v/v	volume/volume
W	Watt
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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 GI, GII, GIII, GIV, GV and GVII clustered in a region of less than 20 cM. The region is flanked by the RFLP marker MWG2099 on the proximal side and the Barley Yellow Mosaic Virus (BYMV) resistance gene *ym4* 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 factors affecting 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 (*Bmy2*) 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 Galleon" and "Chebec X Harrington" populations, respectively, but no effect in the "Clipper X Sahara3771" 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 EII structural locus (*Glb2*), 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 hordein 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, *Es1A* and *Es2A*, 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.

Chapter 1: Introduction and Literature Review

1.1 Introduction

The use of barley to produce malt and beer dates back to Egyptian times (Hardwick, 1977). Some of 10% of the barley crop is devoted to the production of beer today (Hoseney, 1994). The characteristic properties of many beers (for example, their colour, foam and some of their flavor notes) are a direct consequence of the malted barley used in their production (Bamforth and Barclay, 1993). Consequently, barley breeders and maltsters have been striving to identify and improve malting quality. Since Bishop (1934) established the importance of low protein as a malting quality factor, many other parameters have been proposed as criteria for malting quality (see reviews in MacGregor and Bhatty, 1993; Shewry, 1992). These include diastatic power (DP) (Meredith et al, 1942), malt extract (Meredith, 1943), milling energy (Allison et al, 1976), extract viscosity (Morgan and Gothard, 1977), sedimentation (Reeves et al, 1979), acid extract composition (Smith and Briggs, 1980), gelprotein (Van den Berg et al, 1981) and 1,3-1,4-β-glucanase (Fincher et al, 1986). The major quality parameters in common use now are malt extract, barley and malt protein, soluble protein, the ratio of soluble protein to malt protein (Kolbach index), diastase, wort viscosity and apparent attenuation limit (MacGregor and Bhatty, 1993), but these characteristics have different levels of importance for the malt industry. According to the European Brewery Convention (EBC), the respective empirical weightings are: malt extract (0.45), wort viscosity (0.25), apparent final attenuation (0.15), Kolbach index (0.10) and diastatic power (0.05) (Molina-Cano, 1987). Besides these biochemical criteria, some morphological traits are also considered to be important quality factors in the

malting and brewing industry. These include seed dormancy, seed colour, kernel hectoliter weight and % plump grains (as assessed using 2.2 or 2.5 mm screens).

The biochemical process of malting and brewing includes degradation of cell walls and conversion of starch, protein and other reserves into fermentable sugars to provide nutrition for the yeast. Thus hydrolytic enzymes play a central role in malting and brewing. Three kinds of hydrolytic enzymes develop in sequence during germination. They are the cell wall degrading enzymes, endo-peptidases and amylases. The activity of hydrolytic enzymes directly affects malting quality; for example, the activity of 1,3-1,4-β-glucanase is closely related to malt extract and wort viscosity (Henry, 1989; Stuart et al, 1986). Higher levels of hydrolytic enzymes mean higher malting quality and shorter mashing periods and allow for the addition of other sources of starch (adjuncts) to the mashing process (Kasha et al, 1993).

All malt characters have complicated genetic control influenced through environmental interactions (Ellis et al, 1989). Consequently, it is very difficult to dissect and manipulate these characters in a breeding program. Although great efforts have been made to improve malt quality, the advance to date is not satisfactory to the malting and brewing industry. Reiner *et al.* (1984) studied the improvement of barley malt quality in 16 European countries from 1951 to 1980. The results showed that malt extract increased at a rate of between 0.01 and 0.02% per annum, Kolbach index between -0.2 and 0.0% and diastatic power between -0.6 and 4.7% per annum. Although selection for low protein content has taken place, the protein content has still increased in the past sixty years due to the low heritability and complexity of this character and possibly the advent of more intensive agriculture with higher nitrogen. At the current rate of progress, 150 to 300 years would be needed to increase the malt extract from the present level of 80% to the breeding target of 83%. Therefore, there is an urgent need to develop new methods to improve malt quality.

Development of micro-malting has accelerated progress in the improvement of malt quality. However, this is a slow and laborious process and the results vary with the environmental conditions. Thus the major challenge in breeding is to identify desirable genetic variability and then to select for it genotypically rather than phenotypically (Cubitt, 1991). The advent of RFLP (Restriction Fragment Length Polymorphism) (Botstein et al, 1980), PCR (Polymerase Chain Reaction) (Saiki, 1988) and RAPD (Random Amplified Polymorphic DNA) (Williams et al, 1990) provides the possibility of a more precise genetic approach to meet this challenge. Molecular markers would allow breeders to trace specific alleles through the breeding generations, decrease the size of populations and increase selection efficiency in breeding programs (Blake et al, 1991).

1.2 Literature review

1.2.1 The factors which influence malt quality

1.2.1.1 The cell wall and its degradation

The main components of the cell wall in barley grains are arabinoxylan and 1,3-1,4-β-glucan. The cell walls of the aleurone layer consists of 67% arabinoxylan and 26% glucan, and that of endosperm contains 70% glucan, 20% arabinoxylan and 6% protein (Ballance and Manners, 1978; Fincher, 1975, 1976; MacGregor, 1990). As the major starch and protein reserves are contained within the endosperm cell wall, while the hydrolytic enzymes are predominantly synthesised and secreted from the aleurone or scutellum, the cell wall is a physical barrier between the hydrolytic enzymes and their substrates (Fincher and Stone, 1993). Incomplete cell wall degradation in the malting and brewing processes results in lower malt extract, causes filtration difficulty and contributes to certain hazes or precipitates that form in stored beer (Bamforth and Barclay, 1993).

The speed of degradation of the cell walls depends on both the content of β-glucan and arabinoxylan and the activities of related enzymes. The content of gum (glucan and arabinoxylan) is controlled by a simple additive genetic system and the number of genetic factors is between 3 and 5 (Lance et al, 1987; Powell et al, 1985), but the location of these factors and how they may be manipulated are still unknown. However, Henry (1989), Loi *et al.* (1987, 1988) and Aastrup and Munck (1981) showed that malt extract is not highly correlated with total glucan in ungerminated barley grain, but highly negatively correlated with the residual glucan in malted barley. That means the enzymes involved in the degradation of the cell wall are more important than the gum content for malting quality.

1.2.1.1.1 Endo 1,3-1,4-β-Glucanase

The most important enzymes in the depolymerisation of the cell wall are the endo-1,3-1,4-β-glucanases (EC 3.2.1.73) (Fincher and Stone, 1993). Two 1,3-1,4-β-glucanases designated as isoenzymes EI and EII were purified and characterised from extracts of germinated barley (Woodward and Fincher, 1982). These isoenzymes showed different isoelectric points, glycosylation patterns and mobility during SDS gel electrophoresis, although they exhibited essentially identical substrate specificities, pH optima and action patterns (Woodward and Fincher, 1982). Two cDNAs of 1,3-1,4-β-glucanase have been cloned from barley, which correspond to EI and EII. The deduced amino acid showed 91% positional identity (Fincher et al, 1986; Slakeski et al, 1990; Wolf, 1991), therefore it is assumed that these two genes probably arose by duplication of a common ancestral gene (Woodward and Fincher, 1982).

Southern blot analysis demonstrated that there exist two genes of 1,3-1,4-β-glucanase in the barley genome. The EI gene locates on the long arm of chromosome 5 (1H) and EII on the long arm of chromosome 1 (7H) (Loi et al, 1988; MacLeod et al, 1991; Slakeski et al, 1990). The structure of the two genes

have been well demonstrated (Wolf, 1991) and they are regulated by GA and auxin (Slakeski and Fincher, 1992). Examination of 13 barley cultivars using Southern blot analysis revealed that the EII gene was highly polymorphic, but the EI gene is conserved (Slakeski et al, 1990). This polymorphism may be used as a molecular marker to select the EII gene (Beckman and Soller, 1986).

The 1,3-1,4-β-glucanase genes are expressed during seed germination. In quiescent barley grain, no 1,3-1,4-β-glucanase mRNA or active protein were detectable, but one day after the initiation of germination, very high levels of 1,3-1,4-β-glucanase were found in the scutellar epithelium; at this stage no expression could be detected in the aleurone (McFadden et al, 1988). Subsequently, the 1,3-1,4-β-glucanase mRNA in the aleurone increases progressively from the proximal to the distal end of the grain, whereas the levels in the scutellar epithelium decrease rapidly. The enzyme activity reaches a maximum five to six days after the initiation of germination (Balance et al, 1976; Stuart and Fincher 1983). Using the 3' untranslated region of 1,3-1,4-β-glucanase as a gene specific probe, Northern blot analysis confirmed that EI expresses in the scutella and aleurone of germinated seed and developing leaves and roots, whereas EII expression predominates in the aleurone (Slakeski et al, 1990; Slakeski and Fincher, 1992). Thus EII appears to be aleurone-specific. Considering that most hydrolytic enzymes are synthesised and secreted from the aleurone (Fincher and Stone, 1993), the EII gene could be more important than EI for malting.

Quantitative Western transfer analysis indicated that isoenzyme EI and EII develop in approximately equimolar amounts with EI accumulation slightly preceding that of isoenzyme EII in the malting barley variety Clipper (Stuart and Fincher, 1983). However, considerable variation of the ratio was observed in other varieties and in the grains produced under different environmental conditions (Henry, 1990). These results suggested that the activity of 1,3-1,4-β-glucanase is controlled by multiple loci. Recently, attempts have been made to map the QTLs

controlling the activity of the enzyme (Han et al, 1995). The details of these studies will be discussed in the following chapter.

1.2.1.1.2 Endo 1,3-β-Glucanases

In addition to 1,3-1,4-β-glucanases, high levels of 1,3-β-glucanase activity were found in the endosperm of germinated barley (Høj et al, 1988, 1989). 1,3-β-Glucanases (EC 3.2.1.39) can depolymerise 1,3-β-glucans to laminaribiose and glucose in an endohydrolase action pattern. Thus it has been suggested that they may participate in cell wall degradation through an ability to hydrolyse regions of contiguous 1,3-β-linked glucosyl residues in the cell wall (MacGregor and Bhatty, 1993). However, the long sequences of 1,3-β-links do not occur in the polymers of barley endosperm cell walls and purified 1,3-β-glucanases are unable to hydrolyse water-soluble barley 1,3-1,4-β-glucans at 40°C (Woodward and Fincher, 1983; Høj and Fincher, 1995). Therefore, 1,3-β-glucanases are not likely to be involved in the degradation of the endosperm cell walls, but they may function to degrade the small deposits of 1,3-β-glucan that are scattered through the starchy endosperm (MacGregor et al, 1989) and also to open the aleurone cell walls (Bamforth and Barclay, 1993). However, it seems that the activity of 1,3-β-glucanases is much higher than that required to depolymerise the cell wall deposits. The apparent paucity of endogenous substrate, together with their ability to hydrolyse the 1,3- and 1,3-1,6-glucan that are major cell wall constituents in some classes of fungal pathogens of plant, has led to speculation that these enzymes may participate in a general, non-specific strategy to protect grain against pathogen invasion (Fincher, 1989). Indeed, barley leaves infected by powdery mildew increase expression of 1,3-β-glucanase (Xu et al, 1992; Malehorn et al, 1993).

Three 1,3-β-glucanases have been purified from germinated barley (Høj et al, 1988, 1989; Wang et al, 1992), which were designated as isoenzymes GI, GII

Table 1.1 The properties of 1,3-β-glucanases

Isoenzyme	Number of amino acids	MW	pI	Transcription site
GI	310	33 KD	8.6	Young roots and leaves
GII	306	32.3KD	9.5	Aleurone
GIII	305	32.4KD	9.8	Young roots and leaves, shoots
GIV	327	35KD	10.7	Aleurone
GV	312	34KD	7.5	Young roots and leaves
GVI	315	32.9KD	4.6	Not known
ABG2	336	32.6KD	4.9	Roots

Note: The data from Xu *et al.*(1992) and Malehorn *et al.* (1993)

and GIII respectively. They are all basic proteins with a molecular mass of 32 kDa, but showed differences in their isoelectric points. Comparison of the 40 NH_2-terminal amino acids of 1,3-β-glucanase isoenzyme GI and GII with that of 1,3-1,4-β-glucanase isoenzyme EI and EII indicated that the two kinds of β-glucanases share 58% amino acid positional identity (Høj et al, 1989). Using a 734 bp Hinf I fragment of the barley 1,3-1,4-β-glucanase cDNA as a probe (Fincher et al, 1986), a cDNA encoding 1,3-β-glucanase isoenzyme GII was isolated (Høj et al, 1989). Subsequently, six members of the 1,3-β-glucanase gene family have been cloned using GII as a probe, which were designated as GI, GIII, GIV, GV, GVI and ABG2 (Xu et al, 1992; Wang et al, 1992; Malehorn et al, 1993). The properties of 1,3-β-glucanases are listed in Table 1.1.

The amino acid sequences of the mature barley 1,3-β-glucanases show 45% to 81% positional identity among the seven members of this gene family and they share 47% to 51% similarity with 1,3-1,4-β-glucanase isoenzyme EI and EII (Xu et al, 1992; Malehorn et al, 1993). Consequently, it was suggested that all the 1,3-β-glucanase and 1,3-1,4-β-glucanase genes originate from a common ancestral gene (Høj et al, 1989; Høj and Fincher, 1995; Xu et al, 1992).

The activity of 1,3-β-glucanases is located to the embryo in ungerminated grain but increase significantly in the endosperm during germination and is enhanced by gibberellic acid (Ballance et al, 1976). Isolated barley aleurone layers are also capable of secreting 1,3-β-glucanases (Taiz and Jones, 1970). However, little is known about the genetics of the enzyme activity and their importance for seed germination and malting quality is not well demonstrated.

1.2.1.1.3 Other hydrolytic enzymes degrading cell walls

There also exist other enzymes involving in depolymerisation of β-glucans during barley germination. These include cellulases, exo-β-glucanases and β-glucosidases. Cellulases degrade adjacent 1,4-linkages in the cell wall, but their

activity is low and variable, with much of the activity attributable to micro-organisms that reside on the surface of the grain (Hoy et al, 1981). Exo-β-glucanases and β-glucosidases can hydolyse 1,3-β-glucans, 1,3-1,4-β-glucans and oligo-1,3-1,4-β-glucans (Fincher, 1992). Recently, two exo-β-glucanases and two β-glucosidases have been purified from the geminated barley (Hrmova et al, 1995). The exo-β-glucanases hydrolyse polymeric 1,3-1,4-β-glucans at a significant rate. Thus they may play a role in degradation of cell walls.

In addition to the 1,3-β-glucans, one-fifth of the cell walls of barley starchy endosperm is arabinoxylan (Fincher, 1975). Endo- and exoxylanases, α-arabinofuranosidases and β-xyllosidases jointly depolymerize the arabinoxylan during barley germination (Preece and MacDougall, 1958; Taiz and Honigman, 1976). However, only recently have detailed studies commenced on the enzymes responsible for arabinoxylan degradation. Three endo-1,4-β-xylanases (EC 3.2.1.8) have been purified from 5-day-germinated barley (Slade et al, 1989). Their molecular weights are about 41,000 daltons and they have identical NH_2-terminal amino acid sequences. Recently, two cDNAs encoding 1,4-β-xylanases have been isolated from barley (Banik et al, 1996,1997). These enzymes increase in activity during germination and their accumulation was regulated by gibberellic acid (Slade et al, 1989; Banik et al, 1996). However, they appear several days after 1,3-1,4-β-glucanases (Banik et al, 1996). Therefore, it is still unclear whether arabinoxylan degradation is a necessary precursor to proteolysis and amylolysis. Nevertheless, 1,4-β-xylanases may function in the release of hydrolytic enzymes from the aleurone by acting synergistically with endo-β-glucanases (Bamforth and Barclay, 1993). Again, little is known about the mechanisms underlying the activity of these enzymes and their importance for malting quality.

1.2.1.2 Degradation of starch granules

Starch is the main component of the barley grain, composing about 58% to 65% (Briggs, 1992). It is the energy source for seed germination and growth or maintenance in humans and animals or for conversion to fermentable sugars for yeast fermentation such as in the brewing and distilling industries. The starch in barley grain generally contains about 75% amylopectin and 25% amylose (Foster and Prentice, 1987). However, this proportion depends on genetic and environmental factors; 100% amylopectin (Goering and Eslick, 1976) or high levels of amylose (Merritt, 1967) have been reported in barley grains. The different ratio of amylose/amylopectin determines the industrial quality of starch (Kasha et al, 1993).

Starch is stored as starch granules in the endosperm cells. The starch granules are associated with protein and lipids, which appear to contribute to their structural characteristics (Morrison, 1978, 1988). Two kinds of starch granules exist in the endosperm differing in size and shape. The large granules account for 90% of granules by weight (Briggs, 1978). Their relative size and shape are correlated with malt quality (Schildbach et al, 1990; Wallwork and MacLeod, 1991). However, the genetic basis for this is still unknown.

The starch is degraded to simple sugars during germination or the malting and brewing process. Enzymes involved in this process are α-amylase, β-amylase, limit dextrinase (R-enzyme or debranching enzyme) and α-glucosidase (Briggs, 1992). The combined activity of these enzymes determines the diastatic power in malting and brewing industry and directly affects malt yield and quality.

1.2.1.2.1 Alpha-amylases

Alpha-amylases (EC 3.2.1.1) attack α-1,4-linkages within chains of amylose and amylopectin in a random fashion. They initiate the degradation of starch granules. However, α-amylases are unable to hydrolyse the 1,6-α-linkages at the branch points in amylopectin and may vary in their ability to hydrolyse 1,4-α-

linkages close to the branch points (Fincher and Stone, 1993). The activity of α-amylase is positively correlated with malt extract and DP, thus high levels of α-amylase have been suggested as a criterion for good malting quality (Ingversen et al, 1989).

In ungerminated barley, there is no detectable activity of α-amylase and the enzymes are synthesised *de novo* on the rough endoplasmic reticulum of the aleurone during germination (Briggs and Clutterbuck, 1973). It has been shown that the enzymes are transferred from the rough endoplasmic reticulum to the Golgi and directly transported to the plasmalemma of the secreting cell (Fernandez and Staehelin, 1985; Gubler et al, 1986). Gibberellic acid and calcium ions are important regulators for enzyme formation and secretion (Briggs, 1992).

Alpha-amylases show extensive electrophoretic diversity (Hill and MacGregor, 1988; MacGregor, 1983, 1987; MacGregor and Marchylo, 1986). Based on their isoelectric points, four groups of isoenzymes of α-amylase have been detected, which are designated as α-amylase I (low pI, Group A), α-amylase II (high pI, Group B), α-amylase III and α-amylase X (Bertoft et al, 1984; Briggs and Raynes, 1985; Hill et al, 1987; Hill and MacGregor, 1988; MacGregor, 1983, 1987; Muthukrisshnan and Chandra, 1988). Their properties are shown in Table 1.2. Group X is very small component and is not always detectable, which may be due to limited proteolysis of the enzymes (Hayes et al, 1988). Group III is actually comprised of Group II isoenzymes complexed with the α-amylase inhibitors (BASI) (MacGregor, 1987). Therefore, only Groups I and II are directly encoded by the α-amylase genes. Distinct isoforms were also detected within each α-amylase group using isoelectric focusing gels, a total of up to 12 α-amylase isoforms in α-amylase II and 4 isoforms in α-amylase I have been found. Nevertheless, some forms were resulted from post-translational modification of others and can not be considered as true genetic isoenzymes (Aoyagi et al, 1990).

Alpha-amylase I has a low pI and is a minor component of total α-amylases in germinating barley, but its efficiency in degrading starch granules is higher than

α-amylase II (MacGregor, 1987). Immunologically α-amylase I is distinct from α-amylase II although they have the same apparent substrate specificities and action patterns (Bog-Hansen and Daussant, 1974). The two group isoenzymes rise and fall independently during the germination (Callis and Ho, 1983; Chandler and Jacobsen, 1991). It was suggested that α-amylase I predominates in the barley embryo (MacGregor and Marchylo, 1986), whereas α-amylase II is principally in the aleurone layers (MacGregor et al, 1984). Therefore, α-amylase II is the "classical" malt enzyme.

Table 1.2 Properties of different isoenzymes of α-amylase

Enzyme group	pI	Ca^{2+} binding	pH optimum	Electro-phoretic mobility	Proportion in germinated barley
α-amylase I	4.1 - 5.3	Very strong	3.0-5.5	Rapid	Minor
α-amylase II	5.3 - 6.6	Strong	5.0-5.4	Slower	Major
α-amylase III	? - 6.7			Slow	Major
α-amylase x	3.0				Small

Several cDNAs or genes of α-amylase have been isolated from barley (Chandler et al, 1984; Huang et al, 1984; Khursheed and Rogers, 1988; Rogers and Milliman, 1983; Rogers, 1985). Nucleotide sequence analysis of the cDNAs encoding the barley α-amylases show that α-amylase I is 11 amino acid longer than α-amylase II and that amino acid sequence identities between the two groups are approximately 80% (Rogers and Milliman, 1983; Rogers, 1985). Southern blot analysis revealed that the two group α-amylase isoenzymes are encoded by two small gene families located on chromosomes 7H and 6H, designated as *Amy2* and *Amy1*, respectively. The genes in each group are closely linked (Brown and Jacobsen, 1982; Khursheed and Rogers, 1988) and rare recombinations have been detected in the gene family of *Amy1* (Takano and Takeda, 1987; Takano et al, 1988). RFLP analysis revealed at least 3 types of α-amylase II gene families in

barley. However, the isoenzyme banding patterns detected by the IEF method were not directly determined by these three types of structural genes, but affected by a regulatory gene. This regulatory gene is linked with the structural gene and new recombinations could be produced by genetic recombination (Kiribuchi and Ohfuchi, 1989). The structural gene for α-amylase (*Amy1*) has a promoter which is activated by GA but suppressed by ABA. The promoter has two highly conserved sequences: ATACCATGCAGTG and CCTTTT, which are very important for the regulation of gene expression (Lanahan et al, 1992). The GA responsive promoter also exists in the genes of many other hydrolytic enzymes, for example 1,3-1,4-β-glucanase, endopeptidase and carboxypeptidase.

Extensive research has been conducted on the genetic control of α-amylases. Substantial genetic variability exist for both isoenzyme type and activity (Briggs, 1992). The activity of the enzyme was controlled by multiple gene loci (Henry and Johnston, 1991; Lu and Ding, 1991; Zhu et al, 1990) and is significantly affected by environmental factors (Henry and Johnston , 1991). The genetics of the enzyme activity fits an additive-dominance model but dominance is more important (Lu and Ding, 1991; Zhu et al, 1990). These results indicate the difficulty in selecting the barley with high levels of α-amylase.

Gibberellic acids are important factors for controlling the activity of the enzyme. Since the first observation that GA induces α-amylase secretion from barley aleurone cells (Paleg, 1960; Yomo, 1960), much research has been undertaken on the mechanism of GA action. It is now clear that at least two sets of genes are involved in controlling GA-induced gene expression of α-amylase: synthesis of GA and responsiveness to GA (Favret et al, 1978; Kusaba et al, 1991). However, it is still not clear where these genes are located in the barley genome and how these genes may be manipulated in breeding programs.

Another factor affecting the activity of α-amylase is the inhibitors synthesised during seed development. One kind of the inhibitor is the bifunctional α-amylase subtilisin inhibitor (BASI). The inhibitor is a single polypeptide with a

molecular weight of approximately 20 kD (Weselake et al, 1983, 1985). It combines with α-amylase II during seed germination. This results in a reduction of the α-amylase II activity (Hill and MacGregor, 1988; MacGregor, 1983, 1987; Mundy et al, 1983). In brewing, BASI may influence the rate of starch degradation in the early stages, but loses its activity at 60°C to 70°C in the mashing process (Munck et al, 1985). The gene encoding BASI has been cloned and designated as *Isa* (Leah and Mundy, 1989). Large genetic variation has been detected for the activity of BASI in barley. It seems that malting barley has higher concentrations of BASI than feed barley (Jarrett et al, 1995) with variation mainly due to genetic factors (Mundy et al, 1983). However, it is still not clear that how many genes are involved in controlling activity of the inhibitor or how it regulates the activity of α-amylase.

Another kind of inhibitor are the trypsin/α-amylase inhibitors with apparent molecular weight between 12 and 16 KD and encoded by a gene family (Garcia-Olmedo et al, 1992). As some members of this family can be selectively extracted with chloroform/methanol mixtures, they have been classed as CM-proteins and exist as monomeric, dimeric or tetrameric inhibitors in barley (Garcia-Olmedo et al, 1992). Two genes of the trypsin inhibitor have been cloned and designated as *Itr1* and *Itr2*. These two genes are located on chromosomes 3H and 7H and encode protein CMe and CMc (Halford et al, 1988; Hejgaard et al, 1984; Rodriguez-Palnezuela et al, 1988; Salcedo et al, 1984). The tetrameric inhibitor includes one copy of CMa , CMb and two copies of CMd, which are encoded by the genes *Iat1*, *Iat2* and *Iat3* mapped to chromosomes 7H, 4H and 4H, respectively (Salcedo et al, 1984). The monomeric and dimeric inhibitors are encoded by the *Iam1* and *Iad1* loci, respectively. Although their chromosomal locations have not been determined in barley, the genes may be on the chromosomes 6H and 3H, respectively, based on results in wheat (Fra-Mon et al, 1984; Sanchez-Mongle et al, 1986, 1989). However, all these α-amylase inhibitors disappear rapidly during germination and their importance for malting and brewing industries have yet to be determined.

1.2.1.2.2 Beta-amylases

Beta-amylases (EC 3.2.1.2) hydrolyse the penultimate 1,4-α-linkage from the nonreducing end of 1,4-α-glucans to release the disaccharide maltose (Harris, 1962; Briggs and Clutterbuck, 1973), but they are unable to attack intact starch granules (Maeda et al, 1978). This property is presumably important to prevent premature damage of the starch in ungerminated grain. However, β–amylases act synergistically with α-amylases to degrade the starch granules (Maeda et al, 1978). Extensive studies have indicated that β–amylases are the most important hydrolytic enzymes to determine the diastatic power in malting and brewing industries (Arends et al, 1995; Delcour and Verschaeve, 1987; Erdal et al, 1993; Evans et al, 1995; Gibson et al, 1995).

Shewry *et al.* (1988) extensively researched the molecular forms of β–amylases present in developing, mature, germinating and malted grains, and vegetative tissues using SDS-PAGE and Western blot analyses. Five isoforms with different molecular masses have been identified. The major isoform present in mature grain (form B) had a molecular mass of about 60 kD, and was converted to two lower molecular mass isoforms (C and D) during malting and germination. A minor low molecular mass form E, present in whole developing caryopses at 8 to 12 days after anthesis, and a diffuse high molecular mass isoform A, present in small amounts in all seed and vegetative tissues, may correspond to a constitutively expressed "housekeeping" form. However, extensive research on β–amylase has concentrated on the isoforms in mature grain and malt, since they are important for the malting quality.

Beta-amylases are highly heterogeneous in barley grain and malt. At least eight different isoenzyme bands were detected by chromatofocussing (LaBerge and Marchylo, 1983), most formed by post-translational modification, due to formation of intermolecular disulfide bonds (Visuri and Nummi, 1972) or thiol/disulfide

interchange (LaBerg and Marchylo, 1983), association with protein Z (Hejgarrd, 1978), proteolysis (Kruger, 1979; Lundgard and Svensson, 1986) and expression of different genes (Ainsworth et al, 1983). However, only two distinct isoenzyme types exist in cultivated barley grain (Evans et al, 1997a; Forster et al, 1991; Nielsen and Johansen, 1986) and malt (Allison, 1973; Allison and Swanston, 1974) in spite of the multi-isoforms. They were called Sd1 and Sd2 types. The Sd1 type varieties seem to be rare (Allison and Swanston, 1974; Eglinton et al, 1995). These two isoenzyme types showed different immunological property and affinity with the inhibitor protein Z (Allison and Swanston, 1974; Evans et al, 1995; Evans et al, 1997a). The attempt to relate the different isoenzyme type to the enzyme activity was not conclusive (Allison, 1973; Swanston, 1980). Using wheat-barley addition lines, the main isoenzyme polymorphisms were mapped to the long arm of chromosome 4H (Powling et al, 1981).

Two β–amylase genes have been cloned. One was from a variety Hiproly with high protein, high lysine and high levels of β–amylase (Kreis et al, 1987) and another from the malting barley Haruna Nijo with high levels of β–amylase (Yoshigi et al, 1994). However both of these clones encode the Sd2 type isoenzyme (Eglinton et al, 1995). The complete primary structure of β–amylase deduced from the nucleotide sequence of a full length cDNA showed that the enzyme is synthesised in a mature form without an NH_2-terminal signal peptide (Kreis et al, 1987). The calculated molecular mass of the enzyme is about 60 kDa, which matches with the largest isoform purified from the mature grains (Lundgard and Svensson, 1986; Shewry et al, 1988). The protein of β–amylase is characterised by four glycine-rich repeats, each of 11 amino acid residues, at the C-terminus. The repeat regions might be involved in cross-linking β–amylase with other proteins on the surface of starch granules (Kreis et al, 1987). Using the cloned cDNA as a probe, two gene loci were mapped in the barley genome: one on chromosome 2H and two on chromosome 4H (Kleinhofs et al, 1993; Kreis et al, 1988). The relative importance of these loci to the activity of the enzyme has not yet

been demonstrated. The relationship between the isoenzyme and the RFLP locus on chromosome 4H is also unclear, as the isoenzyme and RFLP locus of α-amylase on chromosome 6H appeared different (Takano et al, 1988).

Beta-amylases exist as both free and bound forms in barley grain. The bound form is a hetero dimer between β–amylase and protein Z (Brandt et al, 1990; Evans et al, 1995; LaBerge and Marchylo, 1983; Lundgard and Svensson, 1986). Early research suggested that the combination between β–amylase and protein Z reduced the activity of the enzyme (Bendelow, 1964). Therefore, protein Z is classified as an inhibitor of β–amylase. Bendelow (1964) also classified the ratio of free to bound enzyme as low and high, and suggested that the ratio is controlled by one codominant gene. However, this model seems too simple for the genetics of protein Z. In fact, the variation of free to bound ratio of β–amylase varied quantitatively from 10% to 78% in different barley varieties (Allison, 1973; Allison et al, 1979; Swanston, 1983; Lin et al, 1991). Indeed, one locus on chromosome 4H linked with the different isoenzyme type has been observed to control the free to bound ratio (Allison and Swanston, 1974); at least two loci encoding protein Z were located on chromosomes 4H and 5H (Nielsen et al, 1983; Hejgarrd, 1984); and two trans-acting loci on chromosome 5H also control the content of protein Z (Brandt et al, 1990).

In addition to the inhibitors, a locus on chromosome 4H linked with β–amylase isoenzyme type is significantly correlated with the activity of the enzyme (Allison, 1973; Swanston, 1980), and the polymorphism of the isoenzymes has been suggested as a marker to select β–amylase activity. However, this relationship is significantly affected by nitrogen content (Swanston, 1980). Another locus on the chromosome 2H linked with the gene specifying 2/6 row also affects the enzyme activity (Swanston, 1983). Most importantly, two high lysine mutant genes *lys3a* and *lys1* (Allison, 1978) on chromosome 5H regulate the activity of β–amylase *in trans* (Kreis et al, 1987): *lys3a* increases the enzyme activity several times while *lys1* inhibits β–amylase expression in the barley grain. These results

show that the activity of β–amylase is under control of a complex regulatory mechanisms (Kreis et al, 1987).

1.2.1.2.3 Limit dextrinase

Limit dextrinase (LD) (EC 3.2.1.41), also known as R-enzyme, pullulanase, pullulan 6-glucanohydrolase or debranching enzyme, catalyses the hydrolysis of α-1,6-glycosidic bonds in pullulan, amylopectin and dextrins formed during seed germination (Manners and Yellowlees, 1971). It is the only endogenous barley enzyme that debranches amylopectin and related compounds produced during malting (Kristensen et al, 1993; Sissons et al, 1992a). However, LD has no action on intact starch granules (Maeda et al, 1978) and it hydrolyses α-1,6-linkages in the branched limit dextrin faster than these in the large amylopectin molecules (MacGregor, 1987). These results suggest that LD must act in concert with α-amylase and β-amylase to hydrolyse α-1,6-linkage *in vivo*. In ungerminated grain, small amounts of LD can be extracted in the presence of reducing agents or papain (Lenoir et al, 1984; Manners and Yellowlees, 1973; McCleary, 1992; Yamada, 1981). The activity of LD increases during germination, but it is the last of the hydrolytic enzymes to be synthesised (Lee and Pyler, 1984). Early research showed that LD is synthesised in the aleurone and induced by GA (Hardie, 1975). This result is confirmed in recently research (Zhang et al, 1997).

LD exists in both free and bound forms (Longstaff and Bryce, 1991; MacGregor et al, 1994). In malted barley, most of the enzyme appears to present in the bound form (Serre and Lauriere, 1989). This may explain why most of the α-1,6-linkages in the starch survive the brewing process (Enevoldsen and Schmidt, 1973, 1974; Enevoldsen, 1975). It seems that adequate LD is synthesised for seed germination, but most is stored in a latent form to be fully activated later in germination (after 10 days of germination) (Longstaff and Bryce, 1993; MacGregor et al, 1995; Sissons et al, 1992b). The bound form is a complex between LD and its

inhibitors (MacGregor et al, 1995) and can be released by proteolytic modification during germination (Longstaff and Bryce, 1993). Two inhibitor proteins have been purified and characterised in barley. They are synthesised during seed development (MacGregor, personal communication) but it is still not clear how many genes are involved into control inhibitor content.

Barley starch usually contains 75% by weight of highly branched amylopectin. However, the majority (80%) of the α-1,6-linkages in amylopectin survive the brewing process (Enevoldsen and Schmidt, 1974). With the expansion of the light beer market and the increasing use of starch adjuncts in the brewing industry (Hoseney, 1994), high levels of limit dextrinase is desirable. The high levels of the enzyme activity may improve wort fermentability, increase alcohol production and alter the dextrin profile of the fermented wort (Bryce et al, 1995; Longstaff and Bryce 1991). Therefore, it appears that the most effective method for the manipulation of the dextrin profile in malt and beer is to breed varieties with high levels of enzyme activity (Kasha et al, 1993) or low levels of inhibitor activity (MacGregor et al, 1995).

Isoelectric focusing and gel electrophoresis indicated that 6 isoforms of LD with pI values 4.2-5.0 and molecular mass 80-104 kDa are present in the germinated grain (Lenoir et al, 1984; Sissons, 1992), but the isoenzyme patterns are conserved in cultivated barley (Sissons, 1992). In contrast, significant genetic variation in the activity for both the enzyme and its inhibitors have been observed in barley cultivars and the wild relatives of barley (Lee and Pyler, 1984; Longstaff and Bryce, 1993; Macri et al, 1993; MacGregor et al, 1995; Sissons et al, 1992b). This suggests the possibility of genetic manipulation of the activity of the enzyme. However, little is known about how many gene loci are involved in controlling the activity of the enzyme and what strategy could be implemented to select these genes in breeding programs.

1.2.1.2.4 Alpha-glucosidase

Alpha-glucosidase (EC 3.2.1.20) liberates glucose from glucan polymers and disaccharides exolytically, which has the least well-characterised activity in the amylolytic complex (Bamforth and Barclay, 1993). Activity of the enzyme is present during early seed development and decreases during late maturation (MacGregor, 1987; Stark and Yin, 1987). In ungerminated barley, low amounts of enzyme are detectable in the aleurone, pericarp, and embryo (Jorgensen, 1965; MacGregor, 1987; Stark and Yin, 1987). The activity of the enzyme increases rapidly after germination (Jorgensen and Jorgensen, 1963). This new activity is found in the scutellum, aleurone, endosperm and embryo (Clutterbuck and Briggs, 1973; Hardie, 1975; Jorgensen, 1965; MacGregor, 1987; Stark and Yin, 1987) and is enhanced by GA (Clutterbuck and Briggs, 1973; Hardie, 1975). These suggest that α-glucosidase is synthesised *de novo* during germination.

Two α-glucosidase isoforms have been purified from germinated barley with molecular mass of 65 and 32 kDa. They have different pI, time courses of accumulation in germinating grain and different affinities towards various starch substrates (Stark and Yin, 1987; Sun and Henson, 1990). Sun and Henson (1990) showed that both isoforms are capable of initiating attack on native barley starch granules in the absence of α-amylase. α-Glucosidases also exolytically hydrolyse α-1,2-, α-1,3- and α-1,6-linkages at variable rate in addition to α-1,4-linkage. By combining high pI α-glucosidase with either high or low pI α-amylase, a dramatic synergism, as much as 10.7-fold in native starch granule hydrolysis, occurred (Sun and Henson, 1990). Synergism was also found when low pI α-glucosidase was combined with α-amylases. The mechanism by which α-glucosidases enhance α-amylolysis of starch granules to such a great extent is probably partially due to the removal of inhibiting concentrations of maltose by α-glucosidases, as maltose has been reported to interfere with a noncatalytic, raw starch binding site of α-amylase (Schwimmer, 1950). Another reason may be the ability of α-glucosidase to remove non-α-1,4-glucosidic bonds from the granule surface which were acting as barriers

to α-amylolysis (Sun and Henson, 1990). Sissons and MacGregor (1994) further demonstrated that the two isoforms of α-glucosidase join with α-amylase to hydrolyse intact starch granules, but at a slower rate than α-amylases alone. Synergism was also found between α-amylases and high pI α-glucosidase, but not as great as reported by Sun and Henson (1990). Sun's results may be partially due to the contamination from α-amylases.

Recently, a putative clone of α-glucosidase was isolated from a cDNA library constructed from mRNA of the barley aleurone treated with GA (Tibbot and Skadsen, 1996). The distribution and expression of the mRNA for this gene showed a similar pattern as the α-glucosidase proteins (MacGregor, 1987; Sun and Henson, 1990). However, Southern blot analysis indicated that only a single copy of this gene exists in the barley genome, and the deduced polypeptide had 877 amino acids with a molecular mass of 110 kD. Therefore, additional studies are needed to characterise the gene's possible product with respect to the 65 and 32 kD isoforms previously characterised (MacGregor, 1987; Stark and Yin, 1987; Sun and Henson, 1990).

Although α-amylase from barley has been shown to hydrolyse starch granules, a malt extract containing an equal amount of α-amylase activity hydrolysed the starch granules more rapidly (Maeda et al, 1978). In fact as early as the late 1930s, it was postulated that additional "raw starch factors" are required for efficient breakdown of the native starch granules (Blish et al, 1937). Now it is believed that α-glucosidases may be the long sought "raw starch factor" (Briggs, 1992). Therefore, α-glucosidases are another important factor in malting quality. A recent study showed that α-glucosidase was partially extracted in water but was readily extracted when L-cysteine was included in the extraction buffer (Agu and Palmer, 1997). This suggest that there exist inhibitors *in vivo* to control the activity of α-glucosidase as reported for α-amylase, β-amylase and limit dextrinase. However, little is known the genetic factors controlling the activity of the enzyme.

1.2.1.3 Degradation of the protein

Most proteins of barley grain are present within the endosperm cells and form the matrix in which starch granules are embedded (Pomeranz, 1972). There are four main groups of protein: albumins, globulins, hordeins and glutelins (Baxter and Wainwright, 1979). The albumins and globulins mainly include protein Z, β-amylase and inhibitor protein. Hordein is the main barley grain protein and accounts for 30% to 50% of the total protein. The three hordein genes: *Hor2*, *Hor1*, and *Hor5* which encode hordein B, hordein C and hordein γ were located on the short arm of chromosome 1H. The genes of hordein have some common sequence, especially the -300 element (ATGTAAAGTAAGGTT) (Brandt et al, 1985; Cameron-Mills and Brandt, 1988; Chernyshev et al, 1989; Shewry, 1991; Entwistle, 1988).

The relationship of barley protein and malt quality was first studied early this century (Bishop, 1930). Low protein content is used as a criterion for good malting quality. The effect of protein on malt quality is not simply to decrease the extract by diluting starch. A large amount of protein results in greater malting losses by increasing the rate of root growth and respiration. Further studies by Bishop (1934) showed that the proteins in the barley endosperm interact with some carbohydrates to prevent these carbohydrates being released as extract after malting. These proteins include glycine-rich proteins, threonine-rich glycoprotein, hydroxyproline-rich glycoprotein and some hordein. Consequently, it is very important for the malting industry to have high activity of endopeptidase, so as to release the starch granules.

There are two major groups of proteases in germinating barley: thiol-dependent and metal-activated enzymes. Three metal activated enzymes account for 10% of proteolytic activity and the thiol-dependent enzymes account for 90% (Enari and Sopanen, 1986); these include five acid carboxypeptidases, four neutral aminopeptidases, two alkaline peptidases and one dipeptidase. Most of these

enzymes have been cloned and the genes mapped, but how they affect malt quality and their genetics is yet to be investigated.

Hordein is the most important protein component. Baxter and Wainwright (1979) confirmed that the characteristics of endosperm storage proteins were consistent within a variety and could be utilised to assist in varietal identification, and also suggested that the concentration of hordein B was correlated with the malt quality within a variety. The reason for the apparent importance of the Fraction B protein was not understood, but it was thought probable that this was due to its chemical composition or spatial arrangement. The protein profoundly influenced the accessibility of α-amylase and β-glucanase to their respective substrates (Baxter and Wainwright, 1979; Miflin and Shewry, 1978). There is an inverse relationship between nitrogen content and extract. However, Smith (1990) found that increasing nitrogen mainly results in an increase of hordein C (Smith, 1990). Which one of the hordein B and hordein C proteins is more important for the malt quality or what is it the relationship between hordein B and hordein C in relation to the malt quality?

Gel protein content of barley has been demonstrated to have a significant negative correlation with hot-water extract (Van den Berg et al, 1981; Smith and Simpson, 1983; Smith and Lister, 1983). This suggested that selection for barley with a low gel protein content would not only avoid subsequent wort filtration problems, but also contribute to greater malt extract. Hordein D was found to be highly concentrated in the gelprotein (Barter and Wainwright, 1979), and Smith and Lister (1983) proposed that hordein D forms the backbone of complex three dimensional aggregates with hordein B. Therefore, all hordeins have some effects on malting quality. Although all hordein genes have been cloned and mapped, the genetic basis for the effect of hordeins on malt quality is still not clear.

1.2.1.4 GA induced hydrolytic enzyme expression in germinated barley

Gibberellins (GAs) are a class of plant hormones which regulate cell growth in vegetative tissues, flower and fruit development and seed reserve mobilisation (Hooley, 1994). The observation that GA_3 induces α-amylase secretion from barley aleurone cells (Paleg, 1960; Yomo 1960), coupled with the development of a simple procedure for the isolation of viable aleurone layers that retain hormone sensitivity and the ability to secrete active enzymes (Chrispeels and Varner, 1967), has resulted in the widespread adoption of barley aleurone layers as a model experimental system for *in vitro* investigations of plant hormone action, synthesis and secretion of the hydrolytic enzymes, and regulation of plant gene expression (Fincher, 1989; Jones and Jacobsen, 1991). Alpha-amylase, 1,3-1,4-β-glucanase (Stuart et al, 1986), 1,3-β-glucanase (Taiz and Jones, 1970), 1,4-β-xylanase (Hammerton and Ho, 1986), endopeptidase (Hammerton and Ho, 1986), limit dextrinase (Hardie, 1975; Zhang et al, 1997), α-glucosidase (Tibbot and Skadsen, 1996; Clutterbuck and Briggs, 1973; Sun and Henson, 1990) and nuclease (Brown and Ho, 1986) are all regulated by GA in germinating barley. The ability of barley grain to rapidly synthesise enzymes that hydrolyse the endosperm cell walls and starchy endosperm determines malt extract (Fincher and Stone, 1993) and the length of time needed to convert a specific quantity of starch to sugar. Higher levels of enzymes mean shorter mashing periods and allows for the addition of other sources of starch (adjunct) to the mashing process (Kasha et al, 1993). Therefore, GA_3 is used to augment the kernel's own supply of GA and stimulate the production of the enzymes in the brewing industry (Bamforth and Barclay, 1993).

The mechanism of GA induced gene expression is complex. It may include GA production, perception, transduction and response (Hooley, 1994). Evidence from experiments using GA_4 covalently bound to Sepharose beads and anti-idiotype antibodies suggest that GA is perceived on the plasma membrane in oat aleurone protoplasts (Hooley et al, 1991, 1993; Smith et al, 1993). This is supported by more recent work showing that microinjection of GA_3 in barley aleurone protoplasts fails to induce α-amylase synthesis and secretion. On the other

hand, the protoplasts respond to external application of GA_3. These results indicate that the site of perception of GA is on the external face of the plasma membrane (Gilroy and Jones, 1994). Progress has also been achieved in identifying GA-responsive elements associated with gene promoters. Functional analysis of the barley high-pI α-amylase promoter identified a GA responsive complex consisting of TAACAA and TATCCAC boxes, which are necessary for GA response (Skriver and Mundy, 1990; Skriver et al, 1991; Gubler and Jacobsen, 1992; Rogers et al, 1994). In addition, there are two other sequences that also significantly affect the expression of α-amylase in response to GA (Lanahan et al, 1992). The cAMP and cGMP responsive elements are also involved in the GA responsive complex (Gubler and Jacobsen, 1992; Penson et al, 1996). Similar GA responsive elements are also found in low pI α-amylase (Lanahan et al, 1992), 1,3-1,4-β-glucanase gene (Wolf, 1992), 1,4-β-xylanase (Banik et al, 1997), α-glucosidase (Tibbot and Skadsen, 1996) and limit dextrinase (Zhang et al, 1997) genes. This suggests that there is a common factor that controls the activity of all the hydrolytic enzymes in germinating barley. Recently, a cDNA, *GAmyb*, has been cloned, which can specifically bind with the GA responsive complex in the gene of high pI α-amylase (Gubler et al, 1995). The expression of this gene does not depend on other protein synthesis. Thus, it is believed that *GAmyb* is the sole GA-regulated transcription factor required for transcriptional activation of high pI α-amylase promoter (Gubler et al, 1995). However, little is known about GA production and transduction *in vivo*.

Analysis of dwarf mutants provides an alternative approach to understanding the mechanism of GA-induced gene expression. There are two kinds of dwarf mutants which respond to GA in barley. One group are GA-deficient mutants which have a low level endogenous GA and is very sensitive to exogenous GA_3 application (Boother et al, 1991; Falk and Kasha, 1982; Hentrich et al, 1985; Speulman and Salamini, 1995; Zwar and Chandler, 1995;). Another group are GA-insensitive mutants which showed the normal endogenous GA levels

but do not respond to exogenous GA_3 (Favret et al, 1978). From these results, it was concluded that there are two sets of genes to control GA-induced gene expression: synthesis of GA and responsiveness to GA (Favret et al, 1978; Kusaba et al, 1991). Further analysis of general barley varieties showed that malting grade barleys produced more GA than do feed grade barley (Proudlove and Muller, 1989). The aleurones from different varieties also showed different susceptibilities to GA_3 (Palmer, 1988). This demonstrated the possibility of improving GA production and responsiveness through breeding.

Further analysis showed that dwarf mutants expressed pleiotropic effects. The mutants have shortened internodes, leaf blades and lower activity of the hydrolytic enzymes in germinating barley (Zwar and Chandler, 1995) and all these traits can be recovered through applied GA_3 (Chandler and Musleth, 1990). Thus it has been suggested that GA might exert its effect non-specifically, stimulating the expression of all active genes in a cell rather than specific genes (Baulcombe et al, 1984). This further supports the suggestion that there is a common locus controlling all traits regulated by GA in the barley genome (Henry et al, 1996).

1.2.2 Mapping Genes and Quantitative Trait Loci (QTL) Using Genetic Markers

1.2.2.1 Development of genetic markers

1.2.2.1.1 From morphological markers to RFLP markers

A marker can be defined as "a gene with a known location on a chromosome and a clear-cut phenotype used as a point of reference" (King and Stansfield, 1985). The idea of using markers in genetics and breeding appeared early this century. A typical example is the selection of double flowers using a monogenetically inherited chlorophyll mutant gene as a marker in *Matthiola incana*

(Saunders, 1915 cited from Weber and Wricke, 1994). Until recently, genetic markers used in plant breeding were those affecting morphological characters. However, these mutants have several limitations including (1) low levels of polymorphism; (2) often negative effects on plant growth and viability, such as albinism, sterility and dwarfism.

In the 1960s, biochemical markers (enzyme and other protein markers) were introduced into plant genetics, as a result the observation that plant enzymes can occur in multiple forms. The polymorphisms of plant enzymes are conditioned by genes that may affect subtle secondary characteristics of the enzymes with little or no effect on enzymatic properties, and thus do not impair the viability of mutant organism. Importantly, the inheritance of isoenzyme markers show codominance. During the following years the advantages of using isoenzyme markers were recognised and the techniques were considerably refined. This resulted in the extensive utilisation of isoenzyme markers for multiple purposes in crop improvement (Tanksley, 1983). For example, in barley α-amylase isoenzyme was used to select high kernel weight (Powell et al, 1990). However, the number of isoenzyme markers is quite limited and insufficient to construct a genetic linkage map to cover the whole plant genome.

In 1970s, scientists found that DNA restriction enzymes recognise specific sequences in DNA and catalyse endonucleolytic cleavages, yielding fragments of defined length (see review, Kessler and Manta, 1990). Restriction fragments are displayed by electrophoresis in agarose gels according to their molecular size. The fragments of specific sequences from within a large and complex population of DNA fragments can be detected by hybridisation using the method of Southern (Southern, 1975). Differences among individuals in the length of a particular restriction fragment were defined as Restriction Fragment Length Polymorphisms (RFLP), which may result from loss or formation of a restriction endonuclease cleavage site due to the mutation of one or more bases or from insertion/deletion of blocks of DNA within a fragment.

Botstein *et al.* (1980) developed the basic principle and method to construct a molecular linkage maps with RFLPs. Using random single-copy DNAs as probes, DNA sequence polymorphism can be detected when hybridised to restriction digest of an individual DNA. Each of these probes will define one or more loci. Suitable polymorphic loci can be tested for linkage relationships by established methods and loci can be arrayed into linkage groups to form a true genetic map of DNA marker loci. Pedigrees in which inherited traits are known to be segregating can then be analysed, making possible mapping of the gene(s) responsible for the trait with respect to the DNA marker loci without requiring direct access to a specified gene. Subsequently, a computer package, Mapmaker, was designed for the construction of primary genetic linkage map from molecular marker data (Lander et al, 1987). Subsequently, great efforts have been made to construct RFLP linkage maps for humans and other animals, plant and microrganisms. The relative advantages of RFLP markers over morphological markers for most genetic and breeding application are summarised as follows (see Tanksley et al, 1989; Stuber, 1992):

(1) Abundance: the number of DNA probes used to construct linkage is almost unlimited, and several naturally occurring alleles are available at most RFLP marker loci. Thus, natural variation in existing populations can be used without need to construct special genetic stocks.

(2) Neutral phenotype: RFLP markers have no negative effect on plant phenotype whereas morphological markers frequently cause major alternations in the phenotypes which may be undesirable.

(3) Codominance: Alleles at most RFLP loci behave in a codominant manner whereas dominant-recessive interactions frequently hinder identification of all genotypes associated with morphological traits.

(4) Epistasis: no unfavourable epistatic interactions.

(5) Stable: RFLP markers are not influenced by environment and the specificity of tissues.

1.2.2.1.2 Development of PCR markers

Construction of RFLP linkage map by Southern blot analysis is laborious, costly and may be unsafe due to using the radio-active isotopes (Beckmann and Soller, 1983; Beckmann, 1988). Speed, efficiency and safety considerations have led genome mapping projects to evaluate the polymerase chain reaction (PCR) (Saiki et al, 1985; Mullis and Faloona, 1987) as an alternative to Southern blot analysis. PCR amplifies DNA fragments using a pair of primers flanking a defined sequence, thus PCR markers were also called sequence tagged site (STS) markers (Tragoonrung et al, 1992). PCR can directly distinguish between insertion/deletion and point mutation events. Insertion/deletion polymorphisms can be analysed by sizing of PCR products via electrophoresis (Higuchi et al, 1988; Shin et al, 1990). Point mutation polymorphisms can be detected by hybridising PCR products with allele-specific oligonucleotides (Higuchi et al, 1988; Li et al, 1988; Kurt et al, 1991), DNA sequencing of PCR products (Wong et al, 1987), cleavage of PCR products with a restriction endonuclease (Saiki et al, 1985), and denaturing gradient-gel electrophoresis (Myers et al, 1987; Riedel et al, 1990). A major limitation for PCR analysis is the need for extensive sequence information to synthesise the appropriate primers (Williams et al, 1991). However, this has been simplified enormously by the deposition of more than 300 cereal specific PCR primer sequences in the GrainGenes database (http://wheat.pw.usda.gov). These primers were derived from sequencing the corresponding genetically mapped RFLP probes, and can theoretically be used to develop allele-specific PCR markers at many loci across the barley genome (Powell et al, 1996). However, STS markers remain far from ideal because of the low level of polymorphism frequently detected in the PCR-derived amplicons and the frequent need for a post-amplification processing step to detect polymorphisms.

Different strategies have been developed for using PCR markers, which include reverse PCR (Ochmann et al, 1988), semi-random-PCR (Weining and Langridge, 1991), supported PCR (Rudenko et al, 1993), RAPD (randomly amplified polymorphic DNA) (Williams et al, 1990) or A-PCR (arbitrarily primed PCR) (Welsh and McClelland, 1990) or DAF (DNA amplification fingerprinting) (Caetano-Anolles et al, 1991), simple sequence repeats (SSR) or microsatellite DNA (Weber, 1990) and AFLP (amplified fragment length polymorphism) (Zabeau and Vos, 1993). Of these techniques, RAPD, SSR and AFLP have been extensively used in plant genetics and breeding and may replace RFLP as "second generation of molecular markers" (Henry et al, 1996; Powell et al, 1996).

1.2.2.1.2.1 RAPD markers

The molecular basis of RAPD polymorphism is remarkably simple. Instead of using a pair of carefully designed and fairly long oligonucleotide primers to amplify a specific target sequence, a single, short oligonucleotide primers, which binds to many loci, is used to amplify random sequences from a complex DNA template (Williams et al, 1990; Welsh and McClelland, 1990). Theoretically, the number of amplified fragments generated by PCR depends on length of the primer and size of the target genome. For most plants, primers that are 9-10 nucleotides long are predicted to generate, on average, 2-10 amplification products (Waugh and Powell, 1992). The primers are generally of random sequence, biased to contain at least 50% G + C, and to lack internal inverted repeats. The products are separated by electrophoresis and visualised by UV illumination of ethidium-bromide stained gels. Polymorphisms of RAPD markers result from changes in either the sequence of primer binding site, which prevent stable association with primer, or from changes which alter of the size or prevent successful amplification of a target DNA. However, the size variants are only rarely detected and individual amplification

products represent one allele per locus. Therefore, inheritance of RAPD is expressed as a dominant marker (Rafalski et al, 1991; Waugh and Powell, 1992).

The advantages of RAPD include simplicity, non-radioactive detection, easy access to primers and relative low cost (Powell et al, 1996). The RAPD markers have been used as fingerprinting for identification of varieties and somatic hybrids, evaluation and conservation of genetic resources, targeting agricultural important traits and constructing simple molecular linkage maps (Henry et al, 1996; Powell et al, 1996; Rafalski et al, 1991; Tingey and Del Tufo, 1993; Waugh and Powell, 1992). However, the most useful aspect of RAPD markers is locating and manipulating genes of interest through combination with near-isogenic lines and various DNA and/or genetic pooling strategies (Barua et al, 1993; Giovannoni et al, 1991; Mattin et al, 1991). However, RAPD markers suffer from several disadvantages: relative low level of polymorphism, dominance and lack of identification "alternative" allele, sensitivity to DNA concentration and amplification conditions (Powell et al, 1996). The latter two issues have hindered the wide applicability of RAPD to plant genetics and breeding.

1.2.2.1.2.2 Microsatellite DNA markers

Simple sequence repeats (SSR) or microsatellite DNA markers were developed from the observation that there exists abundant tandem repeated nucleotide motifs which can be as short as 4, 3, 2, and even one nucleotide in eukaryote genomes (Braaten et al, 1988; Hamada et al, 1982; Schafer et al, 1986; Tautz and Renz, 1984; Vergnaud, 1989). Using a pair of flanking unique oligonucleotides as primers, the products amplified by PCR almost invariably show extensive polymorphism due to site-specific length variation, as a consequence of the occurrence of different numbers of repeats (Lonn et al, 1992). Therefore, microsatellite DNA markers share the advantages of the sequence tagged site markers (Olson et al, 1989). Moreover, their hypervariability allows many

Table 1.3 Comparison of different molecular markers (Adapted from Powell et al, 1996)

	RFLP	RAPD	SSR	AFLP
Principle of assay	Endonuclease digestion and hybridization	Amplification with random primers	Amplification of SSRs	Amplification of DNA fragments limited by random nucleotides
Type of polymorphism detected	Single base insertions or deletions	Single base insertions or deletions	Repeat length	Single base insertions or deletions
Dominance	Co-dominant	Dominant	Co-dominant	Dominant
Amount of DNA required	2-10μg	10-25ng	25-50ng	2-50μg
DNA sequence required	No	No	Yes	No
Radioactive detection	Yes/No	No	No	Yes

segregating populations to be used as a reference population for linkage analysis. This makes SSR almost ideal markers. High density maps of microsatellite DNA markers have been constructed in human and mouse genomes as the second generation of molecular markers (Edwards et al, 1991; Garchon and Bach, 1992).

The search of the EMBL and GenBank databases showed that microsatellite DNAs distribute with a frequency of one every 50 kb in plant genome (Morgante and Olivieri, 1993), where AT repeats are the most frequently class of dinucleotide microsatellite. This contrasts to mammalian genomes where the AC repeats are the most abundant (Hamada et al, 1982). However, development of microsatellite DNA markers in plants has been slow. In barley, only 37 primer pairs that amplify polymorphic microsatellite DNA have been mapped (Liu et al, 1996). The main problem of using microsatellite markers is the paucity of the sequences for designing PCR primers. Recently, different enriched microsatellite DNA genomic libraries have been constructed (Rafalski, 1996; Powell et al, 1996) and it is expected that more microsatellite markers will become available. As an alternative approach, random generation of microsatellite DNA polymorphisms have been suggested by Gupta *et al.*(1994), Wu *et al.*(1994), Zietkiewicz *et al.*(1994), and Becker and Heun (1995). The proposed procedures include the random amplification of microsatellite DNAs using a radioactivity labeled microsatellite primer alone or in combination with RAPD (RAMP) or combining RAMP and restriction enzyme digestion.

1.2.2.1.3 Development of AFLP markers

Amplified fragment length polymorphisms (AFLP) are the most recently developed molecular markers (Zabeau and Vos, 1993), which are based on the detection of restriction site variation in conjunction with sequence polymorphism in adjacent sites. The main advantage of AFLP markers is their high multiplex polymorphisms, which means that a large number of amplification products can be

assayed in a single reaction (Powell et al, 1996). This technique has wide applicability in cultivar fingerprinting, linkage mapping, saturation mapping regions of the genome, map-based cloning and backcross conversions. However, most AFLP markers are dominant, which may limit their use in plant breeding.

As mentioned above, different molecular marker techniques have been developed in the past ten years. This provides new opportunities to use molecular markers in plant genetics and breeding, but also places greater emphasis on choosing the appropriate methods for different purposes. The major features of different molecular markers are summarised in Table 1.3.

1.2.2.2 Gene mapping in barley

Barley is among the oldest cultivated crops. It has seven distinct pairs of chromosomes designated by Arabic numbers 1-7 (Nilan, 1964; Ramage, 1985). Due to its diploid nature, relative lower number and large size of chromosomes, self fertility, ease of hybridisation, wide adaptability, high degree of natural and easily induced variation, and relatively limited space requirements, barley has been a model genetic experimental organism since the rediscovery of Mendel's laws of heredity (Kleinhofs and Kilian, 1994). Well over a thousand genes are known and an extensive set of translocation stocks have been developed in barley (von Wettstein-Knowles, 1992). Over the past sixty years, great effort has been made to construct a barley genetic linkage maps. Several authors have reviewed the mapping of the genes in barley (Briggs, 1978; Nilan, 1964; Smith, 1951; Søgaard and von Wettstein-Knowles, 1987; von Wettstein-Knowles, 1992). More recently, a linkage map with 246 morphological genes has been published (Franckowiak, 1996), which makes barley one of the few plant species with a detailed linkage map of morphological loci (Stuber, 1992).

The application of molecular techniques to barley genetic mapping started slowly, perhaps due to its large genome size, $1C=5.3x10^9$ bp (Bennett and Smith,

1976). However, several advantages make barley a model organism among the large genome types for molecular mapping (Kleinhofs and Kilian, 1994). First, doubled haploid lines can be easily developed by two techniques: *Hordeum bulbosum* (Kasha, 1974) and anther culture. Therefore, mapping populations can be conserved. Second, the development of wheat-barley disomic (Islam et al, 1981) and ditelosomic (Shepherd and Islam, 1987) addition lines further facilitates barley genetic studies. These lines contain a normal complement of Chinese Spring wheat chromosomes, and a *cv*. Betzes barley chromosome or chromosome arm. All individual barley chromosome lines, except chromosome 1H (5), are fertile and reasonably stable. Since very high restriction fragment length difference (RFLD) exist between barley and wheat (Shepherd and Islam, 1992), the wheat-barley addition lines have been extensively used to assign markers to linkage groups, organise linkage groups into chromosomes and localise the centromere in barley (Graner et al, 1991; Heun et al, 1991; Kleinhofs et al, 1993, 1994).

The first barley RFLP map published was for the chromosome 6H (6) (Kleinhofs et al, 1988), followed by a partial map of the whole genome incorporating RFLP, morphological, isoenzyme and PCR markers (Shin et al, 1990). Subsequently, significant efforts have been undertaken to construct complete barley molecular linkage map (Graner et al, 1991; Graner, 1994; Heun et al, 1991; Kleinhofs et al, 1993; Kleinhofs and Kilian, 1994; Kasha and Kleinhofs, 1994; Langridge et al, 1995). The population size, number of the markers and length of the map for different mapping projects are summarised in Table 1.4. In addition to these molecular maps covering the entire barley genome, many other populations have also been used to construct partial maps and to determine the location of interesting genes. For example, a Shannon x Proctor population was used to generate a map of chromosome 3H (3) for locating barley yellow dwarf resistance gene *Yd2* (Collins et al, 1996); 120 F_2 plants were obtained from a Betzes x Golden Promise cross and 120 F_2 plants from a Captain x *H. spontaneum* were used to map 5S rDNA genes on the chromosome 2H (2) (Leitch and Heslop-

Table1.4 World-wide populations used to construct barley molecular linkage maps

Population	Population size	Number of markers	Length of map (cM)	Reference
Clipper X Sahara	150	164	1299	Langridge et al, 1995
Haruna Nijo X Galleon	114	271	1494	Langridge et al, 1995
Chebec X Harrington	120	237	1413	Langridge et al, 1995
Vada X *H. spontaneum*	135	251	1453	Graner et al, 1991
Igri X Franka	73	369	1387	Graner et al, 1994
Proctor X Nudinka	113	154	1192	Heun et al, 1991
Harrington x TR306	150	190	1278	Kasha et al, 1994
Steptoe X Morex	150	423	1277	Kleinhofs et al, 1993, 1994

Harrison, 1993); 113 DH lines from cross Magnum x Goldmaker were used to locate the *denso* dwarfing gene on the long arm of chromosome 3H (3) (Laurie et al, 1993); 94 DH lines from Igri x Triumph cross were used to map a photoperiod response gene *Ppd-H1* (Laurie et al, 1994) and a Amamir x *H. spontaneum* population was used to generate a map of the chromosome 4H (Hinze et al, 1991). More recently, comprehensive AFLP maps covering the whole barley genome have been constructed in different populations, including 118 AFLP markers in a Proctor x Nudinka DH population (Becker and Heun, 1995); 249 AFLP markers in a Dicktoo x Morex cross (Hayes et al, 1996); 202 AFLP markers in cross Igri x Franka (Graner, 1996); 329 AFLP markers in the Blenheim x E224/3 DH population (Powell et al, 1996) and 580 AFLP markers in Lina x *H. spontaneum* population (Tuvesson et al, 1996 see Powell et al, 1996). Today, over a thousand the genetic markers which include hundreds of known function genes, have been located on the barley genome by using different populations, and more markers will be developed and mapped in the near future (Franckowiak, 1996; Powell et al, 1996; Qi et al, 1996). These maps provide powerful tools to map genes and quantitative traits loci (QTL), and for map-based cloning of the important genes in barley.

Although exhaustive efforts have been made to construct complete linkage maps in barley, large gaps still exist in individual maps. In order to make fine linkage maps, it then became necessary to utilise as many markers as possible in the region of interest from different mapping populations. The availability of the mapping software program Joinmap (Stam, 1993) made the construction of an integrated barley map possible. Langridge *et al.* (1995) constructed the first barley consensus map with 587 markers by combining the mapping data from seven different mapping populations. More recently, another consensus map was published covering 1060 cM of the barley genome with 898 markers (Qi et al, 1996). Comparison of the consensus map with the individual maps revealed that the overall linear order of markers is in good agreement and that the integrated map is

Table 1.5 Homoeologous chromosomes between rice, wheat and barley

Rice	Wheat	Barley
1	3 (4, 5, 7)	3 (5, 2, 4, 7, 1)
2	6 (1, 3, 7)	6 (2, 3)
3	4 (5)	4, 7 (2, 6)
4	2 (6)	2 (4, 1)
5	1 (3, 6)	5 (7)
6	7	1 (5)
7	2 (5, 6)	2 (3, 1)
8	[1, 2, 3, 4, 6, 7]	[1, 2, 5]
9	5	7 (3, 5)
10	1	[5, 1]
11	[5, 2, 7]	[2, 4, 7, 1]
12	[3, 4, 5]	[2, 4, 7, 1]

Note: Chromosomes enclosed in parentheses () identify additional chromosomes containing comparative loci from specific rice chromosomes. Chromosomes enclosed in backets [] indicate that no obvious syntenous chromosomes were evident for that special rice chromosome (From Saghai-Maroof et al, 1996)

consistent with the component maps. The density of markers on the consensus maps is much higher and the number of the gaps is much lower than that of the individual maps (Langridge et al, 1995; Qi et al, 1996).

The order of genes is highly conserved not only between different barley maps but also between the chromosomes of different cereal crops. Islam *et al.* (1981) provided evidence that barley chromosomes 1, 2, 3, 4, 5, 6 and 7 are homologous to the wheat chromosomes 7, 2, 3, 4, 1, 6 and 5, respectively (Islam et al, 1981; Islam and Shepherd 1990). The syntenous relationships between the chromosomes of barley, wheat, rice, maize and sorghum have also been detected using common markers (Ahn and Tanksley, 1993; Kurata et al, 1994; Saghai Maroof et al, 1996; Whitkus et al, 1992). The syntenous chromosomes of rice, wheat, and barley are summarized in Table 1.5. The conservation in genome structure of related plant species may provide a basis for interpreting genetic information among species, and molecular markers produced in one species may also be exploited by related species with less characterised genetic maps (Saghai Maroof et al, 1996).

1.2.2.3 Mapping quantitative trait loci (QTL) using molecular markers

Many traits in nature and of importance in agriculture exhibit continuous variation in a population of phenotypes which is postulated to be due to independent segregation of several genes, each with a small effect and regulated by environmental variation (Johanssen, 1909; Nilsson-Ehle, 1909; East, 1915). The "polygenes" are usually refereed as quantitative trait loci (QTL). Historically important quantitative genetic parameters, e.g. heritability, additive and dominant genetic variance and epistasis, have been developed to describe the characteristics of a continuous phenotypic distribution (Knapp and Briggs, 1990; Knapp et al, 1992; Knapp, 1994). From these studies several factors could be estimated, including the approximate number of loci affecting the

character in a particular mating, the average gene action, and the degree to which the various polygenes interact with each other and the environment in determining the phenotype (Falconer, 1960; Mather and Jinks, 1977). What was not easy to decipher with this approach was the magnitude of effect, inheritance, and gene action of any specific locus that was affecting the character (Tanksley, 1993). The pioneering work of Sax (1923) provided an approach to deal with these problems. He reported the association of seed size in beans (a quantitatively inherited character) with seed coat pigmentation (a discrete monogenic trait) and interpreted this finding as the linkage of the single gene controlling seed colour with one or more of the polygenes controlling seed size. Subsequent reports also showed linkage of genes controlling quantitative traits with single markers (Breese and Mather, 1957; Thompson, 1975; Thompson and Thoday, 1979). Thoday (1961) put forward the idea of using single markers to systematically characterise and map individual polygenes controlling quantitative traits. The idea was simple. If the segregation of a single marker could be used to detect and estimate the effect of the linked polygene and if single gene markers were scattered throughout the genome of an organism, it should be possible to map and characterise all the polygenes affecting a character. However, putting Thoday's ideas into practice was difficult and fraught with problems due to paucity of suitable markers (Tanksley, 1993).

By the early 1980s isoenzyme markers were employed as a general tool for mapping polygenes and these studies met with considerably more success than previously (Edwards et al, 1987; Tanksley et al, 1982; Vallejos and Tanksley, 1983; Weller et al, 1988). The number of the isoenzyme markers were then and still are not sufficient to cover the entire genome for most crop species. The most successful progress for mapping polygenes followed the development of molecular markers (Botstein et al, 1980). Comprehensive molecular linkage maps have been constructed to cover the entire genome in different species, which provided the possibility to systematically dissect and characterise all the important polygenes (see above review). The next challenge was development of suitable analysis procedures for routinely mapping the polygenes.

Single point regression analyses were employed in the first molecular marker/quantitative genetic studies (Edwards et al, 1987; Tanksley et al, 1982; Weller et al, 1988; Winkelman and Hodgetts, 1992). However, this method normally underestimates the magnitude of the effects of any detected QTL, and can put a QTL in a wrong position due to recombination between the marker locus and the QTL. To take full advantage of linkage maps for QTL mapping, Lander & Botstein proposed the interval analysis method (Lander and Botstein, 1986, 1989). Instead of analysing the population one marker at a time, sets of linked markers are analysed simultaneously with regard to their effects on quantitative traits. By using linked markers for analysis, it is possible to compensate for recombination between markers and the QTL, increasing the probability of statistically detecting the QTL and also providing an unbiased estimate of QTL effect on the character. Today, interval mapping has been used extensively for quantitative trait linkage studies (Tanksley, 1993; Knapp, 1994). However, single point and interval analyses give nearly identical results if the marker density is higher (marker <15 cM apart) (Stuber, 1992).

Recently, much work has been carried out on the theoretical aspects of mapping QTL. In particular, attention has focused on the problems that occur when multiple QTL are mapped one by one using standard interval mapping techniques (Haley and Knott 1992; Martinez and Curno 1992). Jansen (1994) and Zeng (1994) have developed methods where the estimates of a QTL's location and effect are improved by including a number of markers as cofactors to absorb the effects of QTL other than the one under study.

Another consideration of QTL mapping is genotype x environment interaction. Theoretically, QTL x environment interaction would be expressed as:

(1) significant effects detected only in a subset of the total number of environments;

(2) changes in the magnitude of significant effects of QTL across environments;

(3) opposite favorable alleles at a QTL in distinct environments (Hayes et al , 1993).

The significance of QTL x environment interaction can be studied by analysis of variance (Guffy et al, 1989; Paterson et al, 1988; Zehr, 1990; Zehr et al, 1992) or by comparing the frequency of identification of significant marker-QTL associations in different environments (Stuber et al, 1992; Bubeck et al, 1993; Paterson et al, 1991). Tinker and Mather (1995) described a simplified method of composite interval mapping and extended both simple interval mapping and composite interval mapping for analysis of the QTL in multi-environments. Little indication of QTL x environment interaction has been found by Guffy *et al.* (1989) and Stuber (1992). However, Zehr (1990) found more QTL x environment interaction. In contrast, Bubeck *et al.* (1993) and Paterson *et al.* (1991) found little agreement between environments in the markers identifying as having significant QTL. These different results may be a function of traits studied or the method of identifying QTL x environment interaction.

Today, several methods and associated computer software are available for QTL analysis. They are summarised in Table 1.6. Although each method has its advantages, the different methods including single marker analysis provide nearly the same information about the location and effect of the major QTLs (Darvasi et al, 1993; Ronin et al, 1995; Whittaker et al, 1996).

In addition to the analytical methods, several other factors also affect the power to detect QTLs. The first is the heritability of the traits. The higher the heritability, the greater the possibility of detecting all the QTLs controlling the trait (Hyne and Kearsey, 1995). On the other hand, lowering the error could increase the resolution power for mapping the QTL (Soller and Beckmann, 1990). Marker density is another consideration for detecting QTL. Although fine mapping procedures can locate a QTL in a interval as small as 3 cM (Paterson et al, 1990), the result of a simulation study showed that even for QTL with large effect, in experiments with large population, and using an infinite number of markers, confidence interval for the map location of a QTL remains in the order of 10 cM (Darvasi et al, 1993). Therefore, a marker spacing of 20 cM or even 50 cM is suitable for QTL analysis. Finally, the probability level used in QTL mapping determines the numbers of the QTL. In an ideal case all genetic variance of the trait is

explained by detected QTL. However, in practice, a number of QTL may be missed (a type II error) and at a same time a number of false positives may occur, indicating a QTL at a map position where actually no QTL is present (a type I error). Lander and Botstein (1989) suggested using a significance level equivalent to 0.001 in order to reduce the number of false positives. However, reducing the probability of type I error increases the probability of type II error. Edwards *et al.* (1992) and Bubeck *et al.* (1993) used a 0.05 probability level. The appropriate probability level for plant breeding application may depend on the heritability of the traits and the size of the population (Zehr et al, 1992; Jansen, 1994).

However, conventional QTL mapping is time consuming. Different strategies have been developed for more effective QTL mapping. These include the use of near isogenetic lines (Bentolila et al, 1991), recombinant congenic strains (Demant and Hart, 1986), substitution mapping (Paterson et al, 1990) or backcross inbred lines (Beckman and Soller, 1989), all of which are based on definition of the chromosomal segment carrying a given QTL that is common to a number of individuals or lines. Combining the use of RAPD markers and near-isogenic lines provided a route for quickly identifying markers linked to an interesting QTL (Young et al, 1988). However, near-isogenic lines can not be obtained for all traits of interest and developing the near-isogenic lines may be time consuming. Michelmore *et al.* (1991) and Giovanoni *et al.* (1991) established a rapid method for detecting markers linked with a QTL in the specific regions in segregating populations by combining RFLP and RAPD. This provides a effective method to map interesting traits based on a partial molecular linkage map. Selective genotyping (Darvasi and Soller, 1992; Lander and Botstein, 1989; Lebowitz et al, 1987) was also suggested as a design that can reduced the number of individuals genotyped for a given power of detecting QTL, by genotyping only the most informative individuals in the experimental population. However, the influence of selective genotyping on QTL mapping accuracy remains to be investigated (Darvasi et al, 1993).

1.2.2.4 QTL Mapping in barley

Table 1.6 Software packages for QTL analysis

Name	Algorithms	Progeny	Multiple environments
Mapmaker/QTL	ML, Reg	F2, BC, DH	No
Mapmanager QT	Reg	F2, BC, DH, RI	No
qGene	Reg	F2, BC. DH	No
QTL Cartographer	ML, Reg	F2, BC, DH, RI	Yes/No
MapQTL	ML, Reg	F2, BC, DH, RI	Yes/No
Plab QTL	Reg	F2, BC, DH, RI	Yes
MQTL	Reg	F2, BC, DH, RI	Yes

Note: This table is summarised from An alphabetic list of genetics analysis software (http://linkage.rockefeller.edu/soft/list.html) and Quantitative trait locus mapping software (http://s27w007.pswfs.gov/qtl/sofware.html). Reg: Regression analysis; ML: Maximium Likelihood; BC: Backcross population; DH: Double haploid population; RI: recombinant Inbred lines.

Many important traits for barley improvement, such as yield, quality and some types of resistance to biotic and abiotic stresses, are controlled by quantitative trait loci (Hayes et al, 1996). Mapping QTLs using the molecular linkage map started relatively slowly in barley. Nevertheless, barley does have advantages for QTL analysis, which include:

(1) Intraspecific polymorphisms in agronomically meaningful cross combinations (Kleinhofs et al, 1993);

(2) Immortal genetic reference populations of doubled haploids that simplify map construction and QTL estimation in barley.

The first systematic QTL mapping in barley was reported by Hayes *et al.* (1993), in which 62 QTLs underlying 8 traits were mapped. Subsequently, a large number of QTLs have been reported to be mapped in different mapping populations. The main results are summarised in Table 1.7. The QTLs for the same traits may be mapped to different chromosomes or regions by different laboratories. These could be due to environmental factors as well as different mapping populations used for QTL analysis. Another reason could be the difference in the methods of quality assessment adopted (Henry et al, 1996). Furthermore, some traits have no detectable QTL (e.g. kernel thickness) or only few QTL that explain only a small part of the phenotypic variance (e.g. one QTL for seed shape explained 5% of the variance) (Backes et al, 1995). These results demonstrated some of the difficulties and complexity faced by molecular biologists and breeders in QTL mapping.

On the other hand, there are some QTLs which exist for a given trait in different mapping populations. For example, several important malting quality QTLs were always detectable in the similar region of the chromosome 5H in several experiments (Han et al, 1995; Hayes et al, 1993; Oziel et al, 1996; Tinker et al, 1996). Furthermore, some functional loci were found to make a large contribution to an associated QTL (Hayes et al, 1993; Backes et al, 1995; Oziel et al, 1996; Tinker et al, 1996). With the incorporation of more cloned genes into maps, this should assist QTL analysis.

Table 1.7 QTLs for malting quality and agronomic traits in barley

Traits	Populations	Number of QTL	Chromosomes
Malt extract	BXE, 59DH	3-7	1H, 3H, 5H, 7H
	DXM, 100DH	1-2	2H, 5H
	SXM, 150DH	7	1H, 2H, 4H, 5H, 6H, 7H
Diastatic power	SXM, 150DH	9	1H, 2H, 4H, 5H, 6H, 7H
	DXM, 100DH	2-3	4H, 5H, 7H
	BXE, 59DH	5	1H, 2H, 3H, 4H, 5H, 6H, 7H
Grain protein	BXE, 59DH	1-5	1H, 2H, 3H, 5H, 6H
	DXM, 100DH	2-3	1H, 4H, 5H, 6H
	SXM, 150DH	6	2H, 3H, 4H, 5H
Wort protein	SXM, 150	7	1H, 2H, 3H, 4H, 5H, 6H, 7H
	HXT, 146DH	5	1H, 2H, 5H, 7H
Alpha-amylase	SXM, 150DH	9	1H, 2H, 5H, 6H, 7H
	DXM,, 100DH	2	5H, 7H
Milling energy	BXE, 59DH	3-5	2H, 3H, 5H, 7H
Wort β-glucan	DXM, 100DH	1	5H
Malt β-glucan	SXM, 150DH	6	1H, 3H, 4H, 7H
Grain β-glucan	SXM, 150DH	3	1H, 2H
Malt glucanase	SXM, 150DH	3	1H, 4H, 5H
Malt glucanase	SXM, 150DH	5	1H, 5H, 7H
Plump grains	HXT, 146DH	5	1H, 2H, 4H, 5H, 6H
	SXM, 150DH	5	1H, 2H, 3H, 4H, 5H
Test weight	HXT, 146DH	4	1H, 5H, 6H
Specific weight	BXE, 59DH	1-6	2H, 3H, 4H, 5H, 6H
Grain> 2.8 mm	BXE, 59DH	2-4	1H, 2H, 3H, 5H, 6H, 7H
Kernel shape	IXD, 250DH	2	4H, 5H
Kernel length	IXD, 250DH	1	5H
Kernel weight	HXT, 146DH	5	4H, 5H, 7H
	BXE, 59DH	1-4	3H, 6H, 7H
	IXD, 250DH	2	4H, 5H

to be continued

Continue

Traits	Populations	Number of QTL	Chromosomes
Grain yield	HXT, 146DH	4	4H, 5H, 7H
	BXE, 59DH	2-4	2H, 3H, 5H, 7H
	IXD, 250DH	3	2H, 5H, 6H
	SXM, 150DH	6	2H, 3H, 6H, 7H
Heading date	SXT, 79DH	2	2H
	HXT, 146DH	5	3H, 4H, 5H, 7H
	BXE, 59DH	2-7	1H, 3H, 4H, 5H, 6H, 7H
	IXD, 250DH	3	2H, 5H
	WXS, 100DH	6	1H, 2H, 3H, 5H, 7H
	SXM, 150DH	9	2H, 3H, 4H, 6H, 7H
Flowering time	IXT, 94DH	13	1H, 2H, 3H, 4H, 5H, 6H
Ear emergence	BXK, 99DH	8	1H, 2H, 3H, 4H, 5H, 7H
Maturity	HXT, 146DH	4	3H, 4H, 5H, 6H
Plant height	BXK, 99DH	3	3H, 5H, 7H
	HXT, 146DH	3	5H, 7H
	BXE, 59DH	3-5	3H, 5H, 6H, 7H
	IXD, 250DH	3	4H, 5H, 6H
	SXM, 150DH	10	1H, 2H, 3H, 4H, 5H, 7H
Straw length	SXT, 79DH	2	2H
Top internode	SXT, 79DH	3	2H, 6H
Basic internode	SXT, 79DH	2	2H
Ear length	SXT, 79DH	2	2H
Straw diameter	SXT, 79 DH	2	2H
Lodging	HXT, 146DH	4	4H, 5H, 7H
	IXD, 250DH	3	1H, 4H, 6H
	SXM, 150DH	6	2H, 3H, 4H, 6H, 7H
Stem breaking	IXD, 250DH	4	1H, 2H, 4H, 6H
Ear breaking	IXD, 250DH	3	2H, 4H, 6H
Powder Mildew	BXE, 59DH	2-4	1H, 2H, 3H, 5H, 6H, 7H
	IXD, 250DH	1	7H
Net blotch	SXM, 150DH	7	2H, 3H, 4H, 5H, 6H, 7H
Stripe rust	SXM, 150DH	3	2H, 6H
Brown rust	SXM, 150DH	2	2H, 7H
Yellow rust	BXE, 59DH	2	3H, 5H
	BXE, 59DH	3-5	1H, 5H, 6H, 7H

Populations: B x E : Blenheim x E224/3; I x D : Igri x Danilo; W x S : Winter x Spring; H x T: Harrington x TR306; S x M: Stepeo x Morex; D x M: Dcktoo x Morex; I x T: Igri x Triumph; S x T: Six rowed x Two rowed.

This table is summarised from Backes et al, 1995, 1996; Bezant et al, 1996, 1997; Han et al, 1995; Hayes et al, 1993; Kjaeer et al, 1995; Lauries et al, 1993, 1994; Oziel et al, 1996; Pan et al, 1994; Pecchioni et al, 1996; Thomas et al, 1995, 1996; Tinker et al, 1996.

Surprisingly, Hayes *et al.* (1993) showed that QTL x environment interaction only results in differences in the magnitude of QTL effects in barley. Highly significant QTL effects were found for all traits analysed at multiple environments. These results were further supported by Oziel *et al.* (1996) and Tinker *et al.* (1996). Therefore, QTL mapping in barley is an effective tool for improvement of agronomically and economically important traits.

Hayes *et al.* (1996) further suggested that the different QTLs in different populations for a same trait are due to genotype-specificity of the QTLs. This limits straightforward application of the results from one population to another. On the other hand, it implies that not all favourable QTLs are fixed in elite germplasm. An alternative to attempting to extend the QTL results from one population to another is to integrate information from a range of germplasm with the aim of identifying regions of the genome that may affect trait expression. This information can be used to classify germplasm and design matings that will maximise the probability of accumulating favourable alleles (Hayes et al, 1996). Therefore, more populations should be developed for QTL mapping in the future.

1.3 Aims of the present project

Hydrolytic enzymes play a central role during the malting and brewing process. Although the genes for most of the important hydrolytic enzymes have been cloned, it is not known how many loci control the activity of the hydrolytic enzymes. It has become increasingly apparent that the genes controlling activity per se are more important in most case than the actual gene itself. This is one reason why improvement of malting quality has been very slow over the past century. Development of molecular markers and QTL-molecular linkage analysis provides a useful approach to systematically dissect the QTLs controlling the activity of hydrolytic enzymes, and ultimately to utilise this information in a breeding program. Therefore, the aims of the present study include:

(1) Mapping the structural loci of hydrolytic enzymes and determining the contribution of each structural locus to the activity of the enzyme,

(2) Identifying regulatory loci (QTL) controlling the activity of the enzymes,

(3) Identifying common loci controlling the activity of several hydrolytic enzymes,

(4) Molecular characterisition of the two isoenzymes of beta-amylase, the most important hydrolytic enzyme for malting and brewing.

Chapter 2: Materials and Methods

2.1 Materials

Three double haploid (DH) populations including their six parents were used in the present study. One population consisted of 150 DH lines was generated using the *Hordeum bulbosum* method (Hayes and Chen, 1989) from the cross Clipper x Sahara3771 and provided by Dr. AKRM Islam, The University of Adelaide. Two other populations; consisted of 114 DH lines from a Haruna Nijo x Galleon cross and 120 DH lines from a Chebec x Harrington cross, were generated with anther culture and provided by Dr. Sue Logue, The University of Adelaide. The parents for these populations have different genetic background with respect to malting quality. Harrington and Haruna Nijo were the varieties with high malting quality and originated from Canada and Japan respectively; Chebec and Clipper are Australian varieties with fair malting quality; Galleon is an Australian variety with feed quality and Sahara is a landrace from Algeria. Relatively comprehensive molecular linkage maps have been constructed for these populations and shown in Fig. 2.1. The Clipper x Sahara population has 164 markers and a total length of 1299 cM with an average marker density 7.9 cM; the Haruna Nijo x Galleon population has 271 markers, a length of 1494 cM and an average marker density 5.5 cM and the Chebec x Harrington population has 237 markers over a length of 1414 cM with an average marker density 5.9 cM.

All the plant materials were planted in a greenhouse and the seedlings harvested for DNA extraction. The seeds from Clipper x Sahara population were also harvested for analysis of enzymes associated with malting quality. The DH populations from the crosses Haruna Nijo x Galleon and Chebec x Harrington together with their parents were planted at the University of Adelaide's Charlick Experimental Station, Strathalbyn, South Australia in the 1993-1994 growing season. The seeds harvested from this experiment were used to analyse the malting

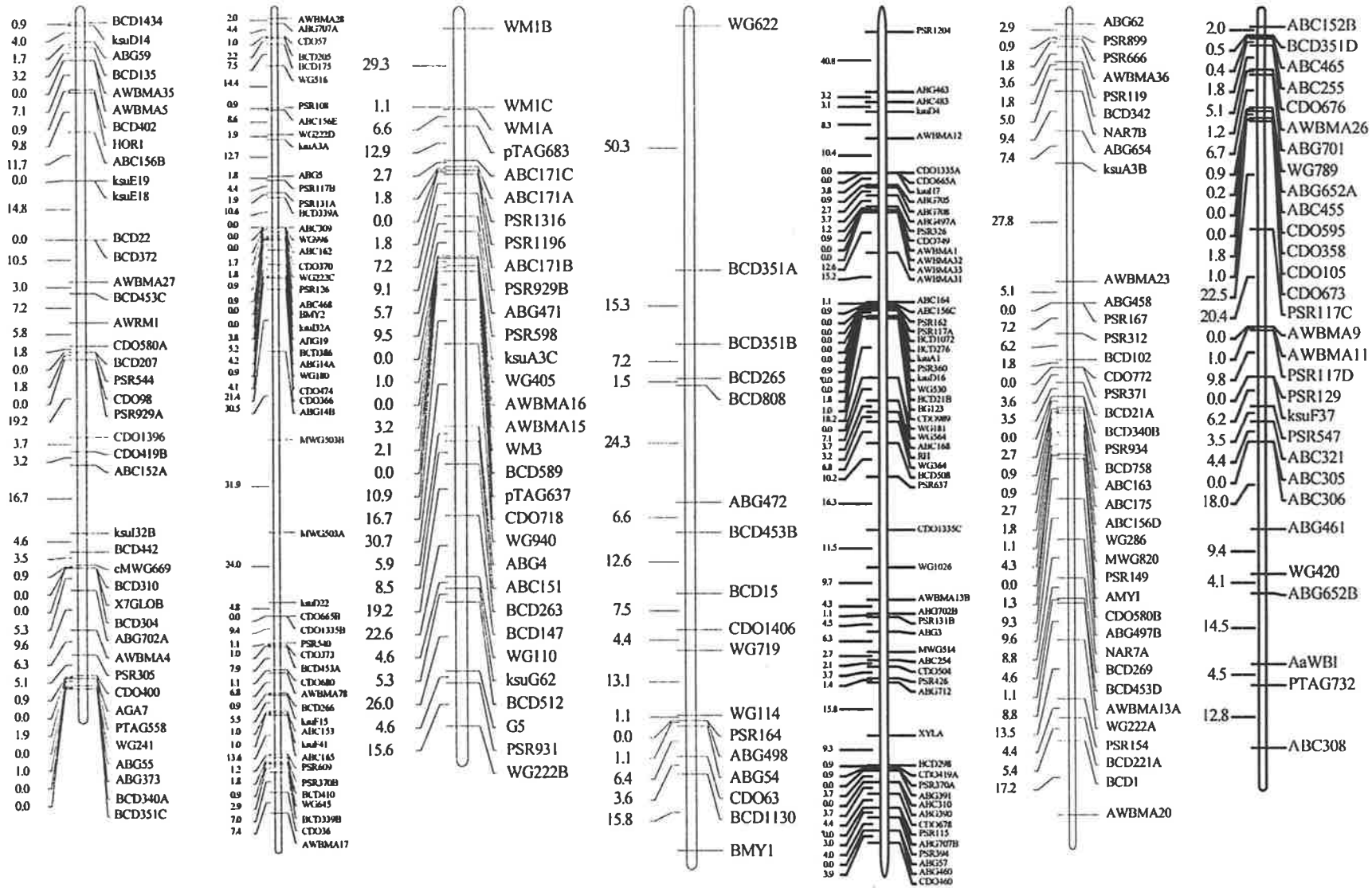

Fig. 2.1a Molecular linkage map of Haruna Nijo x Galleon population

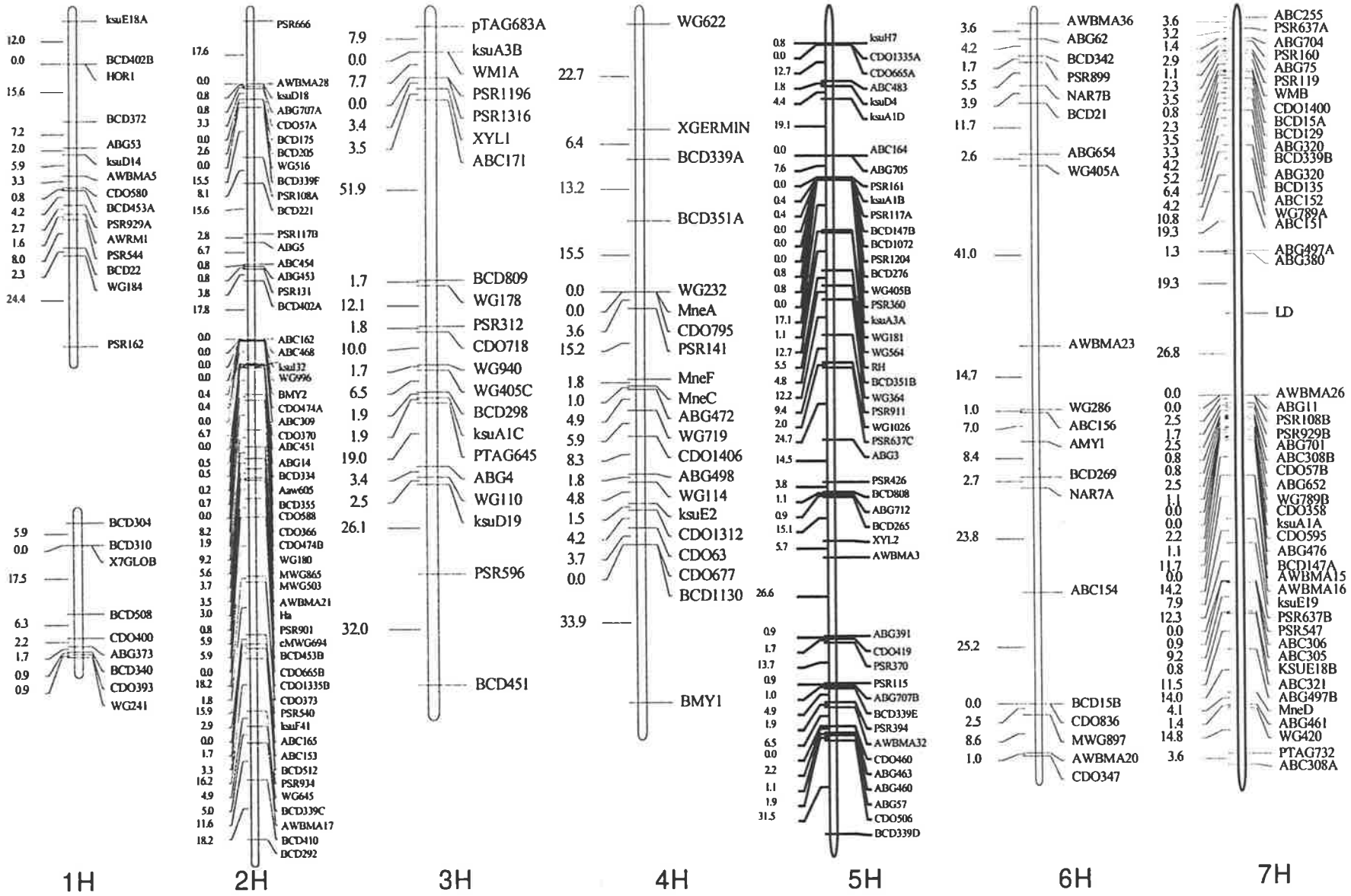

Fig 2.1b Molecular linkage maps of Chebec x Harrington population

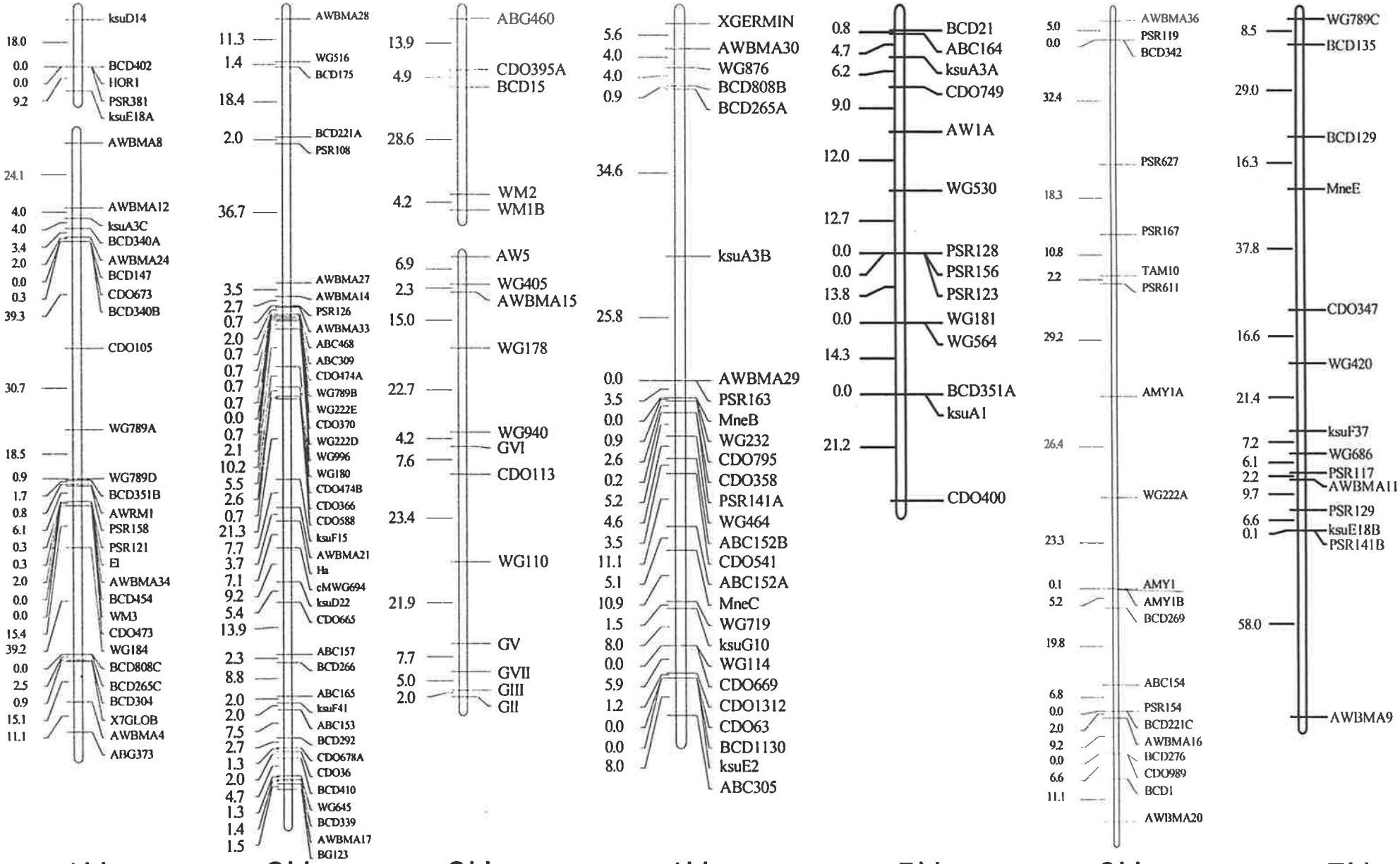

Fig 2.1c Molecular linkage map for Clipper x Sahara population

quality and map the quantitative trait loci (QTL) for malting quality. This material was also planted in the 1994-1995 and 1995-1996 growing seasons and the harvested seeds used for bulked analysis or marker-assisted selection.

Wheat-barley addition lines described by Islam *et al.* (1981) were provided by Drs. KW Shepherd and AKRM Islam; the chromosome 1H addition line, which is a double monosomic addition containing one copy each of chromosomes 1H and 6H (Islam and Shepherd, 1990), was provided by Dr. AKRM Islam. The wheat (*Triticum aestivum* cv. Chinese Spring) and barley (*Hordeum vulgare* cv. Betzes) parents of the addition lines were provided by Dr. KW Shepherd. Throughout this study the numbers of the equivalent wheat homologous group of chromosomes are used.

2.2 Methods

2.2.1 DNA isolation and Southern blot analysis

2.2.1.1 Small scale DNA isolation

Approximately 10 cm of leaf tissues was placed in a 1.5 ml Eppendorf centrifuge tube and frozen in liquid nitrogen. The material was crushed with a knitting needle to a fine powder and homogenised in 600 µl of DNA extraction buffer (1% sarkosyl, 100 mM Tris-HCl, 100 mM NaCl and 10 mM EDTA, pH 8.5). The resulting slurry was extracted with 600 µl of phenol/chloroform/isoamyl-alcohol (25:24:1) for 5 minutes by hand shaking the tube heavily for 20 to 30 seconds. The two phases were separated by centrifugation at 12,000 rpm for 10 minutes. The aqueous phase was transferred to a new tube and the phenol/chloroform/isoamyl-alcohol extraction repeated. The DNA was precipitated by addition of 60 µl of 3 M sodium acetate pH 4.8 and 600 µl isopropanol and centrifuged at 12,000 rpm for 5 minutes. The DNA pellet was washed with 70%

ethanol for 15 minutes in an orbital rotor. Finally, the clean DNA was resuspended in 50 µl of TE buffer (10 mM Tris-HCl pH 7.5, 1 mM Na_2EDTA) containing 40 µg/ml of RNase A.

2.2.1.2 Large scale DNA isolation

Approximately 50 fresh leaves were collected and cut into 10 cm long pieces. The material was frozen in liquid nitrogen and ground to a fine powder using a pre-chilled mortar and pestle. The powder was suspended in 100 ml of DNA extraction buffer and homogenised by gentle grinding. The resulting slurry was transferred to a 250 ml polypropylene centrifuge tube and 100 ml of phenol/chloroform/isoamyl-alcohol was added. The solution was mixed for 1 hour on an orbital mixer at 4 °C and then centrifuged at 3000 rpm at 4 °C for 5 minutes (rotor GSA in a Sorvall centrifuge). The supernatant was poured through 3 layers of cheese cloth and 10 ml of 3 M sodium acetate (pH 4.8) mixed with the solution. The DNA was precipitated by adding 250 ml of 99% ethanol (pre-chilled to -20 °C) and then spooled out with a glass rod. The clump of DNA was washed twice with 10 ml of chilled 70% ethanol and resuspended in 7 ml TE buffer after partial drying. The DNA was further purified via CsCl gradient centrifugation (Sambrook et al, 1989)

2.2.1.3 Digestion of genomic DNA with restriction enzymes

Approximately 5 µg of genomic DNA was digested in a reaction containing 1.1 µl of the appropriate 10x restriction buffer (supplied by the manufacturer), 1 µl of 40 mM spermidine, 1 µl of 1 mg/ml acetylated bovine serum albumin (BSA) and 2 µl (20 units) restriction enzyme and water to 11 µl. The reactions were incubated at 37 °C for 6 hours. The most commonly used enzymes were; *EcoRI*, *EcoRV*,

DraI, *HindIII* and *BamHI*. The other restriction enzymes used were; *SalI*, *SacI*, *XbaI*, *HaeIII*, *DdeI*, *AluI*, *TaqI*, *PstI*, *SphI*, *MspI*, *XmaII*, *XhoI*, *AccI*, *NarI*, *Sau3A*, *RsaI*, *Asp718*, *Asp700*, *NdeI*, *NotI*, *PvuI*, *HinfI* and *BglII*.

2.2.1.4 Agarose gel electrophoresis

Digested DNA was mixed with 2 µl of 6x Ficoll dye (15% Ficoll type 4000, 0.25% brophenol blue and 0.25% xylene cyanol FF) and fractionated on a 1% agarose gel in TAE buffer (40 mM Tris-HCl, 1mM Na_2EDTA) at 20 mA for 16 hours. After electrophoresis, gels were stained in 10 µg/ml ethidium bromide for 20 minutes, visualised under UV light and photographed using Polaroid 667 film.

2.2.1.5 Transfer of DNA onto nylon membranes

The DNA was transferred onto a nylon membrane and fixed with alkali (Sambrook et al, 1989). In brief, the gel was soaked in denaturing solution (1.5 M NaCl and 0.5 M NaOH) for 30 minutes and the DNA was transferred to the nylon membrane (Hybond-N^+, Amersham, UK) using 10x SSC (1.5M NaCl and 0.15 M trisodium citrate) for 6 hours. Upon completion of the transfer, the membrane was rinsed briefly in 2x SSC and blotted dry using paper towels. DNA was fixed by placing the membrane on 3 MM Whatmann paper soaked with 0.4 M NaOH for 30 minutes. Finally, the membrane was rinsed sequentially in neutralising solution (1.5 M NaCl, 500 mM Tris-HCl, 100 mM Na_2EDTA, pH 7.2) and 5x SSC and blotted dry using paper towel.

2.2.1.6 Probe preparation and labeling

DNA fragments cloned into plasmid vector containing the universal M13 forward and reverse sequences were amplified using the polymerase chain reaction

(PCR) and used as probe for hybridisation. Each reaction consisted of 1 µl (50 ng) plasmid vector, 1 µl (0.3 µg/µl) each of forward and reverse primers, 5 µl of 10x Taq reaction buffer, 1.5 µl of 25 mM $MgCl_2$, 4 µl 1.25 mM each of dATP, dCTP, dGTP and dTTP, 1 unit of Taq polymerase (Promega or Boehringer) and water to 50 µl. The PCR reaction consisted of an initial denaturing step of 94°C for 4 minutes followed by 35 cycles of 94°C for 1 minute, 55°C for 2 minutes, 72°C for 2 minutes and finally at 72°C for 5 minutes. The products were separated on 1% TAE agarose gel. The insert band was excised under longwave UV light (302 nm) and purified using Geneclean according to the manufacturer's instructions (Bio 101).

Probes were radioactively labeled using the random oligo-priming method, but a gene-specific priming method was used for the short probes (less than 300 bp). The reaction consisted of 3µl (50 ng) of denatured insert (insert denatured by boiling for 5 minutes and rapid cooling in an ice-water slurry), 12.5 µl of 2x random oligolabeling buffer (40 mM each of dATP, dGTP, dTTP), 100 mM Tris-HCl pH 7.6, 100 mM NaCl, 20 mM $MgCl_2$, 200 µg/ml of acetylated DNase free bovine serum albumin), 3 µl (0.3 µg/µl) of random 9-mer labeling primer, 4 µl [α-^{32}P]-dCTP (30µCi) (Amersham), 1µl of DNA polymerase I (Klenow I) (2 units/µl) (Boehringer) and 2.5 µl water. The reaction was incubated for 1 hour at 37°C and labeled DNA was separated from un-incorporated nucleotides on a G-100 Sephadex column.

2.2.1.7 Hybridisation and autoradiography

Prehybridisation of membranes was performed in hybridisation solution containing 3 ml 5x HSB (3 M NaCl, 100 mM PIPES, 25 mM Na2EDTA pH 6.8), 3 ml Denhardts III (2% gelatin, 2% Ficoll, 2% PVP, 10% SDS, 5% tetrasodium pyrophosphate, filtered through 1 MM Whatmann paper), 3 ml of 25% dextran sulphate, 0.5 ml salmon sperm DNA (5 mg/ml) and 0.5 ml water. The membrane

was first soaked in 5x SSC for 1 minute and placed in a hybridisation bottle (Hybraid) and then the hybridisation solution (pre-warmed to 65°C) added. The membrane was prehybridisation for 6 hours at 65°C. The radio-labeled probe was denatured in boiling water for 5 minutes and rapidly cooled in a tube containing 500 µl salmon sperm DNA (5 mg/ml). It was added directly to the hybridisation bottle containing the membrane and solution. Hybridisation was done at 65°C overnight.

The membranes were then washed in 2x SSC, 0.1% SDS for 20 minutes to remove unbound probe. Washes were repeated in 1x SSC, 0.1% SDS and 0.5x SSC, 0.1% SDS and 0.2x SSC, 0.1% SDS and 0.1x SSC, 0.1% SDS. The membranes were sealed in plastic bag and exposed to X-ray film at -80°C for 5 to 8 days, depending on signal density.

2.2.1.8 Removal of radioactive probe from membranes

Membranes were stripped by pouring 100 °C hot stripping solution (0.2% SDS, 2mM Na_2EDTA, pH 8.0) onto them and then incubating for 30 minutes while shaking. The membranes were re-exposed to X-ray film for two days to be certain of the effectiveness of radioactive probe removal.

2.2.2 PCR amplification of specific genes from the barley genome

For those genes whose clones were not available but the sequences were published, gene specific primers were designed using the computer program Oligo 4.0 to amplify the fragment of the gene from barley genomic DNA. The PCR procedure used is described in 2.1.6, but the annealing temperature was between 45 to 60 °C depending on the primers. The products were separated on a 2 to 3% agarose gel in TAE buffer or on a 6 to 10% polyacrylamide gel in TBE buffer (0.89 mM Tris-borate, 2 mM Na2EDTA) (depending on length of the fragment). If no

polymorphism between the parents of the mapping populations was detected for the PCR products, the DNA band was excised under longwave UV light and purified using Geneclean according to the manufacturer's instructions (Bio 101). The purified DNA fragment was either used as probe as showed in 2.2.1.6 or further digested using different restriction enzymes to check the polymorphism between the parents of the mapping populations.

2.2.3 Gene cloning

2.2.3.1 RNA isolation from developing seeds

Developing barley seeds, (50 seeds) 20 to 23 days post-anthesis were harvested and frozen in liquid nitrogen. The seeds were ground to a fine powder and homogenised in 3 to 4 ml of REB buffer (100 mM Tris-HCl pH8.0, 4% sarcosyl, 10 mM Na_2EDTA). The resulting slurry was transferred to pre-chilled Corex tubes and immediately centrifuged at 5,000 rpm (JA20, Beckman J2-21) at 4 °C for 5 minutes. The supernatant was transferred to a fresh tube and Caesium chloride (1 g CsCl per ml supernatant) was added. The solution was pipetted onto 3 ml of a CsCl cushion (9.65 g CsCl dissolved in TE to a final volume of 10 ml) in ultracentrifuge tubes. The samples were centrifuged at 38,000 rpm (Ty65 rotor, Beckman L8-70) for 16 hours at 4°C. The supernatant was removed and the RNA pellet dissolved in 400 µl REB-buffer. The solution was extracted with phenol/chloroform/isoamyl-alcohol and then the RNA was precipitated with ethanol at -20 °C overnight. The pellet of RNA was washed with 70% ethanol and resuspended in TE buffer.

2.2.3.2 Reverse transcriptase PCR (RT-PCR)

The first strand cDNA was synthesised in a reaction of 20 µl containing 3 µg total RNA (pre-heated to 70°C for 10 minutes and quick chilled on ice), 1 µl of oligo-$(dT)_{18}$, 4 µl of 5 times first strand buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl and 15 mM $MgCl_2$), 2 µl of 0.1 M DTT, 8 µl of 1.25 mM dNTP mix (1.25 mM each dATP, dCTP, dGTP, dTTP) and 5 µl water. The mixture was incubated at 42°C for 2 minutes and then 1 µl (200 units) of SUPERSCRIPT™ II (GIBCOBRL) was added. The reaction was incubated at 42°C for 50 minutes and stopped by heating to 70°C for 15 minutes.

Using the first strand cDNA as template, the target gene was amplified in a PCR reaction of 100 µl containing 4 µl cDNA, 10 µl of 10 times buffer (200 mM Tris-HCl pH8.4, 500 mM KCl), 3 µl of 50 mM $MgCl_2$, 16 µl of 1.25 mM NTP mix (1.25 mM each dATP, dCTP, dGTP, dTTP), 1µl of each gene specific primer (10 µM), 1 µl Taq polymerase (5 units/µl) (Boehringer) and 65 µl water. The thermal cycling consisted of an initial 3 minutes denaturing step at 94°C followed by 30 cycles of 94°C for 1 minute, 53°C for 2 minutes, 72°C for 2 minutes. Final chain extension was allowed to proceed at 72°C for 10 minutes. The PCR product was separated on a 1% TAE agarose gel and the DNA band was excised under longwave UV light and purified using Geneclean according to the manufacturer's instructions.

2.2.3.3 Ligation of DNA-sequences into plasmid T-vector

The ligation was performed in a reaction containing 1 µl of T4 DNA ligase 10 times buffer (300 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP), 1 µl of pGEM-T vector (50 ng) (Promega), 3 µl of the PCR product, 1µl of T4 DNA ligase (1 Weiss units /µl) (Promega) and water to 10 µl. The

reaction was incubated at 13 to 15°C overnight and then kept at 4°C before transformation.

2.2.3.4 Transformation of ligated PCR:pGEM-T vector

Transformation was performed using JM109 high efficiency competent cells (Promega). Five µl of the ligated PCR product:pGEM-T vector reaction was transferred to a sterile 1.5 ml microcentrifuge tube on ice and 50 µl of competent cells added. After gently flicking the tube, the reaction was kept on ice for 30 minutes and then heat shocked at 42°C for 45 to 50 seconds and put back on ice for 2 minutes. The transformed cells were added to 450 µl of LB medium with 0.35% glucose and incubated at 37°C for 1 hour with shaking. The cells were plated out onto LB plates containing 50 µg/ml ampicillin, 0.004% X-gal and 0.1 mM IPTG and incubated at 37°C overnight. Recombinant bacteria were identified as white colonies.

2.2.3.5 Isolation of recombinant plasmids

The alkaline lysis method of Sambrook *et al.* (1989) was used for mini-scale plasmid preparation. Briefly, a sterile culture tube containing 2 ml LB medium with 50 µg/ml ampicillin was inoculated with a single colony and incubating at 37°C overnight with shaking. Cells were pelleted by centrifugation at 5,000 rpm for 5 minutes and the supernatant discarded. The cell pellet was resuspended in 100 µl Plamid I solution (50 mM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA) and put on ice for 10 minutes. Cells were lysed by the addition of 200 µl of Plasmid II solution (0.2 M NaOH, 1% SDS), mixed gently and incubated on ice for 10 minutes. The resulting solution was mixed with 150 µl of Plasmid III (3 M sodium Acetate pH 4.8) and put at -20 °C for 10 minutes. After centrifuged at 12,000 rpm

for 15 minutes, the supernatant was transferred to a fresh centrifuge tube. The plasmid DNA was precipitated with 100% cold ethanol and washed twice with 70% ethanol. After drying under vacuum, the plasmid DNA was resuspended in 30 µl TE buffer.

2.2.3.6 DNA sequencing

Sequencing of inserts cloned into the pGEM-T vector was performed with an ABI automated DNA sequencer (Applied Biosystems). Template preparations and the sequencing reactions were as described by the manufacturer.

2.2.4 Preparation of "green malt"

One hundred seeds per sample were germinated on filter paper in a 9 cm petri dish with 4 ml water at 15°C for 5 days. The germinated seeds were frozen in liquid nitrogen and dried under vacuum at -45°C (green malt). The samples were milled to pass a 0.5 mm mesh screen, stored at 4°C and used for analysis of isoenzymes and activity of the enzymes.

2.2.5 Isoelectric focusing electrophoresis and Western blotting analysis

Enzymes were extracted with glycerol buffer (75 µl of glycerol, 395 µl water and 30 µl ampholyte (pH 3.5-9.5) (Pharmacia). Ultra thin polyacrylamide gel (0.4 mm) (5.5%) containing ampholytes was cast on GEL Bond PAG support film (FMC BioProducts). The pH range was achieved by mixing two ampholytes in the ratio 1:1 (ampholytes for alpha-amylase were pH 3.5-9.5 and pH 5.0-7.0; for beta-amylase were pH 4.5-5.4 and pH 5.0-7.0). Isoelectric focusing was performed at 10°C on a Multiphor II horizontal flatbed electrophoresis unit (LKB). The gel was

pre-focused for 1 hour at 5 W constant power and then run at 2200 V with an upper power limit of 10 W for 2 hours.

A piece of nitrocellulose blotting membrane (Sartorins) was then laid on the gel and proteins were allowed to absorb to the membrane by passive transfer (15 minutes). The immunoblot was developed as described by Harlow and Lane (1988) and detected with HRP labeled goat anti-rabbit antibodies (BioR) with 4-chloro-1-naphthol as the substrate (BioR). All the antibodies were provided by Dr. Evan Evans, The University of Adelaide. After removal of the membrane, the gel was stained for alpha- and beta-amylase activity with starch/iodine stain (Guerin et al, 1992a).

2.2.6 Assay for activity of the enzymes

2.2.6.1 Assay for alpha-amylase activity

Alpha-amylase activity was measured using the CERALPHA method (Megazyme) according to McCleary and Sheehan (1987) and Sheehan and McCleary (1988). The enzyme was extracted from 100 mg of green malt flour with 1 ml extraction buffer (50 mM sodium malate, 50 mM sodium chloride, 2 mM calcium chloride and 0.1% of sodium azide, pH 5.2) for 30 minutes at 40°C. The resulting slurry was centrifuged at 5,000 rpm for 5 minutes and the supernatant was diluted to 1/500 using extraction buffer. Diluted solution (50 µl) was mixed with 50 µl substrate and incubated at 40°C for exactly 10 minutes. The reaction was stopped by adding 750 µl of 1% Trizma base (Sigma). The enzyme activity was measured with a spectrophotometer at 410 nm.

2.2.6.2 Assay for beta-amylase activity

Beta-amylase activity was measured using the Betamyl assay (Megazyme) according to McCleary and Codd (1989). The enzyme was extracted from 100 mg green malt flour with 1 ml extraction buffer (100 mM maleic acid, 1 mM EDTA, 0.1% BSA, 0.02% sodium azide pH 6.2; for extraction of the total enzyme, 143 mM mercaptoethanol was added) for 2 hours at 40°C. The extract was centrifuged at 12,000 rpm for 5 minutes at 4°C. The supernatant was diluted to 1/2000. The diluted solution (50 µl) was mixed with 50 µl substrate and incubated at 40°C for exactly 10 minutes and the reaction was stopped by adding 750 µl stopping solution (1% Trizma base). The enzyme activity was measured with spectrophotometer at 410 nm.

2.2.6.3 Assay for beta-glucanase activity

Beta-glucanase activity was measured with AZO-barley glucan method (Megazyme). The enzyme was extracted from green malt flour (50 mg) with 800 µl extraction buffer (40 mM sodium acetate, 40 mM sodium phosphate and 0.02% sodium azide, pH 4.6) at 30 °C for 30 minutes. The resulting slurry was centrifuged at 12,000 rpm for 5 minutes at 4°C. The supernatant (100 µl) was mixed with 100 µl substrate and incubated at 30°C for exactly 10 minutes. The reaction was inactivated by adding 800 µl precipitant solution (80% methoxyethanol, 4% sodium acetate, 0.45% zinc acetate). The reaction was placed at room temperature for 5 minutes and then centrifuged at 12,000 rpm for 5 minutes. The supernatant was used to measure the enzyme activity with a spectrophotometer at 590 nm.

2.2.6.4 Assay for the activity of limit dextrinase

The activity of limit dextrinase was measured with LIMIT-DEXTRIZYME method (Megazyme) according to McCleary (1992). The enzyme was extracted

from 100 mg green malt flour with 2 ml extraction buffer (100 mM sodium malate and 0.02% sodium azide, pH 5.5; 25 mM DTT was added for extraction of total enzyme) for 6 hours at 40 °C. The extraction was centrifuged at 5,000 rpm for 5 minutes. The supernatant (500 µl) was incubated with the substrate at 40 °C for exactly 10 minutes and reaction was stopped with 1.5 ml of 1% Trizma base. The enzyme activity was measured with a spectrophotometer at 590 nm.

2.2.7 Gene mapping and QTL analysis

A specific gene was incorporated into a molecular linkage map using Mapmaker software (Lander et al, 1987) and further combined into a consensus linkage map using JoinMap software (Stam, 1993). Genetic distance (cM) was calculated by Haldane function (Haldane, 1919). QTL were mapped using QT Manager software with linkage evaluation derived from Silver (1985) and Neumann (1990). The threshold to identify a QTL linked with markers was $P<0.05$. The total variation explained by a given QTL was calculated using stepwise regression with JMP software (SAS).

2.2.8 Reverse bulked segregant-analysis

Bulked-analysis method was developed by Giovannoni *et al.* (1991) and Michelmore *et al.* (1991). The principle is the grouping together of informative individuals so that a particular region of interest can be studied in a random genetic background. Using this method, several molecular markers have been identified linked to a disease resistant gene (Michelmore et al, 1991) or other traits (Giovannoni et al, 1991). In the present study, a known function locus or postulated function locus was used as reference, 10 DH lines were randomly selected with the genotype of one parent to make one bulk and other 10 DH lines with the genotype of the other parent to make the second bulk. Theoretically, the

two bulks have similar random genetic background with different in the marker locus. Any difference in a trait between the two bulks was postulated to be controlled by the marker locus. The principle is the same with bulked analysis, but the process is reversed. Therefore, it is refereed to here as reverse bulked-segregant analysis.

2.2.9 Seed dormancy

Newly harvested seeds (100 seeds) were germinated immediately with 4 ml water at 15°C in a 9 cm petri dish with two Whatman No. 1 filter papers. The number of ungerminated seeds after 48 hours was used as a measure of dormancy .

Chapter 3: Sequence Variation, Isoenzyme Type and Activity QTL of β-Amylase in Barley (*Hordeum vulgare L.*)

3.1 Introduction

During barley germination, α-amylase, β–amylase, limit dextrinase and α-glucosidase jointly degrade the stored starch to simple maltose, glucose and other simple oligosaccharides (MacGregor, 1987, 1990). The ability to mobilise the starch is refereed as diastatic power (DP) in the brewing industry, and the levels of DP affect both malting yield and brewing quality. However, only β–amylase activity is significantly related to DP in most studies (Arends et al, 1995; Delcour and Verschaeve 1987; Evans et al, 1995; Erdal et al, 1993; Gibson et al, 1995), which suggest that in most situations, β–amylase is the limiting factor.

Beta-amylase is synthesised during seed development and stored in mature seeds, thus it has also a storage protein function (Giese and Hejgaard, 1984). This contrasts to α-amylase, limit dextrinase and α-glucosidase which are synthesised *de novo* during germination. In quiescent grain, the enzyme exists in both a free and a bound form in combination with protein Z (Brandt et al, 1990; Evans et al, 1995; LaBerge and Marchylo, 1983; Lundgard et al, 1987). Most of the β-amylase is released during seed germination (Evans et al, 1997b). It is not clear how many genes are involved in this process and how many genes are responsible for the β-amylase activity within mature seed and/or germinated grains.

Beta-amylase is highly heterogeneous. At least 8 different isoenzyme bands have been detected by chromatofocussing (LaBerge and Marchylo, 1983), but most result from post-translational modification. Only two distinct isoenzyme types are evident in cultivated barley (Evans et al, 1997a; Forster et al, 1991; Nielsen and Johansen, 1986) and malt (Allison, 1973; Allison and Swanston, 1974). These isoenzymes are referred as Sd1 and Sd2 type, but Sd1 type varieties are rare (Allison and Swanston, 1974; Eglinton

et al, 1995). These two isoenzyme types show different immunological properties and affinity with the inhibitor protein Z (Evans et al, 1995, 1997a). Using wheat-barley addition lines, the main bands of isoenzyme polymorphism were mapped on the long arm of chromosome 4H (Powling et al, 1981). However, the attempt to relate different isoenzyme type to enzyme activity has been inconclusive (Allison, 1973; Swanston, 1980, 1983).

Two cDNAs encoding β-amylase have been cloned. One was from the high protein, high lysine and high β–amylase activity mutant Hiproly (Kreis et al, 1987) and another from a high β–amylase activity malting barley, Haruna Nijo (Yoshigi et al, 1994). However, both of these clones encode the Sd2 type isoenzyme (Eglinton et al, 1995). Using the cloned cDNA as probe, two loci and three genes were mapped in the barley genome: one gene on chromosome 2H and two genes on chromosome 4H (Kleinhofs et al, 1993; Kreis et al, 1988). The importance of these loci to enzyme activity is yet to be demonstrated. The relationship between the isoenzyme type and the structural genes on chromosome 4H is also not clear.

In the present study, the structural genes and the quantitative trait loci controlling enzyme activity were mapped in the three mapping populations. In addition, two cDNAs, encoding Sd1 and Sd2 types of β–amylase, were cloned using RT-PCR.

3.2 Results

3.2.1 Isolating beta-amylase cDNA

The Sd1 isoenzyme type variety Harrington (with high enzyme activity) and Sd2 isoenzyme type variety Galleon (with low enzyme activity) were chosen for isolating beta-amylase cDNA. Total RNA was extracted from developing seeds at 20 to 23 days post-anthesis and the first strand cDNA was synthesised using SUPERSCRIPT™ II (Life technologies). PCR primers were designed to amplify the whole encoding sequence of β–

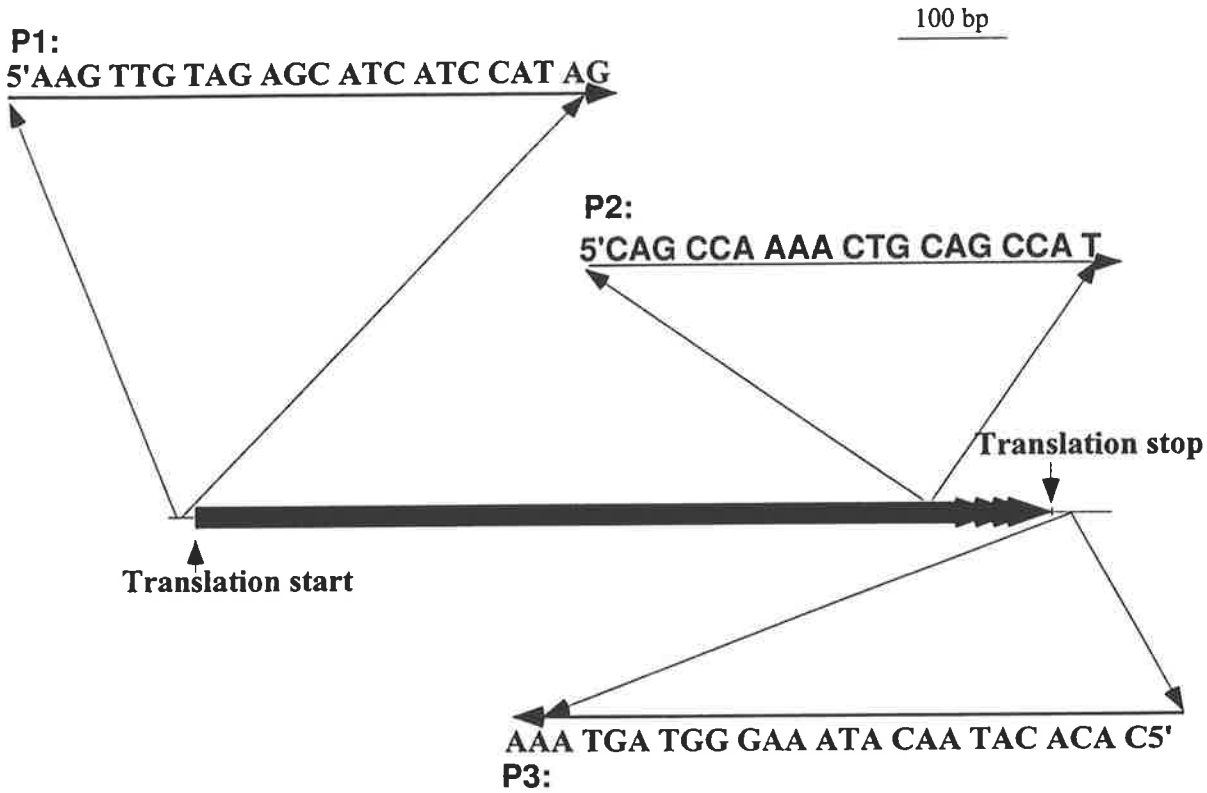

Fig. 3.1 The structure of beta-amylase cDNA and the PCR-primers designed to amplify the translation region (P1 + P3) and the four tandem repeat sequences at the 3'-end (P2 + P3).

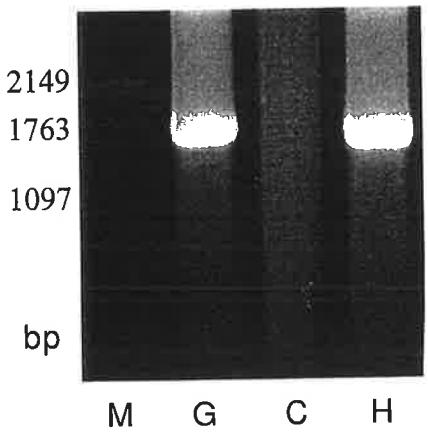

Fig. 3.2 RT-PCR amplified DNA fragment from total RNA of barley developing seeds using beta-amylase specific primers (Fig. 3.1). Lanes M, G, C and H represent DNA marker, Sd2 type variety Galleon, control and Sd1 type variety Harrington.

amylase gene based on the published sequence (Kreis et al, 1987). The primers and their target region in the beta-amylase gene are showed in Figure 3.1.

One major band was amplified from the cDNA of barley developing seeds with RT-PCR (Fig 3.2). There appears to be no difference in size between the two types of β–amylase isoenzyme. The size of the band was about 1.7 kb, which is similar with the cloned β–amylase cDNA (Kreis et al, 1987). This DNA fragment was cloned into a pGEM-T vector. DNA sequence analysis showed that the fragment contained a single open reading frame of 1605 bp and share a high degree of homology with the published β–amylase sequences (Kreis et al, 1987; Yoshigi et al, 1994). Three nucleotides were substituted comparing the low enzyme activity Sd2 type (from Galleon) with the high enzyme activity Sd2 type (from Hiproly) β–amylase gene (data not shown), but only one deduced amino acid has changed (Fig. 3.3). In contrast, the difference between the Sd1 (from Harrington) and Sd2 (from Hiproly) type sequences showed more divergence, 6 nucleotides and 3 deduced amino acids changed (Fig. 3.3). Two asparagines (Asp-266 and Asp-431) and one isoleucine (Ile-527) in the Sd2 type isoenzyme were replaced by serine, threonine and methionine in the Sd1 type isoenzyme, respectively (Fig. 3.3). Three independent clones were sequenced and no difference was found between the clones. It is, therefore, concluded that all differences observed above reflect genetic differences between the varieties and were not due to PCR artifacts. However, in a parallel study (Eglinton, personal communication), protein sequencing of Sd1 type β-amylase (from variety Franklin) has shown that Ser-266 and Thr-431 are not conserved in the Sd1 type β–amylase. Therefore, these two mutations are not related to the isoenzyme type.

The C-terminal sequence of β–amylase protein purified from germinated seed is not available due to proteolysis. For comparing the C-terminal sequences of different isoenzyme types, four Sd1 type varieties (Franklin, Alexis, Carina and Tallon) and two Sd2 type varieties (Chebec and Universe) were further chosen to clone and sequence the gene fragments encoding the C-terminus of β–amylase based on the varietal screening results of Eglinton *et al.* (1995). The Met-527 is conserved in all the Sd1 type

```
Sd1(H)  MEVNVKGNYV QVYVMLPLDA VSVNNRFEKG DELRAQLRKL VEAGVDGVMV 50
Sd2(G)  ---------- ---------- ---------- ---------- ----------
Sd2(Hi) ---------- ---------- ---------- ---------- ----------
        DVWWGLVEGK GPKAYDWSAY KQLFELVQKA GLKLQAIMSF HQCGGNVGDA 100
        ---------- ---------- ---------- ----R----- ----------
        ---------- ---------- ---------- ---------- ----------
        VNIPIPQWVR DVGTRDPDIF YTDGHGTRNI EYLTLGVDNQ PLFHGRSAVQ 150
        ---------- ---------- ---------- ---------- ----------
        ---------- ---------- ---------- ---------- ----------
        MYADYMTSFR ENMKDFLDAG VIVDIEVGLG PAGEMRYPSY PQSHGWSFPG 200
        ---------- ---------- ---------- ---------- ----------
        ---------- ---------- ---------- ---------- ----------
        IGEFICYDKY LQADFKAAAA AVGHPEWEFP NDVGQYNDTP ERTQFFRDNG 250
        ---------- ---------- ---------- ---------- ----------
        ---------- ---------- ---------- ---------- ----------
        TYLSEKGRFF LAWYSSNLIK HGDRILDEAN KVFLGYKVQL AIKISGIHWW 300
        ---------- -----N---- ---------- ---------- ----------
        ---------- -----N---- ---------- ---------- ----------
        YKVPSHAAEL TAGYYNLHDR DGYRTIARML KRHRASINFT CAEMRDLEQS 350
        ---------- ---------- ---------- ---------- ----------
        ---------- ---------- ---------- ---------- ----------
        SQAMSAPEEL VQQVLSAGWR EGLNVACENA LPRYDPTAYN TILRNARPHG 400
        ---------- ---------- ---------- ---------- ----------
        ---------- ---------- ---------- ---------- ----------
        INQSGPPEHK LFGFTYLRLS NQLVEGQNYV TFKTFVDRMH ANLPRDPYVD 450
        ---------- ---------- ---------- N--------- ----------
        ---------- ---------- ---------- N--------- ----------
        PMAPLPRSGP EISIEMILQA AQPKLQPFPF QEHTDLPVGP TGGMGGQAEG 500
        ---------- ---------- ---------- ---------- ----------
        ---------- ---------- ---------- ---------- ----------
        PTCGMGGQVK GPTGGMGGQA EDPTSGMGGE LPATM                535
        ---------- ---------- ------I--- -----
        ---------- ---------- ------I--- -----
```

Fig. 3.3 Alignment of the deduced amino acid sequences of beta-amylase with different isoenzyme types (H: Harrington; G: Galleon; Hi: Hiproly).

isoenzymes but replaced by Ile-527 in all the Sd2 type isoenzymes (data not shown). Therefore, this difference may be related to the isoenzyme types.

3.2.2 Mapping of β–amylase genes with RFLP

DNAs from the six parents used to construct the mapping populations were digested with five different restriction endonucleases *EcoRI*, *EcoRV*, *DraI*, *HindIII* and *BamHI* and probed with the cloned β–amylase cDNA (section 3.2.1). At least one polymorphism could be detected between the parents of each mapping population with each restriction enzyme (data not shown). When the parental DNAs were digested with *DraI*, all three β–amylase genes showed polymorphism in two of the mapping populations (Fig. 3.4). Using Mapmaker software (Lander et al, 1987), one β–amylase gene mapped to chromosome 2H closed to the centromere, and the other two genes mapped to chromosome 4H closed to the telomere. No recombinations were detected between the two genes on chromosome 4H from amongst the 265 DH lines examined (Fig. 3.5). By employing JoinMap software (Stam, 1993), the three β–amylase genes were incorporated into high density consensus linkage maps (Fig. 3.6 and Langridge et al, 1995).

3.2.3 Mapping of isoenzyme polymorphisms

Following IEF and starch/iodine staining (Guerin et al, 1992a), a complicated enzyme banding pattern was shown based on starch degradation in germinated barley (Fig. 3.7a). After transferring the proteins to nitrocellulose and hybridisation with β-amylase polyclonal antibodies, the banding pattern became relatively simple. There were four main β-amylase bands (Fig 3.7b), which showed a very similar pI pattern to the four major β-amylase forms generated with tryptic digestion (Lundgard and Svenson, 1986). Two distinct banding patterns were identified in the 6 parental varieties as described previously (Allison, 1973; Eglinton et al, 1995). The variety Harrington showed the Sd1

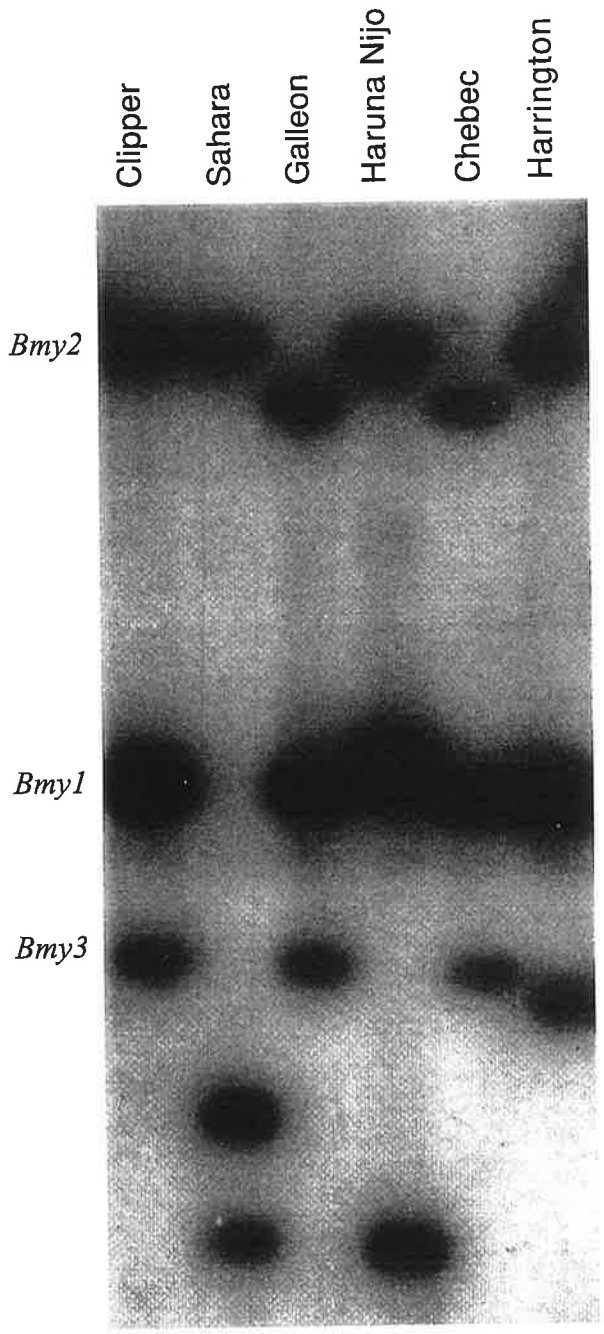

Fig. 3.4 RFLPs between the beta-amylase genes of parents used to construct the mapping populations

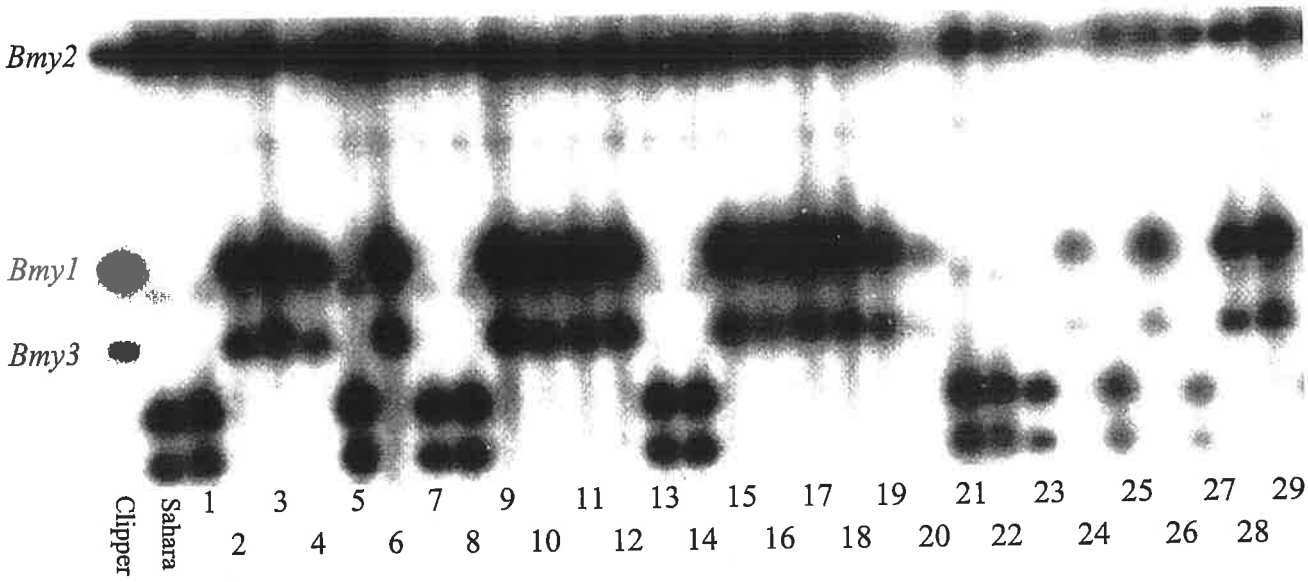

Fig. 3.5 Cosegregation of the two genes of beta-amylase on chromosome 4H in the Clipper X Sahara DH population. The arabic numerals represent the DH lines.

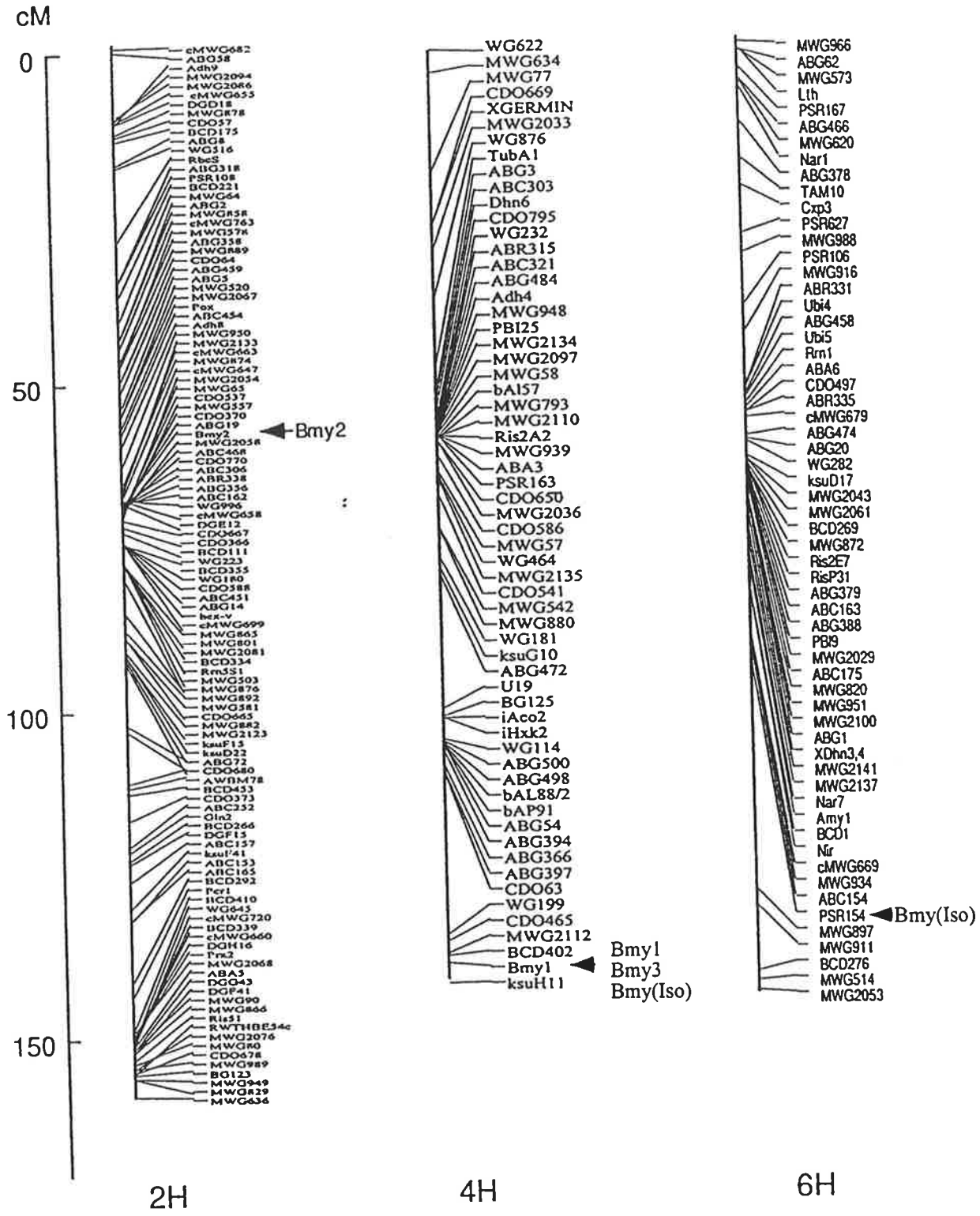

Fig 3.6 Chromosomal locations of beta-amylase genes and the isoenzyme loci on the consensus linkage map (Langridge et al, 1995)

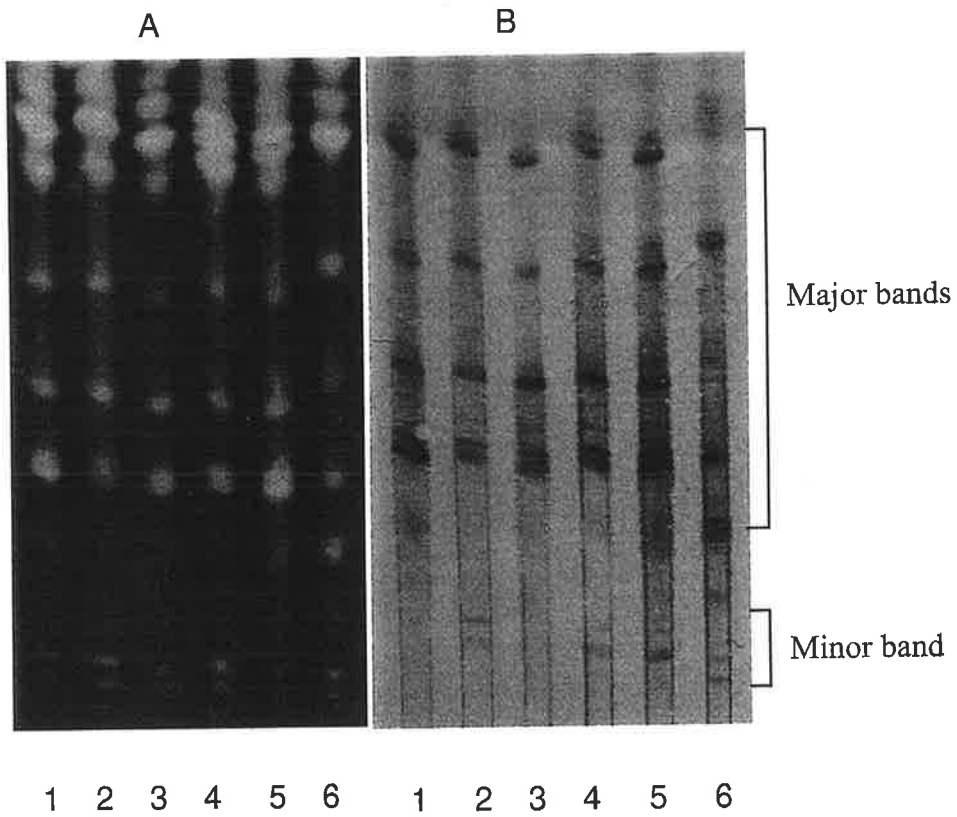

Fig. 3.7 Beta-amylases in IEF gel (A) and immunoblot (B) from germinated barley extract
A: IEF of germinated barley extracts stained with starch/iodine for alpha- and beta-amylase activity; B: IEF immunoblot of germinated barley extracts using anti-Schooner beta-amylase polyclonal sera. Lanes: 1, 2, 3, 4, 5 and 6 represent barley varieties Clipper, Sahara, Galleon, Haruna Nijo, Chebec and Harrington, respectively.

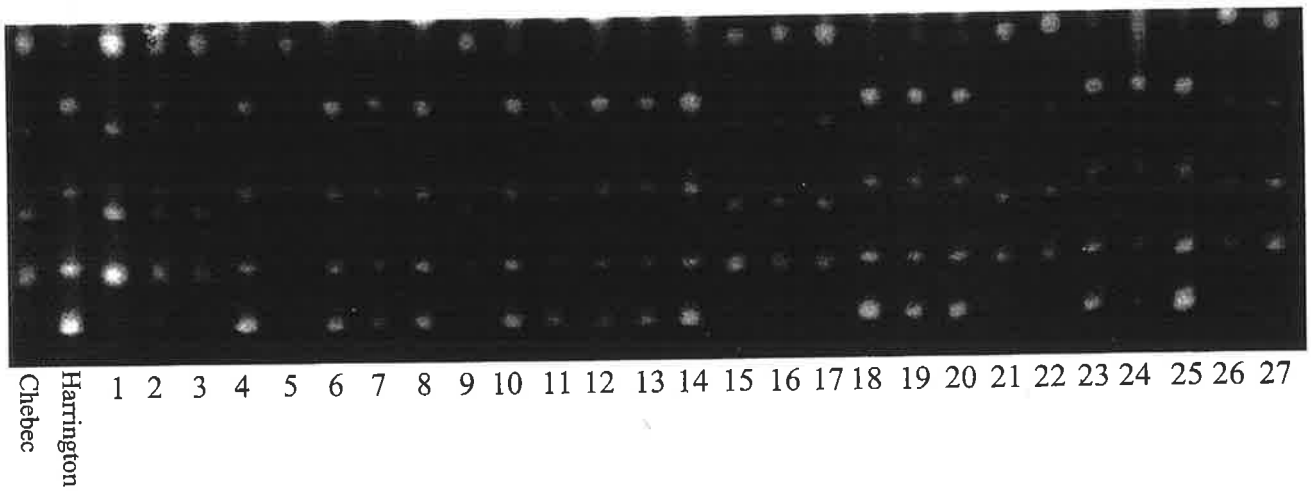

Fig 3.8 Cosegregation of the four isoenzyme bands in the Chebec X Harrington DH population. The arabic numerals represent the DH lines.

isoenzyme type while the five other varieties were of the Sd2 type. In the "Chebec X Harrington" DH population, the segregation ratio of Sd1 and Sd2 was 1:1 with no heterozygote types detected as expected. Using linkage analysis (Lander et al, 1987) and the consensus RFLP map (Langridge et al, 1995), the four major bands were mapped on chromosome 4H, and no recombination was detected between the four bands in 120 DH lines (Fig. 3.8). Furthermore, they cosegregated with the β–amylase genes mapped based on RFLPs to chromosome 4H (Fig. 3.6).

A few minor bands were detected after hybridisation with the β-amylase antibodies (Fig. 3.7B). One polymorphism was mapped on chromosome 6H (Fig. 3.6). Their origin and function are yet to be explained.

3.2.4 Mapping of QTLs controlling the enzyme activity in germinated barley

Most β–amylase in barley grain is converted into the free form during germination (Evans et al, 1997b) and only the free form of the enzyme is useful for the brewing industry. Consequently, only the QTLs controlling free enzyme activity were mapped.

The enzyme activity of the parental varieties Clipper, Sahara, Galleon, Haruna Nijo, Chebec and Harrington were 910±92, 1457±118, 835±126, 1380±96, 1428±37 and 1167±157 U/kg, respectively. Continuous variation of the enzyme activity was observed in all the DH populations and transgressive segregation was found in the "Clipper X Sahara" and "Chebec X Harrington" populations but not in the "Haruna Nijo X Galleon" population (Fig. 3.9). Using QT Manager software and a stringency of $P<0.05$, four, five and seven QTLs controlling the free enzyme activity were identified in the "Chebec X Harrington", the "Clipper X Sahara" and the "Haruna Nijo x Galleon" populations, respectively. The chromosomal location, the closest linked molecular markers, enzyme activity difference and explained total variance for the activity QTLs are listed in Table 3.1. Beta–amylase locus (*Bmy1*) on chromosome 4H was significant related to the enzyme activity in all the three mapping populations, but the locus (*Bmy2*)

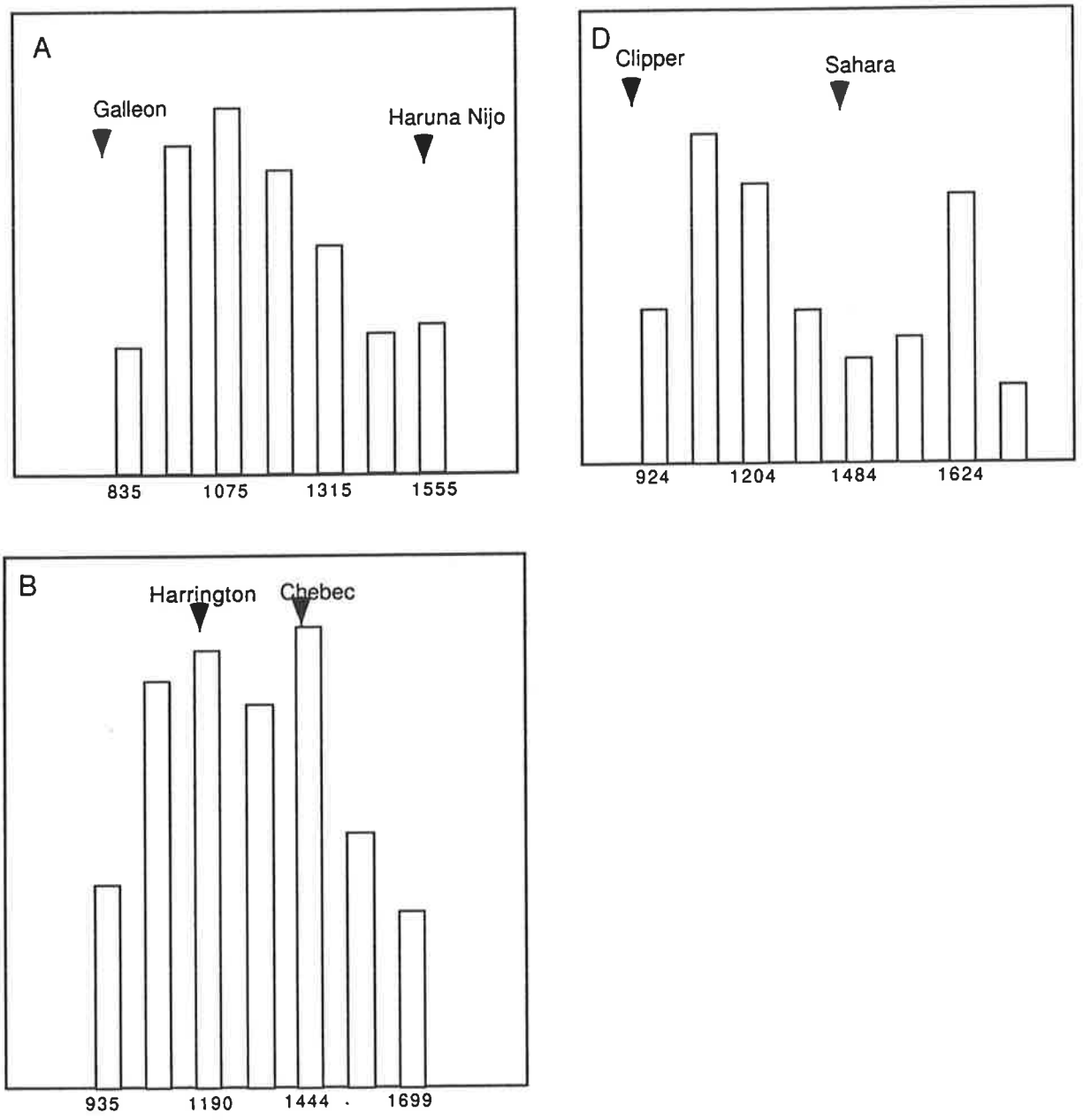

Fig. 3.9 Distribution of the beta-amylase activity in the three mapping populations. The activity is expressed as Unit/kg of malt. A: Haruna Nijo X Galleon; B: Chebec X Harrington; D: Clipper X Sahara

Table 3.1 The chromosomal location, closest linked markers(CM), genetic difference (GD) with the positive effect variety indicated (G: Galleon); HN: Haruna Nijo; C: Chebec; H:Harrington; Cl: Clipper), explained total variation (EV) of the QTLs for β-amylase activity in three doubled haploid populations

Populations	Chromosome	CM	GD%	EV%
Haruna Nijo x Galleon	1H	CDO400	12.2G	5.4
	2H	BCD339A	17.6HN	15.4
	3H	BCD512	19.2HN	11.8
	4H	CDO1406	20.5HN	14.6
	4H	BCD1130	18.7HN	20.4
	5H	ABC254	18.4HN	13.2
	7H	WG789	20.8HN	11.5
Chebec x Harrington	2H	BCD339F	10.2H	2.9
	3H	pTAG683A	9.1H	4.8
	4H	Bmy1	16.1H	11.8
	5H	CDO506	12.6C	6.5
Clipper x Sahara	1H	WG789A	19.2CL	10.6
	2H	BG123	14.6CL	10.1
	3H	GVII	17.3CL	7.3
	4H	Bmy1	16.7CL	13.2
	6H	pTAM10	17.9CL	16.4

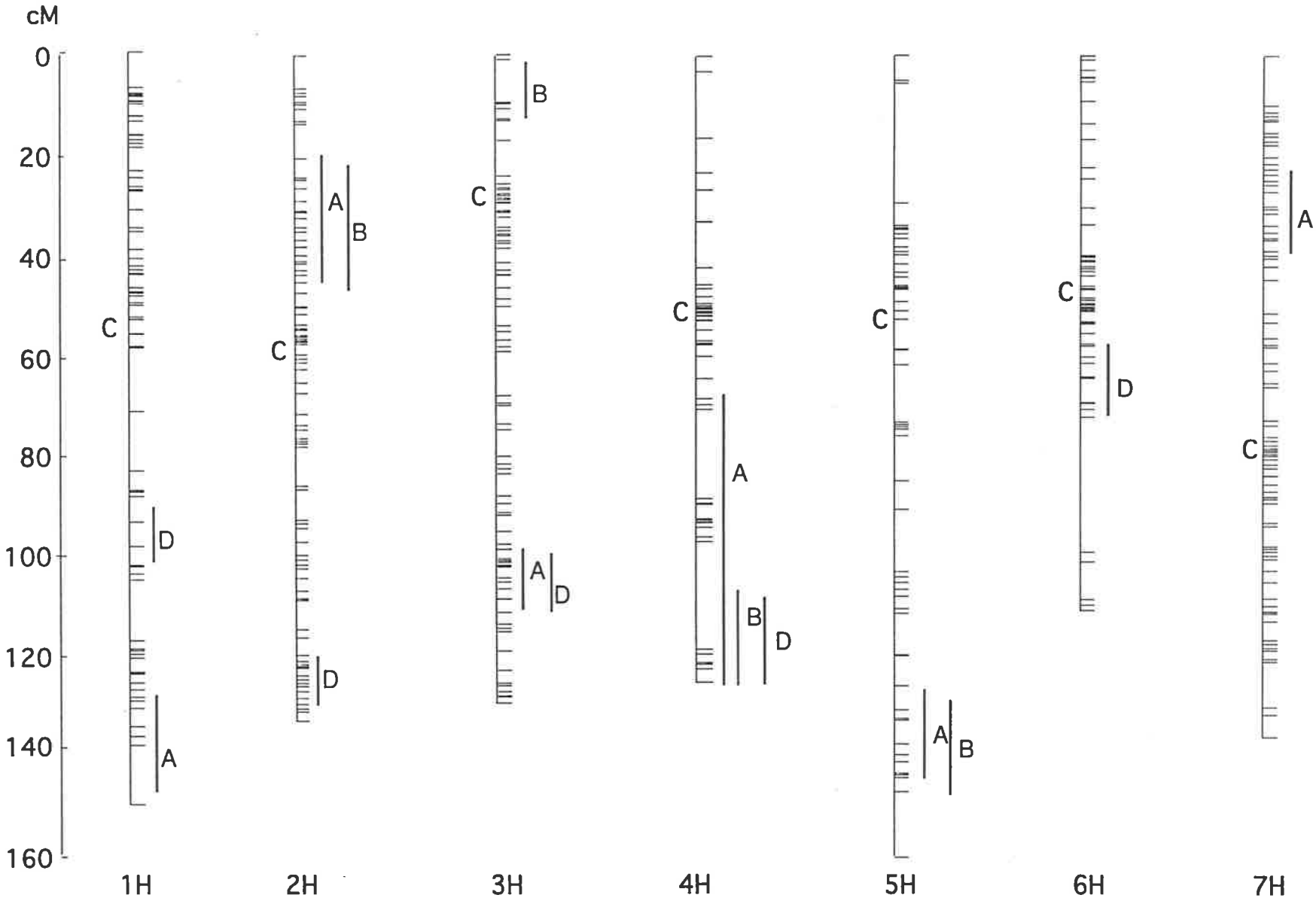

Fig. 3.10 Chromosomal locations of the QTLs for the beta-amylase activity on the barley consensus linkage map (Langridge et al, 1995) The short horizontal lines along the chromosomes indicate the position of markers in the consensus linkage map and the vertical lines show the chromosomal regions significantly linked the QTLs. A: Haruna Nijo X Galleon; B: Chebec X Harrington; D: Clipper X Sahara.

on chromosome 2H had no detectable effect on the enzyme activity. Although one QTL was identified on chromosome 2H, it is well away from the *Bmy2* locus (c.f Table 1 and Langridge et al, 1995). Interestingly, one major QTL detected on chromosome 6H in the Clipper X Sahara population was closely linked with the minor bands of β-amylase isoenzyme. Most other QTLs for the enzyme activity were mapped to similar chromosomal regions in at least two mapping populations (Fig. 3.10). The molecular markers linked with these common QTLs may be useful for marker-assisted selection for high β–amylase activity in a breeding program.

3.2.5 Reverse bulked segregant-analysis of the contribution of the β–amylase locus to the enzyme activity in barley grain

The principle of bulked-segregant analysis is the grouping together of informative individuals so that a particular genomic region of interest can be studied in a random genetic background of unlinked loci (Giovannoni et al, 1991; Michelmore et al, 1991). Using this method, several molecular markers have been identified linked with disease resistant genes (Michelmore et al, 1991) and some morphological traits (Giovannoni et al, 1991). In the present study, the same principle was followed to construct the analysis pool. However, the purpose is not to try to find the markers linked to some traits but to understand the contribution of a given locus to a trait of interest. Thus, this method was called as reverse bulked-segregant analysis.

Two pools with each parental genome type at the *Bmy1* locus were constructed from the "Chebec x Harrington" DH population using the β–amylase locus on chromosome 4H as a molecular marker. These two pools were intended to reflect a random genetic background except for region surrounding the β–amylase locus. The difference of the enzyme activity between the two pools is expected to be associated with the β–amylase gene. There are significant difference of free enzyme activity and the ratio of free to bound enzyme between the two parental varieties, and this difference was also detected between the two pools (Table 3). The total enzyme activity showed a significant

Table 3.2 The free, total β-amylase enzyme activity and the free/bound ratio of the parental varieties and their respective bulks

Samples	Free activity (U/kg)	Total activity (U/kg)	Free/Total ratio (%)
Chebec	1136.7±89.7	1620.9±110.9	70.1±9.4
Harrington	782.7±51.7	1635.2±2.7	47.9±2.9
Chebec bulk	992.2±35.8	1301.1±82.2	76.6±7.6
Harrington bulk	855.7±99.5	1566.5±129.1	54.6±4.4

Note: The parental varieties Chebec and Harrington were harvested from three locations. Chebec bulk: Ten DH lines random selected from the Chebec X Harrington population with genotype of Chebec at *Bmy1* locus; Harrington bulk: Ten DH linesrandom selected from the Chebec X Harrington population with genotype of Harrington at *Bmy1* locus. Three independent bulks were constructed.

difference between the two pools but not between the parental varieties, which may be due to that both parents contained negative QTLs for enzyme activity (Table 3.2). These results demonstrated that the β–amylase locus on chromosome 4H plays an important role for controlling free and total enzyme activity and free/bound ratio of the enzyme (Table 3.2).

3.3 Discussion

3.3.1 Chromosomal location of β–amylase genes

Using the cloned cDNA and wheat-barley addition lines, it has been shown that there are three β–amylase genes in barley genome, one gene on chromosome 2H and two genes on chromosome 4H (Kreis et al, 1987, 1988). Two loci were further mapped on the long arm of chromosome 2H closed to the centromere and the long arm of chromosome 4H closed to the telomere with RFLP linkage analysis (Kleinhofs et al, 1993). In the present study , the chromosomal locations of the two loci mapped were confirmed and incorporated into a high density consensus linkage map (Fig. 3.6). The two β–amylase genes on chromosome 4H were closely linked with no recombination in 264 DH lines. This contrasts to the α-amylase genes on chromosome 6H (Khursheed and Rogers, 1988; Takano et al, 1988), the 1,3-β–glucanase genes on chromosome 3H (Li et al, 1996) and the hordein genes on chromosome 1H (Shewry et al, 1990), in which rare recombinations within the gene clusters have been observed. The copy number of the genes in the β-amylase family were also lower than for the above mentioned gene families. Thus, the β-amylase gene family may represent an early stage in the gene evolutionary process, where the duplicated gene number is still small and tightly linked. This also appears to be the case with the 1,4-β-xylanase gene family (Banik et al, 1997)

Using isoenzyme polymorphisms, one locus of β–amylase has been located on chromosome 4H (Powling et al, 1981). The present study showed that the isoenzyme locus co-segregated with the β–amylase gene. This again contrasts to the α-amylase (*Amy1*) locus on chromosome 6H, in which the recombination between the RFLP and the isoenzyme locus has been observed (Takano and Takeda, 1987; Takano et al, 1988). Interestingly, the minor bands of the β-amylase isoenzyme was mapped to chromosome 6H. The function and nature of these loci are yet to determined.

3.3.2 Genes controlling β–amylase enzyme activity

Extensive research has shown that β–amylase activity is the primary determining factor for high diastatic power in the brewing industry (Arends et al, 1995; Delcour and Verschaeve, 1987; Erdal et al, 1993; Evans et al, 1995; Gibson et al, 1995). However, it was not know how many genes are involved in controlling the enzyme activity or how to effectively manipulate these genes in a breeding program. The isoenzyme polymorphism of β–amylase has been associated with enzyme activity and it has been suggested that it could be used as a marker in breeding programs (Allison, 1973; Swanston, 1980, 1983). The present study shows that the isoenzyme locus previously mapped represents the β–amylase gene on chromosome 4H and it was further confirmed that this locus contributes to the β–amylase enzyme activity (Fig. 3.6; Table 3.1 and 3.2). However, the isoenzyme Sd1 is rarely seen amongst cultivated barley varieties (Allison and Swantson, 1974; Eglinton et al, 1995). Thus, the real utility of the isoenzyme marker may be low in a breeding program. The high polymorphism of the β–amylase gene itself observed in this study demonstrated that the RFLP may be a more useful marker. However, this locus only accounts for 10 to 20% the total variation in the enzyme activity depending on the population. The β–amylase locus on chromosome 2H showed no detectable effect on enzyme activity. This suggested that this gene may not be expressed in the seed, which is consistent with previous observation (Kreis et al, 1988; Shewry et al, 1988). The fact

that all major isoenzyme polymorphisms mapped to the chromosome 4H locus also supports this point.

In addition to the structural gene, three to six other QTLs were identified that control the β–amylase activity in the different mapping populations. Several common loci were detected in at least two mapping populations (Fig. 3.10). Although the function of these loci and how they interact with the β–amylase genes is still unknown, the markers closely linked to these loci are considered to be useful for marker-assisted selection for improvement of the enzyme activity (Paterson et al, 1988). They may also be used as a reference point to undertake map-based cloning of the genes controlling β-amylase activity (Paterson and Wing, 1993). On the other hand, population specific QTLs were found in each population. This indicated the possibility of increasing the enzyme activity by combining loci from different germplasm sources.

One QTL mapped on chromosome 6H was very close to the α-amylase gene. Similarly, one QTL controlling the limit dextrinase enzyme activity was mapped to chromosome 4H close to the β–amylase locus (see Chapter 5). This suggested that various starch degrading enzymes may interact with each other during seed germination. Indeed, the synergistic effect between α-amylase and β-amylase and between α-amylase and α-glucosidase on starch degradation is well accepted (MacGregor and Fincher, 1993; Sun and Henson, 1991). If this is the case, a more complicated strategy may be required for improvement of the diastatic power in a breeding program.

3.3.3 Sequence variation and isoenzyme type

The protein of beta-amylase is highly heterogeneous. Various explanations have been suggested for the occurrence of the multiple isoenzyme forms such as formation of intermolecular disulfide bonds (Visuri and Nummi, 1972) or thiol/disulfide interchange (LaBerge and Marchylo, 1983), association with protein Z (Hejgarrd, 1978), proteolysis (Lundgard and Svensson, 1986) and expression of different genes (Ainsworth et al, 1983). There are three β–amylase genes in the barley genome (Kreis et al, 1987). One

gene on chromosome 2H is not expressed in the endosperm (Kreis et al, 1988; Shewry et al, 1988). In the present study, only one mRNA sequence for β–amylase was detected with RT-PCR in each of eight barley varieties (data not shown). This demonstrates that only one β–amylase gene is expressed in the endosperm or that the two genes on chromosome 4H were of identical sequence. Therefore, the multiple forms of β–amylase must originated from the modification of the product of one gene. Association with protein Z and limited proteolysis may be the main factors determining the multiple forms (Lundgard and Svensson, 1986; Yoshigi et al, 1995).

In spite of the multiple forms, there are only two distinct isoenzyme types in cultivated barley (Allison, 1973; Eglinton et al, 1995; Evans et al, 1997a) and these are controlled by a single locus with incomplete dominance (Allison and Swanston, 1974). The present study further shows that this locus cosegregates with the β–amylase genes on chromosome 4H (Fig. 3.6). This suggested that the different isoenzyme type originated from the β–amylase gene through mutation. By comparing the nucleic acid sequences of β–amylase with different isoenzyme types, six nucleotide substitutions were evident. However, only three deduced amino acids changed (Fig. 3.3). Two asparagines (Asp-226 and Asp-431) and one isoleucine (Ile-527) in the Sd2 type β-amylase were replaced with a serine, threonine and methionine, respectively, in the Sd1 type. However, the calculated pI (5.58) and molecular mass (59611 Dalton) using ExPASy tool (Bjellquist et al, 1994) showed no difference between the different isoenzyme types. This suggested that some post-translational modification determines the isoenzyme type.

The asparagine to serine or threonine differences made suggest that phosphorylation may be involved in determining the isoenzyme types. Alkaline phosphatase was used to treat the purified Sd1 and Sd2 type β-amylase proteins (from Dr. E Evans, University of Adelaide), but this treatment had no affect on the isoenzyme types (data not shown). This suggests that phosphorylation is not involved. Furthermore, protein sequence shows that the changes to Asp-226 and Asp-431 are not conserved between different isoenzyme types (Eglinton, personal communication). On the other hand, the methionine (Met-527) is present in all the Sd1 isoenzyme but is replaced by

isoleucine in all Sd2 isoenzymes (Kreis et al, 1987; the present study) except for the gene from Haruna Nijo (Yoshigi et al, 1994). In Haruna Nijo, methionine is replaced by serine (Ser-347) at different site (Yoshigi et al, 1994). Presently, the change from Met-527 to Ile-527 is the only explanation for the change of isoenzyme type.

Using a dot matrix homology plot, four glycine-rich repeated sequence of 11 amino acids were identified at the C-terminus of β–amylase (Kreis et al, 1987). The consensus sequence is Glu-Gly-Pro-Thr-Gly-Gly-Met-Gly-Gly-Gln-Ala, but its role is unknown. It has been suggested that the sequence is not necessary for *in vivo* activity as Lundgard and Svensson (1986) have shown that limited proteolysis of the C-terminal region with trypsin to remove most of the repeats, generated another catalytically active form of the enzyme with a higher pI. Furthermore, a new form of β–amylase generated during barley malting, has a similar pI and activity with the isoenzyme formed from trypsin treatment (Hejgaard, 1978). This form might be the product of solubilization of bound β–amylase released by proteolysis (Evans et al, 1997a). Therefore, the repeated sequences could be involved in the interaction with protein Z (Hejgaard, 1978; Kreis et al, 1987). In the present study, the free/bound ratio of the enzyme appeared to be affected by the *Bmy1* locus on chromosome 4H. The free/bound ratio of the Sd2 variety Chebec (70.1%) was 22.1% higher than that of Sd1 variety Harrington (47.9%). This difference explained by the *Bmy1* locus is consistent and of the same magnitude in the reverse bulked-segregant analysis (22% difference between the two bulks) (Table 3.2). If this is the case, what is the cause of the higher ratio of bound β–amylase in the Sd1 type isoenzyme (Evans et al, 1997b)? By comparing the repeat sequences between the two isoenzyme types, the only difference is the methionine in Sd1 type being replaced by isoleucine in the Sd2 type in the last repeat (Fig 3.3). Furthermore, methionine is conserved in the four repeat sequences in the Sd1 type isoenzyme and in the first three repeat sequences in the Sd2 type isoenzyme. Therefore, it is possible that each repeat sequence is one binding unit between β–amylase and protein Z and methionine is involved in the binding. The change from methionine to isoleucine in the last repeat sequence would result in the loss of one binding site with protein Z. This is supported by

the fact that Sd1 type β–amylase had one-third more binding activity than that of the Sd2 type (Evans et al, 1997b; Table 3.2). The different binding ratio with protein Z may result in the change of pI of β–amylase and the difference of proteolysis of the enzyme during seed germination, and finally alter the isoenzyme banding patterns.

Chapter 4: Molecular Mapping of Structural Loci for Hydrolytic Enzymes of Germinating Barley

4.1 Introduction

In addition to β-amylases (see Chapter 3), the other hydrolytic enzymes for starch degradation include α-amylases, limit dextrinase and α-glucosidase (Fincher and Stone, 1993; MacGregor, 1990; Manners and Yellowlees, 1971, 1973). All these hydrolytic enzymes are synthesised *de novo* and secreted from the aleurone layers or scutellum during seed germination. However, the starch granules are contained within the cells of the endosperm. Thus the cell wall is a physical barrier between the hydrolytic enzymes and their substrates (Fincher and Stone, 1993). Complete degradation of the cell walls is a prerequisite for the amylases to hydrolyse the starch. Enzymes which degrade the cell walls include endo-1,3-1,4-β-glucanases, 1,3-β-glucanases, exo-β-glucanases, β-glucosidases, cellulases, exo- and endo-xylanases, α-arabinofuranosidases and β-xylosidases (Bamforth and Barclay, 1993; Fincher, 1992; Fincher and Stone, 1993). Due to their commercial and physiological importance, most of these hydrolytic enzymes have been purified and the genes encoding the enzymes have been cloned (Briggs, 1992; Fincher, 1992; Fincher and Stone, 1993). However, little is known about how many genes exist in the barley genome to encode these enzymes, where these genes are located in the genome, how many genes may be involved in the regulation of their respective activities and how these genes could be manipulated in breeding programs.

There has been great interest in mapping specific genes of importance in plant breeding since early this century. However, mapping the genes of the hydrolytic enzymes became possible only after the development of isoenzyme markers (see review in Stuber, 1992). Loci have been mapped for hydrolytic

enzymes including α- and β-amylases (Powling et al, 1981) and 1,3-1,4-β-glucanase (MacLeod et al, 1991). However, the utilisation of isoenzyme markers is quite limited due to low levels of polymorphism. Recently, the development of molecular markers has provided the possibility of systematically mapping all genes (Botstein et al, 1980; Saiki et al, 1985; Saiki, 1988; Williams et al, 1990). In the present study, the genes encoding 1,3-1,4-β-glucanases, 1,3-β-glucanases, β-glucosidase, 1,4-β-xylanases, α-amylases, limit dextrinase and α-glucosidase were mapped.

4.2 Results

4.2.1 Mapping structural loci of hydrolytic enzymes degrading cell walls

4.2.1.1 RFLP and PCR mapping of endo-1,3-1,4-β-glucanase genes

1,3-1,4-β-Glucan is the major constituent of endosperm cell walls, and the most important enzymes in the depolymerisation of the cell walls are endo-1,3-1,4-β-glucanases (Fincher and Stone, 1993; Powell et al, 1985). The two genes encoding 1,3-1,4-β-glucanases, designated EI and EII, have been cloned (Fincher et al, 1986; Slakeski et al, 1990). RFLP and PCR techniques were used to map these two genes in the present study.

The DNAs from the six parental varieties for the mapping populations (Clipper, Sahara3771, Galleon, Haruna Nijo, Chebec and Harrington) were digested with restriction enzymes EcoRI, EcoRV, DraI, HindIII and BamHI and hybridised with probe of the EII cDNA. Two distinct bands were detected with each restriction enzyme digestion (Fig. 4.1) representing EI and EII, as the two genes share 92% positional identity (Slakeski et al, 1990). However, only one

polymorphism was found between "Clipper" and "Sahara" (Fig. 4.1). This indicated that the 1,3-1,4-β-glucanase genes and their immediate regions are highly conserved. The polymorphism was mapped on the long arm of chromosome 1H, where it is flanked on the distal side by the RFLP locus AWBMA34 at a distance of 0.3 cM, and the RFLP locus PSR 121 on the proximal side with a genetic distance of 0.3 cM (Fig. 4.2).

Due to the low level of polymorphism detected in the Southern analysis, PCR primers were designed to assist in mapping the 1,3-1,4 β-glucanase genes. A comparison between the two sequences of EI and EII showed that there is an EI specific region from position -402 to -552 upstream of the TATA box (Wolf, 1992). One primer is located in the EI specific region and the second in the first exon of both EI and EII genes. Polymorphisms were detected in all three mapping populations when the genomic DNA was amplified using this pair of primers (Fig. 4.3). The PCR polymorphism cosegregated with the RFLP locus of EI (Fig. 4.2).

4.2.1.2 Molecular mapping of the 1,3-β-glucanase gene family

High levels of 1,3-β-glucanase activity have been found in the endosperm of germinated barley (Høj et al, 1988, 1989). Their function is largely unknown but they may function in the catabolism of the cell walls of the aleurone (Bamsforth and Barclay, 1993) and also to degrade the small deposits of 1,3-β-glucan that are scattered through the starchy endosperm (MacGregor et al, 1989). Due to their ability to hydrolyse the 1,3- and 1,3-1,6-1,3-glucan that are major cell wall constituents in some classes of fungal pathogens of plant, 1,3-β-glucanases have been speculated to participate in a general, non-specific strategy to protect grain against pathogen invasion (Fincher, 1989).

Seven genes or cDNAs encoding 1,3-β-glucanases have been cloned from barley and designated GI, GII, GIII, GIV, GV, GVI and ABG2 (Xu et al, 1992;

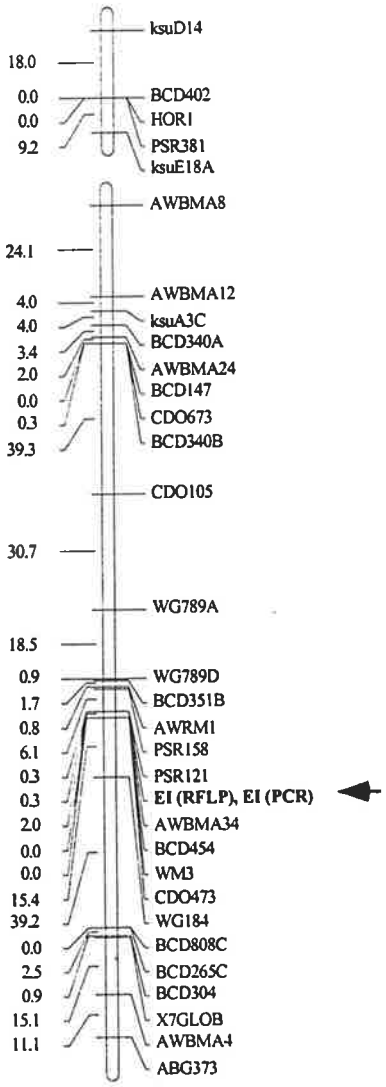

Fig. 4. 2 Map position of the 1,3-1,4-beta-glucanase isoenzyme gene (EI) on chromosome 1H. The gene is flanked by the RFLP markers PSR121 and AWBMA34.

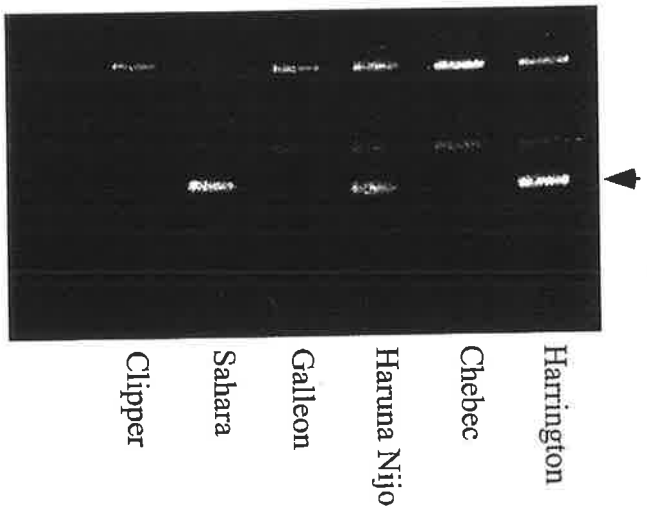

Fig. 4.3 Polymorphism of PCR products amplified by the primer combination (Upper primer 5'ATA CAT GTC ACT TCA CGA; Lower primer 5' AGA GTG AAG ATG GTG GCA). The upper primer is EI specific and the lower primer is located in the first exon of both EI and EII genes (Wolf, 1992). The polymorphism cosegregated with the RFLP locus of EI

Table 4.1 Gene specific probes used to map 1,3-beta-glucanase genes

Isoenzyme	Type of clone	Position of probe *	Length of probe
GI	cDNA	3'-Untranslated region	121 bp
GII	cDNA	3'-Untranslated region	215 bp
GIII	Genomic	8 to 1236 bp	1229 bp
GIV	Genomic	1684 to 2218 bp	535 bp
GV	cDNA	3'-Untranslated region	216 bp
GVI	Genomic	Entire clone	1948 bp

* The base pair numbering system follows Xu *et al.* (1992)

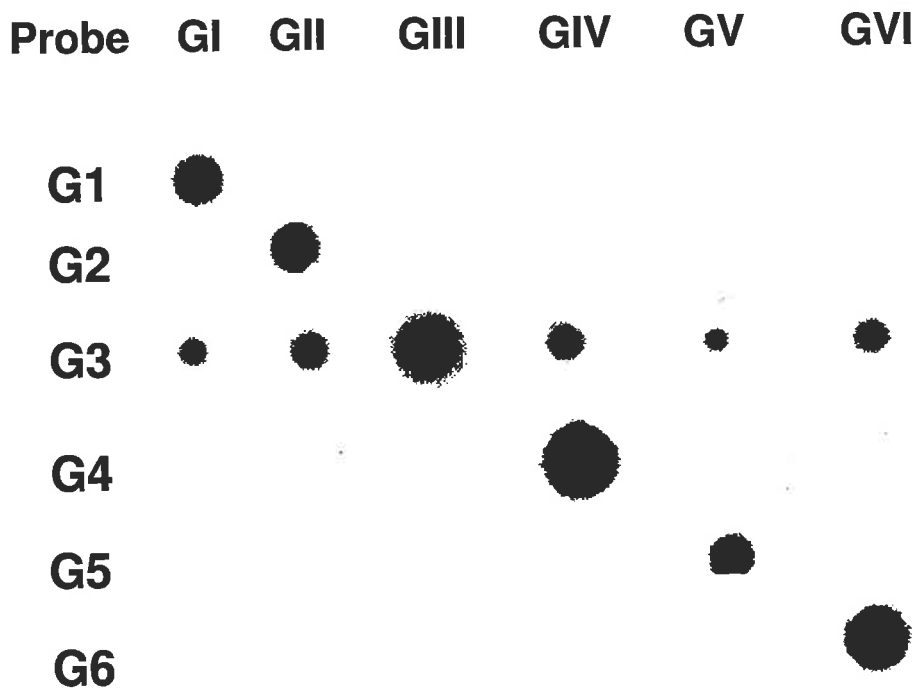

Fig. 4.4 Specificity of probes for mapping the barley 1,3-beta-glucanase genes encoding isoenzyme GI to GVI. DNA from cDNA or genes (Xu et al, 1992) was spotted on nitrocellulose papers strips, and individual strips were probed with gene-specific probes shown in Table 4.1

Malehorn et al, 1993). These seven genes share 44% to 78% sequence identity and also share 47% to 51% similarity with the 1,3-1,4-β-glucanase isoenzymes EI and EII. To map each of the genes independently, gene specific probes were designed by comparing the DNA sequences of the genes. To ensure that the DNA probes (Table 4.1) used to map individual 1,3-β-glucanase genes were specific, each probe was tested against cDNA or genomic DNA dot blots under the same stringency conditions used in the Southern blot hybridisations. The probes for the genes encoding isoenzymes GI, GII, GIV, GV and GVI exhibited no cross-hybridisation with any of the other genes (Fig. 4.4). In the case of the probe used to detect the gene for isoenzyme GIII, some hybridisation with other barley 1,3-β-glucanase genes or cDNAs was evident (Fig. 4.4). Nevertheless, the hybridisation signal with this probe was very much stronger against the isoenzyme GIII gene than against the other cDNAs or genes (Fig. 4.4) and the probe could therefore be used in Southern blot analyses to locate the isoenzyme GIII gene.

The genes encoding isoenzymes GI and GVI were found to be highly polymorphic, with at least two of the six parental varieties from the mapping populations showing polymorphism with each restriction enzyme used (data not shown). The genes for isoenzymes GII and GIII showed polymorphisms when DNA from the varieties "Clipper" and "Sahara" were digested with *Xba*I or *Sal*I, while the gene for isoenzyme GIV showed polymorphism between "Chebec" and "Harrington" digested with *Dra*I and the isoenzyme GV gene showed polymorphism between "Galleon" and "Harana Nijo" digested with *Xba*I (data not shown).

All genes except the gene encoding isoenzyme GIV were detected as single DNA bands after hybridisation with the specific DNA probes. This indicated that the genes for isoenzymes GI, GII, GIII, GV and GVI are present as single copies on the barley genome. In contrast, Southern blots, probed with the isoenzyme GIV DNA, revealed a relatively complex banding pattern; the probe hybridised with 4 genomic DNA fragments, only one of which was polymorphic and could

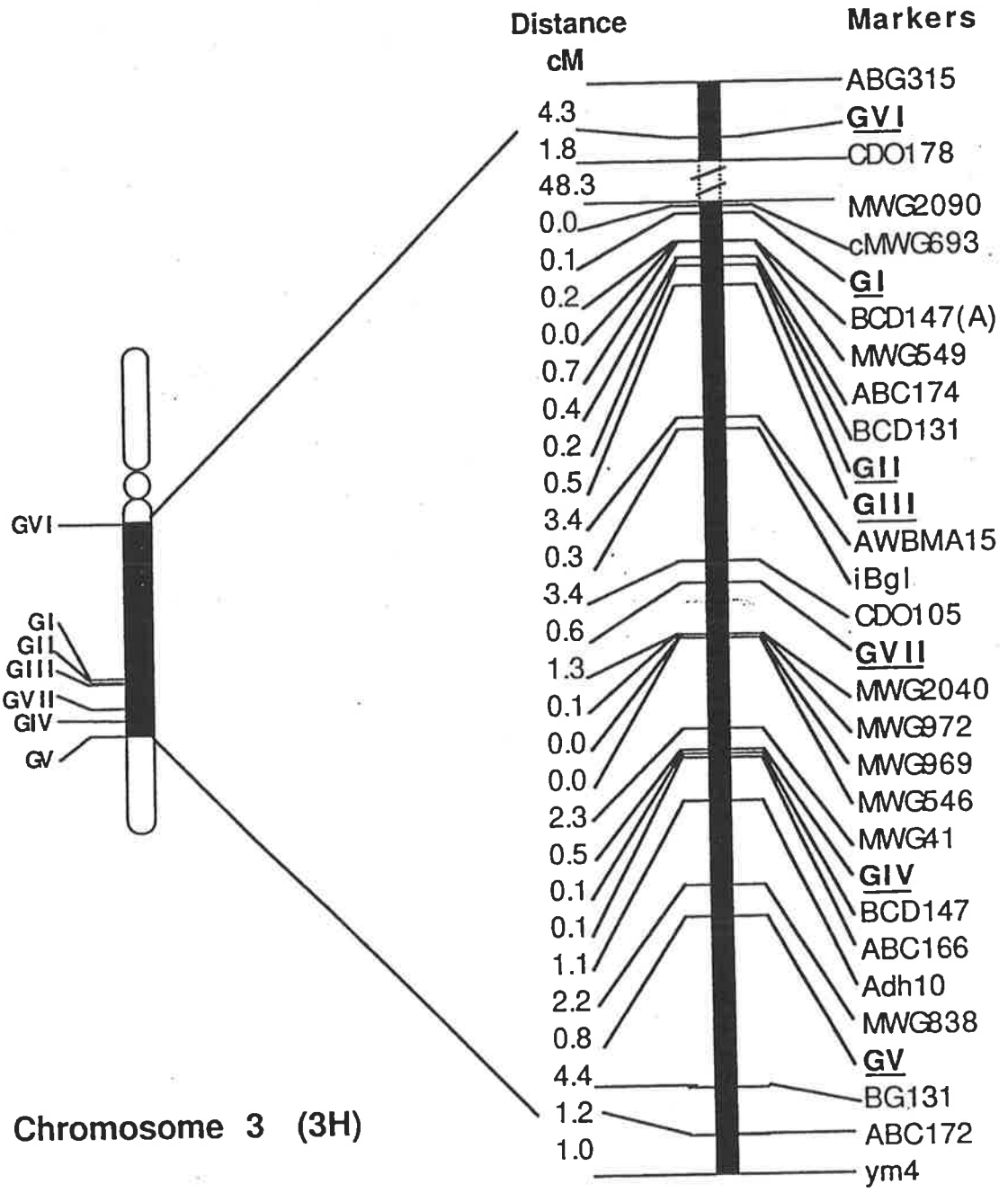

Fig. 4.5 Consensus linkage map of the seven barley 1,3-beta-glucanase genes, encoding isoenzymes GI to GVII, on the long arm of chromosome 3H, in which GI, GII, GIII, GVI and GVII were mapped in the Clipper X Sahara DH population, and GIV and GV were mapped in the Chebec X Harrington and Haruna Nijo X Galleon populations, respectively.

be mapped. When the alternative procedure of PCR mapping was used to amplify the 5'-untranslated region of the isoenzyme GIV gene, several PCR products were again detected (data not shown).

Linkage analysis of the RFLP banding patterns showed that the genes for barley 1,3-β-glucanase isoenzymes GI to GVI are all located on the long arm of chromosome 3H. The positions of the genes in a consensus linkage map are shown in Figure 4.5. The five genes encoding isoenzymes GI to GV lie clustered in a region of less than 20 cM, between the RFLP marker MWG2099 and the *ym4* gene for barley yellow mosaic virus resistance (Fig. 4.5). The distance between the genes for isoenzymes GII and GIII is only 0.5 cM. Of the genes within this cluster the gene for isoenzyme GI is closest to the centromere (76cM) and the most distal gene encodes isoenzyme GV. The isoenzyme GV gene is closely linked with both the *ym4* gene (6.6 cM) and the alcohol dehydrogenase *Adh10* gene (3.0 cM). The RFLP markers most closely linked with the genes encoding barley 1,3-β-glucanase isoenzymes GI to GVI are cMWG693 (0.1 cM), BCD131 (0.2 cM), BCD131 (0.7 cM), MWG41 (0.5 cM), MWG838 (0.8 cM) and CDO178 (1.8 cM). The gene encoding isoenzyme GVI is located outside the cluster, in a position between the centromere and the isoenzyme GI gene (Fig.4.5). The isoenzyme GVI gene is 26 cM from the centromere region and 50 cM from the isoenzyme GI gene.

As the probe for GVII (ABG2) was not available, a PCR method was used. The primers were designed to amplify DNA fragments from the intron of the gene. The isoenzyme GVII gene corresponding to the *ABG2* gene was isolated and characterised by Malehorn *et al.* (1993) and a fragment of 310 bp would be predicted from their sequence data. Using genomic DNA preparations from wheat, barley and the wheat-barley addition lines as templates, a PCR product of approximately 310 bp was amplified from barley DNA and from DNA of the wheat-barley addition line 3H; this product was not detected in DNA from wheat or the other wheat-barley addition lines. These results indicated that the isoenzyme

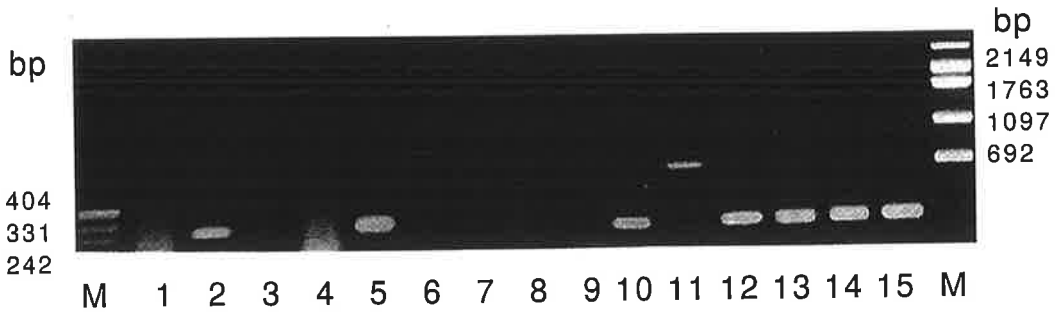

Fig. 4.6 PCR products of wheat, barley and wheat-barley addition lines using isoenzyme GVII primers. Lane 1 Wheat (cv Chinese spring), lane 2 barley (cv Betzes), lanes 3 to 9 wheat-barley addition lines 1H to 7H. Lanes 10-15 barley cvs Clipper, Sahara, Galleon, Haruna Nijo, Chebec and Harrington, respectively. DNA size markers are shown in lanes M.

GVII gene is located on barley chromosome 3H. However, the PCR-amplified product from the parent Sahara3771 used in the "Clipper x Sahara3771" DH lines was approximately 600 bp in length (Fig. 4.6) and this polymorphism allowed the isoenzyme GVII gene to be mapped. Linkage analysis confirmed that the isoenzyme GVII gene is located on chromosome 3H. It is found in the region of the 1,3-β-glucanase gene cluster, 7.7 cM from the isoenzyme GIII gene and 4.2 cM from the isoenzyme GIV gene (Fig.4.5). The RFLP marker nearest the isoenzyme GVII gene is CDO105 (0.6 cM).

4.2.1.3 Chromosomal location of β-glucosidase

Beta-glucosidase is involved in hydrolysis of the short chain β-linked oligosaccharides during germination and plays a role in sugar sink-source movement between endosperm and growing seedling (Essen, 1993; MacGregor and Fincher, 1993; Simos et al, 1994). There is a single β-glucosidase gene in the barley genome (Leah et al, 1995). PCR primers were designed to amplify the 3'-sequence of this gene based on the published sequence (Leah et al, 1995). A single band was amplified from both barley *cv*. Betzes and wheat cv. Chinese Spring and the polymorphism was observed between barley and wheat (Fig. 4.8). The barley specific band was only amplified from wheat-barley addition line 2H (Fig. 4.8). This indicates that the single β-glucosidase gene is located on the barley chromosome 2H. The DNA band was further excised and purified using GeneClean. RFLP analysis showed no polymorphism between the parental varieties of the mapping populations (data not shown) using the purified DNA as a probe. This prevented in the positioning of the β-glucosidase gene into a molecular linkage map.

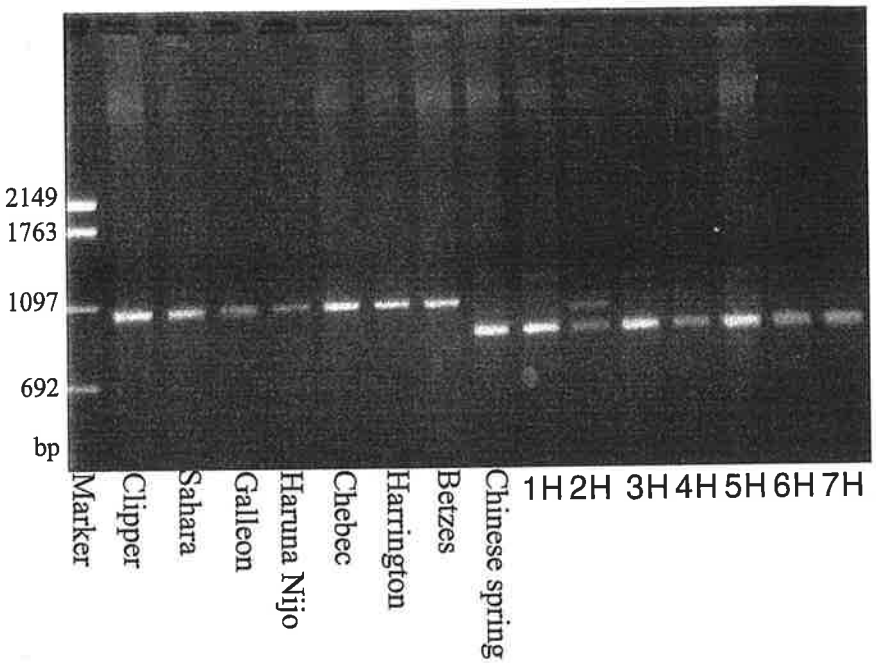

Fig. 4.8 PCR products of wheat, barley and wheat-barley addition lines using beta-glucosidase specific primers. The barley specific band was located on the addition line 2H

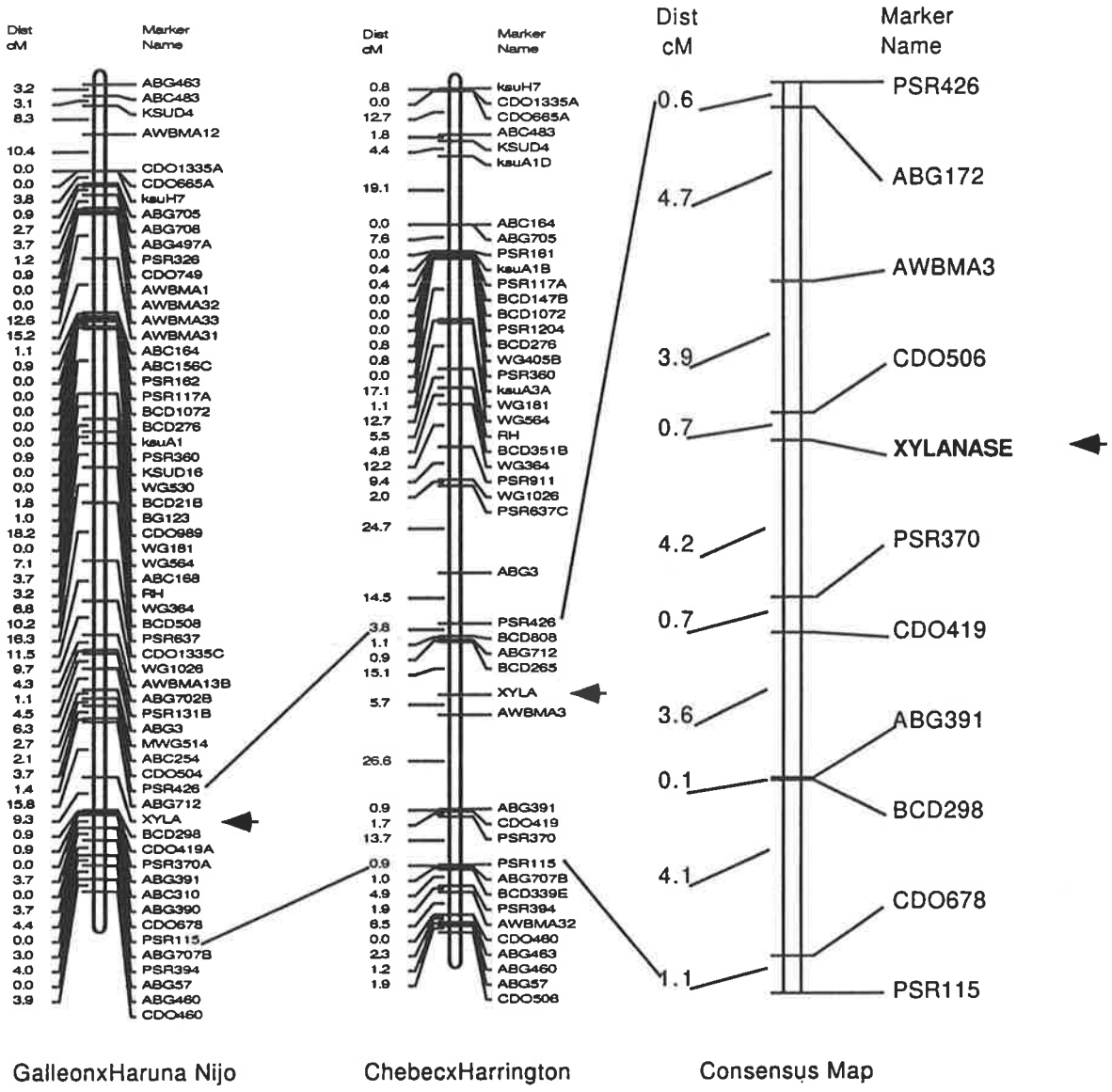

Fig. 4.9 Map positions of the three 1,4-beta-xylanase genes on barley chromosome 7H of the Galleon X Haruna Nijo and Chebec X Harrington DH populations. The consensus linkage map shows the position of the xylanase genes between the RFLP marker CDO506 and PSR370; the centromere is located on the CDO506 side of the genes. No recombination was detected between the three xylanase genes in 234 DH lines.

4.2.1.4 RFLP mapping of the 1,4-β-xylanase genes

In addition to 1,3-1,4-β-glucan, one-fifth of the cell walls of barley starch endosperm is arabinoxylan (Fincher, 1975). Endo- and exo-xylanase, α-arabinofuranosidases and β-xylosidases jointly depolymerise the arabinoxylan during barley germination (Taiz and Honigman, 1976). However, only recently have detailed studies commenced on the enzymes responsible for the arabinoxylan degradation. Two cDNAs encoding 1,4-β-xylanases have been isolated from barley (Banik et al, 1996).

Using the full length cDNA of 1,4-β-xylanases as a probe, Southern blot analysis detected three bands and all bands were highly polymorphic between the parental varieties (data not shown). The three bands corresponding to 1,4-β-xylanase genes were located on the long arm of chromosome 5H (Fig. 4.9). No recombination between the three bands was observed in the 234 DH lines of the two mapping populations. The bands were also incorporated into a consensus linkage map using JoinMap software (Stam, 1993). As shown in Fig. 4.9, the 1,4-β-xylanase gene(s) cluster approximately 0.7 cM distal to the RFLP marker CDO506 and 4.2 cM proximal to the PSR370 marker.

4.2.2 Mapping structural loci of hydrolytic enzymes degrading starch

4.2.2.1 RFLP and isoenzyme mapping of α-amylase genes

Alpha-amylases initiate the degradation of starch. Extensive research has been made on these enzymes. There are two groups of isoenzymes (low pI and high pI), which are encoded by two small gene families located on chromosomes 7H and 6H, respectively (Khursheed and Rogers, 1988; MacGregor, 1987). The aims for mapping the α-amylase genes in the present study include (1) studying

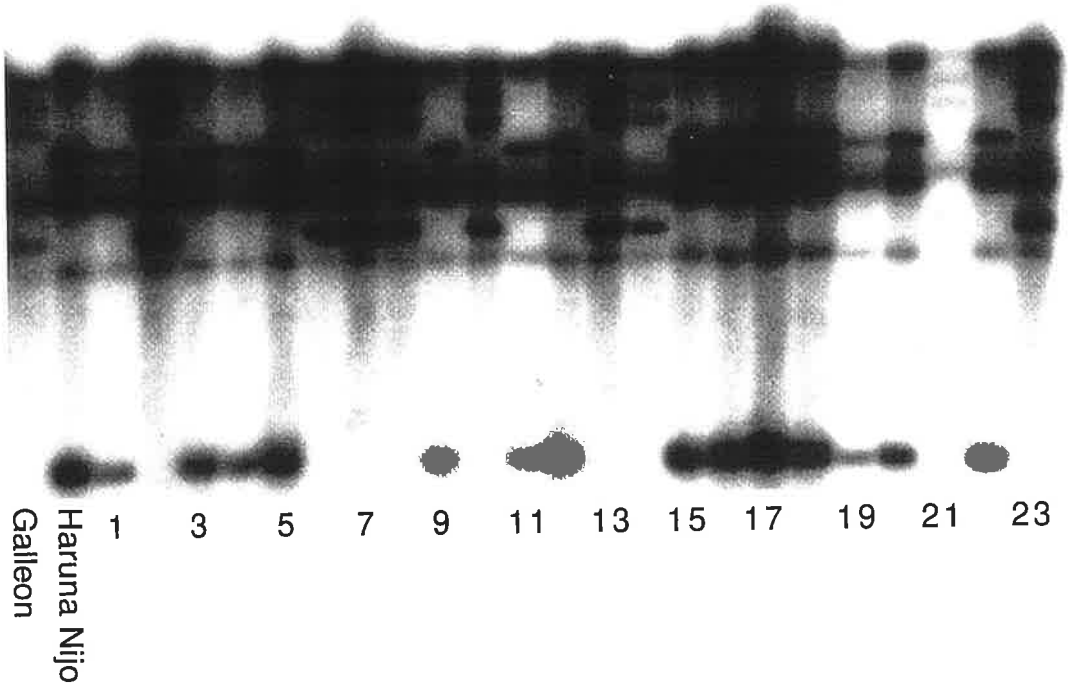

Fig 4.10 Cosegregation of the RFLP bands in the Galleon X Harun Nijo DH population when hybridised with *Amy1* probe. All polymorphic bands were mapped on chromosome 6H

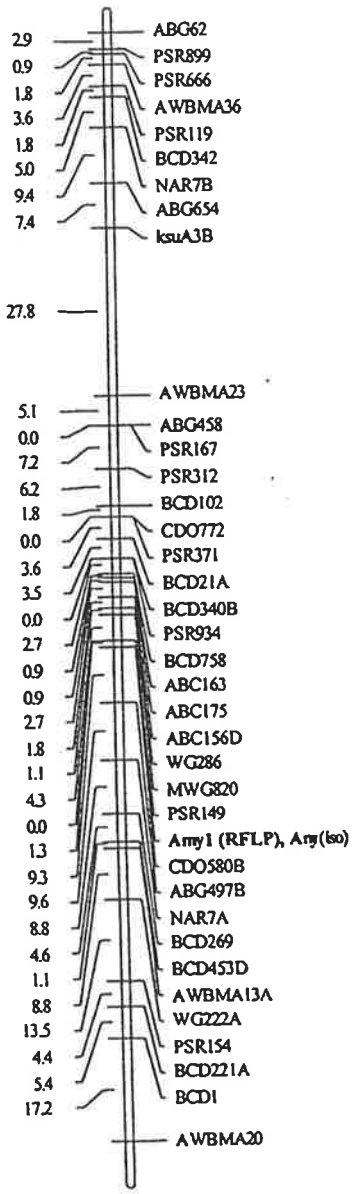

Fig. 4.11 Map position of the alpha-amylase gene (RFLP and isoenzyme) on chromosome 6H

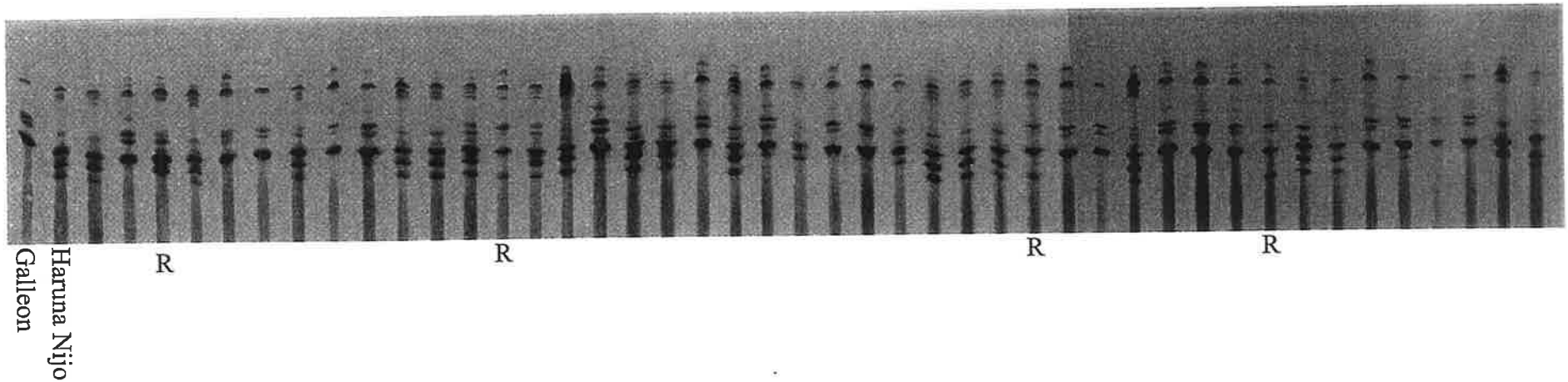

Fig. 4.12 Segregation of the alpha-amylase isoenzyme polymorphism in the Galleon X Haruna Nijo DH population. The polymorphism cosegregated with the RFLP locus of *Amy1* on chromosome 6H. Recombinant lines are designated by an R

the relationship between RFLP and isoenzyme bands; (2) providing a reference to study the genetics of the enzyme activity and interaction of different hydrolytic enzymes during germination.

Six DNA bands were detected by Southern blot analysis using *Amy1* as a probe. However, only three bands were polymorphic (Fig. 4.10). All the polymorphic bands cosegregated and were mapped to chromosome 6H using Mapmaker software (Lander et al, 1987) (Fig. 4.10 and Fig. 4.11). Additionally, IEF and immunoblot analysis showed that most of the isoenzyme bands cosegregated with the RFLP bands, but minor recombining bands were detected (Fig.4.12). However, no corresponding RFLP recombination was found (Fig.4.10 and Fig. 12).

4.2.2.2 RFLP mapping of limit dextrinase gene

Limit dextrinase (LD) is the only endogenous barley enzyme that debranches amylopectin and related compounds produced during germination and malting (Sissons et al, 1992a). Several isoforms of LD with pI between 4.2 and 5.0 and molecular mass between 80 and 104 kD have been purified from geminated barley. However, little is known about the gene(s) and their genetics in barley. Recently, a 670bp cDNA fragment encoding barley LD has been isolated from a cDNA library prepared from the mRNA of GA treated aleurone (X.-Q. Zhang and G.B. Fincher, unpublished data). The cDNA was identified as a LD cDNA because of the correspondence of its nucleotide sequence with the amino acid sequences determined directly from tryptic peptides generated from the purified enzyme (X.-Q. Zhang and G.B. Fincher, unpublished data), and sequence similarity with the rice R-enzyme (Nakamura et al, 1996).

Genomic DNAs from the seven wheat-barley addition lines and their parental varieties were digested with restriction endonucleases *EcoRV* and *HindIII*, and probed with the cDNA fragment of LD. Southern blot analysis

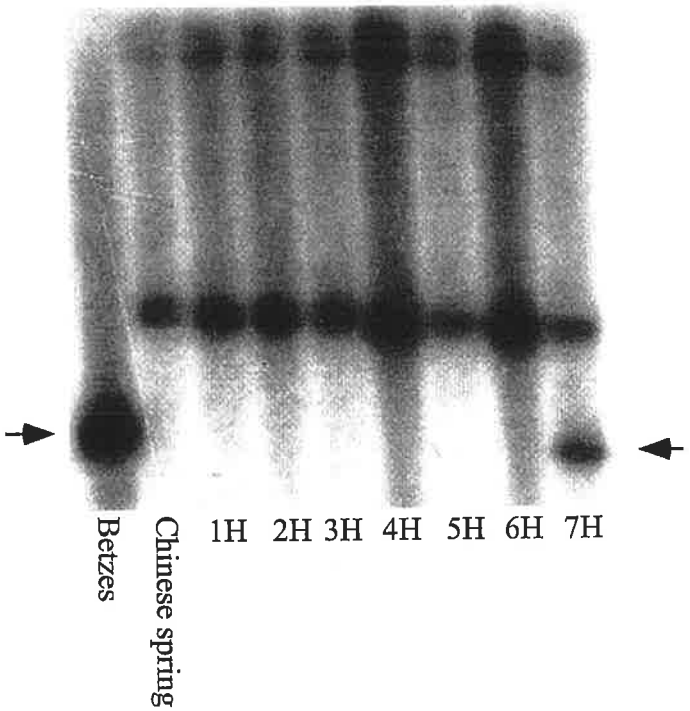

Fig. 4.13 Southern blot analysis of wheat (cv Chinese spring), barley (cv Betzes) and wheat-barley addition lines using LD cDNA as a probe showed barley specific band on chromosome 7H.

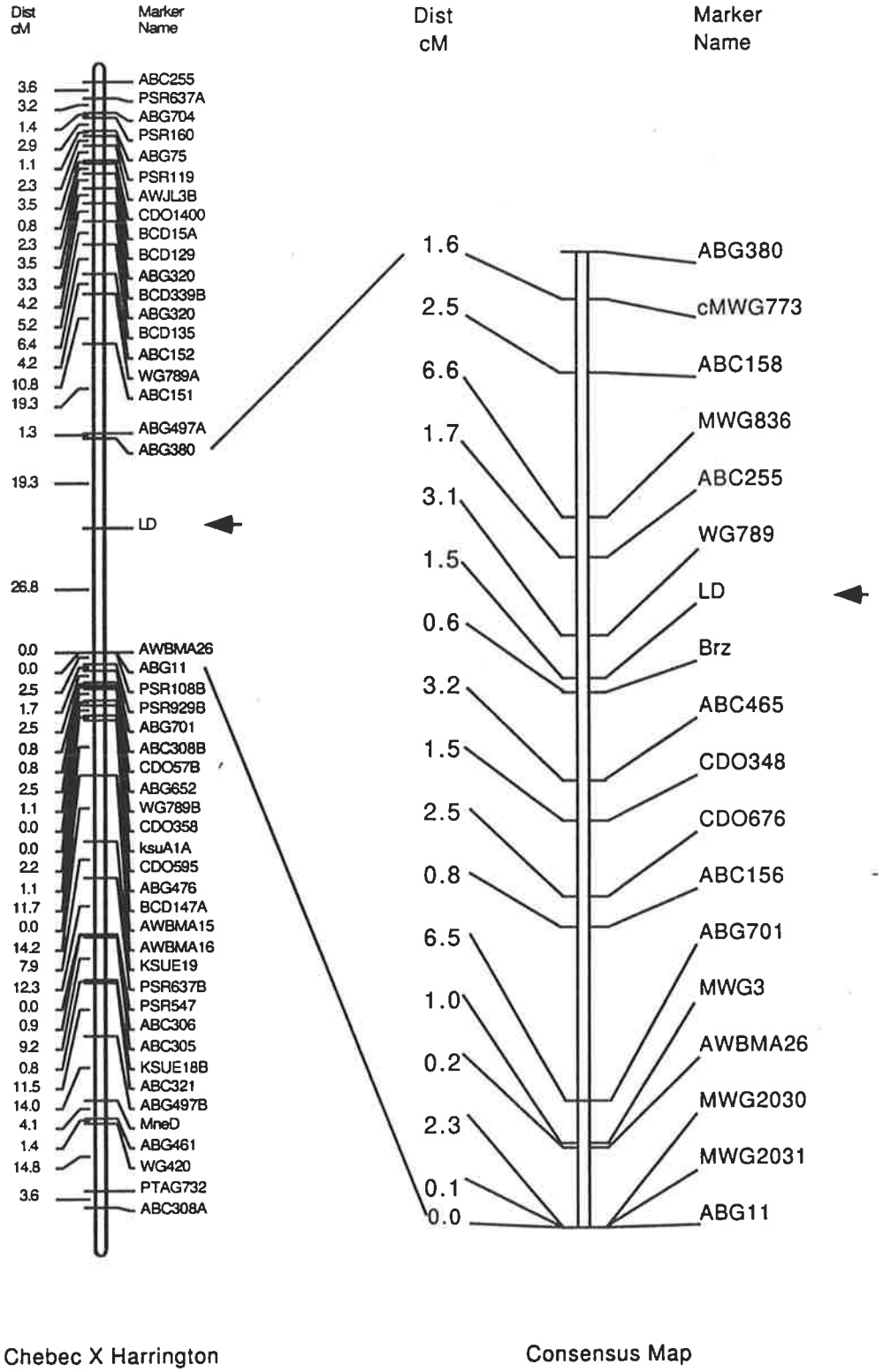

Fig. 4.14 Map position of the limit dextrinase gene on chromosome 7H of the Chebec X Harrington DH population. The consensus linkage map shows the position of the LD gene between the RFLP marker WG789 and glucosyl transferase locus (*Brz*).

showed that a single band in the barley genome hybridised with the LD cDNA fragment while three bands were detected in the wheat genome (Fig.4.13). These results suggested that there is a single LD gene in barley and that one copy of this gene is located on each of the three homoeologous wheat chromosomes. The barley LD fragment was found only in the wheat-barley addition line 7H (Fig. 4.13); this indicates that the gene is located on barley chromosome 7H.

Genomic DNA preparations from the four parents (Galleon, Haruna Nijo, Chebec, Harrington) of the mapping populations were digested with 28 restriction endonucleases to identify polymorphisms at the LD locus. Only one polymorphism was found, when the cultivars Chebec and Harrington were digested with *EcoRV* (data not shown). Using the Mapmaker software (Lander et al, 1987), the polymorphism detected between Chebec and Harrington was mapped on the short arm of chromosome 7H, flanked by the RFLP markers ABG380 on the distal side and AWBMA26 on the proximal side (Fig. 4.14). When the mapping data were combined into the consensus linkage map using the JoinMap software (Stam, 1993), the LD gene was positioned between the RFLP marker WG789 on the distal side and the gene encoding glucosyl transferase (*Brz*) (Wise et al, 1990) on the centromeric side (Fig. 4.14). The genetic distance between the LD locus and the glucosyl transferase gene is 0.6 cM, and 1.5 cM to the WG789 marker (Fig. 4 14).

4.2.2.3 Chromosomal location of α-glucosidase gene

Alpha-glucosidase exolytically liberates glucose from glucan polymers and disaccharides. It is the least characterised enzyme in the amylotic complex in barley (Bamforth and Barclay, 1993). Two isoforms have been purified from germinated barley with molecular mass of 65 and 32 kD. Recently, a putative α-glucosidase clone has been isolated from a cDNA library constructed from mRNA of barley aleurone treated with GA (Tibbot and Skadsen, 1996).

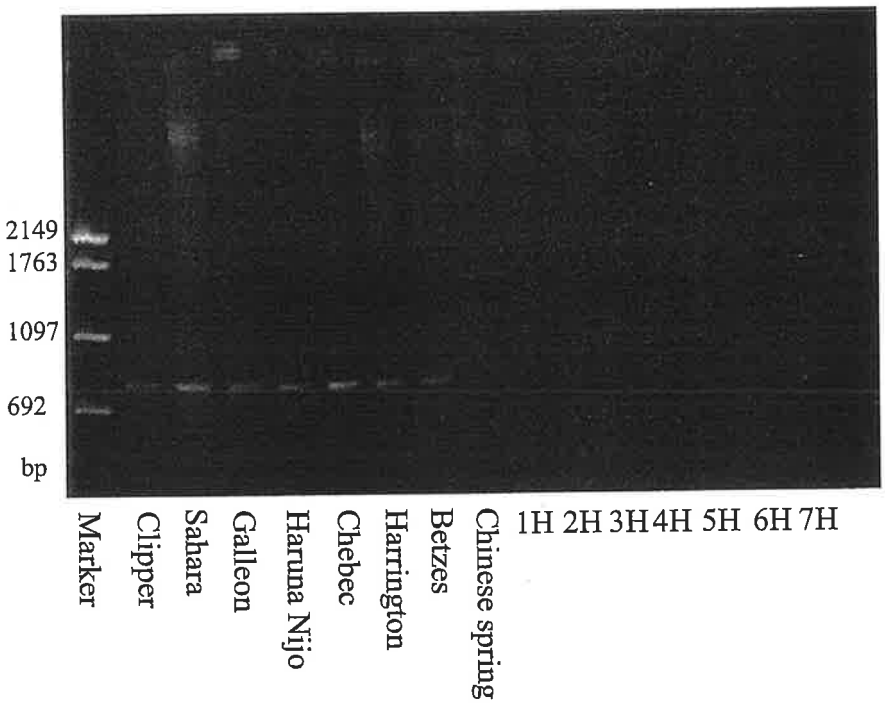

Fig. 4.15 PCR products of wheat, barley and wheat-barley addition lines using alpha-glucosidase specific primers.

PCR primers were designed to amplify the 3'-sequence of this gene based on the published sequence (Tibbot and Skadsen, 1996). A single band was amplified from the barley varieties but no detectable band was produced from wheat *cv*. Chinese Spring DNA as template. The barley specific band was only amplified from wheat-barley addition line 2H (Fig. 4.15). This indicates that the single α-glucosidase gene is located on the barley chromosome 2H. The DNA band was excised and purified using GeneClean. RFLP analysis showed no polymorphism between the parental varieties for the mapping populations using the purified DNA as probe (data not shown). This prevented incorporating the α-glucosidase gene into a molecular linkage map.

4.3 Discussion

4.3.1 Gene locations of 1,3-1,4-β-glucanases

The two genes encoding 1,3-1,4-β-glucanases had previously been mapped on chromosomes 1H and 7H using wheat-barley addition lines or aneuploid and ditelosomic wheat lines (Loi et al, 1988; Slakeski et al, 1990). In the present study, the EI gene was incorporated into a molecular linkage map using RFLP and PCR methods. The EII gene, on the other hand, would appear to be highly conserved and no polymorphism could be detected using either the RFLP or PCR method. This result is supported by isoenzyme mapping (MacLeod et al, 1991) and RFLP mapping from the North American Barley Genome Mapping Project (Kleinhofs et al, 1993), but contradicted with the result of Slakeski *et al.* (1990). As the EII gene is expressed predominantly in the aleurone and is the main enzyme in malt (Stuart and Fincher, 1983; Slakeski and Fincher, 1992), it is more important from the point view of the malting and brewing industries. Therefore, more effort should be made to map the EII gene.

4.3.2 Gene locations of 1,3-β-glucanases

Using RFLP and PCR mapping procedures it was possible to incorporate the genes encoding seven individual 1,3-β-glucanase genes into a high density consensus linkage map for barley (Fig. 4.5). The genes are all located on the long arm of chromosome 3H and six of the genes are grouped within 18.2 cM of each other. The gene encoding isoenzyme GVI is found between the major group of 1,3-β-glucanase genes and the centromere (Fig. 4.5). Each of the genes is represented on the barley genome by a single copy, with the possible exception of the genes encoding isoenzyme GIV, for which multiple DNA fragments were detected during both RFLP and PCR mapping experiments. It is not clear whether this result indicates the presence of additional 1,3-β-glucanase genes which share a high degree of sequence identity with the isoenzyme GIV probe, or whether the isoenzyme GIV gene is a representative of a subfamily. The absence of any detectable polymorphisms in all but one of the DNA fragments that hybridised with the isoenzyme GIV probe precluded the mapping of these other genes and, until these effects are investigated in more detail, it is not possible to make firm conclusions about the number of the 1,3-β-glucanase isoenzyme GIV genes in barley.

In earlier work, Kleinhofs *et al.* (1993) used a near full-length cDNA encoding isoenzyme GII (Høj et al, 1989) to map barley 1,3-β-glucanase genes. Two loci have been mapped to the long arm of barley chromosome 3H and the genes were designated *Glb3* and *Glb4* (Kleinhofs et al, 1993), but the lack of specificity of the probe prevented the assignment of the two loci to individual 1,3-ß-glucanase genes. However, based on the relative map positions (Fig. 4.5 cf. Kleinhofs et al, 1993) and the high degree of sequence identity between isoenzymes GI and GII (Xu et al, 1992), it is likely that *Glb3* and *Glb4* of Kleinhofs *et al.* (1993) correspond to the genes encoding 1,3-β-glucanase isoenzymes GII and GI, respectively.

The use of the designations *Glb3* and *Glb4* by Kleinhofs *et al.* (1993) raises a general problem with nomenclature for β-glucanase genes. Cereal β-glucan endohydrolases can be divided into three major classes: the 1,3-β-glucanases (EC 3.2.1.39), the 1,3-1,4-β-glucanases (EC 3.2.1.73) and the 1,4-β-glucanases (EC 3.2.1.4) or endo-cellulases (Høj and Fincher, 1995). Two 1,3-1,4-β-glucanase isoenzymes, designated isoenzymes EI and EII (Woodward and Fincher, 1982; Slakeski et al, 1990), and seven 1,3-β-glucanase isoenzymes, designated isoenzymes GI to GVII (or in the case of isoenzyme GVII, *ABG2*) (Xu et al, 1992; Malehorn et al, 1993) have so far been characterised. There is every possibility that barley cellulases will be described in the future. Therefore, any systematic nomenclature for the genes encoding these related polysaccharide hydrolases must not only take account of their different specificities, but must also allow for the identification of multiple isoforms within each enzyme class. It is therefore proposed that the 1,3-β-glucanase genes be designated *Glb31* to *Glb3x*, where 3 indicates that the 1,3-linkage is cleaved and x is the number of the individual isoenzyme. In this system the gene for 1,3-β-glucanase isoenzyme GV would therefore be *Glb35*, for isoenzyme GVII *Glb37*, etc. Similarly, the genes for 1,4-β-glucanases, or cellulases, could be designated *Glb41* to *Glb4x*, where the 4 indicates that the 1,4-linkage is hydrolysed and x represents the individual isoform. In the case of the 1,3-1,4-β-glucanases, of which there are only two isoforms in wheat and barley (Slakeski et al, 1990), the simplest designations for isoenzymes EI and EII would be *Glb1* and *Glb2*, respectively; this nomenclature has been adopted in earlier work (Søgaard and von Wettstein-Knowles, 1987; von Wettstein-Knowles, 1992)

4.3.3 Relationship of 1,3-β-glucanase and 1,3-1,4-β-glucanase

Comparative studies on barley 1,3-β-glucanases and 1,3-1,4-β-glucanases suggest that these enzymes are members of a single "super-gene" family and that, in all likelihood, the 1,3-1,4-β-glucanases diverged from the 1,3-β-glucanases during the appearance of the graminaceous monocotyledons (Høj and Fincher, 1995). The evidence is based on similarities in their three-dimensional structures (Varghese et al, 1994), in the topology of their substrate binding domains (Hrmova et al, 1995), in their catalytic mechanisms (Chen et al, 1993, 1995) and in the structure of their corresponding genes (Høj et al, 1989; Slakeski et al, 1990; Xu et al, 1992; Wang et al, 1992). In considering possible evolutionary pathways of the barley β-glucanases, Høj and Fincher (1995) used the algorithms of Hein (1990) to reconstruct a phylogenetic tree, of which a modified version is shown in Figure 4.7. The question arises as to whether the position of individual 1,3-β-glucanase genes on the phylogenetic tree can be reconciled with their positions on the linkage map shown in Figure 4.5. Comparisons of the tree generated by sequence similarity and the linkage distances revealed many similarities (Figures 4.7 and 4.5), in particular the close relatedness of isoenzymes GI and GII, isoenzymes GIII and GVII, and isoenzymes GIV and GV. Furthermore, isoenzyme GVI is placed furthest from the other isoenzymes in both cases (Figures 4.5 and 4.7). The major difference is the relative proximity of the isoenzyme GII/GIII pair in the linkage map analysis (Figure 4.5 cf. Figure 4.7).

The two barley 1,3-1,4-β-glucanases have been classified with the 1,3-β-glucanases in a single gene family (Høj and Fincher, 1995) but the genes encoding the 1,3-1,4-β-glucanase isoenzymes EI and EII are located on the long arms of chromosome 1H and chromosome 7H, respectively (Loi et al, 1988; MacLeod et al, 1991). If the genes encoding 1,3-1,4-β-glucanases are indeed derived from 1,3-β-glucanase genes (Høj and Fincher, 1995), all of which are located on

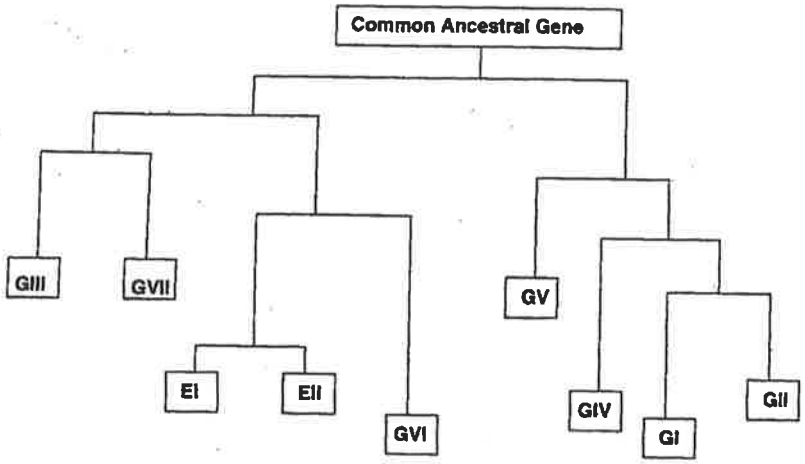

Fig. 4.7 Possible phylogeny of the barley 1,3- and 1,3-1,4-beta-glucanase genes, based on alignments of amino acid sequences and the algorithm of Hein (1990). 1,3-beta-Glucanase genes are designated GI to GVII and 1,3-1,4-beta-glucanase genes are designated EI and EII, in accordance with the corresponding isoenzymes they encode (Xu et al, 1992; Hoj and Fincher, 1995; Malehorn et al, 1993) (from GB Fincher)

chromosome 3HL (Fig. 4.5), it is clear that multiple chromosomal translocations accompanied 1,3-1,4-β-glucanase evolution.

The distribution of 1,3-β-glucanase genes along a single barley chromosome arm implies that duplication of the ancestral gene occurred via a type of illegitimate recombination rather than by an excision, duplication and reinsertion mechanism. It is possible that gene duplication occurred initially by tandem duplication of the ancestral gene. Subsequent dispersal along the chromosome could have been affected by chromosome rearrangements. It is noteworthy that the organisation of the barley 1,3-β-glucanase genes is similar to that of the barley hordein genes on chromosome 1H (Shewry and Miflin, 1982; Bunce et al, 1986; Shewry et al, 1990) and the clustering of α-amylase genes (*Amy1*) on chromosome 6H (Khursheed and Rogers, 1988; Takano and Takeda, 1987; Taknao et al, 1988). The α-amylase genes (*Amy1*) may represent an early stage in the evolutionary process, where the duplicated genes are still closely linked.

4.3.4 Gene location and function of 1,4-β-xylanase

The three bands observed in this study may represent three genes of barley 1,4-β-xylanase (Banik et al, 1996), but they are so tightly clustered that no recombination could be observed between them. If each band represents a gene, then the three genes have most probably arisen by tandem duplication of a single ancestral gene. Several other gene families in barley are also organised as clusters in a limited region of a particular chromosome. The barley 1,3-β-glucanase gene family is distributed along the long arm of chromosome 3H (Section 4.3.3; Li et al, 1996), while barley hordein genes are organised in a similar manner on chromosome 1H (Shewry et al, 1990). However, 1,4-β-xylanase genes may represent an early stage in the evolution of gene families, where tandemly duplicated genes are still closely linked and dispersal by chromosome rearrangement or translocation to other chromosomes has not yet occurred. Recent

duplication of 1,4-β-xylanase genes might also explain the relatively high level of sequence identity (91% at the nucleotide level) between the genes encoding isoenzyme *X-I* and *XII* (Banik et al, 1996). This level of sequence identity can be compared with the values as low as 45% for the seven members of the 1,3-β-glucanase gene family (Xu et al, 1992), six of which are located in a region less than 20 CM on chromosome 3H (Section 4.2.1.2; Li et al, 1996).

In considering the role of 1,4-β-xylanase in cell wall degradation during endosperm mobilisation, it might be anticipated the synthesis and secretion of endohydrolases responsible for hydrolysis of the major cell wall polysaccharides, namely 1,3-1,4-β-glucanases and 1,4-β-xylanases, could be subject to coordinated regulation. However, Slade *et al.* (1989) showed that xylan endohydrolases appeared in extracts of intact, germinated barley grain several days later than 1,3-1,4-β-glucanases. A similar delay is also seen in isolated aleurone layers (Banik et al, 1997). This raises the question of whether arabinoxylan depolymerisation is really necessary for the penetration of cell walls by α-amylases, peptidases, and other hydrolytic enzymes in relation to their respective substrates in the starch endosperm cells. The current mapping of 1,4-β-xylanase genes provides a basis to study the function of 1,4-β-xylanases and their interaction with other hydrolytic enzymes.

4.3.5 Relationship of the RFLP and IEF bands of α-amylase

Takano *et al.* (1988) have suggested that the rare recombination of the isoenzymes resulted from the recombination between α-amylase gene and its regulator gene. In other word, the isoenzyme pattern is controlled by a regulator gene rather than the α-amylase genes. If this is the case, recombining band pattern between RFLP and isoenzyme should be detected. However, in the present study, the isoenzyme band pattern cosegregated with the RFLP locus and the recombination only happened between a few bands rather than the whole band

pattern (Fig 4.12). This suggested that the recombination of the isoenzymes resulted from the recombination within the α-amylase gene family. However, there is no direct evidence to support this suggestion as some of the Southern bands were not polymorphic.

4.3.6 Gene location of limit dextrinase

Barley genomic DNA digested with 28 restriction enzymes was probed with 670 bp cDNA of LD. Only one band was produced after digestion with *BamHI*, *DraI*, *EcoRV*, *HindIII*, *HinfI*, *NarI*, *NcoI*, *PstI*, *SalI*, *SacI*, *SmaI* and *XhoI* (data not shown) and three bands produced for wheat genomic DNA digested with *HindIII* and *EcoRV* (Fig. 4.13). Therefore, LD gene in barley and wheat appears to be present as a single gene per homologous genome. This is consistent with the rice LD data (Nakamura et al, 1996), but different from maize in which three copies of LD gene may be present (Pan and Nelson, 1984).

The structural gene of limit dextrinase was mapped on the barley chromosome 7H using wheat-barley addition lines and linkage analysis. This result was supported by the isoenzyme results (Sissons, 1992). Barley chromosome 7H was thought to be homologous with the rice chromosome 6 (Sherman et al, 1995). However, the R-enzyme gene (limit dextrinase) in rice was mapped on chromosome 4 (Nakamura et al, 1996). This suggests that translocation for LD gene has happened during evolution. Indeed, it has been reported that barley chromosome 7H also shared partial homology with rice chromosome 4 (Saghai-Maroof et al, 1996).

In contrast to a single copy of the gene, six different isoenzyme bands have been revealed by isoelectric focusing analysis (Sissons et al, 1992a). The origin of the isoforms of this enzyme may be due to post translational modification of the main constituent or modification during extraction of the enzyme. The

relationships between the structural gene, the isoenzyme type and the enzyme activity require further study.

4.3.7 Summary

In summary, all cloned genes (except EII and *Amy2*) for degrading cell walls and starch during seed germination were mapped in the present study. These include one gene for 1,3-1,4-β-glucanase, seven genes for 1,3-β-glucanase, three genes for 1,4-β-xylanase, one gene for β-glucosidase, one loci for α-amylase, three genes for β-amylase (see Chapter 3), one gene for limit dextrinase and one gene for α-glucosidase. Their chromosomal locations are shown in Fig. 4.16. These results will provide basis for studying the genetics and function of the hydrolytic enzymes.

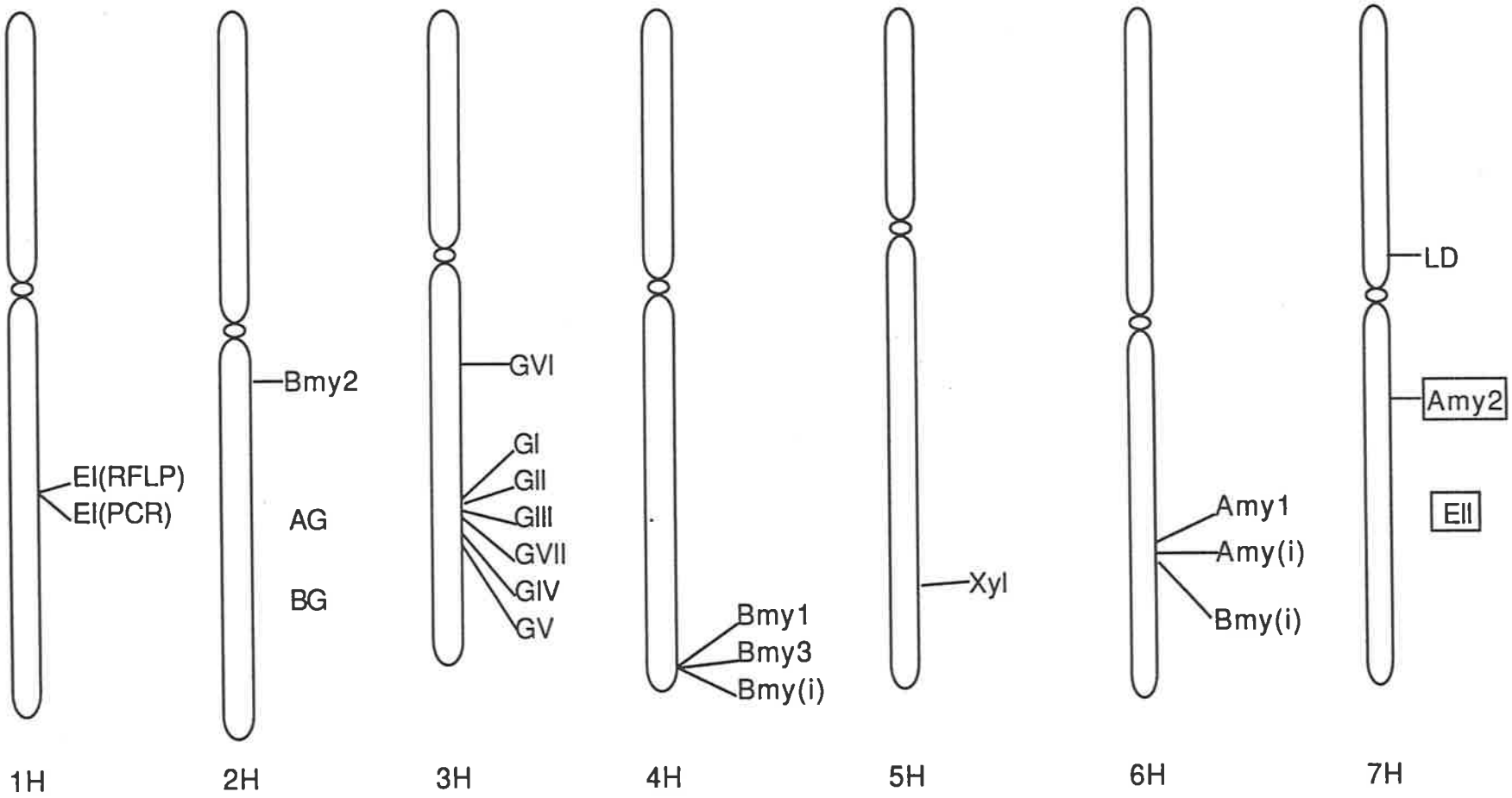

Fig. 4.16 Chromosomal locations of the genes encoding hydrolytic enzymes for degrading cell wall and starch during germination. These genes encode 1,3-1,4-beta-glucanase (EI and EII), 1,3-beta-glucanase (GI to GVII), 1,4-beta-xylanase (Xyl), beta-glucosidase (BG), alpha-amylase (Amy1 and Amy2), beta-amylase (Bmy1, Bmy2 and Bmy3), limit dextrinase (LD) and alpha-glucosidase (AG). The (i) represents isoenzyme. The genes in the box were not mapped in the present study.

Chapter 5: Molecular Mapping of QTLs for the Enzyme Activity of β-Glucanase, α-Amylase and Limit Dextrinase

5.1 Introduction

In the previous chapter, the important genes encoding the hydrolytic enzymes have been mapped. However, it is still not clear i) how important these structural loci are in the determination of enzyme activities, ii) how these genes interact with each other during germination, iii) how many other genes are involved in regulating the enzyme activity, iv) whether there is a common locus controlling the activities of all the enzymes and v) how it may be possible to manipulate these loci in a breeding program. Quantitative trait loci (QTL) analysis may provide an answer to these questions, as it can quantify discrete effects of, and to less extent interaction among the genes determining the enzyme activity in malting and brewing (Hayes et al, 1996).

In the present study, QTL controlling the enzyme activities of β-glucanase (1,3-1,4-β-glucanase), α-amylase and limit dextrinase were mapped and compared for the three mapping populations. Additionally, the contributions of seed dormancy, kernel weight and kernel shape were estimated to determine their effects on enzyme activity .

5.2 Results

5.2.1 QTLs controlling the enzyme activity of 1,3-1,4-β-glucanase

5.2.1.1 Mapping QTLs controlling the enzyme activity

Three DH populations were used to map the QTLs controlling the activity of 1,3-1,4-β-glucanase. The enzyme activity for the parental varieties Clipper, Sahara3771,

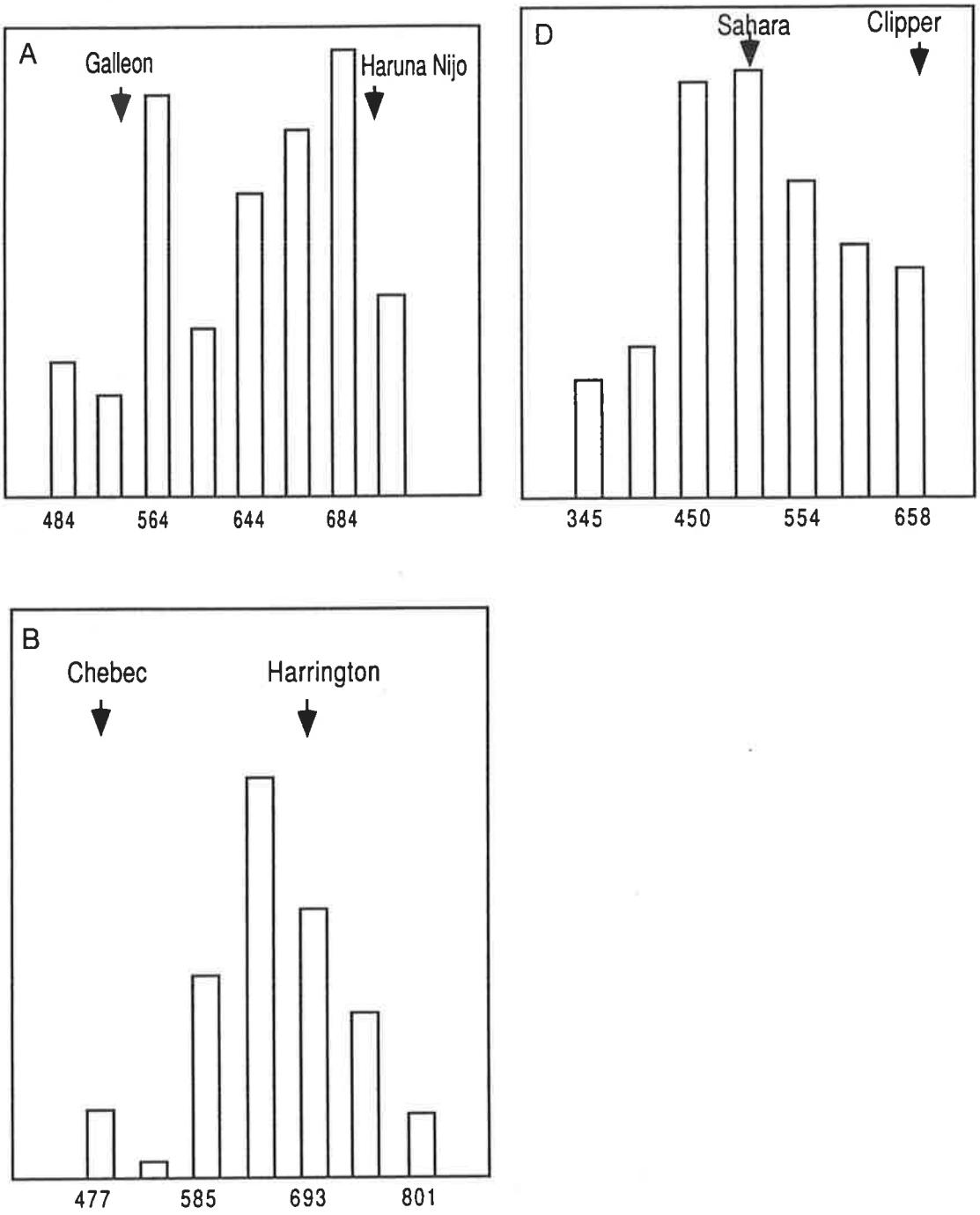

Fig. 5.1 Distribution of the 1,3-1,4-beta-glucanase activity in the three mapping populations. The enzyme activity is expressed as Unit/kg of malt. A: Haruna Nijo X Galleon B: Chebec X Harrington; D: Clipper X Sahara.

Table 5.1 QTL location, genotype difference (GD %) and explained total variation (EV%) and significant level (* P<0.05; ** P<0.01) for 1,3-1,4-β-glucanase activity. The letter suffix indicates the parents contributing the large value allele (G: Galleon; HN: Haruna Nijo; C: Chebec; H: Harrington; CL: Clipper, S: Sahara).

Populations	Chromosome	Closest Marker	GD (%)	EV (%)
Haruna	2H	ABC468	20.0 HN	21.5 **
Nijo X	2H	MWG503B	12.7 HN	9.0*
Galleon	4H	WG622	11.7 HN	20.5**
	5H	WG364	13.4 HN	12.9**
	6H	PSR167	10.3 G	10.1*
	7H	ABC465	17.6 HN	8.3**
Chebec X	3H	BCD451	10.4 H	9.8**
Harrington	5H	KSUD4	3.1 H	8.7**
Clipper X	1H	AWBMA8	17.4 S	8.7*
Sahara	2H	2R/6R	12.2 CL	16.6*
	3H	GVII	60.1 S	6.1*

Galleon, Haruna Nijo, Chebec and Harrington were 650±16.6, 510±38.8, 541±30.2, 715±25.5, 493±21.3 and 674±59.5 U/kg, respectively. The distributions of the enzyme activity in the three populations are shown in Figure 5.1. The enzyme activity exhibited continuous variation in each population, which suggested that the activity of 1,3-1,4-β-glucanase is controlled by multiple loci in the barley genome. Transgressive segregation was observed in all three populations, but it was distorted towards the lower parent in the "Clipper X Sahara3771" population, and to the higher parent in the "Chebec X Harrington" population.

Six QTLs were detected as controlling the activity of 1,3-1,4-β-glucanase in the "Haruna Nijo X Galleon" population. All but one of the positive loci were from the Haruna Nijo parent. The chromosomal location, genotypic difference and variation explained for the QTLs are shown in Table 5.1. Two QTLs were found on chromosome 2H, of which the one linked with the RFLP marker ABC468 had the largest effect on the enzyme activity. One QTL with the second largest effect was located on chromosome 4H. The QTL on chromosome 7H, linked with the RFLP marker ABC465, could be the structural locus of 1,3-1,4-β-glucanase isoenzyme EII (*Glb2*), but no detectable effect was found around the locus of isoenzyme EI (*Glb1*) on chromosome 1H (Loi et al, 1988; MacLeod et al, 1991; Slakeski et al, 1990). The QTL on chromosome 6H was closely linked with the *Amy1* locus of α-amylase (Fig 2.1a).

Three QTLs were detected for the activity of 1,3-1,4-β-glucanase in the "Chebec X Harrington" DH population. All the positive QTLs were from the Harrington parent (Table 5.1). One QTL was located on each of the short and long arms of chromosome 5H. The QTL linked with the RFLP marker BCD451 on chromosome 3H was close to the structural loci of the 1,3-β-glucanase gene family (Li et al, 1996, Fig. 2.1b).

Three QTLs were found in the "Clipper X Sahara3771" DH population as controlling the activity of 1,3-1,4-β-glucanase. The three QTLs were dispersed on chromosomes 1H, 2H and 3H (Table 5.1). Two positive QTLs were from the Sahara3771 parent and one from the Clipper parent. The QTL on chromosome 3H was

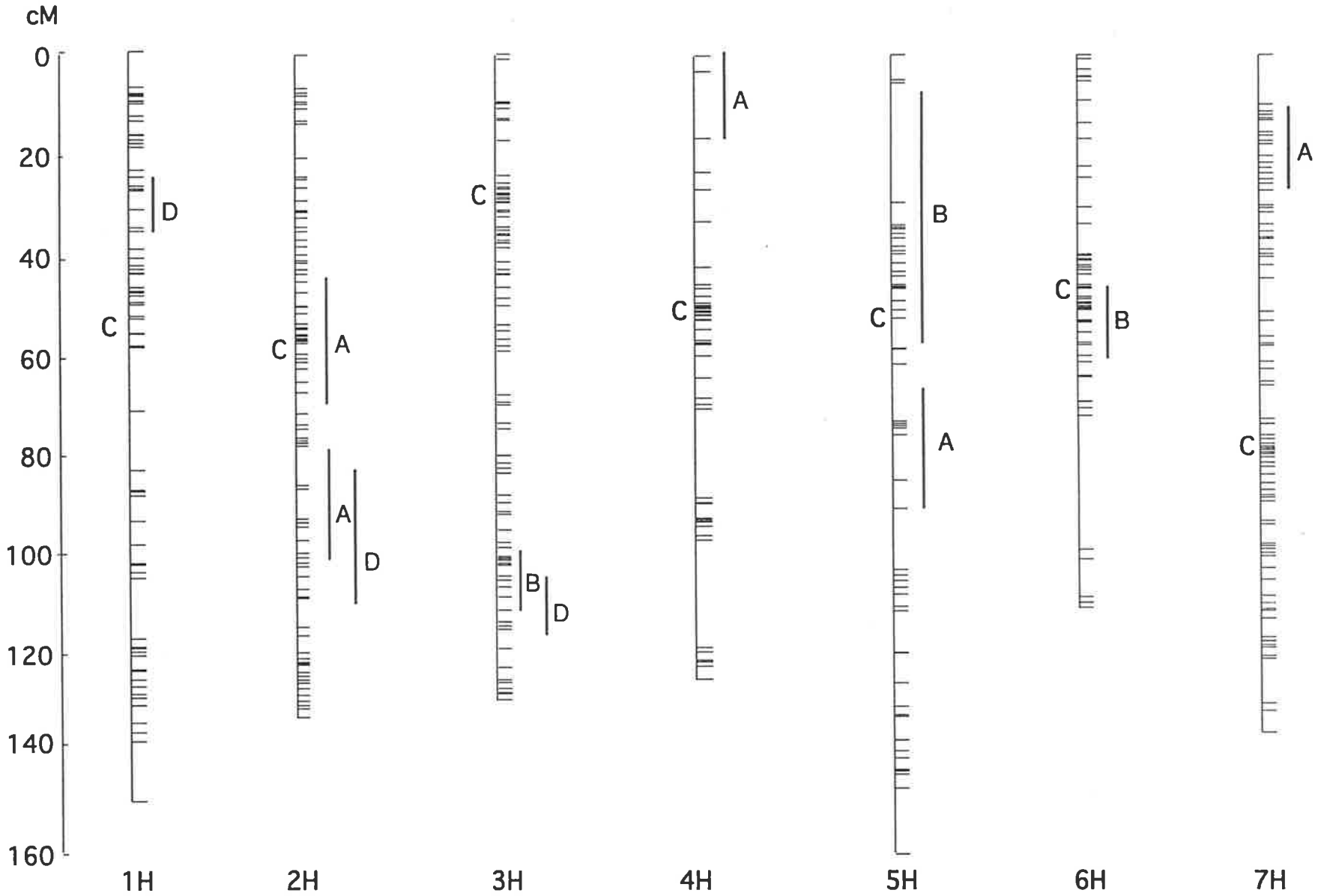

Fig. 5.2 Chromosomal locations of the QTL for the 1,3-1,4-β-glucanase activity on the barley consensus linkage map (Langridge et al, 1995) The short horizontal lines along the chromosomes indicate the position of markers in the consensus linkage map and the vertical lines show the chromosomal regions significantly linked the QTL. A: Haruna Nijo X Galleon; B: Chebec X Harrington; D: Clipper X Sahara; C: centromere.

linked with the 1,3-β-glucanase gene family (Li et al, 1996, Fig 2.1c) and that on chromosome 2H with the two row/six row locus (*V/v*).

5.2.1.2 Comparison of the QTLs in different mapping populations

The common markers used in the different mapping populations and in the construction of the consensus linkage maps (Langridge et al, 1995; Qi et al, 1996) made it possible to compare the QTL mapped in the different populations. By placing the QTL onto the consensus map, it is evident that the QTL mapped to chromosome 2H are located in a similar chromosomal region in all three mapping populations. The QTL on chromosome 5H is found in a similar region in the "Haruna Nijo X Galleon" and the "Chebec X Harrington" populations. The QTL on chromosome 3H is in a similar region in the "Chebec X Harrington" and the "Clipper X Sahara3771" populations (Fig. 5.2, Fig. 2.1). A combination of these results indicated that eight different QTLs were identified as controlling the enzyme activity of 1,3-1,4-β-glucanase in the three mapping populations. The QTLs on chromosomes 5H and 7H in the "Haruna Nijo X Galleon" population were consistent with those mapped in the North American Barley Genome Mapping Project (Han et al, 1995) and the QTL on chromosome 4H coincided with that for the content of malt β-glucan (Han et al, 1995).

5.2.2 QTLs controlling the enzyme activity of α-amylase

5.2.2.1 Mapping QTLs controlling α-amylase activity

Three DH populations were also used to map the QTLs controlling the activity of α-amylase in the present study. The enzyme activity for the parental varieties Clipper, Sahara3771, Galleon, Haruna Nijo, Chebec and Harrington were 343±25.7, 340±24, 207±6.2, 350±28.3, 210±13 and 478±23 U/g, respectively. The enzyme activity

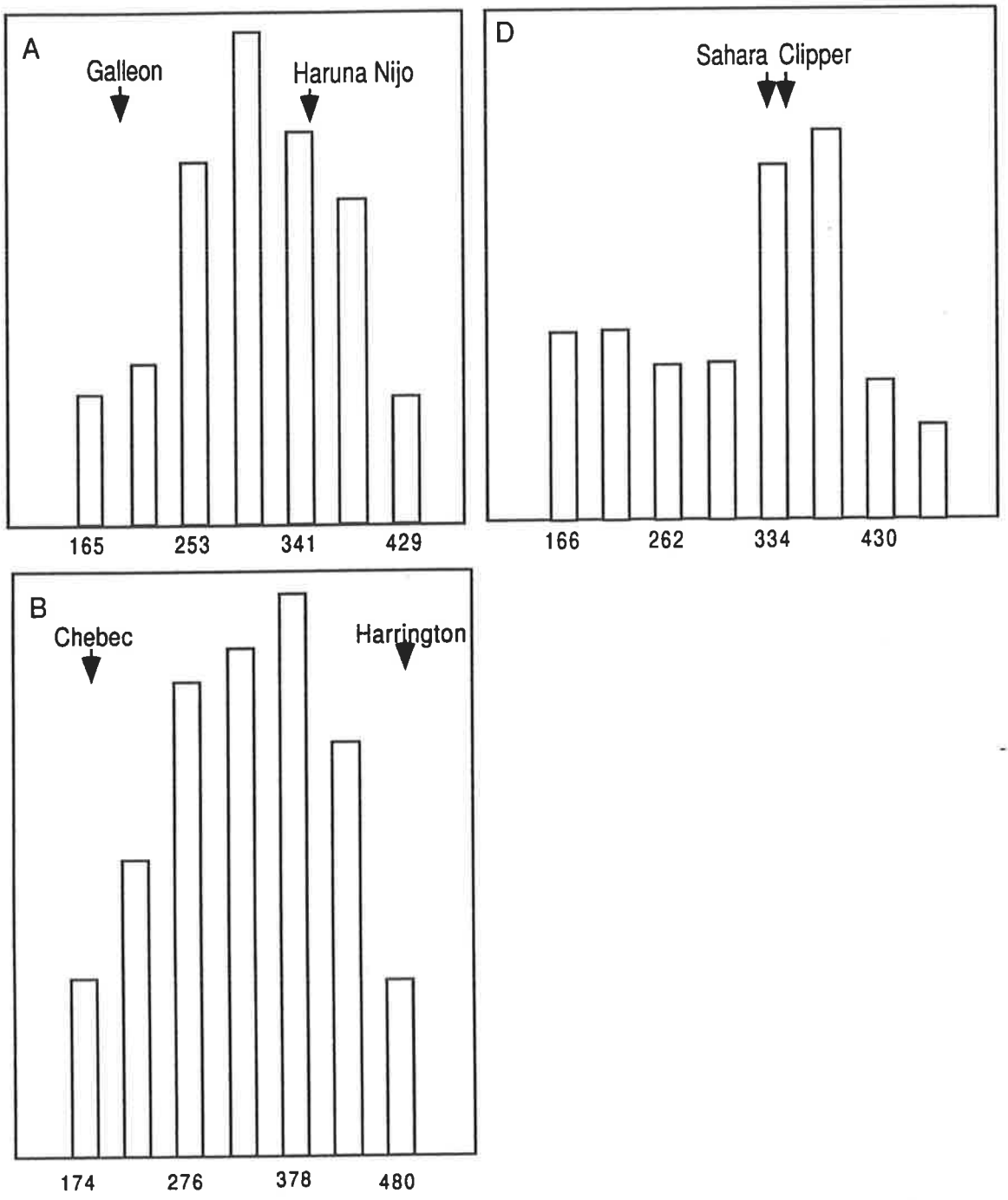

Fig. 5.3 Distribution of the apha-amylase activity in the three mapping populations. The enzyme activity is expressed as Unit/gram of malt. A: Haruna Nijo X Galleon; B: Chebec X Harrington; D: Clipper X Sahara)

Table 5.2 QTL location, genotype difference (GD %) and explained total variation (EV%) and significant level (* P<0.05; ** P<0.01) for α-amylase activity. The letter suffix indicates the parents contributing the large value allele (G: Galleon; HN: Haruna Nijo; C: Chebec; H: Harrington; CL: Clipper, S: Sahara).

Populations	Chromosome	Closest Marker	GD (%)	EV (%)
Haruna Nijo X	1H	AWBMA27	16.5 HN	2.0*
Galleon	2H	ABC468	31.1 HN	18.4**
	2H	MWG503B	24.9 HN	10.1*
	5H	WG364	23.6 HN	14.8**
	5H	ABG702B	26.9 HN	22.3**
	6H	BCD221A	11.9 G	5.3*
	7H	ABC465	23.0 HN	2.3*
Chebec X	1H	PSR121A	15.8 C	13.1*
Harrington	1H	CDO400	14.8 H	18.2*
	3H	BCD451	17.2 H	9.2*
	5H	KSUD4	19.0 H	4.9*
	5H	CDO419	12.4 C	13.6*
	6H	ABC156	16.1 H	12.9*
	6H	AWBA20	14.6 H	4.8*
Clipper X Sahara	1H	Hor1	40.5 S	11.4**
	1H	PSR158	26.7 S	9.5*
	2H	CDO473B	29.6 S	15.6**
	3H	WG405	19.8 CL	6.8*
	5H	WG181	22.5 S	9.1*

showed continuous variation in all the DH populations, and transgressive segregation was observed in the "Clipper X Sahara3771" and "Haruna Nijo X Galleon" populations (Fig. 5.3)

Seven QTLs were detected for α-amylase activity in the "Haruna Nijo X Galleon" population. All but one of the positive alleles were from the Haruna Nijo parent (Table 5.2). Two QTLs were found on each of chromosomes 2H and 5H, which explained most of the variation of the enzyme activity. However, the two structural loci of α-amylases on chromosome 6H and 7H had no significant effect on the enzyme activity (Table 5.2, Fig 2.1a).

Seven QTLs for the activity of α-amylase were identified in the "Chebec X Harrington" DH population. Five positive QTLs were from the Harrington parent and two from the Chebec parent. This result partially explained the transgressive segregation of the enzyme activity (Fig. 5.3). The distribution of the QTLs in the "Chebec X Harrington" population is different from that of the "Haruna Nijo X Galleon" population. Two QTLs were identified on each of chromosomes 1H, 5H and 6H. One QTL on chromosome 6H, linked with RFLP marker ABC156, may represent the structural locus of α-amylase (*Amy1*) (Fig. 2.1b, Fig. 5.4).

Five QTLs for the activity of α-amylase were detected in the "Clipper X Sahara3771" population. There were two QTLs on chromosome 1H, of which one was closely linked with the hordein structural loci (Fig 2.1c, Shewry et al, 1990; Shewry, 1991). The QTL on chromosome 2H had the largest contribution to variation of the enzyme activity. Two other QTLs were located on chromosomes 3H and 5H.

5.2.2.2 Comparison of the QTL for α-amylase in different mapping populations

By placing the QTLs in the consensus map, the QTLs mapped on the short arm of chromosome 1H were located in a similar region in all three mapping populations and closely linked with the hordein gene family (Fig. 2.1, Fig. 5.4, Shewry et al, 1990;

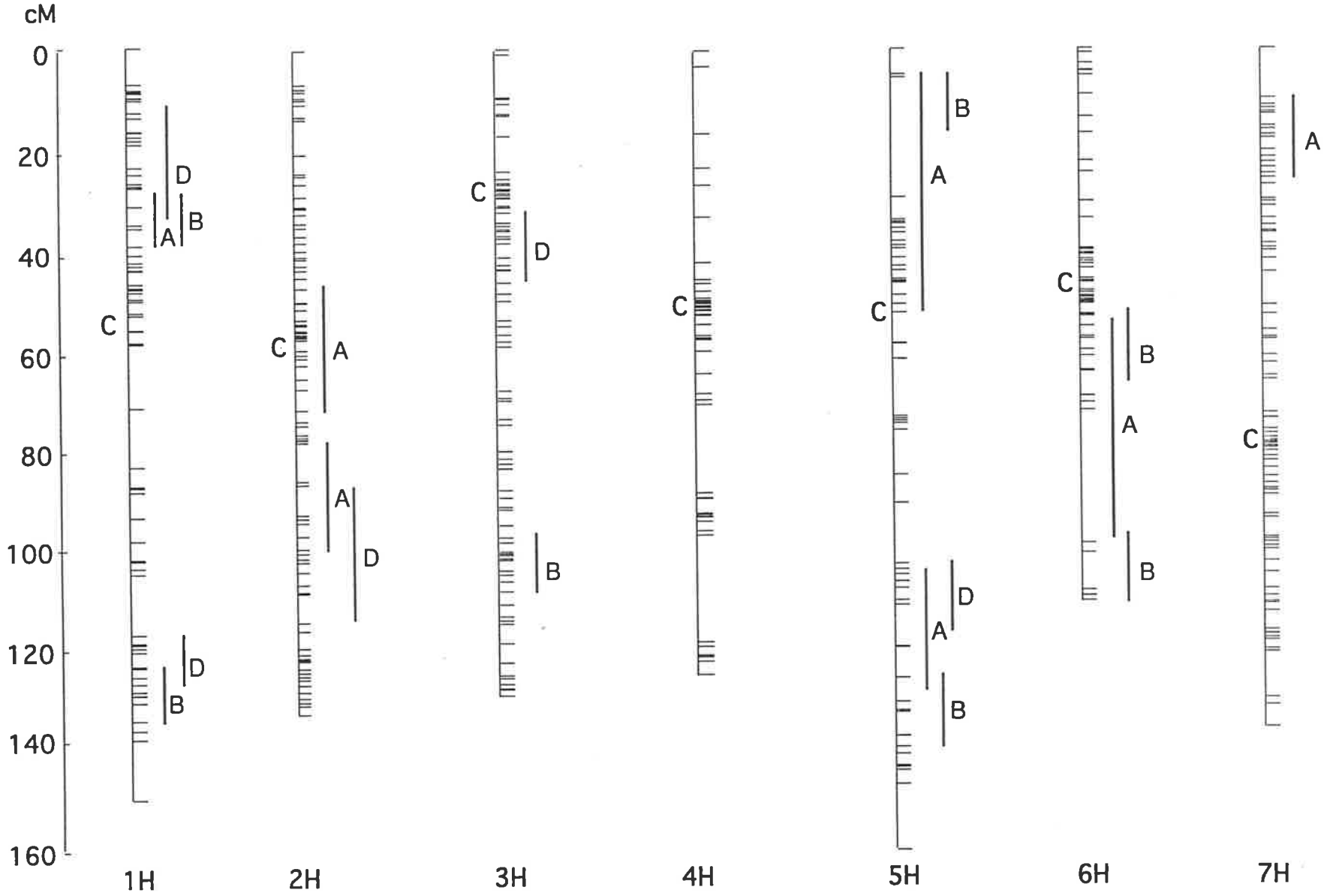

Fig. 5.4 Chromosomal locations of the QTLs for the alpha-amylase activity on the barley consensus linkage map (Langridge et al, 1995) The short horizontal lines along the chromosomes indicate the position of markers in the consensus linkage map and the vertical lines show the chromosomal regions significantly linked the QTLs. A: Haruna Nijo X Galleon; B: Chebec X Harrington; D: Clipper X Sahara; C: centromere.

Shewry, 1991). This may explain the difficulty experienced in selecting for high enzyme activity and low protein content at the same time (Smith, 1990). It is not clear whether this is due to pleiotrophic effects of the hordein genes or the genes for the enzyme activity closely linked with hordein genes. The QTLs mapped on chromosomes 5H and 2H were also in similar chromosomal regions in the three populations (Fig. 5.4). A combination of these results indicated that ten QTLs control the activity of α-amylase in the present study.

Two QTLs for the enzyme activity of α-amylase have been mapped on chromosomes 5H and 7H in the "Dicktoo X Morex" population (Oziel et al, 1996). The QTL with the largest effect is consistent with the common QTL detected on chromosome 5H in the present study. The QTL on chromosome 7H coincides with that mapped in the "Haruna Nijo X Galleon" population (Fig. 5.4, Langridge et al, 1995, Oziel et al, 1996). In another study, nine QTLs related to the activity of α-amylase have been identified in the "Steptoe X Morex" population (Hayes et al, 1993). Except for two QTLs on chromosomes 4H and 7H, all the other QTLs were detected in at least one mapping population in the present study (Fig. 5.4; Hayes et al, 1993).

5.2.3 QTLs controlling the enzyme activity of limit dextrinase

5.2.3.1 Mapping QTLs controlling the LD activity

Two DH populations were used for mapping both total and free LD activity. The parental varieties used to generate the DH populations exhibited different levels of both "free" and "total" LD activity (Table 5.3). When the DH populations were assayed for free and total LD activity, free enzyme levels ranged from 87 to 217 mU/kg in the "Haruna Nijo X Galleon" DH population and from 84 to 464 mU/kg in the "Chebec X Harrington" DH population. Mean levels of free LD in the parental cultivars Galleon, Haruna Nijo, Chebec and Harrington were 112±8.9, 223±9.5, 174±6.4 and 206±38.3 mU/kg, respectively (Table 5.3). Transgressive segregation was observed in the "Chebec X Harrington" DH population, but not in the "Haruna Nijo X Galleon" DH

Table 5.3 The enzyme activity of the parental varieties for the mapping populations

Cultivars	β-Glucanase	α-Amylase	Total LD	Free LD
Clipper	650±16.6	343±25.7		
Sahara	510±38.8	340±24		
Galleon	541±30.2	207±6.2	547±64.2	112±8.9
Haruna Nijo	715.5±25.5	350±28.3	1380±40.8	223±9.5
Chebec	493±21.3	210±13	612±20.3	174±6.4
Harrington	674±59.5	478±23	1857±173.9	206±38.3

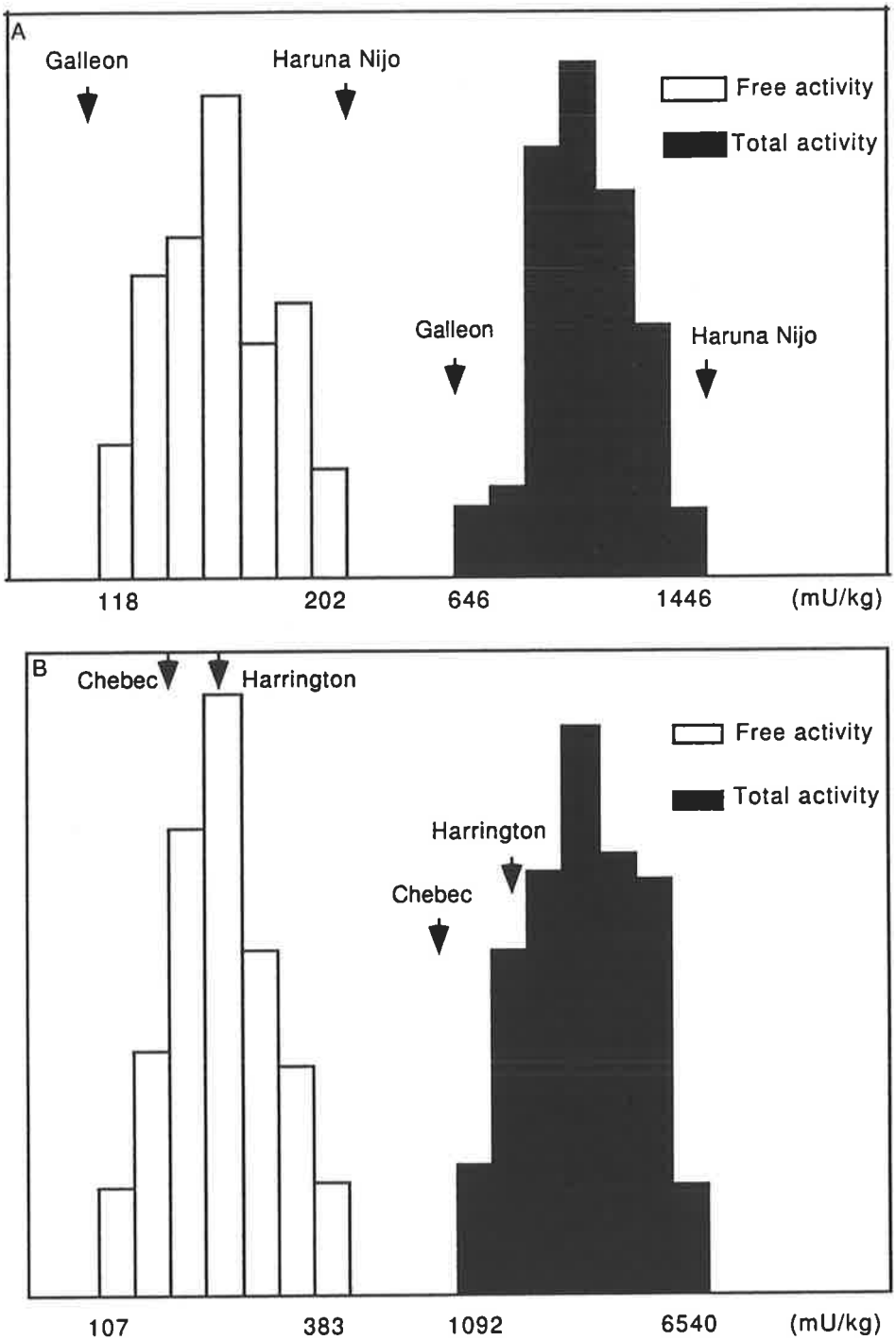

Fig. 5.5 The distribution of total and free enzyme activity of LD in the two mapping populations (A: Haruna Nijo X Galleon; B: Chebec X Harrington)

Table 5.4 QTL location, genotype difference (GD %) and explained total variation (EV%) and significant level (* P<0.05; ** P<0.01). for LD activity. The letter suffix indicates the parents contributing the large value allele (G: Galleon; HN: Haruna Nijo; C: Chebec; H: Harrington). 4A is Haruna Nijo x Galleon population; 4B is Chebec x Harrington population.

5.4a

	Free	enzyme	activity		Total	enzyme	activity
Closest marker	Chromosome	% GD	% Var	Closest marker	Chromosome	% GD	% Var
ABC468	2H	13.1 HN	12.5 **	BCD310	1H	8.1 G	2.5 *
MWG503B	2H	18.4 HN	14.2 **	CDO370	2H	14.5 HN	9.7 **
BMY1	4H	9.5 G	5.4 *	KsuF41	2H	21.3 HN	11.2 **
WG364	5H	13.5 HN	11.4 **	WG181	5H	13.7 HN	8.8 **
PSR115	5H	12.1 HN	7.5 *	AWBMA13B	5H	16.9 HN	9.1 **
PSR154	6H	10.2 G	4.2 *	WG789	7H	20.1 HN	3.8 *
AaWBI	7H	7.4 G	4.8 *				

5.4b

	Free	enzyme	activity		Total	enzyme	activity
Closest marker	Chromosome	% GD	% Var	Closest marker	Chromosome	GD(%)	EV(%)
CDO400	1H	24.9 H	10.1 **	ABG373	1H	14.1 H	5.9 *
ABG453	2H	4.9 H	5.0 *	BCD402A	2H	19.7 H	10.0 *
BCD453B	2H	6.5 C	2.6 *	BCD453B	2H	22.5 H	10.2 *
BCD451	3H	23.9 H	12.3 **	WG114	4H	12.5 C	6.1 *
KSUD4	5H	8.5 C	9.2 *	ABG57	5H	13.7 C	5.3 *

population. Although the free enzyme activities of the two high parents for the two DH populations were not significantly different, the maximum free enzyme activity in the "Chebec X Harrington" population was much higher than that of the "Haruna Nijo X Galleon" population (Fig. 5.5).

Six QTLs for total enzyme activity and seven QTLs for free enzyme activity were detected in the "Haruna Nijo X Galleon" population. The map locations, genotypic differences, the most closely linked markers and variations explained by the QTLs are presented in Table 5.4. Two loci exerting the greatest effect on both total and free enzyme activities were identified on chromosomes 2H and 5H (Table 5.4). The locus linked with the RFLP marker WG789 on chromosome 7H may represent the structural gene of LD (Fig 2.1, Fig. 4.14, Fig. 5.6). However, this locus is only related to the total enzyme activity and has no detectable effect on free LD activity. The QTLs on chromosomes 7H, 4H and 6H were specific for the free enzyme activity, but their effects were minor.

Five QTLs for both total and free activities were identified in the "Chebec X Harrington" DH population (Table 5.4). However, the variation explained by these QTLs was relatively small compared to the "Haruna Nijo X Galleon" population (Table 5.4). The QTLs detected on chromosomes 2H and 5H contribute most of the variation of the enzyme activity (Table 5.4). The QTL on chromosome 3H was only detected in the "Chebec X Harrington" population, but has a major effect on the free enzyme activity.

5.2.3.2 Comparison of QTL locations for limit dextrinase in two mapping populations

The common markers mapped in the two populations enabled a comparison of QTL mapping results from different populations. Such a comparison might be expected not only to identify the common and important regions controlling LD activity, but also to identify regions of the genome that affect LD activity differently in various genetic backgrounds. The QTLs on chromosomes 1H, 2H and 5H, which accounted for most of

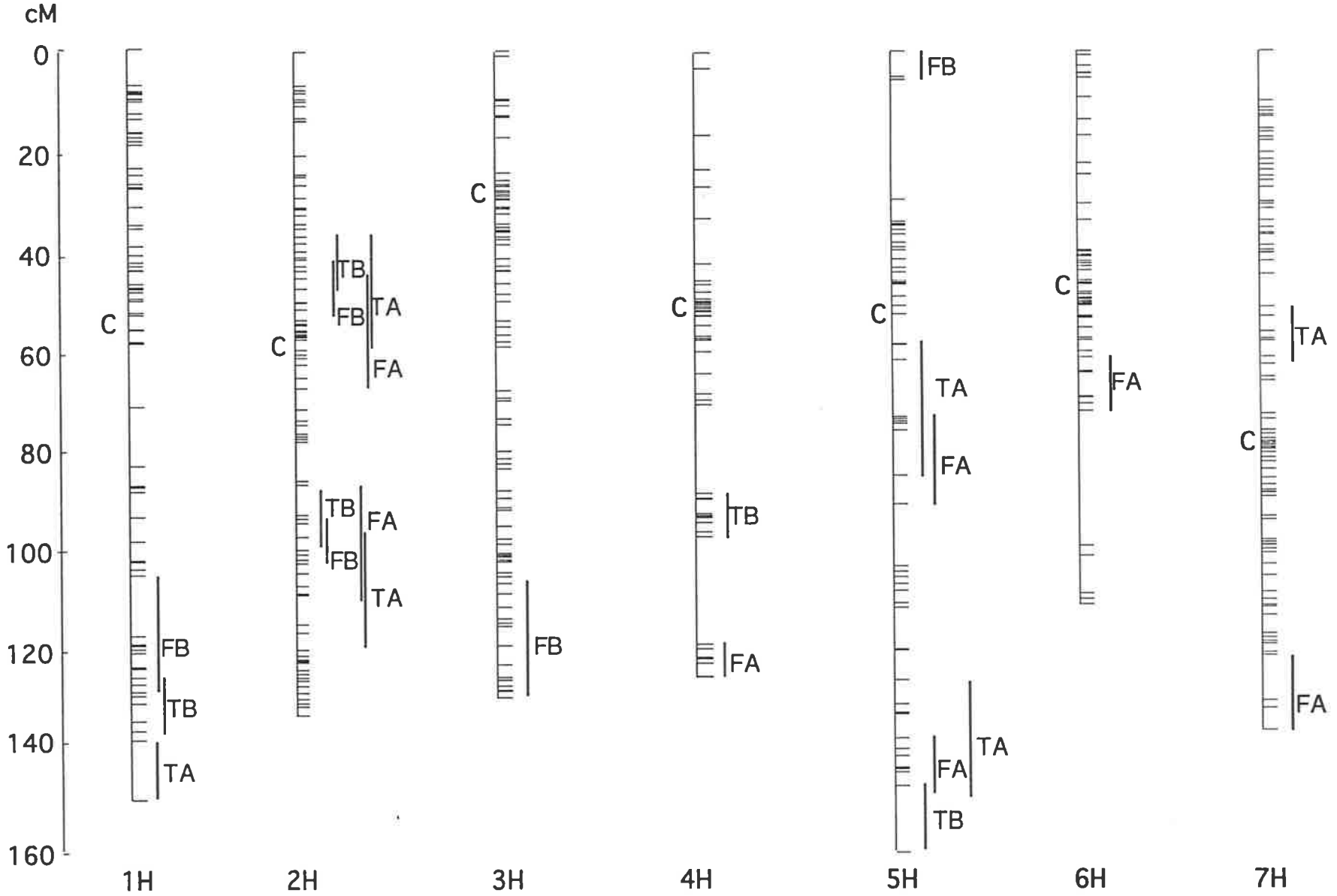

Fig. 5.6 Chromosomal locations of the QTLs for the limit dextrinase activity on the barley consensus linkage map (Langridge et al, 1995) The short horizontal lines along the chromosomes indicate the position of markers in the consensus linkage map and the vertical lines show the chromosomal regions significantly linked the QTLs. A: Haruna Nijo X Galleon; B: Chebec X Harrington; C: centromere; T: total enzyme activity; F: free enzyme activity.

the genetic variations for both free and total activity (Table 5.4), were located in similar positions in the two populations (Fig. 2.1, Fig. 5.6). However, other QTLs on chromosomes 3H, 4H, 5H, 6H and 7H were specific for one of the two mapping populations (Fig. 5.6). A combination of the results suggests that there are eight QTLs for total LD activity and ten QTLs for free LD activity in the varieties examined here. However, the small apparent effect of some of these loci suggests they may not be reliable.

5.2.3.3 Marker-assisted selection for the limit dextrinase activity

The QTLs associated with LD activity were tested for their efficacy in selecting enzyme activity. This was seen as particularly important for these QTLs with relatively minor effect. Using a marker closely linked with a QTL as reference, ten DH lines with each parental genotype were randomly selected to compose two bulks. Theoretically, the two bulks should have a similar but random genetic background except that they differ in the region of the marker locus. All the markers closely linked to the QTLs for the LD activity (Table 5.4) were used for analysis with the seeds harvested in 1995 from the "Haruna Nijo X Galleon" population. The results showed that most of the markers detected previously by conventional QTL analysis can be used to select for elevated enzyme activity (Table 5.5). While there were major differences in the magnitude of the QTL effects between the seed used for the initial QTL mapping and for the bulk-segregant analysis, their direction were not changes in the favourable allele phase. The QTLs on chromosome 2H showed the strongest association with enzyme activity (Table 5.4 and Table 5.5). These results demonstrated that the markers linked to the QTLs are reliable for selecting the enzyme activity.

Table 5.5 The activity of LD selected with molecular markers linked with the QTL (Table 5.4) from "Haruna Nijo X Galleon" DH population. Each bulk was constructed with 10 random selected DH lines with one parent allele at the marker locus (G: Galleon; HN: Haruna Nijo). Three independent bulks were constructed for each locus.

Chromosome	Marker	Bulk	Free activity (mU/kg)	Difference %	Total activity (mU/kg)	Difference %
1H	BCD310	G	205±22		733±33	
		HN	219±11		616±6	
				6.2		18.9
2H	ABC468	G	257±7		644±2	
		HN	298±1		834±20	
				16.3		29.5
2H	CDO373	G	218±7		730±28	
		HN	265±14		880±33	
				21.9		20.5
4H	Bmy1	G	219±3		638±25	
		HN	201±5		674±33	
				8.9		5.6
5H	ABC168	G	219±7		633±11	
		HN	239±1		698±25	
				9.3		10.2
5H	PSR115	G	182±5		700±37	
		HN	187±3		763±27	
				2.4		9.0
6H	Amy1	G	272±15		722±24	
		HN	231±11		740±21	
				17.6		2.5
7H	WG789	G	218±8		611±6	
		HN	241±0		703±1	
				10.5		15.1
7H	BI	G	290±4		813±25	
		HN	243±6		838±26	
				19.3		3

5.2.4 Mapping QTLs controlling seed dormancy, kernel weight and kernel shape

Seed dormancy was determined immediately following harvest. Six QTLs were identified for the control of seed dormancy in the "Chebec X Harrington" population. Both parents "Chebec" and "Harrington" had three positive alleles (Table 5.6). Two QTLs were found on chromosome 5H. The QTL linked with the RFLP marker CDO460 had the major effect on dormancy. These loci for seed dormancy have also been mapped in the North American Barley Genome Mapping Project (Ullrich et al, 1993). The other QTLs were located to chromosomes 2H, 3H, 4H and 7H.

Seven QTLs were detected for kernel weight. The QTLs were distributed on chromosomes 1H, 2H, 3H, 4H, 6H and 7H. The alleles from chromosomes 1H, 2H and 4H had the largest effects (Table 5.6). The QTLs on chromosomes 3H, 4H, 6H and 7H controlling kernel weight have also been identified by other researchers (Backes et al, 1995; Powell et al, 1990; Thomas et al, 1996; Tinker et al, 1996).

Five QTLs were found for kernel shape (ratio of length/width). These QTLs were located on chromosomes 1H, 2H, 5H, 6H and 7H, respectively, with the loci on chromosomes 5H and 7H having the largest effects (Table 5.6). The locus on chromosome 5H has been previously identified (Backes et al, 1995).

By placing the QTLs for seed dormancy, kernel weight and kernel shape in consensus maps, QTLs for kernel weight and kernel shape were located in similar regions on chromosomes 1H, 2H and 7H and those for dormancy and kernel weight in similar regions on chromosomes 3H, 4H and 7H (Fig. 5.7). It is not clear if this is due to the effects of linked genes or pleitrophic effects of single gene.

Table 5.6 QTL location, genotype difference (GD %) and explained total variation (EV%) and significant level (* P<0.05; ** P<0.01) for seed dormancy, kernel weight and kernel shape

Traits	Chromosome	Closest Marker	GD (%)	EV (%)
Seed dormancy	2H	WG645	10.5 C	5.7*
	3H	PTAG683A	15.6 C	9.2**
	4H	CDO63	10.0 H	3.6*
	5H	PSR1240	8.9 H	7.3*
	5H	CDO460	17.8 H	15.4**
	7H	ABC152	14.3 C	14.6**
Kernel weight	1H	BCD402B	5.4 C	15.3**
	2H	CDO57A	4.5 C	12.7**
	2H	WG180	5.1 C	15.9**
	3H	KSUA3B	2.8 H	9.3*
	4H	WG719	4.4C	16.7**
	6H	Amy1	3.7C	4.6**
	7H	BCD129	3.1 C	6.1*
Kernel shape	1H	BCD402B	1.7 H	6.4*
	2H	CDO57A	1.6 H	6.0*
	5H	WG405B	2.6 C	10**
	6H	ABG654	1.6 C	6.6*
	7H	WG789B	2.8 C	8.3**

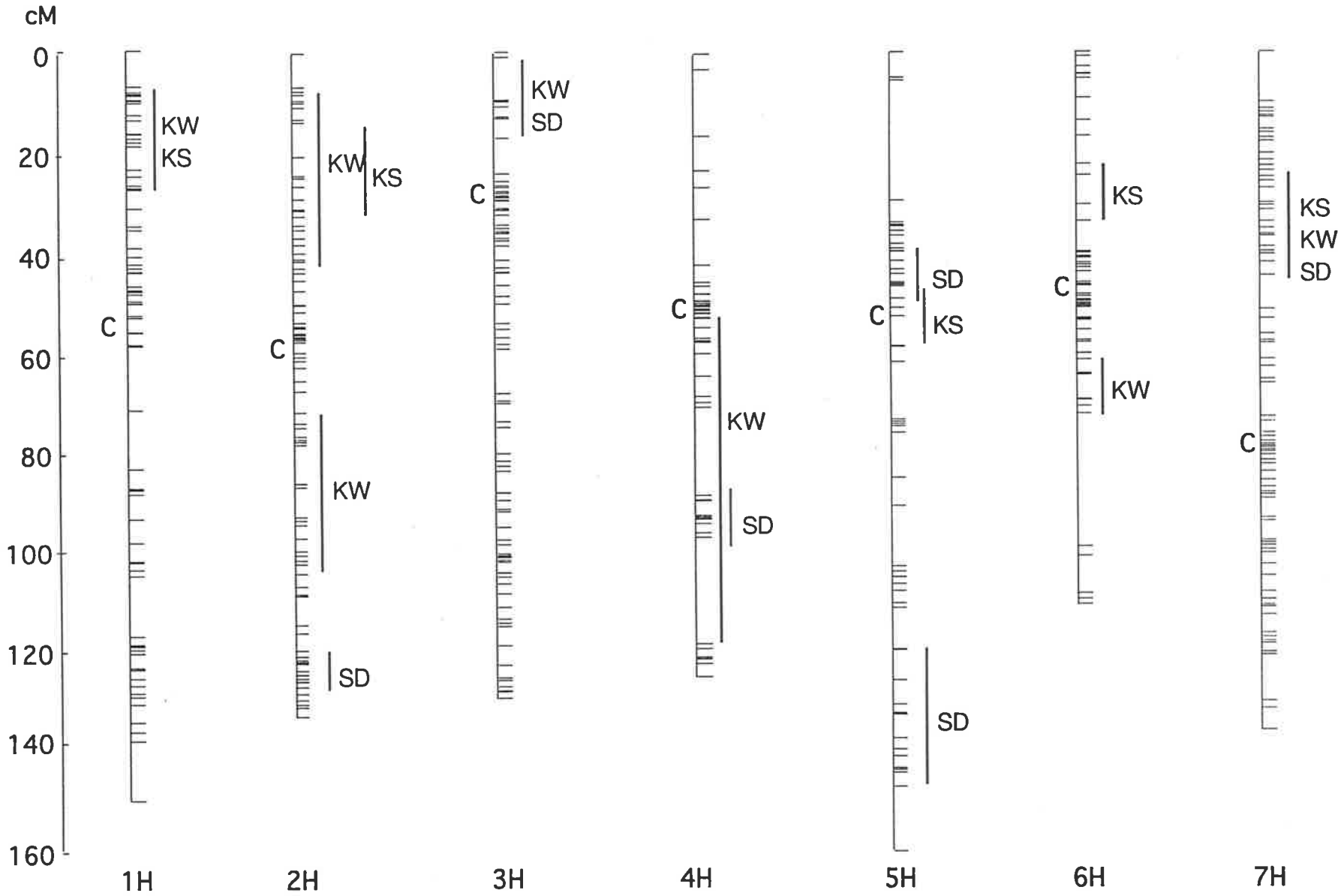

Fig. 5.7 Chromosomal locations of the QTLs for seed dormancy (SD), kernel weight (KW) and kernel shape (KS) on the barley consensus linkage map (Langridge et al, 1995). The short horizontal lines along the chromosomes indicate the position of markers in the consensus linkage map and the vertical lines show the chromosomal regions significantly linked the QTLs. C: centromere.

5.3 Discussion

5.3.1 Relationship between structural genes and enzyme activity

The two structural loci of 1,3-1,4-β-glucanases (*EI* and *EII*) have been mapped to chromosomes 1H and 7H in the barley genome (Chapter 4, Loi et al, 1988; MacLeod et al, 1991; Slakeski et al, 1990). Northern blot analysis has shown that EI is expressed in the scutella and aleurone of germinated seed and developing leaves and roots, whereas EII expression predominates in the aleurone (Slakeski et al, 1990; Slakeski and Fincher, 1992). The present study indicates that the postulated EII locus on chromosome 7H contributed significantly to the enzyme activity of 1,3-1,4-β-glucanase. The EI locus on chromosome 1H was not associated with the QTL controlling activity. These results reinforce the opinion that EII is more important for malting quality. However, the EII gene and its surrounding regions appears to be highly conserved (see Chapter 4). Thus, highly polymorphic markers linked to EII should be developed for selecting high levels of 1,3-1,4-β-glucanase in breeding programs.

High levels of 1,3-β-glucanase activity have been found in the endosperm of germinating barley (Høj et al, 1988, 1989). However, the main component of the endosperm cell walls is 1,3-1,4-β-glucan (MacGregor, 1990), and the purified 1,3-β-glucanases were unable to hydrolyse barley 1,3-1,4-β-glucans (Høj and Fincher, 1995; Woodward and Fincher, 1983). The apparent paucity of endogenous substrate, together with the ability of 1,3-β-glucanases to hydrolyse the 1,3- and 1,3-1,6-β-glucans that are major cell wall constituents in some classes of fungal pathogens of plants, has led to speculation that these enzymes may participate in a general, non-specific strategy to protect grain against pathogen invasion (Fincher, 1989). However, there is no direct evidence to support this point from either quantitative or qualitative disease resistance analysis. The present study showed that one locus around the 1,3-β-glucanase genes

contributed to the enzyme activity of 1,3-1,4-β-glucanase. The substrate used here was purified barley 1,3-1,4-β-glucans, and as such does not contain contiguous 1,3-β-linkages. It is therefore specific for 1,3-1,4-β-glucanases (Megazyme, Sydney, Australia). One possible explanation for the current result may be that 1,3-β-glucanases degrade 1,3-β-glucans in the aleurone cell wall (Bamforth and Barclay, 1993) and consequently accelerate release of 1,3-1,4-β-glucanases from the aleurone layers. The other possibility is that 1,3-β-glucanases hydrolyse 1,3-1,4-β-glucans jointly with 1,3-1,4-β-glucanases. Although 1,3-β-glucanases are unable to degrade the large molecules of 1,3-1,4-β-glucans, they could hydrolyse small fragments of 1,3-1,4-β-glucans after they had been partially hydrolysed by 1,3-1,4-β-glucanases. These speculations call for further study. Nevertheless, this does not exclude the possibility that some isoenzymes of 1,3-β-glucanases take part in the defense response.

Similar to 1,3-1,4-β-glucanases, Two loci encoding α-amylases were located on chromosomes 6H (*Amy1*) and 7H (*Amy2*) and two loci for β-amylases on chromosomes 2H (*Bmy2*) and 4H (*Bmy1*). The present study showed that only one locus for each of the enzymes contributes significantly to controlling the variation on enzyme activity: *Amy1* for α-amylases and *Bmy1* for β-amylases (Tables 3.1, 5.2, 5.3). This means that one structural locus is predominantly expressed in developing or germinating seeds. This result is supported by isoenzyme and Northern blot analysis (Fincher and Stone, 1993; Slakeski et al, 1990; Slakeski and Fincher, 1992; Shewry et al, 1988). However, compared with the locus of β-amylase (*Bmy1*) which is synthesised during seed development, the individual structural loci of α-amylase, 1,3-1,4-β-glucanase and limit dextrinase have relatively minor contributions to the enzyme activities. For those enzymes synthesised during seed germination, the regulatory loci seem more important in controlling enzyme activity (Tables 5.1, 5.2, 5.3).

5.3.2 Genes controlling the activity of LD

Limit dextrinase exists in a free, active form and a latent, bound form (Longstaff and Bryce, 1991, 1993; MacGregor et al, 1994). In malted barley, almost all the enzyme appears to be present in the bound form (Serre and Lauriere, 1989). The proportion of free to bound activity was 5.3% to 28.5% in the present study. The small amount of free LD available in malt may explain why most of the 1,6-linkages survive the brewing process (Enevoldsen and Schmidt, 1974). Sufficient LD is synthesised for seed germination, but most is stored in a latent form. The enzyme is activated at late stages of germination (after 10 days of germination) (Longstaff and Bryce, 1993; MacGregor et al, 1995). This suggests that the amylopectin may be used as a long term stored energy for seed germination, and may explain why the structural gene for limit dextrinase is only related to the total enzyme activity but not the free enzyme activity and the QTLs specific for the free enzyme activity had only minor effects at the early stage of germination in this study. However, the malting industry usually employs a short germination time. It is desirable to select barley cultivars where limit dextrinase is activated quickly during germination. There exists considerable genetic variation for the proportion of free to bound form enzyme in the present study. This provided the possibility to select barley varieties with high free enzyme activity.

The latent limit dextrinase is converted to free enzyme by proteolytic modification during germination (Longstaff and Bryce, 1993). A similar mechanism also controlled the release of bound β-amylase (Grime and Briggs, 1995; Guerin et al, 1992a, 1992b; Lundgard and Svensson, 1986). Several endopeptidases have been isolated (Koehler and Ho, 1988; Phillips and Wallace, 1989), which can activate the bound β-amylase (Guerin et al, 1992a). One endopeptidase gene (*Cep B)* (Guerin et al, 1992b) and two proteinase genes (*EPB1* and *EPB2*) (Mikkomen et al, 1996) have been mapped on chromosome 3H and another endopeptidase gene (*EpHI)* was located on chromosome 7H (Hart et al, 1980). The QTLs on chromosome 3H linked with the marker BCD451 and on chromosome 7H linked with the PCR marker AaWBI only influence the free enzyme activity, but not total activity. Thus it is postulated that these two loci should be involved in the activation of the LD enzyme. The AaWBI is a highly polymorphic microsatellite

DNA marker (Data not shown). Therefore, it is very useful for selecting the high activity of limit dextrinase.

Reducing inhibitor concentration is an alternative to increase free enzyme activity (MacGregor et al,1995), but due to the inability to assay for the inhibitor gene(s), it is difficult to judge the importance of the inhibitor(s) in relation to the free enzyme activity. However, the QTLs specific for free enzyme activity were mapped on chromosomes 3H, 4H, 6H and 7H and these may be candidate LD inhibitor sites.

Several QTLs for the LD activity have been detected in the present study, which provide a tool for marker assistant selection of limit dextrinase. The QTLs on the chromosomes 2H and 5H were similar for both total and free enzyme activities in the two mapping populations. The candidates for these loci may be the gibberellin acid induced genes (Speulman and Salamini, 1995), which have been mapped on chromosomes 2H and 5H (see Chapter 6). It is possible that these loci enhance the free activity by increasing the synthesis of the total enzyme. The most interesting locus is on chromosome 2H linked with the RFLP marker ABC486. This locus also controls the enzyme activity of α-amylase and β-glucanase, plant height and photoperiod response. It is well known that all these traits are regulated by gibberellic acids (Briggs, 1992; Bernier et al, 1993; Faveret et al, 1978; Fincher and Stone, 1993; Hooley, 1994). These provide some indirect evidence to support that limit dextrinase is synthesised following the germination under influence of gibberellic acid (Hardie, 1975; Lee and Pyler,1984). In contrast, the limit dextrinase in rice (*Oryza sativa*) (Yamada, 1981; McCleary, 1992) and pea (*Pisum sativium*) (Vlodawsky et al, 1971) is present in the mature grain and converted to free form after germination.

5.3.3 Interaction of hydrolytic enzymes for degrading starch

Alpha-amylase, β-amylase, limit dextrinase and α-glucosidase jointly hydrolyse starch into simple sugars during germination (MacGregor, 1987). Theoretically, these enzymes may interact with each other to degrade starch. Indeed, dramatic synergism has

been observed between α-amylase and α-glucosidase (Sun and Henson, 1990; Sissons and MacGregor, 1994). However, the relationship between α-amylase, β-amylase and limit dextrinase is not clear. The present study showed that the *Amy1* and *Bmy1* loci contributed to the enzyme activity of limit dextrinase, but in a negative sense (Table 5.3). In other word, high levels of α- and β-amylase repress the activity of limit dextrinase. This observation was supported by a previous study (Sun and Henson, 1991). Nevertheless, the negative regulation appears to result by controlling the release of the enzyme rather than synthesis, as this is only observed in the levels of free enzyme and not for total enzyme (Table 5.3). The mechanism underlying the negative regulation between the enzymes may be due to competition for substrate. If this is the case, it will be difficult to improve DP by increasing the activity of single hydrolytic enzyme. This can also explain why a positive correlation between DP and the activity of single hydrolytic enzyme is not always detectable (Evans et al, 1995; Gibson et al, 1995).

5.3.4 Heritability and QTL mapping

Between five and seven QTLs have been found to control the activity of α-amylase in germinated barley in the three mapping populations. Most of the QTLs were located in similar chromosomal regions in the different populations. A total of ten loci have been identified as controlling the enzyme activity of α-amylase. These loci can explain 36.3% to 45.5% (multilocus r^2) of the total variation of enzyme activity in the different populations. A previous study has shown that the heritability of α-amylase activity ranged from 37% to 65% depending on the cross (Kaeppler and Rasmusson, 1991). Therefore, the QTLs identified in the present study should include most, if not all, of the loci controlling activity of this enzyme. This may also be true for 1,3-1,4-β-glucanase and limit dextrinase, although in these cases the heritability of enzyme activity is not clear.

5.3.5 Common QTLs controlling multiple traits

The localisation of QTLs permit the detection of parallelism between QTL commonly found in many crosses. Two common loci were identified on chromosome 5H, which control seed dormancy and the activity of α-amylase, 1,3-1,4-β-glucanase and limit dextrinase (Figs. 5.4, 5.5, 5.6). These loci have also been identified as controlling seed dormancy (Fig. 5.7; Ullrich et al, 1993), 1,3-1,4-β-glucanase activity (Han et al, 1995), and diastatic power and milling energy (Chalmers et al, 1993; Oziel et al, 1996; Thomas et al, 1996) in other studies. These results are not surprising. Alpha-amylase, β-glucanase and limit dextrinase all contribute to diastatic power and the milling energy QTL in this region may relate to enzyme activity rather than grain structure (Thomas et al, 1996). It has been suggested that seed dormancy, controlled by this locus, was related to the early germination stage of β-glucanase activity and endosperm cell wall breakdown (Han et al, 1995). However, this region also controls activity of limit dextrinase and α-amylase in the present study. It is reasonable to think that the increase of enzyme activity may only result from the breakdown of dormancy rather than be the cause.

Another common locus was identified on chromosome 2H, which controlled the enzyme activity of α-amylase, limit dextrinase and 1,3-1,4-β-glucanase (Table 5.1, 5.2, 5.3) and plant height (data not shown). An equivalent locus has also been found to control DP in a previous study (Hayes et al, 1993). As this QTL is closely linked with the *Bmy2* locus, it has been thought that the QTL for diastatic power on chromosomes 2H is related to the activity of β-amylase (Hayes et al, 1993). However, early research indicated that the *Bmy2* locus is not expressed in developing seed (Shewry et al, 1988). The present study further showed that this locus was not related to the activity of β-amylase (see Chapter 3) but may be linked to a gene controlling the activity of α-amylase, limit dextrinase and 1,3-1,4-β-glucanase. This locus may represent a regulatory gene controlling the activity of all the hydrolytic enzymes during germination or a closely linked gene cluster.

Chapter 6: Molecular Mapping of GA_3-regulated Genes and GA-responsive Loci

6.1 Introduction

Common loci have been identified as controlling the activity of several hydrolytic enzymes (see Chapter 5). It is known that all these hydrolytic enzymes are synthesised during seed germination and are regulated by gibberellins (Banilk et al, 1997; Fincher, 1989; Fincher and Stone 1993; Jones and Jacobsen, 1991; Stuart et al, 1986; Taiz and Jones, 1970; Zhang et al, 1997). It is common practice to stimulate production of the enzymes in the malting industry by using GA_3 (Bamforth and Barclay, 1993). Therefore, the common loci controlling enzyme activity could be related to genes involved in the GA response.

The mechanism of GA induced gene expression is complex. It may include GA production, signal transduction and response (Hooley, 1994). Analysis of dwarf mutants provided a good approach to understand the mechanism of GA-induced gene expression. Two kinds of dwarf mutants responding to GA have been identified in barley. One group are GA-deficient mutants which have a low level of endogenous GA and are very sensitive to exogenous GA_3 application (Boother et al, 1991; Falk and Kasha, 1982; Hentrich et al, 1985; Speulman and Salamini, 1995; Zwar and Chandler, 1995). The others are a GA-insensitive mutants which show normal endogenous GA levels but do not respond to exogenous GA_3 (Favret et al, 1978). From these results, it was concluded that there are two sets of genes controlling GA-induced gene expression: synthesis of GA and responsiveness to GA (Favret et al, 1978; Kusaba et al, 1991). Further analysis of general barley varieties showed that malting grade barleys produced more GA than do feed grade barleys (Proudlove and Muller, 1989) and the aleurone layers from different varieties have different susceptibilities to exogenous GA_3 (Palmer, 1988; Palmer et al,

1989). These results demonstrated the possibility to improve GA production and responsiveness through selection.

Progress has been achieved recently towards understanding the molecular mechanisms by which GA elicits the physiological response in the plant. Two GA_3-regulated cDNAs from a dwarf mutant barley have been cloned (Speulman and Salamini, 1995), which showed significant homology with mammalian epidermal growth factors and the D7 family of late embryogenesis-abundant protein. More importantly, another GA-induced gene, *GAmyb*, has been identified, which is the sole GA-regulated transcription factor required for transcriptional activation of the high-pI α-amylase promoter (Gubler et al, 1995). In the present study, the three GA induced genes were mapped and a GA-responsive locus which controls the activity of α-amylase, 1,3-1,4-β-glucanase and limit dextrinase, plant height and photoperiod response was identified.

6.2 Results

6.2.1 Chromosomal locations of the GA_3 induced genes

Two GA_3 regulated cDNA, *Es1A* and *Es2A*, have been isolated from dwarf mutant dbg567 (Speulman and Salamini, 1995). PCR primers were designed to amplify the partial fragments of these genes from the barley genome. The primers (upper primer: 5'GCC AGC CAT GGA AGG AAA; lower primer: 5' CGA GCA CAC GAA CAC TAC A) for *Es1A* gene amplified the gene from 411 to 643 base pairs and a fragment with 233 bp was expected based on the published sequence (Speulman and Salamini, 1995). The primers for *Es2A* (upper primer: 5' CGA GGC TGT CAC GAA GTC T; lower primer: 5' GAA ACG GAA GGA GGG AAA G) were predicted to amplify a 458 bp fragment from positions 209 to 666 (Speulman and Salamini, 1995). Using the DNAs from the six mapping parents (Clipper, Sahara, Galleon, Haruna Nijo, Chebec and Harrington) as templates, a single DNA band was produced from the two GA_3-induced genes (*Es1A* and *Es2A*). The length of the DNA fragment from *Es1A* was about 230 bp, similar to the

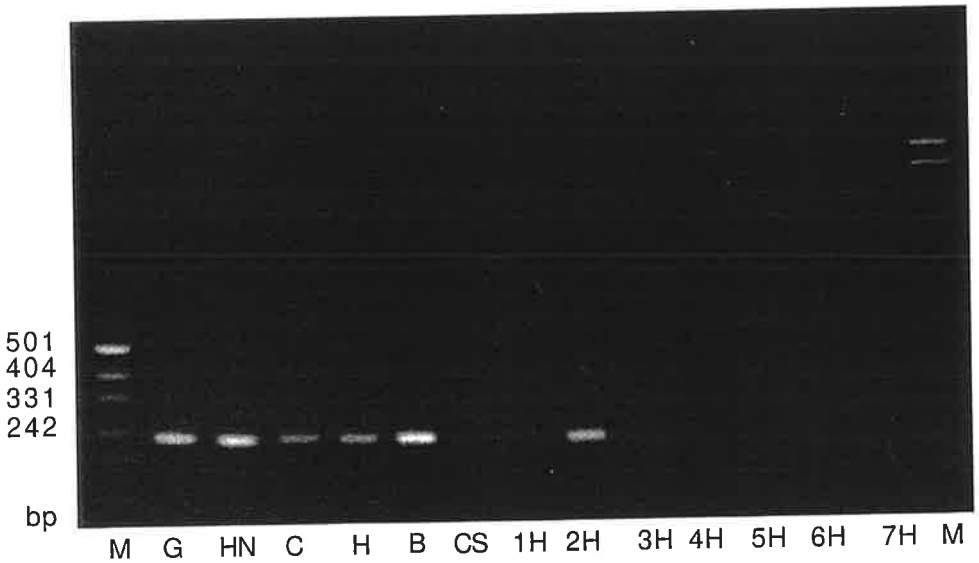

Fig. 6.1 PCR products of wheat, barley and wheat-barley addition line DNA as template using *Es1A* primers (M: DNA marker; G: Galleon; HN: Haruna Nijo; C: Chebec; H: Harrington; B: Betzes; CS: Chinese spring)

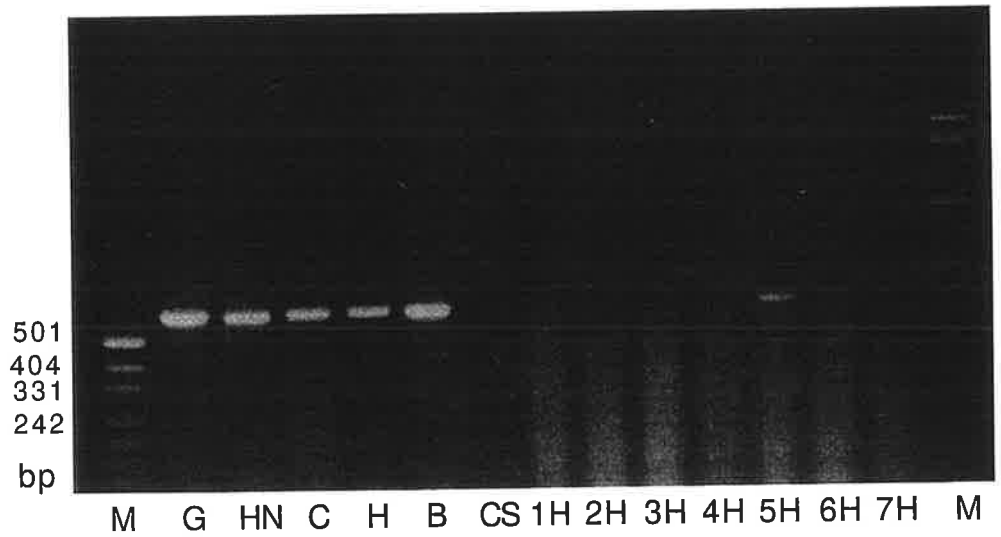

Fig. 6.2 PCR products of wheat, barley and wheat-barley addition line DNA as template using *Es2A* primers (M: DNA marker; G: Galleon; HN: Haruna Nijo; C: Chebec; H: Harrington; B:Betzes; CS: Chinese spring)

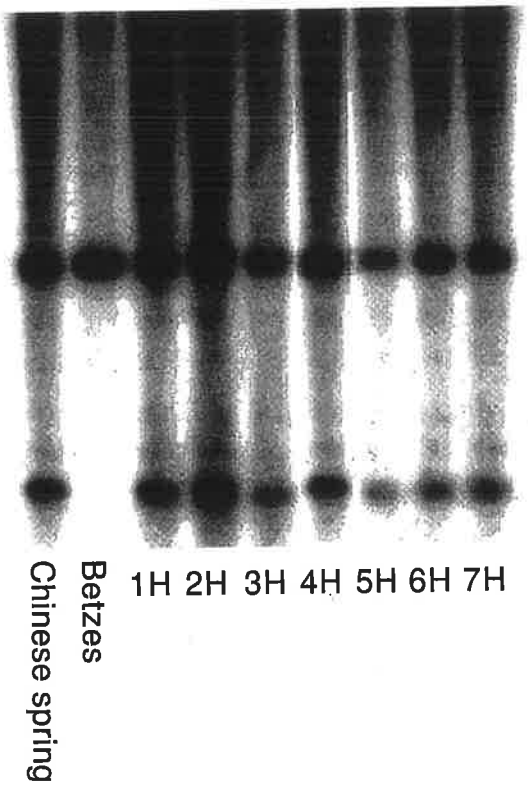

Fig. 6.3 Southern blot analysis showed no polymorphism of *GAmyb* between barley and wheat. The DNAs were digested with *EcoRI* and probed with *GAmyb*.

predicted size (Speulman and Salamini, 1995) (Fig. 6.1). On the other hand, the fragment amplified using *Es2A* primers was about 600 bp, 150 bp longer than the predicted length (Fig. 6.2). This suggests that there is a small intron for the *Es2A* gene.

When the DNAs from the seven wheat-barley addition lines and their parents (wheat *cv* Chinese Spring; barley *cv* Betzes) were used as template, the barley specific band was only detected in the addition line 2H for the *Es1A* primers and on the addition line 5H for the *Es2A* primers (Fig. 6.1; Fig. 6.2). These results demonstrated that *Es1A* and *Es2A* were located on the barley chromosomes 2H and 5H, respectively. However, the DNA fragments from both *Es1A* and *Es2A* showed no polymorphism between the six parental varieties when separated with either agarose or polyacrylamide gel electrophoresis. Therefore, the amplified DNA fragments were further cloned into the pGEM-T vector and used as probe to map the genes by Southern hybridisation.

Another GA_3-regulated cDNA clone, *GAmyb*, was isolated from a barley aleurone cDNA library (Gubler et al, 1995). This gene is able to bind to the promoter sequence of α-amylase and activate transcription of a high-pI α-amylase *in vitro* (Gubler et al, 1995). It has been postulated that *GAmyb* is a part of the GA-responsive pathway leading to α-amylase gene expression in the aleurone cells.

Using *GAmyb* as a probe (kindly provided by Dr. J Jacobson, CSIRO, Canberra), Southern blot analysis showed a single band in both barley and wheat genome when the DNAs were digested with *EcoRI* and *EcoRV* (Fig. 6.3). Lack of polymorphism between barley and wheat prevented the localisation of this gene to a specific chromosome with wheat-barley addition lines. This suggests that *GAmyb* may be highly well conserved in the cereal genome.

6.2.2 Mapping the GA_3-induced genes

The DNAs from the six parental varieties (Clipper, Sahara, Galleon, Haruna Nijo, Chebec and Harrington) for the mapping populations were digested with five different restriction enzymes *EcoRI*, *EcoRV*, *DraI*, *HindIII* and *BamHI*. Southern blot analysis

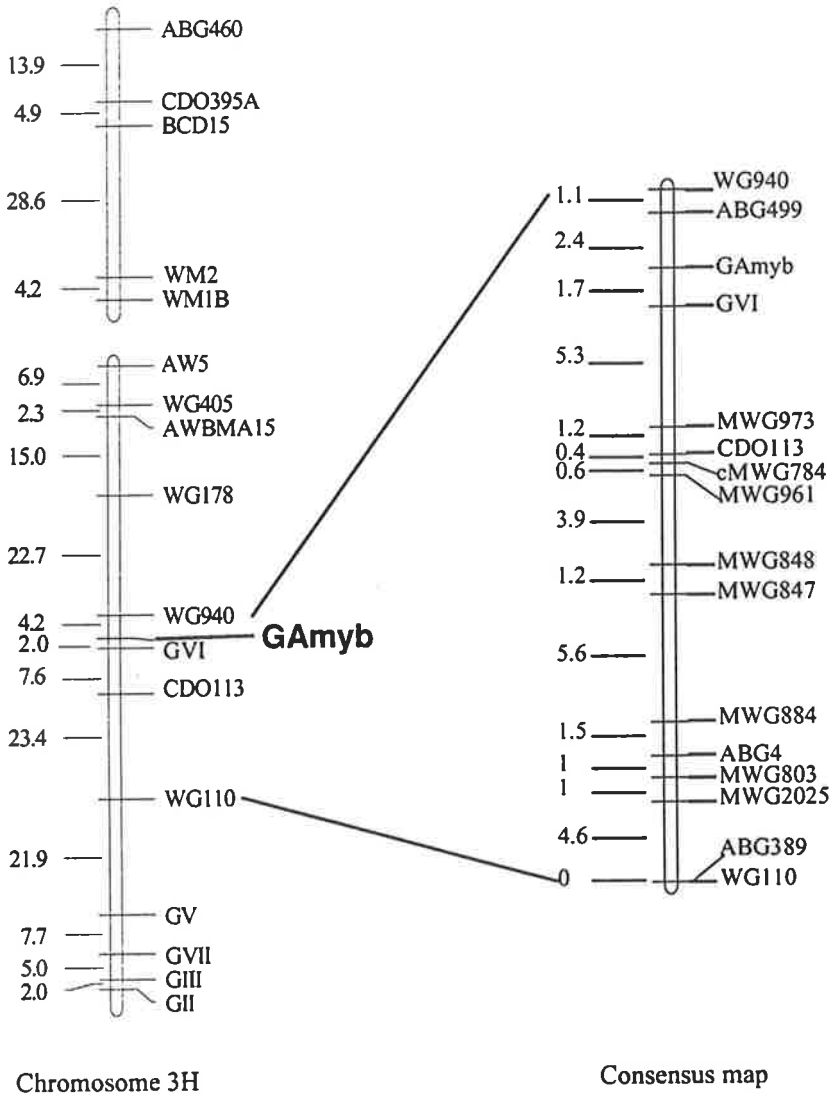

Fig. 6.4 Location of *GAmyb* on chromosome 3H

showed that a single strong band hybridised with the probe of *GAmyb* except the DNAs digested with *BamHI* (data not shown). This suggest that *GAmyb* is a single gene in the barley genome. The polymorphism was only observed between Clipper and Sahara3771, which again demonstrated the conserved nature of this gene. Combining this polymorphism into a linkage map using Mapmaker software (Lander et al, 1987), *GAmyb* was located on the long arm of chromosome 3H, 4.2 cM distal to the RFLP marker WG940 and 2 cM proximal to the 1,3-β-glucanase isoenzyme GVI (Fig 6.4; Chapter 4; Li et al, 1996).

The genes *Es1A* and *Es2A* were extremely conserved. No polymorphism was detected between the six parental varieties when the DNAs were digested with 28 different restriction enzymes and probed with the DNA fragments of *Es1A* and *Es2A* (data not shown). This prevented further mapping of these two genes on chromosomes 2H and 5H.

6.2.3 Reverse bulked-analysis to identify a GA-responsive locus

In the previous analysis (Chapter 5), common loci were identified as controlling the activity of several hydrolytic enzymes during germination. The two loci on chromosome 5H were related to seed dormancy (Chapter 5). Two common loci were also detected on chromosome 2H, in which one locus was not only controlled the activity of the hydrolytic enzymes but also controlled plant height and photoperiod response (Chapter 5). As all these traits are response to GA_3 application (Briggs, 1992; Fincher, 1989; Fincher and Stone, 1993), it was postulated that this locus may be related to a GA control gene. This was tested with a reverse bulked segregant-analysis (BSA).

The principle of BSA showed in section 3.2.5. Using the RFLP marker ABC468 linked closely to the common locus as reference, two analysis bulks were constructed in the "Galleon X Haruna Nijo" population. Theoretically, these two bulks have a similar random genetic background except at the marker locus. Any difference between the two bulks should be attributed to the genes linked closely to the marker locus.

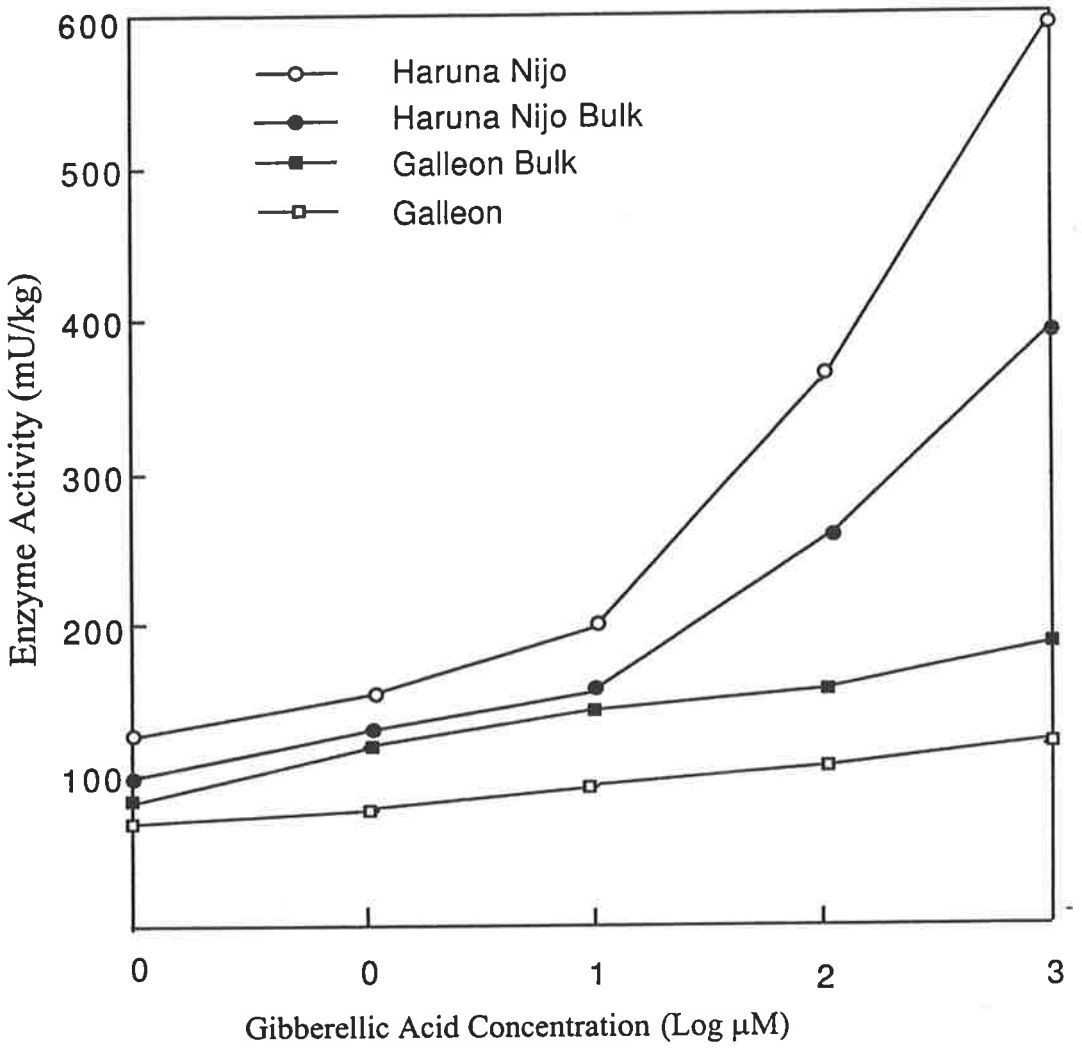

Fig. 6.5 The activity of limit dextrinase induced by GA3 during seed germination. The Galleon bulk consisted of 10 DH lines with the Galleon allele and the Haruna Nijo bulk consisted of 10 DH lines with Haruna Nijo allele at the RFLP ABC468 locus.

The two bulks with their parental varieties Galleon and Haruna Nijo were treated with different concentration GA_3. Then, activity of the hydrolytic enzymes were analysed. The activity of limit dextrinase was used as a indicator to show GA_3-response of different varieties or bulks. The LD activity of Haruna Nijo rose rapidly with increasing GA_3 concentration. On the other hand, the activity of Galleon changed only slowly (Fig. 6.5). Haruna Nijo is a high malting quality variety and Galleon is a feed variety. Therefore, this result is consistent with the previous suggestions that malting grade barleys are more sensitive to gibberellic acid (Palmer, 1988; Proudlove and Muller, 1989). Most interestingly, the bulk with Galleon genotype at the marker locus showed similar response to GA_3 as Galleon and the Haruna Nijo marker genotype was similar to the Haruna Nijo response (Fig. 6.5). This demonstrated that a locus controlling GA-response is closely linked with the RFLP marker ABC468 on chromosome 2H.

6.2.4 Selection for enzyme activity using RFLP marker ABC468

QTL analysis identified a common locus on chromosome 2H controlling the activity of α-amylase, β-glucanase and limit dextrinase (Chapter 5). This locus was further related to GA-response with reverse bulked segregant-analysis. Using RFLP marker ABC468 as reference, 10 DH lines were selected at random with each parental genotype from the "Haruna Nijo X Galleon" DH population. These had been planted at different sites and over two years. The DH lines with a different allele at the marker locus exhibited a significantly different activity for all the hydrolytic enzymes (Table 6.1). 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 Haruna Nijo allele. This locus was predicted to account for 29.5%, 31.4% and 23.4% of the total variation of enzyme activity of α-amylase, β-glucanase and limit dextrinase, respectively. This result is consistent with the conventional QTL mapping result (Chapter 5). Therefore, the markers

Table 6.1 Selection for enzyme activity using the RFLP marker ABC468

Enzyme	Galleon	Haruna Nijo	G Bulk	HN Bulk	Difference (%)
Alpha-amylase (U/g)	95.0±7.0	272.5±29.5	137.3±22.7	189.8±19.8	29.5
Beta-glucanase (U/kg)	230.5±27.5	747.5±137.5	359.3±44.0	521.8±48.0	31.4
Limit Dextrinase (mU/kg)	66.5±11.5	127.5±2.5	79.7±1.7	94.0±2.8	15.2

Note: G Bulk: Ten DH lines random selected from the Haruna Nijo X Galleon population with genotype of Galleon at the marker locus; HN Bulk: Ten DH lines random selected from the Haruna Nijo X Galleon population with the genotype of Haruna Nijo at the marker locus. Three independent bulks were constructed. The parental varieties Galleon and Haruna Nijo were harvested from three locations.

associated with the GA-responsive locus on chromosome 2H could be used to improve the activity of all the hydrolytic enzymes synthesised during seed germination.

6.3 Discussion

Gibberellins (GAs) are a class of plant hormones that exert profound and diverse effects on plant growth and development. They regulate cell growth, flower and fruit development and seed reserve mobilisation during germination (Hooley, 1994; Jacobsen et al, 1995). GAs are of agronomically important in determining plant height (Katsumi and Ishida, 1991), vernalisation (Bernier et al, 1993), dormancy (Bewley and Black, 1982) and malting quality (Fincher and Stone, 1993). Consequently, extensive studies have been made on the chemistry, metabolism, synthesis, transduction and response to gibberellins (Chandler, 1992; Hooley, 1994; Jacobsen et al, 1995). Particularly, the observation that GA induces α-amylase secretion from barley aleurone cells (Paleg, 1960; Yomo, 1960), coupled with the development of a simple procedure for the isolation of viable aleurone layers that retain hormone sensitivity and an ability to secrete active enzymes (Chrispeels and Varner, 1967), has resulted in the widespread adoption of barley aleurone layers as a model experimental system for *in vitro* investigations of plant hormone action, the synthesis and secretion of hydrolytic enzymes, and the regulation of plant gene expression (Fincher, 1989; Gubler et al, 1995; Jones and Jacobsen, 1991). Using this system, a GA-responsive element has been identified in the promoter regions of different genes encoding hydrolytic enzymes (Banik et al, 1997; Gubler and Jacobsen, 1992; Rogers et al, 1994; Skriver et al, 1991; Skriver and Mundy, 1990; Wolf, 1992; Zhang et al, 1997). However, this knowledge has not been incorporated into varietal developing programs, as it is not known how many genes are involved in GA-induced gene expression and where these genes are located on chromosomes. In the present study, one GA-responsive locus was identified on chromosome 2H by QTL and reverse bulked segregant-analysis (Chapter 5 and Fig. 6.5). This locus controls the activities of α-amylase, 1,3-1,4-β-glucanase and limit dextrinase (Chapter 5), plant height and

photoperiod response (data not shown). A controlling gene in the same region also controls diastatic power and α-amylase activity in the other mapping population (Hayes et al, 1993). These results were supported by an mutant analysis (Favret et al, 1978), in which a single mutant gene has been identified as controlling GA-response on chromosome 2H linked to the two/six rowed locus (*V/v*). The mutant also showed short plant height, low activity of hydrolytic enzymes, and insensitivity to light and temperature. Therefore, GA may exert its effects by non-specifically stimulating the expression of wide range of genes rather than specific genes (Baulcombe et al, 1984). Although it is difficult to distinguish the pleiotrophic effects of the identified locus as a single gene or a gene family, this locus could be manipulated as a single gene in a breeding program. High density molecular markers have been located around this locus (Langridge et al, 1995) and wide genetic variation in GA-response has been demonstrated between barley varieties (Palmer, 1988; Palmer et al, 1989; Proudlove and Muller, 1989). Therefore, it may be possible to improve malting quality and adaptiveness of barley varieties by manipulating this locus in a breeding program.

The GA-responsiveness may be related to a GA-receptor. It has been suggested that GA perception is at the external surface of plasma membrane (Gilroy and Jones, 1994). Hormone binding to a cell surface receptor elicits a cascade of intracellular responses, mediated by second messenger molecules or activated enzymes, and culminating in altered patterns of gene expression (Fincher and Stone, 1993). A GA-induced cDNA; *Es1A*, shows significant homology with mammalian epidermal growth factor (Speulman and Salamini, 1995). This gene was mapped on chromosome 2H (Fig. 6.1) and may be a candidate for the GA-responsive gene identified in the QTL analysis.

In addition to the GA-responsive locus on chromosome 2H, two other common loci have been found controlling the activity of α-amylase, 1,3-1,4-β-glucanase and limit dextrinase on chromosome 5H (Chapter 5). These results confirmed work of other researchers (Han et al, 1995; Hayes et al, 1993; Oziel et al, 1996; Thomas et al, 1995). Most importantly, these two loci have been identified as the main genes controlling seed dormancy (Han et al, 1995; Ullrich et al, 1993; Chapter 5). Based on the hormonal theory

of dormancy (Amen, 1968; Bewley and Black, 1982; Jann and Amen, 1977; Wareing and Saunders, 1971), dormancy is held to be controlled by inhibitory and promotive hormones. Inhibitors (mainly ABA) are thought to be involved in the imposition and maintenance of dormancy, and they may decline with disappearance of dormancy; promoters (mainly GA) are responsible for the release from dormancy (Black and Nylor, 1959). Thus, light, chilling and other factors may break dormancy by causing a drop in the inhibitor content and a rise in the level of promoters. This theory was supported by the observations in rice that the inhibitor (ABA) appears in the grains during seed development and is retained into maturity by the dormant varieties but not by those which are non-dormant (Hayashi and Himeno, 1974 cited from Bewley and Black, 1982). It is also clear that GA is involved in grain development and the residual GA in mature, dry grain could be an important source of GA in the germinating grain (Grosselindemann et al, 1991; Raynes and Briggs, 1985). Using specific inhibitors of reactions in the GA biosynthetic pathway, Grosselindemann *et al.* (1991) have concluded that *de novo* GA synthesis begins in the embryo 24 hours after inhibition but that the GA produced is not essential for enzyme induction in the aleurone. They further concluded that any signal from the embryo that causes α-amylase induction in the aleurone must originated either from stored GA precursors or by conversion of physiologically inactive GA into an active form. This result is consistent with the very early appearance of α-amylase in imbibed grain (Fincher and Stone, 1993). Most importantly, a GA-deficient mutant gene (*nld*) was mapped in the similar region of one common QTL for dormancy (Franckowiak, 1996). Based on these findings, it can be postulated that the two loci controlling seed dormancy on chromosome 5H are responsible to GA and ABA synthesis. Higher GA content also means high activity of hydrolytic enzymes. This may explain that these two loci also contribute to the activity of hydrolytic enzymes even when there is no dormancy at all (Oziel et al, 1996).

The gene *GAmyb* is regulated by GA_3 and able to specifically combine with the GA-responsive element and activate expression of high pI α-amylase gene (Gubler et al, 1995). It was suggested that this gene is a part of the GA-response pathway leading to α-

amylase gene expression in the aleurone cells. In the present study, *GAmyb* was mapped on the long arm of chromosome 3H (Fig. 6.4). However, QTL analysis showed that this locus was not related to the activity of α-amylase, or indeed any other hydrolytic enzymes, in the three mapping populations. This conclusion was supported by other research (Hayes et al, 1993; Han et al, 1995; Oziel et al, 1996; Thomas et al, 1995). The possible explanation for this is that *GAmyb* is essential in the GA-responsive pathway and mutation of this gene would be lethal. Thus, this gene is highly conserved in the natural populations. It should be noted that no polymorphism was detected between the barley and wheat genes in Southern analysis. At least two-GA responsive mutants have been reported to be lethal (Favret et al, 1978).

In summary, three GA-regulated genes were mapped on chromosomes 2H, 3H and 5H, respectively. *Es1A* and *Es2A* may be the candidate genes for the common QTL (Chapter 5). The common locus on chromosome 2H is related to GA-response and that on chromosome 5H related to GA-synthesis. These loci control the activities of α-amylase, 1,3-1,4-β-glucanase and limit dextrinase, seed dormancy, plant height and photoperiod response. It seems that GA non-specifically regulates these traits during barley growth and development. Therefore, it is possible that the other hydrolytic enzymes expressed during seed germination and the early vigour for germination are also controlled by these loci.

Chapter 7 General Discussion

7.1 Gene locations and functions of hydrolytic enzymes

One aim of the present study was to map the genes encoding the hydrolytic enzymes involved in mobilising carbohydrate reserves during seed germination. These enzymes are not only physiologically important but also determine malting quality. In the present study, cloned genes of hydrolytic enzymes for degradation of cell wall and starch were mapped, including 1,3-1,4-β-glucanase, 1,3-β-glucanase, 1,4-β-xylanase, β-glucosidase, α-amylase, β-amylase, limit dextrinase and α-glucosidase (Chapter 4). These results will facilitate studying the genetics and interactions of the hydrolytic enzymes and selecting high levels of the enzyme activity in a breeding program.

Two loci have been found encoding each of α-amylase, β-amylase and 1,3-1,4-β-glucanase (Chapter 4; Brown and Jacobsen, 1982; Khursheed and Rogers, 1988; Kleinhofs et al, 1993; Kreis et al, 1988; Loi et al, 1988; Slakeski et al, 1990). However, QTL analysis showed that only one locus for each of the genes contributes to the activity of the enzyme during seed germination: *Amy1* on chromosome 6H for α-amylase, *Bmy1* on chromosome 4H for β-amylase and *EII* on chromosome 7H for 1,3-1,4-β-glucanase. These results were supported by the isoenzyme and Northern blot analysis (Fincher and Stone, 1993; MacGregor et al, 1984 ; Shewry et al, 1988).

The relationship between the coding locus and enzyme activity was particularly important for β-amylase, the key enzyme in determining diastatic power. Comparison of the cDNA sequences of β-amylase with different activity showed little difference. It has been reported that the enzyme activity of β-amylase was controlled by the expression levels of the gene, suggesting that a cis-acting element in the structural gene regulates expression. Identifying this element and the mechanism of tissue-specific expression will facilitate manipulating the enzyme activity by molecular techniques.

7.2 Regulatory loci for the enzyme activity

In addition to the structural loci, three to seven regulatory loci were identified as controlling enzyme activity with several QTLs mapped to similar chromosomal regions in the three mapping populations used. These results may provide tools to understand how the regulatory loci interact with structural loci and may help select high levels of the hydrolytic enzymes in breeding programs. However, most of the populations currently used to map malting quality include one good malting quality parent and one feed quality parent. Typical examples are the "Haruna Nijo X Galleon" population in the present study and the "Morex X Steptoe" population in the North American Barley Mapping Project (Hayes et al, 1993). These populations provide good material to understand the genetic basis of malting quality, but the results may be less useful for improving malting quality, as most of the favoured alleles have already been fixed in one variety by conventional breeding methods (Chapter 5; Hayes et al, 1993; Han et al, 1995). More cross combinations are needed to investigate the QTL controlling malting quality, especially between the varieties with high malting quality but of different origins. This may provide an opportunity to integrate several favourable alleles into one variety.

7.3 Common QTLs controlling the activity of several hydrolytic enzymes

During seed germination, a series of hydrolytic enzymes are synthesised in the aleurone and secreted into the endosperm. Several hydrolytic enzymes were found to be regulated by gibberellic acid (Banik et al, 1997; Fincher and Stone, 1993; Tibbot and Skadsen, 1996; Zhang et al, 1997). Consequently, it has long been postulated that there may exist common loci in the barley genome for controlling the activity of all the hydrolytic enzymes synthesised during seed germination (Chandler, 1992, Fincher and Stone, 1993). If this is the case, these loci would be valuable for improving malting

quality in a breeding program. In the present study, several common loci were identified controlling the activity of α-amylase, 1,3-1,4-β-glucanase and limit dextrinase. The locus on chromosome 2H not only controls the activity of α-amylase, 1,3-1,4-β-glucanase and limit dextrinase, but also plant height and photoperiod response. Another common locus on chromosome 5H controls seed dormancy and activity of α-amylase, 1,3-1,4-β-glucanase and limit dextrinase. Importantly both loci appear to be related to GA response (2H) or synthesis (5H). It is predicted that these loci also control the activity of the other hydrolytic enzymes synthesised during seed germination and related traits (Such as early vigour). The molecular markers linked with these common loci could be used to improve malting quality. Nevertheless, it is also worth noting that improvement of malting quality by these loci may risk reduced dormancy, preharvest sprouting of seed and increased plant height. This may be the reason that some dwarf mutant genes have never been successfully integrated into malting barley varieties, although many dwarf mutants have been found in barley.

7.4 Selecting the enzyme activity by molecular markers

Progress has been achieved recently in mapping the QTLs related to yield, quality and adaptability in barley (Chapter 1). Constructions of consensus linkage maps make it possible to compare the QTLs mapped in the different populations (Langridge et al, 1995; Qi et al, 1996). Figure 7.1 summarised the chromosomal locations of known QTL, including the results of the present study. The QTLs for a wide range of traits are clustered. This may be due to; pleiotrophic effects, multi-locus clusters, or the reduced recombination in regions of the genome with increased marker density (Hayes et al, 1996). In terms of genome organisation, evolution and genetic dissection, these possibilities have very different implications. In terms of breeding strategies, the net effect is that multiple phenotypes will be inherited as a unit or a "block". The availability of markers that define such key regions should be of great utility in classifying

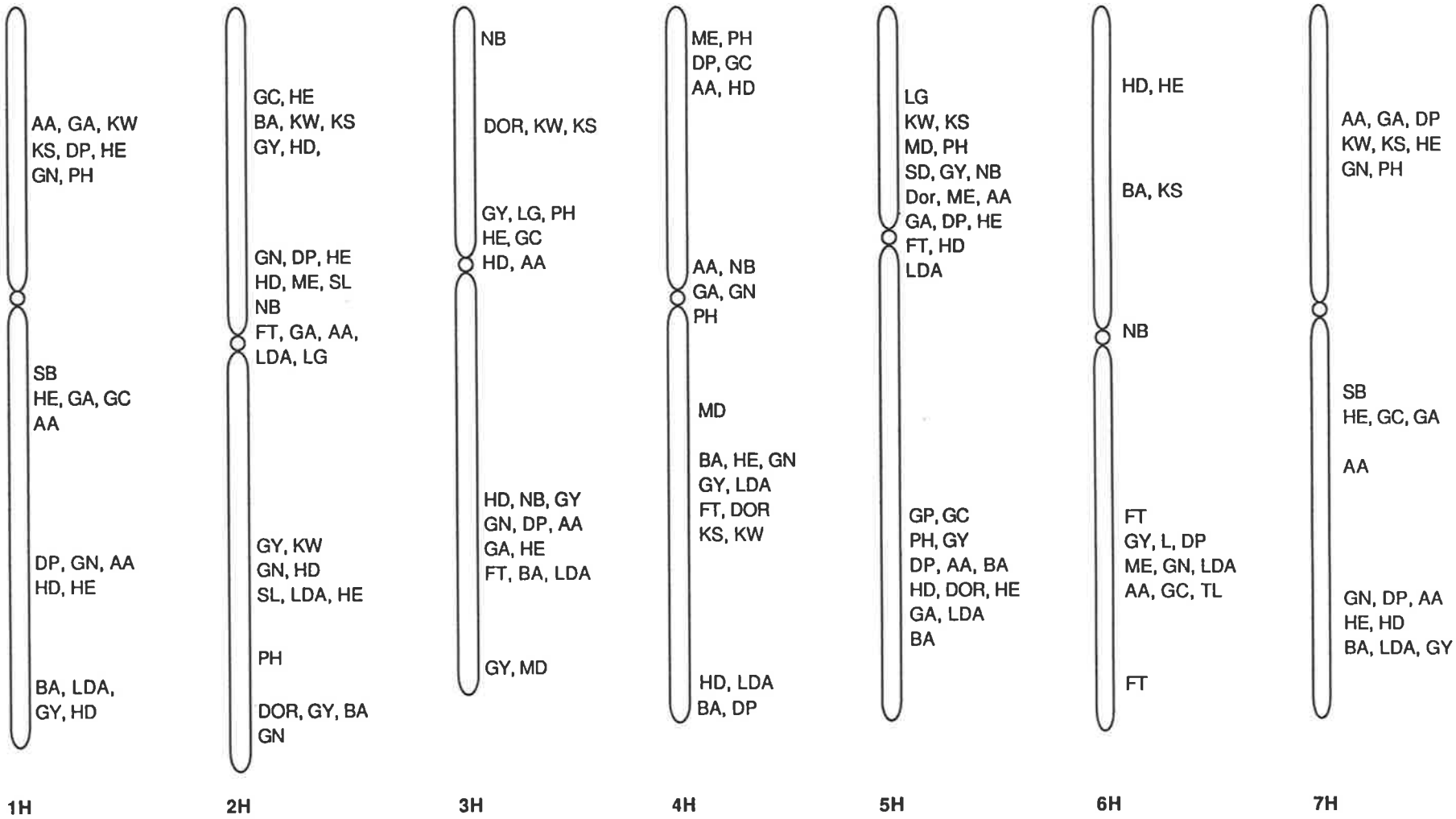

Figure 7.1 Chromosomal locations of mapped QTLs on the barley consensus linkage maps (Langridge et al, 1995; Qi et al, 1996). The QTL results are from Backes et al (1995, 1996), Bezant et al (1996, 1997), Han et al (1995), Hayes et al (1993), Laurie et al (1995), Kjaer et al (1996), Oziel et al (1996), Pan et al, (1994), Pecchioni et al (1996), Steffenson et al (1996), Thomas et al (1995, 1996), Tinker et al (1996) and the present study.

(AA: aplpha-amylase activity; BA: beta-amylase activity; DP: diastatic power; DOR: dormancy; FT: flowering time;GA: glucanase activity; GC: glucan content; GN: grain protein; GY: grain yield; FT: flowering time; HD: heading date; HE: hot water extract; KW: kernel weight; KS: kernel shape; LDA:limit dextrinase activity; LG: lodging; MD: mature date; ME: milling engery; NB: net blotch; PH: plant height; SD:straw diameter; SL: straw length; SB: Stripe blot)

germplasm and designing breeding strategies. Specifically, in relation to malting quality, this has both positive and negative effects. For example, the QTL for high levels of enzyme activity, high DP and high extract were inherited as one unit, which will increase the breeding efficiency for improvement of malting quality. On the other hand, the QTL for high grain nitrogen were consistent with most QTLs for high levels of enzyme activity. Therefore, the use of this information in a breeding program calls for further studies. Furthermore, as multiple QTLs were detected for each trait, the efficient selection of these QTLs at one time in a breeding program, presents a difficult logistical problem and raises further questions.

7.5 Isolating the genes underlying favourable QTL

Gene isolation and transformation provides a powerful tool to improve crop varieties. Ideally, the target gene is isolated; linked with effective promoters, and then transformed back into elite varieties (Kasha et al, 1993). However, progress has been slow in improving malting quality by transformation, although some important genes for malting quality have been isolated (for example, α-amylase, β-amylase, 1,3-1,4- β-glucanase, limit dextrinase). This may be because too many genes are involved in the determination of malting quality. Consequently, it is difficult to improve malting quality by manipulating so many genes. Nevertheless, the present study showed that there are common regulatory loci which control a number of traits related to malting quality. These loci could be useful not only for marker-assisted selection, but also as target genes for transformation. The next challenge is therefore to isolate these regulatory genes.

As the products of the regulatory genes are unknown, map-based or positional gene cloning is the strategy for the isolation (Wicking and Williamson, 1991). Theoretically, a gene underlying a QTL could be isolated by chromosome landing (Tanksley et al, 1995). However, conventional QTL mapping can only locate a QTL in an interval of 10 cM even for QTL of large effect, in experiments with large populations,

and using an infinite number of markers (Darvasi et al, 1993). Fine mapping procedures can locate a QTL in a interval as small as 3 cM (Paterson et al, 1990). However, 3 cM still means millions of base pairs of nuclear DNA. Therefore, more complicated QTL mapping strategy should be developed for isolating a gene underlying a QTL. The present study showed that the chromosomal locations of the common QTLs were consistent with that of the single gene mutation with similar effects. This phenomenon was also observed in other studies (Bezant et al, 1996). As an alternative, the single gene mutants could be used to isolate genes underlying QTL.

In the present study, gene mapping and QTL analysis have been used to show that the structural loci of hydrolytic enzymes are important in controlling the enzyme activities. This method could be expanded to look for the regulatory genes for the enzyme activity by RFLP mapping and large-scale cDNA analysis in the future. If a cDNA library containing cDNAs corresponding to all mRNAs for seed germination is constructed and all these cDNA clones are incorporated into the molecular linkage map, it may be possible to find the candidate gene for controlling the QTLs.

In conclusion, the important genes of hydrolytic enzymes were integrated into molecular linkage maps of barley in the present study. QTL analysis showed that only β-amylase genes (*Bmy1*) have significant and consistent effects on the enzyme activity. *Bmy1* and its closely linked molecular markers could be used to select high levels of the enzyme activity. For the enzymes synthesised during seed germination, the regulatory loci have more contribution to the enzyme activity. Most importantly, there are several common loci controlling the activity of several key hydrolytic enzymes involved in mobilisation of seed reserves. This result will allow a more efficient selection of diastatic power and extract levels.

Bibliography

Aastrup S, Munck L (198l) Genetic and plant breeding studies of barley using new analysis for 1,3-1,4-β-glucan and cell wall breakdown during malting. Barley Genetics IV pp l86-l95

Agu RC, Palmer GH (1997) Alpha-glucosidase activity of sorghum and barley malts. J Inst Brew 103:25-29

Ahn S, Tanksley SD (1993) Comparative linkage maps of the rice and maize genomes. Prec Natl Acad Sci USA 90: 7980-7984

Ainsworth CC, Gale MD, Baird S (1983) The genetics of β–amylase isoenzymes in wheat 1. Allelic variation among hexaploid varieties and intrachromosomal gene location. Theor Appl Genet 66:39-49

Allison MJ (1973) Genetic studies on the β–amylase isoenzyme of barley malt. Genetica 44:1-15

Allison MJ, Swanston JS (1974) Relationships between β–amylase polymorphisms in developing, mature and germinating grains of barley. J Inst Brew 285-291

Allison MJ, Cowe I, McHale R (l976) A rapid test for the prediction of malting quality of barley. J Inst Brew 82: l66-l67.

Allison MJ (1978) Amylase activity and endosperm hardness of high lysine barleys. J Inst Brew 84: 231-232

Allison MJ, Cowie IA, Borzucki R, Bruce F, McHale R (1979) Milling energy of barley. J Inst Brew 85: 262-264

Amen R (1968) A model for seed dormancy. Bot Rev 34: 1-31

Aoyagi K, Sticher L, Wu M, Jones RL (1990) The expression of barley α-amylase genes in *Xenopus laevis* oocytes. Planta 180: 333-340

Arends AM, Fox GP, Henry RJ, Marschke RJ, Symons MH (1995) Genetic and environmental variation in the diastatic power of Australian barley. J Cereal Sci 21:63-70

Backes G, Graner A, Foroughi-Wehr B, Fischbeck G, Wenzel G, Jahoor A (1995) Localization of quantitative loci (QTL) for agronomic important characters by the use of a RFLP map in barley (*Hordeum vulgare L.*). Theor Appl Genet 90: 294-302

Backes G, Schwarz G, Wenzel G, Jahoor A (1996) Comparison between QTL analysis of powdery mildew resistance in barley based on detached primary leaves and on field data. Plant Breeding 115: 419-421

Ballance GM, Manners DJ (1978) Structural analysis and enzymatic solubilization of barley endosperm cell walls. Carbohydrate Res 61: 107-118

Ballance GM, Meredith WOS, LaBerge DE (1976) Distribution and development of endo-β-glucanase activities in germinating barley. Plant Science 56: 459-466

Bamforth CW, Barclay AHP (1993) Malting technology and the use of malt. In: Barley: Chemistry and Technology, MacGregor et al (eds), St. Paul, Minn, American Association of Cereal Chemists, pp 297-298

Banik M, Garrett TPJ, Fincher GB (1996) Molecular cloning of cDNAs encoding 1,4-β-xylan endohydrolases from the aleurone layer of germinated barley (*Hordeum vulgare*). Plant Mol Biol 31: 1163-1172

Banik M, Li CD, Langridge P, Fincher GB (1997) Structure, hormonal regulation, and chromosomal location of genes encoding barley 1,4-β-xylan endohydrolases. Mol Gen Genet 253: 599-608

Barua UM, Chalmers KJ, Hackett CA, Thomas WTB, Powell W, Waugh R (1993) Identification of RAPD markers linked to *Rhynchosporium secalis* resistance locus in barley using near-isogenic lines and bulked segregatant analysis. Heredity 71: 177-84.

Baulcombe D, Lazarus C, Martienssen R (1984) Gibberellins and gene control in cereal aleurone cells. J Embryol Exp Morphol Suppl 83: 119-135

Baxter ED, Wainwright T (1979) Hordein and malting quality. J Am Aoc Brew Chem 37: 8-12

Becker J, Heun M (1995) Mapping of digested and undigested random amplified microsatellite polymorphisms in barley. Genome 38: 991-998

Beckmann JS (1988) Oligonucleotide polymorphisms: a new tool for genomic genetics. Bio/tech 6: 1061-1063

Beckmann JS, Soller M (1986) Restriction fragment length polymorphisms in plant genetic improvement. In: Oxford surveys of plant molecular and cell biology, Miflin B (ed), 3: 196-250

Beckmann JS, Soller M (1983) Restriction fragment length polymorphisms in genetic improvement: methodologies, mapping and costs. Theor Appl Genet 67: 35-43

Beckmann JS, Soller M (1989) Backcross inbred lines for mapping and cloning of loci of interest. In: Development and application of molecular markers to problems in plant genetics, Burr B, Helentjaris T (eds), Brookhaven National Laboratory, New York pp 117-122

Bendelow VM (1964) Inheritance of free beta-amylase in barleys. Can J plant Sci 44: 550-554

Bennett MD, Smith JB (1976) Nuclear DNA amounts in angiosperms. Phil Trans R Soc London 274: 227-274

Bentolila S, Guitton C, Bouvet N, Sailand A, Nykaza S, Freyssinet G (1991) Identification of an RFLP marker tightly linked to the *Ht1* gene in maize. Theor Appl Genet 82: 393-398

Bernier G, Havelange A, Houssa C, Petitjean A, Lejeune P (1993) Physiological signals that induce flowering. Plant Cell 5: 1147-1155

Bertoft E, Andrfolk C, Kulp SE (1984) Effect of pH, temperature and calcium ions on barley malt α-amylase isoenzymes. J Inst brew 90: 298-302

Bewley JD, Black M (1982) Physiology and biochemistry of seeds. Springer-Verlag, Berlin pp 199-264

Bezant JH, Laurie DA, Pratchett N, Chøjecki J, Kearsey MJ (1996) Marker regression mapping of QTL controlling flowering time and plant height in a spring barley (*Hordeum vulgare L*) cross. Heredity 77: 64-73

Bezant JH, Laurie DA, Pratchett N, Chøjecki J, Kearsey MJ (1997) Mapping of QTL controlling NIR predicted hot water extract and grain nitrogen content in a spring barley cross using marker-regression. Plant Breeding 116: 141-145

Bishop LR (1930) The Institute of Brewing Research Scheme. I The prediction of extract. J Inst Brew 36: 421-434

Bishop LR (1934) Prediction of extract. III Application of the carbohydrate regulatory principle. J Inst Brew 40: 75-91

Bjellquist B, Basse B, Olsen E, Celis JE (1994) Reference points for comparisons of two dimensional maps of proteins from different human cell types defined in a pH scale where isoelectric points correlate with polypeptide compositions. Electrophoresis 15; 529-539

Black M, Naylor JM (1959) Prevention of the onset of seed dormancy by gibberellic acid. Nature 184: 468-469

Blake T, Lybeck N, Hays P (1991) Good, bad and untested ideas in RFLP and QTL analysis. Plant Breeding Abstract 61: 1-7.

Blish MT, Sandstedt RM, Mechman DK (1937) Action of wheat amylase on raw wheat starch. Cereal Chem 14: 605-628

Bog-Hansen TC, Daussant J (1974) Immunochemical quantitation of isoenzymes. α-Amylase isoenzymes in barley malt. Analytical Biochem 61: 522-527

Boller T (1987) Hydrolytic enzymes in disease resistance. In: Plant-Microbe Interactions: Molecular and genetic perspectives, Kosuge T and Nester EW (eds) MacMillan, New York. Vol 2 pp. 385-413

Boother GM, Gale MD, Gaskin P, MacMillan J, Sponsel VM (1991) Gibberellins in shoots of *Hordeum vulgare*. A comparison between *cv*. Triumph and two dwarf mutants which differ in their response to gibberellin. Physiol Plant 81: 385-392

Botstein D, White RL, Skolinic M, Davis RW (1980) Construction of a genetic linkage map in man using restriction fragment length polymophisms. Am J Hum Genet 32: 314-331

Braaten DC, Thomas JR, Little RD, Dickson KR, Goldberg I, Schlessinger D, Ciccodicola A, D'Urso M (1988) Locations and contexts of sequences that hybridise to poly(dG-dT)(dC-dA) in mammalian ribosomal DNAs and two X-linked genes. Nucleic Acids Res 16: 865

Brandt A, Montembault A, Cameron-Mills V, Rasmussen SK (1985) Primary structure of a B1 hordein gene from barley. Carlsberg Res Commun 50: 333-345

Brandt A, Svendsen I, Hejgaard J (1990) A plant serpin gene: structure, organisation and expression of the gene encoding barley protein Z4. Eur J Biochem 194 : 499-505

Breese EL, Mather K (1957) The organisation of polygenic activity within chromosome in *Drosophila*. I. Hair characters. Heredity 11: 373-395

Briggs DE, Clutterbuck VJ (1973) Generation of α-amylase in germinating *Hordeum distichon*.. Phytochem 12: 1047-1050

Briggs DE (1978) Barley. Chapman & Hall London, pp 612

Briggs DE, Morrall P (1984) Endosperm breakdown in decorticated grains grown at 25 ^{0}C on a wet substratum. J Inst Brew 90: 363-370

Briggs DE, Raynes JG (1985) α-Amylase isoenzymes of germinated barley. J Inst Brew 3: 67-72

Briggs DE (1992) Barley germination: Biochemical changes and hormonal control. In Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology, Shewry PR (ed), CAB Intern Wallingford pp 369

Brown AH, Jacobsen JV (1982) Genetic basis and natural variation of α-amylase isoenzyme in barley. Genet Res 40: 315-324

Brown PH, Ho THD (1986) Barley aleurone layers secrete a nuclease in response to gibberellic acid. Plant Physiol 82: 801-806

Bryce JH, Kim NJ, Zammit R, McCafferty CA, Pinheiro MGM, Kendall GL (1995) Branched dextrins in wort: is their presence inevitable? EBC Congress, pp 285-292

Bubeck DM, Goodman MM, Beavis WD, Grant D (1993) Quantitative trait loci controlling resistance to gray leaf spot in maize. Crop Sci 33: 838-847

Bunce NAC, Forde BG, Kreis M, and Shewry PR (1986) DNA restriction fragment length polymorphism at hordein loci: application to identifying and fingerprinting barley cultivars. Seed Sci Techn 14: 419-429

Burr B, Burr FA, Thomson KH, Albertson MC, Stuber CW (l988) Gene mapping with recombinant inbreds in maize. Genetics 118: 519-526.

Caetano-Anolles G, Bassam BJ, Gresshoff PM (1991) DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. Bio/Technology 9: 553-557

Callis J, Ho THD (1983) Multiple forms of the gibberellin-induced α-amylase from the aleurone layers of barley seeds. Arch Biochem Biophys 224: 224-234

Cameron-Mills V, Brandt A (1988) A r-hordein gene. Plant Mol Bio 11: 449-461

Chalmers KJ, Barua UM, Hackett CA, Thomas WTB, Waugh R, Powell W (1993) Identification of RAPD markers linked to genetic factors controlling the milling energy requirement of barley. Theor Appl Genet 87:314-320

Chandler PM, Zwar JA, Jacobsen JV, Higgins TJ, Inglis AS (l984) The effects of gibberellic acid and abscisic acid on α-amylase mRNA levels in barley aleurone layers studies using an α-amylase cDNA clone. Plant Mol Bio 3: 407-418.

Chandler PM, Mosleth E (1990) Do gibberellins play an *in vivo* role in controlling α-amylase gene expression? In: Proceed 5th Intern Symp on Pre-harvest Sprouting in Cereals, Ringlund K, Mares DJ (eds), Westview Press, Boulder, CO, USA pp 100-109.

Chandler PM, Jacobsen JV (1991) Primer extension studies on α-amylase mRNAs in barley aleurone II. Hormonal regulation of expression. Plant Mol Biol 16: 637-645

Chandler PM (1992) Gibberellin response in barley. In: Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology, Shewry PR (ed). CAB Int Oxford pp 403-411

Chen L, Fincher GB, Høj PB (1993) Evolution of polysaccharide hydrolase substrate specificity. J Biol Chem 268:13318-13326

Chen L, Garrett TPJ, Varghese JN, Fincher GB, Høj PB (1995) A tetrad of ionisable amino acids is important for catalysis in barley β-glucanases. J Biol Chem 270: 8093-8101

Chernshev AI, , Davletova SK, Bashkivov VI, Shakhmanov SL, Ananiev EV (1989) Nucleotide sequence of B1 hordein gene of barley. Genetics 25: 1349-1355

Chrispeels MJ, Varner JE (1967) Gibberellic acid-enhanced synthesis and release of α-amylase and ribonuclease by isolated barley aleurone layers. Plant Physiol 42: 398-406.

Clutterbuck VJ, Briggs DE (1973) Enzyme formation and release by isolated barley aleurone layers. Phytochem 12: 537-546

Collins NC, Paltridge NG, Ford CM, Symons RH (1996) The *Yd2* gene for barley yellow dwarf virus resistance maps close to the centromere on the long arm of chromosome 3. Theor Appl Genet 97: 858-864

Cowen NM (1988) The use of replicated progenies in marker-based mapping of QTL's. Theor Appl Genet 75: 857-862.

Cubitt IR (1991) The commercial application of biotechnology to breeding. Plant Breeding Abstract 61: 151-158

Darvasi A, Soller M (1992) Selective genotyping for determination of linkage between a marker locus and a quantitative trait locus. Theor Appl Genet 85: 353-359

Darvasi A, Weinreb A, Minke V, Weller JI, Soller M (1993) Detecting marker-QTL linkage and estimating QTL gene effect and map location using a saturated genetic map. Genetics 134: 943-951

Delcour JA, Verschaeve SG (1987) Malt diastatic power. Part II. A modified EBC diastatic power assay for the selective estimation of beta-amylase activity. Time and temperature dependence of the release of reducing sugars. J Inst Brew 93:296-301

Demant P, Hart AM (1986) Recombinant congenic strains-a new tool for analysing genetic traits determined by more than one gene. Immunogenetics 24: 416-422

East EM (1915) Studies on size inheritance in *Nicotiana*. Genetics 1: 164-176

Edwards A, Civitello A, Hammond HA, Caskey TC (1991) DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. Am J Hum Genet 49: 746-756

Edwards MD, Stuber CW, Wendel JF (1987) Molecular marker-facilitated investigations of quantitative traits loci in maize. I. Numbers, genomic distribution and types of gene action. Genetics 116:113-125

Edwards MD, Helentjaris T, Wright S, Stuber CW (1992) Molecular-marker-facilitated investigations of quantitative trait loci in maize. Theor Appl Genet 83: 765-774

Eglinton JK, Evans DE, Brown AHD, Lance RCM (1995) Techniques for the experimentation of isoenzyme variation in malting quality enzymes. Proc 45th Aust Cereal Chem Conf pp530-533

Ellis RP, Swantston JS, Taylor K, Bruce FM (1989) Environmental constraints on the efficiency of selection for malting quality in barley. Ann Appl Biol 114:349-357.

Enari TM, Sopanen J (1986) Centenary review: mobilisation of endospermal reserves during the germination of barley. J Inst Brew 92: 25-31

Enevoldsen BS , Schmidt F (1973) Dextrins in wort and beer. II. Distribution of oligo- and megalo-saccharides during mashing. EBC Congress, pp 135-148

Enevoldsen BS, Schmidt F (1974) Dextrins in brewing: studies on the single-branched and multi-branched dextrins in brewing. J Inst Brew 80:521-533

Enevoldsen BS (1975) Debranching in brewing. EBC Congress, pp 683-697

Entwistle J (1988) Primary structure of C-hordein gene from barley. Carlsberg Res Commun 53: 247-258

Erdal K, Jensen MO, Kristensen M, Krough JJ, Riis P, Vaag P (1993) Total β-amylase in barley used as a screening criterion for combined amylolytic activity in malt. EBC Congress, pp 147-154

Essen A (1993) Beta-glucosidase: Biochemistry and Molecular Biology. ACS Symp Series 533. Am Chem Soc, Washington DC

Evans DE, Lance RCM, Eglinton JK, Logue SJ, Barr AR (1995) The influence of beta-amylase isoform pattern on beta-amylase activity in barley and malt. Proc 45th Australian Cereal Chem Conf, Adelaide. pp357-363

Evans DE, Macleod LC, Eglinton JK, Gibson CE, Zhang X, Wallace W, Skerritt JH Lance RCM (1997a) Measurement of beta-amylase in malting barley (*Hordeum vulgare L.*). Part 1: Development of a quantitative ELISA for beta-amylase. J Cereal Sci. In press

Evans DE, Wallace W, Lance RCM, Macleod LC (1997b) Measurement of beta-amylase in malting barley (*Hordeum vulgare L.*). Part 2 The effect of germination and kilning on beta-amylase. J Cereal Sci. In press

Falconer R (1960) Introduction to quantitative genetics. New York: Ronald Press.

Falk DE, Kasha KJ (1982) A vegetative dwarf mutant in barley. Barley Genet Newslet 12: 69-72.

Favret EA, Favret GC, Malvarez EM (1978) Genetic regulatory mechanisms for seedling growth in barley. Barley Genetics IV pp 37-41

Fernandez DE, Staehelin LA (1985) Structural organisation of ultra-rapidly frozen barley aleurone cells actively involved in protein secretion. Planta 165: 323-334

Fincher GB (1975) Morphology and chemical composition of barley endosperm cell walls. J Inst Brew 81: 116-122

Fincher GB (1976) Ferulic acid in barley cell walls: A fluorescence study. J Inst Brew 82: 347-349

Fincher GB, Lock PA, Morgan MM, Lingelbach K (l986) Primary structure of the 1,3-1,4-β-glucan 4-glucanohydrolase from barley aleurone. Proc Natl Acad Sci USA 3: 2081-2085.

Fincher GB (1989) Molecular and cellular biology associated with endosperm mobilisation in germinating cereal grains. Ann Rev Plant Physiol Plant Mol Biol 40: 305-346.

Fincher GB (1992) Cell wall metabolism in barley. In: Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology, Shewry PR (ed). CAB Int Oxford pp 413-437

Fincher GB, Stone BA (1993) Physiology and biochemistry of germination in barley. In: Barley: Chemistry and Technology, MacGregor AW and Bhatty RS (eds), Am Assoc Cereal Chem, St Paul MN, pp 247-295

Foster AE, Prentice N (1987) Barley. In: Nutritional quality of cereal grains: Genetic and agronomic improvement, Olson RA and Frey KJ (eds). ASA, CSSA and SSSA, Madison. pp 337-396

Forster BP, Thompson DM, Watters J, Powell W (1991) Water-soluble proteins of mature barley endosperm genetic control, polymorphism, and linkage with β–amylase and spring winter habit. Theor Appl Genet 81:787-792

Fra-Mon P, Salcedo G, Aragoncillo C, Garcia-Olmedo F (1984) Chromosomal assignment of genes controlling salt-soluble proteins (albumins and globulins) in wheat and related species. Theor Appl Genet 69: 167-172

Franckowiak JD (1996) Barley morphological linkage maps. http://www.probe.nalusda.gov

Garchon HJ, Bach JH (1992) Mouse map fills the gap. Curr Biol 2: 268-270

Garcia-Olmedo F, Salcedo G, Sanchez-Monge R, Hernandezlucas C, Carmona MJ, Lopezfando JJ, Fernaandez JA, Gomez L, Royo J, Garcia-Maroto F, Castagnaro A, Carbonero P (1992) Trypsin/α-amylase inhibitors and thionins: possible defense proteins from barley. In: Barley: Genetics, Molecular Biology and Biotechnology, PR Shewry (ed), CAB International Oxon. pp335-350

Gibson TS, Solah V, Glennie-Holmes MR, Taylor HR (1995) Diastatic power in malted barley: contributions of malt parameters to its development and the potential of barley grain beta-amylase to predict malt diastatic power. J Inst Brewing 101:277-280

Giese H, Hejggard J (1984) Synthesis of salt-soluble proteins in barley. Pulse labeling study of grain filling in liquid-cultured detached spikes. Planta 161:172-177

Gilroy S, Jones RL (1994) Perception of gibberellins and abscisic acid at the external face of the plasma membrane of barley (*Hordeum vulgare L.*) aleurone protoplasts. Plant Physiol 104: 1185-1192

Giovannoni JJ, Wing RA, Ganal MW, Tanksley SD (1991) Isolation of molecular markers from specific chromosomal intervals using DNA pools from existing mapping populations. Nucleic Acid Res 19:6553-6558

Goering KJ, Eslick RF (1976) Barley starch. VI. A self-liquefying waxy barley starch. Cereal Chem 53: 174-180

Graner A, Jahoor A, Schondelmaier J, Siedler H, Pillen K, Fischbeck G, Wenzel G, Herrmann RG (l99l) Construction of an RFLP map of barley. Theor Appl Genet 83: 250-256

Graner A (1994) Barley Igri X Franka mapping data. greengenes.cit.cornell.edu

Graner A (1996) Molecular mapping of genes conferring disease resistance: The present state and future aspects. Proc V IOC and VII IBGS pp 157-164

Grime KH, Briggs DE (1995) Release and activation of barley β-amylase. J Inst Brew 101:337-343

Grosselindemann E, Graebe JE, Stock D, Hedden P (1991) ent-Kaurene biosynthesis in germinating barley (*Hordeum vulgare cv* Himalaya) caryopses and its relation to α-amylase production. Plant Physiol 96: 1099-1104

Gubler F, Jacobsen JV, Ashford AE (1986) Involvement of the Golgi apparatus in the secretion of α-amylase from gibberellin-treated barley aleurone cells. Planta 168: 447-452

Gubler F, Jacobsen JV (l992) Giberellin-responsive elements in the promoters of a barley high-PI α-amylase gene. Plant Cell 4: 1435-1441

Gubler F, Kalla R, Roberts JK, Jacobsen JV (1995) Gibberellin-regulated expression of a *myb* gene in barley aleurone cells: Evidence for *Myb* transactivation of a high-pI α-amylase gene promoter. Plant Cell 7: 1879-1891

Guerin JR, Lance RCM, Wallace W (1992a) Release and activation of barley beta-amylase by malt endopeptidases. J cereal Sci 15:5-14

Guerin JR, Lance RCM, Wallace W (1992b) Chromosomal location of a barley malt endopeptidase gene. Genet Res C 59:179-181

Guffy RD, Stuber CW, Edwards MD (1989) Dissecting and enhancing heterosis in corn using molecular markers. In: Proc 25th Illinois Corn Breeders School, Champaign, pp 99-120

Gupta M, Chyi YS, Romero-Severson J, Owen JL (1994) Amplification of DNA markers from evolutionary diverse genomes using single primers of simple-sequence repeats. Theor Appl Genet 89: 998-1006

Haldane JBS (1919) The combination of linkage values, and the calculation of distance between linked factors. J Genet 8:299-309

Haley CS, Knott SA (1992) A simple regression method for mapping quantitative trait loci in line crosses using flanking markers. Heredity 69: 315-324

Halford NG, Morris NA, Urwin P, Williamson MS, Shewry P (l988) Molecular cloning of the barley seed protein CMd. Biochimica et Biophysica Acta 950: 435-440

Hamada H, Petrino MG, Kakunaga T (1982) A novel repeated element with Z-DNA-forming potentials is widely found in evolutionary diverse eukaryotic genomes. Proc Natl Acad Sci USA 79: 268-270

Hammerton RW, Ho THD (1986) Hormonal regulation of the development of protease and carboxylase activities in barley aleurone layers. Plant Physiol 80: 692-697

Han F, Ullrich SE, Chirat S, Menteur S, Jestin L, Sarrafi O, Hayes PM, Jones BL, Blake TK, Wessenberg D, Kleinhofs A, Kilian A (1995) Mapping of β-glucan content and β-glucanase activity loci in barley grain and malt. Theor Appl Genet 91:921-927

Hardie GD (1975) Control of carbohydrase formation by gibberellic acid in barley endosperm. Phytochem 14:1719-1722

Hardwick WA (1977) History of brewing in the Aericas. In: The practical brewer. Master Brewers Association of the Americas, HM Broderick (ed), Madison WI, pp 1-12

Harlow E, Lane D (1988) Antibodies. A laboratory manual. Cold Spring Harbour Press, Cold Spring Harbour, New York

Harris G (1962) The enzyme content and enzymic transformations in malt. In: Barley and Malt: Biology, Biochemistry, Cook AH (ed), technology Academic Press, London, pp 583-694

Hart GE, Islam AKMR, Shepherd KW (1980) Use of isoenzyme as chromosome markers in the isolation and characterization of wheat-barley chromosome addition lines. Genet Res Commun 36:311-325

Hayes B, Harmey MA, Byrne H (1988) Multiple forms of α-amylase in malting barley, *var* Emma. J Inst Brew 94: 253-259

Hayes PM, Chen FQ (1989) Genotypic variation for *Hordeum bulbosum L.* mediated haploid production in winter and facultative barley. Crop Sci 29:1184-1188

Hayes PM, Liu BH, Knapp SJ, Chen F, Jones B, Blake TK, Franckowiak G, Rasmusson D, Sorrells M, Ullrich SE, Wesenberg D, Kleinhofs A (1993) Quantitative trait locus effects and environmental interaction in a sample of Noth American barley germ plasm. Theor Appl Genet 87:392-401

Hayes PM, Briceno G, Cerono J (1996) Using QTL in barley. Proc V IOC and VII IBGS pp 182-187

Hein J (1990) Unified approach to alignment and phylogenies. Methods Enzymol 183:626-645

Hejgaard J (1978) "Free" and "bound" β–amylase of barley grain. Characterization by two-dimensional immunoelectrophoresis. J Inst Brew 84:43-46

Hejgaard J (1984) Gene products of barley chromosome 4 and 7 are precursores of the major antigenic beer protein. J Inst Brew 90: 85-87

Hejggard J, Bjorn SE, Nielsen G (1984) Localisation to chromosomes of structural genes for the major protease inhibitors in barley grains. Theor Appl Genet 68: 127-130

Henry RJ (1989) Factors influencing the rate of modification of barleys during malting. J Cereal Sci 10: 51-59

Henry RJ (1990) Quantitative analysis of barley 1,3-1,4-β-glucanase isoenzymes by high performance liquid chromatography. J Cereal Sci 12: 187-192

Henry RJ, Johnston RP (1991) Influence of genotype-environment interaction on malt quality. Barley Genetics VI pp 478-480.

Henry RJ, Weining S, Inkerman PA (1996) Marker assisted selection for quality in barley and oat. Proc V IOC and VII IBGS pp 167-173

Hentrich W, Bergner C, Sembdner G (1985) Characterisation of a gibberellin sensitive dwarf mutant of barley (*Hordeum vulgare L., Mut.* Dornburgy 576). Plant Growth Regul 3: 103-110

Heun M, Kennedy AE, Anderson JA, Lapitan NLV, Sorrells ME, Tanksley SD (1991) Construction of a restriction fragment length polymorphism map for barley. Genome 34:437-441

Higuchi R, Von Beroldingen CH, Sensabaugh GF, Erlich HA (1988) DNA typing from single hairs. Nature 332: 543-546

Hill RD, MacGregor AW (1988) Cereal α-amylases in grain research and technology. Adv Cereal Sci Tech IX: 217-261

Hill RD, MacGregor AW, Weselake RJ, Daussant J (1987) A review of some properties of an endogenous inhibitor of cereal α-amylase. In: Proc 4th Intern Symp Preharvest Sprouting Cereals, Mares DJ (ed), Westview Press, Boulder, CO, USA, pp 474-482

Hinze K, Thompson RD, Ritter E, Salamini F, Schultzelefert P (1991) Restriction length polymorphism-mediated targeting of the *ml-o* resistance locus in barley (*Hordeum vulgare*). Proc Natl Acad Sci USA 88: 3691-3695

Høj PB, Slade AM, Wettenhall REH, Fincher GB (1988) Isolation and characterisation of a 1,3-β-glucan endohydrolase from germinating barley (*Hordeum vulgare*): Amino acid sequence similarity with barley 1,3-1,4-β-glucanase. FEBS Lett 230: 67-71

Høj PB, Hartman DJ, Morrice NA, Doan D, Fincher GB (1989) Purification of (l-3)- β–glucan endohydrolase isoenzyme II from germinated barley and determination of its primary structure from a cDNA clone. Plant Mol Biol 13:31-42

Høj PB, Fincher GB (1995) Molecular evolution of plant β-glucan endohydrolases. Plant J 7:367-379

Hooley R, Beale MH, Smith SJ (1991) Gibberellin perception at the plasma membrane of *Avena fatua* aleurone protoplasts. Planta 183: 274-280

Hooley R, Smith SJ, Beale MH, Walker RP (1993) *In vivo* photoaffinity labeling of gibberellin-binding proteins in *Avena fatua* aleurone. Aust J Plant Physiol 20: 573-584

Hooley R (1994) Gibberellins: perception, transduction and responses. Plant Mol Bio 26: 1529-1555

Hoseney RC (1994) An overview of malting and brewing. Cereal Foods World 39:675-679

Hoy JL, Maculey BJ, Fincher GB (1981) Cellulases of plant and microbial origin in germinating barley. J Inst Brew 87: 77-80

Hrmova M, Garrett TPJ, Fincher GB (1995) Subsite affinities and disposition of catalytic amino acids in the substrate-binding region of barley 1,3-β-glucanases: Implications in plant-pathogen interactions. J Biol Chem 270: 14556-14563

Huang JK, Swegle M, Dandekar AM (1984) Expression and regulation of α-amylase gene family in barley aleurones. J Mol Appl Genet 2: 579-588

Hyne V, Kearsey MJ (1995) QTL analysis: further uses of marker regression. Theor Appl Genet 91: 471-476

Ingversen J, Englyst A, Jorgensen KG (1989) Evaluation of malting quality in a barley breeding programme use of α-amylase and β-glucan levels in malt as preselection tools. J Inst Brew 95: 99-103

Islam AKMR, Shepherd KW, Sparrow DHB (1981) Isolation and characterisation of euplasmic wheat-barley chromosome addition lines. Heredity 46:161-174

Islam AKMR, Shepherd KW (1990) Incorporation of barley chromosomes into wheat. In: Biotechnology in agriculture and forestry, Bajaj YPS (ed), Vol 13 Wheat, Springer-Verlag, Berlin, Heidelberg, pp 128-151

Jackson EA, Ballance GM, Thomsen KK (1986) Construction of yeast vector directing the synthesis and release of barley (1-3, 1-4)-β-glucanase. Carlsberg Res Commun 51:445-458

Jacobsen J, Gubler F, Chandler PM (1995) Gibberellin action in germinated cereal grains. In: Plant hormones, PJ Davies (ed), Kluwer Academic publishers, pp 246-271

Jahoor A, Jacobi A, Schuller CME, Fischbeck G (1993) Genetical and RFLP studies at the *Mla* locus conferring powdery mildew resistance in barley. Theor Appl Genet 85: 713-719

Jann RC, Amen RD (1977) What is germination? In: The physiology and biochemistry of seed dormancy and germination, Khann AA (ed), North Holland Publ Co, Amsterdam, pp 7-28

Jansen RC (1994) Controlling the type I and type II errors in mapping quantitative trait loci. Genetics 138: 871-881

Jarrett SJ, Marschke RJ, Symons MH, Gibson CE, Arends AM, Henry RJ (1995) Determination of the level of biofunctional α-amylase subtilisin inhibitor in Australian barleys using an ELISA. Proc 45th Australian Cereal Chem Conf, Adelaide. pp 444-448

Jayakar SD (1970) On the detection and estimation of linkage between a locus influencing a quantitative characters and a marker locus. Biometrics 26: 451-464

Johansen W (1909) Elemente der exakten Erblichkeitsliehre. Fisher, Jena

Jones RL, Jacobsen JV (1991) Regulation of synthesis and transport of secreted proteins in cereal aleurone. Int Rev Cytol 126: 49-88

Jorgensen BB, Jorgensen OB (1963) Barley malt α-glucosidase with isomaltase activity. Acta Chem Acand 17: 1765-1770

Jorgensen OB (1965) Barley malt α-glucosidase. VI. Localisation and development during barley germination. Acta Chem Scand 19: 1014-1015

Kaeppler HF, Rasmusson DC (1991) Heritability, heterosis, and maternal effects of α-amylase activity in barley. Crop Sci 31: 1452-1455

Kasha KJ (1974) Haploids from somatic cells. In: Haplods in higher plants: advance and potential, Kasha KJ (ed), University of Guelph, Guelph pp 67-87

Kasha KJ, Falk DE, Ziauddin Z (1993) Potential improvement of barley quality through genetic engineering. In: Barley: Chemistry and Technology, MacGregor AW, Bhatty RS (eds), St. Paul, Minn: American Association of Cereal Chemists pp 419-435

Kasha KJ, Kleinhofs A (1994) Mapping of barley cross Harrington X TR306. Barley Genet Newsl 23: 65-69

Katsumi M, Ishida K (1991) Gibberellin control of cereal elongation. In: Gibberellins, Takahashi N, Phinney BO, MacMillan J (eds), Springer-Verlag, New York pp 211-219

Kessler C, Manta V (1990) Specificity of restriction endonucleases and DNA modification methyltransferases - a review (Edition 3). Gene 92: 1-248

Khursheed B, Rogers JC (1988) Barley α-amylase genes. J Biol Chem 263:18953-18960

King RC, Stansfield J (1985) A dictionary of genetics. Oxford Univ Press, New York, Oxford

Kiribuchi TT, Ohfuchi T (l99l) RFLPs analysis of α-amylase genes in barley. Barley Genetics VI pp ll7-ll9

Kjaer B, Jensen J, Giese H (1995) Quantitative trait loci for heading date and straw characters in barley. Genome 38: 1098-1104

Kjaer B, Jensen J (1996) Quantitative trait loci for grain yield and yield components in a cross between a six-rowed and a two-rowed barley. Euphytica 90: 39-48

Kleinhofs A, Cao S, Sharp PJ (1988) Mapping of nitrate reductase genes in barley and wheat. Proc 7th Intal Wheat Genetics Symp pp 541-546

Kleinhofs A, Kilian A, Saghai Maroof MA, Biyashev RM, Hayes P, Chen FQ, Lapitan N, Fenwick A, Blake TK, Kanazin V, Ananiev E, Dahleen L, Kudrna D, Bollinger J, Knapp SJ, Liu B, Sorrells M, Heun M, Franckowiak A, Hoffman D, Skadsen R, Steffenson BJ (1993) A molecular, isozyme and morphological map of the barley (*Hordeum vulgare*) genome. Theor Appl Genet 86:705-712

Kleinhofs A, Kilian A (1994) RFLP maps of barley. In: DNA-based markers in plants, Philips RL, Vasil IK (eds), Kluwer Academic publishers, Dordrecht. pp 163-198

Knapp SJ, Bridges WC (l990) Using molecular markers to estimate quantitative trait locus parameters: power and genetics variances for unreplicated and replicated progeny. Genetics 79: 583-592

Knapp SJ, Bridges WC, Liu BH (1992) Mapping quantitative trait loci using nonsimultaneous and simultaneous estimators and hypothesis tests. In: Plant genomes: Methods for genetic and physical mapping, Beckmann JS, Osborn TS (eds), Kluwer Academic Publishers, Dordrecht, The Netherlands pp 209-237

Knapp SJ (1994) Mapping quantitative trait loci. In: DNA-based markers in plant, Phillips RL, Vasil IK (eds), Kluwer Academic Publishers, London, pp 58-96

Koehler S, Ho THD (1988) Purification and characterisation of gibberellic acid-induced cysteine endoproteases in barley aleurone layers. Plant Physiol 87: 95-103

Koehler SM, Ho TD (1990a) Hormonal regulation, processing, and secretion of cysteine proteinase in barley aleurone layers. Plant Cell 2:769-783

Koehler SM, Ho THD (1990b) A major gibberellic acid induced barley aleurone cysteine proteinase which digests hordein. Plant Physio 94:251-258

Kreis M, Williamson M, Buxton B, Powell W, Hejgaard I, Svendsen B (1987) Primary structure and differential expression of β–amylase in normal and mutant barleys. Eur J Biochem 169:517-525

Kreis M, Williamson MS, Shewry PR, Sharp P, Gale M (1988) Identification of a second locus encoding β–amylase on chromosome 2 of barley. Genet Res Camb 51:13-16

Kristensen M, Svensson B, Larsen J (1993) Purification and characterization of barley limit dextrinase during malting. EBC Congress, pp 37-43

Kruger JE (1979) Modification of wheat β–amylase by proteolytic enzymes. Cereal Chem 56:298-302

Kurata N, Moore G, Nagamura Y, Foote T, Yano M, Minobe Y, Gale M (1994) Conversation of genome structure between rice and wheat. Bio/Technol 12: 276-278

Kurt JH, Bowcock AM, Erlich HA, Nevo S, Carvalli-Sforza LL (1991) Km typing with PCR: application to population screening. Am J Genet 48: 613-620

Kusaba M, Kobayashi O, Yamaguchi I, Takahashi N, Takeda G (1991) Effects of gibberellin on genetic variations in α-amylase production in germinating barley seeds. J Cereal Sci 14: 151-160

LaBerge DE, Marchylo BA (1983) Heterogeneity of the β–amylase enzymes of barley. J Am Soc Brew Chem 41:120-124

Lanahan MB, Ho THD, Rogers SW, Rogers JC (1992) A gibberellin response complex in cereal α-amylase gene promoters. Plant Cell 4: 203-211

Lance RCM, Chevalier P, Bogyo TP, Nilan RA (1987) Genetic studies of the β-glucan content of barley. Barley Genetics V, pp 841-850

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newberg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174-181

Lander ES, Botstein D (1989) Mapping the Mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics 121: 185-199

Langridge P, Karakousis A, Collins N, Kretschmer J, Manning S (1995) A consensus linkage map of barley. Molecular Breeding 1: 389-395

Laurie DL, Pratchett N, Romero C, Simpson E, Snape JW (1993) Assignment of the denso dwarfing gene to the long arm of chromosome 3H of barley by use of RFLP markers. Plant Breed 111: 198-203

Laurie DA, Pratchett N, Bezant JH, Snape JW (1994) Genetic analysis of a photoperiod response gene on the short arm of chromosome 2H of *Hordeum vulgare* (barley). Heredity 72: 619-627

Laurie DA, Pratchett N, Bezant JH, Snape JW (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

Lazaro A, Rodriguez-Palenzuela P, Marana C (1988a) Single peptide homology between the sweet protein thaumatin II and unrelated cereal α-amylase/trypsin inhibitors. FEBS Lett 239 :147-150

Lazaro A, Sanchez Monge R, Salcedo G (1988b) A dimeric inhibitor of insect α–amylase from barley. Europ J Biochem 172: 129-134.

Leach R, Tommerup H. Svendsen I, Muddy J (1991) Biochemical and molecular characterisation of three antifungal proteins from barley seed. J Biochem. 266:1564-1573

Leach R, Mundy J (1989) The bifunctional α-amylase/subtilisin inhibitor of barley: nucleotide sequence and patterns of seed specific expression. Plant Mol Biol 12: 673-682

Leach R, Kigel J, Svendsen IB, Mundy J (1995) Biochemical and molecular characterisation of a barley seed β-glucosidase. J Biol Chem 270: 15789-15797

Lebowitz RJ, Soller S, Beckmann JS (1987) Trait-based analyses for the detection of linkage between marker loci and quantitative trait loci in crosses between inbred lines. Theor Appl Genet 73: 556-562

Lee WJ, Pyler RE (1984) Barley malt limit dextrinase: varietal, environmental and malting effects. J Am Brew Chem 42:11-17

Leitch IJ, Heslop-Harrison JS (1993) Physical mapping of four site of 5S rDNA sequences and one site of the α-amylase-2 gene in barley (*Hordeum vulgare*). Genome 36: 517-523

Lenoir P, MacGregor AW, Moll M, Daussant J (1984) Identification of debranching enzymes from barley and malt by isoelectric focusing. C R Acad Sci ser 298: 243-248

Ling V, Zielinski RE (1989) Cloning of cDNA sequences encoding the calcium-binding protein, calmodulin, from barley. Plant Physiol 90: 714-719.

Lin Y, Zhang Y, Yu Z (1991) Studies on activity and isoenzyme of β-amylase in two-rowed barley and its hybrids. Barley Genetics VI pp 124

Li H, Gyllensten UB, Cui X, Saiki RK, Erlich HA, Arnheim N (1988) Amplification and analysis of DNA sequences in single human sperm and diploid cells. Nature 335: 414-417

Li CD, Langridge P, Lance RCM, Xu P, Fincher GB (1996) Seven members of the 1,3-β-glucanase gene family in barley (*Hordeum vulgare*) are clustered on the long arm of chromosome 3 (3HL). Theor Appl Genet 92:791-796

Li CD, Lance RCM, Zhang XQ, Fincher GB, MacLeod L, Langridge P (1997) Localization of functional and activity loci for limit dextrinase in germinated barley (*Hordeum vulgare)*. Theor Appl Genet

Liu ZW, Biyashev RM, Saghai Maroof MA (1996) Development of simple sequence repeat DNA markers and their integration into a barley linkage map. Theor Appl Genet 93: 869-876

Loi L, Ahluwalia B, Fincher GB (l988) Chromosomal location of genes encoding barley 1,3-1,4-β-glucan 4 glucanohydrolase. Plant Physiol 87: 300-322

Loi L, Barton PA, Fincher GB (l987) Survival of barley β-glucanase isoenzymes during kilning and mashing. J Cereal Sci 5: 45-50

Longstaff MA, Bryce JH (1991) Levels of limit dextrinase activity in malting barley. EBC Congress, pp 593-600

Longstaff MA, Bryce JH (1993) Development of limit dextrinase in germinated barley: evidence of proteolytic activation. Plant Physiol 101: 881-889

Lonn M, Tegelstrom H, Prentice HC (1992) The synthetic (TG)n polynucleotide: a probe for gene flow and paternity studies in wild plant populations? Nucl Acid Res 20: 1153

Lu MQ, Ding SR (l99l) Genetic analysis of some malting quality traits in two-rowed barley. Barley Genetics VI pp 478-480

Lundgard R, Svensson B (1986) Limited proteolysis in the carboxy-terminal region of barley β–amylase. Carlsberg Res Commun 51:487-491

MacGregor AW (1983) Cereal α-amylases: synthesis and action pattern. In: Seed Proteins, Daussant J and Vaughn J (eds), Academic Press, London, pp 1-34

MacGregor AW, MacDougall FH, Mayer F, Daussant J (1984) Changes in the levels of α-amylase components in barley tissues during germination and early seedling growth. Plant Physiol 75: 203-206

MacGregor AW, Marchylo BA (1986) α-Amylase components in excised, incubated barley embryos. J Instit Brew 92: 159-161

MacGregor AW (1987) α-Amylase, limit dextrinase, and α-glucosidase enzymes in barley and malt. CRC Critical Rev Biotech 5: 117-128

MacGregor AW, Ballance GM, Dushnicky L (1989) Fluorescence microscopy studies on 1,3-β-D-glucan in barley endosperm. Food Microstructure 8:235-244

MacGregor AW (1990) Current state of research into barley carbohydrate and enzyme. In: Proc 3th Aviemore Conf Malting Brewing and Distilling, pp 10-33

MacGregor AW, Fincher GB (1993) Carbohydrates of the barley grain. In: Barley: Chemistry and Technology, MacGregor AW and Bhatty RS (eds), Am Assoc Cereal Chem St Paul MN, pp 73-130

MacGregor AW and Bhatty RS (1993) Barley: Chemistry and technology. Am Assoc Cereal Chem St Paul MN

MacGregor AW, Macri LJ, Schroeder SW, Bazin SL (1994) Limit dextrinase from malted barley: extraction, purification, and characterisation. Cereal Chem 71: 610-617

MacGregor AW, Macri LJ, Bazin SL, Salder GW (1995) Limit dextrinase inhibitor in barley and malt and its possible role in malting and brewing. EBC Congress pp 185-192

Macleod LC, Lance RCM, Brown AHD (1991) Chromosomal mapping of the *Glb1* locus encoding (1-3,1-4)-β-D-glucan 4-glucanohydrolase EI in barley. J Cereal Sci 13:291-298

Macri LJ, MacGregor AW, Schroeder SW, Bazin SL (1993) Detection of a limit dextrinase inhibitor in barley. J Cereal Sci 18:103-106

Maeda I, Kiribuchi S, Nakamura M (1978) Digestion of barley starch granules by the combined action of α- and β-amylases purified from barley and barley malt. Agri Biol Chem 42: 259-267

Malehorn DE, Scott KJ, and Shah M (1993) Structure and expression of a barley acidic β-glucanase gene. Plant Mol Biol 22:347-360

Manners DJ, Yellowlees D (1971) Studies on carbohydrate metabolising enzyme. Part XXVI. The limit dextrinase from germinated barley. Starke 23:228-234

Manners DJ, Yellowlees D (1973) Studies on debranching enzymes. Part I. The limit dextrinase activity of extracts of certain higher plants and commercial malts. J Inst Brew 79: 377-385

Martin GB, Williams JGK, Tanksley SD (1991) Rapid identification of markers linked to a pseudomonas resistance gene in tomato by using random primers and near-isogenic lines. Proc Natl Acad Sci USA 88: 2336-23340

Martinez O, Curno W (1992) Estimating the location and the sizes of the effects of quantitative trait loci using flanking markers. Theor Appl Genet 85 :480-488.

Mather K, Jinks SL (1977) Introduction to biometrical genetics. Chapman and Hall, London

Mauch F, Staehelin LA (1989) Functional implications of the subcellular localisation of ethylene-induced chitinase and β-1,3-glucanase in bean leaves. Plant Cell 1:447-457

McCleary BV, Sheehan H (1987) Measurement of cereal α-amylase: A new assay procedure. J Cereal Sci 6: 237-251

McCleary BV, Codd R (1989) Measurement of β–amylase in cereal flours and commercial enzyme preparations. J Cereal Sci 9:17-33

McCleary BV (1992) Measurement of the content of limit dextrinase in cereal flours. Carbohydrate Res 227:257-268

McFadden GI, Ahluwalia B, Clarke AE, Fincher GB (1988) Expression sites and developmental regulation of genes encoding 1,3-1,4-β-glucanases in germinating barley. Planta 173: 500-508

McMillan I, Robertson A (1974) The power of methods for detection of major gene affecting quantitative characterisations. Heredity 32: 349-356.

Meredith WOS, Sallans HR, Rowland H (1942) Prediction of malt diastatic power of hybrid barleys. Sci Agric 22: 761-776

Meredith WOS (1943) Prediction of malt extract of hybrid barley. Sci Agri 23: 355-361

Merritt NR (1967) A new strain of barley with starch of high amylose content. J Inst Brew 73: 583-585

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease-resistance genes by bulked segregatant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA 88:9828-9832

Miflin BJ, Shewry PR (1978) The biology and biochemistry of cereal seed prolamins. Seed Protein Improvements in cereals and Grain Legumes, IAEA Vienna 1: 137-158

Mikkomen A, Porali I, Cercos M, Ho TD (1996) A major cysteine proteinase, *EPB*, in germinating barley seeds: structure of two intronless genes and regulation of expression. Plant Mol Biol 31: 239-254

Molina-Cano JL (1987) The EBC barley malt committee index for the evaluation of malting quality in barley and its use in breeding. Plant Breeding 98: 249-256

Morgan AG, Gothard PC (1977) A rapid, simple viscosmetric technique for indirect estimation of soluble beta-glucan. J Inst Brew 83:37-38

Morgante M, Olivieri AM (1993) PCR-amplified microsatellites as markers in plant genetics. Plant J 3: 175-182

Morgante M, Rafalski M, Biddle A, Tingey P, Olivier AM (1994) Genetic mapping and variability of seven soybean simple sequence repeat loci. Genome 37: 763-769

Moreno-Gonzalez J (1993) Genetic models to estimate additive and non-additive effects of marker associated QTL using multiple regression techniques. Theor Appl Genet 85:435-444.

Morrisom WR (1978) Cereal lipids. Adv Cereal Chem 2: 221-348

Morrison WR (1988) Lipids in cereal starches: a review. J Cereal Sci 8: 1-15

Mullis KB, Faloona F (1987) Specific synthesis of DNA *in vitro* via a polymerase chain reaction. Methods Enzymol 155; 335-350

Munck L, Mundy J, Vaag P (1985) Characterization of enzyme inhibitorsm in barley and their tentative role in malting and brewing. J Amer Soc Brew Chem 43: 35-38

Mundy J, Svendsen I, Hejgaard J (1983) Barley α-amylase/subtilisin inhibitor. I. Isolation and characterisation. Carlsberg Res Commun 48: 81-90

Mundy J, Rogers JC (1986) Selective expression of a probable amylase/protease inhibitor in barley aleurone cells: comparison to the barley amylase/subtilisin inhibitor. Planta 169: 51-53

Muthukrishnan S, Chandra GR (1988) Regulation of the expression of the hydrolase genes in cereal seeds. Adv Cereal Sci Tech IX: 129-159

Myers RM, Maniatis T, Lerman LS (1987) Detection and localisation of single base changes by denaturing gradient-gel electrophoresis. Method Enzymol 155: 501-527

Nakamura Y, Umemoto T, Ogata N, Kuboki Y, Yano M, Sasaki T (1996) Starch debranching enzyme (R-enzyme) from developing rice endosperm: purification, cDNA and chromosomal localisation of the gene. Planta 199:209-218

Neumann PE (1990) Two locus linkage analysis using recombinant inbred strains and Bayes' Theorem. Genetics 126: 277-284

Nielsen G, Johansen H, Jensen J, Hejgaard J (1983) Localisation on barley chromosome 4 of genes coding for β-amylase (*Bmy 1*) and protein Z (*Paz 1*). Barley Genetics Newsl 13: 55-57

Nielsen G, Johansen HB(1986) Proposal for the identification of barley varieties based on the genotypes for 2 hordein and 39 isoenzyme loci of 47 reference varieties. Euphytica 35:717-728

Nilan RA (1964) The cytology and genetics of barley. Monogr Suppl 3, Res Stud Vol 82. Washington State university Press, Pullman

Nilsson-Ehle H (1909) Kreuzunguntersuchungen an Hafer und Weizen. Lund

Ochmann H, Gerber AS, Hartl DL (l988) Genetic application of an inverse polymerase chain reaction. Genetics 120:621-623

Olsen M, Hood L, Cantor C, Doststein D (1989) A common language for physical mapping of the human genome. Science 254: 1434-1435

Oziel A, Hayes PM, Chen FQ, Jones B (1996) Application of quantitative trait locus mapping to the development of winter-habit malting barley. Plant Breeding 115:43-51

Paleg L (1960) Physiological effects of gibberellic acid. II. On starch hydrolysing enzymes of barley endosperm. Plant Physiol 35: 902-906

Palmer GH (1988) Enzyme development in the aleurone and embryos of Galant and Triumph barleys. J Inst Brew 94: 61-63

Palmer GH, Shirakashi T, Sanusi LA (1989) Physiology of germination. EBC Congress Proc pp 63-67

Pan A, Hayes PM, Chen F, Chen TH, Blake T, Wright S, Karsai I, Bedo Z (1994) Genetic analysis of the components of winterhardiness in barley (*Hordeum vulgare L.*) Theor Appl Genet 89: 900-910

Pan D, Nelson ON (1984) A debranching enzyme deficiency in endosperm of the *sugary-1* mutants of maize. Plant Physiol 74: 324-328

Paterson AH, Lander ES, Hewitt JD Peterson S, Lincoln SE, Tanksley SD (1988) Resolution of quantitative traits into Mendelian factors by using a complete linkage map of restriction fragment length polymorphism. Nature 335: 721-726

Paterson AH, Deverna JW, Lanini B, Tanksley SD (l990) Fine mapping of quantitative trait loci using selected overlapping in recombinant chromosomes, in an interspecies of tomato. Genetics 124:735-742

Paterson AH, Damon S, Hewitt JD, Rabinaowitch HD, Lincoln SE, Lander ES, Tanksley SD (1991) Mendelian factors underlying quantitative traits in tomato: comparison across species, generation, and environments. Genetics 127: 181-197

Paterson AH, Wing RA (1993) Genome mapping in plants. Curr Opin Biotechnol 4: 142-147

Paz-Ares J, Ponz F, Rodriguez-Palenzuela P (1986) Characterisation of cDNA clones of the family of trypsin/α-amylase inhibitors in barley. Theor Appl Genet 71: 842-846

Pecchioni N, Faccioli P, Toubiarahme H, Vale G, Terzi V (1996) Quantitative resistance to barley leaf stripe (*Pyrenophora graminea*) is dominated by one major locus. Theor Appl Genet 93: 97-101

Penson SP, Schuurink RC, Fath A, Gubler F, Jacobsen JV, Jones RL (1996) cGMP is required for gibberellic acid-induced gene expression in barley aleurone. Plant Cell 8: 2325-2333

Peterson DM, Forde J, Williamson M, Rhode W, Kreis M (1991) Nucleotide sequence of a chymotrypsin inhibitor-2 gene of barley (*Hordeum vulgare*). Plant Physiol 96: 1389-1390

Phillips HA, Wallace W (1989) A Cysteine endopeptidase from barley malt which degrades hordein. Phytochem 28:3285-3290

Ponze F, Paz-Ares J, Hernandez-Lucas (1986) Cloning and nucleotide sequence of a cDNA encoding the precursor of the barley toxin α-hordothionin. Europ J Biochem 156: 131-135

Pomeranz Y (1972) Scanning electron microscopy of the endosperm of malted barley. Cereal Chem 49: 5-19

Powling A, Islam AKMR, Shepherd KW (1981) Isoenzyme in wheat-barley hybrid derivative lines. Biochem Genet 19:237-254

Powell W, Galigari PDS, Swanston JS (1985) Genetical investigation into β-glucan content in barley. Theor Appl Genet 71: 461-466

Powell W, Ellis RP, Macaulay M, McNicol J, Forster BP (1990) The effect of selection for protein and isoenzyme loci on quantitative traits in doubled haploid population of barley. Heredity 65: 115-122

Powell W, Thomas WTB, Thompson DM, Swanston JS, Waugh R (1992) Association between rDNA alleles and quantitative traits in doubled haploid population of barley. Genetics 130: 187-194

Powell W, Baird E, Booth A, Lawrence P, MacAulay M, Bonar N, Young G, Thomas WTB, McNicol JM, Waugh R (1996) Single locus and multi-locus molecular assays for barley breeding and research. Proc V IOC and VII IBGS pp 175-181

Preece IA, MacDougall M (1958) Enzymic degradation of cereal hemicelluloses. II. Pattern of pentosan degradation. J Inst Brew 64: 489-500

Proudlove MO, Muller RE (1989) Immunological detection of gibberellin in barley during malting. Proc Cong Eur Brew Con, Zurich pp 243-250

Qi X, Stam P, Lindhout P (1996) Comparison and integration of four barley genetic maps. Genome 39: 379-394

Rafalski JA, Tingey SV, Williams JGK (1991) RAPD markers: a new technology for genetic mapping and plant breeding. AgBiotech News and Information 3: 645-648

Rafalski AJ (1996) In: Analysis of Non-Mammalian Genomes-A Practical Guide, Birren E & Lai E (eds), Academic Press

Ramage RT (1985) In: Barley, Rasmusson DC (ed), Amer Soc Agron, Madison Wisconsin, pp 127-154

Rasmussen SK, Brandt A (1986) Nucleotide sequence of cDNA clone for C-hordein polypeptide. Carlsberg Res Commun 51:371-379

Rasmussen SK, Hopp HE, Brandt A (1983) Nucleotide sequence of cDNA for B-hordein polypeptide. Carlsberg Res Commun 48: 187-199

Rasmussen JM (1935) Studies on the inheritance of quantitative characterisations in *pisum*. Heriditas 20: 161-180

Raynes JG, Briggs DE (1985) Genotype and the production of α-amylase in barley grains geerminated in the presense and absense of gibberellic acid. J Cereal Sci 3: 55-65

Reed KC, Mann DA (1985) Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res 20: 7207-7221

Reeves SG, Baxter ED, Martin HL (1979) Prediction of malting quality of barley by a modified Zeleny sedimentation test. J Inst Brew 85: 141-143

Reimann-Philipp U, Schrader G, Martinoia E, Barkholt V, Apel K (1989) Intra-cellular thionins of barley. A second group of leaf thionins closely related to but distinct from cell wall-bound. J Biol Chem 264: 8978-8984

Reiner L, Schmidt F, Geiger H (1984) Brewing barley in Europe, yield and quality 1980. Barley Genetics IV pp 605-611

Riedel GE, Swanberg SL, Kuranda KD, Marquette K, LaPan P, Bledsoe P, Kennedy A, Lin BY (1990) Denaturing gradient-gel electrophoresis identifies genomic DNA polymorphism with high frequency in Maize. Theor Appl Genet 80: 1-10

Rodhe W, Rosch K, Kroger K, Salamini (1990) Nucleotide sequence of a *Hordeum vulgare* gene encoding a glycine-rich protein with homology to vertebrate cytokeratins. Plant Mol Biol 14: 1057-1059

Rodolphe F, Marianne L (1993) A multi-marker model for detecting chromosomal segments displaying QTL activity. Genetics 134: 1277-1288

Rodriguez-Palnezuela P, Pintor-Toro J, Carbonorero P, Garcia-Olmedo F (1988) Nucleotide sequence and endosperm-specific expression of the structural gene for the toxin α-hordothionin in barley (*Hordeum vulgare*). Gene 70: 271-281

Rogers JC (1985) Two barley α-amylase gene families are regulated differently in aleurone layer cells. J Biol Chem 260: 3731-3738

Rogers JC, Milliman C (1983) Isolation and sequence analysis of a barley α-amylase cDNA clone. J Biol Chem 258: 8169-8174

Rogers JC, Lanahan MB, Rogers SW (1994) The *cis*-acting gibberellin response complex in high-pI α-amylase gene promoters. Plant Physiol 105: 151-158

Romagosa I, Ullrich SE, Han F, Hayes PM (1996) Use of the additive main effects and multiplicative interaction model in QTL mapping for adaptation in barley. Theor Appl Genet 93: 30-37

Ronin YI, Kirzhner VM, Korol AB (1995) Linkage between loci of quantitative traits and marker loci: multitrait analysis with a single marker. Theor Appl Genet 90: 776-786

Rudenko GN, Rommens GMT, Nijkamp HJJ, Hille J (1993) Supported PCR: an efficient procedure to amplified sequences flanking a known DNA segment. Plant Mol Genet 21:723-728.

Saghai-Maroof MA, Yang GP, Biyashev RM, Maughan PJ, Zhang Q (1996) Analysis of the barley and rice genomes by comparative RFLP linkage mapping. Theor Appl Genet 92: 541-551

Saiki RK, Scarf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N (1985) Enzymatic amplification of α-globin genomic sequences and restriction site analysis for diagnosis of sickle-cell anaemia. Science 230: 1350-1354

Saiki RK (1988) The design and optimisation of the PCR. In: PCR technology: principles and applications for DNA amplification, Erlich HA (ed), Stockton Press, New York. pp 7-15.

Salcedo G, Fra-Mon P, Molina-Cano JL, Aragoncillo C, Garcia-Olmedo F (1984) Genetics of CM-proteins (A-hordeins) in barley. Theor Appl Genet 54: 61-63

Sambrook J, Fritsch EF, Manniatis T (1989) Molecular Cloning - A Laboratory Mannual. Cold Spring Harbour Laboratory Press, Cold Spring Harbour

Sanchez-Monge R, Gomez L, Garcia-Olmedo F, Salcedo G (1986) A tetrameric inhibitor of insect α-amylase from barley. FEBS Lett 207: 105-109

Sanchez-Monge R, Gomez L, Garcia-Olmedo F, Salcedo G (1989) New dimeric inhibitor of heterologous α-amylases encoded by a duplicated gene in the short arm of chromosome 3B of wheat (*Triticum aestivum L*). Eur J Biochem 183: 37-40

Sax K (1923) The association of size difference with seed coat pattern and pigmentation in *Phaseolus vulgaries*. Genetics 8:552-560.

Schafer R, Ali S, Epplen JT (1986) The organisation of the evolutionarily conserved GATA/GACA repeats in the mouse genome. Chromosoma 93: 502-510

Schildbach R, Burbridge M, Roth F (1990) The endosperm structure of barley varieties and their malting quality. Proc 21st Convention Inst Brewing (ANZ section), New Zealand.

Schwimmer S (1950) Kinetics of malt α-amylase action. J Biol Chem 186: 181-193

Serre L, Lauriere C (1989) Limit dextrinase in cereal seed. Sciences Des Aliments 9: 645-663

Sheehan H, McCleary BV (1988) A new procedure for the measurement of Fungal and Bacterial α-amylase. Biotechnology Tech 2: 289-292

Sherman JD, Fenwick AL, Namuth DM, Lapitan NLV (1995) A barley RFLP map: alignment of three barley maps and comparisons to *Gramineae* species. Theor Appl Genet 91: 681-690

Shepherd KW, Islam AKMR (1987) Cytogenetic manipulation of barley chromosomes in wheat background. Proc 5th Int barley Genetics Symp pp 375-387

Shepherd KW, Islam AKMR (1992) Progress in the production of wheat-barley addition and recombination lines and their use in mapping barley genome. In: Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology, Shewry PR (ed). CAB Int Oxford pp 99-104

Shewry PR, Miflin BJ (1982) Genes for the storage proteins of barley. Qualitas Plantarum, Plant Foods for Human Nutrition 31: 251-267

Shewry PR, Bechman JS (1983) Genetic polymorphism in varietal identification and genetic improvement. Theor Appl Genet 65: 25-33.

Shewry PR, Parmar S, Buxton B, Gale MD, Liu CJ, Hejgaard J, Kreis M (1988) Multiple molecular forms of β–amylase in seeds and vegetative tissues of barley. Planta 176:127-134

Shewry PR, Parmar S, Franklin J, Burgess SR (1990) Analysis of a rare recombination event within the multigenic *Hor2* locus of barley (*Hordeum vulgare*). Genet Res 55:171-176

Shewry PR (1991) Barley seed storage proteins - structure, synthesis and deposition. In: Nitrogen Metabolism of plants, Pilbeam DJ, Mengel K (eds). Oxford University Press, Oxford.

Shewry PR (1992) Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology. CAB Int Oxford

Shin JS, Carpus L, Chao S, Blake TK (1990) A partial map of the barley genome. Genome 33: 803-808.

Silver J (1985) Confidence limits for estimates of gene linkage based on analysis of recombinant inbred strains. J Heredity 76: 436-440

Simpson CG, Sawbridge TT, Jenkins GI (1992) Expression analysis of multigene families by RT-PCR. Nucl Acid Res 20: 5861-5862.

Simos G, Panagiotidis CA, Skoumbas A, Choli D, Ouzounis C, Georgatsos JG (1994) Barley β-glucosidase: expression during seed germination and maturation and partial amino acid sequence. Biochim Biophys Acta 1199: 52-58

Sissons MJ (1992) Studies of barley limit dextrinase. PhD thesis, The University of Adelaide

Sissons MJ, Lance RCM, Sparrow DHB (1992a) Studies on limit dextrinase in barley. I. Purification of malt limit dextrinase and production of monospecific antibodies. J Cereal Sci 16:107-116

Sissons MJ, Lance RCM, Sparrow DHB (1992b) Studies on limit dextrinase in barley II. Application of an ELISA and immunoblotting to studies of genetic variability and malting effects. J Cereal Sci 16:117-128

Sissons MJ, MacGregor AW (1994) Hydrolysis of barley starch granules by alpha-glucosidase from malt. J Cereal Sci 19: 161-169

Sjoholm K, Macri LJ, MacGregor AW (1995) Is there a role for limit dextrinase in mashing? EBC Congress, pp 277-283

Skerritt JH (1988) Hydrolysis of barley endosperm storage protein during malting. I. Analysis using monoclonal antibodies. J Cereal Sci 7: 251-263

Skriver K, Mundy J (1990) Gene expression in response to abscisic acid and osmotic stress. Plant Cell 2: 503-512.

Skriver K, Olsen FL, Rogers JC, Mundy J (1991) *Cis*-acting DNA elements responsive to gibberellin and its antagonist abscisic acid. Proc Natl Acad Sci USA 88: 7266-7270

Slade AM, Høj PB, Morrice NA, Fincher GB (1989) Purification and characterisation of three 1,4-β-D-xylan endohydrolases from germinated barley. Eurp J Biochem 185: 533-539

Slakeski N, Baulcombe DC, Devos KM, Ahluwalia B, Doan DNP, Fincher GB (1990) Structure and tissue-specific regulation of genes encoding barley (1-3,1-4)-ß-glucan endohydrolases. Mol Gen Genet 224:437-449

Slakeski N, Fincher GB (1992) Developmental regulation of 1,3-1,4-β-glucanase gene expression in barley. I. Tissue-specific expression of individual isoenzymes. Plant Physiol 99: 1226-1231

Smith MT, Briggs DE (1980) Externally applied gibberellic acid and α-amylase formation in grains of barley (*Hordeum distichon*). Phytochem 19: 1025-1033

Smith DB, Lister PR (1983) Gel forming proteins in barley grain and their relationship with malting quality. J Cereal Sci 1: 229-239

Smith DB, Simpson PA (1983) Relationships of barley proteins sodium dodecyl sulphate to malting quality and varietal identification. J Cereal Sci 1: 185-197

Smith DB (1990) Barley seed protein and its effects on malting and brewing quality. Plant Varieties and Seeds 3: 63-80

Smith L (1951) Cytology and genetics of barley. Bot Rev 17: 1-51

Smith SJ, Walker RP, Beale MH, Hooley R (1993) Biological activity of some gibberellins and gibberellin derivatives in aleurone cells and protoplasts of *Avena fatua*. Phytochem 33: 17-20.

Søgaard B and Von Wettstein-Knowles P (1987) Barley: genes and chromosomes. Carlsberg Res Commun 52:126-196

Soller M, Beckman JS (1983) Genetic polymorphism in varietal identification and genetic improvement. Theor Appl Genet 65 :25-33

Soller M, Beckmann JS (1990) Marker-based mapping of quantitative trait loci using replicated progenies. Theor Appl Genet 80: 205-208

Southern EM (1975) Detection of specific sequences among DNA fragment separated by gel electrophoresis . J Mol Biol 98: 503-517

Speulman E, Salamini F (1995) GA3-regulated cDNA from *Hordeum vulgare* leaves. Plant Mol Bio 28:915-926

Stam P (1993) Construction of integrated genetic linkage maps by means of a new computer package: JoinMap. Plant J 3:739-744

Stark JR, Yin XS (1987) Evidence for the presence of maltase and α-glucosidase isoenzymes in barley. J Inst Brew 93: 108-112

Steffenson BJ, Hayes PM, Kleinhofs A (1996) Genetics of seeding and adult plant resistance to net blotch (*Pyrenophora terres F*) and spot blotch (*Cochliobolus sativus*) in barley. Theor Appl Genet 92: 552-558

Stone BA, Clarke AE (1993) Chemistry and biology of (1-3)-ß-glucans. La Trobe University Press

Stuart IM, Fincher GB (1983) Immunological determination of 1,3-1,4-β-D-glucan endohydrolase development in germinating barley (*Hordeum vulgare*). FEBS Lett 155: 201-204

Stuart IM, Loi L, Fincher GB (1986) Development of 1,3-1,4-β-glucan endohydrolase in isolated scutella and aleurone layers of barley (*Hordeum vulgare*). Plant Physiol 80: 310-314

Stuber CW (1992) Biochemical and molecular markers in plant breeding.In: J Janick (ed) Plant breeding reviews, John Wiley and Sons Inc, New York, pp 37-61

Sun Z, Henson CA (1990) Degradation of native starch granules by barley α-glucosidases. Plant Physi 94: 320-327

Sun Z, Henson GA (1991) A quantitative assessment of the importance of barley seed α-amylase, β-amylase, debranching enzyme, and α-glucosidase in starch degradation. Arch Biochem Biophys 284: 298-305

Swanston JS (1983) An alternative approach to breeding for high diastatic power in barley. Euphytica 32:919-924

Swanston JS (1980) The use of electrophoresis in testing for high diastatic power in barley. J Inst Brew 86: 81-83

Taiz L, Jones RL (1970) Gibberellic acid β-1,3-glucanase and cell walls of barley aleurone layers. Planta 92: 73-84

Taiz L, Honigman WA (1976) Production of cell wall hydrolysing enzymes by barley aleurone layers in response to gibberellic acid. Plant Physiol 58: 380-386

Takano T, Takeda G (1987) Genetic variation of α-amylase in germinating barley seeds. Barley Genet V:787-792

Takano T, Kiribuchi C, Takeda G (1988) Genetic analysis on banding patterns and activity of α-amylase isoenzymes in barley (*Hordeum vulgare L*). Japan J Breed 38:65-71

Tanksley SD, Medina-Filho H, Rick CM (1982) Use of naturally occurring enzyme variation to detect and map gene controlling quantitative traits in an interspecific backcross of tomato. Heredity 49: 11-25

Tanksley SD (1983) Molecular markers in plant breeding. Plant Mol Biol 1:3-8

Tanksley SD, Bernatzky R, Lapitan NL, Prince JP (1988) Conservation of gene repertoire but not gene order in pepper and tomato. Proc Natl Acad Sci USA 85: 6419-6423.

Tanksley SD, Young ND, Paterson AH, Bonierbale J (1989) RFLP mapping in plant breeding: new tools for an old science. Biotechnol 7: 257-264

Tanksley SD (1993) Mapping polygenes. Annu Rev genet 27: 205-233

Tanksley SD, Ganal MW, Martin GB (1995) Chromosome landing: a paradigm for map-based gene cloning in plants with large genomes. Trends Genet 11: 63-68

Tautz D, Renz M (1984) Simple sequences are ubiquitous repetitive components of eukaryotic genomes. Nucl Acid Res 12: 4127-4138

Thoday JM (1961) Location of polygenes. Nature 191: 368-370

Thomas WTB, Powell W, Waugh R, Chalmer KJ, Barua UM, Jack P, Lea V, Forster BP, Swanston JS, Ellis RP, Hanson PR, Lance RCM (1995) Detection of quantitative trait loci for agronomic, yield, grain and disease characters in spring barley (*Hordeum vulgare L.*) Theor Appl Genet 91: 1037-1047

Thomas WTB, Powell W, Swanston JS, Ellis RP, Chalmers KJ, Barua UM, Jack P, Lea V, Forster BP, Waugh R, Smith DB (1996) Quantitative trait loci for germination and malting quality characters in a spring barley cross. Crop Sci 36: 265-273

Thompson JN Jr (1975) Quantitative variation and gene number. Nature 258: 665-668.

Thompson JN Jr, Thoday JM (1979) Quantitative genetic variation. New York, Adademic

Tibbot BK, Skadsen RW (1996) Molecular cloning and characterisation of a gibberellin-inducable, putative α-glucosidase gene from barley. Plant Mol Biol 30: 229-241

Tingey SV, Del Tufo JP (1993) genetic analysis with random amplified polymorphic DNA markers. Plant Physiol 101: 349-352

Tinker NA, Mather DE, Rossnagel BG, Kasha KJ, Kleinhofs A, Hayes PM, Falk DE, Ferguson T, Shugar LP, Legge WG, Irvine B, Choo TM, Briggs KG, Ullrich SE, Franckowiak JD, Blake TK, Graf RJ, Dofing SM, Saghai Maroof MA, Scoles GJ, Hoffman D, Dahleen LS, Kilian A, Chen F, Biyashev RM, Kudrna DA, Steffenson BJ (1996) Regions of the genome that affect agronomic performance in two-row barley. Crop Sci 36: 1053-1062

Tinker NA, Mather DE (1995) Methods for QTL analysis with progeny replicated in multiple environments. J Quantitative Trait Loci (http://probe.nalusda.gov:8000/otherdocs/jqtl/1)

Tragoonrung S, Kanazin V, Hays PM, Blake TK (1992) STA-facilitated PCR for barley genome mapping. Theor Appl Genet 84:1002-1008

Ullrich SE, Hayes PM, Dyer WE, Blake TK, Clancy JA (1993) Quantitative trait locus analysis of seed dormancy in "Stepoe X Morex" barley. In: Pre-harvest sprouting in

cereals 1992, Walker-Simmons MK, Ried JL (eds). Am Assoc Cereal Chem, St Paul, Minn pp 136-145

Vallejos CE, Tanksley SD (1983) Segregation of isoenzyme markers and cold tolerance in an interspecific backcross of tomato. Theor Appl Genet 66: 241-247

Van De Ven WTG, Powell W, Ramsy G, Waugh R (l990) Restriction fragment length polymorphism as genetic markers in *Vicia*. Heredity 65:329-342.

van den Berg R, Muts GCJ, Drost BW, Graveland A (1981) Proteins from barley to wort. EBC Congress pp 461-469

Van den Bulcke M, Bauw G, Castresana C, Van Montagu M, Vandekerckhove J (1989) Characterization of vacuolar and extracellular ß-1,3-glucanase of tobacco: evidence for a strictly compartmentalised plant defence system. Proc Natl Acad Sci USA 86:2673-2677

Varghese JN, Garrett TPJ, Colman PM, Chen L, Høj PB, Fincher GB (1994) Three-dimensional structures of two plant ß-glucan endohydrolases with distinct substrate specifities. Proc Natl Acad Sci USA 91:2785-2789

Vergnaud G (1989) Polymers of random short oligonucleotides detect polymorphic loci in the human genome. Nucl Acid Res 17: 7623-7630

Visuri K, Nummi M (1972) Purification and characterisation of crystalline β–amylase from barley. Eur J Biochem 28:555-565

Vlodawsky L, Havel E, Mayer AM (1971) The effect of growth regulators and Cu on the activation of amylopectin α-1,6-glucosidase activity in pea seedlings. Plant Phsiol 25:363-368

Von Wettstein-Knowles P (1992) Cloned and mapped genes: Current status. In: Barley: genetics, biochemistry, molecular biology and biotechnology, Shewry P (ed). C. A. B. International, Wallingford, Oxon. pp 73-98

Wallwork MAB, Macleod LC (l99l) Is there a relationship between endosperm structure and malting quality? A study of several Australian barley varieties. Barley Genetics VI pp 489-492.

Wang J, Xu P, Fincher GB (1992) Purification, characterisation and gene structure of (1-3)-β-glucanase isoenzyme GIII from barley (*Hordeum Vulgar*e). Eur J Biochem 209: 103-109

Wareing PF, Saunders PF (1971) Hormones and dormancy. Ann Rev Plant Physio 22: 261-288

Waugh R, Powell W (1992) Using RAPD markers for crop improvement. TIBTECH 10: 186-191

Weber JL (1990) Human DNA polymorphisms based on length variations in simple-sequence tandem repeats. In: Genome analysis: genetic and physical mapping, Dives KE et al (eds). Cold spring Harbour Laboratory Press. pp 159-181

Weber WE, Wricke G (1994) Genetic markers in plant breeding. Paul Parey Scientific Publisher, Berlin & Hamberg pp 1-2

Weining S, Langridge P (l99l) Identification and mapping of polymorphism's in cereals based on the Polymerase Chain Reaction. Theor Appl Genet 82: 209-216

Weller JI, Soller M, Brody T (1988) Linkage analysis of quantitative traits in an interspecific cross of tomato by means of genetic markers. Genetics 118: 329-339

Welsh J, McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucl Acid Res 18: 7213-7218

Weselake RJ, MacGregor AW, Hill RD, Duckworth HW (1983) Purification and characteristics of an endogenous α-amylase inhibitor from barley kernels. Plant Physiol 73: 1008-1012

Weselake RJ, MacGregor AW, Hill RD (1985) Endogenous α-amylase inhibitor in various cereals. Cereal Chem 62: 120-123

Wessels JGH (1993) Wall growth, protein excretion and morphogenesis in fungi. New Phytol 123:379-413

Whitkus R, Doebley J, Lee M (1992) Comparative genome mapping of sorghum and maize. Genetics 132: 1119-1130

Whittaker JC, Thompson R, Visscher PM (1996) On the mapping of QTL by regression of phenotype on marker type. Heredity 77: 23-32

Wicking E, Williamson B (1991) From linked marker to gene. Trends Genet 7: 288-293

Winkelman DC, Hodgetts RB (1992) RFLPs for somatotropic genes identify quantitative trait loci for growth in mice. Genetics 131: 929

Williams JGK, Kubelik AR, Livak KJ (1990) DNA polymorphism's amplified by arbitrary primers are useful as genetic markers. Nucl Acid Res 18: 6531-6535.

Williams MNV, Pande N, Nair S, Mohan M, Bennett J (1991) Restriction fragment length polymorphism analysis of polymerase chain reaction products amplified from mapped loci of rice (*Oryza sativa*) genomic DNA. Theor Appl Genet 82:489-498

Wise RP, Rohde W, Salamini F (1990) Nucleotide sequence of the Bronze-1 homologous gene from *Hordeum vulgare*. Plant Mol Biol 14: 277-279

Wolf N (1991) Complete nucleotide sequence of *Hordeum vulgare* gene encoding (1-3, 1-4)-β-glucanase. Plant Physiol. 96: 1382-1384.

Wolf N (1992) Structure of the genes encoding *Hordeum vulgare* 1,3-1,4-β-glucanase isoenzyme I and II and functional analysis of their promoters in barley aleurone protoplasts. Mol Gen Genet 234: 33-42

Wong C, Dowling CE, Saiki RK, Higuchi RG, Erlich HA, Kazazian HH (1987) Characterization of beta-thalassaemia mutations using direct genomic sequencing amplified single-copy DNA. Nature 330: 384-386

Woodward JR, Fincher GB (1982) Purification and chemical properties of two 1,3-1,4-β-glucan endohydrolases from germinating barley. Eur J Biochem 121: 663-669

Woodward JR, Fincher GB (1983) Water-soluble barley β-glucans. Fine structure, solution behavior and organisation in the cell wall. Brew Dig 58: 28-32

Wu KS, Jones R, Danneberger L, Scolnik PA (1994) Detection of microsatellite polymorphisms without cloning. Nucleic Acids Res 22: 3257-3258

Xu P, Wang J, Fincher GB (1992) Evolution and differential expression of the (1-3)-β-glucan endohydrolase-encoding gene family in barley, *Hordeum vulgare.* Gene 120:157-165

Xu P, Harvey AJ, Fincher GB (1994) Heterologous expression of cDNAs encoding barley (*Hordeum vulgare*) 1,3-β-glucanase isoenzyme GV. FEBS Lett 348:206-210

Yamada J (1981) Conversion of active and inactive debranching enzyme in rice seeds. Agri Biol Chem 45:747-750

Yomo H (1960) Studies on the amylase-activating substance. IV. Amylase-activating activity of gibberellin. Hakko Kyokaishi 18: 600-603

Yoshigi N, Okada Y, Sahara H, Koshino S (1994) PCR cloning and sequencing of the β–amylase cDNA from barley. J Biochem 115:47-51

Yoshigi N, Sahara H, Koshino S (1995) Role of the C-terminal region of β–amylase from barley. J Biochem 117:63-67

Young ND, Zamir D, Ganal MW, Tanksley SD (l988) Use of isogenic lines and simultaneous probing to identify markers tightly linked to the *TM-2a* gene in tomato. Genetics 120: 579-585.

Zabeau M, Vos P (1993) Selective restriction fragment amplification: a general method for DNA fingerprinting. Eur Pat Appl No 92402629.7. Publication No. 0 534 858 A1

Zehr BE (1990) Use of RFLP markers in maize as an aid in selection during inbreeding. PhD diss Uni of Illinos (Diss abstr 91:5651)

Zehr BE, Dudley JW , Chøjecki J, Saghai Maroof MA, Mowers RP (1992) Use of RFLP markers to search for alleles in a maize population for improvement of an elite hybrid. Theor Appl genet 83: 903-911

Zeng ZB (1994) Precision mapping of quantitative trait loci. Genetics 136: 1457-1468

Zhang XQ, Burton R, Fincher GB (1997) High fragmented limit dextrinase gene is regulated by gibberellic acid in germinating barley (*Hordeum vulgare L.*). in preparation

Zhu MY, Xu AB, Yuan MB (l990) Genetic breeding of some components of malt quality in barley. Scientia Agricultura Sinina 23: 15-19

Zietkiewicz E, Rafalski A, Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20: 176-183

Zwar JA, Chandler PM (1995) Alpha-amylase production and leaf protein synthesis in a gibberellin-responsive dwarf mutant of Himalaya barley (*Hordeum vulgare L.*). Planta 197: 39-48