# Marker assisted backcrossing for gene introgression in barley (*Hordeum vulgare* L.)

A thesis submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy at the University of Adelaide

By

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# Declaration

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# Publications arising from thesis

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Jefferies SP, Pallotta MA, Paull JG, Karakousis A, Kretschmer JM, Manning S, Islam AKMR, Langridge P and Chalmers KJ, (2000) Mapping and validation of chromosome regions conferring boron toxicity tolerance in wheat (*Tritcum aestivum*). Theoretical and Applied Genetics (in press)

Jefferies SP, Barr AR, Hunt C, and Wheeler RD (2000) Agronomic and breeding value of genes for resistance to leaf scald (*Rhyncosporium secalis*) in barley (*Hordeum vulgare*) Australian Journal of Agricultural Research volume 51, issue 8

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Jefferies SP, Barr AR, Langridge P, Kretschmer JM, Gianquito P, Karakousis A and Chalmers KJ (1997). Applications of molecular markers in barley breeding. *In:* Proceedings of the 8th Australian Barley Technical Symposium, Gold Coast, September, 1997

Jefferies SP., Barr AR, Islam AKMR, Karakousis A, Kretschmer JM, Manning S, Chalmers KJ and Langridge P (1997). Molecular markers for boron tolerance in barley. Proceedings of the 8th Australian Barley Technical Symposium, Gold Coast, September 1997

Jefferies SP, Barr AR, Langridge P, Chalmers KJ, and Gianquitto P (1999) Backcrossing – a traditional breeding method with a new lease of life. Proceedings of 11$^{th}$ Australian Plant Breeding Conference, Adelaide, April, 1999

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# Table of Contents

1 General Introduction

2 Literature Review: Conventional and marker assisted backcrossing

2.1 Introduction to the application of the backcrossing method in plant breeding

2.2 Early history of the application of the backcrossing method in plant breeding

2.3 Application of the backcross breeding method in the development of wheat and barley cultivars in Australia

2.4 Genetic basis of backcross breeding

2.5 Choice of donor and recurrent parent

2.5.1 General principles

2.5.2 Genetic distance

2.5.3 Backcross variability

2.5.4 Transgressive segregation

2.6 Recovery of the recurrent parent phenotype and genotype with backcrossing

2.6.1 Linkage drag

2.6.2 Progressive reduction in length of donor segment during backcrossing

2.6.3 Selection for small donor segment in recurrent parent background

2.7. Molecular marker assisted backcrossing

2.7.1 Comparison of marker assisted and conventional backcrossing

2.7.2 Marker assisted selection for recurrent parent background

2.7.3 Limitations to marker assisted backcrossing

2.8 Introgression of quantitative characters with backcrossing

2.8.1 Assessment of alternative breeding methods for the introgression of exotic germplasm for the improvement of quantitatively inherited traits

2.8.2 Molecular marker assisted introgression of quantitative traits

2.9. Introgression of exotic germplasm with backcrossing

2.9.1 Assessment of alternative breeding methods for the introgression of exotic germplasm for the improvement of quantitatively inherited traits

2.9.2 Molecular marker assisted introgression of exotic germplasm

2.9.3 Simultaneous mapping and introgression of quantitatively inherited traits from exotic germplasm
2.9.4 Limitations to the backcross method for the introgression of exotic germplasm
2.10. Introgression of transgenes with backcrossing

3. Agronomic and breeding value of genes for resistance to leaf scald (*Rhynchosporium secalis*) in barley (*Hordeum vulgare* )

4. Mapping of chromosome regions conferring boron toxicity tolerance in barley (*Hordeum vulgare* L)

5. Validation and agronomic effects of QTL conferring boron toxicity tolerance in barley
5.1 Introduction
5.2 Materials and Methods
5.2.1 Genetic material
5.2.2 DNA extraction, restriction endonuclease digestion and Southern hybridisation
5.2.3 Population construction
5.2.4 Field experiments 1998 and 1999
5.2.5 Relative root length in solution culture
5.2.6 Flanking markers and additional loci
5.2.7 Statistical analysis
5.3 Results
5.3.1 Concentration of boron in soil
5.3.2 Effect of boron on grain yield and grain size of Sloop control plots
5.3.3 Relationships between boron toxicity symptoms, boron concentration in whole shoots, and grain yield and grain plumpness of Sloop and VB9104 backcross derived lines
5.3.4 Effect of marker alleles at boron tolerance loci on Sloop and VB9104 backcross derived lines
5.4 Discussion
5.4.1 Chromosome 6H locus effects

5.4.2 Chromosome 2H and 4H locus effects

5.4.3 Boron tolerance mechanisms as conferred by chromosome 2H and 4H loci

5.4.4 Deleterious grain yield factors associated with boron tolerance loci

5.4.5 Suitable agronomic backgrounds for boron tolerance QTL in barley grown in dryland areas prone to terminal drought

5.4.6 Implications for marker assisted selection in the breeding of boron tolerant malting quality barley varieties

6 Marker assisted selection for donor gene

6.1 Marker assisted backcross introgression of *Ha 2* conferring resistance to *Heterodera avenae* into a malting quality background

6.1.1 Introduction

6.1.2 Materials and methods

6.1.2.1 Genetic materials and population development

6.1.2.2 CCN Bio-assay

6.1.2.3 DNA extraction, restriction endonuclease digestion and Southern hybridisation

6.1.2.4 Flanking markers

6.1.2.5 Field experiments

6.1.2.6 Malting quality

6.1.2.7 Statistical analysis

6.1.3 Results

6.1.3.1 CCN resistance of $BC_3$ doubled haploid lines

6.1.3.2 Effect of Chebec marker alleles near the *Ha 2* locus on malting quality, grain yield and grain size of $BC_1$ derived lines

6.1.4 Discussion

6.1.4.1 Effects of marker alleles near the *Ha 2* locus on malt quality and agronomic traits

6.1.4.2 Comparison between marker assisted selection for CCN resistance and phenotypic selection

6.1.4.3 Implications for the breeding of CCN resistant malting quality varieties

6.2 Marker assisted backcross introgression of*Yd2* gene conferring resistance to barley yellow dwarf virus in barley (*Hordeum vulgare* L)

6.2.1 Introduction

6.2.2 Materials and methods

6.2.2.1 Genetic materials

6.2.2.2 Population development

6.2.2.3 PCR analysis of barley DNA for YLM genotype

6.2.2.4 Viruliferous aphid management and inoculation

6.2.2.5 Field experiments

6.2.2.6 BYDV northern dot blot hybridisation analysis

6.2.2.7 Malting quality

6.2.2.8 Leaf scald resistance screening

6.2.2.9 Statistical analysis

6.2.3 Results

6.2.3.1 Decimal growth stage

6.2.3.2 Grain yield, grain plumpness and symptom score

6.2.3.3 Malt quality

6.2.3.4 Leaf Scald resistance

6.2.4 Discussion

7. Selection for recurrent parent background

7.1 Accelerated backcrossing strategies for the introgression of the *Ha 2* gene conferring resistance to cereal cyst nematode (*Heterodera avenae*) in barley

7.1.1 Introduction

7.1.2 Materials and methods

7.1.2.1 Genetic materials and population development

7.1.2.2 DNA extraction, AFLP reactions and PAGE

7.1.2.3 Field experiments

7.1.2.4 Malting quality

7.1.2.5 Statistical analysis

7.1.3 Results

7.1.3.1 Effect of cross type and donor parent on the proportion of unique donor parent genome

7.1.3.2 Effect of cross type and donor parent on agronomic and quality performance

7.1.3.3 Relationship between proportion of unique donor parent genome and agronomic and malt quality factors among Chebec derived backcross lines

7.1.4 Discussion

7.1.4.1 Efficiency of selection for recurrent parent background-number of backcrosses

7.1.4.2 Recovery of recurrent parent background

7.1.4.3 Choice of donor parent and recovery of recurrent parent genome

7.1.5 Conclusion

7.2 Implications of genetic distance between donor and recurrent parents on the implementation of accelerated backcrossing strategies

7.2.1 Introduction

7.2.2 Materials and methods

7.2.2.1 Genetic Material

7.2.2.2 DNA extraction and multiflurophore AFLP analysis

7.2.2.3 Statistical analysis

7.2.3 Results

7.2.3.1 Genetic distance between cultivars and breeding lines

7.2.3.2 Population means and genetic variance

7.2.3.3 AFLP analysis of $BC_1$ populations

7.2.4 Discussion

7.2.4.1 AFLP analysis

7.2.4.2 Genetic distance

7.2.4.3 Effect of genetic distance between donor and recurrent parent on recovery of the recurrent parent phenotype.

7.2.4.4 Effect of genetic distance between donor and recurrent parent on genetic variance in $BC_1$ populations

7.2.4.5 Recovery of the recurrent parent genotype

7.2.4.6 Number of marker loci for selection of recurrent parent background

8. General Discussion

8.1 Identification and assessment of donor and recurrent parents

8.2 Identification of markers linked to genes/QTL of agronomic importance

8.3 Validation of markers linked to genes/QTL of agronomic importance

8.4 Implementation of markers linked to genes/QTL of agronomic importance

8.4.1 Indirect selection for donor gene(s)

8.4.2 Selection for recurrent parent background

8.4.3 Linkage drag and marker assisted selection for small introgression segments

8.5 An advanced marker assisted gene introgression strategy

References

Appendix

Jefferies SP, Pallotta MA, Paull JG, Karakousis A, Kretschmer JM, Manning S, Islam AKMR, Langridge P, and Chalmers KJ (2000) Mapping and Validation of chromosome regions conferring boron toxicity tolerance in wheat (*Triticum aestivum*) Theoretical and Applied Genetics (in press)

# Chapter 1

## General Introduction

The average time from cross to commercial release of an Australian malting quality barley variety is 14 years. For a variety to be regarded as acceptable for malting grades in Australia it must show high levels of expression of many complex, often interrelated biochemical and physical quality traits. The methods used to characterise and select for the malting quality traits are also complex and slow. The final stage of commercial malting and brewing evaluation alone, can add more than three years onto the total time required to develop a new variety. Combining wide adaptation, desirable agronomic characteristics and malting quality attributes into a single variety has, therefore, proven to be a highly demanding and time consuming process for breeders.

The backcross breeding method has been used extensively for improving the agronomic characteristics of premium quality wheat cultivars in Australia. The method has led to improvements in the agronomic characteristics and performance of cultivars while conserving the established gene complexes important in elite milling quality types. In contrast, the method has been used rarely in the breeding of malting quality barley cultivars. A major constraint to its adoption has been predicting the commercial life span of the recurrent parent quality type. In the Australian wheat industry, varieties regarded as premium milling quality types in the late 1970's and early 1980's are generally still regarded as premium quality types. The international malting barley market, however, is less conservative, highly competitive, and increasingly quality conscious. A preferred malting quality cultivar can be outclassed in international markets, in only a short period of time. The backcross breeding method is slow and offers little opportunity for genetic gain in non-target (quality) traits carried by the recurrent parent. Molecular markers, however, have the potential to vastly improve the timeliness and efficiency of the conventional backcross breeding method in several ways. These include:

(1). Marker assisted selection (indirect) for the desired trait in the backcross $F_1$ progeny prior to anthesis which allows progression to the next cycle of backcrossing without the need for selfing or phenotyping. Molecular markers have been used extensively to identify the number and location of genes controlling economically important traits in many crop species. Despite this, there are relatively few reports in the literature of the successful adoption of molecular markers for indirect selection of $F_1$ progeny, particularly in a backcrossing strategy.

(2). Marker assisted election for recurrent parent background or against unwanted donor parent genome in backcross progeny. The underlying principle of any backcrossing strategy is that the expected proportion of donor parent genome reduces by fifty percent with each generation of backcrossing. Until recent times, most backcross strategies have focused on this principle and have ignored the genetic variation for the

proportion of donor parent genome that exists around the expected mean. Molecular markers can not only be used for monitoring and selecting for the desired donor parent locus, but also for identifying recombinant individuals that have genome compositions closer to that of the recurrent parent than would be predicted from theoretical expectations. Selection of such individuals should reduce the number of backcrosses required to recover the recurrent parent genotype and phenotype, thereby reducing the time necessary to achieve commercial outcomes. Selection for recurrent parent background in backcrossing strategies has not been widely adopted nor its potential and limitations assessed.

(3) Choice of donor and recurrent parents based on estimates of genetic distance. For some traits it is possible to select from a range of donor parents. It is expected that fewer backcrosses would be required to recover the recurrent parent type if genetically similar donor and recurrent parents are chosen. The implications of genetic distance on accelerating backcrossing strategies, however, have not been assessed.

In light of recent developments in molecular marker technology, Australian barley breeders now need to re-evaluate the application of the backcross method for improving agronomic characteristics of malting quality varieties. Such a re-evaluation will require knowledge of: (1) the availability of suitable donor parents; (2) the number and location of genes/QTL controlling agronomically important traits; (3) the availability of molecular markers linked to genes/QTL controlling agronomically important traits and an assessment of their effectiveness; (4) the agronomic value of, and the potential for deleterious linkage drag and/or pleiotropic effects associated with, genes/QTL controlling agronomically important traits; (5) the practical limitations and benefits of marker assisted backcrossing

This study will aim to address each of these issues in an evaluation of the backcross breeding method for the introgression of agronomically important traits into a malting quality background using molecular markers.

The agronomic traits to be considered in this study will include resistance to cereal cyst nematode (CCN) (*Heterodera avenae*), resistance to barley leaf scald (*Rhyncosporium secali*,), resistance to barley yellow dwarf virus (BYDV), and tolerance to toxic concentrations of soil boron. Molecular markers linked to genes for CCN resistance and BYDV resistance are available. Due to the highly variable nature of the leaf scald pathogen, a number of sources of potentially durable leaf scald resistance genes are being considered with the aim of using molecular markers to pyramid these genes. Several leaf scald gene-mapping initiatives have commenced (NBMMP) but markers are available for only a limited number of loci. Genetic variation for tolerance to high soil boron and a suitable mapping population are both currently available for identifying the number and location of genes/QTL controlling tolerance in barley. This study will include an assessment of potential donor parents for scald resistance, the identification of the number and location of genes/QTL conferring boron

tolerance, and an assessment of the agronomic and breeding value of both the scald resistance genes and boron tolerance QTL.

Chapter 2 will review the literature on the backcross breeding method with particular reference to the application of molecular markers for improving the efficiency of the method. The subsequent chapters will focus, using practical examples, on aspects of each of the major components of a molecular marker assisted backcrossing program (listed above) as outlined in Table 1.

Table 1. Thesis structure

| Component | Chapter | Content/Example |
|---|---|---|
| | Chapter 2 | Review of literature on backcross breeding with particular emphasis on the application of molecular markers |
| Parent assessment | Chapter 3 | Assessment of potential donor parents carrying resistance to barley leaf scald |
| Marker identification | Chapter 4 | Identification of molecular markers linked to QTL conferring boron toxicity tolerance |
| Marker validation | Chapter 5 | Validation of molecular markers linked to QTL conferring boron toxicity tolerance and an assessment of the agronomic and breeding value of these QTL |
| Marker implementation - tracing donor gene/QTL | Chapter 6 | (1) Marker assisted backcross introgression of a single gene conferring resistance to cereal cyst nematode (CCN)<br>(2) Marker assisted backcross introgression of a single gene conferring resistance to barley yellow dwarf virus (BYDV) |
| Marker implementation - selection for recurrent parent background | Chapter 7 | (1) Accelerated backcrossing for the introgression of a single gene conferring resistance to CCN<br>(2) Implications of genetic distance between donor and recurrent parent on accelerated backcrossing strategies |
| | Chapter 8 | General discussion |

# Chapter 2

## Literature Review
## Conventional and Marker Assisted Backcrossing

## 2.1 Introduction to the backcross method in plant breeding

Backcrossing is a breeding strategy commonly used to introgress a single or small number of valuable genes into an elite cultivar or germplasm. The elite cultivar or germplasm often will carry many valuable traits or excels in one or more trait for which genetic progress is slow or difficult.

The backcross method involves crossing between a "recurrent parent" and a "donor parent". The parent contributing the gene or genes that control the desired trait is commonly designated the donor parent. The parent into which genes are to be introgressed is termed the recurrent parent. Recurrent indicates that the parent is used repeatedly.

The conventional backcross method for the introgression of a character controlled by a single dominant gene begins with hybridisation between the donor and recurrent parent followed by successive crosses between selected progeny carrying the desired new trait and the recurrent parent. Following the final backcross, the hybrid is allowed to self to allow homozygosity for the selected trait and non-target loci. For the introgression of a recessive gene, the $F_1$ hybrid is selfed after each backcross to allow for segregation and selection for the homozygous recessive genotype in a backcross $F_2$ population. The number of backcrosses used depends largely on the proportion or extent of recovery of the recurrent parent desired in the final cultivar and the degree of similarity between the donor and recurrent parent. The genetic basis of the backcross method and examples of variations on the method will be discussed in the following sections.

Briggs and Allard (1953) listed three basic requirements for a successful backcross breeding program which remain relevant and appropriate today;
(1) a satisfactory recurrent parent must exist. The principal of the backcross breeding method is based on introgression of one or a few genes of value into a genetic background that otherwise remains unchanged.
(2) it must be possible to maintain a worthwhile intensity or expression of the character under transfer through several backcrosses. To do this, effective selection systems must be available.
(3) the genotype of the recurrent parent must be reconstituted by a reasonable number of backcrosses executed with populations of manageable size.

The backcross method often may form only a component of a broader breeding strategy. Backcrossing can be an efficient method for incorporating useful genes from exotic germplasm into adapted backgrounds. While backcrossing has predominantly been used for the introgression of monogenic or oligogenetic traits it has also been utilised in the introgression of quantitatively inherited traits. A rationale often given by plant breeders for the choice of the backcross method is a perception that there will be a reduced requirement for advanced generation evaluation or commercial quality testing and/or a greater probability of acceptance and more rapid adoption of the resultant cultivar.

Backcrossing is frequently used in genetic and agronomic studies and practical breeding programs requiring the creation of near-isogenic lines and in testcrosses to determine genetic ratios, linkage and crossover percentages.

## 2.2 Early history of the application of backcrossing in plant breeding

The prevailing plant breeding philosophy of the early 1880s was described by Mackey (1963) as a

> "lamarkistic influence on darwinism, which led people to believe that the germplasm of our cultivated plants could be transformed in desirable directions by a systematic and skilled mass selection only" (Mackey,1963).

By the late 1880s agriculturalists believed that nature could not be changed or remoulded at will and that selection was only able to exhibit something previously existing in the population. Early 1900s plant breeding efforts, therefore, concentrated almost entirely on pure line selection. Mackey (1963) described how an improvement plateau through line selection in early 1900s at the Sweedish Seed Association was reached sooner than expected and that lines well balanced in respect to several characteristics were difficult to obtain.

The wheat breeder Sheriff (1873) was one of the first documented to hybridize cereals but he did this in ignorance of Mendelian laws. The classical plant breeding studies of Nilsson-Ehle, however, proved the existence of polygenic factors providing the scientific basis for transgressive segregation and thus laying the foundation for future generations of cross hybridisation breeding.

Mackey (1963) described how early in the 1900's cross hybridisation breeding in wheat undertaken at the Swedish Seed Association focused on combining hardiness and quality from Swedish winter wheat cultivars with the high yield and stiff straw of the English squarehead types. The philosophy was to concentrate on few crosses and on a large scale in order to guarantee the expected ideal combination. This approach gave good early progress but after

some time, genetic variability decreased and consequently genetic progress also slowed. The breeder's persistent focus on restricting the basic material and exhausting the potential of the population through successive recombinations was attributed to frequent failures in previous attempts to broaden the germplasm. With very few exceptions, most crosses between adapted Swedish cultivars and foreign lines were found to produce segregants inferior to the Swedish parent in agronomic performance. It was this general early lack of success in intercrossing exotic and adapted germplasm that gave rise to the first use of backcrossing by plant breeders.

While we can expect that backcrossing was used by plant breeders, either directly or indirectly, prior to the 1920's, the first documented description of the potential of the backcrossing method in cereal breeding was made by Harlan and Pope (1922).

> "The writers feel that there is an important place for backcrosses in small grain breeding that is not now fully appreciated" (Harlan and Pope, 1922).

They illustrated the potential of the backcross method by describing the transfer of a smooth awned trait in the established barley cultivar 'Manchuria' and showed that the smooth awn trait was not modified by repeated backcrossing.

The earliest reported extensive application of backcrossing in a plant breeding program was made by Briggs (1930) who used backcrossing to produce agronomically desirable cultivars of wheat resistant to bunt (*Tilletia tritici* (Bjerk) Wint). Briggs expected that the time required to test the resultant strains against the recurrent parent for agronomic performance would be somewhat less than was usual with a new cultivar and used this as a major justification for adopting the backcross method. Briggs planned to process all new breeding lines, identified for potential commercial release in California, through this backcrossing program. Briggs intended to only release wheat cultivars as resistant to bunt as the donor parent in the future. This or similar philosophy for the control of economically important plant diseases has since been adopted by plant breeding programs throughout the world.

Peterson (1957) studied, by survey, the early trends in backcross breeding among wheat breeding programs in North America, Australia, and South America. He found that in the early 1920's, only a few wheat breeding institutions were using the backcrossing method. During the 1940's, in both North America and Australia, new and highly virulent races of stem and leaf rust were being discovered. It was during this period that an increasing number of wheat breeders made use of the backcross method to transfer rust resistance to adapted cultivars. The backcross method was also used extensively in South America but was not used widely in Europe (with the exception of Svalof, Sweden), Asia and Africa, where cereal rusts were generally less destructive.

## 2.3 Application of the backcross breeding method in the development of wheat and barley cultivars in Australia

Backcrossing has been used extensively by Australian wheat breeding programs since stem rust epidemics caused substantial losses in production during the early 1940s', but particularly since the devastating stem rust epidemic in 1973. Pugsley (1949) initiated a backcross breeding program to introgress stem rust resistance into 10 adapted Australian wheat cultivars. Widely adapted cultivars including Insignia, Bencubbin and Dirk were chosen as recurrent parents. Gabo, which carried the resistance gene from the durum cultivar Gaza, and Eureka, whose resistance broke down early in the program, were chosen as donor parents. As a rule, five successive backcrosses were made in the $F_3$ generation. Some of the new cultivars produced were then used as recurrent parents to add characters such as leaf rust, bunt and mildew resistance. The Gabo source of stem rust resistance broke down shortly after the release of several cultivars including Insignia 49, Bencubbin 48, and Dirk 48. Durable stem rust resistant cultivars were not grown widely in Australia, however, until the release of Kite and Condor in 1973. Despite the loss of rust resistance, Insignia 49 became the most commonly grown cultivar in South Australia during the 1950's.

Table 1 provides details on backcross derived wheat cultivars released in Australia since 1973 to 1995. It should be noted that Condor and Cook, and to a less extent Hartog, have been used regularly as recurrent parents. These cultivars were, for a long time, benchmarks for the premium Prime Hard (Australian Wheat Board) quality classification. Milling and baking quality are complex quantitatively inherited characters which are difficult to recover from wide (exotic by adapted) crosses. The strict backcross breeding method, involving a minimum of five backcrosses, was used intensively during the 1940's and 1950's but since this time wheat breeders in Australia have rarely used more than four cycles of backcrossing.

The strict backcross method was probably seen as over conservative and provided almost no opportunity for transgressive segregation. While a serious stem rust epidemic has not been recorded in Australia since 1974, due mostly to the development of resistant cultivars, Australian wheat breeding programs continue to diversify and pyramid stem rust resistance genes utilising backcross breeding strategies.

**Table 1. Wheat cultivars developed through backcrossing in Australia from 1973 to 1996**

| Cultivar | State of origin/ Year | Recurrent Parent | Donor Parent | Number of BCs[1] | Donor Trait[2] |
|---|---|---|---|---|---|
| Condor | NSW/73 | WW15 | WW80 | 2 | SR |
| Kite | NSW/73 | Falcon | Norin/Brevar//4*Eureka 2 | 3 | SR |
| Oxley | NSW/74 | WW15 | WW80 | 2 | SR |
| Jabiru | NSW/75 | Pinnacle | Mexico 120 | 4 | SR |
| Banks | Qld/79 | Condor | PWTH | 3 | SR |
| Katyil | Vic/81 | Olympic | Aus10894 | 4 | CCN |
| Bass | Qld/83 | Cook | Flinders | 2 | SR |
| Cocamba | Vic/84 | Condor | Aus10894 | 4 | CCN* |
| King | Qld/83 | 7165 | PWTH | 2 | SR |
| Torres | Qld/83 | Condor | 3Ag3 | 3 | SR/LR |
| Osprey | Qld/83 | Condor | WW33B | 2 | winter habit |
| Skua | NSW/84 | Condor | 3Ag14 | 4 | SR/LR |
| Sundor | NSW/84 | Condor | 3Ag14 | 4 | SR/LR |
| Sunelg | NSW/84 | Darf | 3Ag14 | 4 | SR/LR |
| Vasco | Qld/85 | Oxley | 3Ag14/4*Condor | 5 | SR/LR |
| Diaz | Qld/86 | Cook | CombIII/3* Oxley | 3 | SR |
| Grebe | NSW/86 | Egret | Skorospelka | 3 | SR/LR |
| Schomburgk | SA/86 | Aroona | W3589/Oxley//2*Warigal | 2 | SR |
| Sunbird | NSW/86 | Condor | 3Ag14 | 4 | SR/LR |
| Sunco | NSW/86 | Cook | complex | 3 | SR/LR |
| Kiata | Vic/87 | Condor | Aus11577 | 5 | CCN* |
| Moray | Vic/87 | Halberd | Aus11577 | 5 | CCN* |
| Janz | Qld/88 | Condor/Cook | 3AG3 | 4 | SR/LR |
| Molineux | SA/88 | Warigal | Pitic62/Festiguay | 2 | CCN |
| Corrigin | WA/89 | Tincurrin | Gamenya/IAASSUL | | Sep.N/Sep.T |
| Perouse | Qld/89 | Cook | 3AG14/4*Condor/Oxley | 3 | SR/LR |
| Cunningham | Qld/90 | Condor | 3AG3 | 4 | SR/LR |
| Sunbri | NSW/90 | Cook | VPM1 | 5 | SR/YR |
| Angas | SA/91 | Aroona | YR10 | 4 | YR |
| BT-Schomb. | SA/92 | Schomburgk | Halberd/Aroona | 3 | BT |
| Rowan | Qld/92 | Hartog | QT2338 | 4 | Awnless |
| Sunland | NSW/92 | Cook | complex | 3 | SR/LR |

**Table 1 (cont). Wheat Cultivars developed through backcrossing released in Australia from 1973 to 1996**

| Cultivar | State of origin/ Year | Recurrent Parent | Donor Parent | Number of BCs[1] | Donor Trait[2] |
|---|---|---|---|---|---|
| Sunstate | NSW/92 | Hartog | Cook*5/VPM1 | 4 | SR/LR/YR |
| Barunga | SA/93 | Molineux | (Ha*Ar)*Sch#3) | 3 | BT |
| Darter | NSW/93 | Kite | M2293 | 3 | SR/YR |
| Pelsart | Qld/93 | Cook | Potam70 | 4 | RLN |
| Sunvale | NSW/93 | Cook | VPM1 | 5 | SR/LR |
| Trident | SA/93 | Spear | VPM1/5*Cook | 4 | SR/LR/YR |
| Warbler | NSW/93 | Oxley | Karkay/Timgalen | 3 | Winter habit |
| Wellstead | WA/93 | Egret | Karkaz | 4 | Sep |
| Cascades | WA/94 | Aroona | AUSENVII-95 | 3 | YS/Sep.N |
| Datatine | WA/94 | Tincurrin | 3Ag3/3*Halberd | 4 | SR/LR |
| Frame | SA/94 | Dagger | Molineux | 3 | CCN |
| Tern | NSW/94 | Ford | M2293 | 2 | SR/YR |
| Leichardt | Qld/95 | Hartog | CNT2 | 4 | YS |
| Sunbrook | NSW/95 | Hartog | Suneca | 2 | SR/LR/YR |

[1] BC: number of backcrosses

[2] SR: stem rust, LR: leaf rust, YR: stripe rust, CCN: cereal cyst nematode, CCN*: not successful, Sep.N: *Septoria Nodorum,* Sep.T: *Septoria tritici*, YS: yellow leaf spot, BT: boron toxicity tolerance, RLN: root lesion nematode.

The backcross method in Australian barley breeding, in contrast, has been used rarely in the development of Australian commercial barley cultivars. The Tasmanian cultivar Shannon was developed from four backcrosses between a Barley Yellow Dwarf Virus resistant donor parent from North Africa and the recurrent parent Proctor. The South Australian cultivar Galleon culminated from three backcrosses involving a CCN resistant recurrent parent and a donor parent producing grain with long rachila hair. The feed quality cultivar Galleon was selected for long rachilla hair so that it could be differentiated from the commonly grown malting cultivar Clipper at grain delivery points. While not derived from a strict backcross procedure, the South Australian cultivar Chebec was developed from a CCN resistant donor parent and one backcross to Clipper. The first backcross was then crossed to Schooner which itself is 50% Clipper. Backcrossing may not have been used widely in the development of Australian barley cultivars because;

(1) lack of a suitable recurrent parent. This is probably true in recent times where Australian breeding programs have failed to keep pace with malting quality improvements in both Canada

and Europe but it is not true for the 1970's and 1980's where the Australian cultivars Clipper and later Schooner were regarded as international standards for high malting quality.

(2) emphasis on quantitatively inherited traits. Australian barley breeding programs have focused predominantly on improvements in malting quality and grain yield. Until very recently, and with the exception of CCN resistance, little emphasis has been placed on improvement in disease resistance and abiotic stress tolerance.

(3) the backcross method is regarded as slow and offers little opportunity for genetic gain in non-target traits

## 2.4 The genetic basis of backcross breeding

Briggs (1935) provided a means for estimating the proportion of homozygous progeny generated from plants selfing where the proportion of homozygosity = $((2^m -1)/2^m)^n$ ,where $m$ is the number of generations of selfing and $n$ is the number of heterozygous genes. As $m$ increases, the proportion of homozygous individuals will become greater. If a heterozygous population is repeatedly backcrossed to one of the homozygous parents (recurrent parent), homozygosity is attained at the same rate as if the heterozygous population was allowed to self-fertilize. Therefore in the above equation $m$ can also be the number of backcrosses used. Therefore any population obtained by backcrossing will rapidly converge on the genotype of the recurrent parent. This is the simple premise forming the basis of the backcross breeding method. The rate at which donor parent genes are eliminated during backcrossing will, however, be influenced by linkage. Linkage will be discussed in a later section.

## 2.5. Choice of donor and recurrent parent

### 2.5.1 General Principles

Any population derived from backcrossing rapidly converges on the genotype and phenotype of the recurrent parent. The success of a backcrossing program therefore will depend largely on the choice of a suitable recurrent parent.

Crop production in Australia has generally been dominated by only a few cultivars. Over a ten year period, extending from 1982 to 1992, only three barley and four wheat cultivars accounted for more than 70% and 40% of the annual production of barley and wheat in Australia respectively (Clements *et al.*, 1992). These dominant cultivars were characterised by wide agronomic adaptation and superior processing quality (milling, baking, malting). Identifying rare recombinants that successfully combine these complex, highly, quantitative traits is not a simple task and consequently cultivars, which do carry these traits, tend to persist in commercial production for some time. Well adapted, commercially established, cultivars

with superior processing quality, but lacking simply inherited traits, such as foliar disease resistances, are therefore ideal recurrent parent candidates.

The most suitable donor parent is one that carries the alleles needed to improve the recurrent parent but is not seriously deficient in other characters (Fehr, 1987). In reality, many donor parents are very agronomically inferior (eg cytogentic stocks, wild types, landraces). In most cases, the more deficiencies in the donor parent the more backcrosses required to produce an acceptable cultivar. It also follows that the greater the genetic difference between the recurrent and donor parents the greater the number of backcrosses that are likely to be required to recover the recurrent parent phenotype.

### 2.5.2 Genetic distance

Many studies have attempted to determine the phenotypic and genotypic differences, similarities and relationships between cultivars or accessions of the same or related crop species (Wrigley *et al.*, 1982; Cox *et al.*, 1985a; Cox *et al.* 1985b; Cowen and Frey 1987; Souza and Sorrels 1989, 1991a, 1991b; Mesmer *et al.*, 1992 and 1993; Melchinger *et al.* 1994; Neinhuis *et al.*, 1994; Hackenberg and Kohler,1996; and Orden *et al.*,1997). Genetic distance relationships have been derived from;

(1) quantitative characters (eg. Wrigley *et al.*, 1982, Rogers *et al.*, 1983; Souza and Sorrells, 1991a and 1991b)
(2) coefficients of ancestry or parentage based on pedigree information (eg. Cox *et al.*, 1985b; Cowen and Frey, 1987)
(3) polymorphisms in isozyme markers (eg. Price *et al.*, 1984; Hackenberg and Kohler, 1996)
(4) restriction fragment length polymorphisms (RFLPs) (eg. Melchinger *et al.*, 1990; Miller and Tanksley, 1990; and Moser and Lee, 1994)
(5) random amplified polymorphic DNA (RAPDs) (eg. Neinhuis *et al.*, 1994; and Ordon *et al.*, 1997)
(6) micro-satellites or simple sequence repeats (SSRs) (eg. Planchke *et al.*, 1995) and
(7) amplified fragment length polymorphisms (AFLPs) (eg. Russell *et al.*, 1997).

The accuracy of genetic relationships based on quantitative traits and isozyme markers has been found to be low and this has been attributed partly to the lack of characters and poor genome coverage which allowed only loose associations to be determined (Chalmers *et al*, in press).

Several studies have evaluated and compared estimates of genetic distance based on co-ancestry coefficient and RFLP markers. Without exception the studies concluded that RFLP markers provide a more accurate estimate of genetic distance among sets of cereal germplasm (Smith *et al.*, 1990; Mesmer *et al.*, 1993; Siedler *et al.*,1994; Graner *et al.*, 1994; O'Donoughue *et al.*, 1994). AFLPs (Vos *et al.*, 1995) have been shown to be equally as useful as RFLPs for establishing genetic relationships but are considered to be more efficient

to use (Russell *et al.*, 1997; Chalmers *et al.*, in press). Russell *et al.* (1997) showed poor correlation between RAPDs and SSRs , RAPDs and RFLPs and RAPDs and AFLPs and reasonable correlation between SSRs and both RFLPs and AFLPs. Many other studies including Miller and Tanksley (1990); Messmer *et al.*, (1991); Smith and Smith (1992) and Gerdes and Tracy (1994) have also compared measures of genetic distance. In general, agreement between genetic distance measures based on isozyme data and pedigree data were poor. Among RFLP, AFLP, SSR and to a less extent RAPD markers, the number of markers available and coverage of the genome appeared to be more important than the type of marker. The number of markers required to estimate genetic distance with relative accuracy in maize has been estimated to range from 100 (Smith *et al.*, 1991) to more than 250 (Bernado, 1993). Dudley (1994) proposed that a set of 50 to 100 markers covering the maize genome would generally agree with pedigree information. While molecular markers offer improved precision in providing genetic distance estimated, there appear to be no firm guidelines as to the optimum number of markers necessary to provide an accurate assessment.

There is little evidence in the literature of attempts to assess the potential of using genetic distance estimates between parents as a tool for choosing donor and recurrent parents. The aim would be to choose a parent carrying the desired gene or genes which is as genetically similar as possible to the recurrent parent. The theory is that the more similar the donor and recurrent parents, the fewer backcrosses required to recover the genotype and phenotype of the recurrent parent.

#### *2.5.2.1 Genetic distance and backcross variability*

Goodman (1969) indicated that a direct genetic test of the degree of divergence of two parents is the relative variability of their $F_2$. The theory being that the mating of more genetically distant parents should produce a larger than average progeny variance because the number of segregating loci is maximised. Cowen and Frey (1987), using morphological and pedigree data, Souza and Sorrells (1989), using pedigree and both qualitatively and quantitatively inherited morphological and biochemical character data, and Moser and Lee (1994), using RFLP data, showed that genetic distance estimates were relatively poor predictors of genetic variance in oats. The authors concluded that strong associations between molecular marker based genetic distance estimates and genetic variance would only occur under certain conditions and these conditions could be influenced by;

(1) the number of marker loci used in the analysis (only 26 used by Moser and Lee (1994))
(2) linkage between marker loci and QTLs
(3) the origin of identical marker alleles, and
(4) differences in gene expression among populations or crosses.

Moser and Lee (1994) assessed crosses among relatively unrelated parents and they proposed that a wider range and better distribution of pedigree relationships would be required to clearly establish relationships between genetic distance and genetic variance in oats. Manjarrez-

Sadoval *et al.* (1997) studied the potential application of genetic distance estimates for the prediction of genetic variance for soybean yield. In contrast to the results found in oats, the authors found that genetic distance estimates, both marker based and coefficients of parentage based on pedigree information, correctly identified the populations with the highest genetic variance.

Several studies of genetic variance in backcross populations have shown that genotypic variance for quantitatively inherited characters decreases with successive generations of backcrossing but the decreases were often not as expected for an additive gene action (Leininger and Frey 1962; Lawrence and Frey, 1975; Takeda, Bailey and Frey 1985; Carpenter and Fehr 1986). These observations were attributed to dominance or epistasis.

The means and genetic variances of populations of random $F_2$ derived lines from successive backcrosses were also studied by Cox (1984a). The author found that the genetic variance among $F_2$ derived lines depended on the backcross generation, the number of $F_1$ plants crossed and selfed in each backcross generation and the number of $F_2$ derived lines evaluated. Additive genetic variance was shown to decrease linearly with backcrossing when one $BCF_1$ plant was used. However the relationship was curvilinear if more than one $BCF_1$ plant was used. As the number of $BCF_1$ plants increases, the additive genetic variance among $BC_1F_2$ derived lines approached that among $BC_0F_2$ derived lines. The number of $BCF_1$ plants used at each backcross generation is therefore important in determining the genetic variance of $F_2$ generations and has implications on the adoption of different backcross strategies.

Continuous reductions in variability are inherent in the backcross method. To utilise the genetic variability available and to enhance possible gains arising from the regular recombinations inherent in the backcross method, a plant breeder can improve genetic variability through;

(1) choice of donor and recurrent parents based on estimates of genetic distance
(2) control of the number of backcrosses before selection
(3) the size of populations used for selection, including the number of $F_1$ plants crossed and the number of $F_2$ (or later) derived lines selected.

*2.5.2.2 Genetic distance between parents and transgressive segregation*

Transgressive segregation is often observed in wide, interspecific crosses (Lawrence and Frey, 1975; Vega and Frey, 1980). Lawrence and Frey (1975) produced many high yielding transgressive segregants in a backcrossing program involving *A.sativa* and *A.sterilis* germplasm. They attributed the large number of transgressives to the extremely wide exotic x adapted crosses. Despite the high frequency of transgressive segregants found in exotic by adapted crosses, Cowen and Frey (1987) reported that genetic distance estimates were relatively poor predictors of transgressive segregation in oats. Rick and Smith (1953) and de Vicente and Tanksley (1993) proposed two explanations for the occurrence of intraspecific

transgression which could account for this disparity; (1) complementary action of genes from parental species, and (2) unmasking of recessive genes normally held heterozygous in wild species.

de Vicente and Tanksley (1993) and other studies (Tanksley, 1993; and Veldoom and Lee, 1994) have provided evidence that transgressive segregation may be largely due to the accumulation of complementary QTL alleles in the two species and therefore simply choosing parents that are genetically dissimilar will not guarantee transgressive segregation. Mather *et al.* (1997) studied QTL for malt quality traits in a doubled haploid population of an intra-specific cross between two North American two row barley cultivars. They found that where one parent alone, was the major contributor of QTL for a specific malt quality characteristic, the frequency of transgressive segregants for that character was low. In contrast a high frequency of transgressive segregants were identified for malt quality traits for which both parents contributed positive QTL. This provides further evidence that transgressive segregants arise from a combination of complementary QTL.

Transgressive segregation is often observed in wide interspecific crosses, yet the performance of the parents is often a poor predictor of these transgressive segregants (Bramel-Cox and Cox, 1989; de Vincente and Tanksley, 1993; Eshed and Zamire, 1994; and Tanksley *et al.*, 1996). The problem in utilising wild germplasm therefore lies in identifying the sources of complementary QTL.

In a survey of methodology used by wheat breeders throughout the world, Peterson (1957) found that many wheat breeders did not use the full backcross method to reconstitute the recurrent parent, but used only one, two or three backcrosses to retain the benefits of transgressive segregation for agronomic characters such as grain yield. Eaton *et al.* (1986) compared single cross, three-way cross, and one and two backcrosses between unadapted and adapted spring wheat cultivars and looked for differences in the number of transgressive segregants for grain yield. The single cross generated the least number of high yielding transgressive segregants. The first backcross and three-way cross produced a similar number of high yielding lines. The second backcross produced no more high yielding lines than the first backcross or three-way cross and required and additional generation to develop. A greater number of high yielding segregants should have been expected from the three-way cross. If the two adapted cultivars were genetically similar, or did not carry complementary QTLs, then the three way cross would be expected to behave similarly to the first backcross ($BC_1$). While Lawrence and Frey (1975) only compared different backcross generations they found that the early generations ($BC_1$-$BC_4$) were best for selecting high-yielding transgressive segregants in interspecific crosses in oats. It is likely, therefore, that the preference for early generation backcross stages was not directly due to regression of the backcross parents towards the recurrent genotype but to the indirect loss of complementary QTLs as discussed above.

Selection of genetically distant donor and recurrent parents and stopping backcrossing in early generations to optimise opportunities for transgressive segregation would come at a cost of relinquishment of control over the highly regulated genetic forces inherent in the backcross method. Since the basis for transgressive segregation has been proposed to be due to complementary QTL, then compromising the recovery of the recurrent parent genotype and phenotype by selecting genetically distant parents and stopping backcrossing early may not be necessary. Identification of the chromosomal location of these complementary QTL and marker assisted selection for chromosome regions associated with these complementary QTL could overcome this dilemma. The problem in utilising this strategy again lies in identifying sources and chromosomal location of complementary QTL.

#### *2.5.2.3 Genetic distance between parents and heterosis in cross-pollinated crops*

Maize and other hybrid crop breeders often place parents into heterotic groups. A heterotic group is a collection of germplasm that, when crossed to germplasm from a different heterotic group tends to exhibit a higher degree of heterosis than when crossed to a member of its own group (Lee, 1996). The utility of using RFLP-based estimates of genetic distance among elite maize inbreds, for placing lines into heterotic groups, has been demonstrated (Lee *et al.*, 1989; Melchinger *et al.*, 1990; Messmer *et al.*, 1993). Associations between hybrid performance and predicted heterozygosity have, however, been stronger for crosses between lines of similar pedigrees (Lee *et al*, 1989; Smith *et al.*, 1990). Stuber (1994a), Melchinger *et al.* (1990) and Dudley *et al.* (1992) all concluded that marker diversity alone did not appear to be a very satisfactory predictor of hybrid grain yield. Melchinger *et al.* (1990) stated that;

> "it seems necessary to employ specific markers for those segments that significantly affect the expression of heterosis for grain yield".

They implied that heterosis was under the control of specific QTL. Stuber *et al.* (1992) identified QTLs contributing to heterosis in inbred maize lines and found that grain yield was enhanced with marker-assisted backcrossing and selection for the QTL identified. An optimum strategy may, therefore, consist of the application of genetic distance estimates to place cultivars into heterotic groups followed by marker assisted selection for QTL conferring heterosis.

## 2.6 Recovery of the recurrent parent phenotype and genotype with backcrossing

Sunenson, Riddle and Briggs (1941) attempted to show the validity of Brigg's expectation that the yield, quality and adaptation of the recurrent parent could be recovered through backcrossing (Briggs, 1930). They compared the grain yield and adaptation of stem rust resistant White Federation 38 with its recurrent parent, White Federation, and the bunt resistant

Baart 38 with its recurrent parent, Baart. They found no significant difference in grain yield, plant height, date of heading or in reaction to disease other than bunt and stem rust. The authors concluded that Brigg's expectations were correct and the recurrent parent type could be recovered successfully through backcrossing. The authors did not, however, test for differences in physical grain quality, milling and baking quality. While this early study showed that the predicted recovery of the recurrent parent had been achieved for grain yield, maturity and plant height, they did not discuss whether adequate recovery of the recurrent type could have been achieved with fewer backcrosses.

Briggs and Allard (1953) drew attention to the fact that the recovery of the recurrent parent type is primarily a function of the number of backcrosses and that selection in the early backcross generations is effective in directing the population towards the characteristics of the recurrent parent. Leininger and Frey (1962) showed that selection during backcrossing for the recurrent parent maturity and plant height hastened regression towards the phenotype of the recurrent parent. Briggs and Allard (1953) believed that selection for the recurrent parent phenotype, at each backcross stage, if based on moderate-sized populations, was equivalent to one or two additional backcrosses in a continuous series. They provided no theoretical or empirical evidence to support this conclusion, nor any explanation of its genetic basis.

While these early authors were confident that the recurrent parent type could be recovered adequately, all be it not as rapidly as expected, many studies with isolines or near isogenic lines have shown them to deviate significantly in performance from their recurrent parents (eg. Burton *et al.*, 1968; Russell and Eberhardt, 1970; Frey and Browning, 1971; Kohel and Richmond, 1971; Brinkman and Frey, 1977; Kolster *et al.*, 1986; Park and Tu, 1987; Zeven *et al.*, 1986). Most of the early studies reported involved the bulking of a large number of $F_3$ lines to form the final cultivar and, as already stated, this is likely to have reduced the overall observed variation between the backcross derived cultivar and its recurrent parent. Most comparisons have involved relatively simple traits such as plant height and flowering date, many compared grain yield differences while very few compared physical, chemical and processing quality characteristics. Recovery of a suitable quality type is as important as the recovery of an agronomic type for many backcross breeding programs.

The many differences between near-isogenic lines documented in the studies listed above were believed to be largely due to linkage between genes controlling quantitatively inherited traits and the simply inherited donor gene sought in each of the respective backcrossing programs or studies. This phenomena has been termed "linkage drag" (Brinkman and Frey, 1977) and is often defined as a slower than expected rate of elimination of donor parent genes.

### 2.6.1 Linkage drag

The problem of linkage drag in backcrossing programs has been discussed by many authors including Harlan and Pope (1922), Briggs and Allard (1953), Hanson (1959), Allard (1960),

Leninger and Frey (1962), Zeven *et al.*, (1983), Cox (1984a and 1984b) and Brown *et al* (1989a). Zeven *et al.*, (1983) documented many examples of linkage drag in near-isogenic lines of wheat developed through backcrossing. These included one example where red auricles present in the donor parent but not the recurrent parent were carried through five backcrosses with selection for the stem rust resistance gene *Sr6*.

Allard (1960) described a hypothetical situation in which the aim of a backcrossing program was to transfer the allele *A* from an unadapted donor to an adapted recurrent parent and an undesirable allele *b* was linked to *A*. The genotype of the $F_1$ was *Ab/aB* and selection for *A* in the first generation would tend to pull along *b*, making it difficult to obtain the desired recombination *AB*. Since *B* was reintroduced with each backcross, there were a number of opportunities for the crossover to occur. If no selection was practiced, except for *A*, it can be shown that the probability of eliminating *b* is $1-(1-p)^{m+1}$ where *p* is the recombination fraction and *m* is the number of backcrosses.

Table 2 provides the probabilities of eliminating the undesirable gene over a range of recombination fractions after five backcrossings and five selfings. It can be seen that the chance of obtaining the desired recombination is greater with backcrossing than selfing. However, if selection can be practiced against the undesired allele, which is linked to the desired allele, selfing can be more efficient. Effective selection is not always possible, particularly when traits of low heritability are involved.

Table 2. Effect of linkage on the probability of eliminating an undesired gene linked to a desired gene with five backcrosses or with five selfings, Allard (1960).

| Recombination Fraction | Probability that the undesirable gene will be eliminated | |
|---|---|---|
| | With five backcrosses | With five selfings |
| 0.50 | 0.98 | 0.50 |
| 0.20 | 0.74 | 0.20 |
| 0.10 | 0.47 | 0.10 |
| 0.02 | 0.11 | 0.02 |
| 0.01 | 0.06 | 0.01 |
| 0.001 | 0.006 | 0.001 |

It is assumed that selection is practiced for the desired gene only

### *2.6.1.1 Progressive reduction in length of donor segment during backcrossing*

In a backcrossing program, the length of the chromosome segment carrying the donor gene should logically decrease with increasing number of backcrosses. As the size of the donor segment decreases so also should the donor parents contribution to the phenotype decrease.

Bartlett and Haldane (1935) estimated the length of the donor segment in a backcrossing program using the formula, $(2/t)*100$ centimorgans(cM), where $t$ is the number of backcrosses. These criteria were adequate for eight to ten generations of backcrossing only. Hanson (1959a) aimed to develop a distribution of the segment lengths heterozygous about a locus held heterozygous for any number of generations of backcrossing or selfing. Hanson (1959a) calculated the predicted length and length variance of a donor chromosome segment immediately surrounding the desired gene locus. For convenience, Hanson assumed that the donor segment appeared in the exact centre of the chromosome and that the segment containing the desired gene was the only segment introduced on the chromosome carrying the allele of interest.

Stam and Zeven (1981) later modified this method to account for the likelihood that a donor segment is separated from the segment containing the desired gene by one or more segments of recurrent parent genome. They also accounted for the highly probable occurrence that the segment containing the desired gene would not always occur at the exact centre of the chromosome. Both Hanson (1959a) and Stem and Zevens (1981), assumed no interference in crossing over. Table 3 summarises estimates of donor segment length after one to six backcrosses when the total chromosome length is assumed to be 100 centimorgans. They did not, however, take into account variation in recombination frequency across the genome.

Stam and Zeven (1981) took the perceived bias in Hanson's approach into account and concluded that these biases compensated for each other with the overall result of little difference in the predicted donor segment length and variance during backcrossing. From Table 3 it can be seen that the predicted length of donor segment after six backcrosses is still large at 32 cM for a chromosome of 100 cM in total length. After 20 backcross generations a region of 10 cM flanking the target gene is expected to persist. It is therefore highly likely that even after six backcrosses that a large number of donor genes are "dragged" along in the segment containing the desired gene. It can also be concluded, however, that undesirable linkage will eventually be broken with continued backcrossing.

Brown *et al.* (1989a) empirically tested the Stam and Zeven (1981) model for segmental lengths and residual background retention under backcrossing. Brown *et al.* (1989a) used isozyme and seed protein markers introduced from wild barley (*Hordeum spontaneum*) through backcrossing into the commercial cultivar Clipper. The authors used Stam and Zeven's (1981) tables to calculate the isozyme marked segments of the seven chromosomes after three backcrosses as ranging from 52 to 63 cM depending on the total length of the chromosome. The approximate minimum length of the barley chromosome one through to seven was reported by Sogaard and von Wettstein Knowles (1987) to range from 100 to 180 cM. This was later extended by Becker *et al.* (1995), using an approach of combined mapping of AFLP and RFLP markers, to a total chromosome distance ranging from 129 to 362 cM.

Despite the large underestimation of total chromosome length, the results reported by Brown *et al.* (1989a) agreed reasonably with Stam and Zeven's (1981) predictions.

Table 3. Comparison of Hanson (1959a) and Stam and Zevens (1981) predictions of donor segment length, in centimorgans (cM) for a chromosome of 100 cM in length after 1 to 6 backcrosses.

| Number of backcrosses | Prediction of donor segment length (cM) from Hanson (1959a) | | Prediction of donor segment length (cM) from Stam and Zevens (1981) | |
|---|---|---|---|---|
| | mean | variance | mean | variance |
| 1 | 79 | 23 | 78 | 25 |
| 2 | 63 | 26 | 63 | 27 |
| 3 | 52 | 25 | 51 | 26 |
| 4 | 43 | 23 | 43 | 24 |
| 5 | 37 | 22 | 37 | 22 |
| 6 | 32 | 20 | 32 | 19 |

Brown *et al.* (1989a) also found that the total proportion of the genome of the backcross lines that came from the donor parent (*Hordeum spontaneum*) after three backcrosses and selfing to be about ten percent. In each line, about 55% of the "alien" germplasm was in the marked segment, and the remainder recombined independently of this segment. Brown *et al.* (1989a) added that a further round of backcrossing would increase the percentage to 66% and therefore, as backcrossing proceeded, the proportion of alien germplasm within the marked segment would steadily increase. This was attributed to the expectation that the length of the alien segment around the marker would decrease logarithmically, where as that in the background would decrease exponentially (Brown *et al.*, 1989a).

### *2.6.1.2 Selection for small donor segment in recurrent parent background*

The aim of a backcrossing program is often the introgression of a single gene of value from a donor parent into a recurrent parent background while maintaining, as much as possible, of the established gene complexes of the recurrent parent. If the size of donor segment varies amongst individuals at the same stage in a backcrossing program, then selection for those individuals with a small segment will enhance the probability of recovering as much as possible of the recurrent parent gene complexes.

Young and Tanksley (1989) measured the size of introgressed fragments from *Lycopersicon peruvianum* flanking the *Tm-2* locus, confering resistance to tobacco mosaic virus, in several cultivated tomato cultivars (*L. esculentum*) using a high density map of RFLP markers. The authors compared several cultivars derived from different gene introgression programs. The number of backcrosses used to develop the cultivars varied from four to 21. The authors

found wide variation in the size of the segment around the *Tm-2* locus in different cultivars, ranging from 51 cM (cultivar derived from 11 backcrosses) to 4 cM (cultivar derived from 19 backcrosses). The cultivar with the smallest segment (4 cM) was used in an extended backcrossing program consisting of eight further backcrosses. No additional reduction in the size of the segment was achieved. In a similar program, involving a larger segment (50 cM) and a different locus, the segment size was reduced to 26 cM during seven additional backcrosses. Overall, little reduction in linkage drag was observed despite the large number of additional backcrosses. This was proposed to be due to the inability to select for desirable recombinants in proximity to the *Tm*-2 locus. To illustrate this point the authors described a further introgression program of the *Tm*-2 gene in which a visible marker, located two cM away, was used to select for individuals carrying the resistance gene. The result of this marker assisted selection program was to produce a tomato line with an exceptionally small segment of *L.peruvianum* DNA (less than 0.3cM). This study demonstrated evidence of variation in length of donor segment and that backcross breeding without selection was only moderately effective in reducing linkage drag around gene targets and that marker assisted selection had the potential to greatly reduce the problems associated with linkage drag.

## 2.7 Molecular marker assisted backcrossing

In several plant species, comprehensive genetic linkage maps of polymorphic DNA markers have been constructed including barley (Heun *et al.*,1991; Langridge *et al.*, 1995), maize (Helentjaris, 1987), and tomato and potato (Tanksley *et al.*, 1992). Traits of interest can be monitored in a segregating population and trait information on individuals within the population compared with their molecular marker linkage data. This combined analysis can be used to identify linkage relationships between molecular markers and genes and /or quantitative trait loci (QTL) controlling traits of interest. The linked molecular marker or markers can then be used to indirectly trace the gene(s) or QTL of interest in a backcrossing program.

A typical molecular marker assisted backcrossing program commences with the marker screening of several $BC_1$ individuals. Genotypes heterozygous for the desired marker allele(s) are retained and backcrossed to the recurrent parent. Several plants per $BC_2$ family are screened for the desired marker and one marker carrying plant per family is then backcrossed to the recurrent parent. This procedure is repeated for as many backcross cycles as necessary to recover the desired proportion of recurrent parent genotype. In the final generation, one plant of the desired marker genotype per family is evaluated (if possible) for presence of the gene of interest by direct or progeny testing. The optimum number of individuals screened and retained at each backcross generation will depend on linkage between the marker and the gene of interest and whether single marker or flanking markers are used (Melchinger *et al.*, 1990).

### 2.7.1 Comparison of marker assisted and conventional backcrossing

Some of the advantages of marker assisted backcrossing include;

(1) molecular markers are generally silent in their effect on the phenotype (Melchinger, 1990)

(2) some molecular markers show a co-dominant mode of inheritance and therefore heterozygous individuals can be identified. This is of particular benefit in a backcrossing program aimed at introgressing a recessive gene where the phenotype is not expressed in the heterozygote.

(3) the molecular marker genotype can be determined at a very early developmental stage. In a backcrossing program this ability to select early is particularly useful as plants carrying the gene or genes of interest can be identified prior to flowering and backcrossed once they begin flowering substantially accelerating the backcross procedure.

(4) molecular markers can be used for selecting regions of the recurrent parent genome unlinked to the introgressed region

(5) molecular markers can be used to select a small donor segment carrying the gene controlling the trait of interest reducing linkage drag of unwanted donor parent genome near the introgressed regions(s) (Lee, 1996). This provides a means of reducing the number of backcross generations required to recover the recurrent parent genotype and increase the probability of obtaining a suitable introgression product

(6) molecular markers offer the opportunity for simultaneous selection for multiple traits

(7) molecular marker assisted backcrossing can offer improved overall cost efficiency and rate of genetic gain

Lee (1996) provided a list of information that would be necessary to accurately determine the relative costs and rates of gain between marker assisted selection and conventional selection in a practical breeding program;

(1) the total time required to develop a cultivar

(2) variations in cultivar development time depending on quality type (eg malting barley vs feed barley)

(3) expected commercial duration of that cultivar

(4) economic value of these estimates of time from the perspective of the breeder, grower and industry

(5) total financial and genetic costs of developing a cultivar, including the costs of selection for a given breeding method and trait.

Some of this information for some breeding programs, and some traits, is probably available but, within the context of this review, attaining accurate information would be a highly complicated and tedious task. Jefferies *et al.* (1997) compared marker (RFLP) assisted selection and conventional selection for a monogenic trait, cereal cyst nematode (*Heterodera avenea*, CCN) resistance using the bioassay described by Fisher (1982) (Table 4). In this example the bioassay system is a highly efficient (partly mechanised and computerised) service

provided by the Field Crop Pathology Unit of the South Australian Research and Development Institute (SARDI) for cereal breeding programs in southern Australia.

Table 4. Comparison of marker assisted selection and an alternative bioassay for cereal cyst nematode (CCN) resistance (adapted from Jefferies *et al.*, 1997)

| Feature | RFLP marker | Bioassay |
|---|---|---|
| Time | 4 days | 14 weeks |
| Plants needed for reliable assay | 1 | 6-10 |
| Cost per genotype | $4 | $5 |
| Suitability of transplanted seedlings for crossing | Yes | No |
| Potential quantity of seed from selected plants or transplants | High | Low |
| Multiple trait selection | Yes | No |
| Identification of heterozygotes | Yes | No |

As can been seen from Table 4, marker assisted selection involves considerably less time allowing for plants to be screened prior to flowering and with the use of controlled environment conditions, at least three backcrosses are possible in a 12 month period. In addition, plants can be screened with molecular markers as soon as adequate leaf material is available for DNA extraction. In contrast, the optimum growth stage for selecting resistant plants in the bioassay is soon after anthesis. It is estimated that between six and ten plants are required in the bioassay to reliably characterise resistance status.

The bioassay has inadequate precision for $F_1$ generation material and therefore backcross progeny must be allowed to self prior to screening. While the cost per plant is less for the bioassay, the cost per genotype is similar. Because RFLP markers are co-dominant, heterozygote individuals can be identified. This is less relevant in this example as CCN resistance in barley is controlled by either of two single dominant genes (Kretschmer *et al.*, 1997; Barr *et al.*, 1998). However, the RFLP marker can be used to identify CCN resistant homozygous individuals in the $F_2$ and later generations, at the end of a series of backcrosses, eliminating the need for re-selection. In addition to these advantages, marker screened plants can be grown in optimum conditions, suitable for crossing and permitting maximum seed set. Each of the features listed in Table 4 can have an impact on the planning and costing of a breeding strategy. There is no doubt that marker assisted selection can be more efficient and allows the breeder greater planning flexibility than the bioassay. The bioassay is still necessary for final resistance validation.

To demonstrate the potential for increases in genetic gain with marker assisted selection, Beckmann and Soller (1986) calculated the expected frequency of a favourable allele after one

to six backcross generations, with and without selection for a linked marker allele and a pair of flanking markers (Table 5).

Table 5. The frequency of a favourable allele after a given number of backcrosses, with and without selection for a linked marker allele or flanking marker alleles (adapted from Beckmann and Soller, 1986).

| Number of backcross generations | No markers | Single marker[1] | Flanking markers[2] |
|---|---|---|---|
| 1 | 0.25 | 0.81 | 0.92 |
| 2 | 0.12 | 0.73 | 0.88 |
| 3 | 0.06 | 0.66 | 0.85 |
| 4 | 0.03 | 0.59 | 0.82 |
| 5 | 0.02 | 0.53 | 0.78 |
| 6 | 0.01 | 0.48 | 0.75 |

[1] proportion of recombination between marker allele and linked favourable allele, 0.10
[2] proportion of recombination between flanking markers, 0.40

It can be seen from Table 5 that the frequency of the favourable allele is substantially increased when marker assisted selection is used. Therefore, without marker assistance, considerably more backcross lines would have to be screened for the introduced trait. It can be seen that the frequency of favourable alleles is even greater when a pair of markers flanking the gene to be introgressed is used. Flanking markers have the advantage of tighter linkage to the gene of interest but a potential disadvantage is that a larger proportion of the donor parent genome is likely to be linked to flanking markers (Dudley, 1993).

A major advantage of marker assisted backcrossing over conventional backcrossing is the improved efficiency of introgressing several traits in the single program. The most efficient strategy for achieving this is with the use of several backcross streams, introgressing each trait in a separate backcross stream and then merging the streams near or at the final cross. The advantages of marker assisted backcrossing described earlier would equally apply to each of the streams but the major advantage would be in the selection desirable recombinants for several traits almost simultaneously within the final merged population. The ability to introgress several traits with marker assisted selection would be even greater if more than one marker linked trait is carried by a single donor parent and the traits can be selected simultaneously.

At least two studies (Lande and Thompson, 1990; Zhang and Smith, 1992) have assessed the effect of marker-assisted selection on improving the selection accuracy. The results generally showed that significant gains in accuracy were possible for low heritability traits, while there appeared to be little or no significant gain for traits of high heritability.

Comparisons between marker assisted selection and conventional selection for the introgression of quantitative characters will be discussed in a later section.

### 2.7.2 Marker assisted selection for recurrent parent background

The underlying principle of any backcrossing strategy is that the expected proportion of donor parent genome reduces by fifty percent with each generation of backcrossing. Ignoring the effects of linkage drag to the desired donor parent locus under selection, the mean percentage of donor parent genome expected in each backcross generation is calculated as; % donor parent genome=$100(0.5)^{n+1}$ where $n$ is the number of backcrosses.

Until recent times, most backcross strategies have focused on this underlying principle and have ignored the genetic variation for the proportion of donor parent genome that exists around the expected mean. Molecular markers could be used to identify recombinant individuals that have genome compositions closer to that of the recurrent parent than would be predicted from theoretical expectations (Tanksley and Rick 1980).

Powell *et all.* (1996a) genotyped, with 62 polymorphic AFLP products, a $BC_1$ doubled haploid population. They found that the percentage donor DNA composition varied from 8% to 60%. The authors estimated that selection of the genotype with 8% donor DNA in the $BC_1$ would be equivalent to advancing this population to a $BC_3$ generation. It was proposed that the reduction in number of individuals processed in a backcrossing program would substantially reduce the cost associated with further testing and evaluation. This study provides evidence of variation for recurrent parent genome around the theoretical mean.

Table 6 shows how the proportion of recurrent parent genome can rapidly approach 1.0 with marker assisted selection for recurrent parent background. Young and Tanksley (1989) estimated that an introgressed segment could be reduced in two generations of marker-assisted selection for the recurrent parent genotype, to a size, which would require approximately 100 generations of backcrossing without marker-assisted selection.

Openshaw *et al.* (1994) used results from previous literature, computer simulation, and empirical studies to evaluate the application of molecular markers in selection for recurrent parent background in a backcross program. The authors tested several different methods involving different number of progeny, backcross generations, number of markers and number of individuals selected to form the next backcross generation. Selection was based on the presence of the donor allele and high proportion of recurrent parent genome. In the computer study, all methods modelled, greatly increased the speed of recovery of the recurrent parent genome compared to the expected recovery without marker-assisted selection. It was estimated that using markers, the number of backcross generations needed to successfully introgress a single dominant gene into a maize inbred, was reduced from about seven to three.

The number of markers used was found to be important. A small number of markers quickly became un-informative as the marker loci became fixed for the recurrent parent type while the remainder of the genome still carried a significant proportion of donor parent genome. The results of the simulation study compared well with empirical data.

Table 6. The expected proportion of recipient genome recovered after a given number of backcrosses, with and without marker assisted selection against the remaining exotic genome (adapted from Beckmann and Soller, 1986).

| Number of backcross generations | No background selection | Full marker coverage[1] |
|---|---|---|
| 1 | 0.75 | 0.85 |
| 2 | 0.88 | 0.99 |
| 3 | 0.94 | 1.00 |
| 4 | 0.97 | 1.00 |
| 5 | 0.98 | 1.00 |
| 6 | 0.99 | 1.00 |

[1] assuming two markers per chromosome

Hospital *et al.* (1992) investigated, also using computer simulation, the effect of time and intensity of marker-assisted background selection, population size, and the number and position of selected markers on the recovery of the recurrent parent genome. The authors concluded the following;

(1) markers were most useful when their map position was known. The response to selection for recurrent parent background, using mapped markers, was approximately 30% greater than using un-mapped markers.

(2) two to three markers per 100 cM was optimal in early generations. In early generations, few recombination events would have occurred and therefore donor parent segments would be relatively large. More markers would be required in later generations as the number of segments increases but their length decreases. The practical gains in efficiency of increasing marker density in later generations was, however, found to be low as the overall proportion of donor parent genome would also be low.

(3) selection for the introgressed gene should not focus exclusively on proximal markers, particularly in early generations and when population size is low. Selection based only on the most proximal flanking markers may exclude individuals with a large donor segment but low overall donor parent genome. It may be beneficial, therefore, to use distal flanking markers in early generations and more proximal flanking markers in later generations. If a large population size can be managed and thereby a high selection intensity used, the use of more proximal markers would be more efficient

(4) a single generation background selection was most efficient if selection was performed in the last generation. The efficiency of continuos selection depends on the selection intensity

used. Marker allele fixation occurs very rapidly at high selection intensities, and markers become useless after fixation.

(5) It did not seem possible to simultaneously optimise selection both against donor parent genome on non-carrier chromosomes and for a small introgression segment. A selection index that weights markers around the introgression segment equally to those at the remainder of the genome was proposed. The relative weighting would depend on genome size, the generation considered and the genetic distance between donor and recurrent parent

(6) It was possible to save at least two generations of backcrossing with marker assisted selection for recurrent parent background

Frisch *et al.* (1999) used computer simulation to compare marker-assisted backcrossing selection strategies with regard to (1) the proportion of recurrent parent genome recovered and (2) the number of marker loci required to introgress a single gene from the donor parent. They compared a two stage selection strategy, consisting of one introgression gene and one background selection step, with alternative selection strategies consisting of one introgression gene selection step and two or three background selection steps. Simulations were performed with a published maize map consisting of 80 markers. The results of Frisch *et all.* (1999) compared favourably with those of Hospital *et al.* (1992). Frisch *et all.* (1999) found that the backcross procedure could be terminated after four instead of six backcross generations even with small population size and small number of marker loci. With larger populations and more marker loci, marker assisted selection was shown to have the potential to reach, in the $BC_3$ generation, the same proportion of recurrent parent genome as the $BC_7$ generation without marker assisted selection. It was proposed that gains could have been even greater if marker loci evenly distributed throughout the genome were selected. Increasing population sizes from generations $BC_1$ to $BC_3$, compared to a constant population size across all generations, were found to reduce the number of marker loci required by as much as 50%, as many marker loci were already fixed in later generations. Selection for the desired donor gene and a single final background selection stage was shown to be more effective than continuous background selection for a limited backcross program ($BC_1$ or $BC_2$ ). Information on the location of marker loci in early generations was also less important. If reduction in linkage drag is an important issue in the backcrossing program, then a four stage (continuous selection) strategy was recommended.

Results from simulation studies of Hospital *et al* (1992), Openshaw *et al.* (1994) and Frisch *et al.* (1999) all support the conclusion that between two to four generations of backcrossing can be saved with marker assisted selection for recurrent parent background. The relative speed and efficiency of different selection strategies depends largely on population size, selection intensity, number of marker loci, knowledge of the location marker loci, map distance between flanking markers and the target gene and knowledge of the potential for deleterious linkage drag. The relative resources available to the breeder would therefore dictate the most efficient selection strategy that could be adopted. Like simulation studies in general, the results depend on the underlying model and the theoretical assumptions on which the model is based. There

are relatively few reports in the literature, however, on the successful application of marker assisted selection for recurrent parent background in practical backcrossing programs.

Genetic distance between donor and recurrent parent was not considered in these simulation studies. For example, Frisch *et all.* (1999) selected 80 RFLP markers from a published maize linkage map as the basis for their simulations. By default, all markers were polymorphic. In reality, the level of polymorphism would vary considerably, depending on the genetic distance between donor and recurrent parent. The implications of genetic distance between donor and recurrent parent on strategies for marker assisted selection for recovery of recurrent parent background warrants further investigation.

### 2.7.3 Limitations to marker assisted backcrossing

A general assumption used in backcrossing schemes is that the donor genome is inferior to the recurrent parent genome at all regions except for the location of the target gene(s) (Lee, 1996). Studies in oats (Lawrence and Frey, 1976), in maize (Lee *et al.*, 1990) and tomato (de Vincente and Tanksley, 1993) have demonstrated that the donor parent can carry positive factors for traits either unrelated or related to the backcrossing program objective. Regions of the donor parent genome carrying these factors would be routinely eliminated by marker assisted backcrossing unless prior knowledge of their merit was available (Lee, 1996). Under these circumstances, the products of marker assisted backcross could be inferior to those obtained through conventional backcrossing. These potentially negative aspects need to be balanced against the absolute objective of the backcross strategy, that is, recovery of the recurrent phenotype carrying the introgressed trait versus general improvement in the recurrent parent, and also the value of the time and resources saved.

## 2.8 Introgression of quantitative characters with backcrossing

### 2.8.1 Introgression of quantitative traits using the conventional backcross method

Backcrossing has traditionally not been the method of choice for the introgression of quantitative characters into breeding populations. It is often assumed that many genes with small additive effects condition quantitative traits. The probability of recovering a satisfactory number of these genes through backcrossing is often assumed to be very small and not worthwhile attempting as selecting individuals carrying different numbers of QTL is very difficult.

Meredith (1977) assessed the use of the backcross method as a procedure to improve the quantitative traits of fibre strength and lint yield in cotton. These traits were shown to be

negatively correlated. The high lint yield parent 'Deltapine 16' was used as the recurrent parent while the high fibre strength line 'FTA 263-20' was used as the donor parent. While the backcross populations produced from three cycles of backcrossing were not equal in fibre strength to FTA 263-20, fibre strength was maintained at a satisfactory level. It was pointed out, however, that the maintenance of high fibre strength by a 10% selection differential in the $BC_3F_3$ suggested that a relatively small number of genes were conditioning this trait.

Duvick (1974) reported the success of continuous backcrossing for rapid transfer of a quantitative trait, prolificacy (multi-ear trait), from exotic to adapted strains of maize. The lines derived after four backcrosses were more prolific than the recurrent parent but less multiple eared than the original donor parent. No attempt was made to estimate the number of genes involved.

Knott and Talukdar (1971) reported success in transferring high seed weight by backcrossing in spring wheat. While the selected lines, following five cycles of backcrossing, were greater in seed weight than the recurrent parent, none of the lines with increased grain weight equalled the donor parent under drought stress conditions. Sharma and Knott (1964) had previously obtained data indicating that seed weight in the donor parent was controlled by relatively few genes.

Wilcox and Cavins (1995) showed that the quantitatively inherited trait, high seed protein, could be introgressed into adapted soybean cultivars, through backcrossing, without loss of grain yield despite an inverse relationship between these two traits. Selection for the recurrent parent phenotype as a secondary trait to protein in each backcross population was effective in recovering the recurrent parent with only three backcrosses. Wehrmann *et al.* (1987), in similar attempts to improve seed protein in soybeans through backcrossing, were able to recover the grain yield of the recurrent parent but were not completely successful in recovering the seed protein concentration of the donor parent.

Most reported attempts to introgress quantitatively inherited characters with conventional backcross methods have failed to achieve full expression of the donor parent trait. Success in the application of conventional backcrossing as a method for improvement of a quantitatively inherited trait appears to be largely dependent on the number of genes controlling that character, the ease and timing (generation) of selection, and therefore manageable selection intensity and population sizes, the desired level of recovery of the recurrent parent phenotype, the accuracy of screening procedures, and/or the ability to identify individual QTL.

### 2.8.2 Molecular marker assisted introgression of quantitative traits

Paterson *et al.* (1988 and 1990) demonstrated the application of a high density genetic linkage map of RFLP markers and linkage analysis for determining the location of several QTL for three fruit quality traits in tomato. Tanksley *et al.* (1996), identified QTL for enhanced

characteristics in a large number of horticultural traits derived from wild tomato species (*Lycopersicon piminellifolium*). QTL controlling grey leaf spot (*Cercospora zea-maydis*) resistance (Bubeck *et al.*, 1993), QTL influencing several flowering parameters and anthesis silking interval related to drought tolerance (Ribaut *et al.*, 1997) and QTL associated with 24 other agronomic traits (Beavis *et al.*, 1994), have been mapped in maize. Mapping of QTL that affect grain and malt quality has been performed in a North American six-row barley cross (Hayes *et al.*, 1993; Han *et al.*, 1995), a North American two-row barley cross (Mather *et al*, 1997), a European two-row barley cross (Chalmers *et al.*, 1993; Thomas *et al.*, 1995), and two Australian two-row barley crosses (Li *et al.*, 1998). In addition, Tinker *et al.* (1996) reported on QTL that affect agronomic performance in the same North American two-row barley cross while Jefferies *et al.* (1999, and this thesis) reported on the mapping of QTL for boron tolerance in two-row by 6-row barley. These studies are only a small proportion of recent quantitative trait mapping initiatives in crops of agronomic importance.

Since it is possible to identify discrete regions of the genome that account for a significant proportion of variation in a quantitative trait, marker assisted selection for these regions should be effective in improving that quantitative trait. Stuber *et al.* (1982) showed that selections based only on manipulations of allelic frequencies at seven isozyme loci significantly increased grain yield in maize. Stuber and Edwards (1986) showed that marker assisted selection, based on only 15 isozyme marker loci was as effective as phenotypic selection in maize. Stuber (1994b) reported on the backcross introgression of six QTL for enhanced grain yield in maize and, although not all six QTL were successfully introgressed into the recurrent parents, hybrids with grain yield exceeding the recurrent parents by 8-11% were produced. These studies with maize demonstrate that marker assisted selection can be effective in improving quantitative traits, even those as complex as grain yield.

Paterson *et al.* (1991) found that, for a trait with low heritability, the phenotype of the $F_3$ progeny could be predicted more accurately from the genotype of the $F_2$ parent, based on QTL, than from the phenotype of the individual. For a trait with high heritability, in contrast, knowing the QTL genotype of an individual, added little or no predictive value to simply knowing the phenotype. For a trait of low heritability, an individuals phenotype is more greatly influenced by non-genetic factors such as environment. For a mapped QTL, the phenotypic effect is estimated from data on many individuals, thus environmental influences should be reduced (Paterson *et al.*, 1991). Genotypic selection for traits of low heritability should, therefore, be more efficient than phenotypic selection so long as a large proportion of the additive genetic variance is associated with the marker loci (Lande and Thompson, 1990). Lande and Thompson (1990) provided the theory and showed analytically that maximum rate of improvement may be obtained by integrating both phenotypic and marker assisted selection for quantitative traits. This of course would depend largely on a number of factors including (1) the level of linkage disequilibria in the populations which affects the number of marker loci needed; (2) sample sizes needed to detect QTL for traits with low heritability; and (3) sampling

errors and other factors associated with the relative ease of phenotypic selection (Lande and Thompson (1990) and Stuber (1994a)).

Backcrossing was not the breeding method that was used in the QTL selection studies discussed above. In a review of breeding procedures useful for both small and large number of QTL, Dudley (1993) reported that the backcrossing procedure was most appropriate for quantitative traits controlled by a relatively small number of loci.

Using simulation, Hospital and Charcosset (1997) investigated the potential of using both foreground and background marker assisted selection in backcross breeding programs aimed at introgressing one to several QTL. They showed that it was possible to use markers to manipulate and introgress up to four unlinked QTL with population sizes of a few hundred, if; (1) the position of flanking markers is optimised with respect to the probability of the presence of the donor allele at the each QTL and (2) the markers are evenly spaced over the remainder of the genome. It was demonstrated that using at least three markers per QTL allowed good control over several generations. More QTL could be manipulated if; (1) the QTL were linked; (2) very large population sizes were used, and/or (3) if the precision of gene location was high. Compared with a program monitoring several QTL simultaneously, treating QTL one by one in a first step, was as efficient as selecting for recurrent parent background with small population size or higher efficiency with the same population size.

The major limitations to marker assisted backcrossing of QTL compared with qualitative traits is that QTL locations are usually estimated imprecisely and therefore more markers will be required to trace the donor segment and larger donor segments are likely to eventuate. The precision in the identification of the number and location of QTL depends largely on population size, marker density and the quality of phenotypic data. QTL mapping techniques will often have the statistical precision necessary to identify only major loci involved in the control of a given quantitative trait. Marker assisted selection for QTL of such traits is therefore unlikely to fully recover the expression of the desired trait to the level of the donor parent.

The theory and examples outlined above suggest that marker assisted selection can improve the speed of introgression of QTL through backcrossing. Its routine adoption in plant breeding programs will however, depend on the complexity of the trait (number of QTL), heritability of the trait, precision in map location of QTL, availability of polymorphic flanking markers and on the relative costs and expected returns compared to conventional selection.

## 2.9 Introgression of exotic germplasm with backcrossing

An essential component of a modern plant breeding program is the establishment and maintenance of a diverse gene pool.

> "The gene pool must have new germplasm introgressed into it continuously, to permit the occurrence and selection of superior genotypes for commercial use and to maintain the breadth of genetic variability necessary to rapidly accommodate changing patterns of agricultural or commercial practice" (Finlay, 1969).

The introgression of exotic germplasm can provide the necessary genetic diversity and access to new or novel sources of genes for disease resistance and other traits of economic importance. Plant breeders have often been reluctant to use exotic germplasm, however, for fear that undesirable characteristics will be difficult to separate from valuable traits.

As discussed earlier, attempts by wheat breeders to introgress exotic germplasm (in this case within species) at the Swedish Seed Association (Mackey, 1963), using conventional breeding methods (other than backcrossing), failed to produce progeny superior to the adapted Swedish cultivars. It quickly became apparent that the successful incorporation of exotic germplasm relied on minimising the disruption of the gene complexes found in the adapted genotypes. For this reason, backcrossing has become the predominant breeding method for the introgression of exotic germplasm in plant breeding programs.

Anderson and Hubricht (1938) first used the term "introgressive hybridisation" to describe the introduction of germplasm from one species into that of another through hybridisation and repeated backcrossing. While backcrossing by definition involves exotic germplasm (donor parent) the term 'exotic' in most cases in the literature refers to germplasm derived from related species. The term "exotic" can, at times, also refer to distantly related cultivars or landraces within the same species.

Thomas (1952) discussed the introgression of germplasm from *N. glutinosa, N. rustica, N. longiflora, N. gossei* and *N. gluauca* in the breeding for disease resistance in tobacco (*N. tabacum*). Germplasm from at least five different wild relatives of cultivated tomato have been introgressed into cultivated tomato (*L. esculentum*) and shown to contribute many valuable horticultural traits (Paterson *et al.*, 1988; Paterson *et al.*, 1991; de Vincent and Tanksley, 1993; Tanksley *et al.*, 1996 and Fulton *et al.*, 1997). Many sources of exotic germplasm have been successfully introgressed into cultivated potato (*Solanum tuberosum* ssp. *tuberosum)* which led to improvements in several traits, particularly resistance to *Phytophthora infestans* (Debener *et al.*, 1991). Genes for disease resistance have been introgressed from *Thinopyrum intermedium* (Sharma *et al.*, 1995) and *Triticum tauschii* (Cox *et al.*, 1994) into bread wheat (*Triticum aestivum* L.). CIMMYT make large numbers of interspecific hybrids, utilising

backcrossing, between triticale and bread wheat as a major component of its effort to diversify spring triticale germplasm (Barker *et al.*, 1989). Genes for disease resistance derived from *Hordeum spontaneum* have been introgressed into cultivated barley (*Hordeum vulgare* L.) (Brown *et al.*, 1989b). Frey and Browning (1971), Lawrence and Frey (1975) and Takeda *et al.*, (1985) all reported success in generating new genetic variation for grain yield, and other characters such as vegetative growth rate, by backcrossing *Avena sterilis* germplasm into *Avena sativa* backgrounds.

### 2.9.1 Evaluation of alternative breeding methods for introgression of exotic germplasm for the improvement of quantitative traits

Early work with the introgression of exotic germplasm into cultivated soybean drew Schoener and Fehr (1979) to conclude that there was very little potential for using exotic (*Glycine soja.*) germplasm to increase grain yield in cultivated soybeans (*Glycine max*). Only single cross populations between *G. max* and *G. soja* were produced however. Carpenter and Fehr (1986) later studied the genetic variability for agronomic traits in backcross populations containing different percentages of *G. soja* germplasm. They found that the percentage of segregants agronomically equal to the recurrent parent was from zero in the $BC_0$ and $BC_1$ to 65% in the $BC_5$. The later study showed that exotic soybean germplasm could be successfully accessed but only a small proportion of exotic genome could be tolerated in adapted soybean background.

Eaton *et al.* (1986), Helms *et al.* (1993) and Hoffbeck *et al.* (1995) assessed alternative breeding methods for the introgression of exotic germplasm for improvement in quantitative traits. These studies found that means and variances for quantitative traits were very much a function of the parents chosen and the crossing method had only minor effects.

### 2.9.2 Simultaneous mapping and introgression of quantitative trait loci from exotic germplasm

Unadapted or wild species germplasm has been used mainly as a source of major genes for disease and insect resistance which can be readily introgressed into adapted types through backcrossing (examples discussed above). Even with monogenic traits, exotic germplasm introgression has been fraught with problems associated with linkage drag (Tanksley and Nelson, 1996). Problems of linkage drag are likely to be compounded with the introgression of quantitative traits as this would involve more, and potentially larger, donor parent segments. It has been shown that molecular marker assisted selection can substantially reduce linkage drag in an exotic germplasm introgression program (Young and Tanksley, 1989).

Transgressive segregation for quantitative traits is often observed in wide interspecific crosses (Lawrence and Frey, 1975; Vega and Frey, 1980) yet the performance of the parents is often a

poor predictor of these transgressive segregants (Bramel-Cox and Cox, 1989; de Vincente and Tanksley, 1993; Eshed and Zamire, 1994; and Tanksley *et al.,* 1996). While it is relatively simple to identify disease resistance in unadapted germplasm it is difficult to identify accessions that are likely to carry genes for quantitative traits as the unadapted germplasm is almost always inferior to adapted germplasm (Tanksley and Nelson, 1996). Several studies in tomatoes, utilising molecular markers (de Vincente and Tanksley, 1993; Eshed and Zamir, 1994: Tanksley *et al.*, 1996), have demonstrated that QTL isolated from wild accessions, can substantially improve the phenotype of commercial tomato cultivars for most quantitative traits. Regardless of the trait, 10-15% of the QTL detected in the wild species in these studies, improved the trait of interest, even though the wild species phenotype was inferior to that of the cultivated parent (Tanksley and Nelson, 1996).

Traditional QTL mapping techniques have several deficiencies when applied to the detection and introgression of QTL from unadapted germplasm (Tanksley and Nelson,1996);

(1) undesirable alleles occur in high frequency in most unadapted germplasm, making the collection of meaningful data difficult, (eg in barley, lodging, shattering, sterility, large variation for maturity)

(2) Epistatic interactions are statistically difficult to detect, yet are likely to occur in high frequency in conventional ($F_2$, $BC_1$ or recombinant inbred) populations. The most desirable QTLs are those not requiring epistatic interactions.

(3) Subtle (and often negative) pleiotropic effects may go unnoticed in conventional mapping populations due to the large genetic and phenotypic variance created by the segregation of donor alleles in high frequency.

Tanksley and Nelson (1996) proposed that to overcome these problems QTL analysis be delayed to an advanced backcross generation (eg $BC_2$ or $BC_3$). This would overcome agronomic problems associated with individuals within a mapping population which carry a high proportion of wild germplasm. The detection of QTL with epistatic effects would be much reduced. Deleterious effects due to linkage drag would also be less likely.

Typically, conventional mapping populations are used to identify desirable QTL and once identified, marker assisted selection is used to introgress them into elite adapted parents for potential commercial release. The advanced backcross QTL analysis procedure, proposed by Tanksley and Nelson (1996), can be used to simultaneously identify and introgress valuable QTL from unadapted germplasm into elite breeding lines, which can be either, released commercially or used as parents. The potential of this method has been successfully demonstrated in tomatoes by Tanksley *et al* . (1996). The potential in other crop species has not been demonstrated and is likely to be more difficult in more complex genomes than tomatoes. With this in mind, however, the application of this methodology could provide the opportunity to successfully exploit the relatively under-utilised reservoir of genetic variation available amongst wild ancestors and related land races of many crop species.

### 2.9.3 Limitations to the backcross method for the introgression of exotic germplasm

Several studies have found significant interactions between the function of an introgressed chromosomal segment from a wild species and the genetic background of the adapted parent, due mainly to the presence of strong dominance or epistatic interactions (Lawrence and Frey 1975; Eaton *et al.* 1986; Tanksley and Hewitt 1988 and Bramel-Cox and Cox 1989). Consequently the use of a single inbred line as a recurrent parent may limit the potential for extracting favourable specific interactions between adapted and exotic genotypes because it provides a relatively constant genetic background for introgression (Menkir *et al.* 1994). Bramel-Cox and Cox (1989) proposed a broad-based population as an adapted recurrent parent for backcrossing. This approach places each allele or combination of alleles derived from an exotic parent in a broad range of genetic backgrounds which in turn enhances the opportunity to extract favourable epistatic combinations from among the adapted and exotic germplasm sources (Menkir *et al.* 1994). Menkir *et al.* (1994) compared the effectiveness of backcrossing to a broad-based population versus backcrossing to an inbred line for developing improved sorghum lines. Backcrossing to an inbred line gave fewer high yielding segregates, generated less genetic variation and produced less agronomically acceptable lines than backcrossing to a broad-based population.

Backcrossing appears to be an ideal mechanism for the introduction of exotic germlasm into adapted cultivars or germplasm. The number of backcrosses or the specific breeding strategy adopted by the plant breeder will depend on wether the desired trait is qualitatively or quantitatively inherited, wether transgressive segregation is desired, and will depend on the genetic similarity or distance between donor and recurrent parent.

## 2.10 Introgression of transgenes through backcrossing

Most transformation methods exhibit some degree of genotype specificity (Armstrong *et al.*, 1992). Therefore the transgene, in most cases, is likely to be introgressed into non-elite germplasm.

The performance of transgenic cultivars depends not only upon the stable and correctly regulated expression of specific transgenes but also upon the agronomic potential of the background genotype and the potential for it to augment appropriately-regulated expression of the genes and produce the optimum levels of any necessary substrates (Lydiate *et al.*, 1995). The role of the backcross breeding method, at least in the short term, is therefore likely to become more important in the introgression of valuable constructs into appropriate genetic backgrounds. Molecular marker assisted backcrossing has the potential to substantially increase the efficiency of gene introgression through transformation.

As discussed previously, molecular markers can be used to accelerate the backcrossing procedure and therefore the introgression of transgenes. It should also be possible to use molecular markers to select inserts located in more desirable chromosome regions (eu versus hetero-chromatin; centro- versus telomeric regions) (Lee, 1996). It may also be possible to backcross several additional transgene lineages as insurance against unpredictable transgene silencing events in later generations (Lee, 1996).

# Chapter 3

## Agronomic and breeding value of genes for resistance to leaf scald (*Rhynchosporium secalis*) in barley (*Hordeum vulgare* )

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**Abstract**

Scald (*Rhynchosporium secalis*) is one of the most damaging leaf and stem diseases of barley grown in southern Australia. The development of resistant cultivars is the most effective means of controlling scald. However, the highly variable nature of the scald pathogen has often resulted in resistance conferred by single major genes being rendered ineffective. Breeding and selection for non-race specific, durable resistance or the adoption of major gene deployment strategies such as gene pyramiding could largely overcome this problem. Four cultivars (Guardian, Halcyon, Sultan, Waveney) were evaluated as potential sources of scald resistance, suitable for gene introgression and pyramiding programs in southern Australia. Each of these prospective donor parents was backcrossed to the susceptible recurrent parent Sloop through one cycle of backcrossing. All four cultivars were resistant to scald isolates common in southern Australia. No factors, either deleterious or beneficial to grain yield, were associated with scald resistance genes from Guardian, Halcyon, and Waveney. Scald resistance genes carried by Sultan were found to be associated with lower grain yield. Both resistant and susceptible first backcross lines derived from Guardian produced a lower proportion of plump grain than the recurrent parent Sloop. A greater number of backcrosses and large population size may be required to successfully introgress scald resistance genes from Sultan and Guardian into germplasm adapted to southern Australian conditions. The development of molecular markers linked to resistance genes in these parents will allow efficient introgression and pyramiding of scald resistance genes from Waveney, Halcyon, and Sultan.

## Introduction

Scald (*Rhynchosporium secalis*) is one of the most damaging leaf and stem diseases of barley grown in temperate climate areas with winter dominant rainfall and is the most damaging foliar disease of barley in the southern cropping areas of Australia. Annual yield losses in the state of Victoria have been estimated to average 15%, while individual crop losses were reported to be as high as 45% (Brown 1985). Yield losses in excess of 45% and 48% have been reported in experimental plots in Western Australia (Khan 1986) and South Australia (S. P. Jefferies unpublished data), respectively. Scald infection has been found to affect seed weight, percentage of plump grain, test weight (Khan and Crosbie 1988) and malting quality (Basson *et al.* 1990). Recent changes in farming practices including increased cropping intensity, stubble retention, increased use of nitrogenous fertiliser, earlier seeding, and higher seeding rates have contributed to the increased incidence and severity of leaf scald in southern Australia.

Many commercially available fungicides (particularly from the triazole group), applied as a foliar spray, seed dressing or with fertiliser, are effective in the control of leaf scald. While the cost of fungicide and fungicide application in broad-acre crops in Australia is no longer prohibitive, there is increasing concern over fungicide residues in foodstuffs and the impact of crop protection products on the environment. In addition, races of scald resistant to some fungicides have been reported (Taggart *et al.* 1998). The development of resistant cultivars is generally regarded as the most effective means of controlling most plant diseases including scald.

*R. secalis* is a highly variable fungal pathogen. Ali *et al.* (1976) assessed the pathogenicity of 203 isolates (35 pathotypes), collected across southern Australia on 21 barley cultivars including all designated resistant genotypes reported at that time. They found only one genotype, 'Atlas 46', to be resistant to all isolates.

While there are various sources of resistance to scald available in both cultivated (Goodwin *et al.* 1990) and wild barley (Abbott *et al.* 1992), the highly variable nature of the pathogen has often resulted in single major gene resistance being rendered ineffective. The Australian cultivars Skiff and Franklin carried high levels of resistance to scald when released commercially in 1989. By 1993, Skiff was highly susceptible in South Australia while susceptible Franklin crops were first observed in 1997. This problem may be overcome by breeding and selecting for non-race specific, durable resistance (Cselenyi *et*

*al.* 1998) or by the adoption of major gene deployment strategies such as gene pyramiding (Nelson 1978), multi-lines or mixtures (Jensen 1952). Abbott *et al.* (2000) presented evidence, however, to suggest that cultivar mixtures were not an effective strategy for controlling scald, especially when one of the components is susceptible. Gene pyramiding has been shown to be effective in controlling a number of diseases (Burdon 1993) including scald (Abbott *et al.* 2000). The rationale behind the gene pyramiding strategy is, assuming that mutations to virulence at each locus in the pathogen are independent, that the probability of mutation to multiple virulence in the pathogen is very low. Multigenic resistance should, therefore, be more durable than single gene resistance.

The Australian barley cultivars Forrest and O'Connor appear to carry durable resistance to scald. Both varieties have Atlas 57 in their pedigrees. Atlas 57 has been reported to carry two dominant genes, probably *Rh2* and *Rh3* (Goodwin *et al.* 1990). Australian barley breeders need to identify additional sources of scald resistance which are effective against Australian isolates and have either proven to be durable (to this time) in other parts of the world or carry more than one resistance gene proven to be effective on most isolates. The optimum gene deployment strategy would then be to combine or 'pyramid' two or more of these more durable sources of resistance into adapted backgrounds. Australian pathologists and breeders have recently identified a number of suitable scald resistance sources and have commenced mapping initiatives to identify molecular markers that can be used to assist in pyramiding resistance genes into adapted, high quality backgrounds (NBMMP - Record of Discussion 1997). It is important, however, for breeders to have an understanding of the agronomic value and/or pleiotropic effects associated with each gene to be introgressed and pyramided. The aim of this study was to assess the effectiveness, and agronomic and breeding value of scald resistance genes derived from 4 different genotypes important to southern Australian barley breeding programs when introgressed into a similar genetic background.

## Materials and methods

### *Genetic material*

The scald resistant donor parents chosen were the European 2-row winter varieties Halcyon and Waveney, the 6-row North African spring Sultan, and the Canadian 2-row spring Guardian. Penner *et al.* (1996) found the resistance pattern of Guardian was very similar to

that of Turk and pedigree links to Turk were proposed. Habgood and Hayes (1971) reported that Turk contained 3 resistance genes (*Rh*, *Rh5* , *Rh6*.) The genetic basis for scald resistance in Sultan, Halcyon, and Waveney is unknown. Sultan was chosen as one of only two amongst 22 sources of resistance that were resistant to all 7 isolates collected from throughout Australia (Rathjen, 1993). Halcyon and Waveney were chosen because they are winter varieties, their resistance is believed to be polygenic, and they have demonstrated durable resistance in Europe (H. Wallwork, pers comm). Melchinger *et al.* (1994) showed evidence of distinct winter and spring type germplasm pools among European barley cultivars. As most cultivated Australian varieties are derived from the European spring germplasm pool, the winter varieties Halcyon and Waveney may provide Australian breeders with access to different scald resistance genes to those available in current Australian germplasm.

Halcyon, Waveney, and Sultan have been reported to be resistant to scald isolates common to southern Australia while Guardian has been rated as moderately resistant (Australian Barley Diseases Newsletter, 1998).

The Australian Sloop was chosen as the recurrent parent. Sloop is susceptible to scald, is acceptable into malting quality grades in South Australia, and is broadly adapted to southern Australian growing conditions. The 1998 field experiment included the 4 donor parents, the recurrent parent Sloop, and the control cultivars, Skiff (resistant to the some local isolates but very susceptible to others), Franklin (resistant to most local isolates), and Forrest (currently resistant to all local isolates).

*Population development*

$BC_1$ $F_2$-bulk populations were created for each of the four donor parents. $F_2$-bulk populations were grown in an irrigated nursery over the summer of 1996/97 and approximately 100 single plants from each $BC_1$ population were selected. Winter types in the Halcyon and Waveney $BC_1$ populations were avoided. $F_2$ derived lines from each $BC_1$ population were grown as plots in an un-replicated nursery at Turretfield Research Centre, near Gawler, South Australia, in 1997. Sloop was sown as a check every sixth plot. Plots were 4 m long and 2 rows wide and sown at 100-140 seeds/$m^2$. The nursery was inoculated with scald infected straw and a conidial suspension of a mixed scald population at early to mid tillering. Plots were scored for intensity of leaf symptoms (ILS) progressing from lower to upper leaves as described by Couture (1980). The scoring system was based on a

1-9 scale: 1 refers to disease free lower, mid, and upper leaves; 9 refers to severe symptoms on lower, mid, and upper leaves. Plots were scored when Sloop check plots had reached decimal growth stage (DGS) 57 (Zadoks, 1974). Grain was hand harvested from approximately 10 resistant (ILS<4) and 10 susceptible (ILS>6) lines from each $BC_1$ population. Individual lines very divergent from the recurrent parent for flowering time were avoided, otherwise selection of the 20 lines was random.

*Field experiment*

$F_2$ derived $BC_1$ resistant and susceptible lines and control varieties were sown in field plots near Gawler, South Australia in 1998. The experiment was a single replicate, split-plot (+/- fungicide) design with a Sloop split-plot control sown every fifth pair of plots. Wheat plots were sown either side of each barley plot as a disease free buffer. Plots were 8 rows wide (1.5 m) by 5 m in length and seeding rates for each line were adjusted for grain size variation with the aim of establishing 150 plants/$m^2$. The '-fungicide' plots were inoculated at early tillering with infected straw harvested from the 1997 scald nursery at Turretfield. Seed of the '+fungicide' split was treated with 1.5 g Baytan ® (150 g/kg triadimenol) per kg seed and plots were treated when Sloop had reached DGS 25 and again at DGS 50 with 500 mL/ha Tilt ® (250 g/L of propiconazole) fungicide. Untreated plots were scored for intensity of ILS when Sloop plots had reached DGS 65. The field experiment was harvested with a small plot harvester and grain yield and percentage of grain retained over a 2.5 mm screen were determined from the harvested samples.

*Statistical analysis*

Frequency distributions were plotted for ILS data from the 1997 disease nursery for each $BC_1$ population. Chi-squared values were calculated comparing resistance status with expected ratios predicted by various one and two gene models. Population sizes were generally inadequate to test for more than two gene models (Hanson, 1959b). Regression analysis (SAS Institute, JMP® version 3 software) was used to assess relationships between ILS score and decimal growth stage. Means for grain yield and percentage of grain retained over a 2.5mm screen were calculated, allowing for extraneous variation using spatial techniques developed by Cullis and Gleeson (1991). $BC_1$ lines were grouped into classes based on family (donor parent) and also by family and resistance/susceptible phenotype. Least-squares means were calculated on the spatially adjusted grain yield and grain plumpness for individual lines using a single factor ANOVA (SAS Institute, JMP® version

3 software). The means for each class were compared using linear contrasts. Regression analysis was used to relate the factor ILS with grain yield and grain plumpness.

**Results and Discussion**

An epidemic of leaf scald was achieved in both seasons, with terminal severity estimated at 80% of flag leaf area in both 1997 and 1998. ILS scores across common entries in both 1997 and 1998 were highly correlated ($r^2$= 0.81, $P$<0.0001). In the 1998 field experiment, both the control Skiff and the recurrent parent Sloop were rated as highly susceptible (ILS=8) confirming the presence of Skiff attacking isolates. Forrest was rated as resistant (ILS=2), Franklin moderately resistant (ILS=3), Guardian was moderately resistant to moderately susceptible (ILS=4.5), Halcyon, Sultan and Waveney were all rated as highly resistant (ILS=1).

*Genetic control of resistance*

ILS frequency distributions for each $BC_1$ population are provided in Fig 3.1. Frequency distributions for the Guardian and Halcyon $BC_1$ populations were consistent with a single major gene with complete dominance. While a significant inheritance model fit was achieved, making clear delineation between resistant (ILS<4) and susceptible (ILS>5) classes, particularly in the Halcyon $BC_1$ population, was difficult. The lack of clear delineation provides support to the possibility of more complex inheritance. The high frequency of resistant (ILS<4) lines in the Sultan and Waveney $BC_1$ populations and the lack of fit with any of the one and two gene models tested (complete dominance, incomplete dominance, additive and non-additive effects), suggest a more complex mode of inheritance in these populations. The experiments reported here were not designed to accurately determine the inheritance of resistance carried by the potential donor parents assessed and consequently their mode of inheritance can only be speculated on, based on the information available.

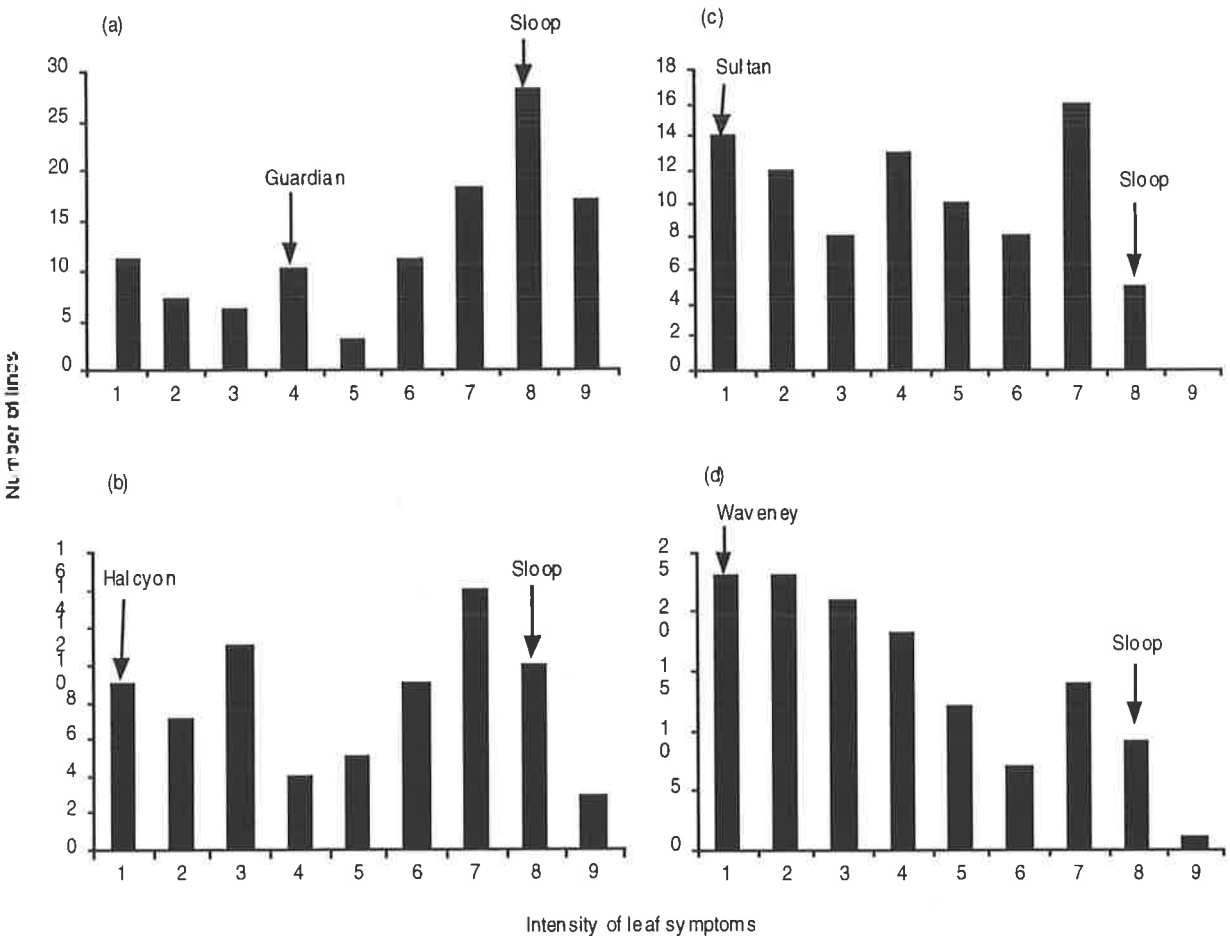


Figure 3.1 Frequency distributions for intensity of leaf symptoms for four $BC_1$ populations (a- Guardian $BC_1$, b- Halcyon $BC_1$, c- Sultan $BC_1$, d- Waveney $BC_1$) grown at Turretfield Research Centre, near Gawler, SA in 1997.

*Flowering time*

Barley varieties with broad adaptation to southern Australian cropping environments generally have very similar rates of development and therefore flowering time. Despite its disease susceptibility, the recurrent parent Sloop has broad adaptation to southern Australian cropping environments. Tight linkage between genes conferring flowering time and scald resistance, or pleitropic effects of scald resistance genes on flowering time, would be disadvantageous in an introgression or pyramiding program. There was no significant relationship between growth stage and ILS score identified in the Guardian, Halycon and Waveney populations in 1997. In contrast, regression analysis revealed a significant ($P<0.001$) positive relationship between growth stage and ILS score in the Sultan $BC_1$ population. However a number of early flowering, highly resistant, Sultan $BC_1$ individuals were identified. This suggests that if one or more scald resistance genes were linked to genes for flowering time then this linkage must not be tight.

*Grain yield and grain plumpness*

Above average spring temperatures coupled with below average spring rainfall in the Lower North cropping districts of South Australia in 1998 resulted in reduced grain yield potential and reduced relative impact of scald on grain yield. Yield losses in excess of 48% have been observed in experimental plots in South Australia (Jefferies unpublished data) in a season with similar scald infection levels to the 1998 experiment but well above average spring rainfall. The grain yield of the susceptible recurrent parent Sloop was 17% higher ($P<0.05$) in plots where scald was controlled (based on the mean of nine split-plot replicates) than in untreated plots (Table 1). While yield loss was only moderate, grain size was severely affected. The fungicide treated Sloop plots retained 83.8% of grain, over a 2.5mm screen compared to the untreated plots which retained only 49.6% (Table 1).

Table 1. Spatially adjusted mean grain yield and grain plumpness (>2.5mm) of Sloop (mean of nine replicates) in scald controlled (-scald) and scald inoculated (+scald) field plots near Gawler, South Australia, 1998. (The probability of a significant difference between means being due to chance alone is provided (*P* value)).

| Trait | + Fungicide | - Fungicide | *P* value |
|---|---|---|---|
| Grain yield (kg/plot) | 3.39 | 2.84 | 0.05 |
| Grain plumpness (% > 2.5mm) | 83.8 | 49.6 | 0.001 |

Plate 3.1 Layout of barley leaf scald inoculated field experiment at Gawler, SA, 1998. barley treatment plots are buffered by wheat plots and a plant-free 2m pathway.

Table 2 provides the mean grain yield and grain plumpness of each of the four $BC_1$ families in the scald controlled plots. There was no significant difference between the grain yield of $BC_1$ families. However, the Sultan $BC_1$ family was significantly lower yielding than the recurrent parent Sloop.

The Sultan $BC_1$ family produced a significantly ($P< 0.05$) greater proportion of plump grain than other families but not significantly greater than Sloop. The Guardian $BC_1$ family produced significantly ($P<0.05$) less plump grain than Sloop. Therefore more than one backcross will be required to adequately recover the grain yield and grain plumpness of the recurrent parent in backcrosses involving the scald resistant donor parents Sultan and Guardian.

Table 2. Least square mean grain yield and grain plumpness (%>2.5mm) of $BC_1$ families in disease controlled field plots (+Fungicide), Gawler, 1998. Means within columns followed by the same letter are not significantly different at P= 0.05

| Family | Number of lines | Mean grain yield (kg/plot) | Mean grain plumpness (% > 2.5mm) |
|---|---|---|---|
| Guardian $BC_1$ | 18 | 3.27ab | 78.0a |
| Halcyon $BC_1$ | 15 | 3.30ab | 81.5ac |
| Sultan $BC_1$ | 18 | 3.13a | 86.9bc |
| Waveney $BC_1$ | 13 | 3.23ab | 80.4ac |
| Sloop | | 3.39b | 83.7bc |

Table 3 compares the mean grain yield and grain plumpness of scald resistant and scald susceptible $F_2$ derived $BC_1$ lines from each family in inoculated and fungicide treated plots. There was no significant difference between the grain yield or grain plumpness of scald resistant and scald susceptible lines of the Guardian, Halcyon and Waveney $BC_1$ families in the fungicide treated plots. These results suggest that the scald resistance genes from these donor parents are not closely linked to genes important in the control of grain yield and grain size or have no obvious pleiotropic affects on grain yield or grain size. In contrast, the scald resistant Sultan $BC_1$ lines were, on average, 9% ($P<0.05$) lower yielding than the scald susceptible lines.

Table 3. Least squares means for grain yield and grain plumpness (>2.5mm) of $BC_1$ families in fungicide treated (+Fungicide) and fungicide untreated (-Fungicide) field plots, Gawler, 1998. Significant differences for pairwise comparisons between resistant (ILS<5) and susceptible (ILS>5) classes for individual families only.

| Family | Resistance class | +Fungicide | Signif. | -Fungicide | Signif. |
|---|---|---|---|---|---|
| | | *Grain yield (kg/plot)* | | | |
| Guardian $BC_1$ | Susceptible | 3.35 | n.s. | 2.46 | 0.05 |
| | Resistant | 3.18 | | 2.77 | |
| Halcyon $BC_1$ | Susceptible | 3.25 | n.s. | 2.61 | 0.05 |
| | Resistant | 3.27 | | 2.86 | |
| Sultan $BC_1$ | Susceptible | 3.29 | 0.05 | 2.53 | n.s. |
| | Resistant | 3.00 | | 2.67 | |
| Waveney $BC_1$ | Susceptible | 3.23 | n.s. | 2.65 | 0.01 |
| | Resistant | 3.23 | | 3.13 | |
| | | *Grain Plumpness (%>2.5mm)* | | | |
| Guardian $BC_1$ | Susceptible | 76.7 | n.s. | 41.1 | <0.0001 |
| | Resistant | 79.1 | | 62.8 | |
| Halcyon $BC_1$ | Susceptible | 77.2 | n.s. | 48.1 | <0.0001 |
| | Resistant | 81.4 | | 76.7 | |
| Sultan $BC_1$ | Susceptible | 86.7 | n.s. | 63.9 | <0.0001 |
| | Resistant | 87.1 | | 80.0 | |
| Waveney $BC_1$ | Susceptible | 80.6 | n.s. | 56.1 | 0.001 |
| | Resistant | 80.4 | | 70.3 | |

n.s., not significant at $P$=0.05

While a positive relationship ($P$<0.001) between growth stage and ILS score was established in the larger 1997 Sultan $BC_1$ population, none of the very late maturing lines were included in the 1998 experiment. Therefore it is unlikely that linkage between minor resistance genes and flowering time genes contributed to differences in grain yield in 1998.

Scald resistant $BC_1$ lines derived from Guardian, Halcyon and Waveney were significantly higher yielding than scald susceptible lines in the inoculated plots (Table 3). There was, however, no significant difference for grain yield between resistant and susceptible lines in

the Sultan $BC_1$ population. The grain yield penalty of resistance genes from Sultan, observed previously, may account for the lack of yield difference between the resistant and susceptible lines in the disease affected plots. Highly significant ($P<0.0001$) differences in grain plumpness were observed between resistant and susceptible $BC_1$ lines in all four populations where disease was not controlled (Table 3).

All grain yield results need to be treated with caution as they were derived from only one site and one season. The ranking of control varieties for grain yield was, however, consistent with long term results at both Turretfield Research Centre and with adjusted statewide (21 experiments in SA) means (Crop Harvest Report, 1999/2000).

*Suitability of Waveney, Halcyon, Sultan and Guardian as scald resistance donor parents in a gene pyramiding program*

The low level of resistance observed in the backcross lines derived from Guardian and the apparently simple inheritance of its resistance suggests that this parent would be less than ideal as a candidate for resistance gene mapping and pyramiding. It has been proposed, however, that low level resistance can be valuable in a gene pyramiding strategy (Nelson, 1978). Nelson (1978) argues that the combined partial effects of several resistance genes should place only limited selection pressure on the pathogen, therefore improving the durability of resistance. In contrast to Guardian, the inheritance of resistance in Waveney, and to a less extent Halcyon, appear complex and the resistance genes found in both parents are not associated with factors deleterious to either grain yield or grain size. Both Waveney and Halcyon would be ideal parents for introgressing and pyramiding resistance into southern Australian germplasm. Due to the likely complexity of inheritance of resistance genes, particularly in Waveney, linked molecular markers will be required for efficient gene pyramiding.

The inheritance of resistance in Sultan also appears complex but this study has shown the resistance genes to be associated with factors deleterious to grain yield. Successful and efficient introgression and pyramiding of resistance genes from Sultan will also require linked molecular markers which can be used to both trace resistance genes through multiple crosses or backcrosses but also to select for small introgressed chromosome segments.

**Summary**

Resistance genes carried by Guardian, Halcyon, Sultan and Waveney are effective on scald isolates common in southern Australia. The level of resistance is greatest in Sultan and Waveney and least in Guardian. The inheritance of resistance in, at least, Sultan and Waveney appears to be complex. Scald resistance genes carried by Sultan, when introgressed into a Sloop background, are associated with factors deleterious to grain yield. Guardian backcross derived lines, both resistant and susceptible to scald, produced a smaller proportion of plump grain than the recurrent parent Sloop. A greater number of backcrosses and a large population size may be required to successfully introgress scald resistance genes from Sultan and Guardian into adapted Australian germplasm. Resistant genes in all four donor parents were not associated with any factors which increased grain yield or grain plumpness. The low level of resistance observed in the backcross lines derived from Guardian and the apparently simple inheritance of its resistance suggests that this parent would be less than ideal as a candidate for resistance gene mapping and pyramiding. Due to the likely complex inheritance of resistance in Waveney, Sultan and Halcyon and the potential for deleterious linkage drag associated with resistance genes from Sultan, identification of linked molecular markers will be required for successful, efficient, gene introgression and pyramiding.

# Chapter 4

## Mapping of Chromosome Regions Conferring Boron Toxicity Tolerance in Barley (*Hordeum vulgare* L)

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## Abstract

Boron toxicity has been recognised as an important problem limiting production in the low-rainfall regions of southern Australia, West Asia and North Africa. Genetic variation for boron toxicity tolerance in barley has been characterised but the mode of inheritance and location of genes controlling tolerance was not previously known. A population of 150 doubled haploid lines from a cross between a boron toxicity tolerant Algerian landrace, Sahara 3771, and the intolerant Australian cultivar Clipper was screened in four tolerance assays. An RFLP linkage map of the Clipper x Sahara population was used to identify chromosomal regions associated with boron tolerance in barley. Interval regression mapping allowed the detection of four chromosomal regions involved in the boron tolerance traits measured. A region on chromosome 2H was associated with leaf symptom expression, a region on chromosome 3H was associated with reduction of the affect of boron toxicity on root growth suppression, a region on chromosome 6H was associated with reduced boron uptake, and a region on chromosome 4H was also associated with the control of boron uptake but was also associated with root length response, dry matter production and symptom expression. The benefits and potential of marker-assisted selection for boron toxicity tolerance are discussed.

## Introduction

Boron (B) is an essential plant micronutrient which can be phytotoxic to plants if present in soils in high concentration. B toxicity to crop plants has been recognised since the early 1930's yet it was not until 1983 that it was first recognised in southern Australia in barley growing under dryland conditions (Cartwright *et al.*, 1984). A 17 percent difference in grain yield of adjacent areas of barley (cv. Clipper) was related to differences in the concentration of *B* in shoots just prior to anthesis (Cartwright *et al.*, 1984). *B* toxicity has also been recognised as a problem in the dry regions of West Asia and North Africa and a problem associated with irrigation water in many other parts of the world (Gupta *et al.*, 1995). Soil amelioration of B toxicity appears to be impractical.

Genetic variation for tolerance of B toxicity exists within a number of crops including wheat and barley (Cartwright *et al.*, 1987, Moody *et al.*, 1988; Paull *et al.*, 1988a) field peas and pasture medics (Paull *et al.*, 1992). Exploitation of this genetic variation through plant breeding in barley may significantly improve productivity and quality of barley growing in areas prone to B toxicity problems.

Using leaf symptom data, Jenkin (1993) studied the inheritance and location of genes conferring B tolerance in barley in three cross combinations and their reciprocals. Jenkin (1993) proposed that B tolerance (reduced leaf symptoms) was controlled by three major gene loci behaving in a largely additive manner. These "genes" were located on barley chromosomes using an RFLP linkage map of 43 doubled haploid lines from a cross between intolerant cultivar Clipper and tolerant Algerian landrace Sahara 3771 (Sahara). Regions on chromosome 2H and 7H were reported to be significantly associated with leaf symptom expression. The associations reported were weak (LOD <3.0) as the number of doubled haploid lines screened (43) were, most likely, insufficient to establish significant relationships with confidence.

The most obvious symptoms of B toxicity in barley are chlorosis and necrosis extending from leaf tips and the formation of brown lesions initially at the leaf margins but extending over the distal half or more of the leaf. While chlorosis and necrosis of leaf tips can occur on new leaves, brown lesions appear on the oldest leaves first and successive leaves become affected in sequence. In severe cases, brown lesions can appear on leaf sheaths, stems and awns.

It appears that B tolerance in cereals involves more than one mechanism. Nable (1988) reported that the sensitivity of wheat cultivars to toxic concentrations of B was governed solely by their ability to exclude B from plant tissue. Cartwright *et al.* (1987) considered that, in addition to a genetic exclusion mechanism, other mechanisms related to root morphology may assist tolerant cultivars to extend roots into B toxic sub soils. Huang and

Plate 4.1 Barley crop grown in the Mid North cropping district of South Australia showing symptoms of boron toxicity.

Graham (1990) demonstrated variation for B tolerance by culturing excised root tips on high B media. They found that tolerant genotypes either produced callus or developed longer root axes than intolerant genotypes. In addition, seedlings of tolerant wheat genotypes grown on filter paper soaked in toxic concentrations of B have been shown to produce significantly longer roots than more sensitive genotypes (Chantachume *et al.*, 1995).

It appears that leaf symptom expression may only be a minor component of the overall tolerance mechanism. Jenkin (1993) assessed differences in both grain yield and leaf symptoms in three populations derived from crosses between tolerant and intolerant parents grown on soil high in B concentration and yet found no consistent relationship between leaf symptom expression and grain yield response. Riley and Robson (1994) also found that severe levels of leaf symptoms could be produced in later stages of plant growth with minimal effect on grain yield. Mahalakshmi *et al.* (1995) found that some barley genotypes produced very few leaf symptoms yet accumulated high concentrations of B in plant tissue. It is therefore likely, that some or all of the mechanisms involved in B tolerance are under separate genetic control. Consequently, attempting to map major genes or quantitative trait loci (QTL) associated with B tolerance based on data from leaf symptoms alone is unlikely to identify all important loci involved in the tolerance mechanism.

Recently, an RFLP map of barley chromosomes was constructed in a doubled haploid population of 150 individuals created from a cross between the B tolerant parent Sahara 3771, a North African landrace, and the intolerant Australian cultivar Clipper (Langridge *et al.*, 1995). The objective of our study was to identify chromosomal regions associated with differential plant response to toxic concentrations of B using this population and RFLP marker data set.

## Materials and Methods

### *Genetic material*

The genetic material used in this study was a population of 150 doubled haploid lines derived from a cross between the B tolerant Algerian landrace Sahara 3771 and a B sensitive Australian cultivar Clipper. The Clipper x Sahara population was produced by the *Hordeum bulbosum* method (Islam and Shepherd, 1981) using embryo culture followed by chromosome doubling with colchicine treatment.

*Screening for B tolerance in solution culture - root length assay*

Treatment levels chosen for the solution culture root length assay were 100 mg B $l^{-1}$ (B100) and 0 mg B $l^{-1}$ (B0). Chantachume *et al.*, (1995) found that seedling root lengths of wheat varieties responded consistently at three concentrations, 50, 100 and 150 mg B $l^{-1}$. A control treatment (B0) was included to account for genetic variation for root length in the absence of B toxicity.

Seeds of each doubled haploid line were surface sterilized with 5.0% sodium hypochlorite and pre-germinated for eight days at 4°C. Three evenly germinated seeds, per doubled haploid line, were placed embryo downwards at a spacing of 2 cm across the middle of a filter paper (Ekwip® 32 x 46 cm grade R6) soaked in either the B0 or B100 solutions. The base solution used in both the control (B0) and high concentration treatment (B100) also included 0.5m*M* $Ca(NO_3)_2.4H_2O$, 0.0025 m*M* $ZnSO_4.7H_2O$ and 0.015 m*M* $H_3BO_3$ following the method of Chantachume *et al.*, (1995). The filter papers were rolled and covered with aluminium foil, then stored upright at 15°C for 12 days. The longest root of each seedling was measured.

The experiment was conducted, as a randomised complete block with two replicates in 1997 and a further two replicates in 1998.

***Screening for B tolerance in soil - leaf symptoms, tissue* B *concentration and dry matter production.***

Methods used for seed preparation followed those described for the solution culture - root length assay.

The soil based assay was conducted in two boxes (2m x 1m x 0.25m) containing soil to which B was applied at 100 mg B $kg^{-1}$. The soil was a bulk sample of silt clay loam from the surface (0-10 cm) of a red-brown earth (Paull *et al.*, 1988b). The concentration of B extractable in hot $CaCl_2$ (Spouncer *et al.*, 1992) was 65 mg B $kg^{-1}$.

Two evenly germinated seeds of each doubled haploid line were planted approximately two centimeters apart, each pair of plants forming a distinct plot. The 150 doubled haploid lines and parents were all sown in a single box, representing a complete replicate with two boxes (replicates) planted in 1997 and a further two in 1998. The parent varieties, Clipper and Sahara, were sown in every alternate seventh plot

Plate 4.2 Seedlings of the Clipper x Sahara mapping population parents grown on filter paper soaked in solutions containing toxic (100 ppm B) and non-toxic (0 ppm B) concentrations of B. Variation in relative root length between tolerant (Sahara) and intolerant (Clipper) parents is presented.

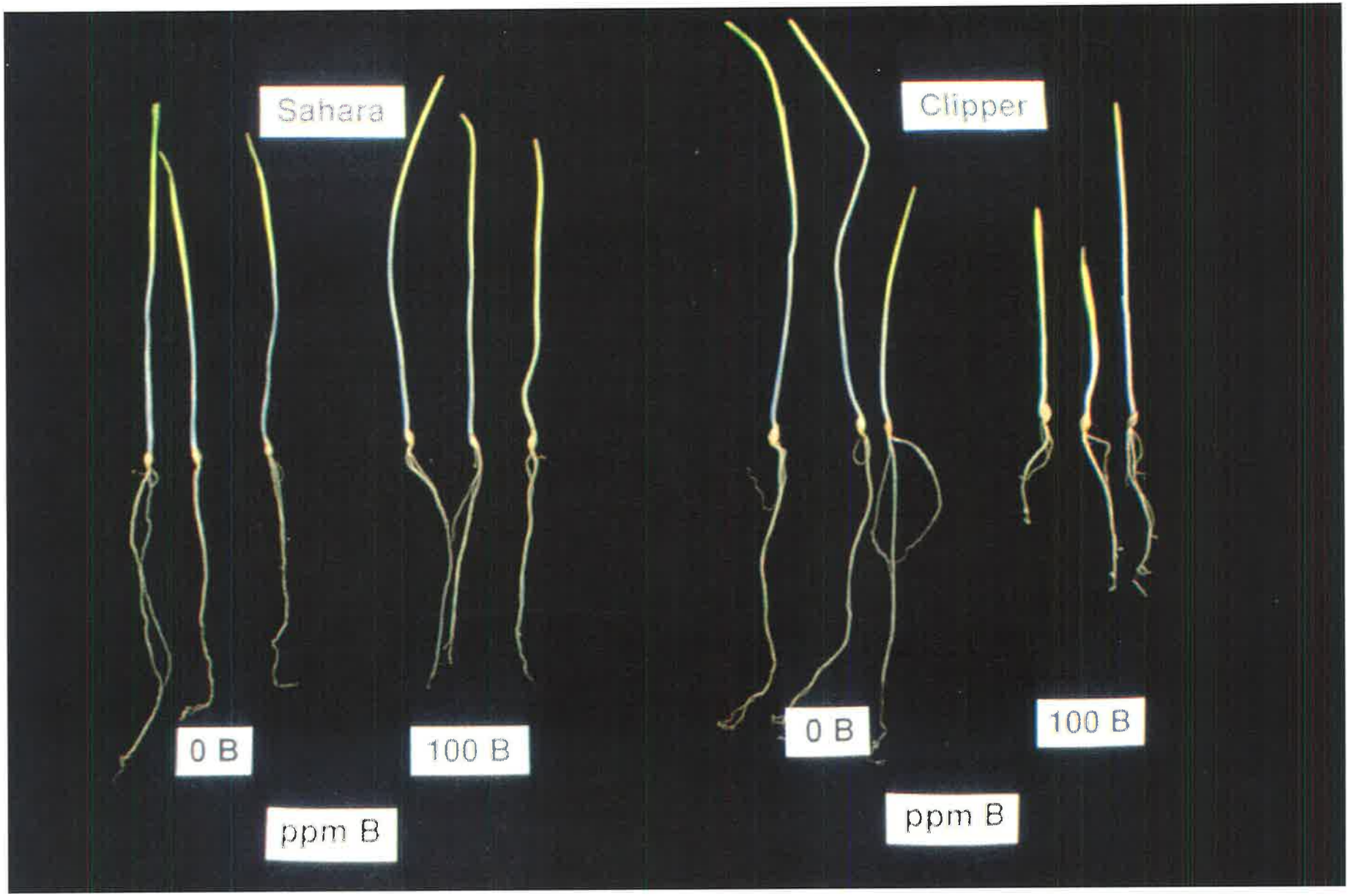
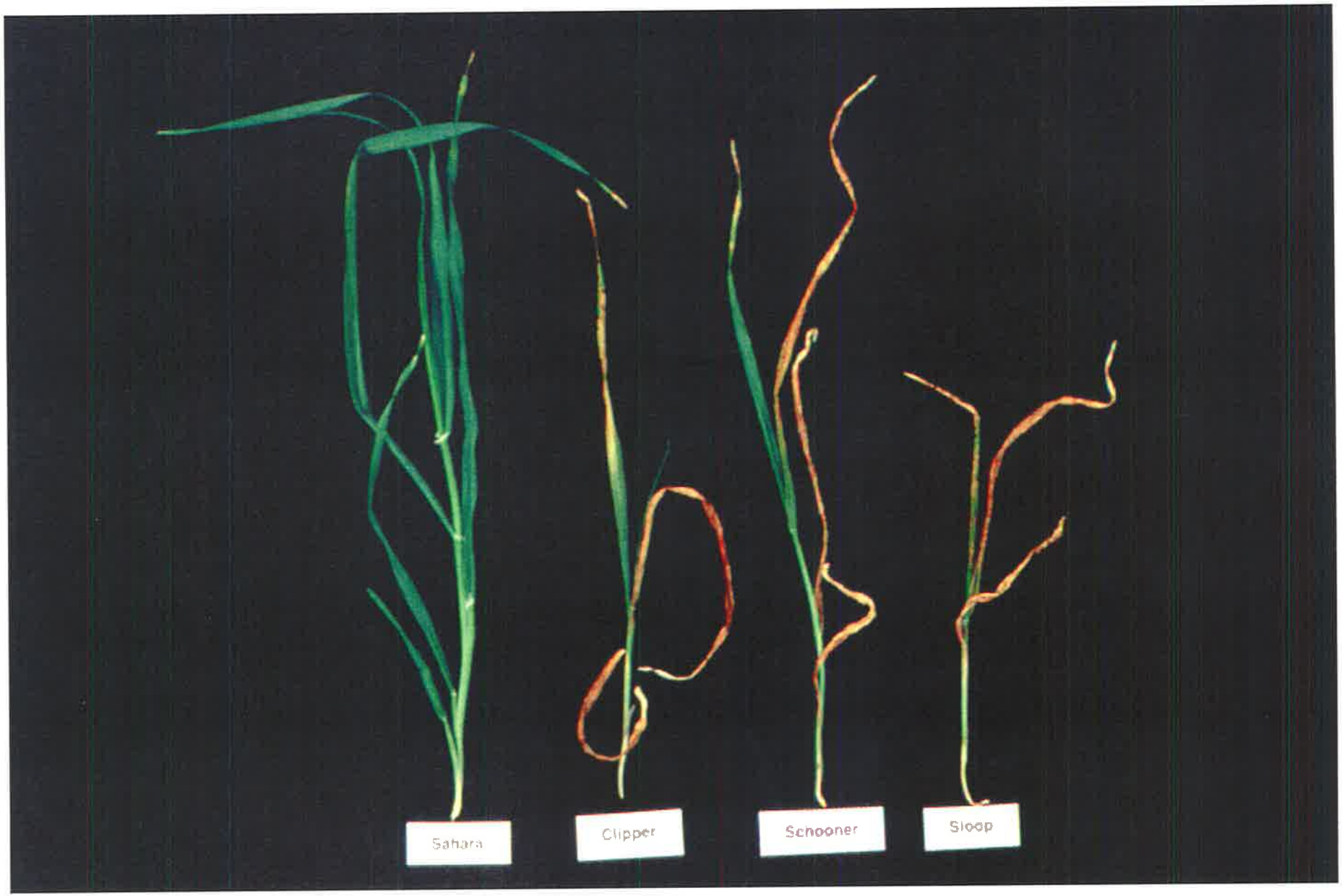

Sahara
Clipper
0 B
100 B
ppm B
0 B
100 B
ppm B

Plate 4.3 Whole shoots of plants of the Clipper x Sahara mapping population parents, and the Australian malting quality cultivars Schooner and Sloop, grown on soil to which toxic concentrations of boron were added. Genetic variation for leaf symptom expression and dry matter production is presented.


Sahara
Clipper
Schooner
Sloop

Four weeks after planting each plant was scored for severity of leaf symptoms on the basis of leaf damage on a scale of 1-6 where 1 was no visual symptoms and 6 was greater than 90% necrosis.

Each plot was harvested one centimeter above ground level five weeks after planting. The plants were dried at 80°C for 48 hours and weighed. Dried shoots were ground, digested in nitric acid and analyzed for concentration of B by inductively coupled plasma spectrometry (ICP) (Zarcinas *et al.*, 1987).

***Statistical analysis of raw data***

All statistical analyses except for interval and multiple regression marker analyses of B response were performed with JMP (v3.0, SAS Institute Inc, 1995) software. The least square means for relative root length (root length at B0 as a percentage of root length at B100), were calculated using ANOVA model. Factors for the ANOVA model were doubled haploid line, replicate and plant number. Least square means for B concentration in whole shoots, leaf symptom score and dry matter production were also calculated using ANOVA model with doubled haploid line and replicate forming the factors for the model. Least square means were calculated for each of the four traits from both the 1997 and 1998 data sets independently and the two years data combined into a single four replicate analysis for each trait. No raw data transformation was required.

Heritability for each trait was estimated from a linear model incorporating data from the 150 doubled haploid lines over four replicates (two in 97 and two in 98). Factors were doubled haploid line and replicate. Heritabilities were calculated from an estimate of the genetic variance component as a proportion of the total variance for each trait.

A total of 168 RFLP markers, covering all barley chromosomes were used for simple and interval regression analysis, the latter by the method of Haley and Knott (1992). A minimum LOD threshold of 3.0 was used. Significant relationships between trait expression and RFLP markers for the 1997, 1998 and combined data sets were compared. A marker locus thought to be associated with a gene or chromosomal region conferring B tolerance was tested for two-way interaction with all other markers in the data set using the method described by Nelson *et al.* (1998). All marker analyses were performed with the computer program 'Qgene' (Nelson, 1997)

***Gene effects***

Each of the 150 doubled haploid lines was scored for the presence of either a Clipper or Sahara marker allele for the RFLP markers most significantly associated with each trait, one

marker representing each unique chromosomal region identified. From this, the marker allele genotype of each doubled haploid line was determined. Lines of identical genotype were grouped into marker allele classes. Least square means for each class were calculated using a single factor (marker class) ANOVA. Means of marker classes were compared using contrasts.

## Results

### *Screening for B tolerance in solution culture - root length assay*

A significant ($P<0.001$) reduction in root growth at B100 was observed in all doubled haploid lines and both parents. Significant ($P<0.001$) genetic variation for seedling root length at both B0 and B100 was observed within the mapping population (Fig 4.1-A and Fig4.1-B). The heritability of root length at B0 was estimated as $h^2=0.51$. The heritability of root length at B100 was significantly greater at $h^2=0.89$. Relative root length (RRL) was chosen as the appropriate variable for mapping as it was expected to provide a measure of tolerance independent of genetic variation for absolute root length. RRL was calculated as root length at B100 expressed as a percentage of root length at B0. The frequency distribution for relative root length is provided in Figure 4.1-C. With the exception of six lines which exceeded the RRL of Sahara, all other lines fell within the parental range.

### *Screening for B tolerance in soil - leaf symptoms, tissue B concentration and dry matter production.*

Significant ($P<0.001$) differences between doubled haploid lines were observed for all three traits measured in the soil-based assay. (Fig.4.1-D, 4.1-E, 4.1-F). The estimated heritability for whole shoot B concentration was 0.89, 0.72 for leaf symptom score and 0.32 for whole shoot dry weight. Frequency distributions for leaf symptom score, whole shoot dry weight and whole shoot B concentration are provided in Fig. 4.1-D, Fig. 4.1-E and Fig 4.1-F respectively. Distributions for leaf symptom score (Fig. 1-D) and whole shoot dry weight (Fig 4.1-E) were slightly skewed towards the lower values.

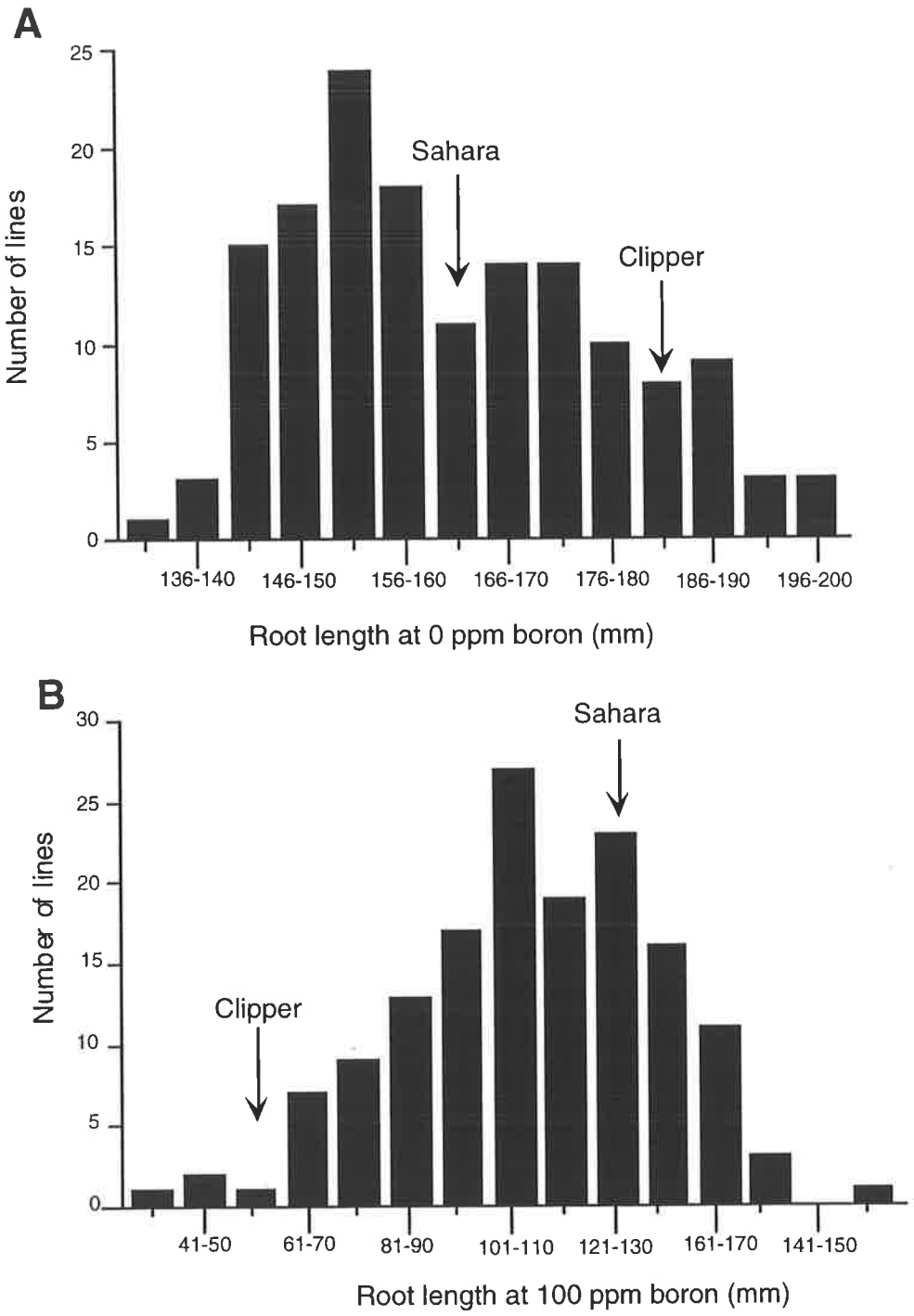



Figure 4.1 (A-B). Frequency distributions for all traits measured on the Clipper x Sahara 3771 mapping population. Data is derived from combined analysis of 1997 and 1998 data sets. **A**; root length at 0 ppm B solution, **B**; root length at 100 ppm B solution.

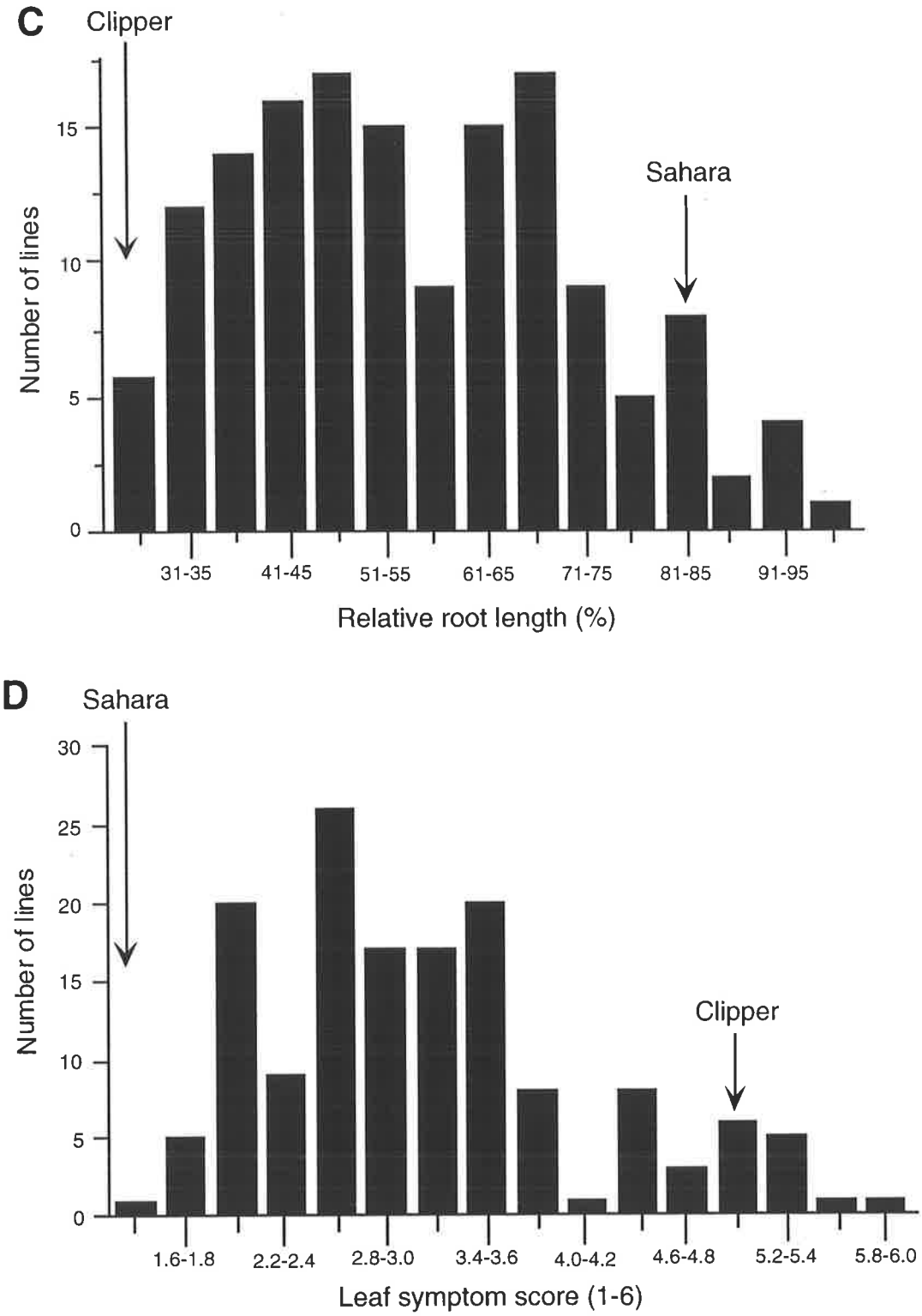



Figure 4.1 (C-D). Frequency distributions for all traits measured on the Clipper x Sahara 3771 mapping population. Data is derived from combined analysis of 1997 and 1998 data sets. **C**; relative root length (RRL) (%), **D**; leaf symptom score (1-6).

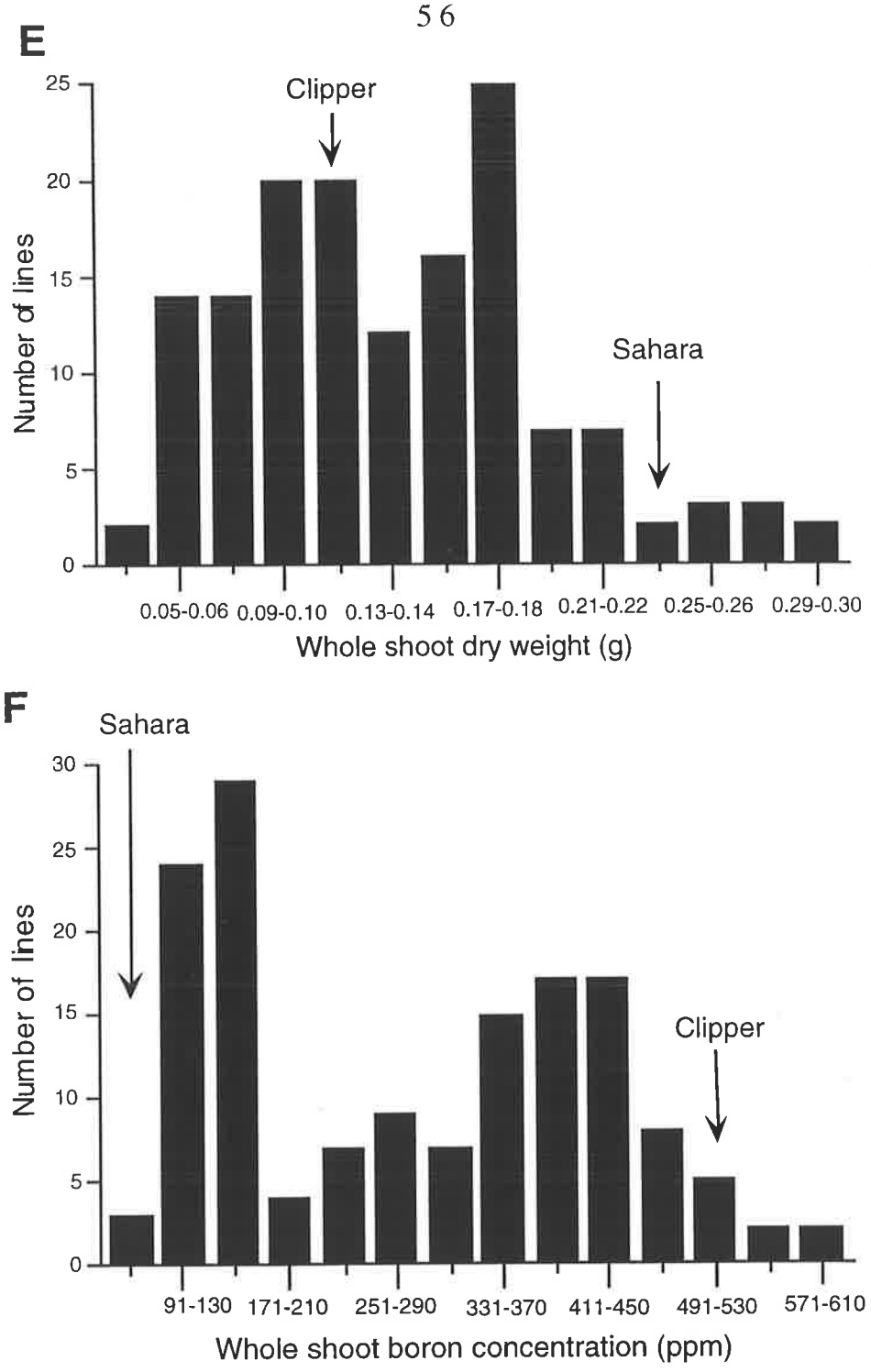



Figure 4.1 (E-F). Frequency distributions for all traits measured on the Clipper x Sahara 3771 mapping population. Data is derived from combined analysis of 1997 and 1998 data sets. **E**; whole shoot dry weight (g), **F**; whole shoot B concentration (%).

Whole shoot dry weight followed a bi-modal distribution (Fig. 1-F) (not tested for fit) consistent with either a single major gene or more than one gene but with a single gene having a major effect. Only two lines significantly ($P<0.05$) exceeded Clipper for leaf symptom score, three lines exceeded Clipper for whole shoot B concentration and two lines were significantly below Clipper for whole shoot dry weight. There was no evidence of statistically significant positive transgressive segregation for any of the traits measured.

***Comparison between B tolerance parameters***

Table 1 details the pairwise correlation coefficients between the four B tolerance traits measured. While all pairwise correlation coefficients were highly significant ($P<0.001$), none exceeded 0.55. Leaf symptom score generally had the lowest overall pairwise correlation coefficients ranging from – 0.27 with whole shoot dry weight to 0.43 with whole shoot B concentration. This is consistent with the lack of association previously found between leaf symptom expression and grain yield (Jenkin, 1993; Riley and Robson, 1994), and leaf symptom expression and concentration of B in plant tissue (Mahalakshmi *et al.*, 1995). Whole shoot B concentration had the overall highest pairwise correlation coefficients ranging from 0.43 with leaf symptom score to –0.55 with whole shoot dry weight.

Table 1. Pairwise correlation coefficients between four B tolerance traits measured on the Clipper x Sahara 3771 mapping population

| B tolerance traits | Whole shoot B concentration | Relative root length | Leaf symptom score |
|---|---|---|---|
| Relative root length | -0.53 *** | | |
| Leaf symptom score | 0.43 *** | -0.30 *** | |
| Whole shoot dry weight | -0.55 *** | 0.37 *** | -0.27 *** |

*** Significant at $P< 0.001$

***Mapping***

*Relative root length*

Marker analysis of the combined (1997 and 1998) data sets for seedling root length at the low (B0) and high (B100) B concentration identified a region on the long arm of chromosome 5H (Fig. 4.2-A) associated with root length *per se* (RL). A region on the long arm of chromosome 4H and the short arm of chromosome 3H were strongly associated with RRL (Fig. 4.2-B and Fig. 4.2-C respectively). Multiple regression analysis showed that these two loci, in combination, accounted for approximately 39% of the variation in RRL. Sahara marker alleles were associated with high RRL. Marker analysis of the low (B0) B

concentration failed to show significant relationships with these same regions on chromosomes 4H and 3H. It can be concluded, therefore, that the regions identified on chromosome 4H and 3H are associated with root length response to high B concentration.

Significant associations between RRL and regions on Chromosome 4H and 3H were identified in both the 1997 and 1998 data sets. There were only minor LOD score variations between the analyses of the 1997 and 1998 data sets. Analysis of the 1998 data sets identified an association between the region conferring RL, previously identified on chromosome 5H, and RRL. The conversion of root length to RRL appears to have failed to account for all variation attributed to RL in the 1998 and combined data sets (Fig. 4.2-A).

RFLP markers on chromosome 3H and 4H most strongly associated with relative root length were *Xawbma15* and *Xwg114* respectively.

*Leaf symptom score*

Markers on regions of chromosome 2H (Fig. 4.3-A) and 4H (Fig. 4.3-B) were associated with the expression of leaf symptoms on plants grown in B toxic soil based on the 1997, 1998 and combined data sets. Based on multiple regression analysis, loci on chromosome 2H and 4H in combination, accounted for approximately 38% of variation in leaf symptom data. The marker on chromosome 4H most strongly associated with leaf symptom score was *Xwg114*. *Xwg114* was also the chromosome 4H marker most strongly associated with RRL (Fig 4.2-B). Sahara marker alleles at this locus were associated with low leaf symptom score.

*Whole shoot dry weight*

Markers on a region of chromosome 4H were strongly associated with whole shoot dry weight. The strongest relationship was again with marker *Xwg114* (Fig 4.4). Based on simple marker regression analysis, the chromosome 4H locus accounted for approximately 34% of variation in dry matter data. Sahara marker alleles at this locus conferred high dry weight.

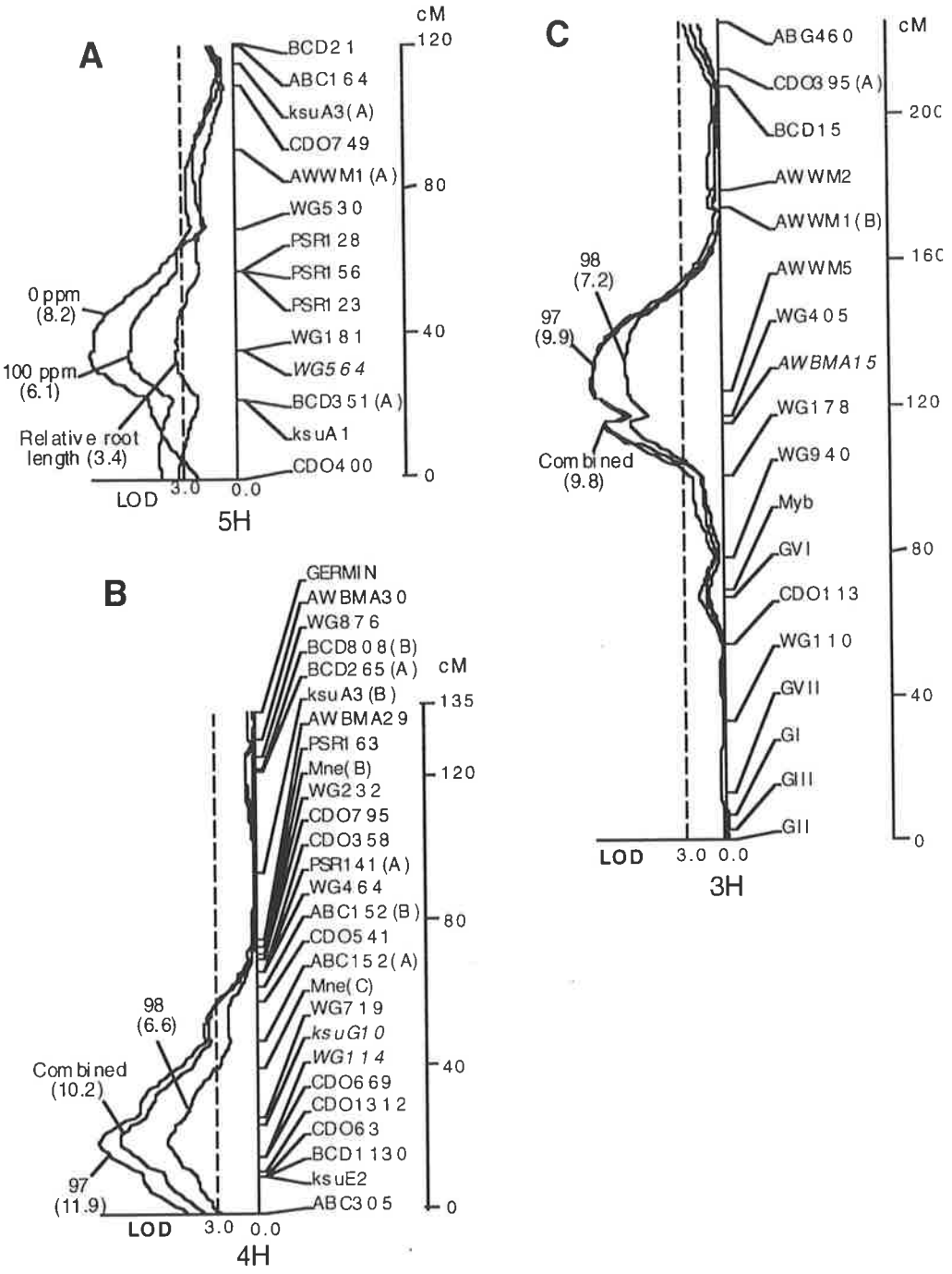



Figure 4.2. Chromosome location of regions associated with relative root length (RRL) expressed in doubled haploid lines from the cross Clipper x Sahara 3771 based on interval mapping of 1997, 1998 and combined data. Short arms are towards the tops of chromosomes. Maximum LOD scores for each peak are provided in parentheses. RFLP marker most significantly associated with trait is presented in italics. Dash lines show LOD 3.0 threshold. Interval map of chromosome 5H based on root length at 0 ppm B, 100 ppm B and relative root length (1997 and 98 combined data)(**A**). Interval map of chromosome 4H (**B**) and 3H (**C**) based on RRL from 1997, 1998 and combined data sets.

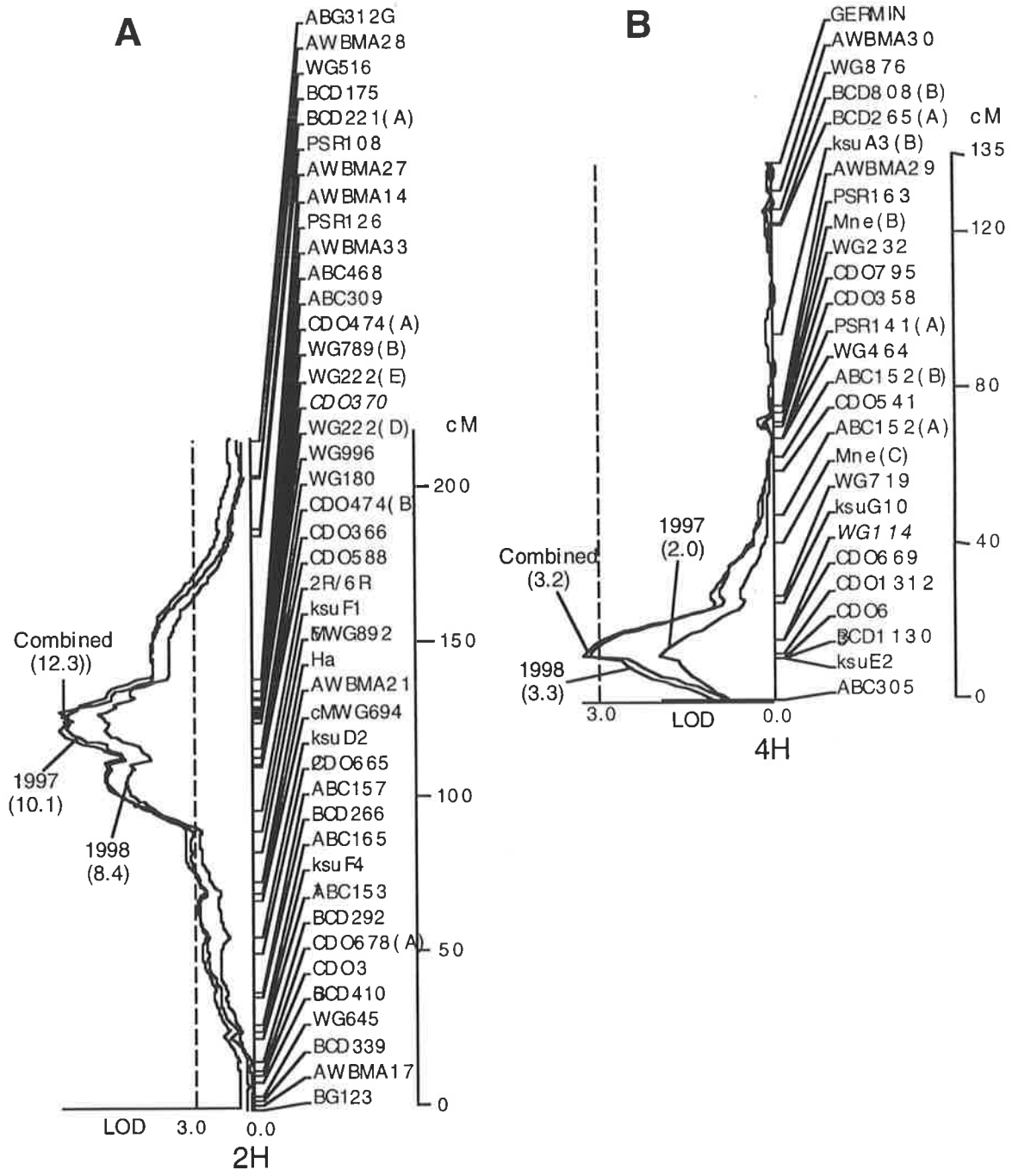



Figure 4.3. Location on chromosome 2H (**A**) and 4H (**B**) of regions associated with leaf symptom score expressed in doubled haploid lines from the cross Clipper x Sahara 3771 based on interval mapping of 1997, 1998 and combined data. Short arms are towards the tops of chromosomes. Maximum LOD scores for each peak is provided in parentheses. RFLP marker most significantly associated with trait is presented in italics. Dash lines show LOD 3.0 threshold.

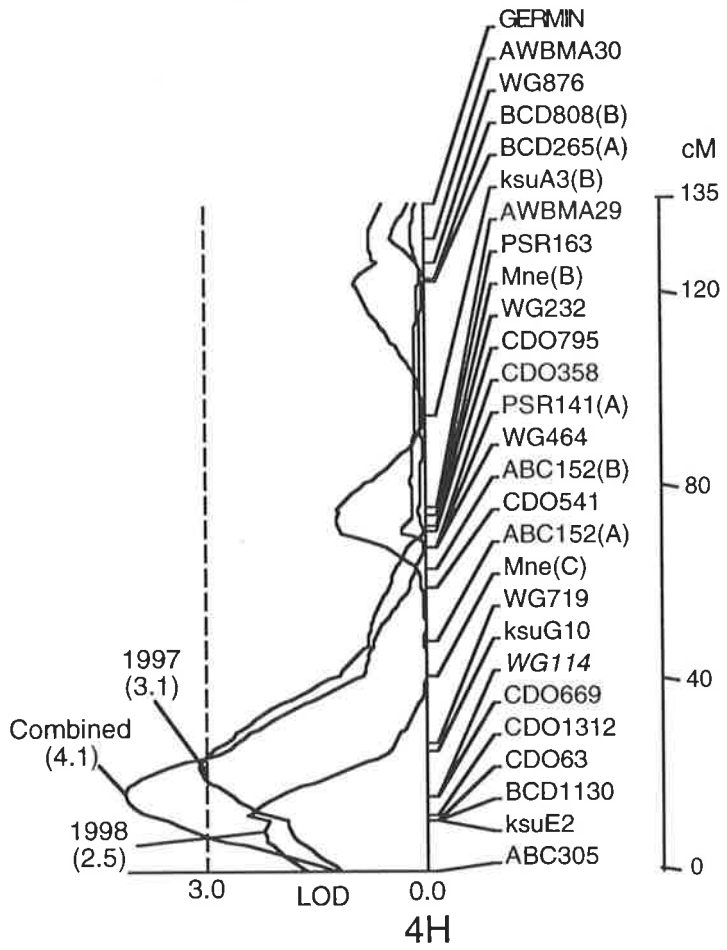



Figure 4.4. Chromosome location of a region associated with dry matter response to toxic concentration of B expressed in doubled haploid lines from the cross Clipper x Sahara 3771 based on interval mapping of 1997, 1998 and combined data. Short arm is towards the top of the chromosome. Maximum LOD scores for each peak is provided in parentheses. RFLP marker most significantly associated with trait is presented in italics. Dash lines show LOD 3.0 threshold.

*Whole shoot B concentration*

Markers on regions of chromosomes 4H (Fig. 4.5-A) and 6H (Fig 4.5-B) were associated with whole shoot B concentration of plants grown in B toxic soil. Based on multiple regression analysis, loci on chromosome 4H and 6H combined, accounted for approximately 53% of variation in whole shoot B concentration data. Sahara marker alleles at these loci conferred low whole shoot B concentration. RFLP markers on chromosome 4H and 6H most strongly associated with leaf symptom score was *Xwg114* and *Xamy-1(A)* respectively.

***Marker interactions***

Marker loci thought to be associated with a chromosomal region involved in the control of any of the four B tolerance traits were tested for two-way interaction with all other markers in the data set. The markers tested for interaction with all other markers were *Xcdo370*(2H), *Xawbma15* (3H), *Xwg114*(4H) and *Xamy-1(A)* (6H). No significant ($P<0.001$) interactions were identified.

***Gene effects***

*Relative root length*

Table 2 details the mean effect of the presence of either a Clipper or Sahara marker allele on RRL. As expected, there was no significant ($P<0.05$) difference between individuals carrying the Clipper or Sahara marker alleles at the chromosome 2H locus, previously identified to be associated with leaf symptom score only. The presence of the Sahara allele at the chromosome 3H locus (*Xawbma15*) conferred an average 13.5% increase in RRL. The presence of the Sahara allele at the chromosome 4H locus (*Xwg114*) conferred an average 19.0% increase in RRL over those individuals carrying the Clipper marker allele only. Individuals carrying the Sahara marker allele at both the 3H and 4H loci produced, on average, 30.7% greater relative root length than individuals carrying the Clipper allele at both loci.

The majority of variation in RRL is controlled by two major loci, on chromosomes 3H and 4H, behaving in a largely additive manner with the chromosome 4H locus having a slightly larger effect.

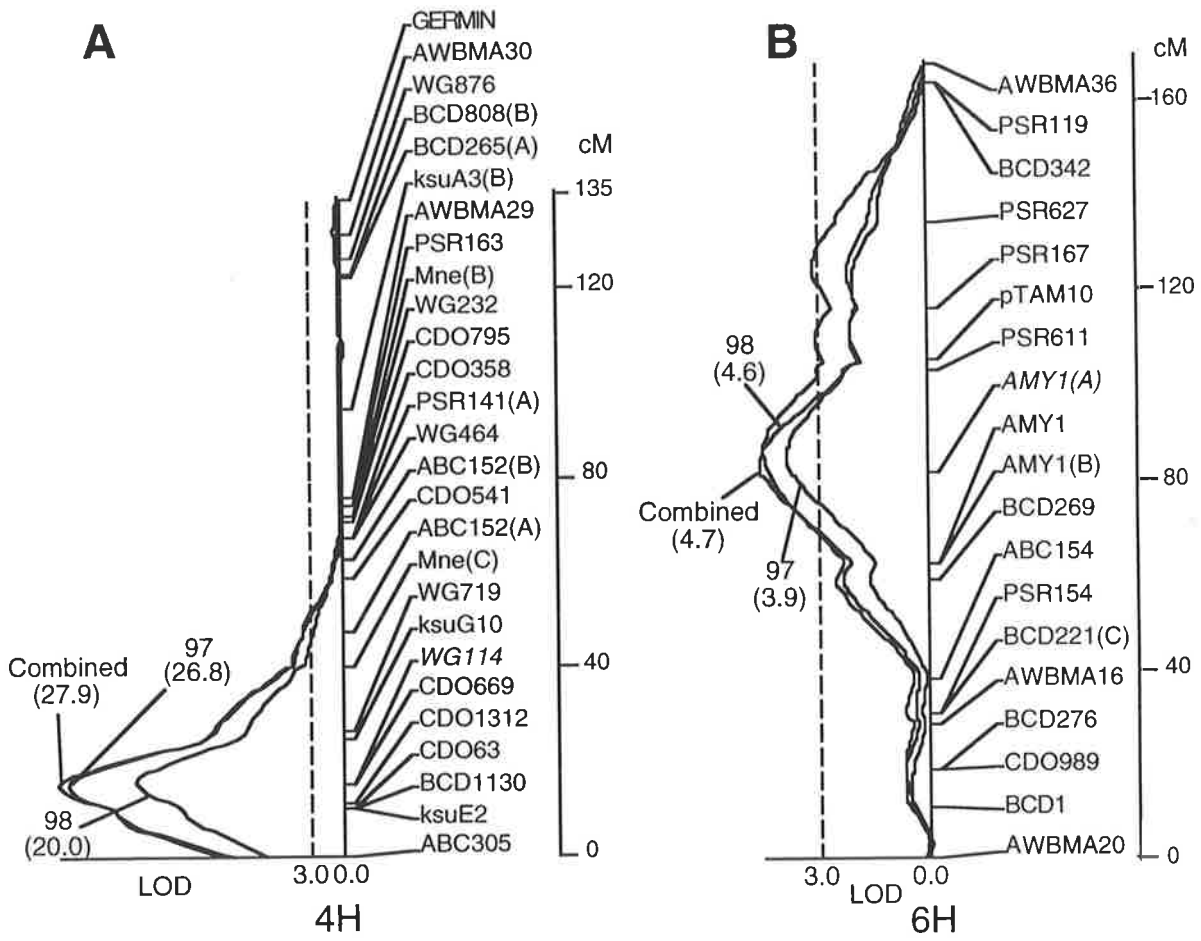



Figure 4.5. Location, on chromosome 4H (**A**) and 6H (**B**), of regions associated with whole shoot B concentration expressed in doubled haploid lines from the cross Clipper x Sahara 3771 based on interval mapping of 1997, 1998 and combined data. Short arms are towards the tops of chromosomes. Maximum LOD scores for each peak is provided in parentheses. RFLP marker most significantly associated with trait is presented in italics. Dash lines show LOD 3.0 threshold.

Table 2. Mean effect of the presence of either Clipper or Sahara marker allele on relative root length (%) of the Clipper x Sahara 3771 mapping population seedlings grown on filter paper soaked in low and toxic concentrations of B.

| Marker | Allele(s)- Clipper (C) Sahara (S) | Number of doubled haploid lines within class | Relative Root Length (Least squares mean (%)) | Standard error of mean |
|---|---|---|---|---|
| *Xawbma15* (3H) | C | 84 | 50.1 a[1] | 1.7 |
| | S | 64 | 63.6 b | 2.0 |
| *Xwg114* (4H) | C | 82 | 47.4 a | 1.5 |
| | S | 64 | 66.4 b | 1.8 |
| *Xawbma15 (3H)/ Xwg114 (4H)* | CC | 48 | 40.9 a,e | 1.8 |
| | CS | 36 | 62.3 b,f | 2.1 |
| | SC | 34 | 56.6 c,f | 2.1 |
| | SS | 28 | 71.8 d,g | 2.4 |

[1] means with letters 'a', 'b', 'c' and 'd" are significantly different at $P<0.05$, means with letters 'e', 'f' and 'g' and significantly different at $P<0.005$, comparisons within major class groups only

*Leaf Symptom Score*

Table 3. details the mean effect of the presence of either a Clipper or Sahara marker allele on leaf symptom score. The presence of the Sahara marker allele at the chromosome 2H locus (*Xcdo370*) conferred an average 1.2 point reduction in leaf symptom score while a Sahara marker allele at the chromosome 4H locus (*Xwg114*) conferred an average 0.6 point reduction. Individuals carrying the Sahara marker allele at both the 2H and 4H loci, on average, received, on average, 1.8 point lower leaf symptom score than individuals carrying Clipper alleles at both loci. It appears that leaf symptom expression is predominantly controlled by two major loci behaving in a largely additive manner with the chromosome 2H locus having a substantially larger effect than the chromosome 4H locus.

Table 3. Mean effect of the presence of either Clipper or Sahara marker allele on leaf symptoms of the Clipper x Sahara 3771 mapping population grown in soil containing toxic concentrations of B

| Marker | Allele(s)- Clipper (C) Sahara (S) | Number of doubled haploid lines within class | Leaf symptom score (Least squares mean) (score 1-6)[1] | Standard error of mean |
|---|---|---|---|---|
| *Xcdo370* (2H) | C | 75 | 3.7 a[2] | 0.1 |
| | S | 72 | 2.5 b | 0.1 |
| *Xwg114* (4H) | C | 82 | 3.4 a | 0.1 |
| | S | 64 | 2.8 b | 0.1 |
| *Xcdo370*(2H)/ | CC | 41 | 4.1 a | 0.1 |
| *Xwg114* (4H) | CS | 33 | 3.2 b | 0.1 |
| | SC | 42 | 2.7 bc | 0.1 |
| | SS | 30 | 2.3 c | 0.1 |

[1] mean of leaf symptom score (1-6 scale; 1 = no symptoms, 6 > 90% leaf necrosis)

[2] means with different letters are significantly different at P<0.005, comparisons within major class groups only

*Whole shoot dry weight*

Table 4 details the mean effect of the presence of either a Clipper or Sahara marker allele on whole shoot dry weight. The presence of the Sahara marker allele at the chromosome 4H locus (*Xwg114*) conferred an average 42% increase in whole shoot dry weight over individuals carrying the Clipper marker allele.

Table 4. Mean effect of the presence of either the Clipper or Sahara marker allele on whole shoot dry weight of the Clipper x Sahara 3771 mapping population grown in soil containing toxic concentrations of B ([1] means a and b significantly different at P<0.001)

| Marker | Allele(s) | Number of doubled haploid lines | Whole shoot dry weight (Least squares mean, g) | Standard error of mean |
|---|---|---|---|---|
| *Xwg114* (4H) | Clipper (C) | 82 | 0.12 a[1] | 0.01 |
| | Sahara (S) | 64 | 0.17 b | 0.01 |

*Whole shoot B concentration*

Table 5 details the mean effect of the presence of either a Clipper or Sahara marker allele on whole shoot B concentration. The presence of the Sahara marker allele at the chromosome 4H locus (*Xwg114*) conferred an average 64% reduction in whole shoot B concentration over those individuals carrying the Clipper marker allele only. The presence of the Sahara allele at the chromosome 6H locus (*Xamy-1(A)*) conferred an average 31% reduction in whole shoot B concentration. Individuals carrying the Sahara marker allele at both the 4H and 6H loci showed, on average, 76% lower whole shoot B concentration than individuals carrying the Clipper allele at both loci. It appears that whole shoot B concentration is predominantly controlled by two major loci behaving in a largely additive manner with the chromosome 4H locus having a substantially larger effect than the chromosome 6H locus.

Table 5. Mean effect of the presence of either Clipper or Sahara marker allele on whole shoot B concentration of the Clipper x Sahara 3771 mapping population grown in soil containing toxic concentrations of B

| Marker | Allele(s)-Clipper (C) Sahara (S) | Number of doubled haploid lines within class | Whole shoot B concentration (Least squares mean (ppm)) | Standard error of mean |
|---|---|---|---|---|
| *Xwg114* (4H) | C | 82 | 372.5 a[1] | 7.4 |
| | S | 64 | 133.2 b | 8.7 |
| *Xamy-1 (A)*(6H) | C | 87 | 311.8 a | 13.9 |
| | S | 61 | 215.7 b | 17.1 |
| *Xwg114/Xamy-1(A)* | CC | 51 | 412.1 a | 7.0 |
| | CS | 31 | 302.4 b | 9.4 |
| | SC | 34 | 158.3 c | 8.8 |
| | SS | 30 | 101.3 d | 9.9 |

[1] means with different letters are significantly different at P<0.001, comparisons within major class groups only

## Discussion

### *Chromosomal locations conferring B tolerance and relationships to proposed tolerance mechanisms*

This study has identified and located four chromosomal locations involved in tolerance to B toxicity as measured by the four traits discussed.

The relative rating of barley genotypes for B tolerance has conventionally been based on expression of leaf symptoms. Genetic variation for B tolerance (leaf symptom expression) was attributed to differences in B concentration in plant tissue and, therefore, proposed to be due to differences in passive B uptake (Nable, 1988: Nable 1991). However, Mahalakshmi *et al.* (1995) found that some barley genotypes showed low leaf symptoms yet accumulated high concentrations of B in plant tissue. In this study, several Clipper x Sahara doubled haploid lines were found to accumulate high concentrations of B in whole shoots yet produced low levels of leaf symptom. Significant ($P<0.001$) correlation between symptom development, tissue concentration, dry weight production and relative root length were found but coefficient values were relatively low (Table 1). This contrasted with the high level of correlation (correlation coefficients ranged from 0.66 – 0.82, $P<0.01$) between root length, symptom score, shoot concentration and dry weight for 14 wheat cultivars reported by Chantachume *et al.* (1995). The strong correlation found in the wheat study could have been due to the relatively small number of genotypes and the preselection of genotypes for extremes in leaf symptom expression, dry matter production and/or grain yield. In addition to discrepancies between leaf symptom score and tissue concentration, poor relationships between grain yield response and leaf symptom expression have also been reported in both wheat and barley (Paull *et al.*, 1988; Jenkin, 1993; Riley and Robson, 1994).

A major locus on chromosome 2H, associated with leaf symptom score, was not associated with other tolerance parameters. In contrast, the chromosome 4H locus was significantly associated with all four tolerance parameters (Fig 4.3, 4.4, 4.5). The chromosome 3H locus was associated with relative root length only. These findings provide evidence that the observed poor relationships between leaf symptom development, tissue concentration (Makalakshmi *et al.*, 1995), and grain yield (Jenkin, 1993; Riley and Robson, 1994) and the relatively low correlation coefficients between tolerance parameters are due to differences in genetic control .

The chromosome 2H locus may be involved in the translocation of B in leaf tissue. It has been shown that there is no difference between roots and shoots in the accumulation of B (Nable, 1991) yet the concentration in leaves increases from young to old leaves and from base to tips (Nable *et al.*, 1990a). A similar pattern is followed in the development of leaf symptoms in barley.

Regions on chromosome 4H and 6H appear to be involved in a B exclusion mechanism which is determining the relative accumulation of B in shoots. The exclusion mechanism is, in turn, likely to reduce the effect of toxic levels of B on root growth (RRL) and dry matter production. Evidence of this was found in the association of RRL (Fig. 4.2) and whole shoot dry weight (Fig. 4.4) with the chromosome 4H region involved in the control of B uptake.

The chromosome 3H locus was not associated with whole shoot B concentration but was involved in RRL response. This locus could be involved in internal physiological compensation resulting in superior root growth in toxic situations.

A B exclusion mechanism plays a major role in overall B tolerance as it has an effect on the expression of other tolerance traits yet other mechanisms have been shown to have an effect. These observations support the conclusions of Cartwright *et al.* (1987), that a genetic exclusion mechanism is important but other mechanisms are likely also to be involved.

***Gene Effects***

The chromosome 2H locus appears to be the most important in controlling leaf symptom expression while the chromosome 4H locus appears to be the most important in controlling B uptake, root length response and dry matter production. Only one chromosome region was associated with response in dry matter production. It is possible that the region on chromosome 6H, associated with control of B uptake, and the chromosome 3H, region associated with the control of RRL, also have an effect but, possibly due to the high error variance in the whole shoot dry weight assay, no additional statistically significant relationships were established.

Further investigations on the role and relative contribution of each region and/or gene, particularly in relation to grain yield response, and the transferability of these regions to a different genetic background are required before an efficient selection strategy can be devised.

***Evolution of B tolerance genes in wheat and barley***

Wheat chromosomes 4A (Paull *et al.*, 1988b), 7B and 7D (Paull, 1990) have all been implicated in the control of B tolerance in bread wheat. A strong association was identified between B tolerance, as measured by the root length assay, and an RFLP marker *Xksug10* on chromosome 4A of wheat (Paull *et al.*, 1993). This location was detected close to the break point between 4AL-7BS on the modern 4AL chromosome (Devos *et al.*, 1995; Mickelson-Young *et al.*, 1995). In this study, a region on chromosome 4H of barley was associated

with all four B tolerance traits measured. On the basis of the consistency in interval analysis outputs from all four traits, a major gene or cluster of genes is located between RFLP markers *Xwg116* and *Xksug10* on chromosome 4H of barley. It is possible that wheat and barley may possess a common gene on chromosome 4A and 4H respectively.

***Marker-Assisted Selection for B Tolerance***

No single glasshouse or laboratory B tolerance bioassay currently available will successfully identify individuals carrying all four QTL. The relative root length and leaf symptom assays are both time-consuming. The assay for whole shoot B concentration is expensive and destructive. The leaf symptom assay is highly subjective and also time-consuming. Field-based grain yield comparisons are prone to experimental error due to within-site variation and errors are compounded by genotype by environment interaction for characters other than B tolerance.

RFLP markers closely linked to genes of agronomic importance have been demonstrated to be useful tools for indirect selection in a barley breeding program (Jefferies *et al.*, 1997). RFLP marker assisted selection is time efficient, non-destructive, and depending on linkage relationships, is characterised by low selection error. In addition, RFLP markers can detect heterozygous individuals and genotypes can be screened for a number of marker linked traits almost simultaneously. Marker assisted selection for B tolerance will provide the plant breeder with an efficient selection tool offering more flexibility than the current bioassay systems. Experiments to validate these markers in different genetic backgrounds are currently underway.

# Chapter 5

# Validation and agronomic effects of QTL conferring boron toxicity tolerance in barley

## 5.1 Introduction

Boron (B) is an essential plant micronutrient which can be phytotoxic to plants if present in soils in high concentration. B toxicity has been recognised as an important problem in the medium to low rainfall regions of southern Australia (Cartwright *et al.*, 1984), West Asia and North Africa and a problem associated with irrigation water in many other parts of the world (Gupta *et al.*, 1995).

The most obvious symptoms of B toxicity in barley are chlorosis and necrosis extending from leaf tips and the formation of brown lesions initially at the leaf margins but extending over the distal half or more of the leaf. Brown lesions appear on the oldest leaves first and successive leaves become affected in sequence. In severe cases, brown lesions can appear on leaf sheaths, stems and awns.

Soil amelioration of B toxicity is generally considered impractical. Genetic variation for tolerance of B toxicity, however, exists within a number of crops including barley (Cartwright *et al.*, 1987, Paull *et al.*, 1988a). Exploitation of this genetic variation, through the development of tolerant cultivars, may significantly improve the productivity and quality of barley growing in areas prone to B toxicity problems.

An RFLP linkage map of an $F_1$ doubled haploid population between B tolerant Algerian landrace, Sahara 3771 (Sahara), and the intolerant Australian cultivar Clipper (Langridge *et al*, 1995) was used to identify chromosomal regions associated with B tolerance in barley (Jefferies *et al*, 1999; and this thesis). Interval regression mapping allowed the detection of four chromosomal regions involved in plant response to B toxicity. A region on chromosome 2H was associated with severity of leaf symptoms, a region on chromosome 3H was associated with root growth response, and regions on chromosome 4H and 6H were associated with reduced accumulation of B in plant tissue. The region on chromosome 4H was also associated with root length response, leaf symptom expression and dry matter production. The two assay systems used to identify these four chromosomal regions areboth time-consuming, expensive, destructive, influenced by environmental factors and/or subjective in assessment. B tolerance is therefore an ideal candidate trait for marker assisted selection as the trait is controlled by relatively few factors and the bioassay systems are complex, inefficient and prone to selection error.

Molecular markers have been demonstrated to be useful for indirect selection in a barley breeding program (Jefferies *et al.*, 1997). Molecular marker assisted selection is time efficient, non-destructive, and depending on linkage relationships, is characterized by low selection error. In addition, co-dominant markers can detect heterozygous individuals and genotypes can be screened for a number of marker linked traits almost simultaneously. Marker assisted selection for B tolerance could provide plant breeders with an efficient selection tool offering more flexibility than the bioassay systems currently available.

The transferability, role and function of the chromosomal regions (QTL) found to be associated with plant response to B toxicity (Jefferies *et al.*, 1999; and this thesis) has not been determined. In addition the B tolerant parent used in mapping, Sahara, is an exotic landrace with very poor adaptation to southern Australian conditions. Selection for Sahara marker alleles at loci conferring B tolerance could, through linkage drag (Brown *et al.*, 1989a), select segments carrying deleterious factors. The planning and execution of an efficient and effective B tolerance introgression strategy will require knowledge of potential deleterious linkage drag and or pleiotropic effects.

Jefferies *et al.* (1999; and this thesis) did not assess grain yield in the mapping experiments as the Clipper x Sahara population had a significant proportion of individuals highly susceptible to lodging, shattering, and head loss, reducing the ability to collect meaningful data from field trials. The relative contribution of major B tolerance QTL to grain yield and grain quality in B toxic field situations needs to be determined.

This study aims to assess the relative transferability of B tolerance QTL from Sahara to alternative genetic backgrounds using molecular markers, determine the potential for deleterious linkage drag and pleitropic effects and determine the relative contribution of QTL to grain yield and grain quality response in B toxic field situations.

## 5.2 Materials and Methods

### 5.2.1 Genetic material

The genetic material used in this study consisted of lines derived from two backcross populations. Backcross populations were used in order to reduce the data collection problems associated with the poorly adapted phenotype of Sahara, that is, it is highly susceptible to lodging, shattering, and head loss. In addition, subtle pleitropic effects may go unnoticed in a more balanced population due to the large genetic and phenotypic variance created by segregation of Sahara alleles in high frequency. The backcross populations used in this study were developed from two recurrent parents, the Australian malting quality cultivar Sloop, and an Australian breeder's line designated as VB9104.

Both recurrent parents are widely adapted to southern Australian cropping environments. VB9104 has a higher general grain yield potential in most environments and consistently produces a greater proportion of plump grain than Sloop. Sloop has some superior malt quality characteristics.

A single individual in the Clipper x Sahara mapping population, designated DH31, was selected as the donor parent for the Sloop backcross population while the breeders line, VB9743 (Sahara/WI2723//Chebec) was chosen as the donor parent for the VB9104 population. Both donor parents were selected on the basis of very low leaf symptoms and above average root mass when grown in soil to which toxic concentrations of B were added. It was later confirmed, from mapping information (Jefferies *et al.*, 1999; and this thesis), that DH31 carried Sahara marker alleles in the region of the 2H, 3H, 4H and 6H B tolerance loci while marker analysis revealed that VB9743 carried Sahara marker alleles at the 2H and 4H loci only. The mapping population parent Clipper is closely related to Sloop, with more than 60% common pedigree links. VB9743 is not closely related to VB9104, but like VB9104, it is relatively well adapted to southern Australian barley growing environments. The choice of DH31 and VB9743 as donor parents, therefore, reduced the need for additional backcross generations.

### 5.2.2. DNA extraction, restriction endonuclease digestion and Southern hybridisation

DNA extraction was achieved using a DNA mini-prep method adapted from Rogowsky *et al.* (1991). Variations to the method were as described below. For the initial extraction, 750 µl of extraction buffer and phenol-chloroform-isoamyl alcohol (25:24:1) were used. The extraction buffer was 0.1 M Tris-HCl (pH 8.5),10 mM EDTA, 0.1 M NaCl, 1% sarkosyl and 2% polyvinyl-polypyrrolidone (insoluble). After the second phenol-chloroform-isoamyl alcohol extraction the aqueous phase was extracted once with an equal volume of chloroform. DNA was precipitated by the addition of 0.1 vol of 3M sodium acetate (pH 4.8) and 1 volume of propan-2-ol. Restriction endonuclease digestion and Southern hybridisation followed standard methods.

### 5.2.3 Population construction

*Development of Sloop backcross derived lines*

The construction of the Sloop recurrent parent backcross population was commenced before the identification and location of QTL conferring B toxicity tolerance was confirmed (Jefferies *et al.*, 1999; and this thesis). A $BC_1F_2$ bulk population was created from crosses between the donor parent DH31 and the recurrent parent Sloop. More than 600 random individuals were grown in the soil based B tolerance assay (Jefferies *et al.*, 1999; and this thesis). Eight single plants were selected on the basis of very low level of leaf symptoms

and high root mass. All eight selected plants were backcrossed to Sloop to form the $BC_2$ population. DNA from 400 random $BC_2F_2$ individuals was probed with RFLP markers closely associated with the regions on chromosomes 2H (*Xcdo370*) and 4H (*Xwg114*) found to confer response to B toxicity (Jefferies *et al.*, 1999; and this thesis). The $BC_2F_2$ population was not initially screened with markers associated with regions on chromosomes 3H and 6H as there was insufficient evidence available, at that time, to suggest that these two regions played an important role in B tolerance. A total of 64 $BC_2F_2$ single plants, homozygous for both marker alleles (either Sahara or Sloop) were selected for seed multiplication. Sufficient seed of 53 of the 64 lines was produced for the first field experiment in 1998. Seed of the 11 remaining $BC_2F_2$ lines were multiplied for field experiments in 1999.

*Development of VB9104 backcross derived lines.*

The breeding lines VB9104 (Europa/IBON#7.148) and VB9743 (Sahara/WI2723//Chebec) were used as recurrent and donor parents respectively, in a limited backcrossing program to develop a series of closely related lines with varying levels of B tolerance. B tolerance in VB9743 was derived from Sahara. The most B tolerant backcross $F_1$ plants were selected for the second backcross using a modification of the filter paper method of Chantachume *et al* (1995). Selected plants possessed the longest root length after 8 days growth using filter papers soaked in a basal nutrient solution amended with 61 ppm B applied as boric acid. Several backcross $F_1$ plants were used in creating the second backcross populations. Progeny assessment, using the method of Moody *et al.* (1988) identified those $BC_2$ $F_1$ derived families from the second backcross with variation in B tolerance. A total of 150 plants were chosen at random from a $F_3$ population derived from a single $BC_2$ $F_1$ plant. A subset of 18 lines, varying in levels of B tolerance, was chosen for examination in field trials.

### 5.2.4 Field experiments 1998 and 1999

Field experiments in 1998 and 1999 were conducted at Minnipa, South Australia, where naturally occurring high concentrations of B are found in the subsoil. Additional field experiments were conducted in 1999 at Pinery, South Australia, and Horsham, Victoria, where B in sub-soils is generally rated as moderate to moderately low. The occurrence of toxicity symptoms in the Pinery and Horsham areas is largely dependent on seasonal conditions while severe toxicity symptoms are observed regularly in barley crops in the Minnipa area. A survey of soils in the Minnipa area was used to identify sites high in subsoil B concentration (Holloway, 1991).

Fifty three $BC_2F_2$ derived lines were sown in field plots in 1998. Sloop backcross lines were sown in plots 8 rows wide (1.4m) by 5m in length and arranged in two complete replicates

Plate 5.1 Layout of field experiment at Minnipa, SA, 1998, showing variation for boron toxicity leaf symptoms amongst $BC_2$ lines derived from backcrosses between the donor parent Sahara (tolerant) and the recurrent parent Sloop (intolerant).

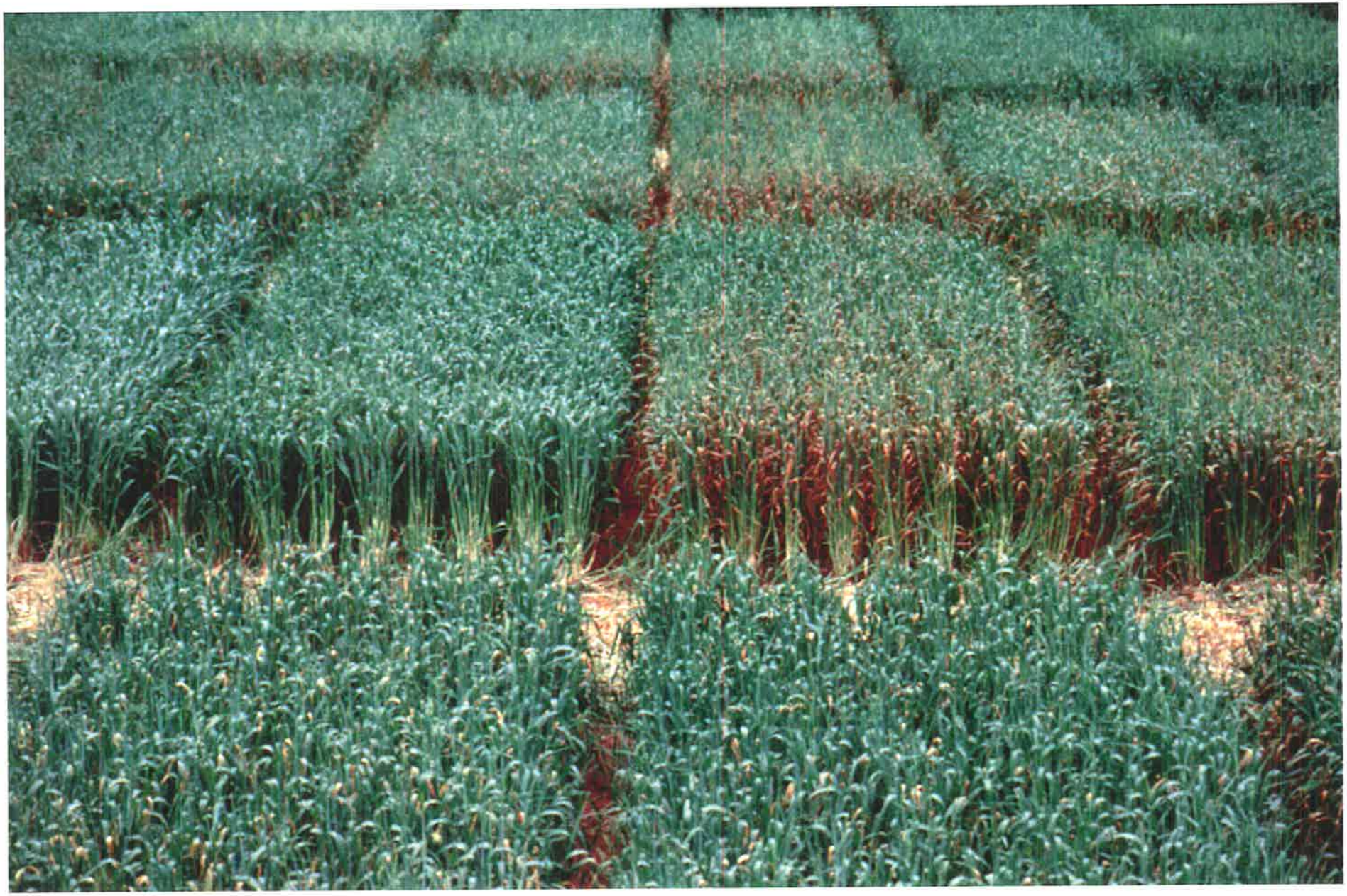


Plate 5.2 Layout of field experiment at Minnipa, SA, 1999, showing variation for boron toxicity leaf symptoms amongst $BC_2$ lines derived from backcrosses between the donor parent Sahara (tolerant) and the recurrent parent Sloop (intolerant).


BORON
BARLEY
6 10 99

with Sloop control plots sown as a grid every fifth plot. The check grid was included to assist in accounting for spatial variation in B concentration in subsoil. Seeding rates were adjusted for grain size variation with the aim of establishing approximately 150 plants/$m^2$. Fertiliser application and weed management followed standard practices for the district. Ten plant shoots per plot were harvested at ground level at decimal growth stage (DGS) 39 (Zadoks, 1974). Whole shoot tissue was dried at 80°C for 48 h, ground, digested in nitric acid and analyzed for B concentration by inductively coupled plasma spectrometry (ICP) (Zarcinas *et al.*, 1987). Each plot was scored for DGS and severity of leaf symptoms when Sloop check plots reached DGS 57. Severity of leaf symptoms was scored on the basis of leaf damage on a scale of 1-9, where 1 related to no visual symptoms and 9 showed greater than 90% necrosis. When plants were at full maturity plots were harvested with a small plot mechanical harvester and grain yield recorded. The percentage of grain retained above a 2.5mm screen in both 1998 and 1999 and 1000 grain weight in 1998 was determined from the machine harvested samples. The number of grains per plot was estimated from the yield per plot and 1000 grain weight. Soil samples were taken from both the 5-15cm and 35-45cm profile in every Sloop check plot after harvest. The concentration of B extractable in hot $CaCl_2$ (Spouncer *et al.*, 1992) was determined for each soil sample.

A further 11 $F_2$ derived, $BC_2$ Sloop backcross lines and 18 VB9104 backcross lines were included in the 1999 field experiments at Minnipa. Replication was increased from two to three in 1999 and the frequency of Sloop check plots reduced from every fifth to every eleventh plot. All measurements taken on the 1998 experiment at Minnipa were repeated in 1999. Grain yield, grain plumpness and relative maturity were measured in field experiments conducted at Pinery and Horsham in 1999. Soil samples were taken from the 5-15cm, 35-45cm and 60-100 cm profiles in every Sloop check plot at Horsham and from the 5-15cm and 35-45cm profile in every second Sloop check plot at Pinery.

### 5.2.5 Relative root length in solution culture

Seeds of each of the 63 Sloop and 18 VB9104 backcross lines and control cutivars were surface-sterilized with 5.0% sodium hypochlorite and pre-germinated for 8 days at 4°C. Four evenly germinated seeds per genotype were placed embryo downwards at a spacing of 2cm across the middle of a filter paper (Ekwip 32 x 46 cm grade R6) soaked in a solution containing either 100 mg B $l^{-1}$(B100) or 0 mg B $l^{-1}$(B0). Full details of the procedure and base solutions were as described in Jefferies *et al.* (1999, and this thesis). The filter papers were rolled and covered with aluminum foil and stored upright at 12°C for 16 days. The longest root of each seedling was measured. The experiment was conducted as a randomised complete block with three replicates (total of 12 seedlings per backcross line). Relative root length (RRL), for each genotype, was calculated from the mean root length at B100 as a percentage of the mean root length at B0.

### 5.2.6 Flanking markers and additional loci

The 63 $BC_2$ Sloop backcross lines were selected on the basis of either Sahara or Sloop alleles for RFLP markers most significantly associated with B tolerance loci on chromosome 2H (*Xcdo370*) and chromosome 4H (*Xwg114*) (Jefferies *et al.*, 1999; and this thesis). DNA from the 63 $BC_2$ Sloop backcross lines was also probed with RFLP markers flanking both of these loci. The chromosome 2H markers chosen were *Xwg180* and *Xabc309*, mapping proximally (10.7cM) and distally (2.1cM) to *Xcdo370* respectively in the Clipper X Sahara mapping population (Langridge *et al.*, 1995)(Fig 5.1). The chromosome 4H markers chosen were *Xksug10* and *Xcdo1312*, located proximally (9.3cM) and distally to *Xwg114* (4.3cM) respectively (Langridge *et al.*, 1995) (Fig 5.1). DNA from the Sloop backcross lines was also probed with RFLP markers *Xawbma15*, associated with the B tolerance locus on chromosome 3H and *Xamy-1* associated with the chromosome 6H locus (Jefferies *et al.*, 1999; and this thesis). Allocation of lines to marker allele class was based on the combined marker information. DNA from the VB9104 backcross lines was probed and genotyped with RFLP markers *Xwg996* (2H), *Xwg114* (4H) and *Xawbma15* (3H) only.

### 5.2.7 Statistical analysis

Means for grain yield, percentage of grain retained above a 2.5mm screen and concentration of B in whole shoots from the Minnipa experiments in 1998 and 1999 were calculated, allowing for extraneous variation using spatial techniques developed by Cullis and Gleeson (1991). Means for grain yield and grain plumpness at Pinery and Horsham in 1999 were estimated using the same methods. A cross-site analysis was conducted using scaled information of estimates of genotype effects from both Pinery and Horsham following the method of Cullis *et al.* (1996). A combined cross-site genotype effect (common effect) was estimated for grain yield and grain plumpness. Regression analysis (SAS Institute, JMP version 3 software) of data from Minnipa (B toxic) on the common genotype effect at Pinery and Horsham (non-toxic) was used to produce fitted values which were subtracted from observed values to provide an adjusted estimate of genotype response to B toxicity in both years.

Lines were grouped into classes based on the likely presence of either a donor or recurrent parent chromosome segment at each of the B tolerance loci following the method described by Jefferies *et al.* (1999; and this thesis). Least-squares class means were calculated using a single factor ANOVA (SAS Institute, JMP version 3 software). Class means were compared using linear contrasts. Regression analysis (SAS Institute, JMP version 3 software) was used to relate soil B concentration in Sloop control plots and backcross derived lines, to grain yield, grain plumpness, leaf symptom score and concentration of B in whole shoots.

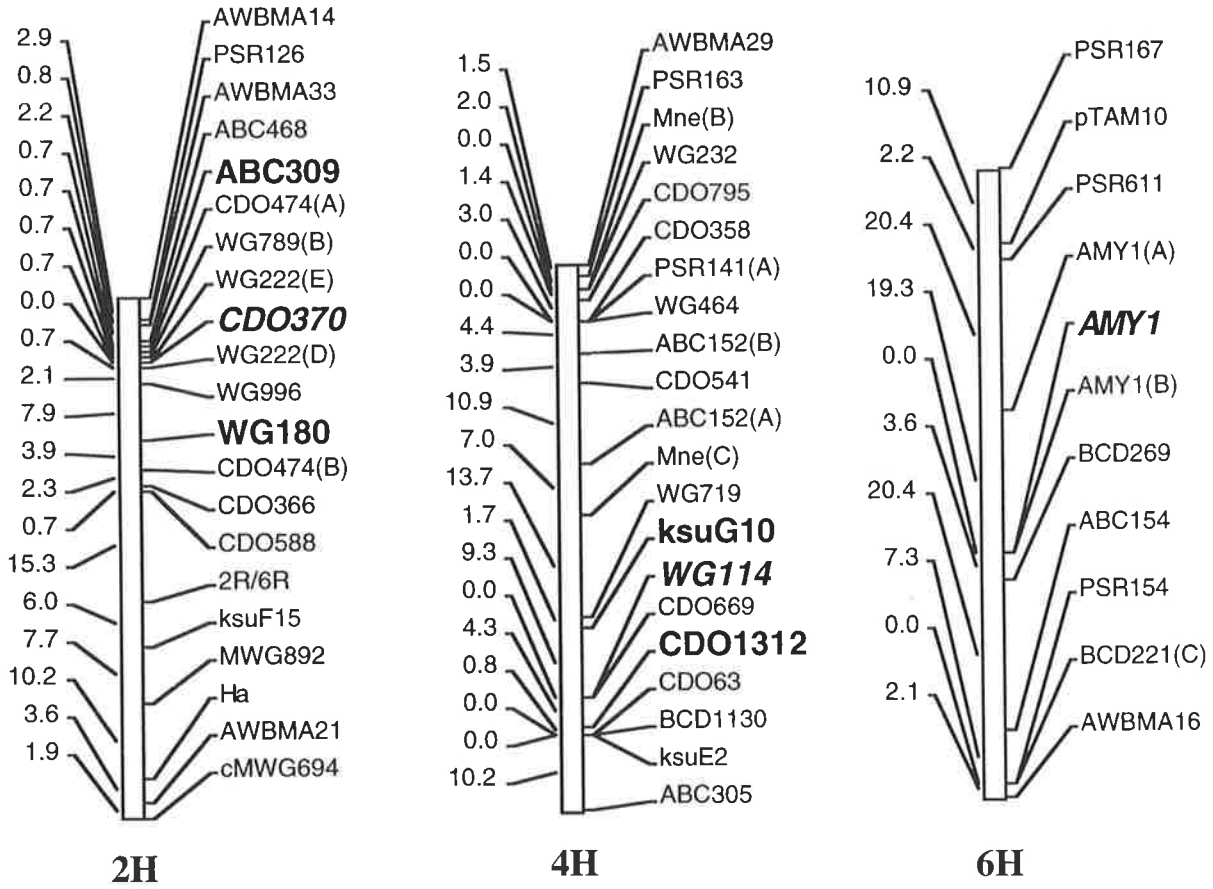



Figure 5.1. Partial linkage map of chromosomal regions surrounding B tolerance loci on chromosomes 2H, 4H and 6H of the Clipper x Sahara linkage map (Langridge *et al.*, 1995). The RFLP marker most significantly associated with plant response to B toxicity is presented in bold italics and flanking markers used to confirm genotype for allocation to classes presented in bold.

## 5.3 Results

Seasonal conditions encountered at Minnipa in 1998 were characterised by above average rainfall during the important early to mid growing season months of June, July and August but were followed by very much below average September rainfall (Fig 5.2), accompanied by above average daily temperatures (data not shown). Above average vegetative growth and high grain yield potential was set during the winter months. Very dry and warm conditions during September resulted in considerably lower grain yield and grain size than expected. In 1999, seasonal conditions were characterised by average rainfall in May followed by very much below average rainfall for the remainder of the growing season (Fig 5.2). Below average summer rainfall prior to both the 1998 and 1999 growing seasons provided no significant soil moisture reserves. Plots in 1999 suffered from moderate to severe drought stress during the majority of the growing season. The mean yield of Sloop control plots was 1.45 t/ha in 1998 and 0.97 t/ha in 1999.

The genetic correlation for grain yield at Pinery and Horsham was highly significant ($r^2$=0.68, P<0.001). There was no significant correlation between grain yield at Pinery or Horsham with grain yield at Minnipa in 1998 and 1999 suggesting that the major factors affecting grain yield at Pinery and Horsham were similar to each other but different to those at Minnipa.

### 5.3.1 Concentration of B in soil

Southern Australian soils generally show considerable horizontal and vertical variability for soil B concentration (Cartwright *et al.*, 1984; Cartwright *et al.*, 1986; and Moody *et al.*, 1993). Typical 'non-toxic' soils range from 0.5-5.0 mg B $kg^{-1}$ (Moody *et al.*, 1993). Soil B concentration in Sloop control plots was generally within this 'non-toxic' range in the 5-15 cm profiles at Minnipa in both 1998 and 1999 but extended well beyond this range in the 35-45 cm profiles in both years (Figure 5.3 and Table 1). Figure 5.3 highlights the considerable spatial variation in soil B concentration typical of B toxic environments. The concentration of B in Sloop control plots at Pinery and Horsham was low in both the 5-15 cm and 35-45 cm profiles but was moderately high in some plots in the 60-100 cm profile at Horsham (Table 1). Control plots with high concentrations of B in the 5 to 15 cm profile at Minnipa also had high concentrations in the 35 to 45 cm profile in 1998 ($r^2$= 0.33, P<0.001) and 1999 ($r^2$ = 0.67, P<0.0001).

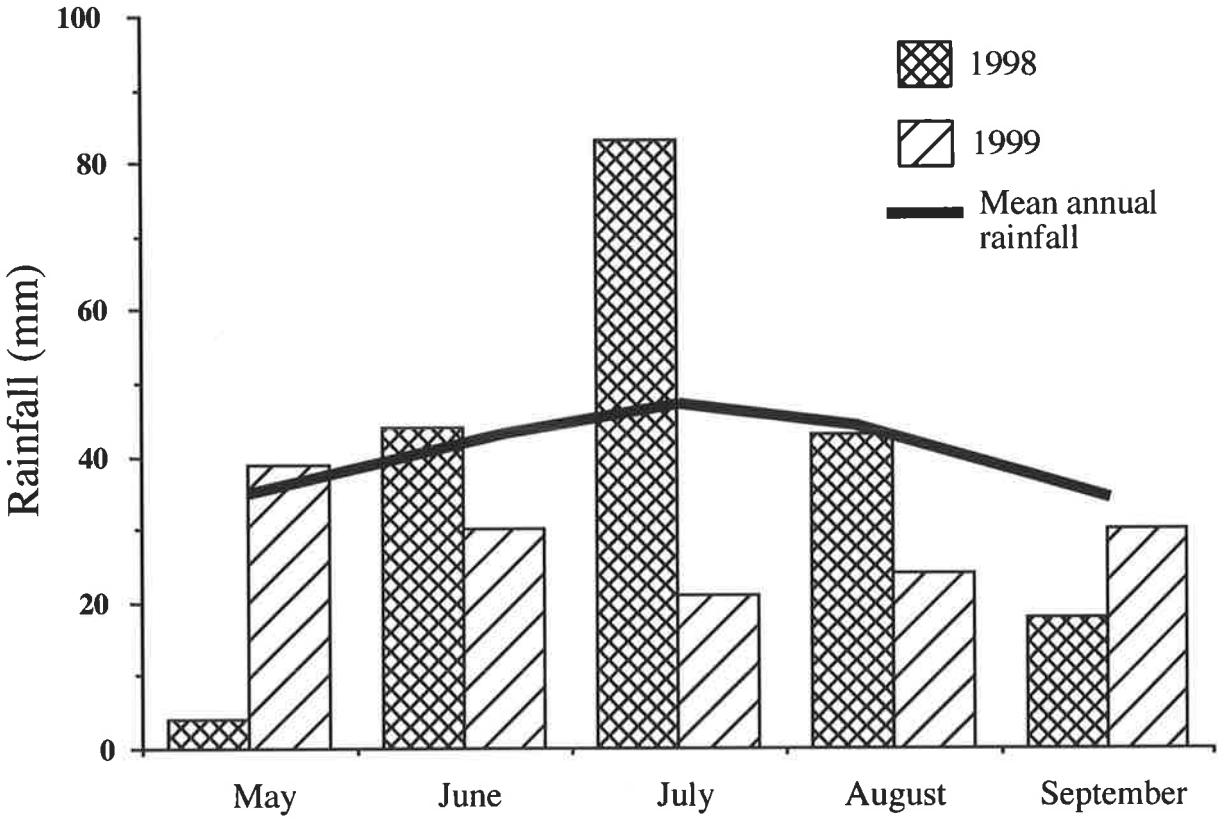



Figure 5.2. Monthly rainfall for the growing period extending from May through to September at Minnipa Research Centre, South Australia, in 1998 and 1999 and the mean annual rainfall (mm) at Minnipa.

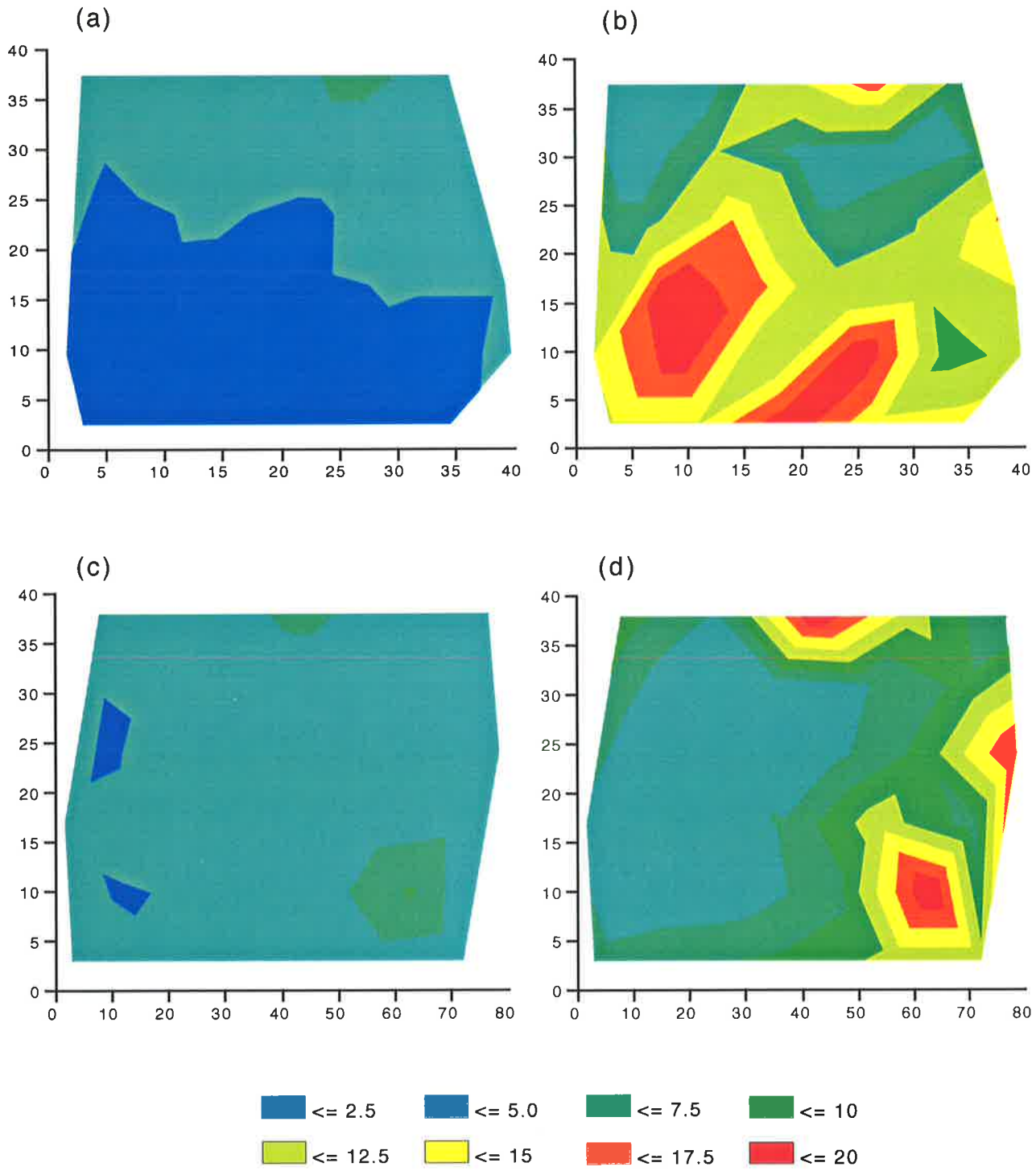



Figure 5.3. Contour map showing concentration of B (mg B kg-1) in soil samples taken from two depths in Sloop control plots in field experiments conducted at Minnipa, SA, in 1998 and 1999. (a) 5-15 cm in 1998, (b) 35-45 cm in 1998, (c) 5-15 cm in 1999, (d) 35-45 cm in 1999.

Table 1. Concentration (mg B $kg^{-1}$) of B (extracted in hot $CaCl_2$) in soil sampled from Sloop control plots in four field experiments at three sites in 1998 and 1999.

| Site | Year | Soil Profile (cm) | Mean (mg B $kg^{-1}$) | Standard deviation | Standard error of mean |
|---|---|---|---|---|---|
| Minnipa | 1998 | 5 - 15 | 2.43 | 0.57 | 0.10 |
| | | 35 - 45 | 10.55 | 5.38 | 0.97 |
| | 1999 | 5 - 15 | 3.46 | 0.91 | 0.17 |
| | | 35 - 45 | 7.83 | 5.21 | 0.99 |
| Pinery | 1999 | 5 - 15 | 1.22 | 0.21 | 0.07 |
| | | 35 - 45 | 2.32 | 1.04 | 0.33 |
| Horsham | 1999 | 5 - 15 | 1.44 | 0.29 | 0.06 |
| | | 35 - 45 | 1.84 | 0.79 | 0.15 |
| | | 45-100 | 9.14 | 4.94 | 0.94 |

### 5.3.2 Effect of B on grain yield and grain size of Sloop control plots

No B toxicity symptoms were observed in Sloop control plots grown at Pinery or Horsham in 1999. No significant relationship was identified between the concentration of B in soil and the grain yield or proportion of plump grain of Sloop control plots at either site. In contrast, B toxicity symptoms were observed in Sloop control plots in both years at Minnipa and ranged from moderate to severe in 1998 and from moderately low to moderately severe in 1999. Similarly, the concentration of B in whole shoots varied considerably and ranged from 14.4 to 57.0 mg B $kg^{-1}$ in 1998 and from 15.8 to 83.3 mg B $kg^{-1}$ in 1999. The concentration of B in soil and whole shoots was strongly ($P<0.001$) associated with B toxicity symptom severity (positive), grain yield (negative) and proportion of plump grain (negative) in both years (Table 2). Multiple regression analysis revealed that soil and whole shoot B levels together accounted for 57% and 61% of variation in grain yield and grain plumpness respectively in 1998, and 39% and 69% of variation in grain yield and grain plumpness respectively in 1999.

Table 2. Correlation matrix ($r^2$) for variables measured on Sloop control plots in field experiments conducted at Minnipa in 1998 and 1999.

| | Year | Grain Yield | Percent plump grain | B toxicity symptom score | Concentration of B in whole shoots | Concentrati of B in soi (5-15 cm) |
|---|---|---|---|---|---|---|
| Percent plump grain | 98 | 0.42 *** | | | | |
| | 99 | 0.48 *** | | | | |
| B toxicity symptom score | 98 | 0.17 * | 0.35 *** | | | |
| | 99 | 0.26 ** | 0.70 *** | | | |
| Concentration of B in whole shoots | 98 | 0.37 *** | 0.42 *** | 0.32 *** | | |
| | 99 | 0.16 * | 0.51 *** | 0.51 *** | | |
| Concentration of B in soil (5-15 cm) | 98 | 0.54 *** | 0.52 *** | 0.45 *** | 0.39 *** | |
| | 99 | 0.26 ** | 0.29 ** | 0.32 ** | NS | |
| Concentration of B in soil (35-45 cm) | 98 | 0.24 ** | 0.33 *** | 0.36 *** | 0.39 *** | 0.33 *** |
| | 99 | 0.26 ** | 0.40 *** | 0.35 *** | NS | 0.67 *** |

NS: not significant: *: P<0.05 **: P<0.01 *** P<0.001

### 5.3.3 Relationships between B toxicity symptoms, B concentration in whole shoots, and grain yield and grain plumpness of Sloop and VB9104 backcross derived lines

Regression analysis revealed a significant positive relationship between the concentration of B in whole shoots and B toxicity symptom severity scores in Sloop (Table 3) and VB9104 ($r^2$=0.46, P<0.01) derived backcross lines in 1998 and 1999 respectively. No significant relationship was established however, between the grain yield of Sloop backcross derived lines and symptom score and only a weak ($r^2$=0.09, P<0.05) relationship was established between grain yield and concentration of B in 1999 only (Table 3). Significant negative relationships were established between adjusted grain yield of VB9104 derived lines and both B toxicity symptom score ($r^2$=0.28, P<0.05) and concentration of B in whole shoots ($r^2$=0.25, P<0.05). In contrast to grain yield relationships, the proportion of plump grain was significantly related to B toxicity symptom score and to concentration of B in whole shoots in 1998 and 1999 in the Sloop derived lines (Table 3). There was no significant relationship between grain plumpness of VB9104 derived lines and whole shoot B concentration or B toxicity symptoms.

Table 3. Correlation matrix ($r^2$) for variables measured on Sloop backcross derived lines grown at Minnipa in 1998 and 1999.

| | Year | Grain Yield | Adjusted grain yield | Percent plump grain | B toxicity symptom severity score |
|---|---|---|---|---|---|
| Adjusted grain yield | 98 | 0.85 *** | | | |
| | 99 | 0.66 *** | | | |
| Percent plump grain | 98 | NS | NS | | |
| | 99 | NS | NS | | |
| B toxicity symptom | 98 | NS | NS | 0.23 *** | |
| severity score | 99 | NS | NS | 0.18 *** | |
| Concentration of B in | 98 | NS | NS | 0.12 ** | 0.10 * |
| whole shoots | 99 | NS | 0.09 * | 0.32 *** | NS |

NS: not significant: *: P<0.05 **: P<0.01 *** P<0.001

### 5.3.4 Effect of marker alleles at B tolerance loci on Sloop and VB9104 backcross derived lines

Presence of B tolerance donor parent marker alleles at chromosome 2H and 4H loci were associated with a significant reduction in severity of leaf symptoms in both Sloop and VB9104 derived lines (Tables 4-6). Donor parent marker alleles at the chromosome 4H locus were significantly associated with both lower concentrations of B in whole shoots and improved relative root length in Sloop and VB9104 derived lines (Table 4, Table 5 and Table 7). DH31 marker alleles at the 6H locus were significantly associated with lower concentration of B in whole shoots of Sloop derived lines in 1999 only (Table 4).

Analysis of data from both the Sloop and VB9104 derived lines at the Horsham and Pinery, non-B toxic sites, revealed significant genetic variation for grain yield. The genetic correlation for grain yield between the Horsham and Pinery sites was high at $r^2$=0.69 (P<0.001). DH31 marker alleles at the chromosome 4H locus were associated with seven percent lower grain yield at the Horsham site, 11% lower yield at the Pinery site and 69 kg/ha lower yield over both sites (Table 8). Grain yield data from Minnipa was adjusted for variation in grain yield at Pinery and Horsham (common effect) so that individual B toxicity response locus effects could be studied independent of other genetic factors relating to grain yield.

Table 4. Mean effect of marker alleles at B response loci on leaf symptoms and concentration of B in whole shoots at Minnipa and relative root length in filter paper assay of Sloop backcross derived lines in 1998 and 1999.

| B response locus | Year | Marker alleles | Leaf symptom score (1-9)[1] | | Concentration of B in whole shoots mg B $kg^{-1}$ | | Relative root length (%) | |
|---|---|---|---|---|---|---|---|---|
| | | | Mean | Standard error | Mean | Standard error | Mean | Standard error |
| 2H | 1998 | Sloop | 6.2 a | 0.2 | 27.3 a | 1.6 | 49.9 a | 1.9 |
| | | DH31 | 3.8 b | 0.3 | 31.0 a | 1.8 | 53.5 a | 2.2 |
| | 1999 | Sloop | 4.5 a | 0.2 | 41.1 a | 1.0 | 41.5 a | 1.7 |
| | | DH31 | 3.6 b | 0.2 | 43.0 a | 1.2 | 42.4 a | 1.9 |
| 4H | 1998 | Sloop | 5.4 a | 0.3 | 33.4 a | 1.2 | 47.8 a | 1.6 |
| | | DH31 | 4.9 a | 0.4 | 20.7 b | 1.6 | 58.9 b | 2.2 |
| | 1999 | Sloop | 4.3 a | 0.2 | 45.8 a | 0.7 | 38.8 a | 1.5 |
| | | DH31 | 3.8 b | 0.2 | 36.3 b | 0.9 | 46.4 b | 1.9 |
| 6H | 1998 | Sloop | 5.3 a | 0.4 | 30.2 a | 1.8 | 47.9 a | 2.3 |
| *(Amy-1)* | | Het. | 5.2 a | 0.6 | 29.0 a | 1.7 | 53.1 a | 2.0 |
| | | DH31 | 4.9 a | 0.3 | 25.7 a | 3.0 | 52.1 a | 3.4 |
| | 1999 | Sloop | 4.4 a | 0.2 | 43.6 a | 1.3 | 40.3 a | 2.2 |
| | | Heterozygote | 4.3 a | 0.3 | 41.8 a | 1.0 | 42.9 a | 3.6 |
| | | DH31 | 3.9 a | 0.2 | 36.8 b | 2.0 | 41.9 a | 1.7 |

[1] mean of leaf symptom score (1-9 scale; 1 = no symptoms, 9 > 90% leaf necrosis)

[2] means with different letters are significantly different at $P<0.01$, comparisons within years and class groups only

Table 5. Mean effect of marker alleles at B response loci on leaf symptoms and concentration of B in whole shoots at Minnipa and relative root length in filter paper assay of VB9104 backcross derived lines in 1999.

| B response locus | Marker alleles | Leaf symptom score (1-9)[1] | | Concentration of B in whole shoots mg B $kg^{-1}$ | | Relative root length (%) | |
|---|---|---|---|---|---|---|---|
| | | Mean | SE | Mean | SE | Mean | SE |
| 2H | VB9104 | 5.6 a | 0.3 | 39.9 a | 1.6 | 37.8 a | 3.0 |
| | VB9743 | 3.2 b | 0.8 | 35.5 a | 4.4 | 43.5 a | 8.6 |
| 4H | VB9104 | 6.5 a | 0.3 | 45.3 a | 1.4 | 29.0 a | 3.2 |
| | VB9743 | 4.4 b | 0.3 | 34.8 b | 1.2 | 46.1 b | 2.7 |
| 2H/4H | VB9104/VB9104 | 6.5 a | 0.2 | 45.3 a | 1.5 | 29.0 a | 3.3 |
| | VB9104/VB9743 | 4.7 b | 0.2 | 34.6 b | 1.4 | 46.8 b | 3.1 |
| | VB9743/VB9104 | - | - | | | | |
| | VB9743/VB9743 | 3.2 c | 0.5 | 35.4 b | 2.8 | 43.5 b | 6.2 |

[1] mean of leaf symptom score (1-9 scale; 1 = no symptoms, 9 > 90% leaf necrosis)

[2] means with different letters are significantly different at P<0.001, comparisons within genotype class groups only

Table 6. Combined effect of marker alleles at the 2H and 4H B response loci on mean B toxicity leaf symptom score on Sloop backcross derived lines at Minnipa in 1998 and 1999.

| Year | Marker alleles (2H/4H) | Leaf symptom score (1-9)[1] | |
|---|---|---|---|
| | | Mean | Standard error |
| 1998 | Sloop/Sloop | 6.6 a[2] | 0.3 |
| | Sloop/DH31 | 4.1 b | 0.3 |
| | DH31/Sloop | 5.5 c | 0.3 |
| | DH31/DH31 | 3.4 b | 0.5 |
| 1999 | Sloop/Sloop | 4.9 a | 0.2 |
| | Sloop/DH31 | 3.8 b | 0.2 |
| | DH31/Sloop | 3.9 b | 0.2 |
| | DH31/DH31 | 3.3 b | 0.3 |

[1] mean of leaf symptom score (1-9 scale; 1 = no symptoms, 9 > 90% leaf necrosis)

[2] means with different letters are significantly different at P<0.01, comparisons within years only

Table 7. Combined effect of marker alleles at the 4H and 6H B response loci on the mean concentration of B in whole shoots in Sloop backcross derived lines at Minnipa in 1998- 99.

| Year | Marker alleles (4H/6H) | Concentration of B in whole shoots (mg B $kg^{-1}$) | |
|---|---|---|---|
| | | Mean | Standard error |
| 1998 | Sloop/Sloop | 34.2 a[1] | 2.1 |
| | Sloop/DH31 | 19.8 b | 3.0 |
| | DH31/Sloop | 31.2 a | 3.3 |
| | DH31/DH31 | 18.8 b | 3.7 |
| | DH31/Heterozygote | 22.1 b | 2.6 |
| | Heterozygote/DH31 | 31.9 a | 1.8 |
| 1999 | Sloop/Sloop | 47.8 a | 1.2 |
| | Sloop/DH31 | 36.2 b | 1.7 |
| | DH31/Sloop | 40.0 bc | 2.2 |
| | DH31/DH31 | 34.3 b | 2.0 |
| | DH31/Heterozygote | 36.7 b | 1.3 |
| | Heterozygote/DH31 | 44.7 c | 0.9 |

[1] means with different letters are significantly different at P<0.01, comparisons within years only

Table 8. Mean effect of marker alleles at B response loci on grain yield of Sloop backcross derived lines grown at two non-B toxic sites in 1999.

| B response locus | Marker alleles | Grain yield (t/ha) at Horsham | | Grain yield (t/ha) at Pinery | | Common genotypic effect (kg/ha) | |
|---|---|---|---|---|---|---|---|
| | | Mean | Standard error | Mean | Standard error | Mean | Standard error |
| 2H | Sloop | 1.87 a | 0.02 | 0.44 a | 0.01 | -3.2 a | 11 |
| | DH31 | 1.91 a | 0.03 | 0.46 a | 0.01 | 10.8 a | 13 |
| | VB9104 | 1.92 a | 0.05 | 0.44 a | 0.01 | 0.1 a | 19 |
| | VB9743 | 1.90 a | 0.13 | 0.43 a | 0.03 | -7.5 a | 54 |
| 4H | Sloop | 1.94 a | 0.02 | 0.47 a | 0.01 | 28.8 a | 11 |
| | DH31 | 1.81 b | 0.03 | 0.42 b | 0.01 | -40.3 b | 13 |
| | VB9104 | 2.01 a | 0.06 | 0.45 a | 0.02 | 31.9 a | 25 |
| | VB9743 | 1.86 a | 0.05 | 0.43 a | 0.01 | -27.6 a | 22 |
| 6H *(Amy-1)* | Sloop | 1.91 a | 0.03 | 0.45 a | 0.01 | 7.8 a | 16 |
| | Het. | 1.86 a | 0.05 | 0.44 a | 0.02 | -10.9 a | 24 |
| | DH31 | 1.88 a | 0.02 | 0.45 a | 0.01 | -2.6 a | 13 |

[1] means with different letters are significantly different at P<0.01, comparisons within backcross populations and class groups only

Despite substantial variation for symptom expression and observed plant growth, particularly in 1998 (not measured), there were no significant B response locus effects on unadjusted grain yield of backcross lines derived from either Sloop or VB9104. There were also no significant chromosome 2H locus effects on adjusted grain yield in 1998. B tolerant donor parent marker alleles at the chromosome 4H locus, however, were related to 8% higher adjusted grain yield in both the Sloop and VB9104 derived lines in 1999 (Table 9 and Table 11). The chromosome 2H locus was associated with 14% higher adjusted grain yield in the VB9104 lines, however, results on effects of chromosome 2H locus in this population need to be treated with care as the VB9743 marker allele class consisted of only two individuals.

Table 9. Mean effect of marker alleles at B response loci on adjusted grain yield and proportion of plump grain of Sloop backcross derived lines at Minnipa in 1998 and 1999.

| B response locus | Year | Marker alleles | Adjusted grain yield (kg/ha) | | Percent plump grain (%>2.5 mm) | |
|---|---|---|---|---|---|---|
| | | | Mean | Standard error | Mean | Standard error |
| 2H | 1998 | Sloop | 1155 a | 24 | 59.1 a | 1.7 |
| | | DH31 | 1148 a | 26 | 65.9 b | 1.9 |
| | 1999 | Sloop | 894 a | 16 | 42.3 a | 1.6 |
| | | DH31 | 871 a | 19 | 46.5 a | 1.9 |
| 4H | 1998 | Sloop | 1151 a | 20 | 60.4 a | 1.6 |
| | | DH31 | 1221 a | 29 | 66.6 b | 2.2 |
| | 1999 | Sloop | 852 a | 16 | 40.3 a | 1.5 |
| | | DH31 | 924 b | 21 | 50.7 b | 1.9 |
| 6H | 1998 | Sloop | 1116 a | 28 | 41.0 a | 2.2 |
| *(Amy-1)* | | Het. | 1128 a | 43 | 46.1 a | 3.4 |
| | | DH31 | 1186 a | 24 | 46.3 a | 1.7 |
| | 1999 | Sloop | 877 a | 21 | 59.7 a | 2.1 |
| | | Het. | 913 a | 32 | 64.4 a | 3.4 |
| | | DH31 | 875 a | 17 | 65.4 a | 1.8 |

[1] means with different letters are significantly different at $P<0.01$, comparisons within years and class groups only

Marker alleles from the B tolerant donor parent at the chromosome 2H locus were strongly associated with improved grain plumpness in the Sloop derived lines in 1998 and the VB9104 derived lines in 1999 (Table 8, Table 9, and Table 10). Marker alleles from the B tolerant donor parent at the chromosome 4H locus were associated with improved grain

plumpness in the Sloop derived lines in 1998 and 1999 but not in the VB9104 derived lines in 1999.

Table 10. Combined effect of marker alleles at the 2H and 4H B response loci on percent of plump grain of Sloop backcross derived lines at Minnipa in 1998 and 1999.

| Year | Marker alleles (2H/4H) | Percent of plump grain (%>2.5 mm) | |
|---|---|---|---|
| | | Mean | Standard error |
| 1998 | Sloop/Sloop | 54.1 a[1] | 2.2 |
| | Sloop/DH31 | 65.2 b | 2.1 |
| | DH31/Sloop | 63.3 b | 2.5 |
| | DH31/DH31 | 68.8 b | 4.2 |
| 1999 | Sloop/Sloop | 36.6 a | 2.1 |
| | Sloop/DH31 | 43.2 b | 2.0 |
| | DH31/Sloop | 48.6 bc | 2.1 |
| | DH31/DH31 | 53.5 c | 3.4 |

[1] means with different letters are significantly different at P<0.01, comparisons within years only

Table 11. Mean effect of marker alleles at B response loci on leaf symptoms and concentration of B in whole shoots at Minnipa and relative root length in filter paper assay of VB9104 backcross derived lines in 1999.

| B response locus | Marker alleles | Adjusted grain yield (kg/ha) | | Percent plump grain (%>2.5 mm) | |
|---|---|---|---|---|---|
| | | Mean | Standard error | Mean | Standard error |
| 2H | VB9104 | 1028 a | 19 | 57.7 a | 1.4 |
| | VB9743 | 1173 b | 54 | 67.6 b | 4.1 |
| 4H | VB9104 | 994 a | 30 | 56.6 a | 2.4 |
| | VB9743 | 1076 b | 24 | 60.2 a | 1.9 |
| 2H/4H | VB9104/VB9104 | 994 a | 27 | 56.6 a | 2.2 |
| | VB9104/VB9743 | 1054 a | 24 | 58.3 a | 2.0 |
| | VB9743/VB9104 | - | - | - | - |
| | VB9743/VB9743 | 1173 a | 51 | 67.6 a | 4.2 |

[1] mean of leaf symptom score (1-9 scale; 1 = no symptoms, 9 > 90% leaf necrosis)

[2] means with different letters are significantly different at P<0.01, comparisons within genotype class groups only

The estimated mean number of grains/plot in Sloop derived lines in 1998 was positively related to grain yield ($r^2$= 0.61, P<0.001) and inversely related to grain plumpness ($r^2$=0.39, P<0.001). DH31 marker alleles at the chromosome 2H and 4H loci individually had no significant effect but in combination the two loci contributed to a 19% reduction (P<0.01) in mean number of grains/plot (Table 12).

Table 12. Combined effect of genotype at the 2H and 4H B response loci on the mean number of grain per plot in Sloop backcross derived lines at Minnipa in 1998.

| Marker alleles (2H/4H) | Number of grain per plot | |
|---|---|---|
| | Mean | Standard error |
| Sloop/Sloop | 42840 a[1] | 1259 |
| Sloop/DH31 | 41123 ab | 1259 |
| DH31/Sloop | 40706 ab | 1565 |
| DH31/DH31 | 34575 c | 2322 |

[1] means with different letters are significantly different at P<0.01, comparisons within years only

## 5.4 Discussion

Jefferies *et al.* (1999, and this thesis) used interval regression mapping techniques to detect four chromosomal regions involved in plant response to B toxicity. They identified a region on chromosome 2H associated with severity of leaf symptoms, a region on chromosome 3H associated with root length response, and regions on chromosome 4H and 6H associated with reduced accumulation of B in plant tissue. The region on chromosome 4H was also associated with root length response, leaf symptom expression and dry matter production.

Only markers at the 2H and 4H loci were used in the development of the Sloop backcross derived lines. Two of the 62 lines selected were found to carry the DH31 allele for *Xawbma15* at the chromosome 3H locus while Sahara marker alleles at the chromosome 3H locus could not be found in VB9743. The small number of lines carrying Sahara marker alleles at the 3H locus precluded field testing for chromosome 3H locus effects.

Results presented in Table 4, 5, 6 and 7 showed that chromosomal regions derived from Sahara, conferring reduced leaf symptom severity (2H and 4H), reduced accumulation of B in whole shoots (4H and 6H) and reduced effects on root growth (4H) were successfully introgressed and expressed in both Sloop and VB9104 genetic backgrounds. Discussion on the relationships between these isolated plant responses and the quantitative and environmentally sensitive plant response of grain yield and grain size follows.

Moderate to severe B toxicity symptoms and relatively high concentrations of B in tissue were observed in Sloop plants grown in control plots in both 1998 and 1999. Concentration of B in whole shoots and severity of leaf symptoms were strongly related to both grain yield and grain plumpness of Sloop (Table 2). These results provided evidence that B toxicity was limiting grain yield and grain size at Minnipa in both years.

### 5.4.1 Chromosome 6H locus effects

Mapping experiments of Jefferies *et al.* (1999, and this thesis) showed the chromosome 6H locus to be associated with accumulation of B in whole shoots only. They proposed that the chromosome 4H and 6H loci were additive in their effect with the 4H locus having, by far, the major role. At Minnipa in 1999, Sloop backcross derived lines carrying DH31 marker alleles at the 6H locus, on average, contained five percent ($P<0.01$) lower concentration of B in whole shoots than lines carrying the Sloop marker allele (Table 4). In contrast, lines carrying the DH31 allele at the 4H locus contained 9.5% lower concentrations in 1999 and 12.7% lower in 1998. There was no significant effect of DH31 marker alleles at the 6H locus on grain yield or grain plumpness at Minnipa in either 1998 or 1999 (Table 9). The 6H locus, therefore, appears to be associated with only minor effects on B accumulation in tissue which, in turn, were not measurable in their effect on grain yield or grain plumpness.

### 5.4.2 Chromosome 2H and 4H locus effects

Considerable genetic variation in the expression B toxicity symptoms and vegetative growth (not measured) was observed, particularly in 1998. While not measured in this study in 1998, the early (pre-anthesis) vegetative growth of Sloop backcross derived lines showing low levels of symptoms appeared to be far superior to those lines with severe symptoms. Surprisingly, no evidence was found of improved grain yield associated with loci involved in the control of both B accumulation (4H) and leaf symptom expression (2H and 4H) (Table 10). DH31 marker alleles at both the 2H and 4H loci were, however, associated with increases in grain plumpness (Table 9 and 10).

Fischer (1983) reported that the total number of florets initiated (tiller number x spikelet number x floret number) per unit area in a wheat crop is generally much greater than the final number remaining at anthesis. The potential grain number is, therefore, never realized because of competition for resources, including water. With the exception of B toxicity, there appeared to be few limitations to plant growth in early stages of development at Minnipa in 1998. This is likely to have contributed to plants, particularly those less affected by B toxicity, establishing high yield potential through the production of a large number of floret primordia. Grain size, however, is widely acknowledged as a major yield component of cereals, yet in 1998 at Minnipa, grain weight and grain yield of Sloop backcross derived

lines were inversely related ($P<0.01$). In addition, the number of grain per unit area in Sloop derived lines carrying DH31 marker alleles at both the 2H and 4H loci was, on average, 19% lower than those carrying the Sloop allele at both these loci. Both moisture stress and high temperature have been shown to reduce the number of competent florets (those with complete floral parts at anthesis) per unit area (Fischer, 1983). Severe moisture stress and above average temperatures encountered during stem elongation and early anthesis, in September of 1998 (Fig 5.2), is likely to have caused abortion of both spikelet and floret primordia. The more vegetative B tolerant lines may have been affected to a greater extent, and this was reflected in a significant reduction in the number of competent florets set. The lower number of competent florets per unit area and the greater photosynthetic capacity (less leaf necrosis) of lines carrying DH31 marker alleles may, through yield compensation effects (Evans, 1993), account for the similar grain yield but superior grain size of these lines compared with lines carrying the Sloop marker alleles.

In 1999, the chromosome 2H locus was associated with a 14% increase in adjusted grain yield in the VB9104 derived lines while the chromosome 4H locus was associated with a 8% increase in adjusted grain yield in both the VB9104 and Sloop backcross derived lines. Significant increases in grain plumpness were associated with B tolerant donor parent marker alleles at the chromosome 2H locus in VB9104 derived lines and at the chromosome 4H locus in both Sloop and VB9104 derived lines. The chromosome 2H-VB9743 class consisted of only two lines, therefore the results from the VB9104 derived lines for the 2H locus must be treated with caution. There was less visual evidence (not measured) of genetic variation for early vegetative growth amongst backcross derived lines in the very dry 1999 season.

A theory has been proposed to explain the lack of yield response associated with B tolerance QTL in 1998. While significant differences in adjusted grain yield and grain plumpness associated with B tolerance QTL were recorded in 1999, the differences were also less than expected. The chromosome 4H locus has been shown to be associated with improved root growth. This coupled with reduced accumulation of B tissue has been proposed as a major advantage to plants grown in low rainfall areas prone to B toxicity. It has been proposed that B tolerant genotypes would be capable of extending their root system further into sub-soils providing greater access to sub-soil moisture and nutrient reserves than would otherwise be possible due to the root growth inhibiting effects of the toxic concentrations of B in the sub-soil. Soil cores taken close to anthesis at Minnipa in both 1998 and 1999 revealed negligible reserves of soil moisture beyond 10 cm in the profile (data not shown). B tolerant genotypes with greater root extension capacity were at no advantage as there was no significant sub-soil moisture reserves in either year.

Relatively high salinity levels have been associated with B toxic sub-soils in the Minnipa area (Holloway, 1991). It has also been proposed that B tolerant genotypes, which extend

root systems into B toxic sub-soils, are more likely to take up toxic concentrations of sodium. No significant ($P<0.01$) relationship was identified between the concentration of sodium in whole shoots and the concentration of B in whole shoots or grain yield or grain plumpness at Minnipa in either backcross population in either year (data not shown).

### 5.4.3 B tolerance mechanisms as conferred by chromosome 2H and 4H loci

It has been shown that there is no difference between roots and shoots of barley and wheat in their accumulation of B (Nable, 1991). The concentration in leaves, however, increases from young to old leaves and from base to tips (Nable *et al.*, 1990a) and is related to transpiration rate (Bowen, 1972). A similar pattern is followed in the development of toxicity symptoms in both barley and wheat. The chlorotic/necrotic patches found on the leaves of some crop species have been shown to contain greatly elevated concentrations of B compared with surrounding leaf tissue (Oertli and Roth, 1969). This pattern of B translocation and symptom development suggests that B is relatively immobile in barley and wheat. It is likely therefore that the chromosome 2H locus is involved in the control of B translocation, particularly since its control is genetically independent of uptake or total plant accumulation (Jefferies *et al.*, 1999; and this thesis). This locus could be inhibiting translocation of B to leaf tissue, increasing B mobility in plant tissue, or internally complexing B. Nable (1991) showed that the pattern of B distribution within tolerant and intolerant genotypes was very similar and independent of total B absorbed. It is more likely, therefore, that the locus is involved in increasing the mobility of B or complexing B in plant tissue. Recent analysis of celery phloem sap and vascular exudate has shown that the majority of B is complexed to the ligands mannitol, sorbitol and fructose (Hu *et al.*, 1997). While little is know about the relative concentration of polyols in barley and their relationship to environmental factors, it is possible that the chromosome 2H locus in barley is involved in the control of concentration and/or movement of B binding sugars such as fructose. Brown *et al.* (1999) reported on transgenically enhanced sorbitol synthesis in tobacco. They found that an increased concentration of sorbitol led to improved phloem B mobility and therefore, tolerance to B deficiency in tobacco. Increased mobility of B in barley, in a B toxic situation, may result in increased concentration of B in more actively growing tissue, potentially creating greater constraint to growth and productivity than effects associated with loss of photosynthetic capacity due to leaf damage. This warrants further investigation.

Nable *et al.* (1990b) showed that B uptake by barley genotypes which differ in their tolerance to B toxicity was directly related to B supply over a range of B concentrations from normal to excessive (1-1000 μM B). The authors proposed passive absorption of B, although genotypes differed in their rates of accumulation. Huang and Graham (1990) who studied B uptake in wheat callus supported these results. In contrast, Bowen and Nissen

(1977) proposed an active uptake mechanism in barley. While the mechanisms underlying differences in B uptake between genotypes, are unknown, Hu and Brown (1997) have put forward a number of theories. They include; (1) differences in the control of an active exclusion mechanism; (2) differences in the control of a root exudation of B complexing compounds restricting B uptake; (3) differences in physical barriers within root cell walls and (4) inherent differences in membrane permeability. This also warrants further investigation.

Studies and theories on B tolerance mechanisms in wheat and barley have, until recently, focussed entirely on B exclusion mechanisms (Nable *et al.*, 1997). This is largely due to the fact that the major known B tolerance sources in barley (Sahara) and wheat (Halberd) carry loci involved in both reduced B accumulation and reduced symptoms (Jefferies *et al.*, 1999, and this thesis; Jefferies *et al.*,2000) and consequently researchers have failed to consider the mechanisms independently. Molecular markers can now be used to facilitate detailed investigations on the independent function of QTL associated with plant response to B toxicity not possible before.

### 5.4.4 Deleterious grain yield factors associated with B tolerance loci

There was no evidence of deleterious grain yield factors associated with B tolerant donor parent marker alleles at the 2H and 6H loci (Table 8). In contrast, Sloop derived lines carrying the B tolerant donor parent alleles at the chromosome 4H locus were associated with reduced grain yield at Horsham and Pinery. The lower grain yield observed could be a function of linkage drag or pleitropic effects of a gene or genes, located on this segment, controlling the accumulation of B in plant tissue.

The mechanisms involved in the control of the accumulation of B in plants proposed earlier may come at some physiological or biochemical cost. The first two proposed mechanisms, active removal of B or the active exudation of a complexing compound, may come at a metabolic cost. The third and fourth proposed mechanisms, cell wall barriers and differential membrane permeability, could also affect the uptake of other elements and or water and could therefore come at a nutritional or water relations cost. Jefferies *et al.*. (2000) identified a region on chromosome 7B of the B tolerant wheat cultivar Halberd that is associated with identical plant responses to B toxicity to that of the chromosome 4H region of barley. Halberd, and related cultivars carrying Halberd marker alleles at the 7B locus, have dominated the area sown to wheat on both B toxic and non-toxic soils in southern Australia for some time (Paull, 1990; and Jefferies *et al*, 2000). Given the very similar function of the 7B locus in wheat and the 4H locus in barley and the dominance of wheat cultivars carrying Halberd alleles at the 7B locus in southern Australia, it is unlikely that the mechanism underlying reduced B accumulation come at a significant metabolic, nutritional or water use efficiency cost.

In the VB9014 derived lines, there were no significant differences for grain yield at Pinery and over both Pinery and Horsham (common effect). This provides some evidence that the consistently lower grain yield effects associated with the chromosome 4H locus in the Sloop backcross derived lines are a function of linkage drag. The estimated proportion of Sahara genome (based on pedigree) in the VB9743 donor parent is 25% compared to 50% in DH31. Stam and Zevens (1981) showed how the size of donor segment surround the introgression gene decreases with increasing number of backcrosses but estimated the average size of this segment to be 51 cM in a chromosome of 100 cm after three backcrosses. Brown *et al.* (1989a) supported these findings in barley using isozyme markers. Sahara chromosome segments around the 4H locus are therefore likely to be larger in the Sloop backcross derived lines than in the VB9104 derived lines as the later population is effectively derived from four rather than three exotic by adapted backcross equivalents. Deleterious factors located on large Sahara chromosome segments surrounding the 4H locus in the Sloop backcross derived lines may, therefore, be responsible for the grain yield differences observed.

### 5.4.5 Suitable agronomic backgrounds for B tolerance QTL in barley grown in dryland areas prone to terminal drought

B toxicity occurs regularly in areas of southern Australia prone to terminal drought stress. Excessive growth and abortion of floret primordia is typical of Sloop and other cultivars of similar phenology, when grown in areas prone to terminal drought. Recent evidence (Coventry, unpublished data) exists of genetic variation among southern Australian commercial cultivars for an ability to produce a lower number of floret primordia but a greater proportion of competent florets and therefore grain set. Cultivars with this type of phenology have improved general adaptation to areas prone to terminal drought than do cultivars with the Sloop type phenology. Cultivars, which combine early maturity, improved osmoregulation and have the ability to sustain a high proportion of floret primordia could be more suitable genetic backgrounds for the introgression of B tolerance QTL in southern Australia.

### 5.4.6 Implications for marker assisted selection in the breeding of B tolerant malting quality barley cultivars

Diastatic Power (DP) is an important parameter determining the suitability of barley cultivars for malting. DP is a measure of total amyolytic enzyme activity including two major enzyme components, α-amylase and β-amylase. Hayes *et al.* (1997) reported the identification of QTL involved in the control of α-amylase activity at a similar region as the B tolerance QTL on chromosome 6H (Jefferies *et al.*, 1999; and this thesis). β-amylase is

typically the largest component of DP. The *Bamy-2* structural gene, which has a major role in the control of β-amylase activity (Kreis *et al.*, 1987), and therefore DP, has been located to chromosome 2H (Kleinhofs *et al.*, 1993) within 5cM of the chromosome 2H B tolerance locus (Jefferies *et al.*, 1999; and this thesis). The chromosome 4H B tolerance locus has been mapped (Jefferies *et al.*, 1999; and this thesis; Langridge *et al.*, 1995) to within 30 cM(distal) of the *Bmy-1* structural gene also associated with β-amylase activity and with QTL for DP (Hayes *et al.*, 1997).

Sahara is a North African landrace that would not have been selected for malting quality. It is likely, therefore, that Sahara alleles at the *Amy-1* (6H), *Bmy-1*(4H) and *Bmy-2* (2H) loci would not confer high α-amylase and β-amylase activity. Several molecular markers located on the long arm of chromosome 4H could be efficiently used to select suitable recombinants combining alleles for high β-amylase activity from an alternative source and improved B tolerance from Sahara. However, QTL for high α-amylase activity on 6H and high β-amylase on 2H would be in repulsion. Given the minor role of the chromosome 6H B tolerance locus, demonstrated in this study, the most efficient breeding strategy would be to ignore B tolerance alleles at this locus and select for alleles conferring high α-amylase activity. Greater precision in the location of the chromosome 2H B tolerance locus in respect to the *Bmy-2* locus will be required to facilitate marker assisted selection for both B tolerance and high β-amylase activity. Alternatively additional sources of high β-amylase activity not associated with the *Bmy-2* locus could be identified and used in crosses aimed at producing B tolerant malting quality cultivars.

A major gene conferring the six row character in Sahara maps to within 33 cM of the B tolerance locus on the long arm of chromosome 2H (Langridge *et al.*, 1995). A major gene conferring resistance to cereal cyst nematode (*Heterodera avenae.*), an important cereal root pathogen in southern Australia and other cereal growing regions of the world (Meagher, 1977), was located to a region 13.7 cM distal of the two row/six row locus in the Clipper x Sahara mapping population (Kretschmer *et al.*, 1997). Marker assisted selection would greatly improve the efficiency of selecting recombinants, likely to be in very low frequency, that carry the chromosome 2H B tolerance and CCN resistance loci from Sahara, and the two row character from an alternative parent.

The chromosome 3H B tolerance locus, not assessed in this study, maps to within 3cM of the *Yd2* gene conferring resistance to barley yellow dwarf virus in barley (BYDV) (Jefferies *et al.* ; and this thesis; Paltridge *et al.*, 1998). Sahara is not likely to be resistant to BYDV (Paltridge *et al.*, 1998) and consequently *Yd2* will be in repulsion with the chromosome 3H B tolerance locus. BYDV is not an important constraint to barley production in areas where

B toxicity is common and therefore it would be more efficient to address these traits in separate breeding strategies.

It has been shown that marker assisted selection for loci conferring plant response to B toxicity can be effective in improving root growth in solution culture, reducing B toxicity symptoms and concentration of B accumulated in whole plants in the field, and in some situations, improving grain yield and grain size of barley grown on B toxic soils. The high degree of environmental interaction associated with grain yield response to B toxicity, discussed earlier, further highlights the advantages of marker assisted selection. In addition, the advantage of marker assisted selection in the introgression of genes/QTL from exotic germplasm, particularly in relation to reduction of deleterious linkage drag, is well documented (Young and Tanksley, 1989) and applies very much to the introgression of B tolerance QTL from Sahara. An improved understanding of the detailed function of genes/QTL involved in the B tolerance mechanism is a high priority for further research.

# Chapter 6

# Marker assisted selection for donor gene

## 6.1 Marker assisted backcross introgression of *Ha 2* conferring resistance to *Heterodera avenae* into a malting quality background

### 6.1.1 Introduction

The cereal cyst nematode (CCN), *Heterodera avenae* Woll., is a major cereal root pathogen, which is endemic throughout the important cereal-growing regions of the world (Meagher, 1977). Yield losses due to CCN have been reported to be as high as 30% in barley and up to 70% in some wheat and oat cultivars (Sparrow and Dube', 1981). CCN can be controlled with cropping sequences involving non-host crop species and with chemical nematicides (Brown and Pye, 1981). Chemical control is very expensive and the compounds used are generally very toxic. Crop rotations involving non-host crops are not always the most economical or feasible, particularly in low rainfall regions. The development of CCN resistant cultivars is therefore the most desirable control strategy.

Studies on the inheritance of resistance to CCN in barley have identified four major sources of resistance genes, *Ha 1*, *Ha 2*, *Ha 3*, all on chromosome 2H, and *Ha 4* on chromosome 5H (Cotton and Hayes 1969; Andersen and Andersen 1970; Kretschmer *et al.*, 1997; Barr *et al.*, 1998). *Ha 3* is allelic to *Ha 2* or closely linked (Cotton and Hayes 1969; Andersen and Andersen 1970). *Ha 1* and *Ha 2* segregate independently and have been used to breed resistant cultivars (Andersen and Andersen 1968). *Ha 1* confers a limited resistance against many of the European pathotypes but is ineffective against the single pathotype found in Australia (Andersen and Andersen 1982). *Ha 2* and *Ha 4* confer effective resistance to the Australian pathotype (Barr *et al.*, 1998). *Ha 4* is present in two related high yielding, broadly adapted Australian cultivars, Galleon and Barque, both possessing very poor malt quality characteristics. *Ha 2* is present in the Australian cultivar Chebec which is widely adapted and, while not accepted into malting grades in Australia, it possess relatively minor malt quality defects. The *Ha 2* gene in Chebec, therefore, is a more desirable donor parent of resistance in breeding strategies aimed at introgressing CCN resistance into malting quality cultivars.

Phenotypic selection for CCN resistance in barley can be undertaken in a relatively efficient manner using a bio-assay system described by Fisher (1982). Molecular markers, however, offer the following advantages over the bio-assay system; (1) screening can be undertaken at early stages of plant development (prior to flowering); (2) the genotype can be reliably identified on a single plant; (3) co-dominant markers such as restriction fragment length polymorphism (RFLPs) can distinguish homozygotes and heterozygotes; (4) plants to be assayed can be grown in optimum conditions suitable for crossing or maximum seed yield; and (5) plants can be assayed for multiple traits simultaneously.

*Ha 2* was mapped in two doubled haploid populations to a region of chromosome 2HL flanked by the RFLP markers *Xawbma21* and *Xmwg694* (Kretschmer *et al.* 1997). The identification of molecular markers linked to the *Ha 2* locus now provides the opportunity for marker assisted selection for CCN resistance in barley. The first aim of this study is to utilise a practical gene introgression breeding strategy to demonstrate and assess the application of molecular markers in the backcross introgression of a single major gene *Ha 2* and confirm the expression of this gene in an alternative genetic background. The second aim is to use molecular markers to assist in determining the extent to which additional agronomic and quality factors are associated with the *Ha 2* gene derived from Chebec. As a consequence, the potential of producing a malting quality CCN resistant variety will be determined.

### 6.1.2 Materials and methods

#### *6.1.2.1 Genetic materials and population development*

This study involved two components integrating data derived from a breeding population developed as part of a practical *Ha 2* introgression program and a population developed specifically to test the extent to which agronomic and quality factors are associated with the *Ha 2* gene derived from Chebec. Chebec is widely adapted to southern Australian barley growing districts and is of marginal malting quality, in particular, it produces lower diastatic power than required by the malting and brewing industry. The recurrent parent was the widely adapted Australian malting quality cultivar Sloop. Sloop is CCN susceptible but has malt quality advantages over Chebec including superior diastatic power, β-amylase activity, viscosity and free amino nitrogen (S Logue, unpublished data).

(1) marker assisted backcross introgression of *Ha 2*

A population of 121 doubled haploid lines derived from three cycles of marker assisted backcrossing between the donor parent Chebec and the recurrent parent Sloop were used to demonstrate and assess the application of molecular markers for the backcross introgression of *Ha 2*. The RFLP marker, *Xawbma21,* mapped within 3 cM of *Ha 2* (Kretschmer *et al.*, 1997), was used to select $BC_1F_1$ individuals carrying the Chebec marker allele. Individuals heterozygous for the marker allele were backcrossed to Sloop to form the $BC_2F_1$ population, which was in turn screened with *Xawbma21* to select individuals for further backcrossing. Approximately 20 $BC_3F_1$ $F_1$ individuals were screened with *Xawbma21* at each backcross stage. At least four $F_1$ plants carrying the Chebec marker allele were backcrossed to Sloop at the second and third backcross stages. $BC_3F_1$ individuals were screened with *Xawbma21* to select doubled haploid donor plants. A total of 121 $BC_3$ fertile doubled haploid plants were produced by Dr P.Davies (SARDI) using the anther-culture method (Finnie *et al.*, 1989).

(2) Malt quality and agronomic factors associated with *Ha 2* derived from Chebec

Thirty six $BC_1$ $F_1$ individuals were produced from backcrosses between the donor parent Chebec and the recurrent parent Sloop. Twenty four $BC_1F_1$ plants were allowed to self to form the $BC_1F_2$ population. DNA from 48 $BC_1$ $F_2$ single plants was probed with *Xawbma21*. Ten plants homozygous for the Chebec marker allele and 10 plants homozygous for the Sloop marker allele were selected for seed multiplication. The underlying principle of bulked segregant analysis (Michelmore *et al.*, 1991), that is, the grouping of individuals into two pools so that a particular genomic region can be studied against a randomised genetic background of unlinked loci, was used to determine the approach and population size for this study.

*6.1.2.2 CCN Bio-assay*

All genotypes used in component (1) of this study were scored for their resistance to *Heterodera avenae* using the bio-assay described by Fisher (1982). Ten seedlings of each genotype were planted in 3 cm tubes filled with sterile soil and inoculated five times at 3 day intervals with 100 second-stage juveniles. After 12 weeks at 15 °C the number of cysts formed on the outside of the root mass of each plant were counted. Doubled haploid lines were separated into two classes, resistant and moderately susceptible to susceptible. Lines with a mean number of cysts per plant of less than 1.0 were rated as resistant while lines with greater than 1.0 cyst per plant were rated as moderately susceptible to susceptible.

*6.1.2.3 DNA extraction, restriction endonuclease digestion and Southern hybridisation*

DNA extraction was achieved using a DNA mini-prep method adapted from Rogowsky *et al.* (1991). Variations to the method were as described below. For the initial extraction, 700 µl of extraction buffer and phenol-chloroform-isoamyl alcohol (25:24:1) were used. The extraction buffer was 0.1 M Tris-HCl (pH 8.5),10 m*M* EDTA, 0.1 *M* NaCl, 1% sarkosyl and 2% polyvinyl-polypyrrolidone (insoluble). After the second phenol-chloroform-isoamyl alcohol extraction the aqueous phase was extracted once with an equal volume of chloroform. DNA was precipitated by the addition of 60 µl of sodium acetate (pH 4.8) and 600 µl propan-2-ol. Restriction endonuclease digestion and Southern hybridisation followed standard methods.

*6.1.2.4 Flanking marker*

*Ha 2* was located 2.9 cM distal to *Xawbma21* and 5.2 cM proximal to *Xpsr901* in the Chebec x Harrington mapping population (Kretschmer *et al.*, 1997). DNA from the 20 selected $BC_1$ Sloop backcross lines were probed with both *Xawbma21* and *Xpsr901*. Lines were allocated into marker allele class based on the combined marker allele information. A partial linkage map of the relevant region of chromosome 2H in the Chebec x Harrington mapping population (Langridge *et al.*, 1995) is provided in Figure 6.1.

*6.1.2.5 Field experiments*

The cultivars Sloop and Chebec and 10 $F_2$ derived $BC_1$ lines homozygous for Chebec and Sloop alleles at both *Xawbma21* and *Xpsr901* were grown in field experiments at Strathalbyn, Pinery and Tuckey, South Australia, in 1999. Plots were six rows wide (1.23m) and 4m in length and arranged as randomised complete blocks with four replicates. Plots were machine harvested and grain yield and percentage of plump grain retained over a 2.5mm screen (Strathalbyn site only) calculated from the harvested samples.

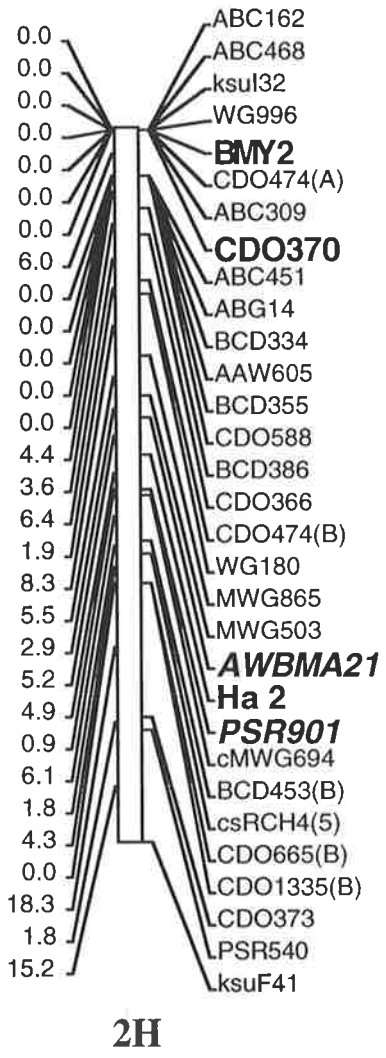



Figure 6.1. Partial linkage of chromosome 2H in the Chebec x Harrington linkage map (Langridge *et al.*, 1995). The *Ha 2* locus is shown in bold with flanking RFLP markers used in population development presented in bold italics. The map location of *Bmy-2* , a β-amylase structural gene and CDO370, an RFLP marker shown to be associated with boron tolerance in barley (Chapter 4) are also presented in bold.

*6.1.2.6 Malting quality*

Samples of grain harvested from two replicates of the field experiment at Pinery were screened on a 2.5 mm screen and 60g sub-samples micro-malted on a Phoenix Automatic Micromalting System (Phoenix Biosystems, Adelaide) without the use of additives. The program comprised the following stages: steep and air rest, 7h, 8h, 9h (wet: dry: wet) at 15°C; germination, 94.5h at 15°C; kilning, 30-40°C, 9h; 40-60°C, 4h; 60-70°C, 2h; 70-80°C, 4.5h; cool to 25°C, 0.5h (total time 138.5h).

Hot water extract (HWE) was determined using a rapid small scale method (MacLeod *et al.* 1991), which has been closely correlated to the EBC fine grind extract method (EBC Analytica, 1998). HWE was expressed as percent on a dry basis. Viscosity was determined on the HWE wort using an AMV 200 rolling ball viscometer (1.7mm capillary tube, 40° angle) and results expressed in centipoise (cP). Wort β-glucan was measured on the HWE wort using a Megazyme kit assay (McCleary and Nurthen, 1986), with results expressed in mg/L. Free amino nitrogen (FAN) was also measured on the HWE wort using a standard (Ninhydrin Colorimetric) EBC method (EBC, 1975) with results expressed as mg/L. Malt protein was assessed using a Technicon 400 Near Infrared (NIR) Spectrometer. This instrument was calibrated using the Kjeldahl method as a reference (IOB Methods of Analysis, 1997: EBC Analytica, 1998). Soluble protein was determined using the American Society of Brewing Chemists (ASBC) spectrophotometric method (ASBC, 1997) which involved measuring the absorbance of a sample of HWE wort, diluted in a sodium chloride solution, at 215nm and 225nm. Soluble protein was then predicted using linear regression against a calibration equation developed using a set of Kjeldahl analyses (Fox *et al.*, 1999a). Kolbach Index was determined as the ratio between soluble protein and total malt protein, expressed as a percentage.

Diastatic power (DP) represents the combined activities of a number of hydrolytic enzymes, including α-amylase and β-amylase. DP was measured on an extract of finely ground malt using a rapid small scale variation of a standard starch digestion followed by measurement of reducing sugars with a para-hydroxybenzoic acid hydrazide (PAHBAH) reagent (Fox *et al.*, 1999b). DP was expressed as micromoles of maltose equivalents released per minute per gram dry weight. Following extraction, an aliquot of the DP extract supernatant was heat treated (65°C for 15 min) to denature the β-amylase component. The sample was re-assayed as for DP to estimate the heat stable α-amylase component. The β-amylase or heat labile

component was calculated from the total DP minus α-amylase. Results were expressed as for DP.

*6.1.2.7 Statistical analysis*

Least-squares means of cysts per root mass for each of the doubled haploid lines were calculated using a single factor ANOVA (SAS Institute, JMP version 3 software). The single factor was doubled haploid line. The observed resistance segregation ratio was tested for significant deviation from that expected from three cycles of backcrossing with and without marker assisted selection using the $\chi 2$ test.

Means for grain yield were calculated allowing for extraneous variation using spatial techniques developed by Cullis and Gleeson (1991). A cross-site analysis was conducted using scaled information of estimates of genotype effects from all four sites following the method of Cullis *et al.* (1996).

Lines were grouped into classes based on marker alleles for *Xawbma21* and *Xpsr901*. Least-squares class means were calculated for all variables measured using a single factor ANOVA (SAS Institute, JMP version 3 software). The single factor was marker allele class. Class means were compared using linear contrasts.

### 6.1.3 Results

*6.1.3.1 CCN resistance of $BC_3$ doubled haploid lines*

Of the 121 doubled haploid plants generated from the marker selected $BC_3$ $F_1$ donor plants, only 114 established adequate number of plants in the CCN bio-assay to provide an assessment of resistant status. Averaged over 40 plants, the control varieties, Chebec and Sloop, produced 0.4 (standard deviation 0.9, standard error = 0.1) and 4.7 (standard deviation = 4.0, standard error = 0.7) cysts per plant respectively. The average number of cysts per plant, among the 114 doubled haploid lines, ranged from 0.0 to 13.2 with a grand mean of 1.7 and standard error of 0.2. The frequency distribution for mean number of cysts is provided in Figure 6.2.

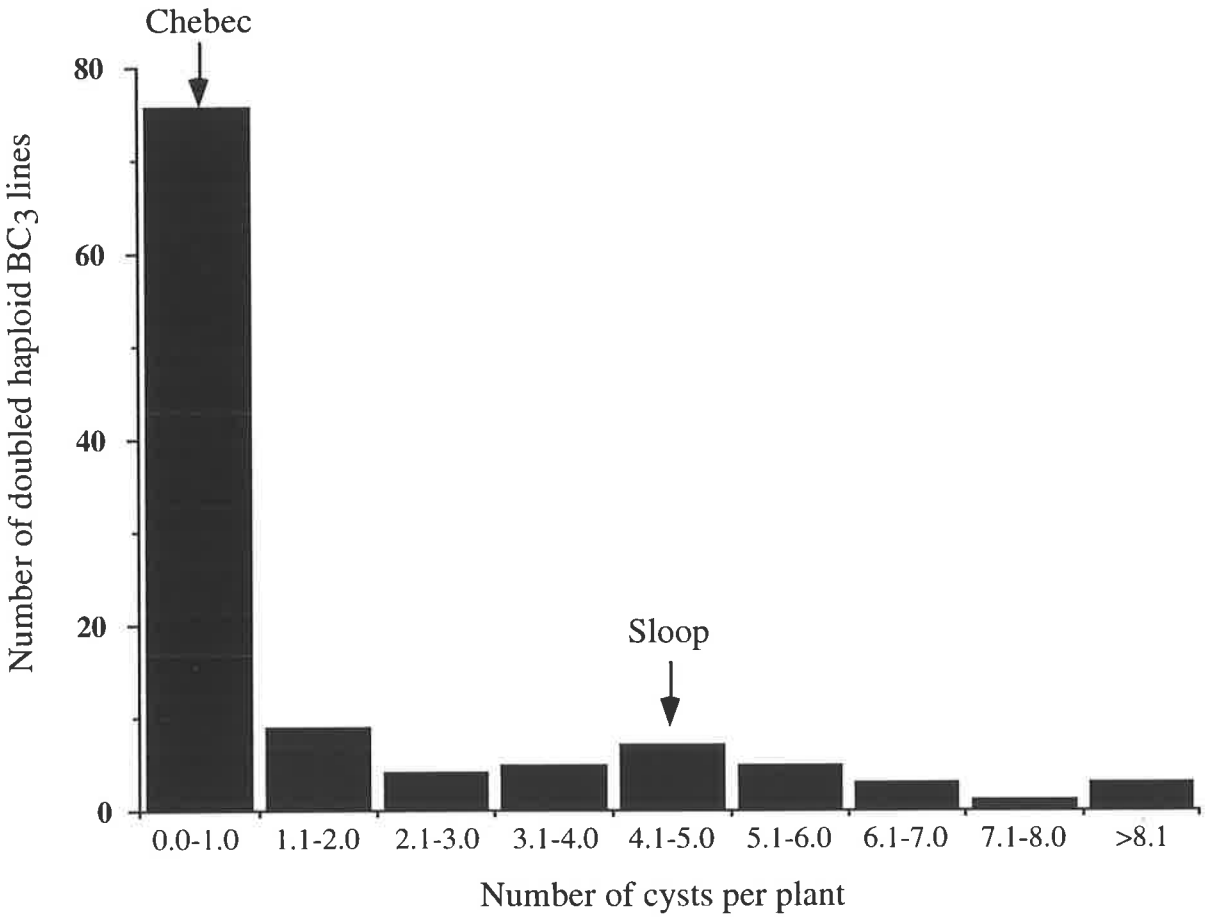



Figure 6.2. Frequency distribution for number of cysts per plant for 114 $BC_3$ doubled haploid lines

Seventy six doubled haploid lines (67%) were rated as resistant and 38 lines (33%) were rated as susceptible. The observed number of resistant doubled haploid lines was significantly (P<0.001) greater than that expected from no active selection (Table 1). The number of observed CCN resistant lines was also significantly (P<0.001) greater than that expected from marker assisted selection, given a recombination frequency between the marker (*Xawbma21*) and *Ha 2*. of three percent.

Table 1. Observed and expected number of lines resistant and susceptible to cereal cyst nematode in a $BC_3$ $F_2$ unselected population and a $BC_3$ $F_1$ derived doubled haploid population for which marker assisted selection was used to select heterozygous $F_1$ at each backcross and to select $BC_3$ $F_1$ doubled haploid donor plants.

| | | Observed | Expected | $\chi^2$ | P |
|---|---|---|---|---|---|
| No selection | Resistant | 76 | 10 | 477.5 | 0.001 |
| | Susceptible | 38 | 104 | | |
| Marker assisted selection | Resistant | 76 | 55 | 15.5 | 0.001 |
| | Susceptible | 38 | 59 | | |

*6.1.3.2 Effect of Chebec marker alleles near the Ha 2 locus on malting quality, grain yield and grain size of $BC_1$ derived lines*

There was no significant difference between the donor and recurrent parents or between backcross lines carrying the Chebec and Sloop marker alleles at the *Ha 2* locus for malt extract, viscosity, free amino nitrogen concentration and α-amylase activity (Table 2a and 2b), wort β-glucan, soluble protein and Kolbach Index (data not shown). The recurrent parent Sloop, was found to produce significantly (P<0.05) greater β-amylase activity and diastatic power than the donor parent Chebec (Table 3a). Similarly, lines carrying Chebec marker alleles at the *Ha 2* locus produced significantly lower β-amylase activity and diastatic power than lines carrying the Sloop marker alleles (Table 3b).

Table 2 (a) Mean malt extract, viscosity and free amino nitrogen (FAN) of donor and recurrent parents grown at Pinery 1999. (Significance (P<0.05) of difference based on pairwise comparison between parents only and represented by different letters).

| Parents | Malt extract (%dry basis) | | Viscosity (cP) | | FAN (mg/l) | |
|---|---|---|---|---|---|---|
| | Mean | Standard error | Mean | Standard error | Mean | Standard Error |
| Sloop | 77.3 a | 0.3 | 1.91 a | 0.05 | 122 a | 4.1 |
| Chebec | 76.7 b | 0.3 | 1.97 a | 0.05 | 128 a | 4.1 |

Table 2 (b) Effect of marker allele on malt extract, viscosity and free amino nitrogen (FAN) of $BC_1 F_2$ derived lines, Pinery 1999. (Significance (P<0.05) of difference based on pairwise comparison between parents only and represented by different letters).

| Marker allele | Malt extract (%dry basis) | | Viscosity (cP) | | FAN (mg/l) | |
|---|---|---|---|---|---|---|
| | Mean | Standard error | Mean | Standard error | Mean | Standard error |
| Sloop | 77.2 a | 0.2 | 1.99 a | 0.03 | 126 a | 3 |
| Chebec | 77.1 a | 0.2 | 1.97 a | 0.02 | 119 a | 3 |

Table 3 (a) Mean diastatic power (DP), α-amylase activity and β-amylase activity (all measured as μM maltose/min/g)of donor and recurrent parents grown at Pinery 1998. (Significance of difference based on pairwise comparison between parents only. Different letters represent significant difference at P<0.05).

| Parents | DP | | α-amylase | | β-amylase | |
|---|---|---|---|---|---|---|
| | Mean | Standard error | Mean | Standard error | Mean | Standard Error |
| Sloop | 436 a | 8 | 93.5 a | 2.4 | 340 a | 7 |
| Chebec | 386 b | 8 | 93.0 a | 2.4 | 293 b | 7 |

Table 3(b) Effect of marker allele on the diastatic power (DP), α–amylase activity and β-amylase (all measured as μM maltose/min/g) activity of $BC_1$ $F_2$ derived lines, Pinery 1998. (Significance of difference based on pairwise comparison between classes only. Different letters represent significant difference at P<0.05).

| Marker allele | DP | | α–amylase | | β-amylase | |
|---|---|---|---|---|---|---|
| | Mean | Standard error | Mean | Standard error | Mean | Standard error |
| Sloop | 419 a | 8 | 91 a | 1 | 335 a | 8 |
| Chebec | 392 b | 8 | 92 a | 1 | 307 b | 8 |

There was no significant genetic variance in grain yield at Pinery and Tuckey in 1999. There was also no significant difference between the grain yield of the donor and recurrent parents at these two sites. Sloop was, however, seven percent higher yielding than Chebec at Strathalbyn (Table 4a). Despite this, there was no significant difference between the grain yield of lines carrying the Chebec and Sloop marker alleles at the *Ha 2* locus at any site (Table 4b).

Table 4 (a). Mean grain yield (t/ha) of donor and recurrent parents grown at Strathalbyn, Pinery, and Tuckey, South Australia 1999. (Significance (P<0.05) of difference based on pairwise comparison between parents only and represented by different letters).

| Parents | Strathalbyn | | Pinery | | Tuckey | |
|---|---|---|---|---|---|---|
| | Mean | Standard error | Mean | Standard error | Mean | Standard error |
| Sloop | 1.19 a | 0.04 | 0.53 a | 0.04 | 0.40 a | 0.06 |
| Chebec | 1.19 a | 0.04 | 0.50 a | 0.05 | 0.40 a | 0.06 |

Table 4(b) Effect of marker allele on the grain yield (t/ha) of $BC_1$ $F_2$ derived lines, at Strathalbyn, Pinery, and Tuckey, South Australia 1999. (Significance (P<0.05) of difference based on pairwise comparison between parents only and represented by different letters).

| Marker allele | Strathalbyn | | Pinery | | Tuckey | |
|---|---|---|---|---|---|---|
| | Mean | Standard error | Mean | Standard error | Mean | Standard error |
| Sloop | 1.19 a | 0.01 | 0.53 a | 0.01 | 0.40 a | 0.01 |
| Chebec | 1.19 a | 0.01 | 0.50 a | 0.01 | 0.40 a | 0.01 |

The recurrent parent Sloop retained 88% of grain over a 2.5mm screen while Chebec retained 70%. Grain samples were taken from the field experiment conducted at Strathalbyn in 1999 (Table 5(a) and (b)). Despite this significant difference between donor and recurrent parent for grain plumpness, there was no significant difference between the grain plumpness of lines carrying the Chebec and Sloop marker alleles at the *Ha 2* locus (Table 5(a) and (b)).

Table 5 (a). Mean grain plumpness (%>2.5 mm) of donor and recurrent parents grown at Strathalbyn, South Australia 1999. (Significance of difference based on pairwise comparison between parents, and between classes only. Different letters represent significant difference at P<0.05).

| Parents | Mean | Standard error |
|---|---|---|
| Sloop | 87.7 a | 5.2 |
| Chebec | 69.5 b | 5.2 |

Table 5 (b) Effect of marker allele on the grain plumpness (%>2.5 mm)of $BC_1$ $F_2$ derived lines, at Strathalbyn, South Australia 1999. (Significance of difference based on pairwise comparison between classes only. Different letters represent significant difference at P<0.05).

| Marker allele | Mean | Standard error |
|---|---|---|
| Sloop | 82.5 a | 0.9 |
| Chebec | 82.7 a | 0.8 |

### 6.1.4 Discussion

Three cycles of marker assisted backcrossing were used to develop CCN resistant, fixed lines in less than three years. No phenotypic selection was used. The bio-assay system described by Fisher (1982) was used to confirm the resistance status of $BC_3$ $F_1$ derived doubled haploid lines. As stated earlier, of the 114 $BC_3$ lines rated for resistance status, 76 (67%) were found to be resistant and 38 (33%) were found to be moderately susceptible to susceptible (Table 1). If we assumed no marker assisted or phenotypic selection over the three cycles of backcrossing, then we would expect the proportion of resistant $BC_3$ $F_2$ plants to be approximately nine percent. The proportion of $BC_3$ doubled haploid lines found to be resistant to CCN was significantly (P<0.001) greater at 67 percent (Table 1). This highly

significant difference between the observed (with marker assisted selection) and expected (no marker assisted selection) proportion of CCN resistant $BC_3$ lines, confirms that marker assisted selection was effective in transferring *Ha 2* from Chebec to Sloop. These results also confirm that *Ha 2*, derived from Chebec was successfully expressed in the Sloop background.

The proportion of $BC_3$ doubled haploid lines found to be resistant to CCN was also significantly ($P<0.001$) greater than that expected (48%) from full marker assisted selection, assuming a recombination frequency between the marker and gene of three percent (Kretschmer *et al.*, 1997) (Table 1). The bias in the observed segregation ratio is clearly seen in the frequency distribution shown in Figure 6.2.

The doubled haploid $BC_3$ lines used in this study were developed from direct regeneration of haploid plants from immature pollen grains (microspores) by the culture of whole anthers followed by diploidisation (Kasha *et al.*, 1990). The population of plants regenerated from culture of $BC_3$ $F_1$ hybrid microspores should, in theory, represent the random segregation from the preceding meiosis. Non-random segregation of gametes during barley microspore culture, however, has been widely reported (San Noeum and Ahmadi, 1982; Powell *et al.*, 1986; Thompson *et al.*, 1991; and Logue *et al.*, 1995).

Differences between genotypes in their ability to respond to anther culture may result in a skewing of the gametic array represented in doubled haploids of $F_1$ hybrids between low and high responding parents towards the high responding parent (Morrison and Evans, 1987, 1988). This type of skewed segregation has been reported in barley by a number of authors (Kao *et al.*, 1983; Foroughi-Wehr and Freidt, 1984; Powell *et al.*, 1986, Graner *et al.*, 1991;Thompson *et al.*, 1991; Logue *et al.*, 1995). Skewed segregation in $F_1$ hybrid doubled haploid populations has also been shown to favour discrete regions of the genome (Graner *et al.*, 1991; Heun *et al.*, 1991; Logue *et al.*, 1995; Tinker *et al.*, 1996). RFLP markers with distorted segregation in favour of the more responsive parent Igri, were clustered in discrete segments on chromosomes 1H, 3H, 5H and 7H in an anther-culture derived population involving Igri and the less responsive Franka (Graner *et al.*, 1991). In contrast, RFLP analysis of a backcross progeny generated conventionally from an $F_1$ between Igri and Franka showed the sexual progeny to segregate at the expected 1:1 ratio (Graner, 1996). These observations suggest that genotypic effects are expressed during *in vitro* regeneration (Graner, 1996). Graner (1996) also proposed that the more two parents differ in their

responsiveness to microspore culture the larger the selection effects, and the more frequent the distorted segregation.

Kretschmer *et al.* (1997) reported significant segregation distortion for both CCN resistance and RFLP marker alleles, in favour of Chebec, on chromosome 2H in the anther-culture derived, Chebec x Harrington mapping population. Marker allele segregation distortion on chromosome 2H in the Chebec x Harrington mapping population is shown in Figure 6.3. Significant segregation distortion is shown over most of chromosome 2H with the greatest level of distortion very close to the *Ha 2* locus (Figure 6.3). The donor parent Chebec is regarded as highly responsive in anther culture while the recurrent parent Sloop is regarded as relatively recalcitrant (P.Davies, pers comm.).

With this in mind, it is possible that similar segregation distortion found in the Chebec x Harrington mapping population could also be evident in the Chebec x Sloop backcross population and this would account for the similar level of CCN resistance segregation distortion found in the backcross population. Cheng *et al.* (1998) proposed that distorted segregation at a marker locus in barley was predominantly due to linkage between the marker and a gametophyte gene (partial lethal factor). Evidence is provided here of the possible presence of a male gametophyte gene on chromosome 2H in the vicinity of the *Ha* 2 locus (Fig 6.3).

A positive gametophytic factor may be associated with Chebec (culture responsive) alleles on chromosome 2H or a negative factor associated with both Harrington and Sloop (relatively recalcitrant in culture) alleles at this locus. A positive gametophytic factor associated with Chebec alleles around the *Ha 2* locus could be a major advantage to breeding strategies aimed at utilising CCN resistance from Chebec. The proportion of anther-culture derived doubled haploid progeny likely to be carrying *Ha2* would be greater than that expected from classical Mendelian segregation and could be used to increase the frequency of CCN resistant individuals in segregating populations. Graner (1996) revealed less or even no distorted segregation, however, in doubled haploid progeny produced according to improved microspore culture protocols. The relative advantages of skewed segregation in favour of desirable alleles needs to be assessed against the low efficiency rates of outdated microspore culture protocols.

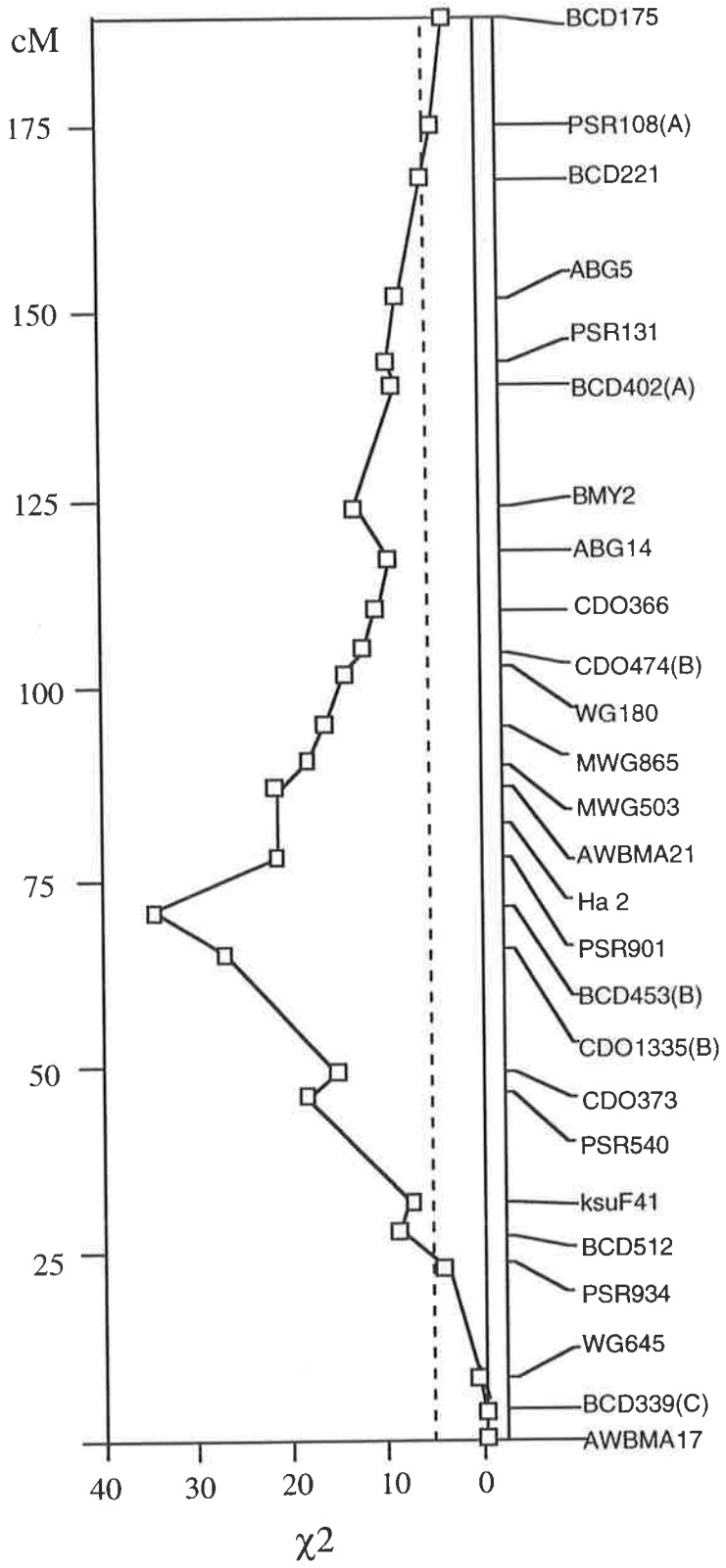



Figure 6.3. $\chi^2$ values for marker allele segregation ratios for markers on chromosome 2H in the Chebec x Harrington mapping population.

### 6.1.4.1 *Effects of marker alleles near the Ha 2 locus on malt quality and agronomic traits*

The only malt quality characteristics found to be significantly associated with donor parent marker alleles near the *Ha 2* locus were diastatic power (DP) and β-amylase activity (Table 3). DP is a measure of the total amylolytic enzyme activity including two major enzyme components, α-amylase and β-amylase. β-amylase is typically the largest component of DP. The *Bmy-2* structural gene, which has a major role in the control of β-amylase activity (Kreis *et al.*, 1987), and therefore DP, has been located to chromosome 2H (Kleinhofs *et al.*, 1993) approximately 40 cM (proximal) from the *Ha2* locus (Kretschmer *et al.*, 1997; Langridge *et al.*, 1995). The lower mean β-amylase activity and DP of lines carrying Chebec marker alleles near the *Ha 2* locus could therefore be partly due to linkage drag. Coventry (1998) showed that Sloop and Chebec carry different alleles at the *Bmy-1* structural gene locus on chromosome 4H. It is therefore unlikely that differences in β-amylase activity and DP between Sloop and Chebec can be attributed entirely to differences at the *Bmy-2* locus. The relatively high density of RFLP markers (Fig 6.1), and the recent addition of several micro-satellite (SSR) markers (Karakousis *et al.*, in prep), in the region between *Ha 2* and *Bmy-2* on chromosome 2H on the Chebec x Harrington linkage map (Langridge *et al.*, 1995) provides ample opportunity for using markers to select recombinants carrying desirable alleles at both loci.

The trait, viscosity, is a measure of the degree of resistance to the flow of wort and is attributed to the combined effect of a number of cell wall materials including β-glucan and arabinoxylan, as well as protein and polyphenols (Meir *et al.*, 1995; Sudarmana *et al.*, 1996). β-glucan content of malt, a major determinant of wort viscosity, depends upon barley β-glucan content and β-glucanase activity. Han *et al.* (1995) identified significant QTL for barley β-glucan content on chromosomes 1H and 2H, the latter close to the*Bmy-2* locus. However, there was no variation amongst backcross derived lines for wort viscosity or wort β-glucan suggesting that the donor and recurrent parents carry similar or identical alleles at this locus or that Sloop carries genes/QTL for enhanced β-glucanase activity.

Numerous studies have located major genes and/or QTL involved in the control of agronomic traits to chromosome 2H, including a major photoperiod response gene, *Ppd-H1* (Laurie *et al.*, 1995). The donor and recurrent parents are closely related and do not differ greatly for most commonly measured agronomic traits including heading date, plant height,

and grain yield and therefore, are likely to carry similar or identical alleles at a large number of loci throughout the genome including chromosome 2H. It is not surprising therefore that there was little evidence of genetic variation amongst the backcross lines for characters such as plant height, heading date (data not shown), grain yield and grain size (Tables 4 and 5).

### *6.1.4.2 Comparison between marker assisted selection for CCN resistance and phenotypic selection*

Phenotypic selection for CCN resistance can be undertaken either in the field or in the laboratory using an assay described by Fisher (1982). Numerous factors can contribute to variation in resistance ratings from field grown plants. For example, plants can escape infection and many factors, including spatial variation in the distribution of the pathogen, can contribute to this. O'Brien and Fisher (1974) showed that considerable loss of disease assessable root material occurred in the field irrespective of the care with which the roots were handled. Variation in the density of the pathogen can also contribute to substantial variation in the expression of symptoms (Fisher, 1982). Other factors such as temperature and fungal infection can also contribute to variation. The laboratory assay system developed by Fisher (1982) overcomes, at least to some extent, many of these problems. The assay system is, however, time consuming (14 weeks) and expensive, and is generally confined to the winter months due to the need for relatively low temperatures (Fisher, 1982). Variation in resistance ratings is much reduced in the bio-assay system but it still remains a problem. Based on the bio-assay resistance ratings of 40 individual plants of Sloop and Chebec, the proportion of Chebec single plants scored as susceptible was relatively low (6%). In contrast, the proportion of Sloop single plants wrongly scored as resistant was considerably higher (23%).

Molecular markers can provide powerful tools for the indirect selection of genes or QTL in a breeding program (Beckman and Soller, 1983). Recent developments in the mechanisation and computerisation of the bio-assay system has led to reductions in the economic cost of phenotyping such that these costs are now comparable, or even less than, that of marker screening given current DNA extraction protocols and RFLP marker screening methods. However, automated DNA extraction methods and PCR based marker systems are likely to greatly enhance the efficiency of marker assisted selection. The application of molecular markers has the potential, therefore, to substantially improve the efficiency of breeding for CCN resistance using backcrossing. For example, DNA can be extracted from tissue sampled from growing plants at very early stages of development. This allows sufficient

time to use linked markers to identify heterozygous resistant, backcross $F_1$ individuals prior to anthesis. In contrast, optimum CCN symptom expression in the bio-assay generally occurs at development stages close to anthesis when crossing should take place. Plants in the bio-assay system are also grown under sub-optimum conditions for crossing and seed set.

The number of plants per genotype required for a reliable assay is of particular importance in a backcross program. The sample size required, to determine the resistance status of a given genotype can be estimated from the following formula;

$$n = Z^2_{\alpha(2)}\, pq/\delta^2$$

where $Z_{\alpha(2)}$ is a 2-tailed normal deviate, $p$ is the probability of a resistance status error, $q$ = 1-$p$ , and $\delta$ is the confidence level (Zar, 1999). Based on the number of cysts per plant achieved on 40 plants of both Sloop and Chebec, the probability of rating Chebec as susceptible and Sloop as resistant, in the bio-assay, is 0.06 and 0.23 respectively. The probability of scoring a susceptible genotype as resistant is considerably greater than the probability of scoring a resistant variety as susceptible because the chance of error associated with infection escape is greater than that of assessment error. The incorrect scoring of a susceptible genotype, however, is the most relevant error in a backcrossing program. Therefore, the number of plants required in the bio-assay to rate a susceptible genotype as susceptible with 95%, 90%, and 80% confidence is 272, 47, and 7 plants, respectively. Based on a recombination frequency between *Ha2* and *Xawbma21*, of less than 3% (Kretschmer *et al.*, 1997) the number of plants required to identify a heterozygote $BC_x$ $F_1$ with 95%, 90% and 80% confidence is 44, 8, and 1 plant respectively. Marker assisted selection using a single marker (*Xawbma21*) can be used to determine the resistance status of a single plant with 80% confidence compared to 62% confidence with the bio-assay. In addition the confidence level of single plant marker assisted selection for CCN resistance would increase substantially with the use of flanking markers (Beckmann and Soler, 1986). Selection on the basis of a single plant is, therefore, considerably more reliable using either a single marker or flanking markers than it is with the bio-assay system.

Any number of phenotypic selection strategies could be adopted for the introgression of *Ha 2*. For example, three broad phenotypic selection strategies may consist of; (1) no selection during backcrossing, using large $BC_x F_1$ (where $x$ is the number of backcrosses) populations that increase as the number of backcrosses increases and a large final population which is screened intensively; (2) selection of resistant $BC_x$ $F_1$ after each backcross, allowing for selection error by increasing the number of individuals selected and backcrossed; and (3)

$BC_x F_1$ allowed to self after each backcross, coupled with phenotypic selection on $F_2$ derived lines at each backcross generation. The time and resources available to the breeder would dictate which strategy is adopted. Similarly, the relative efficiency of marker assisted selection strategies will be dictated by the resources available to the breeder. For example, the number of backcrosses that can be made in a given time could be determined by the availability of and access to, polymorphic, co-dominant single or flanking markers and marker systems, availability of controlled environment conditions for crossing, and/or the capacity to use embryo rescue techniques. Therefore, an accurate quantification of the time and resource savings associated with marker assisted introgression of *Ha 2* over phenotypic introgression, is likely to be highly complicated and not within the aims of this study. The empirical data presented in this study, however, provides evidence that a combined genotypic and phenotypic selection strategy is effective. In this case, marker assisted selection was used to identify suitable $BC_x F_1$ individuals for further backcrossing and the final fixed population phenotyped to confirm resistance. The potential benefits of the application of molecular markers for selecting individuals carrying a greater than expected proportion of recurrent parent genome at any given backcross generation was not considered in this study.

#### *6.1.4.3 Implications for the breeding of CCN resistant malting quality varieties*

Cereal cyst nematode is a major cereal root pathogen, which is endemic throughout the important cereal-growing regions of the world (Meagher, 1977) but is also the most damaging cereal root pathogen in southern Australia (Sparrow and Dube', 1981). Genetic variation for resistance in Australian germplasm was identified as early as the mid 1960's (Sparrow and Dube', 1981), yet only four resistant cultivars have been grown commercially in southern Australia. The breeding of CCN resistant cultivars acceptable for malting quality grades has proven to be difficult. Grain of the four CCN resistant cultivars commonly grown in Australia can only be accepted into feed quality grades. The sources of resistance in these cultivars is derived from poor malt quality landraces (Sparrow and Dube', 1981) and consequently the introgression of this germplasm is likely to have led to disruption of the established gene complexes important in the achievement of high malt quality. Given the multi-factor nature and complex inheritance of malting quality, it is surprising, the backcross method has not been used more extensively in southern Australia for the breeding of malting quality cultivars with improved agronomic traits. The backcross method may have been avoided by Australian barley breeders because of the excessive time required to produce a

desirable outcome and the slow or static rate of genetic gain for malt quality inherent in such a strategy.

This study has shown that marker assisted selection can be effective in the backcross introgression of *Ha 2* conferring CCN resistance and that this can be achieved more efficiently than with phenotypic selection. Despite the fact that the donor and recurrent parents were closely related, and the donor parent carried relatively minor malt quality defects, this study also highlighted the, often underestimated, problem of linkage drag.

Improved knowledge of the number and location of genes/QTL conferring high malt quality will greatly assist the planning and conduct of the marker assisted backcross introgression of desired agronomic traits into malting quality backgrounds by;

(1) providing the opportunity to select for specific recombinants with reduced linkage drag and;

(2) providing the opportunity to simultaneously introgress multiple traits including genes/QTL conferring improved malt quality.

The potential of marker assisted selection for malt quality QTL was demonstrated by Han *et al.* (1997). Marker assisted backcrossing has the potential, therefore, to reduce the time to produce desired agronomic outcomes but also improve the rate of genetic gain for malt quality through the simultaneous introgression of genes/QTL conferring improved malt quality characteristics otherwise fixed in the recurrent parent.

## 6.2 Marker assisted backcross introgression of the*Yd2* gene conferring resistance to barley yellow dwarf virus in barley (*Hordeum vulgare* L)

### 6.2.1 Introduction

Barley yellow dwarf virus (BYDV) is a disease caused by a suite of aphid-transmitted Luteoviruses. BYDV is distributed worldwide and infects most cereals and grasses including barley (Conti *et al.*, 1990). BYDV is recognised as the most economically important and widespread viral disease of small grains in the world (Burnett *et al.*, 1995). In a review of studies on grain yield loss in barley resulting from artificial introduction of viruliferous aphids into field plots, Pike (1990) reported a mean grain yield reduction of 54.5% when plots were infected at the seedling stage, 22.6% when infected at tillering and 18.7% when infected from stem elongation to booting.

It is widely accepted that host-plant resistance is the most efficient control strategy for BYDV, even though losses can be limited by adjusting sowing date (Mann *et al.*, 1997) or through the application of systemic organophosphate insecticide (Johnstone *et al.*, 1990). Suneson (1955) studied the inheritance of field resistance (tolerance) to BYDV in 'Rojo' and found a relatively low level of resistance to be conditioned by a single recessive gene that he named *yd1*. Screening of more than 6,000 accessions of barley identified at least 100 Ethiopian accessions with low BYDV symptom levels (Schaller *et al.*, 1963). The resistance in most of these lines was shown to be due to a single locus, named *Yd2*, (Rasmusson and Schaller, 1959). The *Yd2* gene has been the most effective means of minimising barley yield losses associated with BYDV in the world (Burnett *et al*, 1990). *Yd2* confers resistance to both the PAV and MAV isolates of BYDV (Gill and Buchannon, 1972; Baltenberger *et al.*, 1987) which are the most prevalent in cereal growing areas of the world (Lister and Ranieri, 1995).

Breeding for resistance to BYDV is constrained by the inconvenience and unreliability of biological resistance assays (Paltridge *et al.*, 1998). These resistance assays require a specific isolate of the virus to be carried by a specific aphid vector. In addition, BYDV symptoms are easily confused with other environmental stresses; expression of resistance can be influenced by genetic background (Qualset, 1975) and plant type (Jones and

Cathedrall, 1970); and the *Yd2*-mediated resistance is often expressed in an incompletely dominant or recessive manner (Rasmusonn and Schaller, 1959; Cathedral *et al.*, 1970).

The application of molecular markers associated with the *Yd2* gene could substantially improve the efficiency and precision of BYDV resistance breeding. Using amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995) analysis, Paltridge *et al.* (1998) developed a co-dominant marker (designated YLM) linked (0.7cM) to *Yd2* based on the polymerase chain reaction (PCR) (Saiki *et al.* 1985). Paltridge *et al.*, (1998) tested the YLM marker on a collection of 102 *Yd2* and non-*Yd2* barley lines and found that YLM could be used to correctly identify all 9 lines known to carry *Yd2,* and 85 of the 93 lines which were believed not to carry *Yd2*. Ford *et al.* (1998) reported on the development of an alternative PCR marker designated *Ylp*, which co-segregates with *Yd2*. Two assays were developed at the locus; one was a codominant assay which used post-amplification restriction analysis to differentiate resistance and susceptibility associated alleles; the other a dominant assay, employed allele specific PCR to detect only the resistance associated allele of the *Ylp* gene (Paltridge *et al*, 1998). The YLM assay, in contrast, is co-dominant and requires only a single step. Steyer *et al.* (1999) compared a controlled field system BYDV resistance screening system with the *Ylp* marker system and found relatively good correlation between the assay systems when resistance came from crosses involving Vixen but not WB168-2. There have, however, been no reports in the literature of successful examples of application of the YLM marker for indirect selection for*Yd2*. Comeau and Jedlinski (1990) noted that, when introduced into cultivated varieties, *Yd2* was associated with low grain yield and small grain size. In addition, disease resistance genes other than *Yd2* have been mapped to the same region of barley chromosome 3H as *Yd2*. These include the *Rh* leaf scald (*Rhynchosporium secalis*) resistance gene, presumed to form a resistance gene complex with *Rh3* and *Rh4* (Graner and Tekauz,1996).

The aim of this study is to demonstrate the practical application of YLM in a marker assisted introgression program, to identify and quantify associations between the presence of *Yd2* and agronomic traits including grain yield and grain quality, and to determine whether YLM can be used simultaneously to select for BYDV and leaf scald resistance.

### 6.2.2 Materials and methods

#### *6.2.2.1 Genetic materials*

The *Yd2* donor parent chosen to create a backcross population for this study was the Australian cultivar Franklin. Franklin is derived from a simple cross between Shannon and Triumph. Shannon was derived from a BYDV resistant Ethiopian accession (CI3208-1) backcrossed to Proctor through four cycles of backcrossing. Franklin has been shown to contain the same length variant (amplification product) of YLM as it's BYDV resistant parent Shannon (Paltridge *et al.*, 1998). Franklin flowers eight to ten days later than most of the broadly adapted barley cultivars grown in southern Australia and is consequently adapted only to the longer season areas of this region. Franklin generally produces small grain with excellent malting quality attributes. The Australian variety Sloop was chosen as the recurrent parent. Sloop is susceptible to BYDV and has been shown to contain the same length variant of YLM as Franklin's BYDV susceptible parent Proctor (Paltridge *et al.*, 1998). Sloop is broadly adapted to southern Australian growing conditions and produces moderately large grain with acceptable malting quality attributes.

A second population was used to further test associations between the Franklin YLM variant and grain yield in an alternative genetic background. The population consisted of 78 doubled haploid lines derived from a simple cross between Franklin and the BYDV susceptible Australian malting quality parent Arapiles.

#### *6.2.2.2 Population development*

Forty one $BC_1$ $F_1$ individuals were produced from backcrosses between the donor parent Franklin and recurrent parent Sloop. DNA from 20 of these $BC_1$ $F_1$s was probed with the YLM marker and 12 $F_1$s were found to be carrying the Franklin length YLM variant. Four of these $BC_1$ $F_1$ plants were backcrossed and allowed to self to produce the $BC_2$ $F_2$ population. DNA from fifty randomly selected $BC_2F_2$ plants was probed with the YLM marker. Ten plants homozygous for the Franklin length YLM variant (+YLM) and 10 plants homozygous for the Sloop YLM variant (-YLM) were selected for seed multiplication. Population size was determined following the principle of bulked segregant analysis (Michelmore *et al.*, 1991), as described in Chapter 6.1.

The Franklin x Arapiles doubled haploid population was derived from a single $F_1$ and kindly provided by Dr P. Davies (SARDI) and Mr D.Moody (NRE Victoria) as part of the National Barley Molecular Marker Program (Grains Research and Development Corporation). Arapiles is susceptible to BYDV and has been shown to contain the same length variant of YLM as Franklin's BYDV susceptible parent Proctor (Paltridge *et al.*, 1998). All individuals of the Franklin x Arapiles doubled haploid population were probed with the YLM marker and separated into two groups based on the length of the YLM variant.

*6.2.2.3 PCR analysis of barley DNA for YLM genotype*

DNA extraction was achieved using a DNA mini-prep method adapted from Rogowsky *et al.* (1991). Variations to the method are described below. For the initial extraction, 700 µl of extraction buffer and phenol-chloroform-isoamyl alcohol (25:24:1) were used. The extraction buffer was 0.1 *M* Tris-HCl (pH 8.5),10 mM EDTA, 0.1 *M* NaCl, 1% sarkosyl and 2% polyvinyl-polypyrrolidone (insoluble). DNA was precipitated by the addition of 60 µl sodium acetate (pH 4.8) and 600 µl of propan-2-ol. Oligonucleotide primers were synthesised by Life Technologies. PCR reactions were performed in a 20 µl volume in a PTC-100 Programmable Thermal Controller (MJ Research, USA). Thermal cycling comprised the following steps; 1 minute of denaturation at 94 ° C; 30 seconds of denaturation at 94 ° C; annealing at 65 ° C for 30 seconds; extension at 72 ° C for 30 seconds; the last three steps repeated while reducing the annealing temperature by 0.5 ° C until 55 ° C was reached; denaturation at 94 ° C for 30 seconds; annealing at 55 ° C for 30 seconds; extension at 72 ° C for 30 seconds; the last three steps repeated for 29 cycles; extension at 72 ° C for 5 minutes; and cooled to 25 ° C for 5 minutes. Electrophoretic analyses of the PCR products were performed using 0.75% agarose/2.25% NuSieve GTG agarose (FMC Bioproducts) gels and 1 x TAE electrophoresis buffer.

*6.2.2.4 Viruliferous aphid management and inoculation*

Aphids (*Rhopalosiphum padi* ) for the transfer of BYDV were collected from field-grown wheat at Glen Osmond, South Australia, and their species identity confirmed (Blackman *et al.*, 1990). Virus-free aphid nymphs were taken as they were born from the field-collected aphids and placed on virus-free plants to establish aphid stocks. Aphid and BYDV-$PAV_{adel}$ cultures (donated by Dr Monique Henry, formerly of the University of Adelaide) were maintained separately on plants of the oat (*Avena sativa* ) cultivar Stout in an environmental chamber at 22 ° C days and 18 ° C nights with a 14hr photoperiod. Oat plants were sown in

13cm pots (4 plants per pot) and grown in aphid proof cages made from fine nylon mesh. Three weeks after germination, oat plants were transferred to cages containing viruliferous aphids. Approximately 100 aphid carrying oat plants were harvested at ground level 18 days after introduction into viruliferous aphid cages.

*6.2.2.5 Field experiments*

*(1) Sloop backcross population*

Ten (+)YLM and 10 (-)YLM $BC_2$ $F_2$ derived lines and the donor parent Franklin were sown in a field experiment near Strathalbyn, South Australia in 1998. Plots were six rows wide (1.23m) and 4m in length. The $BC_2$ population was late sown (August) as a split-plot experiment where one split consisted of inoculation with viruliferous aphids and no insecticidal aphid control ('+Aphid') and the other split consisted of no aphid inoculation and treatment with contact and systemic insecticide ('-Aphid'). The split-plots were arranged in randomised complete blocks with two replicates. Sloop check split-plots were sown every fifth pair of barley plots. Wheat plots were sown between every barley plot. Oat straw carrying viruliferous aphids was cut into lengths of approximately 15 cm and spread evenly over all '+Aphid' barley split-plots when Sloop had reached decimal growth stage (DGS) 25 (Zadoks *et al.*, 1974). The '-Aphid' plots were treated with contact and systemic insecticide immediately following inoculation of 'Aphid' split-plots. Wheat buffer plots were not inoculated. All field plots were treated with a contact insecticide 18 days after aphid inoculation to remove all aphids potentially causing feeding damage to plants. Aphid numbers were monitored at approximately 14 day intervals until plants were at full maturity (DGS >90). No significant re-infestation was observed. '-Aphid' plots were scored for decimal growth stage when Sloop had reached DGS 85 and Franklin had reached DGS 50. '+Aphid' plots were given a BYDV damage score based on the visual severity of symptoms on a 1-9 scale. A score of "1" corresponded to a highly tolerant genotype with no obvious visual symptoms and a score of "9" corresponded to very intolerant genotype with severe stunting and chlorosis. Plots were machine harvested, and grain yield and percentage of plump grain retained over a 2.5mm screen calculated from the harvested samples.

*(2) Franklin x Arapiles doubled haploid population*

The Franklin x Arapiles population was grown at Strathalbyn, South Australia in 1999. Plots were scored for decimal growth stage when Arapiles had reached DGS 54. The population of 78 individuals together with control cultivars were sown in plots six rows wide (1.23m) and 4m in length and arranged as a randomised complete block with two replicates. Plots were machine harvested and grain yield calculated from the harvested samples.

*6.2.2.6 BYDV northern dot blot hybridisation analysis*

Individual plants randomly sampled from six Sloop control plots (three per replicate) were subjected to northern dot blot hybridisation analysis to confirm the presence of the PAV isolate and determine if the RPV isolate was also present. A 707 bp DNA probe template was derived from nucleotides 1326 to 2032 of the RNA genome of an Australian BYDV-PAV isolate (Miller *et al.*, 1988; Young *et al.*, 1991). A BYDV-RPV probe template was derived from a fragment of the BYDV-RPV genome between nucelotides 2025 through to 3209 as defined by Vincent *et al.* (1991). *In vitro* transcription was used to generate radioactive probes. The plasmids to be transcribed were linearised by digestion with the appropriate restriction endonuclease at the terminus of the sequence of interest. Linearised DNA was purified by ethanol precipitation. A total of 1-2 µg of linearised DNA was transcribed in a mixture containing 40m*M* Tris-HCL pH7.5, 6m*M* $MgCl_2$, 2m*M* spermidine, 10m*M* NaCL, 10m*M* DTT, 0.5U/µl RNasin (Promega), 0.5m*M* each of dATP, dCTP, and dGTP, 12µ*M* UTP, 50 µCi alpha-$^{32}$ P-UTP, and 20-40 U of T3 or T7 RNA polymerase (Promega) as appropriate. Transcription reactions were incubated at 37° C for 90 minutes, after which transcripts were purified by phenol:chloroform extraction followed by repeated precipitations in 2.5 *M* ammonium acetate and 2.5 volumes of ethanol to remove unincorporated radioactive label. Probes were stored in TE containing 5m*M* β-mercaptoethanol.

Standard extraction procedures were used to prepare cereal nucleic acid samples for northern dot blot hybridisation analysis (Collins *et al.*, 1996). Ten µl samples of nucleic acid were dotted 1.0 cm apart onto Hybond $N^+$ membrane (Amersham, USA). The spots were allowed to dry and the nucleic acid fixed to the membrane by laying the membrane nucleic acid side up onto a pad made from three sheets of Whatmann 3MM paper soaked in 0.05 *M* NaOH. After five minutes, the membrane was rinsed in 2 x SSC for at least five minutes. Pre-

hybridisation was performed at 68° C for a minimum of 8 hours in a bottle containing 20ml of solution consisting of 3ml of 50 x Denhardts Reagent, 5ml of 20 x SSC, 1ml of 10%SDS, 2ml of Carrier DNA (10mg/ml) and 9ml of deionised formamide (BDH). The hybridisation was also performed at 68° C in a bottle containing the pre-hybridisation solution but also including 2ml of Dextran Sulphate (25). Following hybridisation, membranes were washed three times for five minutes in 2.0 x SSC, 0.1% SDS at room temperature, and then two times for 10 minutes in 0.1 x SSC, 0.1% SDS at 68°C. Autoradiography was performed for 24 hours at -80°C with Konica X-ray film and an intensifying screen.

*6.2.2.7 Malting quality*

Sixty gram sub-samples of grain from both replicates of -Aphid treatments was micro-malted and malt quality analysis undertaken on the malted samples as described in Chapter 6.1.

*6.2.2.8 Leaf scald resistance screening*

The twenty $F_2$ derived $BC_2$ lines and Sloop and Franklin controls were sown in plots in a leaf scald disease screening nursery at Turretfield Research Centre, near Gawler, South Australia in 1999. Plots were 4 m long and two rows wide with seed sown at approximately 150 seeds/$m^2$. The experiment was arranged as a randomised complete block with two replicates. The nursery was inoculated with scald infected straw and a conidial suspension of a mixed scald population at early to mid tillering. Plots were scored for intensity of leaf symptoms (ILS) progressing from lower to upper leaves as described by Couture (1980). The scoring system was based on a scale of 1-9 where a score of "1" referred to disease free lower, mid and upper leaves while a score of "9" referred to severe symptoms on lower, mid and upper leaves. Plots were scored when Sloop plants had reached decimal growth stage (DGS) 85.

*6.2.2.9 Statistical analysis*

Means for grain yield, and percentage of grain retained above a 2.5mm screen were calculated, allowing for extraneous variation using spatial techniques developed by Cullis and Gleeson (1991). Lines were grouped into two classes based on the length of the YLM variant, that is, Franklin type or Sloop/Arapiles. Least-squares class means were calculated

for all variables measured using a single factor ANOVA (SAS Institute, JMP version 3 software). The single factor was YLM variant class. Class means were compared using linear contrasts. Simple regression (SAS Institute, JMP version 3 software) was used to derive relationships between BYDV symptom score from the 1998 aphid inoculated experiment and both decimal growth stage and intensity of leaf scald symptom score from the 1999 leaf scald inoculated experiment.

### 6.2.3 Results

An epidemic of BYDV was achieved in the field experiment at Strathalbyn, South Australia in 1998. Severe stunting and chlorosis were observed in all viruliferous aphid inoculated ('+Aphids') Sloop check plots whilst only a trace of symptoms was observed in Franklin plots. The PAV isolate, to which *Yd2* confers tolerance, was identified in plants from all six inoculated Sloop check plots tested. The RPV isolate, however, was identified in only one of the samples taken. The low level of symptoms in Franklin plots (*Yd2* does not confer tolerance to the RPV isolate) and the low frequency of plants carrying the RPV isolate suggests that a late season, low level natural infection occurred at the field site which had very little impact on the experiment. No significant natural aphid infestation occurred at Strathalbyn in 1999. Consequently no BYDV symptoms were observed on susceptible genotypes.

#### *6.2.3.1 Decimal growth stage*

There was no significant difference in decimal growth stage between those plants carrying the Franklin length variant of YLM and those carrying the Sloop YLM variant (Table 1) in the backcross population in 1998 nor between the Franklin variant or Arapiles variant in the doubled haploid population in 1999. In addition, no significant relationship was found between severity of BYDV symptoms and decimal growth stage in 1998.

#### *6.2.3.2 Grain yield, grain plumpness and symptom score*

Viruliferous aphid inoculated (+Aphid) $F_2$ derived $BC_2$ lines carrying the Sloop YLM variant showed a mean grain yield reduction compared to un-inoculated lines of 71% (Table 2). In contrast, lines carrying the Franklin YLM variant showed only a 13% reduction in grain yield. Lines carrying the Franklin YLM variant were over three times higher yielding than lines carrying the Sloop YLM variant in plots inoculated with viruliferous aphids (+Aphids).

Plate 6.1 Barley yellow dwarf virus (BYDV) infected field plots of $BC_1$ lines derived from crosses between the donor parent Franklin and the recurrent parent Sloop, grown at Strathalbyn, South Australia, 1998. The plot showing severe BYDV symptoms (yellowing and stunting) is a $BC_1$ line carrying the Sloop YLM variant while the plot in the foreground showing only minor symptoms is a $BC_1$ line carrying the Franklin YLM variant.

Table 1. Effect of *YLM* variant on decimal growth stage in $F_2$ derived $BC_2$ lines infected with BYDV at Strathalbyn in 1998 and the Franklin x Arapiles doubled haploid population in 1999. Different letters represent a significant difference of pairwise comparison between the variant length classes at P<0.001.

| Experiment and population | YLM variant | Mean Decimal Growth Stage (DGS) | Standard error |
|---|---|---|---|
| 1998/ Sloop $BC_2$ derived | Franklin | 80.5 a | 3.53 |
| lines | Sloop | 79.0 a | 3.16 |
| 1999/ Franklin x Arapiles | Franklin | 47.8 a | 0.9 |
| doubled haploid lines | Arapiles | 47.3 a | 1.1 |

There was no significant difference in the proportion of plump grain (>2.5mm) between lines carrying the Franklin or Sloop YLM variant in uninoculated plots (Table 2). In the presence of viruliferous aphids, lines carrying the Franklin YLM variant retained 40% more grain over a 2.5 mm screen than those carrying the Sloop YLM variant. In addition, viruliferous aphid inoculation of Sloop YLM variant lines resulted in a 40% reduction in the proportion of plump grain whereas there was no significant difference between inoculated and uninoculated lines carrying the Franklin variant.

A highly significant (P<0.000) difference in symptom score was observed between lines carrying Franklin and Sloop YLM variants in viruliferous aphid inoculated plots. The average BYDV symptom score for lines carrying the Franklin YLM variant was 1.7 compared with 5.7 for lines carrying the Sloop variant.

Table 2. Effect of *YLM* variant on grain yield and grain plumpness (%>2.5mm) in $F_2$ derived $BC_2$ lines infected with BYDV at Strathalbyn, SA, 1998 and on grain yield in the Franklin x Arapiles doubled haploid population in 1999. Significance of difference (P) between plots inoculated with viruliferous aphids (+Aphids) and those not (-Aphids) is provided. Different letters represent significant difference of pairwise comparison between YLM variant classes at P<0.001.

| | Year | YLM variant | - Aphids | + Aphids | % of - Aphids | Sign of diff (+/-) (P) |
|---|---|---|---|---|---|---|
| Grain yield (g/plot) | 1998 | Sloop | 1023 a | 316 a | 29 | <0.0001 |
| | | Franklin | 1257 b | 1096 b | 87 | <0.05 |
| | 1999 | Arapiles | 1054 a | | | |
| | | Franklin | 1072 a | | | |
| Plump grain (% > 2.5mm) | 1998 | Sloop | 63 a | 29 a | 34 | <0.0001 |
| | | Franklin | 72 a | 69 b | 3 | N/S |

*6.2.3.3 Malt quality*

There was no significant difference between lines carrying either the Franklin or Sloop YLM variant for all malt quality variables except viscosity where the Franklin YLM variant was associated with 0.04 cP higher viscosity (Table 3).

Table 3. Effect of *YLM* variant on malt quality characteristics in $F_2$ derived $BC_2$ lines grown in uninoculated plots at Strathalbyn, SA, 1998. Different letters represent significant difference of pairwise comparison between YLM variant classes at P<0.001.

| Malt quality variable | YLM variant | Mean | SE |
|---|---|---|---|
| Malt protein (%dry basis) | Sloop | 13.3 a | 0.22 |
| | Franklin | 13.6 a | 0.23 |
| Kolbach Index | Sloop | 39.2 a | 0.63 |
| | Franklin | 38.7 a | 0.66 |
| Diastatic power (μM maltose/min/g) | Sloop | 539 a | 23 |
| | Franklin | 572 a | 25 |
| α-amylase (μM maltose/min/g | Sloop | 132 a | 2 |
| | Franklin | 138 a | 3 |
| β-amylase (μM maltose/min/g) | Sloop | 407 a | 21 |
| | Franklin | 434 a | 23 |
| Hot Water Extract (EBC) (%) | Sloop | 75.4 a | 0.3 |
| | Franklin | 75.2 a | 0.3 |
| Viscosity (cP) | Sloop | 1.52 a | 0.01 |
| | Franklin | 1.56 b | 0.01 |
| Free Amino Nitrogen (mg/l) | Sloop | 153 a | 4 |
| | Franklin | 163 a | 4 |
| Wort β-glucan (mg/l) | Sloop | 176 a | 21 |
| | Franklin | 226 a | 23 |

*6.2.3.4 Leaf Scald resistance*

The intensity of leaf scald symptoms was substantially lower on lines carrying the Franklin YLM variant than the Sloop variant in the leaf scald disease nursery near Gawler, South Australia in 1999 (Table 4). One line carried the Sloop YLM variant but was also resistant to leaf scald (ILS<3). This line was also intermediate (ILS=4) for BYDV symptom score at Strathalbyn in 1998. A very strong relationship ($r^2$=0.80, P<0.001) was established between the intensity of BYDV symptom score in viruliferous aphid inoculated plots in 1998 and the intensity of leaf scald symptoms in 1999 (Fig 6.4).

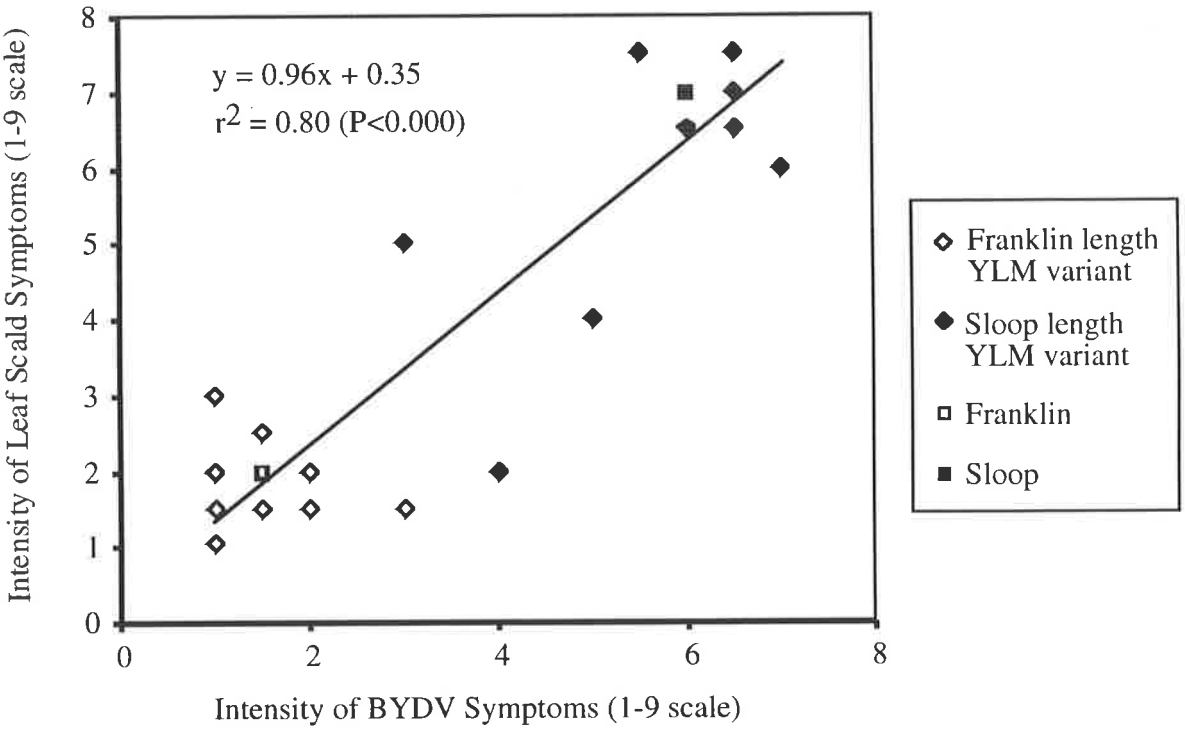



Figure 6.4. Relationship between the intensity of BYDV symptom score in viruliferous aphid inoculated plots at Strathalbyn in 1998 and the intensity of leaf scald symptoms in scald inoculated plots at Turretfield Research Centre 1999.

Table 4. Effect of *YLM* variant on intensity of leaf scald symptoms in $F_2$ derived $BC_2$ lines at Gawler, South Australia, 1999. Different letters represent significant difference of pairwise comparison between the variant length classes at P<0.0001.

| YLM variant | Mean intensity of leaf scald symptom score | Standard error |
|---|---|---|
| Franklin | 1.9 a | 0.4 |
| Sloop | 5.9 b | 0.4 |

### 6.2.4 Discussion

The YLM marker was successfully implemented in marker assisted introgression of the *Yd2* gene conferring resistance to the PAV isolate of BYDV. The YLM marker was used through two cycles of backcrossing with no phenotypic selection undertaken. All $F_2$ derived $BC_2$ individuals identified as carrying the Franklin variant of YLM were shown to be tolerant (ILS<4) of the PAV isolate of BYDV. Tolerance was expressed as substantially reduced leaf symptoms (Fig 6.4), reduced yield loss, and reduced effect on grain size (Table 2). One line designated as carrying the Sloop variant of YLM was rated intermediate for BYDV resistance. DNA for final genotyping was sampled from only three or four plants and therefore it is possible that this line with intermediate resistance was segregating for YLM but only plants homozygous for the Sloop variant were sampled. Alternatively a recombination event may have occurred between the marker and *Yd2* and the line may be homozygous for YLM but segregating for *Yd2*.

In contrast to the findings of Comeau and Jedlinski (1990), no relationship was identified between the presence of *Yd2* and grain yield or grain size under low BYDV pressure (Table 2). The lines carrying the Franklin variant of YLM were 23% ($P<0.000$) higher yielding than those carrying the Sloop YLM variant in the 1998 experiment. Whilst uninoculated plots were treated with systemic insecticide, this would not provide complete protection against BYDV infection. Very low levels of BYDV symptoms were observed in the uninoculated plots. This low level of BYDV in the uninoculated plots is likely to account for a major proportion of the 23% higher yield of the individuals carrying the Franklin YLM variant. In support of this, there was no significant difference in mean grain yield between lines carrying the Franklin YLM variant and lines carrying the Arapiles YLM variant in 1999.

The only malt quality variable found to be associated with the Franklin YLM variant was a small, but significant ($P<0.01$), increase in viscosity (Table 3). There was, however, no significant difference between the parents for this malt quality variable. An epistatic interaction may have occurred between Franklin gene or genes in the region of *Yd2* and one or more chromosome regions of the Sloop background. It is more likely, however, that this result is an anomaly of the small population size. In addition the small increase in viscosity observed would not be considered a significant disadvantage in a conventional commercial brewing processes.

Until recently, Franklin was resistant to all isolates of leaf scald found in southern Australia but new isolates virulent on Franklin have been found since 1997. However, Franklin is still resistant to the predominant isolates found in this region. The *Rh* leaf scald resistance gene, presumed to form a resistance gene complex with *Rh3* and *Rh4* , was mapped to the same region of chromosome 3H as *Yd2* (Paltridge *et al.*, 1998). Lines carrying the Franklin YLM variant were all found to be resistant to leaf scald isolates maintained at the scald resistance screening nursery at Turretfield, near Gawler in South Australia in 1999. The isolates prevalent in this nursery are those currently common to the majority of the southern Australian barley growing region. Resistance has been reported to have broken down in isolated areas (H.Wallwork, pers. comm.). The small population size used in the experiments reported here prevented an assessment of linkage between *Yd2* and scald resistance gene or genes carried by Franklin. It is not clear which, if any, of *Rh*, *Rh3* , *Rh4* or other genes, are carried by Franklin. The adoption of the YLM marker in marker assisted selection for scald resistance derived from Franklin is feasible in southern Australia but not practical because of the likely evolution and spread of new virulent races. The scald resistance associated with the *Yd2* gene conferring BYDV resistance could, however, be beneficial in the building of a pyramid of disease resistances including one or more additional sources of scald resistance.

*Breeding for resistance to BYDV*

Selection for BYDV resistance can rely on natural infection, particularly in areas incurring frequent epidemics, which can be increased by various management methods (Rasmusson and Schaller, 1959). Naturally infected field screening nurseries are highly susceptible to spatial variation error and offer no control over the virus strain or aphid vector present. The most common method used to screen germplasm for resistance to BYDV is viruliferous aphid inoculated nurseries. Highly efficient methods of aphid and plant management have been developed such that very large populations, in excess of 50,000 genotypes, can be effectively screened in a given cropping season (Comeau, 1984; Hewings *et al.*, 1992). Symptom expression in field situations, however, can be highly variable. Variation in time of infection, temperature, plant nutrition, plant maturity, plant height, plant density and tillering ability can all influence the level of symptom expression (Qualset, 1984). Enzyme-linked immunosorbent assay (ELISA) (Lister and Rochow, 1979), serological specific microscopy (SSEM) (Paliwal, 1977), Northern dot blot (method described here) and other biochemical techniques allow quantitative and qualitative detection of BYDV (de Pace *et al.*, 1990). While these methods are not dependent on symptom expression in the field, they

do require accurate inoculation of plants and precise sample extract procedures. Marker assisted selection can overcome all of these problems as it is based on the genotype and not reliant on specific virus strains, aphid vectors and phenotypic expression. Other advantages of marker assisted selection include; (1) screening can be undertaken at early stages of plant development (prior to flowering); (2) the genotype can be reliably (depending on linkage) identified on a single plant; (3) co-dominant markers, such as YLM, can distinguish homozygotes and heterozygotes (*Yd2*-mediated resistance is often expressed in an incompletely dominant or recessive manner (Rasmusonn and Schaller, 1959; Cathedral *et al.*, 1970)); (4) plants to be assayed can be grown in optimum conditions suitable for crossing or maximum seed yield; and (5) plants can be assayed for multiple traits simultaneously. The ability of YLM to identify heterozygotes is of particular benefit in breeding strategies where selection based on $F_1$ material is required. These include strategies for increasing gene frequency in segregating topcross and backcross $F_1$ populations and general backcross methods. This study has demonstrated that marker assisted selection can be used effectively in the indirect selection for BYDV resistance in a backcrossing program.

# Chapter 7

# Selection for recurrent parent background

## 7.1 Accelerated backcrossing strategies for the introgression of the *Ha 2* gene conferring resistance to cereal cyst nematode (*Heterodera avenae*) in barley

### 7.1.1 Introduction

The cereal cyst nematode (CCN), *Heterodera avenae* Woll., is a major root pathogen of cereals in southern Australia and other important important cereal-growing regions of the world. Of the four known sources of resistance to CCN, only *Ha 2* and *Ha 4*, (Cotton and Hayes 1969; Andersen and Andersen 1970; Kretschmer *et al.*, 1997; Barr *et al.*, 1998) confer effective resistance to the single Australian pathotype (Kretschmer *et al.*, 1997; Barr *et al.*, 1998). *Ha 4* is present in two Australian cultivars, Galleon and Barque, while *Ha 2* is present in the Australian cultivar Chebec, providing a comparable level of resistance. The malting quality characteristics of Chebec are considered to be superior to both Galleon and Barque, both of which demonstrate extremely poor malt characteristics. While still of marginal malting quality, with lower diastatic power than required by the malting and brewing industry, Chebec is likely to be a more desirable donor parent in breeding strategies aimed at developing malting quality cultivars with CCN resistance.

Kretschmer *et al.* (1997) located *Ha 2* to a region of chromosome 2HL flanked by the restriction fragment length polymorphism (RFLP) markers *Xawbma21* and *Xmwg694*. The application of the RFLP marker, *Xawbma21,* in the indirect selection for *Ha 2* through three cycles of backcrossing and selection of doubled haploid donor plants was demonstrated in Chapter 6.1 of this thesis.

As described by Beckman and Soller (1986) and demonstrated in Chapter 6.1 and 6.2 of this thesis, molecular markers can be used to monitor and select for introgressed gene(s) in a backcross breeding strategy. The underlying principle of any backcrossing strategy is that the expected proportion of donor parent genome reduces by fifty percent with each generation of backcrossing. Ignoring the effects of linkage drag to the desired donor parent locus under selection, the mean percentage of donor parent genome expected in each backcross generation is calculated as % donor parent genome=$100(0.5)^{n+1}$ where $n$ is the

number of backcrosses. Until recent times, most backcross strategies have focused on this principle and have ignored the genetic variation for the proportion of donor parent genome that exists around the expected mean.

Molecular markers can be used to monitor and select for the desired donor parent locus, and also for identifying recombinant individuals that have genome compositions closer to that of the recurrent parent than would be predicted from theoretical expectations (Tanksley and Rick, 1980). The high multiplex ratio (Powell *et al.*, 1996b) of amplified fragment length polymorphisms (AFLP)(Vos *et al.*, 1996) makes them an ideal marker system for this latter application. Marker assisted selection against donor parent genome, or conversely, selection for recurrent parent background, could provide a means of reducing both the time and number of generations required to adequately recover the recurrent parent genotype and phenotype. The aim of this study is to assess the practical implications of selecting against donor parent alleles in a backcrossing program aimed at introgressing the *Ha* 2 gene conferring CCN resistance into an adapted, malting quality background.

### 7.1.2 Materials and methods

#### *7.1.2.1 Genetic materials and population development*

This study involves phenotypic data derived from breeding populations developed as part of a practical *Ha 2* introgression program. The *Ha 2* donor parent chosen was the Australian cultivar Chebec. Chebec is widely adapted to southern Australian barley growing regions but is of marginal malting quality. The recurrent parent chosen was the widely adapted Australian malting quality cultivar Sloop. Sloop is CCN susceptible but has a number of malt quality advantages over Chebec including superior diastatic power, β-amylase activity, viscosity and free amino nitrogen.

This study involved advanced lines from the following breeding populations;

- ten doubled haploid CCN resistant lines derived from a simple cross between Chebec and Sloop
- fourteen CCN resistant recombinant inbred lines (RILs) derived from a first backcross between the donor parent Chebec and the recurrent parent Sloop
- thirty six CCN resistant doubled haploid lines derived from three marker assisted backcrosses between the donor parent Chebec and the recurrent parent Sloop

- eighteen CCN resistant recombinant inbred lines (RILs) derived from a first backcross between the donor parent Barque (donor parent of *Ha 4*) and the recurrent parent Sloop

A $BC_1$ population involving the donor parent Barque was included for direct comparison with the $BC_1$ population involving Chebec. Barque is also resistant to CCN but carries the *Ha 4* locus on chromosome 5H (Barr *et al.*, 1998). The donor parents Barque and Chebec and the recurrent parent Sloop are related through pedigree links to the cultivars Proctor, Prior and a landrace CI3576. More than 80% of the pedigree of Sloop and Chebec traces to Proctor, Prior and CI3576 while only 30% of Barque's pedigree can be traced to these genotypes. The aim of including Barque derived backcross lines was to compare the recovery of the recurrent parent genotype for two different donor parents.

Selection for CCN resistance involved a combination of molecular (Kretschmer *et al.*, 1997) and the bio-assay system described by Fisher (1982). Since all the genotypes used in the study were advanced breeding lines they had also been selected for flowering time, straw strength, grain size, grain yield, and several malt quality attributes and in this respect, are not random selections from these populations.

Included in a preliminary study of genetic similarity between donor and recurrent parents were other CCN resistant potential donor parents, Galleon (*Ha 4*) and Sahara (*Ha 2*) and cultivars which could be donor parents for other traits including leaf scald (*Rhynchosporium secali*) resistance (Halcyon and Osiris), powdery mildew (*Erysiphi gramini*) resistance (Chariot), boron tolerance (Sahara), *ant-28* mutant (Caminant), semi-dwarf plant type (Skiff), and manganese efficiency (Amaji Nijo).

### *7.1.2.2 DNA extraction, AFLP reactions and PAGE*

DNA extraction followed the methods outlined in Chapter 5. The AFLP method developed by Vos *et al.* (1995) was followed with some modifications. Genomic DNA (1 µg) was digested with the restriction endonucleases *Pst1* and *Mse1*. Double-stranded adapters were ligated to the ends of the restriction fragments followed by suspension in 60 µl of 0.1 M TE. Pre-amplification was performed using primers specific for the *Pst*1 and *Mse* 1 adapters including one selective nucleotide, followed by selective amplification using similar primers *Pst*1 with two and *Mse*1 with three selective bases. The recurrent parent Sloop and donor parents Chebec and Barque were screened with 32 AFLP primer combinations (Table 1).

Ten of the more informative primer combinations were used to screen the 68 simple and backcross derived lines (Table 1).

Table 1. Selective bases for *Pst*1 and *Mse*1 primers used to screen donor and recurrent parents and CCN resistant advanced breeder's lines

| Primer combinations for screening parents | | Primers combinations for population screening (***) | Primer combinations for screening parents | | Primers combinations for population screening (***) |
|---|---|---|---|---|---|
| *Pst*1 selective primer | *Mse*1 selective primer | | *Pst*1 selective primer | *Mse*1 selective primer | |
| AA | CAA | | AG | CAA | |
| AA | CAG | | AG | CAG | *** |
| AA | CAT | | AG | CAT | |
| AA | CCA | | AG | CCA | *** |
| AA | CCT | | AG | CCT | *** |
| AA | CGA | | AG | CGA | |
| AA | CTA | | AG | CTA | |
| AA | CTG | | AG | CTG | |
| AC | CAA | | AT | CAA | *** |
| AC | CAG | | AT | CAG | *** |
| AC | CAT | | AT | CAT | |
| AC | CCA | *** | AT | CCA | |
| AC | CCT | *** | AT | CCT | *** |
| AC | CGA | | AT | CGA | |
| AC | CTA | *** | AT | CTA | |
| AC | CTG | *** | AT | CTG | |

The pre-amplification mix was diluted 1:5 in water before being used in the selective amplification step. Pre-amplification PCR conditions consisted of 20 cycles of 94° C for 30 seconds, 56° C for one minute and 72 ° C for one minute. PCR reaction conditions for selective amplification consisted of one cycle of 94° C for 30 seconds, 65° C for 30 seconds, and 72 ° C for one minute followed by nine cycles over which the annealing temperature was decreased by 1 ° C per cycle with a final step of 25 cycles of 94° C for 30 seconds, 56° C for 30 seconds, and 72 ° C for one minute. Selective amplification was done using γ 32P ATP end-labeled *Pst*1 primers (Feinberg and Vogelstein, 1983).

AFLP fragments were separated by denaturing PAGE (6%) on 36 cm gels. AFLP fragments, polymorphic between Chebec and Sloop and others polymorphic between Barque and Sloop, were scored for the presence or absence of fragments across all individuals.

The proportion of unique donor parent genome in each line was estimated from the number of donor parent polymorphic alleles scored for each line as a proportion of the total number of AFLP products polymorphic between donor and recurrent parent.

*7.1.2.3 Field experiments*

The advanced breeding lines and control cultivars Sloop, Chebec and Barque were sown in field experiments at Brinkworth, Callington, Geranium, Weetulta and Yeelanna, in South Australia in 1998. Plots were six rows wide (1.23m), 4m in length and arranged as randomised complete blocks with three replicates. Field experiments were scored for damage caused by spot form of net blotch (*Pyrenophera teres* f. sp. *maculata* ) at Geranium and Yeelanna, and leaf scald (*Rhynchosporium secalis* ) at Geranium. Disease severity scores were recorded on a scale of 1-9. A score of '1' corresponded to no obvious disease symptoms and score of '9' corresponded to very high density of large lesions with severe necrosis on most plant parts. Plots were machine harvested and grain yield and percentage of plump grain retained over a 2.5mm screen (Yeelanna site only) was calculated from the harvested samples.

*7.1.2.4 Malting quality*

Samples of grain harvested from two replicates of field experiments at Brinkworth and Yeelanna were screened on a 2.5 mm screen and 60g sub-samples micro-malted and malt quality analysis undertaken as described in Chapter 6.1

*7.1.2.5 Statistical analysis*

Means for grain yield at each site were calculated allowing for extraneous variation using spatial techniques developed by Cullis and Gleeson (1991). A cross site analysis was conducted using scaled information of estimates of genotype effects from each of the five sites following the method of Cullis *et al.* (1996). Least-squares means were calculated for all malt quality variables using an ANOVA (SAS Institute, JMP version 3 software) model

with genotype (line), site and rep as main effects, and genotype and site as the interaction effect. Un-replicated data was used for disease scores and grain plumpness.

Lines were grouped into classes based on type of cross (simple cross, $BC_1$ and $BC_3$) and donor parent (Chebec or Barque). Least-squares class means were calculated for all variables measured using a single factor ANOVA (SAS Institute, JMP version 3 software). The single factors were cross type and donor parent. Class means were compared using linear contrasts. Simple regression (SAS Institute, JMP version 3 software) was used to derive relationships between all variables measured and the estimated proportion of unique donor parent genome.

### 7.1.3 Results

Pre-screening of the donor and recurrent parents with 32 AFLP primer combinations produced 72 differentiable AFLP products polymorphic between Chebec and Sloop, and 167 differentiable AFLP products polymorphic between Barque and Sloop. The total number of differentiable AFLP products polymorphic between Chebec and Sloop, and Barque and Sloop, using the ten selected primer combinations were 34 and 39, respectively.

#### *7.1.3.1 Effect of cross type and donor parent on the proportion of unique donor parent genome*

The proportion of unique donor parent genome in individuals from crosses involving Chebec ranged from 8.8% for a $BC_3$ line to 68% for a line derived from a simple cross. The proportion of unique donor parent genome in $BC_1$ individuals derived from Barque ranged from 44% to 65%.

The mean proportion of unique donor parent genome for each type of cross and donor parent is presented in Figure 7.1. There was a highly significant ($P<0.001$) difference in the mean proportion of unique donor parent genome between cross types within Chebec derived crosses. The mean proportion of unique donor parent genome declined with increasing number of backcrosses. There was a highly significant ($P<0.001$) difference in the mean proportion of unique donor parent genome between $BC_1$ individuals derived from the donor parent Chebec and $BC_1$ individuals derived from the donor parent Barque.

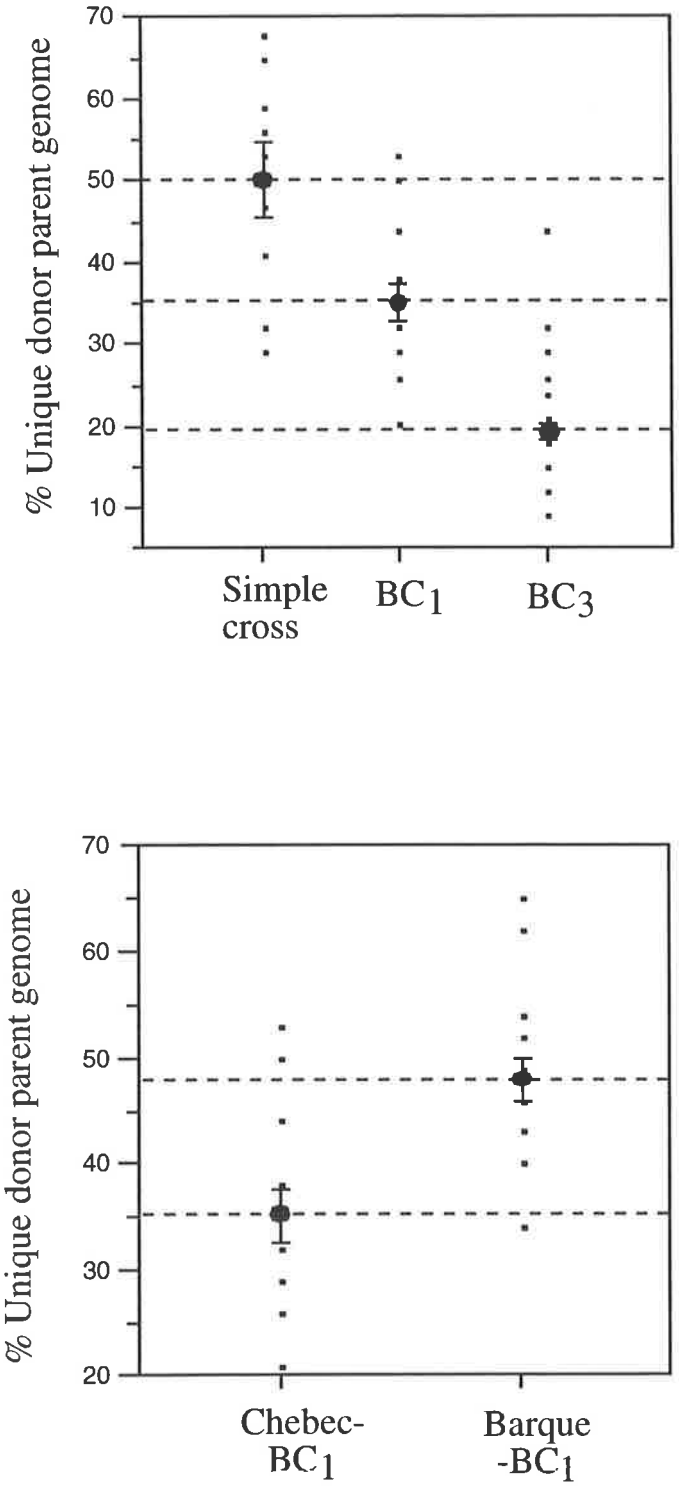



Figure 7.1. The mean proportion of unique donor parent genome for advanced breeders lines derived from simple cross, $BC_1$ and $BC_3$ cross types and two donor parents (Chebec and Barque)

*7.1.3.2 Effect of cross type and donor parent on agronomic and quality performance*

Analysis of variance showed a significant ($P<0.05$) genotype (or line) effect for grain yield, and the malt quality variables DP, α-amylase, β-amylase, hot water extract, FAN, and viscosity. Significant ($P<0.05$) site effects were observed for all malt quality variables, while significant ($P<0.05$) site by genotype interaction effects were observed for DP, FAN, and viscosity (data not shown). There was no significant difference in mean grain yield and proportion of plump grain between lines derived from different cross types (Table 2).

Table 2. Mean grain yield (t/ha) and percentage of plump grain (%>2.5 mm) of advanced breeding lines derived from simple cross and $BC_3$ involving the recurrent parent Sloop and the donor parent Chebec and $BC_1$ lines derived from both Chebec and Barque. Significant ($P<0.01$) differences between the means of cross type and donor parent are indicated by different letters adjacent to means.

| | | | Grain yield (t/ha) | | Plump grain (%>2.5mm) | |
|---|---|---|---|---|---|---|
| Type of cross | Donor parent | Number of lines | Mean | Standard error | Mean | Standard error |
| Simple | Chebec | 10 | 1.08 a | 0.02 | 93.5 ab | 1.2 |
| $BC_1$ | Chebec | 14 | 1.08 a | 0.01 | 90.1 a | 1.0 |
| $BC_1$ | Barque | 18 | 1.10 a | 0.01 | 96.3 b | 0.9 |
| $BC_3$ | Chebec | 36 | 1.06 a | 0.01 | 92.4 ab | 0.6 |

There was also no significant difference in mean grain yield between $BC_1$ lines derived from Chebec and Barque. $BC_1$ lines derived from Barque produced a significantly ($P<0.01$) greater proportion of plump grain.

There was no significant difference between $BC_1$ lines derived from different donor parents for β-amylase and DP (Table 3). $BC_3$ derived lines were, on average, significantly ($P<0.01$) higher in both β-amylase and DP than $BC_1$ or simple cross derived lines. In contrast, the only significant ($P<0.01$) difference in mean α-amylase activity was between $BC_1$ lines derived from the two different donor parents. There was no significant difference in mean hot water extract between lines derived from different cross types or donor parents (Table 4). The $BC_3$ derived lines were significantly ($P<0.01$) lower for viscosity than simple cross derived lines and significantly higher than simple cross or $BC_1$ derived lines for FAN.

Table 3. Mean diastatic power, α-amylase activity, and β-amylase activity of advanced breeding lines derived from simple cross and $BC_3$ involving the recurrent parent Sloop and the donor parent Chebec and $BC_1$ lines derived from both Chebec and Barque. The units for all three variables are expressed as micromoles of maltose released equivalents released per minute per gram dry weight. Significant ($P<0.01$) differences between the means of cross type and donor parent are indicated by different letters adjacent to means.

| Type of cross | Donor parent | Number of lines | Diastatic Power | | α-amylase activity | | β-amylase activity | |
|---|---|---|---|---|---|---|---|---|
| | | | Mean | SE | Mean | SE | Mean | SE |
| Simple | Chebec | 5 | 371 a | 25 | 109 a | 5 | 260 a | 22 |
| $BC_1$ | Chebec | 5 | 412 a | 25 | 116 a | 5 | 294 a | 22 |
| $BC_1$ | Barque | 13 | 375 a | 15 | 97 b | 3 | 276 a | 13 |
| $BC_3$ | Chebec | 18 | 494 b | 14 | 116 a | 2 | 368 b | 12 |
| Controls | Sloop | - | 453 | 38 | 101 | 10 | 352 | 38 |
| | Chebec | - | 378 | 38 | 103 | 10 | 273 | 38 |
| | Barque | - | 389 | 38 | 94 | 10 | 295 | 38 |

Table 4. Mean hot water extract (%dry basis), viscosity (cP) and free amino nitrogen (mg/L) of advanced breeding lines derived from simple cross and $BC_3$ involving the recurrent parent Sloop and the donor parent Chebec and $BC_1$ lines derived from both Chebec and Barque. Significant ($P<0.01$) differences between the means of cross type and donor parent are indicated by different letters adjacent to means.

| Type of cross | Donor parent | Number of lines | Hot water extract (%db) | | Viscosity (cP) | | Free amino nitrogen (mg/L) | |
|---|---|---|---|---|---|---|---|---|
| | | | Mean | SE | Mean | SE | Mean | SE |
| Simple | Chebec | 5 | 78.0 a | 0.4 | 1.62 a | 0.02 | 124 a | 6 |
| $BC_1$ | Chebec | 5 | 77.7 a | 0.4 | 1.60 ac | 0.02 | 133 ac | 6 |
| $BC_1$ | Barque | 13 | 77.4 a | 0.2 | 1.69 b | 0.01 | 112 b | 3 |
| $BC_3$ | Chebec | 18 | 77.9 a | 0.2 | 1.56 c | 0.01 | 143 c | 3 |
| Controls | Sloop | - | 77.9 | 0.8 | 1.55 | 0.04 | 147 | 7 |
| | Chebec | - | 77.8 | 0.8 | 1.62 | 0.04 | 123 | 7 |
| | Barque | - | 75.9 | 0.8 | 1.73 | 0.04 | 100 | 7 |

### 7.1.3.3 *Relationship between proportion of unique donor parent genome and agronomic and malt quality factors among Chebec derived backcross lines*

Simple regression analysis revealed significant ($P<0.01$) relationships between the proportion of unique Chebec genome in individual lines and β-amylase activity ($r^2 = 0.47$), DP ($r^2= 0.49$), viscosity ($r^2= 0.45$), FAN ($r^2= 0.49$), spot form of net blotch symptom severity at Yeelanna ($r^2= 0.34$), and spot form of net blotch symptom severity at Geranium ($r^2= -0.37$) (Fig 7.2). No significant relationships were established between the proportion of unique Chebec genome in individual lines and grain plumpness, hot water extract, α-amylase activity, severity of leaf scald symptoms, and severity of powdery mildew symptoms.

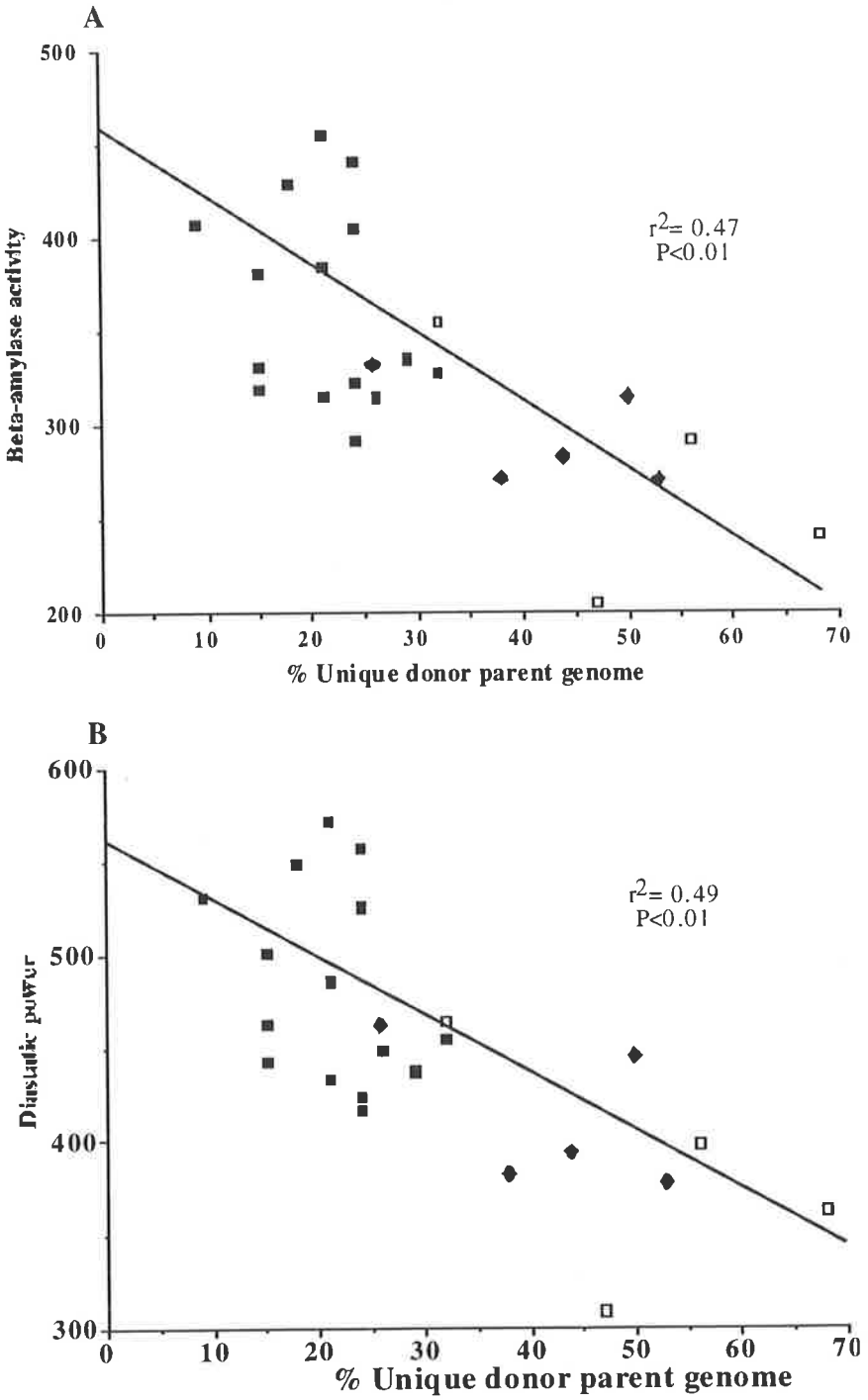



Figure 7.2 (A-B) Relationship between the proportion of unique donor parent genome, based on the number of unique donor parent marker alleles per line as a proportion of the total number of alleles, β-amylase (A) and diastatic power (B). The closed squares relate to $BC_3$ derived lines, the closed triangles relate to $BC_1$ derived lines, while the open squares relate to simple cross derived lines

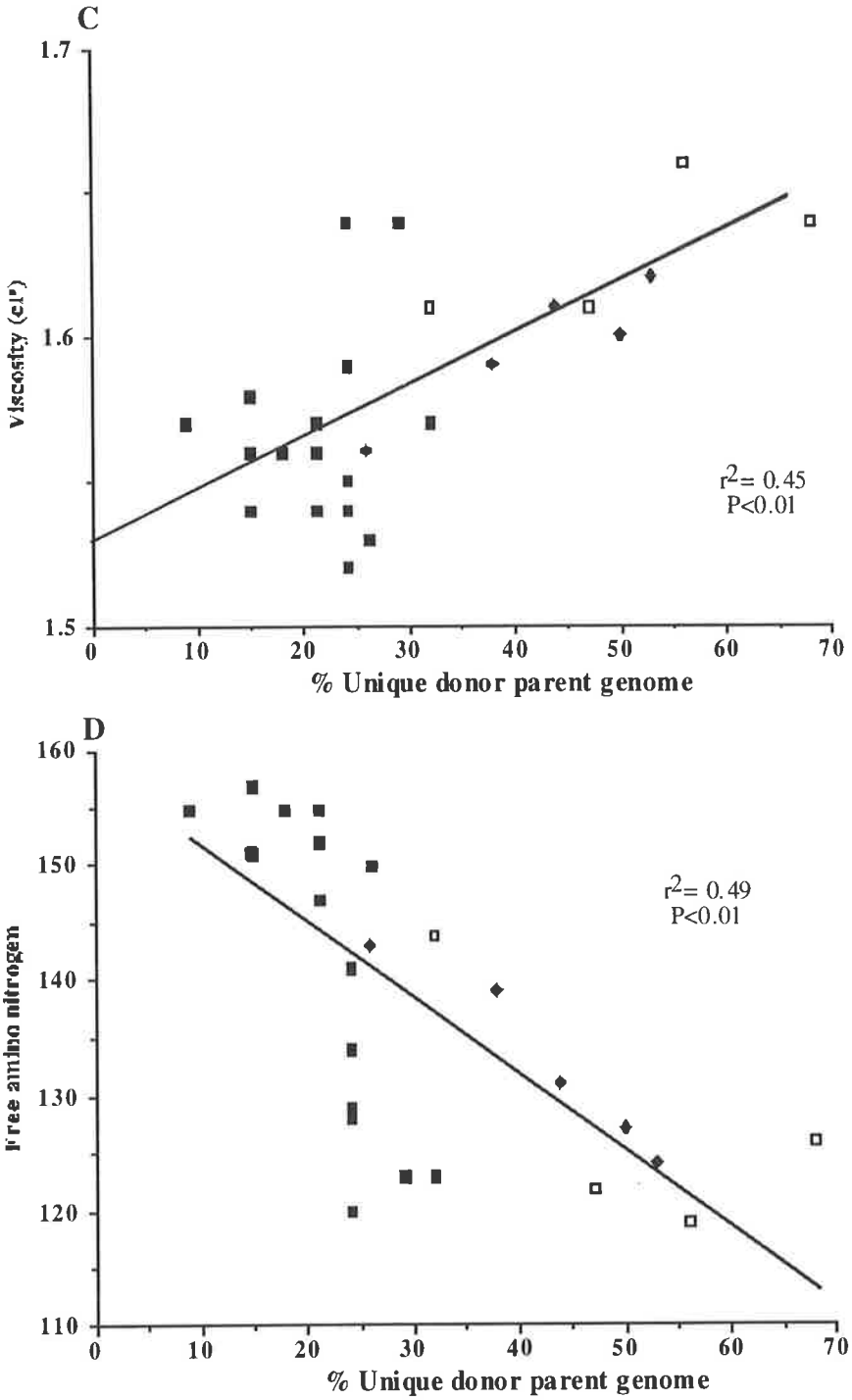



Figure 7.2 (C-D) Relationship between the proportion of unique donor parent genome, based on the number of unique donor parent marker alleles per line as a proportion of the total number of alleles, and viscosity (C) and free amino nitrogen (D). The closed squares relate to $BC_3$ derived lines, the closed triangles relate to $BC_1$ derived lines, while the open squares relate to simple cross derived lines

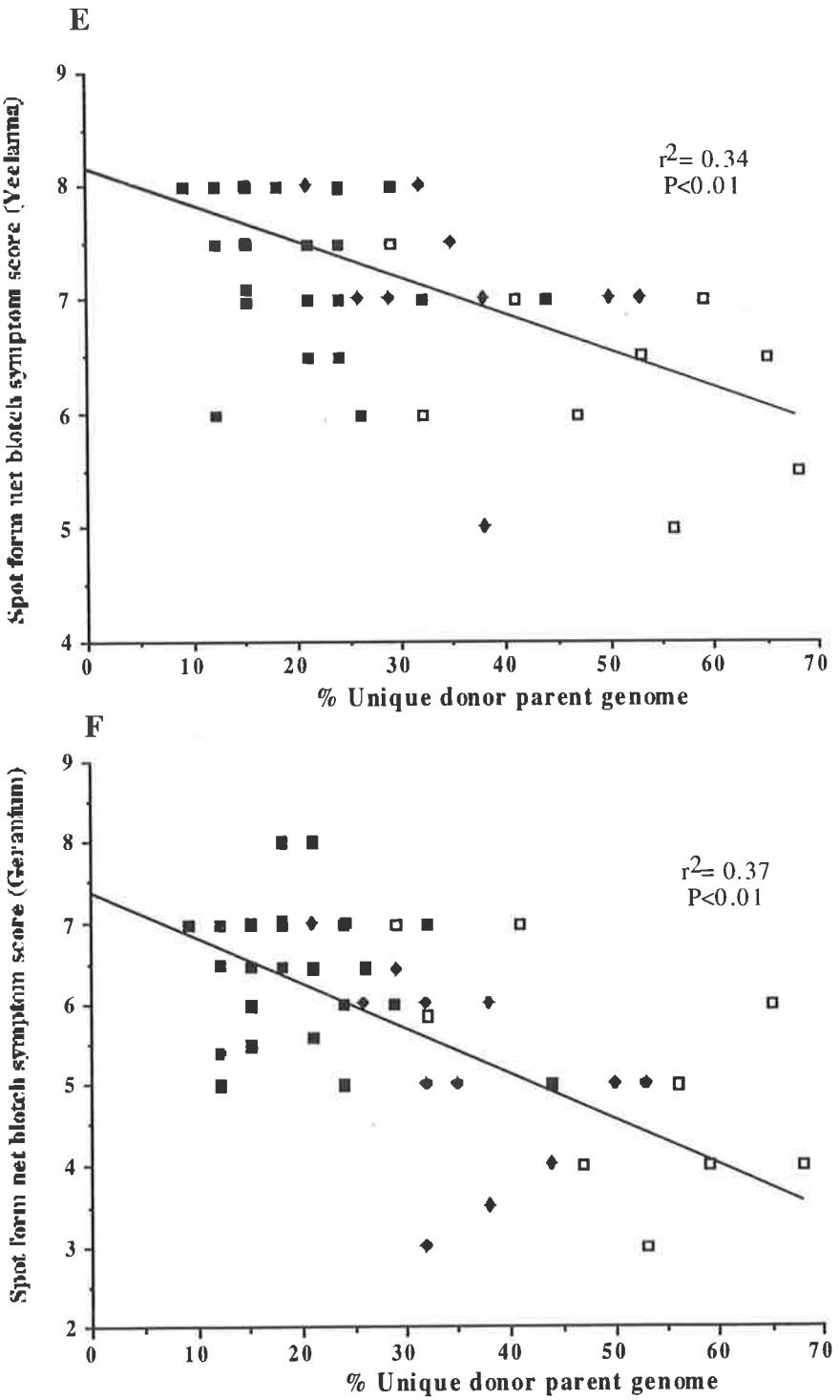



Figure 7.2 (E-F) Relationship between the proportion of unique donor parent genome, based on the number of unique donor parent marker alleles per line as a proportion of the total number of alleles, and susceptibility to spot form of net blotch at Yelleanna (E) and Geranium (F). The closed squares relate to $BC_3$ derived lines, the closed triangles relate to $BC_1$ derived lines, while the open squares relate to simple cross derived lines

### 7.1.4 Discussion

#### *7.1.4.1 Efficiency of selection for recurrent parent background- number of backcrosses*

Openshaw *et al.* (1994) conducted a simulation study on the recovery of the recurrent parent genome in a backcrossing program involving a maize genome model of ten-200 cM chromosomes. In a progeny of 50 selected plants, they found, using one marker loci for every 50 cM of chromosome, individuals carrying 15.5%, 4.8% and 2.4% donor parent genome in the $BC_1$, $BC_2$ and $BC_3$ generations, respectively. In contrast, the lowest proportion of unique donor parent genome found in a single line in the current study was 8.8% in the $BC_3$ generation, and 20.6% in the $BC_1$ generation. Openshaw *et al.* (1994) based selection for recurrent parent background on the presence of donor parent marker alleles. It can therefore be assumed that they also estimated only unique donor parent genome rather than total donor parent genome. Only 34 AFLP marker loci were used to estimate the proportion of donor parent genome in this study. This, however, corresponds to approximately one marker loci for every 30 cM of barley chromosome in comparison to the one marker loci per 50 cM of chromosome Openshaw *et al.* (1994) found to be adequate for efficient marker assisted backcrossing. The populations used in the current study, particularly the $BC_1$ generation, were considerably smaller than in the simulation study. In addition, the $BC_1$ population consisted of advanced RILs, while Openshaw *et al.* (1994) imposed simulation conditions on $F_2$ plants. Donor parent chromosome segments in advanced RILs would be smaller and more frequent than in $F_2$ plants. Hospital *et al.* (1992) and Frisch *et al.* (1999) found that a higher density of markers was required to select for recurrent parent background in advanced generations. Openshaw *et al.* (1994) also assumed even distribution of marker loci. Un-mapped AFLP products were used in the current study and consequently complete and even genome coverage could not be assumed. Map-assigned microsatelittle markers would be of much greater value. Frisch *et al.* (1999) also proposed that even genome coverage was important in optimising the recovery in recurrent parent genome.

In the current study, a single $BC_1$ derived line was identified that carried a proportion of unique donor parent genome not significantly ($P<0.01$) different from the mean of the $BC_3$ generation. Selecting this $BC_1$ individual could therefore have saved two cycles of backcrossing. Frisch *et al.* (1999) performed computer simulations on the recovery of recurrent parent genome in a backcrossing program using a published maize map of 80

markers as the model. Like Hospital *et al.* (1992), and Visscher *et al.* (1996), they found that at least two generations of backcrossing could be saved with selection for recurrent parent background. However, Frisch *et al.* (1999) showed that if a large population size and high marker density were used, then it was possible to recover in the $BC_3$ generation the same proportion of recurrent genome as the $BC_7$ generation without marker assisted selection. Openshaw *et al.* (1994) also suggested that selection for recurrent parent background could reduce the number of backcross generations from seven to three. Empirical results from the current study and those of Powell *et al.* (1996a) showed that at least two generations of backcrossing can be saved.

Results from both simulation and empirical studies support the conclusion that between two to four generations of backcrossing can be saved with marker assisted selection for recurrent parent background. The relative speed and efficiency of different selection strategies, however, depends largely on population size, selection intensity, number of marker loci, knowledge of the location of marker loci, map distance between flanking markers and the target gene and knowledge of the potential for deleterious linkage drag (Hospital *et al.* 1992; Openshaw *et al.* 1994: and Visscher *et al.* 1996; and Frisch et al., 1999). The relative resources available to the breeder would therefore dictate the most efficient selection strategy.

### *7.1.4.2 Recovery of recurrent parent background*

Selection for a low proportion of unique Chebec genome in backcross derived lines would have, theoretically, resulted in the selection of individuals with smaller and less Chebec segments on non-target chromosomes but also smaller donor segments on the target chromosome (2H) around the *Ha2* locus. In this study we showed that the proportion of unique Chebec genome did indeed reduce with each generation of backcrossing and individuals with a substantially lower proportion of unique Chebec genome can be selected. The question remains, however, what proportion of unique Chebec genome is small enough to allow adequate recovery of the Sloop phenotype?

Backcrossing strategies have been used widely to introgress simply inherited traits into elite genetic backgrounds. Elite genetic backgrounds generally carry a combination of desirable traits, many of which are quantitative in inheritance. The desired outcome of the backcross strategy is, therefore, full recovery of the quantitative trait expression of the recurrent parent. The maximum proportion of unique donor parent genome tolerated in backcross progeny

can therefore be estimated from the proportion of unique donor parent genome in progeny that is not significantly different from the recurrent parent in the expression of important quantitative traits.

The recurrent parent chosen for this study, Sloop, combines wide adaptation and several excellent malt quality characteristics including high levels of DP, β-amylase activity, and FAN plus low viscosity. Significant relationships were established between the proportion of unique donor parent (Chebec) genome and the malt quality traits DP, β-amylase activity, FAN and wort viscosity and susceptibility to the spot form of net blotch disease (Fig 7.2 A-F). Using the regression models produced from these significant relationships the proportion of unique Chebec genome in backcross progeny achieving similar malt quality performance to the recurrent parent Sloop was calculated. The maximum proportion of unique Chebec genome carried by backcross progeny which did not differ in malt quality from Sloop, was estimated to be 30.1% for DP, 24.1% for β-amylase activity, 14.5% for FAN, 11.8% for viscosity and 4.1% and 3.0% for spot form of net blotch symptom severity at Yeelanna and Geranium respectively. The wide range in estimated values observed between the various malt quality traits is likely to be a function of the number and location (linkage drag) of genes controlling the trait and the error variance in the assay method for the trait.

DP is a measure of total amylolytic enzyme activity including two major enzyme components, α-amylase and β-amylase. β-amylase is typically the largest component of DP. The *Bamy-2* structural gene, which has a major role in the control of β-amylase activity (Kreis *et al.*, 1987), and therefore DP, has been located to chromosome 2H (Kleinhofs *et al.*, 1993) less than 40 cM (proximal) from the *Ha2* locus (Kretschmer *et al.*, 1997; Langridge *et al.*, 1995). In Chapter 6.1 of this thesis, it was shown that individuals carrying the Chebec allele for *Xawbma21* , located less than 4 cM (proximal) from *Ha2* (Kretschmer *et al.*, 1997), produced significantly ($P<0.01$) lower DP and β-amylase activity than those carrying the Sloop allele. It appears, therefore, that the strong relationships established between the proportion of unique genome and DP and β-amylase activity in the simple cross and backcross derived lines are, at least in part, a function of linkage drag.

Little is known about the number and location of genes controlling FAN and viscosity. No relationship between the presence or absence of the Chebec allele for *Xawbma21* and these two malt quality traits were found in the studies described in Chapter 6.1. FAN is an estimate of the concentration of amino acids, ammonia and the alpha amino nitrogen groups of peptides and proteins in wort. This is related to protein modification during malting,

which in turn is influenced by numerous biochemical and physical processes. A large number of loci behaving either additively, or in interaction with each other, are likely to be important in the expression of high FAN levels in Sloop. Recovery of a high proportion of Sloop genome would therefore be necessary to recover its high level of FAN. While no evidence was found (Chapter 6.1) of linkage drag associated with this trait, the potential of some linkage drag associated with one or more minor genes can not be dismissed.

Viscosity is a measurement of the degree of resistance to the flow of wort and is attributed to the combined effect of a number of cell wall materials including β-glucan, arabinoxylan, protein and polyphenols (Meir *et al.*, 1995; Sudarmana *et al.*, 1996). β-glucan content of malt, a major determinant of wort viscosity, depends upon both barley β-glucan content and β-glucanase activity. Powell *et al.* (1989) reported that barley β-glucan content was controlled by an additive genetic system of three to five "effective factors", but their chromosome locations could not be determined. Han *et al.* (1995) identified significant QTL for barley β-glucan on chromosomes 1H and 2H, the latter approximately 40 cM from *Ha2*. Han *et al.* (1995) also located QTL for malt β-glucan to chromosomes 1H, 3H, 4H and 7H. β-glucanase isoenzymes have been mapped to chromosomes 1H and 7H (Loi *et al.*, 1988), 1H, 4H, 5H, and 7H (Han *et al.*, 1995) and 3H (Li *et al.*, 1996). Recovery of the viscosity levels of Sloop was only achieved by lines with a very low proportion of unique Chebec genome (11.8%). The highly complex nature and inheritance of this trait and the potential for linkage drag between *Ha2* and gene(s) for barley β-glucan content on chromosome 2H, may have contributed to this strong relationship.

No significant relationships were established between the proportion of unique Chebec genome and other complex malt quality traits including hot water extract and α-amylase activity. This lack of relationship was expected given that the parents, Chebec and Sloop, were not significantly ($P<0.01$) different for these traits.

There was no significant relationship found between the proportion of unique Barque genome and the relative performance of the Barque derived $BC_1$ lines for any trait except α-amylase activity. The lack of observed association could be attributed to the small population size and relatively narrow range in donor parent genome amongst this small population, particularly at the low end of the distribution. Nonetheless, a significant relationship was established with α-amylase activity. QTL for α-amylase activity have been located to the short arm of chromosome 5H in three mapping populations and the long arm of chromosome 5H in two mapping populations (Hayes *et al.* 1997). The *Ha4* CCN

resistance gene was located near the centromere of chromosome 5H (Barr *et al.*, 1998). It is likely, therefore, that the significant relationship observed between the proportion of Barque genome and the relative α-amylase activity of Barque $BC_1$ derived lines is due to linkage drag around the *Ha 4* locus on chromosome 5H associated with one or both α-amylase QTL identified.

Sloop is consistently more susceptible than Chebec to spot form of net blotch (H.Wallwork, pers. comm.). A highly significant relationship was identified between the proportion of Chebec genome and susceptibility to spot form of net blotch (Fig 7.2 E-F). The likelihood of linkage between *Ha2* and reduced susceptibility to this diseases in Chebec was not tested in the studies described in Chapter 6.1. It is possible that one or more genes for reduced susceptibility to spot form of net blotch could be located close to the *Ha2* locus on chromosome 2H.

Linkage with genes controlling important traits and the complexity of inheritance of these traits appear to be major factors determining the maximum proportion of donor parent genome tolerated in this backcross strategy. Linkage drag appears to be a significant problem associated with the *Ha2* locus derived from Chebec. The size of the segment surrounding the donor gene is often underestimated (Young and Tanksley, 1989). The potential for significant linkage drag after only three backcrosses is therefore very high.

### *7.1.4.3 Choice of donor parent and recovery of recurrent parent genome*

Only two of the four known sources of CCN resistance, *Ha 2* (carried by Chebec) and *Ha4* (carried by Barque), confer resistance to the Australian pathotype of CCN. Chebec was selected as the donor of CCN resistance in the backcross strategy described in Chapter 6.1 due to its superior malt quality characteristics. If no information was available on the phenotypic performance of either donor parent, then the most suitable donor could be selected on the basis of genetic similarity to the recurrent parent.

On the basis of pedigree information, Chebec is more closely related to Sloop than Barque. This is supported by the substantially larger number of polymorphic AFLP products observed between Barque and Sloop (167), compared with Chebec and Sloop (72), in the screen conducted with 32 AFLP primer combinations. Comparisons of Chebec and Barque derived $BC_1$ populations showed the Barque population to have a significantly ($P<0.01$) greater proportion of unique donor parent genome than the Sloop $BC_1$ population (Fig 7.1).

The higher proportion of unique donor parent genome in the Barque derived $BC_1$ population was reflected in a significantly ($P<0.01$) higher proportion of plump grain, lower α-amylase activity (Table 3), higher viscosity and lower FAN (Table 4) than the Chebec derived $BC_1$ population. Choice of donor parent, therefore, also appears to be an important factor in determining the level and rate of recovery of the recurrent parent phenotype.

### 7.1.5 Conclusion

This study highlighted the problems of linkage drag and recovery of complex quantitative traits in a practical backcrossing strategy. A more effective strategy would be to combine three levels of marker assisted selection;

(1) use of flanking markers covering 10-20 cM around the estimated position of the gene to ensure the donor allele frequency does not decline in later generations of backcrossing (Visscher *et al.* (1996)

(2) use of more distant (30-40 cM) flanking markers to select for small donor segment(s) around the desired gene(s). An alternative approach could be to combine the first two steps by using numerous marker loci saturating the target chromosome, or chromosome arm, and use this marker allele information to select for specific recombinantions around the target gene

(3) select against unwanted donor parent genome using selected mapped marker loci spread approximately 30-40 cM apart, covering the entire genome

The size of backcross populations required, the number of backcross generations required and the probability of achieving a complete backcross conversion with this modified strategy will still depend on a number of factors including;

(1) the degree of linkage drag associated with large donor parent chromosome segments around the target gene(s)

(2) the number, location of, and interaction between genes controlling traits to be recovered from the recurrent parent

(3) genetic and phenotypic distance between donor and recurrent parent

(4) the level of recovery of recurrent parent desired on a trait by trait basis

(5) the likelihood of the donor parent carrying observed or hidden (Tanksley and Nelson, 1996) favourable alleles

Development of an efficient accelerated backcrossing strategy will require precise knowledge of the number and location of genes to be introgressed. In addition effective

markers and efficient marker systems will be required for monitoring and selecting for desired donor parent gene(s) and desirable recombination events in the chromosome region surrounding the introgressed gene. As our understanding of the genetic basis for complex traits and the efficiency of marker systems continue to improve, so will the ability to plan and conduct efficient backcross conversions also improve. However, the potential advantages of genotypic assessment of donor and recurrent parents prior to commencement of a backcross strategy, warrant further investigation.

## 7.2 Implications of genetic distance between donor and recurrent parents on accelerated backcrossing strategies

### 7.2.1 Introduction

As described by Beckman and Soller (1986) and demonstrated in Chapter 6.1 and 6.2, molecular markers can be used to monitor and select for introgressed genes in a backcross breeding programme. Molecular markers can also be used to identify recombinant backcross derived individuals that have genome compositions closer to that of the recurrent parent than would be predicted from theoretical expectations (Tanksley and Rick, 1980). Moreover, results from both simulation (Hospital *et al.* 1992; Openshaw *et al.* 1994: and Visscher *et al.* 1996; and Frisch *et al.*, 1999) and empirical studies (Powell *et al.*, 1996a; and Chapter 7.1 this thesis) support the conclusion that between two to four generations of backcrossing can be saved with marker assisted selection for recurrent parent background. The relative speed and efficiency of different selection strategies, however, depends largely on population size, selection intensity, number of marker loci, knowledge of the location of marker loci, map distance between flanking markers and the target gene and knowledge of the potential for deleterious linkage drag (Chapter 7.1). Not one of the simulation studies on the application of markers in the selection of recurrent parent background, conducted by Hospital *et al.* (1992), Openshaw *et al.* (1994), Visscher *et al.* (1996), and Frisch *et al.* (1999), considered the implications of genetic distance between donor and recurrent parent.

Genetic distance estimates have been used to study relationships amongst cultivars or accessions (Melchinger *et al.* 1994), for predicting genetic variance for quantitative traits (Manjarrez-Sadoval *et al.*, 1997), and for placing parents (cross pollinated species) into heterotic groups (Lee, 1996). They have not, generally, been considered as important factors in accelerated backcrossing strategies. It would be expected, however, that more backcrosses would be required to adequately recover the recurrent parent phenotype and genotype in backcrosses involving genetically distant donor and recurrent parents than with genetically similar parents. The aim of this study, therefore, is to assess;

(1) the impact of genetic distance between donor and recurrent parent on the recovery of the recurrent parent genotype and phenotype, and

(2) the implications of genetic distance on the practical application of recurrent parent non-target genome selection strategies.

## 7.2.2 Materials and Methods

### *7.2.2.1 Genetic Material*

Genetic distance estimates were calculated among 21 cultivars and breeders lines. The cultivars and breeding lines chosen for this study consisted of the recurrent parent Sloop, 12 parents which have the potential to contribute genes of agronomic importance to the recurrent parent, the parents of three mapping populations [including Sloop (recurrent parent) and Franklin (donor parent)], and three other parents important to the South Australian Barley Improvement Program (Table 1).

Four donor parents were chosen for the development of $BC_1$ populations on the basis of genetic and morphological differences and phylogenetic relationships (see Results). The donor parents chosen were;

(3) Chebec, genetically and morphologically similar to recurrent parent Sloop;

(4) Caminant, genetically mid-distant to Sloop, two-row, spring;

(5) Halcyon, genetically mid-distant to Sloop, two-row, winter;

(6) Osiris, genetically distant to Sloop, 6-row, spring

$BC_1$ $F_2$ populations were created from crosses between the four donor parents and the recurrent parent Sloop. Single seed descent was used to progress the $BC_1$ $F_2$ populations to the $F_4$ generation. Seed of the Halcyon $BC_1$ population was germinated on filter paper soaked in water contained within sealed petri-dishes maintained in the dark at 4 ° C for 6 weeks prior to planting at each generation to ensure lines requiring vernalisation were retained. Seed from approximately 100 $BC_1$ $F_4$ single plants from each population was multiplied over the 1998/99 summer to provide sufficient seed for field trials in 1999. The necessity of a summer multiplication precluded the inclusion of winter types of the Halcyon $BC_1$ population in 1999 field trials. Sufficient seed of 41 Halcyon $BC_1$ lines, 95 Caminant $BC_1$ lines, 109 Chebec $BC_1$ lines, and 113 Osiris $BC_1$ lines were produced for 1999 field trials.

Table 1. Cultivars and breeders lines assessed for genetic distance from the recurrent parent Sloop

| | Genotype | Origin | Winter/ Spring | 2 row/ 6 row | Donor trait |
|---|---|---|---|---|---|
| *Donor Parents* | Amagi Nijo | Japan | Spring | 2 row | Manganese efficiency |
| | B1602 | USA | Spring | 6 row | Common root rot resistance |
| | Barque | Australia | Spring | 2 row | CCN resistance |
| | Caminant | Denmark | Spring | 2 row | Ant-free mutant |
| | Chariot | United Kingdom | Spring | 2 row | Powdery mildew resistance |
| | Chebec | Australia | Spring | 2 row | CCN resistance |
| | Franklin | Australia | Spring | 2 row | BYDV resistance |
| | Halcyon | United Kingdom | Winter | 2 row | Leaf scald resistance |
| | Osiris | France | Spring | 6 row | Leaf scald resistance |
| | Sahara | Algeria | Spring | 6 row | Boron toxicity tolerance |
| | Skiff | Australia | Spring | 2 row | Semi-dwarf plant type |
| | Sultan | | Spring | 6 row | Leaf scald resistance |
| | Waveney | United Kingdom | Winter | 2 row | Leaf scald resistance |
| *Mapping Parents* | Alexis | Germany | Spring | 2 row | |
| | Arapiles | Australia | Spring | 2 row | |
| | Kaputar | Australia/ Mexico | Spring | 2 row | |
| | Tallon | Australia | Spring | 2 row | |
| *Important Australia Parents* | Gairdner | Australia | Spring | 2 row | |
| | Keel | Australia | Spring | 2 row | |
| | WI3102 | Australia | Spring | 2 row | |

Table 2. Selective bases for *Pst*1 and *Mse*1 primers used to screen cultivars, breeders lines and $BC_1$ populations

| Primer combinations for screening cultivars and breeders lines | | Primer combinations selected to screen $BC_1$ populations (***) |
|---|---|---|
| *Pst*1 selective primer | *Mse*1 selective primer | |
| AA | CAA | |
| AC | CAT | *** |
| AG | CAA | *** |
| AG | CAG | |
| AG | CAT | |
| AG | CTG | |
| AT | CAA | *** |
| AT | CCA | |
| AT | CTG | |

*7.2.2.2 Field experiments*

The four $BC_1$ populations were sown in adjacent field plot experiments at Strathalbyn, South Australia, in 1999. Field plots consisted of two rows (0.52m), four meters in length, arranged as single replicate nurseries with alternating donor and recurrent parent check plots every fifth plot. Individual plots were rated for Decimal Growth Stage (DGS) (Zadoks *et al.*, 1974) when Sloop check plots were near completion of flowering (DGS 67-68). At the same DGS, plots were also rated for plant height, measured from the base of the plant to the base of the head, and for head type (two-row/six-row). Variation in plant establishment was evident in the Chebec and Halcyon $BC_1$ populations. The total number of plants in one meter of randomly selected row was assessed for each plot in both populations. At full maturity, all plots were harvested mechanically and grain yield and the proportion of grain retained above a 2.5mm screen determined from the harvested sample.

*7.2.2.3 DNA extraction and multifluorophore AFLP analysis*

DNA extraction followed procedures outlined in Chapter 5. Genomic DNA (1 µg) was digested with the restriction endonucleases *Pst1* and *Mse*1. Double-stranded adapters were ligated to the ends of the restriction fragments followed by suspension in 60 µl of 0.1 M TE. Pre-amplification was performed using primers specific for the *Pst*1 and *Mse*1 adapters

including one selective nucleotide, followed by selective amplification using fluorescent-labelled (FAM) *Pst*1 and *Mse*1 primers, *Pst*1 with two and *Mse*1 with three selective bases. The 21 cultivars and breeders lines were screened with nine AFLP primer combinations (Table 2). Three of these primer combinations were used to screen the 370 $BC_1$ lines and control varieties (Table 2).

The pre-amplification mix was diluted 1:5 in water before being used in the selective amplification step. Pre-amplification PCR conditions consisted of 20 cycles of 94°C for 30 seconds, 56° C for one minute and 72 °C for one minute. PCR reaction conditions for selective amplification consisted of one cycle of 94°C for 30 seconds, 65°C for 30 seconds, and 72 °C for one minute followed by nine cycles over which the annealing temperature was decreased by 1 °C per cycle with a final step of 25 cycles of 94° C for 30 seconds, 56°C for 30 seconds, and 72 °C for one minute.

AFLP fragments were separated by PAGE on a ABI Prism 373 DNA sequencer on 24 cm gels using 6% denaturing polyacrylamide. GS-500-TAMRA labelled size standard (Perkin Elmer) was loaded on each lane in order to facilitate the automatic analysis of the gel and the sizing of the fragments. Semi-automated AFLP fragment analysis was performed with GENESCAN 2.1 software. The software was used to estimate detection time, signal peak height and surface for each fragment. Sizing of the fragments was performed by the Genescan software by interpolation to the internal lane standard according to the Local Southern algorithm. Signal peak height and surface was used to determine the presence or absence of a specific sized AFLP fragment which was scored as "1" present or "0" as absent.

*7.2.2.4 Statistical analysis*

AFLP fragments detected by all primer combinations for each cultivar or breeders line were used to make comparisons with all other cultivars or breeders lines. All fragments of the same size derived from the same primer combination were assumed to identify the same allele. Genetic distance was estimated according to the methods of Nei and Li (1979). Genetic distance is defined as the extent of genetic difference between cultivars, as measured by allele frequencies at a sample of loci (Nei, 1987). Genetic distance analysis of AFLP data was carried out using PAUP (Version 4.0b3a), a Macintosh computer software package developed by Swaford (1993). The measure of distance among cultivars was the covariance of allele frequencies summed for all fragments scored. The genetic distance

matrix was used for cluster analysis using the Unweighted Pair Group Method of Averages (UPGMA).

Raw data for each trait from each of the four populations was adjusted for variation in establishment scores (Chebec and Halcyon $BC_1$ populations), and extraneous variation using spatial techniques developed by Cullis and Gleeson (1991). Population means and standard deviations were calculated using SAS Institute, JMP version 3 software. Simple regression analysis (SAS Institute, JMP version 3 software) was used to derive relationships between the phenotypic variables measured and the number of donor parent alleles per line in each population.

### 7.2.3 Results

#### *7.2.3.1 Genetic distance between cultivars and breeding lines*

The nine primer combinations generated a total of 448 polymorphic alleles (over the 21 cultivars and breeders lines), ranging from 34 to 63 polymorphic alleles per primer set. The genetic distance (GD) coefficient between the recurrent parent Sloop and each of the 20 other cultivars and breeders lines ranged from 0.035 for Chebec to 0.123 for Kaputar (Table 3). Pairwise genetic distance estimates among donor parents were also calculated but this data is not presented. The parents found to be the most genetically similar to each other were the Australian cultivars Chebec and Arapiles (GD=0.032) which both have strong pedigree links to Proctor, Prior and CI3576. The most genetically different parents were the Algerian landrace Sahara and the Japanese malting quality cultivar, Amagi Nijo (GD=0.139).

Table 3. Pairwise genetic distance (GD) coefficients between Sloop and 20 cultivars/breeders lines

| | Genotype | Pedigree | GD |
|---|---|---|---|
| *Donor Parents* | Amagi | Complex cross involving Plumage Archer, Prior, Hanna, Golden Melon | 0.057 |
| | Nijo | | |
| | B1602 | | 0.103 |
| | Barque | Triumph/Galleon | 0.058 |
| | Caminant | | 0.078 |
| | Chariot | Dera/CSB626/12 | 0.076 |
| | Chebec | Orge Martin/2*Clipper//Schooner | 0.035 |
| | Franklin | Shannon/Triumph | 0.074 |
| | Halcyon | Warboys/Maris Otter | 0.083 |
| | Osiris | Herta/Pallidum191//Kenia/3/Monte Cristo/Minerva//CI1179/Deba | 0.107 |
| | Sahara | Algerian landrace | 0.116 |
| | Skiff | A.Deba/3/Proctor/CI3576//CPI18197/ Beka/4/Clipper/Diamant//Proctor/CI3576 | 0.064 |
| | Sultan | Balder/3/Agio/Kenia//Arabische | 0.103 |
| | Waveney | Halcyon/Palomino | 0.090 |
| *Mapping Population Parents* | Alexis | St 1622/Triumph | 0.085 |
| | Arapiles | Noyep/Proctor//CI3576/Union/4/Kenia/3/Research//Noyep/Proctor/5/Domen | 0.052 |
| | Kaputar | 5604/1025/3/Emir/Shabet//CM67 | 0.123 |
| | Tallon | Triumph/Grimmett | 0.072 |
| *Important Australian Parents* | Gairdner | Franklin/Onslow | 0.086 |
| | Keel | Clipper/CPI18197//Mari/CM67 | 0.089 |
| | WI3102 | WI2808/3/Skiff/Haruna Nijo | 0.077 |
| *Recurrent Parent* | Sloop | WI2395/Golden Promise// Schooner/Norbet/3/Schooner | |

Phylogenic relationships among the 21 cultivars and breeders lines, based on AFLP data, are represented in Figure 7.4. Phylogenic analysis separated the six-row cultivars B1602, Sultan, Osiris, and Sahara, from the two-row cultivars. Kaputar, a spring two-rowed introduction from CIMMYT, was separated uniquely as was the Australian cultivar Keel and

the single Japanese cultivar, Amagi Nijo. The closely related winter cultivars Halcyon and Waveney formed a distinct cluster. A group of Australian and European cultivars, with pedigree links to Triumph, formed a distinct cluster from the remaining group of Australian cultivars, with most members of this cluster having strong pedigree links to Proctor, Prior and CI3576. From the genetic similarity information provided in Table 3 and the phylogenic relationships represented in Figure 7.4, the parents Chebec, Caminant, Halcyon and Osiris were chosen as donor parents for assessing the impact of genetic distance on recovery of recurrent parent phenotype and genotype. Based on AFLP data, Chebec was genetically the most similar (GD=0.035) and clustered with Sloop amongst other Australian cultivars with strong pedigree links to Proctor, Prior, and CI3576. Caminant was genetically mid-distant from Sloop (GS= 0.078) and clustered with European and Australian cultivars with strong pedigree links to Triumph. Halcyon was also genetically mid-distant from Sloop (GS= 0.083) but clustered uniquely with the other winter variety Waveney. Osiris was genetically distant from Sloop (GS=0.107) and clustered with other six-row cultivars. Table 4 provides the GD matrix for the four donor parents and the recurrent parent Sloop. Caminant and Halcyon had similar pairwise GD coefficients with Sloop but were found to be genetically as distant from each other (GD= 0.073) as they were from Sloop (Table 4).

Table 4. Genetic distance coefficient (Nei and Li, 1979) matrix, based on AFLP data, among selected donor parents and the and recurrent parent Sloop

| | Sloop | Chebec | Caminant | Halcyon |
|---|---|---|---|---|
| Chebec | 0.035 | | | |
| Caminant | 0.078 | 0.071 | | |
| Halcyon | 0.083 | 0.085 | 0.073 | |
| Osiris | 0.107 | 0.113 | 0.125 | 0.123 |

*7.2.3.2 Population means and standard deviations*

The frequency distributions for plant height, decimal growth stage, grain yield, and grain plumpness for each $BC_1$ population are provided in Figure 7.5, Figure 7.6, Figure 7.7 and Figure 7.8 respectively. Table 5 provides the population mean and standard deviation for each trait and each population together with the unadjusted means of the Sloop controls across all four populations. Table 6 provides the trait means for the donor parents and recurrent parent Sloop. In general, population means for the four traits were significantly ($P<0.05$) different from each other with the exceptions of plant height and grain plumpness which were not significantly different for the Chebec and Halcyon $BC_1$ populations. The

means for all four traits measured on the Chebec $BC_1$ population were not significantly different from the mean of the recurrent parent Sloop (Table 5). The Halcyon $BC_1$ population was not significantly different to the recurrent parent for plant height and grain plumpness while the Osiris $BC_1$ population was not significantly different for decimal growth stage. The Caminant $BC_1$ population was significantly ($P<0.01$) different from the recurrent parent for all four traits. For the purpose of comparison, the means and standard error for the four traits measured on the donor parents are presented in Table 6.

Table 5. Population means and standard deviations (Std Dev) for four quantitative traits measured on four $BC_1$ populations involving the donor parents Chebec, Caminant, Halcyon and Osiris, and the recurrent parent Sloop. Means with different letters are significantly different at $P<0.05$

| Population | Plant Height (cms) | | Decimal Growth Stage | | Grain yield (kg/plot) | | Grain Pumpness (%>2.5mm) | |
|---|---|---|---|---|---|---|---|---|
| | Mean | Std Dev | Mean | Std Dev | Mean | Std Dev | Mean | Std Dev |
| Chebec $BC_1$ | 70.4 a | 2.8 | 56.7 a | 0.6 | 466.4 a | 5.6 | 94.0 a | 2.1 |
| Caminant $BC_1$ | 66.6 b | 6.1 | 54.7 b | 4.6 | 459.2 b | 9.6 | 85.1 b | 12.8 |
| Halcyon $BC_1$ | 68.8 ac | 4.2 | 53.0 c | 5.4 | 414.3 c | 38.2 | 91.4 ac | 3.9 |
| Osiris $BC_1$ | 76.5 d | 6.3 | 56.7 a | 3.2 | 446.0 d | 5.6 | 84.7 d | 12.8 |
| Sloop controls | 69.6 ac | 1.7 | 57.0 a | 0.4 | 465.9 a | 40.3 | 93.6 ac | 2.6 |

Table 6. Means and standard error (SE) for four quantitative traits measured on control plots of the donor parents, Chebec, Caminant, Halcyon and Osiris, and the recurrent parent Sloop. Means with different letters are significantly different at $P<0.05$

| Population | Plant Height (cms) | | Decimal Growth Stage | | Grain yield (kg/plot) | | Grain Plumpness (%>2.5mm) | |
|---|---|---|---|---|---|---|---|---|
| | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| Sloop | 69.6 a | 0.4 | 57.0 a | 0.1 | 465.9 a | 6.3 | 93.6 a | 0.7 |
| Chebec | 70.0 a | 0.6 | 56.9 a | 0.2 | 473.0 b | 12.3 | 94.0 a | 1.3 |
| Caminant | 60.8 b | 0.7 | 43.0 b | 0.1 | 439.8 c | 14.9 | 84.2 b | 1.6 |
| Halcyon | 59.4 b | 0.9 | 32.0 c | 0.1 | 285.5 d | 19.0 | 90.0 c | 2.0 |
| Osiris | 91.1 c | 0.4 | 53.4 d | 0.1 | 416.2 e | 12.0 | 64.5 d | 1.3 |

Table 7 presents the percentage of $BC_1$ lines in each population 5% and 10% greater or less than the mean of the recurrent parent. The relative proportion of lines different to the recurrent parent varied for each trait and each population except that the Chebec $BC_1$ population had the lowest proportion of lines either 5% or 10% different from the recurrent parent for all four traits.

The Chebec $BC_1$ population, generally, had the lowest standard deviation for all traits except grain yield, where it was not different to that of the Osiris $BC_1$ population.

*7.2.3.3 AFLP analysis of $BC_1$ populations*

The three AFLP primer combinations used to screen each of the $BC_1$ populations produced 85 polymorphic alleles. Of these, 55 were segregating in the Osiris $BC_1$ population, 22 in the Caminant $BC_1$ population, 24 in the Halcyon $BC_1$ population, and 11 in the Chebec $BC_1$ population (Table 6).

Regression analysis revealed no significant relationship between the number of Chebec or Halcyon alleles and the four traits assessed in the Chebec and Halcyon $BC_1$ populations respectively. A strong negative relationship ($P<0.005$) was established between the number of Caminant alleles and grain yield in the Caminant $BC_1$ population but no significant relationship was established for the other three traits. The number of Osiris marker alleles were significantly ($P<0.01$), negatively, associated with decimal growth stage and significantly ($P<0.001$), positively, associated with plant height but were not related to grain yield or grain plumpness.

Table 7. Percentage of $BC_1$ lines in each population 5% and 10% greater or less than the mean of the recurrent parent

| | Plant Height (cms) | | Decimal Growth Stage | | Grain yield (kg/plot) | | Grain Plumpness (%>2.5mm) | |
|---|---|---|---|---|---|---|---|---|
| | 5% | 10% | 5% | 10% | 5% | 10% | 5% | 10% |
| Chebec $BC_1$ | 16 | 0 | 0 | 0 | 0 | 0 | 3 | 0 |
| Caminant $BC_1$ | 52 | 23 | 37 | 18 | 3 | 0 | 48 | 29 |
| Halcyon $BC_1$ | 29 | 15 | 41 | 20 | 83 | 51 | 17 | 7 |
| Osiris $BC_1$ | 60 | 42 | 32 | 4 | 30 | 0 | 54 | 29 |

The number of donor parent marker alleles per line provides an estimate of the proportion of unique donor parent genome carried in each line. The number of donor parent marker alleles per line ranged from 0 to 9 (mean of 3.3 and standard error of 0.2), for the Chebec $BC_1$ population, and from 5 to 29 (mean of 13.9 and standard error of 0.5) for the Osiris $BC_1$ population (Table 8 and Figure 7.3). The mean and standard deviation for the number of donor parent marker alleles for each population was related to the genetic distance between the parents used in those populations (Table 8 and Fig 7.3). That is, the greater the genetic distance between donor and recurrent parent, the greater the number of polymorphic marker alleles, the greater the mean number of donor parent marker alleles per line, and therefore the greater the proportion of unique donor parent genome carried by each line. The genetic distance between Halcyon and Sloop (0.083) was found to be marginally greater than the genetic distance between Caminant and Sloop (0.078) yet the standard deviation and range was greater for the Caminant $BC_1$ population. The Halcyon $BC_1$ population consisted of only 41 partly selected lines (winter types not included) while the Caminant $BC_1$ population consisted of 95 random lines. These factors combined, are likely to have contributed to lower standard deviation and range in the number of donor parent marker alleles in the Halcyon $BC_1$ population (Fig.7.3 and Table 8).

Table 8. The mean number of donor parent alleles in $BC_1$ populations

| | Genetic distance coefficient[1] | Number of polymorphic AFLP alleles | Number of donor parent alleles per $BC_1$ derived line | | | |
|---|---|---|---|---|---|---|
| | | | Mean | Standard deviation | Standard error | Range |
| Chebec $BC_1$ | 0.035 | 11 | 3.3 | 1.9 | 0.2 | 0-9 |
| Caminant $BC_1$ | 0.078 | 21 | 7.0 | 3.2 | 0.3 | 1-17 |
| Halcyon $BC_1$ | 0.083 | 24 | 10.9 | 2.3 | 0.4 | 6-16 |
| Osiris $BC_1$ | 0.107 | 55 | 13.9 | 5.5 | 0.5 | 5-29 |

[1] Genetic distance coefficient (Nei and Li, 1979) between donor parents and the recurrent Sloop

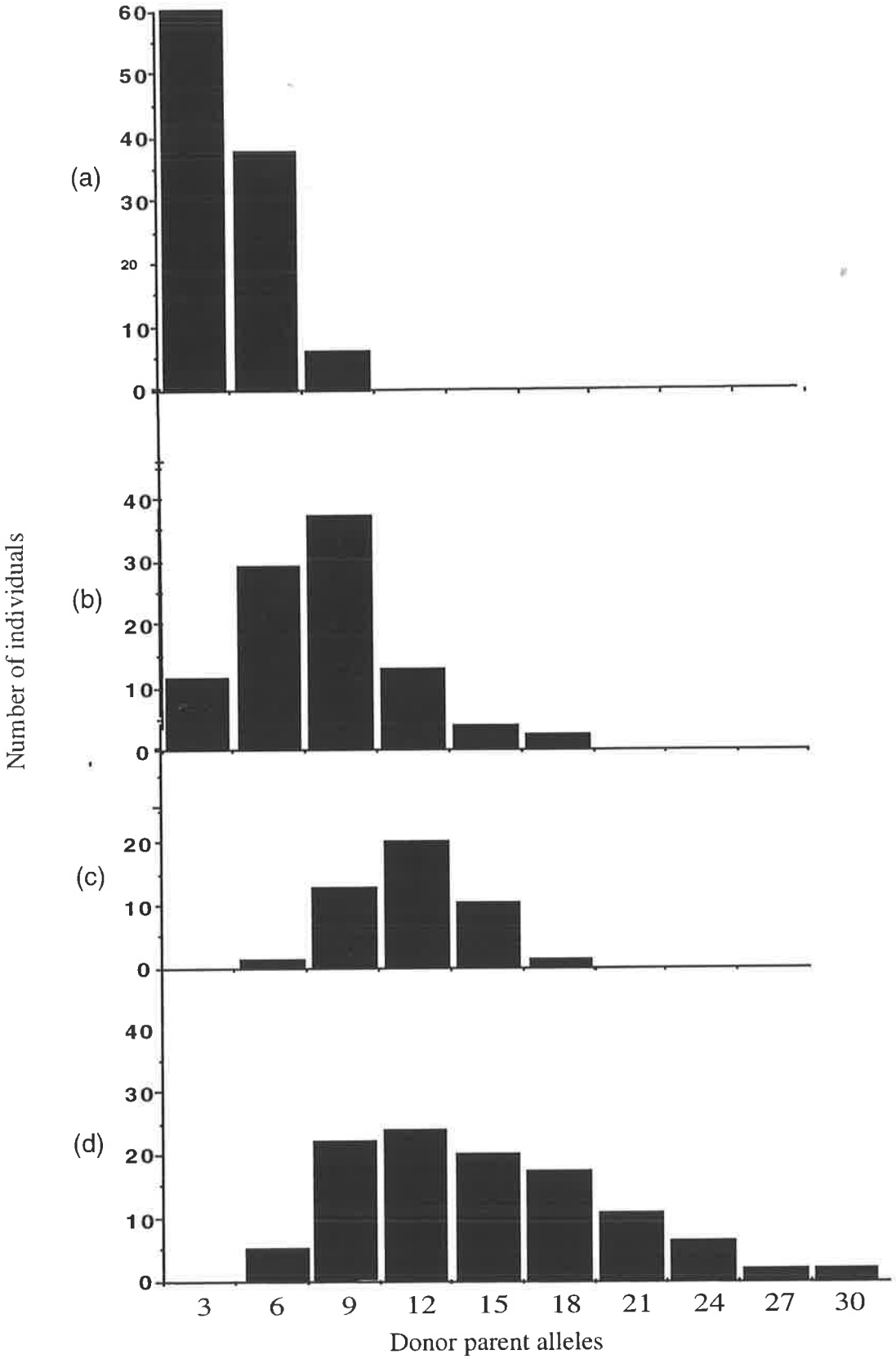



Figure 7.3 Frequency distributions for the number of donor parent marker alleles per line in $BC_1$ populations involving the recurrent parent Sloop and the donor parents (a) Chebec, (b) Caminant, (c) Halcyon and (d) Osiris

### 7.2.4 Discussion

#### *7.2.4.1 AFLP analysis*

The application of AFLP fingerprinting techniques provide a powerful tool in whole genome applications such as mapping and marker assisted backcrossing. The AFLP technique has a major advantage over other marker systems for these applications. A large number of highly polymorphic, randomly distributed, markers are generated in a single reaction which is highly reproducible due to the stringent PCR conditions. Conventional visualization of AFLP fragments by autoradiography or silver-staining, however, has several limitations including; (1) low throughput, as only one sample per gel lane can be analysed; (2) accurate allele typing is often not feasible due to poor resolution of large fragments and/or migration-variability from lane to lane as well as from one run to another; (3) interpretation problems associated with identical, or near identical, sized bands but of different sequence and; (4) visual scoring of segregation data and manual input is time consuming and provides opportunity for increased error (Schwarz *et al.*, 2000). Schwarz *et al.* (2000) recently presented a fluorescence-based, semi-automated procedure for AFLP fragment analysis that overcomes most of these problems. The use of fluorescence dyes with distinguishable wavelength emissions allows electrophoresis of different samples simultaneously in a single lane. In addition, the co-electrophoresis of an internal size standard in every lane, labelled with a different fluorescent dye, provides precise sizing to correct band shifting within and between gel runs (Schwarz *et al.* (2000).

The fluorescence-based AFLP method was applied in this study for establishing genetic distance estimates between potential donor parents and for screening of backcross populations for the proportion of recurrent parent background. Three primer combinations were used simultaneously on any one gel. From each gel, a large number (mean of 149) of polymorphic alleles were generated across the 21 cultivars and breeders lines. Each gel carried 64 lanes and consequently all but one of the backcross populations spanned more than one gel. The number and size of polymorphic alleles identified within a backcross population was found to be highly repeatable between lanes and across gels.

#### *7.2.4.2 Genetic distance*

Russell *et al.* (1997) derived relationships among 18 cultivated barley accessions using RFLP, AFLP, RAPD and SSR data and utilised principal co-ordinate analysis on the

combined data. They found accessions clearly separated into spring and winter types. Among the winter types, the two-rowed and six-rowed cultivars formed two distinct groups with the two-rowed types forming an intermediate group between the spring and six-rowed winter types. Similar arrangements were observed for both AFLP and RFLP data. Melchinger *et al.* (1994), using RFLP data on 48 European barley cultivars, also observed a clear separation between spring and winter types, sub-grouping of two-row and six-row types, and further sub-grouping of cultivars with similar pedigrees. Chalmers *et al.* (in press) also assessed the application of RFLPs and AFLPs for the differentiation and estimation of genetic relationships between 96 barley accessions that have been widely grown in Australia or have played a major role as parents of modern cultivars in Australia. They found that cluster analysis separated accessions at the species level and at the six-row/two-row level. The two-row cultivars were further separated into major geographic/selection environment groups including a North Amercian cluster, a European cluster including some northern Australian cultivars with strong pedigree links to Triumph, and a major Australian cluster with strong pedigree links to Proctor, Prior and CI3576. Chalmers *et al.* (in press) further separated the Proctor/Prior/CI3576 cluster into three groups associated with each of these parents. In the present study, cluster analysis of AFLP data from the 21 selected cultivars and breeding lines, produced similar clustering patterns with the exception that six row cultivars were separated from the two-row cultivars prior to the separation into winter and spring types (Figure 7.4). Like Chalmers *et al.* (in press), this study generally separated Australian breeding germplasm into distinct clusters with strong pedigree links to either Triumph or to Proctor, Prior and CI3576.

The donor parents used in this study were selected on the basis of genetic distance estimates (Table 3), morphological and phenological differences (Table 2), and clustering patterns (Figure 1). In the latter case, an individual was chosen from the six-row cluster (Osiris), the winter-cluster (Halcyon), the Australian and European cluster with strong pedigree links to Triumph (Caminant), and the Australian cluster with strong pedigree links to Proctor, Prior and CI3576 (Chebec).

*7.2.4.3 Effect of genetic distance between donor and recurrent parent on recovery of the recurrent parent phenotype and genotype.*

Due to seed limitations, the field experiments involving the $BC_1$ populations were arranged as adjacent single replicate nurseries and therefore assessing the number, or relative proportion, of lines statistically significantly different to the recurrent parent was not

possible. In this study the number of lines plus or minus 5% different from the mean of the recurrent parent, and the number of lines plus and minus 10% different to the recurrent parent for each of the four traits assessed, was used as an indicator of the degree of recovery of the recurrent parent phenotype.

The only evidence of a relationship between genetic distance and phenotype was the observation that the Chebec $BC_1$ population (Chebec was shown to be genetically most similar to Sloop) had the least proportion of lines 5% and 10% different to the mean of the recurrent parent for all four traits, and the Osiris $BC_1$ population (Osiris was shown to be genetically the most different to Sloop) had the most number of lines 5% or 10% different to the mean of Sloop for two of the four traits (Table 7). Given the statistical limitations of the experimental design, genetic distance between donor and recurrent parent did not appear to be closely related to the recovery of the recurrent parent phenotype. The degree of recovery of the recurrent parent phenotype for each trait appeared to be more closely related to the relative difference between the donor and recurrent parent in the expression of each trait than genetic distance *per se*. For example, the greatest genetic distance between Sloop and each of the donor parents was with Osiris yet the Halcyon $BC_1$ population contained the highest proportion of lines 5% or 10% different to Sloop for decimal growth stage and grain yield. This observation was made despite the small Halcyon BC1 population size and reduced variation due to selection against winter types. The parents Halcyon and Sloop were the most different for grain yield and decimal growth stage (Table 6). The degree of recovery of recurrent parent, therefore, appeared to be related to the relative expression of a few major genes. A stronger relationship between genetic distance and degree of recovery of recurrent parent phenotype could be expected if a greater number of traits were considered, and an overall cross-trait measure of recurrent parent phenotype was adopted. In addition, a more continuous and extensive range in genetic distance between donor and recurrent parent may have facilitated the establishment of stronger relationships.

There was no significant relationship established between the number of donor parent marker alleles and the four traits assessed in the Chebec and Halcyon $BC_1$ populations. The number of donor parent marker alleles for each line was significantly associated ($P<0.01$) with grain yield in the Caminant $BC_1$ population and plant height and decimal growth stage in the Osiris $BC_1$ population. The absence of a significant relationship in the Chebec $BC_1$ population was not surprising given the phenotypic similarity between the two parents for the traits measured. The relatively poor relationships between the number of donor parent marker alleles per line and the four traits measured in the remaining three populations is

more surprising given the significant differences between donor and recurrent parent for these traits (Table 6).

#### 7.2.4.4 *Effect of genetic distance between donor and recurrent parent on standard deviation in $BC_1$ populations*

Goodman (1969) indicated that a direct genetic test of the degree of genetic diversity of two parents is the relative variability of their $F_2$. The theory being that the mating of more genetically distant parents should produce a larger than average progeny variance because the number of segregating loci is maximised. Cowen and Frey (1987), Souza and Sorrells (1989), and Moser and Lee (1994), the latter using RFLP data, showed that genetic distance estimates were relatively poor predictors of genetic variance in oats. Moser and Lee (1994) concluded that strong associations between molecular marker based genetic distance estimates and genetic variance would only occur under certain conditions and these conditions could be influenced by;

(1) the number of marker loci used in the analysis of parents (only 26 used by Moser and Lee (1994))
(2) linkage between marker loci and QTLs
(3) the origin of identical marker alleles, that is, are they identical by descent or by state only?
(4) differences in gene expression among populations or crosses.

In the present study, genetic distance between donor and recurrent parent was also not closely associated with genetic variance as estimated by the standard deviation for each population (Table 5). The standard deviation for each trait in each population appeared to be more closely related to the difference in the level of trait expression between donor and recurrent parent (Table 6) than on genetic distance *per se*. The total number of marker loci used to characterise the genetic distance of the parents in this study exceeded 400. In contrast to Moser and Lee (1994), the number of marker loci and the origin of identical marker alleles is not likely to have influenced the relationship between genetic distance and variance. Linkage between marker loci and QTL conferring trait expression is also not likely to have caused disruption to this relationship due to the large number of marker loci used. Difference in the expression of important genes/QTL appears to be a major factor contributing to this relationship.

It must be noted here that these results are derived from one site in one season only. Consequently the genotype x environment interactions have been restricted to a specific environment and therefore are likely to involve a relatively small number of loci relevant to that site in that season only. For example, seasonal conditions encountered at Strathalbyn, in 1999, were characterised by moderate to severe drought stress extending from anthesis to maturity. Major differences in the flowering time (generally controlled by relative small number of genes) between genotypes, grown under these conditions, is likely to have had a major influence on grain yield. Halcyon, for example, was the slowest developing donor parent (Table 6), and the Halcyon $BC_1$ population produced the lowest mean grain yield (Table 5), the greatest proportion of lines 5% and 10% different to the recurrent parent for grain yield (Table 7), and the highest standard deviation for grain yield (Table 5).

*7.2.4.5 Number of marker loci for selection of recurrent parent background*

Simulation studies on the recovery of recurrent parent background conducted by Hospital *et al.* (1992) and Frisch *et al.* (1999) found that the optimum density of marker loci ranged from 2 to 4 per 100 cM depending on generation stage. In early generations, few recombination events would have occurred and therefore donor parent segments would be relatively large. More markers would be required in later generations as the number of segments increases but their length decreases. Across the four $BC_1$ populations assessed in this study, the three AFLP primer combinations generated 85 polymorphic loci. With reference to the Chebec x Harrington linkage map (Langridge *et al.*, 1995) of approximately 1200 cM, 85 marker loci represents 7 loci/100 cM, significantly more than the reported optimum. However, only 55 of these loci were polymorphic in the Osiris $BC_1$ population. This relates to 5 marker loci/100 cM. The lower level of polymorphism in the Halcyon, Caminant and Chebec $BC_1$ populations (Table 8) meant that the polymorphic marker density was approximately two per 100 cM for the Halcyon and Caminant $BC_1$ populations and one per 100 cM for the Chebec $BC_1$ population. In the simulation study of Frisch *et al.* (1999), the authors selected 80 RFLP markers from a published maize linkage map as the bases for their simulations. By default, all markers were polymorphic. Similarly, Hospital *et al.* (1992) assumed that all marker loci were polymorphic in their simulation studies. The assumption made in these, and other similar studies, is that lack of marker allele polymorphism between genotypes at a given locus implies that the two genotypes have an allele in common at that locus. Therefore the total number of polymorphic loci is the important factor not the genetic distance, or level of polymorphism, between the parents.

Based on this assumption, a more than adequate density of marker loci (seven per 100 cM) was used on the Chebec $BC_1$ population. Despite this, 5% of Chebec $BC_1$ lines were found to consist of 100% recurrent parent background and 20% of individuals consisted of 99% or greater recurrent parent background. Of course, this high proportion of recurrent parent background in a $BC_1$ population is highly unlikely unless the donor and recurrent parent possessed a very high degree of common parentage and this was not the case with these two parents.

In the study reported in Chapter 7.1, 32 AFLP primer combinations were used to identify 72 marker alleles polymorphic in backcross populations involving Chebec and Sloop. Strong relationships between the proportion of unique donor parent alleles and important malt quality traits were established. Together with data presented in Chapter 6.1, this provides evidence of significant deleterious linkage drag in Chebec/Sloop backcross derived lines even after three backcross generations. The 11 polymorphic marker alleles (approximately 1/100 cM), identified in the study reported here, are unlikely to be effective, particularly in late generations, in reducing the proportion of donor parent genome to the extent where no deleterious linkage drag would be evident, even if markers flanking the introgression gene were used.

While many of the results presented here are far from conclusive it does appear that the development of an optimum accelerated backcrossing strategy should consider the implications of genetic distance between donor and recurrent parent on the recovery of the recurrent parent phenotype and the level of marker polymorphism.

Assessment of genetic distance and the evaluation of genetic distance matrices by multivariate statistical procedures represent powerful tools to increase our knowledge of the genetic diversity present in gene banks, the identification of gene pools, and the extent of variability within and between these gene pools. In addition, information on genetic distance and relationships could lead to improved general and specific breeding strategies, such as accelerated backcrossing, which in turn could lead to more efficient exploitation of the genetic diversity available.

# Chapter 8

# General Discussion

As outlined in Chapter 1 and shown in Table 1 (Chapter 2), the backcross breeding method has been used extensively for improving the agronomic characteristics of premium quality wheat cultivars in Australia. In contrast, the method has been used rarely for the breeding of malting quality barley cultivars in this country. A major constraint to the adoption of the method in malting barley breeding is likely to have been the limited commercial life span of the recurrent parent quality type and the slow genetic gain for non-target traits, such as malting quality, inherent in the method. It has been proposed, however, that molecular marker assisted selection has the potential to vastly improve the timeliness and efficiency of the conventional backcross breeding method. In light of this, a re-assessment of its application for the introgression of genes of agronomic importance into malting quality backgrounds was warranted.

The major components of a marker assisted backcrossing program are presented in Figure 8.1 and were considered in this study. The major components consisted of;

(1) identification and assessment of donor parents
(2) identification of markers linked to genes/QTL controlling traits to be introgressed into the recurrent parent
(3) validation of these markers (including an assessment of the agronomic value and the potential for deleterious linkage drag associated with the genes/QTL to be introgressed), and
(4) implementation of molecular markers in backcrossing.

## 8.1 Identification and assessment of donor and recurrent parents

The elite Australian malting quality cultivar Sloop was chosen as the single recurrent parent in all experiments described in this thesis. Sloop produces satisfactory malt and physical quality, is widely adapted to the southern Australian barley growing areas and is deficient in several important agronomic traits for which the genetic control is known to be simple (eg. cereal cyst nematode resistance) or was believed to be relatively simple (eg. boron tolerance).

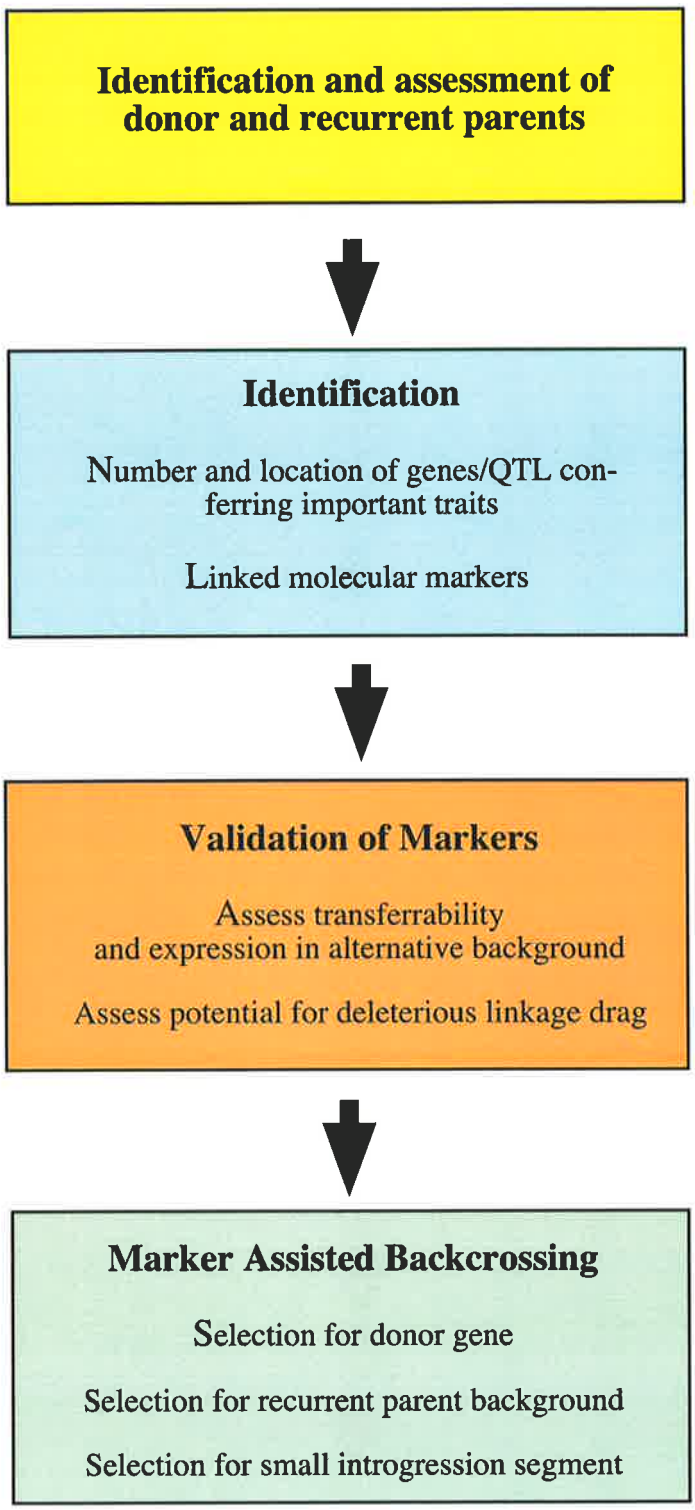



Figure 8.1. major components of a marker assisted backcrossing strategy

Chapter 3 outlines a practical example of a donor parent identification and assessment strategy. In this example, four cultivars Halcyon, Sultan, Guardian and Waveney, carrying resistance to Australian pathotypes of barley leaf scald, were assessed for their potential as donor parents of scald resistance. Three of the these parents, Halcyon, Waveney and Sultan were identified as suitable donor parents. However, scald resistance from Sultan was found to be associated with lower grain yield.

A more comprehensive assessment of the donor parent's potential suitability for gene pyramiding programs would have included more thorough studies on the number and differences between genes carried by each parent. Information on the specific location of resistance genes would have also assisted in the development of efficient introgression strategies into malting quality backgrounds.

## 8.2 Identification of markers linked to genes/QTL of agronomic importance

A successful marker assisted backcrossing strategy relies on the availability of polymorphic markers closely linked to genes controlling traits desired in the recurrent parent. Boron (B) toxicity is an important problem limiting production in the low-rainfall regions of southern Australia. Barley cultivars commonly grown in southern Australia are very intolerant to B toxicity. Genetic variation for B tolerance in barley has been available for some time yet, prior to the commencement of the studies reported here, little was know about the genetic control of this trait. A QTL mapping approach, described in Chapter 4, was successful in both identifying loci (QTL) important in the control of B tolerance in barley, and identifying RFLP markers linked to these QTL that could be used in marker assisted selection. The methodology used to identify the number and location of genes controlling simply inherited traits important to southern Australian barley production, such as CCN resistance, have been reported in the literature (eg. Kretschmer *et al.*, 1997; and Barr *et al.,* 1998).

## 8.3 Validation of markers linked to genes/QTL of agronomic importance

Before marker assisted selection can be applied to a trait of importance it must also be shown that the genes/QTL conferring that trait can be successfully transferred and expressed in an alternative genetic background. In the case of QTL of relatively unknown effect, it is also important to determine the agronomic or breeding value of the respective QTL and the potential for deleterious linkage drag.

Chapter 5 describes an assessment of the transferability and expression of QTL for B tolerance into a Sloop background. Two backcross populations, developed through a combination of phenotypic and marker assisted selection, were used to introgress three of the four QTL conferring B tolerance into an intolerant parent and the function and relative contribution of these QTL were assessed. Marker assisted introgression of these QTL into intolerant backgrounds (Sloop and VB9104) was shown to be effective in improving root growth in solution culture, reducing B toxicity symptoms and reducing concentration of B accumulated in whole plants in the field. In some situations, improvements in grain yield and grain size of barley grown on B toxic soils were achieved. The chromosome 3H B tolerance locus, which accounted for approximately 30% of the variation in relative root length response to B toxicity (Chapter 4), was not assessed as it was not selected in the initial stages of backcrossing. An assessment of the transferability, agronomic value and potential for deleterious linkage drag associated with this locus is still required.

The high degree of environmental interaction shown to be associated with grain yield response to B toxicity and the extent of spatial variation for concentration of B in soil, shown in Figure 5.3 (Chapter 5), highlights the advantages of marker assisted selection over field based phenotypic selection for B tolerance and other complex traits of similar nature.

## 8.4 Implementation of markers linked to genes/QTL of agronomic importance

A successful backcrossing program is also dependent on the ability to select for the donor gene(s)/QTL at each backcross generation and the ability to successfully recover the recurrent parent phenotype. Molecular markers can assist this in at least two ways;
(1) indirect selection for the donor gene(s) at each backcross generation, and
(2) selection for recurrent parent background or against unwanted donor parent genome.

### *8.4.1 Indirect selection for donor gene(s)*

The successful marker assisted introgression, into a malting quality background, of *Ha2* conferring resistance to CCN, and*Yd2* conferring resistance to CCN and BYDV was demonstrated in Chapter 6. As shown in these examples, a major advantage of using molecular markers to trace genes through a backcrossing program is the ability to quickly, and relatively accurately, identify the genotype of a single $F_1$ plant. For example, marker assisted selection for *Ha2* using a single RFLP marker (*Xawbma21*) was shown to be effective in determining the resistance status of a single plant with 80% confidence. This

compares to a single plant selection confidence of 62% for the rigorous, partly automated, CCN resistance bio-assay system. The advantages in single plant selection for CCN resistance would have been even greater if flanking markers were used. While not calculated, the confidence level in selecting a single plant using the YLM marker for *Yd2* would be considerably greater than for phenotypic selection because of;

(1) tight linkage between the donor gene and the marker and,

(2) high potential for error in phenotyping BYDV resistance (Chapter 6.2).

While additional advantages of marker assisted selection over the currently available phenotypic selection methods were discussed for both CCN and BYDV resistance (Chapter 6.1 and 6.2), the absolute gains in efficiency (costs associated with both resources and timing and relative genetic gain) were not determined in this study.

### *8.4.2 Selection for recurrent parent background*

The underlying principle of any backcrossing strategy is that the expected proportion of donor parent genome reduces by fifty percent with each generation of backcrossing. Until recent times, most backcross strategies have focused on this principle and have ignored the genetic variation for the proportion of donor parent genome that exists around the expected mean. Molecular markers can be used to identify recombinant individuals that have genome compositions closer to that of the recurrent parent than would be predicted from theoretical expectations. Selection of such individuals should reduce the number of backcrosses required to recover the recurrent parent genotype and phenotype, thereby reducing the time necessary to achieve commercial outcomes.

The relative recovery of recurrent parent genome in simple cross, $BC_1$ and $BC_3$ derived fixed lines was assessed and reported in Chapter 7.1. A single $BC_1$ derived line was identified with the same proportion of unique donor parent genome ($P<0.01$) as the mean of the $BC_3$ generation. Selecting this $BC_1$ individual would therefore have saved two cycles of backcrossing. These empirically derived results support the findings of Frisch *et al.* (1999), Hospital *et al.* (1992), and Visscher *et al.* (1996), who also found, using computer simulation, that at least two generations of backcrossing could be saved with selection for recurrent parent background. However, Frisch *et al.* (1999) showed that if a large population size and high marker density were used, then it was possible to recover in the $BC_3$ generation, the same proportion of recurrent parent genome as the $BC_7$ generation without marker assisted selection.

Genetic distances between donor and recurrent parents were not considered in the accelerated backcross simulation studies reported in the literature. It was shown in Chapter 7.2 that the mean proportion of unique donor parent genome was greater for $BC_1$ populations involving genetically distant donor and recurrent parents and was less for genetically similar donor and recurrent parents. In fact, the mean number of donor parent marker alleles in the Chebec $BC_1$ population was fewer than the lowest number of donor parent alleles in any single individual in the Osiris $BC_1$ population. Fewer backcrosses would therefore be required to recover the recurrent parent genotype (Sloop) in a backcrossing strategy involving the genetically similar donor parent Chebec than the genetically distant donor parent Osiris.

While the relative extent of recovery of recurrent parent genotype was directly related to genetic distance between donor and recurrent parent, the relationship between genetic distance and phenotypic recovery was not as clear (Chapter 7.2). The recovery of the recurrent parent phenotype appeared to be more a function of major gene differences between parents for traits such as flowering time than genetic distance *per se*. The lack of relationship could have been due to inadequate phenotypic data. A more appropriate assessment of phenotype may have been a phenotypic index that incorporated the relative expression of several quantitative traits measured, preferably, over a number of sites and seasons.

Frisch *et al.* (1999) selected 80 RFLP markers from a published maize linkage map as the basis for simulations studies on the recovery of recurrent parent background during backcrossing. By default all markers loci were polymorphic. A similar approach was taken by Hospital *et al.* (1992), Openshaw *et al.* (1994), and Visscher *et al.* (1996). Given that all marker loci were assumed to be polymorphic, then a lack of polymorphism in a given backcross individual for a given marker locus implied the presence of recurrent parent genome at that locus. The proportion of polymorphic loci would therefore directly reflect the total proportion of donor parent genome. The simulation results, based on marker loci, accurately reflected theoretical expectations based on classical Mendelian segregation. In Chapter 7.2, it was shown that the mean proportion of polymorphic loci, averaged over 85 AFLP marker loci, was 4%, 8%, 13% and 17% for the Chebec, Caminant, Halcyon, and Osiris $BC_1$ populations respectively. The mean number of polymorphic marker loci was directly related to the genetic distance between donor and recurrent parent and was

significantly ($P<0.01$) less than the theoretically expected total proportion of donor parent genome of 25% for all $BC_1$ populations except the Osiris population.

Hospital *et al.* (1992) and Frisch *et al.* (1999) found that the optimum density of marker loci for recurrent parent background selection ranged from 2-4 per 100 cM depending on generation stage. Knowledge of the level of polymorphism, or genetic distance, between donor and recurrent parent would therefore be required to determine the total number of marker loci required to achieve 2-4 polymorphic marker loci per 100cM.

In light of the results presented in Chapter 7.2 and summarised above, genetic distance between donor and recurrent parents should be considered as a critical factor in the design and implementation of accelerated backcrossing strategies.

A major constraint to the adoption of recurrent parent background selection in a practical backcrossing program is the large number of polymorphic marker alleles required to cover the entire genome. Developments in AFLP fingerprinting techniques, however, have provided an improved tool for this application. Frisch *et al.* (1999) showed, with simulation, that the use of marker loci of known location was more efficient than random marker alleles for recurrent parent background selection. This lack of known map location could be a limitation to the routine application of AFLPs for this purpose. Recent evidence suggests, however, that the map location of AFLP markers is highly conserved across barley mapping populations (K.Chalmers, pers. comm.) and therefore AFLP primer combinations can be selected to provide marker loci evenly spread over the entire genome. Despite the various advantages of AFLP markers and particularly the fluorescence-based, semi-automated procedure for AFLP fragment analysis, the time and resources required to visually score fluorescence peaks for many primer combinations over large populations is now a major constraint to routine adoption. In addition, a patent is held on the AFLP technique and this is also a major constraint to routine adoption in a commercially focused breeding program. Microsatellite (SSR) markers are likely to be a more suitable tool for this application.

### *8.4.3 Linkage drag and marker assisted selection for small introgression segments*

A slower than expected rate of elimination of donor parent genes is commonly termed "linkage drag" (Brinkman and Frey, 1977). Often breeders/geneticists underestimate the size of the donor parent chromosome segment surrounding the introgressed donor gene (Young and Tanksley, 1989). Stam and Zevens (1981) estimated the size of an introgression

segment in a chromosome of 100 cM after three backcrosses to be 51 cM. The probability of linkage drag associated with a donor segment of this size influencing the phenotype of backcross progeny after only three backcrosses is, therefore, very high.

Evidence of linkage drag was found frequently in the various backcross studies conducted as part of this thesis. For example;

(1) the chromosome 4H boron tolerance locus derived from Sahara, was associated with lower grain yield in $BC_{2-3}$ derived lines grown in the absence of toxic concentrations of soil boron

(2) the *Ha2* locus derived from Chebec, was associated with lower DP and β-amylase activity in $BC_1$ derived lines

(3) the*Yd2* locus derived from Franklin, was associated with improved barley leaf scald resistance in $BC_2$ derived lines

(4) the *Ha2* locus derived from Chebec, was associated with lower DP and β-amylase activity, higher viscosity, and reduced susceptibility to the spot form of net blotch disease in $BC_1$ and $BC_3$ derived lines.

(5) the *Ha4* locus derived from Barque, was associated with lower α-amylase activity of $BC_1$ derived lines.

The observations described here provide considerable evidence of the problem of linkage drag in a backcrossing program, in particular, they highlight the problems associated with recovery of the recurrent parent quality type.

As discussed earlier, molecular markers can be effectively used to trace one or more donor genes through several cycles of backcrossing and can also be used to identify recombinant individuals that have genome compositions closer to that of the recurrent parent than would be predicted from theoretical expectations. The adoption of these methods will not, however, overcome the problems of linkage drag described above. Several markers located within an area of 30-40 cM surrounding the donor gene could be used to, not only select individuals carrying the target gene, but also, to select individuals carrying a small donor parent introgression segment and thereby potentially reduce linkage drag. Molecular markers could be used to identify individuals with specific, desirable recombination events or chromosomal break points. Although discussed in the review of simulation studies, this facet of marker assisted backcrossing was not addressed in this study. Such a strategy would require numerous, easily assayed, polymorphic markers surrounding the target gene(s) (not available at the time) and large backcross populations. The identification and validation of numerous polymorphic micro-satellite markers, within the close proximity of genes of major interest to

barley breeding programs would, for example, be of considerable advantage. In addition, an improved understanding of the genetic basis of complex traits (number, location and interaction between genes/QTL) to be recovered from the recurrent parent and the allelic variation present in donor parents would greatly assist in determining the extent to which non-target donor parent genome, including that in close proximity to the target gene, needs to be eliminated.

The populations developed as part of this thesis provide an excellent opportunity for further studies on the relative size of donor parent segments surrounding the target gene in backcross populations. The relative impact on phenotypic expression of variations in size of donor segment and residual donor parent genome on non-target chromosomes could also be assessed.

## 8.5 An advanced marker assisted gene introgression strategy

The tools for undertaking marker assisted backcrossing are currently available and have been further developed in the studies presented here. In addition, our understanding of the number and location of chromosome regions important in defining superior malting quality has improved substantially in recent times. In light of these developments more ambitious, or advanced, marker assisted selection strategies can now be attempted. An example of an advanced marker assisted selection strategy of this type follows and is based on;

(1) the germplasm developed as components of experiments described in this thesis

(2) the knowledge base gained from study of the literature and results of experiments presented in this thesis

(3) preliminary (unpublished) information on putative QTL conferring malt quality and agronomic traits in the Alexis x Sloop mapping population (National Barley Molecular Marker Program).

The general aim of this hypothetical strategy is to use markers to assist in the simultaneous introgression of CCN resistance, BYDV resistance, moderate scald resistance, and B tolerance into an elite malting quality background.

*The recurrent parent*

Putative QTL for malt quality traits have been identified on regions of chromosome 5HL (malt extract and FAN), chromosome 1HL (malt extract, viscosity, FAN and DP), chromosome 2HS (malt extract), all derived from Alexis, and on chromosome 4H (FAN and

DP) derived from Sloop. In addition, favourable loci conferring semi-dwarf plant type and the control of heading date were identified on chromosome 3HL (Alexis) and 2HS (Sloop) respectively (National Barley Molecular Marker Program, unpublished data).

An individual in the Alexis x Sloop mapping population was identified which achieved excellent levels of malt extract, DP, FAN, and low viscosity (desirable) and was semi-dwarf in plant type. The graphical genotype display function of Q-Gene (Nelson, 1997) was used to confirm appropriate parental chromosome segments at each locus in this line (Fig 8.2). Unfortunately the unique combination of Alexis and Sloop genome, found in this line, did not include a Sloop segment at the chromosome 2HS heading date locus and consequently it is relatively late flowering (not desirable for southern Australian conditions). This individual was chosen as the recurrent parent because of its outstanding malt quality profile and the availability of detailed knowledge of the number and location of genes/QTL to be recovered.

The traits to be introgressed from donor parents were B tolerance, CCN resistance, BYDV resistance (and linked scald resistance) and a Sloop allele at the chromosome 2H heading date locus. All malt quality QTL were to be conserved from the recurrent parent. The genotype of the recurrent parent and desired outcome is represented in the form of graphical genotypes presented in Figure 8.2.

*Donor parents*

Sahara, the donor parent for B tolerance, is a North African landrace with poor malt quality, poor general adaptation to southern Australian conditions, and is genetically, very distant from both Sloop and Alexis (Chapter 7.2). Given the exotic nature of Sahara, serious linkage drag problems could be expected. Small introgression segments surrounding the B tolerance loci were therefore actively sought in this strategy. Sahara, like Chebec, also carries a CCN resistance allele at the *Ha 2* locus (Kretschmer *et al.*, 1997). A Sahara chromosome segment extending from the B tolerance locus to the *Ha 2* locus would cover more than 50 cM and include the 2-row/6-row locus, for which, the Sahara allele confers the 6 row head type (Langridge *et al.*, 1995). A double recombination even in this region would therefore be required to achieve a B tolerant, CCN resistant 2-row type. Alternatively, a different donor parent for *Ha 2* could be sought. For this reason Chebec, closely related to Sloop, was used as the source of *Ha 2*.

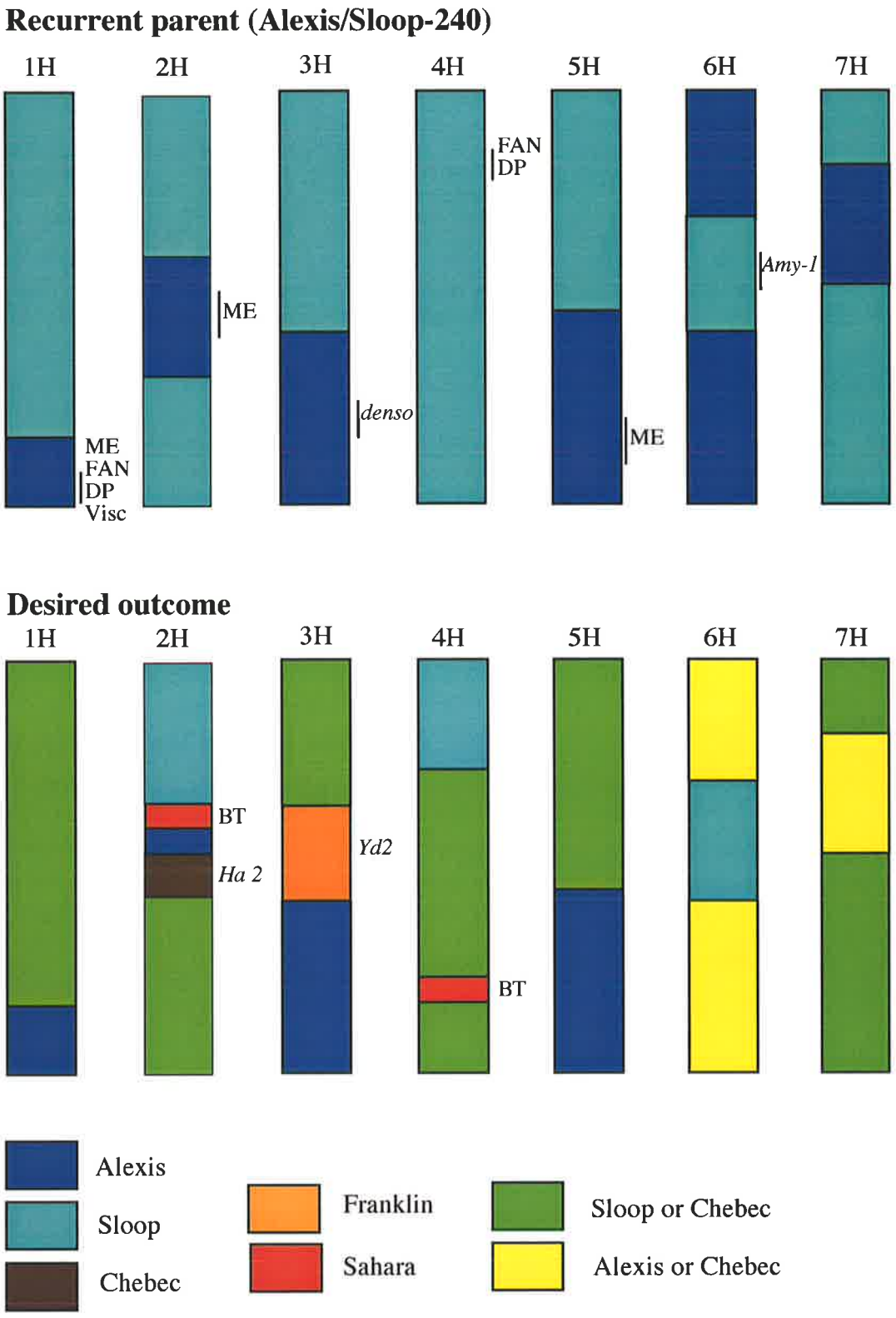



Figure 8.2 Graphical genotype of the recurrent parent (Alexis/Sloop-240) and the desired outcome from an advanced marker assisted backcrossing strategy

As outlined in Chapter 5, the chromosome 3H B tolerance locus, which accounts for approximately 31% of the variation in relative root length response to B toxicity (Chapter 4), is in repulsion with *Yd2* , which confers resistance to BYDV. The contribution of the 3H locus to field B tolerance was not assessed in this study (Chapter 5). Researchers working with Professor R Symons, University of Adelaide, are close to cloning the *Yd2* gene and therefore much is know about the location of this gene. The location of the B tolerance locus is not as precise as it was identified by QTL mapping techniques (Chapter 4). Due to the imprecise location of the B tolerance locus and lack of knowledge regarding its contribution to field tolerance, a Franklin segment at the chromosome 3H locus was preferred over a Sahara segment.

The chromosome 6H B tolerance locus was also found to be in repulsion with the *Amy-1* structural gene involved in the control of α-amylase activity (Chapter 5). It was also shown (Chapter 5) that the contribution of the chromosome 6H locus to field tolerance was not significant. Since the 6H locus appeared to contribute little to B tolerance in the field, a recurrent parent allele for the *Amy-1* locus was preferred.

In the experiments described in Chapter 5, a 2-rowed head type individual from the Clipper x Sahara mapping population (CS-31) was chosen as the donor of B-tolerance. The chromosome 2H and 4H tolerance loci were introgressed into Sloop through two cycles of backcrossing and the presence of the QTL confirmed with both flanking markers and phenotypic assessment. One of these backcross progeny was chosen as the donor parent for B tolerance in this advanced strategy as it was a 2-row type, and a large proportion of unwanted Sahara genome would have already been eliminated.

Franklin was backcrossed to Sloop, through two cycles of backcrossing, in experiments aimed at introgression of *Yd2* (Chapter 6.2). While Franklin produces excellent malt quality, the locations of QTL conferring this are unknown. In addition, Franklin is adapted to the higher rainfall regions of southern Australia only. A high yielding, BYDV resistant, moderately scald resistant, early flowering, backcross derived line was chosen as the donor parent of BYDV resistance. Since no deleterious linkage drag associated with *Yd2* was shown for either malt quality, grain yield or heading date (Chapter 6.2), a small introgression segment on chromosome 3H was not required.

The donor parent of CCN resistance was chosen from the Chebec derived $BC_3$ lines described in Chapter 6.1. While the malt quality of the recurrent parent was not fully

recovered in any of these lines, a CCN resistant individual with high DP, was chosen as the donor parent.

*The strategy*

An outline of the overall breeding and selection strategy is provided in Figure 8.3. The general principle involved the independent introgression of B tolerance, BYDV resistance and CCN resistance, followed by a stepwise merger of the separate streams. The extent to which elimination of donor parent genome was required, dictated the number of backcrosses used in each stream.

Marker assisted selection (MAS) in the CCN stream focussed on selection of individuals with a recombination event close to the *Ha 2* locus on the proximal side, conservation of the recurrent parents extract locus, and a Sloop allele at the heading date locus, all on chromosome 2H. MAS in the B tolerance stream focused on selection for the two B tolerance loci, recovery of the recurrent parent extract locus on 2H, and on elimination of all other unwanted Sahara genome on target and non-target chromosomes. As shown in Figure 8.3, the BYDV and CCN resistance streams were merged first, and the B tolerance stream merged second. Due mostly the specific recombinations sought on chromosome 2H, large populations of $F_1$ plants were used at all MAS stages. The overall desired outcome is presented in Figure 8.2.

Numerous alternative strategies, potentially more efficient than the one presented, could be devised. However, the strategy outlined in Figure 8.3 illustrates how information on the genetics of traits to be recovered in the recurrent parent could be used, in combination with MAS for target genes, recurrent parent background and small introgression segments, to achieve a desired outcome relatively quickly. Other advanced applications of marker assisted selection for the improvement of malting barley could include;

(1) the introgression of chromosome regions responsible for high malting quality into highly adapted, agronomically superior genotypes, and

(2) the pyramiding of genes/QTL conferring superior malting quality

The potential applications of marker assisted backcrossing are only limited by our knowledge of the genetic basis of important traits and the availability of easily assayed polymorphic markers, both in proximity to the genes controlling these traits, and throughout the remainder of the genome.

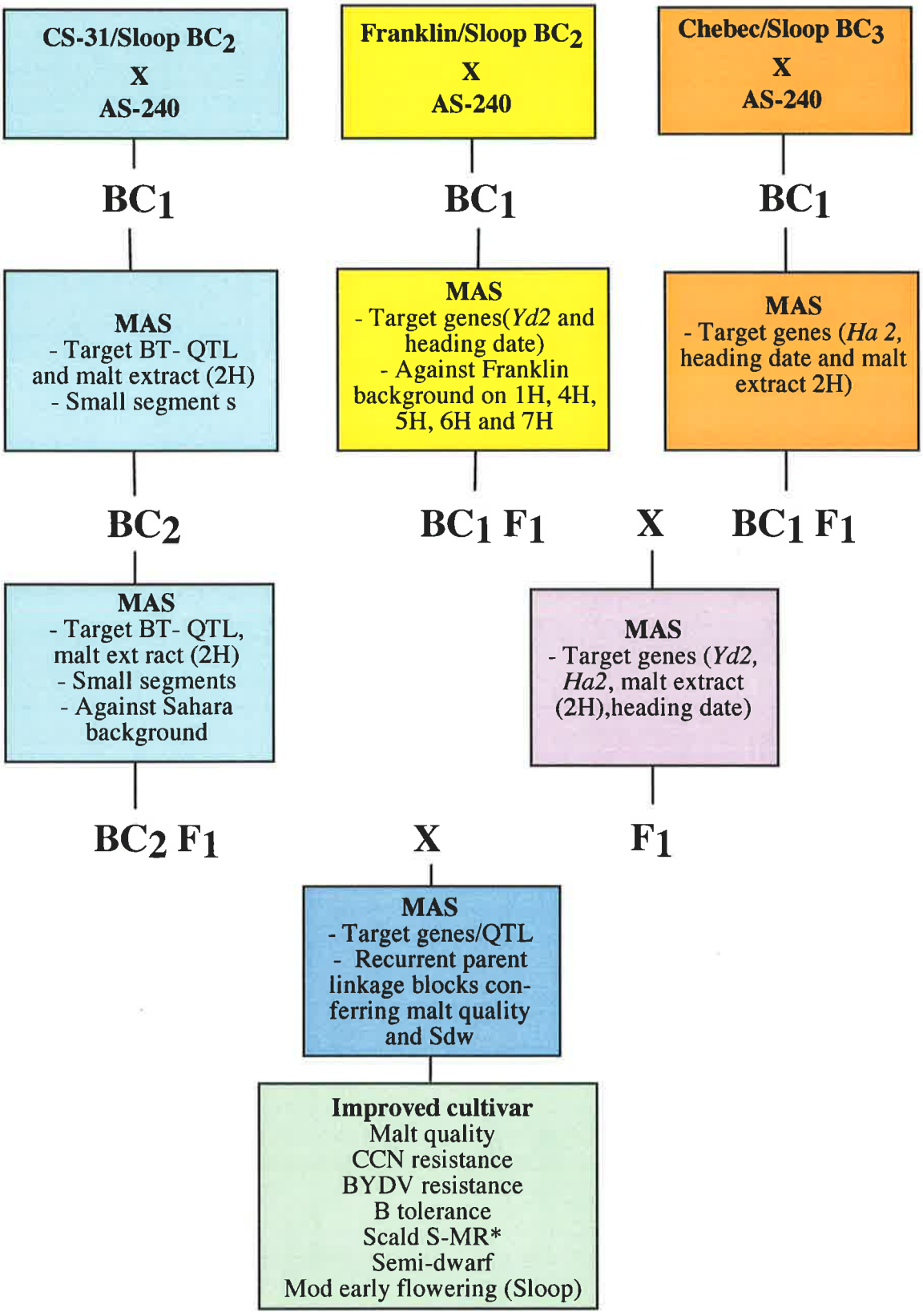



Figure 8.3 Schematic of an advanced marker assisted backcrossing strategy introgressing cereal cyst nematode resistance, barley yellow dwarf virus resistance, boron tolerance and moderate resistance to leaf scald, into a malting quality background

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# Mapping and validation of chromosome regions conferring boron toxicity tolerance in wheat (*Triticum aestivum*)

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**Abstract** Boron is an essential plant micro-nutrient which can be phytotoxic to plants if present in soils in high concentration. Boron toxicity has been recognised as an important problem limiting production in the low rainfall areas of southern Australia, West Asia and North Africa. Genetic variation for boron toxicity tolerance in wheat has been well-characterised. The efficiency of breeding for boron toxicity tolerance could be greatly enhanced by the development of molecular markers associated with QTLs for tolerance in wheat. A population of 161 doubled haploids from a cross between the tolerant cultivar Halberd and the moderately sensitive cultivar Cranbrook was used to identify chromosomal regions involved in boron tolerance. A combined RFLP and AFLP linkage map of the Cranbrook x Halberd population was used to identify chromosomal regions involved in the boron tolerance traits measured. Regions on chromosome 7B and 7D were associated with leaf symptom expression. The region on chromosome 7B was also associated with the control of boron uptake and with a reduction in the effect of boron toxicity on root-growth suppression. RFLP markers at the chromosome 7B and 7D loci were shown to be effective in selecting for improved boron tolerance in an alternative genetic background. Halberd alleles at the chromosome 7B locus were associated with the concentration of boron in whole shoots and grain. The concentration of boron in whole shoots and in grain were both related to grain yield in a field trial conducted on soil containing toxic levels of boron. Implications relating to marker-assisted selection for boron toxicity tolerance in wheat are discussed.

**Keywords** Boron toxicity · Boron tolerance · Mapping · Wheat · Marker-assisted selection

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## Introduction

Boron (B) is an essential plant micro-nutrient which can be phytotoxic to plants if present in soils in high concentration. Boron toxicity to crop plants has been recognised since the early 1930s (Christensen 1934), yet it was not until 1984 that it was first recognised in southern Australia in barley growing under dryland conditions (Cartwright et al. 1984). High concentrations of B have been recorded from soils and plant samples collected from widespread regions of the cereal growing districts of southern Australia (Ralph 1992). Boron toxicity has also been recognised as a problem in the dry regions of West Asia and North Africa and a problem associated with irrigation water in many other parts of the world (Gupta et al. 1995).

A 17% difference in the grain yield of adjacent areas of barley was related to differences in the concentration of B in shoots just prior to anthesis (Cartwright et al. 1984). Moody et al. (1993) estimated that wheat yield losses of up to 11% could be attributed to B toxicity in southern Australia. Paull (1990) found that wheat plants exposed to high concentrations of B, under glasshouse conditions, responded with reduced vigour, delayed development, leaf symptoms which include yellowing of leaf tips of older leaves followed by non-specific necrosis continuing down the leaves and reduced total dry matter and grain yield.

The concentration of B in soils in southern Australia has been shown to increase with depth (Cartwright et al. 1984, 1987). The occurrence of B at depth in the soil profile precludes amelioration through soil modification.

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The breeding of tolerant wheat cultivars, therefore, offers the most feasible option for reducing losses in yield and quality associated with B toxicity.

Genetic variation in tolerance to B toxicity in wheat has been reported by Paull (1985) and Moody et al. (1993). The bread wheat cultivar Halberd is among the more tolerant Australian cultivars identified. Halberd and other related cultivars, tracing back to cvs. Federation and Currawa, dominated wheat production in southern Australia for most of the twentieth century (Paull 1990). Paull (1990) related genetic variation in the concentration of B in grain with the extent and persistence of bread wheat cultivars commercially grown in Australia.

The inheritance of boron tolerance of wheat has been studied (Paull 1990, Chantachume 1995). Tolerance is expressed as a partially dominant character and controlled by at least three major genes acting in an additive manner and named *Bo1, Bo2* and *Bo3* (Paull et al. 1991).

Methods for screening and selection for B tolerance in breeding populations include the growing of plants in pots with soil containing toxic concentrations of B (Paull et al. 1988), by solution culture in filter paper (Chantachume et al. 1995) and in field trials conducted on toxic soils (Moody et al. 1993, Jenkin 1993). Most selection methods are highly labour intensive and susceptible to experimental error. Jefferies et al. (1999) identified four chromosome regions associated with the expression of B tolerance in barley and found that not all four could be readily identified in a single assay system. It is likely that a combination of assay systems, or plant response characteristics, would also be required to select, with relative accuracy, for important chromosome regions associated with B tolerance in wheat. The location of genes conferring B tolerance to chromosomes and then to specific chromosome regions would facilitate the selection of B-tolerant germplasm using linked molecular markers (marker-assisted selection), thereby overcoming many of the limitations associated with alternative assay systems.

Recently, a linkage map was constructed in a doubled haploid (DH) population of 161 individuals created from a cross between the moderately tolerant parent Halberd and the moderately intolerant parent Cranbrook (Chalmers et al. in preparation). The objectives of the study reported here were to use this population and marker dataset to identify chromosomal regions associated with response to toxic concentrations of boron and test the relative expression of those regions when introgressed into an alternative genetic background.

## Materials and Methods

### Genetic material

The genetic material used in the mapping component of this study was a population of 161 DH lines derived from a cross between the moderately B-tolerant cultivar Halberd and the moderately B-sensitive cultivar Cranbrook. The validation studies consisted of two components. The first component was a repeat of the soil-based assay using a population of 98 DH lines derived from a cross between Halberd and the B-sensitive breeders line Warigal/MMC. The mapping population parents, 15 common Australian wheat cultivars and a North African cultivar known to be very sensitive to B toxicity were also included in the assay. The second component involved field trials and marker screening of 25 cultivars important either as commercial cultivars or as parents to southern Australian wheat breeding programmes during the mid-1980s.

### Mapping – plant response to boron toxicity

Two assay systems were used for assessing plant response to B toxicity in the Cranbrook x Halberd mapping population:

1) a filter paper, solution culture assay (Chantachume et al. 1995) in which the relative root length (RRL) of seedlings grown on filter papers soaked in solutions containing toxic and non-toxic concentrations of B was determined.
2) a soil-based assay in which plants were grown in soil to which toxic concentrations of B were added. Leaf symptom expression, total dry matter and whole-shoot B concentration were measured.

Detailed assay methods followed those described by Jefferies et al. (1999). The relative root-length assay was conducted as a randomised complete block with three replicates. Similarly, the soil-based assay was also conducted as a randomised complete block with 3 replicates (2 plants per line, per replicate); each replicate containing within a single large soil box.

### Validation – response to boron toxicity

The response of the Halberd x Warigal/MMC population and selected cultivars to B toxicity was assessed using two replicates of the soil-based assay (Jefferies et al. 1999) alone. Field experiments were conducted at Two Wells (approximately 40 km N of Adelaide) in 1985 and 1986 on a red clay soil with naturally occurring high concentrations of B in the subsoil. The concentration of B in the soil at this site, in 1983, ranged from 3.6 mg $kg^{-1}$ in the first 10 cm, increasing to 101–104 mg $kg^{-1}$ in the 20–40 cm range of the profile (Cartwright et al. 1987). Plots were 4.2 m long and 60 cm wide (4 rows) and sown with approximately 60 kg/ha of seed. The experiments were arranged as a randomised complete block design with six replicates in 1985 and seven replicates in 1986. The B-sensitive genotype Warigal/MMC was grown as a control grid and included every fifth plot. In 1985, five whole shoots per plot were harvested 78 days after sowing when most plants were at or near the boot stage (Zadoks 41–45). Samples were rinsed with deionized water, oven-dried, ground in a stainless steel mill and analysed for concentration of B by nitric acid digestion and ICP-spectrometry (Zarcinas et al. 1987). Field experiments were harvested at maturity and grain yield per plot and the concentration of B in grain were determined for both experiments.

### Statistical analysis

All statistical analyses except for interval and multiple regression marker analyses of B response were performed with JMP (v3.0, SAS Institute Inc, 1995) software. The least squares means for relative root length (root length at 0 ppm B as a percentage of root length at 100 ppm B) were calculated using an ANOVA model. Factors for the ANOVA model were doubled haploid line, replicate and plant number. Least squares means for B concentration in whole shoots, leaf symptom score and dry matter production were also calculated using an ANOVA model with doubled haploid line and replicate forming the factors for the model. No raw data transformation was required as residuals were normally and independently distributed. Comparisons between B tolerance parameters were conducted by calculating simple pairwise correlation coefficients.

Heritability for each trait was estimated from a linear model incorporating data from the 161 DH lines over three replicates. Factors were doubled haploid line and replicate. Heritabilities were calculated from an estimate of the genetic variance component as a proportion of the total variance for each trait.

A total of 545 marker loci, 112 restriction fragment length polymorphisms (RFLPs) and 433 amplified fragment length polymorphisms (AFLPs), covering the majority of the wheat genome (Chalmers et al. in preparation), were used for simple and interval regression analysis, the latter by the method of Haley and Knott (1992). A minimum LOD threshold of 3.0 was used. A marker locus thought to be associated with a gene or chromosomal region conferring B tolerance was tested for two-way interaction with all other markers in the dataset using the method described by Nelson et al. (1998). Initial marker analyses was conducted using an additive regression model with MAP MANAGER QT software (Manly and Cudmore 1997). Interval analysis and marker interaction tests were performed with the computer programme QGENE (Nelson 1997).

### Validation – DNA extraction, restriction endonuclease digestion and Southern hybridisation

DNA extraction was achieved using a DNA mini-prep method adapted from Rogowsky et al. (1991). Variations to the method were as described below. For the initial extraction, 750 µl of extraction buffer and phenol-chloroform-isoamyl alcohol (25:24:1) were used. The extraction buffer was 0.1 *M* TRIS-HCl (pH 8.5),10 m*M* EDTA, 0.1 *M* NaCl, 1% sarkosyl and 2% polyvinylpolypyrrolidone (insoluble). After the second phenol-chloroform-isoamyl alcohol extraction the aqueous phase was extracted once with an equal volume of chloroform. DNA was precipitated by the addition of 0.1 vol. of 3 *M* sodium acetate (pH 4.8) and 1 vol. of propan-2-ol.

Restriction endonuclease digestion and Southern hybridisation followed standard methods. RFLP markers identified as having a significant association with B tolerance in the Cranbrook x Halberd population were subsequently used to genotype the validation population and commercial cultivars. Paull (1990) proposed that boron tolerance genes present in Australian cultivars were of common origin tracing to parents of Halberd. It was assumed, therefore, that Halberd was the only source of B tolerance in the set of Australian cultivars assessed.

### Locus effects

Q-GENE (Nelson 1997) was used to produce "graphical genotypes" (Young and Tanksley 1989) for the entire mapping population. In this function of Q-GENE it is assumed that a marker locus represents a chromosomal segment of the same parental genotype, extending halfway to the next marker locus on either side. Each of the 161 DH mapping population lines were scored for the likely presence of either a Halberd or Cranbrook chromosome segment at the locus most significantly associated with each trait. From this, the predicted genotype of each line was determined. Lines of identical genotype were grouped into chromosome segment classes. Least squares means for each class were calculated using a single factor (segment class) ANOVA. Means of chromosome segment class were compared using contrasts. This method was also used for the validation population except that the genotype of lines were assigned on the basis of the most closely associated RFLP marker allele.

## Results

### Comparison between boron tolerance parameters

Table 1 details the pairwise correlation coefficients between the four B tolerance traits measured (RRL, leaf symptom score, concentration of B in whole-shoots and whole shoot dry matter production). Pairwise correlations between three of the traits, RRL, leaf symptom score, and B concentration in whole shoots were highly significant ($P<0.001$). The concentration of B in whole shoots was highly negatively correlated with RRL (–0.80). Correlation coefficients between leaf score and RRL and B concentration in whole shoots were relatively low at –0.51 and 0.54, respectively. This is consistent with the relatively poor association previously found between leaf symptom expression and grain yield (Jenkin 1993; Riley and Robson, 1994) and leaf symptom expression and concentration of B in plant tissue (Mahalakshmi et al. 1995; Jefferies et al. 1999). In contrast, whole-shoot dry matter production was significantly correlated (-0.17) with whole-shoot B concentration, but only at the $P<0.05$ significance level.

### Mapping

#### *Root-length assay*

A significant ($P<0.001$) reduction in root growth at 100 ppm B was observed in all DH lines and both parents. Significant ($P<0.001$) genetic variation for seedling root length at both 0 ppm B and 100 ppm B was observed within the mapping population (Fig. 1). The heritability of root length at 0 ppm was estimated as $h^2=0.55$, while the heritability of root length at 100 ppm B was significantly greater at $h^2=0.83$. Relative root length was calculated as root length at 100 ppm B expressed as a percentage of root length at 0 ppm B and was used to provide a measure of the root-length response independent of genetic variation for absolute root length. Frequency distributions for root length at 0 ppm B, 100 ppm B, and for relative root length are provided in Fig. 1. The 0-ppm trait approximated a continuous distribution around a mean of 158 mm. The frequency distributions for the 100-ppm trait and RRL were consistent with that of traits controlled by single genes (not tested for fit). With the exception of 1 line which recorded a significantly lower ($P<0.001$) RRL than Cranbrook, the RRL of all DH lines fell within the parental range.

**Table 1** Pairwise correlation coefficients between four boron tolerance traits measured on the Cranbrook x Halberd mapping population

* Significant at $P<0.05$,
*** significant at $P<0.001$

| Boron tolerance traits | Whole-shoot boron concentration | Relative root length (RRL) | Leaf symptom score |
|---|---|---|---|
| Relative root length | –0.80*** | | |
| Leaf symptom score | 0.54*** | –0.51*** | |
| Whole-shoot dry weight | –0.17* | 0.08 | –0.02 |

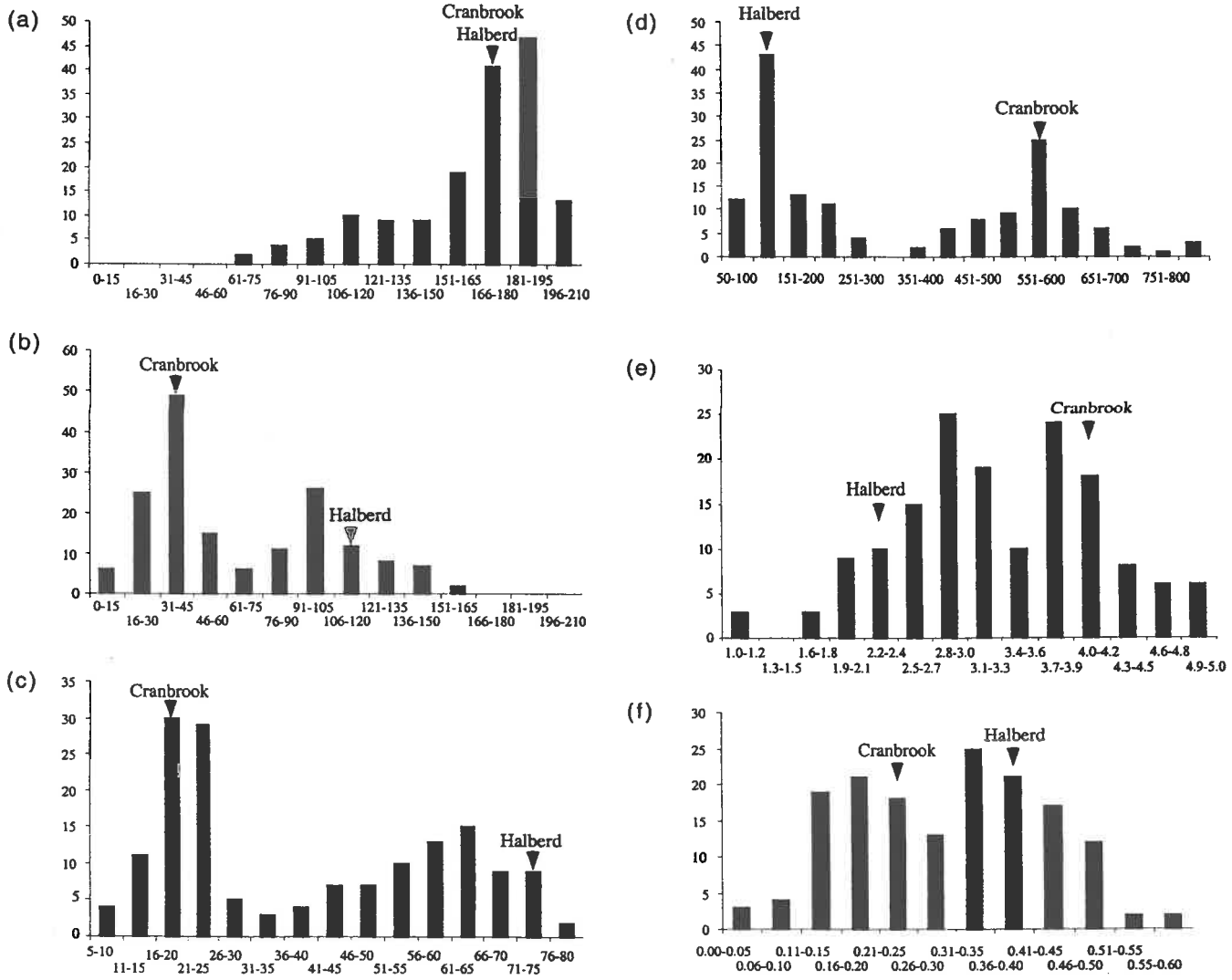


**Fig. 1a–f** Frequency distributions for all traits measured on the Cranbrook x Halberd mapping population. The *y-axis* on all parts represents the number of DH lines. **a** Length (mm) at 0 ppm B, **b** root length (mm) at 100 ppm B, **c** relative root length (%), **d** whole-shoot B concentration (ppm), **e** leaf symptom score (1–6), **f** whole-shoot dry matter (gm)

Marker analysis of seedling root length at 0 ppm B revealed no significant marker-trait association. Marker analysis for seedling root length at 100 ppm B revealed a significant association (LOD=23.6) with markers in a linkage group on chromosome 7B (Fig. 2). A region within this same linkage group was found to be strongly associated (LOD=35.1) with RRL (Fig. 2). Multiple regression analysis showed that this locus accounted for approximately 49% of the variation in root length at 100 ppm B and 64% of the variation in RRL. Halberd marker alleles were associated with high root length at 100-ppm B and high RRL. Marker analysis of the low (0 ppm) B concentration failed to show significant relationships with the region associated with root length at 100 ppm B and RRL (LOD=0.8). It can be concluded, therefore, that the region identified on chromosome 7B linkage group is significantly associated with root-length response to B concentration. The RFLP marker most strongly associated with root length at 100 ppm B and high RRL was *Xpsr680–7B*.

*Soil assay*

In the soil assay, significant ($P<0.001$) differences between mapping population parents and DH lines were observed (by ANOVA) for leaf symptom score, whole-shoot dry matter and concentration of B in shoots. The frequency distributions for the three traits were mostly bi-modal with the DH lines falling within the range of the parents (Fig. 1). The heritabilities of leaf symptom score, whole-shoot dry matter and whole-shoot B concentration were 0.53, 0.23 and 0.81, respectively.

Marker analysis of leaf symptom data revealed significant associations (LOD=8.8) between leaf symptoms and the region on the chromosome 7B linkage group found to be associated with RRL (Fig. 3). A significant association (LOD=4.7) was also revealed with a chromosome 7D linkage group (Fig. 3). Based on multiple re-

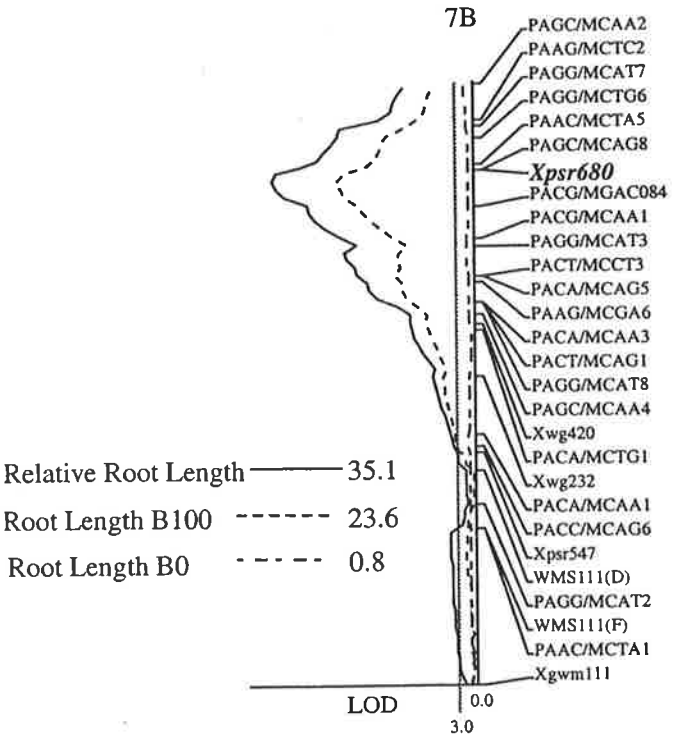


**Fig. 2** Chromosome location of regions associated with root length at 0 ppm B and 100 ppm B and relative root length expressed in DH lines of the cross Cranbrook x Halberd based on a partial interval map of chromosome 7B. The short arm is towards the *top* is the chromosome. Maximum LOD score is provided *adjacent* to the legends. The RFLP marker most closely associated with the traits is presented in *bold italics*. The *finest dashed line* shows the LOD 3.0 threshold

gression analysis, loci on chromosomes 7D and 7B, in combination, accounted for approximately 35% of the variation in leaf symptom data. The RFLP markers most strongly associated with leaf symptom score were *Xpsr680–7B* and *Xpsr160–7D*. Halberd marker alleles at both loci were associated with low leaf symptom scores. Despite a significant difference between parents and DH lines, shown by ANOVA, both regression analysis and interval mapping techniques failed to identify a significant association between markers and whole-shoot dry weight response to B toxicity.

Markers in a linkage group on chromosome 7B were found to be associated (LOD=38.4) with whole-shoot B concentration of plants grown in B toxic soil. Based on multiple regression analysis, the chromosome 7B locus accounted for approximately 69% of variation in whole-shoot B concentration data. Halberd marker alleles at this locus conferred low whole-shoot boron concentration. The RFLP marker on chromosome 7B most strongly associated with whole shoot B concentration was also *Xpsr680–7B*.

## Marker interactions

The 2 marker loci (*Xpsr160–7D* and *Xpsr 680–7B* ) that were significantly associated with chromosomal regions involved in the control of B tolerance traits were tested for two-way interaction with each other and all other markers in the dataset. In addition, each marker in the complete dataset was tested for interaction with all other markers. No significant ($P<0.001$) interactions were identified.

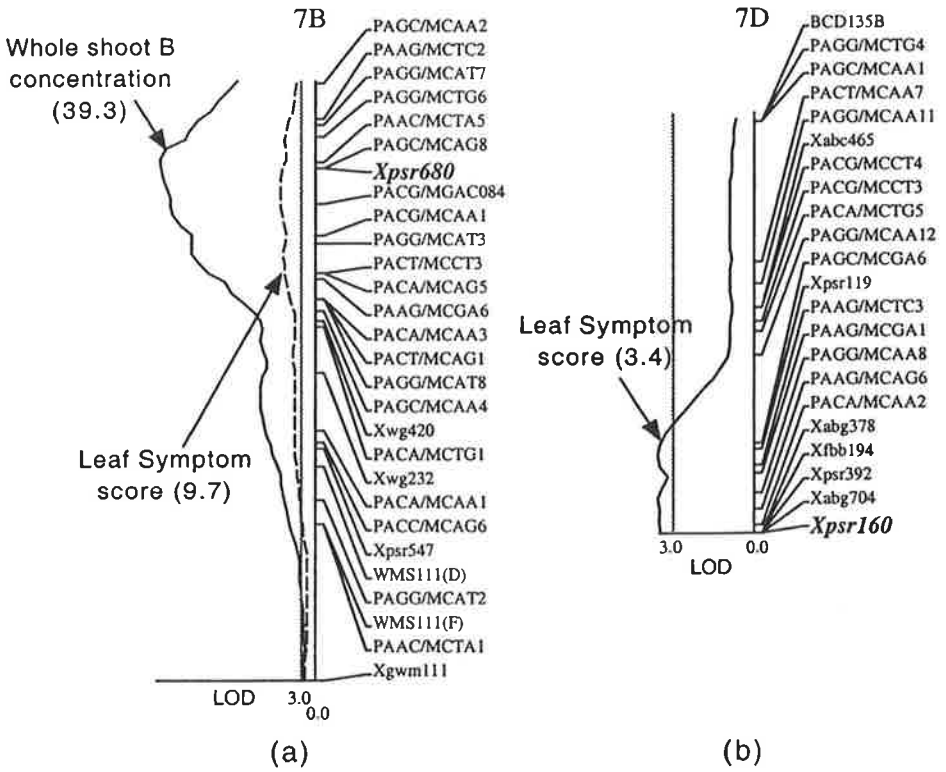


**Fig. 3a, b** Chromosome locations of regions associated with leaf symptom expression and whole-shoot B concentration expressed in DH lines of the cross Cranbrook x Halberd based on a partial interval maps of chromosome 7B (**a**) and 7D (**b**). The short arm is towards the *top* of the chromosomes. Maximum LOD score is provided in *parenhesis*. The RFLP marker most closely associated with the trait is presented in *bold italics*. The *finest dashed line* shows the LOD 3.0 threshold

**Table 2** Mean whole-shoot boron concentration and leaf symptom score in 17 commercial wheat cultivars grown in soil with toxic levels of boron (B toxic soil)

| Cultivar | Halberd (H) or Alternative (A) allele at 2 loci | | Leaf symptom score (1–9) | | Whole-shoot boron concentration (ppm) | |
|---|---|---|---|---|---|---|
| | *Xpsr160–7D* | *Xpsr680–7B* | Least squares mean | Standard error | Least squares mean | Standard error |
| Aroona | H | H | 2.13 | 0.60 | 402.3 | 26.2 |
| BT-Schomburgk | H | H | 3.63 | 0.60 | 229.8 | 26.2 |
| Barunga | H | – | 3.13 | 0.60 | 234.0 | 26.2 |
| Cranbrook | A | A | 4.38 | 0.60 | 517.3 | 26.2 |
| Dagger | H | H | 2.75 | 0.60 | 299.8 | 26.2 |
| Excalibur | A | H | 5.25 | 0.60 | 408.6 | 30.3 |
| Frame | H | H | 4.63 | 0.60 | 244.8 | 26.2 |
| Halberd | H | H | 2.95 | 0.27 | 193.3 | 12.3 |
| Janz | A | A | 3.25 | 0.60 | 447.3 | 26.2 |
| Kenya Farmer | – | – | 8.50 | 0.60 | 487.3 | 26.2 |
| Krichauff | – | – | 2.75 | 0.60 | 212.0 | 26.2 |
| Molineux | H | H | 3.25 | 0.60 | 414.8 | 26.2 |
| Schomburgk | H | H | 3.38 | 0.60 | 419.8 | 26.2 |
| Silverstar | – | A | 5.13 | 0.60 | 494.8 | 26.2 |
| Spear | H | H | 3.25 | 0.60 | 239.8 | 26.2 |
| Stiletto | H | H | 3.88 | 0.60 | 272.3 | 26.2 |
| Trident | H | H | 3.13 | 0.60 | 254.8 | 26.2 |
| WI/MMC | A | A | 7.60 | 0.27 | 528.4 | 12.9 |
| Warigal | H | H | 4.00 | 0.60 | 469.8 | 26.2 |
| LSD ($P<0.05$) | | | 1.65 | | 79.6 | |

**Table 3** Mean concentration of boron in whole shoots and grain of 25 cultivars important to southern Australian breeding programmes, grown in field trials at Two Wells, South Australia, 1985 and 1986

| Cultivar | Halberd (H) or Alternative (A) allele at two loci | | Boron concentration in whole shoots (mg/kg) | Boron concentration (mg/kg) in grain | | Grain yield (g/plot) | |
|---|---|---|---|---|---|---|---|
| | *Xpsr160–7D* | *Xpsr680-7B* | | 1985 | 1986 | 1985 | 1986 |
| Halberd | H | H | 35.6 | 5.84 | 3.32 | 291 | 320 |
| Olympic | A | – | 44.2 | 8.48 | 5.27 | 291 | 229 |
| Spear | H | H | 44.2 | 7.84 | 4.57 | 235 | 288 |
| Aroona | H | H | 46.0 | 7.39 | 6.07 | 214 | 275 |
| Raven | H | H | 51.8 | 9.08 | – | 176 | – |
| Dagger | H | H | 52.4 | 9.03 | 4.95 | 196 | 277 |
| Millewa | H | H | 58.4 | 9.76 | 5.08 | 145 | 316 |
| Sunstar | H | – | 60.0 | 11.60 | 9.15 | 165 | 239 |
| Banks | A | A | 62.0 | 10.51 | – | 154 | – |
| Vulcan | A | – | 64.8 | 13.99 | 9.38 | 206 | 206 |
| Cook | A | A | 64.9 | 11.30 | 10.01 | 190 | 213 |
| Condor | A | A | 68.8 | 12.01 | 8.82 | 181 | 234 |
| Meering | A | A | 69.1 | 11.27 | 6.77 | 186 | 243 |
| Kite | A | A | 73.7 | 15.96 | 10.02 | 152 | 245 |
| Madden | A | A | 73.7 | 14.35 | – | 138 | – |
| Cranbrook | A | A | 74.9 | 12.68 | 6.36 | 171 | 242 |
| Warigal | H | H | 75.5 | 9.72 | 6.56 | 222 | 322 |
| Schomburgk | H | H | 75.7 | 9.89 | 5.80 | 227 | 263 |
| Festiguay | A | – | 78.4 | 12.42 | 7.91 | 177 | 217 |
| Gabo | A | – | 81.9 | 15.84 | – | 126 | – |
| Oxley | H | A | 85.5 | 12.51 | 7.71 | 134 | 231 |
| Bindawarra | H | A | 88.3 | 17.15 | 8.64 | 185 | 266 |
| Egret | H | A | 89.1 | 13.41 | 6.14 | 155 | 268 |
| Machete | A | A | 95.5 | 13.61 | 7.14 | 152 | 276 |
| W1*MMC | A | A | 99.4 | 14.38 | 7.35 | 125 | 208 |
| LSD ($P<0.05$) | | | 30.0 | 4.20 | 1.95 | 62 | 99 |

**Table 4** Pairwise correlation coefficients between concentration of boron in shoots and grain and grain yield of 25 Australian cultivars grown in a field experiment on B toxis soils at Two Wells, South Australia, 1985 and 1986

| | Whole-shoot B concentration 1985 | B concentration in grain, 1985 | B concentration in grain, 1986 | Grain yield, 1985 |
|---|---|---|---|---|
| B concentration in grain, 1985 | 0.76** | | | |
| B concentration in grain, 1986 | 0.54** | 0.77** | | |
| Grain yield, 1985 | –0.43** | –0.50** | –0.38** | |
| Grain yield, 1986 | –0.42** | –0.45** | –0.57** | 0.36** |

** Significant at $P<0.01$

**Table 5** Mean effect of the presence of either the Halberd or the Cranbrook chromosome segment on relative root length (%), leaf symptom score and whole-shoot B concentration of the Cranbrook x Halberd mapping population

| RFLP marker most closely associated with trait | Parent segment-Halberd (H) Cranbrook ( C) | Number of DH lines within class | Least squares mean (%) | Standard error of mean |
|---|---|---|---|---|
| RRL (%) | | | | |
| *Xpsr680–7B* | H | 83 | 54.4 a[b] | 1.5 |
| | C | 75 | 22.3 b | 1.6 |
| Leaf symptom score (1–6)[a] | | | | |
| *Xpsr160–7D* | H | 89 | 2.99 a[b] | 0.08 |
| | C | 68 | 3.49 b | 0.10 |
| *Xpsr680-7B* | H | 80 | 2.83 a | 0.08 |
| | C | 77 | 3.59 b | 0.08 |
| *Xpsr160–7D/ Xpsr680–7B* | HH | 43 | 2.69 a | 0.11 |
| | CC | 34 | 3.88 b | 0.12 |
| | HC | 45 | 3.31 c | 0.11 |
| | CH | 35 | 3.06 a*, c | 0.12 |
| Whole-shoot B concentration (mg/kg) | | | | |
| *Xpsr680–7B* | H | 80 | 171.4 a[b] | 15.4 |
| | C | 75 | 527.9 b | 15.9 |

[a] Mean of leaf symptom score (1–6 scale: 1=no symptoms, 6>90% leaf necrosis)
[b] Means with different letters are significantly different at $P<0.001$; a* is significantly different from a at $P<0.05$

## Validation

Through regression analysis, highly significant ($P<0.0001$) relationships were established between marker allele and both leaf symptom expression and whole-shoot B concentration in the validation population, Halberd x Warigal/MMC. The 2 loci, *Xpsr160–7D* and *Ypsr680-7B*, were estimated to account for 22% and 14% of the variation in leaf symptom data, respectively. Similarly, *Xpsr680–7B* accounted for 84% ($P<0.0001$) of the variation in whole-shoot boron concentration data. As expected, there was no significant relationship between *Xpsr160–7D* and whole-shoot B concentration.

Seventeen wheat cultivars were assessed for differences in leaf symptom score and whole-shoot boron concentration of plants grown on soil containing toxic concentrations of B (Table 2). Significant ($P<0.0001$) relationships were established between the Halberd allele at the *Xpsr680–7B* locus and whole-shoot B concentration and leaf symptom score. Similarly, a significant ($P<0.0001$) relationship was established between the Halberd allele at the *Xpsr160–7D* locus and leaf symptom score.

A sub-set of 25 of the cultivars used in the field study were screened for the Halberd allele at the *Xpsr680–7B* and *Xpsr160–7D* loci. Table 3 presents whole-shoot B concentration for these 25 cultivars grown in the 1985 field experiment and the concentration of B in grain from the 1985 and 1986 experiments. Significant relationships ($P<0.001$) between marker allele and B concentration in both whole shoots and grain were established. There was no significant relationship between *Xpsr160–7D* and B concentration in whole shoots or in grain in 1985, but there was a significant relationship in 1986 ($P<0.005$). Table 4 provides the correlation coefficients between the B concentration in shoots (1985) in grain (1985 and 1986) and grain yield (1985 and 1986).

## Locus effects

The mean effect of the presence of either a Halberd or Cranbrook chromosome 7B segment in the region of *Xpsr680–7B* on RRL, whole-shoot B concentration and the leaf symptom score in the Cranbrook x Halberd map-

**Table 6** Mean effect of the presence of either Halberd or Warigal/MMC marker allele on whole-shoot boron concentration and leaf symptom score of the Halberd//Warigal/MMC population grown in soil containing toxic concentrations of B

| RFLP marker | Marker allele Halberd (H) Warigal*MMC(W) | Number of DH lines within class | Least squares mean | Standard error of mean |
|---|---|---|---|---|
| Leaf symptom score (1–9)[a] | | | | |
| *Xpsr160–7D* | H | 48 | 4.87 a[b] | 0.22 |
| | W | 50 | 5.52 b | 0.20 |
| *Xpsr680–7B* | H | 44 | 4.77 a | 0.22 |
| | W | 54 | 5.57 b | 0.20 |
| *Xpsr160-7D/ Xpsr680–7B* | HH | 25 | 4.30 a | 0.30 |
| | WW | 31 | 5.69 b | 0.25 |
| | HW | 19 | 5.41 b | 0.30 |
| | WH | 23 | 5.26 b | 0.31 |
| Whole-shoot B concentration (mg/kg) | | | | |
| *Xpsr680–7B* | H | 44 | 283.8 a[c] | 16.4 |
| | W | 54 | 432.1 b | 14.5 |

[a] Mean of leaf symptom score (1–9 scale; 1=no symptoms, 9>90% leaf necrosis)
[b] Means with different letters are significantly different at $P<0.05$
[c] Means with different letters are significantly different at $P<0.001$

ping population is provided in Table 5. The presence of the Halberd segment at the *Xpsr680–7B* locus conferred an average 32.1% increase in RRL, a 68% reduction in whole-shoot B accumulation and a 0.76 point reduction .n leaf symptom score over those individuals carrying the Cranbrook segment. The presence of the Halberd segment at the *Xpsr160–7D* locus conferred an average 0.5 point reduction in leaf symptom score. Individuals carrying the Halberd segment at both the *Xpsr160–7D* and *Xpsr680–7B* loci, on average, received a 1.19 point lower leaf symptom score than individuals carrying Cranbrook alleles at both loci. It appears that leaf symptom expression is predominantly controlled by two loci behaving in a largely additive manner, with the chromosome 7B locus having a slightly larger effect than the chromosome 7D locus.

The mean effect of the presence of either a Halberd on Warigal/MMC allele for *Xpsr680–7B* on whole-shoot B concentration and leaf symptoms and either allele for *Xpsr160–7D* on leaf symptom score is presented in Table 6. The presence of the Halberd allele for *Xpsr680-7B* conferred a 74% reduction in whole-shoot B concentration. The presence of the Halberd allele at either or both loci conferred significant reductions in leaf symptom score, but only at $P<0.05$.

## Discussion

Using aneuploid analysis, Paull (1990) identified the chromosomal location of B tolerance genes derived from cvs. Federation and Halberd (moderately tolerant) and cv. G16450 (very tolerant). Chromosomes of homoeologus groups four and seven were found to be involved in the expression of B tolerance. Chromosome 7B was thought to be the most probable location of genes for tolerance to B derived from Federation and, therefore, Halberd, and chromosomes 7D and 4A were the most probable locations of genes derived from G16450. Genetic analysis using a Condor monosomic series [B tolerance in Condor is believed to differ from that of Halberd at 1 locus (Paull 1990)] also located a tolerance gene of Halberd on chromosome 7B (Chantachume, 1995). Similarly, monosomic analysis of G16450, also using the Condor series, supported the location of a major gene for tolerance on chromosome 4A but did not support the hypothesis of a further gene from G16450 on chromosome 7D (Chantachume 1995). In these genetic studies, Paull (1990) used variation in leaf symptoms, while Chantachume (1995) used variation in seedling root length.

While an RFLP marker associated with a gene for B tolerance in the line G16450 has been identified (Paull et al. 1995), any marker-trait association for a gene derived from Halberd, located on chromosome 7B (Paull 1990, Chantachume 1995), had no previously been found. Halberd and a number of related cultivars have been used widely in southern Australian wheat breeding programmes. Identification of a marker closely associated with this gene, or chromosomal region, may have a significant impact on the breeding and selection of B-tolerant cultivars in southern Australia. Additional minor genes conferring B tolerance may be present in Halberd, but the quantitative nature of the traits' expression and the choice of assay system may have inhibited detection using aneuploid analysis.

### Chromosome locations conferring boron tolerance derived from Halberd, and relationships to proposed mechanisms

Nable (1991) found that the pattern of B distribution in plant parts was very similar between tolerant and intolerant barley genotypes despite great differences in the total B accumulated. The chromosome 7B locus identified in this study appears to be involved in the control of boron accumulation in whole plants or, more precisely, the control of a B exclusion mechanism determining the relative accumulation of B in whole plants. The exclusion mechanism is likely to reduce the effect of toxic concentra-

tions of B in solution on root growth, reflected in improved RRL. This is supported by the high correlation ($r$=0.80, $P$<0.001) found between RRL and whole-shoot boron concentration (Table 1).

The chromosome 7D locus was associated with leaf symptom expression only. Nable (1991) found that the concentration of B in leaves of barley increases from young to old leaves and from base to tip. This is the general pattern followed by the progression of leaf symptoms in both barley and wheat. Therefore, it is likely that the chromosome 7D locus is involved in the translocation of boron in leaf tissue, which may contribute to differences in leaf symptom expression.

Nable (1988) attributed genetic variation for leaf symptom expression in wheat and barley to differences in B concentration in plant tissue. Similar relationships between these traits were established in this study, and a genetic basis to this relationship was demonstrated. However this study, and a similar study in barley (Jefferies et al. 1999), identified chromosome regions on chromosome 7D in wheat and chromosome 2H in barley involved in the control of leaf symptom expression independent of boron accumulation. Chantachume (1995) failed to locate genes controlling boron tolerance, derived from Halberd, to chromosome 7D. This investigator used an assay system which only measured variation in root length and hence overlooked the 7D locus. Paull (1990), using leaf symptom scores, proposed that a region on chromosome 7D, derived from G61450, is associated with boron tolerance. It is possible that the Halberd 7D locus identified in the present study and the 7D locus derived from G61450 are common loci.

## Effect of boron tolerance loci on grain yield response

The chromosome 7B and 7D loci identified in this study were shown to have a significant effect on leaf symptom expression and whole-shoot boron concentration in the mapping population, validation population and a number of other, mostly Australian, cultivars. The effect of these loci on grain yield response to boron toxicity was not tested. Significant correlation, however, was observed between the grain yield of a set of Australian cultivars and both whole-shoot B concentration and grain B concentration (Table 4). Paull (1990) proposed that boron tolerance genes present in Australian cultivars are of common origin tracing to parents of Halberd. Australian cultivars carrying the Halberd allele at the *Xpsr680-7B* locus are likely to be carrying the identical chromosome region conferring reduced B accumulation. Concentration of B in whole shoots and grain in 25 Australian cultivars was shown to be strongly related to the Halberd allele for *Xpsr680-7B* (Table 3). There is correlative evidence, therefore, that the *Xpsr680–7B* locus is associated with improved grain yield on boron toxic soils.

The locus conferring improved RRL (chromosome 7B) was transferred from Halberd into cv. Schomburgk to develop BT-Schomburgk through three cycles of backcrossing with selection for vigour in B toxic soil. BT-Schomburgk had a grain yield advantage over Schomburgk of up to 11% in several field trials conducted on soils subject to boron toxicity (Moody et al. 1993). No significant difference was observed between the leaf symptom scores of Schomburgk and BT-Schomburgk, but a significant difference in whole-shoot B accumulation was observed (Table 2). Poor relationships between leaf symptoms and grain yield response have been reported in both wheat and barley (Paull et al. 1988; Jenkin 1993; Riley and Robson 1994). The relative contribution of each major region and/or gene, particularly in relation to grain yield response or 'field tolerance', warrants further investigation.

## Number of loci involved in the control of boron tolerance in wheat

Two regions involved in the control of B tolerance in wheat were identified in the Cranbrook x Halberd mapping population. Jefferies et al. (1999) identified four chromosomal regions involved in the control of B tolerance in barley. Regions on chromosome 4H and 6H were found to be involved in the control of B accumulation in plants, a region on chromosome 2H was found to be involved in the control of leaf symptoms and a region on chromosome 3H was found to be associated with variation in RRL independent of B accumulation. In light of this, it is possible that there are more than two regions in the wheat genome involved in B tolerance.

Exotic wheat germplasm, more tolerant than Halberd, has been identified (Moody et al. 1993). Paull (1990) proposed that genes for B tolerance, derived from an exotic Greek line, GK1450, were located on chromosomes 4A and 7D. Chantachume (1995) supported the location of tolerance genes found in G61450 to chromosome 4A but not 7D.

Paull et al. (1995) tested 110 $F_7$ derived lines of a cross between G61450 (very tolerant) and Kenya Farmer (very sensitive) for segregation in response to B and for 43 RFLP markers. A highly significant association between response to B and an RFLP marker (*XksuG10*) was found. The *XksuG10* locus is located on chromosomes of homoeologous group 4 (Gill et al. 1991). Jefferies et al. (1999) identified a region on chromosome 4H of barley involved in the control of B accumulation also closely associated with *XksuG10*. It is possible that wheat and barley may posses a common B tolerance gene on A4 and 4H, respectively (Jefferies et al. 1999). In addition, transgressive segregation was observed in progeny of a cross between Halberd and G61450 (Paull et al. 1991). This evidence supports the conclusion of at least two different genes controlling B tolerance between the two genotypes.

The cultivar Kenya Farmer and the breeders' line Warigal/MMC produced significantly greater leaf symptom scores than Cranbrook when grouwn on B toxic soil (Table 2). At least 1 additional locus controlling leaf

symptom expression is, therefore, likely to be present in both Halberd and Cranbrook and many other Australian cultivars, but these could not be identified through quantitative trait locus (QTL) mapping of the Cranbrook x Halberd population.

## Marker-assisted selection for boron tolerance

Molecular markers closely linked to genes of agronomic importance have been demonstrated to be useful tools for indirect selection in a barley breeding programme (Jeffereries et al. 1997). Marker-assisted selection is time-efficient, non-destructive and, depending on linkage relationships, characterised by low selection error.

The role and agronomic value of the chromosome 7D locus, derived from Halberd and involved in the control of leaf symptom expression, is uncertain. Selection for B tolerance on the basis of leaf symptom score is also not desirable as the trait is controlled by at least two genes and is subject to high experimental error. Selection on the basis of whole-shoot B concentration is destructive, time-consuming and expensive. The estimated heritability of RRL in the mapping population was very high, exceeding 0.8. The relative time and resource costs of the RRL assay and marker-assisted selection would be comparable. Benefits of marker-assisted selection for the chromosome 7B locus alone would include increased flexibility. The RRL assay is either destructive or requires the transplantation of seedlings. DNA, for marker-assisted selection, can be harvested and extracted from plants grown in the field, glasshouse, or other trait screening assays without significant damage to growing plants.

The major benefit of marker-assisted selection for B tolerance in wheat, particularly in Australia where the frequency of B tolerance genes derived from the same source as Halberd is high, would be in combining, or pyramiding, genes for tolerance from different sources. Transgressive segregation for B tolerance in crosses between Halberd and G61450 has been observed (Paull et al. 1991). Markers associated with important chromosome regions conferring B tolerance in Halberd and G61450 have been identified. The introgression of selected chromosome regions conferring improved B tolerance from both parents into elite quality and agronomic backgrounds could significantly improve the grain yield and quality of wheat grown on soil prone to B toxicity.

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