Characterization of phenotypic and genotypic selection for simple and complex traits of barley

(*Hordeum vulgare* L.)

This thesis is presented for the degree of

Doctor of Philosophy of Murdoch University

July 2009

**REETINDER GILL**

BSc. Agric. (Hons.)
MSc. Vegetable Crops

The School of Biological Sciences,
Division of Science and Engineering,
Murdoch University
I declare that the research presented in this thesis is original and was undertaken and written by myself, except where specifically indicated in the text.

The thesis has been completed during the course of enrolment in a PhD degree at Murdoch University and has not been used previously for a degree or diploma at any other institution.

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Reetinder Gill
Abstract

The challenge to modern day plant breeding is to constantly strive to improve the efficiency and efficacy of utilising the available resources. This requires the integration of conventional breeding technologies together with molecular genetic markers to significantly improve the breeding programs.

Male sterile facilitated recurrent selection (MSFRS) can improve a breeding program by reducing the variety release time to almost half, maintaining continued out-crossing and recombination, thus broadening the germplasm base. A molecular genetic marker system for the male sterile-orange lemma-shrunken endosperm (msg6-rob1-sex1) linkage block was developed during this study, to aid in the differentiation of fully fertile homozygous from fully fertile heterozygous plump individuals in F2 populations. These individuals may be desired for the production of doubled haploids or for use as pollen donor parents in further cycles of MSFRS. A DF578/6*Gairdner BC5 population was chosen for the study and SSR markers were identified in the region of the msg6-rob1-sex1 linkage group on the short arm of chromosome 6H. A linkage map was constructed and it was found that microsatellite markers HVM65, HVM74 and Bmgtttttt1 are tightly linked to msg6-rob1-sex1 linkage block.

Within the MSFRS process, it was found that genotypic selection with SSR markers is an advantage when the phenotype of interest is controlled by a single major gene and the marker is either “perfect” or closely linked. Genotypic selection was found to be very effective in selecting for the aluminium tolerant genotypes with the SSR marker HVM68,
where aluminium tolerance is known to be controlled by \( \text{Alt} \) or \( \text{Alp} \) gene on chromosome 4H.

For the complex trait such as barley scald resistance, genotypic selection was found to be of limited value. Simulation studies demonstrated that multiple genetic factors need to be taken into account while selecting for a complex phenotype. As expected, phenotypic selection method was found to be efficient in selecting for scald resistance as it selected minor genes along with two known major genes for scald resistance on chromosome 4H (\( \text{RhsVlamingh} \)) and 6H (\( \text{RhsWABAR2147} \)).

Simulation studies based on the experimental results provided a guide for the frequency and timing of the use of molecular markers in the breeding program. It was found that markers that are loosely linked to the gene of interest should only be used once early in the breeding program. As in the case of scald resistance, where the markers-gene distances are 5cM or 30 cM, the genes can only be partially fixed. Further cycles of genotypic selection for scald resistance will lead to the selection of the susceptible genotypes instead of resistant genotypes as the phenotype comes in repulsion rather than the coupling phase with the scald resistant genes.

GGT (an acronym of Graphical GenoTypes) software package was extensively used to study genotypic changes in response to selection and to select for the genotypes carrying resistant genes of interest. Based on the allele frequencies and the marker-scald associations carried out using GGT, the SSR marker Bmac213 (1H) was found to be associated with powdery mildew (\( \text{MlaWABAR2147} \)) resistance and SSR markers GBM1221
(4H) and Bmac316 (6H) were found to be associated with scald (\textit{RhsFlamingh} and \textit{RhsWABAR2147}) resistance.

Results presented in this thesis have enabled the identification of mechanisms behind the success of phenotypic selection and its use while selecting for quantitative traits as it incorporates the minor gene effects while selecting for the major genes. Genotypic selection method was found to be efficient in selecting for the desired genotypes but may not give the desired result in terms of phenotype when a complex trait is involved. Genotypic selection will be at par with the phenotypic selection for the complex quantitative traits if the associated markers with the minor genes are included in the selection. Both phenotypic and genotypic selection methods together can be used effectively in the breeding program to increase the rate of genetic gain.
Acknowledgements

During the course of this PhD thesis I have come to believe that interdependence is certainly more valuable than independence as a journey is easier when you travel together. First and foremost I would like to express my special thanks to my husband and best friend Mandeep for helping me to concentrate on completing my thesis and supporting me mentally during the course of this work. I would not be sitting in front of my monitor typing these acknowledgement lines without you. I am very grateful for your love, patience, help and encouragement during the PhD period.

This is a great opportunity to thank all the people who accompanied and supported me during my PhD. I extend my most sincere thanks to my supervisors, Dr. Reg Lance, Prof. Rudi Appels and Dr. Chengdao Li for their advice and support over the period of my candidature. Thank you Rudi for giving me the opportunity to take on this project and for being supportive during all the ups and downs of this journey. I am really grateful to you for all those hours that I am sure you have spent outside of work hours to read my work and respond in the punctual time, which you always do. I wish to thank
Reg for his constant encouragement, great understanding and for the lessons
he gave me on work-life balance - you have always put in a large effort to help
make the fieldwork successful. Internationally, I would like to acknowledge
A/Professor Duane Falk for his help and support whenever I needed it and for
taking time out during holidays to meet me to discuss my project and general
breeding and genetics, when I visited University of Guelph, Canada.

I thank everyone from the Barley group at the Department of Agriculture and
Food, Western Australia and Research station, UWA, for assisting me with
phenotyping experiments. I also appreciate the contributions of Dr. Katia
Stefanova with the statistical analysis for the experiments. My experience at
SABC would not have been such a good one without the presence of all the
'girls' working there. Thanks Dora, Vera, Julie, Esther, Sharon, Fiona, JJ and
Steve for putting up with my questions and for their advice. I have learned a
lot from you all.

Funding for this research was provided by Murdoch University and the
Molecular Plant Breeding CRC. Without this financial support my research
would not have been possible. Financial support for conference attendance has
also been provided by Murdoch University and MPBCRC with additional funds from DAFWA. Additionally, I acknowledge the facilities provided for this research by The Western Australian State Agricultural Biotechnology Centre and the Department of Agriculture and Food, Western Australia.

Special thanks to my friend Yumi, whose friendship I will cherish forever. Over the years I have been grateful for your support and friendship. You made my time at Murdoch good fun, as well as stimulating. I really enjoyed all our wide-ranging discussions and miss them. Many thanks to my friend Dean Deipeveen for always supporting and believing in me which has led to the next step in my scientific career. Thanks are due to my friends Jyoti, Meenu, Neetu, Bobby and Navi for all their support during my PhD candidature. Writing these acknowledgements, I am realizing that never again will I spend the entire weekends working in the lab or thinking about the 'perfect sentences' for the discussion sections in my thesis.

Finally, I would like to thank my parents and my brothers, Puneet and Sumeet, who have always been there for me as an unwavering support.
I wish to dedicate this thesis to my daughter, Raavi - born in March 2009 - who is too young to understand this but I hope this will inspire her to achieve her goals under any circumstances. One of the best experiences that I and Mandeep lived through in this period was waiting for our first child to be born and our little princess has provided an additional and joyful dimension to our life.
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5.3 Allelic codes assigned to the Parents and their different combinations for graphical genotyping display. Different combinations are based on the five parents; A-WABAR2096, B-WABAR2147, C-Birka, D-Skiff and E-Vlamingh.

5.4 Categorizing the alleles into parental alleles according to Identity by Descent. Allele codes are based on Table 5.3.

5.5 Cumulative allele frequencies of all the alleles present in the graphically genotyped population for scald and powdery mildew resistances. For allele codes please see Table 5.3.

5.6 The genotypes with their genotypic frequencies for both phenotypically selected
(Treatment 1) and genotypically selected (Treatment 2) populations, based on Figure 5.8.

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5.8 The frequencies of the favourable alleles after one cycle of selection, observed in the present study and the one simulated using Qu-Gene have been compared. This table is based on Figure 5.4, Figure 5.5 and Table 5.7.
CHAPTER 1

Introduction and Review of Literature

1.1 Introduction

Barley (*Hordeum vulgare*) has played an important role throughout human history. The oldest archaeological remains indicating the use of wild barley, *Hordeum spontaneum*, are from as early as 17000 BC (Zohary & Hope, 1994). When prehistoric man turned to farming, barley was among the first crops to be domesticated. There is relatively little difference between the early domesticated form and the wild forms and it is believed that both were farmed by early settlers. The main difference between the wild form of barley and the early cultivated forms is the ability to retain grains on the ear after maturity. This characteristic is very advantageous in a farming-system and is believed to have arisen soon after agricultural use of wild barley was initiated around 8000 BC (Zohary & Hopf, 1994). Based on genetic markers it is believed that barley was domesticated in the Fertile Crescent somewhere in the Israel-Jordan area (Badr et al., 2000). Today, barley is still one of the most important cereal crops and is cultivated in a wide climatic range of environments, from sub-arctic regions via the tropics to arid semi-deserts.
Barley is a cereal grain, which serves as a major animal feed crop (73% of barley crop) with 19% used for malting (e-malt.com 2007). It is a member of the grass family Poaceae. In 2005, barley ranked fourth in quantity produced and in area of cultivation of cereal crops in the world (560,000 km²).

Domesticated barley (H. vulgare) is descended from wild barley (Hordeum spontaneum). Both forms are diploid (2n=14 chromosome). As wild barley is interfertile when crossed with domesticated barley, the two forms are often treated as one species, divided into Hordeum vulgare subsp. spontaneum (wild) and subsp. vulgare (domesticated). The main difference between the two forms is the brittle rachis of the former, which enables seed dispersal in the wild.

Barley was grown in about 100 countries worldwide in 2005 with Australia being at position seven in production, 6.6 million metric ton (FAO, 2005). In year 2007, the area under barley production in Australia was 1,150,000 hectares with an average yield of 1.65 tonnes/ha and production of 1900 KT (Australian crop report, 2007). Australia exports 1.5 million tonnes of malting barley.

Barley is an important grain industry in Western Australia (WA), second only to wheat. Barley production has doubled over the past 13 years and the industry has established a consistent production base of >1.5 million tonnes/annum.

The barley industry is based on a dry land agricultural system. Barley varieties are 2-row spring types and are sown in rotation with wheat, canola, pulse crops and annual pastures. Barley growers are strongly focused on delivering grain for sale to the malting and brewing
industries, with accredited malting varieties counting for 88% of the acreage sown to barley in 2002. Over 60% of the total amount of barley produced, is selected for use by the malting and brewing industry and the Shochu market in Japan.

Demand for high quality malting barley is increasing both domestically and internationally. The release of high yielding varieties with agronomic management packages are important for WA grain growers to ensure production of barley is competitive against the production of other annual crops including canola, wheat and pulse crops. There is a need of agronomic solutions to help combat the increase of foliar diseases such as scald, powdery mildew, leaf rust etc and the release of new malting barley varieties with improved tolerance to abiotic stresses including acid soil/aluminium and boron toxicities, waterlogging, salinity and spring drought (Jettner et al, 2003).

This project is focused on issues dealing with selecting lines and developing malting barley varieties with biotic and abiotic stress tolerance using male sterile facilitated recurrent selection populations.

The aims of the project are:

- To develop a marker system for the male sterile gene, msg6, so as to aid in the differentiation of homozygote male fertiles and the heterozygote male fertiles in a male sterile facilitated recurrent selection population.
- To compare the characteristics of phenotypic selection versus genotypic selection for aluminium tolerance in a male sterile facilitated recurrent selection population of barley and to select for aluminium tolerant lines from the population.
• To track responses to selection for Scald and Powdery mildew resistances in a male sterile facilitated recurrent selection population of barley and to characterize the phenotypic selection and genotypic selection methods.
• To graphically genotype the male sterile facilitated recurrent selection population of barley to select the scald and powdery mildew resistant lines to be used for further Western Australian breeding programs.

1.2 Review of literature

1.2.1 Male sterile facilitated recurrent selection

1.2.1.1 Male Sterility

Male sterility refers to the absence of functional anthers, pollen or male gametes in otherwise hermaphrodite flowers. Manifestations of male sterility (Kaul, 1988) include:

- Absence or malformation of male organs (stamens) in bisexual plants or no male flowers in dioecious plants.
- Failure to develop normal microsporogenous tissue i.e. anther.
- Abnormal microsporogenesis- the formation of non-viable pollen.
- Abnormal pollen maturation; inability to germinate on compatible stigma.
- Nondehiscent anthers but viable pollen.
- Barriers other than incompatibility preventing pollen from reaching ovule.

Hybrids are produced to improve certain aspects of a plant. This could be to increase the yield, develop disease resistance, create better uniformity of plant and fruit size, high
quality. In order to produce hybrids, two basic requirements need to be satisfied, first, easy emasculation of the female parent and second, effective pollen dispersal from the male parent to ensure satisfactory seed set in the female parent.

The integration of male steriles into a crossing program avoids laborious and time consuming hand emasculation. The traditional manner of producing hybrid plants involves manual emasculation of the female parent, so that self pollination is not possible. This procedure is only practical when it is possible to remove the anthers. Male sterility may be induced by treatment of the plant with a chemical hybridizing agent (CHA) which inhibits the synthesis of viable pollen (Cross and Ladyman, 1991; Hakraborty and Devakumar, 2006). Compared with physical emasculation, CHA treatment is somewhat inefficient and a certain amount of self pollination occurs which results in the harvested hybrid seed containing some seed of the female parent and the separation of this rogue contamination is virtually impossible. A suboptimal dose can result in incomplete male sterility, leading to contamination and excess dose can cause female sterility along with male sterility (Barabas, 1993). Cytoplasmic male sterility (CMS) is another system which can be employed to get the male sterile plants for the production of hybrid seeds. CMS is a maternally inherited trait that is often associated with mitochondrial genomes (Chase and Babay-Laughnan, 2004; Hanson and Bentolila, 2004). Syngenta, a private company, has been releasing F₁ hybrid barley in Europe for the past six years, developed using CMS. A claim has been made by the company for upto 112% improvement in yield in the hybrids developed using this system (www.syngenta.com). The utilization of cytoplasmic male sterility requires three separate plant lines: the male sterile line, an isogenic male fertile “maintainer line” for propagation and a "restorer line" for restoring fertility to the hybrid so
that it can produce seed. The male sterile line is used as the receptive parent in a hybrid cross, the maintainer line which is genetically the male-sterile line except that it lacks the cytoplasmic sterility factors, and the restorer line is any line that masks the cytoplasmic sterility factor. Cytoplasmic male sterile lines, however, can carry associated traits that make them vulnerable to pathogens e.g. the southern corn blight that attacked all corn hybrids made using cytoplasmic male sterility "T" cytoplasm. There is also a problem with loss in yield as high as 10-12%, associated with the use of cytoplasmic male sterility (http://www.wipo.int/pctdb/en/wo.jsp?IA=US2001045756&DISPLAY=DESC). Another drawback is that it is the existence of very few CMS lines and the difficulty in identifying good maintainer and restorer lines (http://www.grain.org/hybridrice/?id=57). The transgenic barnase-barstar system for inducing male sterility has also been investigated where male sterile plants can be obtained by gene manipulation through molecular biology. It works by introducing a gene for a bacterial ribonuclease (barnase gene) in the plants, which includes plant promoters directing the expression through the ribonuclease to degrade mRNA in stamen cells leading to male sterility DNA in stamen cells. Later, male fertility can be restored to the plant with a chimeric fertility restorer gene (barstar gene) coding for male fertility (Mariani et al, 1990, 1997; Frank et al, 2002). A useful alternative procedure which is available to plant breeders and is most commonly used is the phenomenon of genetic male sterility.

Genetic male sterility is ordinarily governed by a single recessive gene, ms. It arises spontaneously or can be artificially induced. A male sterile line can be maintained by crossing it with a heterozygous male fertile plant (MSms) which results in 1:1 segregation of male sterile and male fertile plants. This system is genetically simple, and can be readily
used to promote cross pollination in self-pollinated species to maintain and enhance genetic diversity with minimum labour.

1.2.1.2 Recurrent Selection:

According to Hull (1952), recurrent selection includes reselection generation after generation, with interbreeding of selected lines to provide for genetic recombination. Thus selection among isolates, inbred lines or clones is not recurrent until selected lines are interbred and a new cycle of selection is initiated.

With recurrent selection the most favorable combination of genes contained in a group of plants are continually selected. As the opportunity of recombination is increased, the chances of obtaining satisfactory individuals is increased and it is thus possible to maintain high levels of genetic variability and hence provide for more effective selection over longer period.

1.2.1.3 Barley and genetic male sterility

Barley is primarily a self-pollinated crop species and it is necessary to emasculate or remove the immature anthers to prevent self-fertilization in a crossing program. Since hand emasculation is tedious in barley, it was necessary to devise alternate ways for producing the large volumes of crossed seed needed for the successful growing of commercial hybrid barley (Wiebe, 1965). The first genetic male sterility in barley was reported by Suneson (1940).
Proposals for the production of hybrid barley by Wiebe (1960) and Ramage (1965) by utilizing genetic male sterility emphasize the desirability of locating many different genes to facilitate such approaches (Hockett and Eslick, 1968). A total of 49 male sterile genes were reported in barley, out of which 20 male sterile genes were mapped over seven barley chromosomes (Figure 1.1). In addition to the mapped male sterile genes shown, there are 16 genes which are not mapped to a relative position in barley genome but their tentative association with specific chromosomes is known (Table 1.1).

Figure 1.1: Male sterile genes present and mapped in barley (Franckowiak, 1997)

Eslick and Hockett (1962) published the results of the studies concerning sterility in barley conducted in Montana since 1948, which indicated the presence of various types of sterility including genetic male sterility. Open-pollinated seed-set ranged from 9.2 to 0.6 percent from the results of various studies involving various genetic male sterile genes in 11 varieties grown at 8 locations. Different male sterile genes in a single variety resulted in different levels of the percentage of cross-pollination of male sterile plants. Results
indicated that genetic male sterile genes may be characterized on the basis of anther type, presence or absence of pollen, pollen stainability percentage and in some instances, open-pollinated seed production.

Table 1.1: Information about the male sterile genes which are not mapped but have been linked to the chromosomes per se (Franckowiak, 1997).

<table>
<thead>
<tr>
<th>Genes</th>
<th>Chromosome loc</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>msg20</td>
<td>1H</td>
<td>Hockett <em>et al.</em>, 1971</td>
</tr>
<tr>
<td>msg4</td>
<td>1H</td>
<td>Barley genetic stock database</td>
</tr>
<tr>
<td>msg35</td>
<td>2H</td>
<td>Franckowiak, 1995</td>
</tr>
<tr>
<td>msg48</td>
<td>2H</td>
<td>Franckowiak, 1991</td>
</tr>
<tr>
<td>msg42</td>
<td>3H</td>
<td>Franckowiak, 1995</td>
</tr>
<tr>
<td>msg25</td>
<td>4H</td>
<td>Barley genetic stock database</td>
</tr>
<tr>
<td>msg29</td>
<td>5H</td>
<td>Franckowiak, 1995</td>
</tr>
<tr>
<td>msg49</td>
<td>5H</td>
<td>Franckowiak, 1991</td>
</tr>
<tr>
<td>msg34</td>
<td>6H</td>
<td>Franckowiak, 1991</td>
</tr>
<tr>
<td>msg39</td>
<td>6H</td>
<td>Franckowiak, 1991</td>
</tr>
<tr>
<td>msg40</td>
<td>6H</td>
<td>Franckowiak, 1991</td>
</tr>
<tr>
<td>msg28</td>
<td>6H</td>
<td>Franckowiak, 1991</td>
</tr>
<tr>
<td>msg22</td>
<td>7H</td>
<td>Hockett and Eslick, 1971</td>
</tr>
<tr>
<td>msg26</td>
<td>7H</td>
<td>Franckowiak, 1995</td>
</tr>
<tr>
<td>msg30</td>
<td>7H</td>
<td>Franckowiak, 1991</td>
</tr>
<tr>
<td>msg32</td>
<td>7H</td>
<td>Hockett and Eslick, 1971</td>
</tr>
</tbody>
</table>
1.2.1.3.1 Pollen development in genetic male sterile barley

Male reproductive processes in flowering plants take place in the stamen. This sporophytic organ system contains diploid cells that undergo meiosis and produce haploid male spores called microspores. Microspores divide mitotically and differentiate into multicellular male gametophytes or pollen grains that contain the sperm cells (Bedinger, 1992; Goldberg et al., 1993). Figure 1.2 shows the two morphologically distinct parts of the stamen – anther and filament. The anther contains the reproductive and non reproductive tissues that are responsible for producing and releasing pollen grains so that pollination and fertilization processes can occur in the flower. The filament is the tube of vascular tissue and joins the stamens to the flower and serves as the conduit for water and nutrients.

![Fig 1.2: A generalized overview of anther development (Goldberg et al., 1993). Schematic representation of anther development stages and cross-sections are based on scanning and light microscopy studies of tobacco anther development (Koltumow et al., 1990; Drews et al., 1992). C – connective, CCC – circular cell cluster, E – epidermis, En – endothecium, PG – pollen grain, PS – pollen sac, St – stomium, T – tapetum, Td – tetrads, Th – theca, V – vascular bundle.](image-url)
The development of anthers and pollen of various male sterile mutants in several species has been studied and a close correlation was found between the development of the tapetal cell layer and the development of the pollen grains (Beadle, 1932; Chauhan and Singh, 1966; Joppa et al, 1966; Kaul and Singh, 1966). The tapetum of higher plants was found to play a critical role in pollen development (Pacini et al, 1985). It was generally accepted that the tapetal cells play a nutritive role for the developing microspore (Carneil, 1963; Bedinger, 1992). Although most of the functions of tapetum are still unknown, functions that have been proposed include the tapetal cells releasing the young haploid microspore from the callose wall enclosing the meiotic tetrad by the secretion of a beta-1,3-glucanase or callase (Steiglitz, 1977). The timing of callase secretion by the tapetal cells was found to be critical for normal pollen development. The second main role for the tapetal cells is the production of a precursor for the biosynthesis of the outer pollen wall or exine. The tapetal cells undergo cell death as the microspore becomes vacuolated but the dying tapetal cells still contributes to the pollen development with the deposition of cell remnants (Bedinger, 1992).

Several genetic studies have identified recessive sporophytic mutations at many different loci causing male sterility (Beadle, 1932; Albertsen and Phillips, 1981; Kaul, 1988). Most cytological studies have reported the tapetal cells to be the affected sporophytic cell type (Bedinger, 1992). Several of the maize male sterile mutants (ms7, ms1) were found to be defective in pollen wall biosynthesis which is consistent with the proposed role of the tapetum in exine production. Other ms mutants (ms14, ms13) were found to affect the progression of microspores through mitosis, causing failure of chromosome condensation (Albertsen and Phillips, 1981).
Three types of abnormalities were found in three developmental stages of male sterility in wheat (Chauhan and Singh, 1966). First type was characterized by early degeneration of the tapetal layer. In the second type, the tapetum remained intact beyond the stage when its breakdown should have started. This was accompanied by the increase in nuclear size in tapetum cells. As a result the mature pollen grains were found to be devoid of a germ pore, cytoplasm and nuclei. In the third type, the tapetal cell wall disintegrated at the start of the meiosis.

Kaul and Singh (1966) reported that the tapetal layer in sterile mutants continued to grow beyond the free microspore stage. This resulted in the highly vacuolated tapetal cells while in fertile anthers the tapetal layer degenerated by the time the free microspores were formed. The stages of the breakdown of microsporogenesis in male sterile plants range from the degeneration of the anther tissue during their differentiation through mature pollen abortion or failure. In a majority of heritable cases of male sterility the genetic factors apparently act with utmost precision as to the exact stage and mode of breakdown in normal processes of pollen development (Jain, 1959).

Three barley mutants were chosen by Roath and Hockett (1971) to study the stages of microsporogenesis at which the pollen breakdown occurs. The mutants selected were: Dekap msg7, as it was found to have rudimentary anthers and forms little or no pollen; Betzes msg8, as it showed great variation in anther and pollen development and Heines Hanna msg6, as it had normal appearing anthers and pollen (Roath, 1969). Enough anthers
<table>
<thead>
<tr>
<th>Variety or Mutant</th>
<th>Meiosis</th>
<th>Quartet</th>
<th>Free Microspore</th>
<th>Pollen Dehiscence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sporogenous</td>
<td>Tapetal</td>
<td>Sporogenous</td>
<td>Tapetal</td>
</tr>
<tr>
<td>Betzes fertile</td>
<td>Normal</td>
<td>Binucleate. Reaches maximum size</td>
<td>Normal</td>
<td>Starts to degenerate</td>
</tr>
<tr>
<td>Heines Hanna ms6</td>
<td>Normal</td>
<td>Some cell separation in premeiosis</td>
<td>Normal</td>
<td>Starts to degenerate, cell separation still visible</td>
</tr>
<tr>
<td>Betzes ms8</td>
<td>Normal</td>
<td>Some cell separation in premeiosis</td>
<td>Normal</td>
<td>Considerable cell degeneration and separation</td>
</tr>
<tr>
<td>Dekap ms7</td>
<td>Normal</td>
<td>Normal until telephase 2</td>
<td>Normal</td>
<td>Swelling begins</td>
</tr>
</tbody>
</table>

Table 1.2: Morphological development of male fertile and sterile anthers during anther tissue development (Roath and Hockett, 1971)
were selected so that the samples overlapped each other in the stage of development and provided duplicate samples. The anthers were embedded in paraffin, sectioned stained with the safranin stain fast green stain method. The anther and pollen development of the fertile and mutant lines is summarized in the Table 1.2.

The development and appearance of the pollen and anthers of msg6 were found to be nearly normal. The effect of cell separation of the tapetal layer in pre-meiosis on the pollen development of this mutant is not known. The normal appearance of pollen at anthesis suggested that breakdown of this pollen leading to male sterility probably occurs after anthesis.

In general, the pollen of male steriles had been found to be devoid of cytoplasm and with shrunken walls. The anthers of the sterile types were generally smaller than normal anthers, based on anther size observations in 18 genetic male sterile mutants in barley (Roath and Hockett, 1971). The stains used were 2,3,5-triphenyltetrazolium chloride (TTC), acetocarmine and iodine potassium iodide. Stain uptake with the vital stain TTC was found not a good indication of selfing ability, but non stainability indicated sterility. All anthers from male sterile plants were found to be significantly shorter than their fertile sibs, except for msg1, msg16 and msg6. Mutant msg6 was always found to exhibit filament elongation, anthers with a stomium and an apparently normal dehiscence. In addition, this mutant had stainable pollen, a normal pollen shower and normal anthers; therefore the basis for the pollen not able to produce selfed seeds was not clear.
Another study was conducted on pollen grains of male steriles using the Fast Blue B salt staining method (FBB), which is specific for sporopollenin (a polymer that constitutes the outer wall of spores and pollen grain) containing structures (Ahokas, 1975b, 1976). The aperture sites of pollen grains can usually be demonstrated with FBB staining (Ahokas, 1976). The anthers which opened at anthesis were found to bear undeveloped pollen grains with the stainable ring of the apertural annulus without any actual pore as for msg6. The exine staining was found to be much darker on the mutants. The mutants msg16 and msg-dm were found to produce completely in-aperturate pollen grains and the mutants msg6, msg-dm and msg-dn were classified as actual pollen steriles.

1.2.1.3.2 Genetic male sterility and barley breeding

The use of genetic male sterility has become a widely used tool in barley breeding. In crossing programs, it is being used instead of hand emasculation in producing hybrid seed and also helps to avoid tedious hand crossing procedures and now it is possible to have large numbers of crosses, large number of F1s, large populations and also large numbers of genetic recombinations (Jensen, 1963). Therefore genetic male steriles are used to increase and maintain out crossing and continued recombination in recurrent selection schemes.

The uses of genetic male sterility in plant breeding also include estimations of pollen drift from the desired pollen donors to the male sterile plants. The pollination of the male sterile plants with the pollen from the undesired plants can lead to contamination from unwanted crossing. This can be reduced by planting desired barley parents as pollen donors in close proximity to the male sterile plants and by removing normal plants as soon as they can be identified upon emergence of the heads from the boot (Jensen, 1959).
Composite crosses, based on the use of male sterile plants, have been a valuable genetic resource in barley and the use of genetic male sterility has facilitated crossing and enhanced their utility. Suneson (1959) emphasized the importance of multiple recombinations breeding in the form of composite crosses. Accordingly, they had a high resource value for exploiting selection, either directly or through further recombination. The success with the first multilineal wheat varieties also served as a guide for contemplated composite barley breeding program (Gibler and Borlaug, 1959). A world composite cross involving 6200 spring barleys was reported by Suneson and Wiebe (1962) which included varieties chosen for winter hardiness, disease and insect resistance, agronomic traits and range of adaptation. This “evolutionary” composite cross (XXI) population was constructed as a result of massive intercrossing among male sterile and fertile plants. Hockett and Eslick (1970) observed that enough out-crossing could take place, in barley, to provide for a useful tool in developing Composite Cross populations for use in germplasm improvement and, possibly, direct cultivar development. This approach would be advantageous over conventional crossing by virtue of being able to generate potentially large quantities of hybrid seed, under the right conditions, without much labour input and allow for relatively rapid production of multi-way crosses. Although some breeders argue that multi-way crosses should be avoided if possible so as to preserve the desired linkage block, it is purely a matter of selection, whether the breeder wants to select for the linkage block or wants recombinants within the linkage block.

Since the above landmark study, a large number of composite crosses have been produced and registered in North America in 1980s, using genetic male sterility as a facilitator for recurrent selection to introgress many useful agronomical traits (Table 1.3).
Table 1.3: Composite crosses developed using male sterile facilitated recurrent selection for biotic and abiotic stresses.

<table>
<thead>
<tr>
<th>Composite Cross</th>
<th>Year of registration</th>
<th>Important characteristic</th>
</tr>
</thead>
</table>

Besides disease resistance, composite crosses have pointed the way to more stable yield. They featured plant populations rather than pure lines (Suneson, 1959).

To ensure that male sterile plant or seed can be easily identified, the male sterility genes were linked to some phenotypic character. The use of linkage between a male sterile gene and a gene for the character that can be chemically rogued was first proposed by Wiebe (1960). According to this proposal, the linked genes for male sterility and resistance to DDT would be transferred by backcrossing to the variety that was to be used as the female parent of the hybrid. The strategy was to spray the female rows in the crossing block with DDT, later, to get pure stand of male sterile plants which could be pollinated from the unsprayed plants. This method was found to have leakage in male sterility. DDT is no longer in use due to health and safety reasons.
Any gene for the seed or seedling character that lends itself to economic separation of genotypes can be used in this scheme (Ramage, 1965). Eslick (1969) suggested linkage of male sterile gene with the recessive alleles of dominant, pre-flowering genes so as to make it possible and easy to identify the male sterile plants and rogue any fertile plants before pollination.

1.2.1.4 Linkage of male sterile gene on chromosome 6H with morphological markers; shrunken endosperm and orange lemma

The male sterile gene $msg6$ occurred as a spontaneous mutant in the cultivar Heines Hanna (Hockett et al., 1968) which has complete female fertility (Eslick et al., 1974) and normal (Roath and Hockett, 1971) but non-functional grain because of abnormal aperture development in the pollen (Ahokas, 1975). Ratios and recombination values determined from progeny of $msg6 \times translocation$ crosses, male sterile gene $msg6$ was mapped to chromosome 6. The observed data indicated this gene to be near the centromere or on the long arm of chromosome 6 (Eslick et al., 1974). Lehman and Hagberg (1978) confirmed that $msg6$ gene was located near the centromere of chromosome 6. Kunzel (1982) indicated with the linkage studies of 13 translocations that $msg6$ gene is located genetically in or close to the centromere on the short arm of chromosome 6H. The $msg6$ gene was found to be about 1cM distal from the orange lemma locus, $rob1rob1$ (Eslick et al., 1974). Orange lemma is a spontaneous mutant found in Betzes (Clho5649) (Myler and Stanford, 1942). In this mutant the lemma, palea and rachis have an orange pigmentation that is present in the immature spikes and is retained in the mature grain as well (Buckley, 1930; Myler and Stanford, 1942). Telotrisomic analysis conducted by Shahla et al (1983) concluded that the orange lemma gene $o$ was present on the short arm of chromosome 6H. The various
independant studies conducted by Ramage et al (1961), Kramer and Blander (1961) and Persson (1969) also indicated the presence of orange lemma gene close to the centromere on Chromosome 6H.

The shrunken endosperm gene *sex1* was positioned on the long arm of chromosome 6H at less than 1 cM distance from the orange lemma gene by Eslick and Ries (1976), with the help of translocation studies. Jarvi and Eslick (1975) identified six spontaneous shrunken endosperm mutants in barley and one of them was *sex1 (se6)* which expressed xenia permitting 1:2:1 classification in the F₂. It was collected from a commercial field of Compana (PI539111). The describing factor for this mutant was found to be the depression in the centre of the lemma which it develops after the hard dough stage and is harder than the normal endosperm. Mutant plants were found to have higher lysine content in the endosperm than the normal sibs (Ullrich and Eslick, 1978). Jarvi and Eslick (1975) concluded that *sex1* may be useful in pre-flowering selections and could become an important marker for chromosome 6H. Falk et al (1980) found the male sterile gene *msg6* closely linked (< 1% recombination) to the shrunken endosperm gene *sex1*, on barley chromosome 6. They obtained a recombination between these genes to establish the recessive alleles in the coupling phase and registered the shrunken endosperm male sterile germplasm for the hybridization in barley (Falk and Kasha, 1982). The *sex1; msg6* stock was produced from the crosses with Heines Hanna (*msg6*) and Compana (*sex1*).

With the help of multiple-point linkage tests and flanking markers, Falk et al (1980) determined the gene order of genes near the centromere as *msg6/rob1/sex1*. Two most probable orders of the genes given depending upon the assumption that *msg-bk* is located
on the short arm of chromosome 6 and that \textit{rob1} is very close to the centromere were \textit{sex1/rob1/msg6} and \textit{msg6/rob1/sex1}. The orientation of \textit{alb-q} and \textit{alb-t} influence the arm on which \textit{sex1} will be placed. Falk (1993) created a marked telo 6S trisomic for chromosome 6H by crossing the telo 6S tetrasomic stock to male sterile chromosome 6 marker stock homozygous for \textit{msg6/rob1/sex1/ant13}. The telo 6S trisomic chromosome was found to contain loci complimentary to the homozygous recessive alleles for \textit{msg6} and \textit{rob1}, thus placed them on the short arm of chromosome 6H and the absence of loci complimentary to \textit{sex1} and \textit{ant13} alleles on the normal chromosomes, put them on the long arm of the chromosome 6H. It was found that the gene order near the centromere must be \textit{msg6/rob1/ [centromere]/sex1/ant13}.

It was found feasible to use pre-sowing and pre-anthesis selection of male sterile plants for the production of outcrossed seed by selecting the seeds with shrunken endosperm from a number of F2 populations (Falk, 1981). The linkage block \textit{msg6-rob1-sex1/Msg6-Rob1-sex1} (male sterile allele linked in coupling phase with orange lemma and shrunken endosperm) was used for these selections. The crossing block consisted of two central rows of male sterile plants and one row of males on each side. Plants from the central rows were harvested individually and each plant was then classified for male sterility, orange lemma colour and shrunken seed. It was found that an overall average of 96.21% of the selected seed produced male sterile plants. A total of 2.1% of the plants resulted from the misclassified seeds (\textit{MS-SexSex} and \textit{MS-Sexsex}) and 1.7% were derived from seeds containing crossover gametes (\textit{MS-rob1rob1-sexsex} and \textit{MS-Rob1Rob1-sexsex}). Thus it was stated that since the fertile plants can be readily recognized before anthesis, these can be rogued out and the entire male sterile rows subsequently machine harvested at maturity.
Pre-sowing selection of male sterile lines made the use of male sterile facilitated recurrent selection populations easier and more efficient as a breeding technique.

The use of genetic male sterility to facilitate crossing on a large scale led to the concept of male sterile facilitated recurrent selection populations (MSFRP) (Eslick and Hockett, 1974). Barley HOPE (Hierarchical open-ended pollination enrichment) system based on male sterile facilitated recurrent selection was initiated based on male sterile facilitated recurrent selection (Falk et al, 1990; Kannenberg and Falk, 1991). It was an open-ended system in which germplasm can move upward through the three hierarchical levels and introductions could be added at the first/lowest level.

Figure 1.3: The RIPE system for introgressing new germplasm into the adapted elite core of breeding material (after Kannenberg and Falk, 1995).
The RIPE (Recurrent Introgressive Population Enrichment) method (Kannenberg and Falk, 1995) system was developed to utilize the efficiencies of HOPE along with the genetic male sterility in male sterile facilitated recurrent selection populations. RIPE enabled barley breeders to apply the basic principles of recurrent selection to a normally self pollinated crop, employing specifically the male sterile gene \((msg6)\) and shrunken endosperm gene \((sex1)\), on chromosome 6H for pre-sowing selection of male sterile plants. The RIPE system includes introductions with some trait of proven merit and has four sequential steps contributing introductions into the Elite level (Figure 1.3). Selected lines at each level are crossed to male sterile lines from the Elite level to increase the amount elite germplasm through progressive backcrossing. Thus, the Base level (B) is 50% Elite (E), the Intermediate (I) is 75% E, the third level; High (H) is 87.5% E and at 93.25% E, as the introductions are made. Therefore, the genetic structure of Elite germplasm changes every cycle and E population is maintained through recurrent selection procedures which need two years per cycle.

According to Falk (2004), using RIPE method, nearly 500 crosses can be produced per week by hand crossing/person, compared to 50 crosses per week using traditional methods of emasculation and hand crossing. He reported 50% improvement in yield in 10 years instead of 1% per year, using this system maintaining short-term improvements but keeping long-term potential.

RIPE system developed by Falk and Kannenberg (1995) was definitely a step forward because it simultaneously expanded the genetic base and improved malting and agronomic features so as to keep the elite germplasm intact. A method similar to RIPE is being used in
Western Australia to develop elite germplasm largely using the male sterile gene; \textit{msg6}. RIPE system is very efficient however only for handling small populations where hand crossing is feasible but practically dealing with larger populations in the crossing blocks, the same efficiency is not exhibited and this issue will be discussed later in thesis.

Therrien (2005) published his work based on twenty years of male sterile facilitated recurrent selection employing the hybrid seed produced from the composite cross (CCXXXIII) population. This contained the male sterile gene \textit{msg6} and the elite germplasm containing the linkage block \textit{msg6-rob1-sex1} (the male sterile gene-orange lemma-shrunken endosperm). Along with MSFRS, the conventional (ear to row pedigree) method was also carried out to understand the advantages of using MSFRS over conventional breeding methods. Five hundred lines from each of Standard (Std) and Male sterile derived (MSD) populations were compared for leaf diseases in the field, net blotch and spot blotch in disease nurseries, fusarium head blight and the associated mycotoxin deoxynivalenol, common root rot in the field, two races; Net857 and Net858, of net blotch, one race of scald; Scld1493 and stem rot from laboratory inoculation. The results revealed that except for scald, the male sterile derived population had lower disease incidence scores, in the same environment as Std population. When grain yield was compared for both the populations with the check variety Virden over 14 years, MSFRS lines averaged 25% yield gain over Virden as compared to 8% gain shown by conventional lines and thus it was stated that breeding for disease resistance varieties using MSFRS approach can be advantageous over conventional approaches.
1.2.2 The molecular genetics of barley leaf scald and powdery mildew

Plant disease resistance is the prerequisite for the successful utilization of crop species in modern agriculture. Resistance crops have been produced successfully for many years by conventional breeding techniques. The advent of molecular biology has enabled the genes that confer disease resistance to be analysed at the molecular level, an analysis that is providing insights into the complexity of plant defense and host-pathogen co-evolution as reported by Ayliffe and Lagudah (2004). This knowledge helped in the employment of breeding strategies using marker assisted selection.

Disease can affect the profitability of barley production by reducing the final yield and by lowering the grain quality, which results in the lower financial return to the grower. About 30 diseases and pests have been reported to affect barley (Matre, 1982), though, only few have significant economic impact (Williams, 2003). Among other diseases, two are the major leaf diseases common in Western Australian cereal belt; scald - caused by the fungus *Rhynchosporium secalis* and powdery mildew caused by *Blumeria graminis* f. sp. *hordei*.

Scald and powdery mildew are two of the most economically important foliar diseases (Shipton *et al*, 1974; Balkema-Boomstra and Mastebroek, 1995) that affect barley in many of the major production regions of the crop. The primary loss is reduced yield, which can reach or exceed 30% (Khan, 1986; Vivar *et al*, 1987; Abbott *et al*, 1991). In addition, damage is manifested as a lowering of quality characteristics such as 1000-kernel weight and plumpness (Khan and Crosbie, 1998). This reduction in quality is particularly detrimental for malting barleys, since the grain is size-selected to eliminate the unsuitably small fraction prior to malting. The pathogens, *Rhynchosporium secalis* and *Blumeria*
*graminis* f. sp. *hordei* spreads from plant to plant primarily by air currents and can persist from season to season in crop residue (Shipton *et al.*, 1974).

The occurrence and detrimental effects of disease on barley is dependant on the environment, agronomic practices and the genotype of the pathogen and host. In Australia, barley is grown in a wide range of environments. Powdery mildew is a major disease of barley in the cool and humid agriculture areas. Changing agronomic practices such as minimum tillage are having an effect on disease occurrence and severity, as high levels of stubble-borne inoculum can infect subsequent crops (Wallwork, 2000b). In addition, the use of pesticides as a chemical disease control measure has been reduced due to the community’s concern over the use of pesticides. High pathogen genetic diversity or mutation rates can overcome the effectiveness of chemical treatments or deployment of resistance genes in new cultivars (Williams, 2003). Barley leaf scald pathogen *Rhynchosporium secalis* is highly variable (McDonald *et al.*, 1999) and populations continually contribute to the development of more virulent pathotypes (Watson, 1970). The durability of disease resistance is affected by the evolutionary potential of the pathogen population. Pathogens with a high evolutionary potential are more likely to overcome genetic resistance than pathogens with low evolutionary potential (McDonald and Linde, 2002). The increasing frequency of the virulent strains of the pathogens has contributed to the end of several barley cultivars which were found resistant earlier (Fetch and Steffenson, 1994; Steffenson and Webster, 1992). Twenty three different major genes for resistance against powdery mildew and fifteen major genes for resistance against scald have been reported so far (Chelkowski *et al.*, 2003; Genger *et al.*, 2005).
1.2.2.1 Mapping of scald and powdery mildew resistant loci

Mapping studies have shown that for economically important disease like scald and powdery mildew, few resistant loci exist. Multiple race-specific alleles for both the diseases may be located at these loci but have not yet been identified by host-pathogen differential analysis (Williams, 2003). Cultivated barley and the pathogen causing powdery mildew disease together represent one of the most explored host-pathogen systems in the plants (Falak et al., 1999). There are two explanations for the limited number of resistant genes:

- Greater than expected relatedness in the cultivated germplasm and
- Multiple re-selection of the same genes because of their strong resistant phenotype

Mapping of genetic traits with DNA markers (Beckmann and Soller, 1983; Lander and Botstein, 1989) allows the precise locations of the resistance genes of interest. The source, chromosome location, closest marker(s) and the citation of the all published loci with close DNA based markers for scald and powdery mildew resistant genes are given in Table 1.4.

Mapping studies have shown that in many cases resistance genes tend to cluster and are often genetically linked to the known disease resistance loci. Complex clusters, which contain multiple race-specific genes for resistance to a single pathogen, have been found for both *R. secalis* and *B. graminis f. sp. hordei*. The best studied cluster is at the *Mla* locus on chromosome 1HS which contains 28 named resistance alleles for powdery mildew (Jorgensen, 1994). The *Mla* locus appears to contain multiple specificities organized into multi-gene families (Williams, 2003). In the cultivar Morex, Wei *et al.* (2002) sequenced a 261-kb BAC contig that spans the *Mla* locus and found 15 predicted genes associated with
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Table 14: Mapped genes for resistance to barley leaf scald and powdery mildew
plant defense responses, of which 6 were found associated with defence responses to powdery mildew but which function in different signaling pathways. Homologous sequences, which were amplified using Morex-derived allele-specific primers from Mla-containing lines, co-segregated with Mla6, Mla13, Mla14 and Ml-Ru3 specificities in high resolution mapping populations (Wei et al, 1999).

The lack of standardized testing regimes with defined isolates that can differentiate new genes has made it difficult to determine if a new allele has been identified or a known allele has been re-discovered in the unrelated germplasm (Williams, 2003). The Rrs1 or Rh locus on chromosome 3H conferring resistance to R. secalis, the leaf scald pathogen, was found to be one of these cases, with numerous sources having genes for scald resistance at the same location. More than 10 different alleles for resistance to this fungus have been reported in barley present in this complex locus on chromosome 3H (Graner and Tekauz, 1996; Bjørnstad et al, 2002). The cultivars Johnston and CDC Guardian were found to have scald resistance segregating close to the Rrs1-linked markers on chromosome 3H (Eckstein, 2002). Similarly, the lines B87/14 (Williams et al, 2001), cultivar Sultan and three breeding lines from ICARDA (Genger et al, 2003) were found to have a major scald resistance gene linked to Rrs1. Bjørnstad et al (2002) used near-isogenic lines to map seven alleles for scald resistance to the same locus.

The wild species of cultivated barley, H. vulgare ssp. spontaneum was used in some crosses for scald resistance. Its use helped in the identification of five loci for scald resistance (Abbott et al, 1991, 1995; Garvin et al, 1997, 2000), three of which were later found to be closely linked to PCR-based markers (Genger et al, 2003). Two of the loci in
the wild barley accessions studied, were found to be clustered with *Rrs1* and *Rrs2*, suggesting overlap in scald resistance loci between wild and cultivated barley (Genger *et al*, 2003).

Many major genes have closely linked molecular markers, which can be used for marker-assisted selection, following careful validation of marker-trait linkages (Williams, 2003). Use of multiple validation populations in the marker-assisted selection studies, was found to be better than using one large population, as genetic distances may vary between different crosses (Meseguer *et al*, 1991). The ability to pyramid resistance genes through marker assisted selection has been found to be a powerful tool for the breeders. Brown *et al* (1996) validated this approach by using isozyme markers to combine genes for resistance to leaf scald. The results showed that field disease levels were lower in three of six doubly resistant lines than in backcross lines carrying single resistance gene, which found to be more resistant than the recurrent parent.

1.2.2.2 Quantitative trait loci for scald and powdery mildew resistances

The Quantitative trait loci (QTL) are regions of any genome that is responsible for variation in the quantitative trait of interest. QTL are identified through the statistical analysis of complex traits which are typically affected by more than one gene and also by the environment (Doerge, 2002). Complex traits are more challenging as it is difficult to follow all the genomic regions responsible for the variation of the trait, without knowing the patterns of segregation in that region. Molecular marker techniques which include widely used protein markers such as isozymes and DNA based markers such as Restriction Fragment Length Polymorphism (RFLP), Random Amplification of Polymorphic DNA
(RAPD), microsatellite, Amplified fragment length polymorphism (AFLP) and Single-nucleotid Polymorphism (SNP) have been used extensively to identify quantitative trait loci for malting, brewing and food quality of barley. These markers then can be used to construct detailed genetic maps which provide the foundation for the QTL mapping methodologies which include single marker mapping (Edwards et al, 1987; Beckman et al, 1988; Luo et al, 1989), interval mapping (Lander et al, 1989), composite interval mapping (Zeng, 1993 and 1994; Jansen, 1993 and 1994) and multiple trait mapping (Jiang and Zeng, 1995; Kao et al, 1999; Ronin et al, 1995; Karol et al, 1995) which have provided statistical analysis of genotype-phenotype associations to understand the complex traits.

The availability of extensive linkage maps provide the basis for identifying different genetic backgrounds for individual gene loci (QTL) for disease resistance (Jahoor et al, 2004; Hayes et al, 2005). It has become possible not only to determine and compare the loci involved in a resistance, but also their quantitative effect on the genes, the parent contributing the alleles for resistance and the effectiveness of each locus in various plant development stages or against various pathogen isolates (Qi et al, 1998b, 19999)

Quantitative genetic studies were used to elucidate the genetic control of disease resistance showing continuous variation, before QTL mapping (Geiger and Heun, 1989). According to Williams (2003), QTL mapping had been used:

- To know the number of loci involved in complex resistance
- To know if any race-specific loci are involved in providing quantitative resistance
Table 1.5: Mapped quantitative trait loci for scald and powdery mildew resistances in barley.

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<td>Scald</td>
<td>Keel</td>
<td>3H, 6HS</td>
<td>Cheong et al (2006)</td>
</tr>
<tr>
<td></td>
<td>Chebec</td>
<td>6HL, 7HL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Harrington</td>
<td>6HS, 7HS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>O’Connor</td>
<td>6HS</td>
<td></td>
</tr>
<tr>
<td>Scald</td>
<td>L94</td>
<td>3H, 4H, 6H</td>
<td>Shtaya et al (2006)</td>
</tr>
<tr>
<td>Powdery mildew</td>
<td>E224/3</td>
<td>1H</td>
<td>Thomas et al (1995)</td>
</tr>
<tr>
<td>Powdery mildew</td>
<td></td>
<td>5H, 6H</td>
<td>Falak et al (1999)</td>
</tr>
<tr>
<td>Powdery mildew</td>
<td>WI2291</td>
<td>1H, 2H, 3H, 4H, 5H, 7H</td>
<td>Korff et al (2005)</td>
</tr>
<tr>
<td>Powdery mildew</td>
<td>VB9524</td>
<td>1H</td>
<td>Emebiri et al (2005)</td>
</tr>
<tr>
<td>Powdery mildew</td>
<td>OUH602</td>
<td>1H, 4H</td>
<td>Yun et al (2005)</td>
</tr>
</tbody>
</table>
Many QTL have been identified that contribute to scald and powdery mildew resistances (Table 1.5).

The number of identified QTL involved in complex disease resistance depends on the stringency applied to the analysis, the size of the mapping population and the quality of phenotyping. The numbers of QTL, contributing to resistance can thus differ between separate studies (Williams, 2003).

Thomas et al (1995) found only one QTL with major effect for each disease in more than one environment for adult plant resistance to powdery mildew and scald. On the other hand, Spaner et al (1998), found multigenic control of adult plant resistance to powdery mildew and scald. Resistance was affected by 2-5 QTL, explaining 8-45% of the phenotypic variance/disease.

It has been observed that QTL for disease resistance mostly map to the known major resistance gene loci. Backes et al (1995) reported the QTL for powdery mildew resistance which mapped in the same location as mlt gene. In addition, the QTL at the same location as Mlg was identified by Spaner et al (1998), by visually scoring naturally occurring field symptoms of powdery mildew and Thomas et al (1995) found that the QTL with major effect on adult plant resistance to scald and powdery mildew mapped to the previously identified major resistance genes.

Most of the QTL for scald mapped onto chromosomes 3H, 6H or 7H and some mapped onto chromosomes 1H, 2H or 4H. No resistance to R. secalis has been mapped onto
chromosome 5H (Zhan et al., 2008). There was found to be a clustering of resistance loci on
the barley chromosomes, some of which represent complex loci or co-locations that may be
difficult to disentangle (Yun et al., 2005). Bjornstad et al. (2004) suggested that some QTL
are in fact the alleles of the known major resistant genes. Some QTL independently
identified by different authors may be identical or represent alleles of the same locus.

1.2.3 Molecular genetics of aluminium tolerance

Up to 40% of the arable lands of the world (Kochian, 1995), have aluminium (Al) toxicity
as one of the major constraints on crop productivity on acid soils. Aluminium is the third
most abundant element in the earth’s crust and is toxic to plants at acidic pH (Kochian,
1995). Certain farming practices like application of ammonium-based fertilizers (Kochian
et al., 2002) and accumulation of organic matter (Williams, 1980), are making soils more
acidic. Other factors increasing the soil acidity are the removal of calcium in harvested
products, leaching of nitrates and calcium from the nitrogen producing pastures

According to CSIRO plant industry report (2004), 33 million hectares of Australian
farming land currently have high acidic soils, particularly the productive higher rainfall
country in south-eastern and south-western Australia and Queensland. A further 55 million
hectares of moderately acidic soils are also at risk of severe degradation. Soil acidity has
been identified by the National Land and Water Audit as the most serious land degradation
issue for Australian agriculture, costing about $1 billion in lost production each year. Soil
acidification is a more serious problem than salinity, in terms of both areas affected and
cost to the economy.
National Land and Water Resources Audit has provided detail of the soil surface pHs and agricultural regions over the period 1990-1999. Their spatial distribution is given in Figure 1.4. The total area with a soil pH <5.5 is now 46.7 million hectares (Chartres et al, 1990). Over half the agricultural soils in most of the Australian states, including Western Australia have a surface pH between 4.3-5.5.

Figure 1.4: Surface soil (0-0.1 m) pH distribution in agricultural soils of the intensive Land-use Zone (http://www.deh.gov.au/soc/2001/land/land04-3.html).
Aluminium (Al) tolerance differs greatly among plant species and barley is the most sensitive of the small grain crops (Polle and Konzac, 1985; Bona et al, 1993; Ishikawa et al, 2000). Generally, 6 row cultivars were found to be more tolerant than 2-row and 4-row types, husked more tolerant than naked and winter cultivars more tolerant than spring cultivars (Xu et al, 1991). Many important crop and pasture species lack sufficient Al tolerance within their germplasm to allow effective breeding for this character (Emmanuel et al, 2004).

Al toxicity limits the growth and productivity of barley on acid soils. A description of the types of soils that are acid and their distribution is provided by von Uexküll and Mutert (1995). Al toxicity is manifest by inhibition of root growth resulting in poor uptake of water and nutrients (Emmanuel et al, 2004), followed by swelling and distortion of differentiated cells, as well as root discolouration (Foy et al, 1978; Bergmann, 1992; Hossain et al, 2005). The foliar symptoms in some plants resemble those of phosphorous deficiency manifested by overall stunting, small, dark green leaves, late maturity, purpling of stem, leaves and leaf veins and yellowing and death of leaf tips (Foy et al, 1992).

Relative shoot and root dry weights in tolerant barley cultivars were found to be two fold and three fold higher respectively compared to susceptible cultivars (Foy, 1996). Gallardo et al (1999) reported 50% and 30% reduction in grain yield, respectively for sensitive and tolerant cultivars of barley when they were grown in naturally acidic soil, with pH 4.9 compared to that grown in non-acidic soil with pH 5.8.
Crop production on acidic soils can be sustained by the application of lime. Soil is limed in some areas to improve barley growth and productivity on acid soils, but this practice is often not economically feasible (Alva et al., 1986). The national collaborative project on indicators for sustainability agriculture reported that only a small proportion of agriculture land that would benefit from lime is treated each year. Out of a potential 90 million hectares of agriculture land that are at the risk of severe (pH < 4.8) or moderate (pH 4.8-5.5) acidity, only 600,000 hectares were treated in 1995-96, which is only 2% per year of the most acid agricultural soils received lime. It can take decades to correct acidity at depth because of the slow movement of lime (Foy et al., 1965; Mugwira et al., 1976). Runoff is an undesirable effect of lime application (de la Fuente et al., 1997). Heavy applications of lime may have adverse effects on some crops in the rotation or cause deficiencies of certain nutrients (Whitten, 1997).

An alternative strategy for improving barley productivity on acid soils is to select for cultivars with increased Al resistance or to breed Al-resistant cultivars by introducing Al-resistant genes (Scott and Fisher, 1989). Both the genetic and physiological mechanisms of Al resistance in barley need to be elucidated to achieve this purpose. Al tolerance has been evaluated by different methods around the world (Minella and Sorrells, 1992; Read and Oram, 1995; Maxim and Duta, 1996; Foy, 1996; Ma et al., 1997; Hossain et al., 2005). In Australia, some breeding lines performed significantly better on acidic soils than Al sensitive lines (Oram, 1983) and have been released for commercial cultivation on acidic soils, for example Brindabella, Yambla and Tulla.
Screening of genotypes for Al tolerance has been done in field evaluations, soil bioassays or solution culture methods (Carver and Ownby, 1995). To reduce the dependence on extensive field testing, solution culture methods have been used in genetic studies and in breeding programs (Ma et al, 1997; Minella and Sorrells, 1992; Sibov et al, 1999). Because of the consistent results produced by hematoxylin staining and root re growth measures, they are the most used techniques (Luo and Dvorak, 1996; Camargo, 1998; Tang et al, 2000).

1.2.3.1 Inheritance of Al tolerance in barley

The root staining method using hematoxylin (Minella and Sorrells, 1992; Tang et al, 2000) and eroichrom cyanine (Ma et al, 1997; Wang et al, 2006) were used to assess Al tolerance in barley. The qualitative variations were assessed as stained, unstained and partially stained to represent Al-sensitive, tolerant and intermediate genotypes respectively. Al tolerance on the basis of relative root regrowth in barley was also assessed as a quantitative trait (Raman et al, 2005a). In some cases, continuous quantitative phenotypic variations could be grouped into discrete classes. Raman et al (2002) classified tolerant, sensitive and intermediate genotypes in an F2 segregating population for Al tolerance, evaluated on the basis of root growth rate. Similarly, Ma et al (2004) classified Al tolerant and Al sensitive genotypes by root/shoot ratio.

Research has shown that the Al tolerance in barley is under a single locus control (Wang et al, 2006). The earliest work on barley cultivar Dayton, showed the Al tolerance conferred by a single dominant gene, Alp (Ried, 1970). The results further indicated that the expression of the tolerance (dominant/recessive) was dependant on the Al concentrations.
Al tolerance segregation in F2 populations from the crosses between Dayton (Al tolerant)/Harlan hybrid (Al sensitive), Harrington (Al sensitive)/Brindabella (Al tolerant), Yambla (moderately Al tolerant)/WB229 (Al tolerant) and F6ant28B48-16 (Al sensitive)/Honen, was found to be in a monogenic fashion (Raman et al., 2001; 2002; Tang et al., 2000; Wang et al., 2006).

1.2.3.2 Mapping of gene conferring Al tolerance

Minella and Sorrells (1997) mapped $Alp$ gene, conferring to Al tolerance on the long arm of the chromosome 4H, while working with the population from a cross Dayton with trisomic Shin Ebisu16. This result was later confirmed by Tang et al. (2000) with RFLP mapping analysis, and Raman et al. (2003) with SSR marker linkage analysis. Other Al tolerance loci were mapped to the same chromosomal location on 4H; $Alt$ from WB229 was mapped on 4H with the help of AFLPs, SSRs and with the analysis of wheat-barley chromosome addition lines (Raman et al., 2002). Similarly, $Alp3$ from Brindabella was mapped on chromosome 4H (Raman et al., 2001).

Raman et al. (2005) identified several QTL for the root elongation under Al stress on the chromosomes 3H, 4H, 5H and 6H in a F2 population from Ohichi/F6ant28B48-16.

Similarly, in wheat, aluminium tolerance has been reported to be controlled by one major gene (Delhaize et al., 1993a; Somers and Gustafson, 1995; Somers et al., 1996; Basu et al., 1997; Raman et al., 2005). This major locus conditioning aluminium resistance has been mapped to the long arm of chromosome 4DL (Luo and Dvorak, 1996; Riede and Anderson, 1996; Rodriguez-Milla and Gustafson, 2001; Raman et al., 2005). Yamaguchi et al. (2005)
found a membrane-localised protein encoded by the aluminium tolerance gene in wheat; TaALMT1 (originally ALMT1) which conferred an aluminium activated malate efflux and greater aluminium tolerance to barley plants (Delhaize et al., 2004). It is evident that variation in the expression of the malate transporter on 4D is largely responsible for aluminium tolerance.

1.2.3.3 Association of markers with Al tolerance gene (s)

Molecular markers have been regarded as the efficient tools of selection for the traits linked to Al tolerance (Raman et al., 2002). Table 1.6 shows the results from the studies of molecular markers linked with Al tolerance genes in barley.

Different marker systems like RFLP, AFLP, SSR, STS (sequence tagged sites), SNP (single nucleotide polymorphism) and diversity array platform have been used to identify the Al tolerant alleles in barley. Tang et al. (2000) reported three RFLP markers flanking the Al tolerance gene *Alp*, which was found to be only 2.1cM distal and proximal to the flanking markers. SSR markers; Bmag353, Bmac186 and Bmac310 showed tight linkage with the *Alp* locus (Raman et al., 2003). In another study, SSR markers Bmac310, Bmag353, HVM68 and HVRCABG were found to be linked to the major Al tolerance locus *Alt* in a F2 population from Yambla/WB229 (Raman et al., 2002). These SSR markers were used to distinguish other sources of Al tolerance in different populations derived from Harrington/Brindabella (Raman et al., 2001), Ohichi/F6ant28B48-16 (Raman et al., 2005a) and F6ant28B48-16/Honen (Wang et al., 2006).
Table 1.6: Populations, methods and markers used to map the Al tolerance loci on chromosome 4H

<table>
<thead>
<tr>
<th>Crosses</th>
<th>Al tolerance assessment</th>
<th>Gene and Chr location</th>
<th>Associated markers</th>
<th>Marker type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harrington/Brindabella</td>
<td>Hematoxylin staining</td>
<td>Alp (4H)</td>
<td>Bmac186, Bmac310</td>
<td>SSR</td>
<td>Raman et al (2001)</td>
</tr>
<tr>
<td></td>
<td>and root regrowth</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>and root regrowth</td>
<td>HvMATE</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Molecular mapping with SSR markers allowed the validation of the mechanism of Al tolerance in barley. Ma et al (2004) reported that tightly linked marker Bmag353 explained 51.3% of the phenotypic variance for citrate excretion in a population from a cross Murasakimochi/Morex. This marker also explained the 54% phenotypic variation for blue aleurone colour (Li et al, 2003, Read et al, 2003), indicating the possibility of the tight linkage between the two loci.

Dayton has been found to be one of the most Al-tolerant barley genotypes (Minella and Sorrells, 1992). A single locus; Alp was mapped on chromosome 4HL with the help of different populations derived from Dayton/Harlan hybrid and Dayton/F6ant28B48-16 (Raman et al, 2003; Reid, 1970; Tang et al, 2000). The same locus was found to conditions Al-tolerance in other populations including those generated from Yambla/WB229, Mimosa/WB229 (Raman et al, 2002), Harrington/Brindabella (Raman et al, 2001), Murasakimochi/Morex (Ma et al, 2004), Ohichi/F6ant28B48-16 (Raman et al, 2005a), F6ant28B48-16/Honen (Wang et al, 2006) and Dayton/Zhepi2 and Dayton/Gairdner (Wang et al, 2007). Minor gene effects for Al tolerance have also been suggested (Echart et al, 2002; Raman et al, 2005a).

1.2.4 Simulation studies

Computer simulations have been increasingly used as a methodology for modeling in quantitative genetics. Simulations can be used to understand the genetic relationships behind quantitative traits and the efficiency of the breeding strategies used to select these traits. The genetic improvement of self-pollinated crops such as barley through phenotypic recurrent selection is a proven and successful strategy to increase the production efficiency
within agricultural systems (Allard, 1960). In phenotypic selection, the accurate identification of desirable germplasm depends upon the large genotype by environmental interactions and trait expression, for example for disease resistance, it depends upon the disease epidemic, developmental stage of the plant and the weather conditions. Also, the rate of genetic gain depends upon the time and resources required to reach a desired level of homozygosity (Kuchel et al, 2005).

Simulation studies have examined the potential role for genotypic selection in breeding programs (Hospital et al, 1997; Knapp, 1998; Charmet et al, 1999; Moreau et al, 2000). These studies, based on the theoretical application of genotypic selection, have shown that the adoption of marker assisted selection has the ability to improve selection efficiency over phenotypic selection. Successful application of genotype selection in breeding programs have been reported (Yu et al, 2000; Yousef and Jarvic, 2001; Jefferies et al, 2003; Zhou et al, 2003).

Simulation studies are problem specific and specific scenarios could be investigated when appropriate data for the trait under study is available. According to Cooper et al (2005), simulation studies (a) enable the evaluation of a particular breeding strategy over the alternative scenarios, (b) enable to investigate the otherwise difficult to examine genetic properties and expectation of a wide range of complex genetic models, (c) enable to evaluate the efficiency of any breeding strategy across the levels of trait complexity and heritability.
CHAPTER 2

Mapping and Development of a Marker System for Use in Male Sterile
Facilitated Recurrent Selection in Barley

2.1 Introduction

The recurrent selection process is a breeding strategy based on successive cycles of
selection and recombination to accumulate genes for a particular character in the population
without significantly losing the genetic variability (Ramage, 1992). Most of the recurrent
selection procedures have been employed to improve inbred lines of maize or specifically
to improve yield of cross pollinated crops (Jensen, 1988). But as barley is a self pollinated
crop, desired crosses are not easily made following the maize model, without eliminating
the need for hand emasculation in producing F₁ seeds. Suneson (1945) introduced the use
of genetic male sterility to facilitate crossing in the development of composite crosses of
barley and recurrent selection was used to simultaneously select for the desired trait and the
most favourable background genotype in the composite cross population (Ramage, 1977).
R.F.Eslick suggested that the term ‘male sterile facilitated recurrent selection’ (MSFRS) be
used to describe these procedures (Ramage, 1975).

In barley, genes for genetic male sterility are located throughout the genome (Franckowiak,
1997; see Figure 1.1 and Table 1.1). The genetic male sterile gene msg6 located on short
arm of barley chromosome 6H near the centromere is of particular interest in this study. It is also closely linked (<1% recombination), in a coupling phase to the shrunken endosperm xenia gene sex1 (Falk et al. 1980), synonym - lyz5 (von Wettstein-Knowles, 1993) and orange lemma gene o, synonym - rob (Falk et al., 1981). The recessive alleles rob1-sex1 are used as morphological markers for the recessive msg6 allele (Figure 2.1). The msg6-rob1-sex1/msg6-Rob1-Sex1 linkage allows the pre-sowing selection of male sterile lines to be used in breeding programs to develop male sterile facilitated recurrent selection populations easily and efficiently (Falk et al., 1981).

Figure 2.1: (a) Barley head segregating for male-sterile (shrunken endosperm) and male-fertile (plump) F2 seeds. (b) Male-sterile seeds with orange lemma and shrunken endosperm (msg6-rob1-sex1).

The work in this Chapter has been undertaken to develop a molecular marker system closely linked to the msg-rob1-sex1 linkage block to help in the identification of fully fertile homozygous individuals from fully fertile heterozygous individuals in F2 population.
Fully fertile homozygous individuals would be desirable for production of doubled haploids, in single seed descent or conventional selfed breeding systems or for use as pollen donor parents in further cycles of male sterile facilitated recurrent selection (MSFRS).

### 2.2 Inheritance of male sterility

When the stock containing linkage group \textit{msg6-rob1-sexl} is crossed with a cultivar dominant for these genes, the selfed F\textsubscript{2} seed on the F\textsubscript{1} plant will be approximately 25% shrunken and 75% plump seeds. The progeny of the 75% plump F\textsubscript{2} seed will segregate in the ratio of 1:2 as homozygote male fertiles and heterozygote male fertiles respectively (Figure 2.2 and Figure 2.3).

\[
\begin{array}{c}
\text{Msg6Msg6-Rob1Rob1-Sex1Sex1} \\
(\text{male fertile - normal lemma - plump})
\end{array} \quad \times \quad \begin{array}{c}
\text{msg6msg6-rob1rob1-sex1sex1} \\
(\text{male sterile - orange lemma - shrunken})
\end{array}
\]

\[
\begin{array}{c}
\text{F1} \\
\text{Msg6msg6-Rob1rob1-Sex1sex1} \\
(\text{male fertile - normal lemma - plump})
\end{array}
\]

\[
\begin{array}{c}
\text{Self} \\
\text{F2 Msg6Msg6-Rob1Rob1-Sex1Sex1 : Msg6msg6-Rob1rob1-Sex1sex1 : msg6msg6-rob1rob1-sex1sex1} \\
1 : 2 : 1
\end{array}
\]

Figure 2.2: Flow chart showing the segregation of the genotypes in F\textsubscript{2} population if homozygote male fertile with normal lemma and plump seeds is crossed with the homozygote male sterile with orange lemma and shrunken endosperm.
To retain the male steriles and develop populations, the fertile plants are usually allowed to sib-pollinate the steriles and produce a ratio of 2:1 of plump to shrunken respectively and second cycle of this will give 1:1 ratio (Falk et al, 1980). Thus this coupling permits the breeders to retain, increase or discard male steriles from the breeding populations.

\[
\begin{align*}
\text{Msg6msg6-Rob1rob1-Sex1sex1} & \times \text{msg6msg6-rob1rob1-sex1sex1} \\
(\text{male fertile - normal lemma - plump}) & \quad (\text{male sterile - orange lemma - shrunken}) \\
\downarrow \\
\text{F1 } & \text{Msg6msg6-Rob1rob1-Sex1sex1} \quad \text{msg6msg6-rob1rob1-sex1sex1} \\
1 & : 1
\end{align*}
\]

Figure 2.3: Figure showing the F1 population comprising of 50% heterozygote male fertiles and 50% homozygote male steriles, when a heterozygote male fertile is crossed with the homozygote male sterile.

2.3 Material and Methods

2.3.1 Plant material

Male sterile barley line DF578 (=DF93B578; msg6/5* Zephyr), originally obtained from A/Professor Duane Falk, was crossed six times to Gairdner to obtain the BC5 population. The F1s obtained after every back cross were selfed to generate F2 populations for the next round of back crossing and BC5F2 population was thus generated after six cycles of crossing and selfing. This BC5F2 population was kindly provided by Dr. Reg Lance and Dr. Chengdao Li, to use for this study. Two hundred and five male sterile seeds with orange lemma and shrunken endosperm (msg6-rob1-sex1) were selected from approximately 850 BC5F2s and were grown in the glass house along with Gairdner as the plump, male fertile...
control. After collecting the leaf material for further use, the plants were kept in the glass house to let them grow to the heading stage to phenotype them and to confirm that all of them were male sterile.

2.3.2 Morphological markers and phenotyping
The total 205 shrunken and therefore presumed male sterile seeds were planted in the glass house and were grown to heading stage for phenotyping. The morphological markers shrunken endosperm (sex1) and orange lemma (rob1), closely linked to the male sterile gene, msg6, were used for phenotyping. The plants were phenotyped as orange/white lemma and shrunken/plump endosperm, based on the morphological markers, orange lemma (rob1) and shrunken endosperm (sex1). After phenotyping it was found that three had been misclassified as male steriles at sowing and were phenotyped as plump, green (normal) and fertile seeds. Seeds of six plants were phenotyped as normal lemma with shrunken endosperm; these were the recombinants between orange lemma - shrunken endosperm (rob1-sex1) and normal lemma - shrunken endosperm (Rob1-sex1) (Table 2.1).

2.3.3 DNA extraction and analysis
Leaf tissue of 205 male sterile plants was collected at the three leaf stage for DNA extraction and molecular marker assays. DNA was extracted from leaf tissue of the parent (Gairdner) and BC$_3$F$_2$ sterile progeny using the Mini Prep method adapted from Rogowsky et al (1991).
Table 2.1: Classification of the plants grown from male sterile (shrunken seeds). The seeds were selected from BC$_3$F$_2$ male sterile facilitated recurrent selection population.

<table>
<thead>
<tr>
<th>Category</th>
<th>Number of plants</th>
<th>Genotype</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correctly Classified</td>
<td>186</td>
<td>msg6msg6-rob1rob1-sex1sex1</td>
<td>male sterile – orange lemma - shrunken</td>
</tr>
<tr>
<td>Misclassified$^1$</td>
<td>3</td>
<td>Msg6Msg6-Rob1Rob1-Sex1Sex1</td>
<td>male fertile - normal lemma - plump</td>
</tr>
<tr>
<td>Recombinant$^2$</td>
<td>6</td>
<td>Msg6Msg6-Rob1Rob1-sex1sex1</td>
<td>male fertile– normal lemma - shrunken</td>
</tr>
<tr>
<td>Missing/late</td>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^1$The seeds which were classified as male sterile at the time of planting but were phenotyped as male fertiles at the heading stage.

$^2$The male sterile seeds which were phenotyped as recombinants between normal lemma-shrunken endosperm and orange lemma shrunken endosperm.
2.3.4 Bulk segregant analysis

Bulked segregant analysis (Michelmore et al, 1991) was used to screen DF578/6*Gairdner F₂ population with SSR markers. The bulk was constructed by taking 1µg of DNA from eight male steriles. A total of 48 SSR markers were taken from the region around the centromere of chromosome 6H and were screened against the parent and the bulk of male steriles. The primers that amplified potential polymorphism between the parent and the bulk were retested and those that gave reliable polymorphism were tested against the 205 individual BC₃F₂ sterile plants.

2.3.5 SSR analysis

In all 48 SSRs (Liu et al, 1996 and Ramsay et al, 2000) were tested for polymorphism (Table 2.2) between the parent and the male sterile bulk. SSR loci were amplified in a 10-µl reaction mixture containing 50 ng of template DNA, 1µl 10x PCR buffer, 0.6µl MgCl₂ (25mM), 0.4µl dNTP (10mM), 0.075µl of each primer (5 pM), 0.09µl Taq DNA polymerase (0.5 units) and injection water to make it up to 10µl. Temperature cycling conditions were used as described by Ramsay et al (2000). Amplified products were separated in 7% acryl-amide gels, running them at 80 volts for 16 hrs. Then they were stained with ethidium bromide and visualized using UV light box and photographs of the amplification product were taken using gel documentation system Protean II xi Cell gel system (BioRad, CA, USA). The sizes of the products were estimated using 100 base pair ladder (Figure 2.3).
Table 2.2: Detailed table about the SSR markers screened for polymorphism in a BC$_3$ population. The polymorphic markers were used to map the male sterile-orange lemma-shrunken endosperm (*msg6-robl-sex1*) linkage block around the centromeric region of chromosome 6H.

<table>
<thead>
<tr>
<th>SSR marker</th>
<th>Polymorphic</th>
<th>SSR marker</th>
<th>Polymorphic</th>
<th>SSR marker</th>
<th>Polymorphic</th>
<th>SSR marker</th>
<th>Polymorphic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmac 316</td>
<td>No</td>
<td>Bmac18</td>
<td>Yes</td>
<td>GBM 1274</td>
<td>No</td>
<td>Bmg16t1</td>
<td>Yes</td>
</tr>
<tr>
<td>EBmac 84</td>
<td>No</td>
<td>GMS 6</td>
<td>No</td>
<td>EBmac 28</td>
<td>No</td>
<td>GBM 1356</td>
<td>No</td>
</tr>
<tr>
<td>HVM 22</td>
<td>No</td>
<td>EBmac 639</td>
<td>No</td>
<td>Bmag 867</td>
<td>No</td>
<td>Bmac 40</td>
<td>No</td>
</tr>
<tr>
<td>HVM 11a</td>
<td>No</td>
<td>Bmag 807</td>
<td>No</td>
<td>EBmac 874</td>
<td>No</td>
<td>Bmac 251</td>
<td>No</td>
</tr>
<tr>
<td>HVM 31</td>
<td>No</td>
<td>Bmag 496</td>
<td>No</td>
<td>EBmac 853</td>
<td>No</td>
<td>EBmac 806</td>
<td>Yes</td>
</tr>
<tr>
<td>Bmag 9</td>
<td>No</td>
<td>EBmac 853</td>
<td>No</td>
<td>Bmag 1</td>
<td>No</td>
<td>GBM 1256</td>
<td>No</td>
</tr>
<tr>
<td>HVM 14</td>
<td>No</td>
<td>Bmag 219</td>
<td>No</td>
<td>HVM 65</td>
<td>Yes</td>
<td>GBM 1140</td>
<td>No</td>
</tr>
<tr>
<td>HVM 74</td>
<td>Yes</td>
<td>EBmac 674</td>
<td>No</td>
<td>HVM 22</td>
<td>No</td>
<td>GBM 1319</td>
<td>No</td>
</tr>
<tr>
<td>GBM 1256</td>
<td>No</td>
<td>Bmag 867</td>
<td>No</td>
<td>Bmag 4</td>
<td>Yes</td>
<td>HVM34</td>
<td>No</td>
</tr>
<tr>
<td>GBM 1400</td>
<td>No</td>
<td>GBM 1215</td>
<td>No</td>
<td>Bmac 218</td>
<td>No</td>
<td>GBM 1423</td>
<td>No</td>
</tr>
<tr>
<td>GBM 1389</td>
<td>No</td>
<td>EBmac 602</td>
<td>No</td>
<td>Bmac 297</td>
<td>No</td>
<td>Bmag 571</td>
<td>No</td>
</tr>
<tr>
<td>GBM 1212</td>
<td>No</td>
<td>Bmag 613</td>
<td>Yes</td>
<td>Bmac 144</td>
<td>No</td>
<td>Bmac 175</td>
<td>No</td>
</tr>
</tbody>
</table>
In Figure 2.4, “Gd” stands for the male parent Gairdner and ‘b’ is the genotype sharing the allele with Gairdner. The markers HVM65 and HVM74 were found to be linked with \textit{msg6}, thus selecting for male steriles.

![Figure 2.4: Gel pictures showing the amplification of SSR markers (A) HVM65 and (B) HVM74 for the same lines, where Gd – Gairdner (used as a male parent in the developing this BC$_3$ population and ‘b’ – Gairdner type individual (male fertile-white-plump, \textit{Msg6-Rob1-Sex1}).](image)

2.3.7 Statistical analysis and genetic mapping of \textit{msg6}

Initially, a subset of 46 BC$_3$F$_2$ male sterile individuals with orange lemma and shrunken endosperm (\textit{msg6-rob1-sex1}) were used to develop a linkage map for male sterile gene, \textit{msg6}. Mapping data were obtained by visual scoring of gels as “A” (parent which is a male sterile line) or “B” (Gairdner).
Table 2.3: Segregation pattern of loci and recombination frequency interval between *msg6-rob1-sex1* linkage block, calculated by hand, for a subset of 46 BC$_3$F$_2$ male steriles with orange lemma and shrunken endosperm.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Maternal/paternal</th>
<th>Recombination frequency (p)</th>
<th>Crossover unit (C.U)</th>
<th>Standard error (S.E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmac18</td>
<td>9/31</td>
<td>0.19</td>
<td>19</td>
<td>+/- 0.58</td>
</tr>
<tr>
<td>EBmac639</td>
<td>14/30</td>
<td>0.18</td>
<td>18</td>
<td>+/- 0.57</td>
</tr>
<tr>
<td>msg6-rob1-sex1</td>
<td>46/0</td>
<td>0.17</td>
<td>17</td>
<td>+/- 0.56</td>
</tr>
<tr>
<td>Bmag571</td>
<td>8/38</td>
<td>0.26</td>
<td>26</td>
<td>+/- 0.65</td>
</tr>
<tr>
<td>EBmac602</td>
<td>8/35</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A linkage map was based on the scoring of 4 SSR markers, calculating the recombination frequencies and standard errors (Table 2.3) between the markers using the following formulae:

Recombination frequency (p) = number of recombinants/total number of individuals

Crossover unit (C.U) = recombination frequency (p) x 100

Standard error (S.E) = \[(p (1-p))/N\] $^{1/2}$

The preferred order of the markers was checked by the number of maternal genotypes for each marker and was found consistent with the composite maps by Ramsay *et al*, 2000.
Table 2.4. Segregation pattern of loci and the interval between the adjacent loci, on barley chromosome 6H for 192 BC$_3$F$_2$ male steriles including six recombinants.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Maternal genotypes</th>
<th>Paternal genotypes</th>
<th>Cumulative distance</th>
<th>LOD *</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBmac806</td>
<td>134</td>
<td>48</td>
<td>0</td>
<td>31.5</td>
</tr>
<tr>
<td>Bmag613</td>
<td>145</td>
<td>38</td>
<td>4.6</td>
<td>18.5</td>
</tr>
<tr>
<td>Bmgttttt1</td>
<td>171</td>
<td>10</td>
<td>14.9</td>
<td>37.7</td>
</tr>
<tr>
<td>sex1</td>
<td>192</td>
<td>0</td>
<td>17.8</td>
<td>50.6</td>
</tr>
<tr>
<td>HVM74</td>
<td>182</td>
<td>2</td>
<td>18.3</td>
<td>50.6</td>
</tr>
<tr>
<td>msg6</td>
<td>186</td>
<td>6</td>
<td>18.8</td>
<td>57.8</td>
</tr>
<tr>
<td>o</td>
<td>186</td>
<td>6</td>
<td>18.8</td>
<td>49.7</td>
</tr>
<tr>
<td>HVM65</td>
<td>173</td>
<td>1</td>
<td>19.1</td>
<td>43.2</td>
</tr>
<tr>
<td>Bmac18</td>
<td>171</td>
<td>5</td>
<td>20</td>
<td>13.1</td>
</tr>
<tr>
<td>Bmag4</td>
<td>146</td>
<td>34</td>
<td>33.8</td>
<td></td>
</tr>
</tbody>
</table>

*The LOD score e.g. 31.5 is between the markers EBmac806 and Bmag613 and 18.5 between Bmag613 and Bmgttttt1 and so on.

Later, the larger population of 192 individuals was used to develop a linkage map for mapping data were obtained by visual scoring of gels as “A” (other parent which is a male sterile line) or “B” (Gairdner). The inequality in the numbers in Table 2.4 was caused some failed reactions and hence resulted in missing values. A linkage map was generated using Map Manager QTXb20 (Chmielewicz and Manly 2002). The linkage evaluation was carried out considering the BC$_3$F$_2$ population as Recombinant inbred intercross (self) population (RIX-self). Linkage analysis was performed at the threshold of $p = 0.01$ using
Kosambi map unit function (Kosambi 1944). The order of the markers on the map was found to be consistent with the map given by Ramsay et al (2000) for chromosome 6H (Table 2.4).

2.4 Results

Of the 48 markers tested, 7 SSR markers showed polymorphism between Gairdner and male sterile individuals and these polymorphic markers were then used for genetic analysis in the mapping population of 192 (including 6 recombinants between sex1 (shrunken endosperm) and rob1 (orange lemma)) male steriles derived from this backcross.

For this population, msg6 and the morphological marker for orange lemma, rob1, were found to be present on the same locus because of the lack of any recombinants between them. The microsatellite markers HVM65, HVM74 Bmac18 and Bmgttttttt1 were found to be tightly linked to the male sterile gene, msg6 and of these, HVM65 was the closest at 0.3cM followed by HVM74 and Bmac18 at 0.5 and 0.9cM, respectively (Figure 2.5). With the help of the six recombinants phenotyped between sex1-rob1 and sex1-Rob1, the SSR marker, HVM74 and was mapped in the centre of the linkage block msg6-rob1-sex1, with morphological marker sex1 at 0.5cM on one side and msg6-rob1 at 0.5cM, on the other side.

With the addition of more markers and using the larger population, it was found that the markers EBmac639 and Bmag571 (Table 2.3), which were initially found to be linked with the male sterile-orange lemma-shrunken endosperm (msg6-rob1-sex1) block, in the linkage map developed with a subset of 46 genotypes, were not linked to the male sterile gene in
the larger population. SSR markers which were not tested and tried before on the subset of population came out to be more promising for the larger population.

Figure 2.5: Marker system for msg6-rob1-sex1 linkage block on chromosome 6H, based on 192 individuals using SSR markers (based on the data in Table 2.4)
2.5 Discussion

This morphological marker; orange lemma \((rob1)\) and shrunken endosperm \((sex1)\), are closely linked \((< 1\% \text{ recombination})\) to the male sterile gene \((msg6)\). It is in fact very difficult to find the recombinants between them (Falk 1980). These closely linked morphological markers play an important role in assisting with the logistics of managing crossing blocks in the field where rows of male fertiles are grown adjacent to rows of male steriles (Falk, 1981). The phenotype also aids in harvesting of rows separately. With the morphological markers, it is possible to cull the undesirable plump male fertiles with green (normal) lemma from the female rows in the crossing block before anthesis, so as to avoid any contamination of the females with the undesirable males.

The skewed transmission of alleles was observed in the markers closely linked to the male sterile gene, \(msg6\) (Table 2.4). The explanation for the distorted transmission of the alleles in HVM74, HVM65, Bmggtttttt1, Bmag18 and the morphological marker for orange lemma \((rob1)\), is the selection of the material to be used to develop this marker system. The population selected for this study comprised of male sterile genotypes with orange lemma and shrunken endosperm \((msg6-rob1-sex1)\) which were contributed by the maternal parent, hence lowered the frequency of the alleles from the paternal parent (Gairdner) which is a male fertile with white and plump seeds \((Msg6-Rob1-Sex1)\).

Recombination was found to be suppressed in the centromeric region of chromosome 6H. No recombinants were found between male sterile \((msg6)\) and orange lemma \((rob1)\) and only 6 recombinants were found to be male fertiles with normal lemma and shrunken endosperm, \(Msg6Msg6/Rob1Rob1/sex1sex1\) (Table 2.4 and Figure 2.5). This lack of
recombination is consistent with observations in the literature relating centromeric heterochromatin to poor chiasmata formation in the centromere (Kunzel, 1982). A high recombination in the distal regions and suppressed recombination in the centromeric region of 6H was reported by Kunzel et al (2000) based on studies relating the RFLP maps for barley to translocation breakpoints.

The availability of molecular markers closely linked to the msg6 male sterile gene will aid in differentiating fully fertile homozygous plump individuals from fully fertile heterozygous plump individuals in F2 populations. Furthermore, it will facilitate the selection of male steriles with orange lemma and shrunken endosperm, to be used as females in the crossing block, with the help of SSR marker HVM74. Although the use of markers may seem trivial as progeny testing could be used to get similar results, the molecular markers are more efficient in genetically selecting the required genotypes because progeny test are not required. Marker-assisted selection will enhance the speed and efficiency of removing the breeding lines heterozygous for msg6 from the general breeding program. The selection of fertile heterozygotes is required for the production of male steriles in the segregating populations which can be further used as females in the male sterile facilitated recurrent selection programs. Fully fertile homozygous and pure breeding lines identified with this marker system can be used in the production of double haploid and also as males or pollen donor parent for further cycles of male sterile facilitated recurrent selection programs.

The most tightly linked flanking markers (HVM74, HVM65, Bmac18 and Bmgtttttt1) mapped to less than 5 cM from the male sterile gene, msg6 (Figure 2.5) and thus the
precision of MAS for msg6 with these markers will be adequate in selecting the homozygote fertiles from the heterozygote fertile genotypes which segregate in 1:2:1 ratio (fertile homozygotes: fertile heterozygotes: male sterile respectively) after selfing. Since microsatellite markers are known to reveal high degree of variation (Malyshev and Kartel 1997), it is possible that markers for msg6 can be successfully used across a broad range of male sterile facilitated populations with different genetic backgrounds to select parental material for Double Haploid, Single Seed Descent and conventional breeding programs.
CHAPTER 3

Phenotypic and Genotypic Selection for Aluminium Tolerance

3.1 Introduction
In Australia, about 90 million hectares of arable land is affected by soil acidity every year (http://www.science.org.au/nova/071/071box01.htm) with the estimated costs more than $600 million/year. The predominant form of aluminium, found in acidic soils is Al$^{3+}$. This ion is highly toxic to plant roots (Kinraide, 1991). It inhibits root cell division thus reducing the root growth and consequently the water and nutrient uptake, making the roots susceptible to drought stress and nutrient deficiency (Foy, 1988; Kochian, 1995) which results in poor plant growth and yield (Alam, 1981; Clarkson, 1966; Foy, 1983; Foy et al, 1967; Reid et al, 1969; 1971). A reduction of upto 50% in grain yield has been reported by Gallardo et al (1999), in the aluminium intolerant cultivars of barley. Therefore, development of barley cultivars with higher levels of tolerance to aluminium is one of the strategies for improving barley production in acidic soils.

In barley, Al tolerance has been reported to be controlled by a single major gene (Reid, 1970; Stolen and Anderson, 1978; Minella and Sorrels, 1992, 1997; Tang et al, 2000; Raman et al, 2001, 2002, 2003). The aim of increasing the barley production on acidic soils
could be achieved by introgressing the aluminium tolerant gene into the well adapted genotypes. Furthermore, combining aluminium tolerance with the malt quality traits will provide farmers with an economically viable alternative (Venkatanagappa et al, 2003).

The aim of this study was to characterize phenotypic and genotypic selection methods to select for aluminium tolerance in a male sterile facilitated recurrent selection population of barley where the aluminium tolerance gene from the aluminium tolerant line WB229 (O’Connor/Kaniere) had been introgressed into well adapted commercial malting barley varieties as Baudin and Vlamingh. It was also of interest to select for aluminium tolerant lines from the population under study.

3.2 Material and methods

3.2.1 Plant material

A recombinant F23 population (Figure 3.1) was developed from a complex cross using male sterile aided recurrent selection. The male sterile line, DF168, was kindly provided by Dr. Duane Falk, to be used in the crosses. The material used to monitor the changes in response to selection for aluminium tolerance for this study was kindly provided by Dr. Reg Lance and Dr. Chengdao Li. The pedigree of the cross is:

DF168 (male-sterile line) / Skiff // 2*WABAR2096 /3/ WABAR2094 /4/ Vlamingh /5/ WB229 / 4*Baudin (Figure 3.1, Table 3.1)
Figure 3.1: Showing the pedigree of the complex cross. Each colour represents the parent from and the proportion of each colour corresponds to the proportion of that specific parent in the population.
The aluminium tolerant line WB229 was back-crossed to the elite malting variety Baudin, in order to transfer the aluminium tolerant gene, \textit{Alp} or \textit{Alt}, so as to have aluminium tolerance in a Baudin background.

Table 3.1: The pedigrees of the parents used in the complex cross (Figure 3.1) along with their respective expected frequencies in the population used for this study.

<table>
<thead>
<tr>
<th>Parent</th>
<th>Pedigree</th>
<th>Expected frequency in the F2 population used for this study (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DF168</strong></td>
<td>ms / Birka*2 (88B060Early) / Birka [PPD-INS?]</td>
<td>1.56</td>
</tr>
<tr>
<td><strong>Skiff</strong></td>
<td>Abed Deba /3/ Proctor / CI-3576 // CP118197 // Beka /4/ Clipper / Diamant // Proctor / CI-3576</td>
<td>1.56</td>
</tr>
<tr>
<td><strong>WABAR2096</strong></td>
<td>Yagan / Natasha</td>
<td>9.37</td>
</tr>
<tr>
<td><strong>WABAR2094</strong></td>
<td>Yagan / Prisma</td>
<td>12.5</td>
</tr>
<tr>
<td><strong>Vlamingh</strong></td>
<td>WABAR570 / TR118</td>
<td>25.0</td>
</tr>
<tr>
<td><strong>WB229</strong></td>
<td>O'Connor / Kaniere</td>
<td>3.12</td>
</tr>
<tr>
<td><strong>Baudin</strong></td>
<td>Stirling / Franklin</td>
<td>46.9</td>
</tr>
</tbody>
</table>

3.2.2 Planting the field trials

The F2 progeny developed from this cross were divided into two bulks, one for phenotypic selection (Treatment 1) and one for genotypic selections (Treatment 2). The bulks were further separated into two sub-groups of seeds, male fertile (plump) and male sterile (shrunken) seeds to be planted in the crossing block as males and females respectively. Two crossing blocks with six rows each, each measuring 5m x 2m, were planted in the acid
plots in the paddocks at DAFWA’s Regional Research Station, Wongan Hills, Western Australia, in 2006.

DAFWA’s Regional Research Station, Wongan Hills is located approximately 188 km north of the state capital Perth at 30°45’S, 116°43’E. (http://www.ga.gov.au/bin/gazd). The area has yellow sands which are a mixture of sand and clay with good water holding properties. For the acid paddock, the pH of the soil was checked according to the standard CSBP protocol as the tests were conducted by the same company. A composite sample made of 25 samples from 100m x 60m area was taken. Considering the small trial area, two plots of 5m x 2m, for this study, it could be concluded that the composite sample taken and tested represented the area and that the soil pH (4.5-4.7 at 1-10m) was uniform in the acid plot.

Each crossing block in the acid plot comprised of two rows of females (male sterile, shrunken seeds) in the center with two rows of males (plump, male fertile seeds) on either side, to facilitate natural crossing (Figure 3.2). Fifty seeds were space planted in each row at 10cm distance with 40 cm distance between the rows. In all 400 male fertiles and 200 male steriles were planted in two replicates. Parents were planted as control. The crossing block was set to facilitate random natural crossing between the male fertiles and the male steriles.
3.2.3 Phenotypic selection (Treatment1)

3.2.3.1 Season 2006

The visual assessment of all the plants in the crossing block for aluminium tolerance was carried out at the heading stage. Healthy looking plants with higher number of tillers were selected as aluminium tolerant plants and were tagged in the crossing block. The $F_1$ seeds

---

Figure 3.2: The outline of the crossing block with two rows of females (male steriles) in the centre with two rows of males (fertiles) on either side.
were harvested from the central two rows of male steriles (females) from each crossing block in year 2006 and after threshing, again the selections were made based on the total seed weight. Twenty F1 seeds of each female selected (20 seeds/family) were planted in the summer nursery, in December, 2006 of DAFWA’s Manjimup Horticulture Research Station, Manjimup to obtain the F2 seeds after selfing. Out of 20 F1 plants, only 10 F1/2 plants per family were selected randomly to carry forward to next season (Figure 3.3). The seeds from the male fertile plants selected for aluminium tolerance were harvested and stored to be used as checks for the year 2007 season.

3.2.4 Genotypic selection (Treatment2)

3.2.4.1 Season 2006

Marker assisted selection was carried out on the F2 bulk separated for the genotypic selections (Treatment2). The population was separated into females (male steriles, shrunken seeds) and males (male fertile, plump seeds).

3.2.4.1.1 DNA extraction and SSR marker analysis

The F2 seeds under selection were planted in the trays along with all the six parents, in the glass house. Leaf tissue of 200 males (male fertile, plumps) and 100 females (male sterile, shrunken) plants was collected at a three leaf stage for DNA extraction and molecular marker assays. DNA was extracted from leaf tissue of the parents and the progeny using the Mini Prep method adapted from Rogowsky et al. (1991).

The parents of the composite cross were screened using three SSR markers; Bmag353, Bmac310 and HVM68 which had been reported to be tightly linked to the major
Aluminium tolerance locus *Alt* (or *Alp*) on the long arm of barley chromosome 4H (Raman *et al*, 2001, 2002, 2003 and Wang *et al*, 2006). All three markers used were polymorphic over six parents of the composite cross but SSR marker HVM68 was found to be the only one to distinguish the aluminium tolerant recurrent parent WB229 from the other parents and was used to screen the population of the recombinants to track the WB229 allele in the population.

SSR loci were amplified in a 10-μl reaction mixture containing 50 ng of template DNA, 1μl 10x PCR buffer, 0.6μl MgCl2 (25mM), 0.4μl dNTP (10mM), 0.075μl of each primer (5 pM), 0.09μl Taq DNA polymerase (0.5 units) and injection water to make it upto 10μl. Temperature cycling conditions were used as described by Ramsay *et al* (2000). Amplified products were separated in 7% acryl-amide gels, running them at 80 volts for 16 hrs. Then they were stained with ethidium bromide and visualized using UV light box and photographs of the amplification product were taken using gel documentation system Protean II xi Cell gel system (BioRad, CA, USA). The sizes of the products were estimated using 100 base pair ladder.

The homozygous and heterozygous genotypes for aluminium tolerance were selected with the polymorphic marker HVM68. The selections were transplanted in the crossing block at DAFWA, South Perth, in 2006, with central row of females and outer two rows on either side, of males for random natural pollination (Figure 3.2).
3.2.5 Season 2007 (phenotypic selection and genotypic selection)

Ten Fertile plump seeds/single plant/family (F_{23}) (a total of 138 lines/treatment, from 26 and 19 families from Treatment 1 and 2 respectively) for all the ten plants (F_{1/2}) from both the populations under two treatments (Figure 3.3) were randomly selected to be planted in 1m rows with 10 seeds per row, 20 cm apart in the acid paddock (pH 4.5-4.7 at 1-10 m), at
DAFWA’s Regional Research Station, Wongan Hills, WA, in the 2007 season. For this season, only the homozygote genotypes selected with the marker HVM68 in year 2006 were planted and the heterozygotes were left out so as to match the number of lines per treatment.

3.2.5.1 Traits observed and recorded

The following traits were observed and recorded in the growth season of 2007:

1000-kernel weight:
Grain weight is a measure of plumpness. Using a grain counter, 1000 seeds were counted and weighed. It was expressed in gms/1000 kernel. All the half, shriveled and broken seeds were removed seeds from the grain counter and were replaced with healthy and plump seeds before weighing. High 1000-kernel weight is preferred.

Plumpness (Grain size):
It is a measure of the seed size which is influenced by the amount of starch in the endosperm. 100g of seed sample was taken and shaken for one minute on a series of sieves using Glasblasederei shaking machine fitted with three sieves made of hardened brass; the width of the slots being 2.8mm, 2.5mm and 2.2mm in order from top to bottom. The shaking speed of the shaker was 300-320 revolutions/minute and total length of travel platform was 18-22mm. In Australia, the measurement of grain size is generally based on four fractions: <2.2mm (screenings), >2.2mm, >2.5mm and >2.8mm. Industry standards on plump grain are based on the total of the two largest grain fractions: grains retained on
2.8mm and 2.5mm sieves. Assortments were then calculated as %Plump. High %Plump is preferred.

Number of fertile tillers:
Number of fertile tillers per plot (averaged the tillers of 10 plants in each plot) were counted in the paddock. High number of tillers is preferred.

Yield:
Yield was estimated for each plot based on the yield components as follows.

\[(\text{Tillers/plot}) \times (\text{Grains/tiller}) \times (1000-\text{kernel weight})\]

Data on grains/tiller was not recorded in the field as it was not considered the key issue for this particular study where yield was estimated as a measure for aluminium tolerance. Thus grains/tiller was calculated as:

\[\frac{(\text{Total seed weight})/(1000-\text{kernel weight})}{\text{Total number of tillers per plot}}\]

3.2.5 Experimental design and statistical analysis
A trial with a population of the recombinants (Figure 3.3) from the composite cross was planted in the acid paddock for the season 2007, using spatially balanced rectangular array of rows and columns. Trial with was partially replicated; only 25% of the test lines were replicated and the parents were replicated three times. The design was generated using DiGGer (Coombes, 2002).
Spatial linear mixed models (Gilmour et al, 1997) were used in the analysis (kindly provided by Dr. Katia Stefanova) of each single trial. The approach adopted here identifies three major components of spatial variation in plot errors from field experiments:

- Large-scale (global) variation across the field, such as non-uniformity of pH or plant nutrients in the soil, fertility and moisture trends or linear row and column effects.
- Natural variation or local trend is a small scale variation, reflecting the correlation between the plots.
- Extraneous variation which is usually induced by some experimental procedures e.g. irregularity of water supply, different sowing depth causing different germination rates, wind direction at harvest etc.

The diagnostic tools used in identification of the model (Gilmour et al, 1997; Stefanova et al, 2007) predominantly involve use of plots of the residuals and sample variograms. The best model was chosen by maximizing the REML log-likelihood via the comparison of the deviance terms with a Chi-square distribution. Fixed effects were tested using Wald tests. The lines were fitted as random effects; therefore the results obtained were best linear unbiased predictors (BLUPs). Best linear unbiased estimates (BLUEs) were predicted for the fixed terms. Spatial variation modelling was started with a separable process involving a first order autoregressive model for both rows and columns ($AR1$ (column) $x$ $AR1$ (row)) which accounted for the local trend. The residuals from this model then provided the basis for identification of global and extraneous variation, as well as assessing the adequacy of
Figure 3.4: Variograms presenting the Autoregressive (column) x Autoregressive (row) variations for Yield in the phenotypically (Treatment 1) selected population, (A) with local trend fitted and (B) and random effect added. x-axis represent the absolute plot displacement along the column, y-axis represents the absolute plot displacement along the rows and z-axis represents the sample means of the semi-variances with the same absolute displacement.
the variance structure for local trend. The aim of the analysis was mainly to identify any spatial pattern in the data collected to screen for aluminium tolerance.

There were no significant random effects found for rows and columns for any trait under this study, except for the Yield in phenotypically (Treatment 1) selected population (see Figure 3.4 (A) and (B)), where there was a significant row effect. The row and column autoregressive coefficients for all models are presented in Table 3.2. The models were fitted using statistical software GenStat (version 10.2 http://www.genstat.com). Further, the predicted means and SE of the families (Table 3.3) and correlation coefficient (Table 3.4) were calculated for all the traits observed.

3.3 Results

3.3.1 Year 2006

3.3.1.1 Phenotypic selection (Treatment 1)

The visual assessment of the plants was undertaken in order to select for aluminium tolerance on the acidic soils with pH 4.5 - 4.7.

Figure 3.5 shows the difference in the development of the root system in a aluminium tolerant and aluminium sensitive plants. In all 27 male steriles and 68 male fertiles were selected as aluminium tolerant individuals, out the crossing block in the acidic soil. The 27 male steriles were harvested for summer nursery to develop F₂ seed.
3.3.1.2 Genotypic selection (Treatment 2)

The SSR marker HVM68 linked to the aluminium tolerance gene $Alt$ (Raman et al, 2002, 2003; Wang et al, 2006) was used to screen the population of recombinants (Figure 3.6), to track the aluminium tolerance allele contributed by the recurrent parent; WB229 on chromosome 4H.
Figure 3.6: The acryl-amide gel pictures showing the selections for WB229 allele from the rest of the parents, using a SSR marker HVM68 on chromosome 4H. The white coloured numbers are representing the homozygotes, red coloured representing the heterozygotes for aluminium tolerance and the blue colour represents the aluminium intolerant individuals in the population screened. Where BK – Birka (DF168), Sk – Skiff, 96 – WABAR2096, 94 – WABAR2094, Vlm – Vlamingh, 229 – WB229 and Bdn – Baudin.

In all 36 homozygotes and 40 heterozygotes out of 190 male fertiles and 13 homozygotes and 12 heterozygotes out of 90 male steriles were selected using HVM68 (Figure 3.6) and were transferred to the crossing block so as to derive F1s. The large number of aluminium-sensitive individuals in the population (Figure 3.7) could be a result of the contamination of the male sterile (female) plants with the fertile pollen from the adjacent barley plots while crossing.
Figure 3.7: Showing the number of individuals with different genotypes, selected using the SSR marker HVM68 (Figure 3.5). Encircled homozygote aluminium tolerant individuals (Al-tol (AA)) were planted in the crossing block after selection to develop F₁ seed. Where Al-tol (Aa) – heterozygotes for aluminium tolerance and Al-sensitive – individuals which were found to be sensitive to aluminium.

3.3.2 Year 2007

3.3.2.1 Spatial variations and overview of the analysis for aluminium tolerance

The overview of the models fitted to the data recorded on the traits observed for the indication of aluminium tolerance is presented in Table 3.2. There was no distinctive spatial pattern for aluminium tolerance identified. The only spatial component found in the model was in the small scale variation category (so called natural trend). It could be argued that the main reason for not identifying any global trend was the uniformity of the soil pH in the acidic environment. At p = 0.05, no significant difference was found among the families for 1000-kernel weight and percent plump seeds (screenings). The families were
found to be significantly different for number of tillers and yield for both the treatments. In Table 3.2, autoregressive coefficients have been shown which represent the correlation between the adjacent plots along the rows and columns respectively for the traits under study. The introduction of the natural trend (AR1 x AR1) in the model aims mainly to adjust for the local spatial trends.

Table 3.2: An overview of the autoregressive (AR) coefficients between rows and columns within models fitted to the field variation for aluminium tolerance. The standard errors for the coefficients are given in brackets. Variations are based on the data collected for all the traits for the population under study, with both the treatments. Where AR – autoregressive and col - column. In the screenings, %Plump is the percentage of the seeds retained on or above 2.5mm sieve.

<table>
<thead>
<tr>
<th>Traits observed and recorded</th>
<th>Phenotypic selection (Treatment1)</th>
<th>Genotypic selection (Treatment2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AR1 col (SE)</td>
<td>AR1 row (SE)</td>
</tr>
<tr>
<td><strong>Yield</strong></td>
<td>0.41 (0.17)</td>
<td>0.29 (0.14)</td>
</tr>
<tr>
<td><strong>1000-kernel wt.</strong></td>
<td>0.30 (0.12)</td>
<td>0.10 (0.13)</td>
</tr>
<tr>
<td><strong>%Plump</strong></td>
<td>0.24 (0.11)</td>
<td>0.14 (0.14)</td>
</tr>
<tr>
<td><strong>No. of tillers</strong></td>
<td>0.31 (0.15)</td>
<td>0.45 (0.10)</td>
</tr>
</tbody>
</table>

Mean values, SE and range of variation for the traits studied are shown in Table 3.3. Mean values are significantly different between phenotypic selection and genotypic selections. On average, the lines selected through genotypic selections (Treatment 2) performed better than the ones selected through phenotypic selections (Treatment 1). They yielded higher,
had higher number of tillers, higher screenings of percent plump seeds but had lower 1000-seed weight as compared to the lines under phenotypic selection (Treatment 1). The range and SE of the traits under study were also different for each treatment (Table 3.3).

The correlation coefficients estimated on the 2007 data for the population subjected to phenotypic (Treatment 1) and genotypic (Treatment 2) selections in year 2006 are presented in Table 3.4. In general, the correlation coefficients were found to be positive for both the treatments. The magnitude of the coefficients was found to be larger for phenotypic selection (Treatment 1) as compared to genotypic selection (Treatment 2) between all the traits under study. Highly positive correlations were found between all the traits for the lines selected using phenotypic selection procedures (Treatment 1). This is as expected since most of these traits, 1000-kernel weight and percent plump are the components of yield. The highest correlation ($r = 0.98$) was observed between 1000-kernel weight and the percent plump. For the lines selected using genotypic selections (Treatment 2), positive correlations were found between all the traits under study. The strong positive correlation ($r = 0.85$ and $r = 0.64$) between the number of tillers and the yield, for phenotypic selection and genotypic selection respectively, indicated that the increase in the number of the tillers increases the yield. They are correlated as number of tillers was one of the components, used to estimate yield.
Table 3.3: Mean values, standard errors and range of yield and related traits based on the spatially adjusted results from the analysis of the field experiment conducted at DAFWA’s Wongan Hills Research Station, Wongan Hills, Western Australia.

<table>
<thead>
<tr>
<th>Traits observed and recorded</th>
<th>Phenotypic selection (Treatment 1)</th>
<th>Genotypic selection (Treatment 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SE</td>
<td>Range</td>
</tr>
<tr>
<td>Yield</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000-kernel wt.</td>
<td>5.15 ± 0.59</td>
<td>1.56 – 7.40</td>
</tr>
<tr>
<td>Plumpness (%Plump)</td>
<td>43.15 ± 0.52</td>
<td>38.69 – 51.48</td>
</tr>
<tr>
<td>No. of tillers</td>
<td>81.57 ± 1.6</td>
<td>70.43 – 91.86</td>
</tr>
<tr>
<td></td>
<td>7.2 ± 0.64</td>
<td>5.0 – 10.3</td>
</tr>
</tbody>
</table>

Table 3.4: Correlation coefficients for both the treatments, estimated from the 2007 data based on the field experiment conducted at DAFWA’s Wongan Hills Research Station, Wongan Hills, Western Australia.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>1000-kernel wt vs. %Plump</th>
<th>1000-kernel wt vs. No. of tillers</th>
<th>1000-kernel wt vs. Yield</th>
<th>%Plump vs. No. of tillers</th>
<th>%Plump vs. Yield</th>
<th>No. of tillers vs. Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotypic selection</td>
<td>0.98</td>
<td>0.82</td>
<td>0.79</td>
<td>0.81</td>
<td>0.79</td>
<td>0.85</td>
</tr>
<tr>
<td>Genotypic selection</td>
<td>0.45</td>
<td>0.30</td>
<td>0.31</td>
<td>0.38</td>
<td>0.29</td>
<td>0.64</td>
</tr>
</tbody>
</table>
Table 3.5: Mean values, standard errors and range of the parents used as controls in both the treatments (phenotypic and genotypic) for yield and related traits based on the raw data of the field experiments conducted at DAFWA’s Wongan Hills Research Station, Wongan Hills, Western Australia.

<table>
<thead>
<tr>
<th>Traits observed and recorded</th>
<th>Mean ± SE (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Skiff</td>
</tr>
<tr>
<td><strong>Yield</strong></td>
<td>2.5 ± 0.2 (2.0 – 3.0)</td>
</tr>
<tr>
<td>1000-kernel wt.</td>
<td>37.5 ± 0.5 (36.0 – 39.5)</td>
</tr>
<tr>
<td>%Plump</td>
<td>74.5 ± 1.2 (72.0 – 75.4)</td>
</tr>
<tr>
<td>No. of tillers</td>
<td>4 ± 1.0 (3 – 5)</td>
</tr>
</tbody>
</table>
Mean values, standard errors (SE) and range of variation in the parents used as controls for both phenotypic and genotypic selections for the traits under study are tabulated in Table 3.5. This table is based on the raw field data.

### 3.3.2.2 Results of the spatial analysis

All the lines selected from the phenotypic and genotypic selections were planted at Wongan hills and data on the above mentioned traits was recorded. Predicted means for the families were calculated, for both the selection procedures carried out on F₁s in year 2006, using the Spatial analysis with GenStat (version 10.2).

The distribution of the populations for the 4 main traits, assessed as a measure of aluminium tolerance, are illustrated in Figure 3.8 For yield (Figure 3.8 (A)), more than 50% families were found to have on an average more than 5kg seed/plot (10 seeds each line were planted in 1m rows (plots)) irrespective of their mode of selection; phenotypic (Treatment 1) or genotypic (Treatment 2). 57.7% of the phenotypically (Treatment 1) selected families and 52.6% of the genotypically (Treatment 2) selected families performed equivalent or better than the commercial varieties like Vlamingh and Baudin, for yield. In comparison to the aluminium tolerant parent WB229, 11.5% and 21% of the phenotypically (Treatment 1) selected and genotypically (Treatment 2) selected families respectively, yielded in the same range or even better. 10.5% of the families selected using genotypic selection (Treatment 2) even yielded more than 9 kg/plot. Overall, the genotypic selections (Treatment 2) yielded higher as compared to the phenotypic selection (Treatment 1).
From the predicted means for 1000-kernel weight (Figure 3.8 (B)), it was found that 84.6% of the phenotypically selected (Treatment 1) families and 68.4% of the genotypically selected (Treatment 2) families had 1000-kernel weight greater than 40g. 69.3% and 36.8% of the families from Treatment 1 and 2 respectively, had 1000-kernel weight more than equivalent to the 1000-kernel weight for the aluminium tolerant parent WB229 and 7.7% of
Figure 3.8: Results based on the predicted means of the phenotyping at F2 are shown. The Comparisons between phenotypic (Treatment 1) and genotypic (Treatment 2) selections for aluminium tolerance carried out at F1 stage have been made based on the data collected on the different traits at segregating F2 stage. Comparisons were made based on the data on (A) Yield (B) 1000-kernel weight (C) Plumpness (%Plump, >2.5mm) and (D) number of tillers. Values for the parents of the population are indicated in arrows (see Table 3.5 for details related to the means).
the families from phenotypic selection (Treatment 1) had more than 46g/1000-seed weight/plot. Overall, phenotypic selection (Treatment 1) outperformed genotypic selection (Treatment 2) for 1000-kernel weight.

From the predicted means for Plumpness (%Plump, > 2.5mm), it was found that 65.4% and 63% of the phenotypically selected (Treatment 1) and genotypically selected (Treatment 2) families respectively, had 80% of the total seeds sample, retained on or above the 2.5mm sieve (Figure 3.8 (C)). In comparison to the aluminium tolerant parent WB229, 9.2% of the families from phenotypic selection (Treatment 1) and 21% of the families from the genotypic selection (Treatment 2) had Plump seed more or equivalent; 86%. Overall, families from both the treatments performed more or less equally, with phenotypic selection (Treatment 1) better than genotypic selection (Treatment 2), considering the families having more than 80% of the total seed.

For the data collected on the number of tillers, the predicted means of the families (Figure 3.8 (D)) showed that more than 50% of the families from both the treatments; 53.8% from phenotypic selection (Treatment 1) and 73.8% from genotypic selection (Treatment 2), had more than seven tillers/plant. Considering the performance of the commercial varieties Vlamingh and Baudin and the aluminium tolerant parent in the pedigree of the population, WB229, 28.9% of the families selected with phenotypic selection method (Treatment 1) had tillers numbers between 9-11, similar to WB229, Vlamingh and Baudin, as compared to 5.3% of the families selected with genotypic selection method (Treatment 2). Also, 5.3% of the families selected genotypic selection had more than 11 tillers thus outperformed WB229. Overall, genotypic selection method (Treatment 2) worked better in selecting for
number of tillers but phenotypic selection method (Treatment 1) worked better in matching the performance of the aluminium tolerant parent WB229.

3.4 Discussion
Phenotypic selection and genotypic selection were conducted in this study to characterize breeding system for aluminium tolerance in relation to the agronomical traits such as yield, number of tillers and 1000-kernel weight and grain plumpness.

Genotypic selection method (Treatment 2) was found to be more efficient in shifting the population in the desired direction. Genotypic selection procedure worked efficiently in selecting for the agronomical traits, yield and number of tillers and equals out the phenotypic selection method in selecting for plumpness (Figure 3.8). With this selection method, 5.3% families out performing the aluminium tolerant line WB229 with >11 tillers/plant, were identified for number of tillers. All the families selected through both the selection methods, exhibited >76% plump (Figure 3.8 (C)) seed which is well above the >70% mark specified by the barley industry (Fox et al, 2003).

Phenotypic selection (Treatment 1) performed more efficiently in selecting for 1000-kernel weight. From Figure 3.8, it could be concluded that phenotypic selection method helped in identifying a considerable number of families performing like the aluminium tolerant line WB229, for all the traits under study. Both the selection methods identified the families that out performed WB229 for Yield and 1000-kernel weight, where some families were found to yield >11kg and some families have >46g of 1000-kernel weight.
Considering the pedigree of the cross (Figure 3.1) and the frequency of each parent (Table 3.1) in the population, the individuals under study were basically expected to carry the aluminium tolerant gene \( Alt \) (or \( Alp \)) from WB229 in the malting barley background, mainly contributed by malting varieties, Vlamingh and Baudin with the expected frequencies of 25% and 46.9% respectively. Figure 3.8 highlights the existence of desired and undesired recombinants within the populations which develop as a result of the recombination of genes from a cross between different parents. The progeny developed in this way receive either the more complete favourable genes from the positive parent or a combination of genes from the negative parent (Paterson et al., 1991). Accordingly, it is possible that some progeny within the populations under study carrying the alleles for aluminium tolerance from control parent WB229 had alleles of high yield and superior malting quality from Baudin and Vlamingh. Baudin and Vlamingh were found to group with the aluminium tolerant line WB229 for all the traits under study except for Yield where WB229 as found to be high yielding as compared to Baudin and Vlamingh (Figure 3.8). This could be explained by the aluminium tolerance exhibited by WB229 (Raman et al., 2002) as compared to the two malting varieties. Vlamingh was also found to have lower 1000-kernel weight as compared to Baudin and WB229 which could be attributed to small grain size of Vlamingh.

Many previous studies of seed production have shown that the number of fertile tillers is a major component determining seed yield which is influenced by the traits like 1000-kernel weight and percent plumpness (Wych and Rasmusson, 1983; Lekes, 1989; Grausgruber et al., 2002). It was found that the number of tillers had the largest effect on the yield for both the populations followed by the equal effects exhibited by 1000-kernel weight and percent
plump (Table 3.3). 1000-kernel weight was found to be highly correlated ($r = 0.98$) with percent plump which was found to be correlated ($r = 0.81$) with number of tillers, for phenotypically selected (Treatment 1) population and relatively high (as compared to the others), $r = 0.45$ and $r = 0.38$ respectively, for the genotypically selected (Treatment 2) population. Considering these correlations, it could be suggested that percent plump seed could be used as a major component in selection for yield instead of number of tillers since it is easier to measure.

Aluminium tolerance is thought to be controlled by a single gene mapped on the long arm of chromosome 4H (Tang et al., 2000; Raman et al., 2001; 2002; 2003; Wang et al., 2006; 2007). The high efficiency of genotypic selection method (Treatment 2) in selecting for yield, number of tillers and plumpness could be attributed the fact that the marker HVM68, used to select for aluminium tolerant individuals in the population, was closely associated with the aluminium tolerant gene, $Alt$ or $Alp$, on chromosome 4HL (Raman et al., 2002, 2003; Wang et al., 2006).

From this study it was found that the genotypic selection (Treatment 2) could be the better selection method while selecting for aluminium tolerance. As this study was based on selecting for a trait controlled by a single gene, the availability of the closely linked marker HVM68, made the genotypic selection method (Treatment 2) more efficient in selecting the aluminium tolerant families as compared to the phenotypic selection method (Treatment 1). But if there is more than one gene controlling the trait under study, combined approach would be more efficient and desirable. Genotypic selection could be used as a negative selection tool for the early elimination of all the undesirable lines and then concentrate all
the resources on phenotypic selection among the already selected germplasm pool. This approach may also be able to decrease the cost and the time required for screening the large populations in the field.
CHAPTER 4

Genotypic and Phenotypic Changes in Response to Selection for Scald and Powdery Mildew Resistances

4.1 Introduction

Barley improvement involves selection for many quality traits but disease resistance is also of major interest. Fungal and viral diseases can affect the profitability of barley production by reducing final yield and by lowering grain quality (Chelkowski et al., 2003; Williams, 2003). In order to be profitable, malting barley production requires low input costs, hence expensive chemical control is not economical and the search for and deployment of resistant genes therefore is very important (Emebiri et al., 2005).

In breeding programs, breeders and pathologists select plants or lines (phenotypic selection) with complete or partial disease resistance. The phenotypic selections are either based on the visual assessment of the naturally occurring disease symptoms or on results of the artificially inoculated disease trials. The qualitative classification of the plants or lines, categorize them into resistant or susceptible, thus resistant barley has been produced successfully for many years by conventional breeding techniques.
One of the most efficient tools for improving breeding populations for quantitative traits is phenotypic selection, where the frequency of the favourable alleles is increased within the population over cycles of selection (Hallauer, 1992), provided a good epidemic of the pathogen occurs. Phenotypic recurrent selection has been very successful in improving the disease and pest resistance in several crops (Alsirt, 1993; Barry et al, 1995; Flint-Garcia et al, 2003). Similarly several studies have indicated that genotypic selection (marker-assisted selection) has the potential to be a valuable selection tool. However, only a limited number of studies have provided results comparing phenotypic selection and genotypic selection. The studies on malting quality in barley (Han et al, 1997), drought resistance in common bean (Scheider et al, 1997), flowering time in Arabidopsis thaliana (Van Berloo and Stam, 1999), yield in barley (Romagosa et al, 1999), seedling emergence and kernel quality in sweet corn (Yousef and Juvic, 2001), resistance to south-western corn borer (Wilcox et al, 2002) and stalk strength and European corn borer resistance in maize (Flint-Garcia et al, 2003) are some of the studies which provide us the comparisons between the phenotypic and genotypic selection procedures.

The pathogenic variabilities of Rhynchosporium secalis, the causal agent of scald and Blumeria graminis f. sp. hordei, the causal agent of powdery mildew, continue to pose a challenge to barley breeders. Many resistant genes for scald and powdery mildew have been mapped in different studies with different populations. The phenotypic (Treatment 1) and genotypic selections (Treatment 2) for the resistance to scald on chromosome 4H (RhsVlamingh) and 6H (RhsWABAR2147) and powdery mildew (MldWABAR2147) on chromosome 1H, were carried out in this study.
The aim of this study was to track the genotypic and phenotypic changes in response to selection for scald and powdery mildew resistances in a male sterile facilitated recurrent selection population of barley and to characterize the phenotypic and genotypic selection methods. The selection was based on the reaction types (phenotypic) and the marker types (genotypic) as the responses used as selection tools. While monitoring the changes in response to the selection methods, this study was conducted to assess the value of the genotypic selection in the absence of good epidemic of the disease required for the phenotypic selection to be successful.

4.2 Material and methods

4.2.1 Plant material

Two F$_{2/3}$ populations of recombinants (Figure 4.5) were developed from two complex crosses, using male sterile facilitated recurrent selection and were used to monitor the changes in response to selection for scald and powdery mildew resistant genes present in the barley genome. The pedigree of the crosses was:

- **Cross1**: DF168 (male sterile line) / Skiff // 5*WABAR2096 / 3/ Vlamingh / WABAR2147 (Figure 4.1)
- **Cross2**: DF168 (male sterile line) / Skiff // 2*WABAR2096 / 3/ Baudin /4/ Hamelin /5/ Vlamingh / WABAR2147 (Figure 4.2), where:

DF168 is a male sterile line with pedigree: ms / Birka*2 (88B060 Early) / Birka [PPD-INS?].
Skiff is a feed barley, susceptible to scald and with intermediate reaction to powdery mildew. It is tolerant to high levels of soil boron and has good yield and hectoliter weight. The pedigree is: Abed Deba /3/ Proctor / CI-3576 // CP118197 / Beka /4/ Clipper / Diamant // Proctor / CI-3576.

WABAR2096 was an elite malting quality line which was later not released as a variety principally due to its high susceptibility to scald, poor grain colour and low hectoliter weight. The pedigree of the line is: Yagan / Natasha: where Yagan = WUM 143; a CIMMYT derived line and Natasha (Mla12) = Trumpf (Ml (Ab), Mla7, MlTr3) / Aramir (Mla12).

Baudin is a superior malting quality, plump grained, high yielding barley variety which is adapted to high rainfall area and parts of medium rainfall area. It is intermediate for scald reaction but susceptible to powdery mildew. The pedigree is: Stirling / Franklin: where Stirling = ((Dampier / (Prior / Ymer)) / Piroline and Franklin = Shannon / Triumph (Ml (Ab), Mla7, MlTr3); where Shannon = CI-3208-1 / 4*Proctor.

Hamelin is a superior malting quality, high yielding barley which is susceptible to both scald and powdery mildew. The pedigree of this variety is: Stirling / Harrington: where Striling = ((Dampier / (Prior / Ymer)) / Piroline and Harrington = Klages /3/ Gazelle / Betzes // Centennial.

Vlamingh is a high yielding, plump grained barley variety that is adapted to most barley growing areas of Western Australia. It is moderately resistant to scald and is susceptible to powdery mildew. It is high yielding and has superior malting quality. The pedigree is: WABAR570 / TR118.
Figure 4.1: Showing the pedigree of Cross1. Each colour represents the parent and the proportion of each colour corresponds to the proportion of that specific parent in the population.

235 Double-Haploids were developed and selected for scald resistance at Wongan Hills and Shenton Park. 53 DH were selected and used for crossing.

F₂ population used for this study
Figure 4.2: Showing the pedigree of Cross2. Each colour represents the parent and the proportion of each colour corresponds to the proportion of that specific parent in the population.

235 Double-Haploids were developed and selected for scald resistance at Wongan Hills and Shenton Park. 53 DH were selected and used for crossing.
**WABAR2147** is a high yielding, malting quality line which is moderately resistant to scald and powdery mildew. The pedigree of the line is: Capulet / Franklin: where Capulet = Carnival (Mla6) /4/ Tyra (Mla1) /3/ (Jupiter (Mla6, Ml(La)) // Julia (Ml(CP) / L79 and Franklin = Shannon / Triumph (Ml (Ab), Mla7, MlTr3): where Shannon = (CI-3208-1 / 4* Proctor.

The donor parents Vlamingh and WABAR2147 were crossed to develop F1s. Later a double haploid population of 235 individuals was generated from these F1s, which were selected for scald resistance at two sites, Wongan Hills and Shenton Park in Western Australia. Fifty three double-haploids, which were found moderately resistant to scald at both the sites with different environments, were used as males for Cross 1 (Figure 4.1) and Cross 2 (Figure 4.2). The double haploids were expected to have a frequency of 50% Vlamingh and 50% WABAR2147.

The recipient population crossed to the moderately scald resistant donor double haploid population, comprising of 50% Vlamingh and 50% WABAR2147, was mainly WABAR2096 in Cross 1 (Figure 4.1) and WABAR2096, Hamelin and Baudin in Cross 2 (Figure 4.2).

**4.2.2 Planting and maintaining the disease nursery**

Each F2 population from these two crosses was divided into two bulks, one for phenotypic selections (Treatment 1) and one for genotypic selections (Treatment 2). The bulks were
further separated into male fertile (plump) and male sterile (shrunken) seeds to be planted in the crossing block as males and females respectively. Two crossing blocks, each of 5m x 2m, were planted at Shenton Park, Western Australia, in 2006 as disease nurseries, with six rows each.

Each block comprised of two rows of females (male sterile, shrunken seeds) in the center with two rows of males (plump, male fertile seeds) on either side, to facilitate natural crossing (Figure 4.3). 50 seeds were planted in each row at 10cm distance with 40 cm distance between the rows. A total of 400 male fertiles and 200 male steriles were planted in two replicates. Yagan was planted as control for scald and powdery mildew because of its high susceptibility for scald and moderate susceptibility to powdery mildew. Straw infected with scald spores was spread in between the rows and in the pathways to act as a source of inoculum and to enhance the development of disease, five weeks after planting.

The crossing block was designed to facilitate random natural crossing between the male fertiles and the male steriles. The success of the cross fertilization depends upon the maturity of the plants. If the male steriles (females) mature early, they miss out on the pollen from the males which are late in maturity and vice- versa, resulting in poor or no seed set on some of the male sterile (females) plants. In order to maximize the seed material for the current work, supplementary hand crossing was carried out.

After discarding all the scald and powdery mildew susceptible plants from the disease nursery before anthesis, hand crossing was carried out on each male sterile (female) plant in order to obtain at least 9-10 seeds of each male sterile (female). The heads from the male

97
fertile plants which were moderately resistant to scald and powdery mildew, were chosen randomly and then the pollen were used to hand pollinate the male sterile (female) plants.

Figure 4.3: Crossing block planted in the field (Shenton Park) with two outer rows of males on the either side of the central two rows of male steriles, to enhance pollination. Each row was spaced 40cm apart and the plants in each row were planted at 20cm. Each crossing block measured 5m x 2m.

The pollinated heads of the male sterile plants were then covered with bags so as to avoid any contamination from the parents and checks planted in the same disease nursery and also from the adjacent disease nurseries of barley (Figure 4.4).
4.2.3 Phenotypic selection (Treatment1)

4.2.3.1 Season 2006:

Disease ratings for scald and powdery mildew were carried out three times, for both the populations of Cross 1 and Cross 2, before anthesis and the susceptible plants rated higher than 2 were discarded from the crossing blocks. Yagan was used as the control parent for scald which was rated 4-5 for all the plants. Both the diseases were scored on a whole canopy plot basis with rating scales related to percent disease severity. All the data were in 0-5 scale (0 – resistant and 5 – highly susceptible). The final rating was carried out as the percent leaf area damage on all the residual plants before they started to dry out. The F₁
seeds were harvested from the central two rows of male steriles (females) from each crossing block of year 2006 and 20 F₁ seeds of each female (20 seeds/family) were planted in the summer nursery, in December 2006, at DAFWA’s Manjimup Horticulture Research Station, Manjimup to get the F₂ seeds after selfing. Out of 20 F₁ plants, only 10 F₁/₂ plants per family were selected at random to carry forward to next season’s disease nursery (Figure 4.5).

4.2.4 Genotypic selection (Treatment2)

4.2.4.1 Season 2006:

Marker assisted selection was carried out on the F₂ bulk separated for the genotypic selections (Treatment 2). The population was separated into females (male steriles, shrunken seeds) and males (male fertile, plump seeds). All the five parents in the composite cross were screened using 93 SSR markers (Table 4.1) to track the scald (chromosome 4H and 6H) and powdery mildew resistant (chromosome 1H) loci.

Table 4.1: Number of markers used to screen the parents for powdery mildew (MlaWABAR2147) and scald (RhsVlamingh and Rhs WABAR2147, respectively) resistances.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>1H</th>
<th>4H</th>
<th>6H</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of markers used for screening</td>
<td>15</td>
<td>28</td>
<td>50</td>
</tr>
</tbody>
</table>

The positive allele for scald came from Vlamingh and WABAR2147 on chromosome 4H and 6H respectively. WABAR2147 also contributed for the powdery mildew (Mla) resistance on chromosome 1H.
4.2.4.1.1 DNA extraction and SSR marker analysis

All the male sterile plants studied were subjected to DNA isolation. Leaf tissue was collected at three leaf stage for DNA extraction and molecular marker assays. DNA was extracted from leaf tissue of the parents and the progeny, using the Mini Prep method adapted from Rogowsky et al. (1991).

In all 93 SSRs (Liu et al. 1996 and Ramsay et al. 2000) were tested for polymorphism between the parent. SSR loci were amplified in a 10-μl reaction mixture containing 50 ng of template DNA, 1μl 10x PCR buffer, 0.6μl MgCl2 (25mM), 0.4μl dNTP (10mM), 0.075μl of each primer (5 pM), 0.09μl Taq DNA polymerase (0.5 units) and injection water to make it upto 10μl. Temperature cycling conditions were used as described by Ramsay et al (2000). Amplified products were separated in 7% acryl-amide gels, running them at 80 volts for 16 hrs. The gels were stained with ethidium bromide and visualized using UV light box and photographs of the amplification product were taken using gel documentation system Protean II xi Cell gel system (BioRad, CA, USA). The sizes of the products were estimated using 100 base pair ladder.

Of 93 markers screened for polymorphism, 25 SSR markers were found to be polymorphic over all the five parents but only four SSR markers; Bmac213 (1H), GBM1501 and HVM40 (4H), EBmac874 (6H), were able to distinguish all the recurrent parents from each other in both the composite crosses; 1 and 2. These four markers were used to screen the population of 361 male fertiles (males) and 176 male steriles (females) each for Cross 1 and Cross 2 (Table 4.4). The marker order given by Ramsay et al (2000) was followed in selecting the markers for genotypic selections.
The genotypes selected with the polymorphic markers were homozygotes and heterozygotes for all the three alleles (two for scald and one for powdery mildew) under selection. The selections were planted in the crossing block at DAFWA, South Perth, in 2006, with central row of females and outer two rows on either side, of males for random natural pollination (Figure 4.3).

The hand pollination was carried out on the selected females (male steriles) with pollen randomly selected from the males in the outer rows of the crossing block to enhance random pollination (Figure 4.4). Central two rows of females (male steriles) were harvested and 20 F₁ seeds of each female (20 seeds/family) were planted in the summer nursery, in December, 2006 at DAFWA’s Manjimup Horticulture Research Station, Manjimup to get the F₂ seeds after selfing. Out of 20 F₁ plants, only 10 F₁/₂ plants per family were selected randomly to carry forward to next year’s disease nursery.

4.2.5 Season 2007 (phenotypic (Treatment 1) and genotypic selections (Treatment 2))

The population of recombinants was developed as mentioned in Figure 4.5. Ten Fertile plump seeds/single plant/family (F₂/₃) (a total of 924 lines and 9240 plants for both the crosses and treatments) for all the ten plants (F₁/₂) from both the populations were randomly selected (For Cross 1, 276 individuals each for both the treatments and Cross 2, 186 individuals for each treatment) to be planted as 1m rows with 10 seeds each row, 20cm apart in the disease nursery at Shenton Park, WA, in the 2007 season (Figure 4.3).
Figure 4.5: A schematic presentation of the field trials for phenotypic (Treatment 1) and genotypic (Treatment 2) selection procedures carried out for scald and powdery mildew on both the Cross 1 and Cross 2 populations in years 2006 and 2007.

The infected straw carrying the scald spores was spread for inoculum in the pathways along the columns and rows to facilitate the disease infection process. The scald susceptible
control variety Yagan was planted as a “spreader” to enhance the disease. The disease ratings were carried out three times during the season with the difference of one month between each disease scores. The scale used for rating was based on the CIMMYT scale for assessing the foliar intensity of wheat disease. 0-9 scale was used for both powdery mildew and scald rating, where: 0 – Free from infection, 1 – Very resistant, 2 – Resistant, 3 – Moderately resistant, 4 – Low intermediate, 5 – Intermediate, 6 – High intermediate, 7 – Moderately susceptible, 8 – Susceptible and 9 – Very susceptible.

4.2.5 Experimental design and statistical analysis

Trials with populations from Cross 1 and Cross 2 were replicated using spatially balanced rectangular array of rows and columns. Trials with both the populations were partially replicated, only 25% of the test lines were replicated and the parents and a control variety Yagan were replicated three and eleven times, respectively. All designs were generated using DiGGer (Coombes, 2002).

In the work reported, it was necessary to control and allow for variation in field trials. The experimental design principles; replication (for precision), randomization (to avoid systematic bias) and blocking (to control for extraneous variation) were followed. As the selections were made at each cycle of the breeding trials under study, it resulted in the bias of the data in the direction of the desired genotypes. In order to get the better and realistic estimates of their performance, Spatial linear mixed models (Gilmour et al, 1997) were used in the analysis of each single trial in year 2007. The approach adopted here identifies three major components of spatial variation in plot errors from field experiments as described in Chapter 3, section 3.2.5.
The diagnostic tools used in identification of the model (Gilmour et al, 1997; Stefanova et al, 2008) predominantly involve use of plot of the residuals and sample variogram. The best model was chosen by maximizing the REML log-likelihood via the comparison of the deviance terms with a Chi-square distribution. Fixed effects were tested using Wald tests. Spatial variation modelling was carried out in order to identify the spatial pattern in the spread of scald, to adjust for it and to look for possible inference of powdery mildew spread, where the measurements were taken.

The statistical methodology (kindly provided by Dr. Katia Stefanova) was followed starting by separately carrying out a first order autoregressive model (AR1 (column) x AR1 (row)) for both rows and columns for the trials and the spatial linear mixed models were used for the analysis of each of the trial which excluded any bias. The residuals from this model then provided the basis for identifying global and extraneous variation, as well as assessing the adequacy of the variance structure for local trend. The models were fitted using statistical software GenStat (version 10.2 http://www.genstat.com).

Tables 4.2 and 4.3 present an overview of the models fitted for leaf scald and leaf area damaged data, respectively, in each trial (population). The model fitted for the field variation consisted of an autoregressive (AR1 x AR1) process for the spatial and hence the row and column dependence variation is expressed through the autoregressive correlation coefficients.
Table 4.2: An overview of the models fitted to the leaf scald and powdery mildew data collected three times during the season, for both the crosses and each cross with both the treatments. The numbers represent the autoregressive correlation coefficients for natural spatial variation along with standard errors (SE) for the estimates. Where: T1 – phenotypic selection (Treatment1), T2 – genotypic selection (Treatment2).

<table>
<thead>
<tr>
<th>Population</th>
<th>Time</th>
<th>Genetic variation</th>
<th>Spatial variation</th>
<th>AR1 Col (SE)</th>
<th>AR1 Row (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross1– T1</td>
<td>1</td>
<td>Variety + lin (row) + lin (col)</td>
<td></td>
<td>-0.02 (0.14)</td>
<td>0.37 (0.22)</td>
</tr>
<tr>
<td>Cross1– T1</td>
<td>2</td>
<td>Variety + lin (row)</td>
<td></td>
<td>-0.14 (0.39)</td>
<td>0.54 (0.22)</td>
</tr>
<tr>
<td>Cross1– T1</td>
<td>3*</td>
<td>Variety + lin (row) + lin (col) + PM</td>
<td></td>
<td>0.13 (0.36)</td>
<td>0.004 (0.15)</td>
</tr>
<tr>
<td>Cross1– T2</td>
<td>1</td>
<td>Variety + lin (row)</td>
<td></td>
<td>-0.15 (0.44)</td>
<td>0.14 (0.38)</td>
</tr>
<tr>
<td>Cross1– T2</td>
<td>2</td>
<td>Variety + lin (col)</td>
<td></td>
<td>0.27 (0.30)</td>
<td>0.34 (0.22)</td>
</tr>
<tr>
<td>Cross1– T2</td>
<td>3*</td>
<td>Variety + col</td>
<td></td>
<td>I**</td>
<td>I**</td>
</tr>
<tr>
<td>Cross2– T1</td>
<td>1</td>
<td>col</td>
<td></td>
<td>I**</td>
<td>0.07 (0.20)</td>
</tr>
<tr>
<td>Cross2– T1</td>
<td>2</td>
<td>col</td>
<td></td>
<td>-0.02 (0.16)</td>
<td>0.12 (0.36)</td>
</tr>
<tr>
<td>Cross2– T1</td>
<td>3*</td>
<td>Variety + lin (col)</td>
<td></td>
<td>0.16 (0.39)</td>
<td>0.04 (0.17)</td>
</tr>
<tr>
<td>Cross2– T2</td>
<td>1</td>
<td>Variety + lin (col)</td>
<td></td>
<td>0.03 (0.38)</td>
<td>0.55 (0.21)</td>
</tr>
<tr>
<td>Cross2– T2</td>
<td>2</td>
<td>Variety + lin (col)</td>
<td></td>
<td>0.06 (0.42)</td>
<td>0.50 (0.24)</td>
</tr>
<tr>
<td>Cross2– T2</td>
<td>3*</td>
<td>Variety + lin (col)</td>
<td></td>
<td>I**</td>
<td>I**</td>
</tr>
</tbody>
</table>

* Only for those models where variate PM (powdery mildew) is available.
** Identity was fitted for the column (col), no auto-regression and no trend was observed.
1 lin – linear, col – column and AR - autoregressive.
Table 4.3: An overview of the models fitted to leaf area damage due to scald. Variations are based on the data collected for both the crosses with both the treatments. The numbers represent the autoregressive correlation coefficients for natural spatial variation. Where: T1 – phenotypic selection (Treatment1), T2 – genotypic selection (Treatment2).

<table>
<thead>
<tr>
<th>Population</th>
<th>Genetic variation</th>
<th>Spatial variation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Global/Extraneous</td>
<td>Natural</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AR1 Col</td>
<td>AR1 Row</td>
</tr>
<tr>
<td>Cross1 – T1</td>
<td></td>
<td>0.48</td>
<td>0.23</td>
</tr>
<tr>
<td>Cross1 – T2</td>
<td>Variety + lin (row)</td>
<td>0.06</td>
<td>0.34</td>
</tr>
<tr>
<td>Cross2 - T1</td>
<td>Variety + lin (col)</td>
<td>0.17</td>
<td>0.02</td>
</tr>
<tr>
<td>Cross2 – T2</td>
<td></td>
<td>0.12</td>
<td>0.25</td>
</tr>
</tbody>
</table>

1 lin – linear, col – column and AR - autoregressive.

Since the measurements for leaf scald were taken 3 times, equally spaced in time by one month, it presented a classical repeated-measurements study. A linear mixed model was fitted and unstructured model was used to account for the correlation patterns of the measurements repeated over time on the same plants. Unstructured model for variance-covariance structure was considered as the best among all models. The choice was based on the log-likelihood ratio test.

The GenStat (version 10.2 [http://www.genstat.com](http://www.genstat.com)) procedure REML was employed and the model can be described in a GenStat formula where time is the repeated – measurements factor using the observed level 30 days (the time between measuring periods), given below: .
The model fitted for the response variable scald included Time, Family, Time*Family as fixed part and the Plot.Time as random. In the above model, Time represents the three different times when the data was collected on the incidence of scald, Family represents the families in the trial, Plot represents the 1m rows planted with the seeds of each sib in a family and Time*Family represents the interaction between three scald ratings and Family at three different times. The results from the repeated measurement analysis using the above model are presented in Table 4.6.

Further, the correlation coefficient was calculated for the three scald scores taken at the interval of one month, for both the populations, using the GenStat (version 10.2 http://www.genstat.com) procedure correlation (Table 4.7).

4.2.6 Simulation studies

Simulation studies based on the Cross 1 pedigree (Figure 4.1) were carried in collaboration with Dr. Scott Chapman from the CSIRO, Queensland, Australia. Taking into account the genotype frequency of the recipient parent WABAR2096 (Figure 4.1), Cross 1 could be considered as a Top-cross of WABAR2096 over the selected scald resistant double haploid lines comprising WABAR2147 and Vlamingh. The recovery of the three disease resistant
genes, one for powdery mildew \((Mla_{WABAR2147})\) and two for scald \((Rhs_{Vlamingh} \text{ and } Rhs_{WABAR2147})\), in the F2 population after the Top-cross was of interest.

QU-GENE (developed at the University of Queensland, Australia), a simulation platform for quantitative analysis of genetic models (Wang et al., 2007), was used to carry out the simulation studies for the selection of scald and powdery mildew resistant lines in the population. The program generates populations of genotypes and provides a library of subroutines to develop simulation modules for real-world breeding programs (Podlich and Cooper, 1998). QuLine is a QU-GENE application module that was specifically developed to simulate breeding programs developing inbred lines (Wang et al., 2003).

In this study, the simulations were used to characterize the selection strategies for selecting the scald resistant lines in the population. The simulations were carried out on the phenotypic and genotypic data on the double haploid population, kindly provided by Dr. Reg Lance, Dr. Chengdao Li and Dr. Sanjeev Gupta.

Three simulation runs were set up and repeated for 20 times, initialized from the cross Vlamingh x WABAR2147:

1. Create 235 DH lines and express phenotype
2. Create 235 DH lines and select the best 53 based on phenotype for scald.
3. Create 235 DH lines and select the best 53 based on the marker score for \(Rhs_{Vlamingh}\) and \(Rhs_{WABAR2147}\) only.
From the scald data on the original 235 double haploid lines and phenotypically selected 53 DH lines, population means and variances were calculated and compared with the simulation 1.

Table 4.4: Additive effect (for example if its 0.5, it is accounting for half of the variation) sizes of the genes under study along with the additional minor QTL for scald, for all the genotypes. Where 11 – both the alleles from Vlamingh, 12 – one allele from Vlamingh and one from WABAR2147 and 22 – both the alleles from WABAR2147.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Trait</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mla&lt;sub&gt;WABAR2147&lt;/sub&gt;</td>
<td>PM</td>
<td></td>
</tr>
<tr>
<td>QTL Scald01</td>
<td>Scald</td>
<td></td>
</tr>
<tr>
<td>QTL Scald02</td>
<td>Scald</td>
<td></td>
</tr>
<tr>
<td>QTL Scald03</td>
<td>Scald</td>
<td></td>
</tr>
<tr>
<td>Rhs&lt;sub&gt;Vlamingh&lt;/sub&gt;</td>
<td>Scald</td>
<td></td>
</tr>
<tr>
<td>Rhs&lt;sub&gt;WABAR2147&lt;/sub&gt;</td>
<td>Scald</td>
<td></td>
</tr>
<tr>
<td>QTL Scald07</td>
<td>Scald</td>
<td></td>
</tr>
</tbody>
</table>

Along with the three genes of interest, four extra QTL (additive effect size of each allele = 0.25) for scald were included in the simulations. Donor parent Vlamingh was denoted as allele 1 and WABAR2147 as allele 2 (Table 4.4). It was assumed that in the best combination, this set of genes will result in scald score of 8 (0-9 scale, with 9 as the scald resistant genotype), with \( Rhs_{WABAR2147} = 4 \) and \( Rhs_{Vlamingh} = 2 \) and the four QTL summing to
2. Within family heritabilities of 0.9 and among family heritabilities of 0.8 were applied for this dataset.

4.3 Results

4.3.1 Male sterile facilitated recurrent selections

4.3.1.1 Phenotypic selection (Treatment 1) in year 2006

All the scald and powdery mildew susceptible plants were culled from the disease nursery before anthesis. Percent leaf area damage due to scald data was collected on the remaining resistant to moderately resistant plants in the crossing block, before the plants started to dry off. In Cross 1 population, the 71 individuals that were selected ranged from 0-3.5% for leaf area damage due to (Figure 4.6 (A)) and in Cross 2 population, the 44 individuals selected ranged from 0-5% for leaf area damage due to (Figure 4.6 (B)).
In both Cross 1 and Cross 2 populations, WABAR2147 and Vlamingh were found to be moderately resistant to scald, as expected. Both of these parents ranged from 0.5-1.5% for leaf area damage. The control parent Yagan, which is very susceptible to leaf scald was found to have 80-90% leaf area damage due to scald. Figure 4.7 shows the leaves of the plants with 80-90% leaf area damage with scald (Figure 4.7 (A)) and the leaves of the resistant plant with zero scald infection and leaf area damage (Figure 4.7 (B)).
Figure 4.7: Photographs taken at the end of the season showing (A) the control variety Yagan where the leaf area damage due to scald was between 80-90% and (B) the scald resistant plant with zero leaf area damage and zero scald infection.
4.3.1.2 Genotypic selection (Treatment 2) in year 2006

A marker system was developed to select for the individuals with the desired alleles in the multi-allelic system by grouping the polymorphic markers. The combination of alleles provided a distinctive haplotype for a parent. For example Table 4.5, for chromosome 4H, the combination of GBM1501 and HVM40 (<10cM apart), provided the means for identifying the resistance allele from Vlamingh. The markers were selected based on the criteria that they differentiate between the parents but still have close association with the genes of intent. On chromosome 4H, it was difficult to track and select for the Vlamingh allele from rest of the parents with just one SSR marker.

Table 4.5: Details of the markers used for genotypic selections for powdery mildew \((M_{la}^{WABAR2147})\) resistance and scald \((Rh_{s}^{Vlamingh} and Rh_{s}^{WABAR2147})\) resistance for Cross 1 and Cross 2 populations.

<table>
<thead>
<tr>
<th>Chromosomes</th>
<th>SSR markers</th>
<th>Parental alleles selected against</th>
<th>Parental alleles selected for</th>
</tr>
</thead>
<tbody>
<tr>
<td>1H</td>
<td>Bmac213</td>
<td>Birka (one of the parents of male sterile line DF168), Skiff, WABAR2096 and Vlamingh</td>
<td>WABAR2147</td>
</tr>
<tr>
<td>4H</td>
<td>GBM1501</td>
<td>Birka and WABAR2147 (and Hamelin for Cross 2 population)</td>
<td>Vlamingh, WABAR2096 and Skiff (and Baudin for Cross 2 population)</td>
</tr>
<tr>
<td></td>
<td>HVM40</td>
<td>WABAR2096 (and Baudin for Cross 2 population)</td>
<td>Vlamingh and Skiff (shared the same allele)</td>
</tr>
<tr>
<td>6H</td>
<td>EBmac874</td>
<td>Birka, WABAR2096, Vlamingh</td>
<td>WABAR2147 and Skiff (shared the same allele)</td>
</tr>
</tbody>
</table>
The population was first screened with the SSR marker GBM1501 to subtract Birka and WABAR2147 in Cross 1 population and additional Hamelin like alleles in Cross 2 from the population. The selected individuals were then screened with another SSR marker HVM40 to separate WABAR2096 in Cross 1 and Baudin and WABAR2096 in Cross 2, from the populations from Vlamingh and Skiff alleles (Table 4.5 and Figure 4.8).

Figure 4.8: The acryl-amide gel pictures showing the separation of Vlamingh allele from the rest of the parents except Skiff, using two markers; GBM1501 and HVM40, in the multi-allelic system on chromosome 4H. Where Sk – Skiff, 96 – WABAR2096, Bdn – Baudin, Hml – Hamelin, Vlm – Vlamingh and 47 – WABAR2147.

None of the polymorphic markers selected to track the resistant parent Vlamingh was found to be close enough to GBM1501 and HVM40 to separate Vlamingh from Skiff. Similarly WABAR2147 and Skiff were inseparable with the polymorphic marker EBmac874. Figure
4.8 shows the grouping of parents with two markers on chromosome 4H while tracking Vlamingh.

A total of 48 out of 361 male fertile (male) and 26 out of 179 male sterile (female) individuals from Cross 1 population and 30 of 361 male fertile (male) and 22 out of 179 male sterile (female) individuals from Cross 2 population were selected using the polymorphic markers Bmac213 (1H), GBM1501 and HVM40 (4H) and EBmac874 (6H). These selected individuals may carried all the three resistant genes; \( Mla_{WABAR2147} \) (1H), \( Rhs_{Vlamingh} \) (4H) and \( Rhs_{WABAR2147} \) (6H).

4.3.2 Spatial variation and overview of repeated measurement analysis for scald and powdery mildew

The models fitted to the scald, powdery mildew and the leaf area damage for scald, indicated that the spread of scald for all four trials with two populations with two treatments each (Table 4.2) was predominantly along the columns. There was additional linear row trend only for the population of Cross 1, which may be attributed to a natural conditions like wind direction in the field. No distinctive spatial pattern was identified for the leaf area damage due to scald (Table 4.3).

In year 2006, phenotypic selection (Treatment 1) and genotypic selection (Treatment 2) was carried out and the selected F1 plants were carried forward to next year. It was expected that the F2/3 will segregate for their disease resistance status. The analysis of repeated measurements provided us with the p-values which were found to be highly significant over Time (three scald scorings), Family (families in the trial) and Time*Family.
(interactions between families in the trial and the scald scorings carried out at three different times during the season), for both the crosses and all four treatments with the exception for Time*Family for Cross 1- T1 (Table 4.6). The p-values of <0.001 for Time and Family, indicated that the families and the scald scores taken at three different time periods were significantly different for each cross and treatment.

Table 4.6: Significance (p-values) of Time, Family and Time*Family, in the repeated measurement analysis of both the crosses with two treatments each. Where T1 – phenotypic selection (Treatment1), T2 – genotypic selection (Treatment2), SC1 – scald score 1, SC2 – scald score 2 (taken 30 days after SC1) and SC3 – scald score 3 (taken 30 days after SC2).

<table>
<thead>
<tr>
<th>Population</th>
<th>Time</th>
<th>Family</th>
<th>Time*Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross1 – T1</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.935</td>
</tr>
<tr>
<td>Cross1 – T2</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.031</td>
</tr>
<tr>
<td>Cross2 – T1</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cross2 – T2</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.221</td>
</tr>
</tbody>
</table>

Correlation coefficients (Table 4.7) indicated a strong positive correlation between all the scald data undertaken for all the four populations which indicated the increase in the incidence of scald in time. The increase in the value of one scald score indicated the likely increase in the value of the second and the third scald scores. The correlations were found to be above 80% for all the populations except Cross 2-T2 population, where it was found to be 74% between scald score 1 and scald score 2 (SC1 vs. SC2) and 68% between scald
score 1 and scald score 3 (SC1 vs. SC3). High degree of correlation indicated a good fit to the linear model.

Table 4.7: Correlation coefficients between the three scald scores for both the Crosses and Treatments. Where T1 – phenotypic selection (Treatment1), T2 – genotypic selection (Treatment2), SC1 – scald score 1, SC2 – scald score 2 (taken 30 days after SC1) and SC3 – scald score 3 (taken 30 days after SC2).

<table>
<thead>
<tr>
<th>Population</th>
<th>SC1 vs. SC2</th>
<th>SC2 vs. SC3</th>
<th>SC1 vs. SC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross1 – T1</td>
<td>0.85</td>
<td>0.91</td>
<td>0.8</td>
</tr>
<tr>
<td>Cross1 – T2</td>
<td>0.84</td>
<td>0.9</td>
<td>0.79</td>
</tr>
<tr>
<td>Cross2 – T1</td>
<td>0.8</td>
<td>0.92</td>
<td>0.81</td>
</tr>
<tr>
<td>Cross2 – T2</td>
<td>0.74</td>
<td>0.83</td>
<td>0.68</td>
</tr>
</tbody>
</table>

4.3.3 Phenotypic selection in Year 2007

Predicted means for the families comprising Cross 1 and Cross 2 were calculated, for both the treatments (phenotypic and genotypic selections) carried out on F1s in year 2006, using the Repeated measurement analysis with GenStat (version 10.2). For Cross 1, 79.5% of the lines were found to be moderately resistant (scald score 1-3) to scald for phenotypic selection treatment, including 9.6% of the population with scald score between 0-1 i.e. resistant to moderately resistant. 17.8% were found to be intermediate for scald (scald score 4-6) and 2.8% of the population was found to be susceptible to highly susceptible to scald (scald score 7-9).
Figure 4.9: The comparison between phenotypic selection (Treatment 1) and genotypic selection (Treatment 2) methods based on the predicted means from the phenotyping at F2, carried out in year 2007 are shown. (A) Cross 1 population (see Figure 4.1) and (B) Cross 2 population (see Figure 4.2).
For genotypic selection treatment, 76.9% of the population was found to be moderately resistant (scald score 1-3) to scald with zero resistant lines, 19.2% of the lines were found to be intermediate (scald score 4-6) for scald and 3.8% were found to be susceptible (scald score 7-8) to leaf scald (Figure 4.9A).

For Cross 2, 85.5% of the population was found to be moderately resistant (scald score 1-3) including the 16.7% resistant lines (scald score 0) for phenotypic selection (Treatment 1), 8.3% of the lines were intermediate (scald score 4-6) for scald reaction and 6.3% were found susceptible (scald score 7-9) to scald while for genotypic selection (Treatment 2), 72.7% of the population was found to be moderately resistant (scald score 1-3) with zero resistant lines, 18.2% was intermediate (scald score 4-6) and 9.1% susceptible (scald score 7-9) to scald (Figure 4.9B). The parents WABAR2147, Skiff, Baudin and Vlamingh were found to be moderately resistant to scald while WABAR2096 was found to be intermediate and Hamelin was found to be susceptible. The resistant male fertiles of season 2006, which were used as checks, segregated for scald reaction and were found to be moderately resistant for scald. The control variety Yagan was found to be highly susceptible to scald (Figure 4.9).

4.3.4 Simulation results for scald selection

The mean for scald resistance calculated using the 53 DH lines selected phenotypically at Shenton Park (6.2) was found to be very close to the simulation mean of 7.1. Three simulations (original population of 235 DH lines, phenotypically selected 53 DH lines and genotypically selected 53 DH lines) were carried out in order to characterize the phenotypic and genotypic selection methods used to select the 53 DH lines. It was found that the mean
scald score (on 0-9 scale, 9 being resistant) of the 53 lines selected phenotypically was higher than the original set of 235 DH lines and also from the 53 lines selected genotypically. This indicated the better performance of phenotypic selection in moving the population from mean scald score of 4 to mean scald score of 7 (Figure 4.10).

![Box plot for scald phenotype for each simulation strategy](image)

Figure 4.10: Box plot for scald phenotype for each simulation strategy, 1 – original set of 235 DH lines, 2 – 53 DH lines selected phenotypically and 3 – 53 DH lines selected genotypically. Along x-axis are the three simulations and along y-axis are the scald scores on the scale of 0-9 where 9 represent the most resistant DH lines for scald.

The same three simulations (original population of 235 DH lines, phenotypically selected 53 DH lines and genotypically selected 53 DH lines) were carried out for the three
expected genotypes, 11 (both the alleles from Vlamingh), 12 (one allele from Vlamingh and one from WABAR2147) and 22 (both the alleles from WABAR2147). It was found from the simulation studies that the phenotypic selection was more efficient as it took advantage of the accumulation of the minor QTL. For genotype 11 (both the alleles from Vlamingh), phenotypic selection moved the scald score mean from 4 to 7, in case of genotype 12 (one allele from Vlamingh and one from WABAR2147), the scald score mean was found to be 7 for phenotypic selection as compared to the original population and the genotypically selected individuals, with mean of 6 and for genotype 22 (both the alleles from WABAR2147), phenotypically selected individuals again exhibited a higher mean scald score of 7 (Figure 4.11).

![Box plots for each DH genotype based on the three simulation strategies.](image)

Figure 4.11: Box plots for each DH genotype based on the three simulation strategies, 1 – original set of 235 DH lines, 2 – 53 DH lines selected phenotypically and 3 – 53 DH lines selected genotypically. Along x-axis are the three simulations for each genotype and along y-axis are the scald scores on the scale of 0-9 where 9 represent the most resistant DH lines for scald. Genotype 11- both the alleles from Vlamingh, genotype 12 - one allele from Vlamingh and one from WABAR2147 and genotype 22 - both the alleles from WABAR2147. Black spots represents the scald means of the populations.
4.4 Discussion

4.4.1 Disease epidemic and the statistical analysis

From the statistical analysis (Table 4.2 and 4.3), it was found that the disease spread in year 2007 in the disease nursery was found to have a progression in the direction of the columns which could be due to the fact that the plants are closer to each other in columns and hence facilitate physical spread by contact as compared to rows. This trend of scald spread also explained the reason of low correlation between the first scald score (SC1) and the second scald score (SC2), for Cross 2-T 2 (Table 4.7). The disease progress is driven by the endogenous spread of inoculum from the infected leaves to the healthy leaves. Disease resistance reduces the amount of initial inoculum and suppresses the apparent infection rate. Disease escape also affects the amount of initial inoculum and may delay the first infection of new leaf layers (Cooper, 1999). Infection of a healthy leaf is followed by several cycles of infection and dispersal of inoculum to newly emerged leaves higher in the canopy (Shaw and Royle, 1993). As this population was planted at the end of the disease nursery, the reason for the low first scald rating could be the direction of the disease spread, which was towards the columns and it took longer for the disease to spread.

Another reason for the Cross 2-T 2 population showing lower scald scores at the first rating could be the plant height and the soil moisture, the factors that affect the initial infection (Schroeder and Christensen, 1963), at and after the artificial scald epidemic, induced by scattering the straw. So, many individuals might escape infection and therefore show a pseudo resistance. The epidemic induced by scattering diseased straw on the soil surface was found convenient and effective for identifying scald resistance in a disease selection nursery. Once the scald was spread we found the high correlation between the next
subsequent scald assessments for this population. Overall, there was an increase incidence of scald with time (Table 4.7).

The main effects for *Time* and *Family* (see statistical analysis, section 4.3.2) were found to be highly significant (*p*<0.001) for all the four populations; both the crosses with both phenotypic and genotypic selections (Table 4.6). To compare the efficiencies of the treatments, phenotypic selection and genotypic selection, the predicted family means were considered (Figure 4.9) which was obtained from the replicated trial within the same environment. The selection based on family means was found to have an advantage because the replications reduce the masking effect of environment to a manageable level (Xie and Xu, 1998).

Although the controls indicated a good epidemic, the low powdery mildew ratings in the disease nursery were observed (data not shown). This could be attributed to the selection pressure applied against powdery mildew in the previous generation, in year 2006.

The scald ratings of the parents were found to be similar to the previous reporting. WABAR2147 and Vlamingh were found to be moderately resistant to scald (Chengdao Li, Sanjeev Gupta and Reg Lance, pers. com.), Hamelin was found to be susceptible and Yagan was found to be highly susceptible to scald (Smith *et al*, 2005). The parents Baudin and Skiff, reported to be intermediate to susceptible to leaf scald (Smith *et al*, 2005) were found to be moderately resistant in this study.
4.4.2 Phenotypic selection (Treatment 1) and genotypic selection (Treatment 2)

In this study, the comparison has been made between the efficiencies of phenotypic selection and genotypic selection carried out at F$_1$ stage, prior to the phenotypic family selection at F$_{2/3}$ and to develop an efficient breeding system for resistance to scald with the aid of a recessive male sterile gene, $msg_6$. The results of the present study suggested that both the phenotypic selection and genotypic selection worked well but the phenotypic recurrent selection (Treatment 1) was more effective and feasible in shifting the scald resistance in the desirable direction and enhancing the frequency of resistant individuals (Figure 4.9). The results of this study align with the results obtained from the simulation studies that the phenotypic selection method is more efficient in selection for scald and powdery mildew resistant lines (Figure 4.10 and Figure 4.11).

Resistant to moderately resistant lines for scald, selected in Year 2006, segregated at F$_{2/3}$ level, in Year 2007, as expected. The scald score appeared to be normally distributed for the families in each selection method for both the crosses as expected for a quantitative trait. In both populations, scald susceptible parent WABAR2096 contributed a major component of genotype along with scald resistant parents WABAR2147 and Vlamingh (Figure 4.1, Figure 4.2 and Table 4.1). If we consider the mean between WABAR2147 (scald resistant line) and WABAR2096 (scald susceptible line), in both the populations, it would be in the scald score range of 3-4 in Figure 4.9. So, it is quite clear from the demonstration that both the F$_1$ treatments moved the population in the positive direction. In Cross 1, phenotypic (Treatment 1) and genotypic (Treatment 2) selections carried out at F$_1$ shifted 79.5% and 76.9% of the population respectively, towards a scald score of 4 or less. Similarly, in Cross 2, 85.5% and 72.7% of the population was shifted in the desired
direction with phenotypic (Treatment 1) and genotypic (Treatment 2) selections, respectively, carried out at F1 stage, in year 2006.

For both the crosses, phenotypic selection (Treatment 1) was found to be more efficient in identifying the resistant families (9.6% in Cross 1 and 16.7% in Cross 2) with no resistant lines were identified with genotypic selection (Treatment 2) (Figure 4.9). Overall, for Cross 1, phenotypic selection (Treatment 1) was also found to be better performing in selecting the moderately resistant, intermediate and susceptible families for scald reaction as compared to the genotypic selection (Treatment 2).

For Cross 2, phenotypic selection (Treatment 1) was found to be much more efficient as compared to genotypic selection (Treatment 2) in identifying the moderately resistant, intermediate and susceptible families to scald (Figure 4.9). 85.5% of the families were found to be moderately resistant with phenotypic selection (Treatment 1) as compared to 72.7% with genotypic selection (Treatment 2).

The higher efficiency of the phenotypic selection could be explained by the fact that the scald resistant gene on chromosome 6H (Rhs_{WABAR2147}) expresses itself more strongly (approximately twice as effective, Chengdao Li pers. com.) as compared to the scald resistance gene on chromosome 4H (Rhs_{Vlamingh}). It is observed to have doubled the weighting as compared to scald resistance from Vlamingh. This fact became clearer during this study. It was found that during genotypic selection, the individuals carrying the scald resistant gene on chromosome 6H (Rhs_{WABAR2147}) were double in number than the individuals exhibiting scald resistance on chromosome 4H (Rhs_{Vlamingh}), at F1 level, though
the individuals carrying both the resistant gene were selected for further studies. Secondly, phenotypic selection aids in the selection of the minor genes along with the major genes under study and this could not be achieved with the genotypic selection method.

The reduced efficiency of genotypic selection (Treatment 2) could also be explained by the difficulty in selecting for the lines sharing the alleles with the scald resistant parents WABAR2147 and Vlamingh, using SSR markers. The strategy employed was to track WABAR2147 and Vlamingh with the polymorphic markers within 10 cM to each other, so as to select for the right chromosome segment. The SSR markers EBmac874 and GBM1501 / HVM40 were used to differentiate WABAR2147 and Vlamingh from all other parents except Skiff, on chromosome 6H and 4H respectively (Table 4.5). Due to the lack of the polymorphic SSR markers for differentiating Vlamingh and WABA2147 from Skiff, in 10cM around EBmac874 and GMB1501/ HVM40, the individuals carrying the alleles from Skiff along with the resistant parents were selected for the crossing block in Year 2006 to develop F1 seed. Outcrossing to other sources of resistance did not occur in year 2006 because heads to be crossed were selected and bagged.

4.4.3 Phenotypic and genotypic changes in response to phenotypic and genotypic selections

Resistance to scald is quantitative and in this study, is thought to be based on two major genes; \( R_{hsWABAR2147} \) and \( R_{hsVlamingh} \) and resistance to powdery mildew on \( R_{hsWABAR2147} \) (Li et al., 2006; Chengdao Li, Sanjeev Gupta, Reg Lance pers. com.) and the accumulation of these genes in the lines is highly desirable. In the present study, after a cycle of recurrent selection (excluding the selection of the double haploid lines for scald, prior crossing in
year 2005), the overall increase in the number of lines resistant or moderately resistant to scald in response to phenotypic selection was observed for both the crosses. The resistant and moderately resistant individuals increased by 20.1% for Cross 1 (Figure 4.9) and 30% for Cross 2, in both the populations under study.

The higher gains suggested the selection of the desirable individuals carrying major genes from the parents WABAR2147 and Vlamingh. The reason behind this shift in the desirable direction can be explained by the selection pressure applied while selecting for scald and powdery mildew resistant plants in year 2006. The screening of the crossing block was carried out before anthesis, to cull the plants which exhibited intermediate (scald scores 5-6) and susceptible (scald scores 7-9) reaction to scald and powdery mildew. The resistant male-sterile plants were then allowed to get randomly pollinated by resistant male-fertile plants. The use of parents (both male sterile and male fertile) with at least moderately resistant levels of disease might result in positive transgression for higher resistance during the line development (Snijders, 1990).

To illustrate the genotypic changes in response to genotypic selection separately for all the three genes under study; Mla\textsubscript{WABAR2147} (1H), Rhs\textsubscript{Vlamingh} (4H) and Rhs\textsubscript{WABAR2147} (6H) (Figure 4.12), the data on the Graphical Genotyping from Chapter 5 was combined with the data on the genotypic selections (Treatment 2) carried out in year 2006 during this study. As the Graphical Genotyping was carried out for only Cross 1 population, Figure 4.12 represents the responses to the genotypic selection for only one population. Moreover, the genetic response for year 2007 was based on the Graphical Genotyping (Chapter 5) of only the
resistant genotypes selected in year 2006 and did not include the heterozygotes, as selected and studied for this study.

Figure 4.12: Phenotypic and genotypic changes in response to phenotypic selection (Treatment 1) and genotypic selection (Treatment 2) carried out in year 2006 and 2007 for Cross 1. The data on the genotypic selection for the Year 2007 had been taken from Chapter 5 which was based on the Graphical Genotyping of solely the homozygote scald and powdery mildew resistant genotypes. Where PS – phenotypic selection, GS (1H) - genotypic selection for powdery mildew on chromosome 1H, GS (4H) - genotypic selection for scald on chromosome 4H and GS (6H) - genotypic selection for scald on chromosome 6H.

It was found that there was an increase of 55.6% in response to selection for powdery mildew resistance on chromosome 1H and 28.7% and 11.9 % for scald resistance on chromosome 4H and 6H respectively (Figure 4.12). The lower response to selection for scald could be attributed to the lack of perfectly linked markers used to select for Vlamingh and WABAR2147 on the chromosomes 4H and 6H respectively, where these scald
resistant parents shared the allele with susceptible parent Skiff. Similarly, the higher genetic response to powdery mildew resistance could be due to the availability of a closely linked marker Bmac213, used for the selection of the WABAR2147 like alleles in the population. The high responses to genotypic selection as compared to the phenotypic selection, clearly indicated the fixation of the desired genes and the increasing frequency of the desired genotypes with generations.

4.4.4 Optimal method of selection for scald and powdery mildew

It is clear from this study (Figure 4.9) and also from the simulation results (Figure 4.10, Figure 4.11) that the phenotypic selection (Treatment 1) method worked better than the genotypic selection (Treatment 2) method in selecting for scald resistance considering that there was a good epidemic of scald during the season. The simulation studies demonstrated that this was most likely due to the presence of minor genes which were selected while selecting phenotypically for the major scald resistant genes. At the same time the phenotypic selection (Treatment 1) failed in selecting for powdery mildew resistance in Year 2007 because of the low incidence of disease. This failure of phenotypic selection indicated that genotypic selection is a valuable part of the breeding program.

For simulation studies the possibility of ‘escapes’ for scald was not considered as a key issue in phenotypic selection as all the controls were uniformly spread and randomly replicated in the trial. The controls WABAR2096 and Yagan, which are highly susceptible to scald, were replicated internally and Yagan was also used as a buffer to spread the disease. Moreover, no escapes for scald were observed in the controls. Simulations on
powdery mildew could be an issue because, for example, no disease was observed in year 2007.

The lower efficiency of the genotypic selection method (Treatment 2) could be attributed to the lack of linked or associated markers to select for scald resistance and the limited polymorphism on chromosomes 4H and 6H. But in case of powdery mildew, the frequency of the resistant genotypes increased from 40.2% in Year 2006 to 95.8% in Year 2007, using the genotypic selection method (Figure 4.12), the reason being the presence of the perfectly linked marker, Bmac213, which was used for selection.

Although the phenotypic selection method (Treatment 1) has been considered as the efficient tool in the selections of the quantitative traits (Hallauer, 1992; Flint-Gracia et al., 2003), it works efficiently only in the presence of required incidence of disease infection. In the absence of the epidemic, genotypic selection method (Treatment 2) could be used efficiently if the linked markers are available. Considering the genotypic changes in response to selection for powdery mildew on chromosome 1H, it could be suggested that the genotypic selection method (Treatment 2) would have worked more efficiently as compared to the phenotypic selection method (Treatment 1) in the presence of the linked polymorphic markers.

As in this study, where the selections were carried for three genes, $Mla_{WABAR2147}$ (1H), $Rhs_{Vlamingh}$ (4H) and $Rhs_{WABAR2147}$ (6H) and where the markers used for genotypic selection were far from satisfactory, a combined approach could be more efficient for the selection of the desired genotypes. If we could have a marker system for multi-allelic systems to track
down the desired alleles, it would help in reducing the population size at the early stage (F1/2) by eliminating the undesirable genotypes and thus reducing the number of families for later stages. The time and resources used to identify and optimize molecular markers and employ them could also be significant. Marker-assisted selection (genotypic selection) with less number of families or individual plants could produce substantial increase in the selection responses (Xie and Xu, 1998). In the later stages (F2/3), phenotypic selections could be used on the desirable genotypes and the uncertain genotypes, which were left from the genotypic selection, to select for disease resistance. Genotypic selection followed by phenotypic selection could be considered as a potential alternative selection system.

Another important point of consideration is that the genotypic selections fixes the major genes more rapidly which can prevent the accumulation of the quantitative genes having a small effect on disease resistance. The polygenic response lost in the initial generation, could not be entirely recovered in later generations (Dekkers, 1998). With phenotypic selection, minor gene effects could be observed along with the major gene effects, which help the breeders in selecting the desirable genotypes with more stable resistance.
CHAPTER 5

Graphical Genotyping of a Population for Scald and Powdery Mildew Resistances

5.1 Introduction

Among the many diseases which can severely affect the productivity and quality of barley, there exist two that are the major leaf diseases common in Western Australian cereal belt; Scald, caused by the fungus *Rhynchosporium secalis* and Powdery mildew, caused by *Blumeria graminis* f. sp. *hordei*.

Disease resistant barley has been bred for many years and a large number of disease resistance genes have been introduced by using conventional methods of breeding. Molecular genetic markers assist in analysing the complexity of plant defense and host-pathogen relationships and have contributed to the development of breeding strategies such as marker assisted selection. Molecular genetic markers can be used to select either individual alleles of genes of interest or whole genome haplotypes to monitor genotypic changes in response to selection. The polymorphism between the breeding lines can be detected by a range of molecular methods (Rafalski *et al*, 1996; Russell *et al*, 1997). The recent shift to PCR based assays has increased the throughput of markers for early selection.
For this study previously mapped SSR markers (Liu *et al*, 1996; Ramsay *et al*, 2000; Varshney *et al*, 2007) were used for graphical genotyping of the population to assess and interpret the changes in the alleles and allele frequencies during selection for scald and powdery mildew resistance as a result of either phenotypic or genotypic selection. The advantages of using SSRs are their co-dominant transmission, PCR based detection and multi-allelic nature (Powell *et al*, 1996). A graphic format, called graphical genotyping (Young and Tanksley, 1989) has been utilized to provide a process of rapidly evaluating genome structure between individuals from a population reflecting different phenotypes; for example disease resistant versus disease susceptible. Graphical genotyping provides a rapid method for identifying chromosome regions reflecting population specific allelic configurations (Severson and Kassner, 1995).

This study focused on only one population, Cross 1 (Chapter 4, Figure 4.1) and expanded the number of markers used. The aim of this study was (a) to implement graphical genotyping, (b) to validate the associated markers and (c) to assay recombination to achieve information on the presence of the targeted regions/introgressed segments, so as to monitor genotypic changes in response to selection. From the graphical genotyping analysis, we could select and identify the preferentially selected regions of the genome in the candidate lines which could be further used in the breeding programs.
5.2 Material and methods

5.2.1 Plant material

A F2/3 population (Fig 3) was developed from a complex cross, using recurrent selection and was used for the graphical genotyping of the scald and powdery mildew resistant genes present in the barley genome. The pedigree of the cross is:

\textit{DF168 (male sterile line) / Skiff // 5*WABAR2096 / 3 / Vlamingh / WABAR2147}

(Chapter 4, Figure 4.1)

Table 5.1: Expected genotype frequencies based on the pedigree of the population used for graphical genotyping for scald and powdery mildew resistances.

<table>
<thead>
<tr>
<th>Parents in the pedigree</th>
<th>Expected Frequencies (%) at F2 population, used for this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>DF168</td>
<td>0.8</td>
</tr>
<tr>
<td>Skiff</td>
<td>0.8</td>
</tr>
<tr>
<td>WABAR2096</td>
<td>48.4</td>
</tr>
<tr>
<td>Vlamingh</td>
<td>25</td>
</tr>
<tr>
<td>WABAR2147</td>
<td>25</td>
</tr>
</tbody>
</table>

From the pedigree, it could be concluded that after five backcrosses to WABAR2096, the recipient population crossed to the moderately scald resistant donor double haploid population, comprising of 50% Vlamingh and 50% WABAR2147, became a fixed line with
96.88% WABAR2096. So the F₂ population used to develop F₂/₃ for this study, was mainly WABAR2096, Vlamingh and WABAR2147 with <2% combined frequency of DF168 and Skiff (Table 5.1).

5.2.2 Year 2006

5.2.2.1 Phenotypic selection (Treatment 1)

The disease nursery was planted and maintained as described in Chapter 4. The phenotypic selections were carried out in the Year 2006 and the selected individuals were planted in Year 2007 according to the method described in Chapter 4.

Figure 5.1: The histogram showing the selected genotypes after the phenotypic selections where the leaf area damage (%) ranged from 0-3.5. The genotypes found to be resistant (circled) on the disease scale with 0 leaf area damage (%) were used for the graphical genotyping of the scald and powdery mildew resistances.
To develop the population for graphical genotyping, 11 F1 plants showing resistance phenotypes for scald and powdery mildew, with disease score and leaf area damage (%) 0 (Fig 5.1) were harvested from the central two rows of male steriles (females) and carried forward to develop F2/3 plants to be used for this study as described in Chapter 4.

5.2.2.2 Genotypic selection (Treatment 2)

Marker assisted selection was carried out in the Year 2006 as described earlier in Chapter 4. Thirteen homozygote resistant male steriles (females) out of 26, predicted to carry all the three genes (Fig 2); *MlaWABAR2147* on chromosome 1H, *RhsFlamingh* and *RhsWABAR2147* on chromosomes 4H and 6H respectively, were selected using SSR markers (Chapter 4, Table 4) and carried forward to develop F2/3 population to be used for graphical genotyping, as described in Chapter 4.

5.2.3 Year 2007

5.2.3.1 DNA extraction and SSR analysis

In Year 2007, green leaf material was collected for extracting DNA (as described in Chapter 4) from 480 F2/3 plants developed from the powdery mildew and scald resistant females; 220 individuals from 11 families, selected using the phenotypic (Treatment 1) and 260 individuals from 13 families, selected using genotypic (treatment 2) selection methods in Year 2006, along with the parents.
Figure 5.2: Schematic presentation of gene transfer from the scald and powdery mildew resistant parents to a susceptible population to get the moderately resistant population with all the resistant genes after crossing. In the figure, resistant genes are represented by: A – powdery mildew resistance gene; *MlaWABAR2147* on chromosome 1H, B and C – scald resistance genes; *RhsVlamingh* and *RhsWABAR2147*, on chromosomes 4H and 6H respectively.

In total, 285 SSR markers (Liu *et al*, 1996 and Ramsay *et al*, 2000, Varshney *et al*, 2007) were tested for polymorphism between the five parents comprising the pedigree of Cross1.
SSR loci were amplified in a 10-μl reaction mixture containing 50 ng of template DNA, 1μl 10x PCR buffer, 0.6μl MgCl₂ (25mM), 0.4μl dNTP (10mM), 0.075μl of each primer (5 pM), 0.09μl Taq DNA polymerase (0.5 units) and injection water to make it upto 10μl. Temperature cycling conditions were used as described by Ramsay et al (2000). Amplified products were separated in 7% acryl-amide gels, running them at 80 volts for 16 hrs. Then they were stained with ethidium bromide and visualized using UV light box and photographs of the amplification product were taken using gel documentation system Protean II xi Cell gel system (BioRad, CA, USA). The sizes of the products were estimated using 100 base pair ladder.

Table 5.2: Details of the SSR markers used for graphical genotyping

<table>
<thead>
<tr>
<th>Markers screened (SSRs)</th>
<th>285 (Polyacrylamide gels)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Markers found polymorphic</td>
<td>115 (over five parents)</td>
</tr>
<tr>
<td>Markers used for multiplexing</td>
<td>72</td>
</tr>
<tr>
<td>Markers used for Graphical genotyping</td>
<td>61</td>
</tr>
</tbody>
</table>

One hundred and fifteen SSR markers out of 285 were found to be polymorphic among five parents. Only one polymorphic marker at a given locus was selected and the other co-
segregating markers were discarded in order to obtain as even spread over the entire barley genome (Table 5.2).

After selecting only one marker out of the cluster at a given locus, 72 markers remained for screening the population using the multiplex–ready PCR procedures (Hayden et al, 2007). The SSR genotyping of the barley breeding population was performed using a Biomek3000 liquid handling robot (Beckman Coulter, Fullerton, CA, USA) to automate PCR setup and prepare samples for electrophoresis. Marker panels comprised of SSRs with non overlapping allelic sizes were designed for multiplex PCR using custom-written software, Binner (http://www.genica.net.au). Multiplex–ready PCR assays and post–PCR pooling of multiplexed assays were performed as described by Hayden et al (2007). For this study multi-pooling of multiplexed assays labeled with different fluorescent dyes allowed 17 SSRs to be simultaneously separated in each ABI capillary. Semi automated SSR allele sizing was performed using GeneMapper v3.7 software (Applied Biosystems). The results of the GeneMapper are shown in the Figure 5.3.
Figure 5.3: Representation of the GeneMapper results showing the differences in the allele sizes for SSR markers. (A) – for the marker hv0020, two genotypes; 2 and 3 were found to be homozygotes for the alleles a and b and the genotype 1 was found to be heterozygote, as exhibited both the alleles a and b, (B) – this graph shows the alleles exhibited by three out of five parents for the marker hv0268, all the three parents contained allele a but were homozygotes for allele b, c and d respectively and (C) – for marker hv0614, the first two parents were found to contain allele b and the third parent had allele a.

5.2.3.2 Genotypic analysis

On the basis of the advice from Prof. D. Falk, Biplot analysis software (Yan and Kang, 2003) was examined for the genotypic and statistical analysis. Due to the complex multi-allelic population under study, generated using five parents, GGT (an acronym of Graphical GenoTypes; http://www.dpw.wau.nl/pv/pub/ggt) software (Van Berloo, 1999) was found to be a more suitable option. Graphical genotyping of both the populations, phenotypically selected (Treatment 1) with 203 individuals and genotypically selected (Treatment 2) with
213 individuals was examined using GGT. The marker data from the GeneMapper were arranged in a format commonly used for genetic mapping. The positions of the SSR markers to be used in this study were determined from the previously published mapping studies.

Table 5.3: Allelic codes assigned to the Parents and their different combinations for graphical genotyping display. Different combinations are based on the five parents; A-WABAR2096, B-WABAR2147, C-Birka, D-Skiff and E-Vlamingh.

<table>
<thead>
<tr>
<th>Allele codes</th>
<th>Parents and Parental combinations</th>
<th>Allele codes</th>
<th>Parents and Parental combinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>WABAR2096</td>
<td>N</td>
<td>E-C</td>
</tr>
<tr>
<td>B</td>
<td>WABAR2147</td>
<td>T</td>
<td>E-D</td>
</tr>
<tr>
<td>C</td>
<td>Birka</td>
<td>M</td>
<td>A-C</td>
</tr>
<tr>
<td>D</td>
<td>Skiff</td>
<td>Q</td>
<td>C-D</td>
</tr>
<tr>
<td>E</td>
<td>Vlamingh</td>
<td>I*</td>
<td>A-C-E</td>
</tr>
<tr>
<td>H</td>
<td>Heterozygote</td>
<td>L</td>
<td>C-D-E</td>
</tr>
<tr>
<td>U</td>
<td>Unidentified</td>
<td>W</td>
<td>A-B-C</td>
</tr>
<tr>
<td>P*</td>
<td>B-A</td>
<td>X</td>
<td>B-C-D</td>
</tr>
<tr>
<td>K</td>
<td>B-C</td>
<td>F</td>
<td>B-C-D-E</td>
</tr>
<tr>
<td>J</td>
<td>B-D</td>
<td>S</td>
<td>A-B-C-D</td>
</tr>
<tr>
<td>R</td>
<td>E-A</td>
<td>V</td>
<td>A-C-D-E</td>
</tr>
</tbody>
</table>

* For example: Allele code “P” represents the locus where it was not possible to differentiate between alleles “B” (WABAR2147) and “A” (WABAR2096) and similarly, allele code “I” represent the combination of alleles “A” (WABAR2096), “C” (Birka) and “E” (Vlamingh).
For the graphical genotype display, the parental alleles and all their combinations at a given marker loci were coded (Table 5.3). The proportion of the recipient and donor genome, which is a main selection criterion, was calculated as a summed map length of donor-containing fragments and recipient-containing fragments divided by the total map length. The approach considers distances between markers in such a way that the chromosomal segment flanked by two markers derived from the donor parent is considered to contain 100% donor DNA across the region, while a chromosomal segment flanked by two markers of recipient type is considered as 100% recurrent parent DNA and the chromosomal segment flanked by one donor and one recipient marker, is considered to be a recombinant, with donor DNA extending approximately half way across the interval and recurrent parent DNA across the other half (Young and Tanksley, 1989). The allele frequency calculated by GGT software was used to identify the associated markers.

As the population under study was not constructed as a mapping population, the classical QTL methods were not applied and instead the association mapping was performed in the structured population to identify the linkage between traits assayed and marker region(s). It was determined with the use of GGT by combining the phenotypic data recorded on scald in year 2007 for the study conducted in Chapter 4 with the marker data obtained as a result of this study. The association analysis for this study was based on the GGT calculated squared correlation coefficients ($R^2$ values) between marker data and the trait value and the associated probabilities of the correlation value. As these probabilities become very small, the -10log values are reported. A value of $-10\log (p) = 3$ thus indicates a correlation probability value of 0.001.
5.2.3.3 Simulation studies

In addition to the simulation studies carried out with Qu-Gene, with the help from Dr S Chapman (see Chapter 4), to find the frequencies of the genotypes in the population after the Top-cross of WABAR2096 with 53 Double haploid lines selected for scald. The simulations were based on the gene-marker associations observed in this study. Two gene-marker distances, 30 cM and 5 cM, were studied for their effect on the frequency of the favourable alleles. The process was repeated for 3 cycles of recurrent selection and then the selection strategies were proposed. During the simulations it was assumed that:

- The population developed as a result of four backcrosses to WABAR2096 (Chapter 4, Figure 4.1), is fixed as WABAR2096.

- No selection was carried out for powdery mildew.

5.3 Results

The genotypes of 480 F$_{23}$ (220 from 11 families selected phenotypically (Treatment 1) and 260 from the 13 families selected genotypically (Treatment 2) were determined at 61 SSR loci, spread over the entire barley genome. Microsatellites used for this study were prefixed Bmac, Bmag, EBmac and EBmag (Ramsay et al., 2000), HVM (Liu et al., 1996), GMS (Struss and Plieske, 1998) and GBM (Varshney et al., 2006). The previously mapped markers covered 917.4 cM of the barley genome. The graphical genotypes for all the individuals examined after both the treatments are shown in the Figure 5.4 and 5.5. This method illustrated the complete genome for each individual and provided easily interpertable information for the comparison of both the treatments.
This study was conducted to assess and interpret the changes in the allele frequencies of 2:1:1 for WABAR2096:WABAR2147:Vlamingh (Table 5.1), after a cycle of recurrent selection for scald and powdery mildew resistance as a result of either phenotypic (Treatment 1) or genotypic selection (Treatment 2).

5.3.1 Genetic characterization of the phenotypically selected (Treatment 1) population

The phenotypically selected population (Treatment 1) of 203 individuals from 11 families found resistant to scald and powdery mildew in year 2006 was found to segregate for resistances after a cycle of recurrent selection. The calculated percentage of the donor genome introgressed as homozygous segments into the base population ranged from 0.1 – 21.6% for WABAR2147 (allele code “B”) and from 0.1 – 21.8% for Vlamingh (allele code “E”). The average length of the donor chromosome fragments were found to be 83.8cM accounting for 9.1% from WABAR2147 and 144.7 cM accounting for 15.8% from Vlamingh. The average length of the recipient (WABAR2096) chromosome fragment (allele code “A”) was found to be 93.9 cM accounting for 10.2%.

Allele frequencies of both the donor parents; WABAR2147 and Vlamingh, in this population, ranged from 0-76.4% and 0-76.8% respectively (Figure 5.4), for each marker on each chromosome.
Chromosome 4H

Chromosome 5H

Chromosome 6H
Figure 5.4: Allele frequencies at the marker loci of 203 F2 plants under study to monitor the allelic changes in response to phenotypic selection (Treatment 1). The allele codes are based on Table 5.3. Along the x-axis are the Graphically Genotyped individuals and along y-axis are the SSR markers used for Graphical Genotyping. The allele frequencies of the major alleles at a given locus are given on the right hand side of the figure.

Marker Bmac213 on the short arm of chromosome 1H had a WABAR2147 donor frequency of 76.4% followed by the marker Bmac316 (6H), AWBMS53 (5H), EBmac603 (7H), GBM1501 (4H), Bmac113 (5H), GBM1220 (4H), HVBAMY (4H), GBM1326 (7H), Bmag829 (2H), Bmac126 (2H), HVM36 (2H), EBmag757 (7H) and Bmac298 (4H) with the allele frequencies of 65%, 52.7%, 50.7%, 50.7%, 48.8%, 41.4%, 41.4%, 35.7%, 35.5%, 35.5%, 29.1%, 22.2% and 20.2% respectively. Highest allele frequency from the donor parent Vlamingh was 76.8%, for the marker EBmag496 on chromosome 6H followed by the marker Bmag606 (3H), Bmag223 (5H), HVCMA (7H), GBM1221 (4H), EBmatc3 (5H), GBM1143 (1H), EBmac755 (7H), EBmac708 (3H), Bmag225 (3H), Bmag120 (7H), GMS1 (5H) and Bmac310 (4H), with the allele frequencies of 66%, 60.1%, 59.1%, 58.7%, 53%, 48.8%, 44.8%, 41.4%, 37.4%, 34%, 29.1% and 23.6% respectively (Figure 5.4).
5.3.2 Genetic characterization of the genotypically selected (Treatment 2) population

The genotypically selected (Treatment 2) population of 213 individuals from 13 families found resistant to scald and powdery mildew in year 2006, was also found to segregate for resistances. The calculated percentage of the donor genome introgressed as homozygous segments into the base population ranged from 0.1 – 23.90% for WABAR2147 (allele code “B”) and from 0.3 – 18.9% for Vlamingh (allele code “E”). The average length of the donor chromosome fragments calculated by GGT software was 95.9cM accounting for 10.5% from WABAR2147 and 129.7cM accounting for 14.1% from Vlamingh. The average length of the recipient (WABAR2096) chromosome fragments (allele code “A”) was found to be 78.4cM accounting for 8.5%.

Allele frequencies of both the donor parents; WABAR2147 and Vlamingh, ranged from 0-95.8% and 0-82.9% respectively, for each marker on each chromosome (Figure 5.5). Marker Bmac213 on the short arm of chromosome 1H had the highest frequency of 95.8% followed by Bmac316 (6H) with a WABAR2147 donor frequency of 68.1% followed by GBM1326 (7H) and GBM1220 (4H) with frequency of 65.3% each, Bmag829 (2H), Bmac126 (2H), HVBAMY (4H), HVM36 (2H), AWBMS53 (5H), Bmac113 (5H) EBmac755 (7H), Bmac613 (6H), Bmag173 (6H) and GBM1509 (4H), with the allele frequencies of 61.1%, 53.7%, 53.2%, 50.0%, 49.5%, 44.4%, 43.5%, 28.7%, 26.9% and 23.1% respectively. Highest allele frequency from the donor parent Vlamingh was 82.9%, for the marker Bmag225 on chromosome 3H followed by the marker, EBmac496 (6H), Bmag606 (3H), GMS1 (5H), GBM1221 (4H), Bmag120 (7H), GBM1143 (1H),
EBmac708 (3H), Bmag223 (5H) and EBmate3 (5H) with the allele frequencies of 63.0%, 62.5%, 58.8%, 57.4%, 56.9%, 48.6%, 46.3%, 45.8% and 28.7% respectively (Figure 5.5).
Figure 5.5: Allele frequencies at the marker loci of 213 F2 plants under study to monitor the allelic changes in response to genotypic selection (Treatment 2). The allele codes are based on Table 5.3. Along the x-axis are the Graphically Genotyped individuals and along y-axis are the SSR markers used for Graphical Genotyping. The allele frequencies of the major alleles at a given locus are given on the right hand side of the figure.

5.3.3 Categorizing the alleles according to identity by descent

Apart from identifying and characterizing alleles “B” and “E”; introgressions independently from the donor parent; WABAR2147 and Vlamingh, respectively, there were few other alleles which were considered important while graphical genotyping of this population. During the genotypic selection in year 2006, it was difficult to differentiate between the donor parents, WABAR2147 and Vlamingh from parent Skiff (Chapter 4, Table 4.4), using SSR markers and this resulted in the selection of the genotypes sharing the locus with Skiff.
The lack of enough data to identify the chromosomal segments by state, some of the alleles from this study (Table 5.3) can be categorized for WABAR2096, WABAR2147 and Vlamingh according to identity by descent. Considering the higher expected frequencies of WABAR2096 (allele “A”), WABAR2147 (allele “B”) and Vlamingh (allele “E”) in the population, it can be concluded which allele and the associated chromosomal fragments comes from which of these three parents (Table 5.4).

Table 5.4: Categorizing the alleles into parental alleles according to identity by descent. Allele codes are based on Table 5.3.

<table>
<thead>
<tr>
<th>Allele Codes</th>
<th>Identity by Descent (Parental allele)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M = A-C</td>
<td>A</td>
</tr>
<tr>
<td>K = B-C</td>
<td>B</td>
</tr>
<tr>
<td>J = B-D</td>
<td>B</td>
</tr>
<tr>
<td>X = B-C-D</td>
<td>B</td>
</tr>
<tr>
<td>N = E-C</td>
<td>E</td>
</tr>
<tr>
<td>T = E-D</td>
<td>E</td>
</tr>
<tr>
<td>L = C-D-E</td>
<td>E</td>
</tr>
</tbody>
</table>

For the phenotypically selected (Treatment 1) population, alleles “M”, “K”, “J”, “X”, “N” “T” and “L” were found to cover 13.1 cM (1.4%), 3.5 cM (0.4%), 34.3 cM (3.7%), 21 cM (2.3%), 2 cM (0.2%), 12.2 cM (1.3%) and 17.8 cM (1.9%) of the genome, respectively. For the genotypically selected (Treatment 2) population, alleles “M”, “K”, “J”, “X”, “N” “T”
and “L” were found to cover 24.2 cM (2.6%), 7 cM (0.8%), 23.9 cM (1.9%), 17.6 cM (1.9%), 1.4 cM (0.1%), 12.3 cM (1.3%) and 18.6 cM (2%) of the genome, respectively. Cumulative parental allele frequencies were calculated taking into account the parental allele itself along with the alleles that fall in the same category by identity by descent (Table 5.4).

Table 5.5: Cumulative allele frequencies of all the alleles present in the graphically genotyped population for scald and powdery mildew resistances. For allele codes please see Table 5.3.

<table>
<thead>
<tr>
<th>Alleles in the population</th>
<th>Allele frequencies (%)</th>
<th>Phenotypic Selection (Treatment 1)</th>
<th>Genotypic selection (Treatment 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WABAR2096 A+M</td>
<td></td>
<td>15.1</td>
<td>13.4</td>
</tr>
<tr>
<td>WABAR2147 B+K+J+X</td>
<td></td>
<td>20.2</td>
<td>19.4</td>
</tr>
<tr>
<td>Vlamingh E+N+T+L</td>
<td></td>
<td>23.6</td>
<td>22.2</td>
</tr>
<tr>
<td>DF168 (Birka) C</td>
<td></td>
<td>2.4</td>
<td>3.8</td>
</tr>
<tr>
<td>Skiff D</td>
<td></td>
<td>3.6</td>
<td>3.6</td>
</tr>
<tr>
<td>Undifferentiated Recipient + Donor genome (A-B or A-E)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F+I+P+R+S+W+V+ Heterozygote genome* (H)</td>
<td></td>
<td>31.5</td>
<td>34.3</td>
</tr>
<tr>
<td>Unidentified genome U</td>
<td></td>
<td>3.5</td>
<td>3.3</td>
</tr>
</tbody>
</table>
It was found that 43.8% of the barley genome selected phenotypically (Treatment 1) and 41.6% of the barley genome selected genotypically (Treatment 2), was inherited from the donor parents Vlamingh and WABAR2147, while 31.5% and 34.3% of the genome from phenotypic selection (Treatment 1) and genotypic selection (Treatment 2), remained undifferentiated between the recipient and the donor parents (Table 5.5).

5.3.4 Association analysis
Due to the absence of the required epidemic of powdery mildew in year 2007, sufficient phenotypic data was not collected on the disease. So the marker-trait associations and simulation studies were not carried out for powdery mildew. Association analysis for powdery mildew was only based on the marker data from graphical genotyping.

Marker data from graphical genotyping was combined with the phenotypic data recorded for scald resistance (see Chapter 4), in order to determine the marker–trait associations. The plot display along the chromosomes generated by GGT with thresholds p-value of 0.05 identified the markers associated with scald resistance.

For both the populations under study, SSR markers Bmac213 on the short arm of chromosome 1H (circled yellow in Figure 5.6) and Bmac316 on the short arm of chromosome 6H (circled blue in Figure 5.6 (A) and yellow in Figure 5.6 (B)) were found to be associated with scald resistance. Scald resistance on chromosome 4H was found to be
Figure 5.6: A graphical display of the overview association plots along the chromosome bars developed using GGT. Blue and yellow circled bars show positive association between the marker and scald resistance with threshold p-value of 0.05. (A) Marker-trait association for phenotypically selected (Treatment 1) population. Yellow circled bar is for marker Bmac213 (1H) and blue circled bars are markers GBM1501 (4H) and Bmac316 (6H), similarly, (B) Marker-trait association for genotypically (Treatment 2) selected population. Yellow circled bars are for marker Bmac213 (1H) and Bmac316 (6H) and blue circled bars are markers Bmac310 (4H) and GMS89 (4H). Only chromosomes 1H, 4H and 6H have been considered.
associated with marker GBM1501 for phenotypically selected (Treatment 1) population but markers Bmac298 and GMS89 were found to be associated with scald resistance for genotypically selected (Treatment 2) population (Figure 5.6). Similar results were interpreting from the allele frequency data, calculated using GGT software, for chromosomes 1H and 6H (Figure 5.4 and Figure 5.5). For both the populations, phenotypic selection (Treatment 1) and genotypic selection (Treatment 2), associations were found between allele “B” (WABAR2147) and markers Bmac213 (1H) and Bmac316 (6H). On chromosome 4H, according to the allele frequencies, allele “E” (Vlamingh) was found to be associated with GBM1221 for both the populations. Very weak associations were found with markers Bmac310 (allele frequency – 23.6%) and GMS89 (allele frequency – 17.2%) only in the phenotypically selected (Treatment 1) population.

5.3.5 Selection of the resistant genotypes in phenotypically selected (Treatment 1) and genotypically selected (Treatment 2) populations

Based on the marker-disease association (Figure 5.6) and the results from the allele frequencies (Figure 5.4 and Figure 5.5), as discussed in the previous section, three associated markers were selected to monitor the response to selection against scald and powdery mildew. SSR marker Bmac213 (MlaWABAR2147), GBM1221 (RhsVlamingh) and Bmac316 (RhsWABAR2147) on chromosomes 1H, 4H and 6H respectively were selected to identify the genotypes with required introgressed regions with all the three resistances.

The three selected markers were designated as A, B and C respectively and based on the genotypic data scored and interpreted from Multiplex-ready PCR assays, the genotypes
were identified. The three genes and their respective alleles are shown in Figure 5.7. Punnet-squares (Figure 5.8) were used to identify the different genotypes in both the populations of Cross1. Based on Figure 5.8, the genotypic frequencies were calculated (Table 5.6).

![Diagram of gamete formation](image)

<table>
<thead>
<tr>
<th>1H</th>
<th>4H</th>
<th>6H</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
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<td>B</td>
<td>C</td>
<td>ABC</td>
</tr>
<tr>
<td>a</td>
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<td>e</td>
<td>AbC</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>e</td>
<td>Abc</td>
</tr>
<tr>
<td>B</td>
<td>C</td>
<td>e</td>
<td>aBC</td>
</tr>
<tr>
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<td>b</td>
<td>abC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>e</td>
<td>abc</td>
</tr>
</tbody>
</table>

Bmac213 (A) GBM1221 (B) Bmac316 (C) 8 gametes

Figure 5.7: Forked-line method showing the formation of gametes for markers (A) Bmac213 (*Mla*<sub>WABAR2147</sub>), (B) GBM1221 (*Rhs*<sub>Vlamingh</sub>) and (C) Bmac316 (*Rhs*<sub>WABAR2147</sub>).
<table>
<thead>
<tr>
<th></th>
<th>ABC</th>
<th>ABc</th>
<th>AbC</th>
<th>Abc</th>
<th>aBC</th>
<th>aBc</th>
<th>abC</th>
<th>abc</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
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<td>AABBCc</td>
<td>AABbCc</td>
<td>AABbCc</td>
<td>AaBBCC</td>
<td>AaBBCc</td>
<td>AaBbCC</td>
<td>AaBbCc</td>
</tr>
<tr>
<td>ABc</td>
<td>AABBCc</td>
<td>AABBCc</td>
<td>AABbCc</td>
<td>AABbCc</td>
<td>AaBBcc</td>
<td>AaBBCc</td>
<td>AaBbCc</td>
<td>AaBbCc</td>
</tr>
<tr>
<td>AbC</td>
<td>AABbCC</td>
<td>AABbCc</td>
<td>AAbbCC</td>
<td>AAbbCc</td>
<td>AabCC</td>
<td>AabCc</td>
<td>AabbCC</td>
<td>AabbCc</td>
</tr>
<tr>
<td>Abc</td>
<td>AABbCc</td>
<td>AABbCc</td>
<td>AAbbCc</td>
<td>AAbbCc</td>
<td>AabCC</td>
<td>AabCc</td>
<td>AabbCC</td>
<td>AabbCc</td>
</tr>
<tr>
<td>aBC</td>
<td>AaBBCC</td>
<td>AaBBCC</td>
<td>AaBbCC</td>
<td>AaBbCc</td>
<td>aaBCC</td>
<td>aaBBCc</td>
<td>aaBbCC</td>
<td>aaBbCc</td>
</tr>
<tr>
<td>aBc</td>
<td>AaBBCC</td>
<td>AaBBcc</td>
<td>AAbbCc</td>
<td>AAbbCc</td>
<td>aaBBCc</td>
<td>aaBbCc</td>
<td>aaBbCc</td>
<td>aaBbCc</td>
</tr>
<tr>
<td>abC</td>
<td>AaBbCC</td>
<td>AaBbCc</td>
<td>Aabbc</td>
<td>Aabbc</td>
<td>aaBbCC</td>
<td>aaBbCc</td>
<td>aaBbCc</td>
<td>aaBbCc</td>
</tr>
<tr>
<td>abc</td>
<td>AaBbCc</td>
<td>AaBbCc</td>
<td>Aabbc</td>
<td>Aabbc</td>
<td>aaBbCc</td>
<td>aaBbCc</td>
<td>aaBbCc</td>
<td>aaBbCc</td>
</tr>
</tbody>
</table>

(A)
Figure 5.8: Punnet-square presentation of all the possible 64 genotypes which can be developed from the 8 gametes (Figure 5.7). Each colour represents a genotype present in (A) phenotypically selected (Treatment 1) and (B) genotypically selected (Treatment 2) populations. The numbers in the boxes along the genotypes represent the number the genotypes in the population.
Table 5.6: The genotypes with their genotypic frequencies for both phenotypically selected (Treatment 1) and genotypically selected (Treatment 2) populations, based on Figure 5.8.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Genotypic frequencies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotypically selected population (Treatment 1)</td>
<td>Genotypically selected population (Treatment 2)</td>
</tr>
<tr>
<td>AABBCC/AaBBCC/AABBCC/AAbbCC/AAbbCc/AaBbCC/AaBbCc</td>
<td>24.4</td>
</tr>
<tr>
<td>$Mla_{WABAR2147}$-$Rhs_{Vlamingh}$-$Rhs_{WABAR2147}$ (All three resistant genes)</td>
<td></td>
</tr>
<tr>
<td>AABBcc/AaBBcc/AaBbCc</td>
<td>12.9</td>
</tr>
<tr>
<td>$Mla_{WABAR2147}$-$Rhs_{Vlamingh}$</td>
<td></td>
</tr>
<tr>
<td>AAbbcc/AaBbcc/AbBbCc</td>
<td>19.4</td>
</tr>
<tr>
<td>aaBbCc/aaBbCc/AabBbCc</td>
<td>11.9</td>
</tr>
<tr>
<td>$Rhs_{Vlamingh}$-$Rhs_{WABAR2147}$</td>
<td></td>
</tr>
<tr>
<td>AAbbcc/AaBbCc</td>
<td>9.4</td>
</tr>
<tr>
<td>$Mla_{WABAR2147}$</td>
<td></td>
</tr>
<tr>
<td>aaBbCc/aaBbCc</td>
<td>3.9</td>
</tr>
<tr>
<td>$Rhs_{Vlamingh}$</td>
<td></td>
</tr>
<tr>
<td>aabBCC/aabBbCc</td>
<td>12.4</td>
</tr>
<tr>
<td>$Rhs_{WABAR2147}$</td>
<td></td>
</tr>
<tr>
<td>aabbcc</td>
<td>5.5</td>
</tr>
<tr>
<td>(no resistant genes present)</td>
<td></td>
</tr>
</tbody>
</table>

The desirable genotypes, carrying all the three resistant genes (one powdery mildew; $Mla_{WABAR2147}$ and two scald; $Rhs_{Vlamingh}$ and $Rhs_{WABAR2147}$), accounted for 24.4% and 43.3% of the total populations selected phenotypically (Treatment 1) and genotypically (Treatment 2), respectively. Thus genotypic selection (Treatment 2) method exhibited a
higher percentage of desirable genotypes in the population as compared to phenotypic selection (Treatment 1) method.

Similarly the genotypes exhibiting the powdery mildew resistance on chromosome 1H and a scald resistance on chromosome 4H, were found to be 12.9% and 14.4% of the phenotypically selected (Treatment 1) and genotypically selected (Treatment 2) populations respectively. Powdery mildew resistance (1H) and scald resistance on chromosome 6H was found to be 19.4% (phenotypically selected population) and 27.4% (genotypically selected population) of the genotypes. The genotypes with only powdery mildew resistant gene on chromosome 1H were found to be 9.4% and 13.9% respectively for Treatment1 and Treatment 2 populations (Table 5.6).

5.3.6 Simulation studies
The simulations showed that the recipient parent WABAR2096 persisted at the frequency of 50%, as expected (Table 5.1) and so the Top-cross with WABAR2096 reduced the scald resistance contributed by the donor parents, Vlamingh and WABAR2147, to about half because WABAR2096 contributed negative alleles for scald and powdery mildew resistances.

The simulation was carried out for three markers found associated from the current study, with the three genes under study, Bmac213 associated with $Mla_{WABAR2147}$ on chromosome 1H, GBM1221 associated with $Rhs_{Vlamingh}$ on chromosome 4H and Bmac316 associated
with $Rhs_{WABAR2147}$ on chromosome 6H. Simulations were run assuming the markers at 30 cM and 5 cM from the genes of interest. It was assumed that no selection was carried out for powdery mildew gene on chromosome 1H in year 2007, so that the major focus of the simulations was on the scald resistant genes $Rhs_{Vlamingh}$ and $Rhs_{WABAR2147}$.

Two selection strategies were to select for scald and powdery mildew phenotypically and to screen for two major genes of scald resistance (GBM1221 with favourable allele from Vlamingh and Bmac316 with favourable allele from WABAR2147). The simulations carried out for 3 cycles of recurrent selection showed that the scald scores increase most quickly over cycles for the phenotypic selection compared with the genotypic selection with markers at 30cM or 5cM from the gene for resistance. It was observed that the marker-resistant gene distances did not affect the favourable alleles for the markers GBM1221 and Bmac316, which were fixed by second cycle of recurrent selection, on both the chromosomes 4H and 6H.

It was observed that fixing the two markers not always related to the fixing of the two genes. Figure 5.9(A) and (B) and Table 5.7 show the frequencies of the alleles for markers and genes at 30cM and 5cM distances respectively. Each marker/gene has 4 bars for cycle 0, 1, 2 and 3 of phenotypic selection, and to the right 4 bars for genotypic selection. It was observed from the simulation studies that genotypic selection for GBM1221 was effective in increasing the frequency of the Vlamingh allele from 40 to 100% after 2 cycles of selection. However, because of the 30cM distance between the marker and the gene, the
Figure 5.9 The frequencies of the genes and the associated markers at (A) 30 cM and (B) 5 cM distances. Four bars for each gene/marker represent 0, 1, 2, 3 cycles of selection. Left hand set of four bars for each gene/marker represents the frequencies after phenotypic selection for scald and the right hand set of bars represents the frequencies after genotypic selection for scald. Where: scald-VLM – \textit{Rhs}_{Vlamingsh} and scald-2147 – \textit{Rhs}_{WABAR2147}. It was assumed that no selections were carried out for \textit{Mla}_{WABAR2147} gene on chromosome 1H.
Table 5.7: The frequencies of the scald resistant genes and their associated markers at 30 cM and 5 cM distances, for 0, 1, 2, 3 cycles of selection. The table is based on the frequencies from Figure 5.10. Where: PS – phenotypic selection (Treatment 1) and GS – genotypic selection (Treatment 2).

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Frequency of the favourable allele (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Marker-gene distance = 30 cM</td>
</tr>
<tr>
<td></td>
<td>GBM1221</td>
</tr>
<tr>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>1</td>
<td>49</td>
</tr>
<tr>
<td>2</td>
<td>53</td>
</tr>
<tr>
<td>3</td>
<td>53</td>
</tr>
</tbody>
</table>
allele frequency of Vlamingh only increased from 50 to 68% for the gene $Rhs_{\text{Vlamingh}}$ itself. In contrast, phenotypic selection increased the frequency of the Vlamingh allele to 99% in three cycles at the gene locus (Figure 5.9(A), Table 5.7).

Similar simulation results were observed for marker Bmac316 and $Rhs_{\text{WABAR2147}}$ gene, where the favourable allele is contributed by WABAR2147, while comparing the phenotypic selection and genotypic selection. The marker Bmac316 was found to increase the frequency WABAR2147 allele from 39% to 99%, only after one cycle, though the gene was found to be only 78% fixed (Figure 5.9(A), Table 5.7).

As expected, a greater success rate was seen when the markers were at 5cM from the gene under study but the pattern was the same. Again, the phenotypic selection completely fixed the gene in two cycles of selection and was found to be quite effective even over only one cycle of selection. genotypic selection fixed the known QTL within two cycles of selection but only increased the allele frequency of the associated gene to about 80%, which is due to the marker distances and the lack of selection for the minor QTL (Figure 5.9(B), Table 5.7).

Considering that the scald resistance is contributed by Vlamingh and WABAR2147, simulating the different allele combinations in the Double haploid population and then selecting the favourable alleles with phenotypic selection (Treatment 1) and genotypic selection (Treatment 2), it was found that phenotypic selection method selected the desirable genotype, 12 (with one allele from Vlamingh and one from WABAR2147), along
with selecting the minor gene effects while the genotypic selection method only selected for the desired genotype, 12, thus fixing the genes (Figure 5.10).

Figure 5.10 Histogram of genotype frequencies for three simulations, 1 – original set of 235 DH lines, 2 – 53 DH lines selected phenotypically (Treatment 1) and 3 – 53 DH lines selected genotypically (Treatment 2). Where genotype 11 - both the alleles from Vlamingh, genotype 12 and 21 - one allele from Vlamingh and one from WABAR2147 and genotype 22 - both the alleles from WABAR2147.

5.4 Discussion

For this study, the F$_{23}$ population was used with the elite malting quality line WABAR2147 and variety Vlamingh as the scald and powdery mildew resistances donor parents. The results demonstrated the utility of using graphical genotyping for rapid and concise
assessment of data for large numbers of markers for individuals in a population (Young and Tanksley, 1989, see also Figure 5.4 and Figure 5.5). For this study, this method has been expanded to include whole genome comparisons of two; phenotypically selected (Treatment 1) and genotypically selected (Treatment 2) segregating populations. Graphical genotyping enabled putative associations between genome segments and scald and powdery mildew resistances to be identified. This data can be used to validate the results from the QTL mapping studies or appropriate crosses can be designed to resolve instances where the QTL linked to the resistance gene is suspected (Severson and Kassner, 1995).

In this study, 61 markers were used to graphical genotyping the barley genome covering 917.4 cM. Thus, the shorter introgression regions on some chromosomes are more likely to be due to the larger number of markers used to genotype that chromosome and similarly larger introgressed segments indicated the need of more markers in those regions to get a clear picture of the genome. The main selection criterion for this study was the introgressed donor genome, so as to select for the associated regions. It was found to be 43.8% for phenotypically selected (Treatment 1) and 41.6% for the genotypically selected (Treatment 2) population (Table 5.5). Since the origin of the genome in between the measured marker points is essentially unknown, the estimation errors can happen in the maps of insufficient density. It was assumed that all the crossovers were located midway of the marker locations (van Berloo, 1999, 2001).

Graphical genotyping provides the allelic profiles and the shifts in alleles provides the basis for tracking introgressed donor fragments in the population after selection, helping the
identification of the regions of the genome that have been preferentially selected for. The accuracy, precision and ability to detect alleles, is a key to any efficient whole genome approach to marker assisted selection. The computer program GGT used in this study enables the representation of molecular marker data by simple chromosome drawings differentiating alleles through specific colours. It was noted that it can be difficult to interpret the data as the displays were affected by the number of alleles at the neighbouring SSR loci. These complications would not be there if it could be easy to differentiate between each allele.

Due to the presence of five different parents in the pedigree of the population used for graphical genotyping, large number of colours in Figures 5.4 and 5.5 are the results of lack of discrimination of allelic variation. Bin-mapping could be one of the alternatives where map regions should be considered instead of genetic positions of the markers to be used. Floating bins with a 10 cM span would give the allelic status of the locus or particular region of interest and it would be much easier to interpret the information as compared to GGT where lots of genetic information is hard to interpret due to allele combinations in this case.

5.4.1 Association analysis

 Associations between marker and scald and powdery mildew resistances were examined in two different ways; firstly, associating the markers found to have the higher percentage of the donor parent’s alleles (for both scald and powdery mildew) and secondly, by combining
the phenotypic data to the marker data to identify the marker-trait associations, using GGT (only for scald).

During genotypic selection in year 2006, the markers found associated with resistant genes; \( Mla_{WABAR2147} \) (1H), \( Rhs_{Vlamingh} \) (4H) and \( Rhs_{WABAR2147} \) (6H), were Bmac213, GBM1501 and EBmac874 (Chapter 4, Table 4.4) respectively. The marker-phenotypic association studies carried out in this chapter on chromosome 6H utilized more markers for genotyping resulted in the improved marker association reported in Figures 5.4, Figure 5.5 and Figure 5.6. Bmac316 was found to provide alleles that could be use to track scald resistance, namely, “B” (WABAR2147), with frequencies of 65% and 68.1% for phenotypically (Treatment 1) and genotypically (Treatment 2) selected populations respectively. So for the simulation studies and for finding the genotypic frequencies in the population, SSR marker GBM1221 was selected to be associated with the scald resistance on chromosome 4H based on the favourable allele, “E” (Vlamingh), frequencies of 58.7% and 57.4% for phenotypically (Treatment 1) and genotypically (Treatment 2) selected populations respectively (Figure 5.4 and Figure 5.5).

The powdery mildew resistant gene, \( Mla_{WABAR2147} \) (1H), found to be associated with the SSR marker Bmac213 for both the populations, coincide with the previously mapped powdery mildew resistant Mla locus (Jahoor et al, 1990; Jorgensen, 1994; 2003, Yun et al, 2005, Korff et al, 2005 and Li et al, 2006 pers. com.). Scald resistance contributed by WABAR2147 (\( Rhs_{WABAR2147} \)) on the short arm of chromosome 6H was found to be associated with the SSR marker Bmac316, which is similar to the previous study (Li et al,
2006 pers. com.). The SSR marker GBM1221 and GBM1501, associated with the scald resistant donor segment from Vlamingh \((Rhs_{vlamingh})\), has also been mapped for the scald resistant gene on chromosome 4H in a Vlamingh x WABAR2147 double haploid population (Li et al, 2006 pers. com).

The scald resistant regions of the genome contributed by the donor parents Vlamingh and WABAR2147, in the genotyping of both the populations are similar to several previously described leaf scald resistance loci. Scald resistance QTL have been localized on all the chromosomes; except chromosome 5H in studies of several barley mapping populations, mostly with the help of PCR based markers (Backes et al, 1995; Abbott et al, 1995; Spaner et al, 1998; Garvin et al, 2000; William et al, 2001; Gronnerod et al, 2002; Jensen et al, 2002; Genger et al, 2003; Sayed et al, 2004; Korff et al, 2005 and Yun et al, 2005).

The scald resistance associated with Bmac213, near \(Hor1\) and \(Hor2\) protein regions, on the short arm of chromosome 1H has been reported in previous studies (Garvin et al, 2000; Genger et al, 2003 and Yun et al, 2005), but no QTL for scald resistance was identified on chromosome 1H while mapping the DH population of WABAR2147 x Vlamingh (Chengdao Li, pers. com.). For the marker–trait associations, the phenotypic data collected only for scald was used in GGT, the association exhibited between the marker Bmac213 and scald on chromosome 1H, for both the populations (Figure 5.6) indicates the interaction with either suppressed associated with super-susceptibility in WABAR2096 background or the presence of the minor scald resistant gene from WABAR2147 worth selecting phenotypically or genotypically.
Along with validating the chromosomal locations of the scald and powdery mildew resistant genes, the association study (Figure 5.4 and Figure 5.5) also demonstrated the introgression of the disease resistant fragments on chromosome 5H. But considering the complex population structure, the marker–trait associations found from this study, alone is not sufficient evidence for genetic relationships. However, these data can be used to corroborate results from QTL mapping studies or can be used to assist in establishing QTL mapping populations. This strategy may prove particularly useful for designing appropriate crosses to resolve instances where linked QTL are suspected and to monitor the unlinked parts of the genome.

5.4.2 Selecting for scald and powdery mildew

Another purpose of the study was also to identify the genotypes carrying all the three resistant genes; \( Mla_{\text{WABAR2147}} \) (1H), \( Rhs_{\text{Vlamingh}} \) (4H) and \( Rhs_{\text{WABAR2147}} \) (6H), to be used in further breeding programs.

Genotypic selection (Treatment 1) performed very efficiently as compared to phenotypic selection (Treatment 2), in selecting the desired genotypes, 43.3% and 24.4% respectively, carrying alleles from the donor parents, Vlamingh and WABAR2147, thus carrying all the three resistant genes (Table 5.6). The genotypes selected through both the selection methods containing all the three resistant genes will be able to extend the effectiveness of resistance to powdery mildew and scald. These can be further tested for yield and quality attributes or can be used as scald and powdery mildew resistant lines which can act as the
recipients for more disease resistant genes. These resistant genotypes can be used in the breeding programs focused on pyramiding the disease resistance in one line or cultivar.

With phenotypic selection (Treatment 1), it was possible to identify the genotypes with all the possible combinations of genes, although the genotypic response was found to be higher than the phenotypic response because of the selection of the desirable genotype. The results of this study align with the results of the simulation (Figure 5.10, indicating the fact that genotypic selections (Treatment 2) tend to fix the desirable genes per se and the phenotypic selection (Treatment 1) shifts the population in the desired direction while maintaining the variability in the population at the same time with the selection of the minor QTL along with the major genes.

As found from the simulations, the success of the genotypic selection method (Treatment 2) depends upon the distance of the associated marker from the gene of interest, the distance determines the number of cycles the selection method would take to fix that gene. The simulations studies provided us with the favourable allele frequencies at the associated marker loci, when the marker is 30 cM or 5 cM away from the gene of interest (Table 5.8). For simulations, it was assumed that no selection was carried out for powdery mildew resistance but it is clear from the Table 5.8 that the marker Bmac213 was closely associated with the *MlaWABAR2147* gene on chromosome 1H, thus resulted in the selection of 95.8% of the favourable allele (Figure 5.5).
Table 5.8: The frequencies of the favourable alleles after one cycle of selection, observed in the present study and the one simulated using Qu-Gene have been compared. This table is based on Figure 5.4, Figure 5.5 and Table 5.7.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Favourable allele frequencies (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Observed</td>
<td>Simulation</td>
<td>At 30cM</td>
<td>At 5cM</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phenotypic selection (Treatment 1)</td>
<td>Genotypic selection (Treatment 2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bmac213</td>
<td>76.4</td>
<td>95.8</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GBM1221</td>
<td>58.7</td>
<td>57.4</td>
<td>75</td>
<td>75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bmac316</td>
<td>65.0</td>
<td>68.1</td>
<td>99</td>
<td>98</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The difference in the observed frequencies and the simulated frequencies is due to the complex population structure under study and the inability to differentiate between the 31.5% and 34.3% of the barley genome with phenotypic selection (Treatment 1) and genotypic selection (Treatment 2) respectively (Table 5.5). It is interesting to find that the marker–gene distance did not affect the frequencies of the favourable alleles in the simulations.

Studying the simulation results on the allele frequencies at 0, 1, 2, and three cycles of selection (Table 5.7), it was found that although the markers select upto 100% of the desired alleles only after two cycles of selection, they fixed the associated genes only upto 80%, no matter if the marker is 30cM or 5cM away from the gene of interest. On the other hand the phenotypic selection (Treatment 1) method targets the resistant gene more efficiently (Table 5.7).
The difference in selecting for the desired alleles and fixation of the desired gene with the linked markers, as indicated by simulation studies, provides direction for future selection strategies. It became clear from this study and the results of the simulations that phenotypic selection (Treatment 1) is the method to use while selecting for quantitative traits as it takes care of the minor gene effects while selecting for the major ones. The genotypic selection (Treatment 2) is efficient in selecting for the desired genotypes but may not give the desired result in terms of phenotype when a complex trait is involved (see Table 5.7).
CHAPTER 6

General Discussion

Quantitative traits such as yield, quality or resistance to biotic and abiotic stresses are of great interest in plant breeding. Multiple genes governing these traits are affected by the environment and although selection methods have been developed and implemented to improve these quantitative traits, the methods can be time consuming. Molecular genetic markers can be used to select the individual alleles of genes of interest and also at the whole genome level to monitor genotypic changes in response to selection for specific traits. To improve the barley quality and to develop varieties tolerant to biotic and abiotic stresses, plant breeders are focusing more and more on the genotypic selection along with the phenotypic selection, so as to use the combination of both for higher gains from selection. As barley is a self-pollinated crop, recurrent selection is the approach for selecting, generating recombination and increasing genetic variability in the population. The challenge for the breeders is how to breed self-pollinated crops to get the better handle on the genotypes behind the desirable phenotypes selected using recurrent selection.

The aim of this thesis was to characterize the phenotypic selection and genotypic selection methods in selection for aluminium tolerance and scald and powdery mildew resistance in male sterile facilitated recurrent selection (MSFRS) populations and to track the changes
using Graphical Genotyping of the population under study. A traditional focus of plant breeding program is on phenotypic selection for the traits under study. The genome approach to genetic analysis provides innovative insights into the mechanisms behind the success of the phenotypic selections in male sterile recurrent selection programs. This information can help us in developing strategies which combine the genotypic and phenotypic selections to further improve the recurrent selection thereby increasing the rate of genetic gain along with improving the efficiency of selection process.

Male sterile facilitated recurrent selection (MSFRS) is a well established breeding method. MSFRS has increased efficiency and efficacy of the breeding programs over conventional methods of breeding to reduce the variety release time to almost half while maintaining continued out crossing and recombination thus broadening the germplasm base. In the application employed in this thesis, it aids in population development with pyramiding genes for biotic and abiotic stresses into elite malting quality population.

As genetic male sterility was used as a facilitator in making complex crosses used for this study, the first component of the research was to develop a marker system for improving the efficiency of utilizing the \textit{msg6-rob1-sex1} linkage block and implementing MSFRS system. The markers HVM65, HVM74 Bmac18 and Bmgtttttt1 (Section 2.4), were found to be strongly associated with the male sterile genetic gene, \textit{msg6}, on the short arm of chromosome 6H. Along with the SSR markers, two morphological marker; orange lemma (\textit{rob1}) and shrunken endosperm xenia (\textit{sex1}), are closely linked (< 1% recombination) to the male sterile gene, \textit{msg6} (Falk 1980). Together they comprise a linkage group which on either side of the centromeric region of chromosome 6H. The SSR markers will help the
breeders in differentiating fully fertile homozygous plump individuals from fully fertile heterozygous plump individuals in F₂ populations. The tightly linked markers will also facilitate in setting up the crossing blocks, by selecting the male sterile seed, with orange lemma and shrunken endosperm, to be used as females. This avoids the need of culling heterozygotes from the female rows in the crossing block, before anthesis, to avoid any contamination. The markers for the linkage block could be successfully used across a broad range of male sterile facilitated recurrent selection populations with different genetic backgrounds to select parental material for the production of DH, SSD and conventional breeding programs which will not segregate for msg6.

Using genetic male sterility, selections can be carried out for both male fertile and male sterile plants in the crossing block, before anthesis, letting only the selected plants to randomly cross. Although male sterility aids in the production of large number of crosses as compared to hand crossing, differences in the maturity rate between the male fertile plants and male sterile plants in the crossing block can lead to low seed set. In this thesis hand crossing was also carried out during this study to supplement natural out crossing of male sterile plants and to get the required number of seeds/male sterile plant.

Within the MSFRS process, it was found that the SSR markers are an advantage and were shown to be very effective when a major gene controls the phenotype of interest. Aluminium tolerance is controlled by a single gene, Alp or Alt, mapped on the long arm of chromosome 4H (Minella and Sorrells, 1997; Tang et al, 2000; Raman et al, 2001; 2002; 2003; Wang et al, 2006; 2007). The high efficiency of genotypic selection method (Treatment 2) in selecting for aluminium tolerance (Section 3.3.2.2), could be attributed the
fact that the marker HVM68, used in the early selection of aluminium tolerant individuals in the population (Section 2.3.1.2), was found to be closely associated with the aluminium tolerant gene, Alt or Alp, on chromosome 4HL (Raman et al, 2002, 2003; Wang et al, 2006).

When in retrospect, the phenotype is complex, it was proven that the markers can be misleading (Section 5.3.6) and should be used with caution because the additional genetic factors that affect the genotype can be significant (Figure 6.1). The simulation studies provided clear evidence that for a complex phenotyping such as scald resistance, minor genes had to be included in the model to account for the phenotypic selection (Treatment 1). The results from Chapter 4, Chapter 5 and the simulations on scald resistance characterised the phenotypic selection method (Treatment 1) as more efficient in shifting the population in the desired direction because it included the effects of minor genes as well as the major genes. It was found that the genotypic selection (Treatment 2) focused on the selection of the desired genotypes and even the 100% selection of the desired genotype was not able to fix the 100% phenotype because of its inability to fix the minor genes (Section 5.3.5 and Section 5.3.6). It is evident from the simulation results that if the genotypic selections (Treatment 2) are carried out generation after generation, selecting only the major genes, a stage will come when the phenotype could not be fixed further with the markers and the gene of interest comes in the repulsion phase with the trait under study (Section 5.3.6). This conclusion calls for the need for the molecular biology approaches to have a greater focus on gene-networking instead of the major genes controlling the trait of interest.
Figure 6.1: The distribution of the population without selection (starting population), after phenotypic selection (solid black line) and after genotypic selection (dotted black line) for the major gene and after the genotypic selection of the major gene plus 3-4 minor genes controlling scald resistance. Where R represents the desirable genotypes and S represents the undesirable genotypes in the population. The key Chapters involved in establishing the features of the distributions shown are indicated.
Gene-networks (Systems Biology) affect complex traits such as scald resistance and will vary with the genetic background. This implies that for complex traits, the approach for the use of markers needs to be reassessed. For example, Chapter 5 investigated the whole genome analysis of a complex breeding population for scald resistance with previously mapped markers. The marker-trait associations were validated for the two major scald resistant genes, $Rhs_{Vlamingh}$ on chromosome 4H and $Rhs_{WABAR2147}$ on chromosome 6H and one major powdery mildew resistant gene, $Mla_{WABAR2147}$ on chromosome 1H (Section 5.3.4). Along with these associations, the regions of the genome carrying the fragments from the donor parents, WABAR2147 (allele “B”) and Vlamingh (allele “E”) were identified (Section 5.3.1 and Section 5.3.2). This data itself is not sufficient to provide any evidence for the genetic relationship but could be used to corroborate results from the mapping studies. This could also be useful in designing the crosses to validate the suspected linkage for the hidden minor genes. Based on this data, new regions of interest in the barley genome for scald resistance were defined for increasing the genetic variation in this trait.

The simulation studies characterized the selection based on genotype to be most efficient in predicting a complex phenotype such as scald resistance when a major gene plus 3 minor genes were used in the model. The value of being able to predict the phenotype accurately based on a selection of molecular markers characterizing relevant genome regions is that the DNA analyses can be carried out at times when a field epidemic of the disease does not take hold and a sound phenotypic selection is not possible. In addition the selection can be made earlier in the season, not necessarily at the adult stage of the plant. The simulation studies carried out during this study indicate that genome-wide screen for the major gene
plus several minor genes involved in controlling disease resistance in a particular background needs to be carried out only once during the breeding program (Section 5.3.6, Table 5.5). Following this fewer markers, targeting the regions of interest can be used. Also the efficiency of the genotypic selection (Treatment 2) greatly depends upon the marker-gene associations and how closely the marker is linked to the gene under study. Simulation studies based on the experimental results demonstrated that in selecting the desired gene with the loosely linked marker, as for scald resistance where the markers are 5 or 30 cM distant from the scald resistant genes, the population reaches equilibrium where the genotypes in repulsive phase out numbered the desired genotypes in the coupling phase for the gene under study and the genotypic selection (Treatment 2) would end up in selecting for the scald susceptible genotypes instead of resistant genotypes. Thus for future productivity and sustainability of barley, perfectly linked markers are more desirable for pyramiding disease resistance into elite malting quality barley. The data presented suggests that phenotypic selection is most effective approach. However, genotypic data could be used to maintain marker-gene association. This would be desirable in future breeding efforts where phenotypic selection fails during a given season. Commercial breeders would deploy even loosely linked markers for targets, provided that they can select earlier in the breeding cycle for a target with an efficiency that is greater than purely random selection. Further, for breeders and molecular geneticists focusing on gene-networks, modeling and simulations studies could be beneficial, enabling the selection for parents, design crossing and selection strategies to optimize the use of phenotypic and genotypic selections together. Simulations studies can assist in the development of alternative hypothesis which might uncover new genetic information or variation which could be better that what could be achieved by combining phenotypic and genotypic selection methods. These combined
methodologies should reveal more complex gene combinations and gene interactions than will be revealed with simple analysis of bi-parental populations.

Based on the experience in this thesis it is evident that after making the desired crosses, back-crosses leading to top-cross, to transfer the genes of interest into the recipient parent, the segregating F2 plants should be used to link phenotype to key regions of the genome. A whole genome marker survey could, for example, utilize DArTs (Jaccoud et al, 2001) along with the markers associated considered to be the major gene(s), based on biparental crosses. Another approach could be the use of SNPs. Examples of the successful development of SNPs in cereals include selection of barley beta-amylase alleles (Paris et al, 2002), genotyping the puroindoline b gene for grain hardiness in wheat (Bhave and Morris, 2008) and selecting for polyphenol oxidase genes in wheat (Sun et al, 2005) and SNPs for Imi-tolerance gene (Li et al, 2008). This approach would quantify the percent variation in phenotype and identify the regions of the genome or chromosomal segments showing significant association with the phenotype. Using this approach, breeders would be able to design the crosses to select for the highly variable areas of the genome, along with the major regions of interest, in order to select for both the major and minor gene effects considering that the identification of the minor genes would often be specific to a particular genetic background.


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