Identification and validation of closely linked PCR markers for improving Yellow Spot Resistance in Common Wheat

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Abstract

Yellow spot (*Pyrenophora tritici-repentis*) is a major foliar disease in wheat (*Triticum aestivum*) and it has become more serious in recent years, possibly due to changes in climate or cultural practice. A major QTL located on the short arm of wheat chromosome 2B, which explains 30–40% of the phenotypic variance, has been identified recently. However, the closest marker linked to this QTL was a DArT marker that is not easy to use in large-scale selection; the closest PCR-based marker available (2.7 cM) was too far away to be reliably used for tagging the locus in wheat breeding. For these reasons, studies were undertaken to develop more closely–linked and user-friendly markers for this major QTL. Forty-one new markers either synthesised from DArT markers or identified from the GrainGene database were assessed. From these, a new PCR-based marker (Rfsts1), located 0.3 cM away from the major QTL, was developed for marker assisted selection in yellow spot breeding. This marker linked to the 2BS locus was used to combine with another QTL conferring yellow spot resistance on chromosome 5BL and the effects of gene pyramiding in increasing resistance to this disease were investigated. The results indicated that the combined selection could significantly increase selection efficiency and accuracy and resulted in genotypes with significantly higher resistance to yellow spot.

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Where the thesis includes work to which others have contributed, the thesis must include a statement that makes the student's contribution clear to the examiners. This may be in the form of a description of the precise contribution of the student to the work presented for examination and/or a statement of the percentage of the work that was done by the student.

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Statement of candidature contribution

This thesis was completed during the course of my enrolment in a Master degree in the School of Plant Biology at the University of Western Australia. This thesis contains no experimental material that has been previously presented for any degree at this or any other institution.

This thesis contains two main research chapters. Manuscripts reporting results described in these two chapters have been completed and will be submitted for publication.

Chapter 3:

Ren F, Ji J, Barbetti MJ, Siddique KHM, Liu CJ, Yan GJ (2014) PCR-based markers linked closely to a QTL conferring yellow spot resistance on chromosome arm 2BS in wheat (under preparation)

Contribution of this work is 80% by the candidate and 20% by other co-authors.

Chapter 4:

Ren F, Barbetti MJ, Liu CJ, Yan GJ (2014) Contribution of two known chromosome regions on 2BS and 5BL to the selection of yellow spot resistance in Australian wheat

Contribution of this work is 80% by the candidate and 20% by other co-authors.

Structure introduction

This thesis is presented as two scientific papers. All the work presented has resulted from work done towards this thesis. There are five main chapters in this thesis; *General Introduction*, *Literature Review*, two *Experimental Chapters* and *General Discussion*.

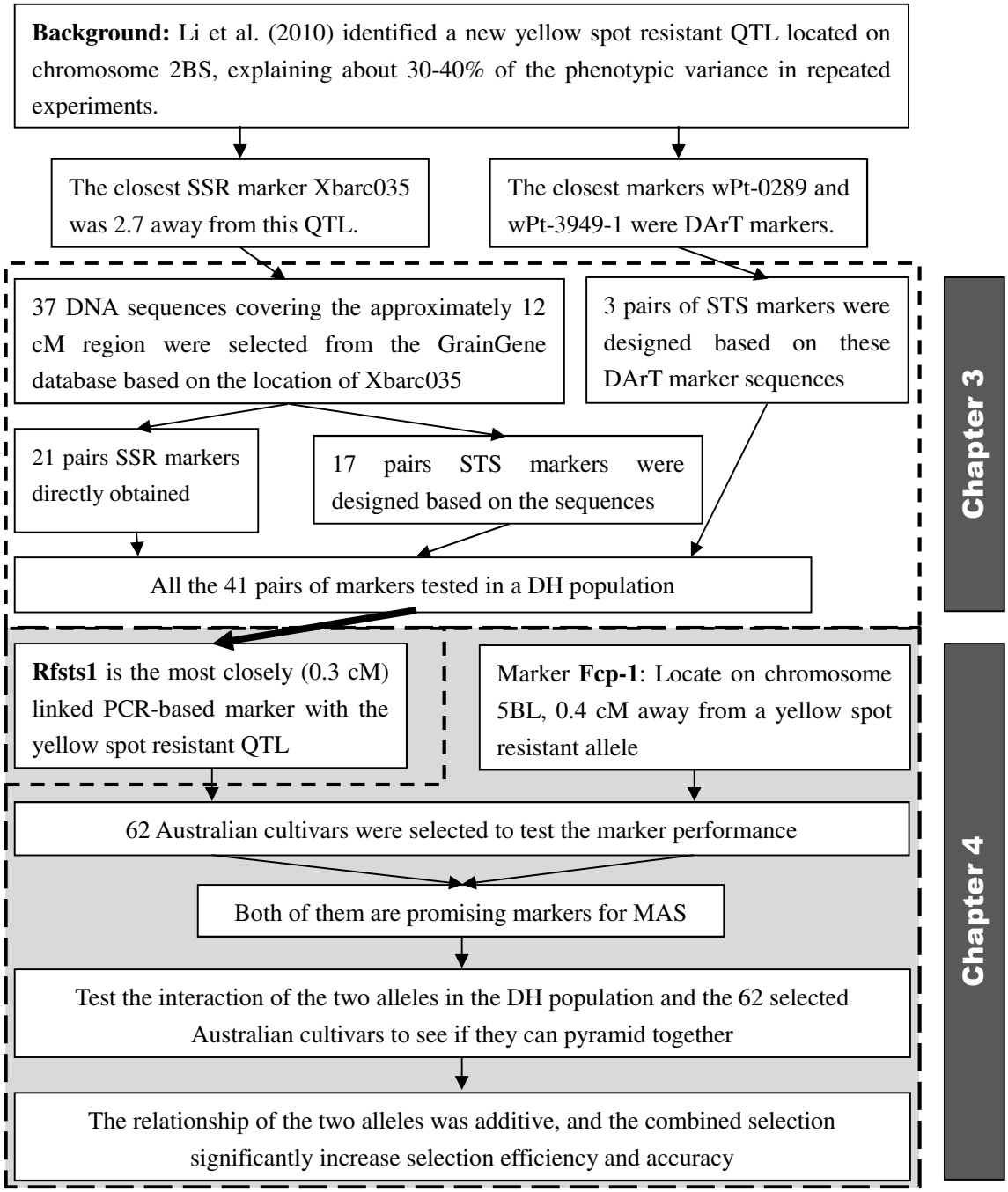
The *Literature Review* covers the broad background for the work presented in the thesis in order to justify the research objectives presented above. A more focused review of literature is presented in the introduction of each experimental chapter.

The two *Experimental Chapters* are presented in the format of scientific papers that can be read individually or as part of the whole thesis. Each *Experimental Chapter* includes the following sections: Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgement and References.

This “thesis-as-a-series-of-papers” format results in some unavoidable repetition, especially in the Materials and Methods sections of Chapters 3 with 4; I have tried to keep such repetition to a minimum.

The references are combined into the back of the thesis with tables and figures inserted into each chapter. Six figures and six tables are included in this thesis.

Concept Map


Background: Li et al. (2010) identified a new yellow spot resistant QTL located on chromosome 2BS, explaining about 30-40% of the phenotypic variance in repeated experiments.
The closest SSR marker Xbarc035 was 2.7 away from this QTL.
The closest markers wPt-0289 and wPt-3949-1 were DArT markers.
37 DNA sequences covering the approximately 12 cM region were selected from the GrainGene database based on the location of Xbarc035
3 pairs of STS markers were designed based on these DArT marker sequences
21 pairs SSR markers directly obtained
17 pairs STS markers were designed based on the sequences
All the 41 pairs of markers tested in a DH population
Rfsts1 is the most closely (0.3 cM) linked PCR-based marker with the yellow spot resistant QTL
Marker Fcp-1: Locate on chromosome 5BL, 0.4 cM away from a yellow spot resistant allele
62 Australian cultivars were selected to test the marker performance
Both of them are promising markers for MAS
Test the interaction of the two alleles in the DH population and the 62 selected Australian cultivars to see if they can pyramid together
The relationship of the two alleles was additive, and the combined selection significantly increase selection efficiency and accuracy
Chapter 3
Chapter 4

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Chapter 1

General Introduction

General Introduction:

As one of the most important staple foods and the major grain crop exported in Australia, wheat satisfies basic nutrient needs for Australians and creates a significant profit for this country. At the same time, wheat is suffering numerous biotic or abiotic stresses. Yellow spot, also known as tan spot, caused by the fungus *Pyrenophora tritici-repentis*, is a disease for wheat that is becoming increasingly serious in recent years. It presents across all wheat growing regions in the world, and causes up to a maximum of 30 - 50% yield loss.

After almost 70 years of study on the disease and the fungus, breeding yellow spot resistant cultivars offers potentially the most effective and the most cost-effective way to achieve control of the disease. And growing resistant cultivars, these methods meet the need for the ultimate and long term control of the disease. Many partially resistant cultivars have been identified and many resistant alleles have been detected. However, traditional breeding methods are slow and expensive, such that how to increase the breeding efficiency becomes the focus for most breeders. Increased selection accuracy is the key to increase the breeding efficiency. After exploring and investigating for many years, breeders identified molecular markers assistant selection (MAS), a method that can be used for indirect selection of a linked trait of interest at any stage of plant growth.

Li et al. (2010) identified a new yellow spot resistant QTL on the short arm of 2B chromosome from an immune cultivar ‘Ernie’. The QTL explained 38.2%, 29.8% and 36.2% of the phenotypic variance for yellow spot resistance, from three independent trials on four structured populations involving Ernie. However, this

promising QTL lacked of a promising molecular marker which can be applied into a large scale breeding program. The target of my study is to make this newly identified QTL applicable for large scale breeding programs, and breeding efficiency and accuracy can be significantly improved by pyramiding it with another resistance allele.

The study is mainly divided into two components: a) to find a closely linked PCR-based marker to this allele by saturating the 2BS region, where a new yellow spot resistant QTL was identified with new markers derived from the available DArT markers or with sequences identified from the GrainGene database; b) pyramiding the new 2BS resistant locus and the traditional 5BL locus through marker assisted selection to test the feasibility that stacking the two loci will significantly increase yellow spot resistance.

Chapter 2

Literature Review

Literature Review:

2.1 Wheat

2.1.1 Origin

Wheat (*Triticum spp.*) is believed to have originated in the Levant region of the Near East and Ethiopian Highlands, and now it is grown on more land area worldwide than any other crop, including rice, maize and potato, with an approximate cultivation area of 240 million ha (Curtis, 2002). Wheat is known as one of the earliest domesticated cereal crops. 'Common wheat', was currently planted, was formed by three different wild plant species (*Triticum monococcum*, *Aegilops speltoides*, and *Aegilops tauschii*) after two times natural events of distant hybridisation during 9000 years of natural selection and artificial selection (Fig. 2.1). It is a hexaploid plant (AABBDD) that arose from a cross of wild Emmer (AABB), which derived from hybrid of wild Einkorn (AA) and wild Spelt (BB), and *Ae. tauschii* (DD).

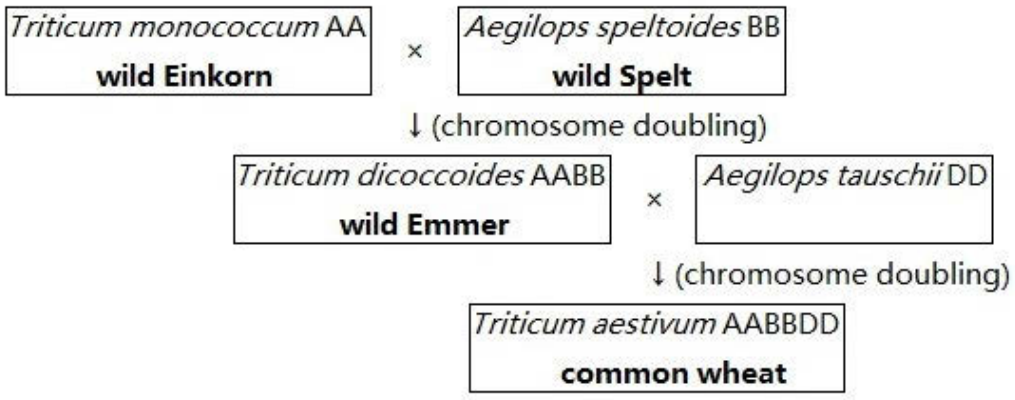

Fig. 2.1: The likely origin of bread wheat

2.1.2 Production

Wheat is the most popular staple food in the world. In the past decade, the world wheat trade fluctuated between 42 - 49% of the total world cereal trade (Food and Agriculture Organization of the United Nations FAO, 2013a). A recent survey shows that the world wheat trade (139.5 million tonnes) constitutes 46% of the total world cereal trade (306.1 million tonnes) in 2012/13 (FAO, 2013b).

In Australia, wheat is the most important crop. During 1998/99 to 2007/08, 14 agro-ecological zones (divided by Grains Research and Development Corporation in 1998) from southern Queensland to Western Australia were recorded for wheat cultivation. The largest wheat production region is the Southern Region. In this decade, the mean wheat area planted was 11.92 million ha, the mean yield was 1.65 tonnes per ha at a value of 4.68 billion dollars per year (Murray and Brennan, 2009). Australia is one of the world's largest wheat exporters, with about 70-80% of the average Australian production exported (Curtis, 2002). In 2010/11, Australia became the third largest wheat exporter in the world, with 18.5 million tonnes of wheat exported to over 52 different countries (Wheat Exports Australia, 2011).

2.1.3 Utilisation

Wheat is an important staple food all over the world. It is easy to grind into flour, easy to store, and rich in various nutrients. Compared with rice, wheat contains 5.5 times more magnesium, 4.1 times more phosphorus, 4.7 times more potassium, 5.4 times more folate, and 4.4 times more fibre. In a recent survey, the total world wheat utilisation was 686.2 million tonnes of which about 70% were used as food, 19% as feed, and remaining 11% in other areas (FAO,

2013b), such as industry adhesive, paper additive, alcohol production, and various other applications (Curtis, 2002).

In addition of daily consumption, wheat is also a focus of extensive research. As mentioned above, wheat is an allopolyploids plant (from natural crossing and natural selection) containing three different genomes. It provides a model system for the study of many areas, such as genetics, cytogenetics, and breeding.

2.1.4 Wheat Breeding

Wheat breeding involves a range of areas and foci of study, including yield increase, nutrition increase (Prasad et al. 1999a), vernalisation response (Galiba et al. 1995), abiotic stress tolerance (Luo and Dvorak 1996) and diseases resistance (Faris et al. 1997). Almost all of the breeding studies were involved in marker assisted selection. And abundant of available loci and markers were identified.

Any future emergence of innovative technologies will affect the development of wheat breeding. However, the work will still largely focus on improving the yield and quality of wheat to meet the global food demand. Specific research methods will be the study of wheat yield potential (Rajaram and Braun 2008), the construction and application of genetic map (Hori et al. 2003), tags and location of target genes (Himi and Noda 2004), finger-printing and identification of varieties (Vaccino et al. 1993), investigating the relationship between the genetic diversity and allied species evolution (Joshi and Nguyen 1993), and molecular marker assisted breeding (Cheong et al. 2004). However, common wheat is a hexaploid plant, which has made the research of molecular breeding more

complex than other diploid plants.

2.2 Yellow Spot

2.2.1 Distribution

Yellow spot, also known as tan spot or yellow leaf spot, was first identified in the United States in 1940s (Ciuffetti and Tuori, 1999), and it quickly spread to almost all wheat growing areas all over the world (Fig. 2.2). The ‘boom’ stage of this disease worldwide was around 1970s (Strelkov and lamari, 2003; Oliver et al., 2008), with the first serious outbreak in 1974 in western Canada (Tekauz, 1976). The reason probably relates to changes in cultural practices (Lamari and Bernier, 1989a; Sutton and Vyn, 1990), continuous wheat cultivation without rotations (Raymond et al., 1985; Sutton and Vyn, 1990), and growing of susceptible cultivars (Rees and Platz, 1992). In Australia, yellow spot was first discovered in 1953 (Oliver et al., 2008), and now it is present across all wheat growing regions (Murray and Brennan, 2009), especially in northern wheat growing areas (Loughman et al., 1998).

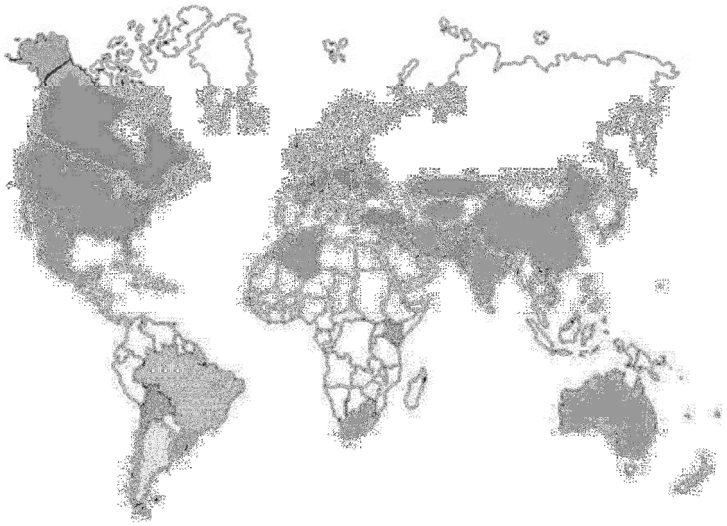

Fig. 2.2: Geographic distribution of yellow spot disease in wheat. Filled regions represent areas where fungus and/or disease were reported (Strelkov and lamari, 2003).

When plants are infected with yellow spot disease, typical disease symptoms consist of yellow to tan-brown spots on both sides of the leaf. Spots will continually enlarge to become irregular lesions. Finally, the plant will express symptoms of necrosis and/or chlorosis (Buhariwalla et al., 2009).

2.2.2 Fungus

Yellow spot is caused by the fungus *Pyrenophora tritici-repentis*. This fungus is a homothallic ascomycete that reproduces both asexually by conidia and sexually by ascospores (Ciuffetti and Tuori, 1999). On the basis of whether the fungus can causes necrosis and/or chlorosis, whether it can infect some previously known resistant lines, and whether it produces particular combinations of the toxins (Table 2.1 & Fig. 2.3), the disease can be classified into 8 races and three

types of toxins – race 1 and race 2 produce toxin Ptr ToxA, race 3 produces Ptr ToxC, race 4 is non-toxic, race 5 produces Ptr ToxB, race 6 combines the virulence of races 3 and 5, race 7 combines the virulence of races 2 and 5, and race 8 combines the virulence of races 2, 3, and 5 (Lamari et al, 1995; Lamari et al, 2003). In Australia, wheat is mainly affected by ToxA, and ToxB is absent (Antoni et al., 2010).

Table 2.1: The classification of yellow spot races

Race	Virulence Pattern	Toxin
1	causes necrosis and chlorosis (nec+ chl+)[1]	Ptr ToxA & Ptr ToxC[2]
2	causes only necrosis (nec+ chl-)[1]	Ptr ToxA[2]
3	causes only chlorosis (nec- chl+)[1]	Ptr ToxC[2]
4	avirulent (nec- chr-), and result in a dark brown spot at the site of infection, without further development of tan necrosis or chlorosis[1]	None
5	causes only chlorosis (nec- chl+), have the potential to overcome resistance of a wheat accession, line 6B662 (which previously known to be resistant to isolates from all pathotypes)[1]	Ptr ToxB[2]
6	combines the virulence of races 3 and 5[2]	Ptr ToxB & Ptr ToxC[2]
7	combined the virulences of races 2 and 5[2]	Ptr ToxA & Ptr ToxB[2]
8	had a virulence pattern that combined those of races 2, 3, and 5[2]	Ptr ToxA & Ptr ToxB & Ptr ToxC[2]

[1] Cited from Lamari et al, (1995) ; [2] Cited from Lamari et al, (2003)

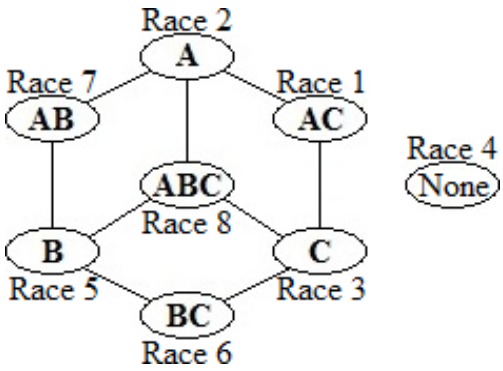

Fig. 2.3: The relationship between toxins and races of yellow spot. Capital letters indicate the different toxins of yellow spot; race 4 is non-toxic

The main hosts of this fungus include bread wheat, durum wheat, rye and wild grasses (Buhariwalla et al., 2009). It can be spread by wind, tolerates low and high temperatures, and readily survives on wheat residues. After a period of dry conditions, the fungus can even survive for up to 18 months on infested stubble (Grains Research and Development Corporation GRDC, 2011), easily re-infest new crops each season. For this reason, yellow spot is often more serious in areas using no-tillage farming systems (Buhariwalla et al., 2009).

2.2.3 Disease hazards

The disease reduces the photosynthetic area of plants, negatively impacting plant growth and grain filling. Yellow spot significantly compromises both the quality and the yield in wheat. While the production loss due to yellow spot may be up to a maximum of 30 - 50% (Riede et al., 1996; Shabeer and Bockus, 1988), more generally losses of between 3 - 15% occur (Buhariwalla et al., 2009). The losses vary depending on the infective period of vegetation (Rees and Platz, 1983); 13% yield reduction may be caused during the early disease infection, 35% during the late disease infection, and 48% from disease throughout the season.

In Australia, from the 1998/99 to the 2007/08 season, the fungus caused approximate annual losses of $212 million. However, the potential average annual loss could be up to $676 million without current control measures (Murray and Brennan, 2009).

2.2.4 Scoring system

Current disease ranking systems are generally based on lesion size (Cox and Hosford 1987), lesion type (Lamari and Bernier 1989b), percent infection (Nagle et al. 1982), cultural management method (National Variety Trials 2013), or some combination of these (Luz and Hosford 1980). In this study, the Lamari and Bernier (1989b) and National Variety Trials (2013) methods are used details of which are provided in Chapters 2 and 3.

2.2.5 Current protective methods

There are four main methods to control yellow spot, cultural practices, chemicals, biocontrol, and host resistance.

Cultural practices

The yellow spot pathogen is stubble-borne fungus, and it can survive on wheat stubble. Minimum and zero tillage practices, which retain crop residues on the soil surface, allow inoculum build up on wheat stubbles over time (Strelkov and lamari, 2003). Hence, any management practices that reduce surface stubble density quantity can be effective in reducing yellow spot infection in the next growing season (Adee and Pfender, 1989). Yellow spot can be largely controlled by using mouldboard plowing (Bockus and Claassen, 1992), which buries or destroys the residue food base of the pathogen (Adee and Pfender, 1989). A

study by Jorgensen and Olsen's (2007) found that the infection level of yellow spot was reduced 4-10 times by using full tillage.

Although yellow spot can be effectively controlled by full tillage or residue destruction, the overall management practice remains towards utilising reduced tillage and no-tillage, as this limits soil erosion, conserves soil moisture, reduces fuel use, and reduces the investment of both time and money (Bockus, 1998). For this reason, rotation with non-host crops to produce a break from wheat is also an effective control of yellow spot (Duveiller and Dubin, 2002; Bockus and Claassen, 1992).

Chemicals

Application of fungicides is the most expeditious means to reduce yield loss due to yellow spot. Prosaro®, Amistar Xtra® and products containing strobilurins together with tebuconazole and propiconazole give the best control of yellow spot (Duveiller and Dubin, 2002, Jorgensen and Olsen, 2007). The efficacy reached 36–97% depending on the number of treatments and doses of fungicides (Bockus, 1998, Jorgensen and Olsen, 2007).

Changes in cultivars, cropping conditions, environment, fungicides, fungicide application time, and many other factors (Duveiller and Dubin, 2002) can all influence the control efficiency. Previous reports showed that the optimum time to apply fungicides was at the boot stage (Bockus and Claassen, 1995), or between boot and fully-headed stage (Bockus et al., 1997). However, fungicide applications between early tillering and stem elongation also can effectively reduce the level of yellow spot infection (GRDC, 2011).

Application of fungicides is costly and in some situations can be considered harmful to both the environment and human health. Further, fungicide application does not always guarantee good economic returns. The cost benefits of fungicide application are dependent on the current price of wheat, the cost of the fungicide and its application and the percentage yield increase from spraying. If also taking into consideration in the instable of fungicide control effect, application of fungicides may not be the best or most cost-effective means to control yellow spot.

Biocontrol

Pfender et al. (1993) found *Limonomyces roseipellis* to be antagonistic fungus to *P. tritici-repentis*. It is a mycoparasite that attacks the mycelium of *P. tritici-repentis*, suppressing the formation of pathogen fruiting bodies on wheat stubble, and preventing the pathogen reproduction. However, there are no follow-up reports to confirm this earlier finding.

Host resistance

Breeding resistant cultivars offers potentially the most effective and also the most cost-effective way to achieve control of yellow spot, and is the ultimate long term solution needed for the control of yellow spot.

For successful breeding for resistance, three criteria must be met (Bockus 1998): 1) the resistance level must be high enough for effective control; 2) a source of resistance must be found, incorporated into a desirable genotype, and deployed; and 3) the resistance should be durable.

To yellow spot disease resistant breeding, clear and significant differences in the

level of yellow spot disease attack among sensitive and resistant varieties have been observed in many areas, such as Denmark, Latvia, Lithuania, and Kansas (Jorgensen and Olsen 2007, Bankina and Priekule 2011, Brown et al. 1996, Cox et al. 1992, Raymond et al. 1985). To date, a total of 16 quantitative trait loci (QTL) and major genes for yellow spot resistance have been identified (Faris et al. 1996, Faris et al. 1997, Friesen and Faris 2004, Faris and Friesen 2005, Chu et al. 2008, Singh et al. 2008, Li et al 2010), located on chromosomes 2AS, 3AS, 4AL, 5AL, 1BS, 2BL, 2BS, 3BS, 3BL, 2DS, 2DL, 7DS, and 1AS, 2BS, 5BL, 3DS (Table 2.2). Studies have shown that resistance of yellow spot can be inherited to the next generation (Nagle et al., 1982; Elias et al., 1989; Anderson et al., 1999), in a Mendelian fashion (Strelkov and lamari, 2003). The narrow sense heritability of the disease is about 0.73, making this a suitable approach for selection for resistant cultivars in wheat (Elias, 1989).

Table 2.2: Yellow spot resistant alleles identified so far

Yellow spot resistant alleles locate on chromosome	Parents	reference
5BL	-	(Faris te al. 1996)
4AL, 2DL, 1AS	W-7984×Opata85	(Faris et al. 1997)
2BS	-	(Anderson et al. 1999)
1AS	W-7984×Opata85	(Effertz et al. 2002)
2AS, 4AL, 2BL, 2BS	W-7984×Opata85	(Friesen and Faris 2004)
1BS, 3BL, 3BS, 5AL, 2DS, 4AL, 7DS	BR34×HRSW Grandin	(Faris and Friesen 2005)
3DS	Langdon×XX41 Langdon×XX45 Landon×XX110	(Tadesse et al. 2007)
2AS, 4AL, 5AL, 5BL	TA4152-60×ND495	(Chu et al. 2008)
3AS	WH542×HD29	(Singh et al. 2008)
2BS	Erine×Batavia	(Li et al. 2010)

2.3 Molecular marker assisted breeding

2.3.1 Introduction

Traditional breeding is mainly based on plant phenotypic selection. However, environmental condition, genetic interaction, and other situations can all affect the expression of the phenotype, in turn affecting the efficiency of selection. Generally, it takes decades to breed a new cultivar. For this reason, increasing the selection efficiency is the key to successful and more rapid breeding.

Breeders found that use of genetic markers to select the desired parameter can increase breeding efficiency and outcomes, and genetic markers utilized to date have mainly included morphological markers, cytological markers, biochemical markers, and molecular markers. The first three marker types had been applied in wheat, corn, and cotton breeding programs. However, they cannot meet the need of large scale breeding programs because of their low polymorphism and the strong influence by plant development stage and environmental conditions. Molecular markers, based on genotypic variation, can be used for indirect selection of a linked trait of interest. This method has the advantage of not being influenced by environmental conditions and can be utilised at any age of plant development (Gupta et al. 1999). Currently, breeders are more focused on the development and application of molecular markers.

2.3.2 Characteristics of suitable molecular markers

Markers suitable for molecular marker assistant selection (MAS) should satisfy

at least three characteristics: 1) be closely linked to the target gene or QTL, 2) be simple PCR-based, and 3) be implementable to a large-scale selection/breeding platform (Li et al, 2011). When these kinds of markers are available, only a small amount of DNA is required; the procedure is simple and fast; the results are more reliable and reproducible than conventional method; and it is much more cost-efficient (Singh et al, 2010).

2.3.3 Molecular marker systems

According to technical characteristics, molecular markers can be divided into three categories (Gupta et al. 1999): 1) DNA marker technology based on molecular hybridisation, such as the restriction fragment length polymorphism (RFLP); 2) DNA fingerprint technology based on polymerase chain reaction (PCR), such as random amplification polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), and sequence tagged site (STS); 3) new-type molecular markers, such as single nucleotide polymorphism (SNP).

Markers mainly used in molecular marker assisted selection program are RFLP, RAPD, AFLP, SSR, STS and SNP (Table 2.3).

RFLP was the first molecular marker discovered. It was developed around 1970s, based on the theory of restriction digestion. However, for the high cost, weak signal, and low sensitivity, breeders are working to convert this into a PCR-based marker (Beckmann and Soller 1983).

RAPD is based on random PCR amplification. Generally, the primer is 10 bp, low cost, can be applied in different corps, and can be converted into STS markers.

The biggest drawback of this marker is its poor reproducibility (Guthrie et al. 1992).

AFLP is based on restriction digestion and PCR amplification. It combines the benefit of RFLP and PCR technique. It does not need to know the sequence information, and can be apply in any crops. The polymorphisms of AFLP are higher than other markers. However, the cost is high, and it has a high requirement of DNA and enzyme quality (Althoff et al. 2007).

SSR is a group of repetitive DNA sequence that represents a significant portion of higher eukaryote genomes, which is based on PCR amplification. It is a co-dominant marker, which can identify heterozygous and homozygous individuals. The results are repeatable and reliable. And the reaction requires less DNA amount and low DNA quality. However, the cost is high (Powell et al. 1996).

STS is a unique sequence that identifies a specific locus and can be amplified by PCR. The practical value is very attractive, for easy transformation from different molecular markers by only knowing the DNA sequence in advance. It can be used in many aspects of genetic analysis, such as marker assisted selection, QTL mapping, positional cloning, gene flow analysis, mating system analysis and genetic diversity studies. However, again, the cost is high (Paglia et al. 1998).

SNP is a third generation of genetic markers with properties of inheritance and detectability. Sensitivity and accuracy is high, cost is low, and can be applied in large scale screening. Currently, it is an ideal genetic marker. However, the efficient use of the marker in large polyploid genomes, such as wheat, needs

more research because of the potential presence of multiple homoeologues for each gene (Lai et al. 2012).

After comprehensive consideration, SSR and STS markers were widely chosen as the best choice of molecular markers for large-scale breeding program. However, SNP markers are also being developed. There is great potential for the SNP marker as the cost for sequencing reduces and the automation of microarrays develops further.

Table 2.3: Comparison of six different markers

Name	Restriction fragment length polymorphisms	Random amplification polymorphism DNA	Amplified fragment length polymorphisms	Simple sequence repeats	Sequence tagged sites	Single nucleotide polymorphism
Abbreviation	RFLP	RAPD	AFLP	SSR	STS	SNP
Principle	restriction digestion	random PCR amplification	restriction digestion and PCR amplification	PCR amplification	Specific PCR amplification	single nucleotide variations
Marker dominance	co-dominant	dominant/co-dominant	dominant/co-dominant	co-dominant	dominant/ co-dominant	co-dominant
DNA required	5-30 µg	10-100 ng	50-100 ng	10-100 ng	50-100 ng	-
Sequence information needed	No	No	No	Yes	Yes	Yes
Run duration	long	short	long	short	short	-
Cost	high	low	high	high	high	low
Reliable	high	medium	high	high	high	high
Primer length	-	10bp	-	16-24bp	16-24bp	-
Richness	medium	very high	high	high	medium	very high
Polymorphism level	medium	High	very high	high	medium	high
Reference	(Beckmann and Soller 1983)	(Guthrie et al. 1992)	(Althoff et al. 2007)	(Powell et al. 1996)	(Paglia et al. 1998)	(Lai et al. 2012)

Chapter 3

Experimental Chapter One

PCR-based markers linked closely to a QTL conferring yellow spot resistance on chromosome arm 2BS in wheat

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With 1 figure and 1 table

3.1 Abstract

Yellow spot (*Pyrenophora tritici-repentis*) is a major foliar disease in wheat (*Triticum aestivum*) that has become more serious in recent years possibly due to climate change. A major QTL located on the short arm of wheat chromosome 2B which explains 30–40% of the phenotypic variance has been identified recently. However, the closest marker linked to this QTL is a DArT marker that is not easy to use in large-scale selections; the closest PCR-based marker available (2.7 cM) is too far away to be reliably used for tagging the locus in wheat breeding. For these reasons, we undertook studies to develop more closely–linked and user-friendly markers for this major QTL. Forty-one new markers either synthesised from DArT markers or identified from the GrainGene database were assessed. From these, we developed a new PCR-based marker (Rfsts1), located 0.3 cM away from the major QTL, suitable for marker-assisted selection for yellow spot resistance in wheat breeding programs.

Key words: wheat — yellow spot — QTL — *Pyrenophora tritici-repentis* — breeding

3.2 Introduction

Yellow spot, also known as tan spot, caused by *Pyrenophora tritici-repentis*, is a major foliar disease on wheat (*Triticum aestivum*) that has become increasingly serious in recent years. *P. tritici-repentis* spreads by wind and is present across all wheat growing regions in Australia (Murray and Brennan 2009). Typical symptoms of yellow spot include yellow or tan spots on both sides of the leaves

that enlarge to become irregular lesions until substantial necrosis and/or chlorosis occurs (Friesen and Faris 2004). Yellow spot reduces the effective photosynthetic area of a plant and negatively impacts on plant growth and grain filling, leading to loss of both quality and yield in wheat. When severe, production losses due to yellow spot can be up to approximately 50% (Singh et al. 2010). In Australia, yellow spot causes estimated annual losses of AUS$212 million, with potential losses of up to AUS$676 million if controls fail or are not effectively utilized (Murray and Brennan 2009). Currently, crop management and rotation, and application of fungicides are the main methods to minimise the impact of yellow spot. However, these methods are both expensive and unpredictable in terms of outcomes. Hence, breeding resistant cultivars offers the most effective and cost-effective way to control yellow spot, particularly in low-input cropping systems such as in Australia.

Numerous studies have shown that yellow spot resistance can be inherited to the next generation (e.g. Nagle et al. 1982, Elias et al. 1989, Anderson et al. 1999). The narrow sense heritability of yellow spot is about 0.73, making breeding a suitable approach to enhance selection for resistance in wheat (Elias et al. 1989). Quantitative trait loci (QTL) for yellow spot resistance have been reported on chromosomes 1AS, 2AS, 3AS, 4AL, 5AL, 1BS, 2BL, 3BS, 3BL, 5BL, 2DS and 2DL (Faris et al. 1997, Friesen and Faris 2004, Faris and Friesen 2005, Chu et al. 2008, Singh et al. 2008). Friesen and Faris (2004) were the first to identify a QTL on chromosome 2BS conferring resistance to yellow spot, and an RFLP marker–Xmwg950–was located 44.3 cM from this QTL. Recent research (Li et al. 2010) identified bread wheat cultivar Ernie as almost immune to yellow spot infection in Australia. QTL analysis on four structured populations involving

Ernie identified a major QTL located on chromosome 2BS explaining 38.2%, 29.8% and 36.2% of the phenotypic variance for yellow spot resistance, from three independent trials. One DArT marker, wPt-0289, was mapped as the closest marker to this QTL. However, DArT markers are complicated to operate in large-scale marker-assisted selection. The closest PCR-based marker identified in that study, Xbarc035, was 2.7 cM from this QTL (Li et al. 2010). The distance between the existing PCR-based marker and the QTL was too far away which hindered its use in wheat breeding. Hence the need to develop a new PCR-based marker more closely linked with this QTL so that it can be fully utilized in breeding programs.

This paper reports the outcomes of studies focused on saturating the 2BS region harbouring the QTL with synthesised new markers based on DArT information or with sequences identified from the GrainGene database (http://wheat.pw.usda.gov/GG2/index.shtml). We report, for the first time, the development of a new PCR-based marker—Rfsts1 located 0.3 cM away from the major QTL—suitable for marker-assisted selection for yellow spot resistance in wheat breeding.

3.3 Materials and Methods

3.3.1 Plant materials

The same DH population used by Li et al. (2010) was used for this study. The population consisted of 78 doubled haploid (DH) lines derived from the cross of American soft red winter wheat cultivar Ernie with Australian cultivar Batavia.

Ernie is immune to yellow spot infection, but Batavia is highly susceptible. Together with their parents, the DH population was grown in an air-conditioned glasshouse at The University of Western Australia, Crawley, Western Australia, for DNA extraction.

3.3.2 Yellow spot assay

An initial yellow spot epidemic environment in a glasshouse was established by artificially inoculating two highly susceptible durum wheat varieties (Wallaroi and Bellaroi) with field-infected plants. The main consideration for using the durum varieties for establishing the initial epidemic environment was that they would produce more ascocarps than with the use of more resistant bread wheat genotypes. This artificial epidemic environment was maintained by repeatedly planting these two susceptible varieties in each corner of the glasshouse maintained at 25/18 (±5)℃ day/night temperature and 65/80 (±10)% day/night relative humidity with natural illumination.

The method used for yellow spot assessment was following the description of Adee and Pfender (1989). First, the infested stems of the two susceptible durum varieties were collected, and the amount of inoculum on the strems was estimated by counting the number of mature ascocarps. Second, stem stubble with similar number of mature ascocarps were cut into 10 cm pieces, and five pieces of the infested stem were placed on the surface of each pot when seedlings reached the three-leaf stage. Third, water was sprayed, to promote yellow spot infection, using a backpack sprayer six times a day for seven consecutive days following inoculation. Last, Yellow spot resistance was evaluated by ranking lines from 1 to 6 using the system developed by Lamari

and Bernier (1989b) that is based on lesion types in wheat in response to *P. tritici-repentis* (6 = no visible lesion without either chlorosis or necrosis: 5 = small dark brown to black spot lesions without chlorosis or necrosis, 4 = small lesions with very little chlorosis or necrosis, 3 = small lesions, generally not coalescing, completely surrounded by a distinct chlorotic or necrotic ring, 2 = small lesions, some coalescing, completely surrounded with chlorotic or necrotic zones; 1 = large lesions consisting of coalescing chlorotic or necrotic zones. Seventy-eight DH lines, each line with two replicates, were evaluated.

3.3.3 DNA extraction and molecular marker analysis

During the growing period, young leaves of the DH population were sampled and DNA was extracted using a standard CTAB protocol (Doyle and Doyle 1990). PCRs for molecular marker analysis were performed in 15 µl volumes containing approximately 100 ng genomic DNA, 400 nM of each primer, 4µl for 5×PCR polymerisation buffer (Fisher Biotec), 4 nM Mg2+, and 1 unit Taq enzyme with the following program: 95℃ for 5 min, followed by 35 cycles of 95℃ for 30 s, annealing for 30 s at different temperatures for different primers, and 72℃ for 45 s, with a final extension at 72℃ for 5min. PCR products were separated on a 3% agarose gel and/or a 6% poly-acrylamide gel. Gels were documented using the gel documentation system (Vilber Lourmat, France).

Based on the position of Barc035 located on the short arm of wheat chromosome 2B, 2.7 cM from the peak of the target QTL (Li et al. 2010) and 3.8 cM distal to the centromere (GrainGene), DNA sequences covering the approximately 12 cM region were selected from the GrainGene database. DNA sequences from Diversity Arrays Technology (http://www.diversityarrays.com)

were also obtained for marker development. Eventually, 41 pairs of SSR and STS markers based on 37 sequences were designed. Primers were designed using DNAstar software (http://www.dnastar.com).

3.3.4 Linkage map construction and QTL mapping

The linkage map was constructed using IciMapping Version 3.3 (Wang et al. 2012). All markers were grouped by LOD threshold and ordered by algorithm. The nearest neighbour was used for tour construction and two-opt was used for tour improvement (nnTwoOpt). Ripped function was used for fine-tuning of the ordered chromosomes using the criterion of sum of adjacent recombination fractions (SARF). The Kosambi function was used to calculate genetic distances.

Mapping of QTL and estimating of their effects were carried out using inclusive composite interval mapping of additive (and dominant) QTL (ICIM-ADD) in IciMapping Version 3.3 (Wang et al. 2012).

3.4 Results

3.4.1 Identification of new markers

Forty one new markers located on chromosome 2BS were evaluated (Table 3.1). Twenty-one of these were directly obtained from the GrainGene database, 17 were newly-designed and synthesised based on the sequences from the GrainGene database, and three were designed based on sequences of DArT

markers. Of all the markers tested, 26 showed polymorphism between the two parents (Ernie and Batavia), and nine were closely linked with the major QTL.

Table 3.1: Details of the 41 markers used in this study: 20 were downloaded from the GrainGene website, while the remaining 21 were newly-designed based on DNA or RNA sequences on the GrainGene website or DArT markers. Twenty six of 41 markers showed polymorphism between the two parents; 9 of those 26 were linked with the major yellow spot resistance QTL on 2BS.

Marker name	Marker type	Forward primer (5' to 3')	Reverse primer (5' to 3')
Xbarc124[1, 4]	SSR	TGC ACC CCT TCC AAA TCT	TGC GAG TCG TGT GGT TGT
Xbarc140[1]	SSR	CGC CAA CAC CTA CCA TT	TTC TCC GCA CTC ACA AAC
Xbarc318[1]	SSR	CGA CTA ACA ATT TTT CAT TT	TGA TTT CGC TAA CAA GGA G
Xbarc045[1, 4]	SSR	CCC AGA TGC AAT GAA ACC ACA AT	GCG TAG AAC TGA AGC GTA AAA TTA
Xcfd267[1, 3, 4]	SSR	GTG CGT CGT GTA GCA GCT C	CTC TCT GTC GTC CAG GTC GT
Xgpw2039[1, 3, 4]	SSR	ACC GGG AGT CCT TGT CTT TT	AGC TTA CCA GGT CGG ACA AA
Xgwm210[1, 3, 4]	SSR	TGC ATC AAG AAT AGT GTG GAA G	TGA GAG GAA GGC TCA CAC CT
Xgwm257[1]	SSR	AGA GTG CAT GGT GGG ACG	CCA AGA CGA TGC TGA AGT CA
Xgwm614[1, 4]	SSR	GAT CAC ATG CAT GCG TCA TG	TTT TAC CGT TCC GGC CTT
Xwmc154[1]	SSR	ATG CTC GTC AGT GTC ATG TTT G	ATG CTC GTC AGT GTC ATG TTT G
Xwmc332[1]	SSR	CAT TTA CAA AGC GCA TGA AGC C	GAA AAC TTT GGG AAC AAG AGC A
Xwmc661[1, 4]	SSR	CCA CCA TGG TGC TAA TAG TGT C	AGC TCG TAA CGT AAT GCA ACT G
Xwmc764[1]	SSR	CCT CGA ACC TGA AGC TCT GA	TTC GCA AGG ACT CCG TAA CA
Xwmc489[1, 3, 4]	SSR	CGA AGG ATT TGT GAT GTG AGT A	GGA CAA CAT CAT AGA GAA GGA A
wPt-7970-2[2]	STS	AGT AAA AGC CCA AAC AAA CAC G	GCA GCG GCG AAA CAG TAG A
wPt-3388-2[2, 3, 4]	STS	GCT ACA AGT GCC CCT AAT GAC A	AGA AGG AAT CGC TAA GGG TGA C
Rfsts1[2, 3, 4]	STS	ATC GGA GAG GAC TTT CAT TG	GAG AGG GGG TGT TTG TCA G
Xbarc101-1[1, 4]	SSR	GCT CCT CTC ACG ATC ACG CAA AG	GCG AGT CGA TCA CAC TAT GAG CCA ATG
Xbarc101-2[2, 4]	STS	ACA TGG CAT TAG CAG GTT	TCG AGG ATA TTA AGT TGA TGG
Xbarc200-1[1, 4]	SSR	GCGATATGATTTGGAGCTGATTG	GCGATGACGTTAGATGCGGAATTGT
Xbarc200-2[2, 4]	STS	TTA TTA TTA TTG AAG GGT GTC C	ATC AGT CTC TTT GGG CAT TTT A
Xwmc025[1, 4]	SSR	TCT GGC CAG GAT CAA TAT TAC T	TAA GAT ACA TAG ATC CAA CAC C
Xwmc314-1[1, 3, 4]	SSR	ACA CGG GGT CTG ATT GCT TTA C	ATC GCT TTT TGA CAA GTG AGG C
Xwmc314-2[2, 3, 4]	SSR	AGG GCA TAT TTC CAA CAC CAG ATT	CAC GCC GCA CAC CCA CAG
Xwmc382-1[1]	SSR	CAT GAA TGG AGG CAC TGA AAC A	CCT TCC GGT CGA CGC AAC
Xwmc382-2[2]	SSR	TGC CAG CGG AAT ACA AAA ACA G	CGC CGC CGG AAC AGA AGC
Xmag3807[1, 4]	STS	CGA GCT TGG TTG GTG ATC TT	CTT GGC TAC CCT GAT GTC GT
Xmag3930[2, 3, 4]	STS	CCT CCA AAG AGA AGC CAT GA	ATG CCC TTG AGG ACG AAC T

Whe0279[2, 4]	STS	CCC GTC CGC CCG TCC CAA CC	GCA CCG CCC TTC TTC ACC CAG CAG T
Whe0367[2, 4]	STS	CCT GTG CGG GCC AAT CAA GAA G	ACC GCC ACC ACC GCC CCT GTA
Whe0442[2, 4]	STS	ATC CCG CAC CAA CTG TTC ATC TTC	ATT CTT CCC CGA GTT TCT TCA CAG C
Whe0488[2]	STS	GCC CCC GCC GTG CTC GTG	GCG GCG CTG GTT GTA CTC GGT GTT
Whe0804[2]	STS	TTG AAC ATC GCG AAC CCC ACC AC	ACT TCC TCC GGC GCT CAC CAA CAC G
Whe0815[2, 4]	STS	GTT CTC CGA CTT CCC TCA GC	GAC AGC GCC ACT TCA TTC TC
Whe0821-0824[2, 4]	STS	CCG CGA GTT GGA CGA GGT GTT C	CAA GAC GGC GCC GAT GGT GT
Whe1713-1716[2, 4]	STS	GGC GAC CAA CCA TTC TC	ATT TTA TAG CAT CAA CCA ACT TCT
Whe1769[2]	STS	TTG CCT CTA TCT CTG CTG GTA ACG	GCA AAT GTA GCC TCG GTC AAT CTC
Whe2118[2]	STS	GGC GCC AGC ATC TTC CCT ATC TCC	GTG CCC CAG TCG CCA TTG TTG C
Whe2340[2]	STS	AGA CCC ATC ACC GCT TTG TTC	ACC GGT GTT TGA TCC CTT CCT C
Whe2952[2, 4]	STS	GCT CCG TTT ACT GCC CTT TCT AT	CCC AAT TCC ATC ACC GTC CAC
Whe2960[2]	STS	TTC TAC GCC AAG GCC TTC AAC TAC A	AGC GCG TCC GTC TCC CTC TG

[1]Markers from GrainGene.

[2]Newly-designed markers based on DNA or RNA sequences on the GrainGene website or DArT markers.

[3]Markers linked with the yellow spot resistance QTL.

[4]Markers showing polymorphism between parents.

3.4.2 Construction of the linkage map

A linkage map of 72 marker loci, including previous PCR based marker Barc035, 45 DArT markers (Li et al. 2010) and 26 new markers, was constructed on the chromosome region of 2BS. The mapped markers spanned a genetic distance of 160.0 cM on chromosome 2B. The chromosome region surounding the major QTL (the short arm of chromosome 2B) harboured Barc035, six DArT markers and nine newly designed SSR or STS markers (Fig. 3.1).

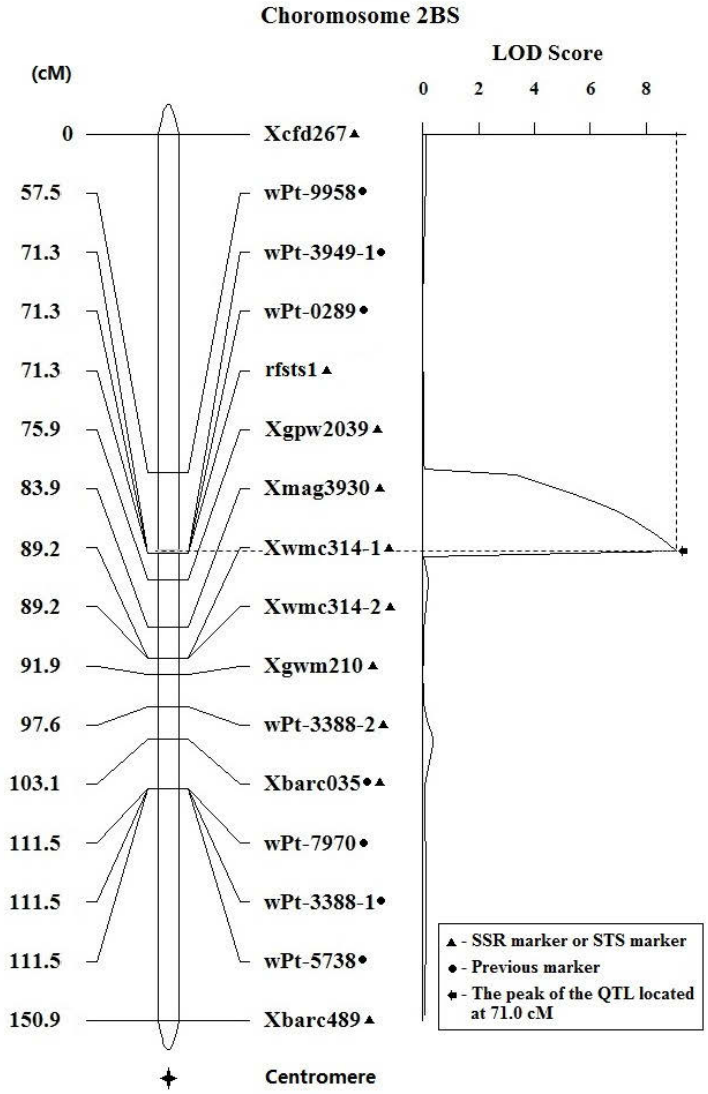

Fig. 3.1: Linkage map of wheat chromosome 2BS harbouring a yellow spot resistance QTL with 7 previous markers (Li et al. 2010) and 9 newly-added PCR-based markers. The peak of the QTL is located at 71.0 cM, and the interval between the closest PCR-based marker, Rfsts1, and the QTL is 0.3 cM.

3.4.3 Markers linked to the QTL

As shown in Fig. 3.1, the major QTLs for yellow spot resistance were mapped between STS marker Rfsts1and DArT marker wPt-9958. Rfsts1 was a newly designed PCR-based marker with a 0.3 cM interval to the peak of the QTL

proximal to the centromere. DArT marker wPt-9958 was 13.44 cM from the QTL towards the distal end of the chromosome. There were no suitable simple PCR-based markers closely linked to the QTL towards the distal side of the chromosome. Two DArT markers wPt-3949-1 and wPt-0289 were located at the same position as Rfsts1.

In addition to Rfsts1, six other newly-designed SSR or STS markers were closely linked to the QTL proximal to the centromere. Linkage distances of SSR markers Xgpw2039 and Xmag3930 were 5.0 cM and 12.9 cM from the QTL proximal to the centromere; SSR marker Xwmc314-1 and STS marker Xwmc314-2 were located at the same locus 18.2 cM from the QTL; and SSR marker Xgwm210 was 21.0 cM from the QTL close the centromere.

3.5 Discussion

Yellow spot has become increasingly serious in recent years, yet current prevention and control methods are less than satisfactory. This has led to an urgent need to breed new cultivars with effective resistance. However, conventional disease screening for yellow spot resistance in the field is both unreliable and time consuming (Brule-Babel and Lamari 1992, Singh et al. 2010) and infection under natural conditions can be compounded by the presence of other foliar diseases (Gilbert and Woods 2001). Therefore, a more reliable and effective method is needed so that the selection of yellow spot resistance can occur with greater breeding efficiency.

Marker-assisted selection offers the most efficient means for identifying yellow spot resistance and is used widely in plant breeding programs targeting other diseases in wheat (Gupta et al. 1999). In particular, molecular markers, based on genotypic variation, can be used for indirect selection of a linked trait of interest; such methods are not influenced by environmental conditions and can be applied at any age of plant development (Gupta et al. 1999). Markers suitable for selection should at least satisfy three characteristics: closely linked to the target gene or QTL, simple PCR-based, and implementable to a large-scale selection/breeding platform (Li et al. 2011). Such markers only require a small amount of DNA, the procedure is simple and fast, the results are more reliable and reproducible than conventional methods, and it is much more cost-efficient (Singh et al. 2010).

In our study, of 41 new markers were evaluated and nine of these, together with seven previously-known markers were linked with the yellow spot resistant QTL on chromosome arm 2BS. All former marker positions agreed with previous maps (Li et al. 2010). Previously, the closest markers were wPt-0289 and wPt-3949-1, which linked to the QTL at interval of 0.34 cM, and remained the closest markers in our study. Unfortunately, both wPt-0289 and wPt-3949-1 are DArT markers, making them difficult to implement in any large-scale marker-assisted selection platform for wheat breeding. Unfortunately, the closest PCR-based marker, Xbarc035, was 2.7 cM from the QTL, hindering its exploitation in wheat breeding. The seven newly-designed PCR-based markers were more closely linked to the QTL than Xbarc035. Of these, the closest was Rfsts1at a distance of 0.3 cM. As Rfsts1 is a PCR-based marker closely linked to the yellow spot resistance QTL, it can be easily implemented in a large wheat

breeding population. A preliminary survey of this marker in Australian cultivars indicated that it fits well with 22 of 25 cultivars screened (F. Ren et al., unpubl.).

The phenotypic variance explained by this QTL is only about 40% and there are other genes/QTLs controlling the trait. Various QTLs for yellow spot resistance have been identified (Faris et al. 1997, Friesen and Faris 2004, Faris and Friesen 2005, Chu et al. 2008, Singh et al. 2008). One of these was located on the long arm of chromosome 5B and has been significantly associated with resistance to *P. tritici-repentis* race 1 explaining 27% of the phenotypic variation (Singh et al. 2008). Marker Xfcp 1 was delimited a 0.4 cM interval to this QTL. We also detected this QTL on chromosome 5B in our Ernie/Batavia DH population, and the percentage of phenotypic variance explained by this QTL was 11.9% and 24.4%, in the two trials conducted (Li et al. 2010). We are now attempting to combine the QTL from chromosome 5BL with that from chromosome 2BS, as together this will enhance both the accuracy and the efficiency of marker-assisted selection in future yellow spot resistance breeding programs.

3.6 Acknowledgements

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Chapter 4

Experimental Chapter Two

Enhancing yellow spot resistance by pyramiding two large-effect alleles in wheat

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With 2 figure and 2 tables

4.1 Abstract

Yellow spot (*Pyrenophora tritici-repentis*) is a major foliar disease in wheat (*Triticum aestivum*) that has become more serious in recent years possibly due to cultural practice change. A major QTL located on the short arm of wheat chromosome 2B which explains 30–40% of the phenotypic variance has been identified, and recently the marker Rfsts1 that is closely (0.3 cM) linked to this QTL was developed. This marker could be applied to a wide range of germplasm. In order to increase the selection accuracy and efficiency, we undertook studies to use this QTL together with an earlier identified yellow spot resistant allele located on the long arm of chromosome 5B to build up a resistance pyramid. The results indicated that the combined selection significantly increase selection efficiency and accuracy.

Key words: wheat — yellow spot — *Pyrenophora tritici-repentis* — resistant allele — germplasm screen — additive — breeding

4.2 Introduction

Yellow spot, caused by the fungus *Pyrenophora tritici-repentis*, is a major foliar disease of wheat (*Triticum aestivum*). It spreads by wind and is present across all wheat growing regions in Australia (Murray and Brennan 2009). The disease may cause up to 50% production losses (Singh et al. 2010a). Host resistance has been shown to be the most reliable, economic and environmental-friendly method to control this disease. Numerous quantitative trait loci (QTL) and major genes for yellow spot resistance have been identified (Faris et al. 1996, Faris et

al. 1997, Friesen and Faris 2004, Faris and Friesen 2005, Chu et al. 2008, Singh et al. 2008, Li et al 2010), including 2AS, 3AS, 4AL, 5AL, 1BS, 2BL, 2BS, 3BS, 3BL, 2DS, 2DL, 7DS, and 1AS, 2BS, 5BL, 3DS (Table 2.2). A gene on chromosome 5BL was the one of the first alleles identified for resistance to yellow spot disease (Faris et al. 1996) and it originated from an immune cultivar W-7976. In 2010, Faris et al. (2010) reported the cloning and characterisation of the Tsn1 gene, which was found to have yellow spot disease resistance gene-like features, including a nucleotide binding site and leucine-rich repeat or serine/threonine protein kinase domains. A marker on 5BL, Xfcp1, that delimited a 0.4 cM interval to the allele, was developed and has been used for MAS during the past few years (Lu et al. 2006). The 2BS chromosome QTL is a newly identified allele by Li et al. (2010) from an immune cultivar Ernie. The QTL explained 38.2%, 29.8% and 36.2% of the phenotypic variance for yellow spot resistance, from three independent trials on four structured populations involving Ernie. An STS marker, Rfsts1, which is located 0.3 cM away from this QTL, was developed (Ren et al. 2014). It is a PCR-based marker closely linked to the yellow spot resistance QTL and it can be easily implemented across a large range of wheat germplasm.

We report studies to build up a resistant pyramid by combining selection of 2BS and 5BL markers together in marker assisted selection breeding, as a means to significantly increase selection accuracy and efficiency for yellow spot resistance. We also report studies to determine if the two markers could produce a banding pattern consistent with the phenotypes of a wide range of germplasm.

4.3 Materials and Methods

4.3.1 Plant materials

The DH population (Li et al. 2010) used for this study consisted of 78 doubled haploid (DH) lines derived from the cross of American soft red winter wheat cultivar Ernie with the Australian cultivar Batavia. Ernie is immune to yellow spot infection, but Batavia is highly susceptible. Together with their parents, the DH population was grown in an air-conditioned glasshouse (maintained at 25/18 (±5) ℃ day/night temperature and 65/80 (±10)% day/night relative humidity with natural illumination) at The University of Western Australia, Crawley, Western Australia, for DNA extraction.

A total of 115 Australia wheat cultivars were screened based on their yellow spot resistance from the National Variety Trials (NVT) (http://www.nvtonline.com.au). According to the information, 62 cultivars (Table 4.1) were selected based on their frequency in different NVT reports and their disease resistance characteristics. Six of the cultivars were highly resistant; 26 were medium resistant; 24 were medium susceptible; and the remaining six were highly susceptible. All these cultivars were germinated in petri dishes in a 25℃ incubation chamber. Seedlings were grown in pots in the same air-conditioned glasshouse until one to two-leaf stages before DNA extraction.

Table 4.1: Australia wheat cultivars used in this research and their resistance level to yellow spot disease

Serial No.	Cultivar	Average Resistance[1]	Serial No.	Cultivar	Average Resistance	Serial No.	Cultivar	Average Resistance
01	Zippy	1.0	22	Calingiri	4.0	43	Lang	5.0
02	Machete	2.0	23	Derrimut	4.0	44	Yandanooka	5.0
03	Yitpi	2.0	24	Drysdale	4.0	45	Yenda	5.0
04	Correll	2.1	25	EGA 2248	4.0	46	Westonia	5.3
05	Frame	2.4	26	GBA ruby	4.0	47	Young	5.8
06	Carinya	2.8	27	Guardian	4.0	48	Cascades	6.0
07	Barham	3.0	28	sunco	4.0	49	EGA Bonnie rock	6.0
08	Camm	3.0	29	sunzell	4.0	50	Espada	6.0
09	EGA Wentworth	3.0	30	Tincurrin	4.0	51	Excalibur	6.0
10	Fang	3.0	31	Catalina	4.3	52	H46	6.0
11	Pugsley	3.0	32	Hartog	4.6	53	Sentinel	6.0
12	rosella	3.0	33	Bumper	5.0	54	Wollaroi	6.0
13	scythe	3.0	34	Carnamah	5.0	55	Ellison	6.1
14	Janz	3.0	35	Datatine	5.0	56	H45	6.3
15	Diamondbird	3.3	36	EGA Bounty	5.0	57	Wyalkatchem	7.0
16	AXE	3.5	37	EGA Burke	5.0	58	King rock	7.0
17	Mitre	3.6	38	EGA Jitarning	5.0	59	LRPB Lincoln	7.0
18	Chara	3.6	39	Fortune	5.0	60	Mace	7.0
19	Annuello	4.0	40	Gladius	5.0	61	Magenta	7.0
20	Arrino	4.0	41	Krichauff	5.0	62	Jandaroi	9.0
21	Bolac	4.0	42	Kukri	5.0			

[1] average resistant value was scored following the reports of NVT. (1.0—Very Susceptible, 2.0—Susceptible to Very Susceptible, 3.0—Susceptible; 4.0—Moderately Susceptible to Susceptible; 5.0—Moderately Susceptible; 6.0—Moderately Resistant to Moderately Susceptible; 7.0—Moderately Resistant; 8.0—Resistant to Moderately Resistant; 9.0—Resistant.)

4.3.2 Disease assessment of a DH population

An initial yellow spot epidemic environment in a glasshouse was established by artificially inoculating two highly susceptible durum wheat varieties (Wallaroi and Bellaroi) with field-infected plants. The main consideration for using the durum varieties for establishing the initial epidemic environment was that they would produce more ascocarps for pathogen sporulation than with the use of more

resistant bread wheat genotypes. This artificial epidemic environment was maintained by repeatedly planting these two susceptible varieties in each corner of the glasshouse maintained at 25/18 (±5)℃ day/night temperature and 65/80 (±10)% day/night relative humidity with natural illumination.

The method used for yellow spot assessment was the description of Adee and Pfender (1989). First, the infested stems of the two susceptible durum varieties were collected, and the amount of inoculum on the stems was estimated by counting the number of mature ascocarps. Second, stems with similar number of mature ascocarps were cut into 10 cm pieces, and five pieces of the infested stem stubble were placed on the surface of each pot when seedlings reached the three-leaf stage. Third, water was sprayed, to promote yellow spot infection, with a backpack sprayer six times a day for seven consecutive days following inoculation. Last, Yellow spot resistance was evaluated by ranking lines from 0 to 5 using the system developed by Lamari and Bernier (1989b) that is based on lesion types in wheat in response to *P. tritici-repentis* (6 = no visible lesion without either chlorosis or necrosis, Very Resistant. 5 = small dark brown to black spot lesions without chlorosis or necrosis, Resistant. 4 = small lesions with very little chlorosis or necrosis, Moderately Resistant. 3 = small lesions, generally not coalescing, completely surrounded by a distinct chlorotic or necrotic ring, Moderately Resistant to Moderately Susceptible. 2 = small lesions, some coalescing, completely surrounded with chlorotic or necrotic zones, Moderately Susceptible. 1 = large lesions consisting of coalescing chlorotic or necrotic zones, Susceptible.) Seventy-eight DH lines and their parents, each with two replicates, were evaluated.

4.3.3 Disease assessment of selected Australia wheat cultivars

The resistance level of selected Australia wheat cultivars for yellow spot was provided by NVT. The rating system used was the NVT disease rating definitions (2013) as follows:

“R” -Resistant- Disease may be found but will be at such a level that no economic management is required, even in instances of high disease pressure;

“MR” -Moderately Resistant- Disease may be observed but no economic management decisions will be required. Preventative sprays not necessary but disease should be monitored. Management of seed quality may be required;

“MS” -Moderately Susceptible- In the presence of inoculum and in seasons conducive to disease, the disease will be seen more readily when inspecting the crop. If the disease appears early in the season then an economic management decision (preventative spray) may be appropriate. Later occurrence of the disease may not require any action. Management of seed quality will be required;

“S” -Susceptible- The disease will be easily found in the crop. Management decisions will be required to reduce yield loss and will most probably be economic to do so. Management of seed quality will be required.); and

“VS” -Very Susceptible- Do not grow this cultivar if the disease in question is a regular occurrence or risk. The cultivar in question can be a complete loss if sown and no disease management is applied.

We assigned the disease resistant levels into 1.0 - 9.0 (1.0—VS, 2.0—S/VS,

3.0—S, 4.0—MS/S, 5.0—MS, 6.0—MR/MS, 7.0—MR, 8.0—R/MR, and 9.0—R) for our data analysis. The higher of the disease index, the more resistant was the cultivar to yellow spot infection.

As two different sets of data were used in this study to show the difference between individuals in the populations and the cultivars, we used six ranking levels: “Very Resistant”, “Resistant”, “Moderately Resistant”, “Moderately Susceptible”, “Susceptible” and “Very Susceptible” to combine the two systems for data analysis.

4.3.4 DNA extraction and molecular marker analysis

During the growing period, young leaves of the target genotypes were sampled and DNA was extracted using a standard CTAB protocol (Doyle and Doyle 1990). PCRs for molecular marker analysis were performed in 15 µl volumes containing approximately 100 ng genomic DNA, 400 nM of each primer, 4µl for 5×PCR polymerisation buffer (Fisher Biotec), 4 nM Mg2+, and 1 unit Taq enzyme with the following program: 95℃ for 5 min, followed by 35 cycles of 95℃ for 30 s, annealing for 30 s at different temperatures for different primers, and 72℃ for 45 s, with a final extension at 72℃ for 5min. PCR products were separated on a 3% agarose gel and/or a 6% poly-acrylamide gel. Gels were documented using the gel documentation system (Vilber Lourmat, France).

Two PCR-based markers were used, viz. Rfsts1, a STS marker 0.3 cM away from a yellow spot resistance QTL located on chromosome 2BS close to the centromere; and, Fcp1, a SSR marker 0.4 cM away from a yellow spot resistance allele located on chromosome 5BL (Lu et al. 2006).

4.3.5 Data analysis

All data were analyzed by Minitab 16 (Minitab Inc., 2010). A one-way analysis of variance (ANOVA) was conducted to detect if there were significant effects for marker assistant selection.

4.4 Results

4.4.1 Marker performance in the DH population (resistant level ranking from 1 to 6)

When the Rfsts1 marker was used, the resistant allele was detected in 33.3% of individuals of the 78 DH populations, and the mean resistance score was 4.9 (Table 4.2 & Fig. 4.1-a); the remaining individuals showed the susceptible allele with the mean resistance score at 4.0. When the Fcp-1 marker was used, the resistant allele was detected in 28.2% individuals of the DH population, and the mean resistance score was 4.7; the remaining individuals showed the susceptible allele with the mean resistance score at 4.2. The mean resistance scores for resistant and susceptible individuals were significantly different ($p<0.05$) for both markers.

The two resistance alleles were simultaneously detected in 15.4% individuals of the DH population with the mean resistance score at 5.4 (a); either one or another resistant allele was detected in 30.8% individuals, and the mean resistance score was 4.2 (b); and the remaining individuals showed both the susceptible alleles with the mean resistance score at 4.0 (c). The mean resistance scores between (a) and (b)/(c) were significantly different ($P<0.01$), however,(b) and (c) were not significantly different ($P>0.05$) (Fig. 4.1-b).

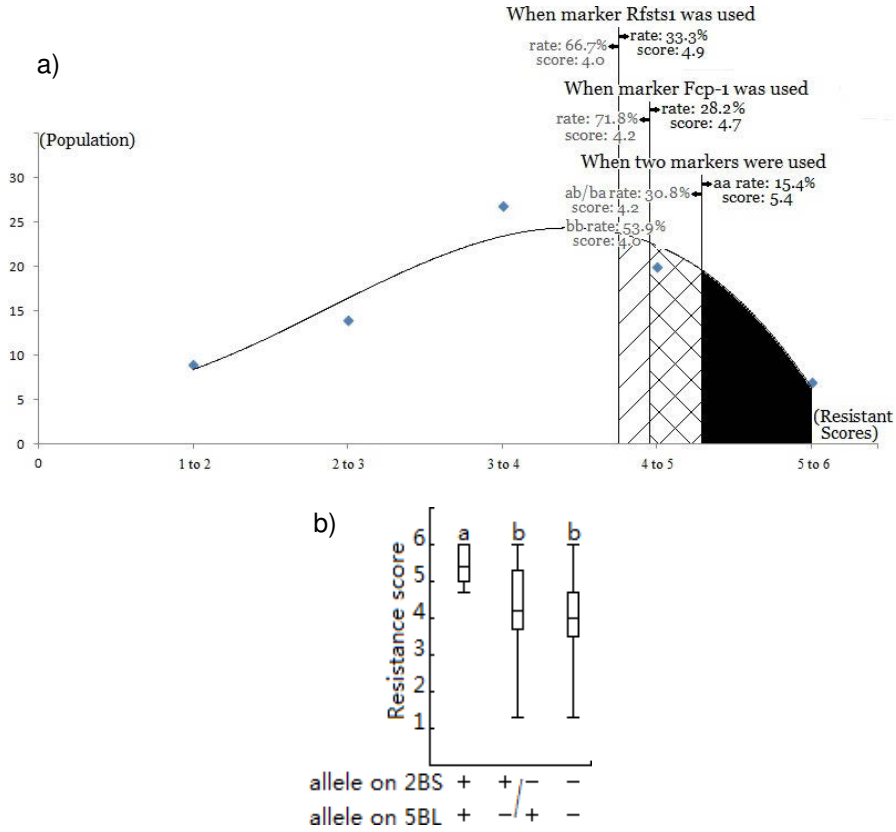

Fig. 4.1: Marker performance in the DH population: **a)** When the Rfsts1 marker was used, the resistant allele was detected in 33.3% and the mean resistance score was 4.9; the rest individuals showed the susceptible band and the mean resistance score was 4.0. When marker Fcp-1 was used, the resistant allele was detected in 28.2% and the mean resistance score was 4.7; the remainder showed the susceptible band and the mean resistance score was 4.2. When two markers were used, both resistant alleles (aa) were detected in 15.4% and the mean resistance score was 5.4; either one or the other resistant allele (ab/ba) was detected in 30.8% and the mean resistance score was 4.2; the remainder showed the susceptible band (bb) and the mean resistance score was 4.0. 'Rate' means the selection rate and 'Score' means the resistance score under this selection rate. The characters in bold indicate the resistant rate and score, which the/both banding pattern(s) is the same as resistant parent 'Erine'; and the characters in gray indicate the susceptible rate and score, where at least one banding pattern is the same as susceptible parent 'Batavia'. **b)** Box plot distributions of resistance scores for yellow spot resistance among lines possessing various combinations of the two targeted alleles. Boxes indicate the 25 and 75 percentiles; the average scores are

indicated by the solid horizontal line. Vertical lines represent the range. The different letters above reticle or bar denote statistically significant at P<0.05 with One-Way ANOVA test.

Table 4.2: The resistant scores and the detection percentage of the DH population and 62 Australian wheat cultivars when one marker or two markers were used for selection

		Only one marker was used				Two markers were used		
		Rfsts1		Fcp-1		RR[3]	RS/SR[4]	SS[5]
		R[1]	S[2]	R	S			
DH population	resistant scores	4.9 a[6]	4.0 b	4.2 a	4.7 b	5.4 a	4.2 b	4.0 b
	percentage	33.3%	66.7%	28.2%	71.8%	15.4%	30.8%	53.9%
62 Australian wheat cultivars	resistant scores	5.1 a	4.2 b	5.9 a	4.4 b	6.7 a	4.9 b	4.0 c
	percentage	43.6%	56.5%	11.3%	88.7%	4.8%	45.2%	50.0%

[1] the marker detected the resistant alleles; [2] the marker detected the susceptible alleles; [3] both resistant alleles were detected; [4] either resistant allele was detected; [5] both susceptible alleles were detected; [6] the different letters behind the scores denote statistically significant at P<0.05 with One-Way ANOVA test.

4.4.2 Marker performance in Australian wheat cultivars (resistant level ranking from 1 to 9)

Markers Rfsts1 and Fcp-1 delineated clear allelic differences among the 62 Australia wheat cultivars tested (Table 4.2 & Fig. 4.2a).

When Rfsts1 was used, the resistant allele was detected in 43.6% cultivars of the 62 Australia wheat cultivars, and the mean resistance score was 5.1; the remaining cultivars showed the susceptible allele and the mean resistance score was 4.2. When Fcp-1 was used, the resistant allele was detected in 11.3% cultivars of the 62 Australia wheat cultivars, and the mean resistance score was 5.9; the remaining cultivars showed the susceptible allele and the mean resistance score was 4.4. The mean scores for resistant cultivars and susceptible cultivars were all significantly different (P<0.01) for when using either

marker.

The two resistant alleles were simultaneously detected in 4.8% cultivars of the 62 Australia wheat cultivars, and the mean resistance score was 6.7; either one or another resistant allele was detected in 45.2% cultivars, and the mean resistance score was 4.9; and the remaining cultivars showed the susceptible alleles and the mean resistance score was 4.0. Overall, there were significant differences ($P<0.05$) across the resistance scores (Fig. 4.2b).

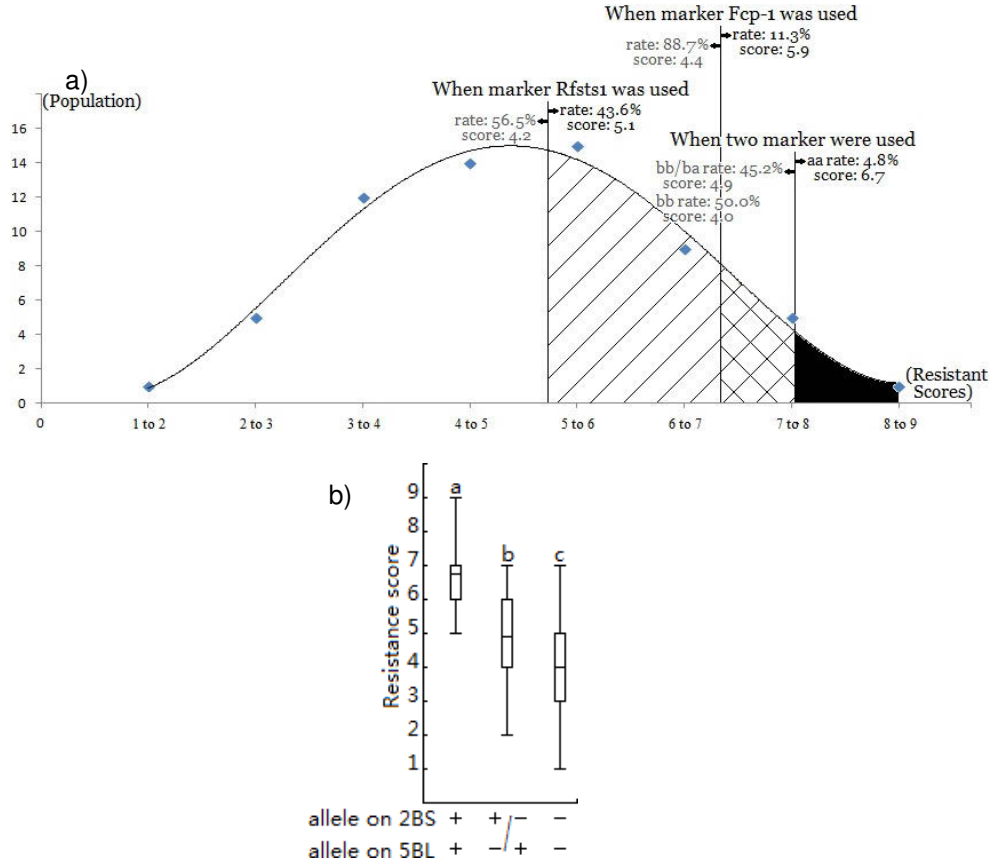

Fig. 4.2: Marker performance in the 62 Australian wheat cultivars: **a)** when the Rfsts1 marker was used, the resistant allele was detected in 43.6% and the mean resistance score was 5.1; the remainder showed the susceptible allele and the mean resistance score was 4.2. When marker Fcp-1 was used, the resistant allele was detected in 11.3% and the mean resistance score was 5.9; the remainder showed the susceptible allele and the mean resistance score was 4.4. When two markers were used, both resistant

alleles (aa) were detected in 4.8% and the mean resistance score was 6.7; either one or the other resistant allele (ab/ba) was detected in 45.2% and the mean resistance score was 4.9; the remainder showed the susceptible band (bb) and the mean resistance score was 4.0. 'Rate' means the selection rate and 'Score' means the resistance score under this selection rate. The characters in bold indicate the resistant rate and score, which the/both banding pattern(s) is the same as resistant parent 'Erine'; and the characters in gray indicate the susceptible rate and score, where at least one banding pattern is the same as susceptible parent 'Batavia'. **b)** Box plot distributions of resistance scores for yellow spot resistance among lines possessing various combinations of the two targeted alleles. Boxes indicate the 25 and 75 percentiles; the average scores are indicated by the solid horizontal line. Vertical lines represent the range. The different letters above reticle or bar denote statistically significant at $P<0.05$ with One-Way ANOVA test.

Significant variation was found in yellow spot resistant value for lines containing the alleles on chromosome 2BS and 5BL from the two assessed (the DH population and the selected 62 Australian cultivars). Comparing with the lines without any of the resistance alleles, the lines with both alleles performed better in yellow spot resistance than those with a single resistant allele.

4.5 Discussion

4.5.1 Suitablility of markers Fcp-1 and Rfsts1 for marker assisted selection

Markers Fcp-1 and Rfsts1 were shown to be suitable markers for marker assisted selection and the most efficient way to select target traits of interest. Marker assisted selection is not influenced by environment and can be used at

any age of plant development (Gupta et al. 1999). A suitable marker for selection should closely link with the target gene or QTL, PCR-based, and implementable to a large-scale selection (Li et al, 2011). Fcp-1 is a SSR marker and 0.4 cM away from the 5BL yellow spot resistant allele. Rfsts1 is a STS marker, which was designed from the sequence of DArT marker wPt-3949-1 (Ren et al. 2014), and 0.3 cM away from the 2BS yellow spot resistant allele. Both markers are PCR-based, and closely linked to the yellow spot resistance alleles. These markers can be easily implemented in a large-scale wheat breeding programs. After the examination of the 62 selected Australian cultivars, it was evident that both markers can amplify PCR product and obtain a clear banding pattern generally consistent with the phenotype in all cultivars. This indicated that these markers perform well with a wide range of germplasm. Hence, markers Fcp-1 and Rfsts1 are suitable for marker assisted selection.

4.5.2 Introduction of the resistance allele on chromosome 2BS with the resistance allele on chromosome 5BL

The allele on chromosome 2BS and the allele on chromosome 5BL are additive. When both the yellow spot resistance allele on chromosome 2BS and that on chromosome 5BL were detected together among the 62 Australian wheat cultivars, the mean resistance score was the highest. However, when neither the resistant allele was detected, both the susceptible alleles were detected and the mean resistance score was the lowest. The mean resistance score was in between, when only one of the yellow spot resistance alleles was detected (Fig. 4.2-a). The exact same conclusion can be made based on the outcome of the DH population (Fig. 4.1-a). The outcome also suggests that additional gene loci are also contributing to the yellow spot resistance as the two known alleles could

not fully explain the phenotypic variation observed. Historically, genetic loci have explained smaller genotypic variations that have been detected, such as the yellow spot resistant alleles located on chromosome 1A and 6A (Li et al. 2010). While our study shows that more research is needed to identify other genome loci responsible for yellow spot resistance to enable further improvements in accuracy and efficiency in MAS to pyramid additional, hopefully all, resistance alleles.

4.6 Acknowledgement

We thank Australia NVT for the yellow spot resistance data.

Chapter 5

General Discussion

Yellow spot has become increasingly serious in recent years, yet current prevention and control methods are less than satisfactory. However, without current prevention and control, the potential average annual loss could be up to $676 million in Australia (Murray and Brennan, 2009). Breeding new effective resistant cultivars offers the best opportunity to manage this disease. To achieve this, molecular marker assistant selection breeding is needed, as conventional breeding methods are both unreliable and time consuming.

5.1 Importance of marker assistant selection and the requirements for markers used in MAS

Molecular markers, based on genotypic variation, can be used for indirect selection of a linked trait of interest at any age of plant growing, and it will not influenced by environmental conditions (Gupta et al. 1999). Markers suitable for selection should at least satisfy three characteristics: be closely linked to the target gene or QTL, be simple PCR-based, and be implementable to a large-scale selection/breeding platform (Li et al. 2011). When applying these kind of markers, only a small amount of DNA will be required, the procedure is simple and fast, the results are reliable and reproducible, and it is much more cost-efficient than conventional methods (Singh et al. 2010).

5.2 Rfsts1 marker is a promising marker for marker assistant selection in yellow spot resistant breeding program

Forty-one new markers were screened; nine of these, together with seven previously-known markers, were linked with the yellow spot resistance QTL on the short arm of chromosome 2B. All former markers are in the same positions

as in previous maps (Li et al. 2010). Historically, the closest PCR-based marker, Xbarc035, was 2.7 cM from the QTL, hindering its exploitation in wheat breeding. All the seven newly-designed PCR-based markers were more closely linked to the yellow spot resistant QTL than Xbarc035. A newly designed STS marker Rfsts1 located at the same position as for what previously consituted the closest markers, wPt-0289 and wPt-3949-1, was linked to the QTL at interval of 0.34 cM, and remains the closest marker in our study. Unfortunately, in previous study both wPt-0289 and wPt-3949-1 are DArT markers, making them difficult to implement in any large-scale marker-assisted selection platform for wheat breeding. However, Rfsts1, at a distance of 0.34 cM, is a PCR-based marker closely linked to the yellow spot resistance QTL; it can be easily implemented into a large wheat breeding population.

After identifying the Rfsts1 marker, 62 selected Australian cultivars were utilised to test the marker's germplasm performance. Rfsts1 amplified the PCR product and produced a clear banding pattern generally consistent with the phenotype across all the cultivars. This indicated that the Rfsts1 marker performs well with a wide range of germplasm.

The Rfsts1 marker was suitable for molecular marker assistant selection and generally satisfies three important characteristics, viz. a) it was closely linked to the target QTL, with a distance of 0.34 cM; b) it was a STS marker, which is a PCR-based marker; and c) it was suitable for implementation into a large-scale selection/breeding platform. Hence, the Rfsts1 marker is a promising marker for marker assistant selection in yellow spot resistant breeding programs.

5.3 The Fcp-1 marker is a promising marker for marker assistant selection in yellow spot resistant breeding program

The Fcp-1 marker was shown to be a promising marker for marker assistant selection in yellow spot resistant breeding program. Fcp1 is a SSR marker, 0.4 cM away from a yellow spot resistance allele located on chromosome 5BL (Lu et al. 2006). After screening with 62 selected Australian cultivars to test the marker's performance across a wide range of germplasm, Fcp-1 successfully amplified PCR product and produced a clear banding pattern generally consistent with the phenotype in all cultivars.

The Fcp-1 marker is clearly suitable for molecular marker assistant selection, and it also satisfied three important characteristics, viz. a) it was closely linked to the target allele, with a distance of 0.4 cM; b) it was a SSR marker, which is a PCR-based marker; and c) it was suitable for implementation into a large-scale selection/breeding platform. Hence, like the Rfsts1 marker, the Fcp-1 marker is also a promising marker for marker assistant selection in yellow spot resistant breeding programs.

5.4 The allele on chromosome 2BS and the allele on chromosome 5BL are additive

Our results strongly suggest that the two resistant alleles, viz. the allele on chromosome 2BS and the allele on chromosome 5BL, are additive. When the yellow spot resistance alleles on both chromosome 2BS and chromosome 5BL were detected together among the 62 Australian wheat cultivars, the mean resistance score was the highest. In contrast, when neither the resistant allele

was detected, rather both the susceptible alleles were detected, the mean resistance score was the lowest. The overall mean resistance score was intermediate to those two extremes, as expected when only one of the yellow spot resistance alleles was detected. The exact same conclusion could be made based on the outcome of the DH population. The outcome also suggests that there are further additional gene loci that also contribute to the yellow spot resistance as these two known alleles on chromosomes 2BS and 5BL cannot fully explain the phenotypic variation observed. Based on earlier research, genetic loci explaining smaller genotypic variation have been detected such as the yellow spot resistant alleles located on chromosome 1A and 6A (Li et al. 2010).

The reports of Boer et al. (2014) and Salgotra et al. (2012) describe studies to combine several resistant alleles to explore the interactions of those alleles in lettuce and rice. Both studies indicate that the effects of resistances from those alleles in lettuce and rice were not all show additive in the contrast to our study, where the effects of the resistances on chromosome 2BS and 5BL alleles were clearly additive. More research is needed to more fully define the interactions between different yellow spot resistant alleles, and also to identify further additive genome loci responsible for yellow spot resistance. In this way, there is still significant additional scope to further improve the accuracy and efficiency in MAS from pyramiding a greater number of additive resistance alleles in the future.

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