Genetic Characterisation and Fine Mapping of Sources of Durable Resistance to Stripe Rust in Selected Wheat Genotypes

By

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DECLARATION

"I, Gloudina Maria Agenbag, declare this thesis hereby submitted by me for the degree Doctor of Philosophy at the University of the Free State is my own independent work and has not previously been submitted by me to another university for any degree."

"I cede copyright of this thesis in favour of the University of the Free State."

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Gloudina Maria Agenbag Date

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Abbreviations

3’	three prime
5’	five prime
X^2	chi-square goodness of fit
AFLP	amplified fragment length polymorphism
$AgNO_3$	silver nitrate
ANOVA	analysis of variance
APR	adult plant resistance
APS	ammonium persulphate
ARC-SGI	Agricultural Research Council Small Grain Institute
avr	virulence
Avr	avirulence
BARC	Beltsville Agriculture Research Center
BC	back-cross
BLAST	Basic local alignment tool
BLAT	BLAST-Like Alignment Tool
bp	base pair
CC	coiled-coil
cDNA	complementary DNA
CFA	Clermont-Ferrand A genome
CFD	Clermont-Ferrand D genome
CIM	composite interval mapping
CIMMYT	International Maize and Wheat Improvement Centre, Mexico
cM	centiMorgan
CTAB	cetyltrimethylammonium bromide
cv.	cultivar
DAFF	Department of Agriculture, Forestry and Fisheries Market Value Chain Profile
DArT	Diversity Arrays Technology
ddNTP	2',3'-di-deoxynucleoside 5'-triphosphate
-dF/dT	first derivative of fluorescence over temperature
DH	doubled haploid
dH_2O	distilled water
dHPLC	high pressure liquid chromatography
DNA	deoxyribonucleic acid
DGGE	denaturing gradient gel electrophoresis
dNTP	2’-deoxynucleoside 5’-triphosphate
dATP	deoxyadenoside triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
dUTP	deoxyuridine triphosphate
E-value	expect value
EDTA	ethylene-diaminetetraacetate
EST	expressed sequence tag
eSTS	expressed sequence tagged site
ETI	effector triggered immunity
f. sp.	*formae specials*
FHB	*Fusarium* head blight
FRET	fluorescence resonance energy transfer
F_x	generation
GDM	Gatersleben D genome Microsatellite
gDNA	genomic DNA
GLR	general linear regression
GWM	Gatersleben Wheat Microsatellite
HRM	high resolution melt
HTAP	high-temperature adult plant

ICIM	inclusive composite interval mapping
IDT	Integrated DNA technologies
IL-1R	interleukin-1 receptor
IM	interval mapping
indel	insertion/deletion
ITMI	International Triticeae Mapping Initiative
IWGSC	International Wheat Genome Sequencing Consortium
LAI	leaf area infected
LNA	locked nucleic acid
LOD	logarithm of the odds
Lr	wheat leaf rust resistance gene designation
LR	likelihood ratio
LRR	leucine-rich repeats
LTN	leaf tip necrosis
LZ	leucine zipper
MALDI-TOF	matrix-assisted laser desorption ionisation-time of flight
MAMP	microbe-associated molecular patterns
MAS	marker-assisted selection
Mb	mega base pairs
mer	oligomer
$MgCl_2$	magnesium chloride
MIM	multiple interval mapping
ML	maximum likelihood
MQM	multiple trait/QTL mapping
NaCl	sodium chloride
$NaCO_3$	sodium carbonate
NBS	nucleotide-binding site
NBS-AFLP	nucleotide-binding site amplified fragment length polymorphism
NCBI	National Centre for Biotechnology Information
NH_2	amino group
NIL	near isogenic line
PAMP	pathogen-associated molecular patterns
PCR	polymerase chain reaction
Pm	wheat powdery mildew resistance gene designation
PRR	pattern recognition receptor
Pst	*Puccinia striiformis* f. sp. *tritici*
PTI	pathogen-associated molecular patterns triggered immunity
qPCR	quantitative PCR
QTL	quantitative trait loci
r	correlation coefficients
R gene	functional resistance gene
r gene	non-functional resistance gene
R^2	phenotypic variance
RAPD	random amplified polymorphic DNA
RCF	relative centrifugal force
RFLP	restriction fragment length polymorphism
RFU	relative fluorescent units
RGA	resistance gene analogue
RIL	recombinant inbred line
RNA	ribonucleic acid
RT	reaction type
SAGL	South African Grain Laboratory
SDS	sodium dodecyl sulphate
SNP	single-nucleotide polymorphism
Sr	wheat stem rust resistance gene designation
SRAP	sequence-related amplified polymorphism
SSCP	single-strand conformation polymorphism
SSR	simple sequence repeat
STS	sequence tagged site
T_A	annealing temperature

TA	transcript assembly
TBE	tris/borate/EDTA
TEMED	tetramethylethylenediamine
TIR	toll and interleukin-1 receptor
T_M	melting temperature
Tris base	tris(hydroxymethyl)aminomethane
TTE	tris/taurine/EDTA
t-test	student t-test
UK	United Kingdom
UNG	uracil-DNA glycosylase
USA	United States of America
v.	version
VNTR	variable number tandem repeat
WMC	Wheat Microsatellite Consortium
Yr	wheat stripe rust resistance gene designation

International system (SI) units used for quantity and concentration measures

Large wrote in 1940, “The greatest single undertaking in the history of plant pathology was to be the attack on rust in cereals”. This effort continues.

Large EC (1940) The advances of the fungi. Jonathan Cape, London

1. OVERVIEW AND OBJECTIVES

This study focussed on the genetic analysis of durable stripe rust resistance sources and the characterisation of quantitative trait loci (QTL) which can be incorporated into South African wheat cultivars. Two wheat genotypes with adult plant resistance to stripe rust were chosen, namely the cultivars Kariega and Cappelle-Desprez, and the genetic components responsible for their resistance were investigated using a QTL mapping strategy. The project is divided into two main sections and will be presented in separate chapters: 1) Identification of adult plant resistance to stripe rust in the wheat cultivar Cappelle-Desprez (Chapter 4) and 2) Fine mapping stripe rust resistance QTL in a Kariega X Avocet S population (Chapter 5). These chapters will be preceded by the General Introduction (Chapter 2) which will give the reader an overview of wheat breeding in South Africa, stripe rust as an internationally important disease and the use of DNA marker technology for the identification of stripe rust resistance QTL. The application of markers in breeding programs will also be discussed. This introduction will be followed by the General Materials and Methods (Chapter 3) containing the protocols applicable to both the Cappelle-Desprez and Kariega studies from Chapters 4 and 5. Chapters 4 and 5 each include an Introduction to the particular study, Materials and Methods, Results and a Discussion of the results. These chapters will lead into a General Discussion (Chapter 6) on the relevance of the findings to stripe rust resistance and wheat breeding, with comments on the specific objectives set out initially when the study commenced. The thesis will be concluded with some general remarks (Chapter 7). Complete records for the literature referred to throughout Chapters 2 to 7 can be found in the References section (Chapter 8). Conference contributions resulting from work done for this study are listed in Appendix I and a paper submitted to a peer reviewed journal on the Cappelle-Desprez QTL study is included as Appendix II.

2. GENERAL INTRODUCTION

2.1 Domestication of wheat

The earliest signs of cereal domestication appeared around 8 000 years B.C. in a small core area near the upper reaches of the Tigris and Euprates rivers, in present-day southeastern Turkey and northern Syria. This region, referred to as the Fertile Crescent, is considered to be the cradle of cereal agriculture (Lev-Yadun *et al.* 2000). Wheat crops cultivated today descended from the einkorn and emmer wheat that grew wild in the Fertile Crescent (Dvorak *et al.* 1998). The cultivated tetraploid wheat *Triticum turgidum* (AABB), is derived from the wild emmer tetraploid *Triticum dicoccoides* (AABB), which in turn was derived from the natural hybridisation between a diploid wild einkorn wheat (diploid AA; closest present day relative *Triticum monococcum*) and an ancestor of *Aegilops speltoides* (diploid BB). A chance hybridisation between cultivated emmer wheat (AABB) with the wild grass *Aegilops tauschii* (diploid DD) gave rise to the progenitor of modern bread wheat, *Triticum aestivum* (hexaploid AABBDD) (Snape and Pánková 2006; Matsuoka 2010) (Fig. 2.1). Each of the A, B and D genomes contains seven chromosomes. Even though *T. aestivum* is an allohexaploid it behaves as a diploid during meiosis. Pairing of chromosomes is governed by the genes *Ph1* (chromosome 5B) and *Ph2* (chromosome 3D) which ensure pairing of chromosomes only occurs between homologues of the same genome, and not between homoeologues across genomes (Gill *et al.* 2004). Hexaploid wheat has the largest genome among agricultural crops, estimated to be 16 000 Mb, with an average of 810 Mb per chromosome (Gupta *et al.* 1999), approximately eight times the size of the maize genome and forty times the size of the model crop rice (Arumuganathan and Earle 1991). The haploid wheat genome contains 17 pg DNA of which 80% is repetitive DNA sequences believed to have been created mainly as a result of extensive duplication events (Devos and Gale 2000). Genetic polyploidisation leads to genes becoming inactive or diverting to a new function. Alternatively dosage compensation allows for duplicated gene functions. This happens through the accumulation of spontaneous mutations and different methylation patterns (Levy and Fieldman 2002).

The tribe *Triticeae* is economically the most important group of the family *Gramineae*, containing cereal crops as well as lawn and forage grasses cultivated and growing around the world (Kellogg 1998), of which wheat is the leading cereal grain consumed and traded in the world today.

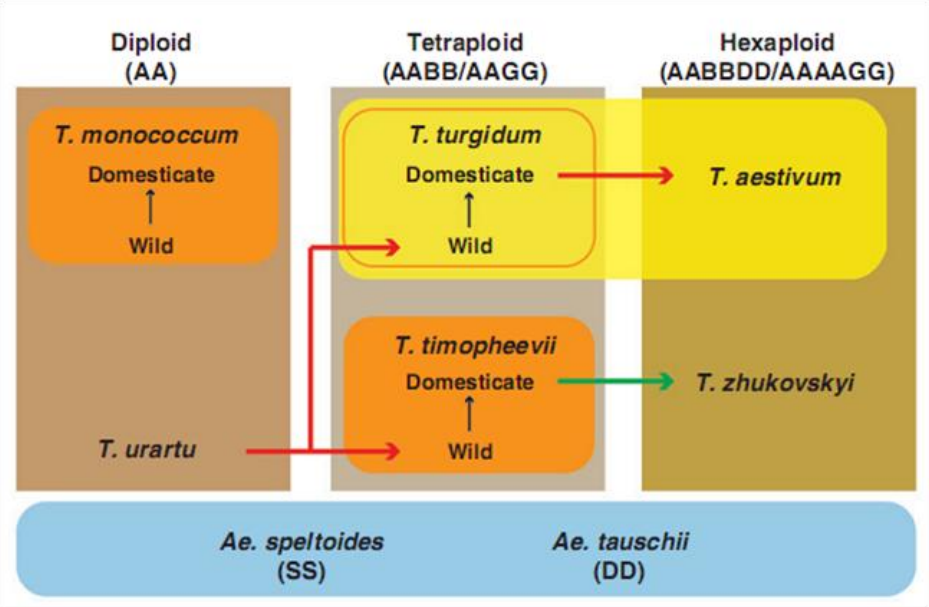

Figure 2.1 An overview of the evolution of polyploidy wheat. The red arrow indicates the allopolyploidisation events that involved the *Aegilops* species, whereas the green arrow indicates the allopolyploidisation event that involved the *Triticum* species, with the vertical arrows denoting the domestication events (figure reproduced from Matsuoka 2010).

2.2 Wheat breeding in South Africa

Wheat has been cultivated in South Africa since 1652 when the first Dutch settlers came ashore on the southern tip of Africa, to establish a refuelling station for passing ships at the Cape of Good Hope (Du Plessis 1933). The new community expanded north and wheat became a significant crop under production. A wider array of genotypes was now in demand due to the different climatic conditions, soil characteristics and altitude. Wheat production areas in South Africa can be considered a microcosm of what is encountered in the rest of the world. European cultivars therefore had to be adapted to local growing conditions. The first wheat breeding program was established in 1891 (Smit *et al.* 2010). Attempts to produce varieties adapted to the many ecological niches in South Africa historically led to the release of a large number of cultivars. Van Lill and Purchase (1995) reported a yield improvement of 87% and improvements in baking quality of 20% between 1930 and 1990. Today wheat breeding is conducted and coordinated by three entities. The Agricultural Research Council Small Grain Institute (ARC-SGI) (previously known as the Small Grain Centre) was established in 1976 as a merger of four provincial breeding programs (Van Niekerk 2001). There are currently two private wheat breeding institutions operational in South Africa. PANNAR SEED began wheat breeding program in the early 1990s, while Sensako (Pty) Ltd was established in the 1960s, functioning as part of Monsanto, South Africa for a few years before becoming independent in 2009.

South Africa has three distinct wheat production areas. Winter wheat is sown under dryland (rain-fed) conditions in the Free State province on stored soil moisture accumulated during the preceding

summer and autumn. Dryland spring wheat is grown in the Mediterranean climate of the Western Cape province, while irrigated spring wheat types are grown adjacent to major rivers in summer rainfall areas. Each of these areas presents their own challenges and specific requirements. The largest quantity of wheat is produced in the Western Cape (36%), Free State (32%) and Northern Cape (14%) provinces with the remaining 18% collectively produced in the Eastern Cape, North West, Mpumalanga, Limpopo, Gauteng and KwaZulu-Natal [Department of Agriculture, Forestry and Fisheries Market Value Chain Profile (DAFF) 2010/2011)]. Wheat is therefore grown to some extent in all nine provinces. The yield in the main production areas ranges from 6.5 tons/ha in the Northern Cape irrigation area down to 2.0 tons/ha in the Free State summer rainfall region. South African wheat farmers produced just under 2 million tons of grain in 2009 on approximately 650 000 ha, of which 80% is derived from dryland and 20% from irrigation wheat cultivars (DAFF 2010/2011). Over the past decades there has been a significant decline in the total wheat area planted, from about 1 million ha in 2000 to only 642 000 ha in 2009 (Bureau for Food and Agricultural Policy 2010). This decline can be directly attributed to reduced financial profits experienced by wheat farmers. Relatively low yields, in particular for wheat cultivated under rain-fed conditions, concurrent with lower wheat prices and steadily increasing input costs have had a negative impact on wheat production in South Africa. The local wheat production is not sufficient to meet the domestic requirements of approximately 2.9 million tons per year. Wheat is therefore imported to meet this demand [South African Grain Laboratory (SAGL) 2008/2009].

The three most important characteristics to release superior bread making wheat cultivars are high yield, good bread baking quality and disease and pest resistance, which ultimately relates to yield and quality. However, a myriad of other criteria are also considered, including vernalisation requirement, plant height, tillering ability, growth period, lodging resistance, drought and mineral stress tolerance, and preharvest sprouting. The development of adapted wheat cultivars for South African growing conditions has been the main priority of research initiatives for the past 25 years (Smit *et al.* 2010). Wheat farmers are tormented by a vast array of pests and diseases; the most common diseases occurring in South Africa include *Stagonospora nodorum* glume blotch, crown rot, take-all, eyespot, *Fusarium* head blight (FHB) and Karnal bunt, and the most common pest being the Russian wheat aphid. At the top of this list are the rust diseases, which are currently receiving a great deal of attention due to the recent epidemics world wide. Commonly diseases are managed through the farming practices of crop rotation and the application of pesticides.

2.3 Stripe rust disease

The economically important rust diseases of wheat include stem (or black) rust caused by *Puccinia graminis*, leaf (or brown) rust caused by *Puccinia triticina* and stripe (or yellow) rust caused by *Puccinia striiformis*. Stripe rust was first described by Gadd in 1777 (Eriksson and Henning 1896). Initially it was named *Puccinia glumarum*, but Hylander *et al.* (1953), followed by Cummins and

Stevenson (1956) revived the name *Puccinia striiformis*. *Puccinia striiformis* has its hosts only in the *Poaceae* (*Gramineae)* family and in the cereal group, wheat and barley being the principal hosts. Stripe rust is an important disease of bread wheat in areas where cool and moist environmental conditions prevail (McIntosh 1980; Stubbs 1985; Danial 1994). Park (1990) defined days as being stripe rust favourable when the mean temperature falls within the range of 12.4 to 18.4°C and the minimum between 7.3 and 14.6°C. Environmental conditions appear to be more critical for the development of stripe rust in comparison to the other rusts. Stripe rust infection leads to reduced plant vigour and limited grain filling (Russel *et al.* 2000) and it is most damaging before and after flowering.

2.3.1 Biology of the fungus

Rust fungi are obligate biotrophic basidiomycetes that are dependent on the host plant to complete their life cycle. Stripe rust can be further subdivided into *formae specials* (f. sp.) depending on the specific host to which a particular form is adapted and on which it is able to complete its asexual lifecycle. *Puccinia striiformis* f. sp. *tritici* (*Pst*) is capable of infecting wheat. *P. striiformis* is not homogenous and within each *formae speciales* there are specific pathotypes (also referred to as physiologic races or strains) that are able to propagate on particular wheat genotypes (Anikster 1984). Pathotypes are differentiated by the infection types produced on a set of selected wheat genotypes or single resistance gene lines referred to as differentials.

The life cycle of wheat rusts involves five spore stages, on two unrelated hosts (Kolmer *et al.* 2009). Teliospores germinate at the beginning of the new growing season, from where it oversummered on dead host tissue in structures called telia. The diploid teliospores undergo meiosis and produce haploid basidiospores, which are released into the air. Basidospores will only infect the alternate host, producing haploid colonies called pycnia. Pycniospores on the host's surface undergo plasmogamy, whereby two cells combine to form a single cell. The rust then forms the aecium, producing large numbers of dikaryotic aeciospores capable of travelling long distances. Aeciospores germinate and invade host tissue through the stomata. This infection results in a pustule called an uredinium which contains urediniospores. This growth stage is asexual and can repeat on the same hosts within one season. At the end of the growing season, uredinia convert to telia producing more hardened teliospores. Stem and leaf rust are considered to be heteroecious and their alternate host and sexual stages are known. The life cycle of stripe rust (Fig. 2.2) was believed to be autoecious, having only a gramineous host upon which its reproduction was exclusively asexual. However, the production of teliospores on cereals indicated a possible sexual cycle (Roelfs *et al.* 1992). Recently evidence was found showing that *Berberis* species can act as an alternative host for the wheat stripe rust pathogen (Jin 2011).

Parallel stripes of yellow/orange coloured pustules on the leaf surface of adult wheat plants give stripe rust its common name. *P. striiformis* urediniospores germinate at low temperatures and high relative humidity on the surface of wheat leaves (Fig. 2.3). A germ tube forms, which enters the leaf directly

through a stoma, without forming an appressorium as occurs with leaf and stem rust (De Vallavieille-Pope *et al.* 1995). A substomatal vesicle forms at the tip of the germ tube from which up to three infection hyphae differentiate. Upon contact with a plant cell a haustorium mother cell differentiates at the tip of the infection hypha, a penetration peg breaches the plant cell wall and a haustorium feeding body forms within the living plant cell. Nutrients are taken up by the haustoria and hyphae form long branches growing up and down the length of the leaf. Some 10 to 14 days after spore germination pustules are produced on the leaf surface containing urediniospores, which are subsequently released and dispersed by the wind. Stripe rust develops systemically in host tissue, whereas stem and leaf rust only produce one new pustule from each infection site (Singh *et al.* 2003). The complete asexual life cycle can take as little as ten days under ideal conditions. Therefore, the disease cycle can be repeated many times in a single growing season. At the end of the growing season telia bearing teliospores are sometimes produced on leaf tissue.

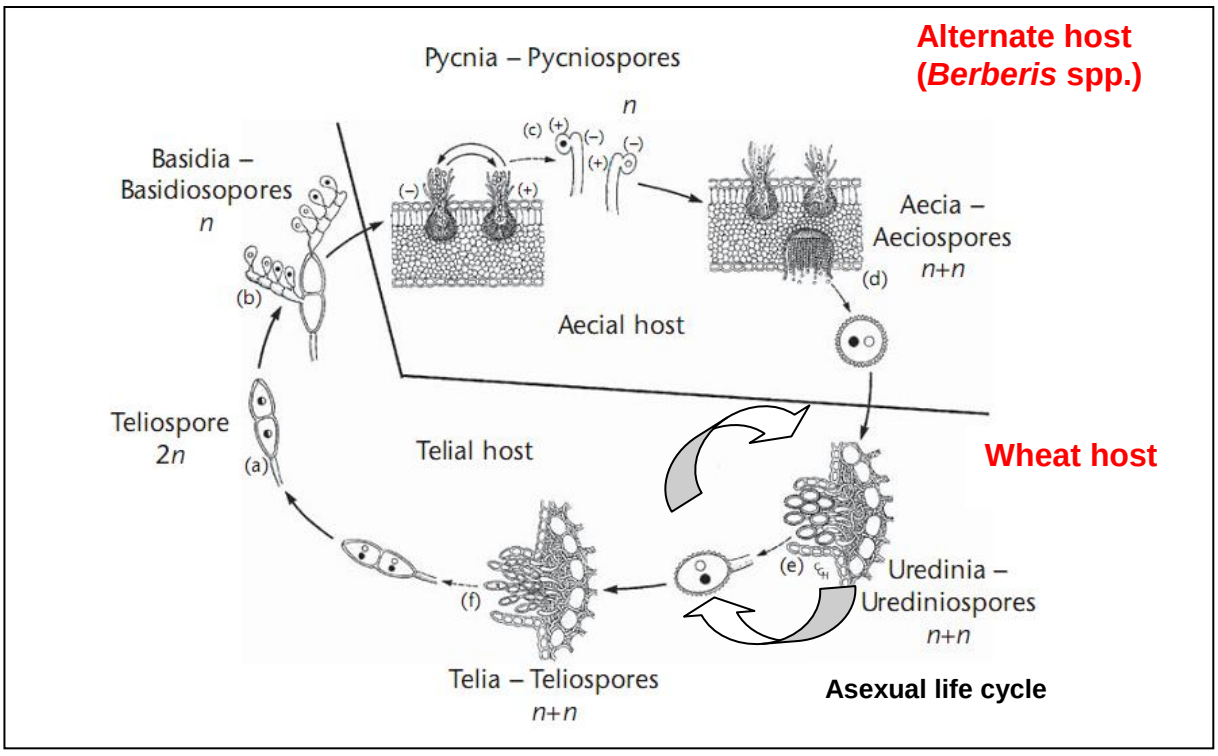

Figure 2.2 The life cycle of *Puccinia striiformis.* (a) Mature, diploid teliospore, (b) basidia with basidiospores, (c) pycnial (sprermogonial) stage, (d) aecial stage, (e) uredinial stage and (f) telial stage (figure adapted from Kolmer *et al.* 2009).

The ability of the uredinial and mycelial stages of the stripe rust pathogen to survive near wheat fields through the non-cropping season plays an important role in disease onset and the increase of inoculum during early growth stages. The summer and autumn survival of the stripe rust fungus is dependent on susceptible volunteer or self-sown wheat plants and, to a lesser extent, on grass species (Wellings and McIntosh 1981; Stubbs 1985). Due to the higher elevation, wheat is frequently

grown in summer in Lesotho, providing a green bridge for stripe rust survival in South Africa (Pretorius *et al.* 2007).

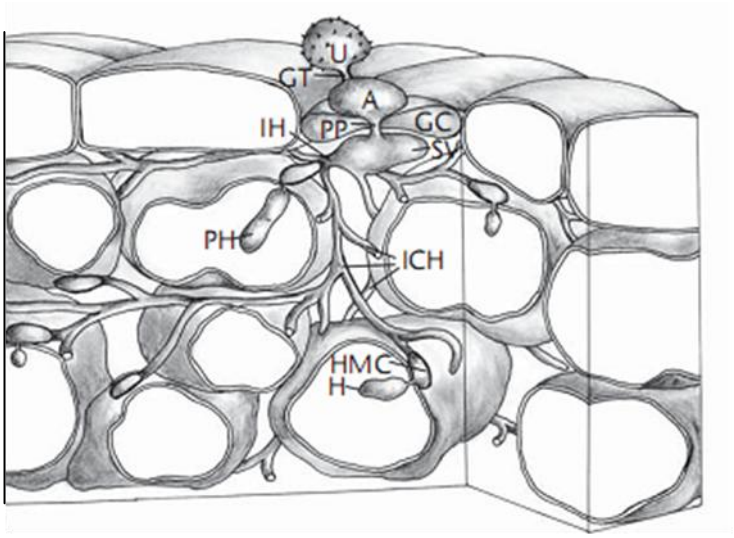

Figure 2.3 Diagrammatic representation of infection structures of a rust fungus. Uredinial infection structures; U, urediniospore; GT, germ tube; A, appressorium (rarely formed by *P. striiformis*); GC, stomatal guard cell; PP, penetration peg; SV, substomatal vesicle; IH, infection hypha; PH, primary haustorium; ICH, intercellular hyphae; HMC, haustorial mother cell; H, additional haustorium (figure from Kolmer *et al.* 2009).

2.3.2 World distribution and virulence

The rust fungi are highly specialised pathogens and significant variation exists in their populations (Johnson 1992). New races have previously been thought to be formed only by mutation and somatic recombination among existing genotypes, followed by selection (Stubbs 1985). But with the sexual stage of *P. striiformis* now identified (Jin 2011), other mechanisms are available which allows for changes in the pathogen population to occur. However, single step mutations remain the predominant cause of variability (Justesen *et al.* 2002; Chen 2005; Wellings 2007; Chen *et al.* 2009). Aggressiveness among isolates is determined by infection efficiency, which is determined by differences in latent period, spore production (length of lesion after point of inoculation of leaf) and how the host and environment influence these factors (Pariaud *et al.* 2009).

In Mexico stripe rust was detected for the first time on *Hordeum jubatum* in 1896 and in Chile on *Hordeum chilense* in 1919 (Hassebrauk 1965). In 1915 stripe rust was documented in the United States of America (US) (Line 2002; Boyd 2005), but epidemics were scarce until serious outbreaks were reported in the Western US and China in the 1950s (Line 2002; Chen *et al.* 2009). Initially stripe rust was found only in the western states, until 1941 when it was also observed in the central plains of Texas. The most widespread epidemic in US history was experienced in 2000 (Milus *et al.* 2009). Its first appearance on wheat was observed in Argentina in 1929. Stripe rust was first reported in Egypt

in 1904, but in central Africa, stripe rust was first reported in northern Zambia in 1958 (Angus 1965). Stripe rust did not occur in Australia until 1979 (O'Brien *et al.* 1980) when it was probably introduced on the clothing of a traveller from Europe (Wellings *et al.* 1988). Wind dispersal then carried the disease to New Zealand a year later (Wellings and McIntosh 1990). A new exotic introduction of stripe rust was detected in Western Australia in August 2002 (Wellings *et al.* 2002). Stripe rust was observed in South Africa in 1996 for the first time (Pretorius *et al.* 1997).

The distribution of stripe rust is therefore now global, being common in Northern Europe (Jagger 2009), the Middle East (Akar *et al.* 2007), China (Chen *et al.* 2009), Eastern and Southern Africa (Pretorius *et al.* 1997), Australia (Wellings *et al.* 2002), New Zealand (Wellings and McIntosh 1990) and the US (Line 2002). More than 20 different stripe rust pathotypes have been detected in Australia and New Zealand since the original introduction in 1979, adding virulence for several resistance genes (Wellings and McIntosh 1990; Wellings *et al.* 2000). Stripe rust is also becoming increasingly important in the US where 42 pathotypes, of which 21 are new virulence combinations, were detected in 2000 (Chen *et al.* 2002). Regions most at risk for stripe rust epidemics in future, determined from data over the period 2000-2010, include the US (Pacific Nort West), East and South Asia (China and Nepal), Oceania (eastern Australia) and East Africa (Kenya) (Wellings 2011). Epidemics have been experienced in these regions in most seasons, with expected regional losses ranging from 5-25%. It is important to continue to monitor the stripe rust population for pathotype changes so that new pathotypes, with the potential to overcome resistance genes currently deployed can be detected early. Stripe rust has been continually extending its geographic range over the past decades and the appearance of more aggressive strains is alarming (Chen 2005; Hovmøller *et al.* 2008; Milus *et al.* 2009).

2.3.3 Introduction to South Africa

The occurrence of stripe rust in the former Transvaal province was mentioned by Verwoerd in a 1935 document, but the accuracy of this is questionable and no official plant disease records describe stripe rust in the early years of wheat breeding (Pretorius *et al.* 2007). Stripe rust was formally observed for the first time in South Africa near Moorreesburg, in the Western Cape during August 1996 (Pretorius *et al.* 1997). It was initially identified in this winter rainfall region under rain-fed conditions on the spring wheat cv. Palmiet. Subsequent surveys at the time of detection showed that the disease was well established in wheat fields in the western and northern parts of the Western Cape, as well as at Nieuwoudtville in the Northern Cape Province (Boshoff *et al.* 2002a). Further evidence of the disease was found in southern parts of the Western Cape, the Eastern Cape and as far north as Rietrivier, in an irrigated summer rainfall region near Kimberley. The initial location and time of the introduction, therefore, could not be determined. Lower than average minimum and maximum temperatures, exacerbated by above-average rainfall recorded during August and September 1996 contributed significantly to the establishment, spread, and subsequent epidemic outbreak of stripe rust in the Western Cape. The fact that almost all of the cultivars grown in this area in 1996 were susceptible to the pathotype that was initially identified contributed to the epidemic. This

necessitated extensive and often repeated applications of fungicides. During 1997 stripe rust spread to the remaining wheat producing regions, including the provinces of KwaZulu-Natal, Gauteng, the North West and Northern province. Within two years *P. striiformis* therefore became endemic to all the major wheat producing areas of South Africa (Boshoff *et al.* 2002a). The wheat stripe rust fungus is well-known for its pathogenic variability and its ability to acquire virulence for previously effective resistance genes (Wellings 2007). In South Africa this called for the development of coordinated control strategies, including studies of pathogen diversity and host plant resistance.

Only one pathotype, 6E16A- (virulent to *Yr2*, *Yr6*, *Yr7*, *Yr8* and *Yr17*) was detected in 1996 (Pretorius *et al.* 1997; Boshoff *et al.* 2002b). Pathotype 6E16A- was previously detected in East and North Africa, southern Europe, the Middle East, and Western Asia (Badebo *et al.* 1990; Louwers *et al.* 1992; Enjalbert *et al.* 2005). Since 2004, similar isolates have been repeatedly detected in northern Europe (Hovmøller and Justesen 2007). During 1998 the presence of a second pathotype, 6E22A- with additional virulence to *Yr25* was recorded. In 2002 a new variant, 7E22A-, virulent to *Yr1*, was detected on the cv. Chinese 166 in a trap nursery in the highlands of Lesotho (Pretorius *et al.* 2007). The pathotype 6E22A+ was identified in 2005, defeating *YrA* (showing virulence to *Yr2*, *Yr6*, *Yr7*, *Yr8*, *Yr17*, *Yr25* and *YrA)* (Pretorius ZA, unpublished). These four pathotypes are the only races detected thus far in South Africa. Hovmøller *et al.* (2008) provided evidence that South African isolates of *P. striiformis* f. sp. *tritici* clustered with those from Europe, and Central and Western Asia.

2.3.4 Resistance in local cultivars

At the time when stripe rust arrived in South Africa, wheat breeders and producers were confronted with a new disease, one they did not have to consider in the past. This called for new management decisions to be made, as great losses in yield and quality were suddenly experienced. Breeding programs had to discard 30-60% of early-generation breeding material which was susceptible to stripe rust and thus had fewer cultivars available for release (Pretorius *et al.* 2007). The quest therefore started to identify and incorporate stripe rust resistance into the South African germplasm.

Several of the available, locally bred cultivars were resistant to stripe rust, resistance having been incorporated inadvertently, as no breeding or selection for resistance was conducted prior to detection of the disease in 1996 (Ramburan *et al.* 2004). However, current indications are that resistance in many of these cultivars is monogenic (Bender and Pretorius 2001) and may have limited potential for durability. Evidence in this regard was provided by the early breakdown of monogenic resistance in the local cvs Hugenoot and Carina in 1998 (Boshoff and Pretorius 1999). In areas at risk of rust infection, producers should be advised to diversify their selection of cultivars, with the intention of deploying different, yet durable sources of resistance (discussed in following sections). Resistance expressed by South African cultivars, evaluated at five different locations during 1998 (Boshoff *et al.* 2002b) appeared stable over different environments.

2.3.5 Economic impact

Stripe rust is considered one of the most damaging global diseases of wheat, causing yield and quality losses as a consequence of shrivelled grains and decreased tillering (Wellings 2011). Most wheat producing areas have seen yield losses ranging from 10-70% (Chen 2005), but under severe epidemic conditions yield losses as high as 84% have been recorded (Murray *et al.* 1994). Fungicide trials in South Africa with spring and winter wheat cultivars infected with stripe rust revealed grain yield losses as high as 65% in the untreated control plots (Boshoff *et al.* 2003). Yield loss is influenced by the growth stage of the crop at the time of infection, environmental factors, as well as the amount of inoculum present. High day temperatures and dry conditions have been observed to result in low disease levels (Boshoff *et al.* 2002b). The application of fungicides (Ash and Brown 1990; Gaunt and Cole 1992; Jørgensen and Nielsen 1994) and to a lesser extent the use of cultivar mixtures (Finckh and Mundt 1992; Mundt *et al.* 1996; Akanda and Mundt 1997) have been successfully deployed in controlling stripe rust outbreaks. Genetic resistance is considered to be an efficient, cost-effective and environmentally-friendly control strategy. Increasing the yield potential of wheat is a target for wheat breeders globally. Disease clearly remains a major constraint to wheat production, making resistance breeding a target of fundamental importance.

2.4 Resistance genes

2.4.1 Mechanisms of resistance

Plant defence mechanisms work either through disease avoidance/escape, tolerance or resistance (Parlevliet 1993). The host plant fights off attack by fungal pathogens through a number of biological pathways resulting in pathogen growth arrest at various stages of infection and with different levels of success (Heath 1981). Flor (1971) studied the inheritance of plant resistance as well as pathogen virulence and formulated the classic “gene-for-gene” model which proposes that for resistance to occur, complementary pairs of dominant genes from both the host (resistance genes) and pathogen (avirulence or *Avr* genes) are required (incompatibility). An alteration or loss of the plant’s resistance gene or of the pathogen’s avirulence gene (*Avr* changing to *avr*) leads to disease (compatibility) (Hammond-Kosack and Jones 1997) (illustrated in Fig 2.4).

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Pathogen-associated molecular patterns (PAMPs), also referred to as microbe-associated molecular patterns (MAMPs) since they also occur in nonpathogenic microorganisms (Boller and Felix 2009), are conserved across microbial genera and/or species. PAMPs contribute to microbial survival and procreation (Hammond-Kussack and Kanyuka 2007). Effectors are species or race specific and contribute to pathogen virulence (Thomma *et al.* 2011). Both types of molecule can trigger plant immunity, designated PAMP-triggered immunity (PTI) and effector triggered immunity (ETI), respectively. The distinction cannot strictly be maintained as effectors may elicit defense responses

and PAMPs may be required for virulence. A continuum is therefore seen between PTI and ETI. Jones and Dangl (2006) introduced the zigzag model for innate immunity in plant pathogen interactions. According to this model, the first line of plant defense is formed by pattern recognition receptors (PRRs). These are cell surface receptors that activate innate immune responses (PTI) upon detection of PAMPs. Successful pathogens are able to overcome PTI by means of secreted effectors that suppress PTI responses, resulting in ETI susceptibility.

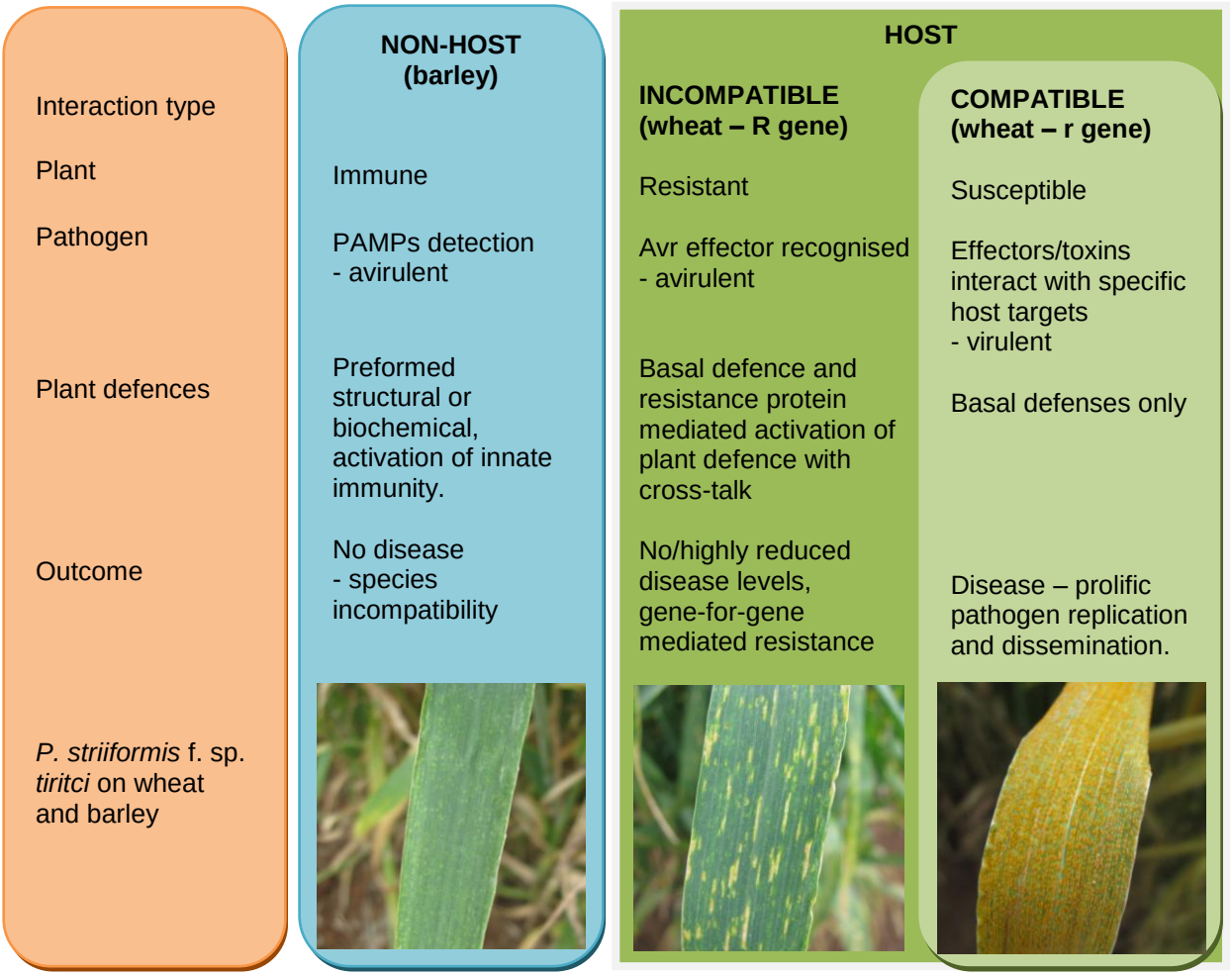

Figure 2.4 The different interaction types and layers of plant resistance. PAMPs – pathogen-associated molecular patterns, R – functional host resistance, r – non-functional host resistance (figure adapted from Hammond-Kosack and Kanyuka 2007).

The protein structure has been investigated for many plant resistance genes. These resistance genes can be divided into five classes based on the domains within the predicted amino acid sequence (reviewed in Aarts *et al.* 1998; Dangl and Jones 2001; Martin *et al.* 2003).

1. Serine/threonine kinase region and a myristoylation motif at the N-terminus (Martin 1999).
2. Nucleotide-binding site (NBS), a stretch of leucine-rich repeats (LRR) and an N-terminal putative leucine zipper (LZ) or coiled-coil (CC) sequence (Martin *et al.* 2003).

3. Similar to class two, except the CC sequence contains a protein region of similarity to the toll and interleukin-1 receptor (IL-1R) known as the TIR region. These genes lack transmembrane domains and are therefore located intracellularly.
4. NBS regions are replaced by a transmembrane domain which locates the LRR region extracellularly and the protein contains a small cytoplasmic tail.
5. NBS regions are replaced by a transmembrane domain which locates the LRR region extracellularly and the protein contains a serine/threonine kinase region (Jones *et al.* 1994; Dixon *et al.* 1996).

Although more than one mechanism may be active at different infection sites in the same plant-fungus interaction, resistance of host plants is typically expressed as pre- or post-haustorial resistance, with post-haustorial resistance often leading to a hypersensitive reaction (Niks and Rubiales 2002). NBS-LRR genes have been shown to cluster within the genomes, usually in the highly recombinant subtelomeric ends (Meyers *et al.* 1999; Bai *et al.* 2002) and they comprise the majority of the seedling resistance genes that have been cloned to date [e.g. *Lr21* (Huang *et al.* 2003), *Lr10* (Feuillet *et al.* 2003) and *Pm3b* (Yahiaoui *et al.* 2004)] (Cloutier *et al.* 2007).

2.4.2 Wheat resistance genes

To date 49 stripe rust resistance genes have been designated an official *Yr* number (*Yr50, Yr51, Yr52* and *Yr53* are pending), while more than 30 stripe rust resistance genes have temporary assignments in the Catalogue of Gene Symbols for Wheat (McIntosh *et al.* 2011). Resistance genes are put into categories based on 1) the plant developmental phase during which it is expressed, 2) the range of pathogens recognised and 3) the resistance pathway it triggers. Seedling resistance is expressed throughout the plant's life (also termed all-stage resistance), whereas adult plant resistance (APR) is expressed only after heading, in mature plants. Resistance can further be classified as race-specific (also termed vertical resistance) or race-nonspecific (horizontal resistance). As the term implies, race-specific resistance provides protection against certain stripe rust pathotypes and not others. Race-specific resistance genes conform to the gene-for-gene hypothesis (Flor 1971) and therefore tend to be short-lived. Once the pathogen adapts and becomes virulent, a new pathotype is produced, the corresponding resistance gene no longer being effective. Race-nonspecific resistance is mostly controlled by genes with minor to intermediate and additive effects and provide protection against a broad spectrum of pathotypes. Resistance genes from this class are considered to be durable. Durable resistance was defined by Johnson (1981; 1984) as resistance in a crop variety that remains effective despite widespread cultivation of the crop for a prolonged period of time in an environment favourable to the disease.

2.4.2.1 Seedling resistance

Seedling resistance genes confer a complete resistant phenotype which is easy to select for in breeding programs and can easily be detected in greenhouse tests. This type of resistance is however normally race-specific. Seedling resistance is often transient once exposed in commercial cultivars due to genetic shifts in the pathogen population (Johnson 1992; Boyd 2005). Most resistance genes classified belong to this group.

2.4.2.2 Adult plant resistance (APR)

In wheat currently 13 APR genes for stripe rust have been formally classified (Table 2.1), although many more APR quantitative trait loci (QTL) have been reported. Individually APR genes express a partially resistant phenotype, but when combined they can provide adequate crop protection (Singh *et al.* 2005). The complex quantitative inheritance of APR makes genetic analysis a challenging task. APR is often difficult to distinguish and better expressed in the field (Boshoff 2000). The environmental sensitivity of the pathogen, including the ensuing interaction with its host, may negate consistent disease assessment. A statistical QTL analysis is often used to predict the number and chromosomal location of QTL contributing to APR, and the proportion of the phenotypic variance accounted for by each QTL. Genetic analysis of cultivars with APR in general finds the resistance to be conferred by the additive effects of several minor genes (Navabi *et al.* 2004; Singh *et al.* 2005). When APR is based on the additive effect of several genes and thus of a quantitative nature, it has been proposed as a source of durable resistance (McIntosh 1992; Boyd 2005; Navabi *et al.* 2005).

Two of the most used APR genes in wheat breeding are *Lr34/Yr18/Pm38* and *Lr46/Yr29/Pm39.* Both these resistance loci provide race-nonspecific resistance to stripe rust, leaf rust and powdery mildew. *Lr34/Yr18/Pm38,* located on chromosome 7D, is associated with the expression of post-flowering leaf tip necrosis (LTN) in some environments (Singh 1992). This gene will be discussed in more detail in Chapter 5. *Lr46/Yr29/Pm39* is located on chromosome 1B (Singh *et al.* 1998; William *et al.* 2003) and was first identified in the CIMMYT-derived Mexican variety Pavon 76. *Lr46/Yr29/Pm39* is also associated with slight post-flowering LTN.

2.4.2.2.1 Slow-rusting, partial resistance

Most of the characterised APR genes for stripe rust are nonspecific, however, a proportion are clearly race-specific (Lagudah 2011). Race-nonspecific APR are associated with a slow rusting phenotype. Slow-rusting resistance is characterised by a slow development of the disease in the field, despite a susceptible infection type and usually confers only partial resistance (Caldwell 1968). This type of resistance usually does not include the expression of a hypersensitive response, hence a susceptible infection type score. Typically, slow-rusting resistance shows longer latent periods, smaller uredinium sizes and reduced sporulation within the first two weeks post inoculation when compared to fully

susceptible plants. Slow-rusting varieties play an important role in the control of rust disease on cereal crops (Shaner *et al.* 1978), effectively reducing infection rate and disease intensity in the field (Nelson 1978; Parlevliet 1979). Danial *et al.* (1995) showed that a progression towards more complex races of stripe rust is inevitable for the wheat rust pathosystems when the selection is for complete (immunity) or near-complete resistance. Slow-rusting resistance genes have proven to be durable,

Table 2.1 Stripe rust APR genes assigned a *Yr* designation.

***Yr* gene**	**Chromosome**	**Source cultivar/species**	**Reference**
11	not known	Joss Cambier	Priestley 1978
12	not known	Mega	Priestley 1978
13	not known	Maris Huntsman	Priestley 1978
14	not known	Hobbit	Priestley 1978
16	2D	Cappelle-Desprez	Worland and Law 1986
18	7DS	Frontana	Suenaga *et al.* 2003
29	1BL	Pavon 76	Bariana *et al.* 2001
30	3BS	Opata 85	Singh *et al.* 2001
36	6BS	*Triticum dicoccoides*	Uauy *et al.* 2005
39	7BL	Alpowa	Lin and Chen 2007
46	4D	Compair	Chen and Zhao 2007
48	5AL	PI610750	McIntosh *et al.* 2010
49	2DS	Chuannai18	McIntosh *et al.* 2011

e.g. *Lr34/Yr18/Pm38.* This philosophy of combining several adult plant partial resistance genes to achieve high levels of resistance is actively pursued by CIMMYT (International Maize and Wheat Improvement Centre, Mexico) as an integral part of achieving durable resistance to rust diseases globally (Singh *et al.* 2011).

2.4.2.2.2 High-temperature adult plant (HTAP) resistance

The expression of stripe rust resistance genes at higher temperatures has been documented, however relatively few reports have been published characterising high-temperature adult plant (HTAP) resistance in wheat (Chen and Line 1995; Uauy *et al.* 2005; Lin and Chen 2007; Santra *et al.* 2008; Carter *et al.* 2009; Lin and Chen 2009). This type of resistance proves most effective during day temperatures of 25-30°C and night temperatures that average 10°C (Line and Chen 1995). That is, high diurnal temperatures followed by cool nights. The level of resistance conferred by HTAP resistance sources are usually rated as moderate and is affected by plant growth stage, temperature, and humidity. Unlike slow-rusting, HTAP resistance involves some degree of hypersensitivity. Although the trait is polygenic, studies have shown that single major genes can account for a significant portion of the variation in HTAP resistance (Börner *et al.* 2000; Bariana *et al.* 2001;

Suenaga *et al.* 2003). Examples of HTAP resistance genes include *Yr36* on chromosome 6BS, *Yr39* on chromosome 7BL and *Yr46* on chromosome 4D.

2.4.3 Alien resistance sources

Wild and domesticated relatives of wheat possessing partly homologous genomes provide abundant sources of rust resistance genes (Dundas *et al.* 2007). Genes which have successfully been transferred to commercial wheat cultivars include *Sr22* (from *Triticum monococcum*), *Sr24, Lr24* and *Sr26* (from *Agropyron elongatum* and *Thinopyrum ponticum)*, *Sr31*, *Lr26* and *Yr9* (from *Secale cereale*), *Sr36* (from *T. timopheevii*) and *Sr38, Lr37* and *Yr17* (from *T. ventricosum*). Unfortunately translocations from wild relatives are often associated with linkage drag, resulting in undesirable characteristics being transferred with the gene of interest. Recombination is suppressed in the region of the introduced alien chromosomal segment (linkage block) and it is therefore difficult to reduce the size of the translocation. Worland *et al.* (1989) reported the existence of one or more yield-reducing genes on an alien chromosome with the eyespot resistance gene *Pch1* from *Aegilops ventricosa.* Another example is the yellow-pigmented flour in bread wheat, which is linked to *Lr19* from *Th. ponticum* (Knott 1980; Marais *et al.* 1992; Prins *et al.* 1996). By inducing homeologous recombination between the alien chromosome segments and normal wheat chromosomes, shortened alien chromosome segments and lines with altered positions of the alien chromatin in the wheat genomes may be produced (Dundas *et al.* 2007). The first interspecies crosses made in South Africa were documented in 1912, when rust resistance genes were introduced into bread wheat from emmer wheats (Pakendorf 1977).

2.5 Breeding for resistance

Deploying cultivars containing the same seedling resistance genes, which are then grown over large areas will lead to resistance breakdown and large scale epidemics (Rosewarne *et al.* 2006). Pyramiding several race-specific genes is only partially more effective (Parlevliet 1995). The resistance is still vulnerable to breakdown, as the pathogen continues to mutate for virulence against multiple resistance genes (McDonald and Linde 2002a, McDonald and Linde 2002b). Minor genes are difficult to detect with conventional methods in mapping populations, but they play an important role in enhancing the effectiveness of slow-rusting genes with large effect in achieving high levels of resistance comparable to immunity (Singh *et al.* 2000). The combined effect of four to five resistance genes has been shown to be more stable across environments (Singh *et al.* 2011), and this is the approach used by CIMMYT (Navabi *et al.* 2003, Navabi *et al.* 2004; Zhang *et al.* 2008; Singh *et al.* 2011). Examples demonstrating interactions between moderately effective race-specific and slow-rusting APR genes include combinations with *Lr34/Yr18/Pm38* (German and Kolmer 1992), *Lr16*'s moderate level of resistance and its near immune level of resistance when combined with additional

slow-rusting genes (Singh and Huerta-Espino 1995), and the slow-rusting gene *Sr2* that enhances the expression of *Sr25* (Njau *et al.* 2010).

It is difficult to select for several minor genes in a single background phenotypically. Means to pyramid genes are therefore usually molecular marker-based and rely on back-crossing or convergent crosses. Primary advantages of this methodology are its suitability for combining genes in a relatively short period of time. Unfortunately diagnostic markers are only available for a selection of resistance genes and to allow for recombination an excess of lines have to be evaluated. However, several markers for rust resistance genes in wheat have been published (Langridge *et al.* 2001; Hoisington *et al.* 2002; Boyd 2005; MASwheat database http://maswheat.uc.davis.edu). Marker development in South Africa has focused primarily on the *Thinopyrum ponticum*-derived leaf rust resistance gene *Lr19* (Prins *et al.* 1997; Prins *et al.* 2001) and stripe rust resistance in the cv. Kariega (Ramburan *et al.* 2004; Prins *et al.* 2011).

2.5.1 The application of biotechnology in wheat improvement

The progress towards efficient cereal transformation has been slow, mainly due to difficulties encountered in the establishment of embryogenic cell culture methods and a lack of efficient DNA delivery systems (Patnaik and Khurana 2001). Several different methods have been attempted for transforming wheat. Direct transfer of DNA into protoplasts mediated by polyethylene glycol (Maas and Werr 1989; Potrykus 1990) and electroporation (Larkin *et al.* 1990; Zhou *et al.* 1993) have proven to be ineffective. A more versatile method for cereal transformation is microprojectile bombardment or biolistics. Usually, the insertion events are random and characterised by multiple copies, as well as a certain degree of rearrangements (Jenes *et al.* 1993). The most recent cereal transformation method involves the use of a naturally occurring bacterium, *Agrobacterium tumefaciens* (Tinland 1996). The possible durability of disease resistance introduced by biotechnology will remain to be challenged by widespread use of cultivars possessing it, and to date classical breeding is still used to achieve host resistance in wheat.

2.6 DNA marker technology

2.6.1 Properties of DNA markers

DNA markers are relatively simple tools that are completely independent of environmental conditions and can be detected at any stage of the plant's development. DNA markers are phenotypically neutral, have high allelic variation and are not influenced by epistatic, pleiotropic or methylation effects. The introduction of DNA based molecular markers paved the way for constructing detailed genetic maps. Restriction fragment length polymorphism (RFLP) was the first DNA marker technology and it relied on sequence variations which created or obliterated restriction enzyme recognition sites (Botstein *et al.* 1980). The emersion of the polymerase chain reaction (PCR) led to the development

of several novel genetic assays based on selective amplification of DNA. Random amplified polymorphic DNA (RAPD) markers soon followed (Rafalski and Tingey 1993). Polymorphisms arise when a genotype has lost or gained a primer recognition site or if the amplified sequence contains deletions or insertions in the sequence changing the size of the fragment. Amplified fragment length polymorphism (AFLP) was developed in 1993 (Vos *et al.* 1995) and is still used to generate genomic fingerprints for species for which limited sequence information is available. It combines sequence variations resulting from alterations in restriction enzyme recognition sites and nucleotide variation in the 2-3 base pairs flanking the restriction enzyme site with PCR. Simple sequence repeats (SSR), also known as microsatellites or variable number tandem repeats (VNTR) has been the marker of choice since the mid 1990s (Röder *et al.* 1995). More recently single-nucleotide polymorphism (SNP) markers have dominated the genomics scene as they provide locus specific information and can be screened with a number of detection techniques. Selected marker systems will be discussed in more detail.

2.6.1.1 Simple sequence repeats (SSRs)

SSRs are stretches of DNA consisting of short (mono-, di-, tri-, tetra- or penta-nucleotide) tandem repetitive units that are found throughout the genome. These regions are amplified by PCR using primers matching the flanking regions. SSRs are abundant, highly polymorphic, locus-specific and adaptable for high-throughput screening. The co-dominant nature of SSRs allows homozygote and heterozygote loci to be distinguished. The nature of microsatellite markers permit them to be used successfully not only for gene mapping purposes, but as diagnostic markers for target genes. Unfortunately the development of new SSR markers is labour intensive and expensive. Several hundred SSR markers are already available for wheat in the public domain in the GrainGenes database (http://wheat.pw.usda.gov).

2.6.1.2 Diversity Arrays Technology (DArT)

The molecular technique Diversity Arrays Technology (DArT) (Triticarte P/L and Diversity Arrays Technology P/L, Australia) has been developed for several crops, including rice (Jaccoud *et al.* 2001), barley (Wenzl *et al.* 2004) and wheat (Akbari *et al.* 2006; Semagn *et al.* 2006). DArT is a microarray hybridisation technology that involves the initial development of a discovery array. This array is used to identify polymorphic DArT markers in genetically distinct wheat genotypes. Selected markers are then assembled into a genotyping array (Gupta *et al.* 2008). Genotypes are screened by hybridising restriction fragments to these array chips and polymorphisms are distinguished based on differential fluorescent hybridisation intensities. Diversity arrays generally detect polymorphisms due to single base-pair changes (SNPs) at the restriction sites of endonucleases (*Pst*I and *Taq*I for the wheat array), and indels or rearrangements within restriction fragments (Jaccoud *et al.* 2001). *Pst*I is a methylation-sensitive restriction enzyme, which will also identify polymorphisms due to DNA methylation (Kilian *et al.* 2005). DArT markers are of a dominant nature. The development of DArT

markers overcame many of the limitations of other markers systems and has developed into a valuable tool for the generation of polymorphic markers in crop species (Jaccoud *et al.* 2001). Several thousand loci are typed in a single assay, thereby generating a whole-genome DNA fingerprint, with several hundred data points in one assay.

2.6.1.3 Single-nucleotide polymorphisms (SNPs)

SNPs represent sites where the DNA sequence differs by a single base (Gupta *et al.* 2001) and such polymorphisms are abundant within the wheat genome (Koebner and Summers 2003). Numerous approaches for SNP discovery have been described. The simplest way to detect SNPs is to perform direct sequencing of PCR products or by pyrosequencing (Langaee and Ronaghi 2005). Alternatively, less direct screening methods could be used to type this marker. Several methods may be employed, including denaturing high pressure liquid chromatography (dHPLC) (Underhill *et al.* 1997), conformation-sensitive gel electrophoresis (CSGE) (Ganguly *et al.* 1993), denaturing gradient gel electrophoresis (DGGE) (Fischer and Lerman 1983), single-strand conformation polymorphism (SSCP) (Orita *et al.* 1989), matrix-assisted laser desorption ionisation0time of flight (MALDI-TOF) (Corona and Toffoli 2004), high resolution melt (HRM) with real-time PCR analysis (Wittwer *et al.* 2003) or other chemical or enzymatic cleavage methods (Cotton *et al.* 1988). More recently, whole-genome sequencing on next-generation sequencing platforms have also been utilised as a screening strategy. Homoeologous ⁄ paralogous and varietal SNPs have previously been studied in wheat (Akhunov *et al.* 2009; Bernardo *et al.* 2009; Bundock *et al.* 2009; Edwards *et al.* 2009; Imelfort *et al.* 2009). However, these studies have shown that distinguishing intervarietal markers from intergenomic polymorphisms is complicated and prone to error. A global effort is now made to develop and validate SNP markers for breeding applications (Allen *et al.* 2011). The mutation detection technologies selected for this study are discussed in more detail in the following sections.

2.6.1.3.1 Single-strand conformation polymorphism (SSCP)

SSCP is a PCR-based mutation scanning technology which detects sequence differences which do not necessarily result in size differences between alleles. During electrophoresis, single-stranded DNA folds into a three-dimensional shape according to its primary sequence (Orita *et al.* 1989). The electrophoretic mobility of separation then becomes a function of the structure of the folded, single-stranded molecule. A number of factors can influence sensitivity in an unpredictable way, such as electrophoretic conditions; including temperature during electrophoresis and matrix composition. The fragment length and the location of the sequence variation along the DNA fragment should also be considered (Nataraj *et al.* 1999). SSCP analysis can be performed by means of polyacrylamide gel electrophoresis or capillary array electrophoresis.

2.6.1.3.2 Real-time PCR high resolution melt (HRM) analysis

HRM analysis involves the amplification of fragments, in the presence of a DNA double-strand intercalating dye followed by a melt cycle whereby the temperature is slowly increased resulting in the separation of double-stranded fragments. The decrease in fluorescence caused by the release of the intercalating dye is recorded in real-time and a melting temperature (T_M) is calculated from the melt curve (Gundry *et al.* 2003; Reed and Wittwer 2004). SYBR Green I is a first generation dye and as it may inhibit PCR it is used at low concentrations (Gundry *et al.* 2003). The low concentrations equals sub-saturation levels which decreases the sensitivity as the dye will relocate in partly melted double-stranded DNA. New generation dyes such as LC Green, LC Green Plus, ResoLight, EvaGreen, Chromofy and SYTO9 are now successfully employed in HRM analysis with more sensitive detection of sequence variants (Reed *et al.* 2007). HRM analysis can therefore be used for detecting both known and new sequence variations.

Alternatively, probes can be designed to recognise sequence variants (indel or SNPs). The amplification step is not necessarily followed by an HRM step. Only known sequence changes can be detected with probes.

2.7 QTL mapping strategies

Most traits of interest in plant breeding show quantitative inheritance, termed quantitative trait loci (QTL). Quantitative traits are more difficult to select for and transfer to selected genotypes. The genetic variation of a quantitative trait is effected by epistasis (interaction between QTL), the environment, and interaction between QTL and environment (Semagn *et al.* 2010). Exploiting molecular markers in breeding requires the identification of markers associated with the QTL responsible for the trait of interest. Linkage analysis and association mapping are the two most commonly used methods for QTL mapping. Association mapping relies on the availability of a large number of different genotypes. The statistical analysis is hampered by the inherent structure present between populations as selection is an unavoidable feature of wheat breeding. Linkage analysis will be discussed in the following section.

2.7.1 Mapping populations

Mapping populations are created to evaluate the segregation of a trait of interest. It is therefore important to choose parents for the population which are polymorphic for the trait and where one is able to phenotype the trait accurately. The reproductive mode of the plant, the time available and the mapping resolution required will determine the population type. Most commonly used are doubled haploid (DH) and recombinant inbred line (RIL) mapping populations. Developing a DH mapping population requires technical skills and tissue culture facilities, but complete homozygosity is achieved within one generation after the F_1, which represents the first generation produced from crossing two parental lines. RIL mapping populations are created by advancing random F_2 seed

(second generation produced by selfing F_1 plants) to further generations. This process is less technically demanding, but several generations are needed to achieve complete homozygosity. Both DH and RIL populations allow for repetitive, multi-trait analysis, no genotypes are lost and both dominant and co-dominant markers are informative. Germplasm can be replicated over years and locations which results in an improved environment variation measure. DH populations are derived from female gametes after one meiosis, while RIL populations are from male and female gametes through many generations of selfing. Thus differences in the genetic distance between the DH and RIL populations may reflect differences in recombination rates. He *et al.* (2001) found uniform marker orders between two rice DH and RIL populations.

2.7.2 Construction of linkage maps

A linkage map is created by typing the segregation of molecular marker alleles in the progeny of a designed cross. The data is used to order the markers in linkage groups. Marker ordering is a very important step in genetic mapping as even if the QTL mapping methods are appropriate the results may be misleading if the markers are poorly grouped or ordered. The order of markers is affected by the addition of new markers, the chosen log-likelihood criteria, the linkage phases, segregation distortion, and chromosomal reorganisation (Wu *et al.* 2003). Typing errors and missing data points lead to unexpected crossovers, which result in large increases in map length (Hackett and Broadfoot 2003). Mapping functions are used to convert recombination frequencies into map distances in centiMorgan (cM). Haldane's mapping function (Haldane 1919) assumes that there is no interference which would increase or decrease the proportion of double crossovers. Kosambi's mapping function (Kosambi 1944) is based on empirical data regarding the proportion of double crossovers. As the physical distance varies this function adjusts the map distance based on interference of neighbouring crossovers.

The wheat genome can tolerate major deletions and rearrangements, or even the loss of complete chromosomes, which has facilitated the isolation of aneuploid genetic stocks. These stocks are valuable tools for determining the chromosomal location of new markers. Several consensus maps, containing mainly SSR and AFLP markers are also available (Röder *et al.* 1998; Appels 2003; Somers *et al.* 2004; Song *et al.* 2005). The mapped SSR markers aid the assignment of linkage groups to specific chromosomes.

2.7.3 QTL mapping

Various QTL mapping methodologies are available for locating QTL on linkage maps. These techniques include single-marker mapping, interval mapping (IM), composite interval mapping (CIM) and multiple trait/QTL mapping (MQM), which allow statistical analyses of the association between phenotype and genotype for the purpose of dissecting the genomic regions that affect complex traits (Doerge 2002). Simple, single-marker tests are done by simple linear regression, analysis of variance (ANOVA) and t-tests, which assess the segregation of a phenotype with respect to a marker genotype

to establish which markers are associated with the QTL. Interval mapping fixes the location of the QTL and estimates the QTL effect between intervals of markers. This way, both the QTL location and effect can be determined. The results of interval mapping are expressed as logarithm of the odds (LOD) scores, which compare the the null hypothesis of no QTL, with the hypothesis of a QTL at the position tested. CIM (Zeng 1993) and MQM mapping (Jansen 1993) extend interval mapping to include additional markers outside a defined window as cofactors with the aim of removing the variation that is associated with other QTL present within the genome. These models therefore allow for the simultaneous detection of multiple QTL which may either be independent, or linked.

2.7.4 Mapping software

Various software packages are available for constructing linkage maps and/or to compute QTL locations and effects (Semagn *et al.* 2010). Although there are a vast number of programmes available, the more commonly used and referenced are QTL Cartographer (Wang *et al.* 2011), MAPMAKER (Lincoln *et al.* 1992), Map Manager QTX (Manly *et al.* 2001), RECORD (Van Os *et al.* 2005), QGene (Nelson 1997), PlabQTL (Utz and Melchinger 2003), JoinMap (Van Ooijen 2009) and MapQTL (Van Ooijen 2006). Map Manager QTX, MAPMAKER, PlabQTL, QGene, RECORD and QTL Cartographer are distributed as freeware.

2.7.5 Fine mapping

Due to practical considerations the size of mapping populations are usually limited to 200-300 individuals and the marker density most frequently obtained in preliminary QTL mapping studies equals approximately 10-20 cM (Yan *et al*. 2006; Semagn *et al*. 2010). To be of value in MAS these QTL intervals have to be reduced, and even more so for map-based cloning of the genes. Successful fine mapping of QTL (also termed high-resolution mapping) that resulted in the isolation of the underlying genes has been reported in several plant species, including hexaploid wheat. Some of the wheat genes cloned through map-based cloning include *Lr10* (Stein *et al*. 2000), the domestication gene *Q* (Faris *et al.* 2003), *VRN2* (Yan *et al.* 2004), *Pm3b* (Yahiaoui *et al.* 2004), *Gpc-B1* (Uauy *et al*. 2006) and Lr34/Lr18/Pm38 (Krattinger *et al.* 2009). These genes mostly locate to recombination hotspots (Xue *et al.* 2010) as recombination-poor regions are problematic for map-based cloning (Erayman *et al.* 2004). Akhunov *et al.* (2003) found that the distal chromosomal regions of the wheat genome generally exhibit a higher recombination rate.

2.7.5.1 Choice of mapping population

Smaller segregating mapping populations are adequate for preliminary QTL analysis. F_2 and back-cross (BC) populations are the simplest types since they are easy to construct and they only require a few generations to develop. RIL populations take longer to generate, but they have the advantage of most alleles being fixed, depending on the generation. DH populations have the same advantage, but

require tissue culture facilities and they are more difficult to produce. Both RIL and DH populations are considered to be eternal resources, as they can be propagated indefinitely through seed.

Fine or high-resolution mapping studies depend upon a significant number of recombination events to define the QTL to a small interval, which is ideally about 1 cM. This can be achieved by either significantly increasing the population size or by increasing the number of meiosis (generations), or both. F_2 or F_3 derived families, RILs and NILs are often used in these studies (Cuthbert *et al.* 2007; Khlestkina *et al.* 2007; Röder *et al.* 2008; Alfares *et al.* 2009; Liu and Bai 2010; Xue *et al.* 2010). F_2 populations are more powerful for detecting QTL with additive effects and can also be used to estimate the degree of dominance for the QTL detected (Semagn *et al.* 2010). Although these lines are mortal, they can be reconstructed by typing the F_3 families. RILs have undergone several rounds of meiosis, these multiple generations increasing the potential number of recombination events and improving the map resolution. However, F_2 mapping populations can be produced in far less time. Selection of the target trait, while fixing the genetic background, is required for the generation of NILs. NILs allow for the construction of high-resolution maps within the target trait interval only. These lines are also ideal for more direct, hypothesis-driven biological experiments (Semagn *et al.* 2010).

2.7.5.2 Molecular marker density

Due to the low level of genome polymorphism between wheat cultivars (Bryan *et al.* 1999) the ability of SSR markers to saturate the wheat genome is limited. In general only between 20-40% of SSR markers are polymorphic between any two cultivars (Somers *et al.* 2004). DArT analysis enables the addition of a large number of markers to a genetic map in a short space of time. Although DArT markers are generally distributed across the genome (Akbari *et al.* 2006), they show a higher frequency of clustering compared to other marker types (Semagn *et al*. 2006). DArTs cannot be pre-selected for a genomic interval of interest and they also require further modification and adaptation for use in high-throughput cost-effective MAS screens. Other conventional markers, like AFLPs are laborious to type and cannot be targeted to a specific genomic region either. With the advent of wheat genome sequencing projects, sequencing data has become available which facilitates improved marker discovery.

2.8 Sequencing of the wheat genome

Extensive aneuploid stocks have been developed, including nullisomic-tetrasomic and ditelosomic lines (Sears 1954; Sears 1966; Sears and Sears 1978). Additionally, a collection of overlapping deletion lines involving all chromosome arms has been identified and characterised (Endo and Gill 1996; Qi *et al.* 2003). The breakpoints of these deletions define physical segments or bins. The deletion lines allow for the physical mapping of genetic markers. Expressed sequence tags (ESTs) are short sequenced fragments of expressed genes. Typically ESTs are single-pass sequenced to

yield 200-700 bp sequences that can be used to search DNA and protein databases for homologues (Adams *et al.* 1991).

The International Wheat Genome Sequencing Consortium (IWGSC) was established by researchers dedicated to sequencing the wheat genome (Feuillet *et al.* 2007; Moolhuijzen *et al.* 2007). It was decided that the chromosomes will be separated by flow-cytometry to ease assembly of the large, complex, hexaploid genome of wheat. This will be done in a coordinated fashion, with laboratories taking responsibility for different chromosomes. Thus far the most progress has been made with chromosome 3B (Paux *et al.* 2008), but a BLAST-searchable sequence that yielded a 1X genome coverage of the cv. Chinese Spring complete genome is already available (http://www.cerealsdb.uk.net). This sequence was obtained with a next-generation whole genome sequencing platform. Spin-off projects from the IWGSC are providing more insight into the evolution, structure and organisation of the wheat genome.

2.9 Comparative genomics

The grass family *Poaceae* includes the major cereals, namely rice, wheat, maize, barley, rye and millet. Members of the grass family diverged evolutionary around 60 million years ago resulting in a variety of genome sizes and structures (Gale and Devos 1998). Despite this diversity a remarkable degree of genome conservation has been detected by comparative genetic mapping studies (Moore *et al.* 1995; Gale and Devos 1998). Comparative mapping has demonstrated that plants have retained different levels of conservation in their genomes during evolution, depending on their phylogenetic separation (Paterson *et al.* 2000). Syntenic or orthologous regions have co-linear gene contents when compared genetically or physically. Macrosyntenic studies compare the order of genes and genetic markers whereas microsyntenic studies look at the order and orientation of coding sequences as well as non-coding DNA sections. The emerging data suggest that the extent of colinearity observed between the *Triticeae* crops and rice may vary between chromosome regions and that even in regions in which gene orders are highly conserved, colinearity is not absolute (Devos and Gale 2000). Multi-copy genes breach the wheat-rice colinearity more than single-copy genes (Singh *et al.* 2007). Wheat telomeric regions have a low physical to genetic ratio and these regions are more prone to structural alterations. Small local rearrangements and duplications are a common feature of genome evolution with differential gene loss following polyploidisation being responsible for much of the deviation in colinearity among closely related grass species (Paterson *et al.* 2003).

2.10 Marker-assisted selection (MAS)

Conventional cereal breeding is time consuming and it can typically take between eight to ten years to breed a new cultivar and even then its commercial release is not guaranteed. Constant improvement of marker detection systems and the identification of markers linked to useful traits can aid the

introgression of more favourable genes into new cultivars and improve on selection strategies. MAS has made a significant contribution in efforts to efficiently screen for resistance early in the breeding process without the need for phenotyping. However, it is crucial that marker loci are tightly linked to the gene of interest, be diagnostic in a wide range of germplasm and usable in a high-throughput system, are reproducible and a cost effective screening procedure (Gupta *et al.* 1999). Pyramiding of race-specific resistance genes is now possible where conventional breeding methods failed because suitable races of the pathogen were not available to test for the presence of each resistance gene (Kumar *et al.* 2008). MAS does, however, not replace, but complements conventional breeding schemes.

The susceptibility of several wheat cultivars, favourable climatic conditions in the most important wheat growing areas, and the additional costs of fungicide application qualify stripe rust as an important disease with significant impact on South African wheat production. Breeding strategies therefore aim to combine sources of durable resistance within high yielding, adapted cultivars with desirable quality characteristics. Two cultivars, namely Cappelle-Desprez and Kariega, have been selected for their durable stripe rust resistance under South African growing conditions. The genetic basis of this resistance has been studied through QTL mapping of the resistance components. The aim of this study was to improve the characterisation of the resistance expressed by these cultivars to the extent where it can be integrated and tracked in new breeding material. The methods used for the identification and development of molecular markers associated with the Cappelle-Desprez and Kariega APR QTL are described in the following chapters.

3. GENERAL MATERIALS AND METHODS

3.1 Genomic DNA (gDNA) extraction

Different protocols were used for extracting gDNA from leaf tissue. A CTAB protocol was followed for obtaining the high yields needed for analysing a large number of SSR markers. Large gDNA fragments (i.e. mostly intact DNA) without RNA contamination is required for DArT markers. The Zymo Research ZR-96 Plant/Seed DNA Kit^{TM} proved to deliver gDNA most suitable for this application. In addition, an SDS protocol was optimised as an alternative, less time consuming method. However, the SDS protocol produces lower yields. Preferentially extractions were done from fresh or freeze-dried leaf cuttings to reduce enzymatic degradation caused by native DNAases. Alternatively seed was germinated in a Petri dish and the coleoptile used for DNA extractions. Autoclaved distilled H_2O was used to prepare all buffers and solutions, which were then autoclaved again to prevent introduction of foreign DNAases. Extracted DNA was stored at 4°C, or at -20°C for long term storage.

3.1.1 Cetyltrimethylammonium bromide (CTAB) protocol

The CTAB gDNA extraction protocol was adapted from Doyle and Doyle (1990). Although this protocol is laborious and time consuming, it produces high yields of pure DNA.

Sample material (leaf segments of about 20 mm X 5 mm) was placed in 2 mL Eppendorf safelock microtubes together with two 3 mm stainless steel ball bearings. CTAB extraction buffer [100 mM Tris (pH 8.0); 20 mM ethylene-diaminetetraacetate (EDTA); 1.4 mM NaCl; 10% (w/v) CTAB; with the addition of 0.2% ß-mercaptoethanol just before needing the buffer] was added either before the sample was ground or straight after. Samples were ground in a Qiagen TissueLyser (Retsch GmbH, Germany) for 3 min at a frequency of 30 revolutions/s, rotating the microtubes after 90 s. Subsequently the samples were incubated in a pre-heated water bath for 1 h at 65°C. The tubes were inverted several times during this period. 500 µL chloroform : isoamylalcohol (24:1 v/v) was added and the contents mixed through inversion. The tubes were centrifuged for 5 min at 12000 RCF in a microcentrifuge where after the supernatant was transferred to a new 1.5 mL microtube, leaving the precipitated proteins behind. Next, 500 µL isopropanol was added and the samples precipitated at room temperature for 20 min. Following precipitation the samples were centrifuged for 5 min at 12000 RCF and the supernatant discarded. The DNA was washed with 500 µL ice-cold 70% (v/v) ethanol. The microtubes were then left to stand at room temperature for 20 min after which they was centrifuged for 5 min at 12000 RCF and the supernatant poured off. Once the pellet had dried completely, it was resuspended in 200 µL TE buffer [10 mM Tris-Cl (pH 8.0); 1 mM EDTA (pH 8.0)] and stored overnight in the fridge (4°C). The following day the tubes were briefly centrifuged and 20 µL ammonium acetate (7.5 M) and 200 µL chloroform : isoamylalcohol (24:1 v/v) added. The solution

was mixed by inversion and centrifuged for 5 min at 12000 RCF. The resulting supernatant was transferred to a new 1.5 mL tube. Subsequently the DNA was precipitated with 500 µL ice-cold 100% ethanol and left for 2 h in the freezer (-20°C). The samples were centrifuged for 15 min at 12000 RCF and the supernatant discarded. The pellet was washed with 500 µL ice-cold 70% (v/v) ethanol and centrifuged for 10 min at 12000 RCF. This wash step was repeated a second time. Finally, once the pellet had dried completely it was resuspended in 50 µL TE buffer [10 mM Tris-Cl (pH 8.0); 1 mM EDTA (pH 8.0)].

An optional RNase treatment could be included by adding 10 µL RNase A (10 mg/mL) to the sample and incubating the sample at room temperature for 30 min before adding the ammonium acetate on the second day of extraction.

3.1.2 Sodium dodecyl sulphate (SDS) protocol

The SDS gDNA extraction protocol is intended for extracting large numbers of samples in 96-well plates (Pallotta *et al.* 2003), but the protocol was adapted to also extract smaller numbers of samples in individual tubes.

Sample material (leaf segments of about 20 mm X 5 mm) was placed in 1.1 mL 8-strip mini tubes (Axygen Incorporation) together with one 3 mm stainless steel ball bearing. The strip tubes were arrayed in 96-well holding plates. Alternatively, a similar quantity of sample material was placed in 2 mL Eppendorf safelock microtubes together with two 3 mm stainless steel ball bearings. To each sample, 600 µL SDS-extraction buffer [0.1 M Tris-HCl (pH 7.5); 0.05 M EDTA (pH 8.0); 1.25% (w/v) SDS] was added. The microtubes were closed and the strip tubes were closed tightly with mini tube 8-strip caps (Axygen Incorporation). The leaf tissue was ground in a Qiagen TissueLyser (Retsch GmbH, Germany) for 3 min at a frequency of 30 revolutions/s, rotating the microtubes/plates after 90 s. Samples were incubated in a pre-heated water bath at 65°C for 30 min. Subsequently samples were briefly centrifuged in a microcentrifuge and placed in the freezer (-20°C) for 15 min to cool them down. Then 300 µL ammonium acetate (6 M) was added after which the microtubes/plates were shaken vigorously. The samples were then left to stand in the fridge (4°C) for 15 min. Plates were centrifuged at 2250 RCF (microcentrifuge with a plate rotor fitted) for 15 min to collect the precipitated proteins and plant tissue. Single microtubes were centrifuged for 5 min at 12000 RCF (microcentrifuge with microtube rotor fitted). About 600 µL of the supernatant was recovered into a new set of microtubes/plates that contained 360 µL iso-propanol. The microtubes/plates were mixed by inversion and left to stand for 5 min at room temperature to allow the DNA to precipitate. The plates were then centrifuged for 15 min at 2250 RCF (single microtubes for 5 min at 12000 RCF) in order to collect the DNA. The supernatant was tipped off and the remaining fluid allowed to drain off the DNA pellet by inverting the tubes onto a piece of paper towel. The pellet was washed with 400 µL ice-cold 70% (v/v) ethanol. The plates were then centrifuged again for 15 min at 2250 RCF and the microtubes for 5 min at 12000 RCF, after which the supernatant was discarded. The pellets were allowed to dry at room temperature before 120 µL TE buffer [10 mM Tris-Cl (pH 8.0); 1 mM EDTA (pH

8.0)] was added. The DNA was left to dissolve overnight in the fridge (4°C). The following day the pellet was resuspended in the solution before centrifuging the plates for 15 min at 2250 RCF and the microtubes for 5 min at 12000 RCF. The supernatant was transferred to a new microtubes/8-strip 1.1 mL tubes, leaving any undissolved debris behind.

An additional protein removal stage was applied with the single tube extractions, by adding 600 µL chloroform : isoamylalcohol (24:1 v/v) after precipitating the proteins with ammonium acetate. The samples were then centrifuged for 5 min at 12000 RCF and the supernatant transferred to a new tube before adding iso-propanol.

3.1.3 Zymo Research DNA extraction Kit

The Zymo Research ZR-96 Plant/Seed DNA Kit™ (Zymo Research Corporation, USA) was used according to manufacturer's instructions to extract high molecular weight gDNA from wheat coleoptiles.

3.2 DNA quantification and dilution

DNA was quantified on the Nanodrop Spectrophotometer ND-1000 and diluted with sterile SABAX H_2O for injection to 50 ng/µL. The molecular weight and quality (degree of degradation and RNA contamination) was checked for a subset of samples. This was done by loading 1 µL undiluted DNA on a 0.8% agarose gel (SeaKem LE agarose, FMC BioProducts) with 0.5X TBE (tris/borate/EDTA) running buffer [5X stock solution: 0.445 M tris(hydroxymethyl)aminomethane (Tris base); 0.445 M boric acid; 0.01 M ethylenediaminetetraacetic acid (EDTA) pH 8.0], which was subjected to electrophoresis for 1 h at 50 V. A molecular weight marker (GeneRuler™ 100 bp DNA Ladder, Fermentas) as well as a lambda concentration standard (Promega Corporation) was included. Gels were stained with 0.5 µg/mL ethidium bromide and visualised on a Uvipro Platinum gel documentation system. DNA was further diluted to 25 ng/µL and arrayed in 96-well plates (master plates) from which replicates were made (PCR plates) containing 2 µL of 25 ng/µL DNA (50 ng total quantity), and stored at -20°C.

3.3 Marker analysis

3.1.1 Simple sequence repeats (SSRs)

SSR primer sequences were obtained from the GrainGenes database (http://wheat.pw.usda.gov) and included markers from the WMC (Wheat Microsatellite Consortium, Gupta *et al.* 2002), CFA (Clermont-Ferrand A genome, Sourdille *et al.* 2003), CFD (Clermont-Ferrand D genome, Guyomarc'h *et al.* 2002), GWM (Gatersleben Wheat Microsatellite, Röder *et al.* 1998), GDM (Gatersleben D

genome Microsatellite, Pestsova *et al.* 2000), PSP (Bryan *et al.* 1997) and BARC (Beltsville Agriculture Research Center, Song *et al.* 2005) series. Primers were synthesised by Applied Biosystems (UK) and forward sequences were labelled with FAM, VIC, NED or PET fluorophores.

3.3.1.1 Polymerase chain reaction (PCR)

A generic PCR mix was used, with each 10 µL reaction containing 50 ng gDNA, 5 pmol fluorescently labelled forward and unlabelled reverse primer (synthesised by Applied Biosystems, UK), 1X GoTaq colourless buffer (Promega Corporation), 0.2 mM of each dNTP (dATP, dCTP, dGTP and dTTP) (KapaBiosystems), 1.5 mM $MgCl_2$, and 0.25 U GoTaq DNA polymerase (Promega Corporation). Thermocycling parameters were standardised, only varying the annealing temperature. Cycling commenced with an initial denaturing at 94°C for 3 min, followed by 40 cycles consisting of 10 s at 94°C, 30 s at the optimised annealing temperature and 30 s at 72°C, completing the PCR with a final extension for 5 min at 72°C. The GeneAmp PCR system 9700 and the Veriti 96-well thermocycler (Applied Biosystems) were used. Amplicons were pooled, combining fluorophore labels which could easily be analysed together.

Another protocol was optimised as a precautionary method, for dealing with PCR-product contamination. Uracil-DNA glycosylase (UNG) (USB® Molecular Biology Reagents and Biochemicals) was incorporated at a concentration of 0.2 U per reaction. The dNTP composition was changed to 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.05 mM dTTP (Promega Corporation) and 0.15 mM dUTP. The standard thermocycling protocol was preceded by a 10 min hold at 37°C The initial denaturing step at 94°C was extended to 10 min to denature the UNG enzyme before cycling commences. Two additional cycles were also added to compensate for the less efficient incorporation of the nucleotide dUTP in comparison to dTTP.

3.3.1.2 Electrophoresis

Amplified products were desalted and primers, nucleotides and proteins removed with the NucleoSpin® 96 Extract II PCR purification Kit on a Tecan Evo150 robotic system. A 3730*xl* Genetic Analyzer (Applied Biosystem) was used according to manufacturer's instructions for electrophoresis. Samples were combined with GeneScan™- 500 LIZ® size standard (Applied Biosystems) and injected with Hi-Di™ formamide. On occasion amplification was first confirmed by agarose gel electrophoresis (1.5% agarose (SeaKem LE agarose, FMC BioProducts) with 0.5X TBE running buffer [5X stock solution: 0.445 M tris(hydroxymethyl)aminomethane (Tris base); 0.445 M boric acid; 0.01 M ethylenediaminetetraacetic acid (EDTA) pH 8.0].

3.3.1.3 Data analysis

Data were analysed using the GeneMapper v.4 software (Applied Biosystems). Different alleles are represented by different amplification sizes for tandem repeats. Two alleles were considered identical when they showed the same fragment size. Genotypes were exported in tab-delimited text format. Microsoft Office Excel 2007 was used to convert allele calls to the format needed for downstream statistical analysis.

3.3.2 Diversity Arrays Technology (DArT)

A DArT marker service is offered by Triticarte P/L, Diversity Arrays Technology (Pty) Ltd (DArT P/L), Yarralumla, Australia (http://www.triticarte.com.au). Good quality gDNA of high molecular weight extracted with the Zymo Research ZR-96 Plant/Seed DNA Kit™ was quantified and diluted to 50 ng/µL. The DNA was arrayed in 96-well plates, closed with 8-strip caps and couriered to Triticarte (Pty) Ltd to be genotyped with the selected DArT array. Data from Triticarte was received electronically. Microsoft Office Excel 2007 was used to convert allele calls to the format needed for downstream statistical analysis.

3.4 Single-strand conformation polymorphism (SSCP)

EST derived markers were analysed by means of SSCP analysis.

3.4.1 Polymerase chain reaction (PCR)

Different protocols were used for the amplification of EST sequences for SSCP analysis. The reactions were done 1) with both primers in a set unlabelled for analysis with acrylamide gel electrophoresis, 2) with the forward primer fluorescently labelled for analysis with capillary array electrophoresis or 3) using M13-tailed sequence specific forward primers and universal forward primers which were fluorescently labelled (Schuelke 2000) for detection with capillary array electrophoresis. Primer sets involving fluorescent labels were synthesised by Applied Biosystems (UK). Unlabelled primer sets were synthesised by Sigma Aldrich (UK) or IDT (Integrated DNA technologies).

3.4.1.1 Gel electrophoresis - PCR protocol 1

PCR was done in 25 µL reaction volumes, containing 50 ng gDNA, 10 pmol unlabelled forward and reverse primer, 1X GoTaq colourless buffer (Promega Corporation), 0.2 mM of each dNTP (Invitrogen), 1.5 mM $MgCl_2$, and 0.5 U GoTaq DNA polymerase (Promega Corporation). Thermocycling parameters were standardised, only varying the annealing temperature. Cycling

commenced with an initial denaturing at 94°C for 3 min, followed by 35 cycles consisting of 1 min at 94°C, 45 s at the optimised annealing temperature and 30 s at 72°C, completing the PCR with a final extension for 5 min at 72°C. The MJ Research PTC-200 DNA Engine was used for thermocycling.

3.4.1.2 Capillary array electrophoresis – PCR protocol 1

PCR was done in 20 µL reaction volumes, containing 50 ng gDNA, 5 pmol fluorescently labelled forward and unlabelled reverse primer, 1X GoTaq colourless buffer (Promega Corporation), 0.2 mM of each dNTP (KapaBiosystems), 1.5 mM $MgCl_2$ and 0.5 U GoTaq DNA polymerase (Promega Corporation). Thermocycling parameters were standardised, only varying the annealing temperature. Cycling commenced with an initial denaturing at 94°C for 3 min, followed by 40 cycles consisting of 10 s at 94°C, 30 s at the optimised annealing temperature and 30 s at 72°C, completing the PCR with a final extension for 5 min at 72°C. The GeneAmp PCR system 9700 and the Veriti 96-well thermocycler (both from Applied Biosystems) were used for thermocycling.

3.4.1.3 Capillary array electrophoresis – PCR protocol 2

PCR was done in 20 µL reaction volumes, containing 50 ng gDNA, 1X GoTaq colourless buffer (Promega Corporation), 0.2 mM of each dNTP (KapaBiosystems), 1.5 mM $MgCl_2$, and 0.5 U GoTaq DNA polymerase (Promega Corporation). The sequence specific M13-tailed forward primer was used at 0.6 pmol and the fluorescently labelled universal forward primer and reverse primer at 10 pmol per reaction. Thermocycling parameters were standardised, only varying the annealing temperature. Cycling commenced with an initial denaturing at 94°C for 3 min, followed by 40 cycles consisting of 10 s at 94°C, 30 s at the optimised annealing temperature and 30 s at 72°C, completing the PCR with a final extension for 5 min at 72°C. The GeneAmp PCR system 9700 and the Veriti 96-well thermocycler (both from Applied Biosystems) were used for thermocycling.

The M13-tailed sequence specific forward primers were used at critically low concentrations. The exact rate-limiting concentration was determined by testing a concentration gradient with real-time PCR, without the use of the fluorescently labelled universal primer. This is to avoid the formation of an unnecessary large number of unlabelled products, which will ultimately compete with the labelled products. Optimisation was done in 25 µL reaction volumes, containing 50 ng genomic DNA, 1X SensiMix^{TM} *Plus* SYBR Kit (Quantace) with added $MgCl_2$ and the M13-tailed sequence specific and reverse primers at varying concentrations. A Rotor-Gene 6000 Real-time Rotary Analyzer (Corbett Life Science) was used for thermocycling and amplification was viewed and analysed in real-time. Cycling conditions involved an initial denaturing step at 94°C for 10 min to activate the hot-start *Taq* polymerase, which was followed by 40 cycles of 20 s at 94°C, 30 s at 60°C and 30 s at 72°C.

3.4.2 Gel electrophoresis

Gel matrices consisted of 0.5X MDE® Gel Solution (Lonza Rockland Incorporated) and 9% (v/v) glycerol, with 0.6X glycerol tolerant tris/taurine/EDTA (TTE) (National Diagnostics) as the running buffer. Gels (39.4 cm X 33.3 cm) were cast with 0.35 mm combs. Polymerisation of the matrix was achieved by adding ammonium persulphate (APS) (Amersham Biosciences) and tetramethylethylenediamine (TEMED) (Amersham Biosciences). Before casting, glass plates were prepared by treating one plate with Repelcote VS silicone (VWR International) and the other with Bind Silane (Amersham Biosciences). PCR products were denatured in a thermocycler in 1:1 formamide loading buffer [95% (v/v) formamide, 10 mM EDTA pH 8.0, 0.05 % (w/v) xylene cyanole and 0.05% (w/v) bromophenol blue] at 95°C for 5 min and snap-cooled on ice before loading. Electrophoresis took place in a 4°C cold room at 4 W for 12-16 h, depending on the fragment sizes. Gels were stained with silver nitrate on one of the casting plates. The staining process involved fixing with 10% (v/v) glacial acetic acid for 30 min, washing the gel for 20 min with dH_2O and staining for 30 min with a staining solution [0.6% (v/v) 1.0 N $AgNO_3$; 0.15% (v/v) formaldehyde (40%)]. After another dH_2O rinse the gel was developed with 0.36 M $NaCO_3$ containing 3:30 sodium thiosulphate solution (0.100 N) : formaldehyde (40%) added just before needed. Once the bands were visible the process was stopped by adding the previously used fixing solution.

3.4.3 Capillary array electrophoresis

PCR products were purified with the NucleoSpin® 96 Extract II PCR purification Kit on a Tecan Evo150 robotic system and diluted as necessary. The products were combined with GeneScan™ 600 LIZ® or GeneScan™ 1200 size standard (Applied Biosystems) as the internal molecular size marker and Hi-Di™ formamide. Denaturing took place at 95°C for 5 min in a thermocycler after which samples were snap-cooled on ice. A 3130*xl* Genetic Analyzer (Applied Biosystems) was used according to a modified protocol suggested by Valesco *et al.* (2007) for electrophoresis. A separation matrix of 5.5% CAP polymer (Applied Biosystems) containing 5% (v/v) ultrapure glycerol was prepared. The polymer was mixed by inversion and filtered through an Acrodisk 32 mm syringe filter with a 0.45 µm membrane and left to stand overnight at 4°C. A 10X TBE stock solution (Sigma Aldrich) was filtered through a Durapore membrane with 0.45 µm pore size and 47 mm diameter. A running buffer of 1X TBE containing 5% ultrapure glycerol was prepared. Products with different fluorescent labels can be combined, but to limit the run time, it is important to combine fragments of similar size ranges.

A spatial calibration was done on the Genetic Analyzer for a 50 cm capillary array. The spectral calibration was carried out with the DS33 matrix standard (Applied Biosystems) using the Any5Dye dye set with the POP-4 or POP-6 protocol as template. A run module was created following the parameters in Table 3.1. Three modules were created for each of the oven run temperatures 18°C,

25°C and 35°C with the same template used for the spectral calibration module. The run modules were created according to the parameters in Table 3.2.

Table 3.1 Run parameters for spectral calibration on the ABI3130*xl* for SSCP analysis.

Parameters	**Value**
Oven_Temperature	30°C
Poly_Fill_Vol	7,300 steps
Current_Stability	5 µA
PreRun_Time	180 s
Injection_Voltage	1,6 KV
Injection_Time	22 s
Voltage_Number_Of_Steps	30
Voltage_Step_Interval	15 s
Data_Delay_Time	250 s
Run_Voltage	13.4 KV
Run_Time	2,500 s

Table 3.2 Run parameters for the standard run modules on the ABI3130*xl* for SSCP analysis.

Parameters	**Value**
Oven_Temperature	*18^0 C, 25^0 C, 35^0 C
Poly_Fill_Vol	7,300 steps
Current_Stability	5 µA
PreRun_Voltage	10 KV
PreRun_Time	180 s
Injection_Voltage	1,6 KV
Injection_Time	15 s
Voltage_Number_Of_Steps	30
Voltage_Step_Interval	15 s
Data_Delay_Time	680 s
Run_Voltage	11 KV
Run_Time	4,400 s

* Different modules for each temperature.

The injection time was adjusted as needed (longer for faint PCR products) and the run time adjusted depending on the length of the fragments analysed. Electrophoresis was considered complete once all the PCR fragments have passed the read window.

3.4.4 Data analysis

Acrylamide gels were scored manually by visual inspection. Electropherograms generated by the Genetic Analyzer was analysed with the GeneMapper v.4 software. A SNPlex™ size standard was created using the basic/advanced peak detection algorithm, which was used in conjunction with an advanced SNPlex™ analysis method. Scan numbers were recorded and used as size standard labels instead of the usual base pair sizes. Default values were retained for the analysis method, except for the peak detection range (analysis window) and peak detection level [intensity measured as relative fluorescent units (RFU)] which were both determined empirically.

3.5 QTL mapping

3.5.1 Linkage map construction

Marker data for the individuals of the mapping population were used to construct a linkage map. Non-parental alleles were regarded as data scoring errors and were discarded. Allele sizes were converted to parental types (A = female genotype; B = male genotype). Heterozygous genotypes were replaced by a missing data score. The markers were ordered with RECORD (Van Os *et al.* 2005a). Data were imported into MapManager QTXb20 (Manly *et al.* 2001) and markers were ordered according to the RECORD output. Linkage groups were assigned to chromosomes according to a consensus map (Appels 2003). LOD linkages were evaluated at $P = 0.001$ and map distances were calculated using the Kosambi mapping function from MapManager QTXb20. The linkage map was visually inspected for double recombinants involving a single marker and these data points were re-considered as they represent improbable genotypes. Redundant loci were hidden. Excluding these loci will cause a minor reduction in map distance. All linkage groups, excluding unlinked markers, were exported. Linkage maps were prepared using map distances from MapManager QTXb20 (Manly *et al.* 2001) in MapChart v.2.1 (Voorrips 2002).

3.5.2 Field trial phenotypic data evaluation

Field trials were conducted at the PANNAR Research Station, Greytown, KwaZulu-Natal, South Africa. Field trials were infected with a spore suspension of pathotype 6E22A+ of *P. striiformis* f. sp. *tritici* (avirulent for *Yr1*,*3a*,*4a*,*4b*,*5*,*9*,*10*,*15*,*27*,*Sp* and virulent for *Yr2*,*6*,*7*,*8*,*17*,*25,A*). Plants in the field trials were closely monitored for the development of stripe rust infection. Stripe rust infection was evaluated at different time points within each growing season. Phenotyping was performed once the first signs of stripe rust disease were visible on the susceptible parent, until the reaction type classes could no longer be accurately distinguished. The is generally between growth stages 41 (flag leaf sheath extension) and 69 (anthesis completed) (Zadoks *et al.* 1974).

A quantitative estimation of disease severity was achieved by using the modified Cobb scale (Peterson *et al.* 1948; McIntosh *et al.* 1995). Infected plants were scored for the percentage leaf area affected (0-100%) by the disease (LAI, leaf area infected), either presenting as sporulating uredinia in a susceptible plant or as chlorotic or necrotic tissue produced by a resistant plant in response to infection. A qualitative score that reflects the defence response expressed by the plant was measured as the host reaction type (RT). McIntosh *et al.* (1995) described the RT scoring system for analysis of adult plant phenotypes, with the categories resistant (R), moderately resistant (MR), moderately susceptible (MS) and susceptible (S). The intermediate categories, resistant-moderately resistant (RMR), moderately resistant-moderately susceptible (MRMS) and moderately susceptible-susceptible (MSS) have been added to reflect the whole range of possible reaction types (Table 3.3). These categories were converted to an ordinal scale of 1 (resistant) to 7 (susceptible) to allow statistical analysis. A score of 30MR therefore indicates that 30% of the leaf area is infected, with an associated moderately resistant host reaction type.

Table 3.3 Criteria for evaluating host reaction type.

Reaction Type	Visible symptoms	Phenotype
Resistant (R)	No symptoms to necrotic tissue with no visible uredia	
Resistant-moderately resistant (RMR)	Necrotic tissue with or without small non-sporulating pustules	
Moderately resistant (MR)	Pustules surrounded by necrotic tissue	
Moderately resistant-moderately susceptible (MRMS)	Pustules surrounded by necrotic and chlorotic tissue	
Moderately susceptible (MS)	Pustules surrounded by chlorotic tissue	

Table 3.3 continued Criteria for evaluating host reaction type.

Moderately susceptible-susceptible (MSS)	Pustules surrounded by chlorotic and green tissue	
Susceptible (S)	Pustules surrounded by green tissue, no chlorosis or necrosis visible	

Photographs provided by ZA Pretorius

3.5.3 QTL identification

The predicted means across replicates were calculated using the linear regression function of the statistical package Genstat for Windows, release 12 (Genstat 5 Committee 2005; Payne *et al*. 2009).

LAI data were transformed with the arcsin function, also called an angular transformation.

$$LAI\ transformed = arcsine\ \sqrt{LAI} = sin^{-1}\sqrt{LAI}$$

and RT data was transformed with the log function,

$$logit(RT) = \log\left(\frac{RT}{1-RT}\right)$$

to ensure a normal distribution of the residuals. CIM was performed with Windows QTL Cartographer v.2.51 (Wang *et al.* 2011) using a forward regression model with a window size of 10 cM and a walk speed of 1 cM. Both the transformed and untransformed trait data were included in the analysis to increase confidence in the obtained QTL.

The LOD score was calculated from the likelihood ratio (LR) test statistic computed by Windows QTL Cartographer,

$$LOD = \frac{1}{2}(log_e) \times LR = 0.217\ LR$$

To determine the significance LOD value for detecting QTL, 1000 permutations were performed (*P* = 0.05) (Doerge and Churchill 1996) for the LAI and RT traits at each score date. QTL graphs were reconstructed in Microsoft Office Excel 2007 from data obtained from Windows QTL Cartographer.

4. IDENTIFICATION OF ADULT PLANT RESISTANCE TO STRIPE RUST IN THE WHEAT CULTIVAR CAPPELLE-DESPREZ

4.1 Introduction

4.1.1 The French wheat pool

The history of wheat breeding in France was reviewed by Bonjean *et al.* (2001). In the early years of wheat cultivation in France wheat populations consisted of bulks selected from native land races (the so-called wheat population *de pays*), the d'Aquitaine group being derived from imported Ukranian wheat and English wheats. In 1883 Henry de Vilmorin (1843-1899) distributed the first modern French wheat, Dattel (Chiddam d'automne à epi rouge X Prins Albert). Other crosses were made between the d'Aquitaine group and English wheats, giving rise to the series of Vilmorin varieties. Originating in the Parisian basin, the Vilmorin dynasty was the founder of the French school of wheat breeding which developed at the end of the 19th and the beginning of the 20th century. Vilmorin wheats occupied 30% of the French acreage sown to wheat, the d'Aquitaine wheats 20% and the wheat de pays 50%. From 1945, after several years of using the same parents and yielding varieties which had a number of characteristics in common, breeders started to introduce parents from other parts of the world in their crosses. Related wild and cultivated species provided another important reservoir of usable genes for breeders. Attention was paid to increasing the stability of yield, resistance to abiotic stress (cold, shrivelling, etc.) and diseases, and later baking quality.

The period 1945-1960 was marked by two famous varieties, Cappelle-Desprez and Etoile de Choisy, which had very different pedigrees. The two varieties subsequently played a major role in the genetic composition of French wheat and gave birth, directly or indirectly, to varieties such as Champlein, Capitole, Hardi, Top, Talent Arminda, Fidel and Camp-Rémy, which dominated the period until the 1980s (Bonjean *et al.* 2001).

4.1.2 Cultivation of Cappelle-Desprez

Cappelle-Desprez (pedigree: Vilmorin-27 X Hybride de Joncquois), released in France in 1946 (Bonjean *et al.* 2001) and marketed in the UK since 1953 (White *et al.* 2008), was the positive result of combining d'Aquitaine and English wheats (Fig. 4.1). The cultivar comprised the majority of acreages sown to wheat in Western Europe until the 1970s (Lupton and Macer 1962; Worland and Law 1986). Cappelle-Desprez is of conventional height and carries the wild-type, tall allele for the dwarfing gene *Rht8*, located on chromosome 2D (Worland and Law 1980; Korzun *et al.* 1998). The cultivar also possesses the alleles for sensitive photoperiodic response (*ppd1*, *ppd2* and *ppd3*) favoured by Western European wheats, where an extended growing period increases yield (Martinić 1975, according to Worland and Law 1986). Cappelle-Desprez has vernalisation requirements (alleles

vrn1, *vrn2* and *vrn3*) (Korzun *et al.* 1998; Worland and Law 1986). Cappelle-Desprez carries the reciprocal centromeric translocation, 5BL-7BL and 5BS-7BS (Riley *et al.* 1967; Seal 1982) which involves the entire long and short arms of the respective chromosomes (Law and Worland 1996). The cultivar also carries the less frequently occurring 3B-3D translocation (Riley *et al.* 1967). Upon release, Cappelle-Desprez exhibited good resistance to stripe rust as well as eyespot disease (Law *et al.* 1978; Thiele *et al.* 2002), much of which was maintained throughout its commercial life-span.

4.1.3 Disease resistance displayed by Cappelle-Desprez

4.1.3.1 Eye spot resistance

Eyespot is caused by two fungal species, *Oculimacula yallundae* and *Oculimacula acuformis*. The disease is widespread in areas where mild and damp autumns promote the growth and spread of the pathogen (Burt *et al.* 2010). The best characterised resistance available to breeders are the genes *Pch1* and *Pch2*. *Pch1* was introduced from *Aegilops ventricosa* and *Pch2,* which is less effective, was originally identified in Cappelle-Desprez. *Pch2* has been mapped to chromosome 7AL, flanked by the SSR loci *Xgwm346*, *Xgwm525* and *Xcfa2010* (Chapman *et al.* 2008). In the UK, the resistance from Cappelle-Desprez became the basis of an extensive programme of breeding eyespot-resistant wheat varieties (Bingham 1981).

4.1.3.2 Leaf rust resistance

Under controlled environmental conditions, adult plants of Cappelle-Desprez showed partial (slow-rusting) resistance to leaf rust, in the field, as well as in the greenhouse (Poyntz and Hyde 1987; Denissen 1993). This observation raised the question as to whether Cappelle-Desprez possessed *Lr34/Yr18/Pm38,* which provides protection against both leaf and stripe rust (McIntosh 1992; Bossolini *et al*. 2006). Lagudah *et al.* (2009) used gene specific markers to show that *Lr34/Yr18/Pm38* is not present in Cappelle-Desprez.

4.1.3.3 Stripe rust resistance

Cappelle-Desprez is known for its resistance to stripe rust. Although fully resistant when released in 1946, it has maintained a partial resistance throughout the period of its cultivation in Europe. This resistance is still evident in descendants cultivated today, like Flinor (released 1974) (Feng *et al.* 2011), Camp Rémy (released 1980) (Jagger *et al.* 2011) and Claire (released 1999) (Powell 2010) (Fig. 4.1).

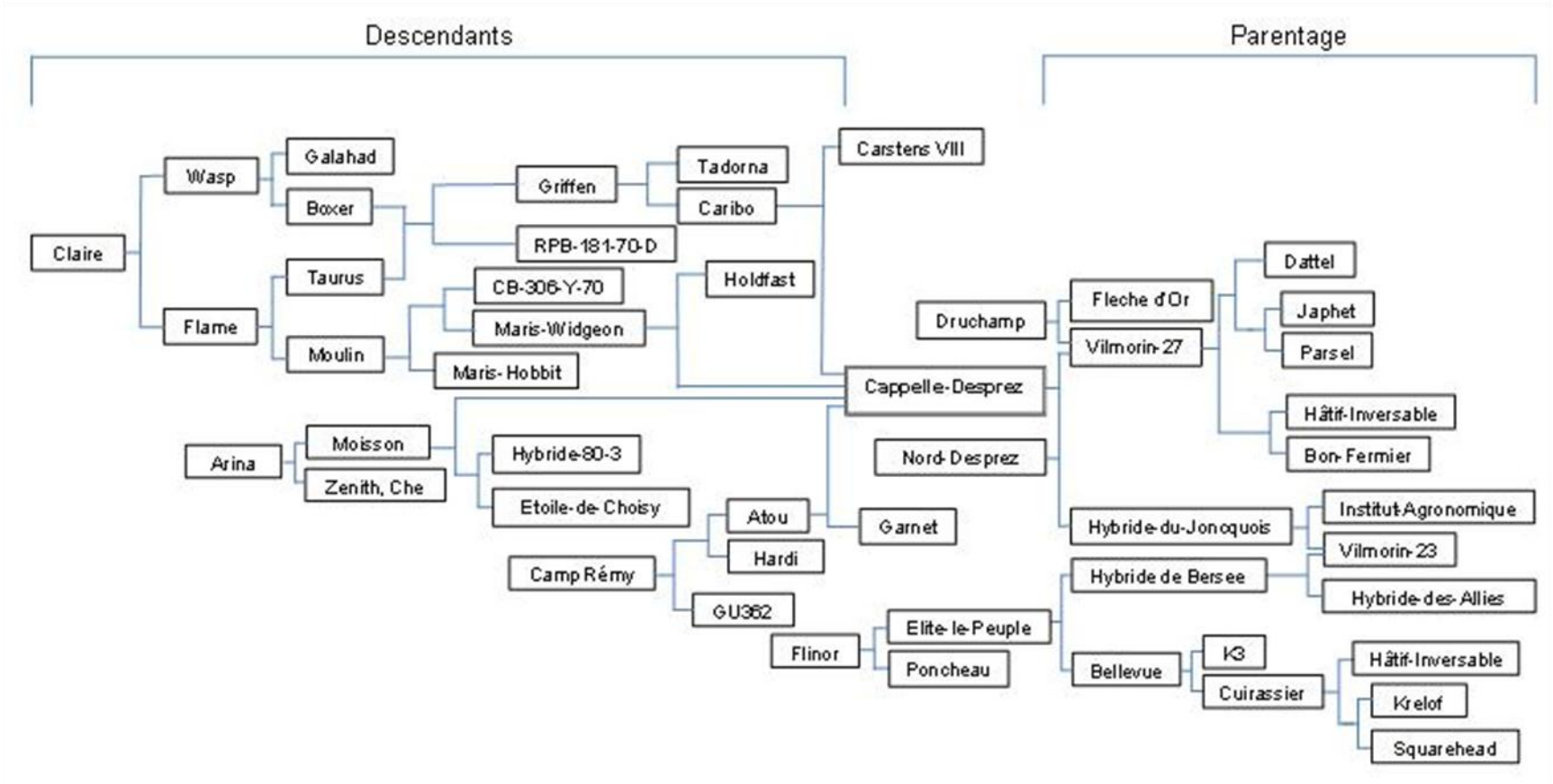

Figure 4.1 The pedigree of Cappelle-Desprez, including the parentage of descendants like the cultivars Arina, Camp Rémy, Claire and Flinor (reconstructed from Bonjean *et al.* 2001; Powell 2010; Feng *et al.* 2011; the Wheat Pedigree Online Database, http://genbank.vurv.cz/wheat/pedigree and the USDA Germplasm Resources Information Network, http://www.ars-grin.gov).

4.1.3.3.1 Seedling resistance

A small number of known race-specific resistance genes are widely distributed in French wheat due to the mostly accidental nature of the introduction of specific resistance factors found in commercial cultivars (De Vallavieille-Pope *et al*. 1990). Cappelle-Desprez possesses the seedling stripe rust resistance genes *Yr3a* and *Yr4a* (De Vallavieille-Pope *et al*. 1990). *Yr3* was located to chromosome 5BL and *Yr4* to chromosome 3B by Worland (1988) and are listed as such in the Wheat Rust Atlas (McIntosh *et al*. 1995). However, Chen *et al*. (1996) determined their positions to be on chromosome 1B and 6B, respectively. These locations have not been validated. Virulence for *Yr3a* and *Yr4a* has been detected, but not in South Africa (personal communication, Pretorius ZA).

4.1.3.3.2 Adult plant resistance (APR)

Reports on the APR in Cappelle-Desprez are not definitive. Early genetic and cytogenetic studies implicated a locus on chromosome 2D, designated *Yr16* (Worland and Law 1986), and the 5BS-7BS translocation (Law and Worland 1997). *Yr16* and the 5BS-7BS translocation are however, unlikely to be the only contributors to stripe rust APR in Cappelle-Desprez. While the identity of the full complement of resistance in Cappelle-Desprez remains unclear, it is believed to have been incorporated into many European varieties. Law and Worland (1996) studied the contribution of each of the Cappelle-Desprez chromosomes by substituting Bezostaya 1 chromosomes into Cappelle-Desprez monosomics. Eleven of the 20 chromosomes assayed showed significant effects on the level of APR, six increasing and five reducing resistance. These predictions for stripe rust resistance have highlighted the very important point that as more and more chromosomes for resistance are brought together, a major part of the potential variation for this character will remain undetected. Although APR is generally believed to be more durable, instances have been found in which such resistance was highly race-specific (Zadoks 1961; Johnson and Taylor 1972; Johnson 1978). APR can be assessed using races selected as virulent at the seedling stage.

4.1.3.3.2.1 Chromosome 2D and *Yr16*

Chromosome 2D is known to carry several genes of economic importance. These include a gene for day length insensitivity, *Ppd1,* and *Rht8*, a gibberellic acid responsive dwarfing gene (Worland and Law 1986). *Yr16* was located to the centromeric region of chromosome 2D in Cappelle-Desprez through cytogenetic analyses (Worland and Law 1986; Worland *et al*. 1988). The gene was placed 9.3 cM from the centromere between RFLP marker loci *Xpsr641-2D* and *Xpsr681-2D* on a consensus map, Ta-Gale-2D (http://wheat.pw.usda.gov/GG2/index.shtml; Devos *et al*. 1993). Worland and Law (1986) reviewed recombination frequencies and determined the gene order to be *Rht8-Ppd1-Yr16-D4*. *D4* is another hybrid dwarfing gene (Hermsen 1967). The chromosome 2B short arm corresponds to 2DL and 2BL to 2DS (Sears and Sears 1978). Worland and Law (1986) suggested that *Ppd1*, *Yr16* and *D4* are homoeoallelic to *Ppd2*, *Yr5*/*Yr7* and *D2* on 2B.

4.1.3.3.2.2 5BS-7BS translocation

The centromeric reciprocal translocations 5BL-7BL and 5BS-7BS were prevalent in West European wheats in the 1960s and 1970s (Riley *et al*. 1967). This translocation can be traced to the variety Noé, which appeared in France in the latter part of the 19th century and probably has a Russian origin (Lupton 1987).

Law *et al.* (1978) used aneuploid lines to study stripe rust resistance conferred by the 5BS-7BS translocation in Cappelle-Desprez. Ditelosomic 5BS was indistinguishable from the euploid, indicating that this arm of the chromosome contained the major part of the translocation resistance effect. Ditelosomic 7BS was phenotypically identical to nullisomic lines. They found no evidence that the significance of this chromosome in disease control was due to the translocation itself. Thus, the resistance determined by the chromosome does not appear to be due to a major complementary interaction between genes on the short arms of chromosomes 5B and 7B. Both the parents of Cappelle-Desprez carry this translocation. However, Vilmorin-27 has a high level of resistance while Hybride de Joncquois is susceptible. Law and Worland (1997) found the translocations from resistant and susceptible varieties to be identical, which lead them to conclude that background chromosomes were mainly responsible for the differences between varieties (Law and Worland 1996; Law and Worland 1997).

4.1.3.4 Stripe rust suppressor genes

Cappelle-Desprez chromosome 6B has been shown to carry a stripe rust suppressor gene (Law and Worland 1996). Substitution of this chromosome resulted in a line which is more resistant than Cappelle-Desprez. Datta *et al.* (2008) observed immune type seedlings (infection type 0;) in F_3 and advanced generations of a Cappelle-Desprez cross, yet Cappelle-Desprez seedlings showed a high infection type (;2) for the Indian pathotypes tested. This phenomenon could only be explained by the presence of a suppressor of seedling resistance in Cappelle-Desprez. Stripe rust suppressor loci have not been reported in more recent publications, but warrants further investigation.

4.2 Study objectives

Capelle-Desprez has shown excellent stripe rust resistance since it was first evaluated in South Africa in 1998 (Boshoff *et al*. 2002b; Pretorius ZA, unpublished). The focus of this study was to confirm the presence and location of *Yr16* on chromosome 2D and the role of the 5BS-7BS translocation in stripe rust resistance, while investigating whether additional stripe rust QTL, derived from Cappelle-Desprez, are effective under field conditions in South Africa. *Yr16* has not been used in breeding for stripe rust resistance in South Africa as it resides in European cultivars mostly adapted to Western European conditions. If the genes can be isolated in an acceptable background, they would provide

additional sources of resistance to breeders. To accelerate the diversification of the South African stripe rust resistance gene pool, Cappelle-Desprez was introduced through a cross to the susceptible cv. Palmiet. By means of a selection process an adapted breeding line, Yr16DH70 was developed which retained Cappelle-Desprez APR, but lacked the seedling stripe rust resistance genes, *Yr3a* and *Yr4a*.

4.3 Materials and methods

4.3.1 Mapping population development

An initial cross was made between the French winter wheat cultivar Cappelle-Desprez (pedigree: Vilmorin-27/Hybride-du-Joncquois) and the South African cultivar Palmiet (pedigree: SST3*//Scout*5/Agent). Palmiet is a stripe rust-susceptible spring wheat carrying the stem rust resistance genes *Sr2* and *Sr24,* as well as the leaf rust resistance gene *Lr24* (Smit *et al*. 2010). Cappelle-Desprez X Palmiet F_2 seedlings were infected with spores of *P. striiformis* f. sp. *tritici* pathotype 6E16A- (avirulent/virulent for *Yr1*,*3a*,*4a*,*4b*,*5*,*9*,*10*,*15*,*25*,*27*,*A*,*Sp* / *Yr2*,*6*,*7*,*8*,*17*) in greenhouse tests. Primary leaves of 7-day-old seedlings were inoculated with urediniospores suspended in light mineral oil. Seedlings were then placed in a dew chamber at 10°C for 48 h. After incubation seedlings were dried off and transferred to a greenhouse cubicle at 15-20°C. Seedling infection types were scored on a 0 to 4 scale (McIntosh et al. 1995) 14 days after inoculation. Susceptible seedlings were kept and plants expressing APR were advanced to the F_4 generation through selfing. One F_4 plant was selected for back-crossing to Palmiet based on its high level of stripe rust resistance and other favourable agronomic characters. The pathotype 6E22A- (avirulent/virulent for *Yr1*,*3a*,*4a*,*4b*,*5*,*9*,*10*,*15*,*27*,*A*,*Sp* / *Yr2*,*6*,*7*,*8*,*17*,*25*) was used to establish field infections. DH lines were produced from the resulting F_1 plants. One DH line, Yr16DH70 was subsequently selected (Fig. 4.2). Palmiet was once again chosen as the adapted, stripe rust-susceptible parent to cross with Yr16DH70. A RIL mapping population, consisting of 204 individuals, was derived from the Palmiet X Yr16DH70 cross by advancing random individual F_2 plants to the F_7 generation by single seed descent.

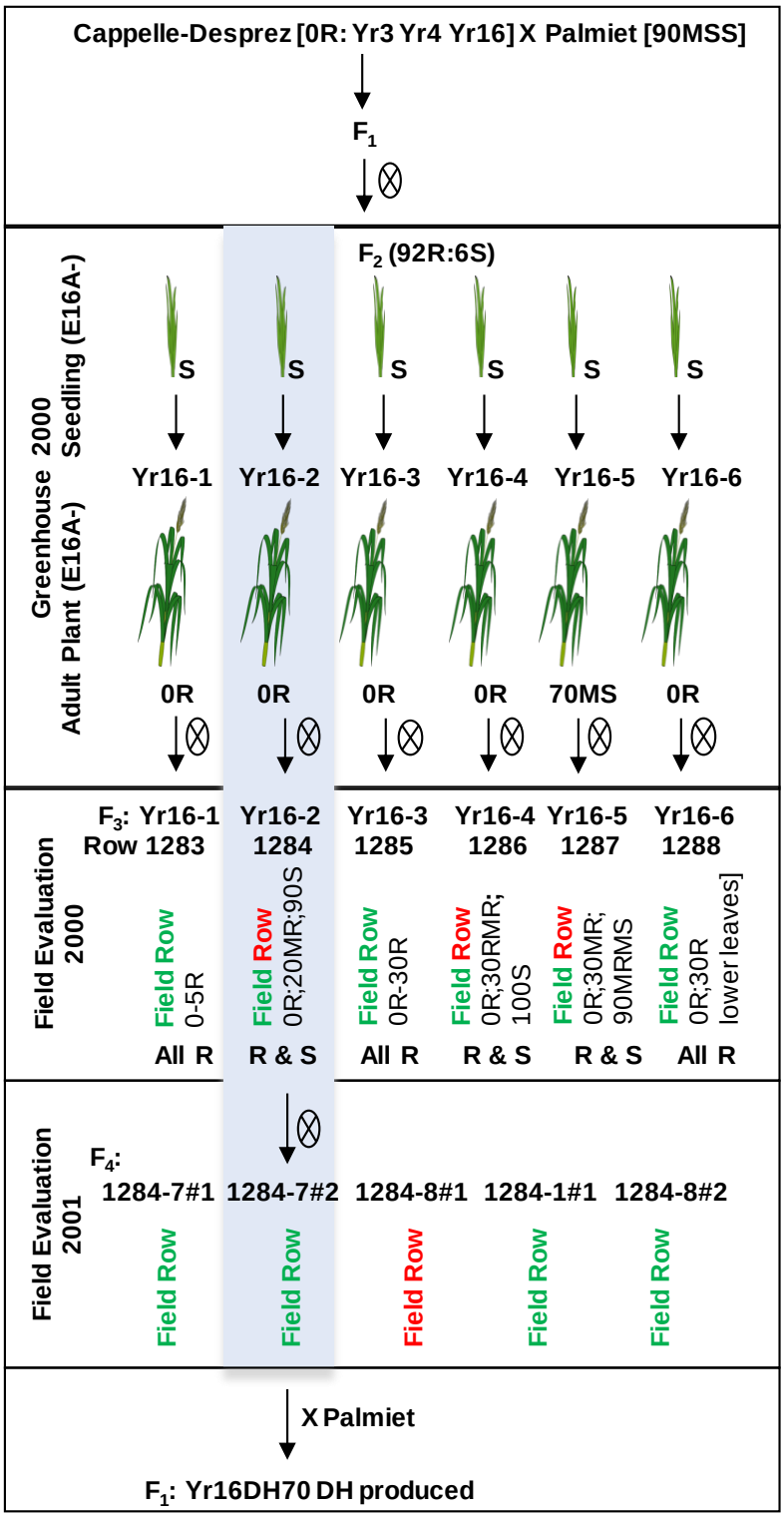

Figure 4.2 Diagram explaining the development of the DH breeding line Yr16DH70. The lines selected with this strategy is shown are the shaded column. The red text ("Field Row") represents susceptible plants, the green text represents resistant plants and a combination of red and green text represents rows segregating for resistance. R – resistant; MR – moderately resistant; MRMS – moderately resistant-moderately susceptible; MS – moderately susceptible and S – susceptible.

4.3.2 Disease evaluation in field trials

Field trials were conducted during 2009 and 2010 at the PANNAR Research Station, Greytown, KwaZulu-Natal, South Africa. The 204 RILs were planted in May 2009 and May 2010 in two randomised replicates. Entries were planted in 1 m row plots spaced 90 cm apart. Each block contained several Palmiet and Yr16DH70 parental controls. The highly susceptible cv. Morocco was planted in spreader rows bordering the trial area, in all pathways perpendicular to plots, and as every tenth entry within the trial. Control plots of Cappelle-Desprez were also included. Field trials were infected with a spore suspension of pathotype 6E22A+ of *P. striiformis* f. sp. *tritici* (avirulent/virulent for *Yr1*,*3a*,*4a*,*4b*,*5*,*9*,*10*,*15*,*27*,*Sp* / *Yr2*,*6*,*7*,*8*,*17*,*25,A*). Stripe rust epidemics in both years were initiated by spraying a suspension of fresh urediniospores in light mineral oil (approximately 2 mg spores per ml) onto rust spreader rows. Following inoculation certain Morocco rows were covered with plastic sheeting overnight to ensure saturated conditions required for spore germination and infection. Stripe rust severity was scored as LAI and RT (described in section 3.5.2). LAI was scored at the Greytown site on 14 September 2009 (LAI14Sept09), 23 September 2009 (LAI23Sept09), 5 October 2009 (LAI5Oct09), 27 September 2010 (LAI27Sept10) and 11 October 2010 (LAI11Oct10). RT was scored on 14 September 2009 (RT14Sept09), 23 September 2009 (RT23Sept09) and 27 September 2010 (RT27Sept10).

A field trial was also planted in May 2010 at the Sensako Research Station, Napier, Western Cape, South Africa, with the RIL population. Layout of the trial plots was similar to those at Greytown. Each block contained Palmiet and Yr16DH70 parental controls and the cv. Morocco was planted in spreader rows. The trial was infected with a spore suspension of pathotype 6E22A+ and the trial was scored for LAI on 21 September 2010 (LAI21Sept10) and 29 September 2010 (LAI29Sept10). RT was scored only on 21 September 2010 (RT21Sept10).

4.3.3 Statistical analyses

All statistical analyses were performed using the statistical package Genstat for Windows, release 12 (Genstat 5 Committee 2005; Payne *et al*. 2009). The LAI and RT scores were transformed to achieve near normality and independence of the means and variances. LAI data were converted to proportions and transformed to arcsin (Sokal and Rohlf 1995), while the RT data were transformed with the log function (refer to section 3.5.3). Comparisons between field phenotypic data sets were carried out for both LAI and RT scores using a 2-sided test of correlation. Analysis of variance (ANOVA) was carried out using the general linear regression (GLR) model in Genstat v.12, which supports unbalanced designs and missing data. The effects of field replicates and genotypes were accounted for in the model. Predicted means for each RIL, for all LAI and RT data sets, were extracted and used in further analyses to minimise error variation in mean values. To identify significant effects between different QTL combinations the GLR model compared RILs within QTL

groups and between groups using t-test comparisons, with all the QTL groups being compared to each other (Jagger *et al*. 2011).

4.3.4 Marker analysis

Genomic DNA was isolated from the parents and a representative individual from the Palmiet X Yr16DH70 F_6 RIL generations using the CTAB DNA extraction method for SSR marker analysis. Genomic DNA was isolated from the F_7 RIL generation for DArT analysis with the Zymo Research Plant/Seed DNA kit™. DNA concentrations were determined with a NanoDrop Spectrophotometer ND-1000 and normalised. The quality and molecular weight were ascertained by means of agarose gel electrophoresis.

The parents lines, Palmiet, Yr16DH70 as well as Cappelle-Desprez were screened with 465 fluorescently labelled SSR markers. These markers were selected to be uniformly distributed across the three wheat genomes, with each of the 21 chromosomes being well represented. Polymorphic SSR markers and DArT markers (using the PstI/TaqI v3.0 array enriched for the D-genome, Triticarte P/L and Diversity Arrays Technology P/L, Canberra, Australia) were typed in the RIL population. One sequence tagged site (STS) marker for the gene *Pina-D1* (US public MAS wheat breeding programs, http://maswheat.ucdavis.edu) and one converted DArT marker, *wPt-0600* (Yu *et al*. 2009) were also screened in the population. The SSR marker WMC525 linked to the eyespot resistance gene from Cappelle-Desprez, *Pch2* (Chapman *et al.* 2008), was included in the parental screen. Marker data were used to construct a linkage map.

4.3.5 QTL identification

Marker data were used to construct a linkage map. Markers were ordered according to the RECORD output (Van Os *et al*. 2005a). Map distances were calculated using the Kosambi map function from MapManager QTXb20 (Manly *et al.* 2001) and linkage groups were declared based on map distances and linkages between markers at a significance level of $P = 0.001$. Chromosomes were assigned to linkage groups by referring to a consensus map (Appels 2003). Heterozygote scores were replaced with a missing data score. Redundant loci were hidden and unlinked markers were excluded for the purpose of QTL analysis.

For QTL analyses CIM was performed with Windows QTL Cartographer v.2.51 (Wang *et al*. 2011) as described in section 3.5.3. Both transformed and untransformed LAI and RT phenotypic data were analysed to increase confidence in the QTL identified. Predicted means obtained for each RIL from the GLR analyses in Genstat v.12 were also used in the QTL analysis. To determine the LOD threshold value above which a QTL is considered significant, 1000 permutations were performed ($P = 0.05$) (Doerge and Churchill 1996) for the LAI and RT traits at each score date. Maps were prepared using MapChart v.2.1 (Voorrips 2002) and Microsoft Office Excel 2007. RIL were assigned to groups

depending on which combination of QTL they carried. The presence of a QTL was based on the genotype of the QTL-associated markers with the highest LOD value in the QTL interval. The predicted means for the LAI and RT phenotypes were calculated for each QTL group.

4.3.6 Cappelle-Desprez identity validation from other sources

DNA from parental plants used by Nazari *et al.* (2005) to study stripe rust resistance in a Cappelle-Desprez X Avocet S mapping population was requested from C Wellings (University of Sydney, Australia). Selected SSR markers flanking the QTL identified in this study were typed on the DNA supplied for Cappelle-Desprez and Avocet S to confirm identity to the Cappelle-Desprez parent used to derive Yr16DH70.

4.3.7 Relationship between QTL detected in the cv. Claire

QTL have been identified in a Claire X Lemhi DH mapping population (Powell *et al.* 2008; Powell 2010). Claire descended from Cappelle-Desprez. The EST markers identified in the proximity of *QTLAPR.2Db* on chromosome 2D, (EST6, EST10 and EST22) were typed in the Palmiet X Yr16DH70 RIL mapping population after confirming the markers to be polymorphic in this population (Table 4.1). The forward primers for these markers were fluorescently labelled, amplified and separated by means of agarose gel electrophoresis to optimise amplification. Once optimised, these fragments were analysed through capillary array electrophoresis SSCP. The EST markers were added to the linkage map with the “Find” function of MapManager QTXb20 (Manly *et al.* 2001). In addition, DNA from the parents, as well as four individuals from the Claire X Lemhi DH mapping population was screened with selected SSR markers flanking the QTL identified in this study.

Table 4.1 Intron flanking EST-derived markers for chromosome 2D as designed by Powell (2010).

EST marker	EST accession	Forward primer (5’→3’)	Reverse primer (5’→3’)	Predicted amplicon size
EST6	BE498622	GCC AAG TCT GGA GGA TCA AG	TGG AGT TGA AGC TCT CTG TTG	258
EST10	BE490491	TCC AGC GAC CCC TAC GTC	AGA GGG TCA GCT CGT CGT T	374
EST22	BE403768	GGG AAA GGC ATT CTT CAC AA	CCT CCC AAT GCG TTG TTA T	543

4.4 Results

4.4.1 Mapping population development

To eliminate seedling stripe rust resistance F_2 seedlings from the cross Cappelle-Desprez X Palmiet were screened in greenhouse tests with the pathotype 6E16A-. These tests yielded 98 resistant and six susceptible plants, giving a good fit to the expected 15:1 ratio for the segregation of the two dominant resistance genes, *Yr3a* and *Yr4a* from Cappelle-Desprez ($X^2 = 0.041$; $P = 0.84$). Adult plant

ratings of the susceptible seedlings were 0R (five plants) and 70MS (one plant). The resistant plants were advanced to the F_4 generation through selfing, with F_3 and F_4 progeny being tested for APR in the field. One F_4 plant, displaying complete stripe rust field resistance and other favourable agronomic characters was crossed to Palmiet, a series of DH lines being produced from the resulting F_1 seed to obtain a pure breeding line. The DH line Yr16DH70 was subsequently selected for backcrossing to Palmiet. Yr16DH70 did not display seedling resistance against the South African pathotypes tested (infection type 2+3), but retained APR which was effective under field conditions in South Africa (30R at the end of the growing season). A RIL mapping population (204 lines) was derived from the cross Palmiet X Yr16DH70.

4.4.2 Field assessment of stripe rust resistance

Good stripe rust establishment was seen at the PANNAR Research Station, Greytown in both years, the highly susceptible cv. Morocco displaying 100S midway through the scoring seasons (Fig. 4.3). Although Palmiet is fully susceptible to stripe rust in the winter rainfall regions of South Africa (Boshoff *et al*. 2002a), severe infection is associated with chlorosis and necrosis in the warmer Greytown environment. In 2009 Palmiet scored 70-80MS, while the resistant parent, Yr16DH70 scored 20R at the last assessment (Fig. 4.3). Less infection was seen in the dryer and warmer 2010 season, with Palmiet scoring 40-50MR and Yr16DH70 10-15R (Fig. 4.4). In general, Cappelle-Desprez expressed marginally higher levels of resistance, having scores of 0R through to 20R in field tests from 1999 to 2011. From the original 204 RILs, three lines were excluded from further analysis as they displayed variable agronomic phenotypes and stripe rust reactions between plants within the same line.

Although Palmiet was not fully susceptible in these field trials, ANOVA showed clear segregation of resistance in the RIL population for all LAI and RT data sets (variability between RIL, F value probability < 0.001; Fig. 4.4; Table 4.2). Some variation between replicates was seen (Table 4.2), although this was only significant for the data sets LAI23Sept09 and RT27Sept10 (F probability < 0.001). Transgressive segregation was seen in both years, with some lines being more resistant than Yr16DH70 and others more susceptible than Palmiet (Fig. 4.4). Comparisons of phenotypic data sets (Table 4.3) showed high, positive correlations for LAI scores, both within and across years (r = 0.61 to 0.93). For RT the correlation between the two score dates in 2009 (r = 0.74) was greater than between years (r = 0.53 and 0.58), although all correlations were significant (P < 0.001).

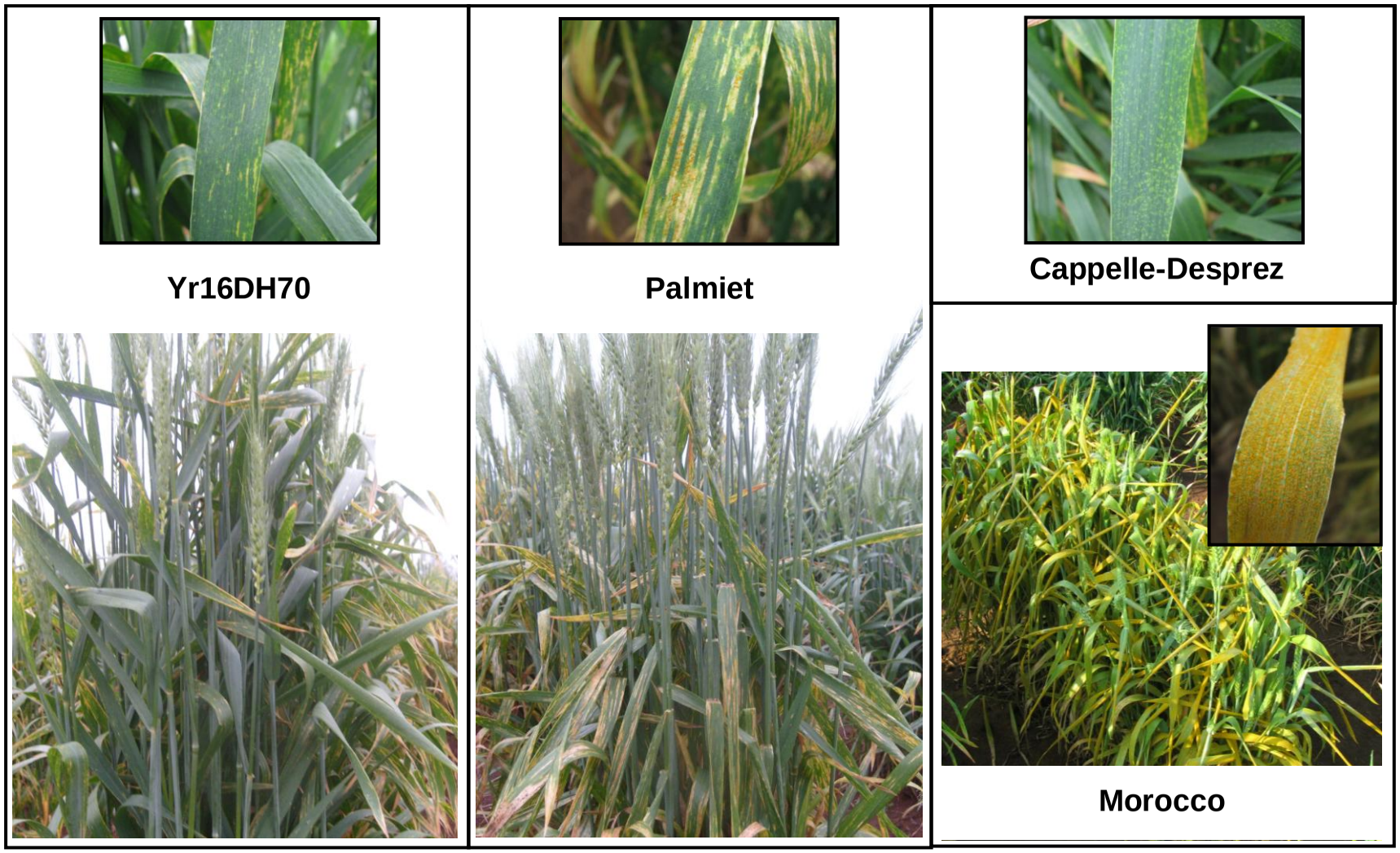

Figure 4.3 Examples of the parental phenotypes as scored in 2009 at the Greytown field trial. Palmiet scored 70-80MS, while the resistant parent, Yr16DH70 scored 20R. Cappelle-Desprez, the cultivar Yr16DH70 was developed from, has a resistant phenotype under South African growing conditions. The susceptible response of Morocco is included for comparison of stripe rust reaction types. (Some photographs provided by ZA Pretorius).

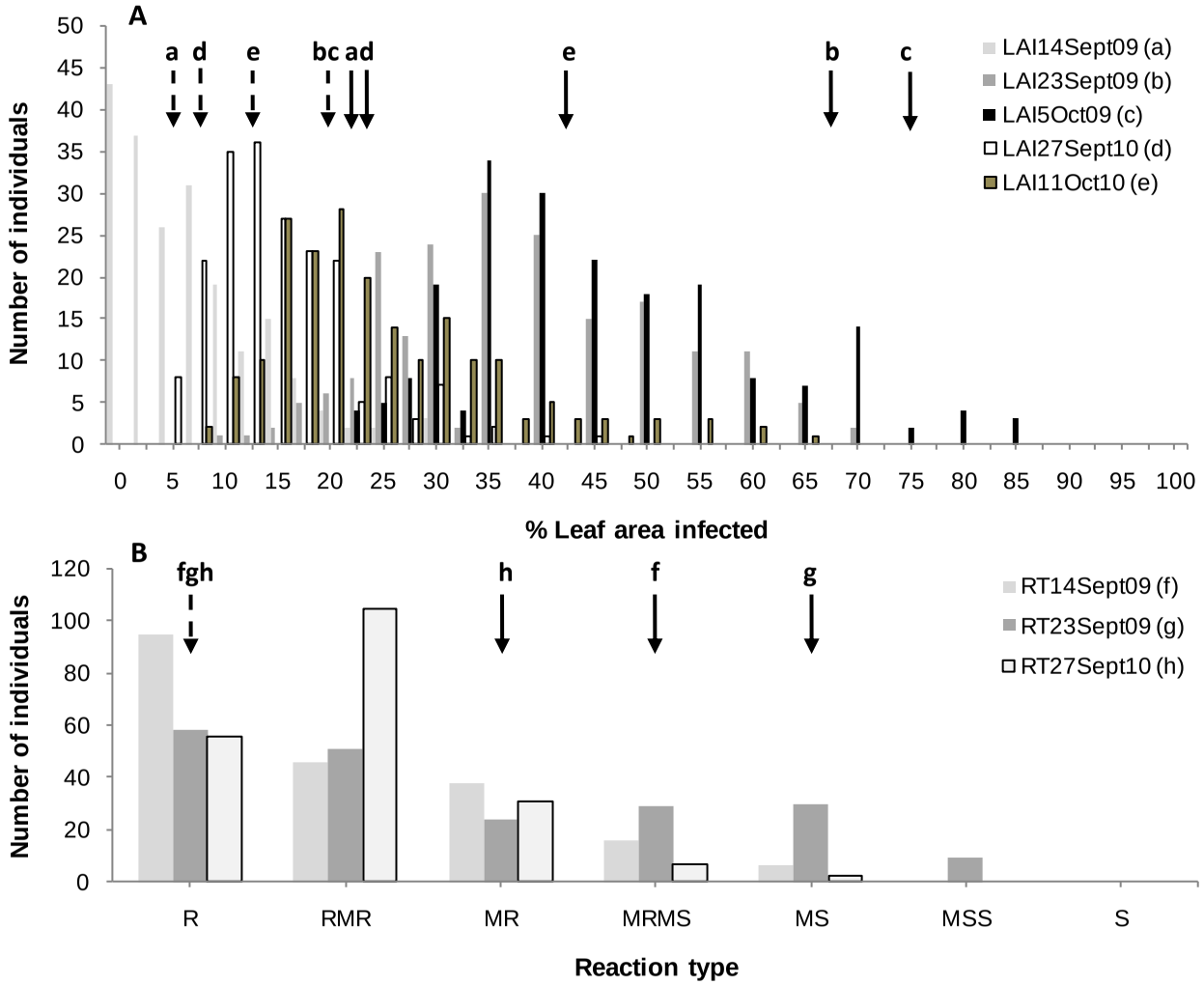

Figure 4.4 Segregation of leaf area infected (A) and reaction type (B) phenotypes in the Palmiet X Yr16DH70 RIL mapping population for the 2009 and 2010 seasons. The broken arrow represents the disease scores for Yr16DH70 and the solid arrow the scores for Palmiet for the eight phenotypic data sets as indicated by lowercase letters.

Table 4.2 Analysis of variance calculated using a general linear model for stripe rust LAI and RT disease scores in the Palmiet X Yr16DH70 RIL mapping population.

Trait	Source	*df*	Mean Square	*F*-value	*P*-value
LAI14Sept09	Replicate	1	149.32	3.55	0.061
	Genotype	200	87.05	2.07	<.001
	Residual	200	42.07		
LAI23Sept09	Replicate	1	1516.21	26.03	<.001
	Genotype	200	325.35	5.59	<.001
	Residual	197	58.24		
LAI5Oct09	Replicate	1	85.09	1.29	0.258
	Genotype	200	426.99	6.46	<.001
	Residual	199	66.13		
LAI27Sept10	Replicate	1	42.06	2.32	0.129
	Genotype	201	102.85	5.68	<.001
	Residual	200	18.1		
LAI11Oct10	Replicate	1	136.5	3.6	0.059
	Genotype	201	245.14	6.47	<.001
	Residual	200	37.9		
RT14Sept09	Replicate	1	3.7836	4.51	0.035
	Genotype	200	2.0038	2.39	<.001
	Residual	200	0.8386		
RT23Sept09	Replicate	1	0.0492	0.07	0.785
	Genotype	200	4.5366	6.85	<.001
	Residual	197	0.6623		
RT27Sept10	Replicate	1	7.1681	20.07	<.001
	Genotype	201	1.1622	3.25	<.001
	Residual	200	0.3571		

df – degrees of freedom

The 2010 field trial at the Sensako Research Station, Napier, was grown under dryland conditions. Due to high day temperatures and low precipitation levels the Palmiet X Yr16DH70 RIL mapping population did not perform well. Although adequate levels of stripe rust infection were obtained the plants were stressed and consistent disease readings could not be obtained.

Table 4.3 Correlation coefficients (*r*) determined with a two-sided test for the Palmiet X Yr16DH70 RIL population of LAI and RT traits over two years, at various dates during each season.

	LAI14Sept 2009	LAI23Sept 2009	LAI5Oct 2009	LAI27Sept 2010	LAI11Oct 2010	RT14Sept 2009	RT23Sept 2009	RT27Sept 2010
LAI14Sept2009	-							
LAI23Sept2009	0.73	-						
LAI5Oct2009	0.68	0.93	-					
LAI27Sept2010	0.61	0.73	0.71	-				
LAI11Oct2010	0.61	0.72	0.71	0.89	-			
RT14Sept2009	0.68	0.72	0.71	0.58	0.59	-		
RT23Sept2009	0.59	0.76	0.75	0.63	0.64	0.74	-	
RT27Sept2010	0.55	0.59	0.53	0.75	0.78	0.53	0.58	-

Significance are shown at 0.01% ($P < 0.001$) (i.e. all values are significant)

4.4.3 Marker analysis

Different genomic DNA extraction protocols were considered for the DArT analyses as high molecular weight DNA is required with no more than trace amounts of RNA present. The Zymo Research Plant/Seed DNA Kit™ was decided upon for the DNA quality produced (Fig. 4.5). It was also determined that leaf tissue is extremely sensitive to freeze-thaw cycles as this leads to enzymatic degradation of DNA, therefore only fresh tissue was used.

Seventy-three of the 465 (15.7%) SSR markers screened were polymorphic between Palmiet and Yr16DH70. These were mapped in the RIL population along with 336 DArT markers and two STS markers (one converted DArT and the *Pina-D1* quality marker). The SSR marker WMC525, associated with the eyespot resistance gene *Pch2* on chromosome 7A (Chapman *et al.* 2008), showed that the gene was not retained in Yr16DH70 as it carried the Palmi*et* allele at this locus. At the F_6 generation an average of four heterozygotes (2%) per SSR marker could still be detected in the RIL population. Fifteen linkage groups, containing three or more loci were identified, representing 12 chromosomes (Fig. 4.6). All the DArT markers could be incorporated into linkage groups, but four SSR markers (WMC173, GDM111, WMC285 and BARC110) remained unlinked at a significance level of $P = 0.001$.

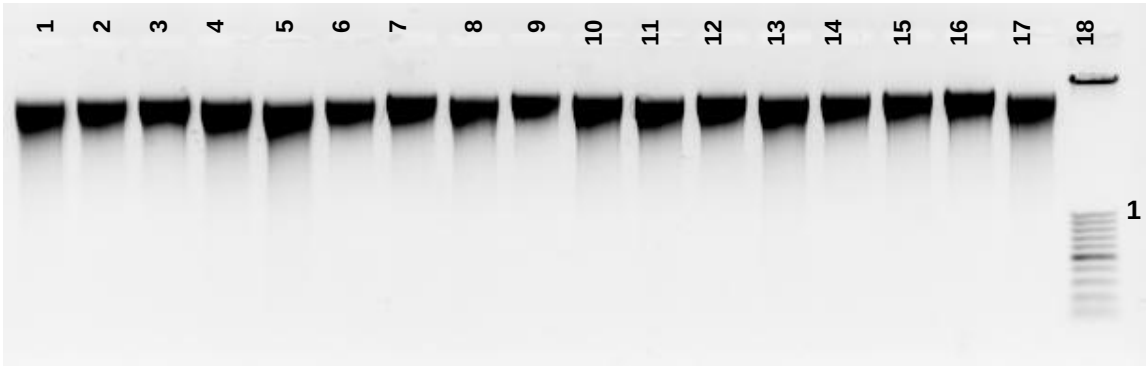

Figure 4.5 Genomic DNA extracted with the Zymo Research Plant/Seed DNA Kit™ resolved on 0.8% (w/v) agarose with 0.5X TBE buffer. Lanes 1-17 show representations of the undiluted extracted DNA and lane 18 shows a 100 bp molecular weight marker (GeneRuler™, Fermentas) as well as a 50 ng lambda DNA standard (Promega Corporation). After electrophoresis at 50V for 1 hour bands were visualised under fluorescent light and captured.

Particularly good marker coverage was obtained for chromosomes 2A (Fig. 4.7) and 2D (Fig. 4.8). The linkage group representing chromosome 2A contained 32 markers with a total map distance of 138.7 cM, while the 2D linkage group contained 96 markers and covered a distance of 158.2 cM (Table 4.4). Chromosomes 5B and 7B were both represented by two, unlinked linkage groups, representing the short and long arms of each chromosome (Fig. 4.6). This would be expected if Yr16DH70 had inherited the 5BS-7BS translocation from Cappelle-Desprez. Chromosome 5D was also represented by two linkage groups (Fig. 4.6). Chromosomes 1A, 1D, 2B, 3A, 4A, 4D, 6A, 6B and 7D were not represented by linkage groups, indicating chromosomes represented primarily by Palmiet DNA. The high number of chromosomes fixed for Palmiet DNA was supported by the lower level of polymorphism found for SSR markers between Palmiet and Yr16DH70 (15.7%), as compared to Palmiet and Cappelle-Desprez (68.0%).

Table 4.4 Summary of marker distribution as mapped in the Palmiet X Yr16DH70 RIL population. Chromosome genetic lengths are compared to consensus maps.

Chromosome / linkage group	**Number of SSR markers**	**Number of DArT markers**	**Total number of markers mapped**	**Genetic length (cM)**		
				PalmietXYr16DH70	Somers *et al.* 2004	Appels 2003
1B	4	40	44	28.7	111	114.4
2A	9	23	32	138.7	143	133.4
2D	21	75	98	158.2	123	143.0
3B	3	23	26	62.2	148	103.7
3D	2	85	87	9.7	79	121.3
4B	10	10	20	75.4	59	89.3
5A	2	3	5	57.5	184	116.3
5B-1	0	10	10	2.0	173	165.0
5B-2	3	9	13 [a]	38.8		
5D-1	3	14	17	7.7	120	119.6
5D-2	2	6	9 [a]	68.6		
6D	5	1	6	40.2	110	202.8
7A	2	10	12	24.3	131	223.0
7B-1	1	18	19	13.9	151	126.3
7B-2	2	9	11	16.0		
TOTAL	**73**	**336**	**407**	**741.9**	**1532**	**1658.1**

[a] Including the STS marker *wPt-0600* on the 5B-2 and *PinaD1* on 5D-2 linkage groups.

4.4.4 QTL analysis of stripe rust resistance

QTL were named according to convention, with “ufs” representing the University of the Free State. CIM analysis detected QTL on chromosomes 2A (*QYr.ufs-2A*), 2D (*QYr.ufs-2D*), 5B (*QYr.ufs-5B*) and 6D (*QYr.ufs-6D)* from Yr16DH70 and one QTL on chromosome 4B (*QYr.ufs-4B*) from Palmiet. Between 33.6% and 75.2% of the total phenotypic variation could be explained by the identified QTL, depending on the phenotypic data set analysed (Table 4.5). QTL analysis was carried out using both transformed and untransformed phenotypic scores, identifying the same QTL, with very similar LOD scores. Only the analyses using the untransformed data are reported (Table 4.5).

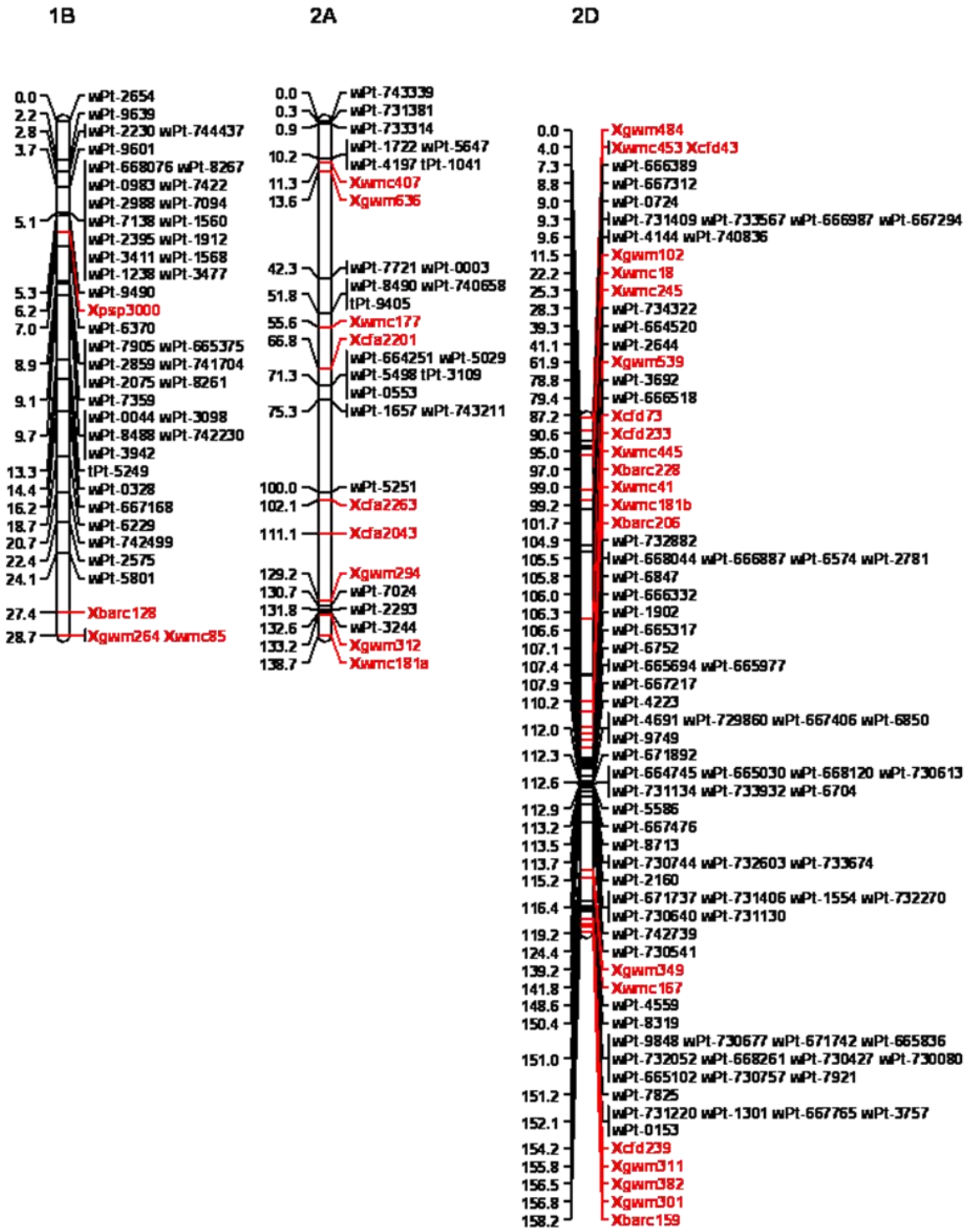

Figure 4.6 Linkage map calculated for the Palmiet X Yr16DH70 RIL mapping population showing 15 linkage groups representing 12 chromosomes. SSR markers are in red and DArT markers and other markers types in black. Distances between markers are in centiMorgan.

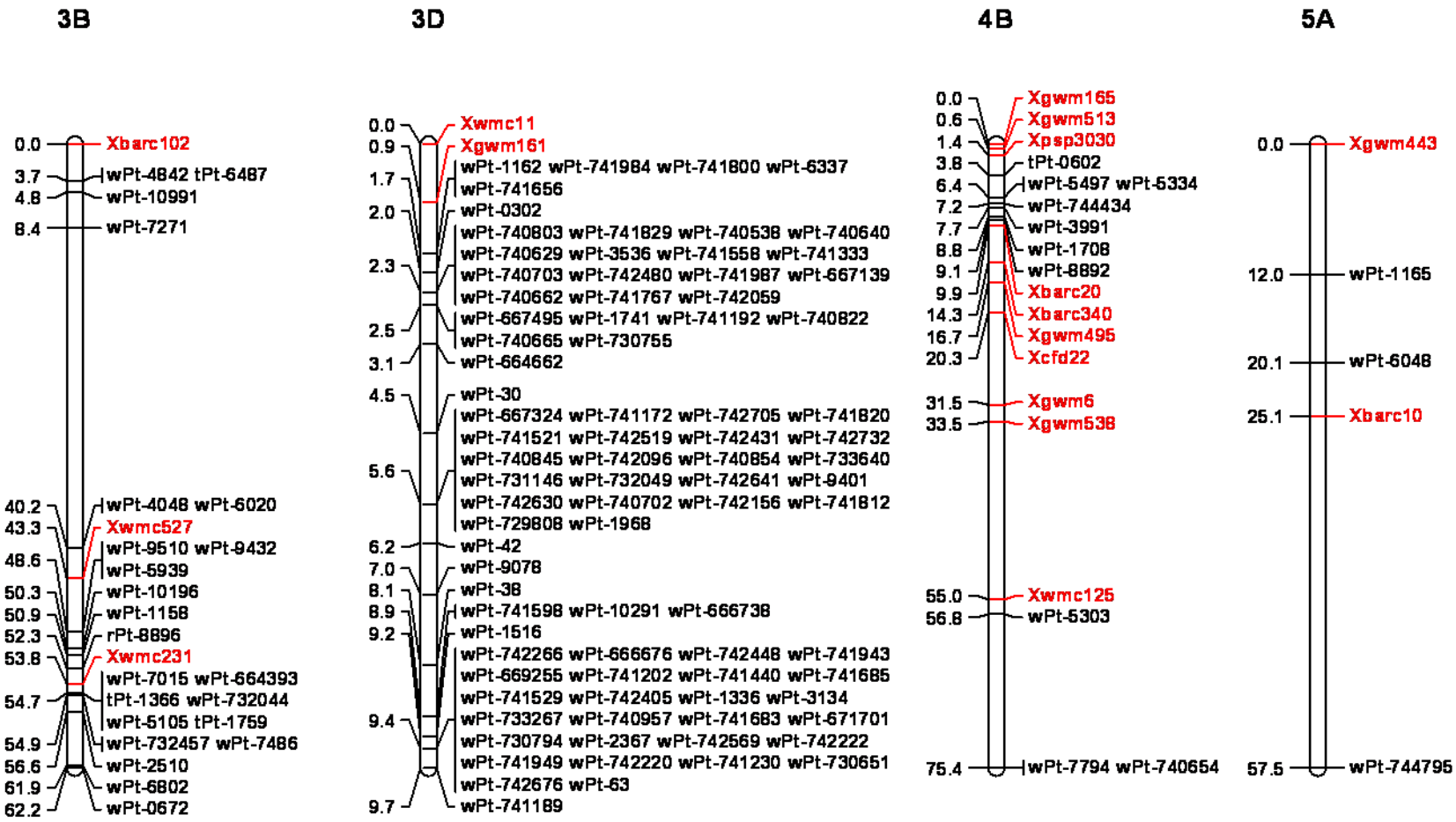

Figure 4.6 continued Linkage map calculated for the Palmiet X Yr16DH70 RIL mapping population showing 15 linkage groups representing 12 chromosomes. SSR markers are in red and DArT markers other marker types in black. Distances between markers are in centiMorgan.

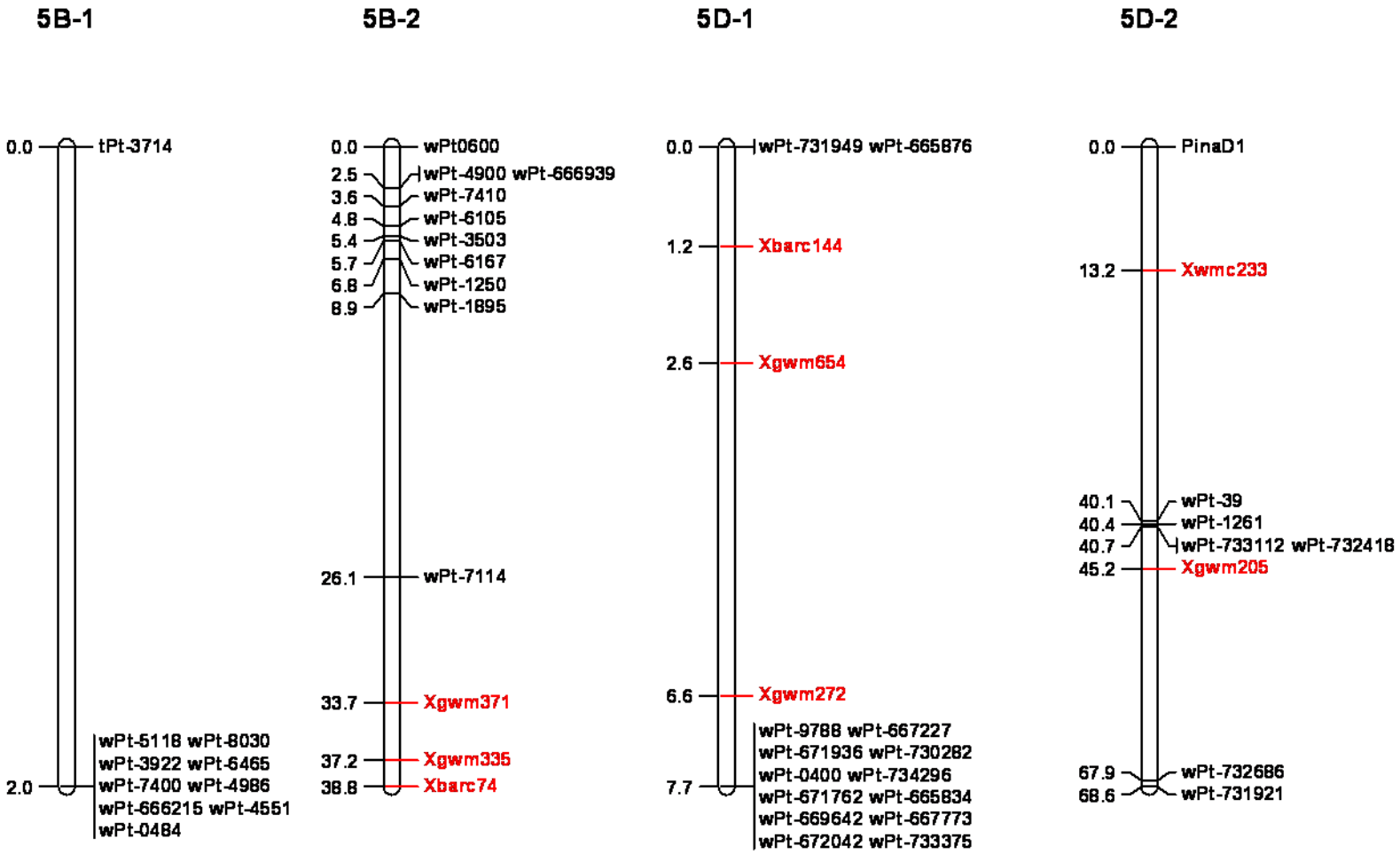

Figure 4.6 continued Linkage map calculated for the Palmiet X Yr16DH70 RIL mapping population showing 15 linkage groups representing 12 chromosomes. SSR markers are in red, DArT markers and other marker types in black. Distances between markers are in centiMorgan.

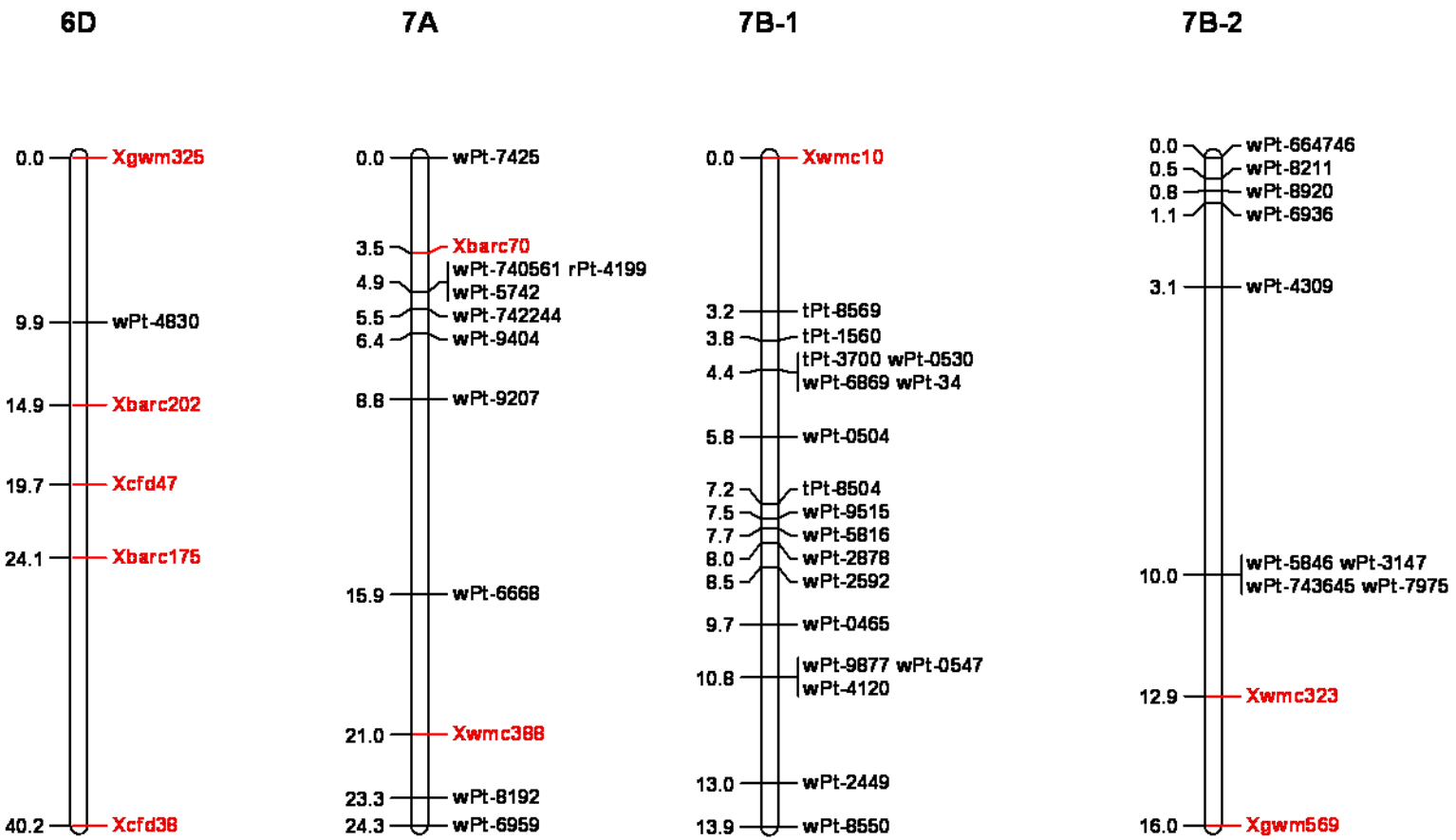

Figure 4.6 continued Linkage map calculated for the Palmiet X Yr16DH70 RIL mapping population showing 15 linkage groups representing 12 chromosomes. SSR markers are in red, DArT markers and other marker types in black. Distances between markers are in centiMorgan.

Table 4.5 Summary of stripe rust APR QTL detected with CIM in the Palmiet X Yr16DH70 RIL mapping population using LAI and RT phenotypic data sets.

			Percentage leaf are infected (LAI)					Host reaction type (RT)			Or oigin
QTL Interval[a]	**Chr[b]**		14Sept09	**23Sept09**	**5Oct09**	**27Sept10**	**11Oct10**	**14Sept09**	**23Sept09**	**27Sept10**	
QYr.ufs-2A	2A	LOD	10.7	18.8	13.7	11.6	13.8	10.6	16.2	8.6	Yr16DH70
wPt-733314-wPt-0003		%VAR[c]	28.8	47.8	39.7	32.6	36.9	30.5	53.2	23.4	(CD)
QYr.ufs-2D	2D	LOD	-	-	-	2.9	5.4	-	6.6	6.1	Yr16DH70
Xgwm102-wPt-664520		%VAR[c]				4.7	8.4		10.3	9.9	(CD[d])
QYr.ufs-4B	4B	LOD	-	-	-	-	-	4.7	7.6	-	Palmiet
Xgwm165-Xgwm495		%VAR[c]						7.0	11.7		
QYr.ufs-5B	5B	LOD	3.2	3.4	2.8	-	-	3.8	-	3.5	Yr16DH70
wPt-7114-Xbarc74		%VAR[c]	4.8	4.9	4.3			5.7		5.3	(CD)
QYr.ufs-6D	6D	LOD	-	4.2	2.8	4.2	-	-	-	-	Yr16DH70
Xgwm325-Xbarc175		%VAR[c]		7.6	4.5	6.2					(CD)
Total variance explained			33.6%	60.4%	48.4%	43.5%	45.3%	43.2%	75.2%	38.6%	

[a]Only QTL with LOD values above the significance levels that were determined after 1000 permutations ($P = 0.05$) are shown

[b]Chromosome

[c]Percentage phenotypic variance explained (R^2) with '-' meaning not significant

[d]Derived from Cappelle-Desprez

A major effect QTL, *QYr.ufs-2A* was detected on the short arm of chromosome 2A with all the phenotypic data sets, explaining up to 53.2% of the phenotypic variation for RT and 47.8% of the variation for LAI (Fig. 4.7; Table 4.5). The QTL mapped between the marker loci *wPt-733314* and *wPt-0003* with the peak located between *Xgwm636* and *wPt-0003*. With the LAI phenotype data sets a potential QTL was also detected at the opposite end of the chromosome 2A linkage group, having a maximum LOD value of 3.2 (Fig. 4.7A). However, the presence of this second QTL on 2AL requires further investigation to provide confirmation. In addition, three, minor QTL were derived from Yr16DH70. *QYr.ufs-2D* was detected on the short arm of chromosome 2D flanked by the marker loci *Xgwm102* and *wPt-664520* and explained up to 10.3% of the phenotypic variation, being detected by both the LAI and RT phenotypic data sets (Fig. 4.8; Table 4.5). *QYr.ufs-5B* explained up to 5.7% of the phenotypic variation, and was detected by both the LAI and RT phenotypes (Fig. 4.9; Table 4.5). *QYr.ufs-5B* spanned an interval of 11.1 cM and was flanked by marker loci *wPt-7114* and *Xbarc74*, placing the QTL near to the centromere. *QYr.ufs-6D* was detected only with the LAI phenotype (Fig. 4.10; Table 4.5). It explained up to 7.6% of the phenotypic variance and was located between the SSR marker loci *Xgwm325* and *Xbarc175*, placing the QTL on the long arm of chromosome 6D. A minor QTL was also detected in Palmiet on chromosome 4B flanked by marker loci *Xgwm165* and *Xgwm495* (Fig. 4.11; Table 4.5). *QYr.ufs-4B* explained up to 11.7% of the phenotypic variance, and was only detected using the RT phenotypic data.

Each RIL was placed in one of 32 groups depending on which of the five QTL it carried. The markers for which the highest LOD values were obtained are *Xgwm636* for *QYr.ufs-2A*, *wPt-734322* for *QYr.uf-2D*, *wPt-8892* for *QYr.ufs-4B*, *Xgwm335* for *QYr.ufs-5B* and *Xgwm325* for *QYr.ufs-6D.* RILs were grouped primarily based on the genotypes of these markers, however, adjacent markers were also considered. The predicted means of each QTL group were obtained from an ANOVA, using the GLR model in Genstat v.12, for all eight LAI and RT datasets (Fig. 4.12, only QTL groups carrying Yr16DH70 QTL are shown). With both LAI and RT phenotypes significant differences were found between QTL groups (F probability < 0.001), while no differences were found between RIL within QTL groups.

Following the ANOVA t-test comparisons of the RILs within each QTL group were undertaken, comparing all 32 QTL groups to each other. *QYr.ufs-2A* alone is more effective than all three Yr16DH70 minor QTL (*QYr.ufs-2D*, *QYr.ufs*-5B and *QYr.ufs-6D*) combined, which in turn is more effective than only combing two (of any combination). A significant effect on stripe rust phenotypes was seen for *QYr.ufs-2A* in combination with the small QTL derived from Yr16DH70, removal of one or more small effect QTL altering the level of resistance (t-test probability < 0.01 to 0.05; Fig. 4.12). Lower disease scores were also seen for both LAI (t-test probability < 0.05) and RT (t-test probability < 0.001 to 0.05) when the minor QTL *QYr.ufs-2D* and *QYr.ufs-5B* were combined. Lines without Yr16DH70 derived QTL (Fig. 4.12 – none) had LAI and RT mean scores statistically similar to Palmiet.

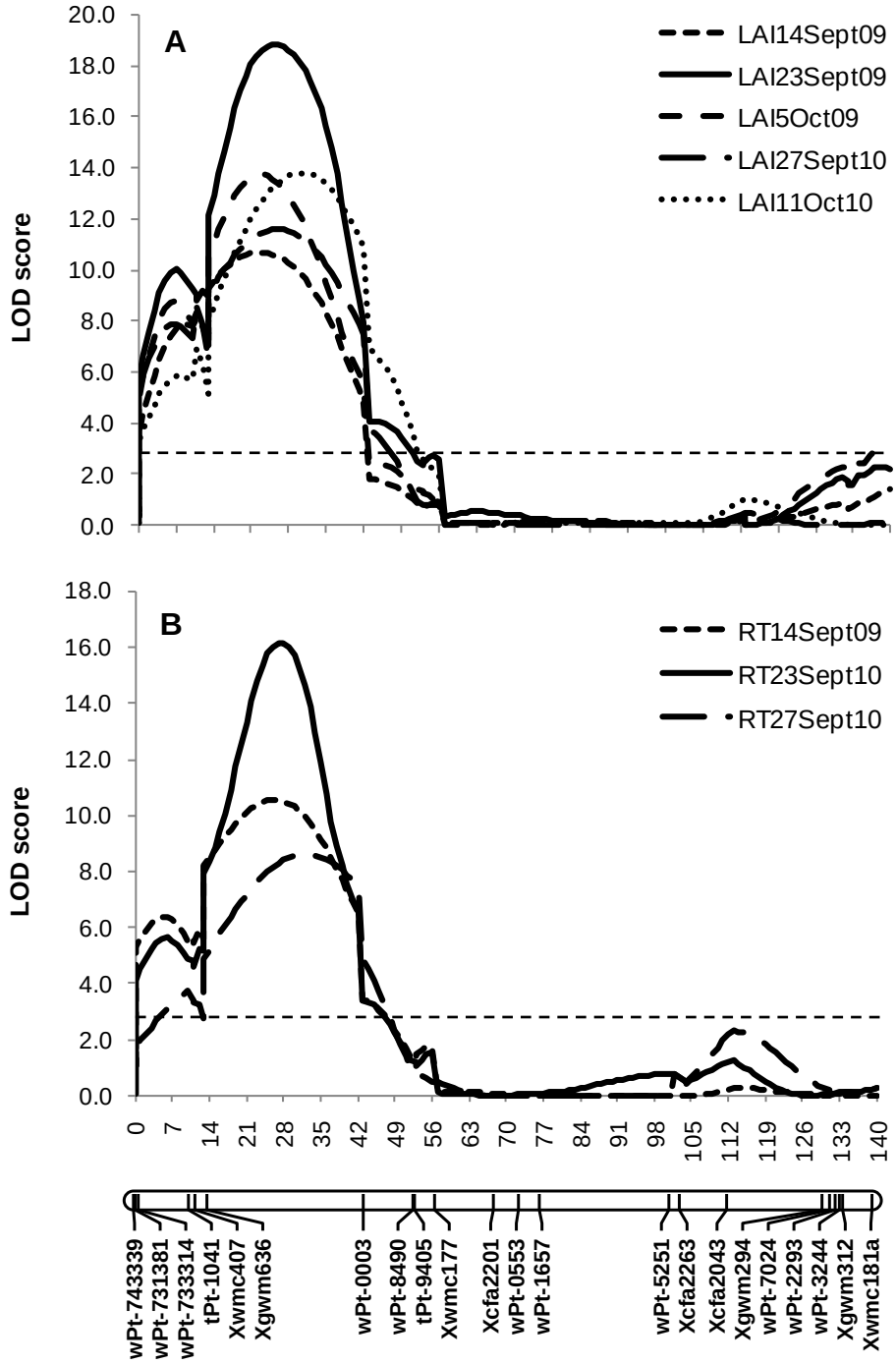

Figure 4.7 Stripe rust resistance QTL, *QYr.ufs-2A* on chromosome arm 2AS identified with the leaf area infected (A) and reaction type (B) data sets. The distances between markers are shown in centiMorgan. The LOD threshold of 2.6-2.7 depending on the trait, as determined from 1000 permutations using Cartographer v.2.51 (Wang *et al*. 2011) is shown.

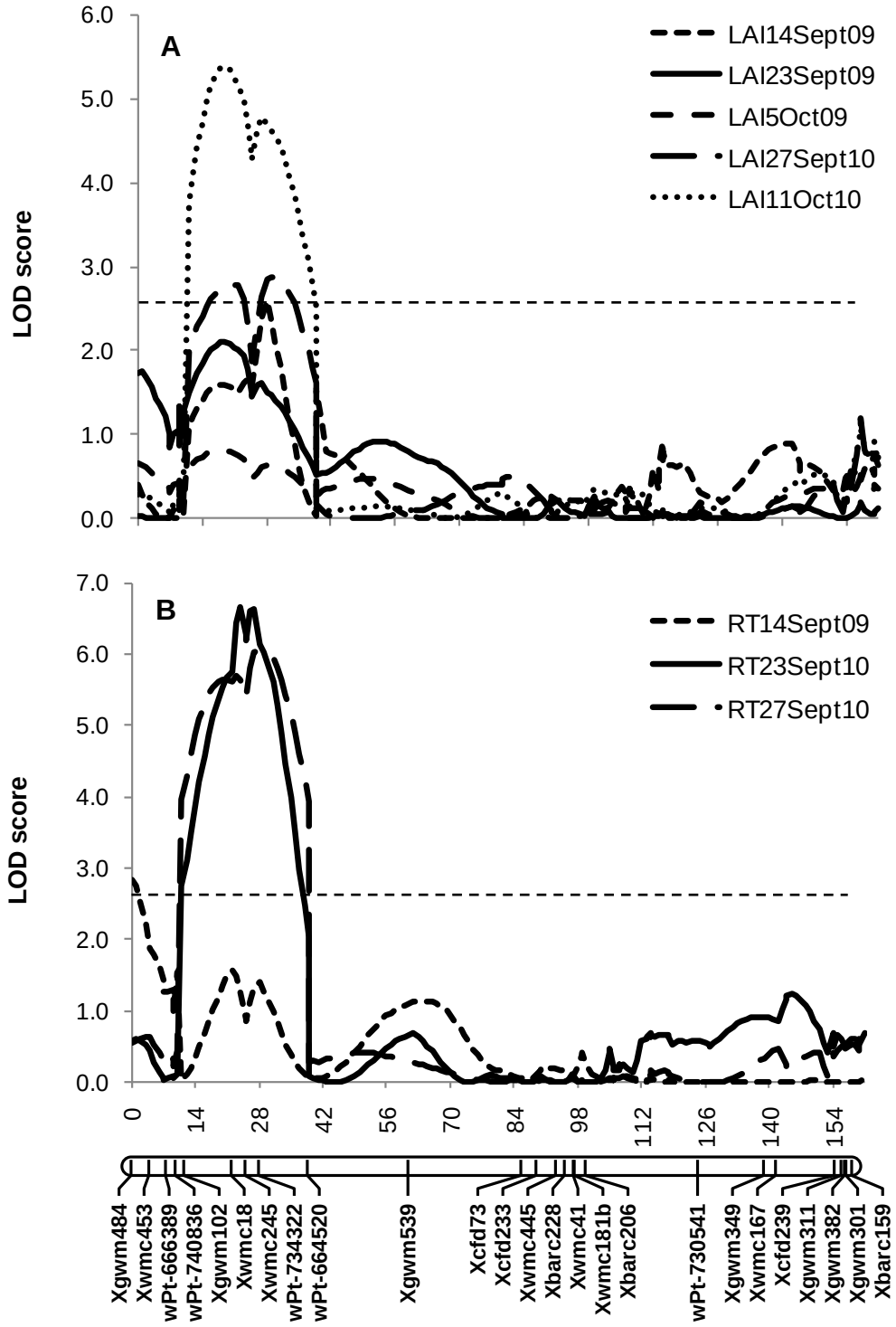

Figure 4.8 Stripe rust resistance QTL, *QYr.ufs-2D* on chromosome arm 2DS identified with the leaf area infected (A) and reaction type (B) data sets. The distances between markers are shown in centiMorgan. The LOD threshold of 2.6-2.7 depending on the trait, as determined from 1000 permutations using Cartographer v.2.51 (Wang *et al*. 2011) is shown.

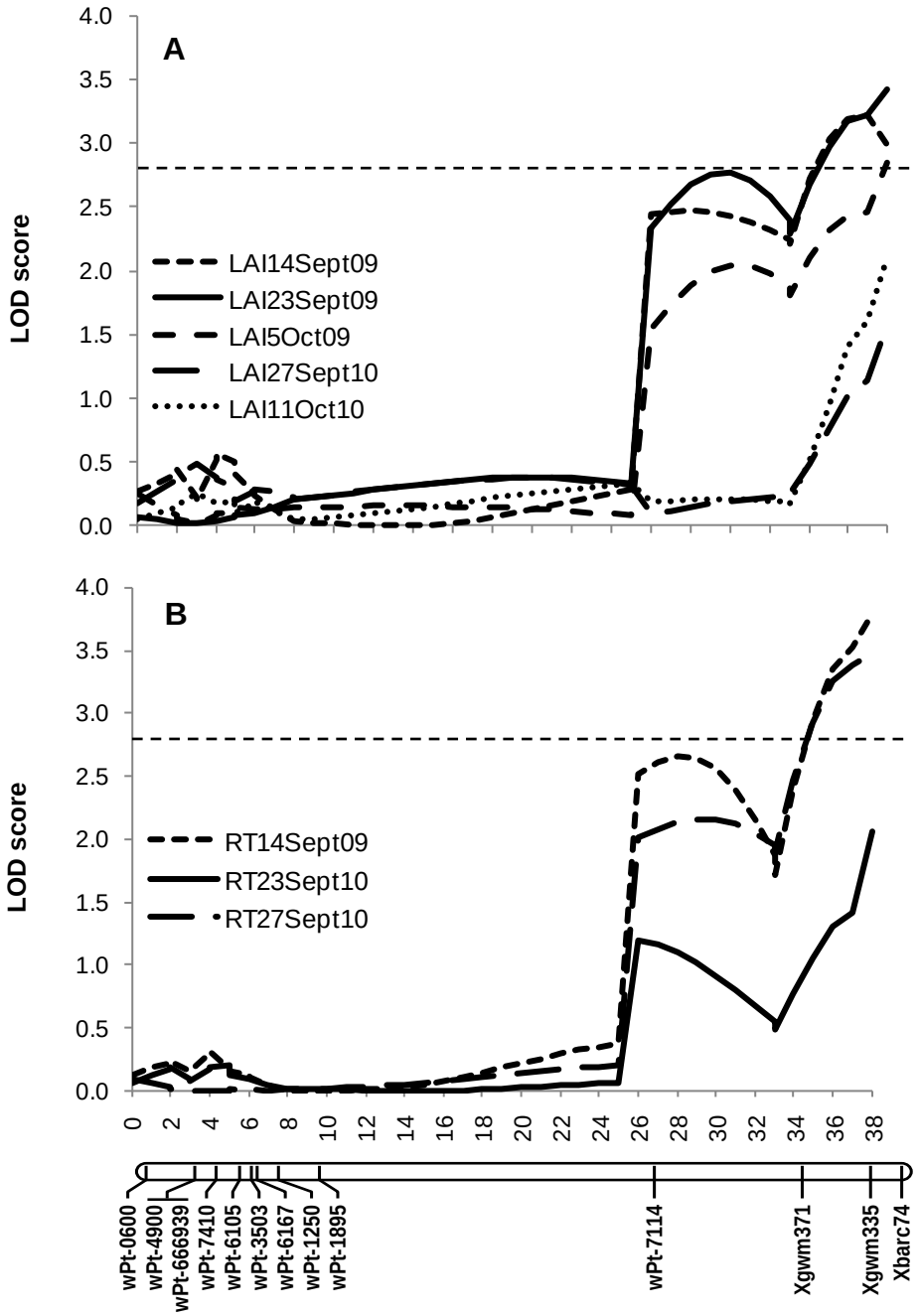

Figure 4.9 Minor stripe rust resistance QTL, *QYr.ufs-5B* on chromosome 5B identified with (A) with the leaf area infected and (B) reaction type data sets. The distances between markers are shown in centiMorgan. The LOD threshold of 2.6-2.7 depending on the trait, as determined from 1000 permutations using Cartographer v.2.51 (Wang *et al.* 2011) is indicated with a dashed line.

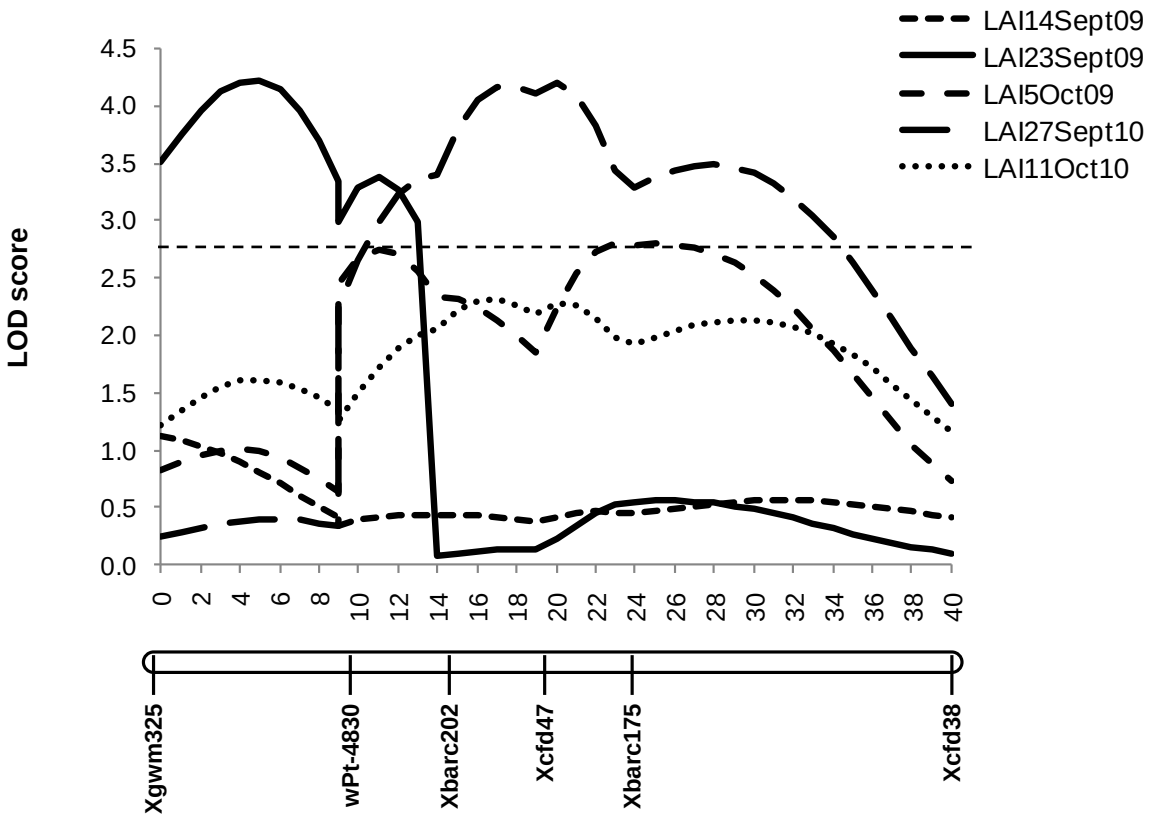

Figure 4.10 Minor stripe rust resistance QTL, *QYr.ufs-6D* on chromosome 6D identified with leaf area infected data sets. The distances between markers are shown in centiMorgan. The LOD threshold of 2.6-2.7 depending on the trait, as determined from 1000 permutations using Cartographer v.2.51 (Wang *et al.* 2011) is indicated with a dashed line. Reaction type traits were not significant.

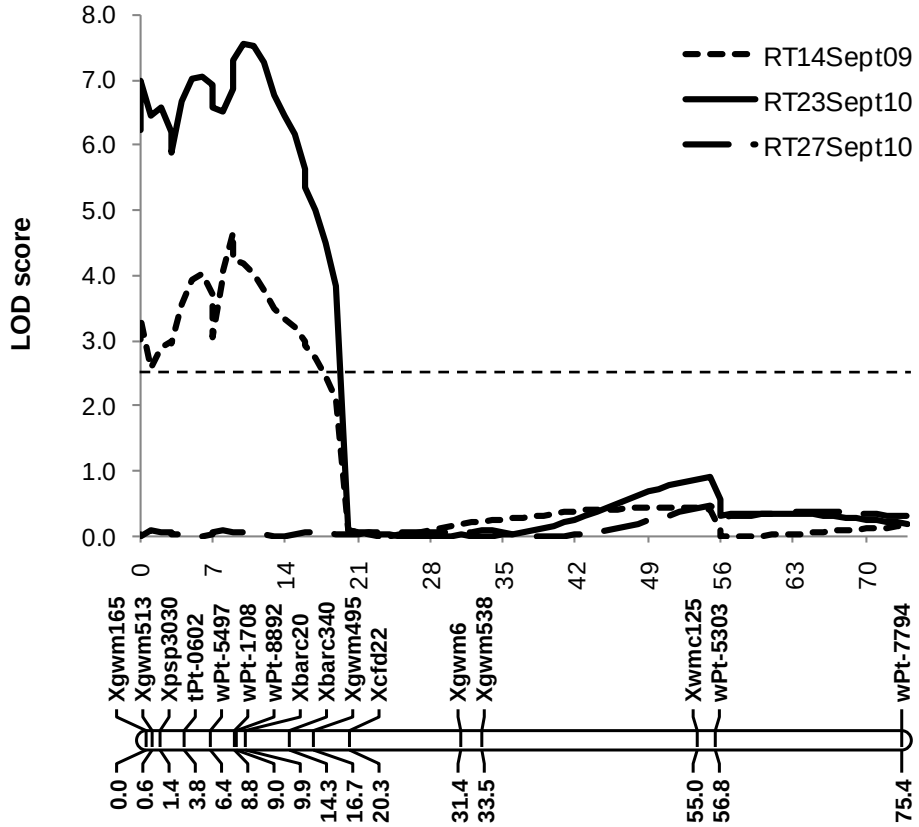

Figure 4.11 Minor stripe rust resistance QTL, *QYr.ufs-4B* on chromosome 4B identified with reaction type data sets. The distances between markers are shown in centiMorgan. The LOD threshold of 2.6-2.7 depending on the trait, as determined from 1000 permutations using Cartographer v.2.51 (Wang *et al.* 2011) is indicated with a dashed line. Leaf area infected traits were not significant.

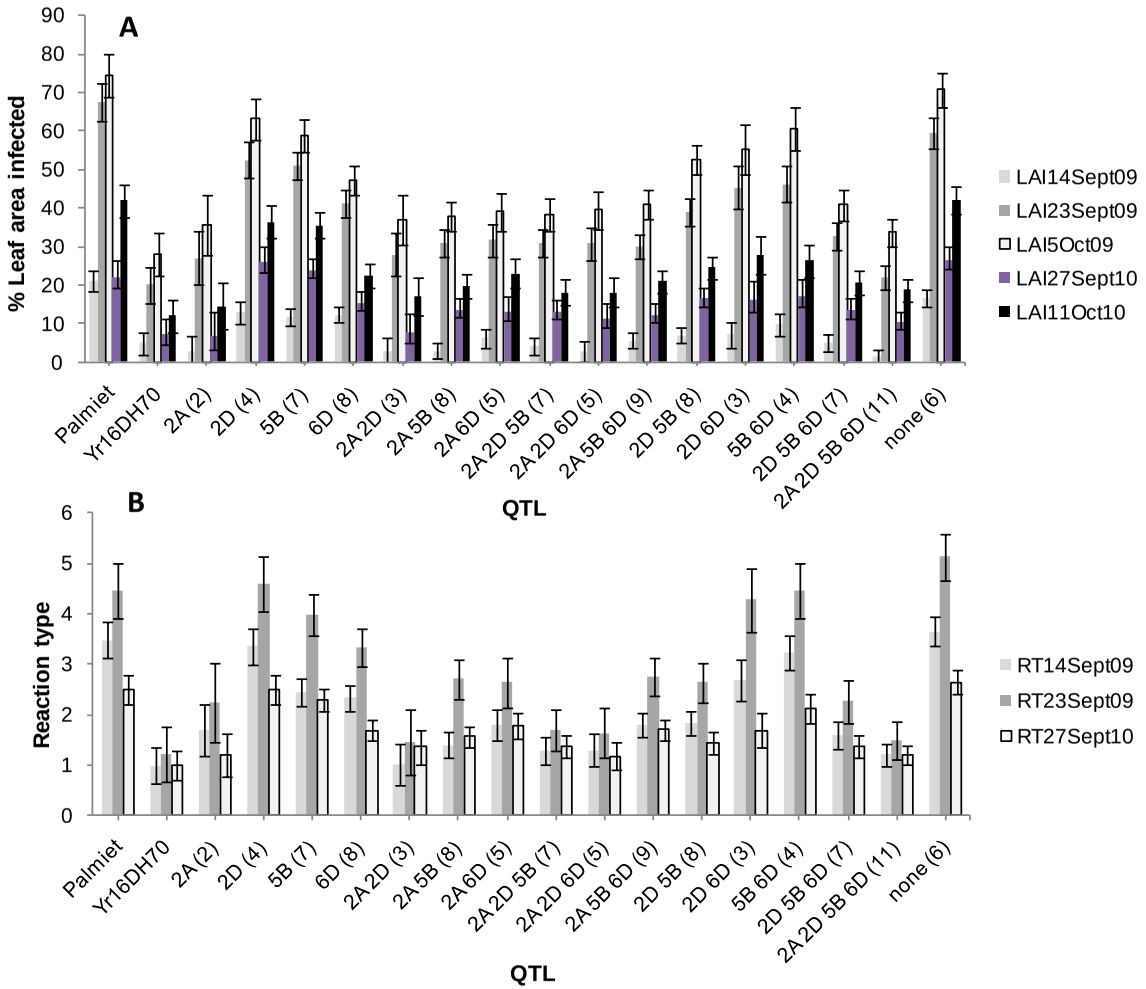

Figure 4.12 Mean percentage leaf area infected (A) and reaction type (B) of the RIL defined by the four stripe rust APR QTL derived from Yr16DH70. QTL groups containing *QYr.ufs-4B* from Palmiet are not shown. The phenotypes of the individual QTL *QYr.ufs-2A, QYr.ufs-2D, QYr.ufs-5B* and *QYr.ufs*-6D are shown, as well as the phenotypes of QTL combinations. The number of genotypes in each group is indicated in brackets. The reaction type scores are represented on an ordinal scale. Error bars show standard error of the mean.

4.4.5 Cappelle-Desprez identity validation from other sources

SSR markers flanking two QTL derived from Yr16DH70, *QYr.ufs-2A* and *QYr.ufs-2D,* were typed on the Cappelle-Desprez parent used to derive Yr16DH70 and Cappelle-Desprez DNA received from the stripe rust QTL mapping study by Nazari *et al.* (2005). The allele sizes indicate that the two Cappelle-Desprez lines are identical for the selected regions (Table 4.6).

Table 4.6 Allele information (bp sizes) for markers typed in an alternative Cappelle-Desprez source.

QTL	Marker	Palmiet	Yr16DH70	Cappelle-Desprez (this study)	Cappelle-Desprez (Nazari *et al.*)	Avocet S (Nazari *et al.*)
QYr.ufs-2A	*Xwmc407*	132	136	136	136	132
	Xwmc636	18	109	109	109	81
	Xwmc181a	259	257	257	257	255
QYr.ufs-2D	*Xwmc245*	148	150	150	150	148
	Xwmc181b	null	193	193	193	null
	Xwmc539	137	135	135	135	131
	Xwmc18	245	222	222	222	245

4.4.6 Relationship between QTL detected in the cv. Claire

The EST-derived markers mapped by Powell (2010) in a Claire X Lemhi population, EST6 (BE498622), EST10 (BE490491) and EST22 (BE403768) were tested on Palmiet and Yr16DH70 with CE-SSCP for polymorphisms. EST6 and EST10 proved to be polymorphic (Fig. 4.13) and could be mapped in the RIL mapping population. EST22, although polymorphic, did not amplify consistently and was not robust enough to be screened on the capillary system. This marker was therefore discarded. Both EST6 and EST10 mapped to the expected interval on chromosome 2D, completely linked with each other (Fig. 4.14). The SSR markers typed in Claire, Lemhi and four individuals from the DH mapping population from this cross included SSR markers in the *QYr.ufs-2A* and *QYr.ufs-2D* QTL intervals (Table 4.7). The UK cv. Claire does not appear to have inherited the 2A QTL (*QYr.ufs-2A*) from Cappelle-Desprez as both markers within the interval, *Xwmc407* and *Xgwm636*, have different alleles. With regards to the chromosome 2D QTL (*QYr.ufs-2D*), Claire has an allele in common with Cappelle-Desprez for *Xwmc245* (150 bp allele), but none of the other marker alleles agree, including *Xwmc18* which maps close to *Xwmc245* within the QTL interval.

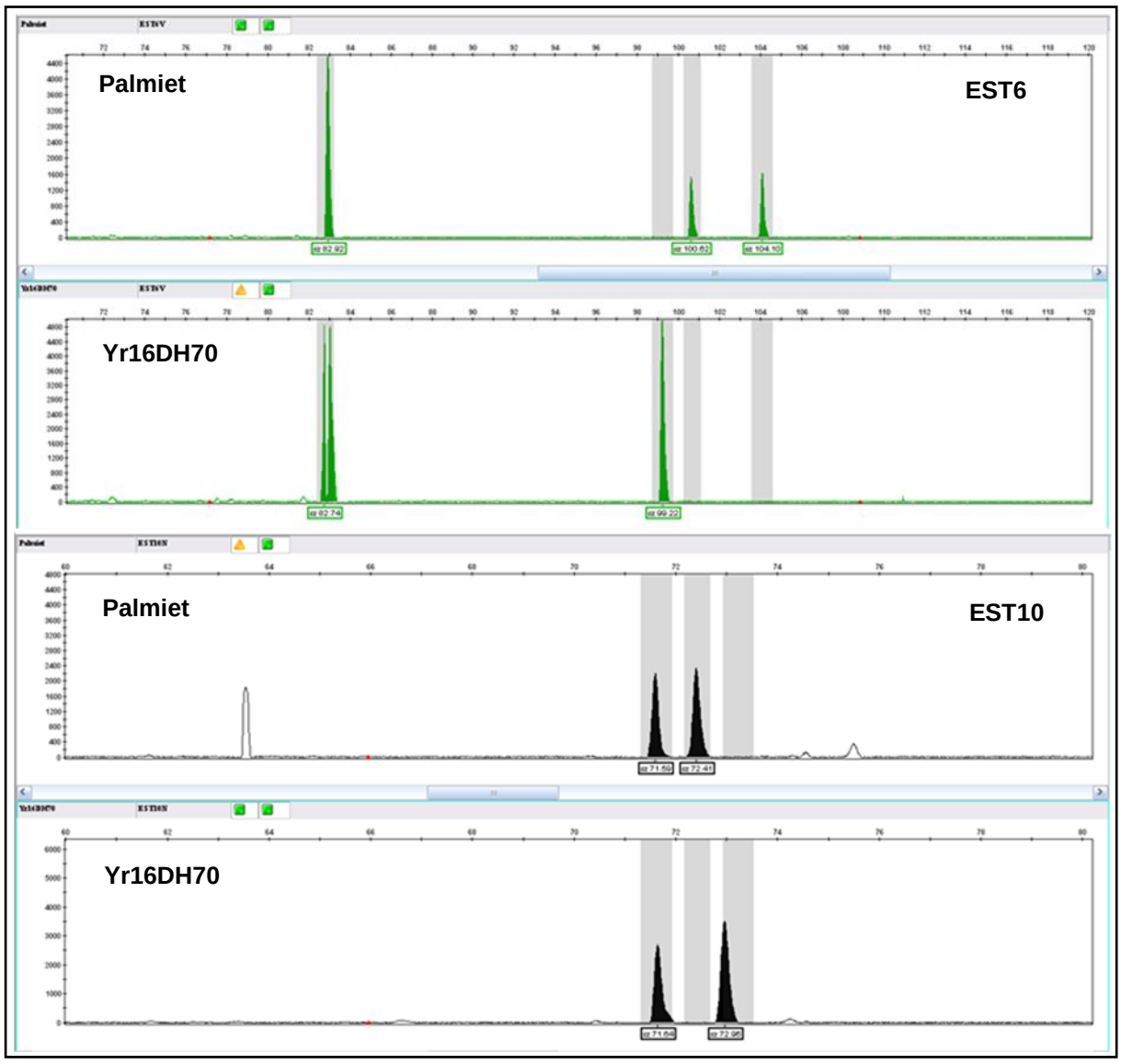

Figure 4.13 Electropherograms for Palmiet and Yr16DH70 with the EST-derived markers, EST6 and EST10, as produced after GeneMapper v.4 analysis. The allele sizes shown have been converted to scan numbers. The relative fluorescent units (RFU) as detected by the 3130*xl* Genetic Analyzer (Applied Biosystems) are indicated on the Y-axis.

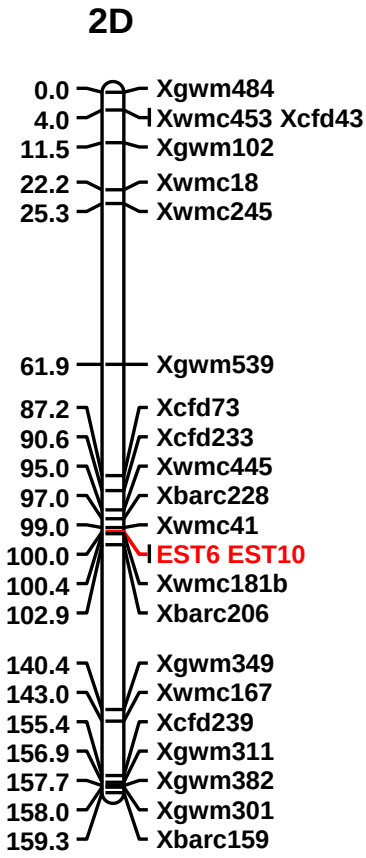

Figure 4.14 Positions of the Claire X Lemhi EST-derived markers, EST6 and EST10, mapped in the Palmiet X Yr16DH70 RIL population (red text), in relation to the SSR markers previously mapped to chromosome 2D.

Table 4.7 Allele information (bp sizes) for markers typed in the parentals and individuals from the Claire X Lemhi cross. Cappelle-Desprez alleles are shaded in grey.

	QYr.ufs-2A				*QYr.ufs-2D*		
	Xwmc407	***Xgwm636***	***Xwmc181a***	***Xwmc181b***	***Xgwm539***	***Xwmc245***	***Xwmc18***
Cappelle-Desprez	136	109	257	193	135	150	222
Yr16DH70	136	109	257	193	135	150	222
Palmiet	132	81	259	null	137	148	245
Claire	132	103	257	193	133	150	233
Lemhi	138	*	248	null	131	148	233
ClaireXLemhi DH1	132	103	248	193	133	150	233
ClaireXLemhi DH2	138	*	257	193	133	150	233
ClaireXLemhi DH3	138	*	257	193	133	150	233
ClaireXLemhi DH4	138	*	248	193	133	150	233

* Similar alleles according to peak pattern but difficult to size.

4.5 Discussion

Cappelle-Desprez was first released in France in 1946 and occupied a considerable part of the wheat acreage across Western Europe up until the late 1970s (Lupton and Macer 1962; Worland and Law 1986; Bonjean *et al.* 2001). The longevity of this cultivar was exceptional, which in part was due to its high levels of resistance to major diseases such as eyespot and stripe rust. Consequently Cappelle-Desprez was used extensively in breeding programs within the UK and Western Europe (Angus 2001; Bonjean *et al.* 2001; Porche 2001). Cappelle-Desprez is still recognised as a good source of stripe rust APR and in South Africa has maintained high levels of resistance since being tested for the first time in 1998 (Boshoff *et al.* 2002b).

4.5.1 Mapping population and marker analyses

Previous studies of stripe rust resistance in Cappelle-Desprez have suggested several APR loci (Worland and Law 1986; Law and Worland 1997) in addition to the seedling resistance genes *Yr3a* and *Yr4a* (Lupton and Macer 1962; De Vallavieille-Pope *et al*. 1990). To increase the sensitivity of detection of minor APR loci derived from Cappelle-Desprez the line Yr16DH70 was developed, whereby the race-specific, seedling resistance genes *Yr3a* and *Yr4a* had been removed. The development of Yr16DH70 resulted in much of the genome of this line consisting of Palmiet DNA. Twenty-three percent of all polymorphic SSR markers between Cappelle-Desprez and Palmiet (316 SSR markers) were also polymorphic between Yr16DH70 and Palmiet (73 SSR markers). This would fit with the expected genetic structure of Yr16DH70, where 25% of the genome should be derived from Cappelle-Desprez and 75% from Palmiet. Genetic mapping of the Palmiet X Yr16DH70 RIL population therefore resulted in the identification of 12 of the potential 21 chromosomes of hexaploid wheat.

4.5.2 QTL mapping

Subsequent QTL analyses identified a major effect stripe rust resistance QTL on the short arm of chromosome 2A (*QYr.ufs-2A*) and three minor QTL on chromosomes 2D (*QYr.ufs-2D*), 5B (*QYr.ufs-5B*) and 6D (*QYr.ufs-6D*). In addition, a minor QTL was detected in the South African cv. Palmiet on chromosome 4B (*QYr.ufs-4B*). While up to 75.2% of the variation seen for RT was explained by the QTL identified, only 60.4% of the variation seen for LAI was explained. Although heavy selection pressure for adult plant stripe rust resistance was imposed during the development and selection of Yr16DH70, minor QTL and QTL where expression is heavily influenced by environmental factors (Boukhatem *et al*. 2002), may not have been retained in Yr16DH70. This may account for the consistently, but marginally higher disease rating obtained for Yr16DH70 compared to Cappelle-Desprez in each year of testing.

4.5.2.1 *QYr.ufs-2A*

QYr.ufs-2A was responsible for a significant proportion of the stripe rust resistance displayed by Yr16DH70 (up to 53.2%) and was consistently detected by both LAI and RT scores. The association between low LAI and low RT scores would indicate a QTL for stripe rust resistance exhibiting a necrotic phenotype typical of race-specific resistance. Statistical analyses of the LAI and RT scores of the RIL within each QTL group indicated significant interactions between *QYr.ufs-2A* and the three smaller QTL derived from Yr16DH70, indicating that although *QYr.ufs-2A* conferred a significant level of stripe rust resistance this could still be enhanced by small effect QTL. SSR markers defined the location of *QYr.ufs-2A* to the short arm of chromosome 2A. The stripe rust seedling resistance gene *Yr17* was transferred to chromosome 2AS from *Triticum ventricosum* (Helguera *et al*. 2003) and maps to the same region as *QYr.ufs-2A* (Christiansen *et al*. 2006). However, the detection of seedling resistance genes is improbable as the population was developed from the seedling susceptible, Cappelle-Desprez derived line Yr16DH70. Furthermore, the release of Cappelle-Desprez predates the introduction of the *Yr17* translocation. In addition, the 2NS-specific marker VENTRIUP/LN2 (Helguera *et al*. 2003) showed that the *Lr37*/*Yr17*/*Sr38* segment is not present in Cappelle-Desprez or Yr16DH70 (Prins R, personal communication). This does not exclude *QYr.ufs-2A* from representing an adult plant race-specific resistance locus which has not as yet been overcome by South African pathotypes.

Boukhatem *et al*. (2002) detected an APR QTL (*QYR2*) in the cv. Camp Rémy, a descendant of Cappelle-Desprez which is also described as having durable stripe rust resistance. *QYR2* was located to a region on the long arm of chromosome 2A which is defined by the SSR marker loci *Xgwm356* and *Xgwm382*. Mallard *et al*. (2005) also detected an APR locus in Camp Rémy on chromosome 2A and designated the QTL, *QYr.inra-2AL*. However, the designation of this QTL to the long arm of 2A may be in question, as all the SSR markers in this QTL interval, except one, GWM382, have been assigned to the short arm of chromosome 2A (Appels 2003). As defined by the chromosome 2AS SSR marker loci, *QYr.inra-2AL* and *QYr.ufs-2A* would lie within a common interval. Mallard *et al*. (2005) attributed between 20-40% of the phenotypic variance to *QYr.inra-2AL,* which is comparable to the phenotypic variance explained by *QYr.ufs-2A*. In the study of Boukhatem *et al*. (2002) *QYR2* accounted for only 15.4% of the phenotypic variation. Bariana *et al*. (2010) identified an APR QTL proximal to the region defining *QYr.ufs-2A* in the cv. Kukri*,* linked to the DArT marker *wPt-0003*, a locus flanking *QYr.ufs-2A* interval. In contrast to *QYr.ufs-2A* this QTL explained only 12-15% of the phenotypic variance and was not detected across years. Recently Lowe *et al*. (2011) identified a small effect QTL, *QYr.ucw-2AS*, contributing 2.3% to the stripe rust resistance expressed by a synthetic derivative (Croc/*Aegilops tauschii* (Synthetic 205)//Kauz). *QYr.ucw-2AS* was located between the markers *wPt-3896* and *Xwmc177*, placing it in the same region as *QYr.ufs-2A.* Yet another QTL, *QYr.uga-2AS,* was detected in the *QYr.ufs-2A* interval in the cv. Pioneer 26R61, flanked by the markers *Xbarc124* and *Xgwm359* (Hao *et al*. 2011). This QTL explained up to 56.0% of the

phenotypic variation and was consistently expressed across different environments. The short arm of chromosome 2A is therefore a region of interest in terms of stripe rust resistance.

4.5.2.2 *QYr.ufs-2D*

A gene for stripe rust resistance, *Yr16* was located to the centromeric region of chromosome 2D in Cappelle-Desprez through cytogenetic analyses (Worland and Law 1986; Worland 1988). The gene was placed 9.3 cM from the centromere between RFLP marker loci *Xpsr641-2D* and *Xpsr681-2D* on a consensus map, Ta-Gale-2D (http://wheat.pw.usda.gov/GG2/index.shtml; Devos *et al*. 1993). Mallard *et al*. (2005) identified a QTL in the cv. Camp Rémy near the centromere on 2DS, *QYr.inra-2DS*. *QYr.inra-2DS* was located between the loci *Xgwm102* and *Xgwm539* and was responsible for 24-69% of the observed phenotypic variance. In this study, *QYr.ufs-2D* mapped to the same region on 2DS, although defined by a smaller interval (*Xgwm102-wPt-664520*), but contributed significantly less to the phenotypic variance (maximum of 10.3%). *QYr.inra-2DS* and *QYr.ufs-2D* probably represent the same QTL, the differences in phenotypic variance indicating the influence of genetic background and/or environmental conditions. *QYr.ufs-2D* appears to perform less well under heavy disease pressure, not being detected late in 2009, but still being effective late in the drier, low stripe rust year of 2010. The sensitivity of expression of *QYr.ufs-2D*/ *QYr.inra-2DS* would be supported by the fact that Boukhatem *et al*. (2002) did not identify a QTL on chromosome 2D in their Camp Rémy X Michigan Amber study.

Various other stripe rust resistance QTL have been mapped to chromosome 2DS within the same region as *QYr.ufs-2D*. However, the ancestral relationship between these resistance sources and Cappelle-Desprez is not as clear as for the Cappelle-Desprez Camp Rémy relationship. Temperature-sensitive seedling resistance, *YrCK* was reported on chromosome 2D in the Australian cv. Cook and a derivative, cv. Sunco (Park *et al*. 1992; Bariana *et al*. 2001), contributing 13-19% of the phenotypic variance for stripe rust and 9-13% of the phenotypic variance for leaf rust resistance (Navabi *et al*. 2005). A stripe rust resistance QTL was identified in the Italian cv. Libellula, *QYr.caas-2DS* explaining 8.4-12.1% of the phenotypic variance (Lu *et al.* 2009), while Suenaga *et al*. (2003) detected a stripe rust resistance QTL in this region in the cv. Oligoculm which explained less than 10% of the phenotypic variance. In the UK cvs Guardian (Melichar *et al*. 2008) and Claire (Powell 2010), both of which have Cappelle-Desprez in their pedigrees, stripe rust resistance QTL were found that mapped close to the *QYr.ufs-2D* region on the short arm of chromosome 2D. SSR markers in the *QYr.ufs-2D* QTL interval was tested in the cv. Claire, but no clear allelic pattern of inheritance could be identified between Yr16DH70 (or Cappelle-Desprez) and Claire. However, one SSR mapping within the QTL interval, *Xwmc245*¸ had the same alleles for Cappelle-Desprez, Yr16DH70 and Claire with recombination having occurred in the breeding of Claire, between markers futher removed from the QTL. These results therefore suggest that the three lines share *QYr.ufs-2D* (*Yr16*).

4.5.2.3 *QYr.ufs-5B*

Cappelle-Desprez carries the reciprocal, centromeric translocations 5BL-7BL and 5BS-7BS (Riley *et al*. 1967; Badaeva *et al*. 2007) which were common in Western European wheat cultivars in the 1960s and 1970s (Riley *et al*. 1967). The 5BS-7BS chromosome was previously shown to substantially contribute to stripe rust resistance in Cappelle-Desprez. The parents of Cappelle-Desprez, Vilmorin-27 and Hybride de Joncquois, both possess this translocation (Law *et al*. 1978; Law and Worland 1997). However, while Vilmorin-27 had a high level of stripe rust resistance, Hybride de Joncquois was susceptible. Ditelosomic 5BS and 7BS Cappelle-Desprez lines subsequently showed the resistance in Cappelle-Desprez to be located on the 5BS arm, close to the chromosomal breakpoint, while the genetic background in which the 5BS resides also appeared to influence the resistance (Law *et al*. 1978; Law and Worland 1997). The presence of the 5BS-7BS translocation in Yr16DH70 was confirmed by C-banding data (Pretorius ZA, personal communication). The marker data placed *QYr.ufs-5B* near to the centromeric region on the long arm of chromosome 5B. It has not been defined exactly where the physical breakpoint of the 5BS-7BS and 5BL-7BL translocations are positioned with respect to the centromeres of each chromosome, therefore *QYr.ufs-5B* may represent the stripe rust resistance located on 5BS in Cappelle-Desprez. *QYr.ufs-5B* did not make a large contribution to the stripe rust resistance observed in Yr16DH70, however, lower disease scores were seen for both LAI and RT when *QYr.ufs-5B* was combined with *QYr.ufs-2D.*

Mallard *et al*. (2005) detected two QTL (*QYr.inra-5B.1* and *QYr.inra-5B.2)* in Camp Rémy which mapped to the telomeric region on the long arm of chromosome 5B. Based on the known map location of the markers defining these QTL (Appels 2003) the authors suggested that the telomeric end of 5BL contained a translocation of a fragment of 5BS. However, this proposal was not confirmed by cytological data. Boukhatem *et al*. (2002), in their study of the cv. Camp Rémy did not identify QTL on 5B. Stripe rust resistance QTL on chromosome 5B have also been detected in the Italian cvs Libellula and Strampelli (Lu *et al*. 2009), the Israeli cv. Oligoculm (Suenaga *et al*. 2003), the Australian cv. Janz (Bariana *et al*. 2010) and the French cv. Flinor (Feng *et al*. 2011). In Flinor two QTL were detected on 5B which are expressed at the seedling stage and at higher temperatures (*QYr-tem-5B.1* and *QYr-tem-5B.2*; Feng *et al*. 2011). Flinor shares ancestry with Cappelle-Desprez and one of these QTL, *QYr-tem-5B.1,* overlaps with the region defining *QYr.ufs-5B. QYr-tem-5B.1* explained up to 33% of the phenotypic variation in Flinor which is substantially greater than the resistance phenotype explained by *QYr.ufs-5B.* It has yet to be determined if there is a relationship between *QYr-tem-5B.1* and *YrDru* from the cv. Druchamp (Chen *et al*. 1996).

4.5.2.4 *QYr.ufs-6D*

No stripe rust APR QTL have been mapped to chromosome 6D, although the seedling resistance genes *Yr20* and *Yr23* were located on this chromosome (Chen *et al*. 1995). *QYr.ufs-6D* represents a small effect QTL, only detected with the LAI scores and then not consistently across years.

4.5.2.5 *QYr.ufs-4B*

A small effect QTL was found on chromosome 4B, *QYr.ufs-4B*, derived from Palmiet. Depending on environment and disease pressure a low level of stripe rust resistance was observed for Palmiet, supporting the detection of this QTL. QTL for stripe rust APR have been reported on chromosome 4B in the cvs Alcedo (Jagger *et al*. 2011), Oligoculm (Suenaga *et al*. 2003), Avocet S (William *et al*. 2006), Guardian (Melichar *et al*. 2008) and cvs Libellula and Strampelli (Lu *et al*. 2009). The stripe rust QTL from Avocet S was also reported to have an effect on leaf rust (William *et al*. 2006). Except for the QTL from Alcedo, which explained up to 29% of the phenotypic variance, none of the above mentioned QTL accounted for more than 15% of the phenotypic variance. This is in line with the relatively small effect seen for *QYr.ufs-4B.* However, the large interval defining *QYr.ufs-4B* does not allow direct comparison to these other published chromosome 4B QTL.

4.5.3 QTL effects

The RIL were assigned to 32 genotype groups depending on which of the five QTL each line contained. Statistical analyses of the LAI and RT scores of the RIL within each QTL group indicated significant additive interactions between combinations of QTL. In general QTL intervals were fairly large, therefore QTL assignment to RILs was based on the marker closest to the QTL peak (highest LOD). Selection based on markers flanking the QTL interval would have eliminated several lines from each QTL group, this being most evident for the major QTL *QYr.ufs-2A. Xgwm636* was used to select for the QTL, having the highest LOD value with all eight traits, whilst the LOD value associated with the flanking marker *wPt-0003* sharply decreases below three for most traits. More variation was generally seen in the QTL interval between traits for the minor QTL, with the highest LOD marker differing between trait data sets, adjacent markers that mapped within the QTL interval were therefore also considered in the assignment of groups. For *QYr.sgi-2D*, the alleles for *wPt-734322* was evaluated together with *Xwmc245*, which is the marker with the highest LOD. The intervals *wPt-8892-Xbarc20* and *Xgwm335-Xbarc75* were analysed for *QYr.ufs-4B* and *QYr.ufs-5B*, respectively. In the case of the problematic region on chromosome 6D, the *Xgwm325-Xbarc202* interval was considered. Selection criteria for each QTL were thus determined by the quality of the QTL interval, but consistently applied for a specific QTL. The grouping of RILs into QTL groups using this method was supported by the similar stripe rust resistance phenotypes seem between the RILs within each QTL group. Clearly the QTL interval regions need to be delimited to smaller intervals in order to facilitate using an interval for QTL assignment instead of using only single markers. This will improve accuracy of RIL assignments to QTL classes as the selection based on single markers are prone to the incorrect inclusion of lines where the actual QTL has been removed via recombination.

Additive effects were seen when combining *QYr.ufs-2A* with the three smaller QTL derived from Yr16DH70, indicating that although *QYr.ufs-2A* conferred a significant level of stripe rust resistance this could still be enhanced by small effect QTL. Lower disease scores were also seen for both LAI and RT when the minor QTL *QYr.ufs-2D* and *QYr.ufs-5B* were combined. A significant reduction in

disease was not consistently seen with any other pairwise combinations of the Yr16DH70 derived QTL.

4.5.4 Conclusion

In South Africa Cappelle-Desprez and the Cappelle-Desprez derived breeding line, Yr16DH70, selected to retain the stripe rust APR found in Cappelle-Desprez, exhibit complete resistance to stripe rust under field conditions. In the UK stripe rust resistance in Cappelle-Desprez is now only partial (Boyd LA, personal communication). While the *Yr16* (*QYr.ufs-2D*) resistance is still believed to be effective in the UK, as seen in the UK cvs Guardian (Melichar *et al*. 2008) and Claire (Powell 2010), it may well be that the major effect QTL, *QYr.ufs-2A* detected in Yr16DH70 is no longer effective against UK pathotypes of *P. striiformis* f. sp. *tritici*. *QYr.ufs-2D*, which is in all likelihood the *Yr16* locus previously reported in Cappelle-Desprez is therefore more likely to be of long term value. The value of *QYr.ufs-5B* and *QYr.ufs-6D* is less clear and an improved linkage map of these QTL intervals would help define their value as stripe rust APR genes. The stripe rust APR QTL from Cappelle-Desprez have already been transferred into a desirable South African spring wheat background through the development of the breeding line, Yr16DH70. This study has now defined these QTL and provides markers by which each QTL can be identified as this breeding line is taken forward in the development of new wheat cultivars

4.6 Future prospects

Markers have been identified which demarcate the Cappelle-Desprez QTL present in the line Yr16DH70. Although these markers are already implemented in selecting for these QTL in breeding material, the QTL intervals are still fairly large and one therefore has to allow for a significant number of recombinants. The low level of polymorphism between Palmiet and Yr16DH70 will make it difficult to find additional SSR markers mapping in the QTL intervals, considering the large number tested already. EST and other SNP based markers have not been explored in this study and will provide additional sources of variation.

The proposed race-specific nature of the large effect QTL, *QYr.ufs-2A,* should be confirmed with European stripe rust pathotypes virulent on Cappelle-Desprez. This QTL, as well as the minor QTL identified in this study should be characterised further by increasing the marker density to improve the map resolution. A Palmiet X Yr16DH70 DH mapping population (85 lines) have been developed and this population will be typed with the QTL flanking SSR markers and segregation of resistance will be recorded at the Greytown field trial site. By identifying individuals from the RIL or DH population carrying single QTL, near isogenic lines (NILs) could be developed for pathogen interaction studies which will lead to a better understanding of the resistance mechanisms conferred by each QTL. Leaf rust resistance has been observed in Cappelle-Desprez (Poyntz and Hyde 1987; Denissen 1993) and this should be investigated, as well as possible broad-spectrum resistance to other fungal pathogens.

5. FINE MAPPING STRIPE RUST RESISTANCE QTL IN A KARIEGA X AVOCET S POPULATION

5.1 Introduction (pre-existing data)

In 1999 a project was approved by the ARC-SGI to use molecular techniques, in combination with DH populations to detect QTL responsible for stripe rust resistance. The wheat cv. Kariega was selected as it expressed complete APR to stripe rust in South Africa. A Kariega X Avocet S DH mapping population was used to create a linkage map and to perform QTL analysis (Ramburan *et al.* 2004; Prins *et al.* 2005; Prins *et al.* 2011). Once identified, the QTL effects were further characterised by means of histological investigations and expression analysis (Moldenhauer *et al.* 2006; Moldenhauer *et al.* 2008).

5.1.1 Kariega X Avocet S DH mapping population

Kariega (pedigree: SST44//K4500.2/SapsuckerS), a hard red spring wheat with good bread baking qualities, was released in 1993 by the ARC-SGI for both dryland and irrigated regions (Smit *et al.* 2010) and is still commercially produced. Kariega exhibits LTN and is resistant to all the stripe rust pathotypes currently detected in South Africa, but it is susceptible to stem rust. An expansion of the pedigree of Sapsucker reveals the presence of Frontana (Payne *et al*. 2002), a known source of the *Yr18/Lr34/Pm38* gene (McIntosh *et al.* 1995). In addition, Kariega is believed to carry the seedling leaf rust resistance genes *Lr1* and *Lr3a* (Pretorius *et al.* 2007), but this has not been confirmed. Avocet S (pedigree: Thatcher-*Ag. elongatum*/3*Pinnacle//WW15/3/Egret) is a white-seeded Australian spring wheat susceptible to stripe rust. The cultivar carries the rust resistance genes *Sr5*, *Sr26*, *Lr10* and *Lr13* (Wellings *et al.* 2004; Prins *et al.* 2005). A DH mapping population was developed from a Kariega X Avocet S cross, using the wheat-maize technique (Pienaar *et al.* 1997). The population originally consisted of 150 individuals (Prins *et al.* 2005) but was later increased to 254 lines (Prins *et al.* 2011).

5.1.2 Marker analysis and linkage map

SSR, DArT, AFLP, sequence-related amplified polymorphism (SRAP), resistance gene analogue (RGA) and quality markers published in the public domain were typed in the DH mapping population and a linkage map was constructed spanning 1322 cM. The genetic map consisted of 463 loci distributed across 31 linkage groups. Chromosome 2A was the only chromosome not represented in the map, while chromosomes 1A, 1D, 2B, 3B, 4A, 5B, 5D, 6B, 7A, 7B and 7D were represented by two linkage groups each. (Prins *et al.* 2011)

5.1.3 Stripe rust QTL identified

Field trials were conducted for stripe rust resistance evaluation in 2000 and 2006 at the PANNAR Research Station, Greytown, KwaZulu-Natal, South Africa. LAI and RT were recorded at different times during the season once the first signs of disease were seen. Greenhouse-based tests were also performed to analyse APR. Windows QTL Cartographer v.2.51 (Wang *et al.* 2011) was used to perform CIM using a forward regression model, a window size of 10 cM and a walk-speed of 1 cM. A LOD score threshold was calculated from 1000 permutations for each trait to declare significance. While Kariega seedlings were stripe rust susceptible, major APR QTL were detected on chromosomes arms 2BS, 7D and 4AL. Minor QTL were also detected on chromosomes 7A and 6B in Kariega. QTL was named according to convention, with *sgi* representing the Small-Grain Institute (Agricultural Research Council).

5.1.3.1 *QYr.sgi-2B*

Previously, two QTL intervals, *QYr.sgi-2B.1* (linked to *Xgwm148*) and *QYr.sgi-2B.2* (linked to *Xpsp3030*), were detected on the short arm of chromosome 2B (Ramburan *et al.* 2004). The importance of the *QYr.sgi-2B.1* region was confirmed in the study Prins *et al.* (2011) (Fig. 5.1A). This QTL is characterised by a trait-specific larger interval (*Xbarc200-wPt-6278*) and a smaller interval (*wPt-5556-wPt-6278*, designated *QYr.sgi-2B.1a*). The *QYr.sgi-2B.1* interval involves about 23 cM and explained up to 45.7% of the field RT variance, being associated with a hypersensitive response. *QYr.sgi-2B.1* explained 33.6% and *QYr.sgi-2B.1a* 37.0% of the LAI variance. For the purpose of this study, the chromosome 2BS QTL (*QYr.sgi-2B.1* and *QYr.sgi-2B.1a*) will only be referred to collectively as *QYr.sgi-2B.*

5.1.3.2 *QYr.sgi-4A*

Qyr.sgi-4A.1 on the long arm of chromosome 4A was described as a minor QTL by Ramburan *et al.* (2004), but was associated with 28.4% of the LAI variation and 13.5% of the RT variation in the larger mapping population from Prins *et al.* (2011) (Fig. 5.1B, *ks12m34A-wPt-5003*). In addition to the larger population size, the improved detection power could be attributed to the introduction of an earlier scoring date and by the addition of markers in the QTL interval. Henceforth *QYr.sgi-4A.1* will be referred to *QYr.sgi-4A.* The QTL interval stretched over about 18 cM of the chromosome 4A map.

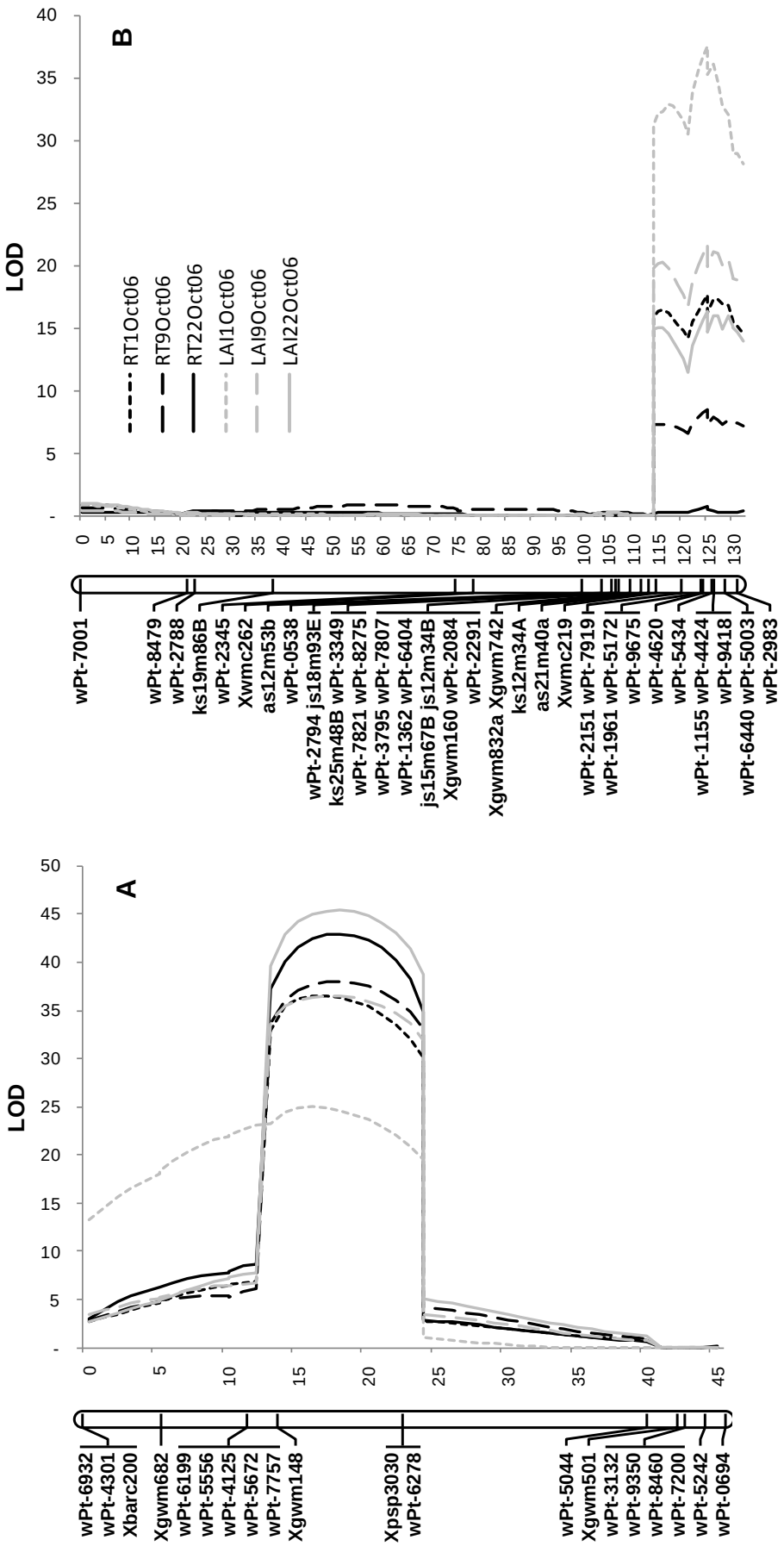

Figure 5.1 *QYr.sgi-2B* (A) and *QYr.sgi-4A* (B) QTL intervals for the Kariega X Avocet S DH mapping population, as published by Prins *et al.* (2011). Reaction type (RT) and leaf area infected (LAI) data are shown for three scoring dates during the 2006 season. The distances between markers on the X-axis are in centiMorgans. LOD values above 2.5-2.9 were declared significant based on a permutation test.

5.1.3.3 *QYr.sgi-7D*

A third major QTL, *QYr.sgi-7D,* was detected on the short arm of chromosome 7D. It contributed up to 35.0% of the LAI and 32.0% of the RT variance and was mapped to the region known to possess the pleiotropic *Lr34/Yr18/Pm38* gene. Lagudah *et al.* (2006) developed a marker, csLV34, to select for this gene. The marker was reported to be located 0.4 cM from *Lr34/Yr18/Pm38*. The presence and thus the identity of *QYr.sgi-7D* was confirmed to be *L34/Yr18/Pm38* with this marker.. *Lr34/Yr18/Pm38* (cloned by Krattinger *et al.* 2009) belongs to the pleiotropic drug resistance sub-familiy of ABC transporters. Two sequence differences have been predicted to be responsible for expression of the stripe and leaf rust resistant phenotype, a three base pair (TTC) insertion/deletion (indel) in exon 11, and a SNP in exon 12. The indel in exon 11 results in the addition of the amino acid phenylalanine in the susceptible allele and the exon 12 SNP causes an amino acid change (tyrosine to histidine). Both amino acid changes affect the first transmembrane domain connecting the two nucleotide-binding domains and may alter the structure and substrate specificity of the transporter (Krattinger *et al.* 2009). Diagnostic markers have been develop for *Lr34/Yr18/Pm38* based on these sequence changes (Lagudah *et al.* 2009).

5.2 Fine mapping of the Kariega QTL

Intervals for the Kariega stripe rust resistance QTL previously identified (Ramburan *et al.* 2004; Prins *et al.* 2011) are large and DNA marker technology can therefore not be employed to its full potential in selecting for the QTL in marker-assisted breeding. The QTL on chromosomes 2B (*QYr.sgi-2B*) and 4A (*QYr.sgi-4A*) were fine mapped in a two-step process. The marker density was first increased for the Kariega X Avocet S DH mapping population (254 lines). First, additional SSR markers were mapped and new marker types were investigated, including EST and nucleotide-binding site amplified fragment length polymorphism (NBS-AFLP) markers. EST and NBS-AFLP markers are used for a more targeted approach in increasing the marker density in selected regions, and selecting for sequences associated with expressed resistance genes, respectively. Real-time PCR markers were considered for future application. Secondly, a much larger mapping population was created, consisting of 1020 Kariega X Avocet S F_2 plants. DArT and EST markers were adapted for high-throughput screening and these markers, together with selected SSR markers, were typed in the F_2 mapping population to increase the map resolution of the QTL intervals. This process of obtaining a high-resolution map for the Kariega QTL are described in the following sections.

5.2.1 Expressed sequence tags (EST)-derived markers

ESTs represent partial complementary DNA (cDNA) sequences from genes expressed in different tissues, at different stages of development and as a result of different stimuli. In 2000 only nine wheat EST sequences were publicly available (www.ncbi.nlm.nih.gov) (Lazo *et al.* 2004). However this number had escalated to more than a million in 2011. By using ditelosomic and nulli-tetrasomic lines

or deletion stocks Qi *et al.* (2004) mapped 16 000 wheat ESTs to individual chromosomal regions or bins. A bin is defined as the region delimited by two adjacent deletion breakpoints in the same chromosome arm. For all three genomes, a total of 159 deletion bins, together with 21 centromeres make up 180 regions where an EST can be cytologically mapped using the selected panel of lines (La Rota and Sorrells 2004). Data on mapped ESTs in the public MySQL relational database wEST is accessible from the GrainGenes database (http://wheat.pw.usda.gov/wEST). SNPs or small indels detected in EST sequences can be utilised to develop markers that discriminate between cultivars, and subsequently be mapped in a population.

5.2.2 Nucleotide-binding site amplified fragment length polymorphism (NBS-AFLP) markers

Identification of numerous functional resistance (R) genes from model and crop species has revealed that the majority of these genes encode cytoplasmic proteins with NBS and LRR domains, and that they often belong to complex loci comprised of arrays of related genes (reviewed in Martin *et al.* 2003). Conserved motifs have been identified within the NBS domain of R-genes. Examples of these include the P-loop and kinase-2 domains (Fig. 5.2). A PCR-based marker system has been developed to utilise these conserved domains using degenerate primers as a strategy to identify R-genes (Van der Linden *et al.* 2004). These degenerate primers, in combination with restriction site sequence variation, as used in AFLPs, generate NBS-AFLP profiles, hence the term NBS-profiling.

Figure 5.2 Schematic representation of the nucleotide-binding site (NBS) domain of functional resistance (R) genes. The position of the P-loop, kinase-2 and leucine-rich repeats (LRR) are shown. (Figure from Van der Linden *et al.* 2004).

5.2.3 Sequence tagged site (STS) markers

DArT markers and EST sequencing are not appropriate for high-throughput screening in a MAS setup. It is therefore required that such markers, when linked to a trait of interest, are converted to PCR-based STS markers. STS markers can then be screened by means of agarose gel electrophoresis or capillary array electrophoresis. The process involves obtaining the DArT or EST reference sequence, as well as the sequence of the resistant and susceptible parents, and designing new primers that can discriminate between the resistant parental allele and any other alleles not coupled to resistance. The new markers need to be validated in a large selection of genotypes to ensure reliable detection of the relevant trait.

5.2.4 Real-time PCR markers

Real-time PCR, also referred to as quantitative PCR (qPCR), enables the visualisation and estimation of the number of amplification products produced by PCR as the cycling progresses. This is accomplished with DNA double-strand intercalating dyes (e.g. SYBR green and EvaGreen™) or fluorescently labelled sequence-specific hybridisation probes. The instrument used for PCR is able to measure and report a fluorescence signal in different channels (set at specific wavelengths). Although the application of real-time gene expression analysis is better known in wheat host response to disease (Coram *et al.* 2008; Bozkurt *et al.* 2007; Tufan *et al.* 2009; Bozkurt *et al.* 2010; Yu *et al.* 2010) it is of particular interest for high-throughput screening of SNP markers (Bagge and Lubberstedt 2008). Unfortunately assay design for detecting point mutations is still technically a challenging task and detection may be complicated by the presence of genetic variation not previously accounted for (Bagge and Lubberstedt 2008). The most popular chemistry systems for mutation detection with real-time PCR are listed and described below. Various modifications of these systems are available.

1. TaqMan probes are based on hydrolysis (Holloway *et al.* 1999) and carry a fluorescent reporter molecule at the 5' end and a quencher molecule at the 3' end. The 5' exonuclease activity of *Taq* polymerase will cleave the probe during PCR. This will bring about a physical distance between the reporter and quencher, causing the reporter dye to fluorescence.
2. Hybridisation, fluorescence resonance energy transfer (FRET) based probes, involve two sequence specific probes, one labelled with a donor fluorophore and the other with an acceptor fluorophore. The probes are designed to bind in close proximity on a target amplicon. When bound, energy is transferred from the donor to the acceptor probe, which emits a fluorescent signal (Okamura *et al.* 2000).
3. HRM involves the precise monitoring of the change in fluorescence caused by the release of an intercalating dye as double-stranded PCR products dissociate with increasing temperature. Alleles are distinguished by melting temperature (T_M) shifts representing sequence variations between amplicons (Krypuy *et al.* 2006).
4. SNaPshot® (Applied Biosystems) is based on single-base extension of the amplicon with fluorescent labelled di-deoxynucleotides (ddNTPs). The final amplicon size is the size of the probe/primer plus the fluorescent base. Detection is done using capillary array electrophoresis (Törjék *et al.* 2003).

The speed and cost involved in evaluating plant material in MAS breeding are critical. The factors that will determine which marker system is most appropriate include the required throughput, labour involved, the reagent cost and access to specialised equipment, either through a large capital investment or as an external user at a service facility.

5.3 Study objectives

Three major QTL contributing to stripe rust APR in the cv. Kariega have been identified (Ramburan *et al.* 2004; Prins *et al.* 2011). These QTL were detected in a Kariega X Avocet S DH mapping population with conventional molecular markers, i.e. SSR, DArT and AFLP markers. *QYr.sgi-2B* on chromosome 2B and *QYr.sgi-4A* on chromosome 4A were limited to large intervals. The third QTL, *QYr.sgi-7D*, corresponded to the cloned resistance gene *Lr34/Yr18/Pm38*.

The current map can only be improved upon by increasing the marker density and screening selected markers in a much larger mapping population. Additional SSR markers were added to the DH map (254 individuals) and alternative markers were also considered. EST-derived markers targeted to the QTL interval were developed and mapped in the population. Conversion of DArT and EST markers to STS markers were investigated as an approach for generating new markers suitable for MAS. In an attempt to improve the resolution in the QTL regions a larger F_2 mapping population was developed from a Kariega X Avocet S cross and selected markers were screened in this population. Molecular data were used with phenotypic data from stripe rust field trials for QTL analysis. This fine mapping approach was implemented with the aim of increasing the map resolution for the *QYr.sgi-2B* and *QYr.sgi-4A* intervals. This process will allow for the identification of closely linked markers which could be implemented in MAS programs for transferring Kariega QTL to new breeding material.

5.4 Materials and Methods

5.4.1 DH mapping population: Increasing the marker density

5.4.1.1 Additional simple sequence repeat (SSR) markers

To increase the marker density of the Kariega X Avocet S DH map additional SSR markers were selected for the *QYr.sgi-2B* and *QYr.sgi-4A* intervals from consensus maps (Appels 2003; Somers *et al.* 2004). These markers were tested in Kariega and Avocet S and the polymorphic markers were typed on the DH mapping population.

5.4.1.2 Development of expressed sequence tags (EST) markers

In addition to the markers developed in this study, five EST markers published by Xue *et al.* (2008) for the telomeric region of chromosome 4A were tested on the parental lines (Table 5.1).

Table 5.1 Primer sequences for chromosome 4A EST markers developed by Xue *et al.* (2008) included in the parental screen.

Deletion bin	EST	Forward primer (5'->3')	Reverse primer (5'->3')	[a]Screening method
4AL4-0.80-1.00	[b]BE446304	TGA CCT ACT GGC TTG CTG TG	GTT CTC CAC CTT GCA TTT CC	CE
4AL4-0.80-1.00	[b]CN010068	AGG TTC CAT CCC AAA CAG TC	GGTGTCTCAGATTGCCAAAGC	Gel
4AL4-0.80-1.00	[b]CV768787	AGC AAT GGA CCA CGC AAC	TCACTCAGGTTTTCGGTTAGC	Gel
4AL4-0.80-1.00	[b]BQ903300	ATA TCG GAC CCA GTC ATT GC	ACGTGCCGAAGAGGAACTTA	Gel
4AL4-0.80-1.00	[b]BI751252	TAG CCA ACC AGG GAC CTA GA	GATTGTTGTGGCGGTTCTG	Gel
4AL4-0.80-1.00	[b]CN011028	AGA AAT CTT CCA GGG GCT GT	TGTGGAGCGTATTCGACAAG	Gel

[a]Gel – acrylamide gel electrophoresis; CE – capillary array electrophoresis

5.4.1.2.1 Bioinformatics search

Physical maps have been constructed which contain SSR markers mapped to the wheat deletion bins (Sourdille *et al.* 2004). These maps (http://wheat.pw.usda.gov/ggpages/SSRclub/GeneticPhysical) were compared with different consensus maps (Röder *et al.* 1998; Appels 2003; Somers *et al.* 2004) and the currently available Kariega X Avocet S genetic map to establish to which deletion bins (Fig. 5.3) the Kariega QTL are located. Once the target deletion bins were identified the GrainGenes wEST database (http://wheat.pw.usda.gov/cgi-bin/westsql/map_locus.cgi) was searched for EST sequences mapped, through Southern hybridisation, to the appropriate deletion bins. These EST sequences were sorted into three categories, those which mapped to multiple-loci, those which only mapped to one homoeologous group and single locus ESTs. The quality of the Southern hybridisation images was evaluated and the deletion bins confirmed. A selection of EST sequences were searched on the TIGR database (Childs *et al.* 2007, http://plantta.tigr.org/search.shtml) to establish which of these had been assembled into the same transcript assemblies (TAs) to avoid the duplication of loci. The TAs were downloaded, and together with the ESTs not assembled into contigs, organised in a single FASTA format file. The complete rice genomic sequence (*Oryza sativa* cv. Japonica) was downloaded from the NCBI database (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov) as a FASTA file.

The BLAT (BLAST-Like Alignment Tool, Kent 2002) algorithm was used to align the EST nucleotide and translated sequences to the rice genome. BLAT works by keeping an index of an entire genome in memory. The index consists of all non-overlapping 11-mer nucleotide sequences (4-mers for proteins) except for those heavily involved in repeat sequences. BLAT requires less computing power, is faster and is more suitable for aligning expressed sequences with genomic sequences, as it allows for large gaps representing intronic sequences. The algorithm will identify sequences longer than 40 bases of 95% or more homology. Protein sequences of more than 20 amino acids and with more than 80% homology will be reported. In contrast, BLAST (Altschul *et al.* 1990) is more suitable for finding shorter, more divergent sequence alignments. ESTs were identified which were either not interrupted by introns (according to the alignment with rice), or of which the predicted intron does not exceed 500 bp.

5.4.1.2.2 Primer design

Primer3Plus software (Untergasser *et al.* 2007, http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) was used to design primer pairs. Primer lengths were selected to be between 18-22 bp, with optimal annealing temperatures (T_A) of 60°C and GC content between 40 and 60%. Default values were retained for all other parameters. The stringency of these criteria were slightly relaxed if they did not find any suitable primers. Primer sequences obtained were visually inspected and primers with polynucleotide stretches were avoided where possible. Primer pairs were selected with a predicted amplicon length of 100 to 700 bp. Oligonucleotides were ordered from IDT

with the addition of universal M13 sequences (M-13 tails, 5'-CAC GAC GTT GTA AAA CGA C) at the 5' end of the forward primer. The M13 tails were ordered separately, from Applied Biosystems, labelled with FAM, VIC, NED or PET fluorophore labels. This is to enable products to be visualised on the ABI3130*xl* Genetic Analyzer (Schuelke 2000). Alternatively, unlabelled primers, without M13 tails, suitable for gel electrophoresis were ordered from Sigma Aldrich (UK).

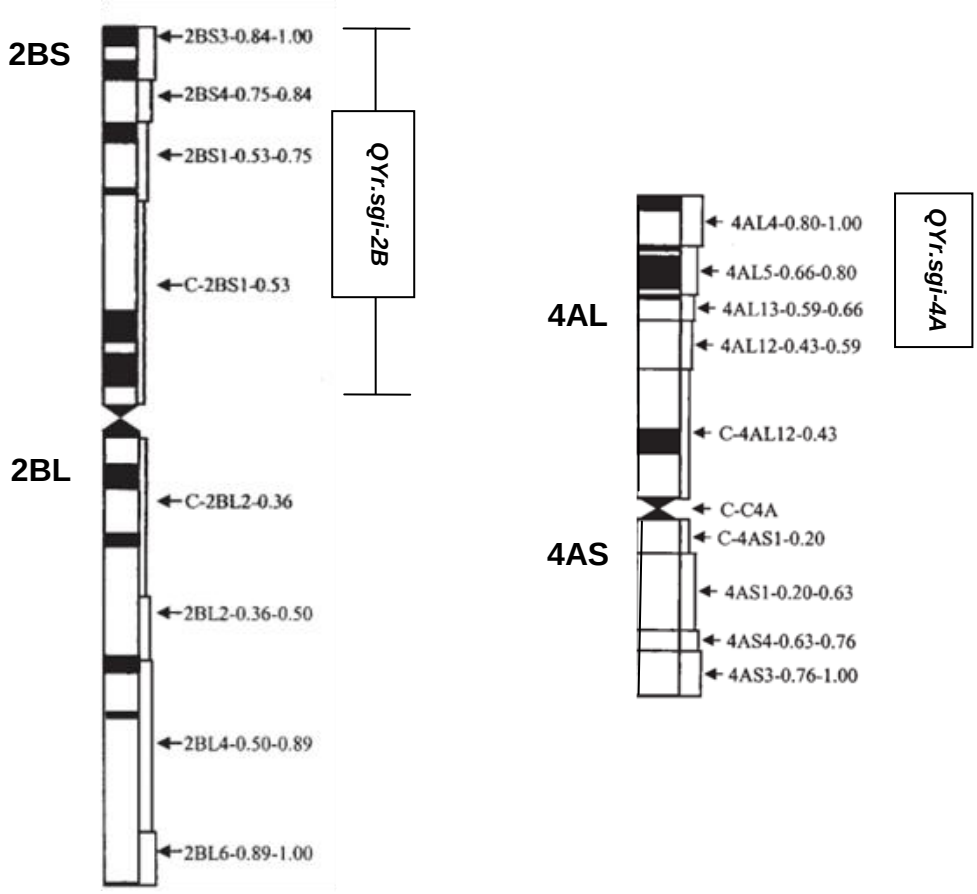

Figure 5.3 Deletion bin maps for chromosomes 2B (Conley *et al.* 2004) and 4A (Miftahudin *et al.* 2004). *QYr.sgi-2B* is located on the short arm of chromosome 2B and *QYr.sgi-4A* on the long arm of chromosome 4A, as indicated.

5.4.1.2.3 Marker optimisation and screening

Genomic DNA for Kariega and Avocet S was amplified, and the expected product size confirmed on 1.5% (w/v) agarose gels (as described for SSR markers in section 3.3.1.2). Annealing temperatures were adjusted for the amplification of a single fragment size. Primer concentrations and thermocycling parameters were optimised as described in section 3.4.1. Once this was achieved, a selection of individuals was amplified from the DH mapping population to establish repeatability. Initially a set of primers was designed for screening on MDE® gels (Lonza Rockland Incorporated) for SSCPs (technical details described in section 3.4.2). Additional primers were designed for the *QYr.sgi-2B* and

QYr.sgi-4A QTL intervals and these markers were screened on a capillary array system to enable high-throughput screening by means of SSCP analysis (technical details in section 3.4.3). Polymorphic primers were screened on the complete mapping population with the appropriate electrophoresis detection system.

5.4.1.2.4 Comparison of screening techniques

Polyacrylamide gel electrophoresis was compared with capillary array electrophoresis for the analysis of SSCPs. Selected EST markers were screened on both systems and the results were compared, considering time, cost and ease of use.

HRM analysis was investigated as an alternative method for analysing EST markers. Genomic DNA (50 ng) from Kariega and Avocet S was amplified for selected markers in 20 µL reaction volumes, containing 1X SensiMix HRM™ (Quantace) (with EvaGreen™ as the intercalating dye and added $MgCl_2$) and 10 pmol unlabelled forward and reverse primer. A Rotor-Gene 6000 Real-time Rotary Analyzer (Corbett Life Science) was used for thermocycling, starting with an initial denaturing step at 95°C for 10 min to activate the hot-start *Taq* polymerase, followed by 40 cycles of 15 s at 95°C, 15 s at 60°C and 15 s at 72°C. The subsequent HRM step involved ramping from 73°C to 90°C, at a rate of 0.1°C per cycle. HRM profiles were analysed with the software provided for the instrument.

5.4.1.2.5 Characterisation of EST sequences

Selected EST sequences were BLAST (Altschul *et al.* 1990) searched against the nucleotide collection in the NBCI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with the megablast program for highly similar sequences. If no significant similarities were found in the database, the discontinuous megablast program was used. Matches with significant identity to putative or characterised resistance genes in other plant species are described. Values for the percentage of nucleotide bases shared by the query (EST) and the subject (database match) (% identity), considering a fragment of the query with homology (% coverage), are reported. E-values < 10^{-20} were considered to be a significant match, E-values < 10^{-10} a very good match and E-values = 1 were considered a perfect match (Lazo *et al.* 2004).

5.4.1.3 Nucleotide-binding site amplified fragment length polymorphism (NBS-AFLP) markers

The degenerate NBS-AFLP markers NBS2, NBS5, NBS3 and NBS7 (as described in Van der Linden *et al.* 2004 and Calenge *et al.* 2005) as well as four new primers which resembles these sequences, but have been adapted to be more specific to cereal genomes (NBS2cer, NBS3cer, NBS5cer and NBS7cer) (Sayar-Turet *et al.* 2011) (Table 5.2) were considered. Unlabelled primers and adapters were synthesised by Sigma Aldrich (UK) and fluorescently labelled primers were synthesised by Applied Biosystems (UK).

Table 5.2 NBS-AFLP primer and adapter sequences.

Primers/ Adapter	[a]Sequence (5'-3')	Targeted NBS motif / orientation of primer binding
NBS2	GTW GTY TTI CCY RAI CCI SSC AT	p-loop / towards 5'end of the motif
NBS3	GTW GTY TTI CCY RAI CCI SSC ATI CC	p-loop / towards 5'end of the motif
NBS5	YYT KRT HGT MIT KGA TGA TGT ITG G	kinase 2a / towards 3'end of the motif
NBS7	ATT GTT GGR ATG GGM GGI MTI GG	p-loop / towards 3'end of the motif
NBS2cer	GTI GTY TTI CCH RII CCH SC	p-loop / towards 5'end of the motif
NBS3cer	GTİ GTY TTI CCH RII CCH SCI VII CC	p-loop / towards 5'end of the motif
NBS5cer	YYT IIT BVT HHT IGA TGA TIT ITG G	kinase 2a / towards 3'end of the motif
NBS7cer	GSD GGI IYD GGI AAR ACI AC	p-loop / towards 3'end of the motif
Adapter primer	ACT CGA TTC TCA ACC CGA AAG	n/a
Adapter upper	ACT CGA TTC TCA ACC CGA AAG TAT AGA TCC CA	n/a
Adapter lower	TAT GGG ATC TAT ACT T	n/a

[a]IUPAC notation

n/a – not applicable

The protocol starts with a restriction digest of 500 ng genomic DNA with 0.5 U *Mse*I and REACT1 buffer (New England Biolabs), in a final volume of 40 µL (Fig. 5.4). The reaction was incubated in a water bath at 37^0C for 3 h, after which the enzyme was heat-inactivated (10 min at 65^0C using a thermocycler). This was followed by the ligation reaction in a volume of 60 µL with 1 U *T4* ligase and 1X ligase buffer (Fermentas), 1 mM ATP (Fermentas) and 50 µM adapter. The adapter was synthesised according to Fischer *et al.* (1995), combining 1.25 nmol of the upper and lower adapter strand (Table 5.2) in a final volume of 75 µL (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$). The short adapter strand (lower adapter) was 5' phosphorylated with an amino group (NH_2) added to the 3' end to prevent extension of this strand by *Taq* polymerase. Both adapter strands were dHPLC purified. The reaction was maintained at 90^0C for 3 min, after which the water bath was turned off and allowed to cool to room temperature before removing the adapters. The ligation reaction was incubated overnight at room temperature and the reaction was terminated in a thermocycler (15 min at 70^0C). An asymmetric PCR (first PCR) was done with 50 ng of digested DNA in a 25 µL reaction volume, using 0.5 U of either Kapa2GFast Hot Start Taq, 1X Buffer A with 1.5 mM $MgCl_2$ (Kapa Biosystems), or Roche Taq, 1X Buffer with $MgCl_2$ (Roche Applied Science), 0.2 mM dNTPs (ABgene) and 15 pmol NBS specific primer. Cycling conditions consisted of an initial denaturing step at 95^0C for 2 min, followed by 30 cycles of 95^0C for 30 s, $55/60^0$C for 1 min 40 s and 72^0C for 2 min. A linear PCR (second PCR) followed using 1 µL of PCR product, 0.5 U of either Kapa2GFast Hot Start Taq, 1X Buffer A with 1.5 mM $MgCl_2$ (Kapa Biosystems) or Roche Taq, 1X Buffer with $MgCl_2$ (Roche Applied Sciences), 0.2 mM dNTPs (ABgene) and 15 pmol NBS specific as well as 15 pmol adapter primer, in a volume of 25 µL. Cycling conditions were identical to those used for the asymmetric PCR. The GeneAmp PCR System 9700, Veriti 96-well thermal cycler (Applied Biosystems) and the MJ Research PTC-200 DNA Engine were used for amplification.

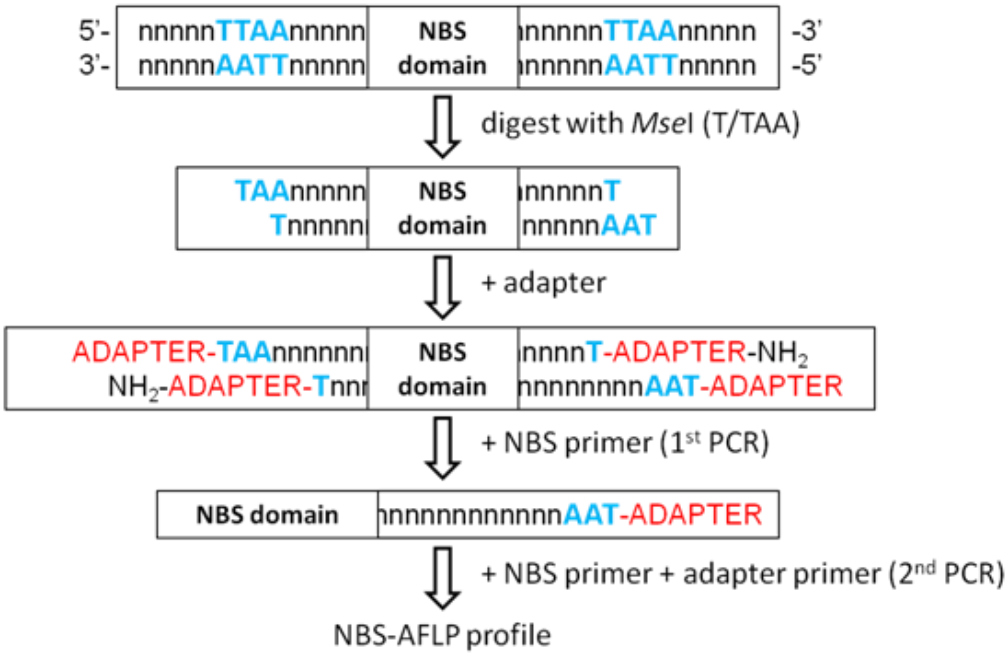

Figure 5.4 Process for generating an NBS-AFLP profile.

A combination of NBS-AFLP and conventional AFLP was also tested. Universal primers for the *Eco*RI restriction recognition site (G/AATTC) were used to amplify *Eco*RI digested gDNA. Only one PCR was done, with two or three selective nucleotide bases in the AFLP primer sequence (5'-GTA GAC TGC GTA CCA AT TC+selective bases) and an NBS-AFLP primer.

Initially electrophoresis was carried out on acrylamide gels and visualised after staining with silver nitrate. The gel matrix consisted of UreaGel 6 and UreaGel Complete buffer solution (National Diagnostics), which polymerise to a 6% polyacrylamide gel after the addition of APS (Amersham Biosciences). 1X TBE (10X stock, Severn Biotech Ltd) was used as the running buffer. Gels (39.4 cm x 33.3 cm) were cast with 0.35 mm combs. Before casting, glass plates were prepared by treating one plate with Repelcote VS silicone (VWR International) and the other with Bind Silane (Amersham Biosciences). PCR products were denatured in a thermocycler in 1:1 formamide loading buffer [95% (v/v) formamide, 10 mM EDTA pH 8.0, 0.05% (w/v) xylene cyanole and 0.05% (w/v) bromophenol blue] at 95°C for 5 min and snap-cooled on ice before loading. Electrophoresis took place at room temperature at 80 W for 1½ to 2 h. Gels were stained with silver nitrate on one of the casting plates. Silver staining was performed as described in section 3.4.2.

Capillary array electrophoresis was tested as a high-throughput alternative fragment separation system. Amplification was first confirmed on 1.5% (w/v) agarose gels (as for SSRs in section 3.3.1.2). Thereafter products were combined with GeneScan™ 500 LIZ® size standard (Applied Biosystems) and injected with Hi-Di™ formamide on a 3130*xl* Genetic Analyzer (Applied Biosystems).

Electrophoresis parameters corresponded to the default SSR/AFLP module. Data were analysed with GeneMapper v.4 software (Applied Biosystems).

5.4.1.4 Linkage and QTL map construction

The order of markers on chromosomes 2B and 4A, after the inclusion of the new SSR and EST markers, were determined with RECORD (Van Os *et al.* 2005a). Map distances were calculated with the Kosambi mapping function from MapManager QTXb20 (Manly *et al.* 2001), with linkage evaluation set at $P = 0.001$ for determining the significant LOD. Linkage maps were prepared with MapChart v.2.1 (Voorrips 2002). QTL analysis was performed for the 2006 datasets with CIM in Windows QTL Cartographer (refer to section 3.5.3).

5.4.2 Marker conversion

Both DArT and EST markers screened by means of SSCP analysis are not suitable for high-throughput screening. These markers were therefore converted to STS markers to enable typing of these markers in the larger F_2 mapping population and also to allow for their potential use in MAS in future.

5.4.2.1 Primer design

Primers used to screen the ESTs by means of SSCP analysis were used to directly sequence the fragments from both parents. If the sequencing results indicated a mixed amplicon, primers were redesigned on the resulting sequence with the aim of obtaining a single amplified fragment. If this failed the initial PCR product was cloned and the clones sequenced. SSCP bands analysed on acrylamide gels were also excised from the gel, cloned and sequenced.

Primers were designed on DArT marker sequences obtained from Triticarte P/L, Diversity Arrays Technology (Pty) Ltd (DArT P/L), Yarralumla, Australia). Primer3Plus software (Untergasser *et al.* 2007, http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) was used for primer design. Primer lengths were selected to be between 18 and 22 bp, optimal annealing temperatures (T_A) of 60°C and GC content between 40 and 60%. Polynucleotide stretches were limited. Default values were retained for all other parameters. The stringency of these conditions were progressively relaxed if primers could not be identified. Primer pairs were selected to amplify fragments of up to a 1000 bp.

5.4.2.2 Sequencing

Amplification was done in 25 µL reactions containing 50 ng gDNA, 10 pmol unlabelled forward and reverse primer, 1X GoTaq colourless buffer (Promega Corporation), 0.2 mM of each dNTP (KapaBiosystems), 1.5 mM $MgCl_2$, and 0.25 U GoTaq DNA polymerase (Promega Corporation).

Thermocycling parameters were standardised, only varying the annealing temperature. Cycling commenced with an initial denaturing at 94°C for 5 min, followed by 40 cycles consisting of 10 s at 94°C, 30 s at the optimised annealing temperature and 30 s at 72°C, completing the PCR with a final extension for 5 min at 72°C. Amplification was confirmed on 1.5% (w/v) agarose gels (as described in section 3.3.1.2). Unincorporated primer, primer dimers and salt were removed from PCR products with the GFX PCR DNA and gel band purification kit (Amersham Biosciences). The concentration of cleaned PCR products were determined with the Nanodrop Spectrophotometer ND-1000 and diluted to 3.3 ng/µL (fragments smaller than 500 bp) or 6.6 ng/µL (fragments larger than 500 bp). Separate sequencing reactions were done for the forward and reverse primers (3.3 pmol primer concentration) using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to manufacturer's instructions. A 3130*xl* or 3730*xl* Genetic Analyzer (Applied Biosystems) was used for electrophoresis.

5.4.2.3 Cloning

PCR products (as described for sequencing in the previous section) were purified with the GFX PCR DNA and gel band purification kit (Amersham Biosciences) and the concentration determined with the Nanodrop Spectrophotometer ND-1000 and diluted to achieve a 5:1 insert:vector ratio. The pGEM-T Easy vector system (Promega Corporation) was used in conjunction with *E. coli* chemically competent cells (Lucigen). Ligation reactions were left overnight at 4°C. Transformed cells were grown overnight at 37°C on *E. coli* FastMedia LB Agar plates with ampicillin and IPTG/X-Gal (Fermentas). White colonies were screened through direct colony-PCR using T7 (5'-TAA TAC GAC TCA CTA TAG GG) and SP6 (5'-ATT TAG GTG ACA CTA TAG) universal primers (Applied Biosystems). Selected transformation positive amplified products were cleaned with the GFX PCR DNA and gel band purification kit (Amersham Biosciences) and sequenced as described in the previous section.

5.4.2.4 Sequence analysis

Generated sequences were viewed and aligned with the BioEdit sequence alignment editor (Hall 1999). The parental alleles (from Kariega and Avocet S) were compared with the reference EST or DArT sequences. SNPs which discriminate between the two parental alleles were incorporated into primer sites to bring about allele specific amplification. The new primers, chosen by visual inspection of the aligned sequences, were evaluated with Primer3Plus according to the criteria described in section 5.4.2.1.

5.4.2.5 Marker validation

Genomic DNA from Kariega and Avocet S were amplified with the new DArT-STS and EST-STS markers. A similar PCR protocol was used as described in section 3.3.1.1 for SSR markers. The annealing temperatures were optimised and products were separated on 1.5% (w/v) agarose (refer to

section 3.3.1.2). Following optimisation, additional cultivars were tested with the EST-STS markers. SSR markers in the QTL intervals were also typed in these cultivars to validate the results obtained with the STS markers. The pedigree available for Kariega was also considered to establish the QTL origins. The DArT-STS markers were screened in a subset of the Kariega x Avocet S DH mapping population and the marker positions confirmed in the relevant linkage groups.

Cultivars from the Kariega pedigree were selected for marker analysis of the *QYr.sgi-4A* interval. SSR markers, DArT-STS and an EST-STS marker were typed in these lines to validate the converted markers and to gain insight into the origin of chromosome 4A QTL.

5.4.3 Developing real-time PCR markers for *Lr34/Yr18/Pm38*

5.4.3.1 Published sequence tagged site (STS) markers

Lagudah *et al.* (2009) developed markers for the *Lr34/Yr18/Pm38* gene's exon 11 deletion that is present in resistant cultivars. Their cssfr5 multiplex marker was used to screen for the presence of the resistant *Lr34/Yr18/Pm38* alleles on agarose. As a high-throughput alternative the cssfr6 co-dominant marker was fluorescently labelled and lines were screened on the 3730*xl* Genetic Analyzer (Applied Biosystems) with the GeneScan™ 1200 LIZ® size standard (Applied Biosystems) as the internal size marker. The original protocol for this marker was adapted by excluding the enzyme digestion with *Fnu4*HI of the amplicon before electrophoresis. The three base pair indel was therefore used to discriminate between the alleles and not the SNP in exon 12 which the restriction enzyme digest identifies.

5.4.3.2 Sequence confirmation

Primers, published in Lagudah *et al.* (2009) were selected. L34DINT9F and L34DINT13R2 (one of the forward and reverse primers from the cssfr5 multiplex marker) were used to generate a PCR product of 1228 and 1231 bp for Kariega and Avocet S, respectively. This primer combination allows for the amplification of a larger fragment. Sequencing was as described in section 5.4.2.2 with the primers L34DINT9F, L34DINT13R2 and cssfr6R, which binds 226 bp upstream from L34DINT13R2. These primers were chosen to obtain adequate coverage of the region, including the exon 11 indel and exon 12 SNP, in both the sense and anti-sense direction (Table 5.3). Alignment with the Chinese Spring consensus sequence (Genbank FJ436983.1) was done with the BioEdit Sequence Alignment Editor software v7.0.9.0 (Hall 1999).

5.4.3.3 Real-time PCR markers

The Chinese Spring reference sequence was used to design new primers and FRET hybridisation probes with the LightCycler® Probe Design v.2.0 software (Roche Applied Sciences) (Table 5.3). The

new *Lr34/Yr18/Pm38* primers amplified a short fragment (170 bp). Once bound to the target sequence, the sensor (donor) probe, labelled with fluorescein, transfers energy to the anchor (acceptor) probe, labelled with CAL Fluor Red 635 or Cy3.5, enabling it to emit a fluorescent signal. Increasing the temperature causes the probes to melt off again. Once separated and unbound the fluorescence decreases as energy is no longer transferred. The anchor probe would melt from the different alleles at exact, but distinct temperatures. This feature is used to generate distinct profiles to discriminate between the *Lr34/Yr18/Pm38* resistant and susceptible alleles. The fluorescein-CAL Fluor Red 635 probe combination was analysed on the LightCycler v.2.0 (Roche Applied Sciences) and the fluorescein-Cy3.5 probe combination was analysed on the Rotor-Gene 6000 Real-time Rotary Analyzer (Corbett Life Science).

Table 5.3 Primer and probe sequences for the detection of the *Lr34/Yr18/Pm38* alleles.

Marker	Primer	Sequence (5'->3')	Product size	Phenotype
[a]cssfr5	L34SPF	GGG AGC ATT ATT TTT TTC CAT CAT G	751 bp	Resistant
	L34DINT13R2	ACT TTC CTG AAA ATA ATA CAA GCA		
	L34DINT9F	TTG ATG AAA CCA GTT TTT TTT CTA	523 bp	Susceptible
	L34MINUSR	TAT GCC ATT TAA CAT AAT CAT GAA		
[a]cssfr6	cssfr6F	FAM-CTG AGG CAC TCT TTC CTG TAC AAA G	649/652 bp	Co-dominant
	cssrf6R	GCA TTC AAT GAG CAA TGG TTA TC		
Lr34	Lr34F	TAT TCC TTC GAA CTC GC	170 bp	n/a
	Lr34R	TGA TGA ATA GAA ATA GTA GCT CTT		
Probes	Sensor	TTT CCA TCT ACA TGA TTA TGT TAA ATG GC–Fluorescein	n/a	Co-dominant
	Anchor	Acta.Fluor 635/Cy3.5 – ACC AGA GAT GAG CAT GCA G–Phosphate		

[a]Markers published by Lagudah *et al.* (2009)

n/a – not applicable

5.4.4 F_2 mapping population: Fine mapping

5.4.4.1 Disease evaluation in field trials

Field trials were conducted during 2009 at the PANNAR Research Station, Greytown, KwaZulu-Natal, South Africa. F_2 seed of a Kariega x Avocet S cross were space-planted on 26 May 2009 in 1 m row plots spaced 90 cm apart. Each row plot contained up to ten plants. Control plots of Kariega and Avocet S were also included. The highly susceptible cv. Morocco was planted in spreader rows bordering the trial area, in all pathways perpendicular to the plots, and as every tenth entry in the trial. Field trials were infected with a spore suspension of pathotype 6E22A+ of *P. striiformis* f. sp. *tritici* (avirulent/virulent for *Yr1*,*3a*,*4a*,*4b*,*5*,*9*,*10*,*15*,*27*,*Sp* / *Yr2*,*6*,*7*,*8*,*17*,*25,A*). A stripe rust epidemic was initiated by spraying a suspension of fresh urediniospores in light mineral oil (approximately 2 mg spores per ml) onto rust spreader rows. Following inoculation certain Morocco rows were covered

with plastic sheeting overnight to ensure saturated conditions required for spore germination and infection. Stripe rust severity was scored in 1020 F_2 plants using the modified Cobb scale for LAI and RT. Stripe rust was rated on 14 September 2009 (LAI14Sept09 and RT14Sept09) and 23 September 2009 (LAI23Sept09 and RT23Sept09). Plants scored for stripe rust were identified by an entry number. Trait data were transformed as described in section 3.5.3.

5.4.4.2 Marker analysis

Genomic DNA was extracted according to the SDS protocol for 96-well plates from the 1020 numbered F_2 plants in the field trial. DNA concentrations were determined with the NanoDrop Spectrophotometer ND-1000 and diluted to 50 ng/µL. SSR markers were selected based on their position in relation to the *QYr.sgi-2B* and *QYr.sgi-4A* intervals on the map generated for the Kariega X Avocet S DH mapping population. Due to the lack of SSR markers in the *QYr.sgi-4A* interval, DArT markers mapped by Prins *et al.* (2011) were converted to STS markers (section 5.4.2) and these were typed by means of capillary array electrophoresis in the F_2 population if they proved to be polymorphic. The *Lr34/Yr18/Pm38* markers csLV34 (Lagudah *et al.* 2006) and cssfr6 (Lagudah *et al.* 2009), and one chromosome 7DS SSR marker was typed in the population. Three markers were selected to obtain a short linkage group for the region. To reduce costs the multiplexing of two to four primer sets was tested. The PCR reaction volume was increased to 20 µL, the $MgCl_2$ concentrations to 3 mM and the GoTaq DNA polymerase (Promega Corporation) to 0.5 U. Thermocycling parameters were not altered.

5.4.4.3 Linkage and QTL mapping

A linkage map was constructed using the complete F_2 population. The marker order was determined with the JoinMap v.4.1 (Van Ooijen 2006; Van Ooijen 2011) maximum likelihood independence LOD algorithm. The QTL positions were confirmed in the F_2 mapping population with CIM (section 3.5.3) of the transformed trait data using Windows QTL Cartographer v.2.51 (Wang *et al.* 2011). Flanking markers were selected for the *QYr.sgi-2B* and *QYr.sgi-4A* intervals. These markers were used to select recombinants within the intervals. Two data sets were created, one for the *QYr.sgi-2B* recombinants and one for the *QYr.sgi-4A* recombinants. This approach was followed to retain information for the alternative QTL and *Lr34/Yr18/Pm38*. The marker order was determined for the recombinant datasets with JoinMap v.4.1 using the same model as before. QTL analysis was performed for these datasets with CIM in Windows QTL Cartographer (refer to section 3.5.3) and MQM in MapQTL v6 (Van Ooijen 2009).

A sequential procedure was used for QTL detection with MapQTL. IM was used to select markers significantly associated with the trait. These were used as an initial set of cofactors. The backwards elimination procedure was applied to the initial set of cofactors. After the selection of cofactors, MQM analysis was performed. A mapping step size of 2 cM was used for both IM and MQM analyses, The

P- value of 0.02, which is the default value in MapQTL, was used as a cut-off value for the elimination of cofactors. The LOD critical values for MQM analysis were empirically determined using the permutation test (10 000 iterations) (Doerge and Churchill 1996). According to Van Ooijen (2009) two different LOD thresholds can be considered, the chromosome wide significance threshold and the genome wide significance threshold. Both thresholds are established at a significance level of 5%. The genome wide significance level was used to declare QTL in MQM.

5.5 Results

5.5.1 DH mapping population: Increasing the marker density

5.5.1.1 Additional SSR markers

Thirteen chromosome 2B and four chromosome 4A specific SSR markers proved to be polymorphic and were typed in the Kariega X Avocet S DH mapping population. These markers were added to the existing map (refer to section 5.5.1.4).

5.5.1.2 Development of EST markers

Due to the lack of SSR markers in the *QYr.sgi-2B* and *QYr.sgi-4A* intervals, the relevant deletion bins could not be identified and EST sequences were selected for a number of deletion bins on the chromosome arms (Table 5.4 and Table 5.5). Somers *et al.* (2003) estimated a homoeologous sequence variant to occur every 24 bp, but the frequency of SNPs within wheat genotypes to be only 1 SNP/540 bp. More than one primer set were therefore designed for each EST/TA, with longer available sequences improving the chances of finding polymorphisms.

Polymorphic EST markers were screened in a subset of the mapping population (16 lines) to confirm consistent amplification and scoreable profiles. Several putative markers which displayed differences between the parents did not meet these criteria in the mapping lines. Bulking of five to ten resistant and susceptible individuals was tested to fast-track the identification of markers linked to the QTL. These individuals were selected based on the existing marker information. Due to the complex SSCP profiles obtained, it was decided not to follow this approach.

Table 5.4 Characteristics and primer sequences for chromosome 4A EST markers included in the parental screen.

Deletion bin	EST	Transcript assembly	Forward primer (5'->3')	Reverse primer (5'->3')	[a]Screening method
4AL13-0.59-0.66	BE585750	TA63943_4565	CTA TCC GGT TTG TTG CCA TC	AAC GGA CCA TGC TTG AGC	Gel
4AL13-0.59-0.66	BE585750	TA63943_4565	CGA AGT CCT CCT GTG TTA CG	ATT GCT GGA AAG ATG AAT GG	Gel
4AL13-0.59-0.66	BE404468	TA66785_4565	CGA CCT CGT CAT CCA GTT CC	CAT GTG TTT GCG GCG ATG	Gel
4AL13-0.59-0.66	BE398593	TA82163_4565	CAG AGG CAT TCC AAG TGT	CTG AAC AAT GAA ATC TAT GAG C	Gel
4AL13-0.59-0.66	BE398593	TA82163_4565	ACG TCG AGA GGC ACA ACA AG	TTC AGC AGA AGT TCA GGG CTA G	Gel
4AL13-0.59-0.66	BE426090	TA92172_4565	CTA GGG CTT CTC CCA CAT	AGG TGA GCC AGC TTG AGT A	Gel
4AL13-0.59-0.66	BE585750	TA63943_4565	GGA CCA TGC TTG AGC TTC TAA	CTA TCC GGT TTG TTG CCA TC	Gel
4AL13-0.59-0.66	BE446304	n/a	TGA CCT ACT GGC TTG CTG TG	CTT GGT TCT CCA CCT TGC AT	Gel
4AL4-0.80-1.00	BE443444	TA87244_4565	AGG TAC GCC AGG AGA TGT TG	GAC AAT GCA GAA TAC CGG AAG	Gel
4AL4-0.80-1.00	[b]BE403796	TA79709_4565	CCT CGC CTC AAA CAA CAC TAC	GAG AGA TCG ACC CAG GCT AC	CE
4AL4-0.80-1.00	[b]BE403856	TA92073_4565	CAA GCC GGG ACA AAA TTA AG	GCA TCC TTG ATG ACC AAA TG	CE
4AL4-0.80-1.00	[b]BE403796	TA79709_4565	AGT AGC CTG GGT CGA TCT CTC	CAT ATT GTG GCC TTC CAA CC	CE
4AL4-0.80-1.00	[b]BE403856	TA92073_4565	AAG CCT ATG GAT GCA ACT GG	AAA GGC ACA ATG ATT CAC AGG	CE
4AL4-0.80-1.00	[b]BE403796	TA79709_4565	GTG TCA CAG CTC AGC CAA C	TTT GGA GAT CCT ACT TGA ATC G	CE
4AL4-0.80-1.00	[b]BE403856	TA92073_4565	AAG TTT GGA GAA ACC ATA CA	GGT GTA GAC AGA ACT AAA CAT T	CE
4AL4-0.80-1.00	[b]BE443412	TA95127_4565	GTG CAG CCA AGG ATG ATA GG	TCA ATG CCG TAA ATC CCT TC	CE
4AL4-0.80-1.00	[b]BE446304	n/a	TGA CCT ACT GGC TTG CTG TG	GTT CTC CAC CTT GCA TTT CC	CE
4AL5-0.66-0.80	BG606378	TA51367_4565	TAC CTC TCC TCC CTC ACC AC	CAC GGA CAC ATA CAC CTT GC	Gel
4AL5-0.66-0.80	BF473887	TA86544_4565	TAT CTT CCC GAG AGC AAT CC	CCA ATT AGG CGG CAC ATC	Gel
4AL5-0.66-0.80	BG606378	TA51367_4565	GCC CGA GAT ACG GTT CAT C	TTC TCT CTG CTC CCT TTC CTC	Gel

[a]Gel – acrylamide gel electrophoresis; CE – capillary array electrophoresis

[b]Forward primer preceded by M13-tail sequence (5'-CAC GAC GTT GTA AAA CGA C)

n/a – not applicable

Table 5.5 Characteristics and primer sequences for chromosome 2B EST markers included in the parental screen.

Deletion bin	EST	Transcript assembly	Forward primer (5'->3')	Reverse primer (5'->3')	[a]Screening method
2BS1-0.53-0.75	BE494209	TA1567_4550	AGA TCC AGC CAA CGA CCT G	CCT ACC TGG TGA CGC TCT TC	Gel
2BS1-0.53-0.75	BG607608	TA2636_4568	AGG AAT CAT GGT CCG ATG TC	TTA ACG AGG TGG GAG AGG TC	Gel
2BS1-0.53-0.75	BE500443	TA52748_4565	GTA TGG CGA CGA CGA TTA CC	GCA CCA CCA CTA GCA TCC TC	Gel
2BS1-0.53-0.75	BE498371	TA66771_4565	TGGA GAT GCT GTA GGT CAC G	CTT CCT CTG GAT GCC GTT C	Gel
2BS1-0.53-0.75	BE517987	TA74702_4565	AGA TCA AGG ACC TGG TGA CG	TGC ACT GGT ACA CCT GGA AC	Gel
2BS1-0.53-0.75	[b]CD453099	n/a	CTA GAA CCC CAC ACC AGT GC	GGC TGA ACC CAG ACT ACG AG	CE
2BS1-0.53-0.75	[b]BE497494	TA61425_4565	TCA CCT TCA TTC AGC ACA GG	TTG GTA AAC GAG GCA ACA TC	CE
2BS1-0.53-0.75	[b]BF201533	TA77223_4565	AGC TCC CTACGATGACGGTA	GGA AGC CGT GGT GGA GAT	CE
2BS1-0.53-0.75	[b]BE445628	TA85532_4565	GCA GGC TGA AAG GGA TCT TAG	CGA GAG CAA TCT CCT CAA GC	CE
2BS1-0.53-0.75	[b]BM138067	TA50786_4565	ACA AGA GCT TGA CCC GTT TC	CTG TTG GTG CCC CAG ATA AT	CE
2BS1-0.53-0.75	[b]BF428792	TA73767_4565	GCT AAG AGC TTT GGG AGC AG	GCA CCT TCC TGA GTT CCA TC	CE
2BS1-0.53-0.75	[b]BE442903	TA77765_4565	GCA TTC AGG AAC CTT TGA GC	TCG AAG AAG CTC ACG AGA AAC	CE
2BS1-0.53-0.75	[b]BE494262	n/a	GCC CCT TTG TAC ATA ATA ACT C	TTT CCT TCT GTT GGG AAA TC	CE
2BS1-0.53-0.75	[b]BE488865	TA56185_4565	CAG CGT CAA GCT CAT CCA C	TGC TTC GCA TAG TCG TGA AC	CE
2BS1-0.53-0.75	[b]BF478837	TA74911_4565	AAA GAA GAT TTG GCA GAA GAT G	CTT ATG GGA CGA TGT GCA G	CE
2BS1-0.53-0.75	[b]BE499478	TA78480_4565	ATA AAA TGG CGC TGT GAA CC	GGG ATT TTC ATG TTG TTG ACG	CE
2BS1-0.53-0.75	[b]BG314532	n/a	TGCA CTT CAT CTG TTT TCC AC	GAA TGA TTT CCT AGC TGT CAC C	CE
2BS1-0.53-0.75	[b]BE500206	TA59174_4565	GCA GAG ATA GGC AAT GCA CTC	TCG AGT GGT TTC AGT TCG AC	CE
2BS1-0.53-0.75	[b]BE604844	TA75106_4565	GCC AAA CCC TCT GGA CTA CC	CCT CGA ACC AGA AGA AGA CG	CE
2BS1-0.53-0.75	[b]BE498254	TA80364_4565	GCT TGG ATG GAA GTT TAC CG	GTT GCC TCT CCA TGT GGT TC	CE
2BS3-0.84-1.00	BG263366	TA106514_4565	GTA GGC GTT GAG CAT GAA GG	CGG CAC GAG GTT GCT TTA C	Gel
2BS3-0.84-1.00	BE444659	TA55005_4565	TGA TCC TTG GCC GTA ACT TC	CTC TCC AAT GAA GGC AAA GC	Gel
2BS3-0.84-1.00	BF292052	TA655_4573	TAA CCT CCT CCA CCT CGT TG	TGA GGG CAT CAA GAA GTT CG	Gel
2BS3-0.84-1.00	BF485266	TA78187_4565	AAT CAG TTG TCC CAG CAA CC	GCA AAG GTG GTT GTT AAT GC	Gel
2BS3-0.84-1.00	BE426083	TA81790_4565	ACT ACG GGA TGT TCC GGT TC	TCG TAG ACT GGG CAC GTT CG	Gel

Table 5.5 continued Characteristics and primer sequences for chromosome 2B EST markers included in the parental screen.

Deletion bin	EST	Transcript assembly	Forward primer (5'->3')	Reverse primer (5'->3')	[a]Screening method
2BS3-0.84-1.00	BE471132	TA87318_4565	AGC AGC AGC GAG AAG AGA AG	AGC AAG TTC TTT GGC GTA GC	Gel
2BS3-0.84-1.00	BG263347	TA82997_4565	TGG CAC CCT TGG TAC TCT TG	CAC CGC TTT GTT CAT GTC AC	Gel
2BS3-0.84-1.00	BE591604	TA82317_4565	TGT CGA ACT TCC CAC TTT TG	TTT GGG CTG TGA CAA TAA GC	Gel
2BS3-0.84-1.00	BE517745	TA81421_4565	GAT CTG TTT CCC CAC ACC TC	ACC TCT CGA AAA GCA TGA CC	Gel
2BS3-0.84-1.00	BE498396	TA80535_4565	ACC ACT ACG CCA GTC CTT TG	AGG AAT GAT GCC AGC CTA TG	Gel
2BS3-0.84-1.00	BF474028	TA80052_4565	CCT GAA AAT ACC GGA AGC AC	CAT TAT CTG GAC TAG CTT GGT GTG	Gel
2BS3-0.84-1.00	BE591555	TA79131_4565	GTG GTC TCA GGG TCA AGG AC	GAA TGG AGC AAC ACA GCA AG	Gel
2BS3-0.84-1.00	BE444541	TA76762_4565	TGC ATG TAA TCA GCG AGT CTG	TCG AGT GAT CGC CTC CTA AG	Gel
2BS3-0.84-1.00	BE488719	TA76503_4565	GCC TTG TAG ACA TCA TCC ACA G	GGG GCA CAG AAA GTG ATC C	Gel
2BS3-0.84-1.00	BE407057	TA68988_4565	CGA CAT GCT TCC GTT AAT TG	GCG GAG TTC AGA TGA TGA TG	Gel
2BS4-0.75-0.84	BG263432	TA97545_4565	CGA GAT TGC AGC CTA GAT CC	GCA GGT CAA CAT CAT TGT CG	Gel
2BS4-0.75-0.84	BF146221	TA1988_4550	CCA GAA CAG CAT CAT CTT CC	TGG AGG TGT CGT CCT TGT G	Gel
2BS4-0.75-0.84	[b]BG275030	TA73490_4565	TCC TGA CAA AGC CTC AGT CC	TTA TTC AGG GCG TTT TCG AG	CE
2BS4-0.75-0.84	[b]BG604518	TA71058_4565	ACA GCG AGA ATG TTG TTT GG	AGT CGG CAT GTG TTT TGT TG	CE
2BS4-0.75-0.84	[b]BG604518	n/a	TTC CAG AAC CTC CTT GAC AAC	CAC CAA ATC TAA ACC AAC AAC C	CE
2BS4-0.75-0.84	[b]BG604518	TA71058_4565	AGA AGT CCC CTG TCC TCC AG	GTC GGG GCC TCT TTT GTA G	CE
2BS4-0.75-0.84	[b]BG604518	TA71058_4565	TTG CTG ATG CTA CCA CCT TC	AGT CCA AAC TCT CGA ACA CCT C	CE
2BS4-0.75-0.84	[b]BG604518	TA71058_4565	GGA GGT GTT CGA GAG TTT GG	TTT GCT TGC TTC AAG GTC AG	CE
C-2BS1-0.53	BF473321	TA74581_4565	GTA CAG CGG GTT GTT CTG	GGA AAC ATG ATC GTC TGC	Gel
C-2BS1-0.53	BF484119	TA72182_4565	GCT GGC CGC TTG ATA GTT AC	TGA CAA CTG CTG AGG TTT GG	Gel
C-2BS1-0.53	BG274976	TA63467_4565	TGG CTT CAC ATG CTC TCA TC	AGG ATG CCC TGT GAG GAA G	Gel
C-2BS1-0.53	BE425962	TA98726_4565	GCT CGA ACC AGT TGA AAA CAC	TTCA GTC TTG AAC GGC ACA C	Gel
C-2BS1-0.53	BE586093	TA85677_4565	GCT CTG GTT GGG CAC TTA AC	TCC AAG GCC AGG TCA TAT TC	Gel
C-2BS1-0.53	BF201328	TA84557_4565	TTT TCA GCC AGT TCA CAT CG	GCA CAC ACT TGA TGC CAG AC	Gel
C-2BS1-0.53	BE442876	TA78558_4565	TCA GAA ATG CAG CAC AGA GG	GGG ATG AAT TGG TCA ACT GG	Gel

Table 5.5 continued Characteristics and primer sequences for chromosome 2B EST markers included in the parental screen.

Deletion bin	EST	Transcript assembly	Forward primer (5'->3')	Reverse primer (5'->3')	[a]Screening method
C-2BS1-0.53	BF483010	TA70722_4565	TTC TTG TGG ATG GTG GAA TG	CCC GCC AAA ATG TAA GAT TG	Gel
C-2BS1-0.53	BF484029	TA65284_4565	TAC AAT GGA GGC ATC AGC AC	TCC TTG GAG CAG CTT GTT G	Gel
C-2BS1-0.53	BF201348	TA62568_4565	TTG TTT ATG GCA AGC CCA AG	AGG ACA CGA AGA CCA CCA AG	Gel
C-2BS1-0.53	BG314068	TA2392_4568	TCAA CCT GTC CCT GGA GGA G	AGA CGG TAG AGC GTG GTG AG	Gel
C-2BS1-0.53	BE406808	TA55656_4565	TAC ATC TGG CAC AGC AAA GC	CCG TTT GTG GTG GTA ATG G	Gel
C-2BS1-0.53	BE398439	TA61480_4565	TGTG AAT GAG CGT CTG AAC C	AAA CTC GCA AAC CAT TCT GG	Gel
C-2BS1-0.53	BG274976	TA63467_4565	AAC TGC ACC ACC AGC TAA CC	CTG ACA ATG CCA TGC AAG AG	Gel
C-2BS1-0.53	BE443112	TA66130_4565	AAA TCA GTG GCC TTG AGC AC	ATC TGG GCA GCC AAA CAT AG	Gel
C-2BS1-0.53	BE403404	TA69079_4565	GTC GAT GTT ACC CTT GTG GAG	GTG GAA CCC TGT GGC TAA TG	Gel
C-2BS1-0.53	BE497171	TA81770_4565	CTG GAA GCA GTG TCA TGG TG	AGA ATC AGC GTC GAA GAT CG	Gel

[a]Gel – acrylamide gel electrophoresis; CE – capillary array electrophoresis

[b]Forward primer preceded by M13-tail sequence (5'-CAC GAC GTT GTA AAA CGA C)

n/a – not applicable

5.5.1.2.1 Characterisation of EST markers

SSCP analysis using gel electrophoresis identified nine polymorphic EST markers which were screened in the DH mapping population (summarised in Table 5.6). Four of the seven ESTs identified on chromosome 2B, mapped to chromosome 2B. Two linkage groups were reported for chromosome 2B, 2B.1 and 2B.2, by Prins *et al.* (2011). Two EST markers, TA1988 and TA52748, mapped to the main linkage group (2B.1), containing the QTL, and two to the second linkage group (2B.2), which mainly contains DArT markers. The position of the second linkage group relative to the main 2B linkage group has not been determined. Although EST markers were selected based on their physical map location, two ESTs on chromosome 2B mapped to chromosomes 1B and 5B/7B, while the third EST marker remained unlinked. The two ESTs selected for chromosome 4A mapped to chromosome 4A. The chromosome 2B markers, TA52748 and TA1988, and the chromosome 4A marker, CV768787 [expressed STS (eSTS) marker MAG1604 from Xue *et al.* 2008], were selected for further analysis. SSCP analysis using capillary array electrophoresis identified three markers which were suitable for mapping, TA61425, CD453099 and TA59174 (Table 5.6). TA59174 proved also to show polymorphism when analysed by agarose gel electrophoresis and due to cost considerations was screened on 1.5% (w/v) agarose. Two markers, CD453099 and TA59174, mapped to the chromosome 2B.1 linkage group, while one marker could not be incorporated into the map, and remained unlinked.

ESTs which mapped to the regions of interest were BLAST searched against the NCBI nucleotide database for homologies to known resistance proteins or protein domains, postulated to be involved in plant defence response. The best matches [expect value (E-value) = 0] for both TA1988, a rye (*Secale cereale)* EST, and TA52748 were with barley (*Hordeum* vulgare) predicted, yet uncharacterised proteins. However, for TA1988 matches with characterised proteins had lower scores (E-value < 2e-38) and these included rice (*Oryza sativa*) and maize (*Zea mays*) partial coding sequences for decarboxylases and an ABC transporter from rice (85-86% identity, 71% cover). TA52748 shared homology with *Phyllostachys edulis* (a bamboo species), rice and sorghum (*Sorghum bicolor*) uncharacterised proteins (83-85% identity, 59-93% coverage). TA61425 showed similarity to rice, maize and soybean (*Glycine* max) ferredoxin-dependent glutamate synthases as well as sorghum and barley hypothetical proteins. These matches were at an E-value = 0, with identities of 77-96% (up to 99% coverage of the query). The rye EST CD453099 showed similarities with rice, barley and sorghum mRNA and gDNA clones. The best match with a characterised protein is with a GTP-binding protein from maize and *Arabidopsis thaliana* (85% identity, 49% coverage). TA59174 only had hits with barley, maize, rice and sorghum predicted proteins (85-87% identity, 57-79% coverage). None of the chromosome 2B ESTs were therefore related to characterised resistance genes. However, the *Lr34/Yr18/Pm38* is a good example of a resistance gene transcribing a protein with an unexpected function, i.e. an ABC transporter. Chromosome 4A EST, CV768787 did not have any highly significant hits, but showed homology to barley powdery mildew

Table 5.6 Summary of EST markers mapped in the Kariega X Avocet S DH mapping population, including the map location. The annotation and values for the identity of the ESTs to characterised sequences from other plant species, as reported in the TIGR database (http://plantta.tigr.org/search.shtml), are included.

Deletion bins	Transcript assembly	Taxon	[a]Screening method	[b]Amplicon size (bp)	Annotation from TIGR database	Identity; Coverage	Linkage group
C-2BS1-0.53	TA84557	*Triticum aestivum*	Gel	197 bp	Putative DNA repair protein rhp16 [*Oryza sativa*]	95.43%; 69.91%	1B
2BS1-0.53-0.75	TA59174	*Triticum aestivum*	CE	236 bp	Hypothetical protein OJ1200_C08.123 [*Oryza sativa*]	89.4%; 57.41%	2B.1
2BS1-0.53-0.75	TA52748	*Triticum aestivum*	Gel	261 bp	Hypothetical protein [*Triticum aestivum*]	99.22%; 61.71%	2B.1
2BS1-0.53-0.75	CD453099	*Secale cereale*	CE	211 bp	Putative GTP-binding protein DRG related cluster	92.93%; 48.21%	2B.1
2BS4-0.75-0.84	TA1988	*Secale cereale*	Gel	257 bp	TPP-binding enzymes [*Medicago truncatula* (Barrel medic)]	88.89% ;71.27%	2B.1
2BS3-0.84-1.00	TA79131	*Triticum aestivum*	Gel	169 bp	OSJNBb0060M15.2 protein [*Oryza sativa*]	97.56%; 97.56%	2B.2
2BS3-0.84-1.00	TA87318	*Triticum aestivum*	Gel	238 bp	OSJNBa0073L13.10 protein [*Oryza sativa*]	84.4%; 84.4%	2B.2
4AL13-0.59-0.66	TA82163	*Triticum aestivum*	Gel	289 bp	Putative actin-related complex protein [*Oryza sativa*]	97.04%; 48.66%	4A
4AL4-0.80-1.00	CV768787	*Triticum aestivum*	Gel	559 bp	RGH1A related cluster	69.29%; 87.9%	4A
2BS1-0.53-0.75	TA1567	*Secale cereale*	Gel	270 bp	Putative nodulin 3 [*Oryza sativa*[	84.23%; 64.32%	5B/7B
2BS1-0.53-0.75	TA61425	*Triticum aestivum*	CE	170 bp	Fd-GOGAT protein [*Oryza sativa*]	94.4%; 99.67%	unlinked
2BS3-0.84-1.00	TA76503	*Triticum aestivum*	Gel	195 bp	Lactoylglutathione lyase [*Cicer arietinum*]	85.23%; 52.85%	unlinked

[a]Gel – polyacrylamide gel electrophoresis; CE – capillary array electrophoresis

[b]Amplicon sizes as calculated from reference EST sequences and wheat-rice alignments; true amplicon sizes vary

resistance *Mla* loci (70-74% identity, 59-60% coverage) with E-values < 3e-65. This EST is therefore a good candidate for further investigation considering its resemblance to characterised resistance genes.

5.5.1.2.2 Comparison of screening techniques

Although in theory, acrylamide gel electrophoresis is more time consuming and labour intensive compared to high-throughput capillary array electrophoresis systems, gel electrophoresis yielded more consistent results. The MDE® gel matrix, which is specifically designed for mutation detection, provided a sensitive detection system without any modifications to the suggested protocol. The polymer matrix used for capillary electrophoresis was not altered for the SSCP analysis, due to the high costs involved with polymer changes on the instrument. However, different oven temperatures (18°C, 25°C and 35°C) at which the run was performed were tested. Although lower temperatures provided better resolution, it slowed electrophoresis down significantly and complicated sizing of the fragments. GeneMapper v.4 software, designed for fragment analysis, does not have standard analysis settings suited for SSCP analysis. Even though the default SNP or SSR analysis methods and size standards could be adapted, it was still not optimal. SSCP analysis with acrylamide gel electrophoresis led to less repetitions and re-analysis, saving both time and cost.

The two systems were further compared with selected markers. After analysis on the gel system, fluorescently labelled universal M13 primers were used in conjunction with M13-tailed sequence specific forward primers to amplify markers TA52748, TA1988 and CV768787 for analysis on the capillary system. Two of the markers, TA52748 and CV768787, were polymorphic on both systems. TA1988 produced very faint peaks on the capillary system and appeared to be non-polymorphic. Different fluorophore labels were tested for the universal M13 primers, as variation in fluorescent signals were seen (e.g. VIC labels had a higher level of fluorescence than PET labels). Despite extensive optimisation on the capillary system, polymorphism could not be detected and the marker could therefore not be typed in the mapping population (Fig. 5.5).

EST markers TA52748, TA1988 and CV768787 were also analysed with HRM. The melting profile for CV768787 is shown (Fig. 5.6). This screening technology was not appropriate for analysis of these markers. HRM is more suited to the analysis of smaller fragments (preferably less than 200 bp) and the amplicons, as designed for SSCP analysis, were too large to allow for accurate detection of sequence variations. Another problem encountered was related to the complexity of the wheat genome, as homoeologous copies are often co-amplified (Ishikawa *et al.* 2007). SSCP relies on sequence differences (not size differences) to cause mobility shifts in a set matrix. In addition to SNPs or indels in the target interval, various polymorphisms were also present in the homoeologous sequences. This resulted in a complex melting profile which disguised polymorphisms in the target sequence. Melting profiles can therefore not be scored reliably.

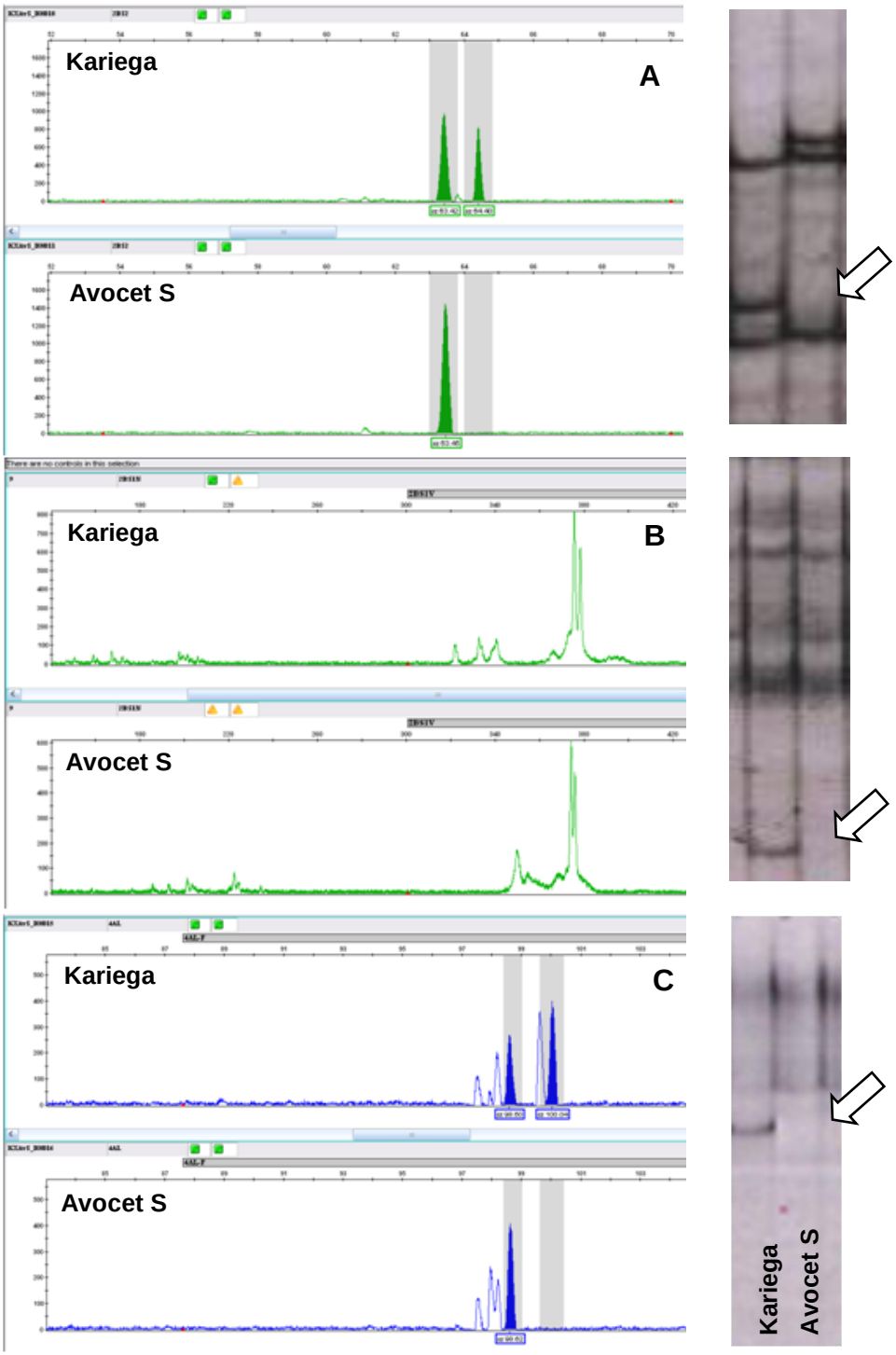

Figure 5.5 Capillary array electrophoresis electropherograms (as obtained with GeneMapper v.4 software) and acrylamide gel images for EST markers (A) TA52748, (B) TA1988 and (C) CV768787. Polymorphic bands are indicated with an arrow and peaks are shaded. Kariega is shown in lane 1 and Avocet S in lane 2. TA1988 (B) was not polymorphic on the capillary system.

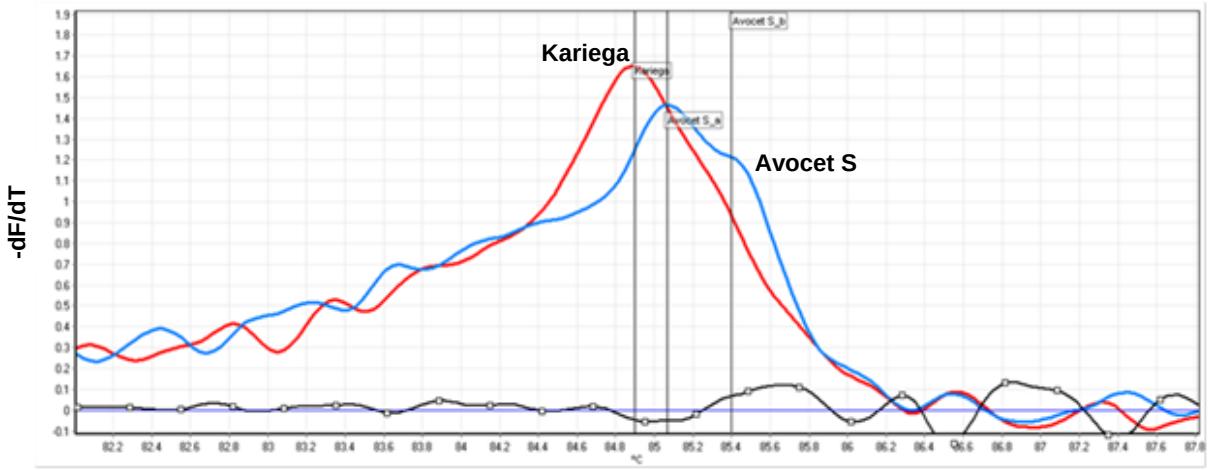

Figure 5.6 High resolution melt (HRM) analysis of the EST marker CV768787 on the Rotor-Gene 6000 Real-time Rotary Analyzer. The melting curve is transformed to the first derivative of fluorescence over temperature (-dF/dT). The melting temperature (T_M) for Kariega (red) is 84.90°C, while Avocet S (blue) melts at 85.09°C and 85.40°C. The non-template control is represented by the black line.

5.5.1.3 NBS-AFLP markers

Enzymatic digestion of gDNA was confirmed with agarose gel electrophoresis. Amplified products created with the asymmetric first PCR, as well as amplicons from the second PCR, were also confirmed with agarose gel electrophoresis before continuing. Examples of the profiles obtained are shown for the NBS-AFLP general (Van der Linden *et al*. 2004), and cereal specific primers (Sayar-Turet *et al.* 2011) (Fig. 5.7). Different NBS-AFLP primer combinations were tested, as well as NBS-AFLP primers in combination with various universal AFLP primers for *Eco*RI restriction enzyme recognition sequences. The generic AFLP primers were tested on gDNA digested with *Eco*RI with the addition of the relevant adapters. Clearly distinguishable and scoreable profiles were obtained with acrylamide gels, but the results were not reproducible (Fig. 5.8).

Due to practical considerations and also with the aim to adapt the NBS-AFLP profiling system for high-throughput screening, capillary array electrophoresis was investigated. Both the adapter and NBS-specific primers were fluorescently labelled to allow detection of both single strands of the amplified fragments. From the electropherograms it could be confirmed that the amplified product consisted of NBS-adapter sequences and not adapter-adapter sequences, therefore the amplicons contained NBS

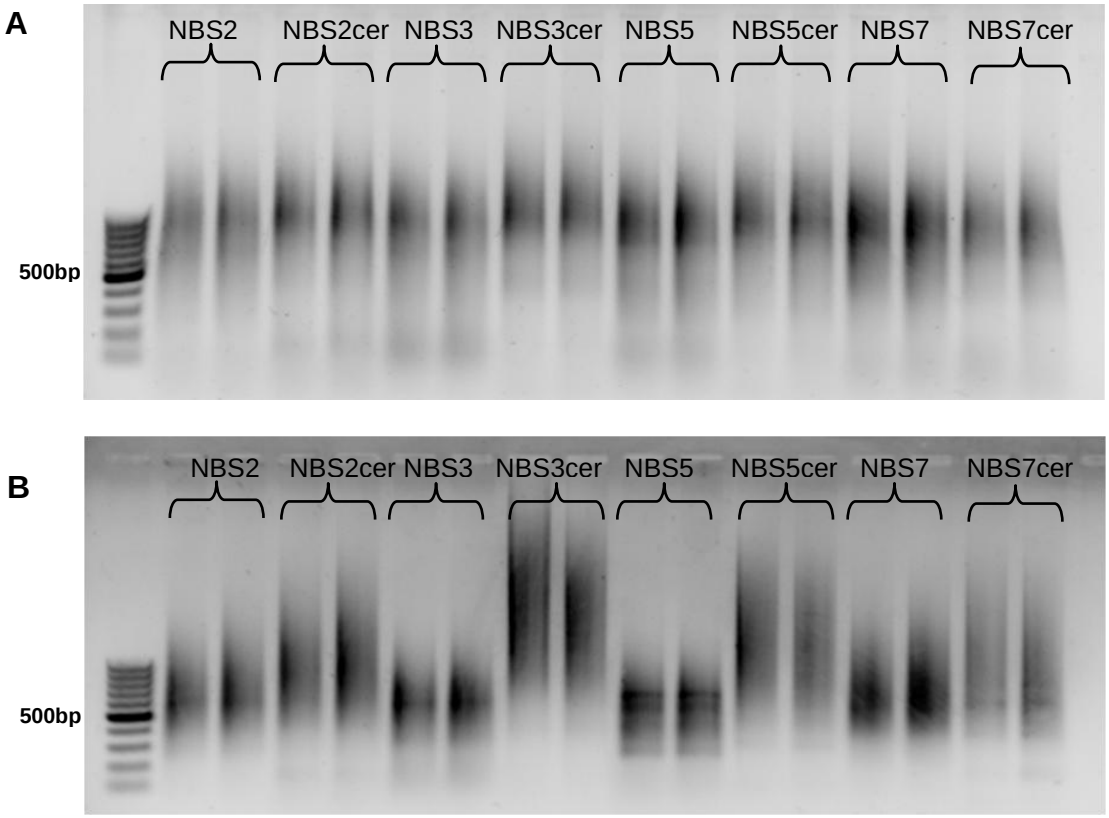

Figure 5.7 Separation of A) 10 µL amplified product generated with the first PCR and B) 5 µL amplified product generated with the second PCR of the NBS-AFLP protocol (van Linden et al. 2004), on 1% (w/v) agarose (0.5XTBE). Both the general (NBS2, NBS3, NBS5 and NBS7) and cereal specific primers (NBS2cer, NBS3cer, NBS5cer and NBS7cer) were tested. The 500 bp fragment of the 100 bp molecular marker (GeneRuler^{TM}, Fermentas) in lane 1 is indicated. The DNA profiles shown are for alternating samples of Kariega and Avocet S.

domains. Different profiles for each NBS-AFLP primer could be distinguished, which also corroborated the participation of the NBS primers in the reaction. Electropherograms, as viewed with GeneMapper v.4

after capillary array electrophoresis are shown in Fig. 5.9. A high level of background noise and a much reduced size range (< 500 bp), from what was expected based on the fragment sizes produced with agarose gel electrophoresis, were observed (Fig 5.7). A dilution series of the first PCR was tested, but this did not have an effect. A selection of NBS-AFLP primers were tested at a range of annealing temperatures (48-60°C) and at an increased $MgCl_2$ concentration (3 mM instead of 1.5 mM), but neither of these variables changed the pattern produced with capillary array electrophoresis. Very few peaks were visible, of which only two were polymorphic and of very low intensity (Fig. 5.9). It was therefore decided not to pursue this type of marker technology as a way of increasing the marker density of the DH map.

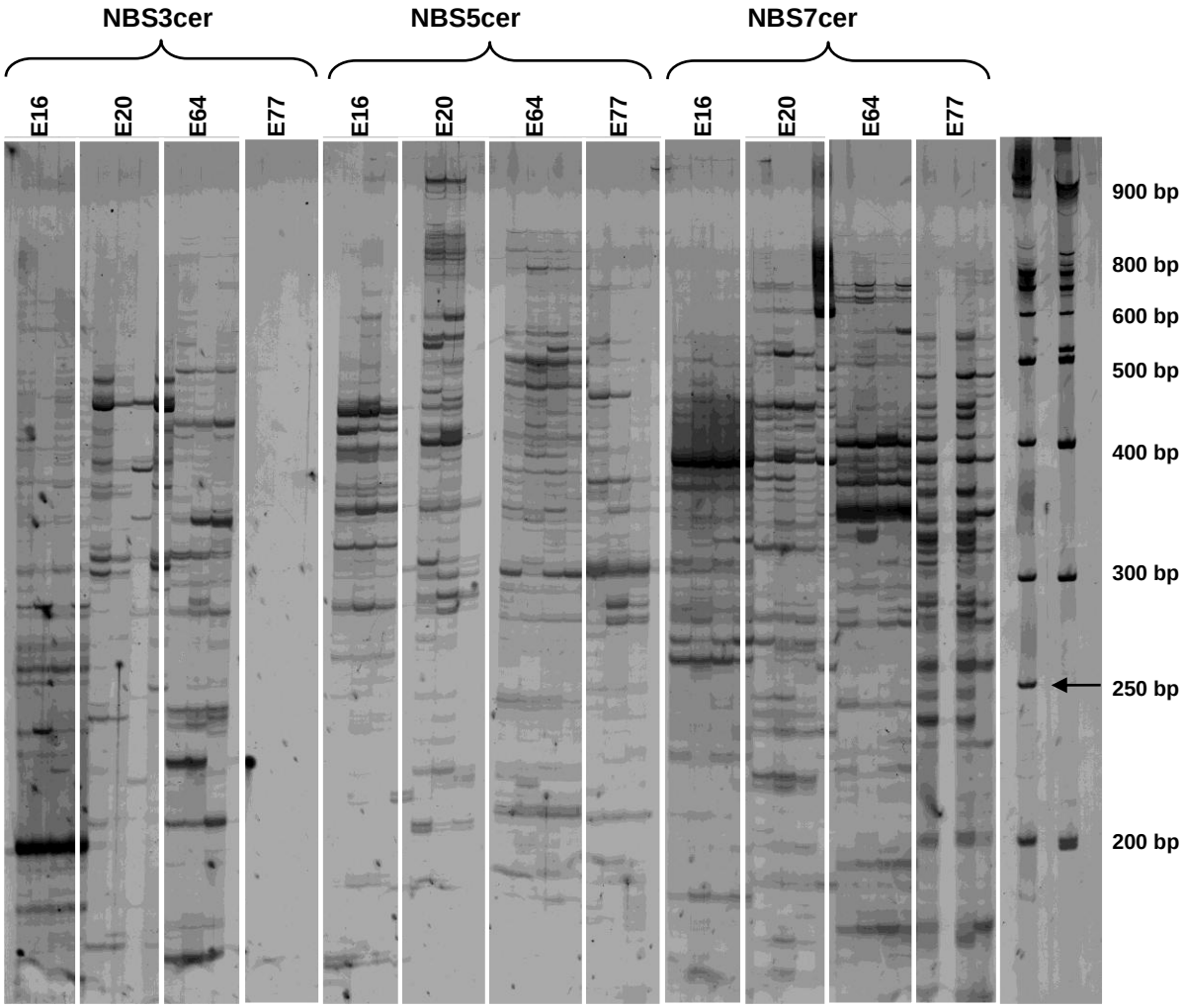

Figure 5.8 Resolution of NBS-AFLP amplified fragments with acrylamide gel electrophoresis and visualised by means of staining with silver nitrate. Alternating amplicons of Kariega, Avocet S and two unrelated (Cappelle-Desprez and Palmiet) cultivars are loaded, with a 100 bp molecular marker (GeneRuler^{TM}, Fermentas) in the last lane. *Eco*RI AFLP primers E16 (+CC), E20 (+GC). E64 (+GAC) and E77 (+GTG) in combination with three NBS-AFLP cereal specific primers (NBS3cer, NBS5cer and NBS7cer) are shown.

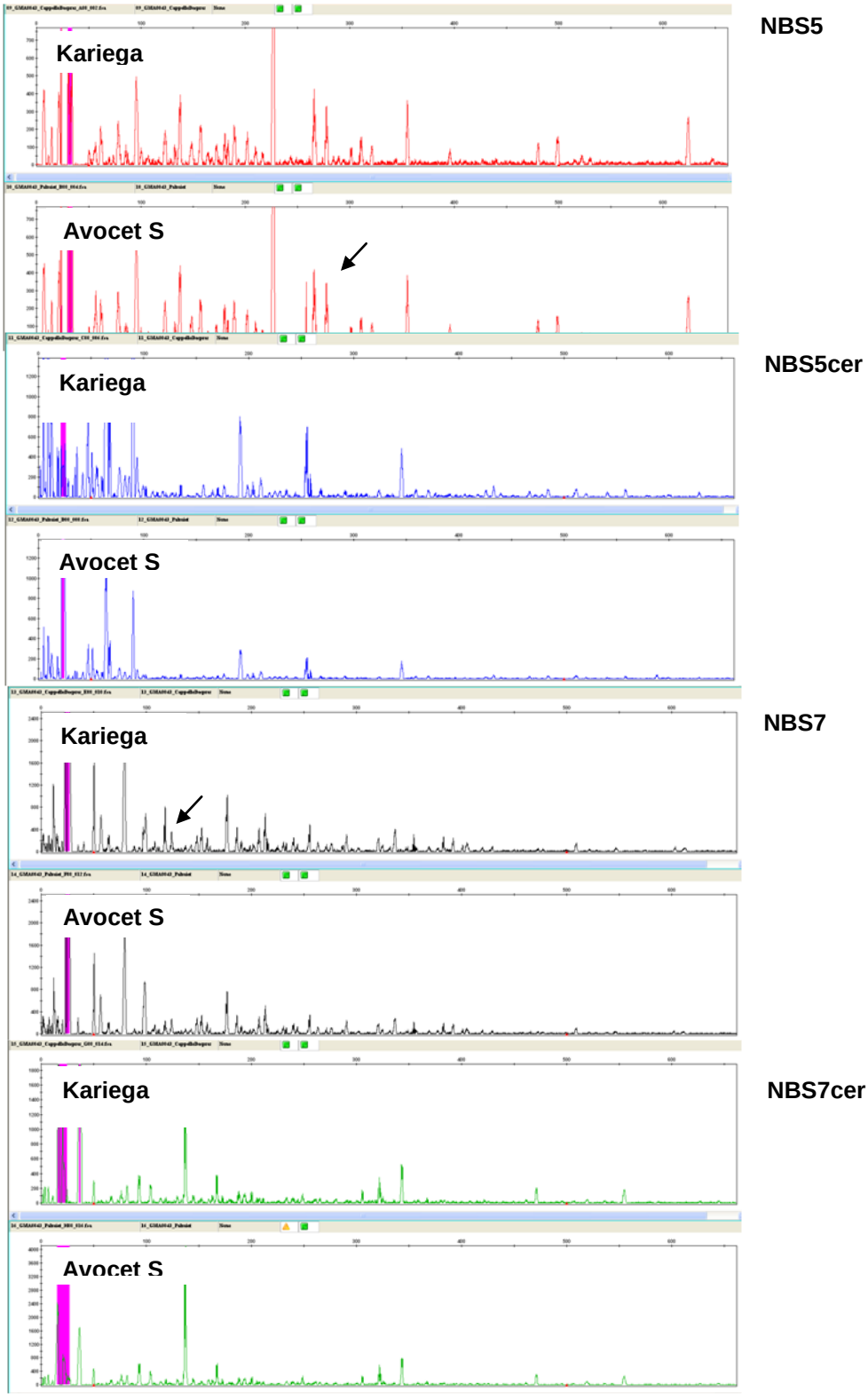

Figure 5.9 Capillary array electrophoresis of NBS-AFLP profiles generated with four selected NBS primers. Polymorphic bands are indicated with an arrow for NBS5 and NBS7. These were the only polymorphic peaks detected above a fluorescent threshold of 100 relative fluorescence units (RFU).

5.5.1.4 Linkage and QTL map

The additional SSR markers and new EST markers were added to the Kariega X Avocet S DH map. This resulted in a denser marker distribution in the QTL intervals, especially for *QYr.sgi-2B* (Fig. 5.10).

5.5.2 Marker conversion

5.5.2.1 DArT to STS markers

Eight DArT markers were selected from the *QYr.sgi-4A* interval. The sequences were obtained from Triticarte (Pty) Ltd (Yarralumla, Australia) and primers were designed to amplify the corresponding fragments (Table 5.7). Two markers, wPt-1961 and wPt-9675 had the same consensus sequence, only one primer set was therefore designed. The marker for wPt-4620 produced multiple bands which did not discriminate between the parental lines. The marker for wPt-5172 amplified a fragment of approximately 500 bp in both lines. These fragments were sequenced for the parental lines. Sequencing results showed co-amplification of homoeologous copies in the wheat genome. Allele specific primers can therefore only be designed for DArT marker wPt-5172 by designing primers that selectively amplify only the chromosome 4A copy. The markers for wPt-4424, wPt-5434, wPt-7919 and wPt-1155 were optimised to yield a single band from Avocet S and no product from Kariega (i.e. a null allele). The marker for wPt-1961 (and wPt-9675) produced fragments of different sizes from the two parental lines. Although DArT markers are dominant, this DArT-STS marker behaves in a co-dominant manner.

Table 5.7 STS primers designed for selected DArT marker sequences from the *QYr.sgi-4A* interval. The expected amplicon size is included, as determined based on the reference sequence, as well as the parent harbouring the dominant allele for the original DArT marker, as mapped by Triticarte (DArT P/L), is included in the table.

DArT marker	STS forward primer (5'->3')	STS reverse primer (5'->3')	Amplicon size	Dominant allele
wPt-4424	GCT TCT GAA TCT CTG CTA CCA C	TGC CGA CAG GAT AGA AAG ATG	352 bp	Avocet S
wPt-5434	GCC AAG TCA TTG TTC GTG	TGC ACT TCT CTC AGA TTT GTC	401 bp	Avocet S
wPt-4620	CTA CAC AGG CTG CTG CAA AG	ATG CCG TCA GTG ATA GCT TG	357 bp	Avocet S
wPt-7919	TTC TAG CGC CGA TTT TCT TC	TGC AGA CGG AGA AAG AGG AG	543 bp	Avocet S
wPt-5172	TTT CCA AGG GGA TAT GGT ATT C	GTG CAG GCA GTT GAA GAA GG	501 bp	Kariega
wPt-1155	CGA CAG GAT AGA AAG ATG TTG G	CAC CGA CAG CCA ATT ACA TC	316 bp	Avocet S
[a]wPt-1961	AAC AGC TAC TCC CTC CGT TTC	GCG CTG TGG TTA TCA GAT TG	278 bp	Kariega

[a]And wPt-9675

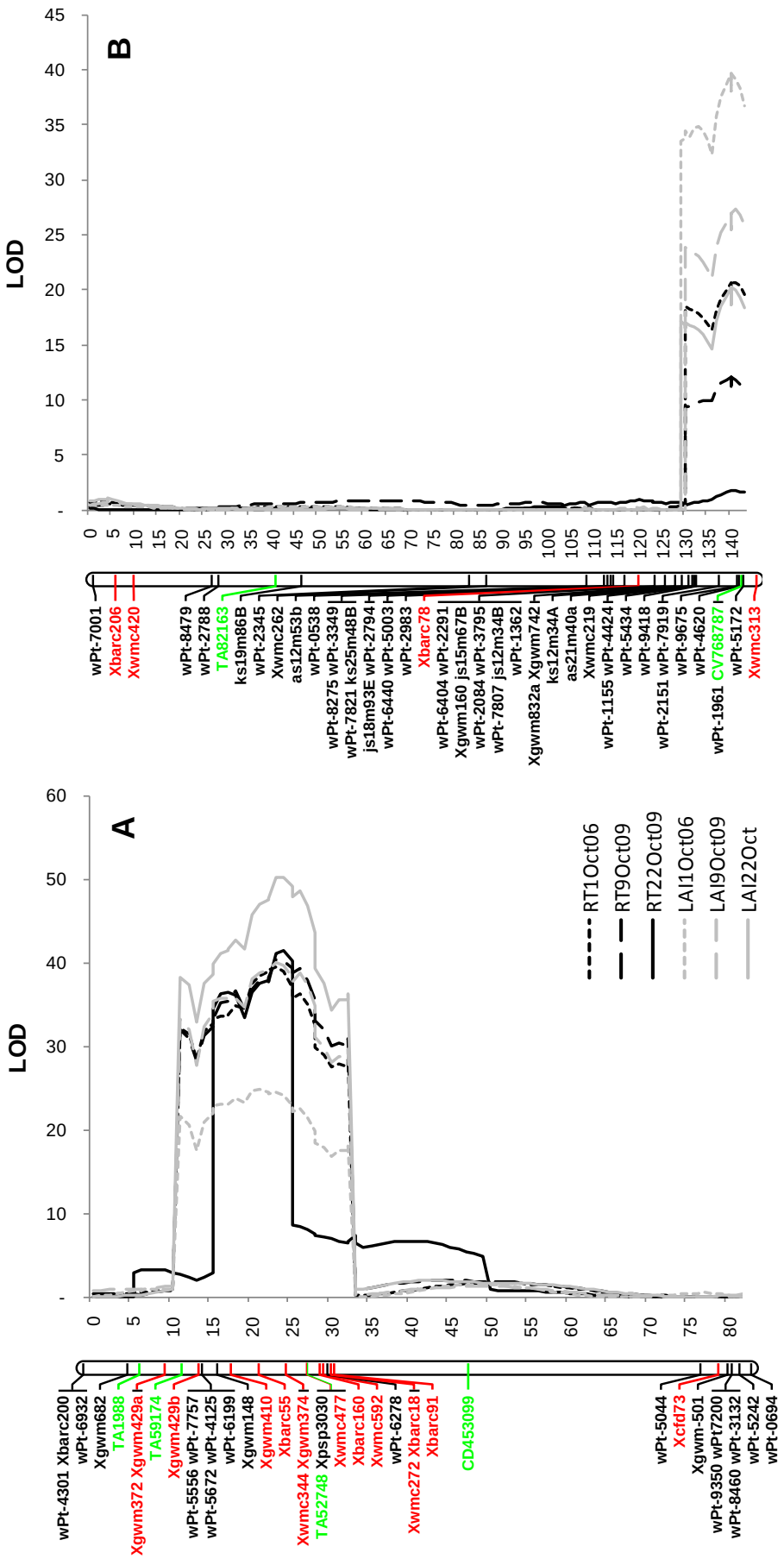

Figure 5.10 (A) Chromosome 2B (2B.1 linkage group) and (B) 4A linkage groups for the Kariega X Avocet S DH mapping population. The distances between markers on the X-axis are in centiMorgan. New SSR markers are in red text and EST markers in green. QTL intervals, as determined with CIM (Windows QTL Cartographer v.2.51) are shown. *QYr.sgi-2B* (A) and *QYr.sgi-4A* (B), as identified with the leaf area infected (LAI) and reaction type (RT) data sets (from Prins *et al.* 2011) are shown. A 1000 permutations determined LOD values between 2.8 and 3.0 to be significant.

5.5.2.2 EST-STS markers

The EST markers TA52748 (261 bp), TA1988 (257 bp) and CV768787 (559 bp) were amplified from Kariega and Avocet S, and the PCR products directly sequenced. The sequencing data showed the presence of homoeologous fragments of the same length. Additional primers were designed to these sequences to select for the different constituting fragments. This approach only allowed discrimination between some of the homoeologous fragments. Sequencing of TA1988 revealed the presence of three fragments from each parental line. TA52748 appeared to amplify three fragments from Kariega, but only one from Avocet S, whereas CV768787 amplified three fragments from Kariega and two fragments from Avocet S. Several polymorphisms were present in all of the fragments co-amplified by the EST markers. Primers were also designed to the original consensus sequence to amplify larger fragments, with the aim of evaluating the priming sites and potentially allow for the amplification of locus specific fragments. However, these primers amplified the same fragments as before. Therefore the original EST primer sets were used for amplification and the PCR products were cloned, after which five clones were sequenced for each EST marker. Sequencing data confirmed the previous conclusion about the number of alleles. Due to the presence of several polymorphisms across the various alleles, it could not be determined which of the copies represented the chromosome 2B and chromosome 4A specific alleles. The gel bands scored in the mapping of these markers were therefore extracted from polyacrylamide SSCP gels, cloned and sequenced. This led to a better understanding of the fragments most likely associated with the QTL (Table 5.8). An allele specific primer pair was designed for EST marker CV768787 (forward 5'-CGA GGT CAG AGA TCT TTC ATA CAT, reverse 5'-TCC GAT GGC ATG CCG T). This marker was optimised to amplify the chromosome 4A sequence present in Kariega (160 bp), but not in Avocet S (i.e. null allele). Stringent conditions, including a high annealing temperature (T_A = 68°C) and a reduced number of cycles, were essential for allele specific amplification.

5.5.2.3 Marker validation

The DArT-STS markers, wPt-4424, wPt-5434, wPt-7919 and wPt-1155 were typed in a subset of the DH mapping population (16, 16, 96 and 16 lines, respectively) and wPt-1961 in all individuals (254 lines) to confirm the identity of the amplicon and the map position (Fig.5.11). The genotypes obtained with the DArT-STS markers corresponded to the genotypes obtained for the original DArT markers and mapped to the same position. These markers can therefore be utilised for screening other mapping populations in future.

The EST-STS marker CV768787, along with SSR markers from the *QYr.sgi-4A* QTL interval (I*Xwmc219* and *Xwmc313*) were typed in selected South African genotypes (Smit *et al.* 2010) (Fig. 5.12). This marker proved to be diagnostic for the QTL in germplasm related and derived from the Kariega gene pool, based on the SSR marker data. Due to the partial resistance (slow-rusting) phenotype expressed by this QTL (Prins *et al.* 2011), it is not possible to unequivocally confirm its presence in the material phenotypically.

Table 5.8 Haplotypes determined from sequencing three EST markers. The haplotypes are represented vertically as SNPs for each of the marker sequences. Sequences identified to most likely be associated with the QTL are bordered with red, whereas similar sequences have the same shading.

	TA1988						TA52748				CV768787				
SNP#	Kariega			Avocet S			Kariega			AvS	Kariega			Avocet S	
	H1	H2	H3	H1	H2	H3	H1	H2	H3	H1	H1	H2	H3	H1	H2
1	**A**	A	A	**A**	A	G	G	**G**	**A**	G	G	A	**G**	G	A
2	**G**	G	G	**G**	A	G	C	**C**	**G**	C	A	A	**G**	A	A
3	**C**	C	T	**C**	C	C	G	**G**	**A**	G	A	T	**A**	A	T
4	**G**	C	G	**G**	G	G	G	**C**	**C**	G	T	T	**C**	T	T
5	**A**	C	C	**A**	C	C	A	**G**	**G**	A	A	A	**G**	A	A
6	**G**	G	G	**G**	G	A	T	**T**	**C**	T	CF	T	**C**	C	T
7	**C**	A	C	**C**	C	C	A	**G**	**C**	A	G	G	**A**	G	G
8	**C**	C	G	**C**	G	G					G	T	**G**	G	T
9	**G**	A	A	**G**	G	A					CF	A	**C**	C	A
10	**C**	C	T	**C**	C	C					G	G	**A**	G	G
11	**C**	C	T	**C**	C	C					A	A	**G**	A	A
12	**A**	A	C	**A**	C	A					CF	C	**T**	C	C
13	**G**	G	G	**G**	A	G					G	G	**T**	G	G
14	**C**	C	C	**C**	C	G					G	G	**A**	G	G
15	**G**	**G**	**G**	**G**	**G**	**G**					A	C	**T**	A	C
16	**G**	G	A	**G**	A	G					A	A	**G**	A	A
17	**C**	C	G	**C**	G	C					G	G	**T**	G	G
18	**G**	**C**	**G**	**T**	**G**	**G**					T	A	**A**	T	A
19											G	G	**T**	G	G
20											T	C	**C**	T	C
21											G	A	**A**	G	A

H – Homologues or homoeologues

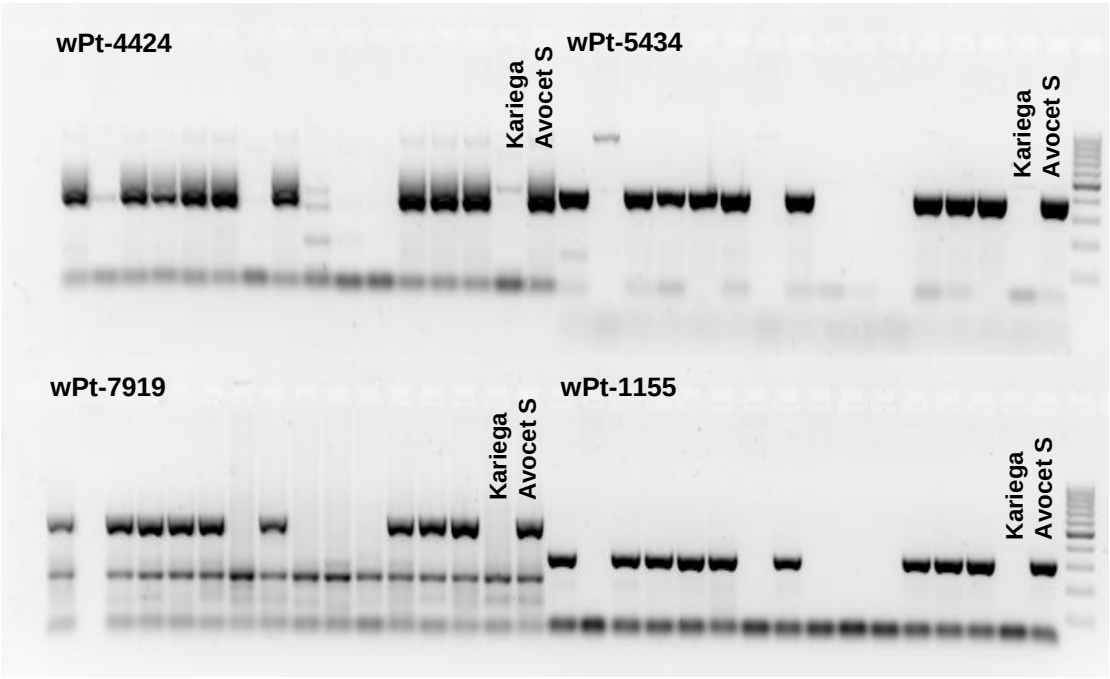

Figure 5.11 Amplicons obtained for four of the DArT-STS markers in a subset of the Kariega X Avocet S DH mapping population. Products are resolved on 2% (w/v) agarose (0.5X TBE). A 100 bp molecular size standard is included in the last lane of each gel (GeneRuler^{TM}, Fermentas).

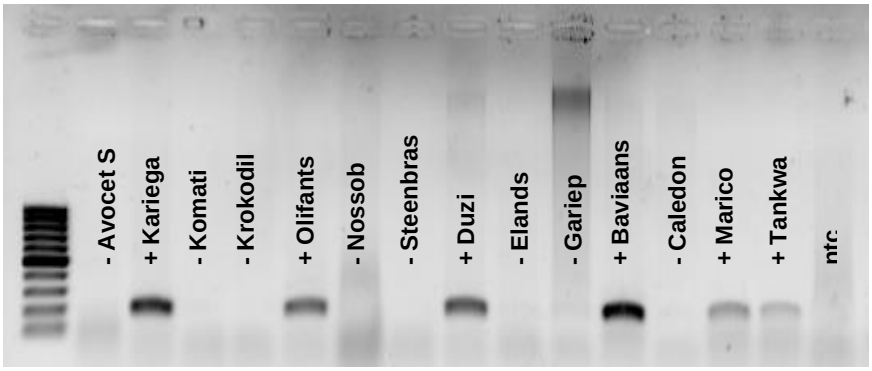

Figure 5.12 EST-STS marker CV768787 amplified in South African cultivars and resolved on 1% (w/v) agarose (0.5X TBE). Lane 1 shows a 100 bp molecular marker (GeneRuler^{TM}, Fermentas). Kariega is the positive control and Avocet S the negative control for the allele (160 bp) associated with the QTL. The cultivars represented were released by the ARC-SGI. The non-template control is loaded in the last lane (ntc).

Cultivars from the Kariega pedigree (SST44//K4500.2/SapsuckerS) were selected for marker analysis. SST44 (pedigree: CI13523(Agent)/3*T4(Anza) (reported by another source, Payne *et al.* 2001 as SST16*3//T4*5/S-67336/3/SST16*4/Eagle) and T4 (pedigree: Lerma-Rojo64/Norin10-Brevor//3*Andes-Enano) harbour the same alleles as Kariega for SSR markers in the *QYr.sgi-4A* QTL

interval, *Xwmc219* and *Xwmc313*, as well as for the DArT-STS markers, *wPt-4424* and *wPt-1155*. Inia66 tested positive for the QTL with the EST-STS marker, CV768787. The *QYr.sgi-4A* resistance QTL could therefore have originated from Inia66 through SST44, originating from SST16 (pedigree: Inia66/Calidad). Alternatively, the QTL could have been transferred to Kariega through SST44, originating from T4.

5.5.3 *Lr34/Yr18/Pm38* real-time markers

Kariega is known to carry the *Lr34/Yr18/Pm38* gene and this was confirmed with the diagnostic marker published by Lagudah *et al.* (2009), *cssfr6*. Additional primers reported by Lagudah *et al.* (2009) were used to amplify and sequence gDNA from both Kariega and Avocet S. The two sequence variations associated with the resistant/susceptible *Lr34/Yr18/Pm38* alleles, the three base pair indel in exon 11 (Fig. 5.13) and the SNP in exon 12, were confirmed in both cultivars.

Primers and FRET hybridisation probes were designed based on sequence data for Kariega and Avocet S and the indel in exon 11 (Fig. 5.14). Derivative profiles were plotted and the T_M for the Kariega allele was determined to be 54.1°C and 57.9°C for Avocet S (Fig. 5.15). HRM analysis with SYBR green/EvaGreen™ has been investigated, but due to the small T_M difference (< 0.1°C according to a theoretic calculation) between the Kariega and Avocet S amplicons, detection would be almost impossible without extensive optimisation.

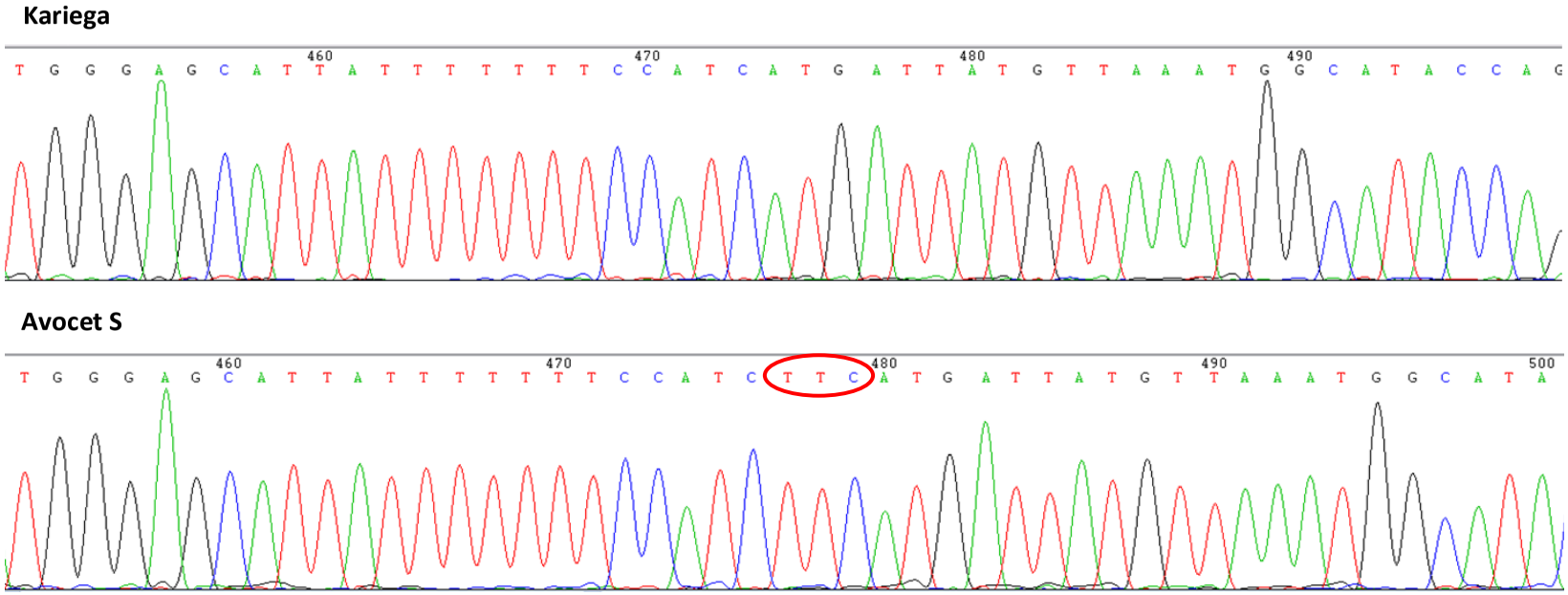

Figure 5.13 Sequencing electropherograms showing the resistant and susceptible alleles for the TTC indel in *Lr34/Lr18/Pm38,* detected in Kariega and Avocet S, respectively.

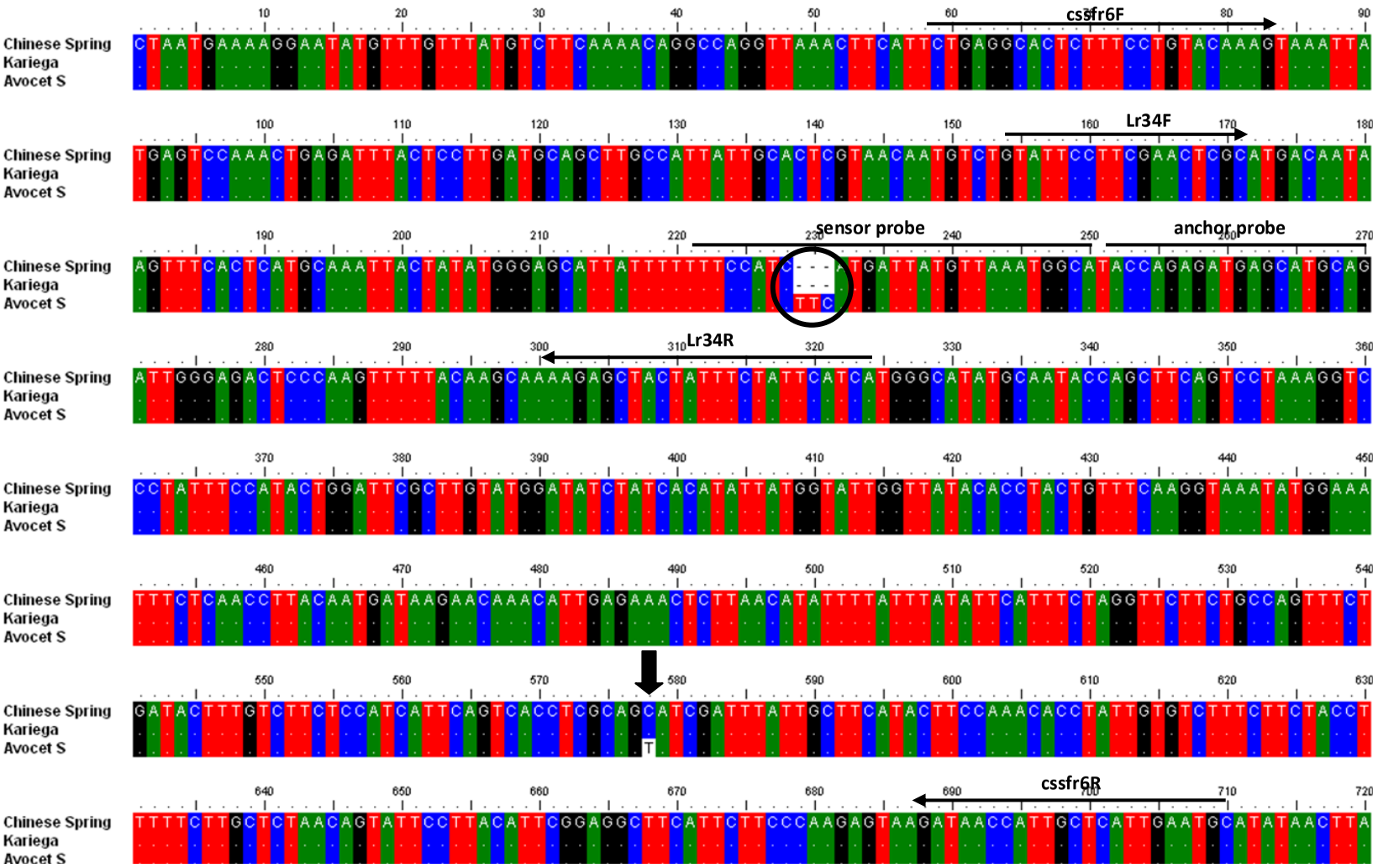

Figure 5.14 Primer and probe positions on sequence alignments done with BioEdit Sequence Alignment Editor for exon 11 and exon 12 of the *Lr34/Yr18/Pm38* gene. The reference sequence for Chinese Spring (GenBank: FJ436983) (*Lr34/Yr18/Pm38* positive) is included with the sequence for Kariega and Avocet S. The arrow indicates the position of the exon 12 SNP (C>T) and the exon 11 indel is circled.

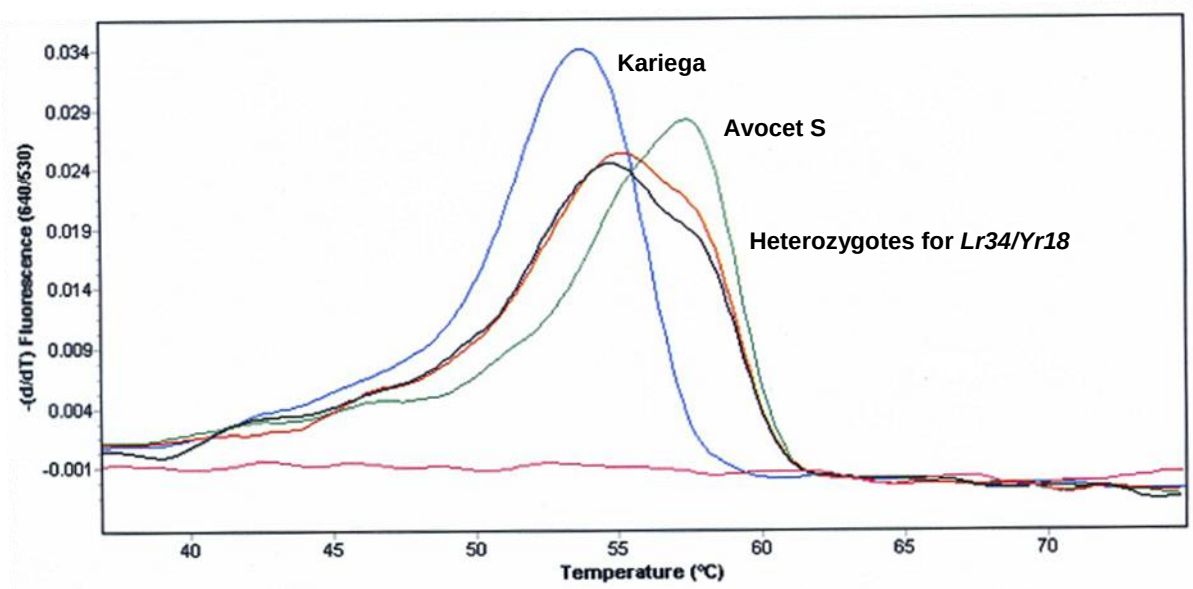

Figure 5.15 Melting profile for the *L34/Yr18/Pm38* FRET hybridisation probe assay on the LightCycler® v.2.0 (Roche Applied Sciences) for Kariega, Avocet S and a heterozygous sample. The non-template control is represented by the black line. The melting curve is transformed to the first derivative of the fluorescence over temperature (-dF/dT).

5.5.4 F_2 mapping population: Fine mapping

A large mapping population of 1020 Kariega X Avocet S F_2 plants were created to enable fine mapping of the *QYr.sgi-2B* and *QYr.sgi-4A* stripe rust resistance QTL from Kariega.

5.5.4.1 Disease evaluation in field trial

Adequate levels of stripe rust infection were achieved in the 2009 field trial, with Avocet S scoring 60S at the early scoring date (14Sept09) and 80S at the late scoring date (23Sept09). Kariega showed a complete resistant reaction throughout the growing season (0R). Segregation of resistance in the F_2 lines was uniform across the trial. In general, the LAI data had a normal distribution, however, an excess of resistant individuals was seen early in the season (Fig. 5.16A). This phenomenon was more pronounced for the RT traits (Fig. 5.16B), and transformation of the data did not negate this effect.

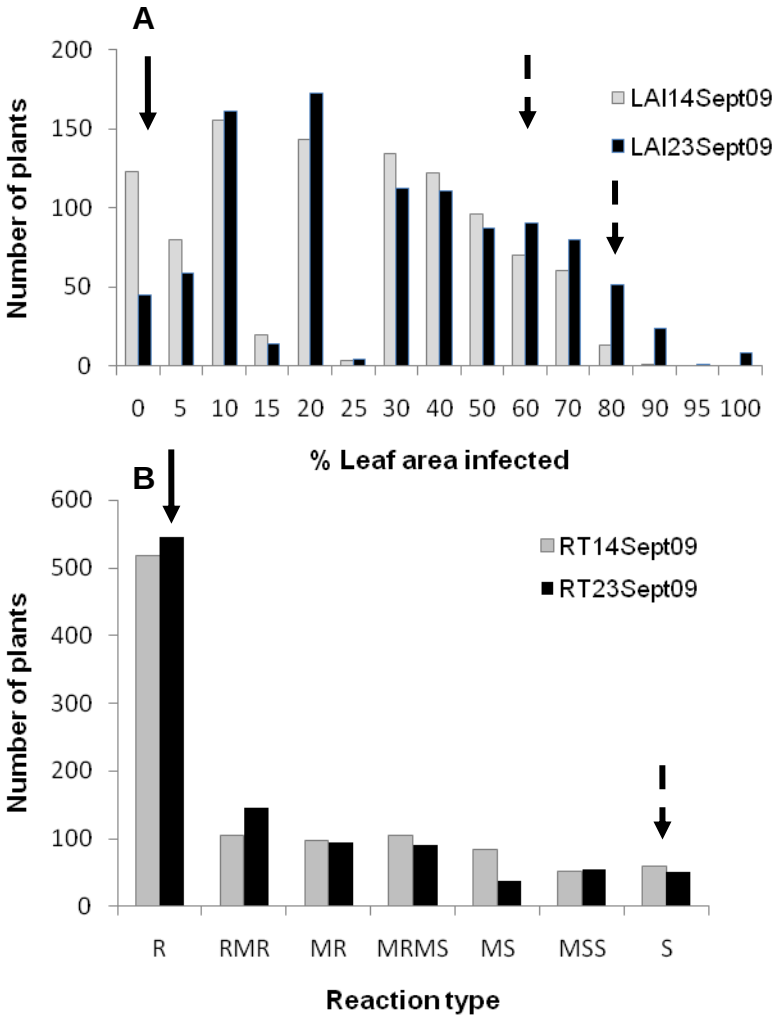

Figure 5.16 Phenotypic distribution of F_2 lines from a Kariega X Avocet S cross for (A) leaf area infected and (B) reaction type. The solid arrow shows the score for Kariega (0R at both scoring dates) and the broken arrow the score for Avocet S (60S and 80S, at the early and late scoring dates, respectively).

5.5.4.2 Marker analysis

Based on the Kariega X Avocet S DH linkage map, ten SSR markers were selected for chromosome 2B (*Xbarc160*, *Xbarc200*, *Xbarc55*, *Xbarc91*, *Xgwm148*, *Xgwm429*, *Xgwm501*, *Xwmc344*, *Xwmc592* and *Xwmc272*) and three SSR markers for chromosome 4A (*Xbarc78*, *Xwmc313* and *Xwmc219*). In addition, three DArT-STS markers (*wPt-4424*, *wPt-1155* and *wPt-5434*) were selected for chromosome 4A to map in the F_2 population. These markers, together with three markers linked to the *Lr34/Yr18/Pm38* gene on chromosome 7D (*Xgwm295*, *csLV34* and *cssfr6*) were typed in the 1020 F_2 plants. They spanned 76.0 cM, 139.2 cM and less than 2 cM on the chromosome 2B, 4A and 7D DH linkage maps, respectively (refer to Fig. 5.10 for the chromosome 2B and 4A linkage groups).

Multiplexing of the four DArT-STS markers using standard Taq was tested. No more than two markers could be combined successfully and multiplexing was not investigated further. Smaller fragments amplified preferentially, despite the fact that the annealing temperatures for all primer sets were the same (Fig. 5.17). Alternatively, up to 10 markers were pooled after amplification and analysed together on the 3730*xl* Genetic Analyzer.

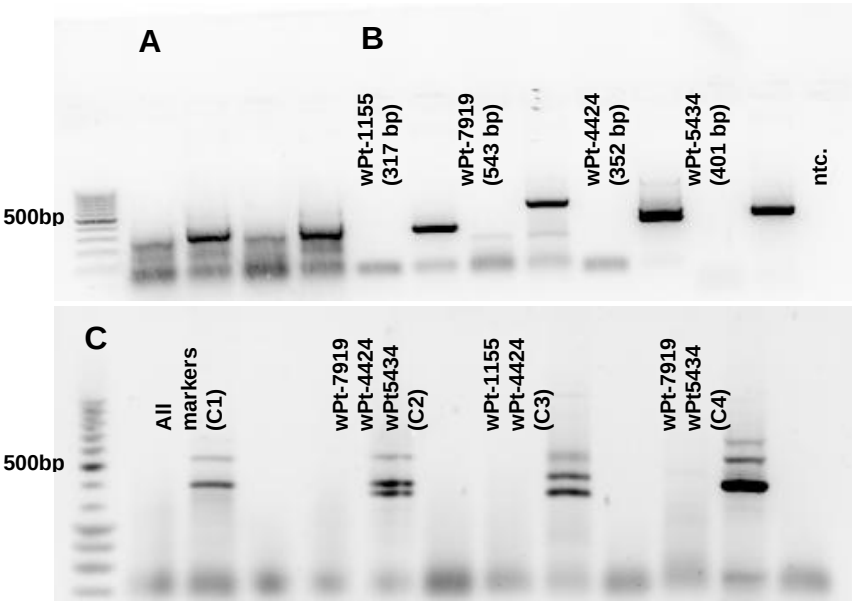

Figure 5.17 Multiplexing results for the DArT-STS markers on 1.5% (w/v) agarose (0.5X TBE). A) All markers combined in one multiplex, B) single reactions for each primer set and C) different combinations of these primer sets, C1 – four markers, C2 – three markers, C3 and C4 – two markers. Fragment sizes are included in brackets. A 100 bp molecular marker (GeneRuler^{TM}, Fermentas) is loaded in the first lane of each gel, (the 500 bp fragment is indicated), followed by alternating Kariega (null allele) and Avocet S amplicons and a non-template control (ntc.).

5.5.4.3 Recombinant mapping

The maps for the Kariega X Avocet S DH, complete F_2 and recombinant only F_2 populations for chromosomes 2B and 4A were compared, as calculated with RECORD (Van Os *et al.* 2005a) (DH) and JoinMap v.4.1 (Van Ooijen 2006; Van Ooijen 2011) (F_2). As expected, the F_2 and F_2 recombinant population produced longer maps compared to the maps produced for the DH mapping population as a result of the higher number of recombination events. A similar marker order was obtained for the populations.

CIM for the F_2 mapping population determined the QTL interval for chromosome 2B to be *Xgwm148-Xbarc160* (Fig. 5.18A; Table 5.9). SSR marker loci *Xgwm429* and *Xwmc592* flanked the QTL and chromosome 2B recombinants (436 genotypes) were selected between these markers. CIM of the recombinants determined *QYr.sgi-2B* to be located in a reduced interval between loci *Xbarc5*5 and

Xwmc344 (Fig. 5.19A; Table 5.9). The interval spanned 6.1 cM on the map produced for the complete F_2 mapping population (1020 individuals) (3.2 cM on the DH map), explaining up to 8.2% of the LAI variance and 22.5% of the RT variance. The chromosome 4A QTL interval was flanked proximally by *Xbarc78* and extended to the distal end of the linkage group (Fig. 5.18B; Table 5.9). Recombinants (459 genotypes) were selected across all markers typed. *QYr.sgi-4A* mapped on the distal end of chromosome 4AL, in the interval *Xbarc78-Xwmc219* (Fig. 5.19B; Table 5.9), reducing the interval from 24.8 cM on the DH map to 16.2 cM on the map produced for the complete F_2 mapping population. The QTL explained 13.0% of the LAI and 9.7% of the RT variance. *Lr34/Yr18/Pm38* explained up to 32.5% of the LAI variance and 21.1% of the RT variance, as calculated for the complete F_2 data set.

MQM analysis with MapQTL v.6 (Van Ooijen 2009) confirmed the QTL intervals obtained with CIM (Fig. 5.20). Marker loci *Xbarc55* and *Xwmc344* for chromosome 2B, *wPt-4424* and *Xwmc313* for chromosome 4A and *cssfr6* were selected for chromosome 7D as cofactors. The maximum phenotypic variance explained by the LAI traits was 7.0% (LOD 15.7) and 10.3% (LOD 25.8) for *QYr.sgi-2B* and *QYr.sgi-4A*, respectively. The RT traits explained 17.3% (LOD 24.8) and 3.6% (LOD 7.2) for *QYrs.gi-2B* and *QYr.sgi-4A*, respectively. The phenotypic variance explained was consistent with the CIM results for the F_2 recombinant population.

Table 5.9 QTL summary for stripe rust resistance in the Kariega X Avocet S F_2 mapping population for both the complete and reduced recombinant data sets obtained with CIM analysis.

QTL	Interval		LAI14Sept09	LAI23Sept09	RT14Sept09	RT23Sept09
F_2 complete population						
QYr.sgi-2B	*Xgwm148-Xgwm160*	LOD[a]	18.9	43.5	55.7	60.1
		%VAR[b]	4.5	10.0	19.0	19.5
QYr.sgi-4A	*Xbarc78-wPt-4424*	LOD	96.9	85.2	29.8	29.5
		%VAR	26.5	20.6	9.0	8.3
Lr34/Yr18/Pm38	*Xgwm295-cssfr6*	LOD	96.6	140.7	44.5	72.5
		%VAR	23.4	32.5	13.5	21.1
F_2 recombinant population						
QYr.sgi-2B	*Xbarc55-Xwmc344*	LOD	7.2	13.1	25.7	24.8
		%VAR	4.5	8.2	22.5	20.8
QYr.sgi-4A	*Xbarc78– Xwmc219*	LOD	26.4	20.2	5.7	16.9
		%VAR	12.9	8.4	3.4	9.7

[a]Calculated from the likelihood ratio (LR)

[b]Percentage phenotypic variation explained (R^2)

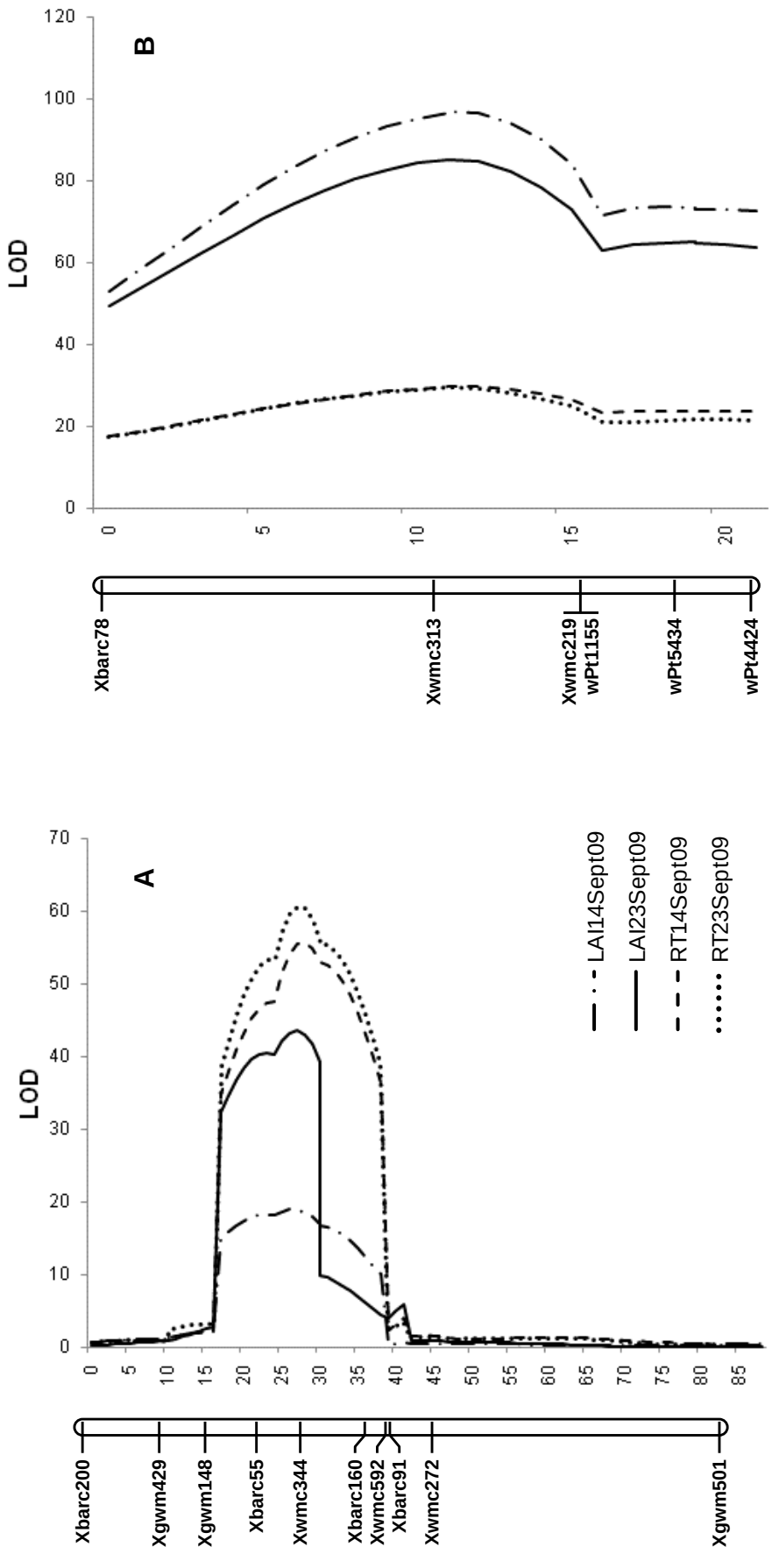

Figure 5.18 QTL intervals for the Kariega X Avocet S complete F_2 mapping population (1020 lines), as determined with CIM (Windows QTL Cartographer v.2.51). *QYr.sgi-2B* (A) and *QYr.sgi-4A* (B), as identified with the leaf area infected and reaction type data sets are shown for both the early and late scoring date. The distances between markers on the X-axis are in centiMorgan. A 1000 permutations determined LOD values above 2.5 to be significant.

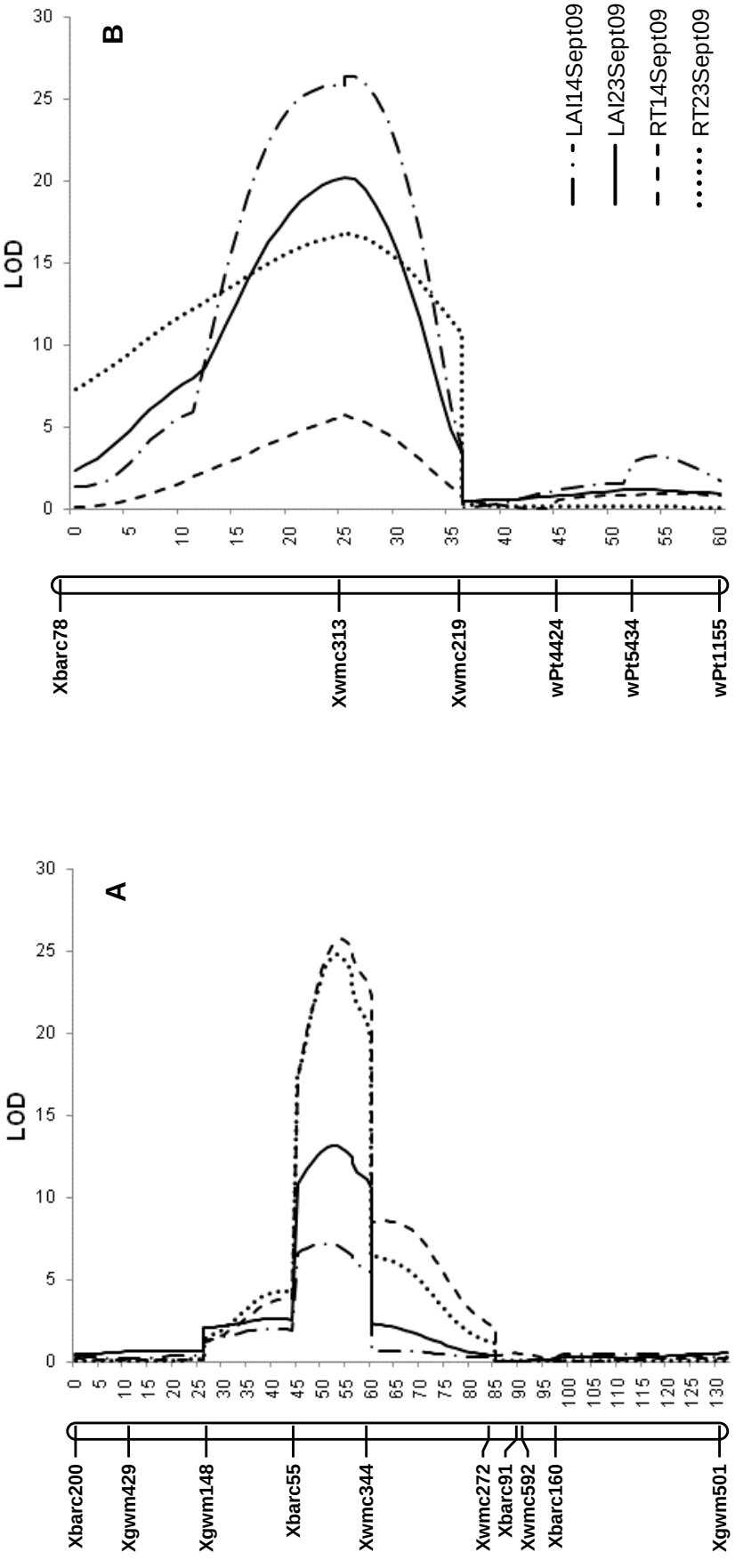

Figure 5.19 QTL intervals determined with CIM (Windows QTL Cartographer v.2.51) for the chromosome-specific recombinants selected from the Kariega X Avocet S F_2 mapping population. *QYr.sgi-2B* (A) and *QYr.sgi-4A* (B), as identified with the leaf area infected and reaction type data sets are shown for both the early and late scoring date. The distances between markers on the X-axis are in centiMorgan. A 1000 permutations determined LOD values above 2.5 to be significant.

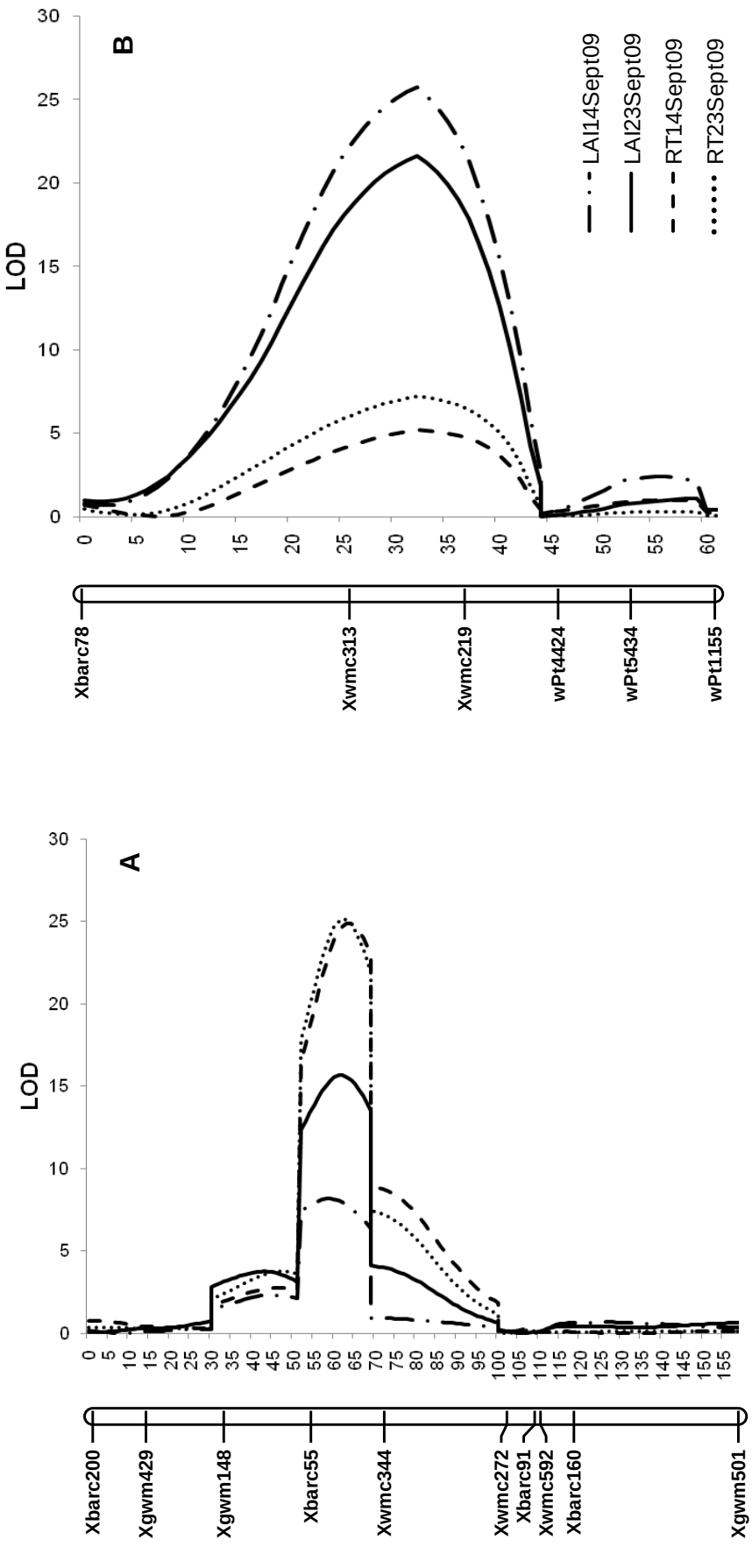

Figure 5.20 QTL intervals determined with MQM (MapQTL v.6) for the chromosome-specific recombinants selected from the Kariega X Avocet S F_2 mapping population. *QYr.sgi-2B* (A) and *QYr.sgi-4A* (B), as identified with the leaf area infected and reaction type data sets are shown for both the early and late scoring date. The distances between markers on the X-axis are in centiMorgan. LOD values above 3.0 were determined to be significant based on 10 000 permutations.

5.6 Discussion

In addition to *Lr34/Yr18/Pm38* (chromosome 7D), two major APR QTL, *QYr.sgi-2B* (*QYr.sgi-2B.1*) and *QYr.sgi-4A* (*QYr.sgi-4A.1*) were reported for the South African stripe rust resistant cv. Kariega (Ramburan *et al.* 2004; Prins *et al.* 2011). The QTL intervals spanned up to 23 cM and 15 cM, respectively. The objective of this study was to improve the characterisation of these QTL. This was accomplished by 1) increasing the marker density of the Kariega X Avocet S DH mapping population and by 2) fine mapping the QTL in a larger, Kariega X Avocet S F_2 population through recombinant mapping. Different marker technologies were investigated for improving the marker density on the chromosome 2BS and 4AL arms, including EST and NBS-AFLP markers. EST and DArT markers were converted to STS markers, which are more practical for high-throughput screening applications. Finally, real-time PCR was investigated as a screening method for both uncharacterised sequences with HRM and allele discrimination of known sequence variations with probes using the *Lr34/Yr18/Pm38* gene present in Kariega, as an example.

5.6.1 Increasing the marker density

5.6.1.1 SSR markers

A limited number of SSR markers, which is the marker type currently favoured in MAS, were mapped in the Kariega X Avocet S DH mapping population in the *QYr.sgi-2B* and *QYr.sgi-4A* intervals. This was a concern, particularly for chromosome 4A, as this QTL interval previously contained only one SSR marker (*Xwmc219*). SSR markers in published maps of these regions were extensively tested. Four additional markers mapped to chromosome 4AL, but only one SSR marker, *Xwmc313*, mapped in the QTL interval. Thirteen SSR markers were mapped to chromosome 2BS, nine of which represented loci in the QTL interval.

5.6.1.2 EST markers

To date, apart from ESTs, only SSR markers have been mapped to the physical wheat map (deletion bins) (Sourdille *et al.* 2004). It is crucial to link markers on genetic maps to markers on the physical map if relevant QTL deletion bins are to be determined. When this study commenced, few SSR markers were mapped to the QTL chromosomal regions and direct comparisons were difficult. Markers had to be developed for ESTs physically mapped to deletion bins across the chromosome 2BS and 4AL arms before it could be determined that *QYr.sgi-2BS* is located in the bin 2BS1-0.53-0.75 (from the position of mapped EST marker TA52748) and *QYr.sgi-4A* in the bin 4AL4-0.80-1.00 (from EST marker CV768787). The deletion bins to which the QTL physically map are therefore now known. This knowledge will enable us to target these deletion bins, which means that EST marker development can be fast-tracked in future. Comparative mapping in related species (e.g. rice and *Brachypodium distachyon*) will facilitate the identification of additional EST markers in the QTL intervals.

5.6.1.3 Comparison of screening technologies

SSCP analysis was utilised for screening EST markers. The sensitivity for detecting sequence variations with this technique is limited. Modifications to the protocol may increase the sensitivity, but this will have implications for the cost and time involved. EST markers were initially screened using polyacrylamide gel electrophoresis, but capillary array electrophoresis was investigated as a high-throughput alternative. Capillary electrophoresis has been reported to be more sensitive for detecting sequence variations, especially in larger fragments (Ren 2000; Anderson *et al.* 2003; Doi *et al.* 2004). However, it was found not to be as robust as gel electrophoresis and the analysis was time consuming. The running cost for the capillary system was much higher, but the system was more sensitive, as nine out of 56 (6.2%) EST markers tested on acrylamide gels were polymorphic compared to three out of 29 (10.3%) markers tested using the capillary system. Direct comparisons were made between the two systems for three EST markers, TA52748, TA1988 and CV768787. Only two of these, TA52748 and CV768787, could be screened with capillary array electrophoresis. These markers were also tested with a third system, real-time PCR with high resolution melt (HRM) analysis. Due to the unpredictable co-amplification of homoeologous sequences, this technique was found not to be suitable for analysing EST markers.

5.6.1.4 NBS-AFLP markers

The NBS profiling marker system could not be implemented successfully and needs further optimisation. It is important to have knowledge of the resistance mechanism of the QTL in question, as NBS-AFLP markers will only be appropriate for mapping genes with NBS domains.

5.6.2 Development of STS markers

5.6.2.1 EST-STS markers

Direct sequencing of PCR products is difficult for the wheat genome. The presence of homoeologous sequences often results in the co-amplification of fragments. This necessitates cloning of the PCR products before sequencing, adding both time and cost. Unfortunately, sequencing is an unavoidable step in the designing of STS markers for ESTs. One EST-STS marker was developed from CV768787 for *QYr.sgi-4A.* This marker was shown to reliably detect the QTL in South African germplasm. The ability of this marker to detect the QTL in diverse genetic backgrounds remains to be established. Limited sequence variation was available for designing allele specific primers, but the marker could be improved upon, for more robust allele discrimination, by modifying the primer sequence with the addition of extra (non-template) mismatches, but the effect will have to be determined experimentally (Zhang *et al.* 2003). Alternatively, locked nucleic acid (LNA) primers may increase the specificity of the primers (Levin *et al.* 2006).

5.6.2.2 DArT-STS markers

Several DArT markers from the *QYr.sgi-4A* interval were successfully converted to STS markers. DArT markers for which the dominant allele is associated with the resistant cultivar (Kariega in this study), should be prioritised, however, it was shown that it is possible to obtain a co-dominant DArT-STS marker from a dominant DArT marker (wPt-1961/wPt-9675). However, this will have to be determined empirically since the outcome cannot be predicted without sequence data for both parental genotypes. DArT converted STS markers have proved to be a valuable marker resource for QTL mapping, however, DArT-STS markers were only tested for DArT markers which were already mapped in the population. The practical value of this approach for increasing the markers density in selected regions, with the selection of DArT markers from consensus maps, remains to be determined.

5.6.3 Real-time markers

The cost involved with screening SSR and STS markers with capillary array electrophoresis is relatively high, as fluorescently labelled primers and electrophoresis on specialised equipment are needed in addition to the normal PCR consumable and reagent cost. Multiplex PCR would decrease the cost, but this was found to be complex for wheat markers. Further optimisation with new generation *Taq* polymerases designed for this application may, however, yield better results. As an alternative, real-time PCR was investigated for the purpose of discriminating between characterised sequence variations (SNPs and small indels). This proved to be a fast and cost-effective way to screen Kariega and Avocet S for the *Lr34/Yr18/Pm38* gene. By using sequence specific probes, the probability of false positives is reduced as the presence of co-amplified products and other PCR artefacts will not cause a change in fluorescence. As for all genetic markers used for selecting genes of interest, care should be taken when evaluating diverse germplasm as uncharacterised sequence polymorphisms or mutations could influence gene expression, without any changes to the marker genotype. Real-time PCR can be adapted for high-throughput screening. However, the success of converting other gene-specific or gene-associated markers to real-time markers, especially SSR markers, will have to be determined.

5.6.4 Fine mapping of the Kariega QTL

Successful fine mapping of QTL that resulted in the isolation of the underlying genes have been reported in several plant species, including *CRY2* in *Arabidopsis* (El-Din El-Assal *et al.* 2001), *Gpc-B1* in wheat (Uauy *et al.* 2006) and *Gw-2* in rice (Song *et al.* 2007). These studies developed a secondary mapping population derived from NILs, and mapped the loci in recombinants, a form of selective genotyping (Chi *et al.* 2010). Selective genotyping is done to reduce the genotyping burden by excluding non-informative loci (Tanksley *et al.* 1995; Ronin *et al.* 2003; Jander 2006). The mapping population should yield an adequate number of recombinants for the QTL interval to allow for a descent sample size needed for accurate QTL mapping. Most fine mapping studies focused on RIL or

NIL populations to isolate the QTL as a Mendelian factor (Cuthbert *et al.* 2007; Alfares *et al.* 2009; Xue *et al.* 2010) or variations of these, e.g. an within-family segregation strategy (Liu and Bai 2010) or advanced BC populations (Röder *et al.* 2008). We decided to use a large F_2 mapping population, which provides an increased number of meiotic events for identifying recombinants, without the time-consuming process of developing advanced generation RILs or NILs, which also relies on accurate genotyping and phenotyping with each back-cross selection. Several thousand meiotic events are needed to narrow a QTL down to an interval small enough for positional cloning (Chi *et al.* 2010). To have a 95% chance of finding at least one crossover in a 0.1 cM interval would require about 3000 meiotic events (Durrett *et al.* 2002). Reddy *et al.* (2008) managed to delimit a gene involved with *Stagonospora nodorum* infection, *Snn1*, to a 0.46 cM interval in a F_2 mapping population by selecting resistant individuals based on phenotype (recessive resistance) from 16 000 lines. Resistance was inherited as a recessive trait in this case. However, for dominant genes, F_3 families are normally phenotyped to determine the composition of F_2 heterozygotes (Blair *et al.* 2003; Bulgarelli *et al.* 2004; Wang *et al.* 2007).

Generally, reverse selection is done to exclude additional QTL not under investigation (Xue *et al.* 2010), as the expression of an individual QTL may be confounded by the genetic background, i.e. other QTL. This is mainly done to simplify the phenotypic assessment and allows for the identification of different phenotypic classes. The borders of an interval flanking the QTL are then identified by comparing the homozygous recombinants that carry contrasting alleles of the two parents and phenotypic data of these recombinants. This approach has been used successfully in mapping QTL in wheat (Cuthbert *et al.* 2007; Xue *et al.* 2010; Liu *and Bai* 2010). Due to the presence of three resistance genes/QTL in Kariega, selecting against the two QTL not targeted, would have resulted in very small sample sizes (27 lines for evaluating *QYr.sgi-2B* and 39 lines for evaluating *QYr.sgi-4A*). This will have serious implications for the map quality considering genotyping and phenotypic errors, as well as environmental effects and the presence of minor, uncharacterized QTL. It was therefore decided to use two recombinant data sets, one for fine mapping *QYr.sgi-2B* and one for *QYr.sgi-4A.* Recombinants were therefore selected for each data set and the QTL under investigation, without any consideration of the genetic background.

The marker order for chromosomes 2B (*Xbarc200-Xgwm501*) and 4A (*Xbarc78-Xwmc313*) produced for the DH mapping population, as determined by RECORD (Van Os *et al.* 2005a) and map distances calculated with MapManager QTXb20 (Manly *et al.* 2001), and the F_2 mapping population, as calculated with JoinMap v.4.1 (Van Ooijen 2006; Van Ooijen 2011), was compared for the specific marker intervals.

The linkage map for chromosome 2B spanned 76.0 cM in the DH population (deduced from the QTL interval reported in Prins *et al.* 2011 and Fig. 5.10) and 88.9 cM in the F_2 population. Both populations underwent one meiosis, therefore the 12.9 cM difference can be ascribed to the larger F_2 population (1020 vs.254 lines) which would yield more recombination events, expanding the map. The marker

order was exactly the same, except for loci *Xwmc272* and *Xbarc91* for which the order was reversed. These two markers are separated by only 0.4 cM on the DH map, and missing data or genotyping errors could easily explain the discrepancy. The F_2 recombinant map (marker interval *Xbarc200-Xgwm501*) had a 133.1 cM map distance, expanding the Kariega X Avocet S map. The recombinant F_2 sub-population therefore allowed for the effective selection of a highly recombinant population. By increasing the distances between the markers within the QTL interval, the QTL can be better positioned on the map, ultimately reducing the marker interval. In the F_2 population, *QYr.sgi-2B* spanned five markers (*Xgwm148, Xbarc55, Xbarc160, Xwmc592* and *Xbarc91*). The QTL have now been demarcated between only two markers, *Xbarc55* and *Xwmc344*, with the aid of the F_2 recombinant population. The marker order between the complete F_2 population and the F_2 recombinant population changed, but since the QTL has been located between two markers, the same results would have been obtained for a different map order.

The *QYr.sgi-4A* interval spans 24.8 cM on the DH map (deduced from the QTL interval reported in Prins *et al.* 2011 and Fig. 5.10) and 22.1 cM on the F_2 map. Considering the relatively small interval it was expected that the map distances would be this similar. This distance was almost three times larger for the F_2 recombinant population, emphasizing the effect of selecting for recombinant individuals. The marker order differed between the maps, but this is also not unexpected since the markers are fairly close together, with the DArT markers forming a cluster on the DH and F_2 maps. By increasing the marker density in this region in the recombinant population, the QTL could be demarcated to a smaller interval, involving only three markers, *Xbarc78, Xwmc313* and *Xwmc219.*

Both CIM (Windows QTL Cartographer v.2.51) and MQM (MapQTL v.6) resulted in the identification of the same QTL interval for *QYr.sgi-2B* and a similar interval for *QYr.sgi-4A* (Figs. 5.19 and 5.20). The phenotypic variances explained by *QYr.sgi-2B* and *QYr.sgi-4A* QTL were less in the F_2 mapping population compared to the values calculated for the DH mapping population (Prins *et al.* 2011). The slow-rusting phenotype of *QYr.sgi-4A* could be confirmed, with the LAI traits having a larger contribution than the RT traits to the resistance response. The opposite was confirmed for *QYr.sgi-2B,* which has a larger effect on the RT traits, and is consistent with the hypersensitive reaction associated with this QTL. *QYr.sgi-4A* spanned a larger marker interval using MQM mapping (*Xbarc78-wPt-4424*). LOD values of more than 25 were obtained with both algorithms, for both QTL. Both CIM and MQM can therefore be recommended for the identification of major effect QTL. The strength of MQM is dependent on the accurate selection of cofactors. The effect of selecting alternative markers as cofactors was tested, but the backwards elimination process suggested by Van Ooijen (2006) proved to be adequate. The efficient identification of minor QTL and the degree of reporting false positives have not been investigated. The focus of this study was the Kariega major QTL, a whole genome linkage map was not constructed for the Kariega X Avocet S F_2 mapping population to allow for this type of analysis.

The F_2 recombinant selection strategy was therefore a successful approach for fine mapping the Kariega QTL. *QYr.sgi-2B* (*QYr.sgi-2B.1*, which includes the *QYr.sgi-2B.1a* interval) was delimited to a 6.1 cM interval between the SSR marker loci *Xbarc55* and *Xwmc344* in the complete F_2 mapping population, recombinant mapping reducing the interval by at least 17 cM. *QYr.sgi-4A* was delimited to a 16.3 cM interval between *Xbarc78* and *Xwmc219.* The intervals are larger on the F_2 recombinant map, as only recombinant individuals were selected for calculating this map. The identification of closer linked, flanking markers will facilitate more successful selection for this QTL in MAS breeding. Both QTL contributed less to the phenotypic variance of the F_2 mapping population, in comparison to the DH population. This could be attributed to the phenotypic data, which is less accurate for the F_2 lines, considering the scoring of single plants in comparison to DH rows in two replicate trials. QTL analysis in the recombinant F_2 mapping population was conducted with both CIM and MQM. The outputs obtained with the two algorithms were in agreement.

5.6.4.1 *QYr.sgi-2B*

Several stripe rust resistance gene have been reported for chromosome 2B, *Yr5/Yr7* (Zhang *et al.* 2009)*, Yr27* (McDonald *et al.* 2004)*, Yr31* (McIntosh *et al.* 2008) and *Yr41* (previously *YrCN19*, Luo *et al.* 2005), all of which confer seedling resistance. Additional seedling stripe rust resistance genes, with temporary designations have been identified for this chromosome, *YrSp* (McIntosh *et al.* 1995), *YrSte* (Chen *et al.* 1998) and *YrV23* (Chen *et al.* 1998). *YrSp* have been shown to be allelic to *Yr5* and *Yr7* (Zhang *et al.* 2009). None of these loci have been defined on a genetic map. *YrP81*, another seedling resistance gene, was identified in the line P81 and mapped 1.8 cM from SSR marker *Xgwm429* (Pu *et al.* 2010). Mallard *et al.* (2005) identified a QTL on chromosome 2BL, *QYr.inra-2BL*, which is flanked by markers associated with the short arm of chromosome 2B (Appels 2003). Although the *QYr.inra-2BL* interval coincides with *QYr.sgi-2B*, it is expressed as seedling resistance. *QYr.sgi-2B* has been well characterized as an APR QTL and therefore represents a different locus from the seedling genes/QTL reported for chromosome 2BS. APR QTL reported for chromosome 2B include, *QYr.ipk-2B* in cv. Opata85 (Börner *et al.* 2002), YQR3 in cv. Opata85 (Boukhatem *et al.* 2002), *QYr.inra-2BS* in cv. Camp Rémy (Mallard *et al.* 2005), *QYrlu.cau-2BS1* and *QYrlu.cau-2BS2* in cv. Luke (Guo *et al.* 2008), *QYrlo.wpg-2BS* in cv. Louise (Carter *et al.* 2009), *QYr.inra-2BS* in cv. Renan (Dedryver *et al.* 2009), *QYr.caas-2BS* in cv. Pingyuan 50 (Lan *et al.* 2010) and *QYrid.ui-2B.2* in the line IDO444 (Chen *et al.* 2011). The marker intervals in relation to *QYr.sgi-2B* are discussed (Fig. 5.21).

- A large interval has been reported for *QYr.ipk-2B* (Börner *et al.* 2002), which includes the interval *Xbarc55-Xwmc344*. The RFLP marker associated with all the stripe rust resistance traits, *Xbcd152*, maps 2 cM from *Xwmc344* (flanking marker for *QYr.sgi-2B*) on a consensus map (Appels 2003).
- QYR3 (Boukhatem *et al.* 2002) was located in the RFLP marker interval *Xcdo405–Xbcd152*, a 7.4 cM interval (4 cM on the consensus map from Appels 2003), which is the same locus as *QYr.sgi-2B.* This QTL was responsible for 30.7% of the phenotypic variance.

- The APR identified in the cv. Camp Rémy by Mallard *et al.* (2005) located to the centromeric region of the short arm of chromosome 2B in the interval defined by marker loci *Xgpw3032-Xcfd50a* , explaining up to 70% of the phenotypic variance observed. However, this QTL interval is proximal to *QYrsgi-2B.*
- Both *QYrlu.cau-2BS1* (*Xwmc154-Xgwm148*) and *QYrlu.cau-2BS2* (*Xgwm148-Xabrc167*) were associated with stripe rust resistance in high-temperature field and greenhouse environments, being responsible for 36.6% and 41.5% of the phenotypic variance, respectively (Guo *et al.* 2008). *QYrlu.cau-2BS1* is distal to *QYr.sgi-2B*, but *QYr.lu.cau-2BS2* overlaps with the *QYr.sgi-2B* interval.
- Carter *et al.* (2009) identified a major QTL (*QYrlo.wpg-2BS*) associated with HTAP resistance flanked by *Xgwm148* and *Xwmc474*, an interval which includes the *QYr.sgi-2B* locus. The QTL was found to be expressed consistently across environments and years.
- Dedryver *et al.* (2009) reported a QTL, *QYr.inra-2BS*, contributing up to 12.3% to the phenotypic variance, between the markers *Xfba70* (*Xgwm210*) and *Xfbb70*. This QTL is located in the telomeric region of chromosome 2BS.
- The cv. Pingyuan 50 was reported to harbour *QYr.caas-2BS*, a QTL responsible for 15.7% of the phenotypic variance for stripe rust resistance (Lan *et al.* 2010). This QTL locus (*Xbarc13-Xbarc230*) includes the Kariega interval.
- Chen *et al.* (2011) published two HTAP QTL, *QYrid.ui-2B1* and *QYridui-2B.2,* on chromosome 2B. The location for one of these, *QYrid.ui-2B2 (Xgwm429-Xbarc91*), coincides with the Kariega QTL. In contrast to *QYr.sgi-2B* this QTL was shown to contribute more to the phenotypic variance explained by the disease severity (i.e. LAI) traits than to the infection type (RT) traits. The QTL was responsible for up to 31% of the disease severity and 10% of the infection type variance.

Considering the published material discussed above, it can be concluded that the stripe rust QTL identified in the cvs Opata85 (Börner *et al.* 2002; Boukhatem *et al.* 2002) and Pingyuan 50 (Lan *et al.* 2010) may be the same QTL as, or closely linked to *QYr.sgi-2B* (Fig. 5.21). The relatedness of these cultivars to Kariega remains to be established. Apart from field identification, *QYr.sgi-2B* was detected in adult plant growth chamber and greenhouse tests at day temperatures of 20°C and 14°C, respectively (Ramburan *et al.* 2004; Prins *et al.* 2011). HTAP resistance is however considered to only be expressed at temperatures above 25°C (Line and Chen 1995). Temperature sensitivity will have to be investigated for *QYr.sgi-2B* before conclusions can be made about the Kariega QTL and the HTAP resistance identified in

the cvs Louise (*QYrlo.wpg-2BS,* Carter *et al.* 2009), Luke (*QYrlu.cau-2BS2,* Guo *et al.* 2008) and IDO444 (*QYrid-ui-2B2,* Chen *et al.* 2011). An association mapping study identified two regions of chromosome 2BS contributing to stripe rust resistance (Crossa *et al.* 2007), one linked to marker loci

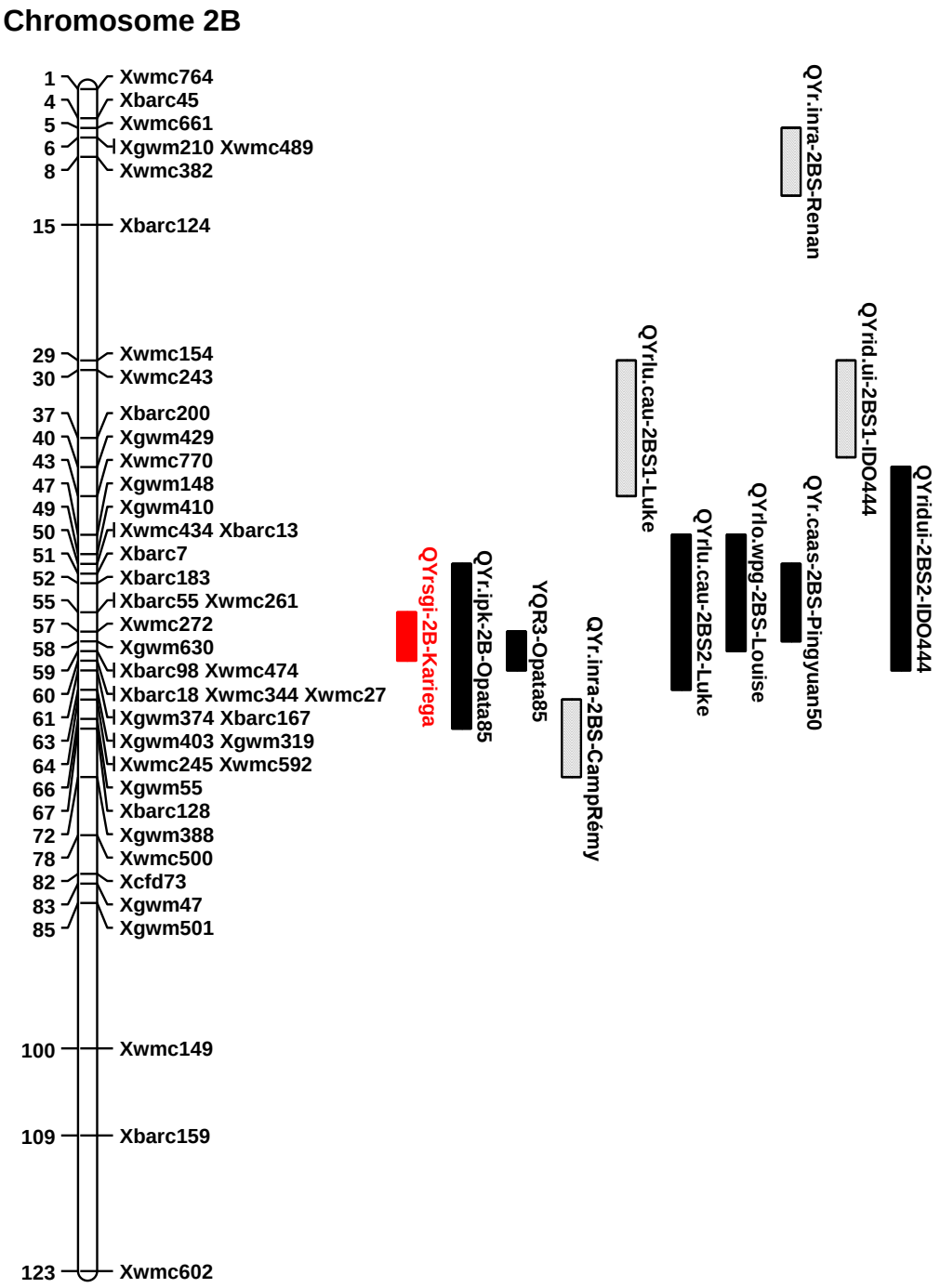

Figure 5.21 Chromosome 2B linkage group (adapted from Somers *et al.* 2004) showing the relative inferred positions for QTL published for the short arm. The *QYr.sgi-2B* from Kariega is highlighted in red and the QTL corresponding to the interval indicated with solid black bars, whereas the QTL mapped outside the interval is indicated with diagonal hatch bars.

Xgwm148/Xgwm410 and marked by the three DArT markers *wPt-5672*, *wPt-4125* and *wPt-7757*, and the other flanked by *Xgwm154* and *Xwmc661*. The former interval coincides with the location of *QYr.sgi-2B*.

5.6.4.2 *QYr.sgi-4A*

QYr.sgi-4A was initially reported to be a minor QTL (Ramburan *et al.* 2004), but it was determined to contribute appreciably to the resistance expressed by Kariega (28% for LAI and 14% for RT) in an extended population (Prins *et al.* 2011). However, the QTL has a limited effect in the greenhouse (Moldenhauer *et al.* 2008; Prins *et al.* 2011). The importance of *QYr.sgi-4A* and its slow-rusting phenotype was confirmed in the F_2 mapping population (26.5% LAI and 9% RT) and the interval was significantly reduced by at least 6 cM. Markers were identified flanking the QTL distally, whereas a drop in the LOD values could not be detected before.

The only chromosome 4A stripe rust resistance genes with Yr designations are the seedling resistance genes *YrHVII* (Chen *et al.* 1996), *YrMin* and *YrND* (Chen *et al.* 1996). These genes have not been mapped to chromosome arms. Another stripe rust resistance gene identified in Australian germplasm, *Yr51*, is pending publication in the Catalogue of Gene Symbols for Wheat (communicated by McIntosh RA, University of Sydney, Australia). Crossa *et al.* (2007) found the 4AL region to contain APR resistance factors in an association study. The only QTL reported thus far is by Chen *et al.* (2011). *QYrid.ui-4A* is associated with HTAP and was located between the DArT markers *wPt-2151* and *wPt-8275*, explaining 22% of the disease severity and 26% of the infection type in the germplasm IDO444. This interval corresponds to *QYr.sgi-4A.*

As a result of an ancient translocation event, chromosome 4A contains translocated parts of chromosomes 5AL and 7BS (Krattinger *et al.* 2011). The most distal part of chromosome 4AL (previously 7BS) therefore shows homology with chromosomes 7AS and 7DS and the middle part (previously 5AL) aligns with chromosome 5BL and 5DL (Krattinger *et al.* 2011). A pericentric inversion resulted in the centromeric region of chromosome 4AL (previously 4AS) to be homologous with chromosome 4BS and 4DS (Naranjo *et al.* 1987; Devos *et al.* 1995; Mickelson-Young *et al.* 1995). It therefore follows that the chromosome 4AL arm may contain resistance genes homoeologous to genes on chromosomes 7AS and 7DS. However, it has been determined that *QYr.sgi-4A* does not share homoeology with *Lr34/Yr18/Pm38* (Krattinger *et al.* 2011; personal communication, W Spielmeyer, CSIRO, Australia).

Kariega is partially resistant to leaf rust, but resistance is governed by *Lr34/Yr18/Pm38* and possibly the seedling genes *Lr1* (chromosome 5D) and *Lr3a* (chromosome 6B) postulated to be present in this cultivar. Neither chromosome 2B (*QYr.sgi-2B*) nor chromosome 4A (*QYr.sgi-4A*) was found to be associated with leaf rust resistance in greenhouse and field trials with the DH mapping population (Prins *et al.* 2011). Leaf rust resistance QTL published for chromosomes 2B and 4A were therefore not considered for comparison.

5.6.5 Conclusion

Kariega not only exhibits complete APR to stripe rust in South Africa, but also has desirable quality characteristics combined with high yield (Barnard *et al.* 2002). The resistance is therefore already available in a suitable background. Two major effect QTL, *QYr.sgi-2B* and *QYr.sgi-4A*, together with the *Lr34/Yr18/Pm38* gene, are responsible for the resistance phenotype. The cultivar's resistance has been incorporated into many South African lines. The identification of molecular markers linked to the QTL has allowed for a more efficient selection strategy in the form of MAS. This study has paved the way for improved QTL selection with the identification of markers flanking smaller QTL intervals. Valuable information has also been obtained that has improved the map resolution of the QTL, moving the study towards positional cloning of the genes behind *QYr.sgi-2B* and *QYr.sgi-4A* .

5.7 Future Prospects

By increasing the map resolution for the Kariega QTL, the genetic intervals were significantly reduced. However, the intervals are still too large for map-based cloning. A further attempt should therefore be made to saturate the regions with molecular markers. Marker types which have not been considered in this study, and which may aid this process include SNP (Allen *et al.* 2011) and insertion site-based polymorphism (ISBP) markers (Paux *et al.* 2010). Screening of lines with real-time PCR needs to be investigated further, as this can reduced cost and speed up genotyping of the mapping population. Additional EST markers could also be considered, as the relevant deletion bins have now been identified. Mapped EST markers will facilitate comparative mapping and possibly uncover proteins associated with resistance, identifying candidate genes underlying the QTL.

NILs are being developed to study the expression of *QYr.sg-2B* and *QYr.sgi-4A* individually in a stripe rust susceptible background (Avocet S). NILs containing only a single QTL are ideal for dissecting individual QTL effects, studying interactions between the QTL and different environments, and for determining breeding potential of an individual QTL (Liu and Bai 2010).

6. GENERAL DISCUSSION

Stripe rust epidemics are typically the result of interactions between susceptible hosts, conducive environmental conditions and sufficient quantities of pathogen inoculum (Wellings 2011). Cereal rust population structure is determined by the introduction of foreign isolates, mutation and more rarely somatic hybridisation and sexual recombination (Park 2008). Considering these factors, wheat stripe rust has the potential to continue to be a major limitation to world wheat breeding (Wellings 2011). That said, management of wheat rusts is one of the best examples of an internationally-coordinated research effort with the most significant investment in cereal producing countries (Chakraborty *et al.* 2011).

Breeding efforts have focussed on incorporating resistance in commercial cultivars and much thought is going into the selection of durable resistance genes. Although the concept of durability (Johnson 1981; Johnson 1984) does not make any conditional implications about the genetic control, mechanism, degree of expression or race-specificity of resistance, experience has taught that APR, in particular race-nonspecific resistance, is more durable. Of the 49 stripe rust resistance genes included in the Catalogue of Gene Symbols for Wheat with formal *Yr* designations, 13 are classified as conveying APR (McIntosh *et al.* 2008 and supplements). Ultimately, in evolutionary terms, no resistance lasts forever (Parlevliet 2002) and the quest for additional resistance genes have to be continued. The probability of a rust pathotype to acquire simultaneous mutations in several avirulence genes is small (Schafer and Roelfs 1985; Parlevliet 2002), thus the reasoning that durability can be achieved with multiple effective (i.e. quantitative) resistance genes. Polygenic resistance is therefore more important than the resistance mechanism employed, and major resistance genes should not be neglected as they can still play and important role in durable resistance (Jacobs and Parlevliet 1993).

Climate change has brought about added concerns. Little is known about the potential effect on the biology of cereal rusts, rust resistance or host-pathogen interactions. Changes in atmospheric composition, temperature, rainfall and humidity will affect the economic importance, geographic distribution and management of diseases effecting crop production and food security (Chakraborty and Newton 2011; Chakraborty *et al.* 2011). Risks which we need to consider include 1) the increased loss from wheat rusts, 2) new rust pathotypes evolving faster and 3) reduced effectiveness of control measures, including the use of fungicides and rust resistance. *Puccinia striiformis* has already demonstrated its ability to adapt to warmer temperatures to cause severe disease in previously unfavourable environments (Pretorius *et al.* 1997; Milus *et al.* 2006; Markell and Milus 2008; Milus *et al.* 2009) and with its ability to disperse over long distances (Nagarajan and Singh 1990), stripe rust will easily keep up with any changes in the distribution of wheat producing regions.

The holistic approach of enhancing the prediction of phenotype from genotype using genomics approaches has been termed genomics-assisted breeding and has already shown its potential for

crop improvement in several cereal species (Varshney *et al.* 2005; Varshney *et al.* 2006). DNA marker technology has become an indispensable tool in studying the genetics of resistance to stripe rust in wheat. Molecular markers have allowed for the development of sufficiently dense genetic maps to initiate the dissection of quantitative traits. This association of quantitative trait expression with mapped markers offers a number of possibilities; this information can serve as the basis for making decisions regarding manipulation of breeding populations, it can be used for molecular marker-assisted selection, or it may serve as the basis for map-based cloning. An understanding of the chromosomal locations of such genes and their biological effects are important in order to ensure they are suitably deployed in elite germplasm.

The identification of rust resistance QTL involves 1) the development of a mapping population segregating for the trait of interest, 2) the construction of a genetic linkage map with adequate marker coverage of the genome, 3) challenging the mapping population with rust isolates and phenotyping the resistance response and 4) the identification of QTL from phenotypic data imposed on the linkage map.

DH and advanced generation RIL mapping populations are particularly well suited for QTL analysis. The fact that the individuals are homozygous and can be propagated by seed makes the populations especially useful for replicated experiments, allowing the population to be assessed over many sites and seasons in order to limit the influence of the environment in detecting QTL. The cost involved with developing the population may be high (e.g. DH production) and the greenhouse and field trial labour and space needed for increasing seed and phenotyping could impose a restriction on the population size. Ferreira *et al.* (2006) evaluated the effect of population size for F_2 (with both dominant and co-dominant markers), BC, RIL and DH mapping populations. The populations with 50 and 100 individuals were the ones unable to reconstitute the original (true) genome and they presented the less accurate genetic maps. Larger populations resulted in lower stress, a function of the average deviation of the distances both between adjacent markers in the linkage group and those in the original genome, and therefore provided more accurate estimates of the genome size. Liu (1998) demonstrated that for a population of 100 individuals, the confidence in the ordering is 90% but drops to 60% when the population size is 50. Simulations have shown that 200 individuals are enough for the construction of reasonably accurate genetic maps with the most efficient populations being the F_2 with co-dominant markers and the RIL population. F_2 populations with dominant markers produce the least accurate maps. In the presence of high proportions of dominant markers and smaller sample size, the probability of repulsion linkage increased between markers and this compromises the marker order (Mollinari *et al.* 2009). It is therefore accepted that DH or RIL populations of 100-200 individuals are sufficient to detect most major QTL (Lynch and Walsh 1998). The primary mapping populations for the current study (Kariega X Avocet S DH and Palmiet X Yr16DH70 RIL) were selected with the considerations discussed above in mind. Both populations consisted of more than 200 individual lines.

The importance of a good trial design cannot be understated, because without sound design, reliable phenotypic information cannot be obtained and QTL information cannot be relied upon (Gilmour *et al.* 1997; Eckermann *et al.* 2001). To control and allow for variation the design of field trials include replication (for precision), randomisation (to avoid systematic bias), and blocking (to control for extraneous variations). In addition to a proper trial design, it is essential to have a reliable and reproducible method of recording infection levels to limit subjectivity. Disease responses can be assessed either qualitatively, quantitatively or a combination of both. Stripe rust severity (LAI) and reaction (RT) were the measures used in this study to evaluate the development of stripe rust infection in adult plants. It was found that a particular QTL could either be associated with both LAI and RT, suggesting that the QTL effects both pathogen growth and plant response or it can affect one phenotype more than the other, suggesting that different QTL may inhibit pathogen infection by different mechanisms, *QYr.sgi-2B*, for example, is explained better by the RT scores as it causes a hypersensitive response characterised by necrosis and chlorosis. Working with leaf rust of wheat, Singh *et al*. (1998) reported that early and late disease ratings are required for accurate ranking of lines. The present study also emphasised the importance of monitoring the progression of the disease by scoring the disease reaction at more than one time during the growing season. The phenotype of *QYr.sgi-4A*, in particular, is more pronounced early in the season.

Although there are various published maps for hexaploid wheat, the wheat composite map (Appels 2003), the wheat consensus map (Somers *et al.* 2004) and additional maps for the International Triticeae Mapping Initiative (ITMI) reference mapping population (synthetic wheat W784 X Opata85 RILs) (Röder *et al.* 1998; Song *et al.* 2005) are the most detailed maps for comparative purposes (Semagn *et al.* 2006). Still, there are various contradictory locus orders present among these maps. For the construction of framework linkage maps, markers that are spaced every 10-20 cM are adequate for QTL mapping (Darvasi *et al.* 1993). Marker order is more important than estimated map distances as map distance estimates may vary across mapping studies by several centiMorgan (Ruiz and Asins 2003; Van Os *et al.* 2005b). Incorrect maps used in map-based cloning and comparative mapping may have serious consequences. Collectively, genotyping errors, missing values and segregation distortion may affect the quality of maps produced, and these elements will be discussed.

SSRs are the marker type most frequently used for constructing linkage maps. They are mainly genome-specific (Röder *et al.* 1998; Harker *et al.* 2001) and allow for direct comparison of map order and distances between studies. The development of DArT overcame limitations of other markers systems and has been demonstrated to be a powerful tool in generating large numbers of polymorphic markers in species with complex genomes such as wheat (Jaccoud *et al.* 2001; Akbari *et al.* 2006). However, DArTs show a higher frequency of clustering in comparison to SSR markers (Semagn *et al.* 2006). In general, the clustering of markers is regularly observed around the centromere where recombination is suppressed (Strommer *et al.* 2002). It is advantageous to use markers with different properties to ascertain good coverage of the genome when constructing linkage maps.

Different approaches were investigated for improving the marker density within QTL intervals. Once the deletion bins to which the QTL map have been determined, EST sequences provide an opportunity for marker development. Due to the modest level of polymorphism within these markers, the identity of the deletion bin is crucial to allow for efficient, targeted marker design. Screening methodologies are still a limiting factor in employing these markers in a high-throughput setup. SSCP analysis is not 100% sensitive, further decreasing the level of polymorphisms detected. Both acrylamide gel electrophoresis and capillary array electrophoresis were shown to be a laborious task requiring specialised equipment. Real-time PCR with HRM was demonstrated not to be suitable for complex genomes such as hexaploid wheat without extensive primer optimisation. Fluorescent probes would provide an alternative but is only cost-effective for typing polymorphic markers in the mapping population and not to pre-screen markers for polymorphisms between parental lines. Converting mapped EST markers to STS markers provided a solution, but not without a significant investment in sequencing and cloning of the amplicons. As a large number of DArT markers are generally mapped in a population, they represent an additional source of markers for mapping and also for MAS if the markers were to be located reasonably close to the QTL. However, DArTs need to be converted to markers which allow for high-throughput and routine screening. It was shown that DArT markers can successfully be converted to STS markers, which could be screened in various systems, without a large investment in time and cost.

Troublesome data points are most likely caused by inaccurate scoring, but some data points that cause ambiguities in the marker order can also be caused by true double recombination events, gene conversions, mutations and other biological phenomena (Van Os *et al.* 2005b). A singleton, as defined by Nilsson *et al.* 1993, is a single locus in one plant that appears to have recombined with both its directly neighbouring loci, and often indicates the misclassification of a marker genotype. The occurrence of double-recombinants is more probable for advanced generation populations, e.g. the Palmiet X Yr16DH70 RIL population which was advanced to the F_7 generation through selfing. Several rounds of meiosis will result in more cross-over events along the linkage groups. In contrast, DH and F_2 populations have only gone through one round of meiosis and double cross-over events, especially over shorter map distances, are highly improbable. Genotyping errors lead to unexpected crossovers which will inflate the map length and are therefore especially detrimental in denser maps. Markers separated by distances of about 10 cM are more robust in the presence of typing errors (Hackett and Broadfoot 2003). In a study by Lehmensiek *et al.* (2005), experimental data were used with a curated linkage map (removing of double recombinants resulting from genotyping errors) to show that although both the original and reviewed maps detected a similar group of QTL, the QTL peak was more sharply located in the re-examined map for one chromosome and the LOD scores were higher for another. A modest number of missing values have less effect than typing errors, but they reduce the number of correctly ordered maps, especially for a marker separation of 2 cM or less, and they will shorten map lengths for more widely spaced markers (Hackett and Broadfoot 2003; Van Os *et al.* 2005b). The removal of correct data points in the vicinity of recombination causes a local decrease of the effective population size (Van Os *et al.* 2005b).

Segregation distortion is the deviation from allele and genotype frequencies expected under the Hardy-Weinberg Law, which states that population frequencies remain in equilibrium across generations unless disturbed by some phenomenon. It is a common feature of wide crosses (Semagn *et al.* 2006) but can also be a function of heterogeneity within parents, unintentional selection in the DH production process or admixture of seed or errors in genotypic data (Semagn *et al.* 2006). Alien translocations in one of the parents or recombination hotspots could also have this effect. Segregation distortion will lead to spurious linkages, clustering of markers and a reduced estimate of recombination frequency (Cloutier *et al.* 1997; Jansen *et al.* 2001; Kammholz *et al.* 2001). However, for the purpose of QTL analysis, this is less of a concern as QTL will have the same segregation ratios as closely linked markers (Lehmensiek *et al.* 2005). Hackett and Broadfoot (2003) showed segregation distortion to have little effect on marker ordering and map distances.

As discussed before, the accuracy of any genetic map estimation method relies on the distribution of recombination frequencies, the proportion of missing data and the quantity of noise due to genotyping errors (Cheema and Dicks 2009). Linkage grouping is best performed through the setting of less stringent rules based on a heuristic approach and on the researcher's experience and knowledge of consensus maps (Cheema and Dicks 2009). Locus ordering on a linkage map requires a criterion that defines the best (resembling the true) map and an algorithm to find the optimal sequence of loci. The locus ordering criteria most often employed include 1) weighted least squares, 2) maximum likelihood (ML) and 3) minimum sum of adjacent recombination fractions criteria (Hackett and Broadfoot 2003). The ML method produces the most accurate marker orders but is very sensitive to typing errors (Hackett and Broadfoot 2003; Cheema and Dicks 2009). Various computer packages for linkage mapping have implemented these criteria, combined with different search algorithms. Jansen (1993) and Zeng (1994) proposed similar methods of QTL mapping that allow for other QTL while looking at a particular interval within a genome, termed composite interval mapping (CIM). The effects of other QTL are removed by regressing on markers not flanking the QTL. Then interval mapping can be performed and inference made using ML. Both forward and backward stepwise selection procedures can be used to select the interval markers. Direct modifications of CIM include multiple interval mapping (MIM) (Kao et al. 1999) and inclusive CIM (ICIM) (Li et al. 2008), which allow for simultaneous estimation of additive and epistatic effects.

Linkage maps were generated for this study with RECORD (Van Os *et al.* 2005a) and managed in MapManager QTXb20 (Manly *et al.* 2001), alternatively maps were generated with JoinMap v.4.1 (Van Ooijen 2006). RECORD (REcombination Counting and ORDering) has been shown to produce more accurate maps than both MapManager QTX (Collard *et al.* 2009) and JoinMap (Van Os *et al.* 2005a). The accuracy of MapManager QTX maps decrease with larger maps (> 30 markers per linkage group) with small marker spacing (< 5 cM). The number of crossovers forms the basis for RECORD since the algorithms minimise the total number of recombination events (Van Os *et al.* 2005a). RECORD was developed for constructing dense maps, such as those with over 500 markers per linkage group. The algorithm is faster and less sensitive to missing observations and scoring

errors than JoinMap, since the optimisation criterion is less dependent on the position of the erroneous markers and it performs better in regions of the map with high marker density. Scoring errors provide RECORD with ordering ambiguities only when they occur near recombination events, on the other hand, pair-wise distance estimates are always affected by errors, independent of their position (Van Os *et al.* 2005a). The SMOOTH procedure was developed, as an accompaniment to the RECORD algorithm, for the detection of suspect data points (Van Os *et al.* 2005b). Unfortunately RECORD only produces marker orders, and not map positions in centiMorgan and it does not assign markers to linkage groups. RECORD is said to be able to deal with any generation obtained by repeated selfing of a hybrid between homozygous parents e.g. DH, BC, F_2, F_3 and RIL mapping populations (Van Os *et al.* 2005a). However, RECORD was not found to be able to deal with heterozygous individuals, and for this reason JoinMap was used for marker ordering in the Kariega X Avocet F_2 mapping population. Furthermore, RECORD does not appear to be under further development (Cheema and Dicks 2009), and newer algorithms will ultimately out compete RECORD. Windows QTL Cartographer v.2.51 (Wang *et al.* 2011) was primarily used for QTL mapping, however, the MQM function of MapQTL v.6 was also investigated for the Kariega X Avocet S F_2 mapping population after the markers were ordered with JoinMap. MapQTL has a more user-friendly interface and produced very similar results to Windows Cartographer.

Features of each software tool may be important to different researchers, e.g. speed, visualisation tools and ease of use. In practice, it is almost certainly best to use a combination of approaches in developing and refining a map, not only because they each bring something unique to the analysis but also because we do not know which approach will succeed best for a new dataset. Map estimation is an iterative process, where researchers should first grasp the global pattern of their dataset before reevaluating and revising the grouping and ordering of markers rather than performing rigid, linear three stage methodology of grouping, ordering and spacing (Cheema and Dicks 2009).

The resolution of QTL mapping depends on population size and marker density surrounding the target region in addition to the genetic background. QTL that contribute less than 10% to the phenotypic variance are particularly susceptible to environmental variances and are difficult to detect across years and environments (Boukhatem et al. 2002). This implies that the genetic variation caused by the QTL should be significantly larger than the variation caused by the environmental factors and experimental errors. Variation not accounted for may be attributed to the presence of undetected minor QTL in regions of the genome not adequately covered by markers, epistasis and QTL interactions, QTL-environment interactions or error variation. This highlights the need for linkage maps with more complete genome coverage, as well as adequately replicated field trials over many environments, and effective QTL mapping software to detect QTL-interaction effects. The chances of detecting minor QTL is improved by 1) increasing the marker density as the closer a QTL is to a marker, the smaller the QTL effect detected statistically, and 2) by increasing the sample size. Larger populations enable a greater resolution to be achieved in the positioning of the QTL, while uniform genome coverage is required to detect all contributing loci (Chalmers *et al.* 2001). In experiments with

small sample sizes and higher probability thresholds, QTL that explain less than 3% of the phenotypic variance are not normally detected. The larger the environmental effect on the character, i.e. low heritability, the less likely a QTL will be detected. Higher probability thresholds for declaring a QTL effect significantly reduce the chances of spurious QTL being reported. An accurate indication of the QTL peak and a significant LOD score are both essential for the identification of markers associated with a QTL.

The genetic map not only underpins studies of QTL, but it also provides a scaffold for genome sequence assembly. Physical mapping data and in particular complete genome sequences, will also present a marker order (Cheema and Dicks 2009). However, the genome sequence is itself an estimate gained from a sequence assembly process and may not be highly accurate for up to several years following initial sequencing (Cheema and Dicks 2009). This should be taken into account when comparing genetic and physical marker orders. The International Wheat Genome Sequencing Consortium (IWGSC) has launched the whole genome sequencing and assembly of the hexaploid wheat genome. Much progress has been made in the construction of physical maps, obtaining survey sequences and high coverage assembled contigs (http://www.wheatgenome.org). Naturally occurring nucleotide diversity is a treasure trove for investigating and harnessing quantitative variation which will be utilised more efficiently with the availability of sequencing data. This will encourage marker development and fast-track map-based cloning of genes.

By definition, quantitative traits refer to phenotypes that vary in degree, and can therefore be attributed to polygenic effects and their interaction with the environment. Identifying QTL for traits such as yield, disease resistance and protein content, has important consequences in breeding programs as this allows for the introduction of MAS. The time to release of a variety for commercial use can be decreased substantially when molecular markers are utilised to supplement conventional phenotypic selection methods (Eagles *et al.* 2001).

7. CONCLUSION

The appearance of the stripe rust pathogen in South Africa necessitated the development of adapted, resistant lines and breeders are now making a conscious effort to introduce resistance genes in new breeding material. A targeted research effort has identified rust resistance QTL in the South African cv. Kariega (Ramburan *et al.* 2004; Prins *et al.* 2011). The two important QTL, *QYr.sgi-2B*, *QYr.sgi-4A* and the *Lr34/Yr18* gene present in Kariega, are already utilised in breeding programs. Another cultivar which has shown excellent stripe rust resistance since it was first evaluated in South Africa in 1998 (Boshoff *et al.* 2002b; Pretorius ZA, unpublished), is the European winter wheat Cappelle-Desprez.

Mapping in the Kariega X Avocet F_2 population of recombinants was a sensible approach for improving the resolution of QTL. Since a DH mapping population segregating for the QTL is also available, it takes away the need to advance the population to the F_3 generation for further selection of individuals to develop NILs for further study. The QTL markers identified in the F_2 population could be taken back to the DH population to selected individuals carrying the QTL. The Kariega QTL were demarcated to smaller intervals thereby improving the characterisation of the QTL, enabling improved QTL selection in breeding material.

The development of Yr16DH70 was shown to be a successful strategy to target APR genes without the masking effect of the seedling resistance genes present in Cappelle-Desprez. This also ensured targeting of chromosomes containing resistance QTL, shortening the time needed to construct a whole genome linkage map. QTL which have not been utilised in South Africa before can now be integrated in new breeding material for improved APR resistance to stripe rust.

Not only were the aims set out for this study achieved by improving the characterisation of the Kariega QTL and identifying stripe rust APR in Cappelle-Desprez, but new marker technologies and mapping approaches were investigated. Furthermore, valuable knowledge was gained for successful implementation in this study and future projects.

The results of this study will enable further characterisation of major QTL regions and the development of genetic material for breeding programs. Germplasm containing each of the major QTL and a combination of them will be developed, using a marker-assisted backcross approach. These can be used as reference gene/QTL stocks, for crossing parents in breeding programmes, for comparison of the effectiveness of the major QTL in different genetic backgrounds and it may aid further characterisation of the QTL regions with more markers. Finally, this study coincided with the establishment of a significant industry investment in MAS for rust resistance in South African wheat breeding programs. As such it provided timely and valuable information in applying marker technology in actual wheat improvement.

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National Center for Biotechnology Information (NCBI), www.ncbi.nlm.nih.gov

Primer3Plus software, http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi

The Institute for Genomic Research (TIGR), http://plantta.tigr.org/search.shtml

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Windows QTL Cartographer v.2.5 software, http://statgen.ncsu.edu/qtlcart/WQTLCart.htm

SUMMARY

Stripe rust, caused by the fungus *Puccinia striiformis* f. sp*. tritici*, is one of the most important diseases of bread wheat (*Triticum aestivum*). In recent years the disease has reached a global distribution, also causing significant crop losses in South Africa since 1996. The deployment of disease resistant cultivars is recognised as an effective approach to minimise crop losses without the higher input costs associated with the application fungicides. The need for new sources of durable resistance has become apparent with the regular changes in the pathogen population and the emergence of more virulent races. Quantitative trait loci (QTL) mapping is a powerful tool for unravelling the genetic components responsible for disease resistance. For the purpose of this study, two cultivars have been identified with complete adult plant resistance (APR) to stripe rust. Previously, the South African spring wheat Kariega has been studied and the *Lr34/Yr18/Pm38* gene and two major QTL, *QYr.sgi-2B* and *QYr.sgi-4A*, were identified in a Kariega X Avocet S doubled haploid (DH) mapping population. Cappelle-Desprez is an old European winter wheat for which resistance genes/QTL have been postulated. The objective of this study was to dissect the APR for stripe rust in these cultivars using a QTL mapping approach. The Kariega QTL were further characterised by increasing the DNA marker density in a more targeted approach. Additional simple sequence repeat (SSR) markers were incorporated in the genetic map and expressed sequence tag (EST) markers were developed for screening by means of single-strand conformation polymorphism (SSCP) analysis. The conversion of EST and Diversity Arrays Technology (DArT) markers to sequence tagged site (STS) markers were investigated to allow for high-throughput screening. In addition, selected SSR and DArT-STS markers were screened in a large Kariega x Avocet S F_2 mapping population to improve the genetic map resolution in the QTL intervals. The *QYr.sgi-2B* and *QYr.sgi-4A* intervals have been delimited to 6.1 and 16.2 cM respectively, in the F_2 mapping population with the aid of recombinant mapping. Resistance displayed by Cappelle-Desprez was studied in a Palmiet x Yr16DH70 recombinant inbred line (RIL) mapping population. The breeding line Yr16DH70, a Cappelle-Desprez derivative, is a more suitable parent in a spring wheat background. SSR and DArT markers were typed in the population. QTL were identified, and up to 75.2% of the phenotypic variance could be accounted for. A major QTL, *QYr.ufs-2A,* explaining up to 53.2% of the phenotypic variance was identified on chromosome 2A. The presence of the *Yr16* gene on chromosome 2D was confirmed (*QYr.ufs-2D*) and additional minor QTL were detected on chromosomes 5B (*QYr.ufs-5B*) and 6D (*QYr.ufs-6D*). A minor QTL, *QYr.ufs-4B* was derived from Palmiet. Stripe rust resistance QTL from Kariega and Cappelle-Desprez provide valuable sources of resistance in adapted, spring wheat backgrounds to South African breeders. Improved characterisation of the QTL has led to the identification of QTL-associated markers, allowing for more efficient selection in marker-assisted breeding schemes, and it also paves the way for map-based cloning.

OPSOMMING

Streeproes word deur die fungus *Puccinia striiformis* f. sp. *tritici* veroorsaak, en dit is een van die mees belangrike siektes wat broodkoring (*Triticum aestivum*) bedreig. Die siekte het oor die laaste jare wêreldwyd versprei en het ook vanaf 1996 tot groot opbrengsverliese in Suid-Afrika gelei. Die ontplooiing van siekte-bestande kultivars word erken as 'n effektiewe benadering om verliese in opbrengs te beperk, sonder die hoër kostes wat met die gebruik van swamdoders gepaard gaan. Met die gereelde veranderinge in die patogeen populasie en verskyning van meer virulente rasse, het dit duidelik geword dat daar 'n behoefte aan nuwe bronne van duursame weerstand is. Kwantitatiewe kenmerk-loci (QTL) kartering is 'n kragtige instrument om genetiese komponente wat vir siekteweerstand verantwoordelik is, te ontrafel. Vir hierdie studie is twee kultivars wat volledige volwasse plant weerstand (APR) teen streeproes het, geïdentifiseer. Die Suid-Afrikaanse lentekoringkultivar Kariega is voorheen bestudeer en die *Lr34/Yr18/Pm38*-geen en twee hoof effek QTL, *QYr.sgi-2B* en *QYr.sgi-4A*, is geïdentifiseer in 'n Kariega X Avocet S verdubbelde haploïed (DH) karteringspopulasie. Cappelle-Desprez is 'n ou Europese winterkoring waarvoor weerstandsgene/QTL gepostuleer is. Die doel van hierdie studie was om die APR vir streeproes van hierdie kultivars te dissekteer deur 'n QTL-kartering benadering te volg. Die Kariega QTL is verder gekarakteriseer deur die DNA merkerdigtheid in 'n meer geteikende benadering te verhoog. Addisionele eenvoudige-volgorde-herhaling (SSR) merkers is geïnkorporeer in die genetiese kaart en uitgedrukte-volgorde-etiket (EST) merkers is ontwikkel wat met behulp van enkelstring-konformasie-polimorfisme (SSCP) geanaliseer kan word. Die omskakeling van EST en "Diversity Arrays Technology" (DArT) merkers na volgorde–gemerkte-setel (STS) merkers is ondersoek om hoë-deurset analise moontlik te maak. Bykomend is geselekteerde SSR- en DArT merkers in 'n groot Kariega X Avocet S F_2 karteringspopulasie getoets om die resolusie van die genetiese kaart in die QTL intervalle te verbeter. Die *QYr.sgi-2B* en *QYr.sgi-4A* intervalle is onderskeidelik tot 6.1 en 16.2 cM in die F_2 rekombinante populasie verkort. Cappelle-Desprez se weerstand is in 'n Palmiet X Yr16DH70 rekombinante ingeteelde lyn (RIL) karteringspopulasie bestudeer. Die lyn Yr16DH70, geteel uit Cappelle-Desprez, is 'n meer geskikte ouer in 'n lentekoring agtergrond. SSR en DArT merkers is in die populasie getoets. QTL is geïdentifiseer en tot 75.2% van die fenotipiese variasie kon verklaar word. 'n Hoof effek QTL, *QYr.ufs-2A* wat tot 53.2% van die fenotipiese variasie verduidelik is op chromosoom 2A geïdentifiseer. Die teenwoordigheid van die *Yr16*-geen is op chromosoom 2D (*QYr.ufs-2D*) bevestig en addisionele kleiner effek QTL is op chromosome 5B (*QYr.ufs-5B*) en 6D (*QYr.ufs-6D*) gevind. 'n Klein effek QTL, *QYr.ufs-4B*, is van Palmiet afgelei. Streeproesweerstand QTL van Kariega en Capelle-Desprez verteenwoordig waardevolle bronne van weerstand in aangepaste lentekoring agtergronde wat vir Suid-Afrikaanse telers beskikbaar is. Verbeterde karakterisering van die QTL het gelei tot die identifisering van QTL-geassosiëerde merkers wat meer effektiewe selekie moontlik maak in merkerbemiddelde teling skemas. Dit lê ook die fondasie vir kaart-gebasseerde klonering van gene.

APPENDIX I

Conference contributions

46th Congress of the Southern African Society for Plant Pathology. Gordon's Bay, South Africa, 25-28 January 2009
Poster: Agenbag GM, Boyd CM, Pretorius ZA, Prins R. Fine mapping of durable resistance to stripe rust in the South African wheat cultivar, Kariega, using an expressed sequence tag (EST) marker strategy

18th Plant and Animal Genome Conference. San Diego, US, 9-13 January 2010
Poster: Agenbag GM, Boyd LA, Pretorius ZA, MacCormack R, Prins R. Fine mapping of durable resistance to stripe rust in the South African wheat cultivar, Kariega, using an expressed sequence tag (EST) marker strategy

8th South African Plant Breeding Association Symposium. Stellenbosch, South Africa, 15-17 March 2010
Oral presentation: Agenbag GM, Pretorius ZA, Boyd LA, Bender CM, Prins R. Identification of quantitative trait loci (QTL) associated with durable resistance to stripe rust derived from the wheat cultivar Cappelle Desprez

21st Biennial Congress of the South African Genetics Society. Bloemfontein, South Africa, 9-11 April 2010
Oral presentation: Agenbag GM, Pretorius ZA, Boyd LA, Bender CM, Prins R. High resolution mapping of stripe rust resistance QTL in the wheat cultivar Kariega

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Oral presentation: Agenbag GM, Pretorius ZA, Boyd LA, Bender CM, MacCormack R, Prins R. Dissecting stripe rust resistance in wheat through QTL mapping strategies

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Poster: Agenbag GM, Pretorius ZA, Boyd LA, Bender CM, Prins R. Dissecting adult plant stripe rust resistance in the wheat cultivar Cappelle Desprez

21st International Triticeae Mapping Initiative and the International Wheat Genome Sequencing Consortium Workshop, Mexico City, Mexico, 4-9 September 2011
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APPENDIX II

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ORIGINAL PAPER

Identification of adult plant resistance to stripe rust in the wheat cultivar Cappelle-Desprez

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Abstract Following the appearance of stripe rust in South Africa in 1996, efforts have been made to identify new sources of durable resistance. The French cultivar Cappelle-Desprez has long been considered a source of durable, adult plant resistance (APR) to stripe rust. As Cappelle-Desprez contains the seedling resistance genes *Yr3a* and *Yr4a,* wheat lines were developed from which *Yr3a* and *Yr4a* had been removed, while selecting for Cappelle-Desprez derived APR effective against South African pathotypes of the stripe rust fungus, *Puccinia striiformis* f. sp. *tritici.* Line Yr16DH70, adapted to South African wheat growing conditions, was selected and crossed to the stripe rust susceptible cultivar Palmiet to develop a segregating recombinant inbred line mapping population. A major effect QTL, *QYr.ufs-2A* was identified on the short arm of chromosome 2A derived from Cappelle-Desprez, along with three QTL of smaller effect, *QYr.ufs-2D, QYr.ufs-5B* and *QYr.ufs-6D. QYr.ufs-2D* was located within a region on the short arm of chromosome 2D believed to be the location of the stripe rust resistance gene *Yr16*. An additional minor effect QTL, *QYr.ufs-4B,* was identified in the cv. Palmiet. An examination of individual RILs carrying single or combinations of each QTL indicated significant resistance effects when *QYr.ufs-2A* was combined with the three minor QTL from Cappelle-Desprez, and between *QYr.ufs-2D* and *QYr.ufs-5B.*

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Introduction

Stripe rust, caused by the fungus *Puccinia striiformis* f. sp. *tritici,* was first observed in South Africa in 1996 in the winter rainfall region of the Western Cape Province (Pretorius et al. 1997). Subsequent surveys at the time of detection showed that the disease was well established in the Western, Northern and Eastern Cape Provinces, with trace amounts present on irrigated wheat further north in the summer rainfall region. The above-average rainfall, exacerbated by lower than average temperatures recorded during August and September 1996 contributed significantly to the establishment, spread and subsequent epidemic outbreak of stripe rust (Pretorius et al. 1997). The spread of stripe rust in 1997 led to the disease becoming endemic in all major wheat producing areas of South Africa within 2 years (Boshoff et al. 2002a). Only one pathotype, 6E16A- (virulent to *Yr2*, *Yr6*, *Yr7*, *Yr8* and *Yr17*) was detected in 1996 (Pretorius et al. 1997; Boshoff et al. 2002b). This pathotype was previously detected in East and North Africa, the Middle East, and Western Asia (Badebo et al. 1990; Louwers et al. 1992). Using DNA marker data, Hovmøller et al. (2008) confirmed similarities between South African stripe rust pathotypes and some Central and Western Asian and European isolates. During 1998, the presence of a second pathotype, 6E22A- with

added virulence to *Yr25,* was recorded. In 2001 a new variant, 7E22A-, virulent to *Yr1*, was detected in a trap nursery in the highlands of Lesotho (Pretorius et al. 2007). Pathotype 6E22A+, defeating *YrA,* was identified in 2005 (Pretorius ZA, unpublished data), resulting in four stripe rust pathotypes in total to date.

Internationally, the emergence of new, more aggressive strains of the stripe rust fungus is of concern to the wheat industry (Chen 2005; Hovmøller et al. 2008). In addition to increased aggressiveness of the pathogen, several previously effective resistance genes are now failing to provide adequate disease protection (Chen 2007; Germán et al. 2007; Hovmøller and Justesen 2007; Wellings 2007, 2011). It is therefore crucial to identify additional sources of durable resistance, and improve the characterisation of known sources, to facilitate gene pyramiding in breeding programs. In contrast to seedling resistance genes, which are normally expressed throughout the lifespan of the host plant, i.e. all-stage resistance, adult plant resistance (APR) genes are predominantly expressed later in plant development. APR that falls in the race-nonspecific class (Lagudah 2011) usually results in partial resistance or slow-rusting phenotypes, and is considered to be durable. The incorporation into new cultivars of a combination of APR genes, often resulting in a high level of resistance, is deemed an effective management strategy (Singh et al. 2011).

In view of the importance of stripe rust, South African wheat breeders are making a conscious effort to introduce durable stripe rust resistance genes into new breeding material. A targeted research effort has identified rust resistance QTL in the South African cv. Kariega (Ramburan et al. 2004; Prins et al. 2011). The major QTL, *QYr.sgi-2B* and *QYr.sgi-4A,* and the *Lr34/Yr18* gene present in Kariega are currently being utilised in breeding programs. However, to extend the diversity of stripe rust resistance QTL available to South African wheat breeders, other sources of resistance are continuously being investigated. One of these sources, with a history of durable resistance, is the European winter wheat cv. Cappelle-Desprez.

After its release in France in 1946, Cappelle-Desprez was widely cultivated in Western Europe through to the 1970s (Lupton and Macer 1962; Worland and Law 1986; Bonjean et al. 2001). When released, the cultivar exhibited complete resistance to stripe rust. Cappelle-Desprez is known to possess the seedling resistance genes *Yr3a* and *Yr4a* (De Vallavieille-Pope et al. 1990) on chromosome 1B and 6B, respectively (Chen et al. 1996), but reports on the APR in Cappelle-Desprez are less definitive. Early genetic and cytogenetic studies implicated a locus on chromosome 2D, designated *Yr16* (Worland and Law 1986), and the 5BS-7BS translocation (Law and Worland 1997) as conferring stripe rust APR. *Yr16* and the 5BS-7BS translocation are, however, unlikely to be the only contributors to stripe rust APR in Cappelle-Desprez. While the identity of the full complement of stripe rust resistance in Cappelle-Desprez remains unclear, its resistance is believed to have been incorporated into many European varieties. Under controlled environmental conditions, adult plants of Cappelle-Desprez also showed resistance to leaf rust (Poyntz and Hyde 1987), which raised the question as to whether Cappelle-Desprez possessed *Lr34/Yr18* (McIntosh 1992; Bossolini et al. 2006). However, Lagudah et al. (2009), using gene specific markers, showed that *Lr34/Yr18* is not present in Cappelle-Desprez.

Capelle-Desprez has shown excellent stripe rust resistance since it was first evaluated in South Africa in 1998 (Boshoff et al. 2002b; Pretorius ZA, unpublished). To diversify the South African stripe rust resistance gene pool, Cappelle-Desprez was introduced through a cross to the susceptible South African cv. Palmiet. By means of a selection process an adapted breeding line, Yr16DH70 was developed which retained Cappelle-Desprez APR, but lacked the seedling stripe rust resistance genes *Yr3a* and *Yr4a*. The objective of this study was to confirm the presence and location of *Yr16* on chromosome 2D and the role of the 5BS-7BS translocation in stripe rust resistance, while investigating whether additional stripe rust resistance QTL, derived from Cappelle-Desprez, are effective under field conditions in South Africa.

Materials and methods

Plant material

An initial cross was made between the French winter wheat cv. Cappelle-Desprez (pedigree: Vilmorin-27/Hybride-du-Joncquois) and the South African cv. Palmiet (pedigree: SST3*//Scout*5/Agent). Palmiet is a stripe rust susceptible spring wheat carrying the stem rust resistance genes *Sr2* and *Sr24,* as well as the leaf rust resistance gene *Lr24* (Smit et al. 2010). Cappelle-Desprez × Palmiet F_2 seedlings were infected with spores of *P. striiformis* f. sp. *tritici* pathotype 6E16A- (avirulent/virulent for *Yr1*,*3a*,*4a*,*4-b*,*5*,*9*,*10*,*15*,*25*,*27*,*A*,*Sp*/*Yr2*,*6*,*7*,*8*,*17*) in greenhouse tests. Susceptible seedlings were kept, and those plants expressing APR in the field after infection with the stripe rust pathotype 6E22A- (avirulent/virulent for *Yr1*,*3a*,*4a*,*4-b*,*5*,*9*,*10*,*15*,*27*,*A*,*Sp*/*Yr2*,*6*,*7*,*8*,*17*,*25*) were advanced to the F_4 generation through selfing. One F_4 plant was selected for backcrossing to Palmiet based on its high level of stripe rust resistance and other favourable agronomic characters. Doubled haploid (DH) lines were produced from the resulting F_1 plants. One DH line, Yr16DH70, was subsequently selected and crossed to Palmiet to generate a recombinant inbred line (RIL) mapping population,

consisting of 201 individuals, by advancing random individual F_2 plants to the F_7 generation by single seed descent.

Disease evaluation

Field trials were conducted during 2009 and 2010 at the PANNAR Research Station, Greytown, KwaZulu-Natal, South Africa. The 201 RILs were planted in 2 randomised replicates. Entries were planted in 1-m row plots spaced 90 cm apart. Each replicate contained several Palmiet and Yr16DH70 parental controls. The highly susceptible cv. Morocco was planted in spreader rows bordering the trial area, in all pathways perpendicular to plots, and as every tenth entry within the trial. Control plots of Cappelle-Desprez were also included. Field trials were infected with a spore suspension of pathotype 6E22A+ of *P. striiformis* f. sp. *tritici* (avirulent/virulent for *Yr1*,*3a*,*4a*,*4b*,*5*,*9*,*10*,*15*,*27*,*Sp/Yr2*,*6*,*7*,*8*,*17*,*25*,*A*). Stripe rust severity was scored using the modified Cobb scale (0–100% leaf area infected, LAI) as a quantitative measure of disease infection (Peterson et al.1948; McIntosh et al. 1995). Host reaction type (RT) was scored on an ordinal scale as resistant (R); moderately resistant (MR); moderately susceptible (MS) and susceptible (S) (McIntosh et al. 1995), augmented with resistant–moderately resistant (RMR); moderately resistant–moderately susceptible (MRMS) and moderately susceptible–susceptible (MSS). These seven classes of reaction types were assigned a numerical value of 1 (resistant)–7 (susceptible). Phenotyping was performed once the first signs of stripe rust infection were visible on Palmiet, and until the reaction type classes could no longer be accurately distinguished. For both years, this was between plant growth stages 41 (flag leaf sheath extending) and 69 (anthesis completed) (Zadoks et al. 1974). LAI was scored at the Greytown site on 14 September 2009 (LAI14Sept09), 23 September 2009 (LAI23Sept09), 5 October 2009 (LAI5Oct09), 27 September 2010 (LAI27-Sept10) and 11 October 2010 (LAI11Oct10). RT was scored on 14 September 2009 (RT14Sept09), 23 September 2009 (RT23Sept09) and 27 September 2010 (RT27Sept10).

Statistical analyses

All statistical analyses were performed using the statistical package Genstat for Windows, release 12 (Genstat 5 Committee 2005; Payne et al. 2009). The LAI and RT scores were transformed to achieve near normality and independence of the means and variances. LAI data were converted to proportions and transformed to arcsin (Sokal and Rohlf 1995), while the RT data were transformed with the log function (ln(score + 1)) (Ramburan et al. 2004). For the seedling stripe rust resistance tests, segregation ratios were used to test gene postulations by Chi-square goodness-of-fit. Comparisons between field phenotypic data sets were carried out for both LAI and RT scores using a 2-sided test of correlation. Analysis of variance (ANOVA) was carried out using the General Linear Regression (GLR) model in Genstat v12, which supports unbalanced designs and missing data. The effects of field replicates and genotypes were accounted for in the model. Predicted means for each RIL, for all LAI and RT data sets, were extracted and used in further analyses to minimise error variation in mean values. To identify significant effects between different QTL combinations, the GLR model compared RILs within QTL groups and between groups using *t* test comparisons, with all QTL groups being compared to all other groups (Jagger et al. 2011).

Marker linkage analysis and QTL identification

Genomic DNA was isolated from the parents and a representative individual from the Palmiet × Yr16DH70 F_6 and F_7 RIL generations using the cetyltrimethyl ammonium bromide (CTAB) DNA extraction method (Doyle and Doyle 1990). This DNA was used for simple sequence repeat (SSR) marker analysis. For diversity arrays technology (DArT) analysis, DNA was extracted using the Zymo Research Plant/Seed DNA Kit™. DNA concentrations were determined with the NanoDrop Spectrophotometer ND-1000 and diluted to 50 ng/μL. The SSR marker technique described by Röder et al. (1998) was followed.

The SSR primers used included markers from the WMC (Wheat Microsatellite Consortium, Gupta et al. 2002); CFA (Clermont-Ferrand A genome, Sourdille et al. 2003); CFD (Clermont-Ferrand D genome, Guyomarc'h et al. 2002); GWM (Gatersleben Wheat Microsatellite, Röder et al. 1998); GDM (Gatersleben D genome Microsatellite, Pestsova et al. 2000); PSP (Bryan et al. 1997) and BARC (Beltsville Agriculture Research Center, Song et al. 2005) series. Fluorescently labelled primers were synthesised by Applied Biosystems. Markers were amplified and subsequently analysed on the 3730*xl* Genetic Analyzer (Applied Biosystems) using GeneScan™ 500 LIZ® as the internal size standard. Data were analysed using GeneMapper v4.0 (Applied Biosystems). The parental lines, Palmiet and Yr16DH70, were screened with 465 SSR markers. These markers were uniformly distributed across the three wheat genomes, with each of the 21 chromosomes being well represented. Polymorphic SSR markers and DArT markers (using the *PstI*/*TaqI* v3.0 array enriched for the D-genome, Triticarte P/L and Diversity Arrays Technology P/L, Canberra, Australia) were typed in the RIL population. One sequence tagged site (STS) marker for the gene *Pina-D1* (US public MAS wheat breeding programs, http://maswheat.ucdavis.edu) and one converted DArT marker,

wPt-0600 (Yu et al. 2009) were also screened in the population.

Marker data were used to construct a linkage map. Markers were ordered according to the RECORD output (Van Os et al. 2005). Map distances were calculated using the Kosambi map function from MapManager QTXb20 (Manly et al. 2001) and linkage groups were declared based on map distances and linkages between markers at a significance level of $P = 0.001$. Chromosomes were assigned to linkage groups by referring to a consensus map (Appels 2003). Heterozygote scores were replaced with a missing data score. Redundant loci were hidden and unlinked markers were excluded for the purpose of QTL analysis.

For QTL analyses, composite interval mapping (CIM) was performed with Windows QTL Cartographer v2.51 (Wang et al. 2011), using a forward regression model with a window size of 10 cM and a walk speed of 1 cM. Both transformed and untransformed LAI and RT phenotypic data were analysed to increase confidence in the QTL identified. Predicted means obtained for each RIL from the GLR analyses in Genstat v12 were also used in the QTL analysis. To determine the LOD threshold value above which a QTL is considered significant, 1,000 permutations were performed ($P = 0.05$) (Doerge and Churchill 1996) for the LAI and RT traits at each score date. Maps were prepared using MapChart v2.1 (Voorrips 2002). RILs were placed in QTL groups depending on the combination of QTL they carried. The presence of a QTL was based on the genotype of the QTL-associated markers with the highest LOD values in the QTL interval. The predicted means for the LAI and RT phenotypes were calculated for each QTL group.

Results

Development of the mapping population

In order to eliminate seedling stripe rust resistance, F_2 seedlings from the cross Cappelle-Desprez × Palmiet were screened in greenhouse tests with the pathotype 6E16A-. These tests yielded 98 resistant and 6 susceptible plants, giving a good fit to the expected 15:1 ratio for the segregation of the two dominant resistance genes, *Yr3a* and *Yr4a* from Cappelle-Desprez ($X^2 = 0.041$; $P = 0.84$). Adult plant ratings of the susceptible seedlings were 0R (five plants) and 70MS (one plant). The resistant plants were advanced to the F_4 generation through selfing, with F_3 and F_4 progeny being tested for APR in the field. One F_4 plant, displaying complete stripe rust field resistance and other favourable agronomic characters was crossed to Palmiet, a series of DH lines being produced from the resulting F_1 seed to obtain a pure breeding line. The DH line Yr16DH70 was subsequently selected for backcrossing to Palmiet. Yr16DH70 did not display seedling resistance against the South African pathotypes tested (infection type 2+ to 3), but retained APR which was effective under field conditions in South Africa (30R at the end of the growing season). A RIL mapping population (201 lines) was derived from the cross Palmiet × Yr16DH70.

Field assessment of stripe rust resistance

Good stripe rust establishment was seen in the field in both years, the highly susceptible cv. Morocco displaying 100S midway through the scoring seasons. Although Palmiet is fully susceptible to stripe rust in the winter rainfall regions of South Africa (Boshoff et al. 2002a), severe infection is associated with chlorosis and necrosis in the warmer Greytown environment. In 2009 Palmiet scored 70–80MS, while the resistant parent, Yr16DH70 scored 20R at the last assessment (Online Resource 1). Less infection was seen in the dryer and warmer 2010 season, with Palmiet scoring 40–50MR and Yr16DH70 10–15R (Fig. 1). In general, Cappelle-Desprez expressed marginally higher levels of resistance, having scores from 0R through to 20R in field tests from 1999 to 2011.

Although Palmiet was not fully susceptible in these field trials, ANOVA showed clear segregation of resistance in the RIL population for all LAI and RT data sets (variation between RIL, F value prob. < 0.001; Fig. 1). Some variation between replicates was seen, although this was only significant for the data sets LAI23Sept09 and RT27Sept10 (F prob. < 0.001). Transgressive segregation was seen in both years, with some lines being more resistant than Yr16DH70 and others more susceptible than Palmiet (Fig. 1). Comparing phenotypic data sets showed high, positive correlations for LAI scores, both within and across years ($r = 0.61$–0.73). For RT, the correlation between the two score dates in 2009 ($r = 0.74$) was greater than between years ($r = 0.53$ and 0.58), although all correlations were significant ($P < 0.001$). Significant correlations were also observed when comparing LAI and RT phenotypes between the RIL, with correlations being greater within years ($r = 0.59$–0.76) than between years ($r = 0.52$–0.64).

Genetic linkage map construction

Seventy-three of the 465 (15.7%) SSR markers screened were polymorphic between Palmiet and Yr16DH70. These were mapped in the RIL population along with 336 DArT markers and 2 STS markers (one converted DArT and the *Pina-D1* quality marker). At the F_6 generation, an average of four heterozygotes (2%) per SSR marker could still be detected in the RIL population. Fifteen linkage groups, containing 3 or more loci were identified, representing 12 chromosomes (Online Resource 2). All the DArT markers

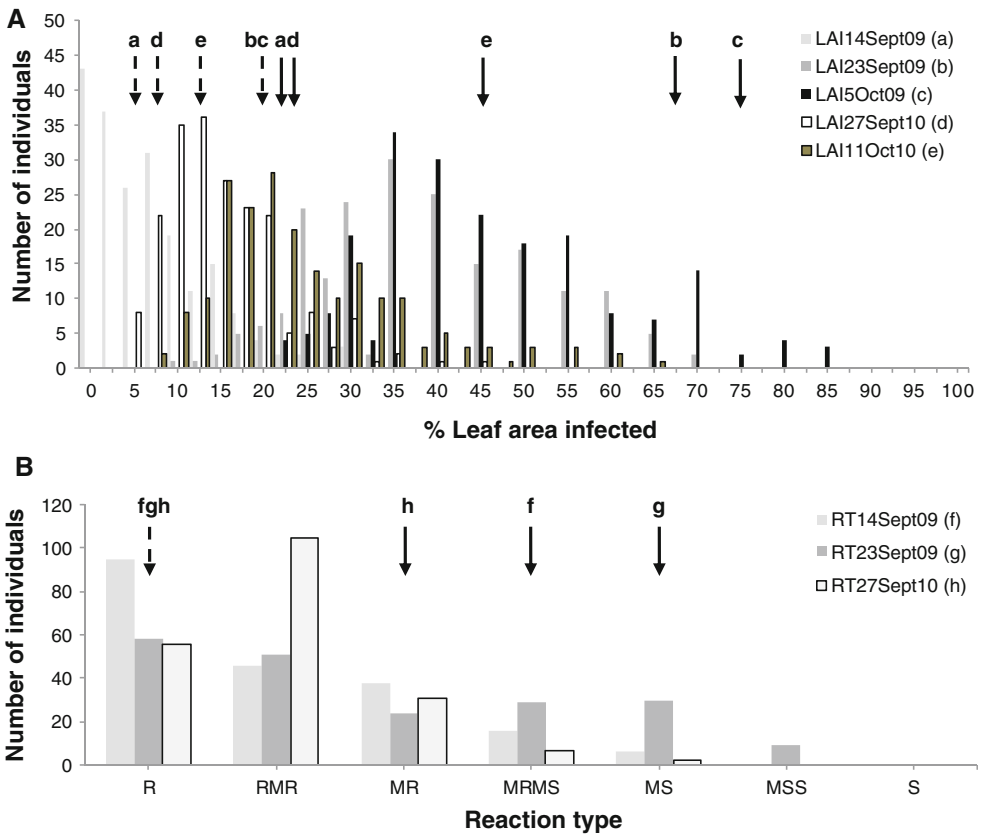

Fig. 1 Segregation of leaf area infected (LAI) (**a**) and reaction type (RT) (**b**) phenotypes in the Palmiet × Yr16DH70 RIL mapping population for the 2009 and 2010 seasons. The *broken arrow* represents the disease scores for Yr16DH70 and the *solid arrow* the scores for Palmiet for the eight phenotypic data sets as indicated by *lowercase letters*

could be incorporated into linkage groups, but four SSR markers (WMC173, GDM111, WMC285 and BARC110) remained unlinked at a significance level of $P = 0.001$.

In particular, good marker coverage was obtained for chromosomes 2A and 2D (Online Resource 2). The linkage group representing chromosome 2A contained 32 markers with a total map distance of 138.7 cM, while the 2D linkage group contained 96 markers and covered a distance of 158.2 cM. Chromosomes 5B and 7B were both represented by two, unlinked linkage groups, representing the short and long arms of each chromosome (Online Resource 2). This would be expected if Yr16DH70 had inherited the 5BS-7BS translocation from Cappelle-Desprez. Chromosome 5D was also represented by two linkage groups (Online Resource 2). Chromosomes 1A, 1D, 2B, 3A, 4A, 4D, 6A, 6B and 7D were not represented by linkage groups, indicating chromosomes represented primarily by Palmiet DNA. The high number of chromosomes fixed for Palmiet DNA was supported by the lower level of polymorphism found for SSR markers between Palmiet and Yr16DH70 (15.7%), as compared to Palmiet and Cappelle-Desprez (68.0%).

QTL analysis of stripe rust resistance

CIM analysis detected QTL on chromosomes 2A (*QYr.ufs-2A*), 2D (*QYr.ufs-2D*), 5B (*QYr.ufs-5B*) and 6D (*QYr.ufs-6D*) from Yr16DH70 and one QTL on chromosome 4B (*QYr.ufs-4B*) from Palmiet. Between 33.6 and 75.2% of the total phenotypic variation could be explained by the identified QTL, depending on the phenotypic data set analysed (Table 1). QTL analysis was carried out using both transformed and untransformed phenotypic scores, identifying the same QTL, with very similar LOD scores. Only the analyses using the untransformed data are reported (Table 1).

A major effect QTL, *QYr.ufs-2A* was detected on the short arm of chromosome 2A with all the phenotypic data sets, explaining up to 53.2% of the phenotypic variation for RT and 47.8% of the variation for LAI (Fig. 2). The QTL mapped between the marker loci *wPt-733314* and *wPt-0003* with the peak located between *Xgwm636* and *wPt-0003*. With the LAI phenotype data sets, a potential QTL was also detected at the opposite end of the 2A linkage group, having a maximum LOD value of 3.2 (Fig. 2a). However, the presence of this second QTL on 2AL requires further investigation to provide confirmation. Three further, minor QTL were derived from Yr16DH70. *QYr.ufs-2D* was detected on the short arm of chromosome 2D flanked by the marker loci *Xgwm102* and *wPt-664520* and explained up to 10.3% of the phenotypic variation, being detected by both the LAI and RT phenotypic data sets (Fig. 3; Table 1). *QYr.ufs-5B* explained up to 5.7% of the

Table 1 Summary of stripe rust APR QTL detected with CIM using LAI and RT phenotypic data sets

QTL interval[a]	Chr[b]		Percentage leaf are infected (LAI)					Host reaction type (RT)			Origin
			14 Sept 09	23 Sept 09	5 Oct 09	27 Sept 10	11 Oct 10	14 Sept 09	23 Sept 09	27 Sept 10	
QYr.ufs-2A	2A	LOD	10.7	18.8	13.7	11.6	13.8	10.6	16.2	8.6	Yr16DH70 (CD[d])
wPt-733314–wPt-0003		%VAR[c]	28.8	47.8	39.7	32.6	36.9	30.5	53.2	23.4	
QYr.ufs-2D	2D	LOD	–	–	–	2.9	5.4	–	6.6	6.1	Yr16DH70 (CD)
Xgwm102–wPt-664520		%VAR				4.7	8.4		10.3	9.9	
QYr.ufs-4B	4B	LOD	–	–	–	–	–	4.7	7.6	–	Palmiet
Xgwm165–Xgwm495		%VAR						7.0	11.7		
QYr.ufs-5B	5B	LOD	3.2	3.4	2.8	–	–	3.8	–	3.5	Yr16DH70 (CD)
wPt-7114–Xbarc74		%VAR	4.8	4.9	4.3			5.7		5.3	
QYr.ufs-6D	6D	LOD	–	4.2	2.8	4.2	–	–	–	–	Yr16DH70 (CD)
Xgwm325–Xbarc175		%VAR		7.6	4.5	6.2					
Total variance explained (%)			33.6	60.4	48.4	43.5	45.3	43.2	75.2	38.6	

[a] Only QTL with LOD values above the significance levels that were determined after 1,000 permutations ($P = 0.05$) are shown

[b] Chromosome

[c] Percentage phenotypic variance explained (R^2) with '–' meaning not significant

[d] Derived from Cappelle-Desprez

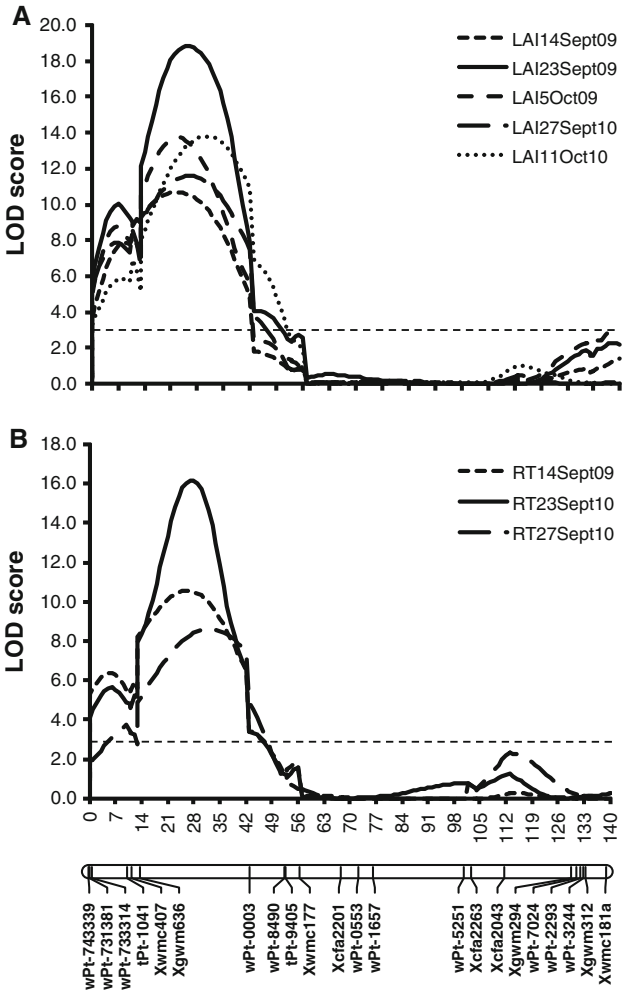

Fig. 2 Stripe rust resistance QTL, *QYr.ufs-2A* on chromosome arm 2AS identified with the leaf area infected (**a**) and reaction type (**b**) data sets. The distances between markers are shown in centiMorgans. The LOD threshold, as determined from 1,000 permutations using Cartographer v2.51 (Wang et al. 2011) is shown

phenotypic variation, and was detected by both the LAI and RT phenotypes (Table 1; Online Resource 3). *QYr.ufs-5B* spanned an interval of 11.1 cM and was flanked by marker loci *wPt-7114* and *Xbarc74*, placing the QTL near to the centromere. *QYr.ufs-6D* was detected only with the LAI phenotype (Table 1; Online Resource 3). It explained up to 7.6% of the phenotypic variance and was located between the SSR marker loci *Xgwm325* and *Xbarc175*, placing the QTL on the long arm of chromosome 6D. A minor QTL was also detected in Palmiet on chromosome 4B flanked by marker loci *Xgwm165* and *Xgwm495* (Table 1; Online Resource 3). *QYr.ufs-4B* explained up to 11.7% of the phenotypic variance, and was only detected using the RT phenotypic data (Table 1).

Each RIL was placed in one of 32 groups depending on the five QTL they carried (Online Resource 4). The predicted means of each QTL group were obtained from an

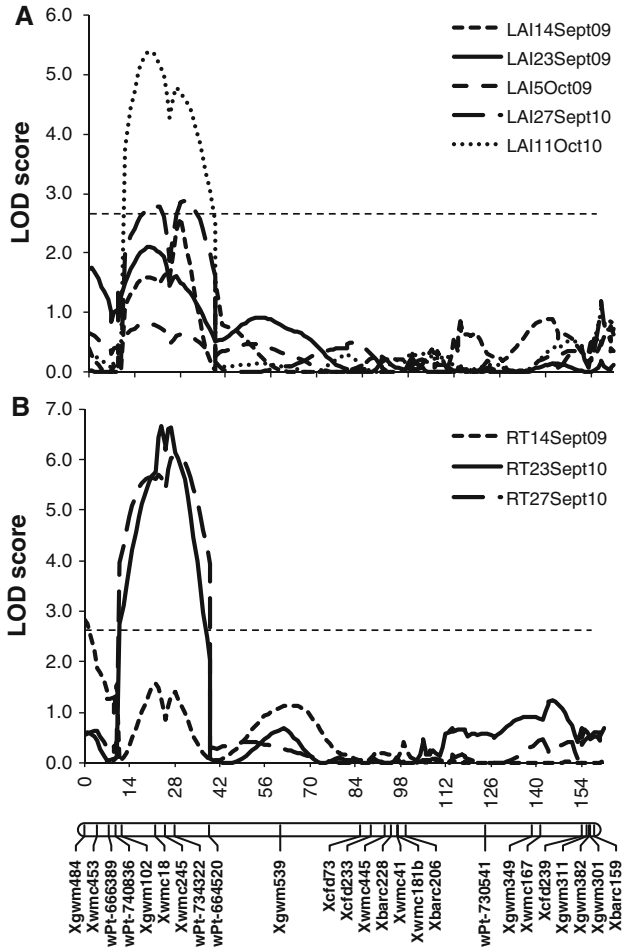

Fig. 3 Stripe rust resistance QTL, *QYr.ufs-2D* on chromosome arm 2DS identified with the leaf area infected (**a**) and reaction type (**b**) data sets. The distances between markers are shown in centiMorgans. The LOD threshold, as determined from 1,000 permutations using Cartographer v2.51 (Wang et al. 2011) is shown

ANOVA, using the GLR model in Genstat v12, for all eight LAI and RT datasets (Fig. 4; only QTL groups carrying Yr16DH70-derived QTL are shown). With both LAI and RT phenotypes, significant differences were found between QTL groups (F prob. < 0.001), while no differences were found between RILs within QTL groups. Following the ANOVA, t test comparisons of the RILs within each QTL group were undertaken, comparing all 32 QTL groups to each other. A significant effect on stripe rust phenotypes was seen when *QYr.ufs-2A* was combined with the small QTL derived from Yr16DH70, removal of one or more small effect QTL altering the level of resistance (t test prob. < 0.01–0.05; Fig. 4). Lower disease scores were also seen for both LAI (t test prob. < 0.05) and RT (t test prob. < 0.001 to 0.05) when the minor QTL *QYr.ufs-2D* and *QYr.ufs-5B* were combined. A significant reduction in disease was not consistently seen with any other pairwise combinations of the Yr16DH70-derived QTL.

Discussion

Cappelle-Desprez was first released in France in 1946 and occupied a considerable acreage across Western Europe until the late 1970s (Lupton and Macer 1962; Worland and Law 1986; Bonjean et al. 2001). The longevity of this cultivar was exceptional, which in part was due to its high levels of APR to a number of major diseases, including stripe rust. Consequently, Cappelle-Desprez was used extensively in breeding programs within the UK and Western Europe (Angus 2001; Bonjean et al. 2001; Porche 2001). Cappelle-Desprez is still recognised as a good source of stripe rust APR and in South Africa has maintained high levels of resistance since being tested for the first time in 1998 (Boshoff et al. 2002b).

Previous studies of stripe rust resistance in Cappelle-Desprez have suggested several APR loci (Worland and Law 1986; Law and Worland 1997) in addition to the seedling resistance genes *Yr3a* and *Yr4a* (Lupton and Macer 1962; De Vallavieille-Pope et al. 1990). To increase the sensitivity of detection of minor APR loci derived from Cappelle-Desprez, the line Yr16DH70 was developed, whereby the race-specific, seedling resistance genes *Yr3a* and *Yr4a* had been removed. The development of Yr16DH70 resulted in much of the genome of this line consisting of Palmiet DNA. Twenty-three percent of all polymorphic SSR markers between Cappelle-Desprez and Palmiet (316 SSR markers) were also polymorphic between Yr16DH70 and Palmiet (73 SSR markers). This would fit with the expected genetic structure of Yr16DH70, where 25% of the genome should be derived from Cappelle-Desprez and 75% from Palmiet. Genetic mapping of the Palmiet × Yr16DH70 RIL population therefore resulted in the identification of 12 of the potential 21 chromosomes of hexaploid wheat.

Subsequent QTL analyses identified a major effect stripe rust resistance QTL on the short arm of chromosome 2A (*QYr.ufs-2A*) and three minor QTL on chromosomes 2D (*QYr.ufs-2D*), 5B (*QYr.ufs-5B*) and 6D (*QYr.ufs-6D*). In addition, a minor QTL was detected in the South African cv. Palmiet on chromosome 4B (*QYr.ufs-4B*). While up to 75.2% of the variation seen for RT was explained by the QTL identified, only 60.4% of the variation seen for LAI was explained. Although heavy selection pressure for adult plant stripe rust resistance was imposed during the development and selection of Yr16DH70, minor QTL and QTL where expression is heavily influenced by environmental factors (Boukhatem et al. 2002), may not have been retained in Yr16DH70. This may account for the consistently, but marginally higher disease rating obtained for Yr16DH70 compared to Cappelle-Desprez in each year of testing.

QYr.ufs-2A was responsible for a significant proportion of the stripe rust resistance displayed by Yr16DH70 (up to

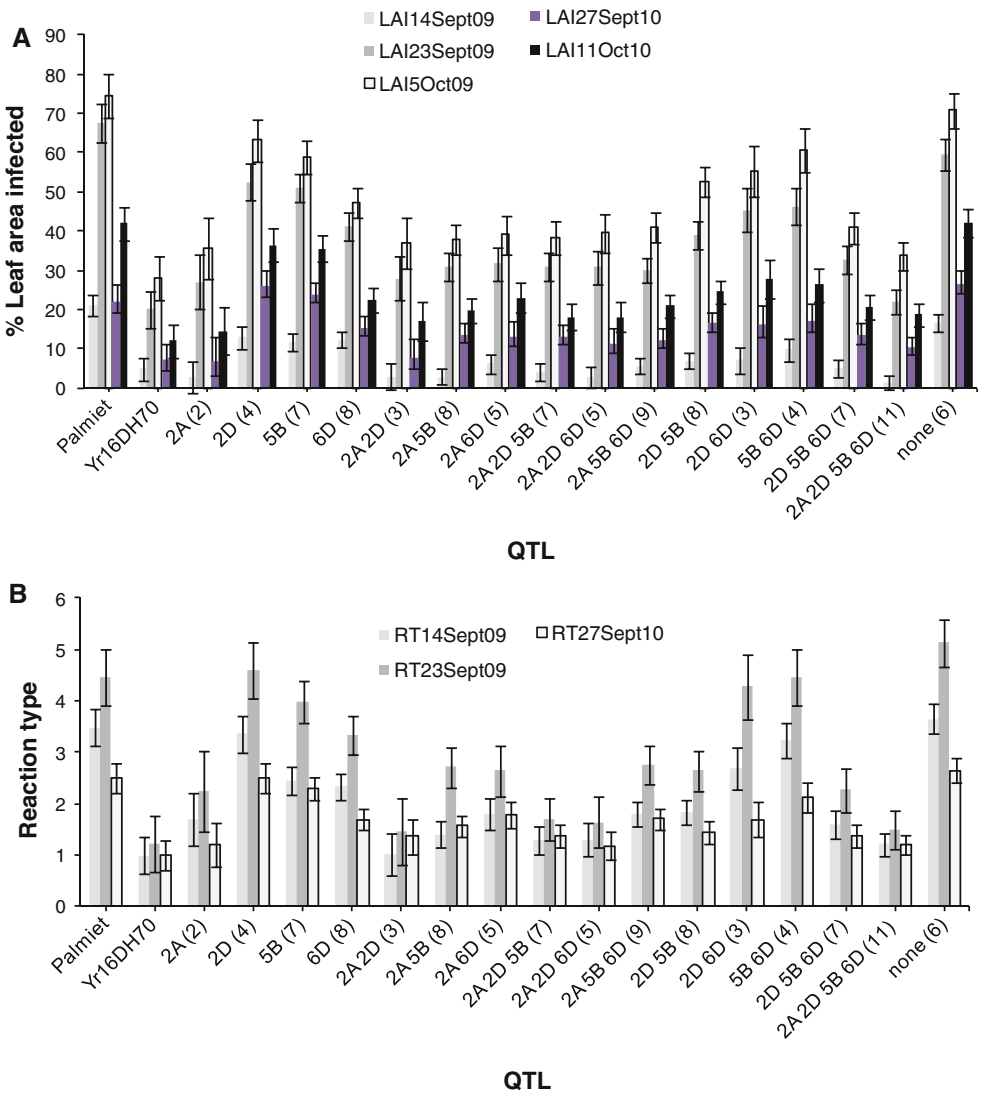

Fig. 4 **a** Mean percentage leaf area infected (LAI) and **b** reaction type (RT) of the RIL defined by the four stripe rust APR QTL derived from Yr16DH70. Groups containing the Palmiet QTL (*QYr.ufs-4B*) are not shown in the figure to simplify the display. The phenotypes of the individual QTL *QYr.ufs-2A, QYr.ufs-2D, QYr.ufs-5B* and *QYr.ufs*-6D are shown, as well as the phenotypes of selected QTL combinations. The number of genotypes in each group is indicated in *brackets*. The reaction type scores are represented on an ordinal scale with *1* resistant, *2* resistant–moderately resistant, *3* moderately resistant, *4* moderately resistant–moderately susceptible, *5* moderately susceptible and *6* moderately susceptible–susceptible. *Error bars* show standard errors of the means

53.2%) and was consistently detected by both LAI and RT scores. The association between low LAI and low RT scores would indicate a QTL for stripe rust resistance exhibiting a necrotic phenotype typical of race-specific resistance. Statistical analyses of the LAI and RT scores of the RIL within each QTL group indicated significant interactions between *QYr.ufs-2A* and the three smaller QTL derived from Yr16DH70, indicating that although *QYr.ufs-2A* conferred a significant level of stripe rust resistance this could still be enhanced by small effect QTL. SSR markers defined the location of *QYr.ufs-2A* to the short arm of chromosome 2A. The stripe rust seedling resistance gene *Yr17* was transferred to chromosome 2AS from *Triticum ventricosum* (Helguera et al. 2003) and maps to the same region as *QYr.ufs-2A* (Christiansen et al. 2006). However, the detection of seedling resistance genes is improbable as the population was developed from the seedling susceptible, Cappelle-Desprez derived line Yr16DH70. Furthermore, the release of Cappelle-Desprez predates the development of the *Yr17* translocation. In addition, the 2NS-specific marker VENTRIUP/LN2 (Helguera et al. 2003) was used to show that the *Lr37/Yr17/Sr38* segment is not present in Cappelle-Desprez or Yr16DH70 (Online Resource 5). This does not exclude *QYr.ufs-2A* from representing an adult plant race-specific resistance locus which has not as yet been overcome by South African pathotypes. Boukhatem et al. (2002) detected an APR QTL (*QYR2*) in the cv. Camp Rémy, a descendant of Cappelle-Desprez which is also described as having durable stripe rust resistance. *QYR2* was located to a region on the long arm of chromosome 2A which is defined by the SSR marker loci *Xgwm356* and *Xgwm382*. Mallard et al. (2005) also detected an APR locus in Camp Rémy on chromosome 2A and designated the QTL, *QYr.inra-2AL*. However, the designation of this QTL to the long arm of 2A may be in question, as all the SSR markers in this QTL

interval, except one, GWM382, have been assigned to the short arm of chromosome 2A (Appels 2003). As defined by the 2A chromosome short arm SSR marker loci, *QYr.inra-2AL* and *QYr.ufs-2A* would lie within a common interval. Mallard et al. (2005) attributed between 20 and 40% of the phenotypic variance to *QYr.inra-2AL,* which is comparable to the phenotypic variance explained by *QYr.ufs-2A*. In the study of Boukhatem et al. (2002), *QYR2* accounted for only 15.4% of the phenotypic variation. Bariana et al. (2010) identified an APR QTL proximal to the region defining *QYr.ufs-2A* in the cv. Kukri, linked to the DArT marker *wPt-0003*, a locus flanking the *QYr.ufs-2A* interval. In contrast to *QYr.ufs-2A* this QTL explained only 12–15% of the phenotypic variance and was not detected across years. Recently Lowe et al. (2011) identified a small effect QTL, *QYr.ucw-2AS*, contributing 2.3% to the stripe rust resistance expressed by a synthetic derivative [Croc/*Aegilops tauschii* (Synthetic 205)//Kauz]. *QYr.ucw-2AS* was located between the markers *wPt-3896* and *Xwmc177*, placing it in the same region as *QYr.ufs-2A*. Yet another QTL, *QYr.uga-2AS,* was detected in the *QYr.ufs-2A* interval in the cv. Pioneer 26R61, flanked by the markers *Xbarc124* and *Xgwm359* (Hao et al. 2011). This QTL explained up to 56.0% of the phenotypic variation and was consistently expressed across different environments. The short arm of chromosome 2A is therefore a region of interest in terms of stripe rust resistance.

A gene for stripe rust resistance, *Yr16* was located to the centromeric region of chromosome 2D in Cappelle-Desprez through cytogenetic analyses (Worland and Law 1986; Worland et al. 1988). The gene was placed 9.3 cM from the centromere between RFLP marker loci *Xpsr641-2D* and *Xpsr681-2D* on a consensus map, Ta-Gale-2D (http://wheat.pw.usda.gov/GG2/index.shtml; Devos et al. 1993). Mallard et al. (2005) identified a QTL in the cv. Camp Rémy near the centromere on 2DS, *QYr.inra-2DS*. *QYr.inra-2DS* was located between the loci *Xgwm102* and *Xgwm539* and was responsible for 24–69% of the observed phenotypic variance. In this study, *QYr.ufs-2D* mapped to the same region, although defined by a smaller interval (*Xgwm102-wPt-664520*), but contributed significantly less to the phenotypic variance (maximum of 10.3%). *QYr.inra-2DS* and *QYr.ufs-2D* probably represent the same QTL, the differences in phenotypic variance indicating the influence of genetic background and/or environmental conditions. *QYr.ufs-2D* appears to perform less well under heavy disease pressure, not being detected late in 2009, but still being effective late in the drier, low stripe rust year of 2010. The sensitivity of expression of *QYr.ufs-2D/QYr.inra-2DS* would be supported by the fact that Boukhatem et al. (2002) did not identify a QTL on 2D in their Camp Rémy × Michigan Amber study. Various other stripe rust resistance QTL have been mapped to the short arm of chromosome 2D within the same region as *QYr.ufs-2D*. However, the ancestral relationship between these resistance sources and Cappelle-Desprez is not as clear as for the Cappelle-Desprez Camp Rémy relationship. Temperature-sensitive seedling resistance, *YrCK* was reported on chromosome 2D in the Australian cv. Cook and a derivative, cv. Sunco (Park et al. 1992; Bariana et al. 2001), contributing 13–19% of the phenotypic variance for stripe rust and 9–13% of the phenotypic variance for leaf rust resistance (Navabi et al. 2005). A stripe rust resistance QTL was identified in the Italian cv. Libellula, *QYr.caas-2DS* explaining 8.4–12.1% of the phenotypic variance (Lu et al. 2009), while Suenaga et al. (2003) detected a stripe rust resistance QTL in this region of 2DS in the cv. Oligoculm which explained less than 10% of the phenotypic variance. In the UK cvs Guardian (Melichar et al. 2008) and Claire (Powell 2010), both of which have Cappelle-Desprez in their pedigrees, stripe rust resistance QTL were found that mapped close to the *QYr.ufs-2D* region on the short arm of chromosome 2D.

Cappelle-Desprez carries the reciprocal, centromeric translocations 5BL-7BL and 5BS-7BS (Riley et al. 1967; Badaeva et al. 2007) which were common in Western European wheat cultivars in the 1960s and 1970s (Riley et al. 1967). The 5BS-7BS chromosome was previously shown to contribute substantially to stripe rust resistance in Cappelle-Desprez. The parents of Cappelle-Desprez, Vilmorin-27 and Hybride du Joncquois, both possess this translocation (Law et al. 1978; Law and Worland 1997). However, while Vilmorin-27 had a high level of stripe rust resistance, Hybride du Joncquois was susceptible. Ditelosomic 5BS and 7BS Cappelle-Desprez lines subsequently showed the resistance in Cappelle-Desprez to be located on the 5BS arm, close to the chromosomal breakpoint, while the genetic background in which the 5BS chromosome arm resides also appeared to influence the resistance (Law et al. 1978; Law and Worland 1997). The presence of the 5BS-7BS translocation in Yr16DH70 was confirmed by C-banding data (Pretorius ZA, unpublished data). The marker data placed *QYr.ufs-5B* near to the centromeric region on the long arm of chromosome 5B. It has not been defined exactly where the physical breakpoint of the 5BS-7BS and 5BL-7BL translocations are positioned with respect to the centromeres of each chromosome, therefore *QYr.ufs-5B* may represent the stripe rust resistance located on the short arm of chromosome 5B in Cappelle-Desprez. *QYr.ufs-5B* did not make a large contribution to the stripe rust resistance observed in Yr16DH70, however, lower disease scores were seen for both LAI and RT when *QYr.ufs-5B* was combined with *QYr.ufs-2D*.

Mallard et al. (2005) detected two QTL (*QYr.inra-5B.1* and *QYr.inra-5B.2)* in Camp Rémy which mapped to the telomeric region on the long arm of chromosome 5B.

Based on the known map location of the markers defining these QTL (Appels 2003) the authors suggested that the telomeric end of 5BL contained a translocation of a fragment of 5BS. However, this proposal was not confirmed by cytological data. Boukhatem et al. (2002), in their study of the cv. Camp Rémy, did not identify a QTL on chromosome 5B. Stripe rust resistance QTL on chromosome 5B have been detected in the Italian cvs Libellula and Strampelli (Lu et al. 2009), the Israeli cv. Oligoculm (Suenaga et al. 2003), the Australian cv. Janz (Bariana et al. 2010) and the French cv. Flinor (Feng et al. 2011). In Flinor, two QTL were detected on chromosome 5B which are expressed at the seedling stage and at higher temperatures (*QYr-tem-5B.1* and *QYr-tem-5B.2*; Feng et al. 2011). Flinor shares ancestry with Cappelle-Desprez and one of these QTL, *QYr-tem-5B.1,* overlaps with the region defining *QYr.ufs-5B*. *QYr-tem-5B.1* explained up to 33% of the phenotypic variation in Flinor which is substantially greater than the resistance phenotype explained by *QYr.ufs-5B*. It has yet to be determined if there is a relationship between *QYr-tem-5B.1* and *YrDru* from the cv. Druchamp (Chen et al. 1996).

No stripe rust APR QTL have been mapped to chromosome 6D, although the seedling resistance genes *Yr20* and *Yr23* were located on this chromosome (Chen et al. 1995). *QYr.ufs-6D* represents a small effect QTL, only detected with the LAI scores and then not consistently across years. A small effect QTL was also found on chromosome 4B, *QYr.ufs-4B*, derived from Palmiet. Depending on environment and disease pressure, a low level of stripe rust resistance was observed for Palmiet, supporting the detection of this QTL. QTL for stripe rust APR have been reported on chromosome 4B in the cvs Alcedo (Jagger et al. 2011), Oligoculm (Suenaga et al. 2003), Avocet S (William et al. 2006), Guardian (Melichar et al. 2008) and cvs Libellula and Strampelli (Lu et al. 2009). The stripe rust QTL from Avocet S was also reported to have an effect on leaf rust (William et al. 2006). Except for the QTL from Alcedo, which explained up to 29% of the phenotypic variance, none of the above-mentioned QTL accounted for more than 15% of the phenotypic variance. This is in line with the relatively small effect seen for *QYr.ufs-4B*. However, the large interval defining *QYr.ufs-4B* does not allow direct comparison to these other published 4B QTL.

The RILs were assigned to 32 genotype groups depending on which of the 5 QTL they contained. In general, QTL intervals were fairly large, therefore QTL assignment to RILs was based on the marker closest to the QTL peak (highest LOD) (see Online Resource 4). Selection based on markers flanking the QTL interval would have eliminated several lines from each QTL group, this being most evident for the major QTL *QYr.ufs-2A*. *Xgwm636* was used to select for this QTL, having the highest LOD value with all eight traits, the LOD value associated with the flanking marker *wPt-0003* sharply decreases to below 3.0 with most trait data sets. More variation was generally seen in the QTL interval between traits for the minor QTL. Where the marker locus with the highest LOD differed between trait data sets, markers that flanked this locus were also considered in the assignment of RILs to groups. In the case of the problematic region on chromosome 6D, the *Xgwm325–Xbarc202* interval was considered when assigning RILs to QTL groups. The assignment of each QTL was therefore determined by the resolution of the QTL interval, but for each QTL the same selection criteria were consistently applied. The assignment of RILs into QTL groups using this method was supported by the similar stripe rust resistance phenotypes seen for the RILs within each QTL group. Clearly, the QTL intervals need to be delimited to smaller regions in order to facilitate the use of an interval for QTL identification. This would improve the accuracy of QTL assignment and avoid the incorrect assignment of QTL which have been removed via recombination.

In South Africa, Cappelle-Desprez and the Cappelle-Desprez derived breeding line, Yr16DH70, selected to retain the stripe rust APR found in Cappelle-Desprez, exhibits complete resistance to stripe rust under field conditions. In the UK, stripe rust resistance in Cappelle-Desprez is now only partial (Boyd LA, unpublished data). While the *Yr16* (*QYr.ufs-2D*) resistance is still believed to be effective in the UK, as seen in the UK cvs Guardian (Melichar et al. 2008) and Claire (Powell 2010), it may well be that the major effect QTL, *QYr.ufs-2A* detected in Yr16DH70 is no longer effective against UK pathotypes of *P. striiformis* f. sp. *tritici*. *QYr.ufs-2D*, which is in all likelihood the *Yr16* locus previously reported in Cappelle-Desprez is therefore more likely to be of long term value. The value of *QYr.ufs-5B* and *QYr.ufs-6D* is less clear and an improved linkage map of these QTL intervals would help define their value as stripe rust APR genes. The stripe rust APR QTL from Cappelle-Desprez have already been transferred into a desirable South African spring wheat background through the development of the breeding line, Yr16DH70. This study has now defined these QTL and provides markers by which each QTL can be identified as this breeding line is taken forward in the development of new wheat cultivars.

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Online Resource 1

Article title: **“**Identification of adult plant resistance to stripe rust in the wheat cultivar Cappelle-Desprez”

Journal: Theoretical and Applied Genetics

Authors: Agenbag GM, Pretorius ZA, Boyd LA, Bender CM, Prins R

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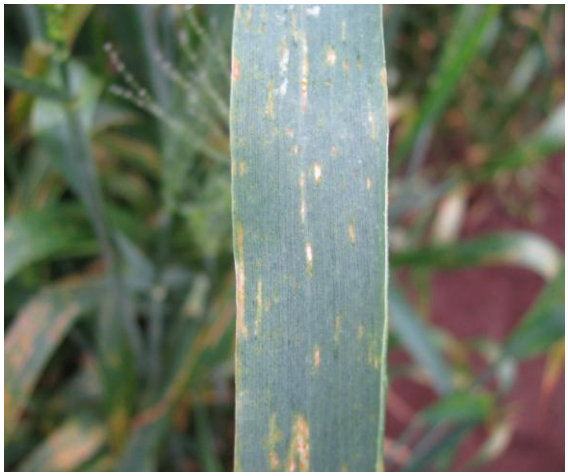
Parental phenotypes

Examples of the parental phenotypes as scored in 2009 at the Greytown field trial. Palmiet scored 70-80MS, while the resistant parent, Yr16DH70 scored 20R.

Palmiet

Yr16DH70



Online Resource 2

Article title: “Identification of adult plant resistance to stripe rust in the wheat cultivar Cappelle-Desprez”

Journal: Theoretical and Applied Genetics

Authors: Agenbag GM, Pretorius ZA, Boyd LA, Bender CM, Prins R

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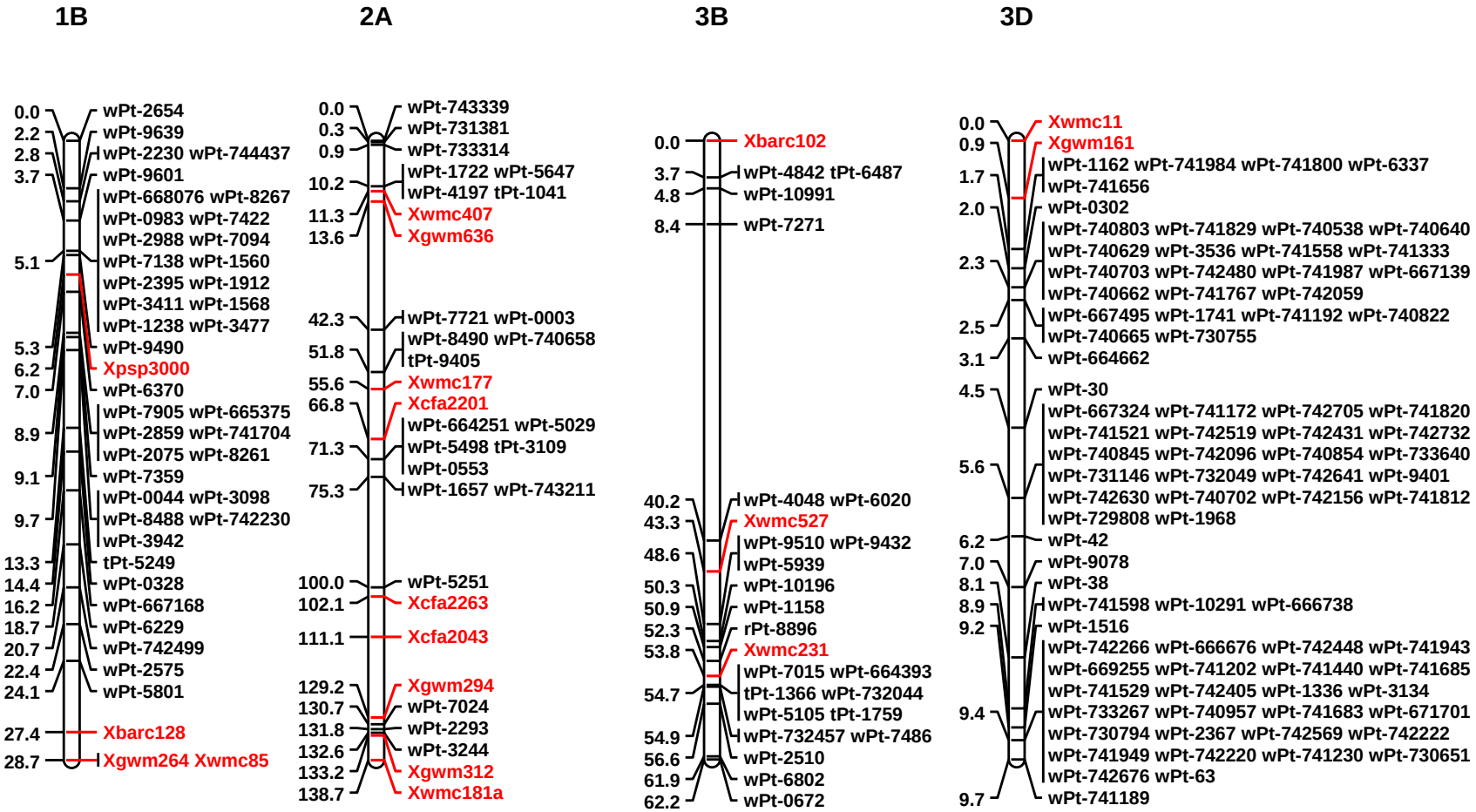
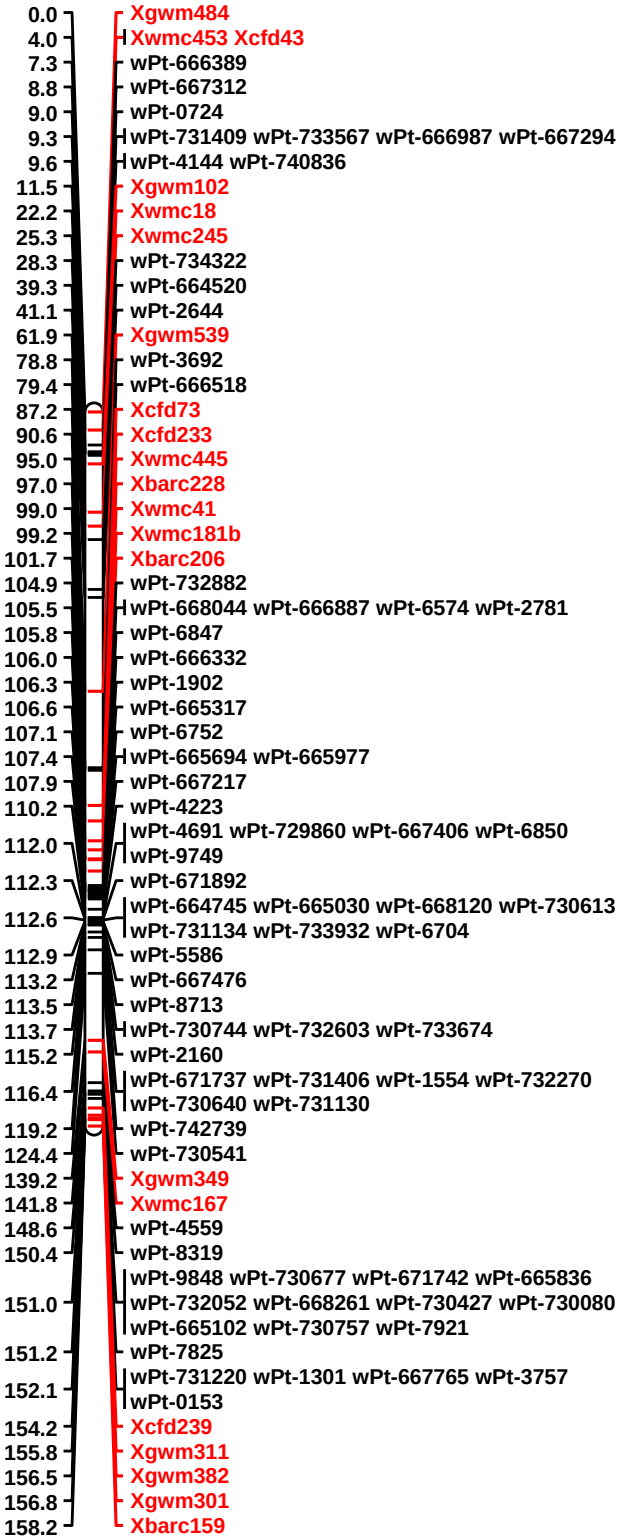
Palmiet X Yr16DH70 linkage map

Linkage map calculated for the Palmiet X Yr16DH70 RIL mapping population showing 15 linkage groups representing 12 chromosomes. SSR markers are in red and DArT markers and other markers types in black. Distances between markers are in centiMorgans.



2D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8.8 wPt-667312
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9.3 wPt-731409 wPt-733567 wPt-666987 wPt-667294
9.6 wPt-4144 wPt-740836
11.5 Xgwm102
22.2 Xwmc18
25.3 Xwmc245
28.3 wPt-734322
39.3 wPt-664520
41.1 wPt-2644
61.9 Xgwm539
78.8 wPt-3692
79.4 wPt-666518
87.2 Xcfd73
90.6 Xcfd233
95.0 Xwmc445
97.0 Xbarc228
99.0 Xwmc41
99.2 Xwmc181b
101.7 Xbarc206
104.9 wPt-732882
105.5 wPt-668044 wPt-666887 wPt-6574 wPt-2781
105.8 wPt-6847
106.0 wPt-666332
106.3 wPt-1902
106.6 wPt-665317
107.1 wPt-6752
107.4 wPt-665694 wPt-665977
107.9 wPt-667217
110.2 wPt-4223
112.0 wPt-4691 wPt-729860 wPt-667406 wPt-6850
wPt-9749
112.3 wPt-671892
112.6 wPt-664745 wPt-665030 wPt-668120 wPt-730613
wPt-731134 wPt-733932 wPt-6704
112.9 wPt-5586
113.2 wPt-667476
113.5 wPt-8713
113.7 wPt-730744 wPt-732603 wPt-733674
115.2 wPt-2160
116.4 wPt-671737 wPt-731406 wPt-1554 wPt-732270
wPt-730640 wPt-731130
119.2 wPt-742739
124.4 wPt-730541
139.2 Xgwm349
141.8 Xwmc167
148.6 wPt-4559
150.4 wPt-8319
151.0 wPt-9848 wPt-730677 wPt-671742 wPt-665836
wPt-732052 wPt-668261 wPt-730427 wPt-730080
wPt-665102 wPt-730757 wPt-7921
151.2 wPt-7825
152.1 wPt-731220 wPt-1301 wPt-667765 wPt-3757
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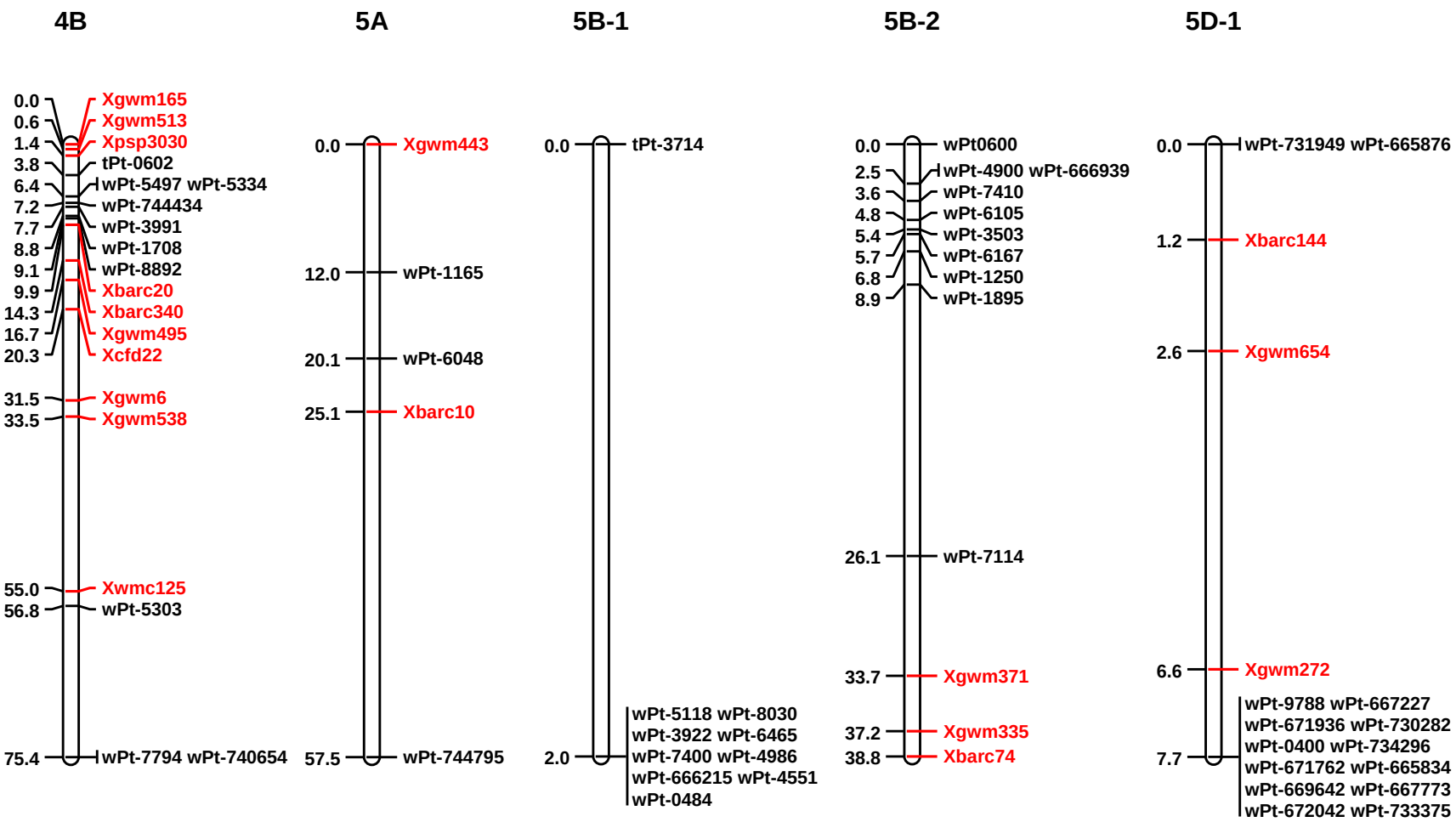
158.2 Xbarc159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1.4 Xpsp3030
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6.4 wPt-5497 wPt-5334
7.2 wPt-744434
7.7 wPt-3991
8.8 wPt-1708
9.1 wPt-8892
9.9 Xbarc20
14.3 Xbarc340
16.7 Xgwm495
20.3 Xcfd22
31.5 Xgwm6
33.5 Xgwm538
55.0 Xwmc125
56.8 wPt-5303
75.4 wPt-7794 wPt-740654
5A
0.0 Xgwm443
12.0 wPt-1165
20.1 wPt-6048
25.1 Xbarc10
57.5 wPt-744795
5B-1
0.0 tPt-3714
2.0 wPt-5118 wPt-8030
wPt-3922 wPt-6465
wPt-7400 wPt-4986
wPt-666215 wPt-4551
wPt-0484
5B-2
0.0 wPt0600
2.5 wPt-4900 wPt-666939
3.6 wPt-7410
4.8 wPt-6105
5.4 wPt-3503
5.7 wPt-6167
6.8 wPt-1250
8.9 wPt-1895
26.1 wPt-7114
33.7 Xgwm371
37.2 Xgwm335
38.8 Xbarc74
5D-1
0.0 wPt-731949 wPt-665876
1.2 Xbarc144
2.6 Xgwm654
6.6 Xgwm272
7.7 wPt-9788 wPt-667227
wPt-671936 wPt-730282
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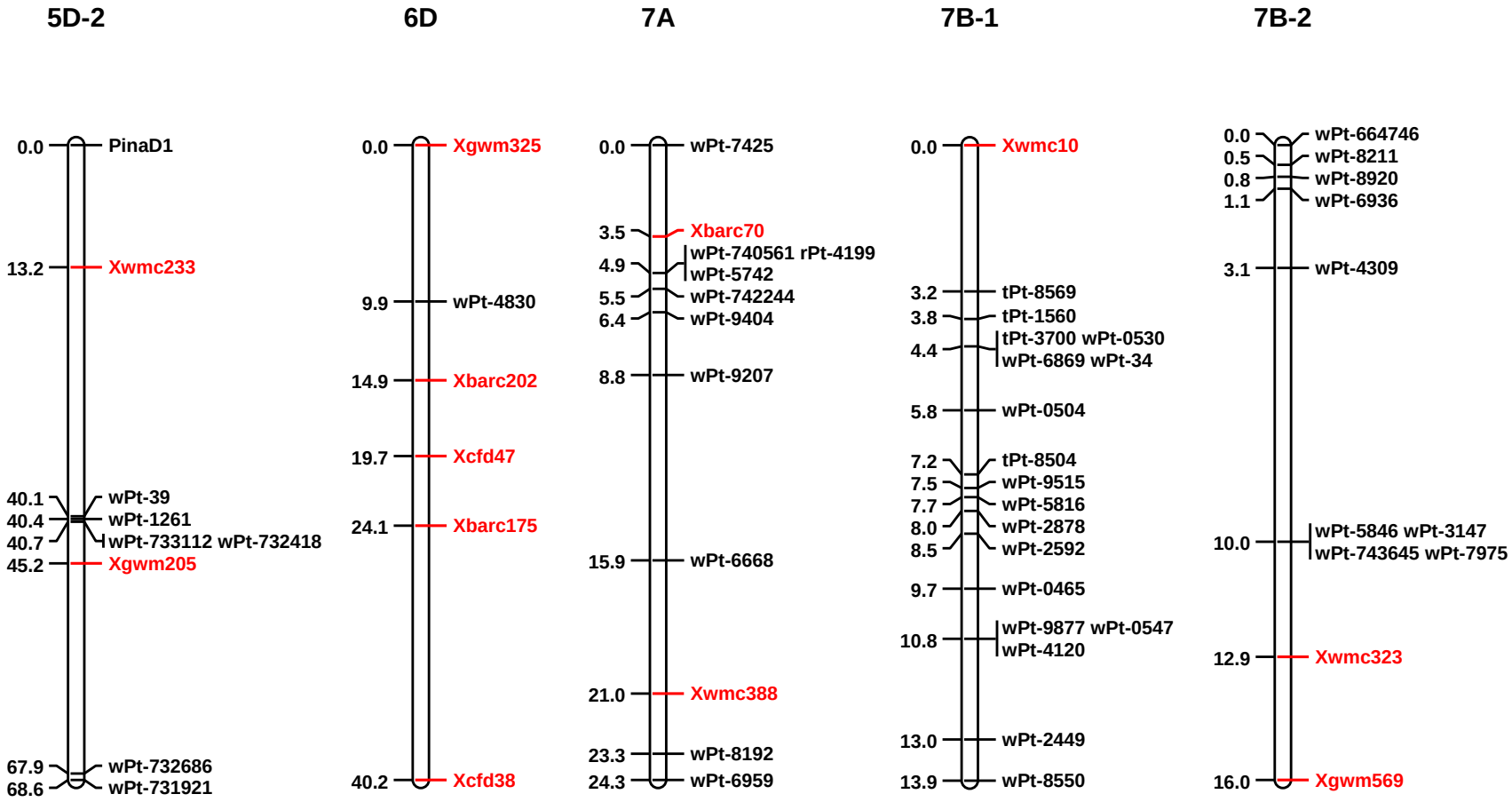
wPt-669642 wPt-667773
wPt-672042 wPt-733375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0.0 PinaD1
13.2 Xwmc233
40.1 wPt-39
40.4 wPt-1261
40.7 wPt-733112 wPt-732418
45.2 Xgwm205
67.9 wPt-732686
68.6 wPt-731921
6D
0.0 Xgwm325
9.9 wPt-4830
14.9 Xbarc202
19.7 Xcfd47
24.1 Xbarc175
40.2 Xcfd38
7A
0.0 wPt-7425
3.5 Xbarc70
4.9 wPt-740561 rPt-4199
wPt-5742
5.5 wPt-742244
6.4 wPt-9404
8.8 wPt-9207
15.9 wPt-6668
21.0 Xwmc388
23.3 wPt-8192
24.3 wPt-6959
7B-1
0.0 Xwmc10
3.2 tPt-8569
3.8 tPt-1560
4.4 tPt-3700 wPt-0530
wPt-6869 wPt-34
5.8 wPt-0504
7.2 tPt-8504
7.5 wPt-9515
7.7 wPt-5816
8.0 wPt-2878
8.5 wPt-2592
9.7 wPt-0465
10.8 wPt-9877 wPt-0547
wPt-4120
13.0 wPt-2449
13.9 wPt-8550
7B-2
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0.5 wPt-8211
0.8 wPt-8920
1.1 wPt-6936
3.1 wPt-4309
10.0 wPt-5846 wPt-3147
wPt-743645 wPt-7975
12.9 Xwmc323
16.0 Xgwm569

Online Resource 3

Article title: “Identification of adult plant resistance to stripe rust in the wheat cultivar Cappelle-Desprez”

Journal: Theoretical and Applied Genetics

Authors: Agenbag GM, Pretorius ZA, Boyd LA, Bender CM, Prins R

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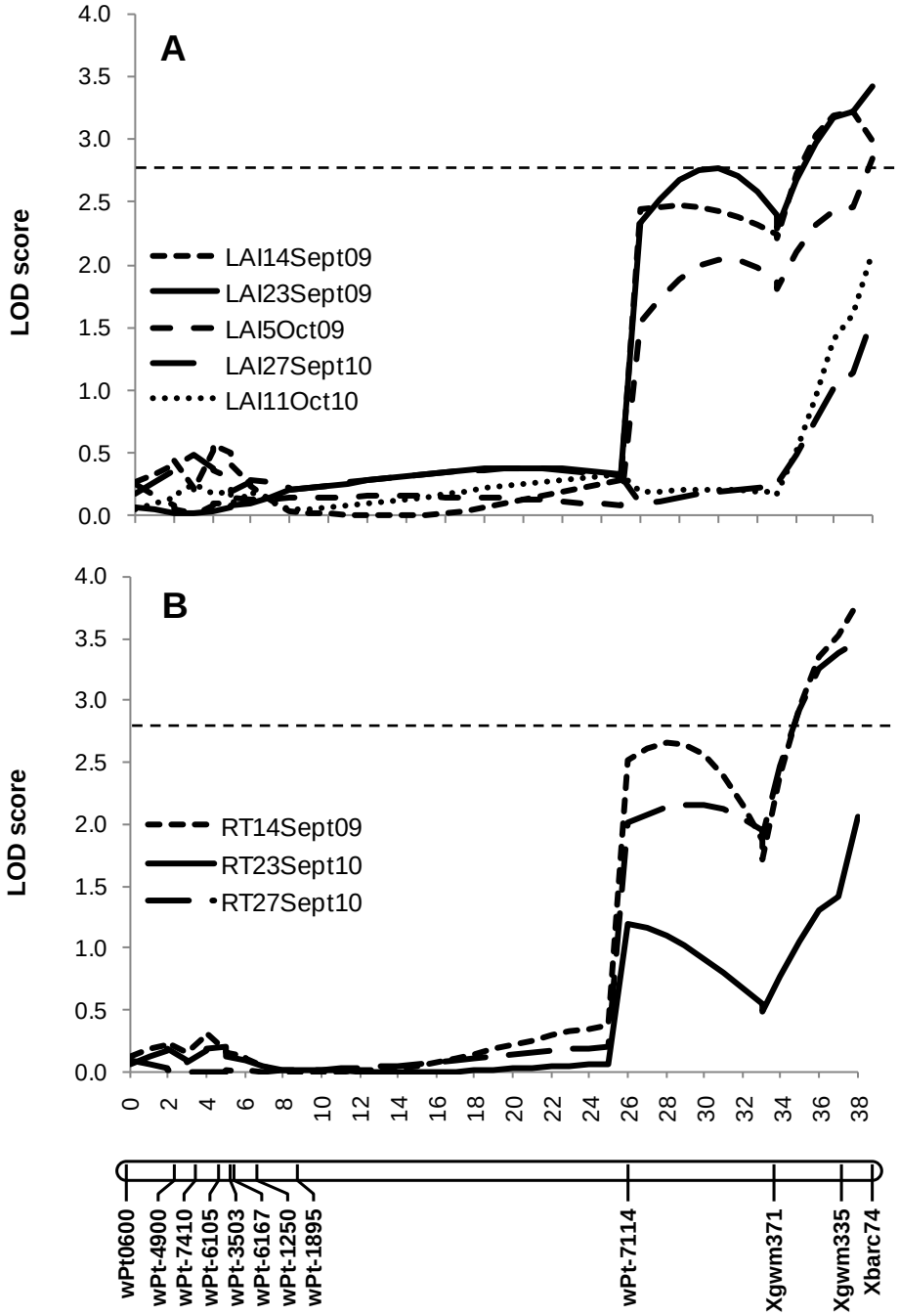
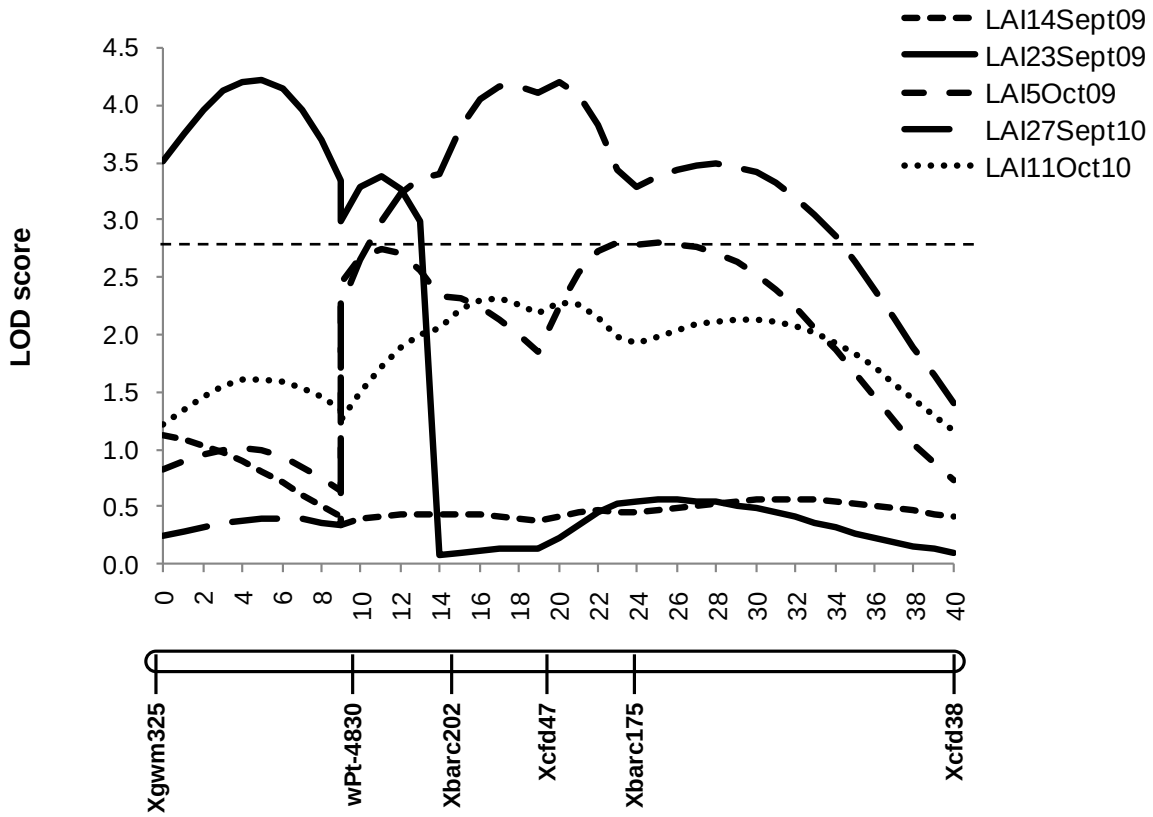
QYr.ufs-5B

Minor stripe rust resistance QTL, *QYr.ufs-5B* on chromosome 5B identified with (A) leaf area infected (LAI) and (B) reaction type (RT) data sets. The distances between markers are shown in centiMorgans. The LOD threshold, as determined from 1000 permutations using Cartographer v2.51 (Wang et al. 2011) is indicated with a dashed line



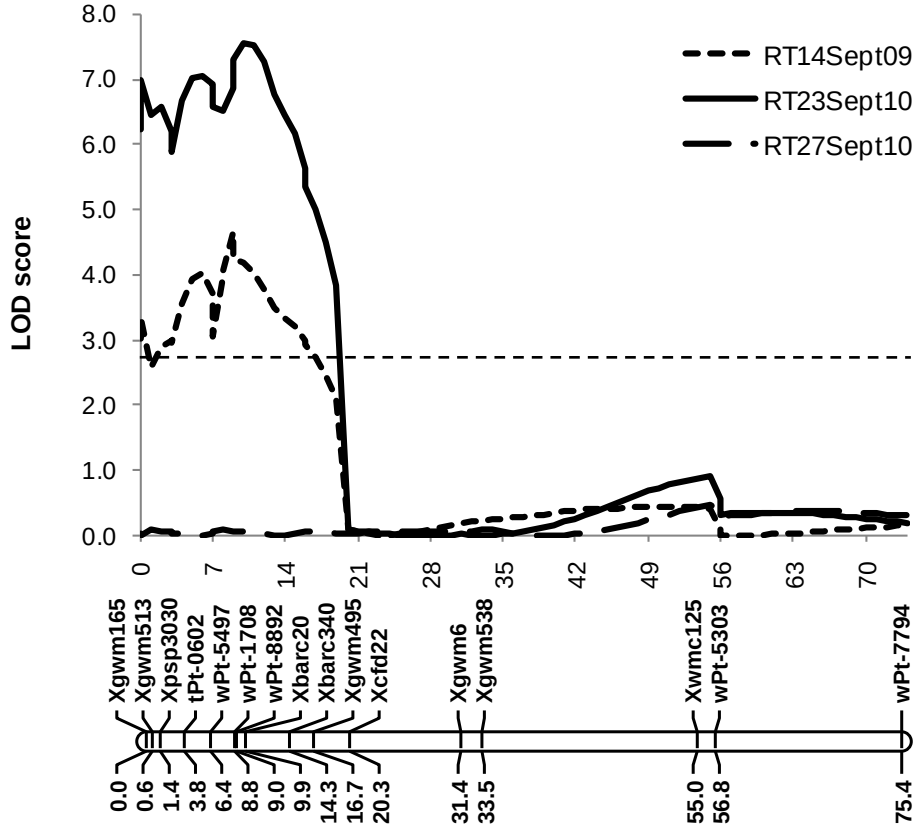
QYr.ufs-6D

Minor stripe rust resistance QTL, *QYr.ufs-6D* on chromosome 6D identified with leaf area infected (LAI) data sets. The distances between markers are shown in centiMorgans. The LOD threshold, as determined from 1000 permutations using Cartographer v2.51 (Wang et al. 2011) is indicated with a dashed line. RT traits were not significant



QYr.ufs-4B

Minor stripe rust resistance QTL, *QYr.ufs-4B* on chromosome 4B identified with reaction type (RT) data sets. The distances between markers are shown in centiMorgans. The LOD threshold, as determined from 1000 permutations using Cartographer v2.51 (Wang et al*.* 2011) is indicated with a dashed line. LAI traits were not significant



Online Resource 4

Article title: "Identification of adult plant resistance to stripe rust in the wheat cultivar Cappelle-Desprez

Journal: Theoretical and Applied Genetics

Authors: Agenbag GM, Pretorius ZA, Boyd LA, Bender CM, Prins R

Corresponding author: Renée Prins
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Distribution of RIL in QTL classes

The phenotypic data for the eight traits are shown for each RIL. In general, the QTL each RIL carries was determined based on the genotype of the marker with the highest LOD (in red), but markers in close vicinity were also considered.

	Traits									QTL				
									QTL markers	Xgwm636	Xwmc245-wPt-734322	wPt-8892-Xbarc20	Xgwm335-Xbarc74	Xgwm325-barc202
RIL	LAI14Sept09	LAI23Sept09	LAI5Oct09	LAI27Sept10	LAI11Oct10	RT14Sept09	RT23Sept09	RT27Sept10	QTL group	2A QTL	2D QTL	4B QTL	5B QTL	6D QTL
75	2.5	30	35	7.5	13	1	1	1	1	2A	2D	4B	5B	6D
79	2.5	28	35	10	7.5	1	1	1	1	2A	2D	4B	5B	6D
101	5	35	40	7.5	10	1	1	1.5	1	2A	2D	4B	5B	6D
113	7.5	25	30	5	7.5	1	1	1	1	2A	2D	4B	5B	6D
9	18	40	45	18	33	2	3	2.5	2	2A	2D	4B	5B	not
88	0	30	30	7.5	10	1	1	1	2	2A	2D	4B	5B	not
92	0	35	40	10	15	1	1	1	2	2A	2D	4B	5B	not
95	0	25	30	7.5	13	1	1	1.5	2	2A	2D	4B	5B	not
122	2.5	35	40	18	25	1.5	2.5	1.5	2	2A	2D	4B	5B	not
134	5	40	45	18	30	1.5	3	3	2	2A	2D	4B	5B	not
179	2.5	15	25	5	13	1.5	1	1.5	2	2A	2D	4B	5B	not
200	7.5	40	50	15	23	1	1	1.5	2	2A	2D	4B	5B	not
201	5	20	23	10	18	1	1	1.5	2	2A	2D	4B	5B	not
98	2.5	23	28	7.5	15	1.5	1	1.5	3	2A	2D	4B	not	6D
128	7.5	45	50	10	18	1	1	1	3	2A	2D	4B	not	6D
147	0	28	30	7.5	10	1	1	1.5	3	2A	2D	4B	not	6D
156	0	25	30	13	20	1	1.5	1	3	2A	2D	4B	not	6D
5	2.5	50	55	10	13	1	1	1	4	2A	2D	4B	not	not
18	0	30	40	7.5	15	1	1.5	1	4	2A	2D	4B	not	not
50	10	35	40	18	23	2.5	1.5	1.5	4	2A	2D	4B	not	not
151	10	40	45	18	25	2	1.5	2	4	2A	2D	4B	not	not
166	10	50	55	13	20	1	1	1	4	2A	2D	4B	not	not
1	2.5	20	30	15	23	1	1	1.5	5	2A	2D	not	5B	6D

	Traits									QTL				
									QTL markers	Xgwm636	Xwmc245-wPt-734322	wPt-8892-Xbarc20	Xgwm335-Xbarc74	Xgwm325-barc202
RIL	LAI14Sept09	LAI23Sept09	LAI5Oct09	LAI27Sept10	LAI11Oct10	RT14Sept09	RT23Sept09	RT27Sept10	QTL group	2A QTL	2D QTL	4B QTL	5B QTL	6D QTL
8	0	10	28	7.5	15	1	1	1	5	2A	2D	not	5B	6D
20	2.5	20	30	5	15	1.5	1	1	5	2A	2D	not	5B	6D
84	7.5	35	40	15	25	2	3.5	2	5	2A	2D	not	5B	6D
110	0	25	40	7.5	13	1	1	1	5	2A	2D	not	5B	6D
148	0	20	28	7.5	20	1	1	1	5	2A	2D	not	5B	6D
161	0	25	35	10	15	1	1	1	5	2A	2D	not	5B	6D
177	0	18	35	10	15	1	1	1	5	2A	2D	not	5B	6D
194	0	23	30	13	23	1	2	1	5	2A	2D	not	5B	6D
195	0	25	35	7.5	18	1	1	1	5	2A	2D	not	5B	6D
196	5	40	50	15	20	2	5	2	5	2A	2D	not	5B	6D
26	10	35	40	18	20	2.5	3	2	6	2A	2D	not	5B	not
28	5	25	30	10	13	1	1	1.5	6	2A	2D	not	5B	not
29	7.5	40	45	15	23	1	3	1	6	2A	2D	not	5B	not
39	2.5	35	35	13	18	1.5	1.5	1	6	2A	2D	not	5B	not
49	0	25	30	7.5	15	1	1	1.5	6	2A	2D	not	5B	not
81	2.5	25	35	10	15	1	1	1.5	6	2A	2D	not	5B	not
94	0	30	50	18	23	1	1	1	6	2A	2D	not	5B	not
3	10	35	45	13	23	2.5	1.5	1	7	2A	2D	not	not	6D
32	2.5	40	55	13	20	1	3	1.5	7	2A	2D	not	not	6D
34	2.5	35	45	18	23	1	1.5	1.5	7	2A	2D	not	not	6D
142	0	25	33	10	18	1	1.5	1	7	2A	2D	not	not	6D
167	0	20	23	5	10	1	1	1	7	2A	2D	not	not	6D
10	2.5	25	35	5	18	1	1.5	1	8	2A	2D	not	not	not
60	5	25	35	13	20	1	1	1.5	8	2A	2D	not	not	not
74	2.5	35	45	7.5	15	1	2	1.5	8	2A	2D	not	not	not
7	5	30	35	23	25	1	1.5	1.5	9	2A	not	4B	5B	6D
61	0	28	35	15	35	1	2	2.5	9	2A	not	4B	5B	6D
90	7.5	35	40	13	20	1	2.5	1.5	9	2A	not	4B	5B	6D
141	0	13	23	7.5	10	1	1	1	9	2A	not	4B	5B	6D
22	5	35	40	10	20	1	1	2	10	2A	not	4B	5B	not
93	2.5	25	35	10	18	1	2	1.5	10	2A	not	4B	5B	not
203	0	23	25	15	23	1	1	1	10	2A	not	4B	5B	not
6	2.5	28	30	13	20	2	2	1.5	11	2A	not	4B	not	6D
43	2.5	28	35	13	20	2	1.5	2	12	2A	not	4B	not	not
57	18	65	85	15	45	3	5.5	2	12	2A	not	4B	not	not
132	7.5	60	60	20	40	2.5	4.5	3	12	2A	not	4B	not	not
12	0	18	28	7.5	15	1	2	1	13	2A	not	not	5B	6D
33	0	28	40	13	20	1	2	2	13	2A	not	not	5B	6D
51	10	30	40	13	20	2.5	3.5	1.5	13	2A	not	not	5B	6D
55	2.5	28	35	13	30	1	3	2.5	13	2A	not	not	5B	6D
97	0	18	28	10	13	1	2	1	13	2A	not	not	5B	6D
118	7.5	25	30	10	20	2.5	1.5	2	13	2A	not	not	5B	6D
121	15	60	70	18	30	3.5	5	2	13	2A	not	not	5B	6D
145	2.5	23	35	13	18	1.5	1.5	1.5	13	2A	not	not	5B	6D
173	13	40	55	13	18	2	3.5	1.5	13	2A	not	not	5B	6D

	Traits									QTL				
									QTL markers	Xgwm636	Xwmc245-wPt-734322	wPt-8892-Xbarc20	Xgwm335-Xbarc74	Xgwm325-barc202
RIL	LAI14Sept09	LAI23Sept09	LAI5Oct09	LAI27Sept10	LAI11Oct10	RT14Sept09	RT23Sept09	RT27Sept10	QTL group	2A QTL	2D QTL	4B QTL	5B QTL	6D QTL
64	5	30	40	13	18	2	3	1.5	14	2A	not	not	5B	not
73	5	35	45	20	30	1	3.5	2.5	14	2A	not	not	5B	not
96	0	28	40	13	18	1	4	1	14	2A	not	not	5B	not
119	0	30	35	13	23	1	2	1.5	14	2A	not	not	5B	not
184	7.5	35	40	10	15	1.5	4	1.5	14	2A	not	not	5B	not
187	0	28	33	15	20	1	1.5	1.5	14	2A	not	not	5B	not
188	2.5	35	35	15	20	2.5	1.5	1.5	14	2A	not	not	5B	not
197	0	25	30	10	15	1	1.5	1.5	14	2A	not	not	5B	not
76	2.5	30	45	10	25	1	3.5	2	15	2A	not	not	not	6D
82	7.5	25	28	7.5	13	2	1	1.5	15	2A	not	not	not	6D
87	2.5	28	35	13	15	1	3	1.5	15	2A	not	not	not	6D
144	2.5	23	35	10	15	2	2	1.5	15	2A	not	not	not	6D
155	18	55	55	28	50	3	4	2.5	15	2A	not	not	not	6D
112	5	25	35	10	20	1.5	1.5	1.5	16	2A	not	not	not	not
178	2.5	30	40	5	10	2	3	1	16	2A	not	not	not	not
16	10	45	65	20	28	1	1	1	17	not	2D	4B	5B	6D
100	5	30	40	13	18	1	1	1	17	not	2D	4B	5B	6D
138	20	45	50	30	50	1	3	3	17	not	2D	4B	5B	6D
139	2.5	18	25	10	18	1	1.5	1	17	not	2D	4B	5B	6D
181	15	35	40	13	25	1.5	3	1.5	17	not	2D	4B	5B	6D
191	5	35	35	15	23	2.5	2	2	17	not	2D	4B	5B	6D
204	10	45	50	7.5	18	1	1.5	1	17	not	2D	4B	5B	6D
111	0	40	50	13	20	1	1	1	18	not	2D	4B	5B	not
115	2.5	20	25	7.5	15	1	1	1	18	not	2D	4B	5B	not
136	7.5	50	60	13	18	1	1	1	18	not	2D	4B	5B	not
158	7.5	45	45	18	28	1.5	1	1.5	18	not	2D	4B	5B	not
172	20	45	45	13	20	2	1.5	1	18	not	2D	4B	5B	not
199	10	35	40	18	30	1	2	1.5	18	not	2D	4B	5B	not
23	7.5	40	50	15	33	3	1	2.5	19	not	2D	4B	not	6D
44	2.5	30	30	13	10	1	1	2	19	not	2D	4B	not	6D
102	5	30	45	15	25	1.5	3	1	19	not	2D	4B	not	6D
120	13	45	60	15	28	2	4	1.5	19	not	2D	4B	not	6D
48	7.5	28	35	10	20	2	1.5	2.5	20	not	2D	4B	not	not
114	0	30	35	10	13	1	1	1.5	20	not	2D	4B	not	not
54	0	25	30	7.5	15	1	1.5	1.5	21	not	2D	not	5B	6D
71	15	35	35	15	25	2.5	2	1.5	21	not	2D	not	5B	6D
124	7.5	45	55	15	20	2.5	3.5	1	21	not	2D	not	5B	6D
129	0	30	40	10	15	1	1	1.5	21	not	2D	not	5B	6D
130	0	40	50	23	33	1	4.5	2	21	not	2D	not	5B	6D
131	7.5	30	40	13	18	2	1.5	1	21	not	2D	not	5B	6D
186	2.5	23	33	10	18	1	1.5	1	21	not	2D	not	5B	6D
35	5	55	70	25	30	1.5	4.5	1.5	22	not	2D	not	5B	not
38	10	45	55	20	30	3.5	3.5	2	22	not	2D	not	5B	not
78	5	28	30	10	15	1	1	1.5	22	not	2D	not	5B	not
80	7.5	33	45	15	18	1.5	1	1	22	not	2D	not	5B	not

	Traits									QTL				
									QTL markers	Xgwm636	Xwmc245-wPt-734322	wPt-8892-Xbarc20	Xgwm335-Xbarc74	Xgwm325-barc202
RIL	LAI14Sept09	LAI23Sept09	LAI5Oct09	LAI27Sept10	LAI11Oct10	RT14Sept09	RT23Sept09	RT27Sept10	QTL group	2A QTL	2D QTL	4B QTL	5B QTL	6D QTL
117	7.5	40	55	20	30	1.5	3.5	2	22	not	2D	not	5B	not
126	5	30	40	10	20	2	2.5	1	22	not	2D	not	5B	not
153	0	35	70	15	28	1	1	1	22	not	2D	not	5B	not
182	13	45	50	15	25	2.5	3.5	1.5	22	not	2D	not	5B	not
13	7.5	55	70	20	40	4	5	2.5	23	not	2D	not	not	6D
17	7.5	33	45	13	23	2.5	3.5	1	23	not	2D	not	not	6D
162	7.5	50	55	18	23	1.5	4.5	1.5	23	not	2D	not	not	6D
4	5	50	65	25	28	2.5	5	2	24	not	2D	not	not	not
15	23	65	80	40	60	3.5	4	4.5	24	not	2D	not	not	not
47	13	45	55	23	28	3	4.5	2	24	not	2D	not	not	not
190	13	50	55	18	33	4.5	5	1.5	24	not	2D	not	not	not
37	5	40	45	18	25	1.5	3	2	25	not	not	4B	5B	6D
40	13	60	75	13	35	2.5	4	1.5	25	not	not	4B	5B	6D
53	10	40	50	20	43	2.5	2	2.5	25	not	not	4B	5B	6D
56	10	40	55	20	35	2	3	2	25	not	not	4B	5B	6D
59	5	35	40	18	33	1	4	2.5	25	not	not	4B	5B	6D
68	2.5	40	40	15	23	1	4	2	25	not	not	4B	5B	6D
140	2.5	23	30	13	20	1	1	1.5	25	not	not	4B	5B	6D
152	10	40	33	20	40	2.5	2	3	25	not	not	4B	5B	6D
171	7.5	40	40	10	15	1.5	1.5	1.5	25	not	not	4B	5B	6D
180	15	55	55	20	40	2.5	4.5	2	26	not	not	4B	5B	not
198	0	30	35	10	13	1	1.5	1	26	not	not	4B	5B	not
11	15	40	50	20	30	1.5	2.5	2.5	27	not	not	4B	not	6D
137	10	35	35	18	33	1	2.5	3	27	not	not	4B	not	6D
175	30	50	65	7.5	20	2	3.5	1.5	27	not	not	4B	not	6D
185	10	40	45	35	55	1	3	4	27	not	not	4B	not	6D
2	20	70	85	28	55	4.5	5.5	3	28	not	not	4B	not	not
21	30	55	65	45	55	3	4.5	4	28	not	not	4B	not	not
36	7.5	55	80	35	65	3.5	5	3	28	not	not	4B	not	not
83	13	50	65	30	45	2	3.5	4.5	28	not	not	4B	not	not
86	25	60	70	23	45	3	3	4	28	not	not	4B	not	not
154	15	40	45	20	33	2.5	2	2.5	28	not	not	4B	not	not
45	10	50	70	20	30	3.5	4.5	2	29	not	not	not	5B	6D
62	10	40	55	18	23	3	3.5	1.5	29	not	not	not	5B	6D
99	5	35	50	13	20	2	4	1.5	29	not	not	not	5B	6D
170	15	60	70	20	35	4.5	6	3.5	29	not	not	not	5B	6D
30	20	65	80	30	40	4	5.5	2	30	not	not	not	5B	not
31	15	45	55	20	35	2.5	5	2.5	30	not	not	not	5B	not
58	2.5	40	50	25	35	1	1	1	30	not	not	not	5B	not
108	15	60	70	25	43	4	5	2.5	30	not	not	not	5B	not
109	5	35	35	15	23	1	1	2	30	not	not	not	5B	not
135	7.5	50	50	20	38	2	4.5	3	30	not	not	not	5B	not
163	15	60	70	30	35	2.5	5.5	3	30	not	not	not	5B	not
24	10	50	55	20	28	2.5	4	2.5	31	not	not	not	not	6D
63	25	45	50	15	18	3	3	1.5	31	not	not	not	not	6D

	Traits									QTL				
									QTL markers	Xgwm636	Xwmc245-wPt-734322	wPt-8892-Xbarc20	Xgwm335-Xbarc74	Xgwm325-barc202
RIL	LAI14Sept09	LAI23Sept09	LAI5Oct09	LAI27Sept10	LAI11Oct10	RT14Sept09	RT23Sept09	RT27Sept10	QTL group	2A QTL	2D QTL	4B QTL	5B QTL	6D QTL
85	18	35	45	10	15	1.5	2.5	1.5	31	not	not	not	not	6D
116	5	23	35	10	15	2.5	2	1	31	not	not	not	not	6D
149	18	50	60	20	30	3.5	4	2	31	not	not	not	not	6D
157	15	55	55	18	25	3	4.5	1.5	31	not	not	not	not	6D
165	0	25	28	13	20	1	1.5	1.5	31	not	not	not	not	6D
193	5	45	45	15	28	1.5	4.5	2	31	not	not	not	not	6D
46	23	55	70	20	35	3	4.5	2	32	not	not	not	not	not
52	30	60	70	30	48	3.5	5.5	3	32	not	not	not	not	not
66	7.5	55	65	30	60	3	5	3	32	not	not	not	not	not
103	18	70	85	33	50	4.5	6	3	32	not	not	not	not	not
146	10	65	75	28	38	4	5	3.5	32	not	not	not	not	not
159	13	50	55	18	23	4	5	1.5	32	not	not	not	not	not

Online Resource 5

Article title: “Identification of adult plant resistance to stripe rust in the wheat cultivar Cappelle-Desprez”

Journal: Theoretical and Applied Genetics

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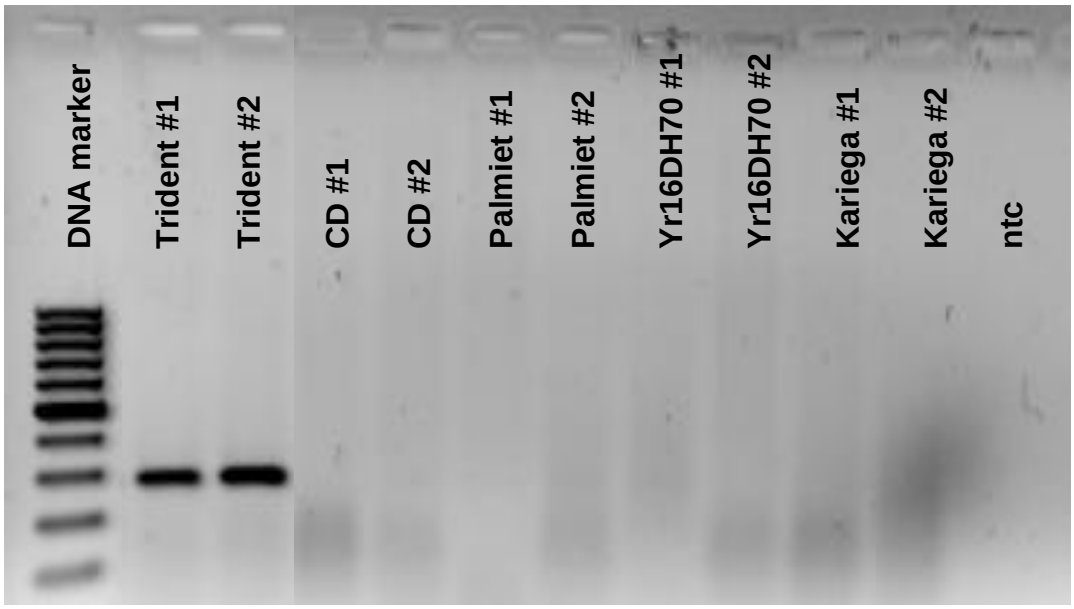
Yr17 marker screen

Although the presence of *Yr17* was not expected, the 2NS-specific marker *VENTRIUP/LN2* (Helguera et al. 2003) was tested in Cappelle-Desprez and Yr16DH70. The PCR protocol for *VENTRIUP/LN2*, described by Helguera et al. (2003), was modified and products were separated on agarose. The cv. Trident served as the positive control for the gene (Seah et al. 2001) and cv. Kariega as the negative control.

PCR amplification with the 2NS-specific marker *VENTRIUP/LN2* in Trident, Cappelle-Desprez (CD), Palmiet, Yr16DH70 and Kariega, separated on 1% (w/v) agarose (0.5X TBE). Cultivar lines were amplified in duplicate. A 100 bp DNA marker (Fermentas) is shown in the first lane and a non-template control (ntc) in the last lane. The 2NS-specific amplicon of 259 bp from *Ae. ventricosa* was present in the control (cv. Trident), but absent in Cappelle-Desprez, Yr16DH70, Palmiet and the negative control, cv. Kariega.



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