Molecular Marker assisted Selection for Crown Rot Resistance in *Triticum turgidum* ssp. *durum*

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy at the University of Southern Queensland

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Abstract

Multicolour fluorescence *in situ* hybridisation (MCFISH), Simple Sequence Repeat markers (SSR) and Diversity Arrays Technology (DArT) markers were employed in this study to analyse six generations of interspecific wheat crosses between a Crown Rot (CR) resistant hexaploid wheat (*Triticum aestivum* L. var. 2-49) and three tetraploid durum wheat lines (*T. turgidum* L. spp. *durum* (Desf.) var. M= 961111 (Bellaroi); N= 950329; O=971179), over the years 2005-2009.

In the F_2 progeny from a 2-49/Bellaroi cross, 82 out of 83 F_2 plants investigated with DArT analysis carried some D genome material, principally as entire chromosomes, while 40 plants included at least one complete copy of all seven D genome chromosomes. Twelve plants containing partial D chromosomes were identified. MCFISH analysis of 26 additional F_2 plants of the same cross showed that all 26 plants contained varying amounts of D genome material of which three carried single A-D translocations. In addition, two telocentric D genome chromosomes were detected. The D genome content of each line and the breakpoint positions of the three A-D translocations were confirmed with DArT marker analysis. Overall results indicate a random recombination of A and B genome loci from the hexaploid female parent and the tetraploid male parent in this F_2 population and a significant retention of the maternal D genome material.

SSR markers linked to CR seedling resistance in 2-49 in previous studies were useful for the prediction of CR resistance in the crosses. DArT analysis of 191 F_7 plants revealed significant QTL for CR on chromosomes 1A and 4B. D chromatin was not responsible for CR resistance, as it was progressively eliminated throughout successive generations.

This study illustrates that the combined application of SSR markers, MCFISH and DArT techniques provides a powerful approach for the analysis of crosses between cereal genotypes of different ploidy.

Certification of Dissertation

I certify that the ideas, experimental work, results, analyses and conclusions reported in this dissertation are entirely my own effort, except were otherwise acknowledged. I also certify that the work is original and has not been previously submitted for any other award, except where otherwise acknowledged.

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Signature of Candidate Date

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Signature of Supervisors Date

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Publications

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Martin A., Simpfendorfer S., Hare R.A., Eberhard F.S., Sutherland M.W. (2011) Retention of D genome chromosomes in pentaploid wheat crosses. Heredity 107: 315-319.

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Glossary of Abbreviations

AA, BB, DD, JJ, GG	Wheat genomes
ABA	Abscisic acid
AFLP	Amplified fragment length polymorphism
BAC	Bacterial artificial chromosome
BARC	Name of Molecular marker
BLAST	Basic local alignment search tool
Bp	Base pairs
cDNA	complementary Deoxyribonucleic Acid
CFD	Name of Molecular Marker
CIMMYT	Centro Internacional de Mejoramiento de Maíz y Trigo
CR	Crown Rot
DART	Diversity arrays technology
DAS	Diacetoxyscirpenol
DL, DS	Long arm, short arm (of a chromosome)
DHN	Dehydrin gene
DON	Deoxynivalenol
EST	Expressed sequence tag
F_1, F_2	Generations
Fa	*Fusarium avenaceum*
Fc	*Fusarium culmorum*
Fg	*Fusarium graminearum*
FHB	*Fusarium* head blight
Fp	*Fusarium pseudograminearum*
FX	FusarenonX
GISH	Genome In situ hybridization
GLU	Glutenin
GWM	Gatersleben Wheat Microsatellie marker
LOD	Logarithm (base 10) of odds
LRS	Likelihood ratio statistics

M1	First division in meiosis
MAS	Marker assisted selection
MCFISH	Multicolour fluorescent in situ hybridization
MJ	Methyl Jasmonate
NIV	Nivalenol
PCR	Polymerase chain reaction
PMC	Pollen mother cell
QTL	Quantitative trait loci
RAPD	Random amplification of polymorphic DNA
RFLP	Restriction fragment length polymorphism
Rht1	Dwarfing gene
RNA	Ribonucleic acid
SSR	Simple sequence repeats
STEDV	Standard deviation
STS	Sequence tagged site
T-chromosome	Translocation chromosome
t-chromosome	Telocentric chromosome
TE	Tris EDTA Buffer
TRI5	Trichodiene synthase
TW	Test weight
WMC	Wheat Microsatellite Consortium marker
ZEA	Zearalenone

1 LITERATURE REVIEW

1.1 INTRODUCTION

1.1.1 IMPORTANCE OF DURUM IN AUSTRALIA

Two major wheat species are currently grown commercially in Australia: bread wheat (*Triticum aestivum* L.) and durum wheat (*Triticum turgidum* ssp. *durum* L). Bread wheat has the chromosome constitution AABBDD (2n= 6x = 42), implying it is hexaploid, whereas the tetraploid durum wheat has the chromosome constitution AABB (2n= 4x= 28). Durum wheat is mainly used for pasta and semolina production. Durum was first tested for use in Australia by the pioneering wheat breeder William Farrer at the beginning of the last century (Kingsbury et al. 2009), but not much demand existed then, as most pasta was made from bread wheat. Even today little durum is grown in Australia compared to other countries. The yearly Australian durum production is currently about 500,000 tons, 200,000t of which is being exported, mainly to Italy (70%) and North Africa (NSW Department of Primary Industries, Durum Wheat Report 2008). By comparison, bread wheat production was around 22 million tons in 2008 and 2009 (FAO 2009). Durum wheat originating from Australia is considered to be of the finest quality worldwide. Durum wheat production is very important for the Australian wheat industry as it is priced almost twice as high as bread wheat on world markets. The amount of durum wheat produced in Australia has increased in the last 10 years (Australian Government 2009). But dry weather and Crown Rot disease are constraining further growth. For example, the production in South Australia sank from 220,000 tons in the years 2004- 2005 to just 50,000 tons in 2006– 2007 (AWB Ltd. 2008, Table 1-1).

Table 1-1: Historical durum wheat production in (t) in Australia during the years 1994 -2006.

Historical durum wheat production in Australia						
Season	Qld	NSW	Vic	SA	WA	Total (t)
1994–95	2000	2000	0	33,000	0	37,000
1995–96	6000	55,000	0	65,000	1000	127,000
1996–97	5997	210,000	0	51,000	0	266,997
1997–98	4971	200,600	0	82,601	0	288,172
1998–99	10,737	303,730	0	84,429	984	399,880
1999–00	51,382	527,358	0	142,423	5120	726,283
2000–01	6334	138,696	0	269,524	4009	418,830
2001–02	6033	380,696	0	405,565	4142	796,283
2002–03	8100	55,000	0	162,000	3000	228,100
2003–04	47,700	337,000	2000	217,900	6895	611,495
2004–05	50,000	375,000	2000	220,000	7000	654,000
2005–06	16,230	297,135	6500	117,086	5200	442,151
2006–07	10,000	125,000	10,000	50,000	5000	200,000
5 YearØ	26,406	237,827	5125	153,397	5419	427,149
Source: Australian Wheat Board Ltd. 2008						

In Australia, most durum wheat is grown in northern New South Wales, south east Queensland and South Australia (Figure 1-1), although production is currently expanding in Western Australia. In NSW, most durum is grown in the area from Moree to Narrabri to Inverell close to the Queensland border (SBS food).

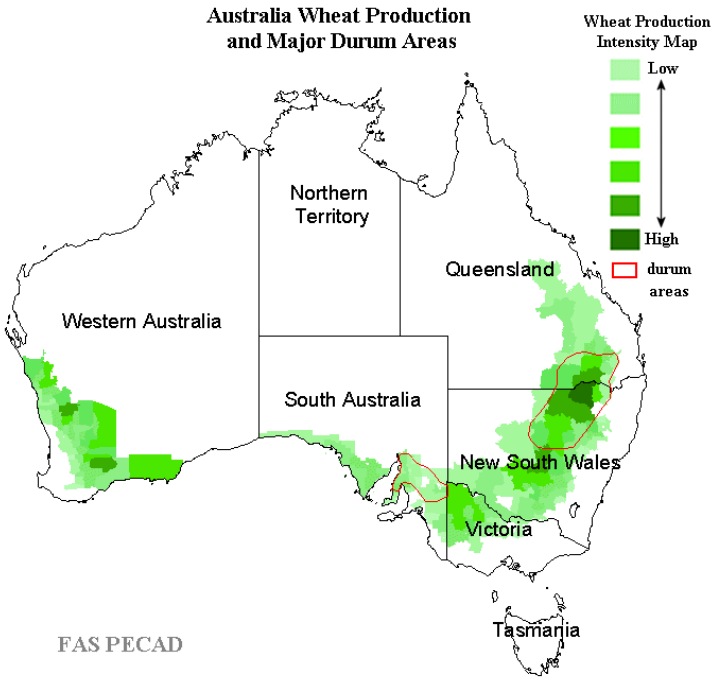


Figure 1-1: Areas in Australia where most durum wheat is grown.

Source: United States Department of Agriculture (Crutchfield 2005).

1.1.2 MAJOR CONSTRAINT ON DURUM PRODUCTION

Crown rot (CR), caused by the fungus *Fusarium pseudograminearum* (*Fp*), is a major restraint on production of durum wheat in Australia. Other biotic and abiotic restraints are briefly described in the following section. More than 50% of the potential yield is reported to be lost due to Crown Rot disease each year (Daniel and Simpfendorfer 2006). In northern New South Wales, CR is estimated to have reduced durum yields by at least 20% in 2005 (Simpfendorfer 2005). A strong trend towards minimum cultivation practices and direct seeding into the previous year's stubble has seen a worldwide, rapid increase in the occurrence of this disease in the last two decades (Lamprecht et al. 2006). Many primary producers in Australia are now reluctant to grow durum wheat at all, despite its high market value. Therefore finding resistance for this disease has high priority. To date, all commercial durum varieties are more or less susceptible to CR and tetraploid sources of resistance are yet to be validated. The reason for the high

susceptibility of durum wheat to CR is the lack of the D genome where one of the major Crown Rot resistance genes is located in hexaploid wheat (Collard et al. 2006).

1.1.3 BREAD WHEAT

Wheat varieties can be traced back as early as the sixth millennium BC. Modern wheats emerged about ten thousand years ago in the Middle East in a process of repeated hybridizations of *Triticum* spp. with related grasses. Natural spontaneous hybridization occurred often, although most *T. Aegilops* grasses are self- fertilizing (Zaharieva and Monneveux 2006). Many of the wild grasses and rare *Triticum* species are significant reservoirs of superior characters which can help improve the current cultivars of wheat (Devos and Gale 1997). An overview of the different wheat species can be obtained from Dvorak et al (1993).

Bread wheat or *T. aestivum*, is the world's main cereal staple food after maize and rice. In 2007 world production of maize was 784 million tonnes, of rice 651 million tonnes and of wheat 607 million tonnes (FAO 2007). Bread wheat has the chromosome constitution AABBDD (2n= 6x = 42), implying that it is hexaploid. It evolved when cultivated tetraploid Emmer *T. turgidum* ssp. *dicoccum* (AABB) hybridised with a diploid *Aegilops* grass (which donated the D genome) in the southern Caspian plains. It has always been a model example of the evolution of a key crop species by allopolyploidization. Petersen et al. (2006) isolated two single copy nuclear genes from each of the three genomes found in hexaploid wheat, (A, B, D), and also from the two genomes of the tetraploid durum wheat progenitor *T. turgidum* ssp. *dicoccum* (A, B). The gene sequences were compared with sequences from representatives of all the diploid *Triticeae* genera. The results provide strong evidence for the D genome to be derived from *Aegilops tauschii*. The A genome was found to be derivative from *T. urartu* (Dvorak et al. 1993) and the highly diversified hexaploid B genome from *Aegilops speltoides* (Petersen et al. 2006). However, some evidence suggests, that the B genome donor could also be *Aegilops searsii* (Liu et al. 2003).

1.1.4 DURUM WHEAT

Durum wheat is the major tetraploid wheat species widely grown today. Durum wheat (*T. turgidum* ssp*. durum* L.) has the chromosome constitution AABB (2n= 4x =28). It has large, hard, yellow-coloured kernels, is high in protein and gluten strength, and is mainly used for pasta. Certain countries also use it for burghul, bread, couscous and frekeh *(*El Haramein et al. 1993*)*. The protein content in durum wheats varies between 9 to 18 per cent. For high quality durum wheat, at least 13% protein is required. Durum varieties with strong gluten properties are very sought after because not only do they provide quality flour for exceptional pasta products (Pfeiffer and Payne 2005), they are also suitable for bread making or mixing with bread wheat flour (Boyacioglu and Dappolonia 1994).

Maccaferri et al. (2003, 2005) have investigated the genetic diversity of durum populations and showed that the genetic basis of durum wheat did not decrease but instead increased over time, despite the high selection pressure exerted in durum breeding programs. These studies analysed 134 durum wheat plants, from geographically divergent sources, and estimated genetic variation by using 70 SSR markers. Cluster analysis showed 6-8 main subpopulations. Only 20% of the molecular variation was due to the geographic source of origin. Mediterranean cultivars were most distant to Northern American cultivars, whereas CIMMYT-ICARDA plants were very similar to Italian accessions. The North American cultivars were also the most homogeneous group. Also, rare alleles in durum were found to be plentiful, which was also shown by Branlard et al. (1989), suggesting that a large number of genetic introgressions contributed to the foundation of the modern germplasm (Maccaferri et al. 2003; 2005).

1.1.5 GLOBAL DURUM PRODUCTION

Durum amounts to only 5% of world wheat production or around 40 million tonnes, but it has high economic importance. Durum wheat is grown especially in southern Russia, North Africa, North America, India, and across Europe and the

Mediterranean. The total world production of durum was 26 million tonnes in 2005/2006, down from the years before because of extreme drought in many growing regions across the globe (Figure 1-2).

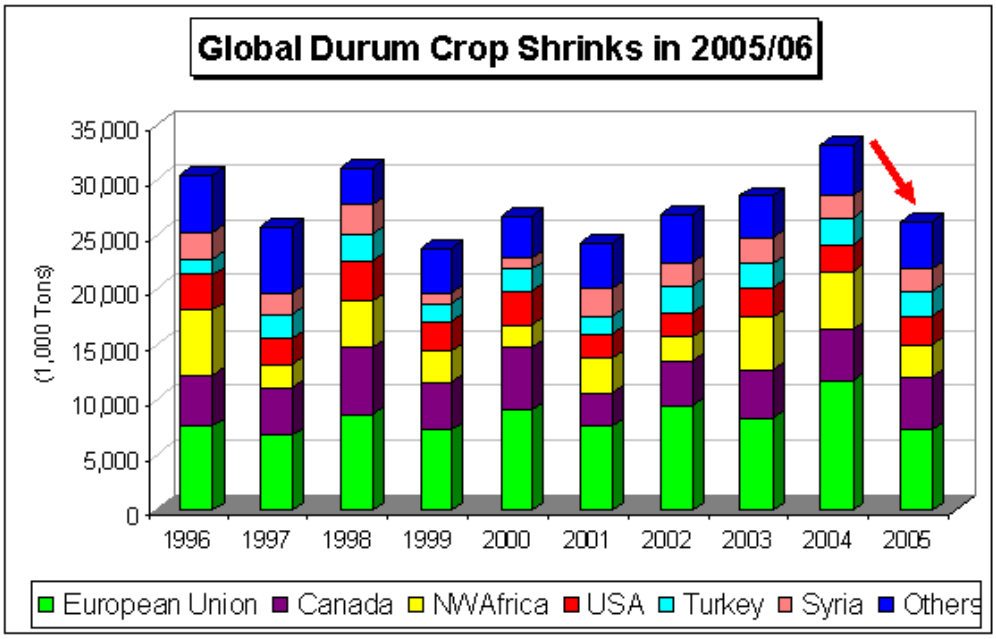


Figure 1-2: Durum wheat production in selected countries;
source: USDA, United States Department of Agriculture (Crutchfield 2005).

Canada is the largest durum producer in the world and pioneers durum research worldwide, along with Italy, USA and other major producers. Durum wheat varieties developed by CIMMYT (Centro Internacional de Mejoramiento de Maíz y Trigo, International Maize and Wheat Improvement Center) in Mexico have also significantly improved durum germplasm (Pecetti and Annicchiarico 1998). In Ethiopia and the Middle East, traditional farming systems have a positive impact on maintaining the genetic diversity of durum wheat (Kebebew et al. 2001). A survey in inaccessible mountain oases in northern Oman has shown a great variation in tetraploid landraces, which often represent a heterogeneous mix between durum and bread wheat (Al-Maskri et al. 2003; Al Khanjari 2007). In Mediterranean farming systems, tall durum varieties are used, because their straw is needed for the feeding of livestock. The value of this durum straw is almost as high as the durum grain itself; therefore local growers are often

reluctant to grow newer dwarf or semi-dwarf durum varieties (Annicchiarico and Pecetti 2003).

1.1.6 ABIOTIC CONSTRAINTS ON DURUM PRODUCTION

Stress tolerance is described as the ability of a plant to maintain constant high yield, regardless of environmental adversity. There are a range of abiotic stress factors that limit durum productivity in Australia, the main being drought, heat, frost, salinity and sub- optimal nutrient availability. Reviews of drought stress in durum and bread wheat can be found in the following studies: (Rawson 1971; Labhilili et al. 1995; Lacerenza et al. 1995; Flagella et al. 1996; Damania et al. 1997; Al Hakimi et al. 1998; El Hafid et al. 1998; Condon et al. 2002; Piro et al. 2003; Borrell et al. 2004; Reynolds et al. 2005; Zarco-Hernandez et al. 2005; Monneveux et al. 2006; Brini et al. 2007). Salt stress studies were conducted for example by Lutts et al. 2004. Stress due to the suboptimal availability of plant nutrients, especially nitrogen, in wheat was investigated by Cook et al. (1980), Cook and Veseth (1991) and Lopez-Bellido et al. (2006).

1.1.7 BIOTIC CONSTRAINTS ON DURUM PRODUCTION

Infection by *Fusarium* species represents the major biotic constraint on durum production in Australia. *Fp* is the most common *Fusarium* species causing CR in Australia, followed by *F. culmorum (Fc*), which is not prevalent in Queensland, as well as a variety of other *Fusarium* fungi. *Fp* is not common in colder areas of the world, where *F. nivale* (*Microdochium nivale*), *F. graminearum* (*Fg*) (teleomorph *Giberella zeae*) or others prevail (Backhouse and Burgess 2002).

Backhouse and Burgess (2002) conducted an analysis of the distribution of *Fp, Fg* and *Fc* on cereals in Australia. *Fg* was found mostly in warm temperate to subtropical areas with moderate to high summer rainfall, while climate did not limit the distribution of *Fp* within the Australian grain belt (Backhouse and Burgess 2002). Backhouse et al. (2004) conducted a survey of *Fusarium* species associated with CR of wheat and barley in eastern Australia. *Fp* was the main species isolated

from crops in Queensland and NSW. It was the most common species in Victoria and South Australia, but *Fc* was also frequently isolated in these states (Backhouse et al. 2004).

Scott et al.(2004) surveyed CR affected wheat crops in northern NSW and southern QLD. Sixteen species of *Fusarium* were isolated from crowns and basal stem nodes. *Fp* was the predominant CR pathogen isolated, followed by *F. crookwellense*, *Fg* and *Fa* (*Fusarium avenaceum*), (Scott et al. 2004). *Fp* was found more often (48%) than *Fg* (28%) in northern NSW, where in many cases it infected the same field as *Fp* (Akinsanmi et al. 2004).

1.1.8 *FUSARIUM* RESEARCH IN AUSTRALIA AND ELSEWHERE

Most of the early *Fp* research was conducted in Australia. For a long time, it was thought that *Fg* Schwabe, which was first recorded in Queensland in 1951, was the pathogen responsible for CR (McKnight and Hart 1966; Purss 1969). Earlier records were made of a head condition of wheat associated with an organism identified as *Fusarium roseum* (Tyron 1918). *Fg* in its perfect form *Gibberalla zeae (*Schw.*)* has been recorded frequently on maize for many years (Simmonds 1966). Wildermuth and Purrs (1971) stated that CR is caused by *Gibberella zeae*, when he tested several partially resistant wheat cultivars in Queensland. At that time, he found the wheat cultivar Gala to be the most resistant cultivar. Burgess and Griffin (1967) described the pathogen as a fungus called *Fusarium roseum 'Graminearum*'.

However, isolates from maize, the main host, did not cause the disease in wheat, whereas isolates from grasses and other gramineous hosts were able to cause the infection. Also, different morphological features, like colony growth rates, raised first doubts about the true nature of *Fp*. In the late 1970's it was agreed that some *Fg* fungi had traits that distinguished them from others in the same species. Burgess, Wearing and Toussoun (1975) found, that most isolates of *Fusarium roseum 'Graminearum*' did not form perithecia in culture and called them *Fg* group 1. Group 2 normally (not always) forms perithecia. They also distinguished them on the basis of morphological characteristics (Burgess et al..

1975). Francis and Burgess (1977) confirmed the difference in perithecia formation and differences in colony growth and morphology, conidial septation and sizes and production of conidia. Now two groups were proclaimed: *Fg* group 1, later called *Fp*, and *Fg* group 2 or *Fg* (Balmas et al. 1995).

Wildermuth and McNamara (1994) acknowledged *Fg* group 1 as a different strain. Aoki and O'Donnell (1999) distinguished *Fp* from *Fg* based on the difference in colony expansion rates, the width of different conidia, dissimilar reactions to near UV black -blue radiation and the distance from end to end of the conidia. *Fp* was also again found to have heterothallic perithecia, which indicated that sexual stages exist. (Aoki and O'Donnell 1999). The sexual stage of the newly discovered *Fp* was called *Giberella coronicola* T. Aoki& O'Donnell.

1.1.9 *FUSARIUM* CROWN ROT

The north-eastern wheat production regions of Australia (Queensland and northern NSW) are especially hard hit by *Fp*. The following table shows the ratings of CR severity in the different regions (Table 1-2).

Table 1-2: CR severity in different regions of Australia (Murray and Brennan 2001); (rating 1-2: low; 2-5: moderate to high and above 5: very high CR severity).

Region	North	South East	South Central	West
CR severity	5.2	3.1	4.3	0.6

The asexual spores and direct contact with asexual hyphae from infected stubble residues are considered the primary causes of infection, since the sexual stage or teleomorph is rarely observed in the field (Paulitz et al. 2002). Initial infection centres on the crown, which is the area just on the soil surface at the top end of the sub-crown internode where the tillers emerge. Molecular

characterisation has confirmed the association of *Fp* with wheat stem bases (Bentley et al. 2006).

In moist, warm conditions *Fp* also infects the upper plant parts, spreading into the leaf sheaths of seedlings, and several nodes up the tillers in adult plants. Infection can occur throughout the season and is characterized by light honey-brown to dark brown lesions on the base of infected tillers. The extent of discoloration is used as an indicator of the severity of infection. Although initial *Fusarium* infection is enhanced in wet conditions, it is believed that CR symptoms are worsened by subsequent drought stress, due to restricted water movement from the roots through the stems, producing white heads on maturing plants that contain either no grain or light-weight shrivelled grain. In warmer, dryer years, when the germination of the seed is delayed, the grain filling period occurs later in the spring, which aggravates the symptoms (Burgess 1978; Burgess et al. 2001).

Most *Fusarium* species survive detrimental conditions as chlamydospores or conidiospores in soil and plant parts, or as mycelium inside the plant. The fungus also survives on many species of grassy weeds. In different regions of the world other survival forms like ascospores can also be observed. Altogether, the mycelium is not as resilient as the spores which can live for far longer periods and travel further (Aoki and O'Donnell 1999).

1.1.10 *FUSARIUM* HEAD BLIGHT

Fusarium head blight (FHB), also known as scab, is caused by the fungus *Fusarium graminearum* (*Fg*), formerly *Fusarium graminearum* group 2. Resistance breeding for FHB was difficult because no resistance genes were known and the resistance was due to a multitude of factors, therefore breeders had to overcome the same problems that are prevalent in CR resistance breeding today. Partially *Fg* resistant wheat and barley cultivars now exist, although complete immunity has never been achieved.

Mitter et al. (2006) investigated the influence of ascospores and conidiospores on FHB infection of wheat both in Australia and the US. They found that

ascospores exhibited a considerably lower pathogenicity than conidiospores, but there were slight differences between the two countries.

Anderson et al. (2001) investigated FHB resistance QTL with molecular methods and found that the main QTL for CR resistance was located on chromosome 3BS.

In durum, partial FHB resistance could be achieved by crossing it with the resistant bread wheat var. Sumai 3 (Rudd et al. 2001; Elias and Manthey 2005).

1.1.11 DISTINCTION OF *FUSARIUM* SPECIES WITH MOLECULAR METHODS

It is very difficult to distinguish *Fusarium* species based solely on morphological features. The use of modern techniques like PCR, AFLP or RFLP greatly enhances the efficiency of classification. A wide variety of *Fusarium* species, including many which are not pathogens of agricultural crops have been investigated in this manner (Phan et al. 2004; Schilling et al. 1996; Williams et al. 2002).

Benyon et al (2000) studied the genetic relationship of 56 isolates of *Fusarium* using the Restriction Fragment Length Polymorphism (RFLP) technique as well as Southern blotting of random genome and mitochondrial DNA probes of *Fusarium* species. They observed a significant genetic relationship between *Fg, Fc* and *Fusarium crookwellense*, whereas mitochondrial DNA analysis revealed that *Fg* and *Fp* have a common ancestor. More recent work has indicated that these two species are not very closely related within the *Fusarium* genus.

Akinsanmi et al. (2003) compared the diversity between *Fp* and *Fg* using species-specific PCR assays. Isolates were obtained from wheat grown in Queensland and northern New South Wales. A high level of diversity was observed within the genes of each species.

1.1.12 TOXINS AND OTHER SUBSTANCES PRODUCED BY *FUSARIUM*

Most of the mycotoxins produced by *Fusarium* species belong to the Trichothecenes, but oxalates and polyketides also play a role (Wang et al. 2006). The discharge of oxalate by fungi benefits their development and the colonization of substrates. There is a significant correlation between virulence, pathogenesis and the secretion of oxalate (Dutton and Evans 1996).

The *Tri5* gene carries the code for the enzyme Trichodiene Synthase. This enzyme is responsible for the first reaction in the Trichothecene pathway. An association exists between the expression of this gene and the increase of Deoxynivalenol (DON). *Fp* is able to produce Diacetoxyscirpenol (DAS) and FusarenonX (FX) (Niessen et al. 2004; Clear et al. 2006; Niessen 2007). Both *Fg* and *Fp* produce Zearalenone (ZEA), Nivalenol (NIV) and Deoxynivalenol (DON). Some *Fusarium* strains also produce Acetyl-nivalenol (Blaney and Dodman 2002). *Fusarium* infection may be dangerous when infected grains are used for human or animal consumption because immune- suppressant toxins and hormone-like substances are produced by the fungus. DON is toxic to animals as well as plants. For example, DON application resulted in a decline of mitotic activity in onions (Rahman et al. 1993). In chickens, a negative effect on nutrient transport in the intestines has been observed (Awad 2008). ZEA causes reproductive trouble in animals because it acts like the hormone estrogen (Lysoe et al. 2006). Another important substance produced by *Fusarium* species is aurofusarin. In one study, it was shown that it negatively affected egg production of adult quail (Dvorska et al. 2001). The red colour of *Fg* species is due to the deposit of aurofusarin in their cell walls. Malz et al. (2005) demonstrated the significance of aurofusarin for the pathogenicity and physiology of the fungus. They proved that a gene cluster, which includes the gene PKS12, is responsible for the synthesis of this substance. Aurofusarin plays no role in the protection of fungi from the sun (Malz et al. 2005).

1.1.13 MECHANISMS OF RESISTANCE

When a fungal spore comes into contact with its host, it can recognize signals from the plant and starts producing specific enzymes and growth pattern which assist its entry into the host. The trigger can be wax or ethylene or other plant signals that activate the formation of appressorii on the fungus (Kolattukudy et al. 1995). Two types of resistance are described, for example by Huisman (1982), Meyers et al. (1999), Delaney et al. 1994 and Meyers et al. (2005).

Vertical (qualitative) resistance, which is inherited via one (monogene) or a few genes and the host generally displays a visible reaction towards the pathogen. Frequently such resistance is effective against some, but not all races of a pathogen.

Horizontal (quantitative) resistance, which is partially effective against all pathotypes of the pathogen and is inherited via many genes (polygenic) of small individual effect. Typically horizontal resistance decreases the severity of disease but generally does not eliminate it (Person et al. 1959; Vallega and Zitelli 1973).

Methyl jasmonate (MJ) plays an important role in the defence against *Fp* by inducing resistance genes. The manipulation of the MJ pathway in wheat could be helpful to induce CR resistance (Desmond et al. 2006).

1.2 CROWN ROT RATING

Scoring of CR disease symptoms is difficult, especially scoring for field resistance, because seasonal conditions can vary widely and have profound effect on visible symptom expression. However, one recent study showed that individual variability in the visual rating of plant disease symptoms does not negatively impact the resulting accuracy of QTL identification (Poland and Nelson 2011).

The process of rating CR infection is time intensive and laborious. Controlled CR infection is also not possible in the field. Therefore many researchers prefer the rating of seedlings. Wildermuth and McNamara et al. (1994) developed a method of assessing CR resistance that is now commonly used. Soil is sterilized with steam

and infected with a wheat-barley grain inoculum of *Fp.* After 22 days, leaf sheaths 1, 2 and 3 are assessed for signs of necrosis. Ten plants are rated together by individually scoring their first three leaf sheaths on a scale of 0-4 (0= healthy, 1= less than 25% necrosis, 2= 25-50% necrosis, 4= greater than 75% necrosis). The individual scores of the three leaf sheaths are then added together. These authors reported a correlation coefficient of 0.78 between the results of seedling screens and field testing (Wildermuth and McNamara 1994). A positive correlation between seedling resistance and resistance of the mature plant to CR was also found by Wildermuth and Purrs (1971), Wildermuth et al. (2001) and Wildermuth and Morgan (2004).

Mitter et al. (2006), by using a droplet of conidia in water to inoculate emerged seedlings, also found that the seedling resistance ratings could be used to predict field resistance. However, greenhouse studies do not always reflect conditions in the field or predict the responses of adult plants, leading some authors to question whether there is a consistent relationship between seedling and field resistance (Balmas et al. 1995).

Wallwork et al. (2004) developed an intermediate approach in an attempt to solve problem of CR scoring of adult plants in the field. Plants were grown in inoculated tubes which were open at both ends. These tubes were put into galvanized baskets containing 100 plants each, that were then placed on sand beds in outdoor terraces. This makes it possible to screen numerous plants for CR resistance under conditions in which water-stress can be applied. This method is currently being used in South Australia to find sources of resistance to CR in durum and bread wheat.

1.2.1 PARTIAL RESISTANCE OF WHEAT TO CROWN ROT

No complete resistance to CR exists in wheat. Partial CR resistance in wheat is clearly of a horizontal nature, because many gene regions were found that contribute to it (Wallwork et al. 2004, Collard et al. 2005 and 2006, Bovill et al. 2006).

Partially CR resistant wheat genotypes have been recognized for some time, the main lines used in breeding being Sunco, Gala and 2/49. This resistance is partially effective against all isolates of the pathogen tested in seedling or field trials. The Bulgarian durum wheat variety Chirpan is reported to carry partial resistance against *Fc* and *Fp* (Lalev 1985). Intensive greenhouse and field screening at CIMMYT, Mexico resulted in the finding of durum wheat variety Sooty_9/Rascon_37, which possibly possesses resistance against *Fp* (Singh et al. 2005).

The American winter wheat varieties Brundage, Gene, and Eltan show tolerance against *Fp* (Paulitz et al. 2002). As these results were obtained in countries with different climatic conditions, they cannot easily be transferred to the Australian conditions. However, in an extensive screening study of 1130 international durum wheat plants, 140 plants showed good tolerance against *Fc* (Nsarellah et al. 2000). This shows that although durum wheat is generally very susceptible to CR infection, it has some inherent CR resistance which is not dependent on the presence of the D genome.

Disomic wheat subsitution lines can be employed to test the impact of individual chromosomes on disease resistance. In one trial, two Langdon durum disomic substitution lines, [LDN(IsraelA-3B) and LDN(PI478742-3B)], where the female Langdon durum chromosome 3B was replaced by 3B from *T. diccoccoides,* had the highest CR score among the set of substitutions series, suggesting that the LDN 3B chromosome may be more important in reducing CR infection than other chromosomes (Zheng et al. 2008, Haobing et al. 2008).

Wildermuth et al. (2001) found partial CR resistance in adult plants grown in soil which was inoculated with CR. The same resistance could also be detected in seedlings in most of the examined plants. It was found that the position of the crown in relation to the soil surface was negatively correlated to partial CR resistance. A resistant plant possibly has a crown that is more shallow in the soil than the crown of a susceptible plant. A higher placement of the seed might therefore be beneficial especially in susceptible plants (Wildermuth et al. 2001).

1.3 SOIL-BORNE PATHOGENS IN CROPPING SYSTEMS

Fusarium pseudograminearum is not a soil-borne pathogen per-se, although it is often treated as such. It is mainly spread by conidiospores and mycelium in the soil *(*Mitter et al. 2006). Therefore crop rotation and tillage have a huge influence on CR development. However, CR can also be spread by air. Sexual stages do exist as heterothallic perithecia (Aoki and O'Donnell 1999).

1.3.1 CROP ROTATION AND TILLAGE

No-till or direct-seeding is now very common in Australia and other countries. The seed is placed directly into the residue of the crop from the previous year, without tillage of the soil prior to seeding. This saves cultivation costs, improves the soil structure and reduces erosion. Crop residues cover the soil, thereby conserving water and reducing plant water stress (Papendick and Cook 1974). Unfortunately it leads to an increase of fungal diseases because crop residues are left on the surface, where they facilitate the development of fungal survival and fruiting structures. In direct-seeding systems infection occurs regularly on stem bases or crowns of emerging seedlings (Paulitz et al. 2002; Paulitz 2006). Smiley et al. (1996) found rising *Fusarium* crown rot levels with increasing crop residue on top of the soil. Whereas wheat grown in ploughed soil had only four per cent white heads, eight per cent of wheat grown in low till soil developed white heads (Smiley et al. 1996). However, minimum tillage does not always increase CR infection of wheat. In a study in northern NSW, stubble retention in wheat significantly decreased *Fp* mycelium, presumably because other fungi displaced it. Compared to this, stubble burning was not considered beneficial for soil health as it reduces other species of saprophytic fungi and bacteria (Donovan et al. 2006). However, it is likely that stubble burning also has a detrimental effect by decreasing soil moisture, thus enhancing yield losses caused by CR (Simpfendorfer 2005).

Monoculture of wheat provides very good conditions for soil borne diseases, whereas wheat-legume rotations hinder their development. The same is true for a rotation rich in cereal crops (Smiley et al. 1996; Lamprecht et al. 2006). Kirkegaard

et al. (2004) found that rotations with *Brassica* species (canola, mustard seed or faba beans being the most effective) were able to counter the effect of *Fusarium* infection and lead to increased yield. The better performance of *Brassica* break crops compared to other broad-leaf break crops like chickpea could be due to either the suppression of *Fp* through root exudates of *Brassicum*, or, more likely, to the faster decomposition of cereal residues which reduces *Fp* inoculum and leads to a more beneficial soil biology, apparent by a much higher percentage of the fungus *Trichoderma* ssp. present in the soil. Leguminous break crops do not form a dense canopy and lead to the accumulation of nitrogen, which can increase *Fp* severity (Kirkegaard et al. 2004).

It was found that the best method to reduce CR inoculum in a no-till farming system was to sow cereal crops in between the rows of previous winter cereal crops. This significantly decreased the severity of CR infection (Simpfendorfer 2005).

1.3.2 ROLE OF PLANT NUTRIENTS

A well balanced fertilization regime is necessary to reduce CR severity. The timing of nitrogen (N) applications is important. Nitrogen application leads to a faster break-down of plant residues but also provides detrimental fungi with nutrients (Lemaire and Jouan 1976). It was shown that higher levels of inorganic nitrogen resulted in higher disease pressure. Lower rates of infection were obtained from manure-treated plots (Smiley et al. 1996). N application should be matched to soil moisture and existing N content in the soil. This prevents too much plant growth at the beginning of the growing season. N fertilization before or at the sowing date should be avoided (Evans et al. 2009). Organic fertilizers and compost can reduce soil borne diseases (Lazarovits 2001; Bailey and Lazarovits 2003).

Copper is an essential element that can increase resistance to fungal diseases (Wood and Robson 1984). Wheat plants fertilized with zinc had a higher yield and earlier maturity in Victoria, Australia (Millikan 1938).

1.3.3 MICROORGANISMS

The soil microflora has a huge effect on the survival and virulence of pathogens, although not necessarily on the CR pathogen, as it survives on the stubble above the soil. As the crop rotation systems change, so does the soil microflora. Different soil fungi, bacteria and endophytic actinomycetes all have beneficial effects on plant health and resistance against fungal diseases. Work in South Australia and Western Australia demonstrated that some of them can increase germination, reduce root infection, promote plant growth and induce resistance against foliar diseases and insects. *Trichoderma koningii* is effective in reducing root disease and promotes plant growth, *Pseudomonas* spp. is also considered as beneficial (Rovira 2001). Many other *Fusarium* species which are only slightly pathogenic or non-pathogenic can also suppress the fungal infection of crops (Henry 1931). Populations of these microorganisms may be reduced when certain pesticides and herbicides are applied to the soil, which may also change the host physiology and the amount and composition of root exudates. The effects of saprophytic microflora on the development of *Fusarium culmorum* was investigated by Liggitt et al. (1997). Winter wheat ears were inoculated with *Alternaria*, *Botrytis* and *Cladosporium* in the greenhouse prior to the inoculation with *Fusarium culmorum,* which lead to a reduction of the severity of *Fusarium* ear blight. The saprophytes produced antibiotics that suppressed the development of *Fc*. It is possible that the fungicides suppress the saprophytic fungi so they cannot perform their normal function of suppressing the mycelia growth of *Fc* (Liggitt et al. 1997). The application of fungicides leads to a change in the ability of the plant to extract nutrients and the predisposition to diseases is increased. On the other hand, an increase in beneficial soil organisms may also be possible when populations of pathogenic fungi are decreased by fungicides. Interactions in the root- soil interface are very complex and not yet well understood (Rodriguez-Kabana and Curl 1980).

1.4 HEXAPLOID WHEAT X DURUM CROSSES

1.4.1 CROSSES TO IMPROVE AGRICULTURAL PERFORMANCE OF DURUM WHEAT

Wild wheat varieties have been successfully used to improve quality characteristics like gluten strength and protein content in durum wheat. Mainly wild emmer, *T. turgidum* ssp. *dicoccoides* was crossed into durum wheat to achieve this goal (Blanco and Porceddu 1983; Blanco et al. 1990, 1996; Gadaleta et al. 2003). Quality traits located on the D genome chromosomes of bread wheat were successfully transferred into durum wheat (Ceoloni et al. 1996), (Pogna and Mazza 1996). Vitellozzi et al. (1997) transferred a 1DL segment containing the Glu-D1d allele into the 1AL arm of a tetraploid line and confirmed it with fluorescent in situ hybridization (FISH) experiments (Vitellozzi et al. 1997). Lanning et al. (2003) crossed durum wheat with hard red spring wheat. The resulting hexaploid wheats had superior bread making traits but much higher levels of the detrimental enzyme polyphenol oxidase, whereas the resulting tetraploid wheats had a colour more suitable for pasta making. Generally, the existence of durum wheat genes in the derived hexaploid lines had only a slight impact on quality traits. The presence or lack of the D genome is likely to have the main influence on quality traits (Lanning et al. 2003).

Zitelli and Vallega (1968) crossed hexaploid wheats from North Dakota carrying resistance to stem rust with the Italian durum varieties Capella and Giorgio. This resulted in durum that was resistant to both stem and leaf rust

Bai and Knott (1992) investigated the influence of D-genome chromosomes on rust resistance in bread wheat. Stem rust resistant *T. dicoccoides* was crossed with different bread wheat varieties. It was found that chromosomes 1D, 2D and 4D suppress stem rust resistance in the *T. dicoccoides* accessions when all of them were present.

In East European countries bread wheat by durum wheat crosses have been employed to improve drought resistance, cold tolerance and head blight resistance in durum wheat (Saulescu 2005).

In 1927 the Russian researcher Kobaltova crossed an unknown *T. durum* spring wheat with *T. aestivum* winter wheat variety Will, demonstrating that durum x hexaploid crosses were being investigated at that time (Kobaltova 1927).

In Romania, durum crosses with *T. aestivum* have made a fundamental contribution to the improvement of winter hardiness of winter durum (Ittu et al. 1998, Saulescu et al. 1998, Saulescu 2001).

Maliani (1964) in Italy increased the number of fertile flowers per spikelet in durum wheat following crosses to hexaploids and was thus able to develop higher yielding varieties. CIMMYT in Mexico conducted a breeding program in 1974, in which short stiff straw character was transferred from the dwarf hexaploid wheat Creso into durum wheat, which resulted in higher yield and quality (Pfeiffer and Payne 2005).

Unfortunately, many of these crosses show lower yield and negative traits. In Morocco crosses between *T. aestivum* and *T. durum* were conducted to improve yield potential and adaptation traits in durum wheat. This approach ended unsuccessfully, as the resulting plants grew poorly and were susceptible to hybrid necrosis and sterility (Nsarellah and Amri 2005). Although its genetic base is not as broad as bread wheat, durum wheat provides traits that can be beneficial for bread wheat improvement, for example resistance to stripe rust (Nachit 1992, Ma et al. 1995, Hussein et al. 2005).

The majority of studies have shown that F_1 seed show higher germination rates when the female parent is the hexaploid wheat, while in reciprocal crosses where the durum parent receives hexaploid derived pollen, higher seed set but poorer germination is observed (Watkins 1928, 1932; Thompson and Robertson 1930; Belea 1969).

1.4.2 CYTOLOGICAL STUDIES

Not many recent articles dealing with crosses between durum and bread wheat are currently available. The bulk of research on the genetic constitution of pentaploid wheat crosses was done in the 1930's. Kihara's book "Wheat Studies-Retrospect and Prospects" (1982), is a recent review of this work. In this book, he gave a profound overview of what happens, when tetraploid wheat is crossed with hexaploid wheat. He investigated the rate of chromosome elimination, the fertility of crosses, their respective disease resistance (such as to rusts), the phenotype of adult plants with gene deficiencies, and the fertility of pollen. When tetraploid wheat is crossed with hexaploid wheat, the A- and B-genome chromosomes pair normally with each other, whereas the seven D genome chromosomes in the hexaploid parent cannot pair in meiosis and only one copy is transferred into the nucleus of the F_1 cells. Therefore F_1 plants from hexaploid x durum crosses always have 35 chromosomes in their nuclei. They consist of 14 bivalents (AA or BB) and seven D genome univalents. The 14 bivalents behave normally throughout meiosis, but the seven univalents separate only in the M1 (first meiotic division) and not in the M2, as they have no partner. For pollen formation, the D genome chromosomes are distributed to the poles erratically. Pollen or ovules may then contain between 14 and 21 chromosomes. If the distribution of univalents were at random, statistically there should be a majority of F_2 plants with intermediate chromosome numbers, but this is not always the case. Many of the plants tend to have either less or more chromosomes than expected because in the first instance some of the D genome univalents fail to reach either pole, often being trapped in the newly-forming cell wall while in the second instance the univalent chromosomes tend to travel in clusters and are not evenly distributed between the two poles. In successive generations of selfing after crosses between hexaploid wheat with tetraploid wheat, Kihara describes a 'declining' group of plants which lose excessive D genome chromosomes and thereby eventually become tetraploid again, and an 'increasing' group of plants,

which, above a certain chromosome number regenerate bread wheat progeny again.

Hence in later generations almost all plants with intermediate chromosome numbers disappear (Kihara 1982). The main feature of the increasing group is that the number of bivalents (in this case AA or BB) plus the number of univalents is at least 21, with every D genome chromosome represented at least once. Plants with less than 21 bivalents plus univalents belong to the declining group. Sterile combinations were observed if the sum of bivalents and univalents was between 15 and 20 (Kihara 1982). The above information was also confirmed by Thompson and other colleagues (Thompson et al. 1925, 1927; Thompson and Cameron 1928; Thompson and Robertson 1930).

In one recent study chromosome numbers were counted in an F_2 population of bread wheat/durum crosses. Most of the F_2 plants showed morphological characteristics similar to the morphological traits of the durum parent. More than half of the plants had 36-39 chromosomes, with F_2 hybrids averaging 36.54 chromosomes per plant (Wang et al. 2005).

Gilbert et al. (2000) crossed hexaploid FHB resistant spring wheat lines Sumai 3, Ning8331 and 93FHB21 with the susceptible tetraploid lines Stewart 63 and DT486. Forty-three plants from the F_2 were analysed with D genome specific microsatellite markers, 24 of them had all D genome chromosomes present, however it is not known if these were bivalents or univalents. Presence or absence of the D genome chromosomes did not influence FHB resistance.

Lanning et al. (2008) investigated the ploidy levels of offspring of *T. aestivum* L. x *T. turgidum* ssp. *durum* L. crosses over several generations. They found that inbreeding hybrids between hexaploid hard red spring wheat and durum wheat to the F_5 generation lead to 54% tetraploid lines, 36% hexaploid lines, and 10% aneuploid lines.

While Kihara revealed much about the fate of the unpaired D genome chromosomes in the progeny of crosses using traditional cytological methods, he could not tell if there were any intergenome translocations between A- and B-genome chromosomes or if any D genome segments were translocated to the A-

or B-genome chromosomes, nor the chromosome type. As I will demonstrate in subsequent chapters, recent advances in chromosome staining (MCFISH) and molecular marker technologies now allow these issues to be determined.

1.5 MCFISH

GISH (Genomic in situ Hybridisation) is a technique used to identify DNA or RNA sequences in the cell. The cell (originating mostly from root-tips) is fixed and then a single stranded nucleic acid probe, which is labeled, is hybridised to the sequence to be investigated. Under the fluorescense microscope this sequence is perceptible as a band (Schwarzacher et al. 1989) .The method is limited, as only the differences in the single-coloured band structures can be used to compare species.It has been difficult in the past to distinguish the three different genomes in hexaploid wheat by genomic in situ hybridization (GISH) (Mukai et al. 1993) because of the close resemblance among the diploid donor species, *T. urartu* the A genome donor (Sax 1922; Kihara 1924), *Ae. speltoides*, the B genome donor and *Ae. tauschii*, the D genome donor (Kihara 1944; McFadden and Sears 1944).

Multicolor Fluorescence in situ Hybridization (MCFISH) is now extensively used in the physical mapping of genes in plants and animals. The hybridization reaction of MCFISH identifies target genomic sequences, thus making it possible to study their size and location. DNA or RNA sequences from suitable chromosome-specific probes are first labelled with fluorescent reporter molecules and then identified through fluorescence microscopy after binding to their target sequence . The slide with the specimen is treated by a series of chemical reactions and washing steps. These steps include RNase treatment, fixation, dehydration and denaturation. The labelled DNA or RNA probe is then hybridized to the interphase nuclei or metaphase chromosomes on a slide, followed by several washing steps. After this the specimen is screened for the reporter molecules by fluorescence microscopy (Trask 1991; Reid et al. 1992; Zhang et al. 2004).

Bacterial artificial chromosomes (BACs) can contain large inserts from *Triticum* and *Aegilops* species, making them suitable for MCFISH mapping. Zhang et al.

(2004) discovered two dispersed repetitive DNA sequences that preferentially hybridize to the A- and D-genome chromosomes respectively. The BAC clone 676D4 from the *T. monococcum* library (Lijavetzky et al. 1999) holds an isolated repeat that preferentially hybridizes to A-genome chromosomes, and two BAC clones, 9I10 and 9M13, from the *Ae. tauschii* library (Moullet et al. 1999) contain an isolated repeat that mostly hybridizes to the D-genome chromosomes. These repeats are helpful in simultaneously differentiating the three different genomes in hexaploid wheat, and in recognizing intergenome translocations in wheat or between wheat and alien chromosomes by showing up in different colors under the fluorescent microscope according to the fluorescent label of the reporter molecules attached to them. Sequencing results indicate that both of these repeats are transposable elements, which demonstrates the significance of transposable elements and particularly retrotransposons, in the genome evolution of wheat (Zhang et al. 2004).

1.6 MOLECULAR MARKERS

"Molecular markers can provide a spectacular improvement in the efficiency of plant breeding" (Langridge and Chalmers 1998). Genetic markers are lengths of DNA sequences from either coding or non-coding regions of chromosomes which vary in the identity of one or more bases or in the number of repetitions of a short sequence motif (and therefore length) between different genotypes of the organism in question. These variations in length and sequence identity can be detected by a number of methods (Smeets et al. 1983). An abundance of marker types have been developed and used for mapping and to find quantitative trait loci (QTL) linked to quality traits or disease resistance over the last decades. Some of them are briefly described in the following section.

Restriction fragment length polymorphisms (RFLPs) are DNA markers that have been used extensively to construct linkage maps (Paterson et al. 1996). However, RFLP analysis is a laborious and time-consuming procedure which requires relatively large amounts of DNA. It requires information on the genome being examined and is not suitable to produce large numbers of DNA markers for

species whose genomes are not at least partially characterised (Powell et al. 1996b).

Random amplified polymorphic DNAs (RAPDs) consist of several base primers, containing 8-12 nucleotides, which are randomly chosen and amplified together with the DNA sample using the Polymerase Chain Reaction (PCR). Amplification only takes place when the primers bind. RAPDs are used to search for variations in unknown DNA sequences. A large, intact DNA sequence is needed (Williams et al. 1990). RAPDs are not completely reproducible (Paterson et al. 1996) and can include artefacts (Ellsworth et al. 1993). They also have limitations because they are dominant markers. However, RAPDs have been employed to produce large numbers of genetic markers that are useful for linkage mapping rapidly and economically (Antolin et al. 1996).

Another marker type is the sequence tagged site (STS). A STS is a short DNA sequence which is 100-500bp long. It occurs only once in the chromosome or genome. Its sequence must be known (Wang et al. 1994). STS can be found in a variety of ways, for example with expressed sequence tags (EST's) or random genome sequences obtained from cloned genome DNA. EST's are short sequences obtained by analysis of complementary DNA (cDNA), which is mRNA that is converted into double-stranded DNA. They provide valuable insights into the sequences of expressed genes (Marra et al. 1998).

Amplified fragment length polymorphisms (AFLPs) are widely used in mapping of wheat as high polymorphism rates are obtained and the procedure is relatively simple and easily reproducible. The technique has proven particularly useful for the improvement of existing maps (Zabeau and Vos 1993). For AFLP, genomic DNA is digested using specific restriction enzymes, adaptors attached to the fragment end, and the restriction fragments selectively amplified by PCR and then visualised by polyacrylamide gel analysis. The adapter and restriction site sequence is used as the target sites for primer annealing. Therefore no knowledge of the nucleotide sequence is necessary. Usually 50-100 restriction fragments are amplified and detected. Only dominant markers can be detected. Polymorphism is based on the

analysis of banding patterns (Vos et al. 1995; Barrett and Kidwell 1998; Ridout and Donini 1999; Gerber et al. 2000).

Sequences of simple sequence repeats (SSRs) are the most common markers used today. The genomes of eukaryotes are made up of SSRs to a significant part. SSR markers are co-dominant genetic markers which provide useful information after amplification of the sequences with PCR. Wheat microsatellites contain about 40 dinucleotide repeats (Roder et al. 1995). The detection of SSRs and other marker types in genomes has lead to a revolution in molecular biology, because it is now possible to assess highly variable regions of the mammalian and plant genomes (Powell et al. 1996a). Not only are SSR markers suitable for the detection of QTL when compared with phenotypic data, they are also a powerful tool for research into plant genomes, pathogen-plant interactions, regulatory genes and their proteins as well as an array of other complex traits (Asins 2002).

For a more detailed discussion of the techniques introduced above see Avise (2004).

In recent times, the understanding of SSRs and their function within the genome greatly increased. Not only are more and more SSRs found within transcribed genetic regions, particularly untranslated regions (UTR's) and introns, the notion that they have no function at all was proven wrong. New data indicates that the distribution of SSRs across protein-coding regions, UTRs, and introns is non-random. SSR expansion or reduction in protein-coding regions can lead to a increase or loss of gene function (Li et al. 2004). They were also found to change with time, getting smaller or larger by way of point mutations, replication slippage or recombination, leading to problems or improvements in gene transcription and regulation via frame shift mutations. Their products (mRNA and proteins) can also prevent splicing or lead to gene silencing. They can even lead to phenotypic changes. SSRs within genes are more likely to cause significant changes than SSRs in other regions, because of higher selection pressure there. It might even be that SSRs are one cause of gene evolution (Gupta et al. 1994; Cuadrado and Schwarzacher 1998; Kantety et al. 2002; Li et al. 2004).

The databases containing genome and expressed DNA sequences are continually growing, with most important crops now represented. As these databases are open to the public, they can be readily used for the detection of markers. The benefit of EST- derived SSRs markers lies in their known position in the genome, compared to randomly generated SSRs (Nicot et al. 2004). To find SSR markers, it is necessary to analyse either EST's or segments of non-coding genomic DNA. Kantety et al. (2002) found that only 1.5% of EST's in maize and 4.7% in rice contained SSRs. Therefore BLAST (Basic Local Alignment Search Tool) analysis was used to effectively improve the search results. SSRs markers are now available for a significant proportion of the grass genera. Eujayl et al. (2002) used EST-derived SSRs to genotype the A and B genomes of durum wheat. EST derived SSR markers from bread wheat can also be used on the A and B genomes of durum wheat. They were found to have high discriminatory power.

Diversity array marker technology (DArT) can drastically improve breeding efficiency because it provides a detailed assessment of the whole genome of any species in a very short time by utilizing hundreds of dominant markers. (Jaccound et al. 2001; Kilian et al. 2003). At first a microarray is constructed from DNA samples that represent the gene pool to be analysed. For this an *E. coli* library is created. The inserts from individual clones are then multiplied and used as molecular probes in the DArT microarrays.

DNA of the sample to be analysed is then hybridized to this microarray with the bases of polymorphism being single nucleotide polymorphisms (SNPs) and insertions and deletions (INDELs) at restriction enzyme cutting sites. DArT markers are bi-allelic, dominant markers (Kilian et al. 2003).

The technology is dependable and not reliant on prior sequence information, has a high throughput and cheaply generates reproducible data. Its applications cover genome profiling and genome background screening, construction of genetic linkage maps, detection of quantitative trait loci (QTL) and the fast introgression of genome regions in backcrossing programs, marker-assisted selection for several traits, evaluation of genetic diversity, identification of the

variety of a crop and determining the composition of microbial and other DNA samples (Wenzl et al. 2004; Akbari et al. 2006).

To understand DArT analysis, an understanding of marker quality is important. DArT marker quality is based on of three components (personal communication from E. Huttner). One of them is represented by the P (or Q) value. P reflects how well the two phases (Present = 1 versus absent = 0) of the marker are separated in a sample set (Akbari et al. 2006). P is based on ANOVA. P values above 77 are reliable, above 80 very reliable. Markers with lower P values may be mapped, although there is a higher risk of scoring errors.

Another factor in marker quality is the call rate, which represents the percentage of markers that can be scored (number of effective scores divided by the number of samples; personal communication from Eric Huttner).

The third parameter is the PIC value. It is a measure of the distribution of 0 and 1 scores in the sample. The highest PIC value is 0.5, which means that the alleles which emit a signal and the null alleles are distributed equally. Such a marker is very useful. Low PIC markers indicate rare alleles. A sufficiently large dataset is necessary to calculate reliable PIC values. For the mapping of populations the PIC value is not necessary, except to confirm the Mendelian nature of the marker (Anderson et al. 1993).

1.6.1 MARKER ASSISTED SELECTION (MAS)

MAS with RFLP markers was first described by: Osborn et al. 1987; Tanksley and Hewitt 1988 and Paterson et al. 1991.

Wheat microsatellites are used to identify genetic variation between wheat plants. A small number of microsatellites is needed to identify cultivars in wheat (Plaschke et al. 1995). SSR markers have been used to screen large segregating populations and to select superior plant material in backcross populations (Ribaut et al. 1997). Single large-scale marker-assisted selection (SLS-MAS) facilitates the selection of plants with a beneficial genetic region at an early developmental stage. Parents from elite plants are crossed to produce segregating populations. From their progeny, plants that are homozygous for beneficial alleles are selected.

A certain number of plants, usually at least 100 or more, is necessary to maintain sufficient allelic variability on the locus of interest as well as outside of the target regions (Lande and Thompson 1990; Ribaut and Bertran 1999). How to conduct an efficient MAS using backcrosses was described by Ribaut et al. (2002).

Today the most common use of MAS in wheat breeding is to backcross major genes into known elite wheat plants. This approach is especially useful for alleles that are difficult to phenotype. They help reduce linkage drag of unwanted genes close to the gene of interest. Fewer generations are needed to achieve breeding results and the recovery of the parental genetic background is fast. MAS is more difficult to deploy for polygenic traits, due to epistatic interactions, the lower phenotypic effect of each of the individual loci involved and genotype by environment interactions. However, when the number of plants analysed is high and the experiment is conducted at multiple sites,this technique can be successful. However, the higher resources needed reduces the cost effectiveness of MAS compared to conventional techniques (Mohan et al. 1997; Holland 2004).

1.6.2 MARKER ASSISTED SELECTION FOR CR

In Australia, where CR is most severe, selection for CR resistance in wheat has suffered from the lack of a fast and reproducible plant assay for CR screening. Several screening techniques have been employed, but none are both fast and reproducible enough to easily screen large amounts of germplasm. All are labor intensive and expensive. Problems include the need for large field plots/ glasshouse space, an even rate of infection, the laboriousness of visual assessment and the accuracy with which any technique reflects field performance (Dodman and Wildermuth 1987; Wildermuth et al. 2001). As CR resistance is a partial resistance, it is difficult to detect due to the number of genes involved and the weak expression of contributing genes (Mitter et al. 2006). Also differences in climate and interaction with other pathogens must be considered. Glasshouse screening often gives different results than field screening, because seedling- and field- resistance are sometimes only weakly correlated (Purss 1966) although

Wildermuth and McNamara (1994) and Klein et al. (1985) found a good correlation. Unfortunately studies which compare field- with seedling resistance are scarce. Wallwork et al. (2004) developed a 'terrace' system, in which seedlings are grown to the adult stage on sand-based terraces in plastic tubes with a layer of inoculum positioned on top of the seed. However this method doesnot generate the severity of symptoms seen in field experiments of highly susceptible durum wheat. Mitter et al. (2006) developed a reproducible high-throughput glasshouse bioassay to detect Crown Rot resistance in wheat germplasm. They claim that the results from their glasshouse experiments correlated in a highly significant way with field resistance to CR.

The discovery of molecular markers for CR resistance has opened the way for faster transfer of resistance into elite commercial wheat varieties, since the selection of resistant wheat plants based solely on their degree of expressed disease symptoms in the field is labour intensive, painstaking, time consuming, approximate (due to varying environmental and operator effects) and expensive (Wildermuth and McNamara 1994; Wildermuth and Morgan 2004).

The first attempts to map CR resistance genes were conducted at the University of Southern Queensland, based on phenotypic evaluation of infected seedlings in glasshouse-based experiments, using doubled- haploid populations in which known partial sources of CR resistance were present.

On the basis of the marker alleles present, Collard et al. (2005) found six genetic regions (QTL) linked to CR resistance in a 2-49 (Gluyas Early/Gala) x Janz doubled haploid seedling population (bread wheat) using a genetic map based on SSR markers. 2-49 is partially CR resistant, whereas Janz is susceptible. The QTL were shown to be on chromosomes 1D, 1A, 2A, 2B, 4B and 7B, confirming the multigenic nature of the resistance derived from 2-49. The most significant QTL was found on 1DL and was inherited from Gluyas Early (Collard et al. 2006). Smaller QTL were mapped on 1AL, 4BL and on 7BS (all from the Gala parent of 2-49). The QTL on 2BS was from Janz. None of these QTL were in the same regions as FHB resistance QTL. This makes it unlikely that there is a connection between

FHB resistance and CR resistance in wheat (Collard et al. 2005). The CR resistance locus on 4B, located not far from the dwarfing gene Rht1, had earlier been detected in the bread wheat cultivar Kukri, based on bulked segregant analysis (Wallwork et al. 2004).

Collard et al. (2006) published a paper which largely confirms the previous findings in a Gluyas Early x Janz doubled haploid population. It was shown that the major QTL for seedling CR resistance was situated on 1DL (from Gluyas Early). The QTL on 1AL, 4BL and 7BS, detected in the 2-49/Janz population, could not be confirmed in the Gluyas Early/Janz population because they were inherited from Gala (Collard et al. 2006). Since the work cited above, other hexaploid sources of resistance to CR have been characterized with molecular markers at USQ. Bovill et al. (2010) investigated a W21MMT70/Mendos DH population for which the W21MMT70 parent shows partial seedling and adult plant resistance while Mendos expresses only adult plant resistance in the field.

Composite interval mapping exposed eight QTL linked to CR resistance. Three of them, located on chromosomes 2B, 2D, and 5D were identified in each of the three seedling trials. Two QTL on chromosomes 1A and 3B were detected in two of the three trials. The 2D, 3B, and 5D QTL were inherited from W21MMT70, whereas the 1A and 2B QTL were inherited from Mendos.

Because these QTL are in different regions to those previously identified, they can be pyramided with other known QTL (Bovill et al. 2006, 2010). Other CR resistance sources include the varieties Sunco, IRN427 and CPI133814. Haobing et al. (2008) found a QTL for CR on chromosome 3BL which explained up to 50% of the variation, and also one on 4B. The 3B QTL has also been detected by researchers at USQ (Bovill et al. 2010).

1.7 GENETIC LINKAGE MAPS

Maps of the whole wheat genome (consensus maps) can be found for example on the Graingenes website: *www.pw.usda.gov (*viewed 11/011/2010). These maps represent a compilation of a variety of existing maps. Molecular maps of bread wheat, which share many genes and markers with other members of the grasses

in varying degrees of synteny, are essential for genetic studies on the molecular level and for breeding. These maps may help to release the valuable genetic resources that are present in the genomes of wild and domesticated plants (Tanksley and McCouch 1997).

For the creation of maps it is essential to have at least two segregating populations which have sufficient genetic differences in a great variety of traits. Especially beneficial are double haploid populations and wide hybridizations with wild wheat varieties. When they are crossed and analysed, cross-over events can be observed and the distance and frequency of these events can be used to determine the position of the markers on the map. Different populations result in various qualities and densities of markers. It is therefore essential to use multiple mapping populations (Chalmers et al. 2001).

The development of plant genome maps started around 1985 with physical mapping, using genome in situ hybridization (GISH). The patterns produced on somatic metaphase chromosomes supply good physical markers, which can be used to identify chromosomes. Repetitive DNA and multicopy gene lines were mapped routinely this way. Especially allopolyploid species like wheat were analysed in regards to their chromosome structure and evolution (Jiang and Gill 1994). Nelson et al. (1995) undertook the mapping of the group 2 chromosomes of wheat (A, B, D) in 1995. By using RFLP's they constructed maps from a synthetic wheat by bread wheat cross. He employed 114 F_7 plants for this purpose. This study used clones from different libraries. He found 57 markers on 2A, 60 on 2B and 56 on 2D, each about 200 centimorgan (cM) apart. Amongst the discovered genes was an abscisic acid responsive locus, an epidermal waxiness inhibitor and several leaf rust and stem rust resistance genes (Nelson et al. 1995).

One of the biggest contributions to wheat genome mapping was made by Roder et al. (1998). The wheat genome naturally displays low levels of intraspecific polymorphism. Procedures that were optimized for the huge wheat genome were employed to obtain highly polymorphic markers. The use of unmethylated regions of the genome in order to isolate clones that contained microsatellites almost doubled the percentage of useful markers. Most of them

(80%) were polymorphic for only one locus (A, B, or D). This made it possible to insert 279 loci, which were amplified by 230 primer sets, into a previously established RFLP map from the International Triticea Mapping Initiative. Ninety-three loci could be plotted to the A genome, 115 to the B and 71 to the D genome. Some groups of loci assembled near the centromere, but most were randomly spread along the map (Roder et al. 1998).

Other authors specifically mapped rust genes in *T. aestivum* by using the above linkage maps. They used crosses between bread wheat and *T. timopheevii*, which is resistant to leaf rust. If gene material from *T. timopheevii* could be detected in the group 2 chromosomes of bread wheat, then the wheat was leaf rust resistant (Salina et al. 2001).

Kammholz et al. (2001) assessed several doubled haploid wheat populations, including Sunco/Tasman, CD87/Katepwa and Cranbrook/Halberd focusing on genetic relationships, quality traits and segregation distortion.
Chalmers et al. (2001) used these populations to construct linkage maps which included RFLP's, AFLP's, SSRs, protein and phenotypic markers.

It was found, that D chromosomes showed lower levels of polymorphism than the A and B genomes. Irregular cross-over events and segregation distortion were also found and some plants inherited one or more complete parental chromosomes in which no crossing over had occurred. This highlights the need for very large populations when attempting to determine the positions of complex quantitative traits (Chalmers et al. 2001).

Lehmensiek et al. (2005) used three doubled haploid populations of Australian origin to demonstrate the importance of map refinement (map curation) to improve the quality of linkage maps and detection of QTL. The marker order was re-evaluated; the genetic maps were checked in relation to a consensus map; the data was corrected for double cross-over by substituting genotypes at perceived double cross-over loci with missing values. Approximate recombination fractions for all marker pairs were established. This resulted in a significantly shorter new map. The new maps resulted in QTL peaks which had higher LOD (log-likelihood)

scores and better defined QTL peaks for the grain quality traits investigated (Lehmensiek et al. 2005).

1.7.1 DURUM MAPS

Durum maps were, up until recently, not as well developed as wheat maps. Chromosomes 6A and 6B of *T. durum* were mapped with restriction fragment length polymorphism markers (RFLP's) by Chen et al. (1994), using recombinant substitution plants from durum cultivar *Langdon* with *T.turgidum* var *'dicoccoides'*.

A map consisting mainly of RFLP's for durum wheat was constructed by Blanco et al. (1998). Sixty-five recombinant inbred plants from a cross between the durum variety Messapia and *T. turgidum* ssp*. dicoccoides* were used. The map was compared with bread wheat maps and this confirmed the known reorganizations of chromosomes 4A, 5A, and 7B and the presence of a translocation between 2B and 6B. The map did not detect the 2BS deletion common in durum wheat (Blanco et al. 1998). Lotti et al.(2000) improved the map of Blanco et al. by adding 80 AFLP markers.

The first to systematically apply genetic markers on a large scale in durum wheat were Nachit et al. (2001). Two durum wheats *Jennah Khetifa* and *Cham 1* were crossed to provide a population of 110 F_9 recombinant inbred lines. The parents showed significant differences in grain quality and resistance to biotic and abiotic stresses. One-hundred and thirty-eight RFLP, 26 SSR, 134 AFLP, three genes and five seed storage proteins (glutenins and gliadins) were put on the map. The map has a length of 3598 cM, the distance between markers is approximately 12 cM and 12% of markers showed segregation distortion. The map closely conformed with the contemporary *Triticeae* consensus map (Nachit et al. 2001).

Elouafi et al. (2001) constructed a genetic linkage map of a durum x *T. turgidum* var. *dicoccoides* backcross population based on SSRs and AFLP markers. The final map was 2289 cM long and consisted of 124 SSRs, 149 AFLP's and six seed storage proteins. Blanco et al. (2004) updated this durum map by extending the number

of loci to 458. This map includes a variety of marker types, which are evenly distributed between the A and B genomes.

DArT and SSR markers were recently used to create a consensus map from durum wheat. Fifty-six durum genotypes were utilized to produce a DArT micro array. A durum wheat recombinant inbred population of 176 plants was used to map SSR and DArT markers. The map retrieved contained 554 loci (162 SSRs and 392 DArT markers) and covers 2022 cM. The average marker distance is 5 cM. The DArT markers performed well in assessing genetic relationships in the durum plants when compared to the SSR markers (Mantovani et al. 2008).

1.8 SCOPE OF THE PROJECT, RESEARCH QUESTIONS

a.) *What is the fate of the unpaired hexaploid derived D chromatin in a hexaploid bread wheat x durum cross, and is any of this material translocated into the A and B- genome chromosomes of the progeny?*

Two major wheat species are currently used commercially, bread wheat or hexaploid wheat (*Triticum aestivum* L.), which has a chromosome constitution AABBDD (2n=6x=42), and tetraploid durum wheat (*T. turgidum* spp*. durum* L.), with a chromosome constitution AABB (2n=4x=28). The bread wheat gene pool provides an excellent resource for durum wheat enhancement, as durum wheat shares the A and B genomes with bread wheat. The gluten quality of durum wheat for example has been significantly improved by the introgression of D genome material from hexaploid wheat (Ceoloni et al. 1996; Pogna and Mazza 1996). Although there are some recent studies on hexaploid wheat by durum crosses (Wang et al. 2005; Lanning et al. 2008), most cytological research into the fate of chromosomes in the progeny of *T. aestivum* x *T. turgidum* spp*. durum* crosses was conducted in the 1920's (Kihara 1982, Sax 1922, Thompson and Hollingshead 1927). In a review of his work, Kihara (1982) gives a profound overview of what happens when tetraploid wheat is crossed with hexaploid wheat. He found that F_1 plants of pentaploid crosses had 35 chromosomes consisting of 14 bivalents and seven univalents. In successive generations, plants divided into an 'increasing group', resulting in plants which became hexaploid wheat and a 'declining group', resulting in plants which lost all D genome chromosomes and became durum wheat (Kihara 1982). While Kihara and other authors revealed much about the fate of the unpaired D genome chromosomes in the progeny of crosses using traditional cytological methods, they could not determine the identity of the chromosomes, nor tell if there were any intergenomic translocations between the A, B and D genome chromosomes. Modern cytogenetic and molecular techniques, such as Multicolour fluorescence in situ hybridization (MCFISH) and Diversity Arrays Technology (DArT) markers can answer these questions.

The objective of this study was to determine the degree to which the A, B and D genome material from a hexaploid parent is inherited by the progeny of a *T. aestivum* (var. 2-49) by *T. turgidum* ssp*. durum* (var*.* Bellaroi) cross over several generations. The genome analysis with SSR and DArT markers was conducted on F_2 - F_7 plants. Root tips from F_2 and F_3 seedlings were analysed with MCFISH (Zhang et al. 2001). Additionally, root tips of a number of F_2 and F_3 plants were stained with 1% acetocarmine, (Singh 2002) or according to the Feulgen Method (Singh 2002) and meiotic chromosomes counted.

b.) *Can the partial resistance to Crown Rot found in some hexaploid wheats be transferred into the progeny of crosses to tetraploid durum wheat?*

Durum wheat is more susceptible to CR infection than bread wheat (Zheng et al. 2008). Given its high economic importance to the Australian wheat industry, finding resistance for this disease in durum has high priority. Some contemporary bread wheat varieties (e.g. Sunco) show significant partial adult plant resistance in field trials. The best seedling resistance is found in the bread wheat line 2-49 (Wildermuth and McNamara 1994). Current trials in New South Wales, (NSW Department of Industry and Investment, Tamworth) indicate that resistance can be transferred into durum wheat from hexaploid wheat. If this proves true, durum wheat plants expressing CR resistance could be rapidly incorporated into durum breeding programs, leading to varieties with improved disease resistance. In this project, CR resistance in the progeny of three crosses between the hexaploid CR resistant line 2-49 and three related durum breeding lines, 961111 (Bellaroi) (M), 950329 (N), 971179 (O), was monitored over several generations (F_4- F_6). CR field scores were provided by the NSW I&I Tamworth.

c.) *Will genetic markers linked to resistance in the hexaploid sources still be useful predictors of resistance in the progeny of hexaploid x durum crosses?*

This project aims to confirm whether the genetic markers for hexaploid seedling resistance already established by other researchers at USQ (Collard et al. 2005; Bovill et al. 2006; Collard et al. 2006), prove to be effective predictors of resistance when transferred into derived durum lines.

Recently Bovill et al. (2010) identified field resistance QTL to CR on chromosomes 1A, 1B, 4B and 7B in a 2-49/Janz doubled haploid population, these overlap, but are not identical with some of the previously discovered seedling QTL identified on chromosomes 1A and 4B. Additional seedling QTL were identified on 1D and 2A. Only the QTL on 1B was solely an adult resistance QTL and not detected in seedlings.

2 GENERATION OF INTERSPECIFIC WHEAT LINES AND CROWN ROT RATING

2.1 INTRODUCTION

It was shown in the literature review, that crosses between hexaploid and tetraploid wheat varieties have been used in the past with more or less success to increase resistance to a variety of diseases. However, this method has not yet been employed to increase CR resistance in durum wheat. This study aimed to investigate the effectiveness of transfering CR resistance into a durum background with the goal of providing plant breeders with advanced durum lines with enhanced CR resistance. To achieve this goal, the CR resistant hexaploid wheat line 2-49 was utilized as the maternal parent and crossed with three related durum wheat lines. The progeny lines were planted in field plots artificially infected with CR. Thousands of resulting adult plants were screened over several generations (until the F_6) for CR resistance in the field. Each year only the most resistant plants were re-planted, which is reasonable from a breeding point of view, but was detrimental for the genetic screening, as the populations were not randomly segregating across the full spectrum of resistance responses anymore.

Almost all of this initial work was done by the plant breeders Dr Steven Simpfendorfer and Dr Ray Hare and their team at the Department of Industry and Investment in Tamworth (I&I), NSW, Australia. They provided all the CR data and have to be given credit for their exceptionally thorough effort which, in the end, led to the success of this study.

As the original crosses, the generation trials and disease data were all generated by research colleagues and not the author, this chapter focusses on the analysis of these data sets. This analysis provides the basis upon which the marker analysis, described in the following chapters, was conducted.

2.2 METHODOLOGY

2.2.1 HEXAPLOID WHEAT X DURUM CROSSES

Three advanced durum lines: Bellaroi [961111 (920405/920274)], (M); 950329 (920196/920357), (N); and 971179 (920777/Kronos), (O), a Jandaroi sib, were selected by Dr Ray Hare for development of subsequent materials.

The six digit numbers represent I&I NSW breeding line codes in which the first two digits indicate the year in which the F_3 progeny from the parental crosses was selected.

Pollen from these three lines was then crossed to the hexaploid wheat line 2-49 (Gluyas Early/ Gala) in 2001. For convenience, the resultant three populations were labelled 2-49/M, 2-49/N and 2-49/O, where M, N and O represent the lines 961111, 950329 and 971179, respectively. The original labelling of the populations by Steven Simpfendorfer was changed from 2-49/A to 2-49/M, 2-49/B to 2-49/N and 2-49/C to 2-49/O in order to avoid confusion with the names of the wheat genomes (AA, BB, DD). Lines from the selfed, subsequent generations of these materials were provided to this project each season by Dr Hare. Selection for durum head type, desirable plant architecture and Crown Rot reaction was conducted at the F_4 and subsequent generations.

The lines were labelled according to the cross and each line was assigned a number in the F_1, for example 2-49/N-15. This F_1 plant became the ancestor of the lines used in the trials, the offspring of this plant formed a family. In the following F_2 generation, the offspring were given the original label and the respective siblings were numbered sequentially, for example 2-49/N 15-5. This number remained with the members of the family over the next generations. In the succeeding F_3, all plants were additionally labelled with a three to four digit entry number. The seed obtained from the F_3 was sown onto different plots, and the plants assigned new entry numbers, one for each plot. These F_4 plants were not genetically identical, but had the same F_1 ancestor. The family name, which remained stable over the generations, as well as the entry number, were needed to identify plant lines. Since the entry numbers did not disclose the year or

generation, for the purposes of readability, entry numbers are not routinely shown in this dissertation but complete records in raw data tables are available (Appendix CD, Chapter 2).

2.2.2 FIELD TRIALS

A completely randomized block design with four replications per family was used for the field trials conducted by Dr Steven Simpfendorfer from the NSW I&I Research Station, Tamworth and field plots near Breeza. Each row was individually inoculated with *Fp* by depositing the inoculum into the furrow before sowing. If possible, disease free paddocks were used which had carried a break-crop in the year before, to enable comparison with un-inoculated plots, which were grown in the same area. Trials were all dryland (i.e. not irrigated). Fertiliser was always 50 kg/ha of Granulock 12Z (11.3% N, 17% P, 4.7% S and 2% Zn) applied with seed at sowing as a starter fertiliser and 100 units of nitrogen applied as granular urea incorporated between seed rows at sowing. Each year around 44-85 plots with 80 Bellaroi parent plants each (3500 -7000 plants) were sown separately on uninoculated plots, (which might have had natural infection), in order to evaluate the variation of CR severity over the years, as well as environmental interactions, and to generate an annual CR severity value for comparison with the breeding lines. The parents 2-49 and durum M, N and O were distributed amongst the progeny plants. Some lines from families with known susceptibility to CR were also included in the trial over subsequent generations in order to overcome the problem that the populations were not naturally segregating, a pre-requisite for QTL analysis. The durum plants underwent selection for durum appearance and CR resistance by the breeders. The trials were replicated over three years from 2004 to 2006. For CR assessment, the complete plants were harvested by hand after ripening, and individually scored for CR, whereas for plant DNA analysis, leaves were harvested at the three leaf stage, and transported to the laboratory on dried ice.

2.2.3 INOCULATION OF FIELD TRIALS WITH *FUSARIUM PSEUDOGRAMINEARUM*

The inoculum consisted of a macro-conidial suspension, a mixture of at least five *Fp* isolates, grown in autoclaved mung-bean broth. The macro-conidial suspension was then used to infect sterilized whole durum grains (Scott et al. 2004). After three weeks they were mixed with viable test seed prior to sowing, at a rate of 2g/m row. The viable and colonised seed was then sown directly into the furrow.

2.2.4 ASSESSMENT OF CROWN ROT SEVERITY IN FIELD MATERIALS

A visual assessment of the extent of basal browning was undertaken on 25 plants per plot after harvest of the mature plants. This was done by the plant pathology team at the Tamworth Research Station led by Dr Steven Simpfendorfer. Twenty-five plants/ plot were assessed. The following parameters were assessed (personal communication from Dr. Simpfendorfer):

a.) total tillers,

b.) tillers with browning,

c.) extent (height) of browning (0-3 scale: 0 = no browning, 0.5 = partial browning of 1^{st} internode, 1 = complete browning 1^{st} internode, 2 = complete browning 1^{st} and 2^{nd} internodes, 3 = complete browning 1^{st}, 2^{nd} and 3^{rd} internodes).

CR Severity = ((b/a x 100)/3) x c.

In addition: white heads were separately assessed as percentage of total tillers.

Plating of individual tillers on potato-dextrose agar (PDA) plates was performed to recover *Fp* and estimate percentage incidence (Akinsanmi et al. 2008).

2.3 RESULTS CROWN ROT FIELD SCORES AND OTHER TRAITS

The average CR scores over the years 2005-2007, as well as data from the other traits are shown in Table 2-1.

Indicated are parameters which were assessed for the determination of CR severity (tillers, brown tillers and brown nodes) as well as the resulting average CR score and height of the plant and head number over three years.

Table 2-1: Average trait scores of three hexaploid wheat x durum crosses, (2-49/M, 2-49/N , 2-49/O), provided by Steven Simpfendorfer, Tamworth, NSW, Australia, over the years 2005-2007.

			F_4			
Popul.	**Tillers**	**Tillers brown**	**Node brown**	**CR score**	**Height (cm)**	**Head No.**
2-49/M	8	7	1	34	52	7
2-49/N	7	6	1	29	59	6
2-49/O	7	7	1	34	53	7
2-49	5	4	1	18	71	6
Dur.M	5	5	1	42	57	5
			F_5			
2-49/M	6	5	1	29	49	4
2-49/N	6	5	1	27	54	4
2-49/O	6	6	1	33	51	4
2-49	5	4	1	16	66	-
Dur. M	4	4	2	60	49	3
Dur. N	11	1	2	58	59	5
Dur. O	5	5	1	45	49	4
			F_6			
2-49/M	8	7	1	34	55	6
2-49/N	8	7	1	34	57	7
2-49/O	7	7	1	35	60	6
2-49	9	8	1	23	65	8
Dur. M	7	7	2	69	52	6
Dur. N	6	6	2	72	57	6
Dur. O	7	7	2	66	54	6

2.4 OVERVIEW OF THE METHODS USED

Figure 2-1 shows an overview of the different methods used in this study for analysis in the different generations of three different hexaploid x durum Wheat crosses. All experiments were conducted by the autor, except the MCFISH

analyses which were done by Dr Peng Zhang from the University of Sydney, Cobbitty, NSW, Australia.

	SSR	DArT	Chrom. Stains	MCFISH
F_2	X	X	X	X
F_3	X	X	X	X
F_4	X	-	-	-
F_5	X	-	-	-
F_6	X	-	-	-
F_7	-	X	-	-

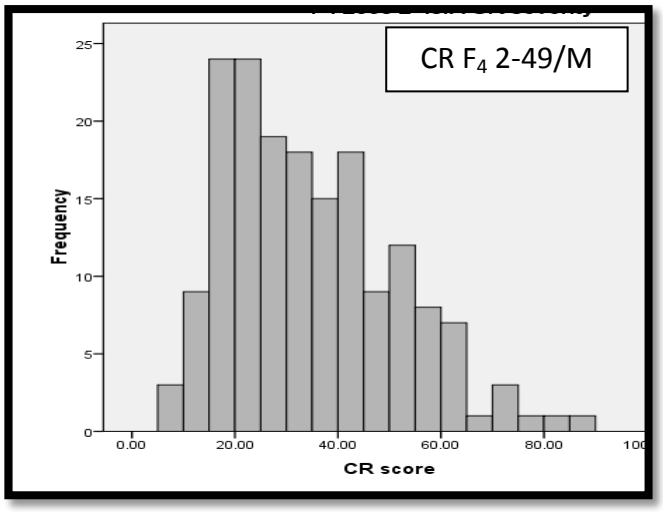
Figure 2-1: Summary of analysis methods used in this study.

2.4.1 F_4 2005

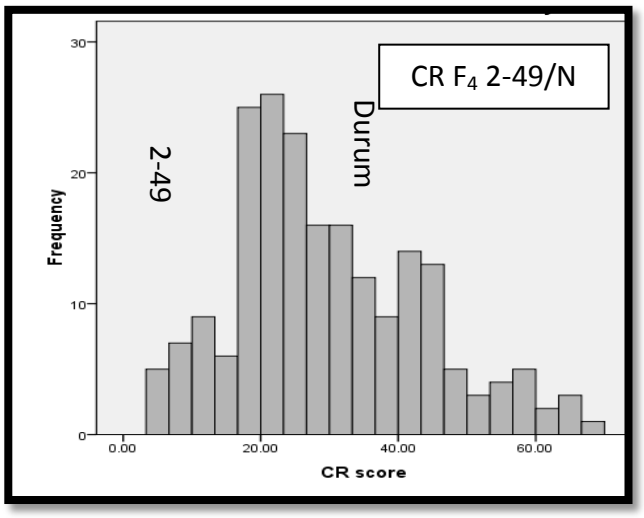
In the F_4 generation (grown in 2005 and analysed in 2006), three hexaploid wheat x durum crosses were analysed: 2-49/M (961111 or Bellaroi), 2-49/N (950329) and 2-49/O (971179). In this study the average CR scores of the whole population of thousands of plants were analysed. The raw data can be obtained from the Appendix CD, Chapter 2, F_4.

Subsequent low CR scores in all three populations imply that the disease pressure was not high in this year, even the susceptible Puseas check variety had a low CR score of 51. The hexaploid wheat line Puseas, which is highly susceptible to CR was used as a reference (Wildermuth and McNamara 1994).

Figure 2-2 shows a graph of the frequency of the CR scores of the three different F_4 populations obtained from the line averages of all field scores. All three populations exhibited a normal distribution of CR scores (Kolmogorov-Smirnov test). There was also no significant difference between the mean CR severity of the entire populations and the respective subsamples taken from them.



STE +/-1.3



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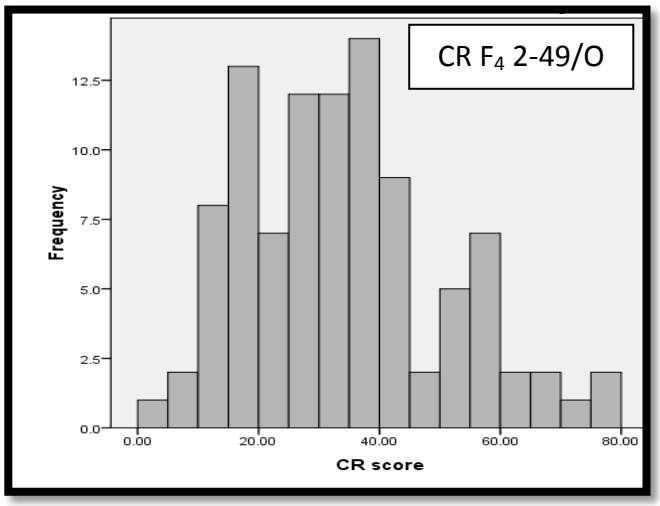


STE +/- 1.6

Figure 2-2: Frequency of Crown Rot scores in the three different analysed F_4 populations (2-49/M, 2-49/N, 2-49/O). The graphs were produced with SPSS.

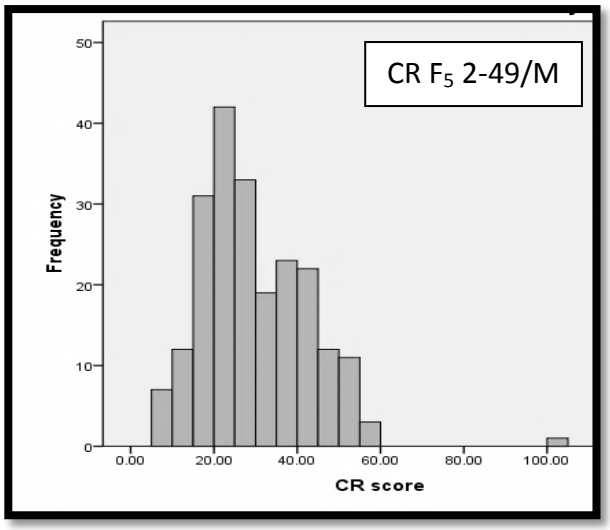
The three F_4 populations exhibited an average CR severity 40- 50% lower than Bellaroi. The 2-49/M population had an average CR score of 74% of the Bellaroi check, the 2-49/N population 66%, and the 2-49/O population 67% of the Bellaroi check . There was no data for the N and O durum parents available in this year.

Because the aim of this trial was to identify CR resistant durum lines, the head type also needed to be considered when selecting suitable lines. Most plants with high CR resistance had long and thin heads, unsuitable for commercial durum production (refer to Appendix CD, Chapter 2). Only two lines with low CR scores in the 2-49/M population possessed durum-like heads (2-49/M 31-1 and 34-3); while in the 2-49/N population, there were four (2-49/N 18-2; 22-1; 22-2 and 25-5) identified. No lines with low CR scores and durum like heads were discovered in 2-49/0.

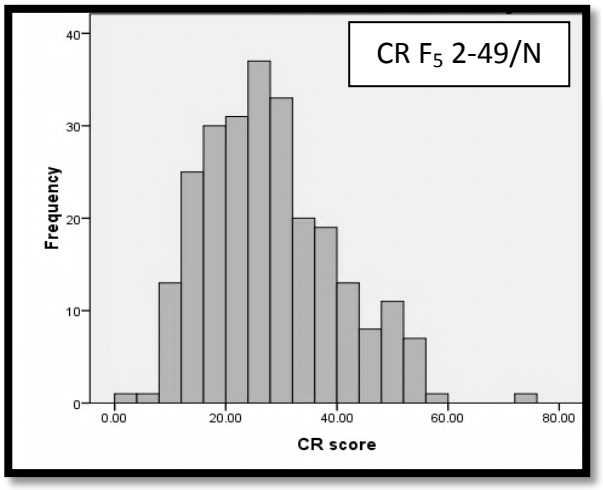
2.4.2 F_5 2006

In the F_5 generation (grown in 2006 and analysed in 2007), three hexaploid wheat x durum crosses were analysed: 2-49/M (961111 or Bellaroi), 2-49/N (950329) and 2-49/O (971179). In this study, the average CR scores of the whole population of 4927 plants (1986 in 2-49/M, 1982 in 2-49/N and 959 in 2-49/O)were analysed. The raw data can be obtained from the Appendix CD, Capter 2, F_5.

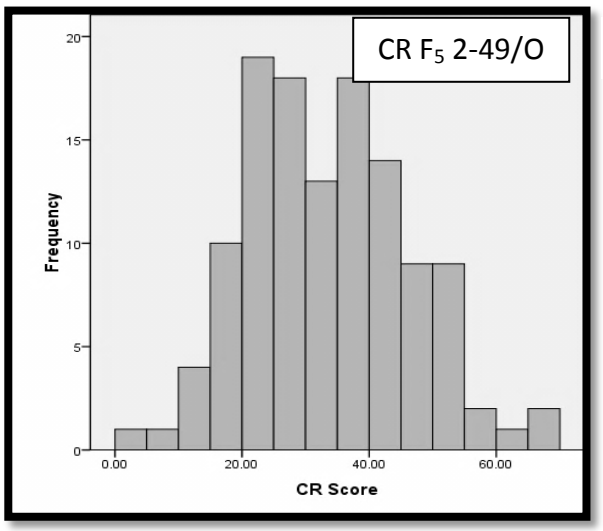
In 2006, the lowest CR scores ranged from 2.6 to 14.2, and the highest from 43.3 to 75. A summary of the average field CR scores is shown in Table 2-1 in the section above, their frequency is shown in Figure 2-3. The CR scores followed a normal distribution.



STE +/-0.9



STE +/- 0.7



STE +/- 1.2

Figure 2-3: Frequency of Crown Rot scores in the three different analysed F_5 populations 2-49/M, 2-49/N and 2-49/O. The graphs were produced with SPSS.

The population 2-49/M had an average CR score of 59% of the Bellaroi check, 2-49/N had 50%, and the 2-49/O population had 58%. In all three populations few plants gave CR scores above 60.

2.4.3 F_6 2007

In the F_6 generation (grown in 2007 and analysed in 2008), three hexaploid wheat x durum crosses were analysed: 2-49/M (961111 or Bellaroi), 2-49/N (950329) and 2-49/O (971179).

In 2007 845: 2-49/M; 1785: 2-49/N and 773: 2-49/O F_6 seed were sown in Tamworth. From these lines, altogether 718 seedlings from the 2-49/M (123), 2-49/N (471) and 2-49/O (125) populations were tagged, eight plants per family. The eight plants were randomly selected from within the plots which contained individual families . The tagging made it possible to trace back each individual plant to an individual CR score. In this study, the average CR scores of the whole population of thousands of plants was analysed. The raw data can be obtained from the Appendix CD, Chapter 2, F_6.

The year 2007 had a very high disease pressure in the field. Due to this, the durum parents had a high average CR score of 66 (Figure 2-4), while 2-49 showed an average disease score of 23. All interspecific lines again had an average CR score far below all three durum parents, with some lines scoring lower than 2-49.

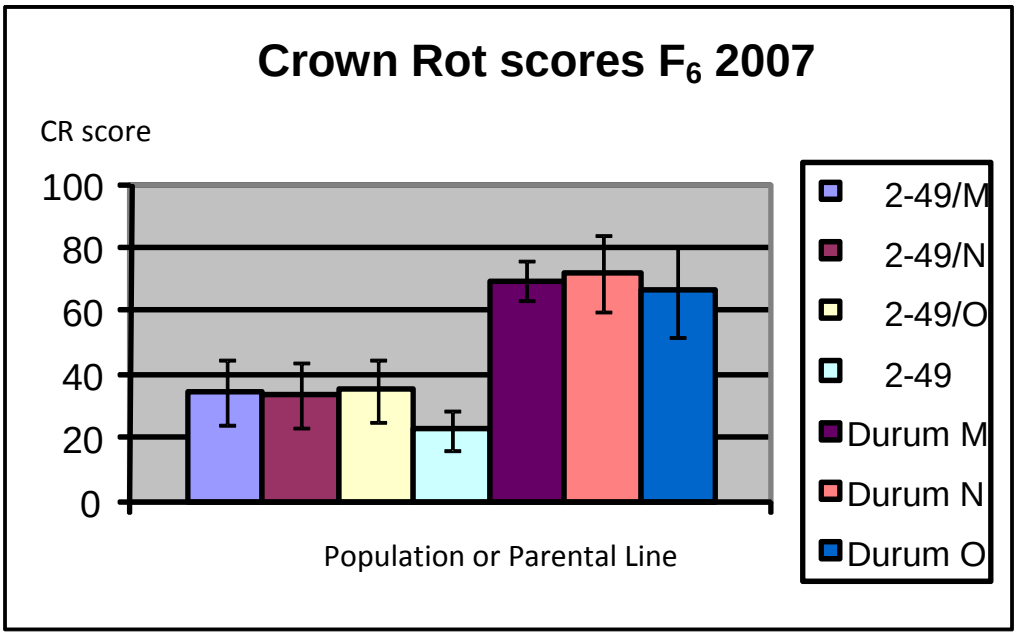


Figure 2-4: Average Crown Rot scores of the three hexapoid x durum wheat populations, (2-49/M, 2-49/N and 2-49/O), and their parents in the F_6.

The CR scores in the F_6 in all three populations varied widely, but despite the high disease pressure in the field in 2007, there were still some lines that had significantly lower CR scores than 2-49.

The three lines with lowest CR scores in the tagged 2-49/M- population were (CR scores shown in brackets): 2-49/M 13-4b (10), 2-49/M 3-3 (11); 2-49/M 25-1a (11). The three lines with lowest CR scores in the tagged 2-49/N population were: 2-49/N 29-10 (8); 2-49/N 7-7a (17); 2-49/N 29- 12b (19). The three lines with lowest CR scores in the tagged 2-49/O- population were: 2-49/O 15-1a (24); 2-49/O 4-2b (25); 2-49/O 6a (26).

Almost all plants with superior CR resistance possessed undesirable head types, only two plants with durum like heads (2-49/M 13 and 2-49/M 4b) had CR scores of 8.3 and 16.7, respectively.

Often the same families consisted of plants with extremely different CR scores. Table 2-2 compares the means of the three different populations, using the Z-test. The tagged plants almost always had a significantly higher CR score than the untagged ones, except in the 2-49/O population. The untagged 2-49/N plants had

a CR score of only 35 whereas the tagged 2-49/N plants had a higher CR score of 45. This could be due to a higher humidity around the stems caused by the plastic tag in the lower stem area.

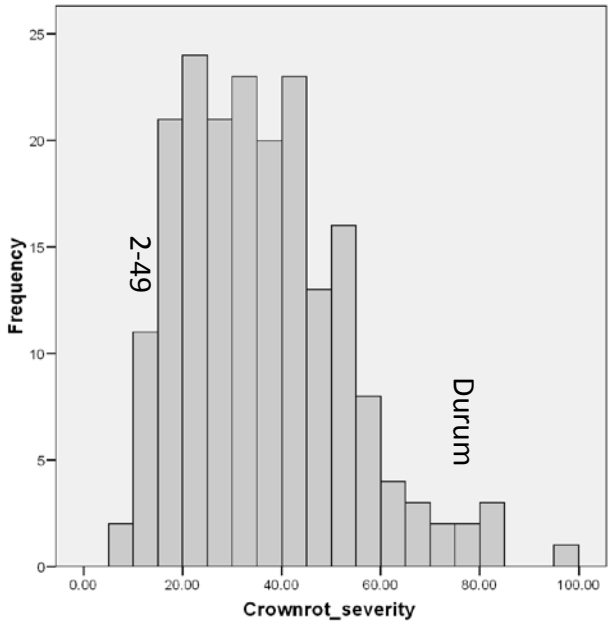
Table 2-2: Differences of Crown Rot means in three F_6- populations of hexaploid x durum wheat crosses, (2-49/M, 2-49/N, 2-49/O).

Compared Cross	Comparison CR means	Difference
2-49/M	tagged/untagged	significant
2-49/N	tagged/untagged	highly significant
2-49/O	tagged/untagged	insignificant

2.4.3.1 F_6 2-49/N

In 2006, due to time constrains, only the 2-49/N population was analysed, because this was the largest population. The average CR score for the entire 2-49/N population was 38. The disease spread ranged from CR scores of under 5 to over 80.

The CR distribution in the F_6 followed a normal distribution (Kolmogorov-Smirnov test). Figure 2-5 shows the spread of the CR scores in the analysed 2-49/N population.



STE +/-1.2

Figure 2-5: Frequency of Crown Rot scores in the F_6 2-49/N population, 2006 (SPSS).

2.5 COMPARISON OF CROWN ROT SEVERITY IN THE THREE YEARS

Clearly, CR resistance from 2-49 was transferred into the progeny of the three different crosses. The three F_4 populations examined in 2004 exhibited a CR severity of 65-73% of the Bellaroi check. The CR severity in the F_5 population compared to Bellaroi was less than 60% of the Bellaroi check, implying an improvement in resistance to CR presumably resulting from active selection in the F_4. By 2007 the mean CR severity was about 45% of the Bellaroi check in the F_6 populations (Figure 2-6).

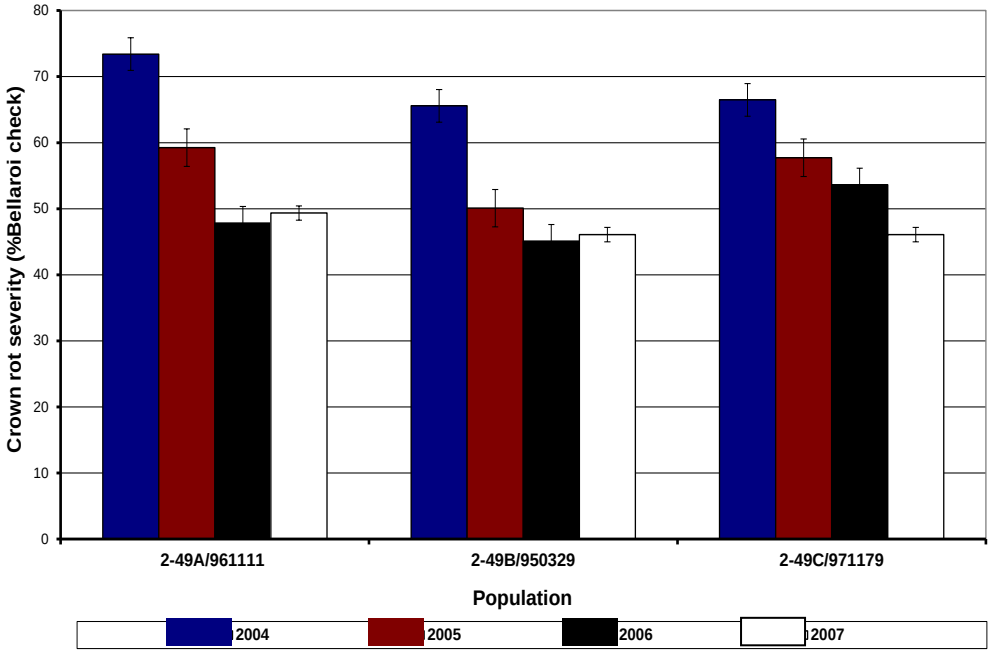


Figure 2-6: Comparison of Crown Rot severity in three durum x hexaploid wheat crosses as a percentage of the susceptible variety Bellaroi in the Years 04-07 (Data provided by Dr Steven Simpfendorfer, NSW, Australia).

Over the years, the populations did not differ significantly in their trait data (Table 2-1). In the F_6 2-49/N population the CR average was higher, because it was a year with very high CR pressure.

2.6 DISCUSSION CROWN ROT RESISTANCE

2.6.1 F_4-F_6 CROWN ROT SCORES FIELD RESULTS

Over the three years, many lines showed lower CR scores than the durum parent and some were even better than 2-49. However, only lines from the families 2-49/M-3; 2-49/M-8; 2-49/M-13; 2-49/N-29 and 2-49/O-4 showed consistently lower CR scores over all three years. Therefore, a genetic (inherited) component can be assumed for this resistance in the crosses. This aspect is very encouraging and shows that improvement of the durum material, which is considered very susceptible to CR, is possible and selection for CR resistance should be continued.

A draw-back to this success was that many of the very resistant lines looked more like the 2-49 parent and not like durum. Backcrosses, currently undertaken at the NSW I&I and USQ aim to correct this problem and incorporate the resistance into a durum background. In 2005, only the Bellaroi (M) parent was available for comparison with the populations. This most probably did not cause much distortion in the F_4 results, as there was no significant difference in CR resistance between the genetically very similar durum parents. For example, in the F_5 trial, durum parent M had a CR score of 60, N had 58 and O had 45. In the F_6 trial, durum parent M had an average CR score of 69, N had 72 and O had 66.

A few other points which may have influenced the results:

Uneven spread of the inoculum in the soil is common in field experiments. The inoculum has to touch the crown directly in order to cause CR. Also, water availability varies between plots but is paramount for evenly distributed inoculation. This point may have lead to some distortion in the CR scores of different plants. The plots were not irrigated.

Tagging with a plastic tag around the lower stem increased the CR scores of the plants significantly, presumably because of higher humidity under the tag which was attached to the lower stem above the crown of the young wheat plants. In the F_6 generation, where plants were tagged, a Z- test was conducted and it was found that the plastic tag increased the CR severity. Tagging around the stem should therefore not be used when assessing fungal infections (Table 2-2).

3 RATE OF ALLELIC FREQUENCIES AND FATE OF THE D GENOME

3.1 INTRODUCTION

Most cytological research into the fate of chromosomes in the progeny of *T. aestivum* x *T. turgidum* spp. *durum* crosses was conducted in the 1920's (Kihara 1982; Sax 1922; Thompson and Hollingshead 1927). One study by Thompson and Hollingshead (1927) quotes the expected theoretical frequency of plants with the *T. aestivum* chromosome constitution in the F_2 of pentaploid wheat crosses to be 1:16384. At that time phenotypic markers were used, but species distinguishing characters were able to transfer a very accurate picture of the genetic constitution (Thompson and Hollingshead 1927). While Kihara and other authors revealed much about the fate of the unpaired D genome chromosomes in the progeny of crosses using traditional cytological methods, they could not determine the identity of the chromosomes, nor tell if there were any intergenomic translocations between the A, B and D genomes.

There have been two recent studies on hexaploid wheat by durum crosses using either the Feulgen staining method to count root tip chromosomes (Wang et al. 2005) or polymorphic SSR markers (Lanning et al. 2008).

The objective of the work described in this chapter was to determine the degree to which the A, B and D genome material from a hexaploid parent is inherited by the progeny of a *T. aestivum* (var. 2-49) by *T. turgidum* ssp. *durum* cross over several generations. Genome analysis with SSR and DArT markers was conducted on progeny of this cross ranging from the F_2 to the F_7 generations. Root tips from F_2 and F_3 seedlings were analysed with MCFISH as described in Zhang et al. (2001). Additionally, root tips of a number of F_2 and F_3 plants were stained with 1% acetocarmine, (Singh 2002) or according to the Feulgen Method (Singh 2002) and the number of chromosomes in cells undergoing mitosis was counted.

Modern cytogenetic and molecular techniques, such as Multicolour fluorescence in situ hybridization (MCFISH) and Diversity Arrays Technology (DArT) markers are now used to determine the identity of chromosomes and show intergenomic translocations. This is the first study to employ DArT marker and MCFISH techniques in concert.

3.2 METHODOLOGY

3.2.1 PLANT MATERIAL FOR DART ANALYSIS

DNA from leaves of the wheat crosses was send for DArT analysis to Triticarte Pty. Ltd., ACT., Australia, at three time points over the years 2006-2008. They were labelled first, second and third DArT experiment and each trial will be described in the following.

For the first DArT experiment, around 200 F_2 seed from the 2-49/M population described in Chapter 2 (section 2.2.1) were obtained from NSW I&I Tamworth and grown in the USQ glasshouse in 20cm pots in standard potting mixture, watered every two days and fertilized with Osmocote slow release fertilizer. There was a large amount of unviable, shrivelled seeds. Therefore, many seeds did not germinate or the seedlings died soon after germination, probably due to an adverse chromosome constitution. Leaves from the remaining plants were harvested ater three weeks and their DNA extracted. This resulted in 83 DNA samples which were analysed with DArT.

For the two other DArT experiments which examined the F_2 seeds used by Dr Peng Zhang for MCFISH, the F_3 offspring of the F_2/Bellaroi cross analysed in the first DArT experiment and F_7 seeds from the 2-49/M, 2-49N and 2-49/O cross, seeds from all three populations (2-49/M, 2-49/N and 2-49/O) were germinated in petri dishes lined with wet filter paper. The petri dishes were placed in plastic bags in the growth cabinet at 25˚ C and leaves were sampled after 8-10 days. The number of plants used in each population is described in the relevant section. Refer to Appendix CD, Chapter 3, for the lines used in the DArT experiments.

3.2.2 DNA EXTRACTION FOR DART

For the first DArT experiment, DNA was extracted from leaf material using the DNeasy Plant Mini kit (Qiagen Pty Ltd, Vic., Australia). The protocol provided by the supplier was used (www1.qiagen.com/HB/DNeasy96Plant). This kit provides a fast way to purify DNA without the use of phenol or chloroform. High quality DNA, with an A260/A280 ratio of 1.7-1.9, was obtained. The frozen leaves were disrupted in the tubes with lysis buffer by grinding them in the Qiagen TissueLyser for 2x30-60 seconds. RNase, included in the lysis buffer, digested the RNA in the sample. After lysis, the proteins and polysaccharides were separated from salt and cell debris by centrifuging and the precipitate removed. Binding buffer and ethanol were added to promote binding of the DNA to a membrane during centrifuging of the sample. DNA was then dissolved in buffer or water. Simultaneously, for the first DArT experiment, SSR markers were amplified on the DNA to test segregation. The data is shown on the Appendix CD, Chapter 4.

For the 2rd and 3rd DArT experiments the Genome Wizard extraction method (Promega, Madison, USA), was employed instead of the Qiagen DNA extraction kit, because it was cheaper and the resulting DNA quality was equally good. The protocol provided by the suppliers was used.

3.2.3 DART ANALYSIS

For DArT analysis (Akbari et al. 2006; Wenzl et al. 2004), the quality of the purified DNA was tested on a 1% agarose gel. The gel was run on an electric current of 90V for 20min., then analysed with a Bio Rad Gel doc Molecular Imager XR system, using the software Quantity One. Acceptable DNA quality was assumed if the DNA was not fragmented but displayed a marked band. Ten to 20 µl of 50 ng/µl of DNA of sufficient quality was loaded onto a 96 well tray and sent to Triticate Pty. Ltd., Vic., Australia for DArT analysis (www.diversityarrays.com, accessed 23 July 2010). DNA was hybridized to a wheat array, a genomic representation of a mixture of mainly hexaploid wheat cultivars and a small

number of durum cultivars. For each marker a P-value (based on ANOVA) was established, which represents an estimate of marker quality. Only markers with a P-value greater than 77 were considered, as this is the recommended benchmark value (Akbari et al. 2006, Wenzel et al. 2004).

3.2.4 ROOT TIP SQUASHES AND FEULGEN STAINING

Chromosomes counts of approximately 150 mitotic metaphase F_3 cells were conducted at USQ using the Feulgen method of chromosome staining (Singh et al. 2002). Seed was sterilized for 1 min. in 70% alcohol, 5 min. in 30% bleach and rinsed with dH_2O. Seed was grown for three days on heat sterilized filter paper on petri dishes placed in plastic bags, in the growth cabinet, at 25° C. The roots were harvested in the morning and placed in a labelled tube containing filtered tap water. The tube was placed on ice in the fridge for 29 hours. The roots were then fixed in a Methanol : Propionic acid (4:1) solution and placed in dH_2O for 30 min. They were hydrolysed for 6.5 min. In 1N HCL at 60°C, and cooled in fresh dH_2O. They were then stained in fresh Feulgen stain in the dark for at least 60 min. and washed twice in distilled water. Each root was placed on a slide and the tip cut off. A drop of Acetocarmine was placed on the tip and a placeholder cover slide placed on the side of the tip. Another cover slide was placed upon the tip, overlapping the other cover slide, and gently tapped with the back of a dissection needle, until the root dispersed. Then the slide was briefly heated on an alcohol burner and the root tip vigorously squashed between the slides with the thumb. The chromosomes in dividing cells were counted under the light microscope with a 100x oil immersion lens. Cell photography was carried out using the MICROPUBLISHER 5.0 RTV, High resolution IEEE 1394 FireWire™ Digital CCD Color Camera with High Speed Real Time Viewing, the computer program used was Qcapture Pro (www.qimaging.com).

3.2.5 MCFISH

This method uses tagged DNA probes to determine the chromosome constitution of mitotic metaphase cells. The experiments involving MCFISH were

conducted by Dr Peng Zhang at the University of Sydney, Cobbitty NSW. I spent three days in Dr. Zhang's laboratory learning the Feulgen and Acetocarmine staining methods, and observing the specialized MCFISH staining technique applied to these materials.

Seed germination, root-tip pre-treatment, squash preparations, slide pre-treatment and denaturation were performed according to Zhang et al. (2001), the reference for the laboratory protocol is quoted as Zhang et al. (unknown year). Root-tips were collected separately from each seedling and chromosome numbers were counted in root-tip squashes stained with a 1% acetocarmine solution before the slide was used for MCFISH.

A bacterial artificial chromosome (BAC) is an artificial piece of DNA which is replicated inside the DNA of bacteria, usually *E. coli* and can be used for transforming and cloning of DNA. The typical insert size is 150-350 kbp up to, or more than 700 kbp. The bacterial artificial chromosomes BAC 676D4 and 9M13 DNA were isolated using a Qiagen Plasmid Midi Kit (Qiagen). The BAC clone 676D4 from the *T. monococcum* library contains a dispersed repeat that hybridizes to A-genome chromosomes, and 9M13, from the *Ae. tauschii* library contain a dispersed repeat that hybridizes to the D-genome chromosomes. These repeats are useful in discerning the three genomes in hexaploid wheat, and in identifying intergenomic translocations in wheat (Zhang et al. 2004). One microgram of 676D4 and 9M13 was labelled with Tetramethyl-Rhodamine-5-dUTP (Roche Applied Science, Australia) using nick translation and biotin-14-dATP using the BioNick Labelling System (Invitrogen Life Technologies, Australia), respectively. The protocol provided by the supplier was used. The maximum excitation and emission wavelengths for visualizing the Rhodamine were 551nm and 575 nm, respectively. The hybridisation and post-hybridisation washes were conducted as described in Zhang et al. (2004). The biotin-labelled probe was detected with fluorescein-avidin DN (Vector Laboratories, Burlingame, CA. USA). The wavelength settings (max. excitation and emission), were at 495-500 nm and 514-521 nm respectively.

Chromosome preparations were analysed with an epifluorescence Zeiss Axio Imager microscope. Images were captured with a Retiga EXi CCD (charge-coupled device) camera (QImaging, Surry, BC, Canada) operated with Image-Pro Plus 6.2 software (Media Cybernetics Inc., Bethesda, MD) and processed with Photoshop v8.0 software (Adobe Systems, San Jose, CA, USA).

3.2.6 SSR ANALYSIS WITH GEL ELECTROPHORESIS

For SSR analyses of F_4-F_6 plants, single leaves were taken from three week old plants (Zadoks stage 2-3 (Zadoks et al. 1974)) grown in the field at Tamworth and Breeza, NSW. Leaf samples were placed in Eppendorf tubes and transported on dry ice to Toowoomba were they were stored in the freezer until DNA extraction.

DNA was extracted with the Genome Wizard kit (Promega, Madison, USA), as described in section 3.2.2., then mixed with other ingredients in preparation for PCR (Polymerase Chain Reaction) amplification of selected markers.

The PCR reaction mix consisted of:

- 30ng genomic DNA;
- 5 μM of each primer (GWM, WMC, CFD and BARC primers, sequences were obtained from graingenes: http://wheat.pw.usda.gov/gg2/index.shtml);
- 0.25U BioTaq Red polymerase, (Bioplant Pty Ltd., Australia).
- 100 μM of each dNTP;
- 1.5 mM $MgCl_2$;
- 1 x PCR buffer (Bioplant Pty Ltd., Australia).

The total volume of the PCR reaction mix amounted to 10 μl.

The tubes were placed into a thermo cycler (TGradient PCR machine (Biometra, Germany)). The PCR cycle profile was used: 7 min at 95°C, followed by 35 cycles of 30s at 95°C, alternating with the elongation steps of 30 seconds each at 50-60°C (depending on the annealing temperature of the primer) and a final elongation step of 5 minutes at 72°C.

DNA samples from the PCR reaction described in section 3.2.4 were stained with a gel loading buffer (2 μl) consisting of EDTA, Formamide and Bromophenol blue, then visualized with a Corbett Gelscan machine using a 15% denaturing

acrylamide gel. Microsatellite alleles of different length travel at different speeds through the gel and hence pass the laser detector of the machine at different times. The position of the fragment is then compared with a standardised “size” ladder, which is loaded onto the gel with each run to determine the fragment sizes.

3.2.7 CHROMOSOME MAPPING

Linkage maps were produced from the combined marker data (SSR and DArT markers). The marker order was determined with Record (Van Os et al. 2005). All markers were then manually ordered using the “link report” function in MapManger QTX (Manly and Olson 2001). Subsequently some markers were later sorted manually and double-crossovers were removed, resulting in a significant decrease in the map size.

The genetic distances were calculated with the Kosambi mapping function (Kosambi 1944). MapChart 2.1 (Voorrips 2002) was used to produce the map figures.

3.2.8 STATISTICS

A Chi square test (χ^2- test) of independence was conducted to examine the relative abundance of parental markers and to determine regions where segregation distortion was present (Chernoff and Lehmann 1954).

3.3 RESULTS

This section compares the results obtained from DArT and SSR marker analysis with MCFISH chromosome staining and chromosome counting results. Results are divided into sub-sections according to the year in which the analysis was conducted. For a complete list of markers and scoring results refer to the attached appendix CD, Chapter 3 for DArT results, Chapter 4 for SSR marker results).

3.3.1 RESIDUAL VARIATION IN PARENTAL SEED SOURCES

As the uniformity of the genomic constitution within each parental group is of vital importance for the success of any molecular marker work, special consideration was given to the examination of such material. To test this, 120 SSR markers were screened across the durum parents M, N and O (four samples were analysed from each parent) . Significant differences were observed between the parents M, N and O. It was shown that durum O was the most dissimilar from the other two parents. Moreover, some residual polymorphism within the parents themselves was observed. To demonstrate the differences in the N parent, seven of the markers which had shown residual polymorpism were tested again on newly extracted DNA of 18 individual plants. Four of these lines showed a variant allele for the SSR marker wmc471. This low level of variation within wheat varieties is not unusual. DNA from nine individual Bellaroi (durum M) plants was analysed with 500 DArT markers (P>80). The differences in the Bellaroi (M) parent in samples sent to Triticarte for DArT analysis at three different time points (called 1^{st}, 2^{nd} and 3^{rd} experiment in the following) occurred at 6%, 2% and 4% of loci respectively. The genetic differences in the Bellaroi parents were deemed minor and not statistically accounted for in the analysis. However, for mapping, only parental lines that seemed appropriate, i.e. lines that did not have too many outlying alleles, were used.

Parent 2-49 on the other hand showed no polymorphism when 500 DArT markers were screened across nine individual plants.

3.3.2 F_2 DART EXPERIMENT

Around 200 F_2 seeds from the 2-49/M (Bellaroi) cross were obtained from Tamworth. These were sown in the USQ greenhouse. Almost half of the seeds did not germinate, presumably due to a non-viable chromosome constitution. Since the F_2 population is of critical importance for breeding selection, initially the DNA of 109 F_2 plants from this cross and the parents 2-49 and Bellaroi was sent to Triticarte Pty Ltd (ACT, Australia), (*www.diversityarrays.com*, viewed 19/11/2010)

for DArT analysis. Roots from twenty-six of these plants were analysed using MCFISH chromosome stains as described in sub-section 3.3.4.

DNA was hybridised to a wheat array, consisting of a genome representation from mainly hexaploid cultivars with only a few durum varieties included (Wenzl et al 2004).

Five hundred and forty-six markers hybridised on the DNA of the population. Markers which could not be mapped, or had a P value below 77 (48 markers), or were non-polymorphic among the parents, were set aside. An exception was made for twenty markers with P values between 74 and 77 which mapped to the D genome. These were included in the map. A 'missing' score was established for 5.5% of the data. Of the analysed DArT markers, 88% were maternal markers (score of 1) and 12% were paternal markers (score of 0). The high percentage of maternal markers in this experiment was caused by the predominantly hexaploid nature of the wheat array used in DArT.

For the D genome analysis 157 markers out of 546 were removed for various reasons, mainly low P value, but also apparent mismatch of alleles to the places where they mapped. This was determined by comparing the suggested marker position to their respective position on several existing durum and hexaploid wheat maps as well as simple comparison with adjoining markers.

Figure 3-1 displays the frequency of inheritance of alleles in the different chromosomes. Eighty per cent of all markers segregated 3:1 (signal: no signal). This is consistent with Mendelian ratios for a randomly inherited dominant character such as a DArT marker.

Segregation distortion ($P< 0.01$) was only observed in the case of ten closely linked markers on chromosome 5B which favoured inheritance of the durum alleles. This was confirmed with the χ^2 -test, which showed that there were significant differences from the expected ratio. No plant was exactly like either parent; all offspring had differing amounts of both hexaploid and durum alleles, excluding the possibility that selfed F_1 progeny were present in the previous generations.

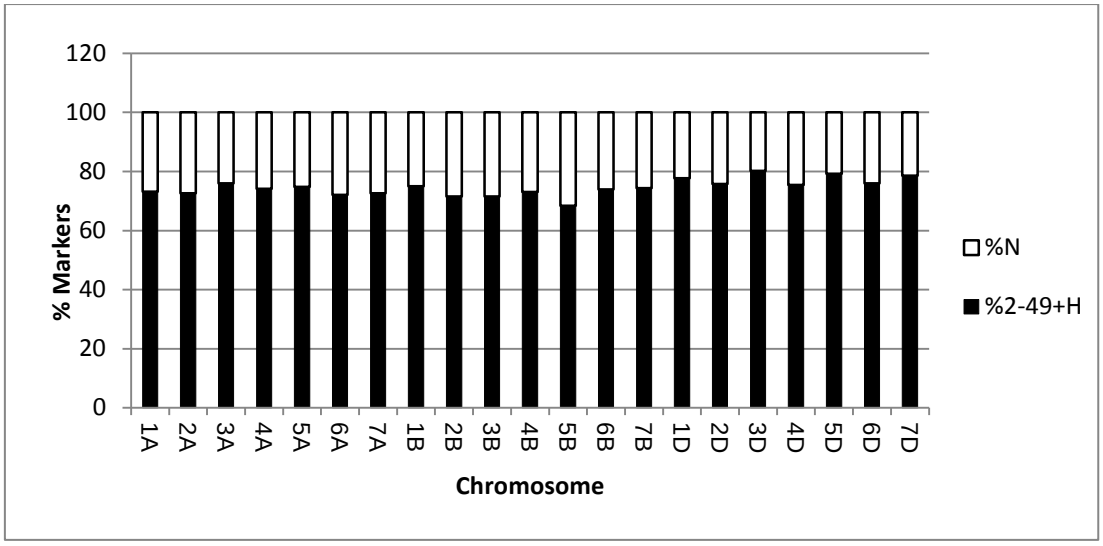


Figure 3-1: Frequency of inheritance of parental alleles in the F_2 generation of a 2-49/Bellaroi cross analysed with DArT markers; 2-49 = homozygous for 2-49 alleles; N = homozygous for N durum alleles, H= heterozygous alleles.

The results after ordering all markers with Map Manager showed, that 7.5% of all markers were unlinked, 12.9% mapped to the A genomes, 29.9% to the B genomes and 49.7% to the D genome chromosomes (Figure 3-2).

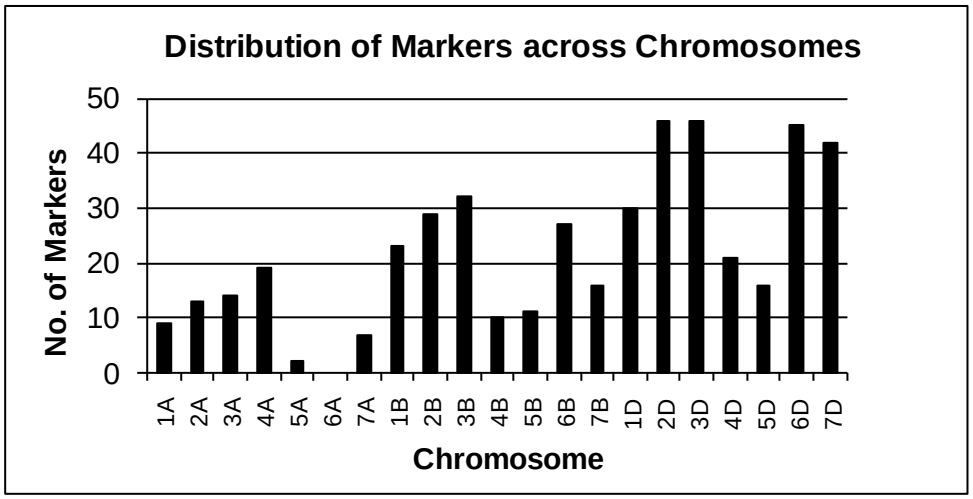


Figure 3-2: Distribution of DArT markers on the chromosomes in the F_2 of a 2-49/Bellaroi cross.

A map was produced by the author based on the DArT markers which were run on wheat plant DNA prepared for DArT by the author. This map is shown in Figure 3-3. For publication, a map from the same data was produced by Dr. Anke Lehmensiek. This map is shown in the Appendix CD, Publications (Martin et al. 2011). Both maps were compared by the author. In most regions, the two maps had the same marker order, but no markers were detected by the author on chromosome 6A. All other figures and tables in this chapter are based on the map produced by the author. Several computer programmes were used for analysis. The obtained marker order was compared with the DArT-based wheat and durum maps available on the Triticarte website (*www.triticarte.com.au*, viewed 19/11/2010). The map length was approximately 1800 cM for the A and B genomes.

Figure 3-3: Genetic map of the A and B genomes of a 2-49/Bellaroi F_2 population. Distances (cM) are indicated on the far left side, markers are given on the right. MapChart 2.1 (Voorrips 2002) was used to produce the map figures.

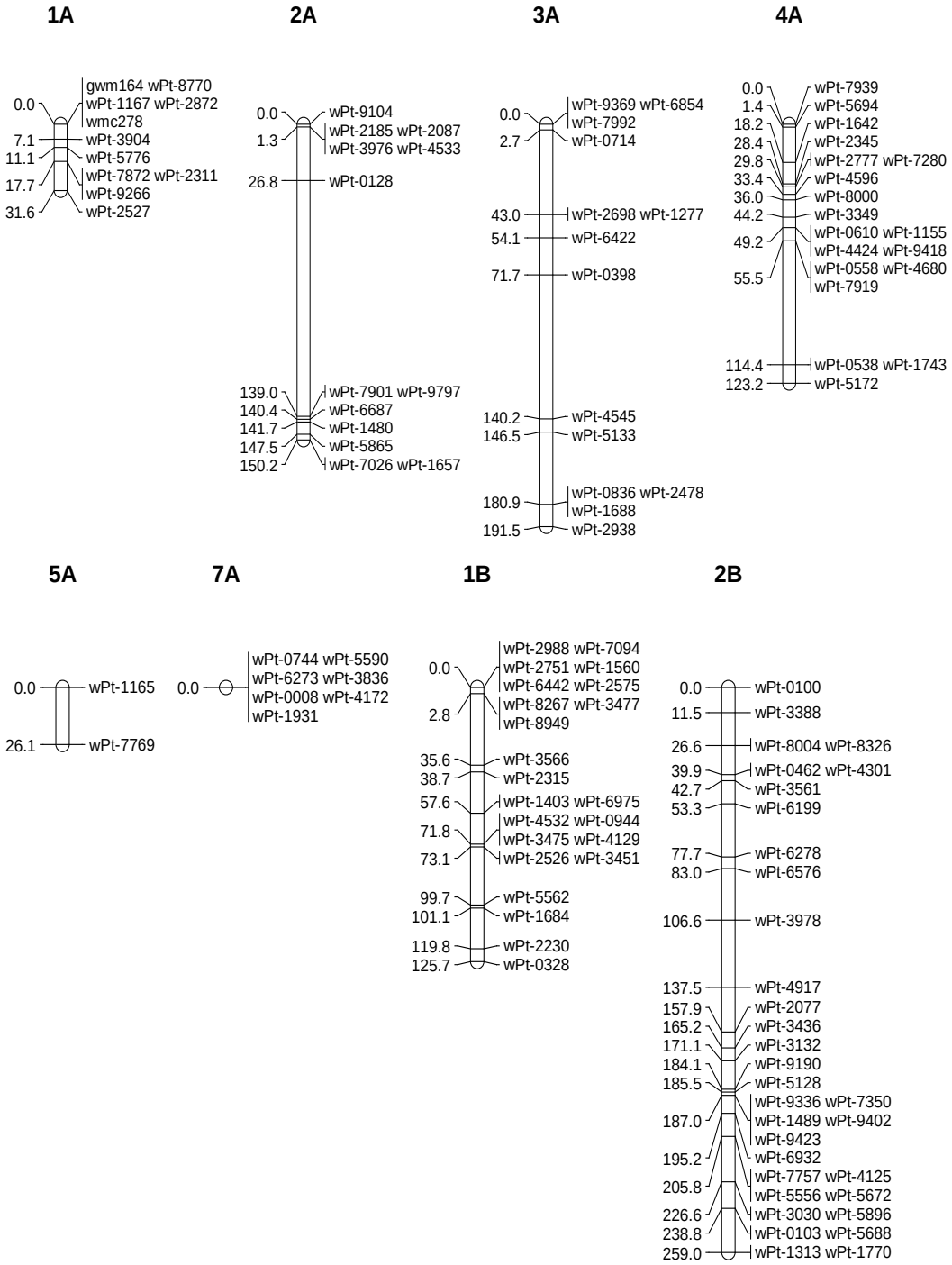
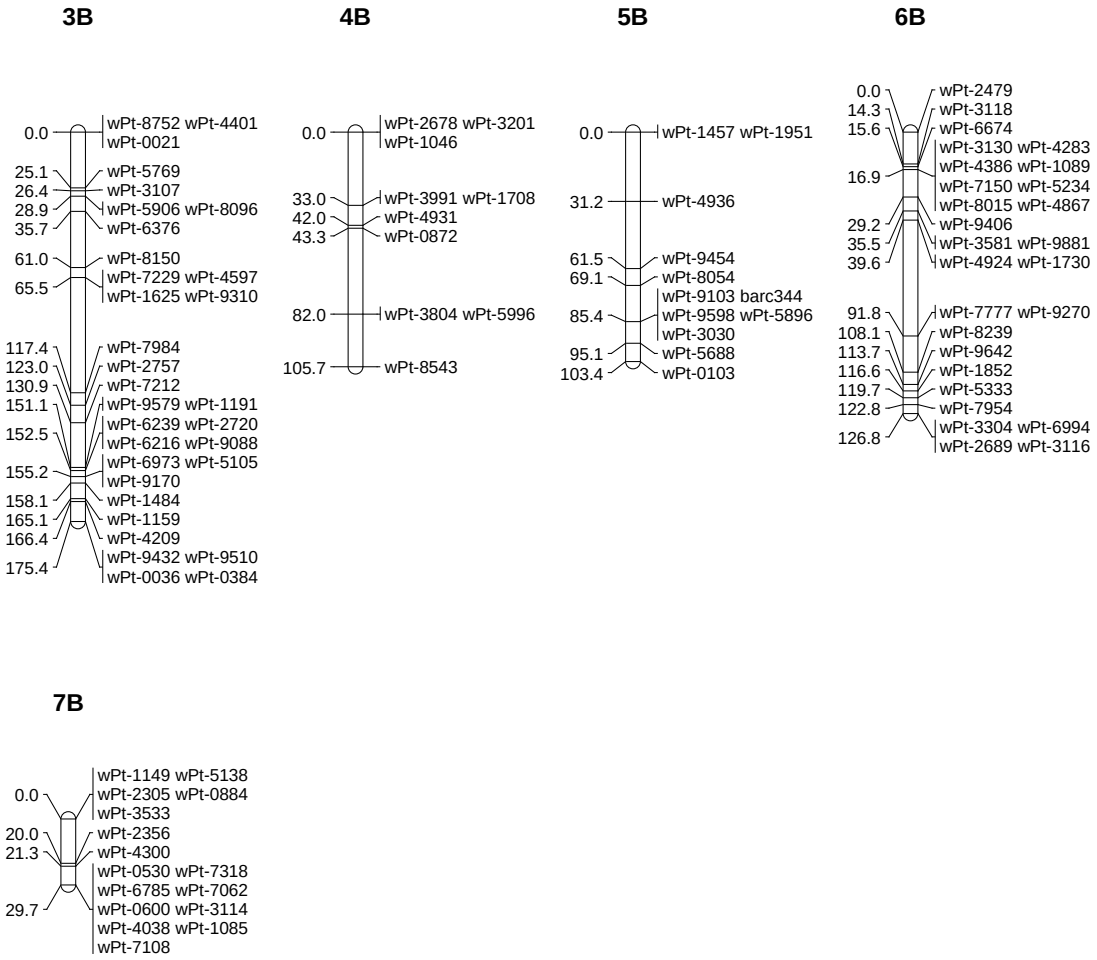


Figure 3-3: Continued



Cross-over events are necessary to determine the length of a chromosome and establish the distances between genes. The further apart two chromosome loci are, the higher the likelihood that cross-over events or recombination between the paired chromosomes occur (Morgan 1911). In the F_1 generation of a hexaploid wheat x durum cross, all plants inherit only one copy of each D genome chromosome. No recombination can therefore take place and a map length cannot be established. However, it is possible to determine the approximate position of a marker. D genome markers, obtained from DArT analysis, were compared with, and matched to, linkage groups of D genome loci from several different genetic maps provided by Triticarte. These maps, which are not yet combined into a consensus map, were generated by Triticarte from other hexaploid wheat crosses. It was found that of the 247 D genome markers that could be placed on the new map, 157 markers were previously classified by Triticarte as unlinked. Seven markers mapped to chromosomes other than originally stated in the DArT report. This was due to the predominantly hexaploid wheat based-array which did not account for the different location of some marker alleles in durum wheats (personal communication with E. Huttner, Triticarte). The markers that mapped to the D genome chromosomes are shown in Figure 3-4.

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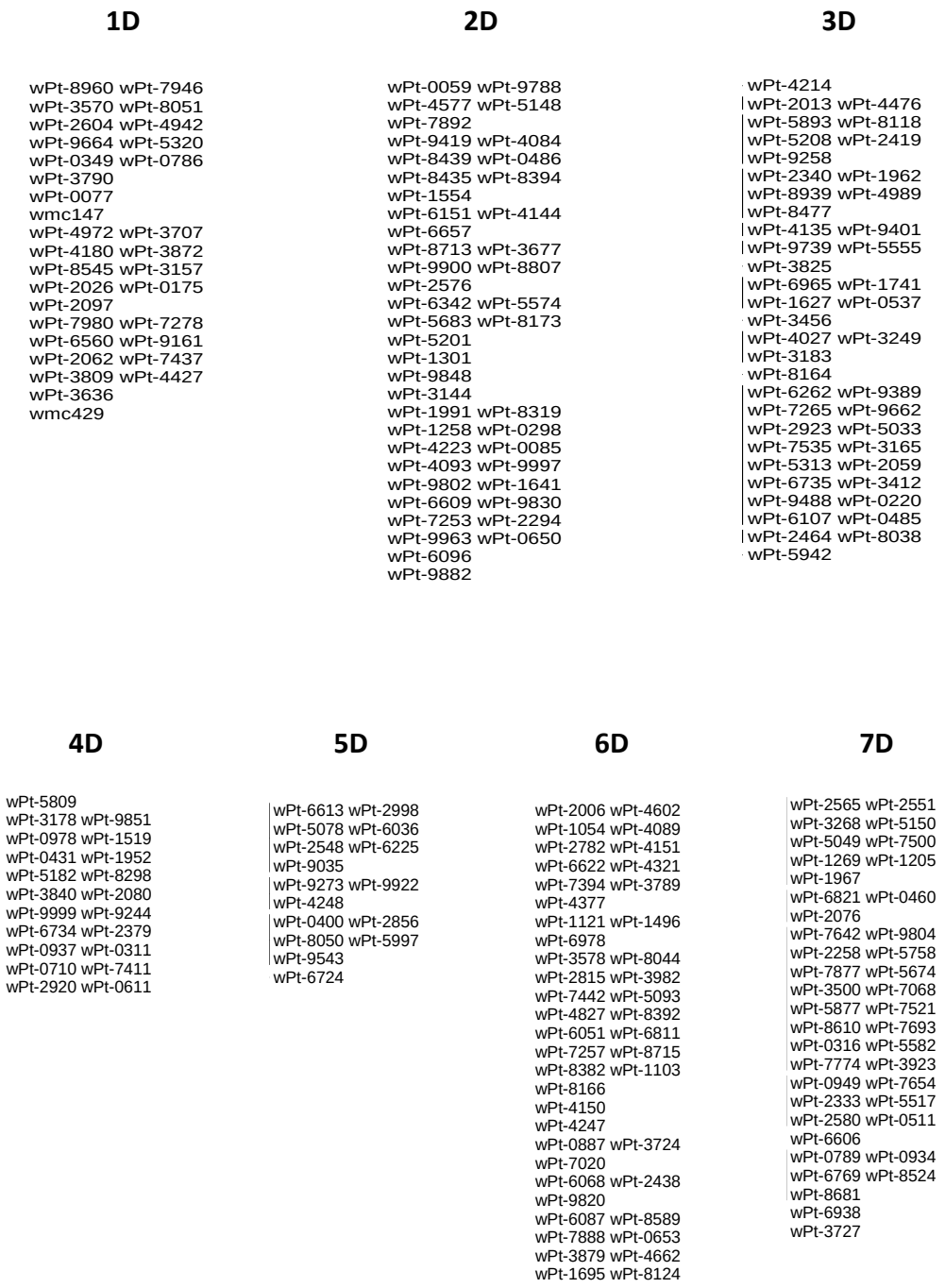
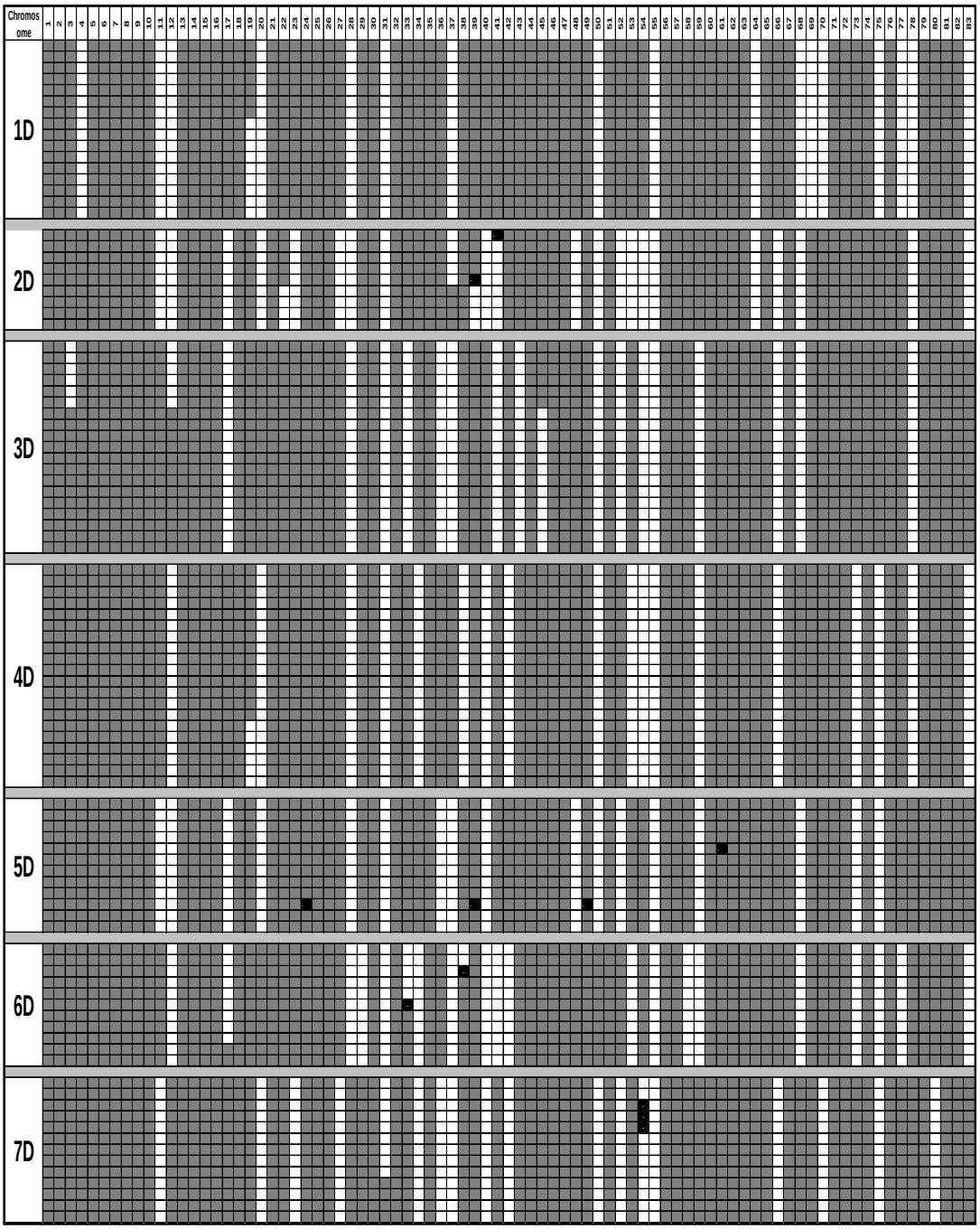


Figure 3-4: Genetic Map of the D genome of the F_2 generation of a 2-49/Bellaroi cross. MapChart 2.1 (Voorrips 2002) was used to produce the map figures.

Table 3-1 provides an overview of the results of DArT for the D genome chromosomes in 83 of the F_2 plants sent for DArT analysis. These were plants that were not analysed by MCFISH. In total 80% of the D chromatin from 2-49 was still present in the F_2 lines from the 2-49/M cross. Eighty-two out of 83 F_2 plants contained at least some D chromatin. Altogether 39 different D chromosome combinations were counted; the majority of combinations were present only once.

Thirty-four out of 83 plants (41%) had a full set of all 7 complete D genome chromosomes, however, due to the dominant nature of DArT markers, it is not known if they consisted of only one copy of the chromosome (univalents), or of both copies (bivalents).

Table 3-1: D chromatin content in 83 F_2 2-49/Bellaroi plants analysed with DArT markers. Each column represents 1 of the 83 F_2 plants analysed. Each row represents a different marker, and different D genome chromosomes (Chrom) are indicated. Alleles from 2-49 are indicated in grey, Bellaroi null alleles in white and missing values in black.



Twelve plants contained one or more possible translocations or partial chromosomes in chromosome 1D (1), 2D (3), 3D (3), 4D (1), 6D (3) and 7D (1) (Table 3-2).

Table 3-2**:** Summary of D genome chromosome numbers in 83 F_2 plants of a 2-49/Bellaroi population analysed with DArT markers.

D Chromo.	1D	2D	3D	4D	5D	6D	7D
complete	65	59	64	64	66	62	65
partial	1	3	3	1	0	3	1
none	17	21	16	18	17	18	17

3.3.2.1 Head Types

F_2 plants from the 2-49/M cross displayed very different head types, as Figure 3-5 shows (all of these plants had at least seven different D genome chromosomes in the DArT analysis).

Figure 3-5: Different head types of interspecific F_2 plants from a 2-49/Bellaroi cross grown in the glasshouse at USQ, Toowoomba, Australia. The numbers are relating to the F_2 DArT analysis numbering.

3.3.3 DART MARKER QUALITY

Most markers used in the DArT experiments displayed a sufficiently informative content as measured by the PIC value (Table 3-3). A description of the PIC value can be found in section 1.6. Two markers with very low, but technically reliable, PIC value were observed (wPt-1272 on 4B and wPt-9382 on 6A).

The average PIC value was higher in the second and third DArT experiment (described later), because seedlings were grown in the growth cabinet instead of the greenhouse, resulting in better qualtity DNA (Mundy, 2005). In the third experiment the average PIC value was the highest with 0.41.

Table 3-3: Polymorphism Information Content (PIC) of DArT markers in three different DArT experiments conducted on different generations (F_2, F_3, F_7) of a 2-49 x durum cross.

PIC value in three DArT experiments			
	PIC value	DArT markers	% of DArT markers
1st experiment	0.5- 0.4	191	35.0
	0.4- 0.3	334	61.2
	0.3- 0.2	16	2.9
	0.2- 0.1	3	0.6
	0.1- 0.0	2	0.4
2nd experiment	0.5-0.4	296	53.5
	0.4-0.3	188	34.0
	0.3-0.2	60	10.8
	0.2-0.1	7	1.3
	0.1-0.0	2	0.4
3rd experiment	0.5-0.4	740	75.7
	0.4-0.3	53	5.4
	0.3-0.2	77	7.9
	0.2-0.1	51	5.2
	0.1-0.0	57	5.8

The relationship between P value, call rate and PIC was analysed in the first experiment (Table 3-4). The average P value for the A genomes was 84.61, for the B genomes 84.35 and for the D genomes 84.78. A small reduction in PIC value and call rate could be observed in the categories with lower P values. This was expected.

Table 3-4: Relationship between P value, Call Rate, PIC value of DArT markers analysing the F_2 generation of a 2-49/Bellaroi cross .

	100 > P > 90	90 > P > 80	80 > P > 70
No. of Markers	80	332	134
Call Rate	97	95	90
PIC	0.41	0.39	0.37

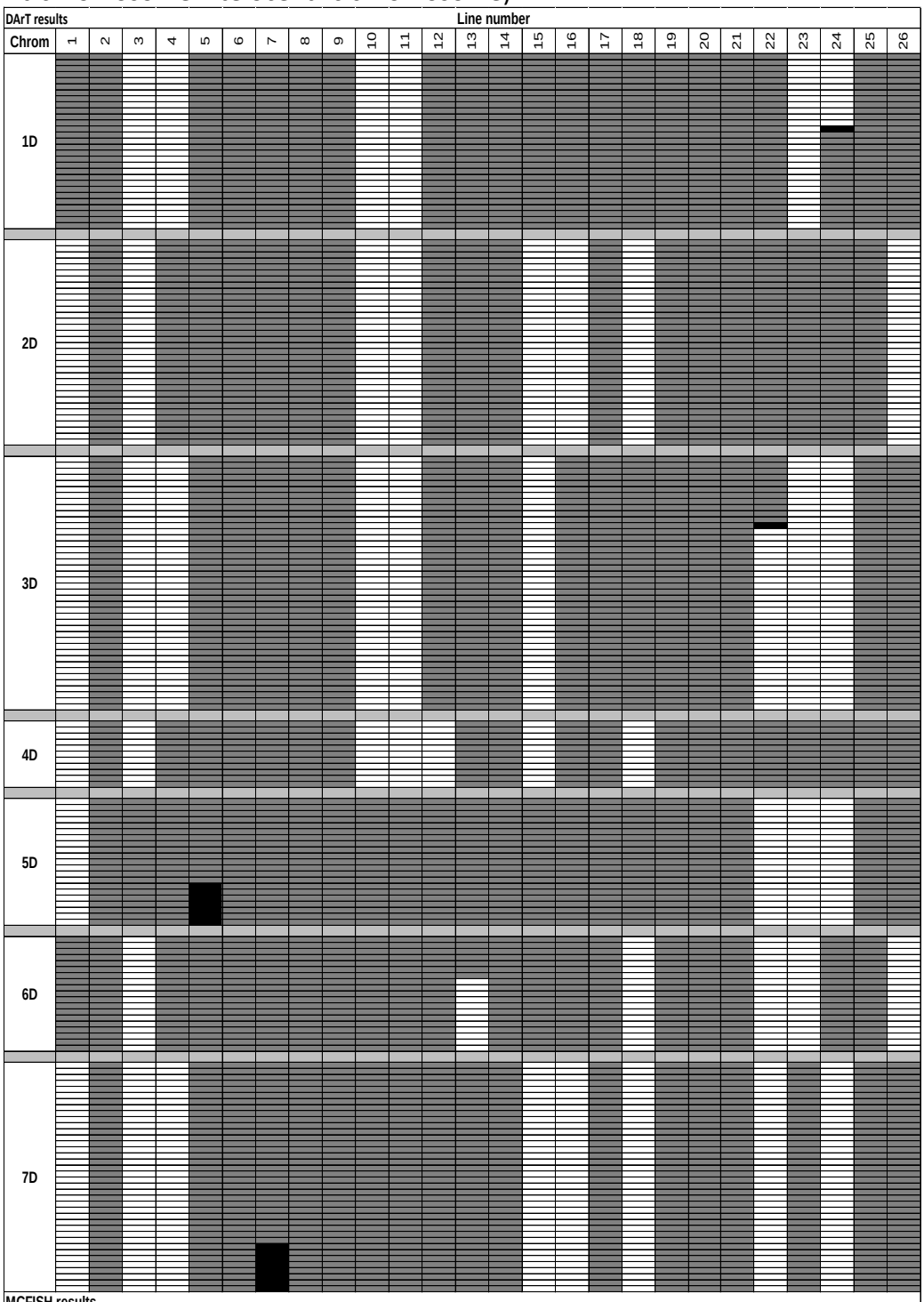
3.3.4 F_2 DART COMPARED TO MCFISH

Twenty–six other F_2 plants from the same 2-49/ Bellaroi cross were simultaneously analysed with MCFISH and DArT. For MCFISH, root squashes were used and the DNA of the same plants was extracted from leaf samples and used for DArT marker analysis. Five-hundred and fifty- three markers hybridized to these samples. One-hundred and sixty-six of these hybridized markers had a P value of < 80. These markers were not excluded. Markers which had no name allocated to them by Triticarte and markers that did not separate the two parents were removed, so that 507 markers remained for analysis**.** There was no significant segregation distortion in this small population(χ^2 -test). Altogether, the D chromatin content of the plants was about the same as in the 86 plants analysed above (80%).

Four-hundred and twenty two markers were maternal and 85 paternal. Paternal markers were not used for comparison with MCFISH. Four-hundred and twenty- two maternal markers remained. The results of both experiments are shown in Table 3-5.

Each column in Table 3-5 represents one of the 26 F_2 plants analysed. In the DArT results each row represents a different marker, and different D genome chromosomes (Chrom) are indicated. Alleles from 2-49 are indicated in grey, Bellaroi null alleles in white and missing values in black. Under theMCFISH results the chromosome counts for the A, B, and D genomes are shown. Translocation- (T) and telocentric (t) chromosomes are also indicated.

Table 3-5: D chromatin content in 26 F_2 plants of a 2-49/Bellaroi cross analysed by DArT markers and MCFISH (T-chromosome = Translocation chromosome; t-chromosome = telocentric chromosome).



MCFISH results																											
A	14	14	14	14	14	14	14	14	14	14	14	14	13+T	14	14	14	14	14	14	14	14	13+T	14	14+T	14	14
B	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14
D	2	12	1	4	14	7	12+t	11	11	3	3	11	9+T	9	4	5	10	3	12	14	7	3+T	4+t	3+T	14	5
Total	30	40	29	32	42	35	40+t	39	39	31	31	39	37	37	32	33	38	31	40	42	35	31	32+t	31	42	33

MCFISH results showed that all of the plants contained varying amounts of D chromatin. Three plants contained the bread- wheat compliment of 42 chromosomes. Fifteen plants had uneven chromosome numbers, which suggests that unpaired D genome chromosomes were present. Figure 3-6 (a) shows a cell of F_2-5 with 42 chromosomes and a cell of F_2-6 with 35 chromosomes (b). Three plants had an A-D translocation each (F_2-13, F_2-22 and F_2-24), this is shown in Figure 3-7. The translocations were from chromosomes 6D, 3D and 1D, respectively These D genome chromosome pieces were translocated into unidentified A genome chromosomes. The translocation in plant F_2-24 is not a whole-arm translocation as in F_2-13 and F_2-22. The translocation is on the long arm of the A genome chromosome where a small D-genome segment partially replaces the A genome chromosome (Figure 3-7b). This is a rare translocation (personal communication Dr Zhang). Two plants (F_2-7 and F_2-23) had telocentric (partial) chromosomes. The translocations are clearly visible in the DArT results, but only one telocentric chromosome (F_2-7) could be observed and was identified as 7D. The telocentric chromosome detected with MCFISH in plant F_2-23 could not be detected with the DArT markers because they are dominant and another copy of the chromosome was probably present. The DArT results do not indicate to which A genome chromosomes the D genome chromatin translocated.

Table 3-6: Summary of D genome Chromosome Number in 26 F_2 Plants analysed with DArT markers.

D Chromosomes	1D	2D	3D	4D	5D	6D	7D
complete	20	18	17	19	23	20	18
partial chromos.	1	0	1	0	0	1	0
none	5	8	8	7	3	5	8

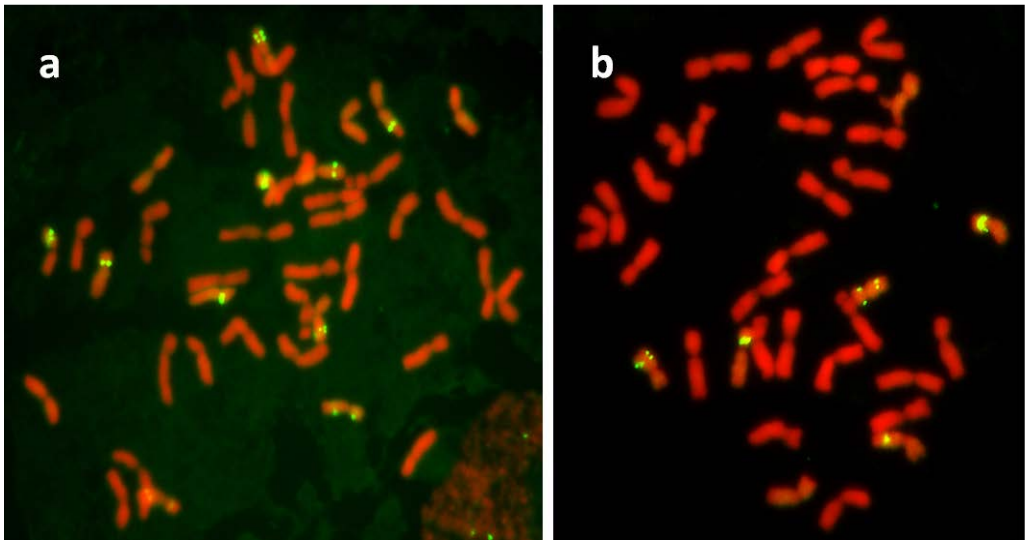


Figure 3-6: Mitotic F_2 root cells from a 2-49/ Bellaroi cross stained with Acetocarmine by Dr Peng Zhang, USYD, NSW, Australia; (a): 42 chromosomes (F_2-5) and (b): 35 chromosomes (F_2-6).

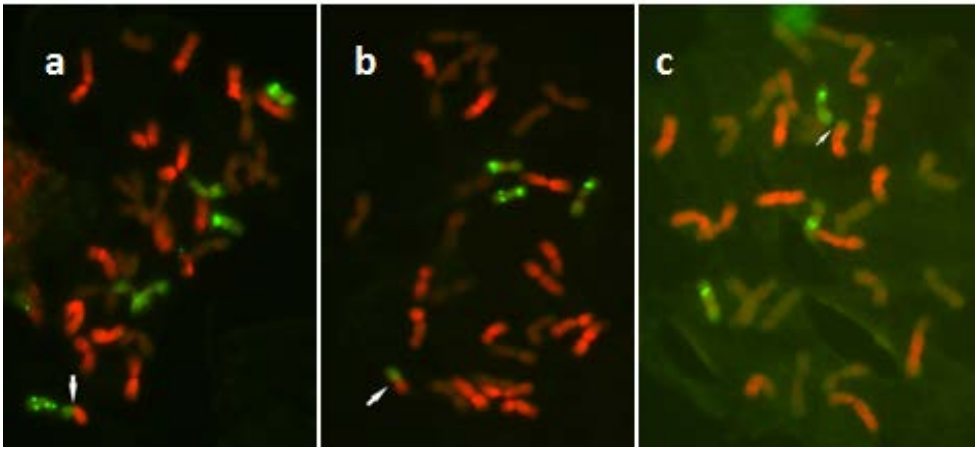


Figure 3-7: Mitotic F_2 root cells analysed with MCFISH by Dr Peng Zhang, USYD, NSW, Australia, showing A-D translocations. A genome chromosomes red; B genome chromosomes brown and D genome chromosomes green; (a): F_2-13; (b): F_2-22, (c): F_2-24.

3.3.5 F_2 POPULATION ANALYSED WITH SSR MARKERS

F_2 seed (176 plants) from the same *T.aestivum* by *T. turgidum* ssp*. durum* (var. Bellaroi) cross, grown in 2006 and 2007, was analysed with SSR markers. This population included the 83 plants analysed by DArT markers and additionally 93 F_2 plants grown in Breeza, Tamworth. Refer to Appendix CD, Chapter 4, SSR summary for results and lines used. Figure 3-8 shows an example for an acrylamide gel with DNA fragments obtained with SSR marker gwm46. The first two lanes (arrow) show the parents. Refer to Appendix CD, Chapter 4 for a summary of all SSR markers used.

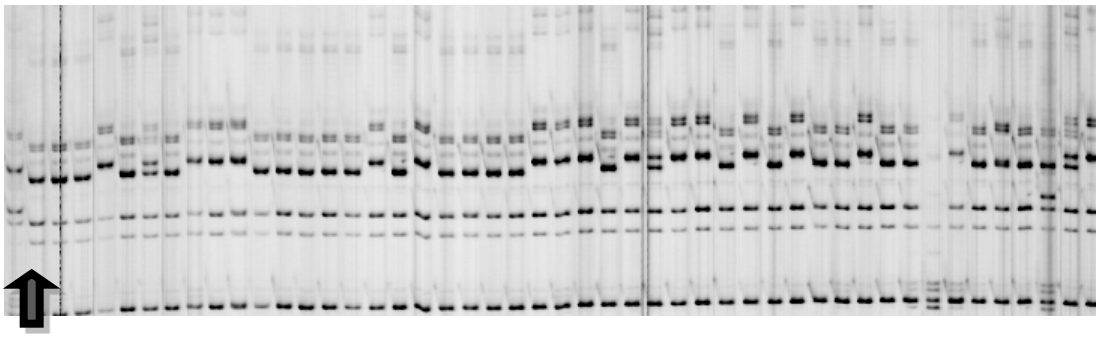


Figure 3-8: Example for an Acrylamide Gel with DNA samples from a 2-49/Bellaroi cross undergoing electrophoresis in a Corbett Gel Scan machine.

Table 3-7 shows that the segregation of the dominant markers generally followed Mendelian principles (75:25). It also shows the different results obtained by dominant markers. Chromosome 5B preferentially inherited durum rather than 2-49 alleles, which was also observed with the DArT markers.

Table 3-7: Segregation ratios of 179 F_2 plants analysed with SSR markers; % 2-49 = % homozygous alleles from 2-49; % M = % homozygous alleles from Bellaroi (M); % H = % Heterozygote alleles; % - = % Missing Data.

Chr	Marker	Type	% 2-49	% M	% H	% -
1A	wmc278	**bi-allelic**	23	22	49	6
1A	gwm164	**domin.**	69	26	0	5
2A	gwm95	**bi-allelic**	26	21	50	3
2A	gwm515	**bi-allelic**	27	26	39	8
3B	barc75	**bi-allelic**	15	22	60	3
3B	gwm566	**domin.**	67	26	0	7
4B	gwm251	**bi-allelic**	24	30	43	3
4B	wmc48	**bi-allelic**	28	19	37	16
4B	gwm113	**bi-allelic**	40	16	29	15
5B,3A	barc69	**bi-allelic**	21	1	71	7
5B	wmc362	**bi-allelic**	12	28	52	8
5B	barc344	**bi-allelic**	16	29	48	7
7B	gwm350	**domin.**	70	20	0	10
7B	gwm46	**bi-allelic**	44	10	40	6
1D	gwm147	**domin.**	76	23	0	1
1D	wmc429	**domin.**	77	21	0	2
1D,3A	wmc147	**domin.**	56	44	0	0
5B	wmc362	**bi-allelic**	12	28	52	8
5B	barc344	**bi-allelic**	16	29	48	7
7B	gwm350	**domin.**	70	20	0	10
7B	gwm46	**bi-allelic**	44	10	40	6
1D	gwm147	**domin.**	76	23	0	1
1D	wmc429	**domin.**	77	21	0	2
1D, 3A	wmc147	**domin.**	56	44	0	0

3.3.6 F_3- DIFFERENT POPULATIONS OVER SEVERAL YEARS

Three different sets of F_3 plants were analysed during the course of this study.

- Fifty- four F_3 plants, representing direct descendants of the 83 F_2 plants used for the first DArT experiment, were analysed with DArT markers (2nd DArT experiment) to establish the amount of D genome loss from one generation to the next. Up to five different offspring from the same F_2 plant were analysed.
- Chromosome counts were conducted on another 50 descendants of these F_2 plants. The F_3 plants were compared with their F_2 parents as well as their F_3 siblings which were sent for DArT analysis. A direct comparison between the data of the F_3 DArT analysis and the F_3 chromosome counts is not possible as different F_3 plants were used in the two methods, although a few root squashes of the same plants which were used for DArT analysis were also used for chromosome counts.
- F_3 plants from all three crosses, whose F_4 offspring contained D chromatin when analysing them with SSR markers (see next chapter) were also analysed with SSR markers. The results were compared with the MCFISH chromosome staining results of the same plants.

3.3.7 F_3 DART AND COMPARISON WITH F_2

The same number of DArT markers as in the previous experiment was tested The expected segregation ratio of dominant markers in the F_3 is 62.5%: 37.5%. This ratio was observed at most loci in this experiment, but there was significant segregation distortion of four markers on chromosome 5B towards durum alleles.

A summary of the D genome chromosome numbers is supplied in Table 3-8. Fifty plants still had D chromatin at the F_3 stage. Four plants had no D chromatin. Fourteen plants had partial chromosomes or translocations.

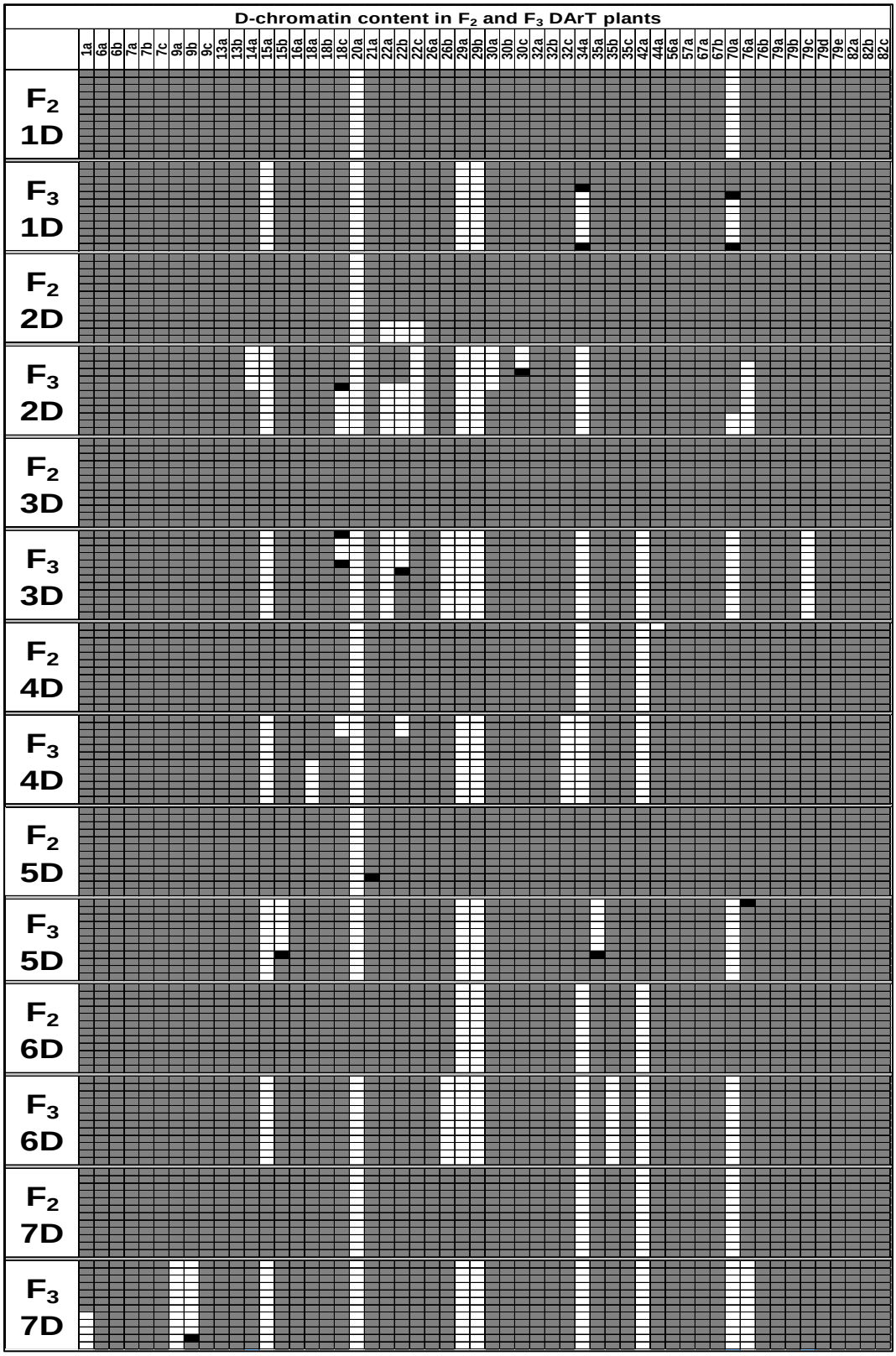
Table 3-8: Summary of D genome chromosome numbers in 54 F_3 plants of a 2-49/Bellaroi cross analysed with DArT markers.

D Chromo.	1D	2D	3D	4D	5D	6D	7D
complete	48	40	42	44	47	45	43
partial	2	8	2	3	2	0	2
none	4	6	10	7	5	9	9

Table 3-9 shows a comparison between the F_2 and the F_3 DArT results. Up to five different offspring of one F_2 plant were analysed. The numbering remains the same as in the F_2. To make the comparison easier, the same F_2 parents are shown several times and are positioned above each F_3 offspring. To make the table shorter, only the same number of representative markers are shown for each D genome chromosome.

The D chromatin loss from the F_2 to the F_3 generation varied among siblings, although some showed an identical loss (For example F_3-9a and 9b both lost only 7D). One plant, F_3-15a, lost all 7D chromosomes, whereas the sibling F_3-15b still had all 7D chromosomes. Around 85% of analysed F_3 plants still possessed either partial or complete D genome chromosomes, the great majority being complete.

Table 3-9: Comparison of D chromatin in 53 F_2 and F_3 plants of a 2-49/Bellaroi cross analysed with DArT markers; 2-49 alleles =dark grey, Bellaroi null alleles =white, missing = black.



3.3.8 CHROMOSOME NUMBERS OF F_3 PLANTS AND COMPARISON WITH DART RESULTS

One to six cells each of 50 F_3 2-49/M plants were analysed at USQ by the author by counting their mitotic metaphase chromosomes which were stained using the Feulgen method. Some microscopy photos are shown in Figure 3-9 as a sample of this work (refer to section 3.2.4. for a description of the methodology). Observed chromosomes were drawn on paper and then counted by ticking them off.

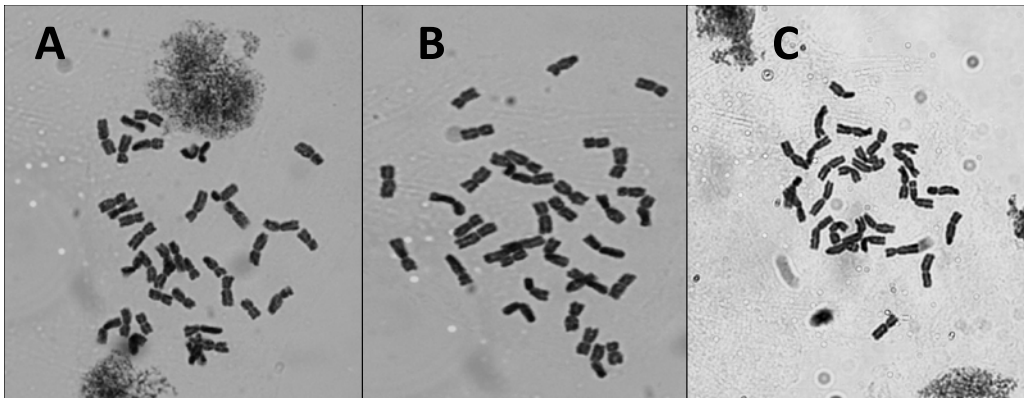


Figure 3-9: Mitotic F_3 cells of a 2-49/Bellaroi cross stained with the Feulgen method at USQ, Toowoomba, QLD, Australia. Different numbers of chromosomes are shown. Photo (A): 38 chromosomes; (B): 36 chr. and (C): 28 chromosomes.

Table 3-10 shows the results for some of the analysed F_3 root tip cells, whose donor plants were subsequently analysed with DArT. All analysed plants were descendants from F_2 plants also analysed with DArT (as described in section 3.3.2.) which contained at least one copy of all seven D genome chromosomes. The root tips were collected when the leaves were harvested for DNA extraction. Although it is not possible to distinguish the chromosome type of Feulgen stained chromosomes directly, it is possible in estimate the D genome chromosome numbers by subtracting the numbers of the A and B chromosomes (28) from the total numbers of chromosomes counted. The remainder of the counted

chromosomes are most likely D genome chromosomes. The counted chromosome numbers corresponded well with the results obtained from DArT, given that DArT analysis does not indicate whether one or two copies of a chromosome are present.

Table 3-10: Chromosome numbers of mitotic F_3 cells of a 2-49/Bellaroi cross stained with the Feulgen method, and results of the F_3 DArT analysis of a 2-49/Bellaroi cross. The last column in the table compares the results of the Feulgen staining method to the DArT results obtained from the F_3.

Parental F_2 Dart line no.	**Counted F_3 chromosomes**	**No. of D chromosomes**	**DArT analysis of F_3 Dchromosomes**
2-49	42	14	na
6	41/ 42	13/14	all 7 detected
7	39	11	all 7 detected
7	40	12	all 7 detected
9	35	7	Chr. 7D missing
35	38	10	Chr. 5D partial
79	35	7	all 7 detected

The other F_3 plants, for which chromosome counts were undertaken, were siblings of the F_3 plants analysed with DArT and therefore not directly comparable to the F_3 DArT results. These results are shown in Table 3-11 and Figure 3-10.

Table 3-11: Counted chromosome numbers of mitotic F_3 cells from a 2-49/Bellaroi cross (Feulgen stained).

Parental F_2 DArT no.	Counted F_3 chromosomes	No. of F_3 D chromosomes
1	35	7
1	34	6
7	33	5
9	40	12
15	34	6
18	41	13
22	37	9
26	37	9
26	35	7
30	40	12
32	38/39	10/11
32	37	9
35	39	11
46	37/38	9/10
46	41	13
46	40	12
57	34?	6
57	38/39	10/11
65	39	11
65	41	13
65	42	14
67	42	14
69	39	11
69	39	11
69	36?	6?
69	41+t?	13+t?
70	30	2
76	38	10
79	38	10

Figure 3-10 shows a summary of the results of the counted mitotic chromosomes in root tips of the F_3 plants. Most plants contained a relatively high

chromosome number (close to 42), with 39 being the most common. The average number was 37.3 chromosomes.

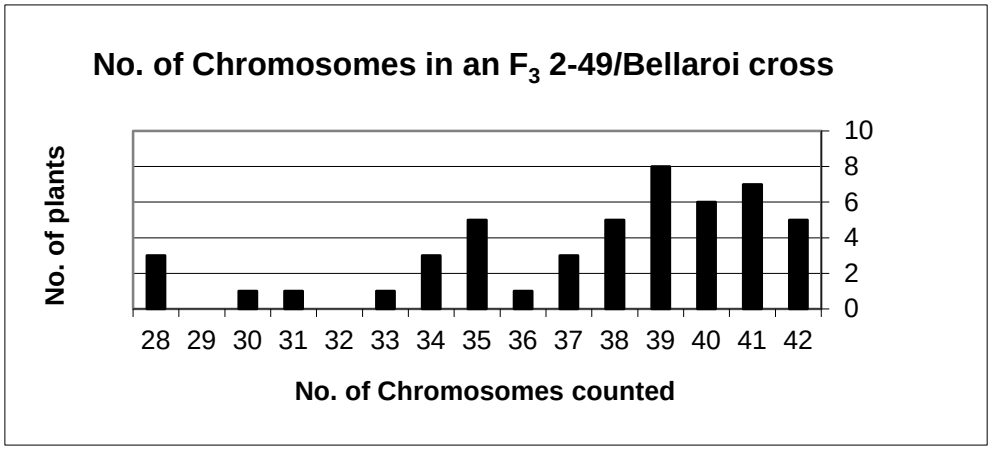


Figure 3-10: Chromosome numbers in mitotic cells of a F_3 2-49/ Bellaroi cross stained with the Feulgen method.

3.3.9 SSR AND MCFISH RESULTS FROM OTHER F_3 MATERIALS

Twenty-eight plants from all three crosses (2-49/M, 2-49/N, 2-49/O) were analysed by Dr. Zhang using MCFISH analysis and simultaneously by the author at USQ using SSR markers. The results from the SSR analysis were compared with the MCFISH chromosome staining results.

Table 3-12 shows the results of the MCFISH chromosome stains performed by Dr Zhang on F_3 plants. Out of 28 plants, 13 carried the normal durum compliment of 14 A and 14 B chromosomes. The remaining 15 lines carried varying numbers of whole D genome chromosomes and fragments. Four cells were not complete, (i.e. not the whole contents of the cell was visible under the microscope, as chromosomes may get lost in the squash process). One plant (O 4/1) carried two A-D translocations (Figure 3-11(a)) and one, (M19/7), (Figure 3-11(b)), carried two B-D translocations . Three plants had a telomeric chromosome piece (t) without a centromere.

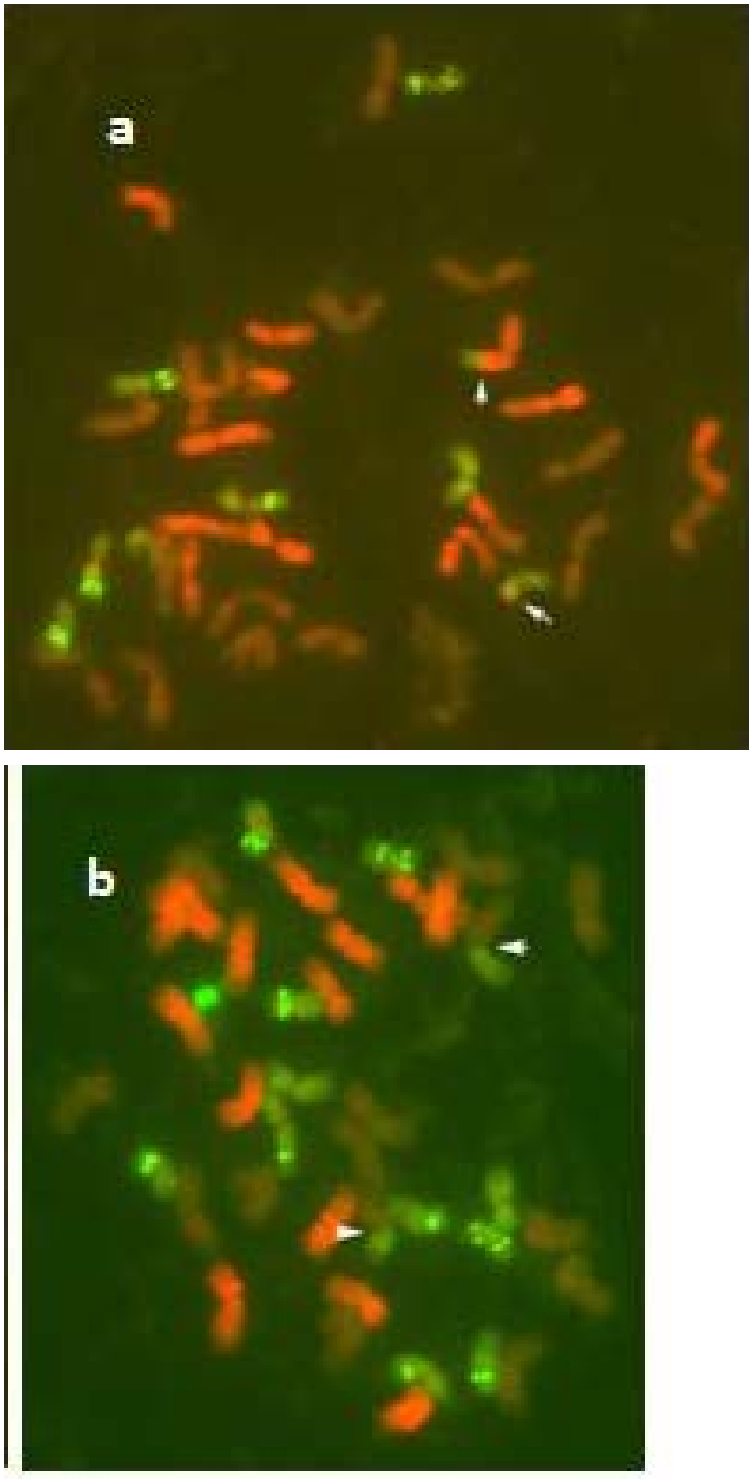


Figure 3-11 : Translocations in F_3 plants of a hexaploid wheat x durum cross analysed with MCFISH by Dr Peng Zhang, USYD, NSW, Australia; (a): A-D translocation in Plant O 4/1, (b): B-D translocation in plant M19/7, (red = A; brown = B; green = D genome chromosome).

The DNA used for MCFISH was also analysed with SSR markers (Table 3-12). The results and lines used are shown in Table 3-12, for all SSR results refer to Appendix CD, Chapter 4. When no D chromatin was detected with MCFISH, in most cases no complete D genome chromosomes were detected with SSR markers, although some partial chromosomes were identified in plants N17/1, N17/2, N25/1, N25/2, O 2/1, O 2/2, O 10/2, O 13/1 and O 13/3. Two of the t- chromosomes are visible, namely in plant M25/1 and N7/2. The A-D translocation in plant O 04/1 is not vdetectable in the SSR results, and the plant with the B-D translocation (M19/7) was not analysed with SSR markers. Plants 2-49/M 14/2; 2-49/N 5/1 and 2-49/N 7/2 had incomplete cells (the complete chromatin content of the nucleus was not visible).

Table 3-12: Results of chromosome counts from MCFISH analysis of meiotic F_3 cells of hexaploid wheat x durum crosses and comparison with SSR marker results from their DNA; P= partial chromosome, C= complete chromosome, t= telocentric chromosome.

	MCFISH results			SSR results from 20 D markers							
line 2-49/	**Chr num.**	**A**	**B**	**D**	**1D**	**2D**	**3D**	**4D**	**5D**	**6D**	**7D**
M11-1	28	14	14								
M11/2	28	14	14								
M12/1	28	14	14								
M12/2	28	14	14				P				
M13/1	41	14	14	13	C	C	C	C	C	C	C
M13/3	40	14	14	12	C	C	C	C	C	-	C
M14/1	38	14	14	10	C	C	C	C	C	P	C
M14/2	31	14	14	3	C	P		C		-	C
M25/1	37+t	14	14	9+t		P	C	C	C	C	C
M25/2	40	14	15	11		C	C	C	C	C	C
N5/1	30	14	14	2			C	P			C
N5/2	30	14	14	2			C				C
N7/1	30	14	14	2	C	C					
N7/2	33+t	14	14	5+t	C		C		P	P	C
N17/1	28	14	14		P	P				P	
N17/2	28	14	14						P		
N25/1	28	14	14		P						
N25/2	28	14	14		P			P	C?		
O 2/1	28	14	14						P	P	
O 2/2	28	14	14					P	P	P	
O 4/1	37	14	15	8	C	C	C	C	C	C	C
O 4/2?	39	14	14	11			C	C	P	P	
O 10/1	29?	14	14	?							
O 10/2	28	14	14						P	P	
O 13/1	28	14	14				P				
O 13/3	28	14	14				P				
O 14/1	32	14	14	4	C		P		C	C	C
O 15/1	41+t	13	15	13+t	C	C	C	C	C	C	C

3.3.10 F_6 2007

In 2007 845: 2-49/M; 1785: 2-49/N and 773: 2-49/O F_6 seed were sown in Tamworth. From these populations, altogether 718 seedlings from the 2-49/M (123), 2-49/N (471) and 2-49/O (125) populations were tagged, eight plants per family. For a description of a family refer to section 2.2.1. The tagging made it possible to trace back each individual plant to an individual CR score (see Chapter 4). From the tagged plants, a selection of 184 plants from 2-49/N was analysed. Refer to the appendix CD, Chapter 4, F_6, for lines and markers used and SSR results.

The allelic frequencies in the A- and B- genome chromosomes of the 184 plants generally favoured the durum alleles. Only chromosomes with regions containing CR resistance QTL were analysed. The rate of heterozygosity was lower than 10% (Figure 3-12).

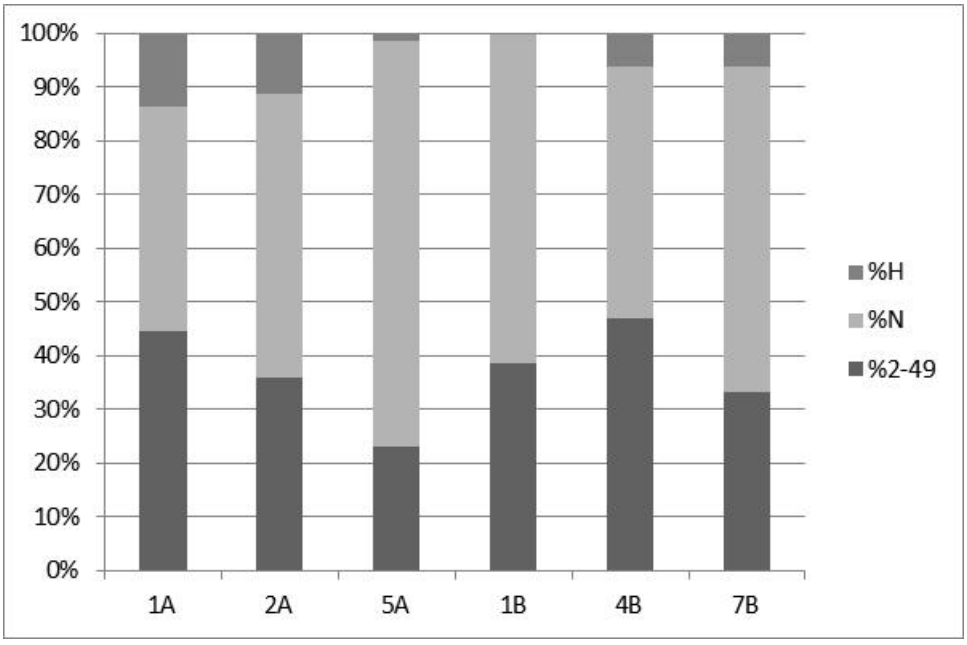


Figure 3-12: Percentage of allelic segregation in F_6 plants from a 2-49/N cross; % 2-49 = % 2-49 alleles; % N = % N durum alleles, % H= % heterozygote alleles.

3.3.11 F_7 2008

DNA samples of 191 2-49/M F_7 lines were sent to Triticarte for DArT analysis. Refer to Appendix CD Chapter 3, DArT report 3. This was the third and last DArT experiment. Each sample consisted of a mixture of leaf DNA from three plants/ line. Nine-hundred and seventy- eight markers hybridized to the DNA. 334 markers were excluded from the analysis, due to either low P values or lack of polymorphism. Of the 644 markers remaining in the analysis, 68% hybridised to the maternal parent allele. Permutation tests were performed on the DArT results (Fisher 1935; Good 2005).

A genetic linkage map from the maternal and paternal markers was produced from the information provided as previously described. This map is shown in Chapter 4. There are much more markers in this map than in the previous F_2 map, which had less than 400 markers, because Triticarte provided us with an additional wheat array for 3B.

The results after ordering the remaining 644 markers with Map Manager showed, that 118 mapped to the A genome chromosomes, 249 to the B genome chromosomes and 211 to the D genome chromosomes. The rest (66) were unlinked. The distribution of markers on the different chromosomes is shown in Figure 3-13.

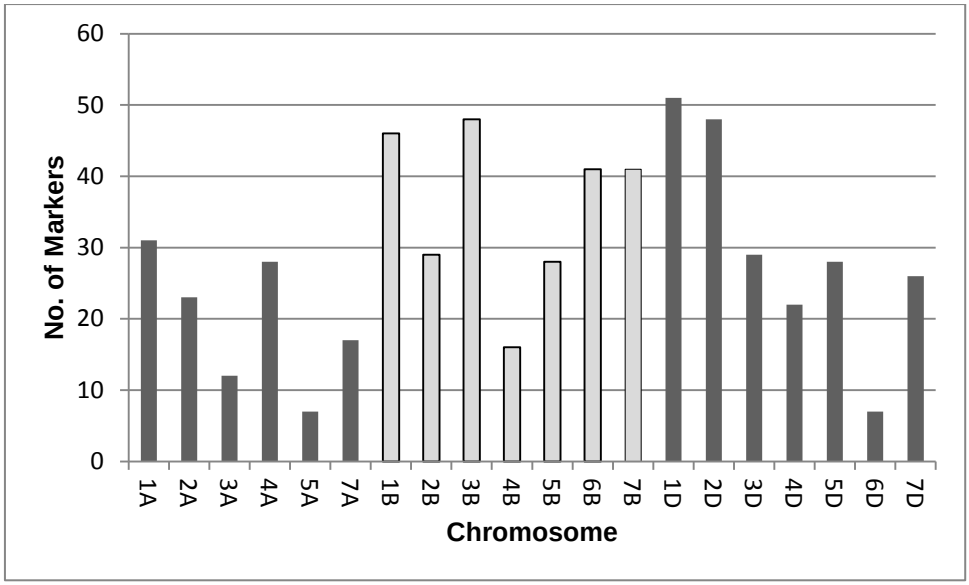


Figure 3-13: Distribution of DArT markers on the chromosomes of the 2-49/N cross in the F_7.

Figure 3-14 shows the segregation ratios of 2-49/N in the F_7. A significant shift towards durum alleles has taken place, compared to the F_2 (refer to Figure 3-1). Around 55% of all alleles were contributed by durum. This can be explained by the strong selection towards durum morphology by the breeders in successive generations.

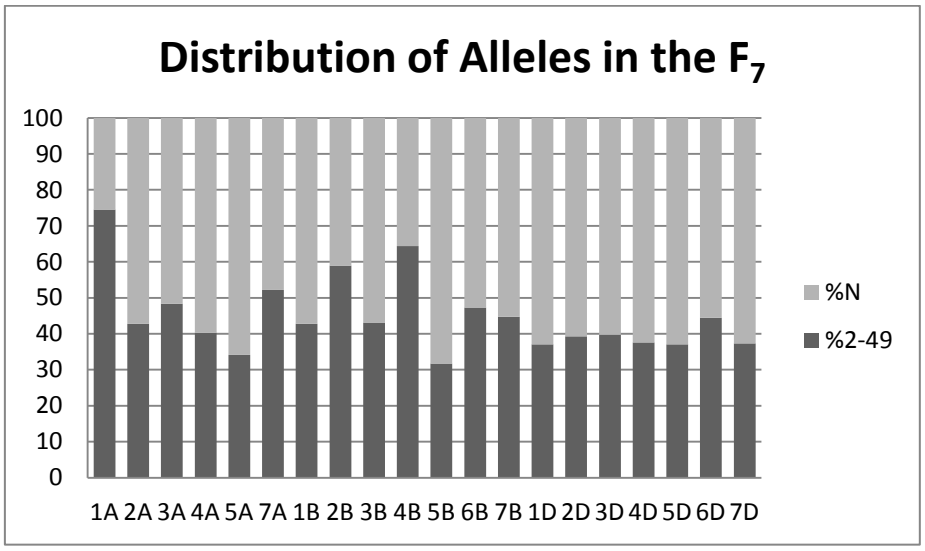


Figure 3-14: Frequency of inheritance of alleles in the F_7 in the 2-49/N cross; (% 2-49 = % 2-49 alleles; % N = % durum N alleles).

By the $F_{7,}$ the overall D chromatin content in the 2-49/N lines still amounted to 36%. The majority of plants had lost all D genome chromosomes completely, whereas the others had at least one copy of all seven complete D genome chromosomes.

Figure 3-15 shows the D chromatin content in 56 2-49/N lines established with DArT analysis. The data shown represents average values from the 191 lines analysed (as stated before, a mixture of leaves from three plants/ line was analysed). Almost all lines of the 2-49/N 29, 12-6, 5-1 and 7-3 families possessed all seven complete D genome chromosomes. Partial D genome chromosomes were rare, with single instances observed in 2-49/N 1-11, 2-49/N 1-7, 2-49/N 5-2a and 2-49/N 7-6 had slightly shortened chromosomes.

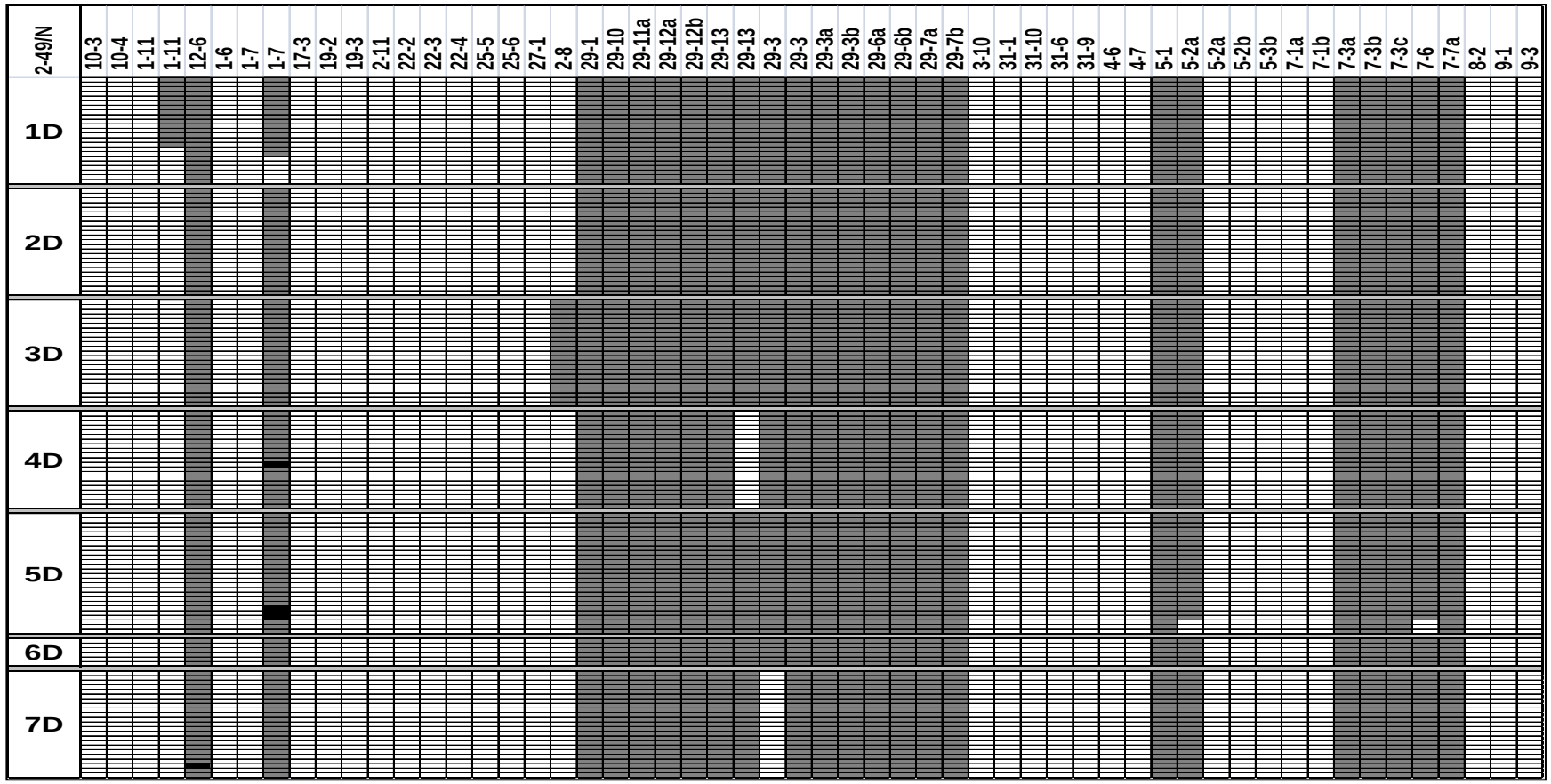


Figure 3-15: D chromatin content in F_7 lines of the hexaploid wheat x durum cross 2-49/N. D genome chromosomes are shown on the left side, lines across the top row; (2-49 alleles = dark grey, durum alleles = white, missing = black).

Figure 3-16 and Table 3-13 show a summary of the D genome material in two of the DArT experiments. A significant reduction in 2-49 material could be observed when comparing the F_2 (set at 100%) 2-49/Bellaroi population to the F_7 2-49/N population (Figure 3-16). Chromosome 1A showed no reduction in 2-49 alleles, but instead a slight increase. Because there have been several selection rounds by the breeder between the two generations, it is possible that the proportion of lines with 2-49 material in the 1A chromosome has been effectively maintained at the same level (or become slightly enriched) compared to that detected in the F_2. This chromosome contains a QTL for CR resistance.

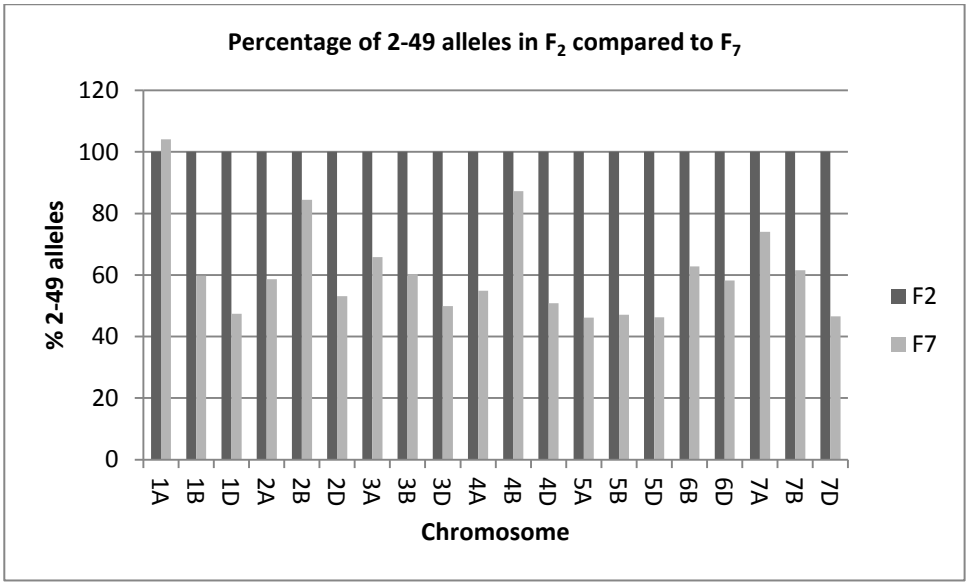


Figure 3-16: Percentage of 2-49 alleles in the F_2 generation of a 2-49/Bellaroi cross compared to the F_7 generation of a 2-49/N cross.

D genome chromosomes were mainly inherited as complete chromosomes, especially in later generations (Table 3-13).

	Chromosome	1D	2D	3D	4D	5D	6D	7D
F_2	**% D chrom.complete**	**78**	**70**	**71**	**75**	**84**	**76**	**75**
	%D chrom. partial	**3**	**2**	**4**	**1**	**0**	**4**	**0**
F_3	**% D chrom. complete**	**90**	**76**	**93**	**81**	**86**	**84**	**79**
	% D chrom. partial	**5**	**14**	**3**	**5**	**5**	**0**	**2**
F_7	**% D chrom. complete**	**41**	**43**	**45**	**41**	**39**	**43**	**41**
	% D chrom. partial	**4**	**0**	**0**	**0**	**4**	**0**	**0**

Table 3-13: Summary of D genome material from all DArT experiments conducted at USQ, Toowoomba, QLD, Australia.

The frequency of partial chromosomes or translocations was lowest in the F_7.

Families 2-49/N- 29 and 2-49/N- 7 retained most D genome material over the generations up to the F_7. By the F_7 the other families had lost all D genome material.

3.4 DISCUSSION

3.4.1 DART ARRAY F_2, F_7 AND SSR ANALYSIS OF F_6

The objective of this study was to trace the fate of alleles from crosses of the hexaploid wheat 2-49 to three durum wheat varieties (M, N, O) using both SSR and DArT markers to examine a range of generations from F_2 to F_7.

In the F_2 generation of the 2-49/N cross, the A and B genome alleles from durum and bread wheat were inherited equally from the parents, this confirms a largely random segregation of parental alleles in this cross. One exception was chromosome 5B, on which some regions contained significantly more durum alleles than expected. Families 2-49/N- 29 and 2-49/N- 7 retained most D genome material over the generations, and by the F_7 the other lines had lost all D genome material. There was a significant decrease in 2-49 alleles along with an increase of durum alleles from the F_2 to the F_7 in the A and B genomes. The decrease in 2-49 alleles was most likely caused by the selection for durum appearance by the breeders.

Segregation distortion is common in wheat and other species and is a variation of the observed genetic ratios from the expected Mendelian ratios in a segregating population (Zhang and Dvorak 1990; Faris et al. 1998; Messmer et al. 1999).

Segregation distortion is often a driving factor of evolution (Sandler 1957). In most cases, male gametes are responsible for the distortion. This can be caused by rivalry among gametes for favoured fertilization. Distortion through the male parent is also dependent on the occurrence of matching genes in the female (Kumar et al. 2007; Taylor and Ingvarson 2003). It has previously been observed that markers on regions of wheat chromosomes 2B, 5B and 6B often do not follow the expected Mendelian ratios of segregation (Cadalen et al. 1997; Kumar et al. 2007).

Group 5 chromosomes in particular are frequently subject to distortion (Faris et al. 1998, 2000). The distorted areas on 5B in this study may be due to favoured transmission of durum alleles over wheat alleles through the male gametes. The female gametes could also have harboured certain alleles that lead to favoured fertilization by gametes carrying durum alleles (Kumar et al. 2007).

The level of distortion towards durum alleles was higher in the F_7 generation than in the F_2, which was also analysed with DArT. This was due to the selection applied by the breeders towards durum appearance.

In the first DArT experiment where 83 F_2 plants were analysed, all plants except one still had D chromatin. The plant with no D genome material also had alleles from both parents, indicating that it was not a selfed durum parent. As the DArT markers are dominant, the precise amount of D genome material is unknown. D genome chromosomes can be present as either univalents or bivalents, single or twin copies of the chromosomes. The amount of D genome material in the entire F_2 popluation of 83 plants contained this study was approximately 80% compared to hexaploid plants. Previous studies suggest that plants that have at least one complete copy of each D genome chromosome are likely to produce, when selfed, bread wheats with a full complement of 42 chromosomes in later generations (Kihara 1982). Subsequent generations from lines with less than 35 chromosomes tend to lose the remaining D chromosomes and become stable tetraploid durums (Kihara 1982). This was confirmed in this study. Although most of the selection was man-made, and only plants with the durum constitution survived, because they were not eliminated, it confirms that pentaploid wheat lines revert to either tetraploid or hexaploid wheat lines in later generations and lose plants with intermediate chromosome numbers (Kihara 1982). Overall, the results also show that D chromatin loss in this specific pentaploid cross happens slowly over the generations.

In one study (Gilbert et al. 2000), hexaploid FHB resistant spring wheat was crossed with susceptible tetraploid wheat (Stewart 63 and DT486). Forty-three F_2 plants were analysed with D genome specific microsatellite markers, 24 of them

had all D genome chromosomes present, however it is not known if these were bivalents or univalents.

In two other studies that used the Feulgen chromosome staining method, the chromosome numbers of hexaploid wheat x tetraploid wheat hybrids were reported. In these studies, 80% and 63% of plants had chromosome numbers above/equal to 36, respectively. Figure 3-17 showes the per cent distribution of chromosome numbers in F_2 hybrids in these two studies (Kihara 1982; Wang et al. 2005).

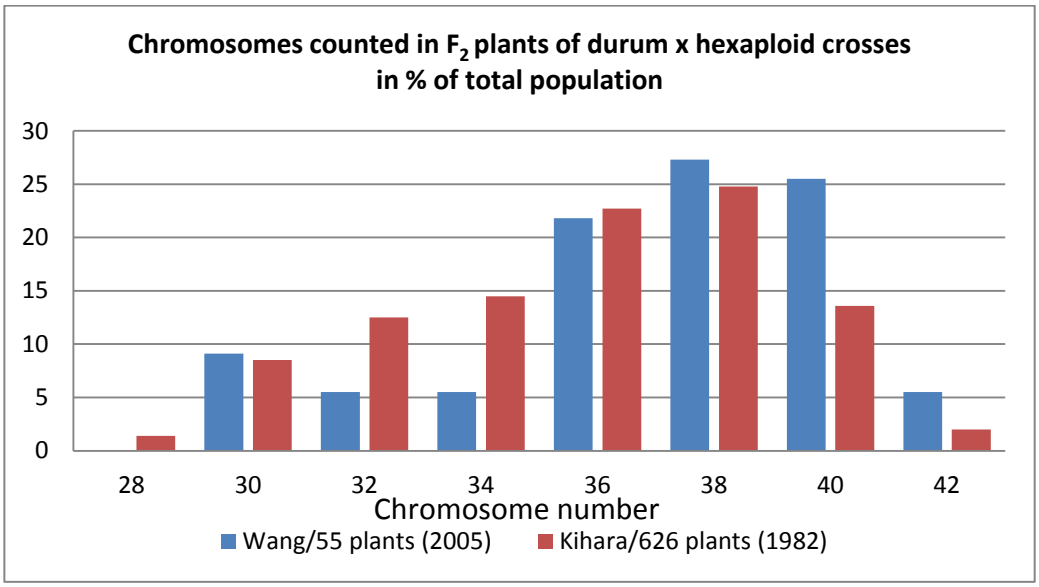


Figure 3-17: Chromosome numbers (%) in F_2 hexaploid wheat x durum hybrids in a study from Wang (2005) compared to a study from Kihara (1982).

The USQ study cannot be directly compared to these earlier studies, because DArT analysis does not show if the chromosome is present in the disomic or monosomic form. However, more than half of the plants (54%) analysed with DArT markers contained at least seven different D genome chromosomes (partial chromosomes included). These plants had chromosome numbers ranging from 35 to 42. Therefore F_2 plants in this study contained a comparable percentage of D genome material.

D genome chromosomes were mainly inherited as whole chromosomes. The overall distribution of D chromatin was irregular and no specific D genome chromosome occured more often than others. Cross-over events (translocations) between the A and D or the B and D genomes were rare.

One-hundred and ninety one 2-49/M lines were analysed with DArT arrays in the F_7 (3.3.11). Heterozygosity in these lines was very low, indicating the stabilisation of the genome material.

3.4.1.1 Reduction of 2-49 Alleles F_2-F_7

Figure 3-16 shows the per cent reduction of 2-49 alleles in the different chromosomes determined by DArT analysis. A significant reduction in 2-49 material could be observed. This is due to the selection towards durum appearance by the breeders. Chromosome 1A had no reduction in 2-49 alleles, but instead a slight increase. This could be an artefact or the result of cross-over events.

3.4.1.2 Genetic Linkage Maps

The map of the F_2 generation was prepared to establish allele segregation patterns in the A and B genomes. The D genome markers revealed the inheritance of the D genome and could be matched to the consensus maps provided by Triticarte without providing mapping distances. It is not possible to determine the mapping distances of D genome markers in pentaploid material, as D genome chromosomes are inherited without the possibility of recombination. A large number of segregating markers (247) in the F_2 DArT experiment could be matched to the D genomes. From them, 157 markers were previously classified by Triticarte as unlinked. They may be of importance for further mapping studies. The F_7 map was also used to establish allele segregation and D genome loss and to compare the results to the F_2, but also to determine CR resistance QTL in the genome. This will be discussed in Chapter 4.

Akbari et al. (2006) found that the D genome is the least diverse wheat genome with fewer polymorphic markers found in this genome. This is due to the relatively

recent introduction of the D genome into bread wheat and its monophyletic origin from only one ancestor (*T. tauschii*). In contrast to this, recent DArT studies of Australian wheat showed that in this country the D genome is more diverse than elsewhere (White et al. 2008).

About twice as many markers mapped to the B- genomes compared to the A-genomes. It has been known for some time that the B- genome contains more polymorphic markers than the A- genome although it is physically shorter (Boeuf et al. 2003). The B- genome donor was long thought to be a so far unknown relative of *Aegilops speltoides* L. (Boeuf et al. 2003). Some evidence also suggests, that the B genome donor may be *Aegilops searsii* (Liu et al. 2003). The difficulty of finding the B- genome donor could be due to the rapidly changing and evolving nature of the B- genome (Boeuf et al. 2003).

PstI restriction enzymes, used to digest the DNA in the DArT procedure, could be another reason why the A genome chromosomes have fewer markers than the other chromosomes. This is a known problem with DArT (Mantovani 2008) . These restriction enzymes prefer hypo- methylated genes which are not numerous on some chromosomes, especially on the group 5 chromosomes, notably 5A (Mantovani 2008).

When paternal maps, derived from the markers emitting a signal for the male parent (in this case durum) and maternal maps, derived from the markers emitting a signal for the female parent (in this case bread wheat), are combined, maternal and paternal markers tend to form clusters in the combined maps. This can cause problems when using the programme Map Manager QTX because when the clusters are, by chance, in the same region, they increase the mapping distance (personal communication from Dr Martin). The programme also cannot differentiate between co-dominant and dominant signals, therefore the combination of dominant DArT markers with co-dominant SSR markers can be problematic. For this reason SSR markers have been excluded from the maps. However, as they provide a cheaper alternative to DArT markers and are sufficient

for QTL mapping, they have been used extensively in this study. They also provide additional insight because they are co-dominant.

3.4.2 MCFISH RESULTS AND COMPARISON TO F_2 DART RESULTS

Twenty-six plants were analysed with DArT markers as well as with MCFISH chromosome staining. The DArT results correspond very well with the MCFISH results. Refer to section 3.3.4 for a description of this experiment.

It is not known whether the D genome chromosomes detected with DArT are homozygous or heterozygous because DArT markers are dominant. However, when analysing the MCFISH results, it was found that fifteen out of 26 plants had uneven D genome chromosome numbers. This indicates that some of the D genome chromosomes were unpaired.

The MCFISH results show that 12 out of 26 plants had chromosome numbers above 36. This result is comparable to a study of Kihara (1982) who found the percentage of F_2 plants with chromosome numbers above 36 to be 63%. According to Thompson and Hollingshead (1927), the expected theoretical frequency of plants with the *T. aestivum* chromosome constitution in the F_2 of pentaploid wheat crosses is only 1:16384. Many intermediate types were found by these authors. The observed amount of D genome chromosomes in hexaploid x tetraploid wheat crosses is always different from the theoretically expected ratio, because some of the plants do not make it to adulthood due to seedling mortality. Also, all seven D genome chromosomes tend to migrate together to one pole in meiosis. Furthermore, some of the wrinkled seed either may not have germinated or died soon after emergence, thus leading to bias towards selecting healthier plants for tissue sampling. Kihara stated that there is a correlation between the ability of a seed to germinate and the outer morphology of the seed (Kihara 1919, 1921, 1924, 1925, 1937, 1982). In our study only around 60% of the sown plants survived, resulting in the genome of the others being lost for analysis. Consistent with this result, Kihara found a seedling survival rate of 60% or less in a *T. aestivum* x *T. turgidum* ssp. *durum* cross (Kihara 1919).

Very recently, our research group (Martin et al. 2011) showed that the identity of both parents in a hexaploid/tetraploid cross can significantly affect both the segregation distortion levels observed in the A and B genomes and the degree of D genome retention in the F_2 progeny as well as the rate of loss of remnant D genome in subsequent generations. This could lead to different chromosome constitutions in different crosses in the F_2 and therefore to the differences between our results and the above mentioned study. This is consistent with the different levels of retention of the D genome observed in earlier studies of particular hexaploid/tetraploid crosses (Kihara 1982; Wang et al. 2005).

The A-D translocations detected with MCFISH in plants F_2-13; F_2-22 and F_2-24 (Table 3-5), can also be traced in the DArT results and involve arms or segments from chromosomes 6D, 3D and 1D, respectively. The translocation in plant F_2-24 is not a whole-arm translocation as in F_2-13 and F_2-22. The translocation is in the long arm of the A genome chromosome with only a small D-genome segment. This kind of translocation is rare (personal communication with Dr. Zhang). The translocations are clearly visible in the DArT results. Plant F_2-22 also had an incomplete A-genome chromosome. This could be an A genome chromosome with the missing 3D piece. Two telocentric chromosomes (t= small chromosome piece without centromere) in plants F_2-7 and F_2-23 were observed with MCFISH, but only one telocentric chromosome (F_2-7) could be identified as chromosome 7D with DArT. The telocentric chromosome detected with MCFISH in plant F_2-23 was not detected by DArT. In the DArT results only some translocations are visible since the presence of an entire homologous chromosome without the translocation will mask its presence in a dominant marker system such as DArT arrays.

Only one of the two telocentric D genome chromosome fragments detected with MCFISH could be identified in the DArT results. In the larger population analysed with DArT only (83 plants), no conclusive statements could be made as to the number of telocentric chromosomes. The reason for this is that the

mapping of the D genome in this case is approximate because the consensus map provided by Triticarte has been used to determine the position of markers. Another reason why telocentric chromosomes cannot be detected easily is the dominant nature of DArT markers which do not distinguish between a null allele signal and a missing signal. As most fragmented D genome pieces were shown to be translocations with MCFISH, it is assumed that a large percentage of those observed in DArT analyses are in fact translocations too.

Based on DArT and MCFISH results, D genome chromosomes were predominantly inherited as complete chromosomes. Plants with translocations were not numerous. In a study from Lukaszewski and Gustafson 1983, investigating four triticale x wheat populations with 785 plants over four generations, about 1/3 of the plants analysed with C banding showed translocations. One-hundred and ninety-five wheat/rye and 64 rye/rye translocated chromosomes were discovered, most of them consisting of complete chromosome arms. The percentage of translocations in this study was much higher than in ours.

One reason may be that the chance for centromere mis-division of the univalent D-genome chromosome is low as the other D chromosome partner is not present.

3.4.3 F_3 DART AND COMPARISON WITH F_2

Segregation distortion was observed on chromosomes 3B and 5B, confirming observations in the F_2 generation.

Around 85% of the analysed F_3 plants still possessed either partial or complete D genome chromosomes, a great majority being complete. Again, it is unknown if these were disomic or monosomic forms of the chromosomes. Compared to that, the F_2 parents of the analysed F_3 plants contained around 95% D chromatin. Although there has been some D chromatin loss, it was not high; the reduction from the F_2 to the F_3 being only 10%.

3.4.4 F_3 PLANTS ANALYSED WITH FEULGEN, DART, MCFISH AND SSR MARKERS

The F_2 and F_3 DArT results corresponded well with the results from the chromosome counts of metaphase meiotic cells of root tips from 51 F_3 plants stained with the Feulgen method. A strong trend towards higher chromosome numbers was observed, with 39 chromosomes being the most common count. This confirms the results of the DArT analysis in the F_2 and F_3, i.e. that a high percentage of D chromatin is still present in these generations, in accordance with the findings of Kihara (1982) discussed above. This also indicates that either method can be used and that they complement each other when the limitations of DArT are taken into account. Although the F_3 chromosomes count results cannot be directly compared to the DArT data (siblings of the DArT plants were analysed except in a few samples where roots from the same plants were analysed), the results of the chromosome counts seem reasonable.

Another F_3 population of 30 plants was analysed with both SSR markers and MCFISH. The two methods were then compared to ensure their accuracy. The SSR marker data again corresponded well with the results from MCFISH, even showing some of the partial t-chromosomes detected with MCFISH. Out of 30 plants analysed with MCFISH, 13 had the tetraploid chromosome constitution of durum, while the others had varying amounts of D chromatin incorporated.

All four analysis methods used in this study, (DArT, SSR markers, Feulgen method and MCFISH) were reliable and compatible tools for chromosome evaluations and should be employed in combination in the future in order to obtain a better understanding of crosses of species with a dissimilar chromosome constitution.

4 QTL ANALYSIS

4.1 INTRODUCTION

On the basis of the marker alleles present, Collard et al. (2005) found six genetic regions (QTL) linked to CR resistance in a 2-49 (Gluyas Early/Gala) x Janz doubled haploid seedling population (bread wheat). The authors used a genetic map based on SSR markers. 2-49 is partially CR resistant, whereas Janz is considered susceptible. The QTL were shown to be on chromosomes 1D, 1A, 2A, 2B, 4B and 7B, confirming the multigenic nature of the resistance derived from 2-49. The most significant QTL, located on 1DL, is inherited from Gluyas Early (Collard et al. 2006). QTL of smaller effect on 1AL, 4BL and on 7BS were inherited from Gala. A single QTL on 2BS was inherited from Janz.

Recently Bovill et al. (2010) detected field resistance QTL to CR on chromosomes 1A, 1B, 4B and 7B in the 2-49/Janz doubled haploid population following field trials. The QTL on 1A, 4B and 7B were coincident with the corresponding QTL revealed in seedling screens. The QTL on 1B was not observed in seedling tests and is considered to be a QTL expressed only in adult plants in the field. Notably the major 1D seedling QTL on the other hand was not detected in field trials, suggesting that it was expressed only in the seedling stages of plant development.

The work described in this chapter is to investigate whether the genetic markers in CR resistance regions already established in the hexaploid genotype 2-49 by other studies at USQ (Collard et al. 2005; Bovill et al. 2006; Collard et al. 2006), prove to be effective predictors of resistance in the analysed progeny of pentaploid durum crosses. Several generations (F_4-F_7) of a cross between the CR resistant hexaploid line 2-49 and three related durum breeding lines (A, B and C) were genetically analysed with SSR and DArT markers for CR resistance QTL.

4.2 METHODOLOGY

Genomic regions previously identified by researchers at USQ as CR resistance regions were analysed by the author with SSR and DArT markers. In the F_4-F_6 generations of pentaploid durum crosses, SSR markers were used because they represent a cheaper alternative to DArT analysis and provide sufficient accuracy to detect strong QTL in target regions. Their co-dominant nature provides additional insight into the genome compared to the dominant DArT markers, because the allelic variation of a given locus can be determined.

The SSR markers which were used were closely linked to the QTL regions on 1D, 1A, 1B, 2B, 2A, 4B, 7B and represented the seedling and/or adult plant resistance loci detected by Collard et al. (2005, 2006) and Bovill et al. (2010). In order to obtain a more thorough overview of the entire genome of the hybrids, whole genome screens were also conducted, utilising the cheaper SSR markers in the F_4 and in the F_5 bulked segregate analysis. DArT markers were used in the F_2 and F_7. In the F_2 generation, this expensive analysis was undertaken because of the importance of this generation for breeding. DArT markers can analyse a huge number of loci at once, thus providing a highly efficient way to obtain an extensive overview of the genome . In the F_7, DArT analysis was undertaken to detect CR resistance QTL and to finish the study with a complete picture of the genome. In theory, almost complete homozygosity should be achieved in the F_7. The methods as well as the populations used for SSR marker and DArT analysis were described in Chapter 3.

Significant linkages between known QTL regions in the hexaploid parents and CR resistance in the derived durum progeny were determined by entering SSR or DArT marker scores into the program MapManager QTL (Manly and Olson 1999) in conjunction with the programme Record (Van Os et al. 2005), accompanied by phenotypic data obtained by Dr Steven Simpfendorfer in field trials at Tamworth, NSW (refer to Chapter 2).

Markers were ordered with the programme Record (Van Os et al. 2005). Other programes used were: QTLmapper 1.60 (A Computer Software for Mapping Quantitative Trait Loci (QTL) with Additive Effects, Epistatic Effects and QTL-Environment Interactions; Department of Agronomy, Zhejiang University, China). The QTL graphs were drawn with Windows QTL Cartographer 2.5, (Statistical Genetics, North Carolina State University, USA).

DNA was extracted from F_7 seedlings germinated from each of three seeds harvested from a single tagged F_6 plant. Seeds were germinated in petri dishes lined with wet filter paper placed in a plastic bag and grown in a growth cabinet at 25˚C in the dark. Leaves were sampled after 8-10 days. The Genome Wizard extraction method (Promega, Madison, USA), was employed for DNA extraction. The protocol provided by the supplier was used (refer to Chapter 3, DNA extraction for DArT).

The number of lines and specific marker names used in the different populations can be obtained from the Appendix CD (Chapter 4). The primer sequences for the markers used can be obtained from GrainGenes, *http://wheat.pw.usda.gov/GG2/index.shtml (*viewed 14/10/2010).

The QTL for plant height on chromosome 4B (Sadeque and Turner 2010), was also determined with MapmanagerQT. Trait data provided by Steven Simpfendorfer was used. Refer to Appendix CD Chapter 4 for SSR markers used.

4.2.1 F_4 2005

In the F_4 generation (grown in 2005 and analysed in 2006), three pentaploid (hexaploid x durum) crosses were analysed: 145 samples from 2-49/M (961111 or Bellaroi), 178 samples from 2-49/N (950329) and 89 samples from 2-49/O (971179). The entire F_4 population was analysed with SSR markers. For SSR scores, populations, markers used and number of plants refer to Appendix CD, Chapter 4). Threshold LRS values for suggestive and significant QTL were >5.0 and > 12, respectively.

Additionally, a bulked segregate analysis was conducted with the same F_4 material. Only the lines with the 15 highest and 15 lowest CR scores (for each of the 2-49/M and 2-49/N populations, and the 12 highest and 12 lowest (for the smaller 2-49/O population) were analysed (Table 4-1).

Table 4-1: Lines used for BSA analysis in the F_4 generation of a hexaploid wheat x durum cross. CR= Crown Rot Score (determined from % brown tillers and brown nodes), provided by Steven Simpfendorfer, Tamworth.

Plants with lowest CR								
2-49/M			2-49/N			2-49/O		
Entry	Family	CR	Entry	Family	CR	Entry	Family	CR
386	2/49M8-10	8	561	2/49N4-5	4	739	2/49O3-2	5
484	2/49M30-4	9	587	2/49N9-1	5	731	2/49O2-5	10
356	2/49M3-6	10	518	2/49N1-2	6	790	2/49O13-2	10
401	2/49M12-3	11	658	2/49N22-3	6	760	2/49O8-2	11
462	2/49M26-8	12	683	2/49N25-2	7	738	2/49O3-1	11
438	2/49M18-13	12	659	2/49N22-4	7	783	2/49O10-12	12
429	2/49M18-4	12	687	2/49N25-6	7	806	2/49O13-18	16
450	2/49M24-1	13	646	2/49N19-6	9	820	2/49O14-11	17
353	2/49M3-3	14	703	2/49N29-7	9	764	2/49O8-6	17
413	2/49M13-4	14	699	2/49N29-3	10	730	2/49O2-4	17
457	2/49M26-3	15	604	2/49N10-12	10	815	2/49O14-6	17
351	2/49M3-1	15	517	2/49N1-1	11	776	2/49O10-5	19
458	2/49M26-4	15	723	2/49N31-11	11			
346	2/49M2-5	15	643	2/49N19-3	11			
427	2/49M18-2	16	632	2/49N17-2	12			
Plants with highest CR								
Entry	Family	CR	Entry	Family	CR	Entry	Family	CR
358	2/49M3-8	58	673	2/49N23-7	50	742	2/49O4-1	52
474	2/49M27-10	59	591	2/49N9-5	52	787	2/49O12-2	56
481	2/49M30-1	59	609	2/49N12-4	53	788	2/49O12-3	56
367	2/49M5-2	60	670	2/49N23-4	53	813	2/49O14-4	57
383	2/49M8-7	62	675	2/49N23-9	55	822	2/49O15-1	57
436	2/49M18-11	64	724	2/49N31-12	55	737	2/49O2-11	58
447	2/49M22-1	65	714	2/49N31-2	56	736	2/49O2-10	63
397	2/49M11-9	65	717	2/49N31-5	58	763	2/49O8-5	65
491	2/49M30-11	65	539	2/49N2-11	58	808	2/49O13-20	67
508	2/49M34-1	70	693	2/49N28-1	59	773	2/49O10-2	72
513	2/49M34-6	70	623	2/49N16-1	60	735	2/49O2-9	75
390	2/49M11-2	70	551	2/49N3-10	61	794	2/49O13-6	76
439	2/49M18-14	73	679	2/49N24-3	65			
348	2/49M2-7	75	527	2/49N1-11	65			
349	2/49M2-8	83	566	2/49N5-3	67			

A sufficient spread of CR scores was present in the plants selected for BSA, thus rendering the population suitable for single marker regression analysis.

4.2.2 BSA F_5 2006

A bulked segregate analysis (BSA) was conducted with the F_5 plant material. Only the 2-49/N population was genotyped in the F_5 generation. A single marker regression analysis for the previously established seedling/ field QTL loci was conducted. 2-49/N lines with the 30 highest and 30 lowest CR scores (refer to 2.2.4) were used for BSA. The lines used are listed in Table 4-2.

Table 4-2: Lines used in the F_5 BSA analysis of the hexaploid wheat x durum cross 2-49/N. Crown Rot scores obtained from Tamworth field trials 2006.

Low CR			High CR		
Entry No.	Entry	CR	Entry No.	Entry	CR
691	2/49N 29-1	2.6	605	2/49N 17-2	43.3
698	2/49N 29-6	6.5	536	2/49N 5-1	43.8
537	2/49N 5-2	8.2	655	2/49N 22-1	44.4
714	2/49N 31-1	9.7	534	2/49N4-7	44.4
617	2/49N 19-2	10.0	640	2/49N 22-3	44.4
693	2/49N 29-3	10.2	614	2/49N 18-7	45.8
675	2/49N 25-6	10.3	590	2/49N 12-6	45.8
620	2/49N 19-3	10.4	495	2/49N 1-1	47.2
566	2/49N 10-2	10.6	689	2/49N 28-4	47.2
572	2/49N 10-6	11.5	718	2/49N 31-9	47.2
645	2/49N 22-4	11.5	546	2/49N 7-3	48.3
720	2/49N 31-1	11.6	715	2/49N 31-6	49.0
672	2/49N 25-5	11.8	571	2/49N 10-5	50.0
638	2/49N 22-2	11.9	860	2/49N 3-10	50.0
555	2/49N 9-1	12.0	685	2/49N 27-3	50.0
852	2/49N 1-6	12.1	601	2/49N 16-2	50.0
502	2/49N 1-7	12.3	541	2/49N 6-3	50.0
682	2/49N 27-1	12.5	520	2/49N 3-13	50.0
857	2/49N 3-2	12.5	621	2/49N 19-3	50.0
532	2/49N 4-6	13.3	716	2/49N 31-6	50.0
870	2/49N 17-3	13.4	577	2/49N 10-7	50.0
719	2/49N 31-9	13.4	854	2/49N 1-11	52.8
493	2/49N 1-1	13.5	569	2/49N 10-4	52.8
710	2/49N 29-1	13.6	511	2/49N 2-8	54.2
583	2/49N 11-1	13.6	591	2/49N 12-6	54.2
535	2/49N 4-7	13.7	584	2/49N 11-1	54.2
500	2/49N 1-3	13.9	859	2/49N 3-10	54.2
559	2/49N 9-3	14.0	550	2/49N 7-6	55.2
856	2/49N 2-11	14.0	644	2/49N 22-4	58.3
642	2/49N 22-3	14.2	676	2/49N 25-6	75.0

4.2.3 F_6 2007

One- hundred and eighty- four 2-49/N plants representing all families, were genotyped with 22 SSR markers in the putative CR QTL regions in the F_6 (refer to appendix CD, Chapter 4).

4.2.4 F_7 2008

Phenotypic data from the tagged F_6 plants was used for QTL analysis of F_7 plants which were analysed with DArT markers. This was done because the field experiment ended with the F_6 generation and phenotypic data was not available for the F_7. The genetic difference between both generations was assumed to be minor due to expected near homozygosity in the genomes of the F_6 and F_7 generations. The significantly higher CR scores of the plants from the tagged population (which represented a sub-sample of the whole population analysed by the team in Tamworth) were not accounted for in the QTL analysis because all tagged plants were equally affected. This should therefore not change the accuracy of the QTL analysis. Refer to section 3.3.11 for a description of this population, number of plants and DArT analysis. In order to confirm the results obtained by this population, and to even out effects of widely varying CR scores in the F_6 plants, another statistical analysis was conducted with a smaller number of 51 selected lines. From each F_6 family, one F_7 seed from the tagged F_6 plant with the highest CR score was selected for genotyping. Plants with the highest CR scores were used in order to eliminate plants which may not have come into sufficient contact with inoculum.

4.3 RESULTS

4.3.1 SEGREGATION AND HETEROZYGOSITY

Segregation ratios describe the relative inheritance of alternative alleles at a single locus and indicate whether alleles assort randomly among the progeny or whether inheritance of one allele is favoured over the other. Segregation of alleles is best determined using co-dominant detection systems such as SSR markers which indicate individuals which are heterozygous at a particulat locus, due to the appearance of both corresponding DNA bands on the gel system employed.

In the F_4 generation heterozygosity was around 30%. In the F_6 a significant decrease to around 5% segregation was observed. The theoretical remaining heterozygosity in the F_6 can be readily calculated. In the F_1 the heterozygosity is always 100%, this is halved in every successive generation, resulting in a theoretical heterozygosity of around three per cent by the F_6. However, due to selection by the breeders, the level of heterozygosity was higher in this population. The difference in ploidy levels of the parental lines may also have influenced (slowed down) the rate at which homozygosity was reached.

Table 4-3 shows the segregation ratios and heterozygosity on selected chromosome regions in the 2-49/N F_4 population.

Table 4-3: Segregation ratios and heterozygosity in the F_4 generation of the hexaploid wheat x durum cross 2-49/N, (% 2-49 = hexaploid alleles from 2-49; % N = tetraploid alleles from N durum; %H = % heterozygote alleles; % - = % missing data).

Chr	Marker	%2-49	%N	%H	%-
1A	wmc278	46	29	24	1
1A	gwm164	56	29	15	0
1A	wmc120	47	34	19	0
1A	wmc312	28	37	34	1
1A	cfa2129	23	31	42	4
2A	gwm515	33	37	30	0
2A	gwm95	30	46	23	1
4B	gwm113	45	31	21	3
4B	gwm251	27	48	22	3
4B	gwm6	23	36	36	5
7B	gwm46	13	58	28	1

Table 4-4 shows the segregation ratios and heterozygosity on selected chromosome regions in the F_6 2-49/N population. There is a significant quantity of missing data due to low quality of extracted DNA, possibly arising from freeze drying the leaf material before grinding it. The two other populations were not assessed in this year.

Table 4-4: Segregation and heterozygosity in the F_6 generation of the hexaploid wheat x durum cross 2-49/N; (% 2-49 = hexaploid alleles from 2-49; % N = tetraploid alleles from N durum; %H = % heterozygote alleles; % - = % missing data).

Chr	Marker	%2-49	%N	%H	%-
1A	wmc120	40	35	9	16
1A	wmc278	33	35	17	15
1A	gwm164	39	30	8	23
2A	gwm95	42	37	4	17
5A	gwm291	16	52	1	31
1B	wmc403	23	37	0	40
1B	wmc134	21	27	1	51
4B	gwm113	47	33	8	12
4B	gwm251	30	42	1	27
7B	gwm471	27	49	5	18

4.3.2 THE QTL FOR PLANT HEIGHT

The QTL for plant height (rht1 gene), determined with the programme Mapmanager on the short arm of chromosome 4B (Sadeque and Turner 2010), was used as an indicator for the suitablility of this population for QTL analysis which was questionable due to the selection towards a durum genotype that had occurred. This QTL is not close to, or related to the putative CR QTL. The trait data for plant height was obtained from Steven Simpfendorfer, Tamworth, NSW, (refer to Appendix CD Chapter 4 for a summary of the results of the QTL analysis conducted with MapmanagerQT). In the F_4, the plant height QTL was detected in

the 2-49/N population with a very significant LRS score of 57.5 on chromosome 4B, explaining 29% of the variation (refer to Appendix CD, Chapter 2 for trait data and Chapter 4 for Mapmanager results and SSR markers and scores). A high LRS score indicates that the statistical likelihood of finding a specific QTL in this specific genomic location (DNA sequence) is high.

In the F_5, this QTL gave an LRS score of 14.5 and in the F_6 an LRS score of 13, verifying the results and confirming that the population was suitable for QTL analysis.

4.3.3 QTL RESULTS FOR F_4- F_6 GENERATIONS

These populations analysed with SSR markers are presented summarised, as linkages to the hexaploid derived resistance loci were not strong in field trials of these generations. The known CR resistance regions on chromosomes 1A (markers wmc278, wmc120 and gwm164) and 1B (wmc222) showed suggestive linkage to CR resistance in all years (F_4-F_6), the QTL on chromosome 4B (gwm251) was detected only in the F_6 (Table 4-5). Marker cfd61 showed linkage to CR resistance in the F_4 and was positioned at the peak of the 1D seedling QTL determined by Bovill et al. (2006) in the doubled haploid wheat population W21MMT70 x Mendos.

We detected a significant linkage to CR resistance (barc165) chromosome 5A in the F_4 in a region not previously known as CR resistance region. Table 4-5 shows an overview of the main markers showing weak linkage to CR resistance found with SSR markers from the F_4 to the F_6 including the bulked segregate analyses of the F_4 and F_5. The bulked segregate analyses were done because no strong QTL could be detected in the analysed populations which were selected by the breeders. The population used for BSA in the F_4 consisted of 15 plants with the highest and 15 plants with the lowest CR scores, the population used for BSA in the F_5 consisted of 30 plants with the highest and 30 with the lowest CR scores. The data for the SSR analyses of these populations is available on the Appendix CD, Chapter 4, summary of all SSR scores. The likelihood ratio statistic (LRS score)

indicates the relationship between variances in phenotypes and variances in particular DNA sequences (refer to the Literature Review for a more detailed description of LRS scores and % variation).

Table 4-5: Summary of LRS scores of chromosomal regions harbouring Crown Rot QTL in 2-49/durum crosses from the F_4 –F_6 generations.

Possible CR QTL regions (from SSR markers) (LRS score /% Variation)					
Chromoso.	Marker	F_4	F_4BSA	F_5	F_6
1A	wmc278	-	-	4/8	-
1A	wmc120	-	-	-	5/4
1A	gwm164	9/5	-	-	5/3
5A	barc165	-	17/50	-	
1B	wmc222	9/5	6/23	4/7	6/14
4B	gwm251	-	-	-	7/5
1D	cfd61	5/5	-	-	-
2D	gwm515	-	-	5/9	-

These weak linkages to CR resistance found with SSR markers were confirmed by the results from the DArT analysis of the F_7 described in the next section.

A regression analysis was performed which related 1D chromatin content in the F_6 population to the average CR scores.The coefficient of determination R^2 was small with 0.012, indicating that there was a lack of correlation between CR scores and 1D content.

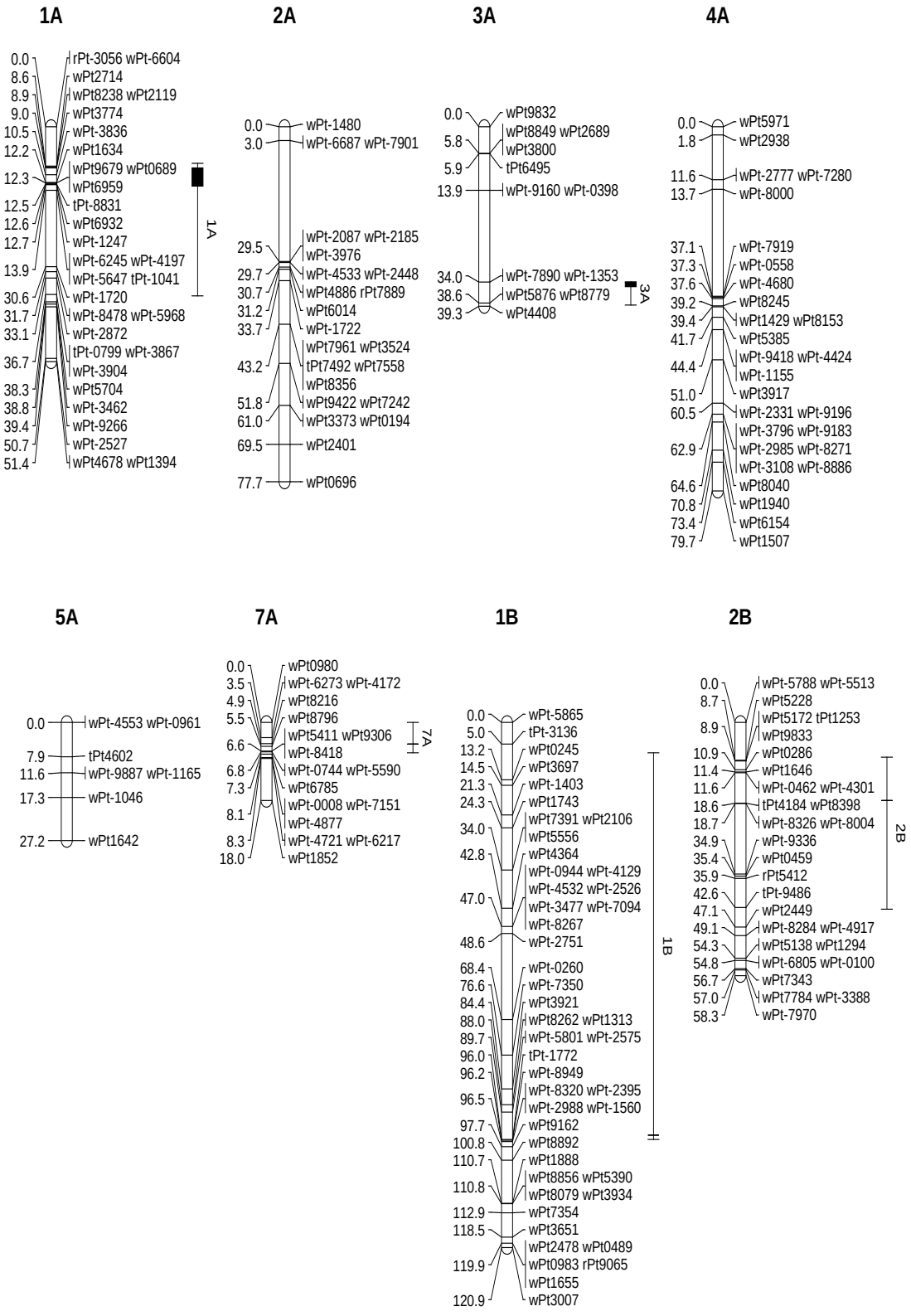
4.3.4 F_7 2008

The plant breeder and pathologist at Tamworth did not conduct field evaluation of these materials after the F_6, since at this stage the lines were largely fixed and no longer segregating. The most resistant lines were taken and used for backcrossing to selected elite durum lines (breeding work that is outside the scope of this project).

DNA from leaf material from germinated F_7 seed from 191 selected F_6 lines were sent to Triticarte for DArT analysis. These samples consisted of one to eight

plants per family, representing families examined in previous generations and covering a wide range of CR scores.

The F_7 QTL analysis was more successful than the analyses in the previous years. Due to the low heterozygosity now present in the lines, marker data was analysed using the phenotypic scores for the F_6 parents in the field. The established CR resistance QTL were confirmed on 1A, 1B, 2B, 4B and 7B. The chromosomes with QTL are shown in the map created from the F_7 DArT data with the programme MapmanagerQT, and QTL mapping was conducted in Win QTL Cartographer 2.5. (Figure 4-1).


1A
0.0 rPt-3056 wPt-6604
8.6 wPt2714
8.9 wPt8238 wPt2119
9.0 wPt3774
10.5 wPt-3836
12.2 wPt1634
12.3 wPt9679 wPt0689
wPt6959
12.5 tPt-8831
12.6 wPt6932
12.7 wPt-1247
13.9 wPt-6245 wPt-4197
wPt-5647 tPt-1041
30.6 wPt-1720
31.7 wPt-8478 wPt-5968
33.1 wPt-2872
36.7 tPt-0799 wPt-3867
wPt-3904
38.3 wPt5704
38.8 wPt-3462
39.4 wPt-9266
50.7 wPt-2527
51.4 wPt4678 wPt1394
1A
2A
0.0 wPt-1480
3.0 wPt-6687 wPt-7901
29.5 wPt-2087 wPt-2185
wPt-3976
29.7 wPt-4533 wPt-2448
30.7 wPt4886 rPt7889
31.2 wPt6014
33.7 wPt-1722
wPt7961 wPt3524
43.2 tPt7492 wPt7558
wPt8356
51.8 wPt9422 wPt7242
61.0 wPt3373 wPt0194
69.5 wPt2401
77.7 wPt0696
3A
0.0 wPt9832
5.8 wPt8849 wPt2689
wPt3800
5.9 tPt6495
13.9 wPt-9160 wPt-0398
34.0 wPt-7890 wPt-1353
38.6 wPt5876 wPt8779
39.3 wPt4408
3A
4A
0.0 wPt5971
1.8 wPt2938
11.6 wPt-2777 wPt-7280
13.7 wPt-8000
37.1 wPt-7919
37.3 wPt-0558
37.6 wPt-4680
39.2 wPt8245
39.4 wPt1429 wPt8153
41.7 wPt5385
44.4 wPt-9418 wPt-4424
wPt-1155
51.0 wPt3917
60.5 wPt-2331 wPt-9196
wPt-3796 wPt-9183
62.9 wPt-2985 wPt-8271
wPt-3108 wPt-8886
64.6 wPt8040
70.8 wPt1940
73.4 wPt6154
79.7 wPt1507
5A
0.0 wPt-4553 wPt-0961
7.9 tPt4602
11.6 wPt-9887 wPt-1165
17.3 wPt-1046
27.2 wPt1642
7A
0.0 wPt0980
3.5 wPt-6273 wPt-4172
4.9 wPt8216
5.5 wPt8796
wPt5411 wPt9306
6.6 wPt-8418
6.8 wPt-0744 wPt-5590
7.3 wPt6785
8.1 wPt-0008 wPt-7151
wPt-4877
8.3 wPt-4721 wPt-6217
18.0 wPt1852
7A
1B
0.0 wPt-5865
5.0 tPt-3136
13.2 wPt0245
14.5 wPt3697
21.3 wPt-1403
24.3 wPt1743
wPt7391 wPt2106
34.0 wPt5556
42.8 wPt4364
wPt-0944 wPt-4129
wPt-4532 wPt-2526
47.0 wPt-3477 wPt-7094
wPt-8267
48.6 wPt-2751
68.4 wPt-0260
76.6 wPt-7350
84.4 wPt3921
88.0 wPt8262 wPt1313
89.7 wPt-5801 wPt-2575
96.0 tPt-1772
96.2 wPt-8949
96.5 wPt-8320 wPt-2395
wPt-2988 wPt-1560
97.7 wPt9162
100.8 wPt8892
110.7 wPt1888
110.8 wPt8856 wPt5390
wPt8079 wPt3934
112.9 wPt7354
118.5 wPt3651
wPt2478 wPt0489
119.9 wPt0983 rPt9065
wPt1655
120.9 wPt3007
1B
2B
0.0 wPt-5788 wPt-5513
8.7 wPt5228
wPt5172 tPt1253
8.9 wPt9833
10.9 wPt0286
11.4 wPt1646
11.6 wPt-0462 wPt-4301
18.6 tPt4184 wPt8398
18.7 wPt-8326 wPt-8004
34.9 wPt-9336
35.4 wPt0459
35.9 rPt5412
42.6 tPt-9486
47.1 wPt2449
49.1 wPt-8284 wPt-4917
54.3 wPt5138 wPt1294
54.8 wPt-6805 wPt-0100
56.7 wPt7343
57.0 wPt7784 wPt-3388
58.3 wPt-7970
2B

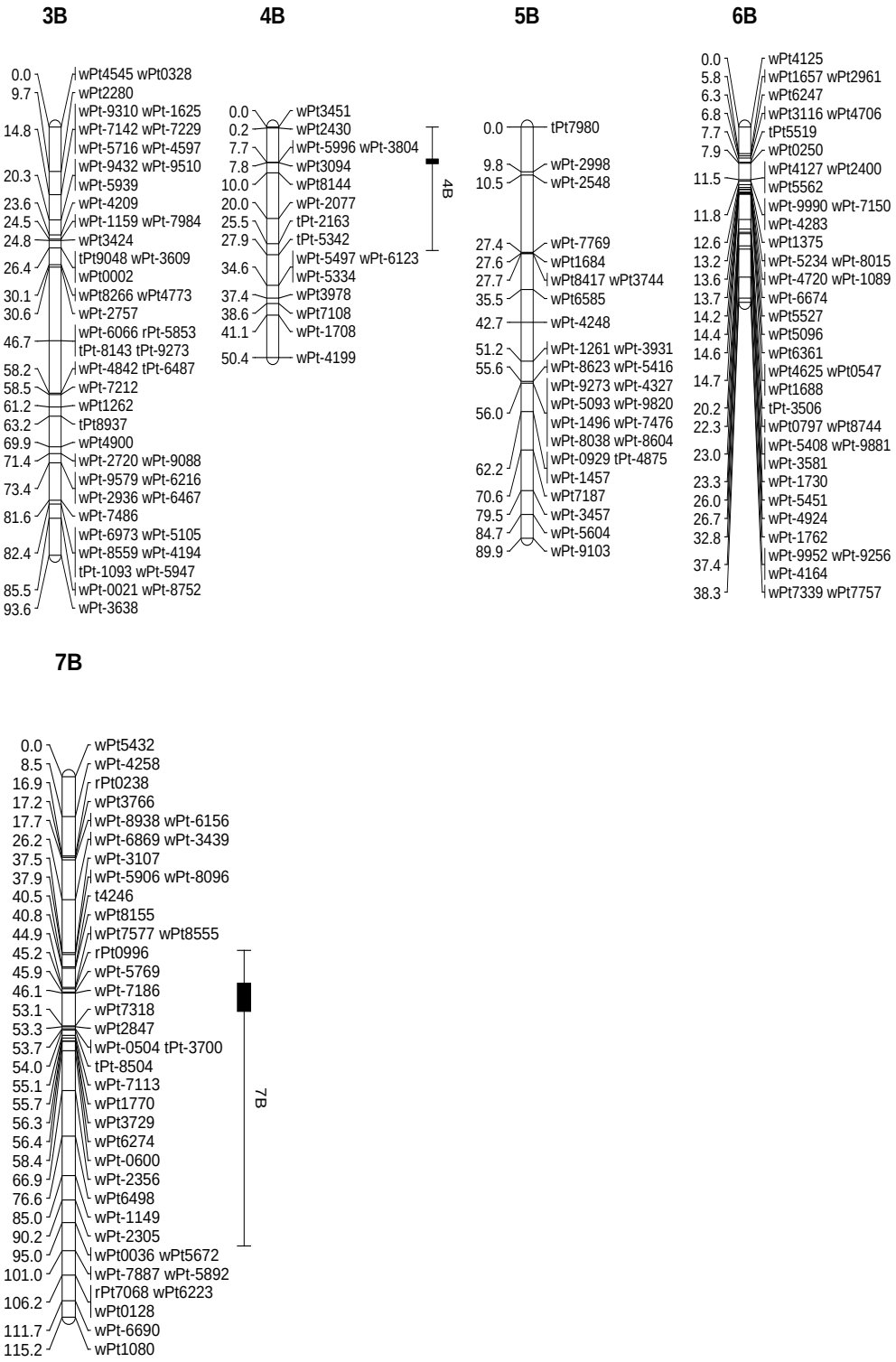


Figure 4-1: Map from DArT data of the F_7 generation of a 2-49/N cross with CR resistance QTL; marker distance in cM given in left colum, areas of QTL peaks = black bars.

The permutation test (1000 permutations, 10cM Intervals) (Churchill and Doerge 1994) was performed with Win QTL Cartographer 2.5 (Statistical Genetics, North Carolina State University, USA). It showed that the threshold for significant QTL was at an LRS score of 43.5, and for suggestive QTL at an LRS score of 17.2. These unusually high thresholds were caused by the fact that some of the analysed plants belonged to the same family and therefore had the same genotype, implying that some repetitive data was used. According to this information, the QTL on chromosomes 1A and 4B were significant QTL (Figures 4-1, 4-2 and 4-3), while the others were suggestive QTL. Figures 4-2 and 4-3 show graphs of the QTL on 1A and 4B and the threshold LRS score of 44. The graphs below show the positive additive effects of the maternal 2-49 alleles which contributed to the QTL.

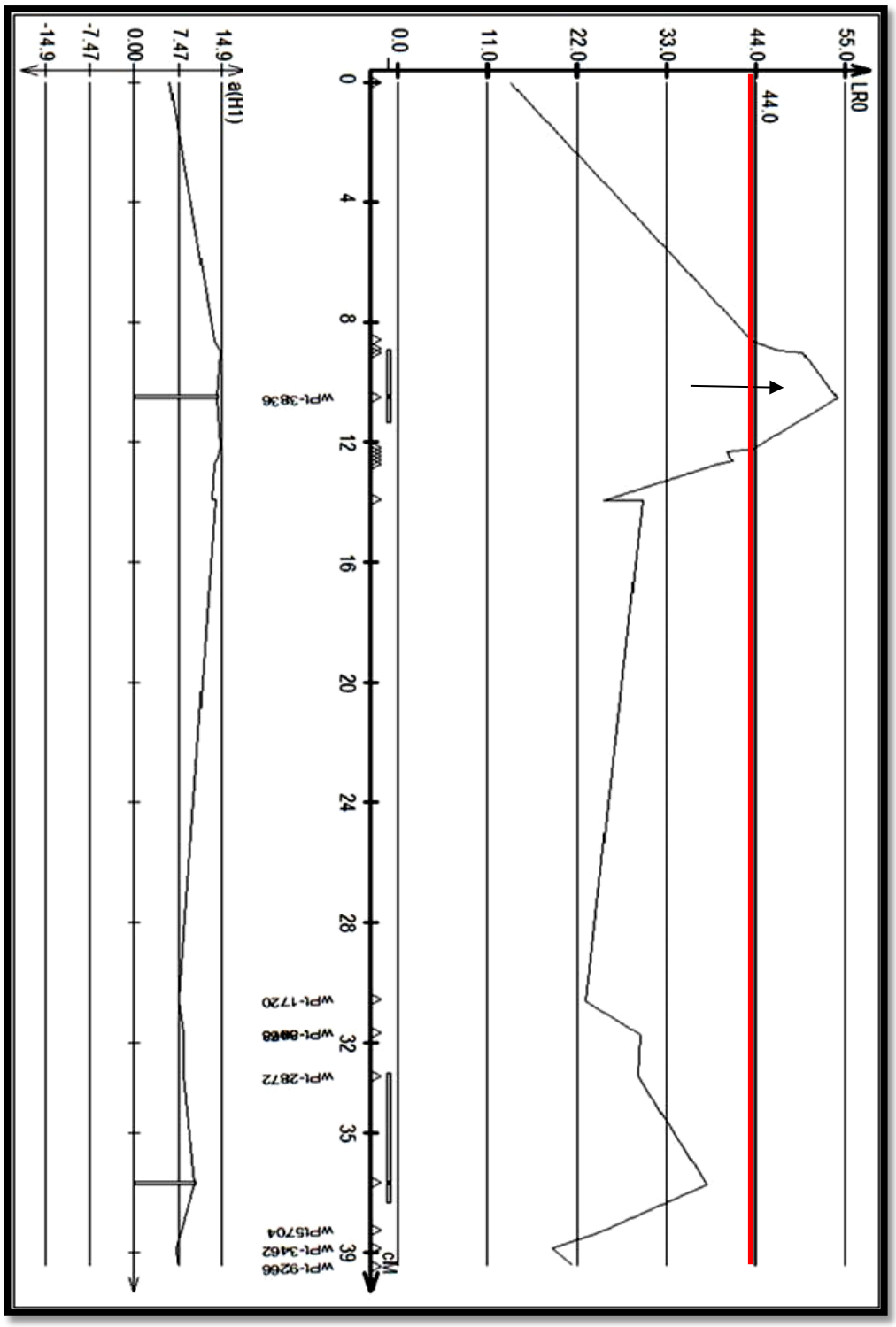


Figure 4-2: Significant Crown Rot resistance QTL on chromosome 1A in a 2-49/N cross, indicated by the arrow, surpassing the threshold of LRS score 44 (Win QTL Cartographer 2.5). The graph below shows the positive additive effects of the maternal 2-49 alleles which contributed to the QTL.

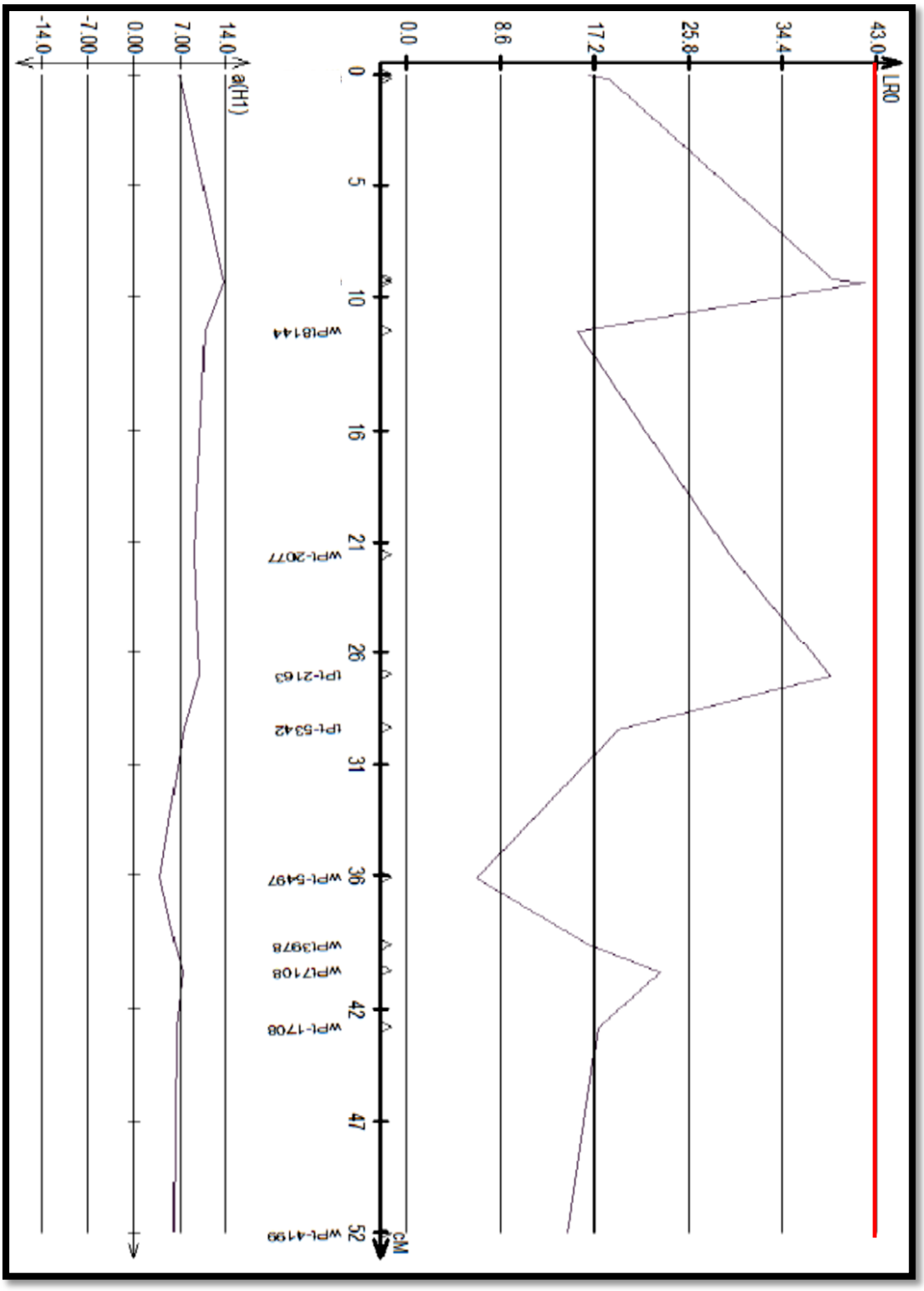


Figure 4-3: CR resistance QTL on chromosome 4B in the F_7 generation of the hexaploid wheat x durum cross 2-49/N. The graph below shows the positve additive effects of maternal 2-49 alleles.

As some plants sent for DArT analysis to Triticarte belonged to the same family and therefore had almost the same genotype, the population tested above was not altogether a normally segregating population.

Accordingly, in order to confirm the results given above, and to even out effects of widely varying CR scores in F_6 parents with the same genotype, another statistical analysis was conducted with a smaller number of 51 selected lines (refer to Methods section 4.2.4). This analysis confirmed that the QTL on chromosomes 1A and 4B were both significant CR resistance QTL, whereas 2B and 7B were only suggestive and the putative QTL on 1B was not detected. The threshold for significance in this experiment was determined at an LRS score of 11.5 (1000 Permutations, 10cM distance).

Significant DArT markers from the test with 51 plants are shown in Table 4-6. There was a slight difference in the marker order and LRS scores compared to the population with 191 plants (refer to Appendix CD Chapter 4, Marker regression).

Table 4-6: Significant DArT markers in the F_7 generation of a 2-49/N cross showing LRS score and % Variation; Add: positive additive effects of the maternal 2-49 alleles contributing to the QTL.

Chromo.	Marker	LRS score	% Var.	Add
1A	tPt-1041	11.8	21	12.9
1A	wPt-5647	13.3	23	15.3
1A	wPt-4197	11.7	20	13.6
1A	wPt-6245	11.7	20	13.6
1A	wPt-1247	12.0	21	13.6
1A	wPt6932	13.2	23	12.8
1A	tPt-8831	14.8	25	14.3
1A	wPt6959	14.8	25	14.3
1A	wPt0689	14.8	25	14.3
1A	wPt9679	14.8	25	14.3
1A	wPt1634	16.2	27	13.9
1A	wPt-3836	20.4	28	15.6
1A	wPt3774	16.2	27	13.3
1A	wPt2119	16.5	28	13.0
1A	wPt8238	15.0	25	12.4
1A	wPt2714	14.6	25	12.7
4B	wPt3451	6.5	13	7.2
4B	wPt2430	5.9	11	7.1
4B	wPt-5996	11.1	20	12.1
4B	wPt-3804	14.8	25	14.3
4B	wPt3094	17.8	29	14.4
4B	wPt-2077	12.6	22	9.9
4B	tPt-2163	13.8	24	9.8
4B	tPt-5342	7.0	13	9.0
4B	wPt3978	4.3	8	5.9
4B	wPt7108	5.3	10	6.7
4B	wPt-1708	6.9	13	7.3
4B	wPt-4199	4.4	9	5.8

4.4 DISCUSSION QTL ANALYSIS

The CR data collected over several generations of the hexaploid x tetraploid crosses was not ideally suited for QTL analysis, as the populations underwent up to six rounds of selection by the breeders in Tamworth, however, the results shown above indicate that the predetermined regions specific for CR resistance were associated with resistance and the CR QTL have been transferred into the progeny.

The F_4- F_6 populations showed significantly higher average CR resistance than the durum parent Bellaroi (A) (refer to section 2.5).

It was initially anticipated that the CR resistance in part originates from the hexaploid 1D chromatin, and our data shows that it seemed to have had some influence on the enhanced resistance of the durum progeny in the earlier generations, as weak linkage to CR resistance could be found on chromosome 1D in the adult plants (Table 4-5). This may have been due in part to the influence of the seedling resistance gene previously identified by Collard et al. (2006) on 1D. However, when comparing the few plants that still contained 1D chromatin in the F_6 with the ones that did not in terms of CR resistance, there was no significant difference. In the F_7, where few lines retained the 1D chromosome, resistance was clearly evident, indicating that the A and B genome loci were responsible.

MCFISH results from the F_2 and F_3 show that translocation events between the A and D or B and D genome were very rare, ruling out the possibility that translocation mechanisms are responsible for transfer of resistance from the hexaploid parent to progeny of the cross with durum.

Results from DArT analysis of the advanced F_7 generation supported results from the earlier generations. One significant QTL was discovered, located on chromosome 1A, in close proximity to the CR seedling and field resistance QTL identified by Collard et al (2005) and Bovill et al. (2010). Another QTL on 4B was very close to the significance threshold LRS of 44 in one experiment, and well

above the threshold LRS of 11.5 in the other (refer to Appendix CD, Marker regression analysis). The other analysed QTL regions (1B, 2B, 7B) were detected as suggestive CR resistance QTL. This confirms that the resistance QTL first identified in the hexaploid parent have been successfully transferred in the progeny of a cross to a tetraploid durum line. This is particularly encouraging in view of the increased possibility of loss of linkage between marker and target gene due to segregation during the multiple meiotic events required to generate F_7 materials.

In all generations analysed, linkage to CR resistance loci inherited from the hexaploid parent were detected (Table 4-5: Summary of LRS scores of chromosomal regions harbouring Crown Rot QTL). The linkages consisted of a mix of suggestive and significant QTL. Several factors may have contributed to the relatively modest LRS scores observed in some generations and that is why some QTL were not observed in every generation examined.

Selection by pathology and breeding staff at Tamworth for both CR resistance and durum plant types.

This was not a random population. The genetic constitution of the excluded plants was lost. For successful genotyping a sufficient spread of susceptible plants as well as resistant plants is needed. In this case, resistant plants with undesirable plant form may have been discarded. At our request a selection of susceptible materials were included each year in selections for the next generation of field trials in order to provide adequate numbers of susceptible lines for analysis.

Environmental influences on CR development (GxE effects).

The number of plants needed to find a QTL is in proportion to the variance of the environmental contributions and inversely proportional to the square of the strength of the QTL (Rebai et al. 1995; Lynch and Walsh 1998, Manly and Olson 1999). In a trait like CR, where environmental influences play a significant role in QTL expression, a large number of plants needs to be analysed, probably a lot more than the number analysed in this experiment.

In the F_6, where residual segregation should be lost, there was still significant variation of trait scores among individual plants of the same genotype which indicates a significant environmental impact on CR development. An example for the wide variety of trait data is shown in Table 4-7.

This family (2-49/N 31-10) showed significant CR resistance in all three years it was assessed (2004-2006).

Table 4-7: Example for spread of Crown Rot scores in different plants of 2-49/N 31-10 in the F_6.

Different F_6 Plants of the Family 2-49/N 31-10															
Plant	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
CR score	33	33	33	33	17	50	50	33	13	50	17	10	33	67	50

This indicates significant environmental effects at play which can cause significant differences within repetitions and plots. Uneven spread of the inoculum in the soil, which has to touch the crown directly, is common in field experiments and possibly played a role here. Water availability also varies between plots, but is paramount for evenly distributed inoculation. The difference in ploidy levels of the parental durum lines may have influenced (slowed down) the rate at which homozygosity was reached.

Average CR scores for the plot were used for the QTL analysis in the F_4 and F_5.

The average CR score of all the plants of the plot, which was provided by Steven Simpfendorfer, was used for the QTL analysis of the individual plants which were selected for analysis. This might have negatively impacted the results in the earlier generations. However, in the F_6, tagged plants with individual CR scores were used for analysis.

CR resistance is based on multiple genes.

A complete set of QTL conditioning CR resistance is hard to capture because the mapping of traits that are controlled by horizontal or quantitative (multiple) gene resistance is generally more demanding than the mapping of single gene traits. Traits that explain 1-10% of the trait variance (moderate QTL) can theoretically be detected when sufficient progeny, (around 300- 500 plants) are analysed, but the detection will lack power. This results in the non- discovery of some QTL while others may be overestimated. It can even lead to the detection of two different sets of QTL for the same trait. The reason for this is that two experiments with an individual power of about 10% or less will each find a different overestimated minority of the whole trait (Manly and Olsen 1999).

MAS is not usually employed for the improvement of polygenic traits like CR, because mapping techniques are not sophisticated enough for this task. Also, extrapolation from one breeding population to another is not easy or impossible (Holland 2004). However, when the number of plants analysed in a population is increased, and the experiment is conducted at multiple sites, the precision of QTL mapping is improved. This on the other hand leads to the need for more resources and reduces the efficiency of MAS compared to conventional techniques. The variety of genes influencing CR resistance also leads to a variation of CR QTL exhibition between years (Bovill et al. 2010).

Tightness of marker linkage.

In this study, flanking markers with a marker spacing of 10-20cM from the gene of interest were deployed (refer to Figure 3-3). For populations smaller than 200 individuals, marker spacing below 10 cM does not improve the power of analysis, as the size of the confidence interval is not significantly improved. Only in situations with high levels of recombination would a closer marker spacing be advisable (Salinas 1998). If the marker spacing is greater than 40 cM, interval mapping is more powerful than single-locus regression analysis. In our study the marker spacing was usually smaller than 40 cM, therefore interval mapping would not provide additional benefits (Darvasi 1998).

5 GENERAL DISCUSSION

This study has attempted to address three research questions as set out in Section 1.8. I will now deal with each of these in turn.

5.1 THE FATE OF THE UNPAIRED D GENOME MATERIAL

"What is the fate of the unpaired hexaploid derived D chromatin, and is any of this material translocated into the A and B- genome chromosomes of the progeny?".

SSR marker and DArT marker analysis have confirmed that the decline in the quantity of D genome material in successive generations derived from the hexaploid x tetraploid crosses examined followed the patterns described in earlier literature (Kihara 1982). In successive generations, lines with an incomplete set of D genome chromosomes were increasingly eliminated, with hexaploid revertants and tetraploid lines predominating.

Very recent work by Martin et al. (2011), in which some of this thesis work is included, shows that the degree of D genome retention in the F_2 generation is cross specific, indicating that some crosses eliminate D genome materials to a greater extent and much more rapidly than others. For example, a Sunco/Bellaroi cross eliminated most of its D genome material in the F_2 generation, whereas the 2-49/Bellaroi cross retained most of the D genome chromosomes in the F_2.

Hexaploid x durum crosses are being increasingly considered as a means of transferring desired genes in either direction (Wang et al. 2005; Lanning et al. 2008). However, the zero or relatively low yield of fertile F_1 seed which results from some crosses, the challenge of establishing the stable recurrent ploidy of choice (28 or 42) in subsequent generations and the potential loss of blocks of desirable alleles from the recurrent parent genome are all potential obstacles to the success of this strategy. In one of the successful hexaploid x durum crosses examined in this study, polymorphic DArT alleles from the hexaploid and tetraploid parents segregated randomly among the progeny across almost all loci on the A and B genomes. Interestingly, twice as many polymorphic DArT markers mapped to the B genome chromosomes compared to the A genome

chromosomes, consistent with previous observations of higher levels of polymorphism in the B genome of modern wheats (Boeuf et al. 2003). Clearly this still applies even to comparisons between hexaploid bread and tetraploid durum wheats, reflecting both their common ancestry and subsequent limited evolutionary divergence. The segregation distortion observed for a group of closely linked markers on chromosome 5B is consistent with studies in hexaploid wheats which have also reported distortion on 5B (Kumar et al. 2007).

With regard to the D genome, we observed a very significant retention of this material in the F_2 progeny of the cross. Of the 83 F_2 lines analysed with the dominant DArT markers, 48% retained at least one apparently entire copy of each D chromosome. Where both copies of a particular D chromosome had been lost, there was no evidence that loss or retention of particular chromosomes (or groups of chromosomes) was favoured. In one previous study, 24 (56%) out of 43 F_2 lines from a Sumai 3 x DT486 pentaploid wheat cross analysed with microsatellites possessed at least one copy of all seven D genome chromosomes (Gilbert et al. 2000). While our MCFISH studies indicated that 14 (54%) out of 26 lines retained 35 or more D chromosomes, Wang et al. (2005) found that 80% of 55 F_2 plants of a As195 (durum) by Chuannong (hexaploid) cross were in this class.

The retention of some D chromatin in all but one of the total of 109 progeny examined indicates successful transfer of many unpaired D chromosomes through meiosis in selfed F_1 plants. Based on comparison of the DArT and MCFISH results for 26 lines, D genome chromosomes were predominantly inherited as complete chromosomes. However, single telocentric chromosomes and translocation events were observed in five of these lines (19%). In the larger sub-population analysed only by DArT analysis, partial chromosomes were observed with a slightly lower frequency (14%). It is not possible, based on DArT analysis alone, to determine whether these are translocations or telocentric chromosomes. Furthermore, in the absence of MCFISH analysis, some incomplete chromosomes will go undetected due to the presence of the entire homologous D chromosome. For

similar reasons, A or B chromosomes involved in translocations of D genome material cannot be identified using DArT markers alone.

All three translocations of D genome material characterised by MCFISH were confirmed by DArT marker analysis, indicating that the homologous D chromosome was absent. This suggests that prior loss of the homologous chromosome may favour a translocation event and deserves further investigation, since translocation of selected D chromosome loci may be desirable. Crosses between species of different ploidy, which favour such losses, could therefore be conductive to such events.

MCFISH is a labour intensive cytogenetic technique, requiring a high level of operator expertise, which does not lend itself to high throughput screening of population lines. Nevertheless, its judicious use along side high throughput marker-based techniques such as DArT screening has begun to reveal much that will be of practical application to the development of hexaploid x durum derived materials in breeding programs.

This study has pioneered the combination of DArT analysis and McFISH, supplemented by SSR (or other co-dominant marker) analysis to reveal the detailed chromosome composition of progeny from these crosses.

5.2 WAS THERE TRANSFER OF PARTIAL CR RESISTANCE FROM HEXAPLOID SOURCES INTO DURUM WHEAT?

"Can the partial resistance to CR found in some hexaploid wheats be transferred into the progeny of crosses to tetraploid durum wheat?"

There clearly was a transfer of resistance from 2-49 into the new crosses. The mean CR resistance in successive selfed populations derived from the three crosses examined increased significantly over the years, recording a 55% drop in disease severity score relative to the Bellaroi susceptible parent in 2007 (F_6). Some families showed consistent resistance over multiple generations.

Improved resistance was therefore not dependent on D genome material being retained. It was shown that especially the CR resistance QTL on chromosome 1A had a significant influence on the resistance of the new crosses. The positive

additive effects of the maternal 2-49 alleles contributed significantly to this QLT. However, it cannot be ruled out that durum wheat has some inherent resistance in these genomic regions as well.

This study shows that the transfer of disease resistance is possible in interspecific crosses between hexaploid wheat and durum wheat and suggests that these crosses can be successfully used in the future.

5.3 WAS THERE STABLE TRANSFER OF MOLECULAR MARKERS FOR RESISTANCE INTO PROGENY OF PENTAPLOID LINES?

"*Will genetic markers linked to resistance in the hexaploid sources still be useful predictors of resistance in the progeny of hexaploid x durum crosses?".*

Despite several rounds of selection based on morphological characteristics and Crown Rot score, markers for CR resistance first identified in the hexaploid populations 2-49/Janz and Gluyas Early/Janz, were still linked to resistance in the F_4 to F_7 generations derived from crosses between 2-49 and a selection of durum backgrounds.

The results from four years of QTL analysis with SSR and DArT markers showed that all previously discovered seedling QTL regions (1A, 1B, 4B) showed linkage to CR resistance in adult plants.

In the F_7 generation, a significant QTL linked to CR resistance was established. This QTL is in the same area as the previously discovered field CR resistance QTL on the short arm of chromosome 1A. Chromosome 4B also features a significant CR QTL. Both these loci have been detected in previous seedling and adult plant trials of hexaploid populations (Collard et al. 2005; Bovill et al. 2010).

For further studies, SSR markers linked to CR resistance that were detected in this study (wmc222 on 1B, wmc120 and gwm164 on 1A) could be employed to detect resistant durum plants in breeding populations. The QTL linked to these markers are effective contributors to resistant phenotypes in a durum background and the markers provide potential tools for selection of resistance in commercial

breeding. However, the transfer of markers from one population to another is not always possible.

6 OUTLOOK

Backcrosses of resistant tetraploid F_6 and F_7 materials to elite durum recurrent parents are currently being undertaken at the New South Wales Department of Industry and Investment and at USQ by Dr Anke Martin. These lines contain molecular markers for the QTL detected in this study.

Commercial durum varieties with enhanced CR resistance may emerge in as little as four years with the assistance of MAS rapid breeding techniques that ensure several generations per year.

In the F_4 and F_5, five markers on the long arm of 5A (gwm291, gwm234, wmc150, barc100 and barc165) were significantly linked to CR resistance and this region overlaps with certain stress tolerance QTL. This region on chromosome 5A should be subject to further studies, especially because a major QTL affecting drought- induced ABA accumulation was mapped on the long arm of chromosome 5A of wheat, in the vicinity of the locus controlling frost resistance and tightly linked to the Dhn1/Dhn2 (dehydrin) locus (Cattivelli et al. 2002). This suggests a genetic linkage between ABA accumulation, stress tolerance (Quarrie et al. 1994) and Crown Rot resistance. This is consistent with the enhancement of Crown Rot symptoms in plants undergoing late season water stress (Simpfendorfer 2006).

In contrast, durum wheat is considered to shows higher levels of drought tolerance than hexaploid wheat (Chandrasekar et al. 2000) but is still more susceptible to CR. Yet, compared to bread wheat durum is generally less salt tolerant (Rahnama et al. 2010). No studies of CR in regards to salt tolerance in wheat exist at the present time. It would be worthwhile to have a closer look at the possible relationship between these genetic traits. A salt tolerant durum variety was developed recently by the CSIRO (ScienceDaily, Apr. 26, 2010, http://www.sciencedaily.com/releases/2010/04/100423094622.htm).

After the first marker assisted breeding programs were introduced, cautioning voices could be heard. It was stated that few real achievements were made for

practical breeding. First concerns were published in 1999, stating that marker-assisted selection was not forceful enough to provide real improvements (Young 1999). However, especially the DArT analysis of the F_7 generation shows that, depending on the setup of the experiment, the generation and number of plants used, marker assisted selection can be very powerful and provide real benefits to plant breeding, as it provides information about useful resistance loci that can be easily utilised.

MCFISH, SSR and DArT analyses proved to be effective methods to analyse the wheat genome and complemented each other well. These results provide important knowledge for enhanced breeding with interspecific wheat crosses and transferring genes from hexaploid parents into durum.

The recent study by Martin et al. (2011) of a range of interspecific crosses between hexaploid wheat x durum wheat, reveals that different proportions of both D genome material and parental hexaploid and durum alleles in the A and B genome are retained in the F_2 generation. These proportions are influenced by both the hexaploid and tetraploid parent in the cross. Hence careful selection of the parents, depending on whether a tetraploid or hexaploid trait is being introgressed, will significantly influence the speed with which transfer of desirable genes into the recurrent background and desirable ploidy is achieved. Research to further illuminate the parental factors controlling the proportions of each genome inherited should be continued.

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