Mapping of chromosome arm 7DL of *Triticum aestivum* L.

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Promoter: Prof G.F. Marais

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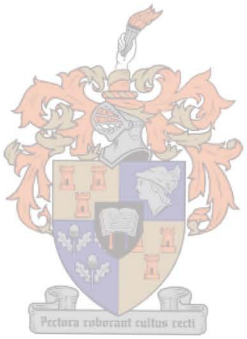
Declaration

I the undersigned hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

Signature: .....

Date: 18/02/05



SUMMARY

The Russian wheat aphid, *Diuraphis noxia* (Mordvilko), is a serious insect pest of wheat and barley. It affects the quality and yield of grain by sucking plant sap from the newest growth whilst toxic substances are injected that destroy plant tissue. The Russian wheat aphid also acts as a vector of plant viruses. The cultivation of aphid resistant cultivars is the preferred control strategy and nine resistance genes, designated *Dn1* to *Dn9*, have been identified. Another undesignated gene, *Dnx*, was found in the wheat accession PI220127. Mapping of the resistance genes relative to known markers will improve their use in breeding programs.

The dominant RWA resistance gene, *Dn5*, was identified in the accession PI294994 and mapped to chromosome arm 7DL. However, recent reports have placed *Dn5* on chromosome arm 7DS. This study was undertaken to confirm the chromosome arm location of *Dn5* and to map it relative to chromosome 7D markers. 92RL28, a near isogenic line of the cultivar 'Palmiet' was used as the source of *Dn5*. Monotelodisomic plants having a normal chromosome 7D (carrying *Dn5*) and either 7DS or 7DL were derived and testcrossed with 'Chinese Spring' nullisomic 7D plants. Monotelosomic TF_1 and ditelosomic TF_2 plants were selected for the 7DL and 7DS groups, respectively, and their progenies tested for Russian wheat aphid resistance. Four microsatellite markers that map to chromosome arms 7DS and 7DL, respectively, as well as endopeptidase analysis were used to verify the telosomes. Three to five TF_2 families that segregated for resistance were found among the 7DL monotelosomic derived progeny, while the TF_3 progeny, ditelosomic for chromosome arm 7DS, were all susceptible. The three resistant families obviously resulted from recombination between 7DL and 7D of 92RL28, thereby confirming that *Dn5* occurs on 7DL. While it is a limited data set, the distance between the centromere and *Dn5* was estimated at 11 – 19 map units, which would suggest linkage with the centromere.

In order to map a gene (*Dn?*) initially believed to be *Dn5* relative to other 7DL loci, a doubled haploid mapping population available in the department and derived from the F_1 of PI294994 and 'Chinese Spring' was used. The parental lines were screened for polymorphisms with 14 microsatellite markers of which 9 proved to be polymorphic. Seven of these markers mapped to chromosome arm 7DL while the remainder mapped to chromosome arm 7DS. A linkage map was created and the results suggested that *Dn?* is loosely linked to microsatellite markers *Xgwm 111* and *Xgwm 44* on chromosome arm 7DS. If

this map position is correct, the gene segregating in the doubled haploid population cannot be *Dn5* but rather one of *Dn1*, *Dn2*, *Dn6* or *Dnx.* However, an alternative explanation of the data is also possible: The map distances calculated may simply be wrong as a result of distorted segregation that was observed in the particular chromosomal region. The study highlights the necessity to repeat mapping experiments done in the past by various laboratories with 7D linked genes, *Dn1*, *Dn2*, *Dn5*, *Dn6* and *Dnx*. These studies often led to contradictory conclusions and generally, the organization of the 7D RWA resistance genes remains unclear. Authenticated single gene sources of the respective genes should be established and a combination of genetic and physical mapping should be employed when characterizing them.

OPSOMMING

Die Russiese koringluis, *Diuraphis noxia* (Mordvilko) is ‘n vername insek pes van koring en gars. Dit beïnvloed graankwaliteit en opbrengs deurdat dit plantsap uit jong plantdele onttrek en terselfdertyd toksiese stowwe inspuit wat lei tot die vernietiging van plantweefsel. Die Russiese koringluis dien ook as vektor vir plantvirusse. Die produksie van luisbestande kultivars is die beste beheermaatreël en nege weerstandsgene, *Dn1* tot *Dn9*, is reeds geïdentifiseer. ‘n Geen sonder simbool, *Dnx*, kom voor in die *Triticum aestivum* landras PI220127. Kartering van hierdie weerstandsgene relatief tot bekende merkers sal hul aanwending in teelprogramme vergemaklik.

Die dominante weerstandsgeen, *Dn5*, is in *T. aestivum* aanwins PI294994 geïdentifiseer en op die lang arm van chromosoom 7D gekarteer. ‘n Meer onlangse publikasie het egter getoon dat *Dn5* eerder op die kort arm van chromosoom 7D voorkom. Die doel van hierdie studie was om die chromosoom arm ligging van *Dn5* te bevestig en die geen te karteer relatief tot ander 7D merkers. 92RL28, ‘n naby isogeniese lyn van die kultivar ‘Palmiet’, is as bron van *Dn5* gebruik. Monteloidisomiese plante met ‘n normale chromosoom 7D (waarop *Dn5* voorkom) plus een van chromosoomarms 7DS of 7DL is verhaal en getoetskruis met ‘Chinese Spring’ plante nullisomies vir 7D. Monotelosomiese TF_1 (7DL) en ditelosomiese TF_2 (7DS) plante is onderwerp aan Russiese koringluis weerstandstoetse. Vier mikrosatelliet-merkers wat onderskeidelik karteer op chromosoomarms 7DS en 7DL, asook endopeptidase analise is gebruik om die betrokke telosome te bevestig. Tussen die 7DL monotelosomies-verhaalde TF_2 nageslagte is drie tot vyf families geïdentifiseer wat segregeer het vir weerstand. Die 7DS ditelosomies-verhaalde TF_3 nageslag was egter almal vatbaar. Die bestande families was die resultaat van oorkruising tussen 7DL en 7D van 92RL28 en bevestig dus die posisie van *Dn5* op chromosoomarm 7DL. Alhoewel dit ‘n beperkte datastel is, kon geraam word dat *Dn5* 11 – 19 kaart eenhede vanaf die sentromeer voorkom.

‘n Dubbel haploïed karterings-populasie, beskikbaar in die department en oorspronklik verhaal uit die kruising: ‘Chinese Spring’ X PI294994, is gebruik om ‘n onbekende geen (*Dn?*) wat aanvanklik vermoed was om *Dn5* te wees te karteer. Veertien mikrosatelliet-merkers is gebruik om die ouerlyne te sif en nege polimorfiese merkers is geïdentifiseer. Sewe hiervan kom op chromosoomarm 7DL voor terwyl die oorblywende twee op chromosoomarm 7DS voorkom. ‘n Koppelingskaart is opgestel en die weerstandsgeen,

Dn?, het geblyk om losweg gekoppel te wees aan die merkers *Xgwm 111* en *Xgwm 44* op chromosoomarm 7DS. Indien aangeneem word dat die kaartposisie korrek is, is die geen wat in die dubbel haploïde populasie segregeer dus nie *Dn5* nie maar eerder *Dn1*, *Dn2*, *Dn6* of *Dnx*. ‘n Alternatiewe verklaring van die data is egter ook moontlik: Die berekende kaartligging mag eenvoudig verkeerd wees vanweë segregasiedistorsie in die betrokke chromosoomgebied. Die studie beklemtoon die noodsaak om ten minste sommige van die eksperimente wat in die verlede met die 7D gekoppelde gene, *Dn1*, *Dn2*, *Dn5*, *Dn6* en *Dnx* uitgevoer is, te herhaal. Hierdie studies het dikwels gelei tot gevolgtrekkings wat mekaar weerspreek terwyl die organisasie van 7D Russiese koringluis weerstandsgene steeds onbekend is. Oorspronklike enkelgeen bronne van die onderskeie weerstandsgene moet verkry word en ‘n kombinasie van genetiese en fisiese kartering moet gebruik word om hulle te karakteriseer.

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CONTENTS

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Wheat is an annual grass that is adapted to a wide range of environments. It can therefore be grown in areas that are not suited for rice and maize production, which do best at intermediate temperatures. The total land area covered by wheat exceeds those of any other crop, including rice and maize, and it ranks first in total production (Briggle and Curtis, 1987). The first evidence of wheat utilization was found in Israel where archaeologists found remains of tetraploid *Triticum dicoccoides* dating back to 17000 BC. The earliest domesticated tetraploid and diploid wheat originated in South Western Asia approximately 8000-7500 BC. Early crop remains of the tetraploid *T. dicoccum* and the diploid *T. monococcum* were found in Syria from where it spread to central and Western Europe (Feldman et al., 1995). Sixty five percent of all grain consumed are used as food for humans due to its significance as a source of energy and protein. Wheat provides approximately 20% of the total food calories of the world and contributes 25% of proteins consumed by humans (Gooding and Davies, 1997).

1.1.1 The cytotaxonomic background of bread wheat

The tribe *Triticeae* Dumort forms part of the family *Poaceae* (Gramineae) and is characterized by a compound spike, laterally compressed spikelets with two glumes, single starch grains and basic chromosome number of x = 7 (Miller, 1987). The subtribe *Triticinae* contains the genera *Aegilops* L., *Secale* L., *Agropyron* Gaertn., *Triticum* L. and *Haynaldia* Schur (Morris and Sears, 1967). The genus *Triticum* L. includes a number of cultivated species, for example bread wheat (*T. aestivum*), durum wheat (*T. turgidum* var. durum), spelt (*T. aestivum* var. spelta), emmer (*T. aestivum* var. dicoccon) and einkorn (*T. monococcum*) – (Morris and Sears, 1967).

Based on chromosome number, cultivated wheat can be divided into diploids, tetraploids and hexaploids containing 14, 28 and 42 chromosomes, respectively. Cultivated diploid (*T. monococcum* L.), tetraploid (*T. turgidum* L.) and hexaploid (*T. aestivum* L.) wheat species contain one, two and three genomes respectively, each consisting of 7 chromosome pairs. The A genome is common to all three ploidy levels, the B genome is present in all tetraploid and hexaploid wheat species and the D genome is unique to hexaploid wheat (McFadden and Sears, 1946). The diploid species *T. monococcum* L. var. *urartu* (2n = 14, AA) was proposed to be the donor of the A genome. The wild tetraploid *T. dicoccoides* (2n = 28, AABB) may have arisen through hybridization of *T. urartu* (AA) and a unknown diploid/diploids with a genome similar to that of the Sitopsis section of *Aegilops* (Miller, 1987). Common bread wheat (*Triticum aestivum* L. em. Thell., 2n = 42, AABBDD) originated approximately 10000 years ago, presumably from hybridization of one or a few genotypes of tetraploid wheat (*Triticum turgidum* L., 2n = 28, AABB) and diploid *Triticum tauschii* (Coss.) Schmal. (syn. *Aegilops squarrosa* L., 2n = 14, DD) (Kihara, 1944; McFadden and Sears, 1946) – (Fig. 1.1).

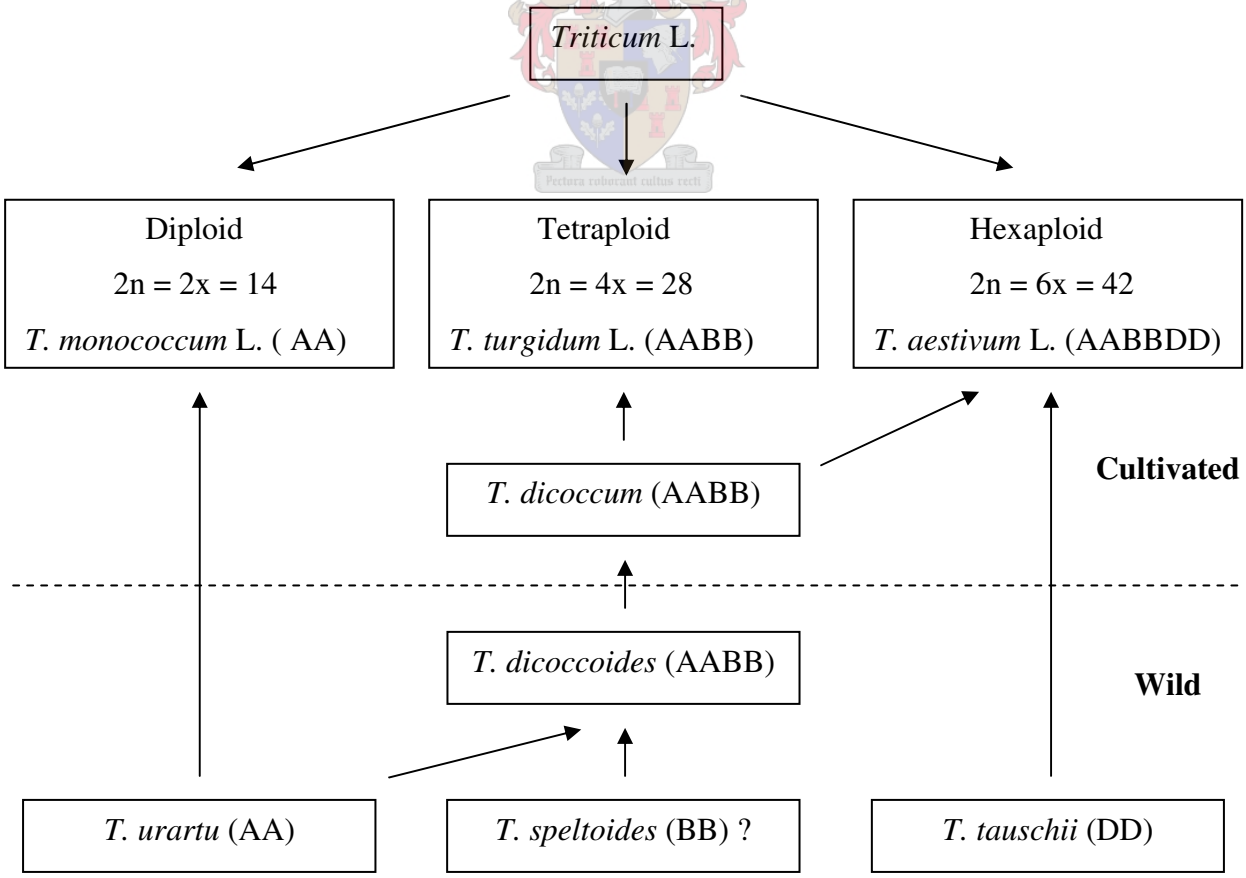


Figure 1.1 The evolution of cultivated wheat (adapted from Miller, 1987).

1.1.2 The genomes of bread wheat

The DNA content of a haploid common wheat nucleus is 18.5 picograms (pg) which is equivalent to approximately 16 billion base pairs (16 x 10^6 kb) - (May and Appels, 1987). More than 70% of the genome consists of repeated DNA sequences with various degrees of repetition while less than 20% consists of low copy number or single copy sequences (Smith and Flavell, 1975). Less than 1% consists of actual coding genes (May and Appels, 1987). The average length of a wheat chromosome is 10 µm with a DNA content which equals one half of the haploid rice genome while three wheat chromosomes are equal to the haploid maize genome (Gill and Gill, 1994). Nishikawa and Furata (1979) showed that the DNA contents of the three genomes in hexaploid wheat is present in a ratio of 1.14 : 1.2 : 1. More than 85% of wheat genes are present in uninterrupted gene-rich clusters, interspersed by gene-poor regions consisting of retrotransposon like repetitive sequences and pseudogenes. Each chromosome arm consists of approximately 6-8 gene-rich regions spanning less than 10% of the chromosome (Barakat et al., 1997; Feuillet and Keller, 1999). Each gene-rich region may be sub-divided into 'mini' gene-rich and gene-poor regions (Sandhu and Gill, 2002) – (Fig. 1.2).

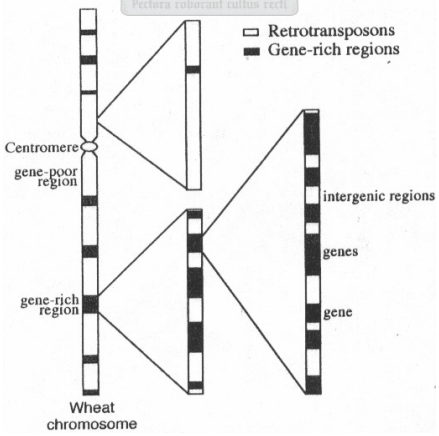


Figure 1.2 Gene distribution in wheat (*Triticum aestivum*) – (Sandhu and Gill, 2002)

Keller and Feuillet (2000) found that the gene density and organization in gene-rich regions are not significantly influenced by the size of the plant genome. The gene-rich regions show some similarity in the physical location, structural organization and gene densities among the three genomes of bread wheat (Keller and Feuilett, 2000;

Sandhu et al., 2001). However, the gene-rich regions vary in the number of genes, gene density and the frequency of recombination (Sandhu and Gill, 2002). The distribution of recombination is highly uneven over the *Triticeae* chromosome and it appears to be limited to distal chromosome regions (Curtis and Lukaszewski, 1991). When comparing physical maps to genetic linkage maps, Gill et al. (1996) found that recombination occurs only in gene-rich regions of the wheat genome. This was confirmed by Sandhu et al. (2001) who found 82% recombination in the 1S0.8 gene-rich region, located on the group 1 short arm. However, the level of recombination varies within the same gene-rich region. A low level of recombination was found in the proximal 20-30% of wheat chromosomes, despite the presence of gene-rich regions due to the presence of the centromere (Gill et al., 1996; Sandhu and Gill, 2002). As a result of the non-random distribution of recombination along the chromosome length, the bp/cM may vary from 118 kb in gene-rich regions to 22 Mb in gene-poor regions (Gill et al., 1996).

Homoeologous chromosome pairing is largely suppressed by the presence of the gene, *Ph1*, located on the long arm of chromosome 5B (Riley and Chapman, 1958). In its absence, not only homologous chromosomes synapse, but also homoeologues, giving rise to very complex meiotic structures. A less effective pairing regulator (*Ph2*) is found on the short arm of chromosome 3D in *Triticum aestivum* (Mello-Sampayo, 1968). Sears (1976) found a number of less effective suppressor genes on various other chromosomes. Feldman (1966) concluded that *Ph1* regulates chromosome pairing in common wheat during premeiotic stages by suppressing premeiotic association which causes the distribution of chromosomes, keeping the homoeologous apart.

Wheat cells also contain chloroplasts and mitochondria. The mitochondria have a circular genome, but the DNA of individual mitochondria may vary in length due to deletions, inversions and large repeats. The chloroplast genome is also circular with a length of approximately 135kb (May and Appels, 1987).

1.1.3 Molecular marker maps in bread wheat

In the past, the complexity of the wheat genome hindered the development and utilization of molecular markers. However, extensive molecular maps have been

developed despite the low variability in wheat and 36 traits have been linked to various molecular markers (Gupta et al., 1999). Wheat is possibly the most difficult of the cereals in which to develop and use molecular markers due to the following:

1) The size of the wheat genome is estimated to be 16 X 10^9 bp (May and Appels, 1987) as compared to the size of the rice genome which is 4 X 10^8 bp.
2) The level of polymorphism is not consistent across the three genomes and the D genome is substantially more difficult to map (Röder et al., 1998).
3) The presence of three related genomes of wheat (A, B and D) adds to the complexity of marker assays and the analysis thereof.
4) Wheat shows a low level of polymorphism due to a narrow genetic base (Chao et al., 1989; Kam-Morgan et al., 1989).

The level of polymorphism in wheat may be increased by crossing it with synthetic hexaploid derived from the hybridization of *T. turgidum* and *T. tauschii*, which are evolutionary related to wheat. The ITMI mapping population (ITMI – International Triticeae Mapping Inisiative), derived from the W7984 X 'Opata' cross, is based on such a mapping population and has been used as an international reference mapping population for wheat (Langridge et al., 2001).

Various molecular maps for all major types of molecular markers have been established in wheat. Detailed RFLP linkage maps have been constructed for all 7 homoeologous groups as summarized by Gupta et al. (1999). In 1996 the International Wheat Microsatellite Consortium (WMC) was formed to concentrate efforts in the search for microsatellite markers for hexaploid wheat. Röder et al. (1998) mapped a total of 279 microsatellites; 93 mapped to the A genome, 115 to the B genome and 71 to the D genome. Fifty-five microsatellites were mapped by Pestsova et al. (2000) and 50 microsatellite loci were mapped by Stephenson et al. (1998). Gupta et al. (2002) mapped 66 new microsatellites (as members of the WMC) while Sourdille (2003) mapped 185 new microsatellite loci. A wheat molecular map based on a total of 325 AFLP and microsatellite markers has been constructed using a doubled haploid population derived from the cross 'Garnet' X 'Sanders' (Penner et al., 1998).

1.2 Mapping of genes and traits in wheat

Mapping may be done physically or genetically. Genetic maps make use of polymorphic markers and the frequency of recombination to determine distances between loci, which are measured in centimorgan (cM). Physical maps are used to show the physical location of a marker on a chromosome. Markers which genetically map near the centromere were however found to be physically located at a considerable distance from it (Werner et al., 1992). This lack of correlation between physical and genetic distances between loci, emphasized the need for more detailed physical maps (Delaney et al., 1995).

Molecular markers have recently become available in animal and plant systems. Such markers are being used extensively in the development of detailed genetic and physical chromosome maps (Gupta et al., 1999). Genetic maps with high genome coverage will facilitate the mapping of genes of interest and provide the framework for understanding the biological basis of complex traits (Chalmers et al., 2001). The mapping of newly acquired genes are important in order to optimize their use in breeding programmes. Genetic maps also define the spatial relationship between loci, the way in which they will segregate and possible allelism. Molecular markers are becoming essential tools for selection in breeding programmes since they offer alternative solutions to many breeding problems resulting from phenotypic traits that are difficult and/or time consuming to select. These traits are usually multigenic or quantitative and their effects are influenced by the environment (Rafalski and Tingey, 1993). The availability of markers closely linked to a trait of interest, simple or quantitative loci (QTL), can now be used in marker assisted selection (MAS) programmes making it possible to select indirectly for a gene without measuring its phenotypic expression, since these markers are not affected by the environment and are present at all stages of plant development. Molecular markers may also be used to study synteny between various grass species (Gupta et al., 1999). In addition, markers and comparative mapping of different species contribute to the understanding of genome organization and function and have allowed the isolation of interesting genes through map based cloning (Hoisington et al., 2002).

1.2.1 Physical mapping

Cytogenetically based physical maps are derived by ordering loci (from a genetic map) using cytological chromosomal landmarks (Werner et al., 1992). The frequency of recombination exponentially increases as the distance from the centromere increases which may result in a 153 fold variation in number of DNA base pairs per cM. A genetic distance of 1 cM may therefore vary from approximately 1,530 kbp in distal chromosome regions to approximately 234,000 kbp for regions close to the centromere (Lukaszewski and Curtis, 1993). When comparing physical and genetic maps of wheat group 6 chromosomes, Gill et al. (1993) found that 1 cM equals 0.44-172 Mb. Physical mapping proved to be more effective in the ordering of proximal loci while genetic analysis is recommended for distally located genes due to the high levels of recombination at the chromosome ends (Werner et al., 1992). The distribution of recombination differs for physically short and physically long arms. The short arms show a higher level of distal recombination and a lower level of proximal and interstitial recombination, which cause the proximal 70 to 75% of short arms to be under represented in genetic maps. The genetic map of a long arm is mostly derived from recombination events in the most distal 20 to 30% of the arm while the interstitial 35 to 40% of their length makes a minor, but identifiable contribution (Lukaszewski and Curtis, 1993). Curtis and Lukaszewski (1991) reported that 88% of recombination occurs in the distal 51.4% of the long arm while the remaining 12% occurs on the proximal half of the long arm.

Markers tagged to chromosomal regions (MTCRs) are used for marker ordering and are resolved through long-range restriction mapping of DNA fragments. Therefore cytogenetically based physical maps are useful for the integration of chromosome and long-range restriction maps (Werner et al., 1992).

The methods used for constructing physical maps can be described as: i) molecular based and ii) cytogenetically based. Molecular methods are useful for fine-structure mapping of small areas of the genome and include the construction of contigs and long-range restriction maps using rare cutting enzymes. Cytogenetically based methods are useful for constructing whole genome physical maps and include *in situ* hybridization, C bands and deletion mapping (Delaney et al., 1995).

1.2.1.1 Cytogenetic mapping: Aneuploidy

Aneuploidy refers to change in the number of chromosomes. Asynapsis, desynapsis and non-disjunction give rise to aneuploids at all levels of ploidy and are the most important causes of aneuploidy in wheat, cotton and maize (Sybenga, 1972). Aneuploids survive more readily in polyploid species. The polyploid nature of wheat allows it to compensate for the loss of a chromosome or part of a chromosome (Law et al., 1987). Sears (1954) developed a wide range of aneuploid lines: monosomics (one chromosome of a homologous pair present, 20'' + 1'), nullisomics (one homologous pair is absent, 20''), trisomics (one additional chromosome of a homologous pair is present, 20'' + 1''') and tetrasomics (an additional homologous pair is present, 20'' + 1''''). Monosomic and nullisomic analysis are mostly used for assigning a gene of interest to a particular chromosome while telocentric chromosomes are used to determine the location of genes on particular chromosome arms, and in some cases to map genes with respect to the centromere (Law et al., 1987). A disadvantage of mapping to chromosome arms using telosomic analysis is that the position of the gene being mapped relative to existing markers is unknown and further molecular mapping will be required in order to determine the exact position.

1.2.1.1.1 Monosomics

Monosomics occur spontaneously at a frequency of approximately 1% in varietal populations and result from n-1 gametes produced by normal individuals as a result of non-disjunction (Riley and Kimber, 1961). Monosomics may also occur through non-disjunction induced by radiation or in meiosis of translocation heterozygotes (Sybenga, 1972). It is therefore possible to isolate a complete set of monosomics through phenotype observation and chromosome counting which prove to be tedious. An alternative method is to use a existing set of monosomics to establish a further set in another cultivar through repeated backcrossing (Law et al., 1987). An existing monosomic F_1 line may be maintained/multiplied through self pollination and the F_2 progeny will consist of approximately 73% monosomics, 24% disomics and 3% nullisomics. The lack of vitality and reduced competitive abilities of pollen lacking a

chromosome reduce the probability of a nullisomic to be formed. Therefore, only 3% nulllisomics are present among monosomic progeny (Sybenga, 1972).

Person (1956) found that the selfed progeny of monosomic plants are not always monosomic for the same chromosome as the monosomic parent. The process is known as 'univalent shift' and occurs when some monosomics undergo partial asynapsis and loss of a different chromosome (Sybenga, 1972). 'Univalent shift' is a constant problem in the development of monosomics through backcrossing and in their maintenance. 'Univalent shift' may be revealed through chromosome morphology or by crossing the appropriate monosomic backcross, as the female parent, with ditelocentric lines in 'Chinese Spring' (CS). Pollen producing progeny containing 19 bivalents, one univalent and one heteromorphic bivalent will be indicative of 'univalent shift' (Law et al., 1987).

Monosomic analysis is done by crossing each of the 21 'CS' monosomic lines with a line homozygous for the gene of interest. Monosomic F_1 progeny are then selected and analyzed. If the gene of interest is recessive (Fig. 1.3), the monosomic hybrids of the critical F_1 line will be hemizygous and will display the recessive phenotype. Monosomic plants of the other 20 non-critical lines will have the dominant phenotype since they are heterozygous (Law et al., 1987). The appearance of recessives in a particular family is a clear indication that the gene is located on the chromosome that is monosomic in that family.

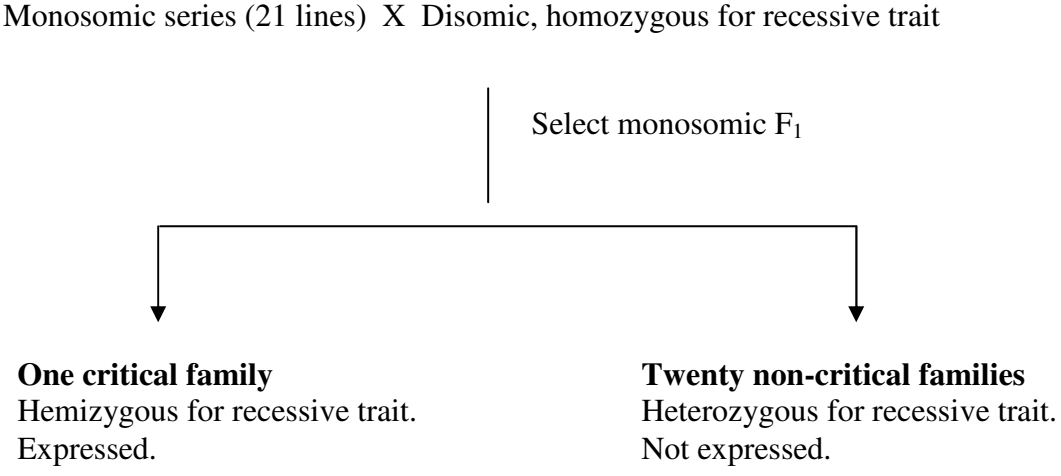


Figure 1.3 Schematic representation of monosomic analysis of a recessive trait.

If the gene of interest is dominant (Fig. 1.4), all the F_1 monosomic progeny will have the dominant phenotype and need to be self pollinated to produce a F_2 that can be analyzed. The 20 non-critical lines will show a 3:1 ratio of segregation. The critical F_2 monosomic line will segregate in a ratio of 97:3 with only the nullisomic (3%) plants expressing the recessive phenotype (Law et al., 1987).

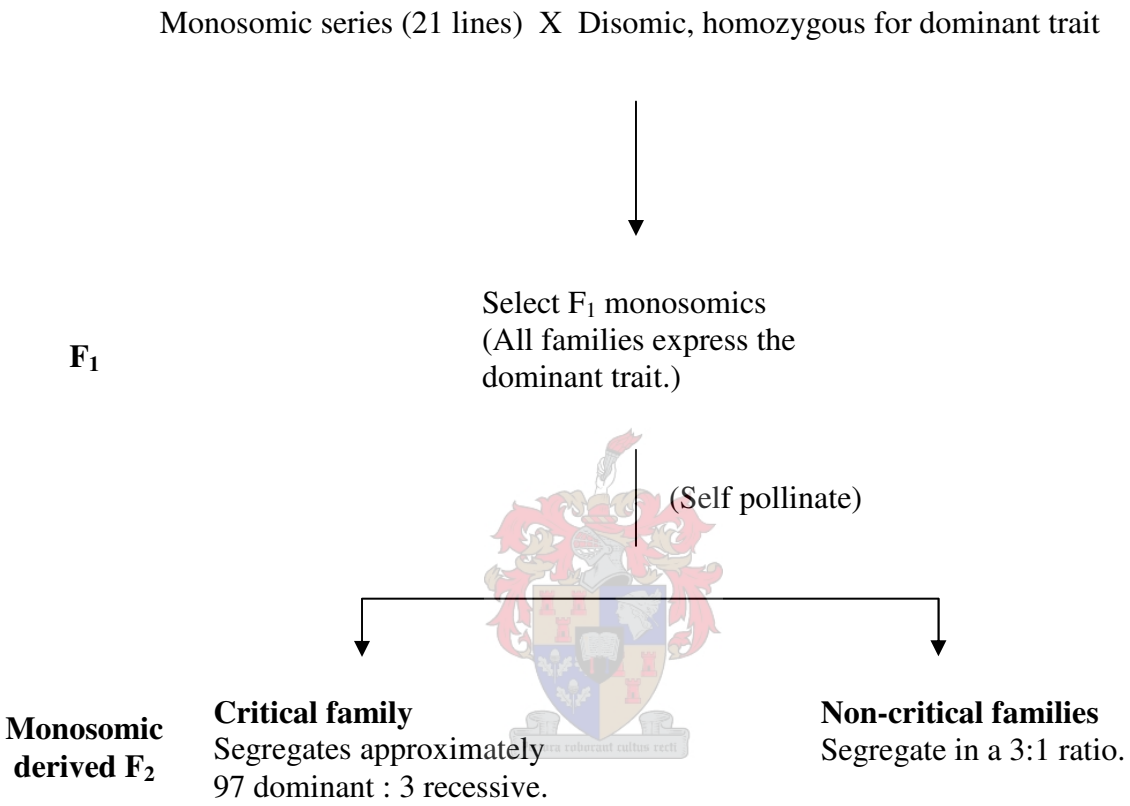


Figure 1.4 Schematic representation of monosomic analysis of a dominant trait.

When two genes are present, giving a 9:7, 15:1 or 9:3:3:1 ratio in the F_2 generation, it is possible to determine their chromosome location through monosomic analysis. However, the analysis is complicated if the two genes are separated by more than 50 map units (Law et al., 1987).

1.2.1.1.2 Telosomics

Telocentric chromosomes are easily obtainable in wheat through the misdivision of monosomes and may be used to assign genes to particular chromosome arms and to determine the gene-centromere distance through the backcross and F_2 methods (Sears,

1962, 1966). The correct chromosome arm location of many genes have been determined, simply by observing the particular phenotype in monotelosomic or ditelosomic lines for a particular chromosome (Sears, 1954).

Telosomic analysis is done by crossing two aneuploid parents with the donor parent, that may be heterozygous or homozygous for the gene of interest. One aneuploid parent is ditelosomic for the long arm and the other ditelosomic for the short arm of the chromosome of interest (Fig. 1.5).

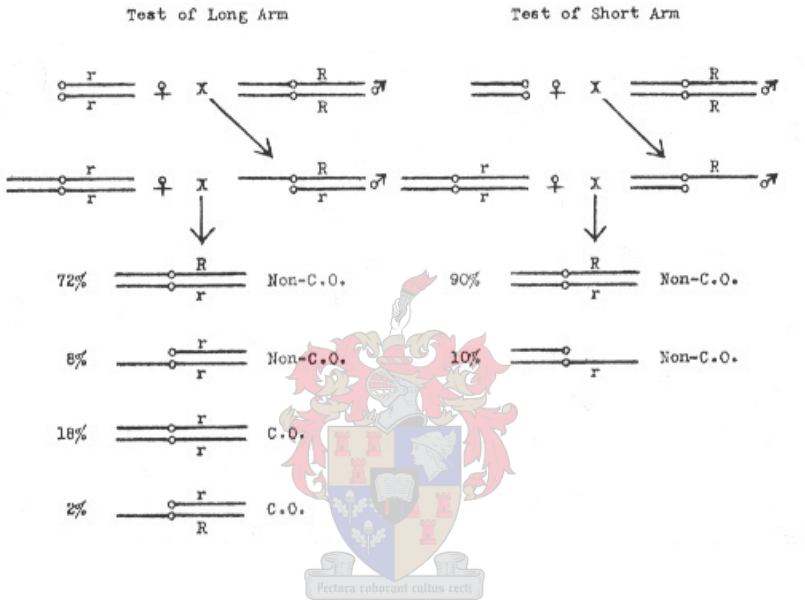


Figure 1.5 Telosomic analysis for locating a gene of interest. The gene is located 20 crossover units from the centromere on the long arm. The occurence of recessives without a telocentric chromosome in the backcross derived F_1 population of the long arm, show that the gene is located on the long arm (Sears and Loegering, 1968).

The resulting hybrid is heterozygous for the gene of interest but hemizygous for the opposite chromosome arm and crossing over is therefore restricted to the arm carrying the gene (Law et al., 1987). Monotelodisomic F_1 hybrids are selected and are testcrossed, as the female parent, with a disomic line, homozygous recessive for the gene of interest (Khush, 1973; Du Toit et al., 1995). However, Sears (1962, 1966) used the monotelodisomic heterozygote as the male parent in testcrosses. Root tip chromosome counts and screening for the trait of interest are done on the testcross derived F_1's to determine linkage between the telosome present and the trait of interest (Du Toit et al., 1995).

Backcross derived F_1 plants are classified as parental or recombinant products. The gene of interest is located on the chromosome arm where recombinant types occur. The frequency of recombination between the gene of interest and the centromere will give a good indication of gene/centromere distance and can be calculated by dividing the number of recombinants by the total number of progeny.

Driscoll (1966) compared the backcross method to the F_2 method in determining gene/centromere distances and found the F_2 method to be equally efficient for genes close to the centromere. Efficiency decreased for genes further away from the centromere. If they segregate independently from the centromere they are further than 50 cM away and distance estimates will be unreliable. The greatest advantage of the F_2 method is its practical usefulness following monosomic analysis. However, it is statistically very complicated and involves a larger standard error.

1.2.2 Genetic mapping

Genetic mapping is based on meiotic recombination between polymorphic markers to determine distances between loci and genetic distances are depicted in cM. A wide range of markers is available to detect DNA sequence variation between individuals and can be divided into three groups (Gupta et al., 1999):

i) Southern hybridization-based DNA markers for example restriction fragment length polymorphisms (RFLPs) and oligonucleotide fingerprinting.

ii) Polymerase chain reaction (PCR) based DNA markers for example randomly amplified polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSRs) or microsatellites and sequence tagged sites (STS).

iii) DNA chip and sequencing based DNA markers for example single nucleotide polymorphisms (SNP)

RFLPs were the first molecular markers developed and were initially used for human genome mapping. RFLPs were later adapted for mapping of the plant genome, including bread wheat but have some limitations due to its laborious and time consuming nature (Gupta et al., 1999). Several new marker types emerged with the

development of PCR technology, which reduced the time, effort and expense required for molecular mapping (Gupta et al., 1999; Hoisington et al., 2002). Other markers that proved to be useful in the detection of polymorphism in wheat are microsatellite primed PCR (MP-PCR), arbitrarily primed PCR (AP-PCR), allele specific PCR (AS-PCR) and DNA amplification fingerprinting (DAF). The relative advantages and disadvantages of some of these markers are summarized in Table 1.1.

Table 1.1 Comparison of the most popular marker systems used in cereals (adapted from Rafalski and Tingey, 1993)

Feature	RFLPs	RAPDs	AFLPs	SSRs	SNPs
DNA required (µg)	10	0.02	0.5-1.0	0.05	9.05
DNA quality	high	high	moderate	moderate	high
PCR-based	no	yes	yes	yes	yes
Number of polymorphic loci analyzed	1.0-3.0	1.5-5.0	20-100	1.0-3.0	1
Ease of use	not easy	easy	easy	easy	easy
Amenable to automation	low	moderate	moderate	high	high
Reproducibility	high	unreliable	high	high	high
Development cost	low	low	moderate	high	high
Cost per analysis	high	low	moderate	low	low

1.2.2.1 Isozymes

The molecular variants of an enzyme are called isozymes (Hart, 1987). Numerous isozyme loci have been mapped in wheat including lipoxygenase, endopeptidase, acid phosphatase, aminopeptidase, alcohol dehydrogenase, α-amylase and β-amylase (Tang and Hart, 1975; Hart and Langston, 1977). Isozyme zymograms can be obtained by electrophoretic separation of crude plant tissue extracts. The enzymes are visualized on the gel by supplying the appropriate substrate and cofactors of which the product is linked to a colour-producing reaction to form a visible band on the gel. Several isozyme structural genes have been allocated to various chromosome homoeologous groups using compensating nullisomic-tetrasomic lines. These genes are then localized to specific chromosome arms using ditelosomic lines. Isozyme zymograms revealed extensive intergenomic variation between homoeologous isozyme structural genes (Hart and Langston, 1977). Isozymes that are closely linked to an agronomically important trait can be used in marker assisted selection since it is

rapid, economical and highly informative as a co-dominant marker. Isozyme loci rarely display epistasis and do not exhibit pleiotropic effects (McMillin et al., 1986). However, the number of isozyme loci mapped are limited and proved to be inadequate to ensure linkage to agronomically important traits. Another disadvantage of isozymes is the variation of expression of certain isozymes in various tissues and at certain stages of plant development (Hart and Langston, 1977).

Since endopeptidase analysis was done in this thesis, some of the isozyme structural genes on the group 7 chromosomes of wheat are discussed. The endopeptidase phenotype of 'CS' consists of three bands of which the intermediate band has been shown to be the product of two isozymes. Hart and Langston (1977) designated the four endopeptidase bands as EP-1, EP-2, EP-3 and EP-4 and the structural genes for these isozymes were assigned to chromosome arms 7DL, 7AL, 7BL and 7BL, respectively, using aneuploids. Hart and Langston (1977) concluded that the three endopeptidase isozymes, EP-1, EP-2 and EP-3 are the products of three homoeologous structural genes and designated them as *Ep-A1*, *Ep-B1* and *Ep-D1*, respectively. The endopeptidase isozyme, EP-4, is the product of the structural gene, *Ep1*. These genes code for isozymes that cleave the peptide band in the synthetic peptide N-α-benzoyl-DL-arginine-β-naphthylamide (BANA) – (Hart and Langston, 1977). Koebner et al. (1988) studied the levels of polymorphism at each of the three loci, *Ep-A1*, *Ep-B1* and *Ep-D1* by screening crude extracts of mature seeds of a range of wheat varieties. They detected three alleles at the *Ep-A1* locus, five at the *Ep-B1* locus and three at the *Ep-D1* locus. While the *Ep-A1* and *Ep-B1* loci are more variable, very limited polymorphism was found at the *Ep-D1* locus. The *Ep-D1a* allele occurs in 'CS' and most common wheats. Worland et al. (1988) found the *Ep-D1b* allele tightly linked to the eyespot resistance gene, *Pch1*, which was translocated to wheat from *Aegilops ventricosa*. A band that corresponds to the *Ep-D1a* product was found to be absent in the wheat 'Synthetic'. Koebner et al. (1988) concluded that the band in 'Synthetic' is the product of the A and (or) B genomes and that the *Ep-D1* (*Ep-D1c*) allele is a null allele in that line. Marais et al. (1998) found that two novel *Ep-1* alleles were expressed in *T. aestivum* accession PI294994 and designated them as *Ep-A1d* and *Ep-D1e*.

1.2.2.2 Microsatellites

Litt and Luty (1989) first demonstrated the highly polymorphic nature of microsatellites in the human cardiac muscle actin gene locus. Microsatellites are tandem repetitive lengths of a core sequence consisting of 1-6 base pairs, flanked by conserved DNA sequences (Weber and May, 1989; Akkaya et al., 1992; Wang et al., 1994). Primers, complementary to the sequences flanking the repeat region, are used in PCR analysis of microsatellites (Lagerkrantz et al., 1993). Polymorphism at a single microsatellite locus is the result of variation in the number of simple sequence repeats (SSRs) and high resolution gels are used to resolve size differences between various alleles (Gupta and Varshney, 2000; Akkaya et al., 1992). Polymorphism at a microsatellite locus is primarily the result of polymerase slippage during DNA replication thereby increasing or decreasing the number of repeats (Schlötterer and Tautz, 1992). Null alleles have been reported at microsatellite loci in the human genome and many plant species. Null alleles refer to the absence of PCR products using locus specific primers due to a mutation within the primer binding site (Gupta and Varshney, 2000) – (Fig. 1.6).

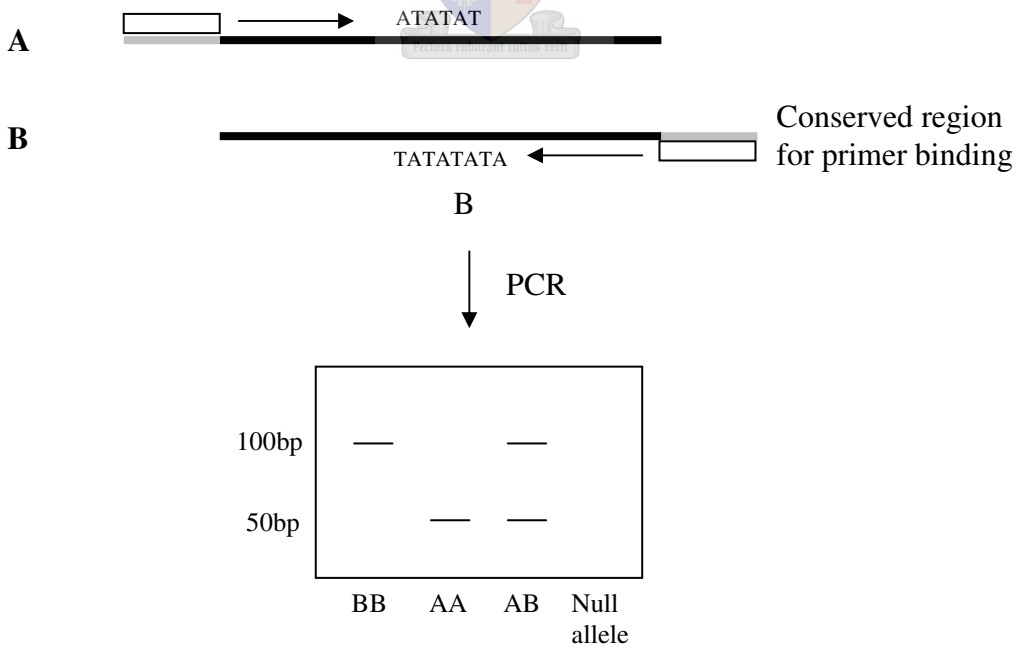


Figure 1.6 Theoretical example of the allelic variation detected following PCR analysis of a microsatellite locus.

Dinucleotides are the most common repeat motif in wheat microsatellites and may involve up to 40 repeats. Röder et al. (1995) found that the dinucleotide repeat, (GA)n/(CT)n occurs every 270kb in wheat. Similarly (AC)n and (GA)n dinucleotide repeats were found every 292kb and 212kb, respectively. The trinucleotide repeats, (TCT)n and (TTG)n, were 10 times less frequent than the two dinucleotide repeats and tetranucleotide repeats were rare (Ma et al., 1996). Wang et al. (1994) and Lagerkrantz et al. (1993) observed that the (AT)n, (AA)n and (AG)n repeats were the most abundant in plants and comprised 75% of all microsatellites with 6 or more repeats. The dinucleotide motif, GT/CA, is the most abundant repeat in the human genome and is found every 30-60kb throughout the genome (Weber and May, 1989).

Microsatellite frequencies have also been studied in organelle genomes. Wang et al. (1994) found that SSRs are highly infrequent in organelle DNA compared to nuclear DNA. The function of microsatellites is unclear, but alternate purine/pyrimidine repeats are able to form the Z-DNA structure that may be involved in genetic recombination, gene regulation or chromosome packing/condensing (Weber and May, 1989; Lagerkrantz et al., 1993).

Microsatellites have emerged as an important source of ubiquitous markers in prokaryotic and eukaryotic genomes and are present in coding and non-coding regions (Wang et al., 1994; Zane et al., 2002). However, microsatellite markers have been developed less rapidly in plants and are five times less abundant than in mammals due to the difference in methylation patterns between plants and animals (Lagerkrantz et al., 1993).

The low level of polymorphic RFLP loci in wheat (Chao et al., 1989; Kam-Morgan et al., 1989; Röder et al., 1998) and the considerable degree of RFLP clustering on genetic maps led to the development of microsatellite markers due to their co-dominant, highly polymorphic, highly informative and locus-specific nature. Furthermore, PCR analysis of microsatellites requires only small amounts of DNA and is easily automated which make them suitable for implementation in a MAS breeding program. Microsatellites are also useful to attain complete genome coverage of the wheat genome since they are evenly distributed along chromosomes with one microsatellite every 50kb which include di- and trinucleotide repeats (Morgante and

Oliviera, 1993). However, the development of microsatellite markers is expensive and time consuming due to the large genome size of wheat. Only 30% of primer sets developed for microsatellite sequences proved to be functional for genetic analysis (Röder et al., 1998). Wheat microsatellites are mainly genome specific and microsatellite primer sets usually amplify only a single locus from one of the three genomes. The absence of homoeologous SSR loci restricts SSRs to intraspecific mapping and makes them inappropriate for comparative analysis and even introgression studies involving wild species related to wheat (Stephenson et al., 1998; Gupta et al., 1999).

1.2.2.2.1 Strategies for microsatellite isolation

Traditionally, digested genomic libraries have been used for the isolation of microsatellites. A large number of clones are screened, using colony hybridization with probes consisting of simple-sequence oligonucleotides or simple sequence polymers (Rassmann et al., 1991). The number of microsatellite containing clones isolated using the traditional method varies between 12% and 0.04% (Zane et al., 2002). The PIMA method (PCR isolation of microsatellite arrays) is a different approach to the traditional method since it uses RAPD primers to amplify the target species genome which is cloned and screened with [^{32}P]-labelled repeat specific primers (Lunt et al., 1999). This technique has an advantage over the traditional method since RAPD fragments are a rich source of microsatellites and other repetitive elements. However, traditional strategies are less useful when dealing with taxa containing a low frequency of microsatellites, such as plants. Alternative strategies have been developed to reduce the time and increase the yield of microsatellites isolated.

The first strategy avoids the construction of libraries or screening of clones for microsatellite sequences. Instead they use a slightly modified RAPD approach involving labelled repeat containing anchored primers (Wu et al., 1994) or PCR amplification, using RAPD primers followed by Southern hybridization with [^{32}P]-labelled microsatellite probes (Richardson et al., 1995). The advantage of these techniques is that no prior sequence information is needed which make them useful for plant studies.

A second strategy involves primer extension for the construction of libraries containing a large number of microsatellite repeat sequences. This theory is based on the production of a circular single stranded, primary library which serves as template for the synthesis of a second strand using $(CA)_n$ or $(TG)_n$ oligunucleotide primers. Highly enriched libraries may contain a 50-fold enrichment in microsatellite repeat sequences (Ostrander et al., 1992).

Another isolation method is based on selective hybridization. After enriching clones with $(CA)_n$ microsatellites, using this approach, Kandpal et al. (1994) found that more than 90% of clones contained CA repeats. They also successfully applied this method to enrich tri- and tetranucleotide repeats. The first step of this method is the recovery of fragmented inserts of a phage library through PCR amplification and ligation to a vector or an adapter. The DNA is hybridized with a repeat containing probe, bound to a nylon membrane (Karagyozov et al., 1993) or biotinylated oligonucleotide bound to a Vectrex-avidin matrix (Kandpal et al., 1994). The enriched DNA is eluted and retained through PCR amplification whereafter it is cloned into an appropriate vector.

The last strategy, called FIASCO (fast isolation by AFLP of sequences containing repeats) is a new method developed by Zane et al. (2002) which relies on the very efficient digestion ligation reaction of the amplified fragment length polymorphism (AFLP) procedure.

1.2.3 Comparative mapping

Comparative mapping provides a basis for genome structure analysis in various species and it enables us to understand the evolution of genomes. Gene synteny is extremely highly conserved among the three genomes of wheat (Hart, 1987; Devos and Gale, 1997) and the colinear organization of genes extends to related and distantly related species. Maize, wheat and rice are very similar in gene order and gene content (Ahn and Tanksley, 1993; Ahn et al., 1993) while the rye genome shows multiple evolutionary translocations relative to the hexaploid wheat genome (Devos et al., 1993). However, while there is conservation of sequences of genes with similar function in many species, a number of disease resistance gene analogous (RGAs) and

resistance genes show a lack of conservation between grass genomes (Keller and Feuillet, 2000). The wheat leaf rust resistance gene, *Lr1*, shows a low level of colinearity when isolated from wheat and rice (Gallego et al., 1998). This may suggest that comparative genome analysis is less useful in the case of rapidly evolving genes.

Rice remains the model plant for grasses and 25 rice linkage blocks were used to establish a consensus grass map which includes the genomes of oats, *Triticeae*, maize, sorghum, sugar cane and foxtail millet (Devos and Gale, 1997). Comparative genetics enables us to isolate genes from larger genomes by using a smaller genome as reference. However, it may not prove to be that simple since rearrangements (inversions, translocations and insertions) at genetic map level decrease the level of microlinearity between different grass species (Keller and Feuillet, 2000). These chromosome re-arrangements may be characteristic of certain taxonomic groups, while others may have developed after species formation.

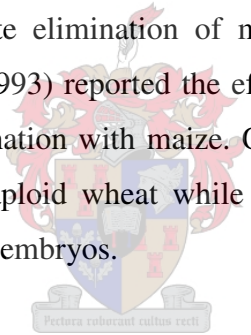
Molecular markers, in particular RFLP markers, play an important role in identifying colinearity through comparative mapping. One hundred and fifty two 'anchor' probes were isolated from cDNA libraries developed from wheat, barley, oats and rice. These probes may be used to examine chromosome structural conservation between different grass species (Van Deynze et al., 1998).

The low level of polymorphism in wheat combined with problems associated with polyploid inheritance, have hindered the development of molecular markers and a complete genetic linkage map (Chao et al., 1989; Kam-Morgan et al., 1989). The D genome of *T. tauschii* (*Aegilops squarrosa*), the diploid progenitor of wheat, shows complete pairing with the D genome of bread wheat (Gill and Raupp, 1987). Genetic analysis and tagging of useful genes may be done in *T. tauschii* due to its genetic diversity of resistance and other agronomically important genes (Lubbers et al., 1991). Furthermore, *T. tauschii* is ideal for RFLP mapping because of its simple diploid inheritance and a high degree of polymorphism (Kam-Morgan et al., 1989). Genetic variation of *T. tauschii* may be transferred to wheat by direct hexaploid X diploid crosses (Gill and Raupp, 1987).

1.2.4 Mapping populations

1.2.4.1 Doubled haploid mapping populations

The production of doubled haploids (DH) has been an important development in wheat breeding since homozygous lines may be obtained in short time (Amrani et al., 1993). Haploid plants have been acquired through anther culture, ovary culture and chromosome elimination in intergeneric crosses (Kisana et al., 1993). The latter include crosses between wheat and *Hordeum bulbosum*, wheat and *Secale cereale* or wheat and maize. Anther culture is restricted by few responsive genotypes, cytological instability and a low haploid recovery rate (Kisana et al., 1993). The wheat and maize cross procedure has the advantage of being stable and genotype independent (Kisana et al., 1993). It may therefore be used to exploit inherently unstable genome combinations and to study the expression of maize genes in wheat plants in the case of incomplete elimination of maize chromosomes (Laurie and Bennett, 1986). Amrani et al. (1993) reported the efficient production of haploids in tetraploid wheat following pollination with maize. On the other hand, anther culture proved to be inefficient in tetraploid wheat while crosses of tetraploid wheat and *Hordeum bulbosum* produced no embryos.



Maize is insensitive to the action of the dominant crossability suppressor genes, *Kr1* and *Kr2*, present in almost all wheat varieties. These genes are located on the long arms of chromosomes 5B and 5A, respectively (Laurie and Bennett, 1987), and suppress fertilization in crosses of wheat with *Hordeum bulbosum* and *Secale cereale* (Falk and Kasha, 1981, 1983). Chromosome substitution studies have shown that the *Kr1* locus results in more dramatic reduction in both rye and *H. bulbosum* crossability than the *Kr2* locus and that these loci have a cumulative effect. The *Kr2* allele does not have a significant influence on *H. bulbosum* crossability compared to a dramatic reduction in crossability with rye (Falk and Kasha, 1981, 1983). The *kr* alleles act as null alleles thereby failing to promote crossability while the *Kr* alleles decrease the level of recombination in rye and *H. bulbosum* (Falk and Kasha, 1983). Crosses with *Hordeum bulbosum* resulted in an average seed set of 0.5% (Sitch and Snape, 1987) compared to crosses with the maize genotype ‘Seneca 60’ which gave fertilization in up to 59% of florets (Laurie, 1989).

In intergeneric crosses with wheat and maize, the maize chromosomes are lost during the first cell division cycles due to poorly defined centromeres that have little affinity for microtubules. This results in embryos containing a haploid complement of wheat chromosomes. The endosperm of such seed is either absent or highly abnormal and need to be rescued to avoid degeneration (Laurie and Bennett, 1986). However, Laurie and Bennett (1988) found that spikelet culture is much more efficient in the recovery of viable embryos. Treating the embryos with colchicine after root formation may produce DH lines (Fig. 1.7).

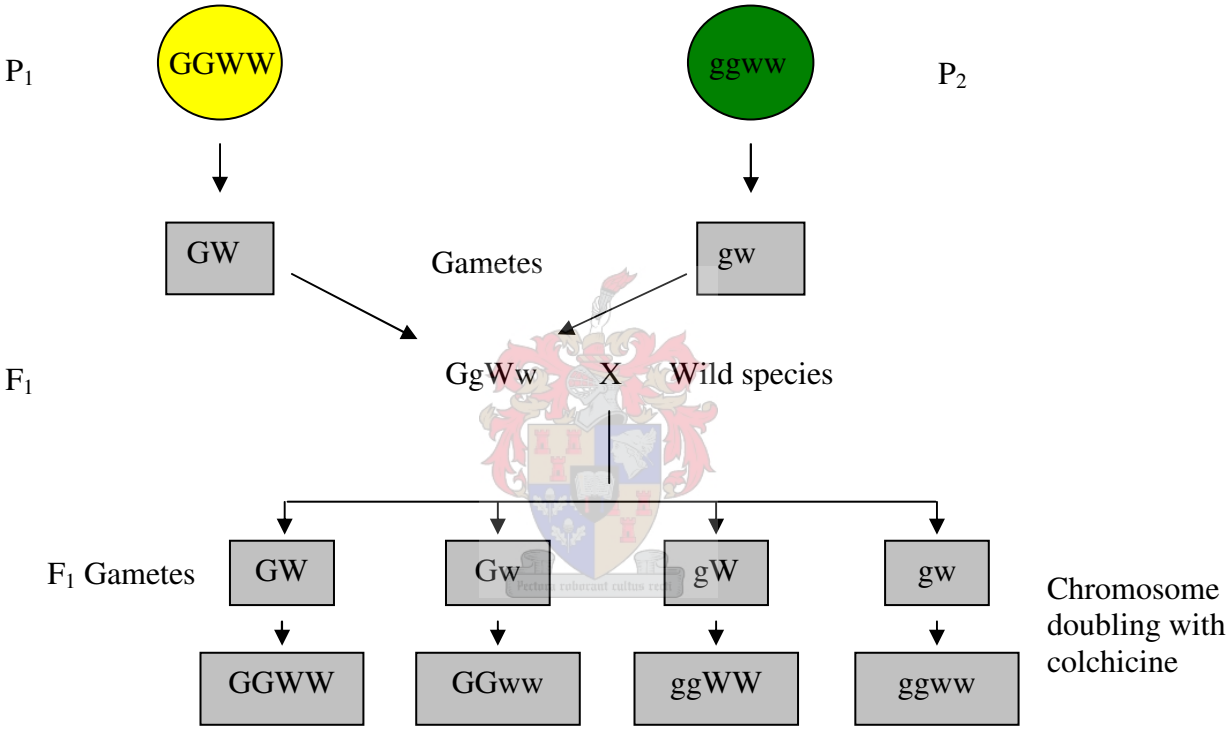


Figure 1.7 A diagramatic representation of the use of wide crosses to obtain double haploid plants.

1.3 The Russian wheat aphid

Aphids rank among the world's major insect pests of crop plants. Aphids form part of a diverse group of arthropods that pierce and suck sap from the leaves, stems and, less frequently, the developing kernels of wheat, thereby affecting the quality of grain. Some inject toxic substances that destroy plant tissue while others are vectors of viruses that may cause widespread losses (Hatchett et al., 1987).

1.3.1 Distribution

The Russian wheat aphid (RWA) is one of the most destructive pests of small grain and have caused substantial losses since the turn of the century. The aphid is indigenous to the Southern Soviet Union (originally collected and named in 1900 from the Caucasus) and countries bordering the Mediterranean Sea, Iran and Afghanistan. It became recognized as a serious pest of wheat in South Africa in 1978 and was identified as the causal agent of a leaf streak virus. It appeared in Mexico in 1980 and by 1986 it had reached Texas in the USA. Since then the RWA has spread north and west and by 1989 it was reported in 17 western states and 3 Canadian provinces. Other countries subsequently affected are the Middle East, Pakistan, China, Ethiopia and Mozambique (Walters et al., 1980; Kindler and Springer, 1989; Robinson, 1992; Elsidaig and Zwer, 1993).

1.3.2 Biology

The RWA belongs to the order *Hemoptera*, the family *Aphididae* and the genus and species *Diuraphis noxia* (Mordvilko) – (Hatchett et al., 1987). It is a small (less than 2mm long) pale green aphid that has an elongated, spindle shaped body that may be covered with a powdery coating of wax. The presence of a supracaudal process and short antennae above the cauda (or tail) and the visual absence of a siphunculi distinguish it from other wheat infesting aphids in South Africa (Walters et al., 1980; Robinson, 1992) – (Fig. 1.8).

Figure 1.8 An adult RWA wingless female feeding on a leaf (Digital Diagnostics - www.ento.okstate.edu/ddd/insects/russianwheataphid.htm).

The RWA is able to feed on a wide range of grasses. Wheat and barley are the most susceptible while rye and triticale are infested to a lesser extent. Oats (*Avena sativa* L.) can be infested as well but little or no damage is observed. RWA survive during the summer months on cool season grasses, such as crested and intermediate wheat grasses (*Agropyron* sp.) and rescue grass (*Bromus wildenowii*) (Walters et al., 1980; Kindler and Springer, 1989).

1.3.3 Life cycle

The RWA is holocyclic in its native country, meaning that both parthenogenetic and sexual reproduction occurs. In the Americas and South Africa, the RWA is anholocyclic, meaning that sexual reproduction is not known to occur. In South Africa, two morphological forms of RWA are found, namely winged (alate) and wingless (apterous) females. Reproduction takes place without mating (parthenogenesis) since males are not found locally. Viviparous winged females are produced under adverse environmental conditions, on depletion of food sources or when host plants are under stress. The winged aphids spread to nearby fields making use of prevailing winds and convection currents. On finding suitable host plants the female immediately starts to feed and gives birth to small nymphs. Nymphs are born live and will mature in about 7 to 14 days to reproducing wingless females. Each female can produce about 3-4 nymphs per day and have a 25-30 day life span. About 20-40 generations may occur per year under favourable conditions. Each female may produce in excess of 70 nymphs and explosive increases in aphid populations may occur due to their high reproduction rates and short maturation times (Walters et al., 1980; Dreyer and Campbell, 1987; Robinson, 1992).

1.3.4 Infestation symptoms

Aphid colonies are found within the tubes of tightly curled leaves and they continually infest the young leaves as soon as they emerge (Walters et al., 1980). Fouché et al. (1984) evaluated RWA damage and found that chloroplasts and cellular membranes were destroyed during feeding due to a phytotoxin that is injected into the leaf tissue causing white, yellow and purple to reddish-purple longitudinal streaks and reduced photosynthetic efficiency (Fig. 1.9). The nature of the toxin is unknown, but may be

similar to other aphid toxins which contain a mixture of cellulases, lipases, pectinases and proteolytic enzymes (Robinson, 1992).

Figure 1.9 Characteristic white/yellow leaf streaks caused by feeding aphids (Prescott et al., 1986).

Aphids probe intercellular with a group of tongue- and groove-connected stylets to feed on plant sap, largely sucrose that act as feeding stimulant for the aphid, from the phloem.

Plant cells are held together by a layer of middle lamella, which is mainly composed of pectin, binding plant cells together. Plant cell walls are also made of pectin but are interlaced with two polysaccharides, hemicellulose and cellulose. Aphids have pectinase in their saliva which is injected into intercellular spaces during probing causing the digestion and depolymerization of the middle lamellar pectin. The pectinase may also cause cell wall destruction causing the death of cells which accounts for chlorosis observed at the site of probing aphids. Aphid saliva may also initiate a second biochemical process which regulates the flow of nutrients in the phloem. Aphid saliva contains 1,3- glucosidase that, if injected, could cause depolymerization of the callose lining of the phloem pores resulting in pore enlargement and increased phloem flow of sugars and amino acids used by aphids (Dreyer and Campbell, 1987).

At low levels of infestation, the RWA is capable of disrupting osmoregulatory processes (Burd and Burton, 1992) and interferes with cold hardening which increases the possibility that the plant may be killed by severe cold (Thomas and Butts, 1990).

Aphid feeding prevents young leaves from unrolling and the heads are often deformed due to awns that are trapped by tightly curled flag leaves. Plants that are heavily infested are stunted and often have a flattened appearance, with the young tillers lying almost parallel to the ground exhibiting typical drought stress symptoms when soil moisture is not limited (Walters et al., 1980; Robinson, 1992). The RWA is also responsible for the transmission of plant viruses and may act as a vector for barley yellow dwarf virus, brome mosaic virus and barley stripe mosaic virus (Rybicki and Von Wechmar, 1984).

1.3.5 RWA management

In South Africa, winter wheat yield losses ranged from 35-60% when 40% of the crop was protected with insecticides (Robinson, 1992). Wheat yield losses of up to 90% for individual plants have been recorded under field conditions (Du Toit and Walters, 1984). Adverse weather conditions play an important role in the survival of the RWA. The high temperatures and rainfall of the Highveld in January may lead to increased mortality and reduction in aphid numbers while low winter temperatures will restrict the increase of aphid populations (Walters et al., 1980; Dreyer and Campbell, 1987). Means to limit the damage done by the RWA include cultural practices, biological control, chemical control and breeding for host plant resistance.

1.3.5.1 Insecticide management

Aphids usually feed deep within rolled leaves which complicates the penetration of contact insecticides. However, chloropyrifos has been effective due to its ability to vapourize, the vapour being able to penetrate rolled leaves. Systemic insecticides, such as disulfoton and dimethoate, may be used with success (90-100%) but proved to be a costly practice (Robinson, 1992; Hill et al., 1993). The direct costs of insecticides, environmental contamination and potential damage to beneficial insects such as pollinators and insect predators involved in biological control, may prove to be a disadvantage (Dreyer and Campbell, 1987). It should also be taken into account that the RWA may develop resistance against insecticides used on a regular basis (Robinson, 1992).

1.3.5.2 Cultural management

The choice of planting date and the control of alternate host plants are two means of cultural management. It is suggested that in the Free State planting of cereals should be done after May, and then only winter and intermediate types, since it may restrict infestation of the young plants. The elimination of oversummering host plants, for example rescue grass (*Bromus wildenowii*), *Agroticum*, a winter pasture grass, barley and triticale may reduce early crop infestation (Walters et al., 1980). Grazing wheat is another popular practice in the USA that may reduce RWA densities by up to 66% through ingestion, trampling and competition. Dense, wealthy, well-fertilized crop stands growing under favourable soil moisture conditions are more resistant to RWA damage. Laboratory research has shown that grain yield loss in nitrogen deficient plants due to RWA can be reduced by increasing the levels of nitrogen (Riedell, 1990). However, the interaction between nitrogen fertilizer application and aphid infestation proved to be not significant (Riedell and Kieckhefer, 1993).

1.3.5.3 Biological management

Parasitoid wasps and aphidophagous coccinellid beetles play an important role in reducing the numbers of the RWA. The population growth of predators and parasitoids tend to lag behind that of the aphid population and control is seldom totally effective. This lag in predator population may be due to the lack of life cycle synchronization and the activities of natural predator and parasitoid enemies (Dreyer and Campbell, 1987; Robinson, 1992). The RWA live and feed in tightly rolled leaves which limits accessibility to predators which are to large to feed within the leaves (Robinson, 1992).

The use of the aphid lethal paralysis virus (ALPV) was studied by Von Wechmar et al. (1990) as a possible RWA control strategy. Various species of aphids were allowed to feed on leaves coated with purified, freshly prepared ALPV. The aphids died soon after feeding and incomplete nymph development was observed. Treating the crop with solutions containing the virus may control RWA infestation but has practical limitations.

Fungi are the only microbiological pathogen that has a significant influence on Homoptera and Hemiptera. Feng et al. (1990) reported significant RWA mortality using the fungal pathogen *Verticillium lecanii* while Vandenberg et al. (1995) found RWA to be extremely susceptible to *Beauveria bassiana* and *Paecilomyces fumosoroseus* (Fig. 1.10).

Figure 1.10 A RWA that died to a fungus infection (www.ppru.cornell.edu/insect_pathology).

1.3.5.4 Breeding for RWA resistance

Plant aphids have caused damage to crops for years and the most effective control strategy, that is financially viable and environmentally safe, is the breeding of resistant cultivars. At the time of outbreak of the RWA in Western countries, wheat cultivars outside central Asia had no resistance to the RWA. An attempt was therefore made to identify resistance genes in wheat germplasm from the aphid's countries of origin (Souza et al., 1991). High levels of antibiosis and antixenosis resistance were found in two wheat introductions from Iran and USSR, respectively (Du Toit, 1987), and subsequently also in a line originating from Bulgaria (Du Toit, 1988). RWA resistance exists in the wild wheat species *Triticum monococcum*, *T. timopheevi*, *T. dicoccoides* and *Aegilops squarossa* (*T. tauschii*) - (Butts and Pakendorf, 1984; Du Toit and Van Niekerk, 1985). Resistance to RWA has also been reported in rye (Nkongolo et al., 1989) and barley (Kindler and Springer, 1991).

Genetic resistance to the RWA was first reported by Du Toit (1987) in two germplasm lines, PI137739, a hard white spring wheat from Iran, and PI262660, a hard white winter wheat from Bulgaria. Du Toit (1989) found that the resistance of

PI137739 and PI262660 is controlled by single dominant genes, *Dn1* and *Dn2* respectively, which are not linked and inherit independently. However, Schroeder-Teeter et al (1994) concluded that resistance in PI137739 was controlled by one major gene located on chromosome 7D and one minor gene located on chromosome 7B. Saidi and Quick (1996) reported that *Dn1* and *Dn2* were probably allelic at the same locus. A recessive resistance gene, *dn3*, was isolated in *Triticum tauschii* (Nkongolo et al., 1991a). Nkongolo et al. (1991b) found a resistance gene in PI372129 which differs from *Dn1* and *Dn2*. This was confirmed by Saidi and Quick (1996) who designated it *Dn4*. The number of resistance genes in PI294994 (a hard red winter wheat from Bulgaria) is still unclear but it has been reported by Marais and Du Toit (1993) that one dominant gene, *Dn5,* derived from PI294994 and located on chromosome 7DL (Du Toit et al., 1995) controls the resistance in 92RL28, a near isogenic line of 'Palmiet'. Saidi and Quick (1996) found a further gene in PI24378 that is non-allelic to *Dn1*, *Dn2* and *Dn4* and designated it *Dn6*. A resistance gene, *Dn7*, was found to be associated with chromosome arm 1RS of the rye accession 'Turkey 77', and was transferred to the wheat cultivar 'Gamtoos' that has the 'Veery' 1BL.1RS translocation (Marais et al., 1994). *Dn8* and *Dn9* were identified in near isogenic wheat lines derived from PI294994, which is also the source of *Dn5* (Liu et al., 2001). They also found that resistance in the wheat accession PI220127 is conferred by a single dominant gene, *Dnx*, which is different from *Dn1*, *Dn2* and *Dn5*. A summary of the RWA resistance genes and markers they are linked to are given in Table 1.2.

After screening various wheat, triticale and rye lines, the rye 'Imperial' was found to carry resistance. Using wheat-rye addition lines, Nkongolo et al. (1990) concluded that 'Imperial' rye chromosomes 1R, 3R, 4R and 7R enhanced RWA resistance in normally susceptible 'CS'. Thus, RWA resistance in 'Imperial' is controlled by a number of genes on various chromosomes. Quick et al. (1993) reported the introgression of a single dominant gene located on chromosome 4R of *Secale montanum*.

It appears that RWA resistance in wheat is primarily controlled by single, major dominant or recessive genes. Single dominant genes can readily be manipulated in breeding and are easy to incorporate in new selections since they express total resistance (Robinson, 1992). Unfortunately, monogenic resistance is soon neutralized

by the development of new RWA biotypes (Robinson, 1992). Biotypes of aphid species are morphologically indistinguishable but differ in their preference for different host plants. The formation of different biotypes allows the aphids to extend their host range and adapt to adverse environmental conditions (Dreyer and Campbell, 1987). New biotypes may arise through sexual recombination under conditions that favour the development of sexual morphs. Mutations may also give rise to new biotypes by inducing translocations, gene duplications and point mutations, thereby changing genes and chromosome number. The process of selection is influenced by environmental conditions and host availability and will favour those biotypes that are able to occupy an empty or new ecological niche (Puterka and Burton, 1990).

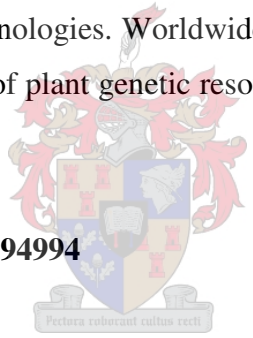
It is important to incorporate multiple resistance mechanisms into a breeding line to produce a cultivar that confers multibiotypic resistance that is durable. However, the RWA is often found in the presence of other aphids that cause additional crop damage. RWA host plant resistance should therefore be combined with resistance to other aphids and the viruses they transmit (Robinson, 1992).

Host plant resistance to insects is determined by allelochemicals, nutritional imbalance and obstructive plant characteristics. These features operate as a unit to make the plant unsuitable for insect infestation. Painter (1951) defined these mechanisms as antibiosis, tolerance and non-preference, which was later changed to antixenosis (Panda and Khush, 1995). Antixenosis describes a plant that is unable to serve as host for the insect due to the presence of morphological or chemical plant factors that influence insect behaviour resulting in alternate host plant selection. Physical barriers such as plant hairs, waxy leaves and stem coatings may influence insects to select alternate host plants. The presence of unique photochemicals in antixenosis may also contribute to repel or deter insects from feeding (Smith, 1989).

Antibiosis affects the biology of an insect by disrupting the normal metabolic processes. Upon feeding on a resistant plant, the insect may suffer a decline in size and weight, reduced metabolic processes and greater larval or pre-adult mortality (Panda and Khush, 1995). The presence of plant allomones or absence of plant kairomones is distinctive to antibiosis while antibiotic resistant cultivars may contain high levels of lignin and silica that reduce insect feeding (Smith, 1989).

Tolerance is the ability of a plant to withstand or recover from damage caused by feeding insects without yield or quality loss. It is the inherent genetic ability of a plant to outgrow insect infestation and to recover after insect feeding. Tolerance is distinct from antibiosis and antixenosis since it is not part of a insect plant interaction but provides an adaptive mechanism for plant survival through the use of plant characteristics (Smith, 1989; Panda and Khush, 1995).

Breeding multibiotypic resistant cultivars are the most practical option for controlling RWA in terms of costs and limiting environmental damage. However, to be effective it should be integrated with other control practices. Due to the scarcity of resistance genes, additional sources of resistance genes are needed. Crop resistance may be acquired through the conservation and use of plant genetic resources, thereby improving crop yield and nutritional qualities. Insect resistance genes from conserved and unadapted germplasm may be introgressed into cultivated genomes using advanced molecular genetic technologies. Worldwide agricultural productivity can be increased with the conservation of plant genetic resources, thereby assuring long term food security.



1.3.6 Gene heterogeneity in PI294994

PI294994, a winter wheat accession from Bulgaria, is the source of the RWA resistance gene, *Dn5*. Elsidaig and Zwer (1993) crossed PI294994 with two susceptible club wheat cultivars 'Moro' and 'Hyak'. Results from F_2 and F_3 families indicated that resistance in PI294994 is conferred by a homozygous recessive allele at one locus and a dominant allele at the second locus. This hypothesis was supported by Dong and Quick (1995) who obtained F_2 segregation data from crosses between PI294994 and six RWA resistance lines.

In a study conducted by Marais and Du Toit (1993) they reported that when PI294994 was crossed with 'CS' the F_2 and backcross data suggested the presence of a single dominant gene, located on chromosome 7D via monosomic analysis. They concluded that the gene found in PI294994 is not allelic to *Dn1* and *Dn2* and designated it *Dn5*. However, they suggested that *Dn5* might be linked to *Dn1* on

chromosome 7D. *Dn5* was later assigned to chromosome arm 7DL via telosomic analysis (Du Toit et al., 1995).

To determine if allelism exists between the various RWA resistance genes, Saidi and Quick (1996) crossed PI294994 with PI137739 (*Dn1*), PI262660 (*Dn2*), PI372129 (*Dn4*) and PI243781 (*Dn6*). The F_2 progeny of these crosses were all resistant, indicating that PI294994 contains at least one gene that is allelic to a gene in other resistant lines carrying *Dn1*, *Dn2*, *Dn4* and *Dn6*. To determine the mode of inheritance in PI294994, it was crossed with the susceptible cultivar 'Carson'. The F_2 data indicated the presence of two dominant genes in PI294994 (Saidi and Quick, 1996). In their search for microsatellite markers linked to RWA resistance genes in wheat, Liu et al. (2001) found at least three resistance genes in PI294994 which they identified as *Dn5*, *Dn8* and *Dn9*.

Thus, researchers differed on the number and types of genes controlling resistance in PI294994. Saidi and Quick (1996) concluded that these conflicting reports may be the result of:

i) heterogeneity in the RWA resistance genes in the original PI294994 accession
ii) variation in parents used for crosses with PI294994
iii) RWA biotypes that differ between researchers

Zhang et al. (1998) studied genetic variation within PI294994 to determine the cause of inconsistent results. They concluded that results from previous studies were not in conflict with each other but were based on genetic variation within PI294994 and suggested that the original PI294994 be regrouped into four sub-accessions based on their resistance to RWA.

1.3.7 Markers linked to RWA resistance genes

A number of RWA resistance genes have been mapped with various types of molecular markers (Table 1.2). Ma et al. (1998) found two RFLP markers, *ABC 156* and *Ksu A1*, to be linked to *Dn4* on chromosome arm 1DS and *Dn2* on chromosome arm 7DL, respectively. Myburg et al. (1998) developed RAPD and SCAR markers linked to *Dn2*, with genetic distances that vary between 3.3 cM and 4.4 cM. In a study conducted by Miller et al. (2001) they found five microsatellite markers, *Xgwm*

44, *Xgwm 111*, *Xgwm 437*, *Xpsp 3113* and *Xpsp 3123* to be closely linked to *Dn2* on chromosome 7D. In a similar study Liu et al. (2002) identified two flanking microsatellite markers, *Xgwm 106* and *Xgwm 337*, linked to *Dn4* on chromosome arm 1DS. They also found that two additional microsatellite markers, *Xgwm 44* and *Xgwm 111*, near the centromere on chromosome 7DS were linked to *Dn6* which proved that *Dn6* is either allelic or tightly linked to *Dn1*, *Dn2* and *Dn5*. Liu et al. (2001) found *Dn1*, *Dn2*, *Dn5* and *Dnx* to be tightly linked to the microsatellite marker *Xgwm 111* near the centromere of chromosome arm 7DS and concluded that *Dn1*, *Dn2* and *Dn5* are either allelic at the same locus or tightly linked to each other. The resistance gene *Dn8* was found to be tightly linked to microsatellite marker *Xgwm 635* near the distal end of chromosome arm 7DS while microsatellite marker *Xgwm 642* was located <3.2 cM from *Dn9* in the centre of chromosome arm 1DL (Liu et al., 2001). In a attempt to determine the location of *Dn5* and *Dn7* on chromosome arms 7DL and 1RS, respectively, Marais et al. (1998) found *Dn5* to be loosely linked to the *Ep-D1b* (32± 5 map units) and *cn-D1* loci (37±6.3 map units). They also mapped *Dn7* at 14.5±3.9 map units from *Lr26* on the 1BL.1RS translocation.

In view of the conflicting results in literature regarding the map position of *Dn5* an attempt was made to confirm the chromosome arm location of the gene using the same source material as used by Du Toit et al. (1995). Secondly, *Dn5* was mapped relative to known chromosome 7D loci using a doubled haploid mapping population derived from the F_1 of PI294994 and 'CS'.

Table 1.2 A summary of information of existing RWA resistance genes.

Resist. gene	Source	First identified by:	Chrom. location	Other
Dn1	PI137739	Du Toit 1987, 1989	7D	Schroeder-Teeter et al., 1994: PI137739 has 2 genes (7B/7D). Saidi and Quick, 1996: *Dn1* is allelic to *Dn2*.
Dn2	PI262660	Du Toit 1987, 1989	7DL	Ma et al., 1998: *Dn2* is linked to *Ksu A1* (7DL). Miller et al., 2001: *Dn2* is linked to *Xgwm 111, Xpsp 3123, Xpsp 3113 Xgwm 437*, *Xgwm 44* and *Xgwm 437*.
dn3	*T. tauschii*	Nkongolo et al., 1991(a)		
Dn4	PI372129	Nkongolo et al., 1991(b) Saidi and Quick, 1996	1DS	Ma et al., 1998: *Dn4* is linked to *ABC 156* (1DS). Liu et al., 2002: *Dn4* is linked to *Xgwm 337* and *Xgwm 106* (1DS).
Dn5	PI294994	Marais and Du Toit, 1993	7DL	Elsidaig and Zwer, 1993 : PI294994 has 1 dominant and 1 recessive gene - confirmed by Dong and Quick, 1995. Saidi and Quick, 1996: *Dn5* is allelic to *Dn1*, *Dn2*, *Dn4* and *Dn6* (F_2 data suggested PI294994 has 2 dominant genes. Liu et al., 2002: Identified three resistance genes, *Dn5*, *Dn8* and *Dn9* (7DS) in PI294994. Genetically mapped *Dn5* to 7DS.
Dn6	PI243781	Saidi and Quick, 1996	7DS	Liu et al., 2002: *Dn6* is linked to *Xgwm 44* and *Xgwm 111*.
Dn7	Rye	Marais et al., 1994	1RS	
Dn8	PI294994	Liu et al., 2001	7DS	Liu et al., 2001: *Dn8* is linked to *Xgwm 635* (7DS distal).
Dn9	PI294994	Liu et al., 2001	1DL	Liu et al., 2001: *Dn9* is linked to *Xgwm 642*.
Dnx	PI220127	Liu et al., 2001	7DS	Liu et al., 2001: *Dnx* is linked to *Xgwm 111*.

CHAPTER 2

MATERIALS AND METHODS

2.1 Confirmation of the chromosome arm location of the Russian wheat aphid resistance gene, *Dn5*

2.1.1 Derivation of tester lines

The line 92RL28 is a near isogenic line, developed in the cultivar 'Palmiet' (Du Toit, 1995 – Personal communication) and was used as the source of *Dn5* in this study. 92RL28 was also the source material used by Du Toit et al. (1995) to map *Dn5* to 7DL. 92RL28 was crossed as the male parent with i) a 'CS' plant that was ditelosomic for chromosome arm 7DS and ii) a segregate from the cross 89M88 that was monotelodisomic for 7DL (Fig. 2.1). Monotelodisomic F_1 plants ($2n = 41 + t^{7D}$) were selected from each cross and were testcrossed with the aphid susceptible 'CS' nullisomic 7D.

2.1.1.1 Derivation of tester lines ditelosomic for 7DS

Monotelosomic TF_1 plants ($2n = 40 + t^{7DS}$) were selected and allowed to self (Fig. 2.1). Ninety TF_2 seeds (ten TF_2 seeds from each of nine TF_1 plants monotelosomic for 7DS) were germinated to identify ditelosomic plants ($2n = 40 + 2t^{7DS}$). Microsatellite analysis was conducted on each selected ditelosomic plant to verify the telosome. Twenty five TF_3 progeny of each ditelosomic family and two controls 'CS' and PI294994 were then tested for resistance to RWA. Endopeptidase analysis was also performed on the ditelosomic TF_3 progeny, since absence of the *Ep-D1a* locus (on 7DL) would confirm the 7DS telosome.

2.1.1.2 Derivation of tester lines monotelosomic for 7DL

Monotelosomic plants ($2n = 40 + t^{7DL}$) were selected from 54 TF_1 seeds germinated (Fig. 2.1). Microsatellite and endopeptidase analyses were done on each

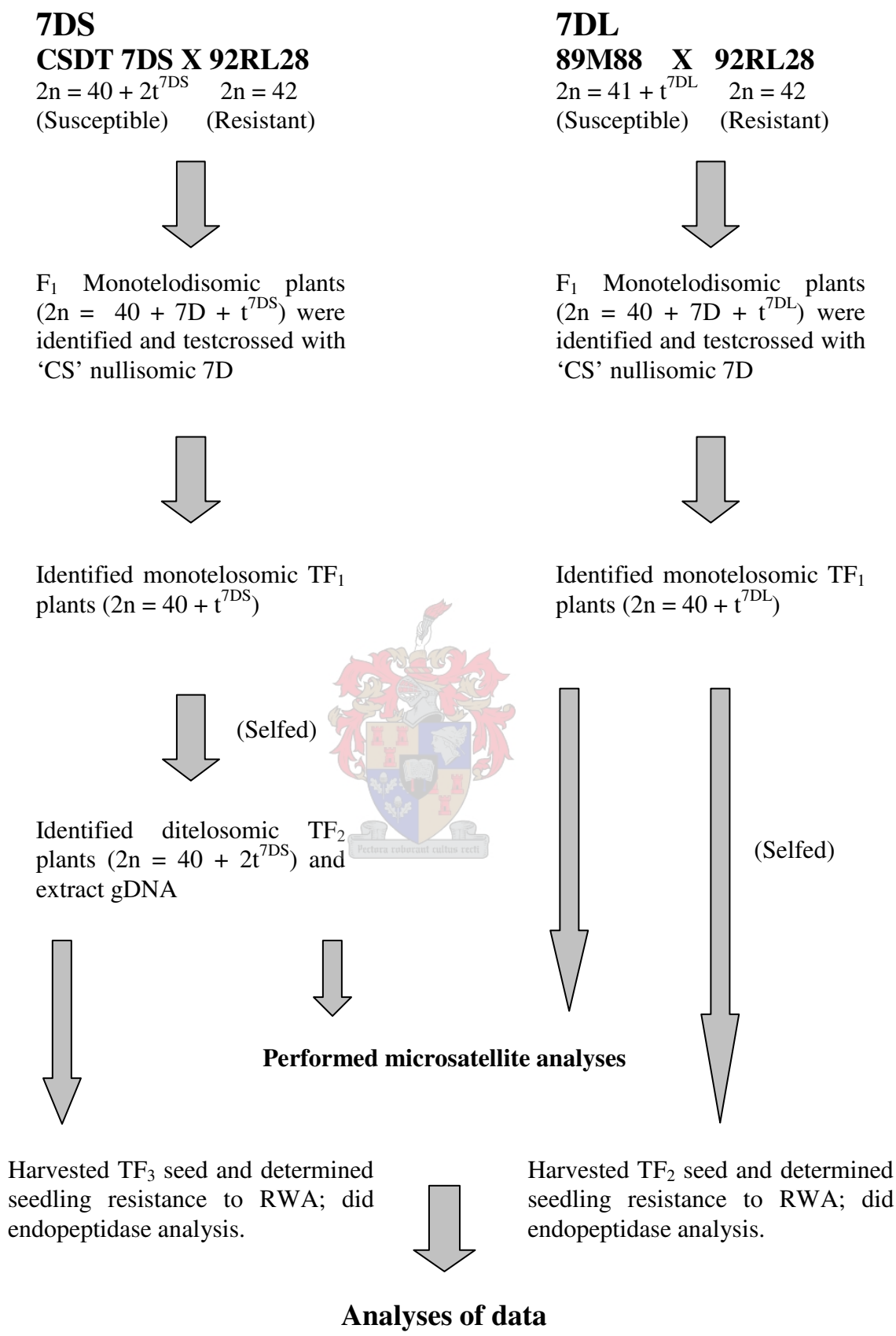


Figure 2.1 Schematic representation of the strategy used to confirm the chromosome arm location of *Dn5*.

monotelosomic plant and the TF_2 progeny of each monotelosomic family, respectively, in order to verify the 7DL telosome. RWA resistance screening was done, using 25 TF_2 progeny of each monotelosomic family and two controls, 'CS' and PI294994.

Du Toit et al. (1995) reported that *Dn5* occurs on 7DL and is not linked to the centromere. If this is correct, some of the testcross progeny with 7DL should have acquired *Dn5* through crossover and will segregate for resistance. Conversely, no 7DS testcross progenies should have been resistant.

2.1.2 Verification of telosomes

Two microsatellite markers, *Xgwm 111* and *Xgwm 44*, that map to chromosome arm 7DS and two, *Xgdm 150* and *Xgwm437*, that map to chromosome arm 7DL (Röder et al., 1998; Pestsova et al., 2000) – (Table 2.2) were used to verify the telosomes. Röder et al. (1998) mapped marker *Xgwm 111* to chromosome arm 7DL but more recently Somers et al. (2004) and Liu et al. (2001) found *Xgwm 111* to be located on chromosome arm 7DS. The markers were applied to five controls, PI294994, 92RL28, 'CS', 'CS' nullisomic 7D and 'CS' ditelosomic 7DS as well as each ditelosomic 7DS TF_2 and monotelosomic 7DL TF_1 plant. Microsatellite amplification and high resolution size separation was done as described in section 2.3.9. The *Ep-D1a* endopeptidase locus was used as marker specific for chromosome arm 7DL and isoelectric focussing was done as described in section 2.3.7.

2.2 Mapping of a *Dn* gene on chromosome arm 7DL

2.2.1 Mapping population

In order to map *Dn5*, use was made of DNA of a doubled haploid population (94 plants) derived from the F_1 of PI294994 / 'CS' by Groenewald (2001) and stored at -80°C. 'CS' is susceptible to the Russian wheat aphid while PI294994 carries the RWA resistance gene, *Dn5*.

2.2.2 Microsatellite analyses

Groenewald (2001) mapped four microsatellite markers, *Xgwm 428*, *Xgwm 437*, *Xgwm 37* and *Xgwm 111* to chromosome 7DL, using a doubled haploid population, and found *Dn5* to be linked to *Xgwm 437* and *Xgwm 111* at 28.6 cM and 25.4 cM, respectively. However, no linkage was found between *Dn5* and *Xgwm 428* or *Xgwm 37*. In order to extend the map, seven additional microsatellite markers, *Xgdm 46*, *Xgdm 67*, *Xwmc 94*, *Barc 172*, *Xwmc 157*, *Barc 26* and *Barc 76* that map to 7DL (Pestsova et al., 2000; Gupta et al., 2002; Ward et al., 2003) were selected to screen the doubled haploid population. Two markers specific for chromosome arm 7DS, *Xgwm 44* and *Xgwm 111*, were used to identify the location of the centromere. The markers were first tested on six controls (PI294994, 92RL28, 'CS', 'CS' nullisomic 7D, 'CS' ditelosomic 7DS and W1378, a line ditelosomic for 7DL and having *Dn5* that was developed earlier (section 2.1.1.2)) to verify their chromosome arm location and to determine whether they are polymorphic in the parents. All individuals in the mapping population were then characterized for the polymorphic markers.

2.3 Methodology

2.3.1 Russian wheat aphid resistance screening

Seedlings were screened for RWA resistance by Dr. F. du Toit (PANNAR, PO Box 17164, 9388 Bainsvlei, South Africa). Aphids cultured in a greenhouse on susceptible wheat seedlings at day/night temperatures of 22/15°C were used to infest plants at the one leaf stage. Twenty one days after infestation each seedling was scored visually for resistance on a empirical scale (Table 2.1) – (Du Toit, 1987).

2.3.2 Germination of seeds for root tip chromosome counts

Seeds were placed on moistened Whatman filter paper in Petri dishes and kept in plastic bags to prevent it from drying out. The Petri dishes were incubated at 21°C for 24 hours whereafter it was transferred to a refrigerator at 2-4°C for 24 hours and returned to an incubator at 21°C to complete germination.

Table 2.1 The rating scale used for RWA seedling resistance screening (Du Toit, 1987).

Rating	Description	Resistance/Susceptibility
1	Small isolated chlorotic spots on leaves	Highly resistant
2	Larger chlorotic spots on leaves	Resistant
3	Chlorotic spots become streaky	Moderately resistant
4	Mild streaks, lengthwise rolling of leaves	Moderately susceptible
5	Prominent white/yellow streaks, leaves tightly rolled	Susceptible
6	Severe white/yellow streaks, leaves tightly rolled and dying from the tips	Highly susceptible

2.3.3 Cutting and fixation of root tips for chromosome counts

Roots from germinated seeds were cut when 1-2 cm in length between 08h00 and 11h00. Two to three roots were cut from each kernel and were placed in cold ddH_2O in numbered 18 mm X 50 mm vials on ice at 4°C in a refrigerator (29 hours). Seedlings were placed in a Sterilin dish with numbered compartments containing moist filter paper and stored at 4°C in a refrigerator until planted. The cold ddH_20 was drained from the vials and replaced with freshly made fixative solution (3 parts methanol : 1 part propionic acid) for approximately one week (at least two days) at room temperature until staining.

2.3.4 Root tip staining

Staining of the root tips was done as follows. The fixative was replaced with distilled water for 30 min. The roots were transferred to vials containing 1N HCL at 60°C for 7½ min whereafter the roots were washed with distilled water for 2 min to stop the hydrolysis of the DNA. The distilled water was replaced with leuco-basic fuchsin (made according to Darlington and La Cour, 1960) and placed in a refrigerator (4°C) for at least 2 hours or overnight. The leuco-basic fuchsin was drained and the roots rinsed twice with distilled water. The roots were removed from the water and rinsed with 7.5 mM sodium acetate buffer (3.16 g sodium acetate and 3.47 ml glacial acetic acid in distilled water, pH adjusted to 4.5) for 3-5 min. The buffer solution was drained and replaced with 1-2 ml filtered 2.5% w/v pecticlear solution (0.5 g pecticlear from Serevac in 20 ml 7.5 mM sodium acetate buffer solution, pH 4.5) at

37°C for 25 min. The pecticlear solution was replaced with water and stored in a refrigerator (4°C) until the roots were mounted on slides.

Root tips were mounted on slides by cutting of the root tip in a drop of Rosner 1% w/v aceto-carmine (1% aceto-carmine : 1 g of carmine is added to 55 ml of boiling water, mixed well and cooled to 50°C. Fourty five ml of glacial acid is added and the solution is slowly boiled in a reflux condensor for 4 hours and filtered when cold). The blunt end of a hardwood peg was used to gently tap the root tip, thereby releasing the cells and a cover slip was placed on top. The slide was placed in folded filter paper and firmly pressed with a rolling action of the thumb to remove excess aceto-carmine and to spread the chromosomes evenly. The chromosomes were counted under an oil-immersion 100X lens of the microscope using a green filter in the filter holder.

2.3.5 gDNA extractions

Plant gDNA extractions were done using approximately five week old seedlings raised in a greenhouse. The protocol was as described by Doyle and Doyle (1990) with slight modifications. Approximately 1 g fresh leaf tissue was ground in 10 ml of CTAB isolation buffer (1.4 M NaCl, 20 mM EDTA (pH 8), 100 mM Tris-Cl (pH 8), 0.2% v/v BME and 2% w/v CTAB) at 60°C using a mortar and pestle followed by incubation in a 60°C waterbath for 1 hour. This mixture was extracted with an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuged for 10 min at 7000 rpm (room temperature). The aqueous upper phase was removed and the nucleic acids precipitated with ⅔ volume of cold isopropanol at 20°C for 1 hour. Samples were then centrifuged for 5 min at 7000 rpm (10-15°C) and the supernatant replaced with 15 ml washing buffer (76% ethanol and 10 mM ammonium acetate) overnight. Samples were centrifuged for 10 min at 10000 rpm (10°C), the supernatant poured off and the pellet allowed to air dry briefly. The pellet was resuspended in 1 ml TE buffer (10 mM Tris-Cl and 1 mM EDTA, pH 8) and left to dissolve. The dissolved DNA was treated with RnaseA (1 mg/µl) for 30 min at 37°C and diluted in 2 volumes distilled water. One ml 7.5 M ammonium acetate (pH 7.6) was then added followed by 2.5 volumes of 100% ethanol. The sample was centrifuged for 10 min at 10000 rpm (10°C) and the supernatant removed. The pellet was air dried briefly and resuspended

in 1 ml dH_2O. The sample was transferred to a 2.2 ml microfuge tube. Five hundred µl phenol and 500 µl chloroform-isoamyl alcohol (24:1) were added followed by centrifugation for 10 min at 12000 rpm (room temperature). The supernatant was transferred to a clean 2.2 ml microfuge tube and 1000 µl chloroform-isoamyl alcohol (24:1) added. The supernatant of each sample was divided into two clean 2.2 ml centrifuge tubes. DNA was precipitated with $^1/_5$ volume of cold 10 mM ammonium acetate and 2 volumes cold 100% ethanol. Samples were gently mixed and left for 1 hour at -20°C followed by centrifugation for 30 min at 12000 rpm (4°C). Each DNA sample was washed twice with 1 ml 70% ethanol and centrifuged at 12000 rpm for 30 min (4°C). The pellets were dried for 30 min in a 37°C oven and resuspended in appropriate volumes of water.

2.3.6 Quantification analyses

DNA concentrations were determined on a 0.8% agarose gel. Gels were run in 1 X TBE (90 mM Tris-Cl, 90 mM boric acid and 2 mM Na_2EDTA, pH 8.3) running buffer stained with ethidium bromide (EtBr). One µl of DNA, 4 µl SABAX water and 10 µl Ficoll Orange G loading dye were loaded together with two lambda DNA concentration standards (0.1 µg/µl and 0.3 µg/µl) and run for one hour at 70 V. The DNA bands were visualized under UV light and the concentrations estimated by comparing band intensity to the lambda concentration standards.

2.3.7 Isoelectric focusing

The *Ep–D1a* locus was used as a marker of the presence of chromosome arm 7DL. Endopeptidase analyses were done according to Koebner et al. (1988) with some modifications. The embryo half of 1-3 mature kernels was incubated in 120 µl dH_2O for at least two hours at room temperature. It was macerated and centrifuged at 12000 rpm for 20 min (2-5°C) after which 30 µl of supernatant was loaded directly on the gel surface at the cathodal end. Gels were prepared consisting of 8% w/v acrylamide : bis-acrylamide (30 : 0.8) solution with 2% w/v ampholyte (14% v/v ampholine solution containing 2 parts Pharmalyte 4.2 – 4.9 and 1 part Pharmalyte 4 – 6.5) and 13% v/v glycerol. The anolyte was 0.5 M acetic acid and the catholyte was 1 M glycine. Electrophoresis was conducted at 4°C using a Hoefer Isobox. After

prefocussing the gel at 13 W for 30 min it was run at the same settings for another 3 hours. Staining was done for 60 min at room temperature according to Tang and Hart (1975). The gel was removed from the glass plate using running water and was spread on a sheet of white paper to dry overnight.

2.3.8 Microsatellite primers

The primers used for microsatellite analysis were synthesized by Inqaba biotec and their sequences and annealing temperatures are listed in Table 2.2.

Table 2.2 Primer details of microsatellites used for telosome verification and mapping of a *Dn* gene.

Microsatellite locus	Forward primer sequence	Reverse primer sequence	Tm (^{0}C)
Xgwm 437 (7DL)	GATCAAGACTTTTGTATCTC TC	GATGTCCAACAGTTAGCTTA	50
Xgdm 150 (7DL)	ACTAGCCTGGCAGTTGATG C	CCGACCGGTTCACTTCC	60
Xgwm 44 (7DS)	GTTGAGCTTTTCAGTTCGG C	ACTGGCATCCACTGAGCTG	60
Xgwm 111 (7DS)	TCTGTAGGCTCTCTCCGAC TG	ACCTGATCAGATCCCACTCG	55
Xgdm 46 (7DL)	TGTGTTGGCCTTGTGGGTG	CTACCCAATGCATCCCCTTA	60
Xgdm 67 (7DL)	AAGCAAGGCACGTAAAGAG C	CTCGAAGCGAACACAAAACA	60
Xwmc 94 (7DL)	TTCTAAAATGTTTGAAACGC TC	GCATTTCGATATGTTGAAGTA A	55
Barc 172 (7DL)	GCGAAATGTGATGGGGTTT ATCTA	GCGATTTGATTTAACTTTAGC AGTGAG	60
Xwmc 157 (7DL)	CTTGATCCAAGTGGTTCTTT CC	TCCAAATGTTTGCGAAACCT GA	50
Barc 26 (7DL)	GCGCTGGGTAAAAAGTGAA ATTC	TGCAAGTGGAGGGGGAGGC GAGAG	53
Barc 76 (7DL)	ATTCGTTGCTGCCACTTGC TG	GCGCGACACGGAGTAAGGA CACC	58

2.3.9 Microsatellite amplification

Microsatellite amplification reactions were performed in 0.2 ml PCR tubes. The 20 µl reaction volume consisted of 70 – 100 ng of gDNA, 0.5 µM of each primer, 200 µM of each dNTP, SABAX water as well as 1 unit of Taq polymerase (Bioline), 2 X PCR buffer and 1.5 mM $MgCl_2$. Amplification was done using an Eppendorf Gradient

cycler and the PCR programme consisted of denaturation at 94°C for 4 min, followed by 30 cycles of 94°C for 30 sec, optimal annealing temperature for 30 sec and 65°C for 1½ min followed by a final extension for 3 min at 65°C and a soak temperature of 4°C. A 2% agarose gel (1 X TBE buffer, 1 hour at 70 V) was used to do a preliminary separation of products in order to confirm that the PCR reaction was successful.

Size separation of various samples was done on a 6% w/v acrylamide : bis-acrylamide denaturing gel containing 6 M urea and 1 X TBE. A gel was prepared by adding 800 µl of ammonium persulphate (10% w/v) and 160 µl N,N,N',N'-Tetramethylendiamine (TEMED) to 160 ml 6% stock solution. The gel was casted using 1.0 mm spacers and combs and allowed to set for one hour before it was placed on a Model S 2001 sequencing electrophoresis apparatus (Life-Technologies$_{TM}$). The gel was pre-run for 30 min at 70 W and the wells flushed with 1 X TBE before loading the samples. Equal volumes of AFLP loading dye (98% formamide, 10 mM EDTA, 0.05% bromophenolblue and 0.05% Xylene cyanol FF) were added to each sample prior to loading, denatured at 95°C for 5 min and then immediately quenched on ice. The volume loaded depended on the PCR yield and varied between 10-15 µl. Two µl of the 100 bp ladder or 30-330 AFLP ladder (Gibco®) was also loaded on the gel. The gel was run for 5½ hours at a constant power of 70 W before silver staining.

2.3.10 Silver staining

Following electrophoresis, the glass plates were separated and the plate containing the gel was transferred to fixative (0.5% v/v glacial acetic acid and 10% v/v ethanol) for 20 min. This was followed by two 5 min washing steps with dH_2O at room temperature. Staining was done in 0.1% w/v $AgNO_3$ for 20 min at room temperature and the gel rinsed in dH_2O for 10 sec to remove excess staining solution. The gel was transferred to a container with developing solution (1.5% w/v NaOH and 0.16% v/v formaldehyde added immediately before use) and was developed at 8°C until the bands appeared. This was followed by a final rinse in dH_2O whereafter the gel was sealed in a plastic bag to prevent it from drying out.

2.3.11 Data analysis with Mapmaker® and Joinmap®

Seven microsatellite markers that map to chromosome arm 7DL and are polymorphic in the parents, 'CS' and PI294994, were used to screen the doubled haploid population. Chi-square tests were performed for each microsatellite locus to determine if the segregation ratio conformed to 1:1. Linkage analysis was performed using a combination of Mapmaker®/Exp version 3.0b (Lander et al., 1987) and Joinmap® version 3.0 (Stam, 1993) software. Both mapping programmes are based on the 'greedy algorithm' strategy whereby a linkage map is constructed by adding one marker at a time. The program starts off with two markers whereafter a third marker is added. The best fitting option is selected and another marker is added. The order in which markers are added depends on the amount of marker information.

Using Mapmaker® a data matrix was constructed with 1 representing the presence of the 'CS' allele and 2 representing the presence of the PI294994 allele. This data matrix was imported into Mapmaker®. Initial grouping of markers was performed using the GROUP command at a LOD threshold of 3.0 and a maximum recombination fraction of 0.40. The ORDER command was employed to determine the most probable marker order within each linkage group. After defining the most probable marker order, the TRY command could be used to assign additional markers to the intervals. Final marker order was checked by the RIPPLE command and maps were constructed using the MAP command. When using Joinmap® for map construction, a minimum LOD score of 3.0 and a maximum fraction of recombination of 0.40 were used to form linkage groups. In both cases recombination frequencies were transformed into genetic distances (cM) using the Kosambi mapping function.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Confirmation of the chromosome arm location of the Russian wheat aphid resistance gene, *Dn5*

3.1.1 Root tip chromosome counts

The chromosome numbers of 54 TF_1 seeds, descended from monotelodisomic plants ($2n = 41 + t^{7DL}$) and pollinated with 'CS' nulli 7D were determined in order to identify plants monotelosomic for 7DL ($2n = 40 + t^{7DL}$) – (Fig. 3.1). Twenty three seedlings had $40 + t^{7DL}$ chromosomes whereas 31 had 41 chromosomes.

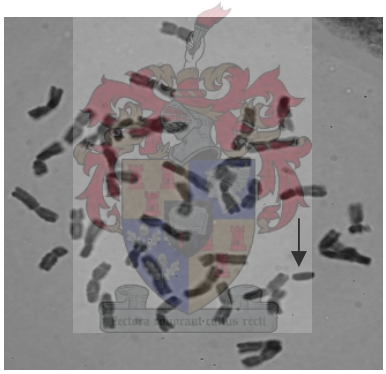


Figure 3.1 A mitotic metaphase chromosome spread of a TF_1 plant monotelosomic for chromosome arm 7DL. The arrow indicates the single 7DL telosome.

Root tip chromosome counts were also performed on 90 TF_2 seeds (Table 3.1) derived from nine TF_1 plants, monotelosomic for 7DS, to identify ditelosomic plants ($2n = 40 + 2t^{7DS}$) – (Fig. 3.2). Four seeds had 39 + t or 41 chromosomes which probably resulted from the presence of structural differences between specific chromosomes of the parental genomes and consequently a degree of non-pairing during meiosis. The chromosome number of several seeds could not be determined due to low mitotic indices. A total of 34 and 37 seedlings with chromosome numbers of 40 and $40 + t^{7DS}$, respectively, were identified while 9 ditelosomic seedlings, one for each of the TF_2 families, were selected and raised in the greenhouse. At least three to five metaphase cells were counted per root tip squash.

Table 3.1 Somatic chromosome numbers of TF_2 (cross: 02M1) segregating for 7DS.

7DS Family	Chromosome numbers						
	40	40 + t	40 + 2t	39 + t	41	Not counted	Total
02 M1 30	4	4	1			1	10
02 M1 32	5	3	1			1	10
02 M1 33	1	8	1				10
02 M1 34	4	4	1	1			10
02 M1 35	3	6	1				10
02 M1 39	4	2	1			3	10
02 M1 41	5	1	1	1	1	1	10
02 M1 42	3	6	1				10
02 M1 44	5	3	1	1			10
Total	34	37	9	3	1	6	90

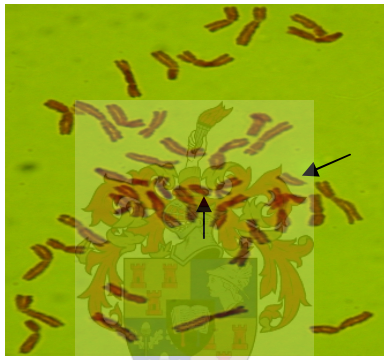


Figure 3.2 A mitotic metaphase chromosome spread of a 7DS ditelosomic TF_2 plant. The arrows indicate the two 7DS telosomes.

3.1.2 RWA seedling resistance screening

Twenty five TF_2 progeny of each 7DL monotelosomic plant ($2n = 40 + t^{7DL}$) were screened for RWA resistance (Table 3.2). One 7DL family produced only a few seeds and could not be tested. Two TF_2 families (03 M 42 – 20 and 03 M 42 – 5) could not be scored unambiguously and these families were also not considered in the interpretation of the data. A total of 17 susceptible and 3 families that segregated for resistance were found. The three families with resistance obviously resulted from recombination between 7DL and 7D of 92RL28. Using the data, a rough estimate of the distance between the centromere and *Dn5* can be derived, i.e. 11 map units. Du Toit et al. (1995) concluded that *Dn5* is not linked to the centromere (distance = 59.3 map units), however, the authors noted very strong segregation distortion in their

population which probably influenced their estimate. If families 5 and 19, did in fact segregate for the presence of *Dn5*, then the distance estimate would have been 19 map units. Thus, keeping in mind that it is a limited data set, the present results suggest linkage with the centromere.

Table 3.2 Results obtained following RWA resistance screening of TF_2 progeny of 7DL monotelosomic plants.

No.	Plant code	Resistant / Susceptible	*Ep-1*	% Resistant seedlings	% Susceptible seedlings
1	03 M 42 - 1	Resistant	Ep-D1a	72.00% (18/25)	28.00% (7/25)
2	03 M 42 - 2	Susceptible	Ep-D1a		100.00% (24/24)
3	03 M 42 - 3	Susceptible	Ep-D1a		100.00% (25/25)
4	03 M 42 - 4	Susceptible	Ep-D1a		100.00% (24/24)
5	03 M 42 - 5	Uncertain	Ep-D1a	8.00% (2/25)	92.00% (23/25)
6	03 M 42 - 6	Resistant	Ep-D1e	68.00% (17/25)	32.00% (8/25)
7	03 M 42 - 7	Susceptible	Ep-D1e		100.00% (25/25)
8	03 M 42 - 9	Susceptible	Ep-D1a		100.00% (25/25)
9	03 M 42 - 10	Susceptible	Ep-D1a		100.00% (24/24)
10	03 M 42 - 11	Susceptible	Ep-D1a		100.00% (24/25)
11	03 M 42 - 12	Susceptible	Ep-D1a		100.00% (25/25)
12	03 M 42 - 13	Susceptible	Ep-D1a		100.00% (25/25)
13	03 M 42 - 14	Susceptible	Ep-D1a		100.00% (24/24)
14	03 M 42 - 15	Susceptible	Ep-D1a		100.00% (25/25)
15	03 M 42 - 16	Susceptible	Ep-D1e		100.00% (25/25)
16	03 M 42 - 17	Susceptible	Ep-D1a		100.00% (25/25)
17	03 M 42 - 18	Susceptible	Ep-D1a		100.00% (25/25)
18	03 M 42 - 19	Susceptible	Ep-D1e		100.00% (25/25)
19	03 M 42 - 20	Poor resistance	Ep-D1e	41,67% (10/24)	58.33% (14/24)
20	03 M 42 - 21	Susceptible	Ep-D1e		100.00% (24/24)
21	03 M 42 - 23	Susceptible	Ep-D1a		100.00% (25/25)
22	03 M 42 - 24	Resistant	Ep-D1a	62.50% (15/24)	37.50% (9/24)

RWA resistance screening was also done on 25 TF_3 progeny of each ditelosomic (7DS) plant. 'CS' (susceptible to RWA) and PI294994 (resistant to RWA) were included as controls (Table 3.3). All the ditelosomic TF_3 progeny were susceptible.

In order to confirm that *Dn5* segregates with chromosome arm 7DL, the chromosome numbers of 50 and 25 seeds, from the resistant F_2 family 03M42-1 were determined and the seedlings screened for RWA resistance by Ms. V. Tolmay (Small Grain Institute, Bethlehem) and Dr. F. Du Toit (PANNAR), respectively. Not all the seedlings containing the 7DL telosome showed resistance. This was probably due to the fact that some of the aneuploid plants had low viability and succumbed to the

aphid even though they carried the resistance gene. However, no seedlings showing resistance were found among those with 40 chromosomes.

Table 3.3 Results obtained after RWA resistance screening of TF_3 progeny of 7DS ditelosomic plants.

No.	Plant code	Resistant / Susceptible	% Resistant seedlings	% Susceptible seedlings
23	02 M1 30	Susceptible		100.00% (25/25)
24	02 M1 32	Susceptible		100.00% (24/24)
25	02 M1 33	Susceptible		100.00% (24/24)
26	02 M1 34	Susceptible		100.00% (24/24)
27	02 M1 35	Susceptible		100.00% (25/25)
28	02 M1 39	Susceptible		100.00% (24/24)
29	02 M1 41	Susceptible		100.00% (25/25)
30	02 M1 42	Susceptible		100.00% (23/23)
31	02 M1 44	Susceptible		100.00% (25/25)
32	PI294994	Resistant	100.00% (16/16)	
33	'CS'	Susceptible		100.00% (15/15)

3.1.3 Microsatellite analyses

Four microsatellite markers, *Xgwm 437* and *Xgdm 150*, specific for chromosome arm 7DL; *Xgwm 44* and *Xgwm 111* specific for chromosome arm 7DS (Röder et al., 1998; Pestsova et al., 2000) were used to verify the telosomes.

Marker *Xgwm 437* amplified a fragment of 112 bp in 'CS' and a fragment of 105 bp in PI294994, while *Xgdm 150* proved to be non-polymorphic in the parents and amplified a fragment of 117 bp in both 'CS' and PI294994. Both markers map to chromosome arm 7DL and was only amplified in 7DL monotelosomic plants (Figs. 3.3, 3.4). Marker *Xgwm 111* amplified a number of loci on chromosome arm 7DS. *Xgwm 111* proved to be polymorphic in the parents and amplified fragments of 215 and 210 bp in PI294994 and 'CS', respectively, and was only amplified in 7DS ditelosomic plants (Fig. 3.5). These results contradict those of Röder et al. (1998) who mapped *Xgwm 111* to chromosome arm 7DL yet agree with those of Liu et al. (2001) and Somers et al. (2004) who placed *Xgwm 111* on 7DS. Marker *Xgwm 44* amplified multiple loci on chromosome arm 7DS and was only amplified in ditelosomic 7DS plants. *Xgwm 44* proved to be polymorphic as it amplified fragments of 180 and 184 bp in PI294994 and 'CS', respectively (Fig. 3.6).

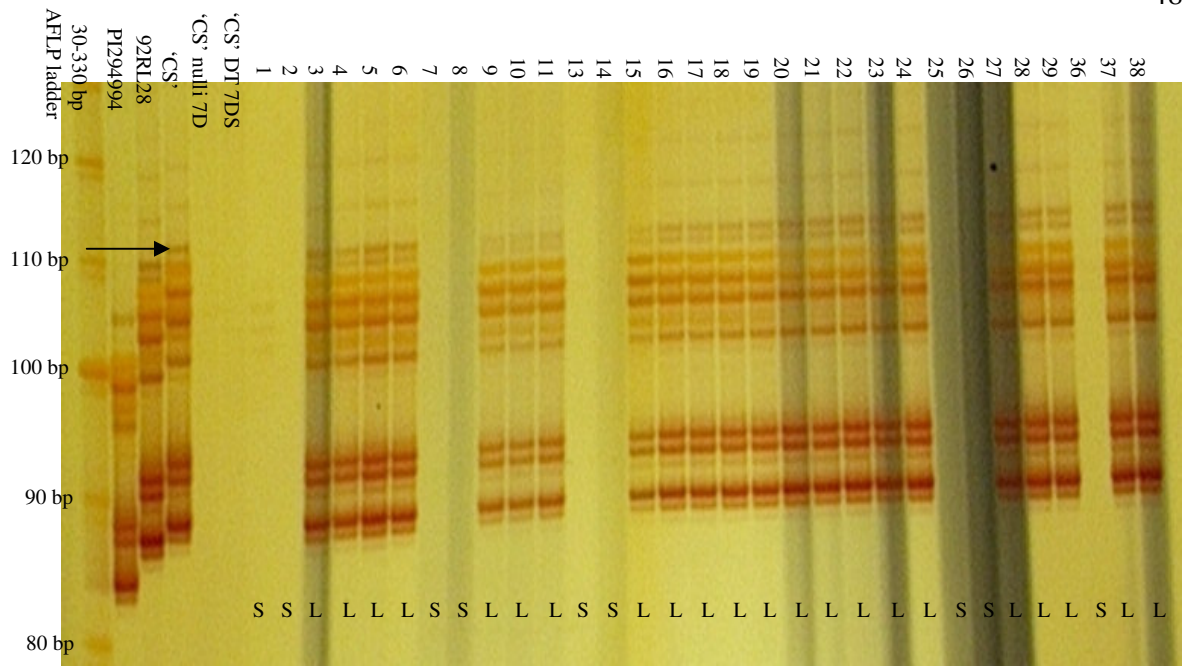


Figure 3.3 Amplification profiles of ditelosomic 7DS and monotelosomic 7DL plants, amplified with microsatellite marker *Xgwm 437* and size separated on a 6% acrylamide : bis-acrylamide denaturing gel visualized by silver staining. The arrow indicates the 112 bp fragment amplified in 'CS' and visible in all plants monotelosomic for chromosome arm 7DL.

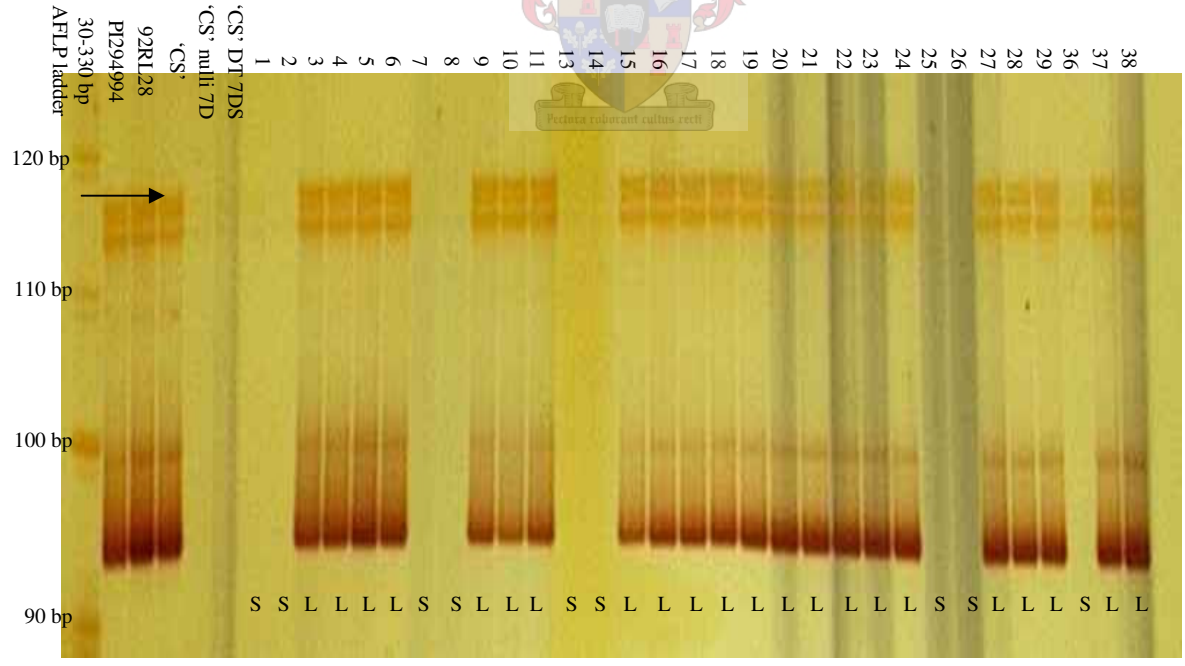


Figure 3.4 Amplification products obtained using DNA of ditelosomic 7DS and 7DL monotelosomic plants in conjunction with microsatellite marker *Xgdm 150*. Products were size separated on a 6% acrylamide : bis-acrylamide denaturing gel and visualized by silver staining. The arrow indicates the 117 bp fragment amplified in 'CS' which proved to be specific for chromosome arm 7DL and therefore is visible in all 7DL monotelosomic plants.

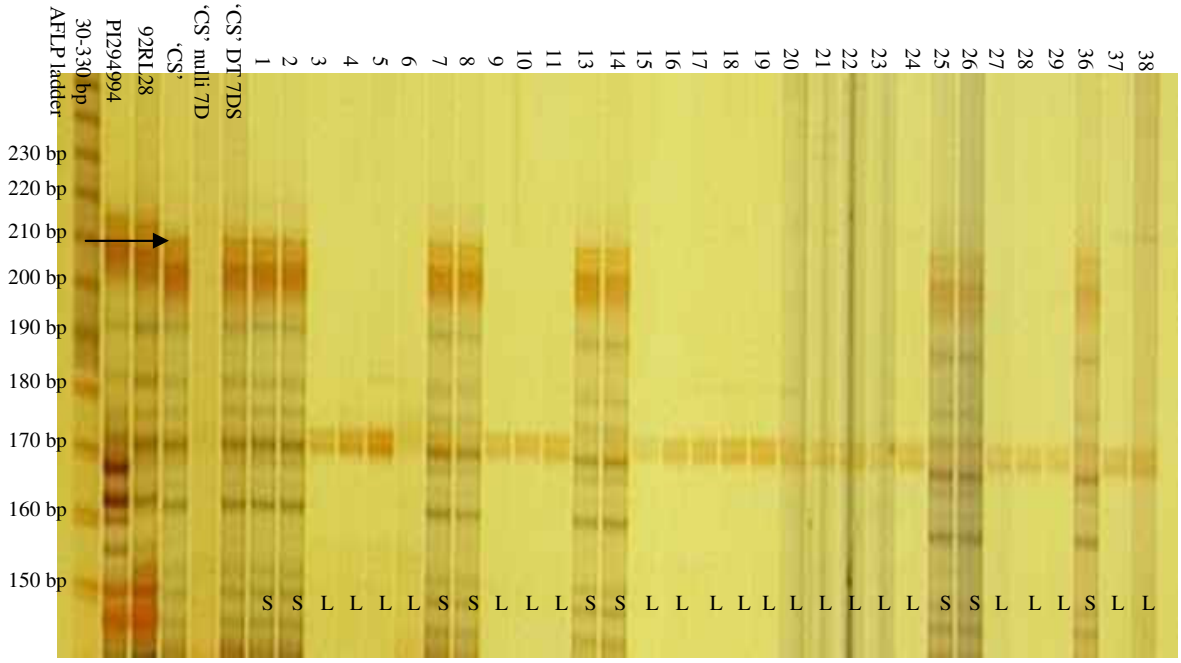


Figure 3.5 Size separation of amplification products obtained using DNA of ditelosomic 7DS and monotelosomic 7DL plants in conjunction with microsatellite marker *Xgwm 111*. Products were separated on a 6% acrylamide : bis-acrylamide gel and visualized by silver staining. Marker *Xgwm 111*, specific for chromosome arm 7DS, amplified a 210 bp fragment (arrow) in 'CS' and plants ditelosomic for chromosome 7DS.

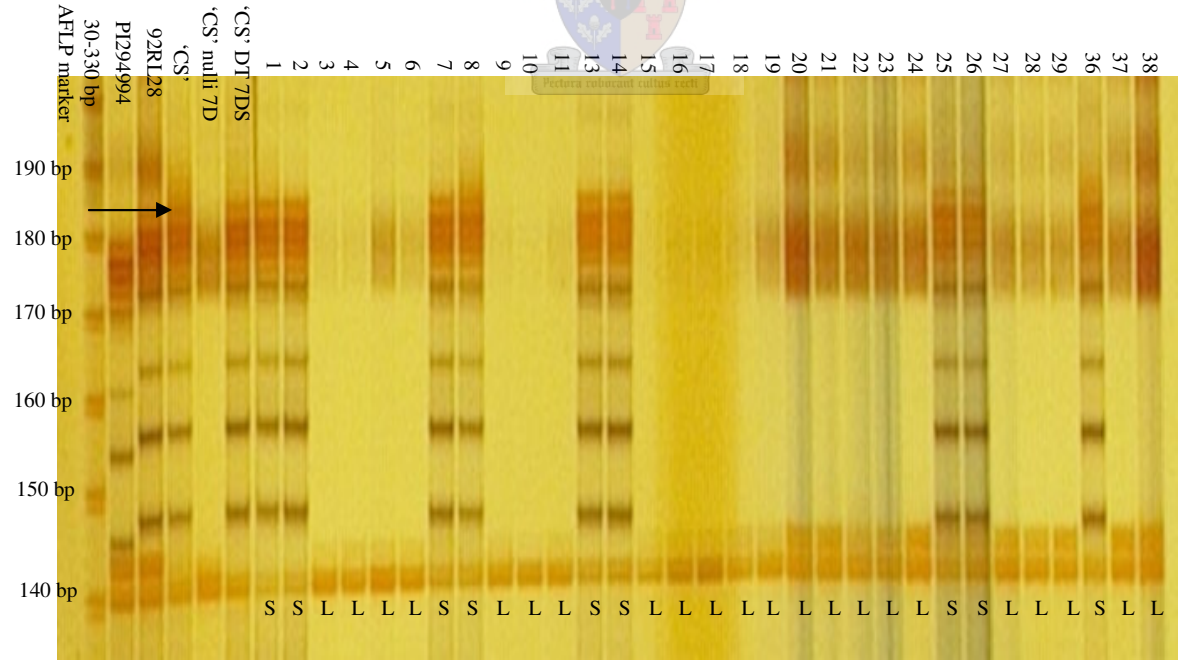


Figure 3.6 Separation of DNA fragments amplified by microsatellite marker *Xgwm 44*, specific for chromosome arm 7DS, in ditelosomic 7DS and monotelosomic 7DL plants. Size separation was done on a 6% acrylamide : bis-acrylamide gel, and bands were visualized by silver staining. A 184 bp fragment was amplified in 'CS' (arrow) and plants ditelosomic for 7DS.

3.1.4 Isoenzyme screening

The *Ep-1* homoeoloci occur on the 7L arms of wheat chromosomes (Hart and Langston, 1977) and the *Ep-D1a* locus was used as marker for the presence of chromosome arm 7DL. The endopeptidase profiles of 'CS' and PI294994 are shown in Fig. 3.7 (reproduced from Marais et al., 1998). The *Ep-D1a* band found in 'CS' and most other common wheat has a distinctly more acidic PI whereas the *Ep-D1e* allele which occurs in PI294994 produces a band with more basic PI.

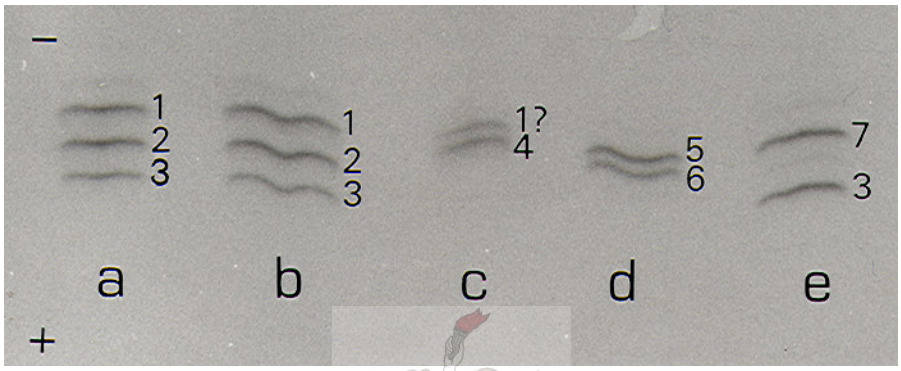


Figure 3.7 The expression of endopeptidases in (a) 'W84/17' (b) 'Inia 66' (c) 'VPM1' (d) PI294994 and (e) 'CS'. The bands are: (1) *Ep-A1b*, (2) *Ep-B1c*, (3) *Ep-D1a*, (4) *Ep-D1b*, (5) *Ep-D1e*, (6) *Ep-A1d* and (7) *Ep-B1a* (Illustration from Marais et al., 1998).

When different plants of 92RL28 were analyzed (Fig. 3.8) it was found that the *Ep-D1a* allele does not occur in all 92RL28 plants and that some of the 92RL28 plants expressed *Ep-D1e* instead.

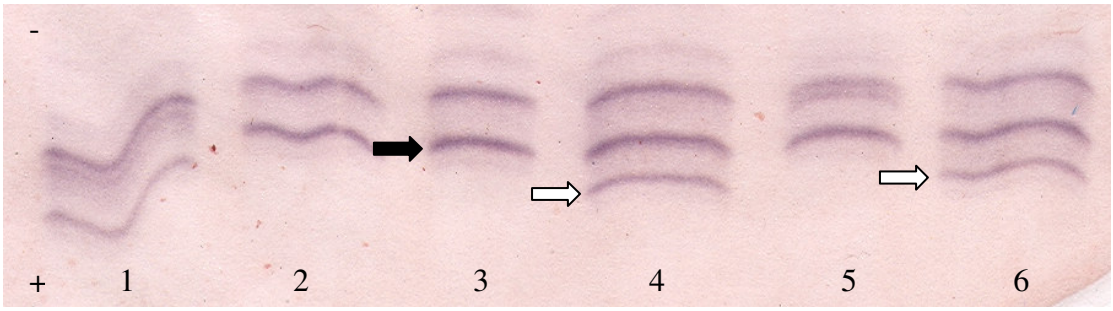


Figure 3.8 Endopeptidase bands produced by (1) 'CS' and (2-6) 92RL28. Plants 4 and 6 express the *Ep-D1a* (⇨) allele which is absent in plants 2, 3 and 5 which express *Ep-D1e*, a band that coincides with an unknown band in 'CS' (➡).

Extracts of 92RL28, PI294994, 'CS' and combinations of 92RL28 and 'CS' and PI294994 and 'CS' were iso-focussed to relate their band profiles to that of TF_2 segregates from plants monotelosomic for 7DL (Fig. 3.9a). An easier to follow

diagrammatic representation of the same bands is given as Fig. 3.9b. As is evident from these figures the *Ep-D1e* allele of PI294994 produces a band with PI very similar to that of an unidentified band that occurs in some of the 92RL28 plants.

When the endopeptidase bands produced by the TF_2 progeny of each 7DL monotelosomic family were determined, it was found that the *Ep-D1a* allele was present in 16 of the TF_2 progeny but absent in the remaining six families which appeared to posses the *Ep-D1e* allele that occurs in PI294994 (Fig. 3.9a). One family was not included in the endopeptidase analysis due to a lack of seed.

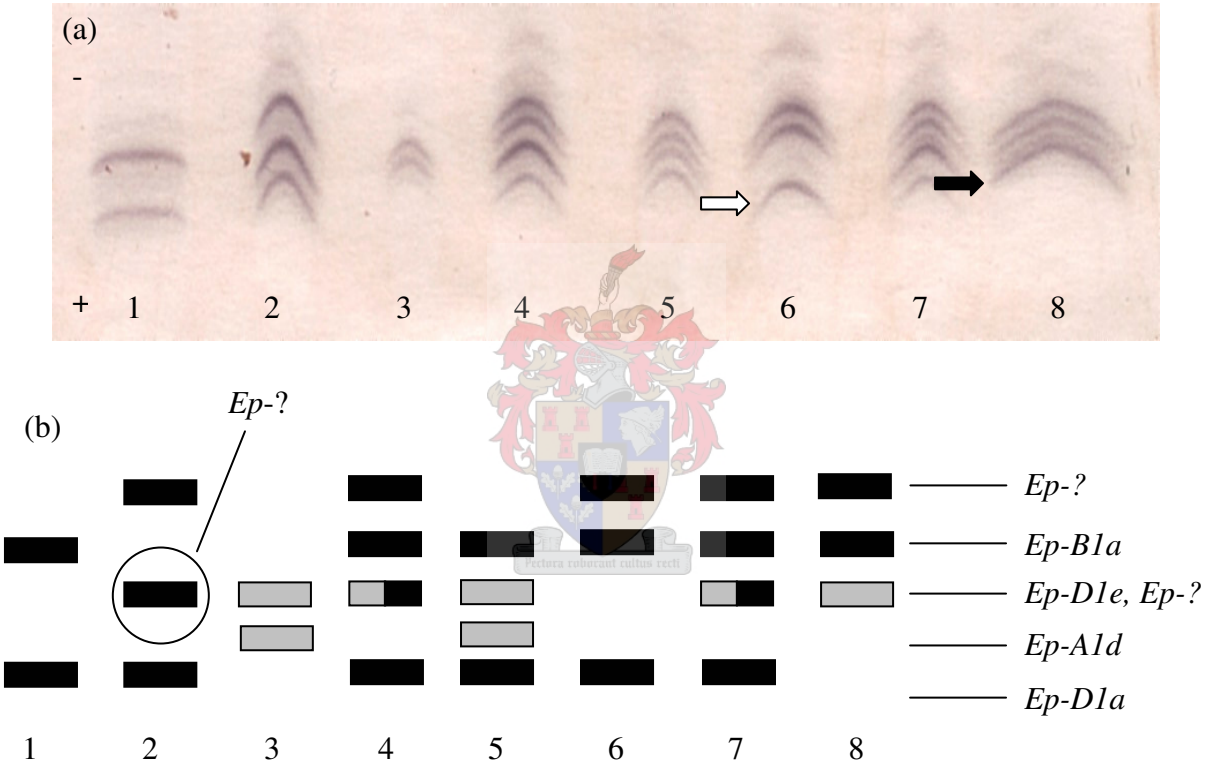


Figure 3.9 (a) Endopeptidase bands present in (1) 'CS', (2) 92RL28, (3) PI294994, (4) 'CS' + 92RL28, (5) 'CS' + PI294994, (6-8) TF_2 segregates from plants monotelosomic for 7DL. The *Ep-D1a* allele (⇨) is present in plants 6 and 7 while plant 8 expresses *Ep-D1e* (➡). (b) A diagrammatic representation of the bands in Fig. 3.9a.

Root tip chromosome counts were done on progeny of the six plants with *Ep-D1e* to confirm the presence of the 7DL telosome. Seeds monotelosomic and ditelosomic for 7DL were identified for each family. The variation in *Ep-D1* alleles among the 7DL TF_2 progeny may be explained by the fact that this locus was not selected for during backcrossing to produce the near isogenic line (92RL28). This resulted in the co-transfer of *Ep-D1e* and *Dn5* during backcrossing in a proportion of

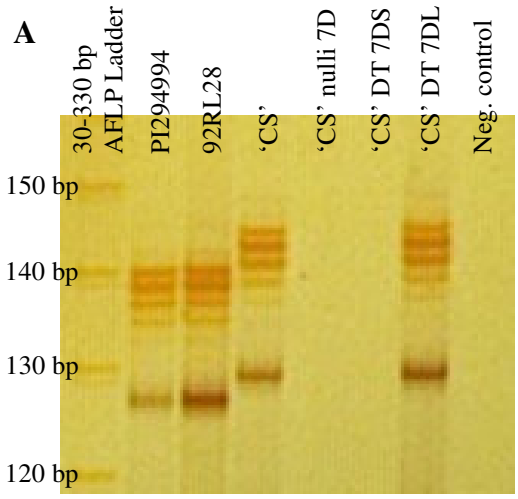
plants. Marais et al. (1998) found *Dn5* to be loosely linked in coupling to *Ep-D1e* with a recombination frequency of 32±5.0%.

Du Toit et al. (1995) mapped *Dn5* to chromosome arm 7DL, whereas Liu et al. (2001) found *Dn5* to be tightly linked to microsatellite *Xgwm 111* on chromosome arm 7DS. The resistant TF_2 families, derived from monotelosomic 7DL plants, confirmed that *Dn5* occurs on 7DL as was proposed by Du Toit et al. (1995). The four microsatellite markers clearly verified the individual telosomes. Microsatellite *Xgwm 111* was confirmed to map to chromosome arm 7DS as was suggested by Liu et al. (2001) and Somers et al. (2004) instead of 7DL as previously reported by Röder et al. (1998). The *Ep-D1a* locus employed as chromosome arm 7DL specific marker was clearly visible in 16 of the 22 TF_2 progeny, derived from monotelosomic 7DL families. The six remaining families apparently possessed *Ep-D1e* although its presence could not always be detected unambiguously.

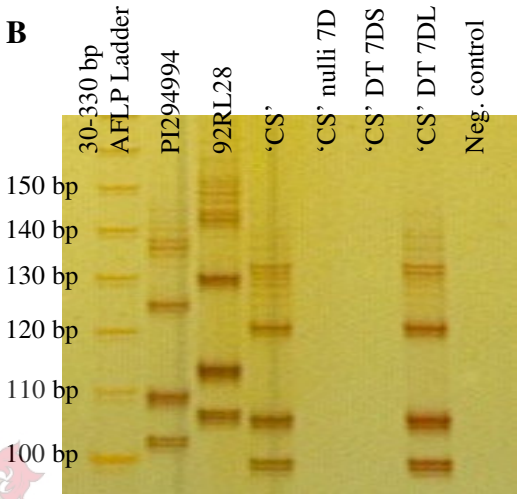
3.2 Mapping of a Russian wheat aphid resistance gene

A total of 14 microsatellite markers were screened against six genotypes (PI294994, 92RL28, 'CS', 'CS' nullisomic 7D, 'CS' ditelosomic 7DS and W1378) to verify their chromosome arm location and to determine if they are polymorphic in the two parents (PI294994 and 'CS'). Of the 14 markers examined, nine (64%) produced clear polymorphic profiles and were mapped using a doubled haploid population (cross: 'CS' / PI294994) segregating for a RWA resistance gene. As PI294994 is a heterogenous source of *Dn*-genes (Elsidaig and Zwer, 1993; Marais and Du Toit, 1993; Dong and Quick, 1995; Saidi and Quick, 1996; Liu et al., 2001) and the *Dn*-gene that segregates in the DH population is unknown, it will be referred to as *Dn?* Two of the markers, *Xgwm 44* and *Xgwm 111*, mapped to chromosome arm 7DS and were used to deduce the location of the centromere. The remaining markers amplified either monomorphic fragments or a smear, which probably resulted from too many fragments amplified and insufficient size separation. Of the 14 markers tested, only one amplified more than one mappable locus, so that the majority of markers used were chromosome specific.

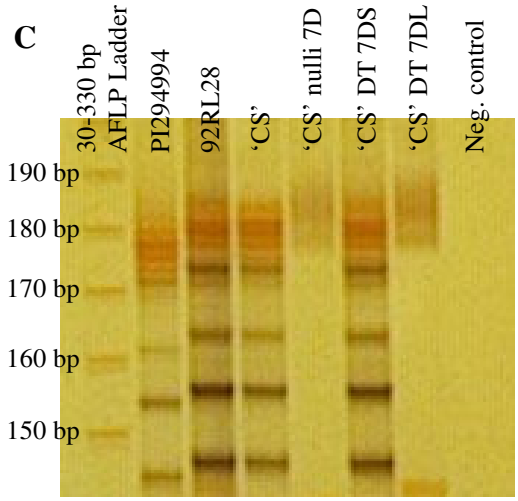
Microsatellites vary in their ease of use and are rated on a scale of 1 (best) to 5 (useful but only with care), based on the degree of stuttering and the number of fragments produced from distinct loci (Stephenson et al., 1998). All the markers used, could be classified according to these criteria and the polymorphic profile of each marker is shown in Fig. 3.10.



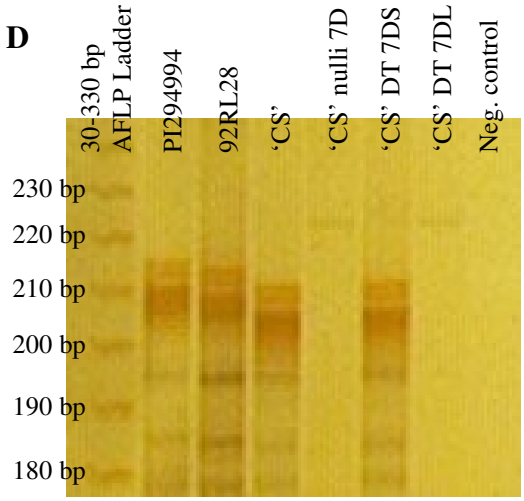
Xgdm 46 (7DL) PI294994 – 140 bp
'CS' – 145 bp
Classification - 3



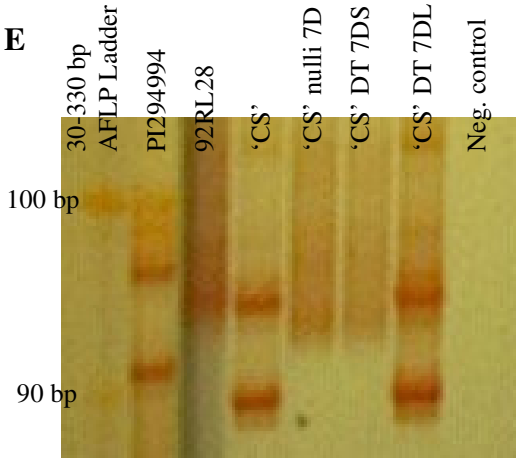
Xgdm 67 (7DL) PI294994 – 138 bp
'CS' – 132 bp
Classification - 3



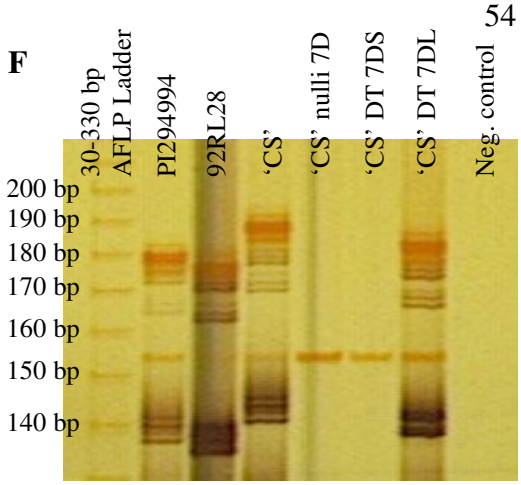
Xgwm 44 (7DS) PI294994 – 180 bp
'CS' – 184 bp
Classification - 3



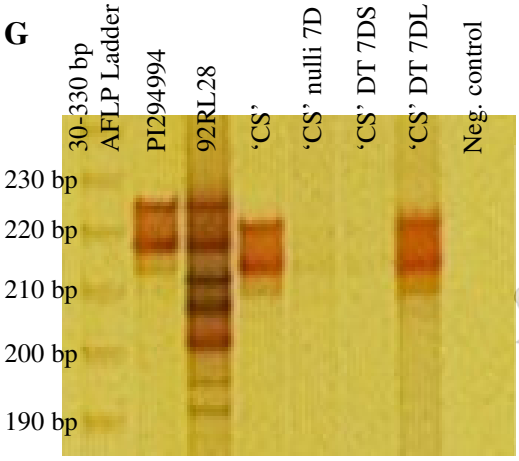
Xgwm 111 (7DS) PI294994 – 215 bp
'CS' – 210 bp
Classification - 4



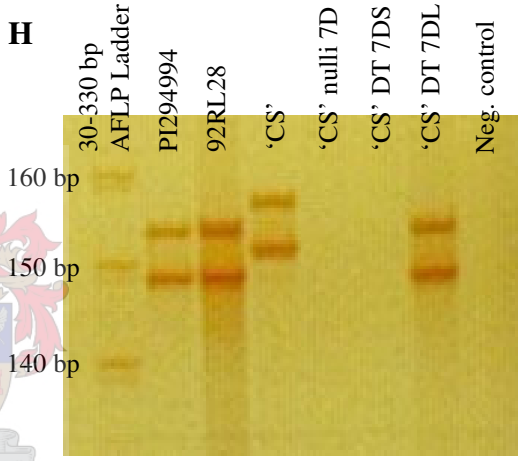
Xwmc 94 (7DL) PI294994 – 97 bp
'CS' – 95 bp
Classification - 1



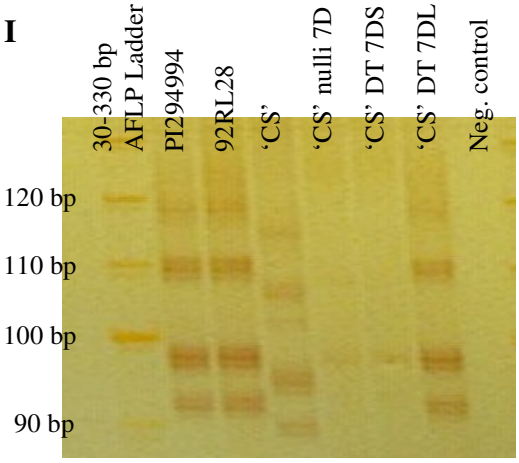
Barc 172 (7DL) PI294994 – 181 bp
'CS' – 190 bp
Classification - 4



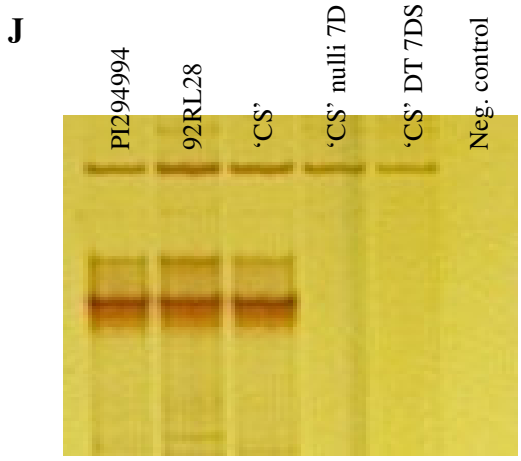
Barc 76 (7DL) PI294994 – 225 bp
'CS' – 220 bp
Classification - 1



Barc 26 (7DL) PI294994 – 154 bp
'CS' – 158 bp
Classification - 1



Xwmc 157 (7DL) PI294994 – 119 bp
'CS' – 115 bp
Classification - 2



An example of a non-polymorphic microsatellite marker specific for 7DL.

Figure 3.10 A summary of profiles of markers used for the mapping of a RWA gene.

A total of 9 markers were used to construct a linkage map with *Dn?* Each marker was tested for conformance to the 1:1 ratio (single gene) of Mendelian segregation. None of the markers deviated significantly from the expected 1:1 ratio ($\alpha = 0.05$), however, the probabilities calculated for markers *Barc 26* (6.3%) and *Dn?* (9.9%) were small and do not provide convincing proof of 1:1 segregation (Table 3.5).

Table 3.5 Chi-square analysis to confirm single gene segregation of markers used in the doubled haploid population.

Marker	Number of progeny having the: 'CS' allele	PI294994 allele	Segregation ratio	Chi-square (χ^2)	P-Value
Xgdm 46	51	43	1:1	0.6805	0.40929
RWA resistance (*Dn?*)	39	55	1:1	2.7234	0.0988
Xgdm 67	49	45	1:1	0.1702	0.6799
Xwmc 94	52	42	1:1	1.0638	0.3023
Barc 172	54	40	1:1	2.0851	0.1487
Barc 26	56	38	1:1	3.4468	0.0633
Xwmc 157	46	48	1:1	0.0425	0.8365
Barc 76	50	44	1:1	0.3829	0.536
Xgwm 111	54	40	1:1	2.0851	0.1487
Xgwm 44	51	43	1:1	0.6808	0.4092

Mapmaker®/EXP 3.0b and Joinmap® 3.0 were used to determine the locus order and distances between markers. A LOD threshold of 3.0 and maximum recombination frequency of 0.40 were used to determine linkage groups. The computer software organized the markers in two linkage groups, since the recombination frequency between *Xwmc 157* and *Xgwm 428* exceeds 40%. All the distances were calculated with a LOD score higher than 3.0 except between *Xwmc 157* and *Xgwm 428* where a LOD score was not determined. Comparative analysis was performed using Joinmap® software. No order irregularities were observed for framework markers optimized with Mapmaker® (Table 3.6).

Table 3.6 Inter marker distances calculated with Joinmap® with a LOD threshold of 3.0 and recombination threshold of 0.40.

Marker	Linkage group	Inter marker distance (cM)	LOD score	Recombination frequency
Dn?	1			
		16.082	8.15	0.2234
Xgwm 44	1			
		10.421	13.46	0.117
Xgwm 111	1			
		1.547	24.1	0.0213
Barc 26	1			
		1.033	25.52	0.0106
Xgwm 437	1			
		6.915	17.02	0.0745
Barc 172	1			
		2.082	24.35	0.0213
Xwmc 94	1			
		1.092	26	0.0106
Xgdm 46	1			
		8.853	16.43	0.0851
Xgdm 67	1			
		30.73	4.69	0.266
Xwmc 157	1			
		\	\	\
Xgwm 428	2			
		28.725	18.53	0.0638
Xgwm 37	2			
		4.255	21.03	0.0426
Barc 76	2			
		6.357	4.28	0.2766
Ep-D1	2			

The marker order (Fig. 3.11) of the first linkage group corresponds with the 7D map given by the 'Komugi' database (www.shigen.nig.ac.jp/wheat/komugi/top/top.jsp) except for markers *Xgwm 428* and *Xwmc 157* that are switched around. When comparing the marker order of the second linkage group to the 'Komugi' map it was found that markers *Xgwm 37* and *Barc 76* are switched around relative to *Ep-D1*. The centromere is located between markers *Xgwm 111* and *Barc 26*. The genetic distances between the markers do not correspond very well to that published by 'Komugi' and these differences may be attributed to the sizes of the mapping populations used or variation in parental genetic background.

According to the marker order calculated by Joinmap® and Mapmaker®, *Dn?* is located on the short arm of chromosome 7D and is loosely linked to microsatellite markers *Xgwm 111* and *Xgwm 44*. Liu et al. (2001) obtained a similar result in a genetic mapping experiment with a PI294994 derived RWA resistance gene which they believed to be *Dn5*. The data obtained with the physical mapping experiment, using the single gene source material employed by Marais and Du Toit (1993) when they named the gene, clearly shows that *Dn5* is situated on 7DL. Thus, *Dn?* is either a gene other than *Dn5* or the linkage map data is inaccurate. Non-Mendelian segregation of *Dn5* was reported by Marais et al. (1998) and Marais and Du Toit (1993) and in the present study the possibility of segregation distortion could not be ruled out. If specific recombination products had reduced viability it may have distorted the genetic map.

In view of the highly contradictory results obtained in the past with the *Dn*-genes on chromosome 7D, it will be necessary to repeat many of the studies while emphasizing the following:

(1) Authenticated single gene sources of the individual *Dn*-genes should be established.

(2) Allelism tests should be redone employing F_2-derived F_3 families rather than F_2 plants for classification, so as to minimize classification mistakes. Chromosome and marker analysis should be done on susceptible segregates in order to rule out aneuploidy as a cause.

(3) Both physical and genetic mapping procedures should be employed when mapping a gene so as to ensure that non-Mendelian inheritance of a chromosomal region will not distort the data.

(4) RWA screening tests should be standardized.

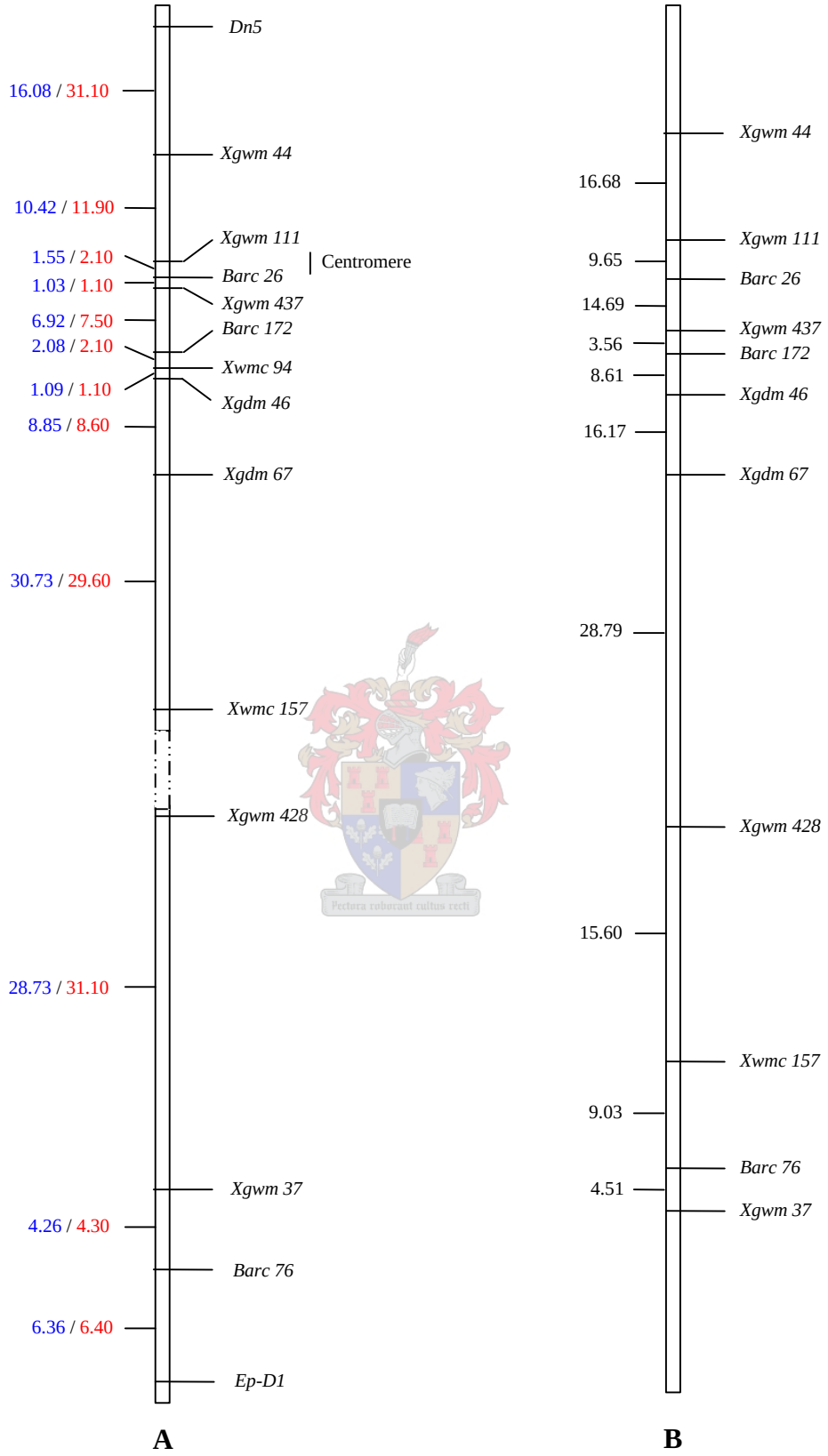


Figure 3.11 (A) Linkage map of 7DL constructed with Joinmap® (blue) and Mapmaker® (red) in comparison with a marker map compiled by 'Komugi' (B).

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ADDENDUM

Microsatellite markers typed on a doubled haploid population segregating for a RWA resistance gene.

Line	*RWA*		*Xgwm 44*		*Xgwm 111*		*Barc 26*		*Barc 172*	
	Dn5	***dn5***	**180**	**184**	**215**	**210**	**154**	**158**	**181**	**190**
PI294994	*Dn5*		180		215		154		181	
'CS'		*dn5*		184		210		158		190
DH 2	*Dn5*			184		210		158		190
DH 3	*Dn5*			184		210		158		190
DH 4	*Dn5*			184		210		158		190
DH 6	*Dn5*			184	215		154		181	
DH 7		*dn5*		184		210		158		190
DH 9	*Dn5*			184		210		158		190
DH 10	*Dn5*		180		215		154		181	
DH 12		*dn5*		184		210		158		190
DH 13	*Dn5*		180		215		154		181	
DH 14	*Dn5*			184		210		158		190
DH 15		*dn5*		184		210		158		190
DH 16	*Dn5*		180		215		154		181	
DH 18	*Dn5*		180		215		154		181	
DH 21	*Dn5*		180		215			158	181	
DH 22		*dn5*		184		210		158		190
DH 23		*dn5*		184		210		158		190
DH 26		*dn5*		184		210		158		190
DH 27	*Dn5*		180		215		154		181	
DH 28	*Dn5*		180		215		154		181	
DH 29		*dn5*		184		210		158		190
DH 31	*Dn5*		180			210		158		190
DH 32	*Dn5*		180		215		154		181	
DH 34		*dn5*		184		210		158	181	
DH 35		*dn5*		184		210		158		190
DH 37		*dn5*		184		210		158		190
DH 39	*Dn5*			184		210		158		190
DH 40		*dn5*		184		210		158		190
DH 43		*dn5*	180		215		154		181	
DH 45		*dn5*		184		210		158	181	
DH 46	*Dn5*		180		215		154		181	
DH 47		*dn5*		184		210		158		190
DH 48	*Dn5*		180		215		154			190
DH 49		*dn5*		184		210		158		190
DH 50	*Dn5*		180		215			158		190
DH 51	*Dn5*		180		215		154		181	
DH 52	*Dn5*			184		210		158		190
DH 53	*Dn5*			184		210		158		190
DH 54	*Dn5*			184		210		158		190
DH 55	*Dn5*		180			210		158		190
DH 56	*Dn5*		180		215		154		181	
DH 57		*dn5*		184		210		158		190
DH 58		*dn5*		184		210		158	181	
DH 59	*Dn5*		180		215		154			190
DH 60	*Dn5*		180		215		154		181	
DH 62	*Dn5*		180		215		154		181	

Continued.

Line	*RWA*		*Xgwm 44*		*Xgwm 111*		*Barc 26*		*Barc 172*	
	Dn5	***dn5***	**180**	**184**	**215**	**210**	**154**	**158**	**181**	**190**
DH 63	*Dn5*		180		215		154		181	
DH 64	*Dn5*			184		210		158		190
DH 65	*Dn5*		180		215		154		181	
DH 66		*dn5*		184		210		158		190
DH 67	*Dn5*			184		210		158		190
DH 68	*Dn5*		180		215		154		181	
DH 70	*Dn5*		180		215		154		181	
DH 73		*dn5*		184		210		158		190
DH 74	*Dn5*			184		210		158		190
DH 76		*dn5*	180			210		158		190
DH 77	*Dn5*		180		215		154		181	
DH 78		*dn5*		184		210		158		190
DH 79		*dn5*		184		210		158	181	
DH 81	*Dn5*			184		210		158		190
DH 82		*dn5*		184		210		158		190
DH 84	*Dn5*			184	215		154		181	
DH 85		*dn5*	180		215		154		181	
DH 86	*Dn5*		180		215		154		181	
DH 87	*Dn5*		180		215		154		181	
DH 88	*Dn5*		180		215		154		181	
DH 89		*dn5*		184		210		158		190
DH 90		*dn5*		184		210		158		190
DH 91	*Dn5*		180		215		154		181	
DH 92		*dn5*	180			210		158		190
DH 93		*dn5*		184		210		158		190
DH 94		*dn5*	180			210		158		190
DH 95		*dn5*		184		210		158		190
DH 96	*Dn5*		180		215		154		181	
DH 97	*Dn5*		180		215		154		181	
DH 99		*dn5*		184		210		158		190
DH 100		*dn5*		184		210		158		190
DH 102	*Dn5*		180		215		154		181	
DH 103	*Dn5*			184	215		154			190
DH 104	*Dn5*		180		215		154		181	
DH 105		*dn5*		184		210		158		190
DH 108		*dn5*		184		210		158		190
DH 109	*Dn5*		180		215		154		181	
DH 110	*Dn5*		180		215		154		181	
DH 111	*Dn5*		180			210		158		190
DH 112		*dn5*	180		215		154		181	
DH 113	*Dn5*			184		210		158		190
DH 114	*Dn5*		180		215		154		181	
DH 116		*dn5*		184		210		158		190
DH 117		*dn5*		184		210		158		190
DH 119		*dn5*	180			210		158		190
DH 120	*Dn5*			184		210		158		190
DH 121		*dn5*		184		210		158		190
DH 125	*Dn5*			184	215		154		181	
DH 129	*Dn5*		180		215		154		181	
	55	39	43	51	40	54	38	56	40	54

Continued.

Line	*Xwmc 94*		*Xgdm 46*		*Xgdm 67*		*Xwmc 157*		*Barc 76*	
	97	**95**	**140**	**145**	**138**	**132**	**119**	**115**	**225**	**220**
PI294994	97		140		138		119		225	
'CS'		95		145		132		115		220
DH 2		95		145		132		115	225	
DH 3		95		145		132		115		220
DH 4		95		145		132	119		225	
DH 6	97		140		138		119			220
DH 7		95		145		132		115		220
DH 9		95		145		132		115		220
DH 10	97		140		138		119		225	
DH 12		95		145		132		115		220
DH 13	97		140		138		119			220
DH 14		95		145	138		119		225	
DH 15		95		145		132		115	225	
DH 16	97		140		138		119			220
DH 18	97		140		138		119			220
DH 21	97		140		138		119		225	
DH 22		95		145		132		115		220
DH 23		95		145		132	119			220
DH 26		95		145		132	119		225	
DH 27	97		140		138			115		220
DH 28	97		140		138			115		220
DH 29		95		145		132	119			220
DH 31		95		145		132		115		220
DH 32	97		140		138			115		220
DH 34	97		140		138			115	225	
DH 35		95		145		132		115		220
DH 37		95		145		132		115		220
DH 39		95		145		132		115	225	
DH 40		95		145		132	119		225	
DH 43	97		140		138		119		225	
DH 45	97		140		138		119			220
DH 46	97		140		138		119		225	
DH 47		95		145		132		115	225	
DH 48		95		145		132		115	225	
DH 49		95		145	138		119		225	
DH 50		95		145		132	119			220
DH 51	97		140		138		119			220
DH 52		95		145	138		119		225	
DH 53		95		145	138		119		225	
DH 54		95		145		132		115	225	
DH 55		95		145		132	119			220
DH 56	97		140		138		119			220
DH 57		95		145		132	119		225	
DH 58	97		140		138		119			220
DH 59		95		145		132		115		220
DH 60	97		140		138			115		220
DH 62	97		140		138		119		225	
DH 63	97		140			132		115	225	
DH 64		95		145		132	119		225	

Continued.

Line	*Xwmc 94*		*Xgdm 46*		*Xgdm 67*		*Xwmc 157*		*Barc 76*	
	97	**95**	**140**	**145**	**138**	**132**	**119**	**115**	**225**	**220**
DH 65	97		140		139		119		225	
DH 66		95		145		132		115		220
DH 67		95		145		132		115		220
DH 68	97		140		138		119		225	
DH 70	97		140		138		119			220
DH 73		95		145		132		115	225	
DH 74		95		145		132	119		225	
DH 76		95		145		132		115	225	
DH 77	97		140			132		115		220
DH 78		95	140		138			115		220
DH 79	97		140		138		119			220
DH 81		95		145		132		115		220
DH 82		95		145		132		115		220
DH 84	97		140		138		119		225	
DH 85	97		140		138			115		220
DH 86	97		140			132		115		220
DH 87	97		140		138		119		225	
DH 88	97		140		138		119		225	
DH 89		95		145		132		115		220
DH 90		95		145		132	119		225	
DH 91	97		140		138		119		225	
DH 92		95		145		132		115	225	
DH 93		95		145		132		115		220
DH 94		95		145		132		115		220
DH 95		95		145		132		115		220
DH 96	97		140		138			115	225	
DH 97	97		140		138		119		225	
DH 99		95		145		132		115	225	
DH 100		95		145		132		115	225	
DH 102	97		140		138			115		220
DH 103		95		145		132	119		225	
DH 104	97		140		138		119		225	
DH 105		95		145		132	119			220
DH 108		95		145		132		115	225	
DH 109	97		140		138			115		220
DH 110	97		140		138		119			220
DH 111	97		140		138		119			220
DH 112	97		140		138			115		220
DH 113		95		145		132		115		220
DH 114	97		140		138		119			220
DH 116		95		145	138		119		225	
DH 117	97		140		138		119		225	
DH 119		95		145		132		115		220
DH 120		95		145		132		115	225	
DH 121		95		145		132	119		225	
DH 125	97		140		138		119			220
DH 129	97		140		138		119			220
	42	52	43	51	45	49	48	46	44	50