NOTE TO USERS

DISSERTATION

MOLECULAR AND GENETIC BASIS OF RESISTANCE TO RUSSIAN WHEAT APHID (*Diuraphis noxia*)

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY VINOD SHARMA ENTITLED MOLECULAR AND GENETIC BASIS OF RESISTANCE TO RUSSIAN WHEAT APHID (*Diuraphis noxia*) BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

MOLECULAR AND GENETIC BASIS OF RESISTANCE TO RUSSIAN WHEAT APHID (*Diuraphis noxia*)

The Russian wheat aphid (*Diuraphis noxia*, RWA) is a pest of wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), and rye (*Secale cereale* L.). It has caused serious economic damage to wheat and barley growers all over the world. RWA infestation results in leaf rolling, streaking of the leaves, leaf and head trapping, stunted growth of the plants and even death of susceptible plants. Under heavy infestation it can cause a yield reduction of 50% or more. The use of resistant cultivars is an efficient, economical and environmentally safe method to protect small grain cereals from losses by RWA. Until spring 2003 there was only one RWA biotype (biotype 1) found in N. America. In spring of 2003 another RWA biotype (biotype 2) was reported from Colorado and other wheat growing states. This biotype was found to be virulent to all known RWA resistance genes except for *Dn7*. *Dn7* is a dominant resistance gene which was introduced into wheat from rye by 1BL.1RS translocation. It provides superior level of resistance against both RWA biotypes.

The objectives of this study were: 1) Saturation mapping of a gene-rich region in rye chromosome arm 1RS, containing a Russian wheat aphid resistance gene *Dn7*, 2) To find out if there is synteny between region of short arm of rye chromosome 1RS containing *Dn7* and rice, and 3) *In-vitro* reaction of wheat to extracts of Russian wheat aphid biotypes 1 & 2.

F_2 derived F_3 families of a cross between wheat line '94M370' (resistant to RWA containing *Dn7*) and wheat cv 'Gamtoos' (susceptible to RWA) were advanced to the F_6 generation by single seed descent. DNA polymorphisms were detected in 98 recombinant inbred lines (RILs) with restriction fragment length polymorphism (RFLPs), expressed sequence tags (ESTs), amplified fragment length polymorphism, simple sequence repeats (SSRs), resistance gene analogs (RGAs) and sequence tagged sites (STS) markers. To find out if there is a synteny between 1RS region containing *Dn7* and rice genome, DNA sequences of the markers linked to *Dn7* were used to do BLAST homology searches with the rice sequence databases. For *in vitro* studies, cut etiolated seedlings of RWA resistant and susceptible cultivars were treated with crude extract, saliva, proteins, and non protein compounds of RWA biotypes 1 and 2.

The present genetic map of 1RS contains twenty six DNA markers covering a genetic distance of 97.5 cM. The markers flanking the *Dn7* are *XHor1* and *Xlb267* which are 2.1 and 3.9 cM from *Dn7*, respectively. Nine PCR-based markers were developed for *Dn7*. One AFLP fragment linked to *Dn7* was successfully converted to high-throughput STS marker. This is the first time PCR-based markers have been developed for *Dn7*. Two PCR-based markers, *Xlb267* and *Xrems1303_1*, flanking the *Dn7* gene had an accuracy of 96% when used for marker assisted selection for RWA resistance. No conserved homology was observed with rice chromosome 5 and 10 which are known to be homoeologous with the group 1 chromosomes of Triticeae when the rice BAC/PACS showing highest homology were selected. But three markers linked to *Dn7* were found to be homologous with rice BAC/PACs from rice chromosome 1 (R1) when all the rice BAC/PACs having homology of $E \leq e^{-10}$ were selected. These BAC/PACs were aligned

in the same order as markers in *Dn7* linkage map. However this synteny between the regions of 1RS carrying *Dn7* and rice genome was not able to provide additional markers for the *Dn7* gene. Crude extract of RWA biotype 1 significantly inhibited leaf unrolling of susceptible cultivar 'Gamtoos' whereas had no significant effect on other two susceptible cultivars 'Carson' and 'Synthetic Hexaploid'. Crude extract of RWA biotype 2 significantly inhibited leaf unrolling of 'Carson' and 'Synthetic Hexaploid' but not of 'Gamtoos'. The data obtained from RWA saliva, proteins and non protein compounds were generally uninformative as few significant differences were observed between treatments and control.

A dense genetic map of 1RS region can facilitate the map-based cloning of important disease and insect resistance genes which are present on short arm of rye chromosome 1RS. Also DNA makers developed for *Dn7* in this study will help in marker assisted selection (MAS) for RWA resistance screening as 96% accuracy can be achieved for selecting a resistant line by using PCR markers flanking the *Dn7*. Although *in vitro* studies were generally uninformative, it lays the ground for further work to develop a bioassay for RWA resistance screening and also to find out the elicitors responsible for expression of resistance in RWA resistant cultivars.

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Introduction and Review of Literature

The Russian wheat aphid (*Diuraphis noxia,* RWA) is a native of Eastern Europe and Western Asia. It was first reported from Russia in early 20th century and since then has been reported from Mediterranean region, South Africa and North America. It was introduced into USA in 1986 in Texas and has spread to all wheat (*Triticum aestivum* L.) growing states of USA.

Russian wheat aphid is small in size (1.4-2.33 mm), lime green in color, spindle shaped, and has short antennae which distinguish it from other small grain aphids. Although both males and females have been found in their native range, only females are found in North America where they reproduce asexually and females gives birth to live young ones which mature and start reproducing within two weeks or more.

RWA is a serious economic pest of wheat and barley (*Hordeum vulgare* L.). Economic damage caused by RWA in USA is around $ 1 billion (Legg and Amosson 1993; Webster *et al.* 2000). RWA infestation causes leaf rolling, streaking of the leaves, leaf and head trapping, stunted growth of the plants, and even death of susceptible plants. Under heavy infestation it can cause a yield reduction of 50% or more.

Many control measures have been recommended for RWA control and for minimizing the damage caused by its infestation. Biological control agents such as lady bird beetles, lace wings, and spiders feed on RWA. Also some exotic RWA enemies have also been recommended for the RWA control. But generally biological control methods have not been found to be very effective in RWA control. Cultural control practices like delayed planting of winter wheat, early planting of spring wheat, and crop rotations have been beneficial in RWA control measures. Chemical control has also been recommended

for the control of RWA but chemical control is expensive and can have long term environmental problems. Moreover, topical spray of insecticides is not effective as the leaves on which aphids feed generally get rolled and this prevents direct contact of insecticides with the aphids. The use of resistant cultivars is an efficient, economical and environmentally safe method to protect small grain cereals from losses by RWA.

There are 11 genes known to provide resistance to RWA, *Dn1*, *Dn2* (Du Toit, 1987), *dn3* (Nkongolo *et al.* 1991a), *Dn4* (Nkongolo *et al.* 1991b), *Dn5* (Marais and Du Toit, 1993; Saidi and Quick, 1996; Zhang *et al.* 1998), *Dn6* (Saidi and Quick, 1996), *Dn7* (Marais and Du Toit, 1993), *Dn8*, *Dn9*, *Dnx*, *Dny* (Liu *et al.* 2000; Smith *et al.* 2004). RWA resistance gene *Dn4,* present on short arm of wheat chromosome 1D, is a dominant gene and originated from Russian bread wheat accession PI372129 (Ma *et al.* 1998). *Dn4* has been deployed by breeders in Colorado for developing RWA resistant cultivars and is in all except one resistant cultivar grown in Colorado (Haley *et al.* 2004). Until spring of 2003 only one RWA biotype (biotype 1) was found in N. America. But a new RWA biotype (biotype 2) was reported in Colorado and wheat growing states of USA (Haley *et al.* 2004). This new biotype is virulent to all known RWA resistance genes except for RWA resistance gene *Dn7*. *Dn7* is a dominant gene from rye (*Secale cereale* L.) and was introduced into a wheat background by 1RS.1BL translocation (Marais *et al.* 1994). *Dn7* provided superior level of resistance against RWA biotype 1 (Anderson *et al.* 2003) and also against RWA biotype 2 (Haley *et al.* 2004).

Dn7 is present on the short arm of rye chromosome 1RS which is an important source of many disease resistance genes, for example *Lr26, Sr31, Pm*8 (Singh *et al* 1990), insect resistance gene for example *Gb2* (Mater *et al* 2004) and *Dn7* (Marais and Du Toit,

1993), and also of quantitative trait loci (QTL) which affects grain yield (Schlegel and Meinel 1994).

Anderson *et al.* (2003) produced a genetic map of *Dn7* containing six RFLP markers. The closest markers were *Xbcd1434* and *Xksud14*, which mapped at distances of 1.4 cM and 7.4 cM respectively. No PCR-based markers have been developed yet that would facilitate marker-assisted selection (MAS) of the gene. *Dn7* is linked to the rust gene complex in 1RS. Marais *et al.* (1998) reported that *Dn7* is linked to *Lr26* gene with a distance of 14.5 ± 3.9 cM. A saturated map for this gene-rich region of 1RS would be useful for map-based cloning studies of genes in this region. Many genomic tools are available now which can be used to saturate 1RS region carrying *Dn7* and other important disease and insect resistance genes. Wheat, barley, rye and oats (*Avena sativa* L.) have extensive similarity in chromosome structures and linkage groups are conserved among them and they belong to group Triticeae. With the help of common markers, homoeology in the gene content and order have been demonstrated between members of Triticeae at a low-resolution genetic map level (Van Deynze *et al.* 1995; Nelson *et al.* 1995a,b). Common DNA markers can be used between members of Triticeae which can be of help in saturating the gene of interest in a chromosome. Microsatellite markers (SSR) have been developed in rye, wheat and barley which can be use for saturating the region. Moreover ESTs have been generated in wheat, rye and barley which can also be used. The use of amplified fragment length polymorphism (AFLP) markers for the construction of genetic map in plants has accelerated genome analysis and genetic improvement (Meksem *et al.* 2001).

Beyond Triticeae, rice (*Oryza sativa* L.) also offers resources that may be useful for saturating particular chromosome regions. Extensive similarities have been demonstrated between genome of the grass species both at the markers level (Ahn *et al.*, 1993; Van Deynze *et al.*, 1995) and at sequence level (Peng *et al.* 2004; Rota and Sorrels, 2004). Rice genetic maps and molecular markers have been used to saturate the *Rpg1* region of barley (Kilian *et al.* 1995). Kato *et al.* (1999) performed comparative mapping of the wheat *Vrn-A1* region with the rice *Hd-6* region. They use a rice cDNA clones which had been previously mapped on to the *Hd-6* region for comparative genetic mapping of the *Vrn-A1* region and reported that four cDNA markers, linked within 2.2 cM in the rice *Hd-6* region, were mapped on the flanking region of the wheat *Vrn-A1*, with a complete correspondence of order.

Rice is an important cereal crop of the many parts of the world. Rice is diploid, has a small genome size (~ 400 Mbp) and its genome contains less amount of repetitive DNA (50%) (Kurata *et al.* 1994) as compared to cereals with complex genomes like wheat (~16000 Mbp, and 80% of repetitive DNA) (Smith and Flavell 1975). Moreover rice genome has been sequenced which makes rice genome an important tool for identifying markers and for positional cloning of genes from cereals like wheat and barley.

Since new RWA biotypes have been found in N. America which are virulent to most of the known RWA resistance genes, fast development of new RWA resistant cultivars has become a high priority for plant breeders. For this there is an urgent requirement of fast and efficient screening methods for RWA so that new germplasm can be screened for finding new resistant genes and which can be incorporated into

agronomically well adapted but susceptible cultivars. Greenhouse seedling screening has been widely used to test RWA resistance, but the high temperature during the summer months prohibit such screening due to intolerance of RWA to high temperature. There is an increased mortality rate of RWA in temperatures above 20°C (Michels and Behle, 1998). Therefore, a simple lab screening method is needed to solve this problem. Rapid screening methods will save time, effort, and greenhouse space for screening of seedlings for resistance even during the time of the year when aphids are not available for greenhouse screening.

Dong *et al.* (1994) used crude aphid extracts to test in-vitro responses of three wheat genotypes, and found out that crude extract of RWA biotype inhibited leaf unrolling in susceptible genotypes but has no effect on the unrolling of etiolated seedlings of RWA resistant genotypes. At that time only one RWA biotype (biotype 1) was present in N. America but now there are many biotypes present and resistance genes for two biotypes (biotype 1and biotype 2) are known.

I divided my thesis work into 3 chapters.

Chapter 1: Saturation mapping of a gene-rich region in rye chromosome arm 1RS, containing a Russian Wheat Aphid resistance gene *Dn7*. The objectives of this study were 1) Saturate the distal region of short arm of rye chromosome 1R containing RWA resistant gene *Dn7*, 2) to develop PCR based markers for *Dn7*, and 3) to find out if there is synteny between short arm of rye chromosome 1R region carrying Dn7 and rice genome.

Chapter 2: Synteny studies between rye chromosome 1RS and rice. The objective of the study to assess the degree of synteny between the distal region of the short arm of rye chromosome 1RS containing *Dn7* and rice, and to determine whether rice genome sequences can be used to find markers to saturate the genetic map of *Dn7*.

Chapter 3: *In-vitro* reaction of wheat to extracts of Russian wheat aphid biotypes 1 & 2. The objective of this study was to develop a simple and efficient bioassay for RWA resistance, which may lead to the identification of elicitors responsible for expression of resistance in RWA resistant cultivars.

Chapter 1

Saturation mapping of a gene-rich region in rye chromosome arm 1RS containing the Russian Wheat Aphid resistance gene *Dn7*

Abstract

The 1BL.1RS translocation chromosome has been extensively used in wheat breeding programs all over the world because the short arm of rye chromosome 1R (1RS) carries many important disease resistance genes, insect resistance genes and genes for agronomically important traits. Most of these resistance genes are located in the distal region of rye chromosome 1RS. A dominant Russian wheat aphid (RWA) resistance gene *Dn7* was also mapped to the short arm of rye chromosome 1R. RWA is a pest of cereals and has caused serious economic damage to wheat and barley growers all over the world. The objectives of the present study were to 1) saturate the distal region of short arm of rye chromosome 1R containing the RWA resistance gene *Dn7*, 2) to develop PCR based markers for *Dn7*, and 3) to determine if there is synteny between the short arm of rye chromosome 1R region carrying *Dn7* and the rice genome. The genetic map of 1RS developed in this study contains twenty six DNA markers covering a genetic distance of 97.5 cM. The markers flanking *Dn7* are *XHor1* and *XIb267* which are 2.1 and 3.9 cM from *Dn7*, respectively. Nine PCR-based markers were developed for *Dn7*. PCR-based markers include one rye microsatellite (SSR) marker, one amplified fragment length polymorphism (AFLP) marker, one AFLP derived (sequence tagged site) STS marker, one wheat expressed sequence tag (EST) homologous to known resistance gene analogs (RGAs), two RGAs, and two STS markers derived from restriction fragment length polymorphism (RFLP) markers. One AFLP fragment linked to *Dn7* was successfully converted to high-throughput STS marker. This is the first time PCR-based markers have been developed for *Dn7*. To do synteny studies with rice, DNA sequences of the markers from rye chromosome 1RS containing *Dn7* were used to do BLAST homology searches

with DNA sequences of rice BACs/PACs. Only three RFLP markers and two wheat ESTs mapped to the distal region and were linked with *Dn7* showed significant homology ($E \leq e^{-10}$) with the rice BAC/PACs. No conserved homology was observed with rice chromosomes 5 and 10 which are known to be homoeologous with the group 1 chromosomes of Triticeae.

Introduction

The short arm of rye (*Secale cereale* L., 2n = 2x = 14) chromosome 1 (1RS) has been extensively used in wheat (*Triticum aestivum* L.) breeding programs for decades through wheat translocation chromosomes (Schlegel and Korzun 1997; Graybosch 2001). The interest in rye chromosome 1RS is based on the plethora of resistance genes it contains against several pathogens and insects, as well as genes for agronomic traits (Mater *et al.* 2004). Chromosome arm 1RS contains genes conferring resistance to the causal agents of powdery mildew (*Pm8*), leaf rust (*Puccinia recondita* f. sp. *tritici*) (*Lr26*), stem rust (*Puccinia graminis* f. sp. *tritici*) (*Sr31*), and stripe rust (*Puccinia striiformis* f. sp. *tritici*) (*Yr9*) (Singh *et al.*1990). Wheat genotypes containing the 1RS translocation generally have higher yield potential, and it has been postulated that a quantitative trait loci (QTL) affecting yield of wheat is present in this chromosome (Schlegel and Meinel 1994). This QTL is associated mainly with an increase in spikelet fertility (Lelley *et al.* 2004). The short arm of rye chromosome 1R also has greenbug (*Schizaphis graminum* Rondani) resistance gene *Gb2* (Mater *et al.* 2004).

The resistance genes are located in the distal region of rye chromosome 1RS which has been designated as 'ISO.8' (Sandhu and Gill 2002). This region is homoeologous to a region in Triticeae group 1 chromosome that also contains similar genes and DNA markers. The name 'ISO.8' is based on the nomenclature given to the deletion line where the genes have been physically mapped. Although most of the Triticeae genomes are very large, the expressed portion of the genome is very small. Expressed genes are present only in 1-5% of the wheat genome (Sandhu and Gill 2002)

and are mostly present in physically small regions called "gene-rich" regions which are physically separated by blocks of repetitive DNA (Gill *et al* 1996 a,b; Sandhu *et al.* 2001). The repetitive DNA consists mainly of retrotransposons and duplicated genes (SanMiguel *et al.* 1996; Wicker *et al.* 2001). '1S0.8 region' is one of the largest and perhaps most important 'gene-rich' region in the short arm of Triticeae homoeologous group 1 chromosome. Although this region is less than 1% of chromosome 1 of wheat still it contains about 31% of the genes (Sandhu and Gill 2002).

A rye gene from 1RS that confers resistance to the Russian wheat aphid (*Diuraphis noxia* Mordvilko, RWA) was transferred to wheat via a 1RS.1BL translocation (Marais *et al.* 1994). The resistance gene, *Dn7,* is a dominant gene that confers a superior level of resistance to at least two RWA biotypes (1 and 2) (Anderson *et al.* 2003; Haley *et al.* 2004). Our laboratory has been working on finding resistance genes for the RWA, which is a serious economic pest of wheat, barley (*Hordeum vulgare* L.) and rye. Anderson *et al.* (2003) produced a genetic map of *Dn7* containing six restriction fragment length polymorphic (RFLP) markers. The closest markers were *Xbcd1434* and *Xksud14*, which mapped at distances of 1.4 cM and 7.4 cM respectively. No PCR markers have been developed yet that would facilitate marker-assisted selection (MAS) of the gene. *Dn7* is also being targeted for map-based cloning and hence there is a need to construct a saturated map for this region. *Dn7* is linked to the rust resistance gene complex in 1RS. Marais *et al.* (1998) reported that *Dn7* is linked to *Lr26* at a distance of 14.5 ± 3.9 cM. A saturated map for 'gene-rich' region of 1RS would be useful for map-based cloning studies of genes in this region.

Recently developed genomics tools in the Triticeae may provide tools for saturating the short arm of rye chromosome 1R. Simple sequence repeats (SSR) or microsatellite markers have been developed in rye (Hackauf and Wehling 2002b; Saal and Wricke 1999). Expressed sequence tags (ESTs) have been developed in rye (http://wheat.pw.usda.gov/genome/) and these ESTs can be a good source of saturating the 1RS region. ESTs can be used directly as RFLP probes or their sequence can be used to mine/isolate SSRs (EST derived SSR markers, eSSR). Hackauf and Wehling (2002a) developed SSR markers using the rye EST database.

With the help of common markers, extensive homoeology in the gene content and order have been demonstrated between members of Triticeae at a low-resolution genetic map level (Van Deynze *et al.* 1995; Nelson *et al.* 1995a, b). A consensus map for the '1S0.8 gene rich region' for the Triticeae group was developed using common markers and sixteen marker loci common between wheat and barley consensus maps, twelve between wheat and rye and six between wheat and oat (Sandhu and Gill 2002). Many disease resistance genes, for example *Lr21*, *Lr26*, *Pm3a*, *Sr31*, *Yr9*, *Pm8*, *Sr21*, *Sr33*, and *Yr9*, have also been placed on the 1S0.8 region of the consensus physical map of short arm of wheat chromosome 1 (Sandhu *et al.* 2001).

Conserved motifs within the resistance genes have been used to isolate resistance gene analogs (RGAs) using various PCR based techniques from many plant species (Maleki *et al.* 2003). RGAs can be used as tightly linked markers for resistance genes or as candidates for resistance genes. RGAs, ESTs, eSSRs, and RFLP markers from Triticeae can be an important source for saturating the short arm of rye chromosome 1R.

Beyond Triticeae, rice also may be useful for saturating the 1RS. Extensive similarities have been demonstrated between genomes of the grass species both at the marker level (Ahn *et al.* 1993; Van Deynze *et al.* 1995) and at the sequence level (Peng *et al.* 2004; Sorrels *et al.* 2003). Rice genetic maps and molecular markers have been used to saturate the *Rpg1* region of barley (Kilian *et al.* 1995). Yan *et al.* (2003) demonstrated the use of orthologous regions in wheat and rice to saturate the genetic map of *Vrn1*, a wheat vernalization gene, which led to the positional cloning of the gene.

The objectives of the present study were to 1) saturate the distal region of short arm of rye chromosome 1R containing the RWA resistance gene *Dn7*, 2) to develop PCR based markers for *Dn7*, and 3) to determine if there is a synteny between the short arm of rye chromosome 1R region carrying *Dn7* and the rice genome.

Materials and methods

Plant materials

F_2 derived F_3 families of a cross between wheat line '94M370' (resistant to RWA containing *Dn7*) and wheat cv 'Gamtoos' (susceptible to RWA) were advanced to the F_6 generation by single seed descent. Both parents contain the 1RS.1BL translocation. These lines constituted a population of 98 recombinant inbred lines (RILs) used in this study. The derivation of '94M370' was described by Anderson *et al.* (2003).

RWA resistance screening and phenotyping

RWA screening and scoring were performed at the Colorado State University greenhouse in the winters of 2002 and 2003. Ninety eight RILs were used for RWA screening and phenotyping. Ten to fifteen seeds were planted in greenhouse flats in a single row and were infested with RWA biotype 1 instars as described by Nkongolo *et al.* (1989). 'Gamtoos' and '94M370' were included in each flat while 'Carson' served as a susceptible control. The RWA damage was rated at 7, 14, and 21 days after infestation and were scored according to Ma *et al.* (1998).

DNA extraction, bulk segregant analysis and Southern blotting

Total genomic DNA was extracted from 98 RILs as well as from the parental genotypes ('94M370' and 'Gamtoos') according to Peng *et al.* (2004). Resistant and

susceptible bulked segregants were constructed by combining DNA from eight resistant and eight susceptible recombinant inbred lines. Four restriction enzymes (*EcoR*I, *Xba*I, *EcoR*V, *Hin*DIII) were used to digest the genomic DNA from parental lines, resistant and susceptible bulks, and 98 RILs. Blotting and Southern hybridizations were as described by Peng *et al.* (2004). The pair of bulk segregants and parents were used to screen 105 molecular markers for polymorphism. The markers showing polymorphism between the resistant and susceptible bulks were then mapped using the 98 RILs. The details of probes mapped in this study are given in Table 1.1.

Polymerase chain reaction (PCR) analysis

All PCR reactions were performed in a 25 µl volume using a PTC-200 MJ Thermocycler (MJ Research, Inc., Waltham, MA). The reaction mixture contained: 250 nM of each primer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1U of *Taq*-polymerase enzyme (Promega Madison, WI, USA) and 100 ng of template DNA. The amplification reactions were carried out using the following profile: 94°C for 3 min, 45 cycles of 94°C for 1 min, 50°C or 55°C or 60°C for 1 minute (depending upon the annealing temperature of the primers), 72°C for 1 min, with a final extension step of 72°C for 10 min. The amplification products were separated on 3% agarose or 7% polyacrylamide gels or on a 5% denatured polyacrylamide gel. The gels were visualized with ethidium bromide (0.5 µg/ml) or silver staining.

Development of AFLP derived markers

AFLP reactions were performed on the resistant and susceptible parents as well as the resistant and susceptible bulked segregants. AFLP analysis was performed using the standard protocol (Vos *et al.* 1995). For selective amplification, *Pst*1 and *Mse*1 primers with three additional nucleotides were used. Silver staining was used to visualize the gel. The primers and adapters sequences used in this study have been described in Peng *et al.* (2000a).

Cloning and sequencing of AFLP fragment

AFLP fragments that were polymorphic between the two parents and between the bulked segregants were carefully excised from the 5% denatured polyacrylamide gel. Gel fragments containing the DNA were re-hydrated in 100 µl of deionized H_2O for three minutes, crushed and incubated in boiling water for 10 minutes. The supernatant was transferred to a new 1.5 ml Eppendorf tube after being centrifuged at 12,000 rpm for 15 minutes. DNA was precipitated overnight at -20°C by adding 10 µl of 3M sodium acetate (pH 5.2) and 2.5 volume of ethanol. The pellet was washed in 70% ethanol and was resuspended in 10 µl of deionized H_2O. 2µl of the supernatant was used as template to re-amplify the fragment using primers and reaction conditions similar to those for the AFLP reaction. The PCR products were separated on a 1.1% agarose gel, excised and purified using QIA quick gel extraction kit (Qiagen, Valencia, CA, USA). The PCR product was cloned into pGEM-T easy vector (Promega, Madison, WI, USA) according to the manufacturer's instructions. Due to the simultaneous migration of the different AFLP fragments of the same size, it is difficult to identify the correct fragment.

Therefore, fifteen clones were selected on the basis of their insert size after being digested with *EcoRI* and were sequenced at SeqWright (Houston, Texas, USA). These clones were divided into four groups based on sequence similarities. Oligonucleotide primers were designed from the each of the two largest groups using the primer3 software program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and were screened on the parents and resistant and susceptible bulks.

Genetic mapping

A linkage map was constructed using MapMaker 3.0 (Lander *et al.*, 1987). A threshold LOD score of 3.0 was used in the mapping analysis. CentiMorgan units were calculated using the Kosambi mapping function (Kosambi, 1944).

Calculation of accuracy and efficiency of marker assisted selection

In order to find out the effectiveness of the PCR markers linked to *Dn7*, accuracy of marker assisted selection (AMAS) and efficiency of marker assisted selection (EMAS) of the markers were calculated using the empirical formula proposed by Peng *et al.* (2000b).

AMAS is the number of homozygous resistant plants among the total number of plants containing the resistant parent-type marker. EMAS refers to the number of plants that are homozygous for the marker among the homozygous resistant plants. AMAS and

EMAS were calculated for a single marker, combination of two markers flanking the gene and combination of two markers on the same side of the gene.

BLAST homology search against rice genomic sequences

To find syntenic regions in rice, DNA sequences of the mapped markers linked to *Dn7* were used for BLASTn (http://www.ncbi.nlm.nih.gov; Altschul *et al.* 1997) homology searches with DNA sequences of rice BACs/PACs (http://tigrblast.tigr.org/euk-blast/index.cgi?project=osa1). Sequences of the clones used to do BLASTn searches were found from NCBI website (http://www.ncbi.nlm.nih.gov/). Similarities between DNA markers sequence and rice BAC/PACs with $E \leq e^{-10}$ was adopted as the standard to claim a significant similarity. DNA sequences were determined for RFLP probes *Xiag95* and *Xscb241* and PCR marker *XIb267*.

Results

1RS genetic map

In order to saturate the ISO.8 region of 1RS, available genomics resources were utilized, including rye SSRs, wheat ESTs, AFLP derived STS markers, RFLP, RGAs and PCR markers from 1RS. The current genetic map of 1RS contains twenty six DNA markers covering a genetic distance of 97.5 cM (Figure1.1). The map has seventeen RFLP markers which includes five wheat ESTs from wheat chromosome 1S, and nine PCR based markers. One more wheat EST BF475048 was mapped via a PCR approach. The markers flanking *Dn7* are *XHor1* and *XIb267* which are 2.1 and 3.9 cM from *Dn7*, respectively. *XIb267* is a PCR-based marker and is also linked to stem rust resistance gene *SrR* (Mago *et al.* 2002).

RFLP markers previously mapped to the short arm of rye chromosome 1R, ESTs and RFLP markers from wheat chromosome 1S, and RFLP markers from the short arm of barley chromosome 1H were screened for polymorphism between resistant and susceptible parents. Out of thirty wheat ESTs from the distal region of 1S screened for polymorphism among the parents, twenty one ESTs (70%) detected polymorphism between the parents with at least one of the four restriction enzymes used and five (23.8%) of them were placed on the 1RS linkage map. Out of 25 RFLP markers screened for polymorphism between the parents and resistant and susceptible bulks, 10 (40%) were placed on the 1RS linkage map.

Development of PCR based markers for Dn7

Only PCR-based markers which were polymorphic between the resistant and susceptible bulks were mapped. Therefore those not close to *Dn7* were eliminated early on. The current map has nine PCR based markers which include one rye SSR marker, one AFLP marker, one AFLP derived STS marker, one wheat EST homologous to known RGAs, two RGAs, and two STS markers derived from RFLP markers. PCR markers linked to *Dn7* along with their primer sequences are listed in Table 1.2. The PCR-based markers flanking *Dn7* are *XIB267* and rye SSR marker *Xrems1303_1*, which are at distances of 3.9 and 11.2 cM, respectively, from *Dn7*. *XIB267* is linked to *Dn7* in repulsion phase and amplifies a DNA fragment of approximately 200 bp in the susceptible parent ('Gamtoos') (Figure 1.2a), whereas *Xrems1303_1* is linked to *Dn7* in coupling phase and amplifies a DNA fragment of 450 bp in the resistant parent ('94M370') (Figure 1.2b).

DNA sequences of the RFLP markers linked to *Dn7* were used to design oligonucleotide primers and were tested for their linkage to *Dn7*. Oligonucleotide primers designed from the DNA sequence of two RFLP markers *Xksud14* and *Xiag95* amplified polymorphic bands between resistant and susceptible parents and their respective bulk segregants and were placed on the *Dn7* linkage map. Oligonucleotide primers were also developed from five wheat ESTs showing homology with known RGAs localized on the consensus map of 1S (Dilbirligi *et al.* 2004). One EST (BF475048) was linked 10.3 cM distal to *Dn7*. This STS marker amplified a band of approximately 1700 bp in the resistant parent.

In order to find out if wheat eSSRs can be used to saturate the 1RS region containing *Dn7*, we tested eleven wheat eSSRs from wheat chromosome 1S (Peng and Lapitan, 2005) for polymorphism between RWA resistant and susceptible parents and their respective bulk segregants. Five eSSRs showed polymorphism between the parents but none showed polymorphism between the bulks. This suggests that those eSSRs are far from *Dn7* or were probably on chromosomes other than 1RS.

Out of the twelve AFLP primer combinations tested, four (33.3%) showed polymorphism between the resistant and susceptible parents but only two primer combinations were linked to *Dn7*. Primer combination PCGG/MCTC amplified a band of approximately 225 bp in the susceptible parent and susceptible bulk (Figure 1.3a). Another primer combination PCGC/MCTT amplified a 170 bp band in the resistant parent and resistant bulk. Sixty RILs were screened with primer combination PCGC/MCTT which was found to be linked to *Dn7* gene at a distance of 11.5 cM.

A 225 bp fragment produced by AFLP primer combination PCGG/MCTC (Figure 1.3a) was carefully extracted from silver stained gel and was cloned. Fifteen clones were selected based on the insert size and were sequenced. The clones were divided into four groups based on identical sequences and oligonucleotide primers developed from the largest group of sequences amplified two polymorphic band of approximately 173 and 175 bp in the susceptible parent and susceptible bulk (Figure 1.3b). The resulting STS marker was thus designated as *XcsuP57225*.

Synteny studies between 1RS and rice chromosome

The *Dn7* linkage map contains fifteen markers with known sequences which were used to do comparative studies between the 1RS distal region and the rice genome. The sequences of fifteen markers linked to *Dn7* which includes RFLP, ESTs and PCR markers, were used to do BLASTn homology searches with the DNA sequences of rice BAC/PACs. All the markers which were used to find homologous rice BAC/PACs are listed in Table 1.3. Only three RFLP markers and two wheat ESTs showed significant similarity ($E \leq e^{-10}$) with the rice BAC/PACs. The rest of the markers did not meet the criteria specified in this study ($E \leq e^{-10}$) to be considered significant. The similarity ratio of 33.33% (out of 15 DNA markers only 5 showed homology with rice BAC/PACs) between the 1RS distal regions and rice genome was much lower than that between wheat and rice genomes (Peng *et al.* 2004).

Discussion

Saturated map for 1RS distal region containing Dn7

The 1RS chromosome of rye is often used in wheat breeding because it contains many genes useful for wheat improvement. RWA resistant gene, *Dn7,* is another gene mapped to this chromosome (Marais *et al.* 1994). This paper presents a dense genetic map of *Dn7* with twenty six DNA markers covering 97.5 cM region on 1RS chromosome. RFLP markers *Xbcd1434*, *Xksud14*, *Xksuf43*, *Xmwg36*, and *Xmwg938*, linked to *Dn7* have been placed in 1S0.8 region of 1S physical map of wheat (Sandhu *et al.* 2001) (Figure 1.1). Five wheat ESTs, BE403717, BE438866, BF575048, BE590674, BE442682, and BE405778 which have been placed on 1S 0.86-1.00 region were also found to be linked to *Dn7*. Marais *et al.* (1998) reported that *Dn7* is linked to the *Lr26* gene with a distance of 14.5 ± 3.9 cM. Many disease resistance genes, for example *Lr21*, *Lr26*, *Pm3a, Sr31, Yr9, Pm8, Sr21, Sr33*, and *Yr9,* have also been placed on the 1S0.8 region of the consensus physical map of short arm of wheat chromosome 1 (Sandhu *et al.* 2001). This therefore suggests that the *Dn7* gene is located on the distal part (1S0.8 region) of the chromosome 1RS. Most of the resistance genes are found to be in the terminal portion of the chromosomes and are clustered together (Akhunov *et al.* 2003). The higher recombination frequencies in this region (Gill *et al.* 1996a,b; Schnable *et al.* 1998; Sandhu *et al.* 2001) may provide a mechanism for a rapid response to new insect biotypes or pathogen races. Indeed, resistance genes are known to undergo frequent rearrangements and exhibit copy number variation (Leister *et al.* 1998). Presence of resistance genes on terminal portions of chromosomes could help plants adapt well to

frequent changes without having any deleterious effects to other genes (Leister *et al.* 1998).

Although I was able to develop a dense genetic map of 1S0.8 region, the closest markers to *Dn7* are still more than 1 cM from the gene. Tightly linked markers for *Dn7* could facilitate map-based cloning of this important RWA resistance gene. But as *Dn7* gene is in an area of 1S0.8 region where many other important disease resistance genes have been mapped, markers linked to *Dn7* can be of help in map-based cloning of those genes. The actual distance between the markers could be much less as what they appear in the present 1RS genetic map containing *Dn7*. This could be due to the fact that the gene rich region 1S0.8 is highly recombinogenic (Gill et al 1996 a,b; Sandhu et al 2001). In the Triticeae group, 80% of recombination in the short arm occurs in the 1S0.8 region and 32% genes are present in this region (Sandhu and Gill 2002).

Five RFLP markers (*Xiag95*, *Xmwg2062*, *Xscb241*, *Xmwg938* and *Xksud14*) in the current map of *Dn7* have also been mapped to chromosome 1RS map by using two rye x rye mapping populations by Korzun *et al.* (2001). The order of these markers is maintained in both maps, but whereas these markers covered a distance of 2.4 cM in a rye background (Korzun *et al.* 2001), in a wheat background these markers spanned a distance of 20.2 cM (Figure 1.1). This increase in distance for this region could be due to the difference in the recombination frequency of 1RS in a wheat background versus a rye background. Sandhu and Gill (2002) reported that localized difference in relative recombination between markers was significant between wheat and rye, for example, in the consensus maps of 1S0.8 region of wheat and rye, two markers, *Xcdo580* and *Tri*, are less than 2 cM apart in wheat but are 16 cM apart in rye. Similarly in oats, markers

Xcmwg645 and *Xksud14* were completely linked, while they were 30 cM apart in wheat. This could be due to major rearrangements (Sandhu and Gill 2002).

Another reason for this inflated distance could be due to the different population used for mapping of these markers. Ma *et al.* (2001) reported a difference of 16.7 cM between two terminal markers (*Xpsr596* and *Xwg241*) on 1R when two different mapping populations were used. Melz *et al.* (1992) also reported variation in genetic distance for common markers in rye.

The linear order of the markers in the current *Dn7* map and the *Dn7* map by Anderson *et al.* (2003) is the same except for two markers *Xmwg36* and *Xwrag2*. In the *Dn7* genetic map by Anderson *et al.* (2003), RFLP marker *Xmwg36* is between *Xksud14* and *Xksuf43*, and *Xwrga2* is between *Xmwg2062* and *Xbcd1434*. This could be due to the reason that current map is more dense as twenty more markers have been mapped to *Dn7* and different mapping populations were used for mapping. Another possible reason could be that population size used for the present study is smaller than used by Anderson *et al.* (2003). Ma *et al.* (2001) also pointed out that by adding more markers, the locus order showed minor differences from other maps in spite of using the same mapping populations.

The 1BL.1RS translocation is associated with bread-making quality defects (Dhaliwal *et al.* 1990; Burnett *et al.* 1995). This is due to the presence of rye secalin (*Sec1*) locus in the translocation (Dhaliwal *et al.* 1990; Graybosch *et al.* 1993; Lee *et al.* 1995) and absence of glutenin and gliadin genes or combination of both factor (Kumlay *et al.* 2003). To ascertain the position of *Dn7* in relation to *Sec1*, we used *Sec1* as an RFLP probe and also designed oligo primers from the *Sec1* sequence. No polymorphism

was observed in either case probably due to the reason that both resistant and susceptible parents contain the same allele of *Sec1* derived from 'Petkus'. *Sec1* has been mapped 19.7 cM proximal to *Xiag95* (Nagy *et al.* 2003). In the present genetic map of 1RS, RFLP probe *Xiag95* and PCR marker *IB267* have been mapped 6.5 cM and 3.9 cM, respectively, distal to *Dn7*. Mago *et al* (2002) have placed *Sec1* proximal to *Xiag95* and PCR marker *XIb267*. This indicates that *Sec1* is proximal to *Dn7* and could be tightly linked to *Dn7*. Therefore cloning of *Dn7* is important so that it can be directly introduced into wheat cultivars without *Sec1*.

Development of PCR markers for Dn7

In order to apply MAS in breeding, it is critical to have high-throughput marker screening capability. PCR-based markers can be used for such purpose as they are less expensive and easy to handle in comparison with RFLPs. PCR markers are valuable tools for MAS as most of them are quite simple to use and can be used as high-throughput markers. SSR markers are highly polymorphic and have been used to develop genetic maps in many crop species like wheat (Stephenson *et al.* 1998; Röder *et al.* 1998), and barley (Liu *et al.* 1996.). Miller *et al.* (2001) identified five SSR markers linked to RWA resistant gene *Dn2*, *Xgwm437* being the closest one at 2.8 cM from *Dn2*. Liu *et al.* (2002) and Arzani *et al.* (2004) mapped two SSR markers *Xgwm106* and *Xgwm337* flanking RWA resistant gene *Dn4*. In this study, I was also able to map a rye SSR marker *Xrems1303_1* to *Dn7* at a distance of 11.7 cM which is a dominant marker and is linked to *Dn7* in coupling phase.

The use of amplified fragment length polymorphism (AFLP) markers for the construction of genetic maps in plants has accelerated genome analysis and genetic improvement (Meksem *et al.* 2001). However, AFLP markers are expensive to generate, too laborious, and are not conducive for high-throughput screening of genotypes which limit their use in MAS. By converting AFLP markers into STS or SCAR markers, they can be used for high-throughput screening. In this study an AFLP fragment was successfully converted into a reliable, inexpensive PCR-based STS marker linked to RWA resistance gene *Dn7*. The conversion of AFLP markers to low cost, high-throughput STS marker will facilitate MAS for RWA and will also help in pyramiding RWA resistant genes for different biotypes present in North America.

Conversion of AFLP marker into high-throughput STS marker is not very straightforward. Generally the problem arises due to the short size of the AFLP fragment which limits designing primers that could amplify a polymorphic band (Bradeen and Simon 1998) and also due to simultaneous migration of the different AFLP fragments of the same size (Prins *et al.* 2001). Guo *et al.* (2003) recommended DNA isolation twice from the gel to solve the problem of contaminating bands. They also find primer from two largest groups of sequences which amplified a polymorphic band between the two parents. Prins *et al.* (2001) identified an STS from the second smallest sequence group. Negi *et al.* (2000) used PCR walking methodology to isolate genomic regions adjacent to the AFLP marker. Inverse PCR method has also been used to isolate flanking regions for conversion to SCAR (Bradeen and Simon 1998; DeJong *et al.* 1997).

Marker assisted selection of Dn7

Traditionally, screening for RWA resistance is done under controlled conditions in the greenhouse. This process is tedious, time consuming and can only be done during certain times in a year. DNA markers developed for *Dn7* will facilitate MAS for RWA resistance as it will be possible to do RWA screening throughout the year instead of only during cooler months of the year when the RWA multiplies. Especially PCR-based markers are fast and convenient to use and hence will facilitate MAS for RWA resistance. In the present study three PCR-based markers were analyzed for their accuracy and effectiveness in MAS based on the AMAS and EMAS values calculated by using empirical equations derived by Peng *et al.* (2000b).

For single markers, *XIb267* gave the highest value for accuracy (86%) and efficiency (88%) (Table 1.4). Even though SSR marker *Xrems1303_1* is 11.3 cM from *Dn7* gene, it is still giving high values (> 79%) of both AMAS and EMAS. For markers in combination flanking the *Dn7* gene, both combinations, *IB267-Xrems1303_1* and *XcsuP57225-Xrems1303_1*, gave high values for AMAS (96%), which means that by using any of these PCR marker combination, the probability of selecting a line with RWA resistance gene *Dn7* is 96%. This can facilitate MAS of lines containing this gene early in selection process.

Although the AMAS values for each of the three markers when analysed individually was greater than 79%, AMAS values increased substantially (> 96%) when these markers were used in combination with other flanking markers. However, EMAS values were greater when MAS is based on single marker (> 80%) as compared to MAS based on two flanking markers (> 75%). Peng *et al.* (2000b) also reported that AMAS

values increase when two flanking markers are used as compared to a single marker, but there is not significant improvement for EMAS values when two flanking markers are used in combination.

Synteny studies with rice

Comparative genomics research has opened the avenues for integration of information on gene location and expression across the species (Sorrells *et al.* 2003). Availability of the rice genome sequence draft provides resources for performing comparative studies between rice and other cereal species (Yuan *et al.* 2003). The rice genome can be exploited for finding genes from cereals with larger genome sizes, for example, wheat and barley. Yan *et al.* (2003) demonstrated the use of orthologous regions in wheat and rice to saturate the genetic map of *Vrn1*, a wheat vernalization gene, which led to the positional cloning of the gene.

In order to determine if there is a synteny between region of 1RS containing *Dn7* and homologous rice chromosomes, DNA sequence of the markers linked to *Dn7* were used to do BLAST homology searches with the rice BAC/PACs database. Only three RFLP markers and two wheat ESTs showed homoeology with the rice BAC/PACs from five chromosomes R1, R5, R6, R7 and R11. Ten markers linked to *Dn7* either didn't have any homology with the rice sequences or showed insignificant homology with the rice BAC/PACs. Four of these markers were wheat ESTs which represent coding sequences. These results suggest that rice genome sequences have limited use for saturating the region of 1RS containing *Dn7*. Expected synteny between rice chromosome R5 and R10 was not observed. Only one marker *Xmwg2062* showed homology with the rice sequence from R5. La Rota and Sorrells (2004) did comparative DNA sequence analysis between

wheat and rice and reported that numerous discontinuities exist in gene order between wheat and rice that would complicate the transfer of information and markers between these species. Comparative analysis of the *Tsn1* locus, which provides resistance against Tan spot caused by fungal pathogen *Pyrenophora tritici-repentis* (Died.) Drechs, on wheat chromosome 5B with rice showed that very low levels of colinearity exists between wheat group 5 chromosomes and rice (Haen *et al.* 2004). Out of fifteen markers, six markers linked to *Tsn1* showed homology with three rice chromosomes whereas nine markers detected no homologous sequences in the rice database. *Dn7* is distally located and the distal portions of wheat chromosome have been shown to contain duplicated copies of loci and rapidly evolving genes (Akhunov *et al.* 2003). Resistance gene families are included in the category of rapidly evolving genes. This could hinder the use of rice genome as a tool to find tightly linked markers to *Dn7*.

Conclusion

In the present study a saturated genetic map of the short arm of rye chromosome 1RS containing RWA resistance gene *Dn7* was generated which includes twenty six DNA markers covering a genetic distance of 97.5 cM. Nine PCR-based markers were developed for *Dn7*. This is the first time that PCR-based markers have been developed for *Dn7*. One AFLP fragment linked to *Dn7* was successfully converted to high-throughput STS marker. PCR-based markers will assist in MAS for RWA resistance as 96% accuracy can be achieved for selecting a resistant line by using PCR markers flanking the *Dn7*. Even though a dense map of *Dn7* was generated, no marker tightly linked to *Dn7* was found which could facilitate in map-based cloning of this important RWA resistance gene. Mapping of *Dn7* by using rye mapping populations might provide tightly linked markers. The use of new molecular mapping techniques like sequence-related amplification polymorphism (SRAP) (Li and Quiros 2001), which uses pairs of primers with AT- or GC- rich cores to amplify intragenic fragments for polymorphism detection, might also be helpful in finding tightly linked markers to *Dn7*. The advantage of this technique is, like AFLP, it also generates multiple fragments in a single PCR reaction. The use of rice genome for saturating the 1RS region containing *Dn7* does not look promising as no significant homology of 1RS region was observed with rice genome.

Although 1RS introgression has resulted in development of wheat cultivars which have shown high adaptability, high yields and resistance to biotic stresses, still wheat carrying 1RS has been reported to have poor end use quality. One of the reasons for poor end use quality could be presence of *Sec1* in rye chromosome 1RS. An ongoing study by

scientists in our research group is to dissociate the *Dn7* gene from *Sec1*. The markers developed in my study will be instrumental in that study.

Table 1.1: List of markers mapped to rye chromosome 1R '1S0.8' region, their source, type of marker and species of origin.

Marker	Type of marker	Species of origin	Reference/Source
Xmwg938	RFLP	Barley (*Hordeum vulgare* L.)	Graner *et al.* (1991)
Xmwg36	RFLP	Barley (*Hordeum vulgare* L.)	Graner *et al.*(1991)
Xmwg77	RFLP	Barley (*Hordeum vulgare* L.)	Graner *et al.* (1991)
Xksud14	RFLP	*Aegilops tauschii*	Gill *et al.* (1991)
Xiag95	RFLP	Rye (*Secale Cereale* L.)	Phillip *et al.* (1994)
Xbcd1434	RFLP	Barley (*Hordeum vulgare* L.)	Huen *et al.* (1991)
XHor1	RFLP	Barley (*Hordeum vulgare* L.)	Anders Brandt (Carlsberg Laboratory, Copenhagen, Denmark).
BE403717	EST	Wheat (*Triticum aestivum* L.)	http://wheat.pw.usda.gov/cgi-bin/westsql/map_locus_rev.cgi
BE438866	EST	Wheat (*Triticum aestivum* L.)	http://wheat.pw.usda.gov/cgi-bin/westsql/map_locus_rev.cgi
BF475048	EST(PCR)	Wheat (*Triticum aestivum* L.)	http://wheat.pw.usda.gov/cgi-bin/westsql/map_locus_rev.cgi
BE590674	EST	Wheat (*Triticum aestivum* L.)	http://wheat.pw.usda.gov/cgi-bin/westsql/map_locus_rev.cgi
BE442682	EST	Wheat (*Triticum aestivum* L.)	http://wheat.pw.usda.gov/cgi-bin/westsql/map_locus_rev.cgi
BE405778	EST	Wheat (*Triticum aestivum* L.)	http://wheat.pw.usda.gov/cgi-bin/westsql/map_locus_rev.cgi
Xscb241	RFLP	Rye (*Secale Cereale* L.)	V. Korzun, Bergen, Germany

Xksuf43	RFLP	*Aegilops tauschii*	Gill *et al.* (1991)
Xrems1303_1R	SSR	Rye (*Secale Cereale* L.)	V. Korzun, Bergen, Germany.
Xlb267	PCR	Rye (*Secale Cereale* L.)	R. Mago *et al.*(2002)
RGAa,b	PCR	Wheat *Triticum aestivum* L.)	
XcsuP57225	AFLP derived STS marker		This study
Xwrga2	RFLP	Wheat (*Triticum aestivum* L.)	Devos and Gale 1993

Table 1.2: List of PCR-based markers linked to *Dn7* along with their primer sequences.

Marker	Type of Linkage	Sequence
XIb267	repulsion	R5'-AATGGATGTCCCGGTGAGTGG-3' L 5'-GCAAGTAAGCAGCTTGATTTAGC-3'
Xrems1303_1	coupling	R5'- TTTCCCATCAGAAAAATCGC-3' L 5'-TGTAAAACGACGGCCAGTTAGCACCA-3'
BF475048	coupling	R5'-AGAGCTCATCCCTCTCCACA-3' L 5'-GCA GCT TGT TCC CTG AAG AC-3'
XcsuP57225	repulsion	R5'- TGGGAACGATGAGTCAGTTG-3' L 5'-CGTCCAGTTCTCGCACCA-3'
Xiag95p	coupling	R5'-CCTAGAACATGCATGGCTGTTACA-3' L 5'-CTCTGTGGATAGTTACTTGATCGA-3'
XPCGC/MCTT	coupling	P00(universal primer) TAGACTGCGTACATGCAG M00 (universal primer) GATGAGTCCTGAGTAA P00 +3 primers CGC M00 +3 primers CTT
Xksud14p	coupling	R5'-CCAAAGAGCATCCATGGTGT-3' L 5'-CGCTTTTACCGAGATTGGTC-3'
XRGAa ,b	Coupling	R5'-TCACCAGGCAAGTCATGGTA-3' L 5'-CTAGGGCTTTCCACAACAGC-3'

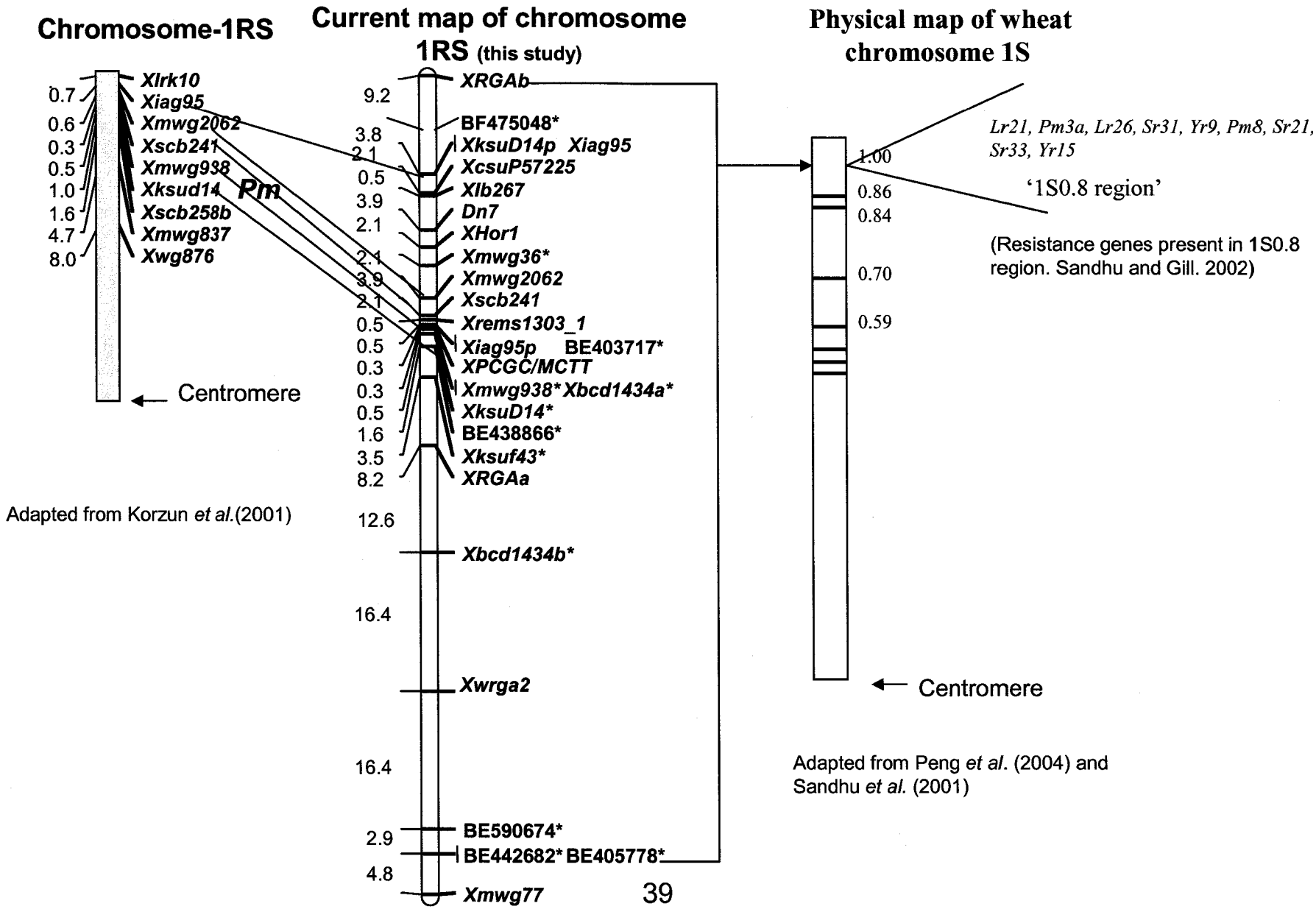
Table 1.3. Clones mapped to Dn7 showing homology with Rice BAC/PACs

Clone	Rice BAC/PAC	E-value	Rice chromosome
Xksud14	OSJNBa009K04	3.00E-68	1
BE403717	OJ1112_H11	8.00E-41	6
Xscb241	P0506C07	1.00E-73	7
Xmwg2062	OJ1008_D04	1.00E-16	5
BF475048	OSJNBa0082N20	e-31	11
BE442682		≥e-10	
BE405778		≥e-10	
BE590674		≥e-10	
Xbcd1434		≥e-10	
Xmwg938		≥e-10	
Xiag95		No hit	
XHor1		≥e-10	
XcsuP57225		≥e-10	
BE438889		≥e-10	
Xlb267		≥e-10	

Table 1.4. The accuracy and efficiency of marker-assisted selection based on single marker on two flanking markers, and on two non flanking markers.

Marker	Map distance (cM)	Accuracy (%)	Efficiency (%)
Xlb267	3.9	86.75	88.59
XcsuP57225	4.4	86.25	88.05
Xrems1303_1	11.2	79.37	80.78
Xlb267-Xrems1303_1	15.1	96.78	75.80
XcsuP57225-Xrems1303_1	15.6	96.69	75.35
Xlb267-XcsuP57225	0.5	89.14	86.23

Figure 1.1. Saturated map of 1RS region (center) in comparison with 1RS genetic map by Korzun *et al.* (2001) and physical map of 1S (adapted from Peng *et al.* 2004 and Sandhu *et al.* 2001). Markers with * have been placed on 1S0.8 region by Peng *et al.*(2004) and Sandhu *et al.* (2001).



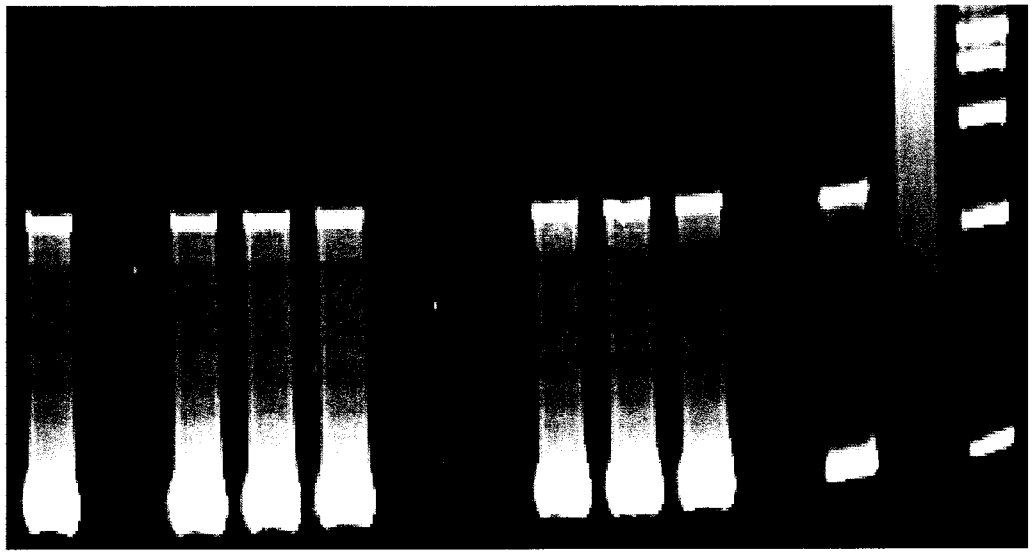
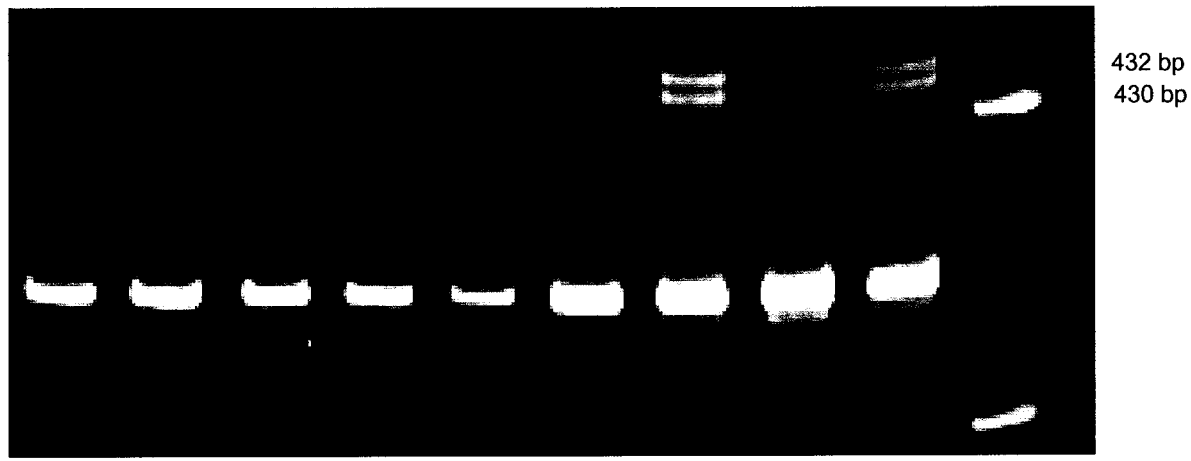


Figure 1.2a. *XIB267* is a PCR-based marker linked to *Dn7*. The gel pictureshows that the 200 bp band is present in susceptible progeny and absent in resistant progeny.



P1- Resistant Parent
P2- Susceptible parent

RB- Resistant parent
SB- Susceptible parent

R-Resistant progeny
S-Susceptible progeny

Figure 1.2b. Rye SSR marker *Xrems1303_1* amplified two DNA fragments of size 430 and 432 bp in resistant parent, resistant bulk, and progenies

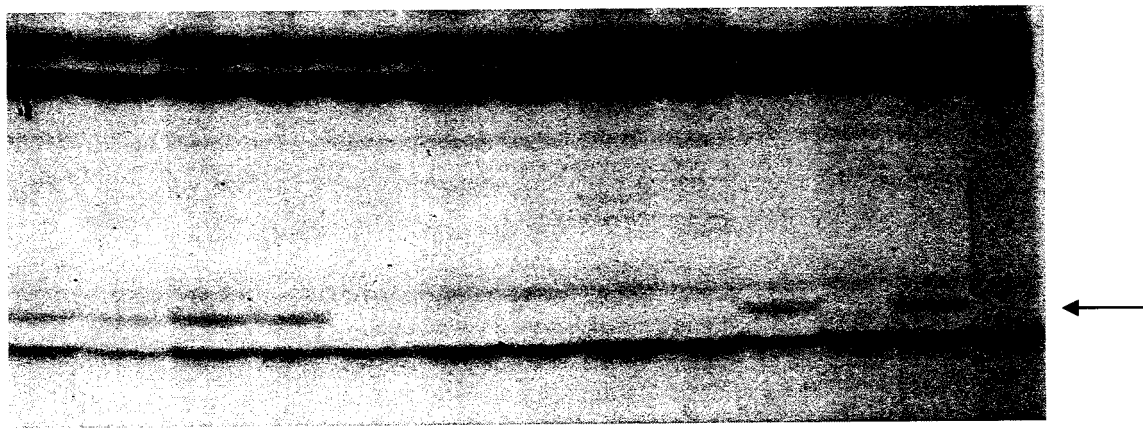
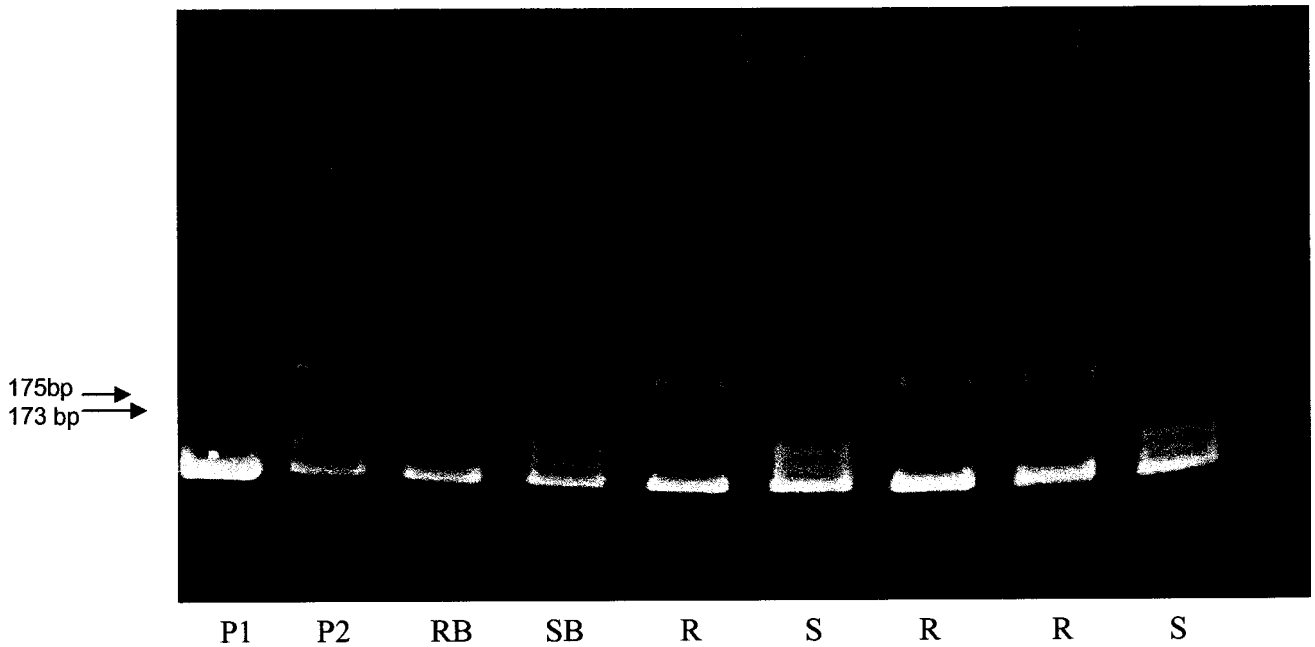


Figure 1.3a. A 225bp band showing polymorphism between the parents –'94M370' and 'Gamtoos' on a 5% denatured gel. This band was cut out from the silver stained gel, DNA was extracted and cloned and sequenced and was developed into a STS marker.



P1- Resistant Parent
P2- Susceptible parent

RB- Resistant parent
SB- Susceptible parent

R-Resistant progeny
S-Susceptible progeny

Figure 1.3b. AFLP derived STS marker *XcsuP57225* amplified 2 polymorphic DNA fragments in susceptible parent, susceptible bulk and progenies.

Chapter 2

Synteny studies between rye chromosome 1RS and rice

Abstract

The 1BL.1RS translocation chromosome has been extensively used in wheat breeding programs all over the world because the short arm of rye chromosome 1R (1RS) carries many important disease resistance genes, insect resistance genes and genes for agronomically important traits. *Dn7*, a rye gene from 1RS, that confers resistance to the Russian wheat aphid (*Diuraphis noxia*, RWA) was transferred to wheat via a 1RS.1BL translocation. The RWA is a pest of cereals and cause serious economic damage to wheat and barley growers all over the world. With remarkable level of synteny between Triticeae and rice genomes established, the rice genome can be used to saturate genetic maps of important genes which would help in map-based cloning of the genes. The objectives of the present work were to assess the degree of synteny between the distal region of the short arm of rye chromosome 1RS containing *Dn7* and rice, and to determine whether rice genome sequences can be used to find markers to saturate the genetic map of *Dn7*. DNA sequences of the markers linked to *Dn7* were used to do BLASTn homology searches against rice genome sequences. Three markers were found to be homologous with rice BAC/PACs from rice chromosome 1 (R1) and these BAC/PACs were aligned in the same order as markers in *Dn7* linkage map. All rice BAC/PACs in the homologous region were used to do BLAST searches with wheat EST database. Two wheat ESTs from group 1S chromosome were polymorphic between the *Dn7* parents and their respective bulks. However no linkage with *Dn7* was observed when these ESTs were tested on 98 recombinant inbred lines (RILs). Problems associated with experimental materials like errors in developing RILs or error in developing bulk

segregants used in mapping cannot be ruled out in not finding linkage with *Dn7*. False positives detected in the PCR reaction and subsequent digestion of the PCR product by restriction enzymes could also be responsible for not finding linkage. To rule out any experimental error, these EST markers will be tested again for linkage on a new *Dn7* F_2 population currently being developed in our lab. New resistant and susceptible bulks will be developed based on the RWA phenotypic score as well as on PCR-based markers developed for *Dn7* to test these markers.

Introduction

Rye (*Secale cereale* L.) is an important cereal crop because of its ability to withstand both biotic and abiotic stress (Mater *et al.* 2004). The short arm of rye chromosome 1RS is an important source of many disease resistance genes (Singh *et al.*1990), genes for insect resistance (Mater *et al.* 2004; Marais *et al.* 1994) and also for quantitative trait loci (QTLs) affecting yield and other important agronomic traits (Schlegel and Meinel 1994). Because of the presence of many important genes, the 1RS.1BL translocation is one of the most frequently used alien introgressions in wheat (*Triticum aestivum* L.) breeding programs throughout the world (Braun *et al.* 1998). *Dn7*, a rye gene from 1RS that confers resistance to the Russian wheat aphid (*Diuraphis noxia*, RWA) was transferred to wheat via a 1RS.1BL translocation (Marais *et al.* 1994). *Dn7* is a dominant gene and provides superior resistance against at least two RWA biotypes (biotypes 1 and 2). RWA is an important economic pest of wheat, barley (*Hordeum vulgare* L), and rye.

With the help of common markers it has been established that there is extensive chromosome arm homoeology between wheat, rye, barley and oats which come under the group Triticeae. Many genes and markers which are present on wheat chromosomes 1A, 1B, and 1D and on barley chromosome 1H have also been localized on rye chromosome 1. Common markers between members of Triticeae have been useful in saturating the genetic maps of this group. A consensus map for the distal '1S0.8 gene rich region' of the Triticeae group was developed using common markers. Sixteen marker loci were common between wheat and barley consensus maps, twelve between wheat and rye and six between wheat and oat (Sandhu and Gill 2002). Disease resistance genes *Lr21*, *Lr26*,

Pm3a, Sr31, Yr9, Pm8, Sr21, Sr33, and *Yr9* have been placed on the 1S0.8 region of the consensus physical map of short arm of wheat chromosome 1S (Sandhu *et al.* 2001) out of which *Lr26, Yr9*, and *Sr31* were placed on the 1S0.8 region of the consensus physical map of the short arm of rye chromosome 1R (Sandhu and Gill 2002).

Beyond the Triticeae, rice (*Oryza sativa* L.) also offers resources that may be useful for saturating particular chromosome regions. Comparative mapping, using a set of cross hybridizing markers, allows an approach to the genetic analysis of wheat by utilizing the advantages of information from other cereal species (Van Deynze *et al.* 1995). The extensive conservation of marker synteny for different chromosomal regions has been reported among cereals, including those of rice and wheat (Ahn *et al.* 1993), barley and rice (Kilian *et al.* 1995) and maize (Ahn and Tanksley 1993) despite differences in chromosome number and genome size. The hexaploid wheat (AABBDD) genome with its large size (1.7×10^{10} bp per haploid genome) and high repetitive DNA sequence content (> 80 %) (Smith and Flavell 1975) would be difficult to sequence. This limits the ability to conduct positional cloning of genes in this species. In contrast, the rice genome is 40 fold smaller (4×10^{8} bp per haploid genome) and consists of 50 % repetitive DNA (Kurata *et al.* 1994). The low level of repetitive DNA in the diploid rice genome also makes it attractive for positional gene isolation techniques. The rice genome sequences are a useful resource for performing comparative studies between rice and other cereal species (Yuan *et al.* 2003).

Yan *et al.* (2003) demonstrated the use of orthologous regions in wheat and rice to saturate the genetic map of *Vrn1*, a wheat vernalization gene, which led to the positional cloning of the gene. Rice genetic maps and molecular markers have been used to saturate

the *Rpg1* and *rpg4* region of barley (Kilian *et al.* 1997). Distelfeld *et al.* (2003) demonstrated a high level of microcolinearity between a region on wheat chromosome 6 having a locus affecting grain protein content (GPC) and 20.4 cM on rice chromosome 2. They mapped 11 colinear genes between wheat and rice after doing BLAST (http://www.ncbi.nlm.nih.gov; Altschul *et al.* 1997) analysis of the rice BAC sequences covering the colinear GPC region against the Triticeae EST database.

In the work described in Chapter 1, a dense genetic map of *Dn7* containing twenty six DNA markers was developed. The *Dn7* linkage map contains fifteen markers with known sequences which were used to do comparative studies between the 1RS distal region and the rice genome. Rice BAC/PACs which showed greatest homoeology with the markers present on the short arm of rye chromosome 1RS were selected. However, some markers showed homoeology with multiple rice BAC/PACs of rice with an e value of $E \leq e^{-10}$. A homoeology value of $E \leq e^{-10}$ is considered to be an indication of significant similarity between the DNA sequences. Therefore, in this study, in order to utilize every opportunity to use rice genome as a tool to saturate the genetic map of *Dn7*, every rice BAC/PAC which showed homoeology of $E \leq e^{-10}$ with DNA markers were taken into consideration to do synteny studies between the short arm of rye chromosome 1RS containing Dn7 and rice genome sequences.

The objectives of the present work were to assess the degree of synteny between the distal region of the short arm of rye chromosome 1RS containing *Dn7* and rice, and to determine whether rice genome sequences can be used to find markers to saturate the genetic map of *Dn7*.

Materials and methods

Plant materials

F_2 derived F_3 families of a cross between wheat line '94M370' (resistant to RWA containing *Dn7*) and wheat cv 'Gamtoos' (susceptible to RWA) were advanced to the F_6 generation by single seed descent. Both parents contain the 1RS.1BL translocation. These lines constituted a population of 98 recombinant inbred lines (RILs) used in this study. The derivation of '94M370' was described by Anderson *et al.* (2003).

Polymerase chain reaction (PCR) analysis

All PCR reactions were performed in a 25 µl volume using a PTC -200 MJ Thermocycler (MJ Research, Inc., Waltham, MA). The reaction mixture contained: 250 nM of each primer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1U of *Taq*-polymerase enzyme and 100 ng of template DNA. The amplification reactions were carried out using the following profile: 94°C for 3 min, 45 cycles of 94°C for 1 min, 50°C or 55°C or 60°C for 1min (depending upon the annealing temperature of the primers), 72°C for 1 min, with a final extension step of 72°C for 10 min. The amplification products were separated on 3% agarose or 7% polyacrylamide gels or on a 5% denatured polyacrylamide gel. The gels were visualized with ethidium bromide (0.5 µg/ml) or silver staining.

Genetic mapping

A linkage map was constructed using MapMaker 3.0 (Lander *et al.*, 1987). A threshold LOD score of 3.0 was used in the mapping analysis. CentiMorgan units were calculated using the Kosambi mapping function (Kosambi, 1944).

Synteny Studies

To find syntenic regions in rice, DNA sequences of the markers linked to *Dn7* were used for BLAST homology searches with DNA sequences of rice BACs/PACs. (http://tigrblast.tigr.org/euk-blast/index.cgi?project=osa1). Sequences of the clones used to do BLAST searches were taken from NCBI website (http://www.ncbi.nlm.nih.gov/). Wheat markers DNA sequences having hits with rice BAC/PACs at $E \leq e^{-10}$ were included in synteny studies.

Rice BAC/PACs from the same rice chromosomes were aligned with the *Dn7* genetic map based upon their physical map location (http://rgp.dna.affrc.go.jp/IRGSP/status.html). Rice BAC/PACs which were placed in the same order as their homologous *Dn7* marker were selected and all BACs/PACs between them were used to do BLAST searches with the wheat EST database (http://www.graingenes.org/cgi-bin/ace/custom/goBlast/graingenes).

Oligonucleotide primers were developed from the wheat ESTs from short arm of group 1 chromosome of wheat (1AS, 1BS, and 1DS) using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

The primers were screened for polymorphism among *Dn7* parents ('Gamtoos' and '94M370') and resistant and susceptible bulks as described in Chapter 1. PCR product of

markers which produced monomorphic bands between parents and bulks were digested with restriction enzymes (*EcoR*1, *Hind 111* and *Xba1*). To the 20 µl of the PCR product, 2.5 µl of 10X buffer (Invitrogen, Carlsbad, CA) and 1 µl (4 u/µl) of restriction enzyme (Invitrogen, Carlsbad, CA), and 1.5 µl of dH_2O was added and reaction mixture was incubated at 37°C for 3 hours. The digested product was run on 5% denaturing polyacrylamide gels and visualized with silver stain. The markers showing polymorphism between the resistant and susceptible bulks were then screened using the 98 recombinant inbred lines (RILs) for their linkage with *Dn7*.

Results

Five DNA markers linked to *Dn7* showed homology with the rice BAC/PACs from rice chromosomes R1, R2, R4, R5, R7, R8, R10 and R11. All rice BAC/PACs showing homology ($e \leq -10$) with markers linked to *Dn7* are listed in Table 2.1. Three DNA markers were found to be homologous with rice BAC/PACs from rice chromosome 1 (R1) and these BAC/PACs were aligned in the same order as markers in *Dn7* linkage map. (Figure 2.1)

Sequence of all rice BAC/PACs between rice BAC/PACs P0043B10 and OSJNBa0090K04 (http://www.gramene.org/Oryza_sativa/SeqTable) (Figure 2.1) were used to do BLAST searches with wheat EST database. Out of 86 rice BAC/PAC in this region, 41 (47.67%) showed homology with wheat ESTs from wheat chromosome 3. For wheat ESTs from chromosome 1, any homology of $E \leq e^{-10}$ was considered to be significant and was used in the present study to find new markers for *Dn7*. Sixteen rice BAC/PACs from rice chromosome 1 showed homology with eleven wheat ESTs from wheat chromosome 1 (Table 2.2). Eight of these ESTs have been localized in the terminal region (1S0.8) of wheat chromosome 1S. Oligonucleotide primers of two ESTs BF292158 and BF483989 out of those eight ESTs amplified a monomorphic band in 'Gamtoos', '94M370' and their respective bulks. After digesting the PCR product with *EcoR1*, BF292158 amplified two polymorphic bands in 'Gamtoos' and susceptible bulks (Figure 2.2). Similarly, BF483989 amplified one polymorphic band in 'Gamtoos' and susceptible bulk after digestion of PCR product with restriction enzyme *Hind111*. However no linkage with *Dn7* was observed for these two ESTs when oligonucleotide primers were screened for 98 recombinant inbred lines (data not shown).

Discussion

Synteny between rye chromosome 1RS containing Dn7 and rice

With remarkable level of similarities at marker and gene levels between the genomes of the different grass species, the genome sequence of rice may be useful for isolating genes from complex genomes like wheat and barley. Hybridization of rice EST with a high level of similarity with N-terminus of gibberellin insensitive gene (*GAI*) with wheat genomic DNA led to the identification of RFLPs cosegregating with the wheat dwarfing genes *Rht-D1* and *Rht-B1* (Peng *et al* 1999).

Markers from homoeologous regions in rice may also be useful in saturating the map of genes of interest in wheat or barley. Kato *et al.* (1999) performed comparative mapping of the wheat *Vrn-A1* region with the rice *Hd-6* region. They used rice cDNA clones which had been previously mapped on to the *Hd-6* region for comparative genetic mapping of the *Vrn-A1* region and reported that four cDNA markers, linked within 2.2cM in the rice *Hd-6* region, were mapped on the flanking region of the wheat *Vrn-A1*, with a complete correspondence of order.

Studies were conducted to find out whether synteny existed between region of chromosome 1RS containing *Dn7* and rice chromosome 5 and 10 known to be homoeologous with the group 1 chromosome of the Triticeae. Three RFLP markers shared homoeology with rice BAC/PACs, the linear order of the sequences were conserved between 1RS and rice, but the rice chromosome which showed synteny with the distal part of rye chromosome 1RS was rice chromosome 1. Expected synteny with rice chromosome 5 and 10 was not observed. The observed synteny between the

sequence of the markers on 1RS and rice chromosome 1 could be due to transposition events or duplication of chromosomal segments before the divergence of wheat and rice. Rostoks *et al.* (2002) and Bennetzen and Ramakrishna (2002) observed a break in synteny between barley chromosome 7H and rice chromosome 6 in the regions flanking the *Wx* locus, where the genes within 10 kb of the *Wx* gene have closer homology to genes on rice chromosome 4 than chromosome 6 (Bellgard *et al.* 2004). Rice chromosome 11 has been reported to have synteny with maize (*Zea mays* L.) chromosome 4 but Tarchini *et al.* (2000) reported that there is a synteny in the *Adh1-Adh2* region between maize and rice except that *Adh1* was located on chromosome 1 of maize compared to the expected chromosome 4 location (Bellgard *et al.* 2004). Transposition events in addition to the classical translocation and inversion events were considered to be the possible reason for the breakdown of synteny (Bellgard *et al.* 2004).

Use of rice genomic sequence to find new markers for Dn7

Although the markers linked to *Dn7* showed homology with the BAC/PACs from rice chromosome 1 and not from rice chromosome 5 and 10 as was expected, I still tried to find out if sequence information of rice chromosome 1 can be used to find new markers for *Dn7*. Although two wheat ESTs showing homology with rice BAC/PACs from rice chromosome 1 showed polymorphism between *Dn7* parents and respective bulks segregants, they were not found to be linked to *Dn7*. Normally if a marker is showing polymorphism between bulks, it has high probability of being linked to the gene of interest (Michelmore *et al.* 1991). In the work described in Chapter 1, these bulks have been successfully used for mapping of the RFLP and PCR-based markers for *Dn7*.

However, problems in experimental materials used in mapping could not be ruled out. An error in developing the RILs, for example, mechanical mixing and out crossing could be a reason for not finding linkage in spite of getting polymorphism in the bulks. Also false positives detected in the PCR reaction and subsequent digestion of the PCR product by restriction enzymes could also be responsible for not finding linkage. The possibility of mis-scoring to be a cause for not finding linkage can be ruled out as sufficient care was taken in scoring of the gels. More work is needed to determine the possible reasons for not finding linkage between these markers and *Dn7*. To rule out any experimental error, these EST markers will be tested again for linkage on a new *Dn7* F_2 population currently being developed in our lab. New resistant and susceptible bulks will be developed based on the RWA phenotypic score as well as on PCR-based markers developed for *Dn7* to test these markers.

In previous work done in my lab it has been found out that the aphids do not feed on rice. Since rice is not a host of this insect, it would not be possible to clone a resistance gene directly from rice. Moreover *Dn7* has been mapped to distal portion of rye chromosome 1RS and distal portions of wheat chromosome have been shown to contain duplicated copies of loci and rapidly evolving genes (Akhunov *et al.* 2003). Resistance gene families are included in the category of rapidly evolving genes.

Although comparative mapping of molecular markers have established high level of similarities in gene order between rice and wheat (Kurata *et al.* 1994, Van Deynze *et al.* 1995, Gale and Devos 1998), there is a frequent disruption in the synteny especially for disease resistance gene due to transposition events, translocation, inversions, and duplication (Bellgard *et al.* 2004). Most of homoeology for gene order between rice and

Triticeae has been found for 'house keeping genes' which have been conserved since these species have diverged.

La Rota and Sorrells (2004) did comparative DNA sequence analysis between wheat and rice and reported that numerous discontinuities exists in gene order between wheat and rice that would complicate the transfer of information and markers between these species. Comparative analysis of the *Tsn1* locus, which provides resistance against Tan spot caused by fungal pathogen *Pyrenophora tritici-repentis* (Died.) Drechs, on wheat chromosome 5B with rice showed that very low levels of colinearity exists between wheat group 5 chromosomes and rice (Haen *et al.* 2004).

Conclusion

Three DNA markers were found to be homologous with rice BAC/PACs from rice chromosome 1 (R1) and these BAC/PACs were aligned in the same order as markers in *Dn7* linkage map. Therefore 1RS region containing *Dn7* showed homology with rice genome but this homology was with rice chromosome 1 and not with rice chromosomes 5 and 10 which are known to be homoeologous with group 1 chromosome of Triticeae. Duplication and translocation events could be the possible reasons for finding homology with non homologous rice chromosome 1. The rice BAC/PACs from rice chromosome 1 showing homology with the wheat DNA markers were used to do BLASTn searches with the wheat EST database. Homologous wheat ESTs from group 1S chromosome were tested for their linkage with *Dn7*. Two wheat ESTs showed polymorphism between the *Dn7* parents and the respective resistant and susceptible bulks, however, no linkage with *Dn7* was observed when those ESTs were screened for *Dn7* population. Problems associated with experimental materials like errors in developing RILs or error in developing bulk segregants used in mapping cannot be ruled out in not finding linkage with *Dn7*. To rule out any experimental error, these EST markers will be tested again for linkage on a new *Dn7* F_2 population currently being developed in our lab. New resistant and susceptible bulks will be developed based on the RWA phenotypic score as well as on PCR-based markers developed for *Dn7* to test these markers.

Table 2.1. Clones mapped to Dn7 showing homology with Rice BAC/PACs

Clone	Rice BAC/PAC	E-value	Rice chromosome
Xksud14	OSJNBa009K04	3.00E-68	1
	Ba0064H09	1.40E-55	11
	OSJNBb0060M15	1.70E-21	4
	P0666D11	7.50E-16	8
	P0456E06	6.00E-15	6
BE403717	OJ1112_H11	8.00E-41	6
	P0435E12	1.00E-19	2
	P0043B10	5.00E-13	1
Xscb241	P0506C07	1.00E-73	7
	OSJNBa0051B13	2.00E-66	11
	OSJNBa0082I13	9.00E-61	4
	OSJNBa0027N19	4.00E-52	5
	P0011G08	E-20	1
Xmwg2062	OJ1008_D04	1.00E-16	5
	OSJNBa0069D17	5.00E-16	4
	OSJNBa0040E17	e-14	10
BF475048	OSJNBa0082N20	e-31	11
	OSJNBa0060B20	3.00E-29	4
	OSJNBb0076h04	5.00E-13	10

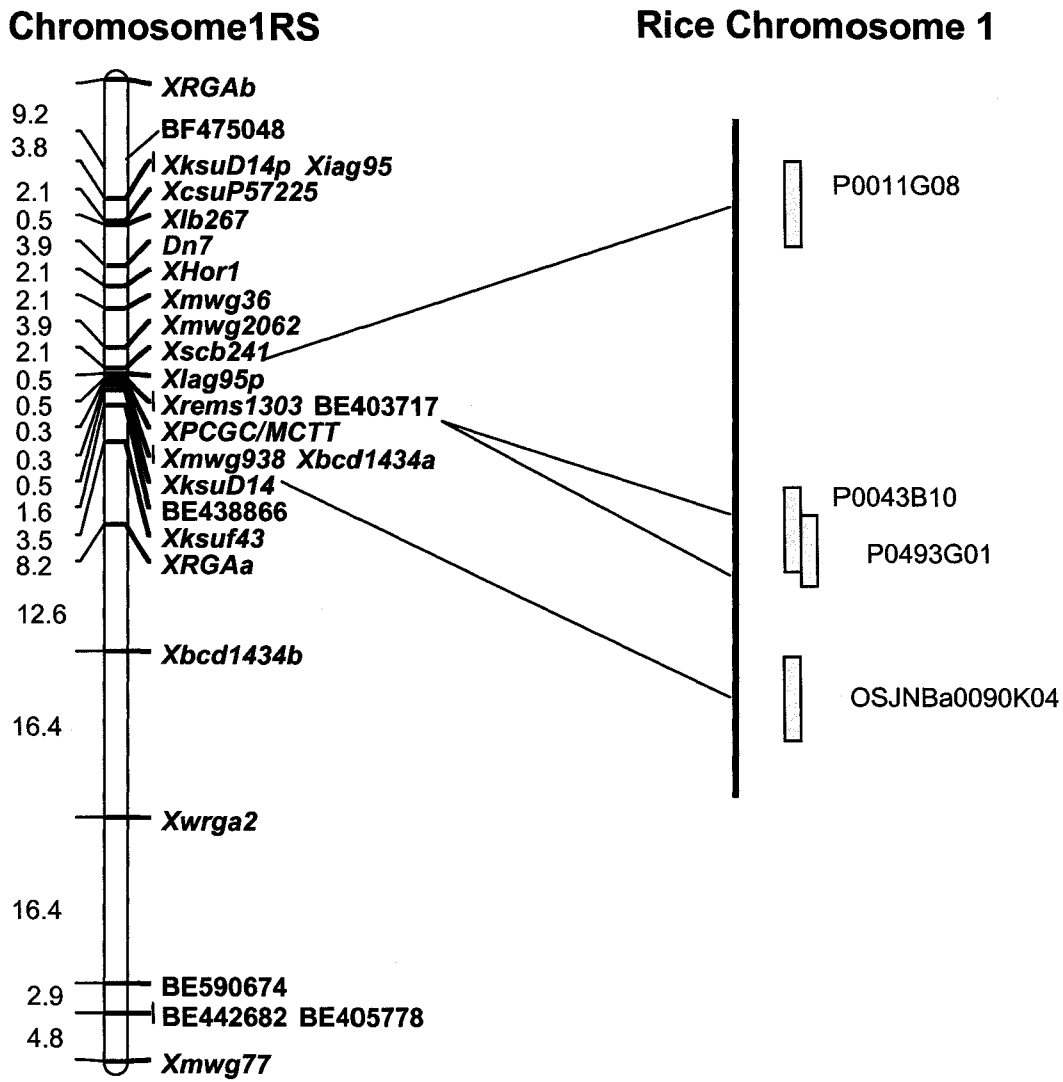
Table 2.2. Rice BAC/PACs from chromosome 1 showing homology with wheat ESTs from group 1 homoeologous chromosomes

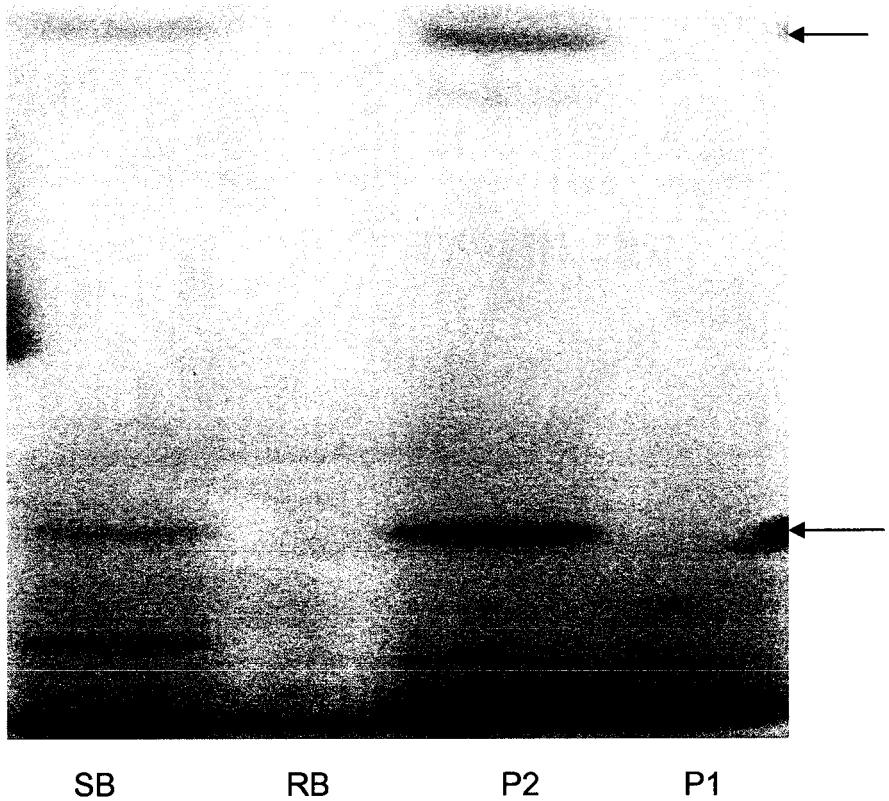
Rice BAC/PACs	Wheat ESTs	Evalue	Wheat Chromosome	Score
P0043B10	BE444890	7.00E-12	1AS 1BS 1DS 4BS	75.8
	BE403717	4.00E-10	1AS 1BS 1DS 7BL	69.9
	BE404135	1.00E-35	5AS 5BS 5DS	155
10P20		≥e-10		
P0493G01	BE444890	7.00E-12	1AS 1BS 1DS 4BS	75.8
	BE403717	4.00E-10	1AS 1BS 1DS 7BL	69.9
	BE404135	1.00E-35	5AS 5BS 5DS	155
B1039D07	BE498820	2.00E-15	1AS 1BS	87.7
B1045D11	BE490765	2.00E-11	3BS 3DS	73.8
B1111C09	BF200791	2.00E-15	1AS 1BS 1DS	87.7
	BE405667	5.00E-68	5D	262
P0416G11	BM138635	2.00E-46	3AL 3BL 3DL	190
B1150E06		≥e-10		
B1096D03	BF474251	9.00E-61	4BL 4DL 5AL	238
OSJNBa0091E23	BF474251	9.00E-61	4BL 4DL 5AL	238
OSJNBb0093M23	BE490153	7.00E-30	4AL 4DS	135
P0676G08	BE405187	2.00E-15	1AS 3DS	87.7
OSJNBb0008D07	BG274402	3.00E-32	4AL 4DS	143
OJ1123_G09	BE405509	8.00E-23	3AS 3BS 3DS	111
OJ1460_H08	BE405509	8.00E-23	3AS 3BS 3DS	111
B1109A06		≥e-10		
OJ1058_D04		≥e-10		
B1061G08		≥e-10		
OSJNBa0093G23	BF292158	2.00E-38	1AS 1DS 7AL	163
OSJNBb0063C17		≥e-10		
OSJNBa0088J04		≥e-10		
OSJNBa0029L04	BF292158	2.00E-36	1AS 1DS 7AL	157
OJ1126_G08		≥e-10		
B1111E11	BF482436	1.00E-22	3AS 3BS	111
B1080D07	BF482462	5.00E-22	3AS 3DL	109
P0507H06	BG605575	1e-99	7AS 7BS 7DS	367
P0436D06	BF473020	7.00E-74	4AL 4BL 4DL	281
OSJNBa0094H06	BF474859	2.00E-15	3BS 3DS	87.7
P0455H03	BF473020	7.00E-74	4AL 4BL 4DL	281
P0520B06	BE443797	2.00E-52	1AS 1BS 1DS	281
	BF473020	5.00E-74	4AL 4BL 4DL	281
OSJNBa0054L14	BE443797	2.00E-52	1AS 1BS 1DS	210
P0504D03	BE443862	3.00E-26	3BS 3DS	123
B1075D06	BE494515	4.00E-25	5AL 5BL 5DL	119
OSJNBb0016A06	BM136947	5.00E-09	7AL 7BL 7DL	65.9
B1108H10	BE498242	4.00E-10	6AS 6BS	69.9

B1003B09		≥e-10		
B1168H06		≥e-10		
P0516D04	BI479637	e-107	3BL	391
OJ1005_B10	BE494280	3.00E-23	2DL	113
B1249E06	BE494280	3.00E-23	2DL	113
B1147B04	BM138225	8.00E-73	3BS 3DS	278
B1074C08	BE443401	1.00E-35	1AS 1BS 6AS 6BS 6DS 7AS 7DS	155
	BM138225	1.00E-72	3BS 3DS	278
B1129H01	BE443401	6.00E-36	1AS 1BS 6AS 6BS 6DS 7AS 7DS	155
P0702H08	BF145855	9.00E-33	3DL	145
OJ1619_F12	BE445154	7.00E-33	3BS	145
OJ1029_F04	BE591684	4.00E-81	3DS	305
OSJNBa0086A10	BE591684	4.00E-81	3DS	305
P0014E04		≥e-10		
B1166B08		≥e-10		
OSJNBa0047D12		≥e-10		
OSJNBa0062A24		≥e-10		
OSJNBa0065J17	BE500072	1.00E-31	3BL	141
OSJNBa0051H17	BE490486	8.00E-12	1BS	75.8
	BG262696	e-115	3DS	418
OSJNBa0026J14	BE490486	7.00E-12	1BS	75.8
	BE442854	3.00E-26	2BS 5DL	123
OSJNBa0042P21		≥e-10		
B1156H12	BE495066	e-107	3AL 3BL 3DL	392
OSJNBa0024F24	BE404899	6.00E-71	7AS 7BS 7DS	272
	BE406903	4.00E-35	3BS 3DL	153
OSJNBa0066C06		≥e-10		
B1110C07		≥e-10		
OJ1014_G12	BG262295	2.00E-74	3BS 3DS	283
B1144D11	BE443276	5.00E-59	3AS 3BS 3DS	232
B1064G04	BE405599	1.00E-84	3AL 3BL 3DL	317
P0496H05		≥e-10		
P0468H06		≥e-10		
OSJNBb0049O23	BE405854	2.00E-17	1AL 1BL 1DL	93.7
P0697C12	BE405854	2.00E-17	1AL 1BL 1DL	93.7
P0002B05		≥e-10		
B1114B07	BE591527	e-175	3AL 3BL 3DL	618
B1129G05	BF483989	3.00E-23	1BS 3AS 3BS	113
B1151A10	BF483989	2.00E-23	1BS 3AS 3BS	113
B1112D09	BG274556	5.00E-34	3AL 3BL 3DL	149
P0022F12	BE517872	9.00E-48	2AS 2DS	194
P0013G02	BE517872	9.00E-48	2AS 2DS	194
P0672C09	BE591590	2.00E-42	3AL 3BL 3DL	176
B1097D05	BF428637	e-178	3BL 3DL	626
OSJNBb0024F06	BM135339	1.00E-31	3AS 3BS 3DS	141
P0712E02	BE517681	1.00E-16	3BS	91.7
P0700A11	BE444380	1.00E-62	3DL	244

P0415C01	BE499982	1.00E-50	3AL 3BL 3DL	204
P0710A02	BF145332	4.00E-44	3AL 3BL 3DL	182
P0451D05	BF292654	3.00E-26	3BL 3DL	123
B1103C09	BF292654	3.00E-26	3BL 3DL	123
P0025A05	BE442805	7.00E-52	3AL 3BL 3DL	208
P0518F01	BG263929	5.00E-25	1BS 3BL 6AS	119
	BE490584	1.00E-71	1AL 5BL 6AS 6BS 6DS	274
	BE442805	7.00E-52	3AL 3BL 3DL	208
P0681B11	BE497804	e-142	3DS	509
P0704D04	BE494807	3.00E-88	3BS	329
OSJNBa0090K04	BE494807	3.00E-88	3BS	329

Figure 2.1. Markers present on short arm of rye chromosome 1R showing homology with rice BAC/PACs from rice chromosome 1





P1- '94M370' , P2- 'Gamtoos', RB- Resistant Bulk SB- Susceptible bulk

Figure 2.2: Oligonucleotide primers developed from sequence of wheat EST BF292158 showing polymorphism between *Dn7* parents and their respective bulks.

Chapter 3

In vitro reaction of wheat to extracts of Russian wheat aphid biotypes 1 & 2

Abstract

The Russian wheat aphid (*Diuraphis noxia*, RWA) is a serious economic pest of wheat and other small grain crops. Several new RWA biotypes have been reported in North America since spring 2003. Rapid and efficient screening methods for RWA resistance are required in order to find new sources of resistance and to develop resistant cultivars. The objective of this study was to develop a simple and efficient bioassay for RWA resistance, which may lead to the identification of elicitors responsible for expression of resistance in RWA resistant cultivars. Cut etiolated seedlings of RWA resistant and susceptible cultivars were treated with crude extract, saliva, proteins, and non protein compounds of RWA biotypes 1 and 2. Crude extract of RWA biotype 1 significantly inhibited leaf unrolling of susceptible cultivar 'Gamtoos' whereas it had no significant effect on two other susceptible cultivars 'Carson' and 'Synthetic Hexaploid'. Crude extract of RWA biotype 2 significantly inhibited leaf unrolling of 'Carson' and 'Synthetic Hexaploid' but not of 'Gamtoos'. The data obtained from RWA saliva, proteins and non protein compounds were generally uninformative as few significant differences were observed between treatments and control. One possible reason for discrepancy in the results could be due to the genetic background effect. Role of intensity of light (illumination) cannot be ruled out in certain discrepancies in results. Contact of RWA crude extracts and proteins with the leaf surface might not be enough for development of RWA symptoms. *In vivo* assay in which RWA extracts and proteins are injected into the leaves of plants might provide with better assay for RWA resistance screening.

Introduction

The Russian wheat aphid (*Diuraphis noxia*, RWA) is a serious pest of cereals in numerous wheat producing countries. RWA was introduced in USA in 1986 and since then has caused economic damage of nearly $1 billion (Legg and Amosson 1993; Webster *et al.* 2000). RWA usually infests young plants and causes leaf rolling, streaking, head trapping, and even death of susceptible plants. Control of RWA by insecticides is expensive and generally, topical spray of insecticides is not effective as the leaves on which aphids feed get rolled and this prevents direct contact of insecticides with the aphids (Miller *et al.* 2001). The use of resistant cultivars is an efficient, economical and environmentally safe method to protect wheat from losses by RWA (Liu *et al.* 2002).

Several sources of resistance to RWA have been found within hexaploid wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), goat grass (*Aegilops cylindrica)* and 11 RWA resistance genes have been identified (Du Toit, 1987; Nkongolo *et al.* 1991a,b; Marais and Du Toit 1993; Saidi and Quick 1996; Zhang *et al.* 1998; Liu at al 2001; Smith *et al.* 2004). RWA resistance gene *Dn4,* present on short arm of wheat chromosome 1D, is a dominant gene and originated from Russian bread wheat accession PI372129 (Ma *et al.* 1998). *Dn4* has been deployed by breeders in Colorado for developing RWA resistant cultivars and is in all except one resistant cultivar grown in Colorado (Haley *et al.* 2004). *Dn7* is a dominant gene from rye and was introduced into a wheat background by 1RS.1BL translocation (Marais *et al.* 1994). *Dn7* confers a

superior level of resistance to biotype 1 (Anderson *et al.* 2003) and biotype 2 (Haley *et al.* 2004).

Until the spring of 2003, there was only one RWA biotype (Biotype 1) known in the USA, but since then several new biotypes have been found which are virulent to RWA resistance gene, *Dn4.* Fast development of new RWA resistant cultivars has become a high priority for plant breeders and for this there is an urgent requirement of fast and efficient screening methods for RWA so that new germplasm can be screened for finding new resistant sources or genes which can then be incorporated into agronomically well adapted but susceptible cultivars.

Greenhouse seedling screening has been widely used to test RWA resistance, but high temperatures during the summer months prohibit such screening due to intolerance of RWA to high temperature. There is an increased mortality rate of RWA in temperatures above 20°C (Michels and Behle 1998). Therefore, a simple lab screening method is needed to solve this problem. Rapid screening methods will save time, effort, and greenhouse space for screening of seedlings for resistance even during the time of the year when aphids are not available for greenhouse screening.

Identification of plant as well as insect elicitors can lead to the development of easy and efficient screening for RWA. Elicitors are molecules that are able to induce physiological or biochemical responses associated with the expression of resistance (Kogel *et al.* 1998).

Dong *et al.* (1994) used crude aphid extracts to test *in vitro* responses of three wheat genotypes which included one susceptible ('Carson') and two RWA resistant (CORWA 1, and PI 294994) genotypes. Crude extracts of RWA biotype 1 inhibited leaf

unrolling in the susceptible genotype but not in RWA resistant genotypes. Brigham (1992) also reported that extracts from homogenized RWA showed very strong inhibition of leaf segment unrolling response. Both studies were done with RWA biotype 1 which was the only RWA biotype present at that time. In spring 2003, RWA biotype 2 was reported from Colorado and adjacent states, which was found to be virulent to all RWA resistance genes except for *Dn7* (Haley *et al.* 2004). In the present work, RWA biotypes 1 and 2 were used to develop a bioassay for efficient and easy screening of RWA resistance. RWA biotypes 1 and 2 were used for this study as resistance genes for both biotypes are known.

The objective of this study was to develop a simple and efficient bioassay for RWA resistance, which may lead to the identification of elicitors responsible for expression of resistance in RWA resistant cultivars.

Materials and methods

RWA biotypes

RWA biotypes 1 and 2 have been separated and maintained in different greenhouses by Jeff Rudolph, Department of Bioagricultural Sciences and Pest Management, Colorado State University. A mixture of susceptible wheat and barley was grown in small pots (10 cm diameter) for aphid colony maintenance.

RWA crude extract

RWAs were collected from the greenhouse just before use, immediately frozen in liquid nitrogen, and then ground to powder using a pre-chilled mortar and pestle. Potassium phosphate buffer (KH_2PO_4, 0.025 M, pH 6.8) was added with an aphid/buffer ratio of 1 g/10 ml in a mortar. Additionally, live aphids were ground in buffer as described by Dong *et al.* (1994). After a preliminary *in vitro* assay, no differences were found between whole extracts from ground live or frozen ground aphids, and I proceeded with the first protocol since grinding frozen aphids was easier than grinding fresh aphids. The buffer-aphid homogenate was centrifuged at 1000 x g for 15 min at 4°C. The supernatant was transferred to a flask and further diluted to 100 ml/1 g live aphids for *in vitro* assay by addition of KH_2PO_4 buffer. The buffer was used as a blank control against the whole extract.

RWA proteins

RWAs were frozen in liquid nitrogen, ground, and then homogenized in buffer KH_2PO_4 (0.1M pH 7.5) buffer as described in Ni *et al.* (2000). After 10 min incubation on ice, the homogenate was centrifuged at 10,000 x g for 15 min. The supernatant was transferred into a flask and crude proteins were precipitated by addition of 56.8 g ammonium sulfate/ 100 ml protein solution following the protocol provided by Coligan *et al.* (2003). The solution was centrifuged at 10,000 x g for 10 min, and supernatant was discarded. The pellet was resuspended in 1-2 pellet volumes of the 0.1M KH_2PO_4 buffer. Any insoluble materials were removed by centrifugation at 10,000 x g for 5 min, while the ammonium sulfate was removed by using an Amicon® Ultra-15 Centrifugal Filter Device. The filtered protein solution (~ 200 µl) was diluted by adding the 0.025 M KH_2PO_4 to a final volume of 100 ml/1.25 g fresh aphids for *in vitro* assay. All steps were performed at 4°C. The average protein concentrations after 1.25:100 dilution were 1.039 and 0.997 mg/ml for RWA biotypes1 and 2, respectively. The blank control for RWA proteins was buffer-only which went through the whole procedure as described for protein precipitation, salt removal, and dilution.

RWA non-protein water-soluble compounds

After isolating the whole extract from the RWAs as mentioned above, the solution of whole extract containing both water-soluble proteins and other compounds was incubated with 50 µg/ml proteinase K (Promega) at 50°C for one hour, and boiled for 15 min to denature the proteinase K and any uncatalyzed proteins. The final volumes were

100 ml/1 g fresh aphids for *in vitro* assay. The 0.025 M KH_2PO_4 buffer-only treated with proteinase K and heat was used as a blank control for non-protein compounds.

RWA saliva

RWA saliva was collected in the lid of a 150 × 15 mm petri dish, to provide a wide surface for aphid feeding. The shallow depth of these Petri dishes also allowed collecting more concentrated saliva. Distilled water was added into the lid which was then sealed with Parafilm "M" (Pechiney Plastic Packaging). Each dish was tightly enclosed with Whatman Filter paper covered with Aluminum foil to prevent escape of the aphids. Live RWAs were placed on top of the parafilm. Once the RWA punctured the parafilm, their saliva was released into the water. After overnight incubation (10-12 hrs) of aphids at room temperature, the water was collected in a beaker for measurement of UV absorption at A_{280}. The average protein concentrations were 0.460 and 0.378 mg/ml for RWA biotype 1 and 2, respectively. The saliva was immediately and directly used for *in vitro* assay. Water was used as blank control for saliva.

Plant materials

The following wheat genotypes were used: 'Carson', 'Synthetic Hexaploid' (S. Hex), 'Gamtoos', which are all susceptible to RWA, and 'Halt' and '94M370', both resistant to RWA. 'Halt' contains the *Dn4* resistance gene, which confers resistance to RWA biotype 1, but is susceptible to RWA biotype 2. '94M370' contains the *Dn7* resistance gene, which confers resistance to both RWA biotypes.

Etiolated seedlings

Seeds were germinated in large petri dishes and grown in the dark at room temperature in the laboratory. After the etiolated seedlings grew to about 10 cm tall, they were used for the *in vitro* assay.

In vitro assay

The experiment was conducted in 60 × 15 mm Petri dishes. Each petri dish contained 5 ml 0.025M KH_2PO_4 buffer control or the buffer with one of the following: RWA whole extract, proteins, and non-protein compounds. The first leaves of etiolated seedlings were cut with a pair of small scissors from just above the coleoptile. Five leaf segments 1.5 to 2.0 cm long were put into a petri dish with the solution or control buffer. Each treatment was repeated five times. These petri dishes were placed on a rotating platform under two 40 W fluorescent light apparatus for about 8 hours at room temperature. The leaf segments were scored according to four degrees of unrolling: 1- completely unrolled (flat), 2- partially unrolled but towards to 1, 3- partially unrolled but towards no unrolling, 4- no unrolling or tightly rolled.

Data analysis

Data accumulated from *in vitro* assays were analyzed as follows: (1) Differences between each treatment and its blank control was analyzed using the nonparametric Kruskal-Wallis ANOVA test (Conover 1980), since the leaf unrolling scores did not show a normal distribution and the experimental design was completely random. (2)

Since different treatments used different blank buffers, data for effects of different treatments and from different investigation dates were not comparable. We therefore standardized the data of leaf unrolling scores as follows:

$$\Delta X_n = X_n - \bar{a}$$

where sample number n = 1, 2, 3.....n, X_n was the observed leaf unrolling score for the n^{th} leaf segment, and $\bar{a}$ was the average leaf-unrolling score of the blank control. Therefore, ΔX_n was the net effect of a treatment on the leaf unrolling of the n^{th} leaf segment. The standardized data were then comparable since the effects of different buffers and possible differences due to temperature or light conditions for different dates had been removed. Positive values indicated inhibition of leaf unrolling compared to its blank control, while the negative values indicated an increase of leaf unrolling over the blank control. Based on the standardized data, ANOVA was used to analyze effects of RWA biotypes, RWA extracts, wheat genotypes, and their interaction. Duncan's multiple range method was used for multiple comparisons of means within each source of effect.

Results

Effects of crude RWA extract on leaf unrolling

Crude extract from RWA biotype 1 significantly inhibited the leaf unrolling of 'Gamtoos' (Kruskal-Wallis ANOVA test: $H_{(1,N=31)} = 4.466$, $P < 0.05$) (Figure 3.1a & Table 3.1). Crude extract from RWA biotype 2 significantly inhibited leaf unrolling of 'Carson' (Kruskal-Wallis ANOVA test: $H_{(1,N=45)} = 14.918$, $P < 0.0005$), and 'Synthetic Hexaploid' ($H_{(1,N=50)} = 10.120$, $P < 0.005$), but has significant leaf unrolling in 'Halt' ($H_{(1,N=40)} = 10.820$, $P < 0.005$) as compared to the control treatment.

Effects of RWA saliva on leaf unrolling

RWA saliva of both biotypes was not found to be very informative in this study. The saliva significantly inhibited leaf unrolling for 'Carson' (RWA-biotype 1: $H_{(1, N = 48)} = 6.877$; $P < 0.01$; RWA-biotype2: $H_{(1, N = 48)} = 4.237$, $P < 0.05$). Saliva of both biotypes did not significantly affect the leaf unrolling for other genotypes (Figure3.1b & Table 3.1).

Effect of RWA proteins

Effects of RWA proteins were not different according to resistant and susceptible genotypes, but varied between the biotypes (Figure 3.1c & Table 3.1). Proteins from RWA-biotype 1 significantly increased leaf unrolling of S. Hex ($H_{(1, N = 42)} = 5.964$, $P < 0.05$); but inhibited the leaf unrolling of susceptible 'Gamtoos' ($H_{(1, N = 41)} = 5.561$, $P <$

0.05). Proteins from RWA biotype 2 significantly increased leaf unrolling of 'Carson' ($H_{(1, N = 50)} = 6.314$, $P < 0.05$) and '94M370' ($H_{(1, N = 31)} = 3.914$, $P < 0.05$).

Effect of RWA non-protein compounds

Non-protein compounds of RWA biotype 1 showed no significant effects on all genotypes. Non-protein compounds of RWA biotype 2 showed significant unrolling effect on 'Carson' ($H_{(1, N = 50)} = 6.344$, $P < 0.05$) and 'Halt' ($H_{(1, N = 44)} = 3.877$, $P < 0.05$) as compared to the control treatment (Figure 3.1d & Table 3.1).

RWA biotype effects

Biotype effects of non-protein compounds were significantly different ($F_{1, 208} = 3.942$; $P < 0.01$), particularly on 'Carson' (Table 3.1). The largest biotype effects were found between biotype proteins ($F_{1, 208} = 19.04$; $P < 0.00005$, Table 3.1).

Genotype effects

Genotype effects on the etiolated leaf unrolling were significant under all treatments including RWA saliva, whole extract, compounds and proteins (Table 3.1). With the treatments of saliva and whole extract, in general, the susceptible genotypes showed a tendency for inhibition of leaf unrolling. In the resistant genotypes, there was no inhibition of leaf unrolling, although there were differences between RWA biotypes. This result indicates that the resistance genes (*Dn4* and *Dn7*) could overcome inhibition from RWA saliva or whole extract.

Wheat genotype x RWA biotypes

Significant interaction effects were observed for whole extract treatment ($F_{4, 221}$ = 24.724; $P < 0.00005$) and RWA protein treatment ($F_{4, 208}$ = 6.153; $P < 0.001$, Table 3.1). The largest effects of whole extract occurred in 'Gamtoos' (0.487) for RWA biotype 1, and in 'Carson' (0.870) and 'Halt' (-0.747) for RWA biotype2.

Discussion

In an effort to develop an *in vitro* bioassay for RWA biotypes 1 and 2, RWA crude extract, RWA proteins, RWA saliva and RWA compounds lacking proteins were used to treat etiolated seedlings of 5 wheat genotypes, both resistant and susceptible to RWA. The data obtained from the study were generally uninformative as few significant differences were observed between treatments and control. However, crude extract of RWA biotype 1 has been reported to have a significant effect on unrolling of etiolated seedlings of the RWA susceptible genotype but no effect on resistant genotypes (Dong *et al.* 1994; Brigham 1992). One possible reason for discrepancy in the results could be due to the genetic background effect. The genetic background in which the resistance genes are present plays a role in the effectiveness of the resistance response (van der Westhuizen *et al.* 1998). Role of intensity of light (illumination) cannot be ruled out in certain discrepancies in results. Brigham (1992) used four 40 W fluorescent light apparatus for 18 hours, whereas we used two 40 W fluorescent light apparatus for 8 hours Macedo *et al.* (2003) reported that development of RWA feeding damage symptoms on susceptible wheat seedlings is a light-activated process.

It has been hypothesized that damage symptoms from feeding by piercing – sucking insects are caused by the injection of salivary phytotoxins into plants (Miles, 1987; Burd *et al.* 1998). Campbell (1986) and Miles (1990) considered pectinases and cellulases as important salivary enzymes contributing to greenbug damage symptom formation on wheat. In this study *in vitro* studies with saliva from both biotypes 1& 2 significantly inhibited leaf unrolling in only one susceptible cultivar 'Carson'. The use of

aphid saliva for bioassay studies does not look promising from this study. It could possibly be due to the lower concentrations of the functional components in RWA saliva than other extracts. Because of dilution the enzymes present in the aphid saliva could have become less effective in inhibiting leaf unrolling in susceptible cultivars.

The results of the present study indicate that crude extract of both biotypes generally inhibited leaf unrolling in susceptible genotypes. But crude extract of RWA biotype 2 didn't inhibit leaf unrolling in cultivar 'Halt' which is susceptible to RWA biotype 2 and resistant to RWA biotype 1. In subsequent studies (Li *et al.* Personnel communication), when crude extract from RWA biotype 2 was injected into leaves of 'Halt' plants, the leaves showed rolling symptoms just as shown by leaves infested by live aphids. This suggests that possible elicitor which is responsible for producing leaf rolling is effective only when injected into 'Halt' plants and not when applied on the leaf surface.

Similarly, when RWA proteins were used as a treatment, the effect of RWA protein was not significant among the resistant and susceptible genotypes. Proteins from RWA biotype 1 inhibited leaf unrolling not only of RWA susceptible genotype 'Gamtoos', but also of RWA resistant genotype '94M370'. Inhibition of unrolling of '94M370' etiolated leaves by proteins extract from RWA biotype 1 is hard to explain but it could be due to low light intensity or could be due to very high concentration of proteins. However, if proteins were highly concentrated, inhibition of unrolling for all of the genotypes should have been observed. More work needs be done to establish standards for concentration of different treatments like aphid saliva and proteins and also

of intensity of light before an effective *in vitro* assay for RWA screening can be developed.

Subsequent *in vivo* bioassay studies done by Li *et al.* (personnel communication) using crude extract, proteins and non-protein compounds of RWA biotypes 1 and 2, have shown encouraging results. Injection of crude extract of both biotypes induced leaf rolling in plants of wheat cultivar 'Gamtoos'. Similarly proteins and non protein compounds induced leaf rolling in 'Gamtoos', but did not have any effect on RWA resistant cultivar '94M370'. Also after injection of RWA crude extract and proteins, there was a significant increase in activity of defense related enzymes (catalases, peroxidases and β-glucanases) in '94M370' than in 'Gamtoos'. Injection of RWA crude extract and proteins into plants appears to be a better method in developing an assay for RWA resistance screening than *in vitro* method. This could be due to the reason that in *in vivo* method, there is a direct contact of RWA extract and proteins with the plant metabolites which leads to either a resistant or susceptible reaction. In *in vitro* method, only contact of RWA extract and proteins is with the outer surface of leaves and maybe it may not be sufficient in inducing the resistance or susceptible reactions which will lead to the presence or absence of the RWA symptoms.

Although no potential elicitors from RWA have been identified until now, glycoproteins isolated from the intercellular wash fluid of the resistant plants infested with RWA were considered to be potential elicitors of plant origins (Mohase and van der Westhuizen 2002). For insects any compound of the saliva or gut contents of insects which can interact with a plant at a cellular level is a potential elicitor (Tjallingii 1995; Miles 1999), such as lytic enzymes (Felton & Eichenseer 1999; Funk 2001), fatty-acid-

amino-acid conjugates in regurgitant (Halitschke *et al.* 2001). In case of pathogens, proteins secreted by the pathogens or the compounds of the cell wall released by the action of the microbial enzymes can set up defense response in plants (Nurnberger 1999; Boiler 1995).

Conclusion

Crude extract of RWA biotype 1 inhibited leaf unrolling of susceptible cultivar 'Gamtoos', but had no significant effect on leaf unrolling of other susceptible cultivars 'Synthetic Hexaploid' and 'Carson'. Crude extract of RWA biotype 2 had significant effect on leaf unrolling of 'Synthetic Hexaploid' and 'Carson' but did not inhibit leaf unrolling of 'Gamtoos' and 'Halt'. The data obtained from RWA saliva, proteins and non protein compounds were generally uninformative as few significant differences were observed between treatments and control. The genetic background effect and illumination effects could be the possible reasons for getting uninformative results. More work is required to set standards for developing bioassays for RWA resistance screening. *In vivo* tests which have showed encouraging results for RWA resistance screening could be used to develop bioassay and also for identifying the elicitors responsible for expression of resistance in RWA resistant cultivars.

Table 3.1. **Effects of crude extract, saliva, compounds and proteins isolated from the two RWA biotypes on wheat leaf rolling**

Wheat genotype	Saliva			Crude extract		
	RWA-1	RWA-2	Biotype effect [2]	RWA-1	RWA-2	Biotype effect
Carson	0.292±0.069a	0.167±0.000a	NS	0.120±0.000b	0.870±0.092a	****
S. Hex	0.042±0.095b	0.077±0.110a	NS	0.125±0.092b	0.560±0.117b	**
Gamtoos	0.008±0.056b	0.008±0.098a	NS	0.487±0.175a	0.040±0.101c	**
Halt	-0.167±0.058b	-0.170±0.080b	NS	0.075±0.064b	-0.747±0.095e	****
94M370	-0.046±0.101b	-0.177±0.119b	NS	0.000±0.000b	-0.305±0.114d	*
Mean	0.031±0.037	-0.010±0.041		0.145±0.038	0.084±0.069	
ANOVA summary:						
Biotype	$F_{(1, 208)}$ = 0.692			$F_{(1, 221)}$ = 1.60		
Genotype	$F_{(4, 208)}$ = 7.330****			$F_{(4, 221)}$ = 28.301****		
Genotype x biotype	$F_{(4, 208)}$ = 0.423			$F_{(4, 221)}$ = 24.724****		

Wheat genotype	Compounds			Proteins		
	RWA-1	RWA-2	Biotype effect	RWA-1	RWA-2	Biotype effect
Carson	0.071±0.135	-0.352±0.084b	*	-0.013±0.095b	-0.359±0.092a	NS
S. Hex	0.077±0.166	-0.025±0.142a	NS	-0.381±0.105c	-0.190±0.131a	NS
Gamtoos	0.250±0.103	0.147±0.102a	NS	0.392±0.099a	-0.355±0.126a	****
Halt	-0.030±0.136	-0.333±0.103b	NS	-0.036±0.101b	-0.042±0.112a	NS
94M370	0.110±0.098	-0.170±0.055b	NS	0.270±0.103a	-0.346±0.133a	****
Mean	0.096±0.056	-0.61±0.045		0.062±0.051	-0.246±0.053	
ANOVA summary:						
Biotype	$F_{(1, 215)}$ = 3.942**			$F_{(1, 208)}$ = 19.04****		
Genotype	F(4, 215) = 3.941***			$F_{(4, 208)}$ = 2.568*		
Genotype x biotype	F(4, 215) = 0.742			$F_{(4, 208)}$ = 6.153***		

Note:

1. Within each column, data with the same letter are not significantly different (P>0.05), but those with different letter are significantly different (P<0.05)
2. Duncan' multiple range test: Significance of differences between two RWA biotypes

* P < 0.05
** P < 0.01
*** P < 0.001
**** P < 0.00005
NS = not significant

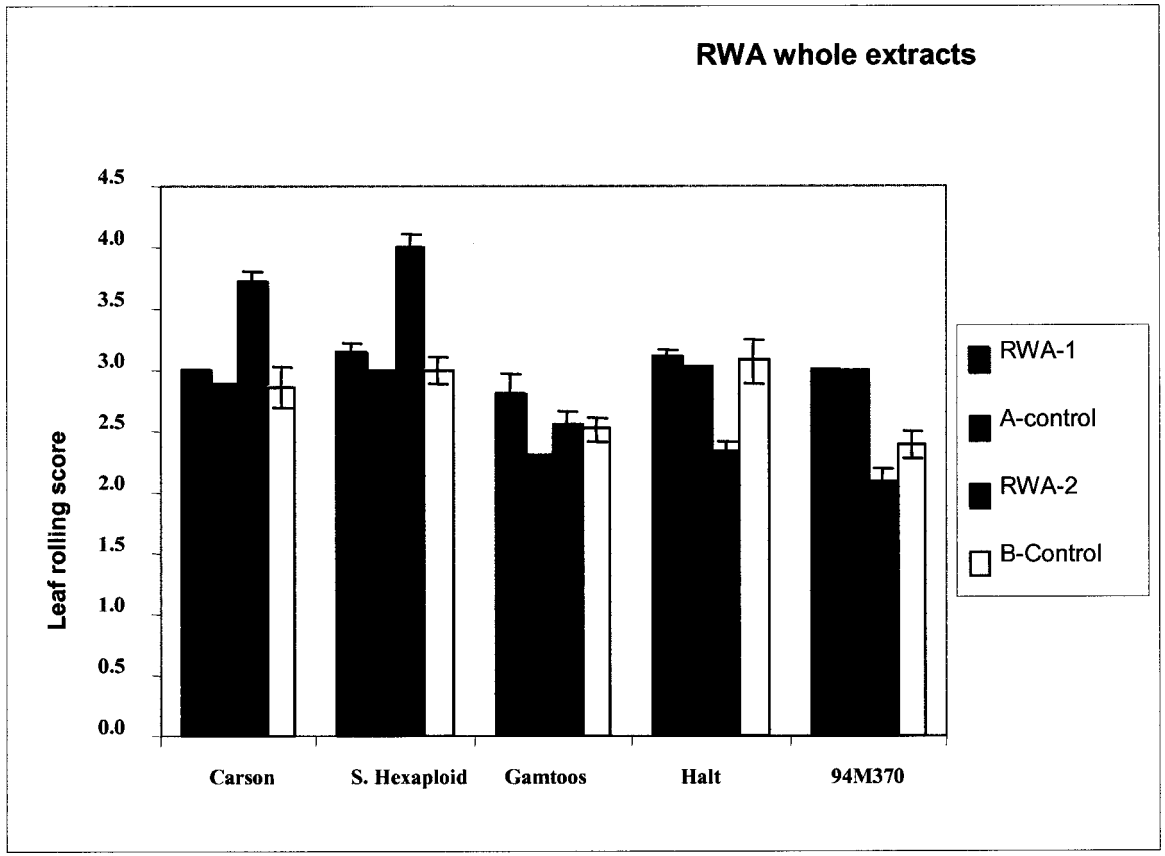


Figure 3.1a: Means (± SE) of the leaf rolling scores of resistant and susceptible wheat genotypes under treatments with RWA whole extracts.

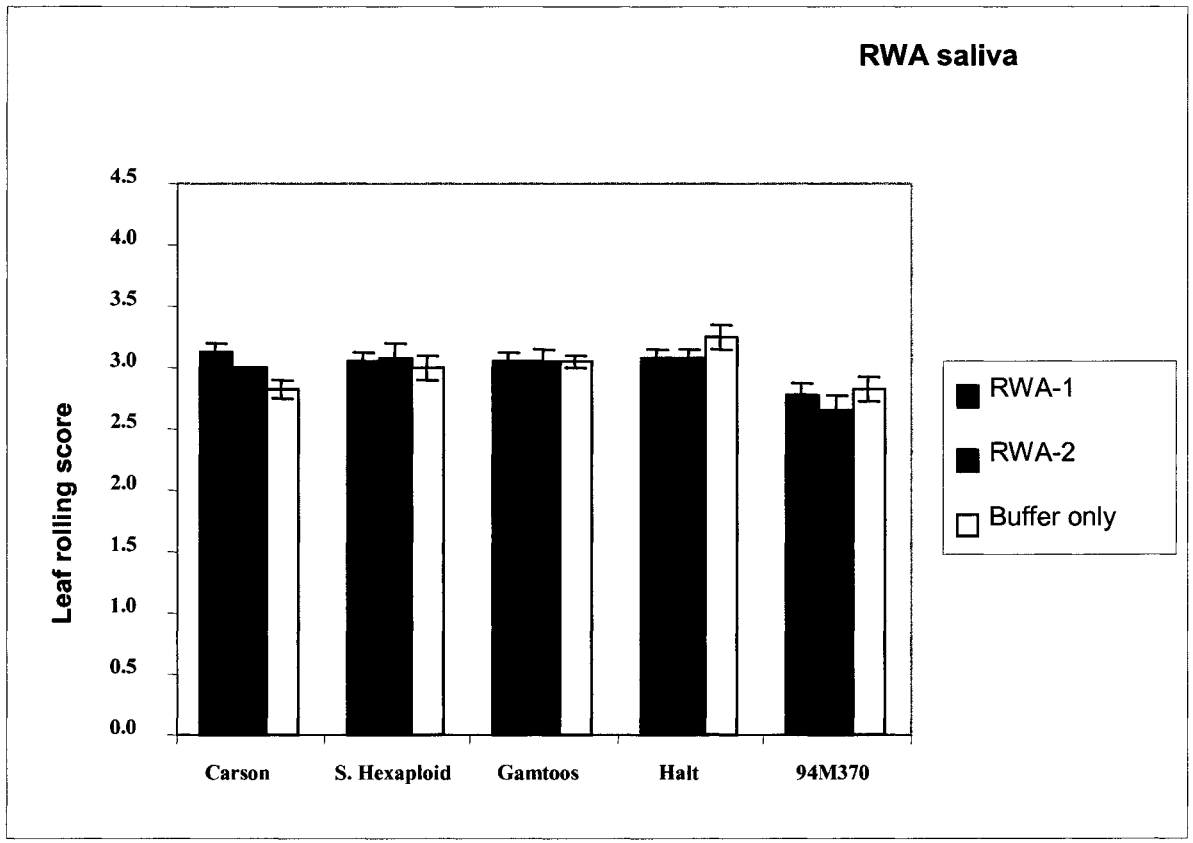


Figure 3.1b: **Means (± SE) of the leaf rolling scores of resistant and susceptible wheat genotypes under treatments with RWA saliva extracts.**

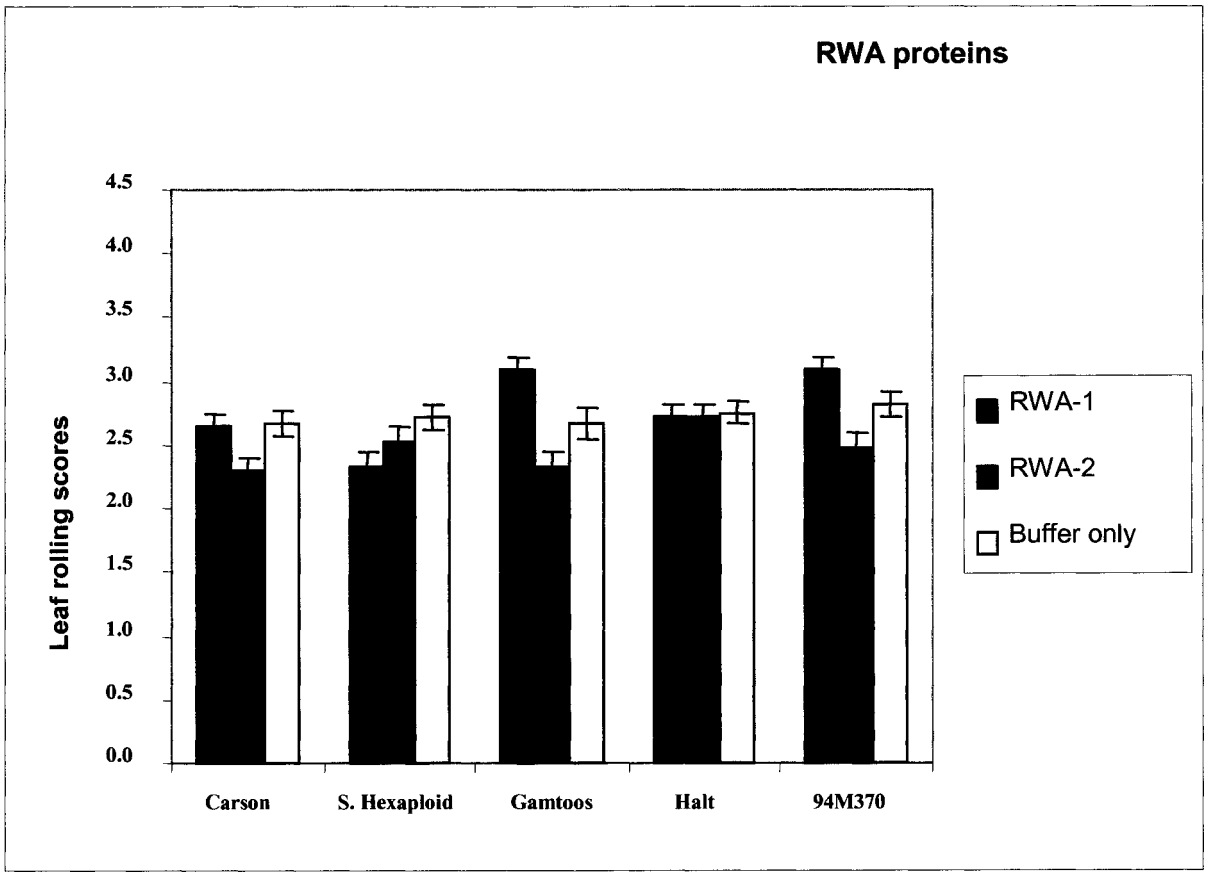


Figure 3.1c: Means (± SE) of the leaf rolling scores of resistant and susceptible wheat genotypes under treatments with RWA proteins extracts.

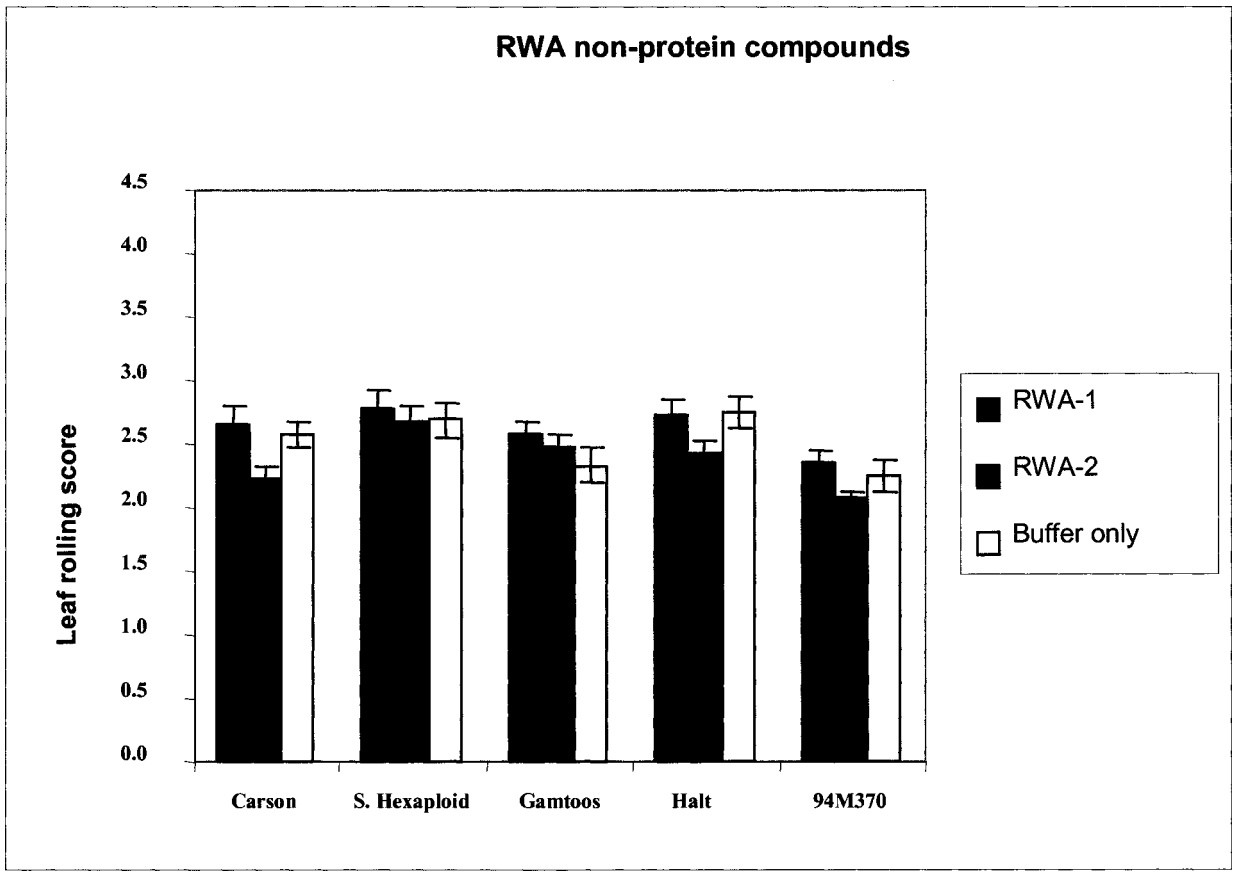


Figure 3.1d. Means (± SE) of the leaf rolling scores of resistant and susceptible wheat genotypes under treatments with RWA non-protein compounds.

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APPENDIX 1: List of markers used for mapping of *Dn7* gene.

S.No.	Marker	Origin	Chromosome Location	Polymorphic Between Parents	Linked To Dn7
1	*Yr10*	RFLP	1A	YES	NO
2	*T+RGA1*	RFLP	1A	YES	NO
3	*Hor1*	RFLP	1H	YES	YES
4	*Xbcd1434*	RFLP	1D	YES	YES
5	*Xksud14*	RFLP	1D	YES	YES
6	BE405778	RFLP	1DS	YES	YES
7	BE498831	RFLP	1DS	YES	YES
8	BE426701	RFLP	1DS	YES	NO
9	*Gliadin*	RFLP	1DS	YES	NO
10	BE403717	RFLP	1DS	YES	YES
11	BG263575	RFLP	1DS	YES	NO
12	BE443071	RFLP	1DS	NO	NO
13	*Xmwg77*	RFLP	1D	YES	YES
14	*Xmwg938*	RFLP	1D	YES	YES
15	*Xmwg 2062*	RFLP	1R	YES	YES
16	*Secalin*	RFLP	1R	NO	NO
17	*Xlag79*	RFLP		NO	NO
18	*Xksuf43*	RFLP	1R	YES	YES
19	BE500570	RFLP	1D	YES	NO
20	*Xksue18*	RFLP	1D	YES	NO
21	*Xmwg837*	RFLP	1R	YES	NO
22	BE403631	RFLP	1D	NO	NO
23	BE444859	RFLP	1D	NO	NO
24	BF474833	RFLP	ID	NO	NO
25	BE500714	RFLP	1D	NO	NO
26	BE426488	RFLP	1D	YES	NO
27	BF473668	RFLP	1D	YES	NO
28	ESTF04	RFLP	1D	YES	YES
29	BE404396	RFLP	ID	YES	NO
30	*Xmwg36*	RFLP	1R	YES	YES
31	*Xlag95*	RFLP	1R	YES	YES
32	BE444846	RFLP	1D	NO	NO
33	BF474677	RFLP	1D	YES	NO
34	*Xabc156*	RFLP	1D	YES	NO
35	BG604768	RFLP	1D	YES	NO
36	BG262410	RFLP	1D	NO	NO

37	BG275046	RFLP	1D	YES	NO
38	BE426257	RFLP	1D	NO	NO
39	*RGA2*	RFLP	1R	YES	YES
40	*Xscb241*	RFLP	1R	YES	YES
41	*Xscb258*	RFLP	1R	NO	NO
42	EST30	RFLP	1D	YES	NO
43	EST31	RFLP	1D	YES	NO
44	EST29	RFLP	1D	YES	NO
45	BE445204	RFLP	1D	YES	NO
46	BE444859	RFLP	1D	NO	NO
47	BE442682	RFLP	1D	YES	YES
48	BE590674	RFLP	1D	YES	YES
49	*Nor(RR1&2)*	RFLP		NO	NO
50	*Xbcd1434*	PCR	1A 1B 1D	YES	NO
51	*Xksud14*	PCR	1A 1B 1D	YES	YES
52	*Xabc156*	PCR	1A 1B	YES	NO
53	*Xmwg337*	PCR	1DS	YES	YES
54	*Sub6*	PCR		YES	NO
55	*XHor1*	PCR	1H	YES	NO
56	*Sub83*	PCR		YES	NO
57	*SSR03*	PCR	1D	YES	NO
58	*Xmwg33*	PCR	1D	NO	NO
59	*Xgwm106*	PCR	1D	NO	NO
60	*Sub52*	PCR		YES	NO
61	*Sub82*	PCR		YES	NO
62	*Xlb267*	PCR	1R	YES	YES
63	*Secalin*	PCR	1R	NO	NO
64	*Xwrga*	PCR	1D	YES	YES
65	*XCM155*	PCR	1R	NO	NO
66	*XCM146*	PCR	1R	YES	NO
67	*XCM94*	PCR	1R	NO	NO
68	*RGA39*	PCR		YES	NO
69	*Xiag95*	PCR	1R	YES	YES
70	*XCM127*	PCR	1R	NO	NO
71	*XCM3*	PCR	1R	NO	NO
72	*XCM1*	PCR	1R	NO	NO
73	*XCM30*	PCR	1R	NO	NO
74	*XCM160*	PCR	1R	NO	NO
75	*XCM21*	PCR	1R	NO	NO
76	*XCM163*	PCR	1R	NO	NO
78	*YR9*	PCR		NO	NO
79	*GLU1*	PCR	1D	NO	NO
80	*Xcwem3*	PCR	1AS	YES	NO
81	*Xcwem6*	PCR	1AS,1BS,1DS	YES	NO
82	*Xcwem8*	PCR	1DS	YES	NO

83	*Xcwem9*	PCR	1BS,1AS	NO	NO
84	*Xcwem10*	PCR	1DS	NO	NO
85	*Xcwem11*	PCR	1DS,1BS	YES	NO
86	*Xcwem12*	PCR	1DS,1AS	NO	NO
87	*Xcwem20*	PCR	1AS	YES	NO
88	*Xcwem25*	PCR	1BS	YES	NO
89	*Xcwem46*	PCR	1DS	YES	NO
90	*Xcwem47*	PCR	1BS	YES	NO
91	*Xcwem54*	PCR	1BS	YES	NO
92	BE590674	PCR	Wheat group1	YES	NO
93	*XKsuk951*	PCR (RGA)	1DS	YES	NO
94	*XKsu946*	PCR (RGA)	1DS	NO	NO
95	BE444890	PCR	Wheat group1	NO	NO
96	BF292158	PCR	Wheat group1	YES	NO
97	BE443401	PCR	Wheat group1	YES	NO
98	BF483989	PCR	Wheat group1	NO	NO
99	BG263929	PCR	Wheat group1	YES	NO
100	BE403717	PCR	Wheat group1	YES	NO
101	BE405778	PCR	Wheat group1	NO	NO
102	BE492937	PCR	Wheat group1	YES	NO
103	BE498831	PCR	Wheat group1	YES	NO
104	BE499561	PCR	Wheat group1	NO	NO
105	BF475048	PCR	Wheat group1	YES	YES