CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS
DEL INSTITUTO POLITECNICO NACIONAL

UNIDAD ZACATENCO

DEPARTAMENTO DE BIOTECNOLOGIA Y BIOINGENIERIA

Molecular mapping of major genes effectives against stem rust-Ug99 in bread wheat

T E S I S

Que presenta

ERIC EUGENIO LÓPEZ VERA

Para obtener el grado de

DOCTOR EN CIENCIAS

EN LA ESPECIALIDAD DE BIOTECNOLOGIA

Directores de la tesis:

Dra. Beatriz Xoconostle Cázares

Dr. Sukhwinder Singh

MEXICO, D.F. FEBRERO, 2014.

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A mi esposa Silvia

A mi hija Liliana

CONTENTS

TABLE INDEX

FIGURE INDEX

RESUMEN

El trigo aporta cerca del 20% de proteínas y carbohidratos en la alimentación humana. Es el tercer cultivo más importante por la superficie sembrada, después del maíz y el arroz. Las enfermedades en trigo disminuyen el rendimiento y por lo tanto el volumen de producción a nivel mundial. Entre las enfermedades, la roya del tallo causada por *Puccinia graminis* pv *tritici* es una de las enfermedades que más afecta la producción de trigo, es un organismo biotrofico que cumple parte de su ciclo asexual en tallos y raíces, en hospederos alternos se lleva a cabo el ciclo sexual del hongo, lo que genera la amplia diversidad para infectar las variedades de trigo. A partir de 1960, la enfermedad causada por la roya del tallo se había mantenido controlada por la utilización de fungicidas y variedades mejoradas; sin embargo, en Uganda se determinó una nueva raza, llamada Ug99 la cual es capaz de infectar plantas con genes de resistencia a roya del tallo. A partir del año 1999, se han encontrado siete nuevas razas que afectan a diferentes variedades de trigo que utilizan materiales que ofrecieron resistencia a la enfermedad. Uno de los esfuerzos por controlar la roya del tallo, es por medio de un programa de mejoramiento genético ayudado por marcadores moleculares. Con el uso de los marcadores moleculares se conoce patrones de bandeo relacionados con un carácter de interés. Se utilizaron marcadores moleculares para conocer la ubicación de regiones de resistencia a roya del tallo en poblaciones F5 de trigo en siete poblaciones F5 desarrolladas por el método de una sola semilla descendiente. Los datos fenotípicos fueron obtenidos de la estación experimental Keniana (KARI) ubicada en Njoro, Kenya. Los marcadores utilizados fueron microsatelites y marcadores polimórficos de un solo nucleótido (SNPs). Para el manejo de los datos se utilizaron los programas ICIMAPPING y MapDisto. Se encontraron regiones de resistencia en 4 poblaciones en el brazo corto del cromosoma 6D, en una población el cromosoma 4A brazo largo y en dos poblaciones más en el cromosoma 2B en los brazos largo y corto. Existen genes reportados previamente en cada uno de los cromosomas que encontramos; sin embargo, la ubicación mostrada por los marcadores moleculares y el tipo de

reacción indican que pudieran tratarse de nuevos genes, aunque se requiere de una prueba de alelísmo para comprobar que son nuevos genes con resistencia a roya del tallo.

SUMMARY

Wheat provides about 20% of protein and carbohydrates in food diet. It is the third largest crop cultivated after maize and rice. Diseases in wheat decrease the yield and therefore worldwide production. Diseases like rusts are destructive to wheat. Stem rust caused by *Puccinia graminis* pv *tritici* affects wheat production, its a obligated biotrophic fungi, part of its asexual cycle in stems and roots is carried out in wheat, the sexual cycle is developed in alternative hosts, which generates the broad diversity to infect wheat varieties containing resistance major genes. Since 1960, the disease caused by stem rust had remained controlled by the use of fungicides and improved varieties; however, in Uganda a new race, called Ug99 which is capable of infecting plants with resistance genes to stem rust was found. Since 1999, there have been reported seven new races affecting different wheat varieties carring, resistance to the disease. One of the efforts to control stem rust is by a breeding program aided by molecular markers. With the use of molecular markers, banding patterns with a character of interest is correlated. In this work, molecular markers were used to find the resistance regions to stem rust in seven populations F5 of wheat, developed by the method of a single seed descent. The phenotypic data were obtained from the experimental station Kenya (KARI) located in Njoro, Kenya. Markers used were microsatellite and single nucleotide polimorphism. To manage the data, Icimapping and MapDisto programs were used. Resistance regions in 4 populations were found on the short arm of chromosome 6D, one more population on the 4A chromosome long arm and two more resistance regions were found in the chromosome 2B both arms, short and long. There are reports of resistance genes effectives to Ug99 in each of the chromosomes we found linked, but the location shown by the molecular markers and the given reaction type might indicate the identification of novel genes, although it requires an allelism test to confirm our findings.

INTRODUCTION

Wheat (*Triticum aestivum L.*) is one of the most important food crops. It is grown in more than 225 million hectares worldwide (FAO, 2009, Figure 1). From approximately 680 million tons produced annually, half is produced in developing countries (Aquino et al. 2002; FAO, 2009). Among biotic stresses, stem rust (*Sr)*, stripe rust (*Yr*) (*P. striiformis* f.sp. *tritici)*, and leaf rust (*Lr*) are important factors limiting yield and quality of bread wheat throughout the world (Figure 2). Regional differences in severity and incidence are pronounced for these diseases.

Figure 1. Wheat (Triticum aestivum L.) is planted worldwide in 225 millions Ha with a production of 680 million tons (50% in developing countries) FAO, 2009.

Stem rust is one of the main threats for wheat production. Since 1999, virulent races of *Puccinia graminis tritici* have evolved, affecting wheat resistant varieties that carry mayor genes for this interaction (*Sr*31, *Sr*38, *Sr*24) (Singh et al, 2006; Singh et al. 2008). Since the identification of stem rust Ug99 in Uganda, new races in Africa capable of infect resistant wheat plants, have been isolated (Visser et al. 2011). Additionally, most of the wheat varieties sown around the world are susceptible; for this reason, this fungus could potentially cause an epidemic (Singh et al. 2008).

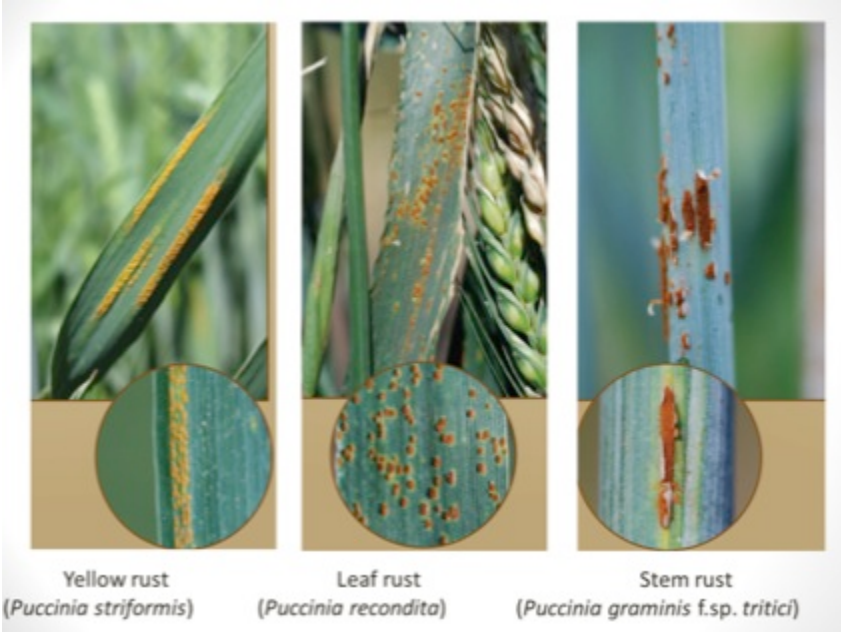

Figure 2 Three kinds of rusts affects wheat: Stripe rust (Puccinia striformis) Yellow rust (Puccinia recondita) and Stem rust (Puccinia graminis tritici).

Stem rust is consider a very important threat because evidences suggest this kind of disease can spread by different hosts and expand at great scale (http://www.wheatrust.cornell.edu/) (Figure 3). Its wide range of hosts along with it facility of dispersion makes a major challenge for the alleviation of this pathogen. Under good conditions for Ug99 race development, 57% yield losses are estimated, however by spraying fungicides these lose are low (Wanyera et al. 2009). In order to make a better management of this pathogen, eradication of alternative hosts have been carried out, due to they are part of diversity of the virulence, although not has been successful at all (Jin, 2010).

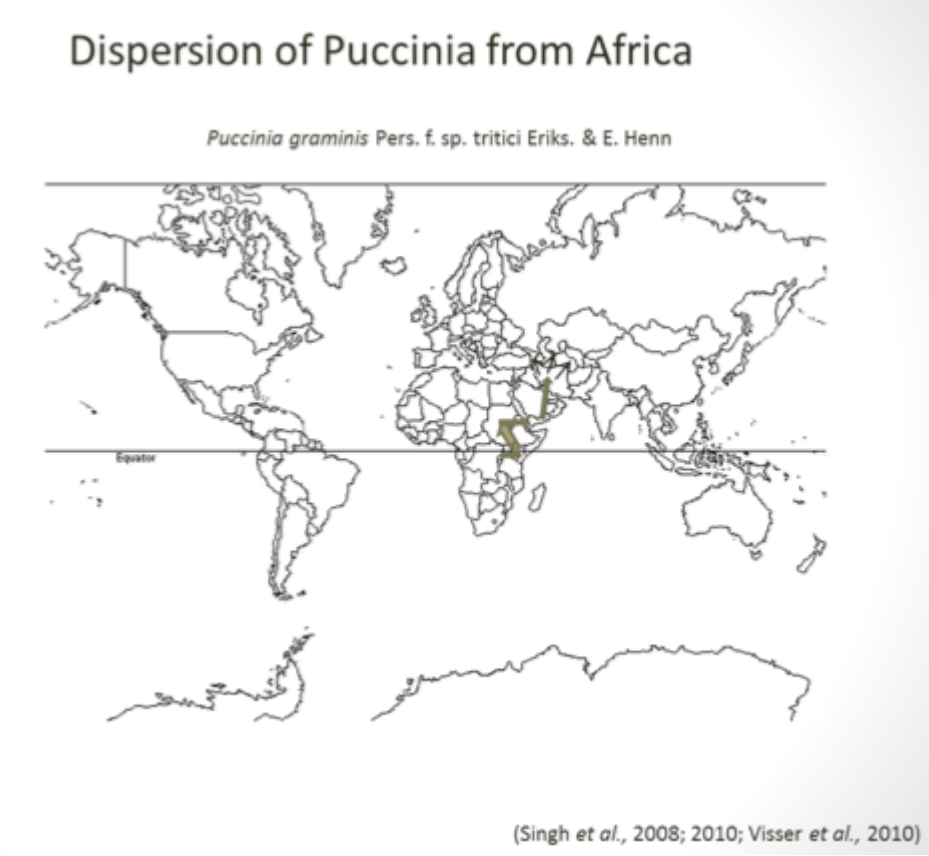

Figure 3 Ug99 race moving from Kenya to several wheat producer areas

The pest management strategy should be based in the use of different control methods. Plant resistance is a friendly environment alternative for diseases management. The deployment of resistance in commercially grown wheat cultivars is the most cost effective method to fight diseases. Most single gene resistances against pathogens of wheat as well as many other crops have proven to be non-durable. Inheritance of resistance for *Sr*, *Yr*, and *Lr*, diseases of wheat is both qualitative and quantitative. However, the later is known to be more durable. Several genes with small additive effect control quantitative resistance, consequently called minor genes and expressed en advanced phonological stages, therefore mentioned as adult plant resistance (APR). APR is considered more durable by slowing (rather than completely prevent) the development of the pathogen (Singh et al. 2008). This is thought to result from the host's ability to

lengthen the time required for the pathogen to colonize and to reduce the pathogen's sporulation capacity (Caldwell in Guo et al. 2008).

Identification and genetic characterization of new sources of resistance and their deployment to adapted genetic backgrounds is of great importance for wheat improvement. The development of molecular markers closely linked to resistance regions offers alternative methods for germplasm selection, which facilitates effective pyramiding of resistance and the possibility of selecting resistant genotypes in the absence of the pathogens (Ramburan et al. 2004; Krattinger et al. 2009; Singh et al. 2007). The availability of DNA markers also provides an additional mean to determine gene uniqueness. Apart from their indirect use in pyramiding resistance genes, markers also help to verify findings of conventional analyses, which become complicated when large numbers of genes are already known. Such a situation is encountered in the case of rusts of wheat where more than 60 resistance genes are named (McIntosh et al. 1995).

Plant traits, such as yield, quality, and disease resistance, among others are controlled by genes. These genes can be associated with a particular trait, in regions of the genome, called quantitative trait loci (QTL). Phenotypic traits do not give us a clue about genome regions due to some of them are environment activated. For this reason, DNA is used to construct linkage maps for simple or quantitative traits (Collard et al. 2005).

Breeding for rust using major and minor genes could be one strategy for improving wheat varieties; there are unknown genes in wheat relatives and even in several elite varieties that can be used to diminish the invasion of stem rust among crop fields (Singh et al, 2006).

Plant-pathogen interaction is carried out gene to gene, thus creating specific and non-specific resistance. The specific interaction can be measured on seedling stage and adult stage of the plant; although, sometimes the effect of this kind of genes is only observed on adult plants. Non-specific resistance is carried out in all

stages of the plant, is durable, present in several environments and years and has broad spectrum against several pathogens.

To accelerate the generation of new, improvement varieties, molecular tools are employed in massive screenings. DNA markers which are linked to important genes are used for marker assisted selection (MAS) or molecular breeding. These markers are not affected by the environmental conditions of plant stages; however, a good phenotypic measurement is needed to make the linkage map. A linkage map gives the relative position and distance between markers and traits in the chromosome. For the construction of a linkage map, phenotypic data, identification of polymorphism between markers and a mapping population are needed and for genetic mapping using molecular markers, the accurate measurement of disease index (phenotypic data) provided by standard scales is quite important (Collard et al. 2005).

Many efforts have carried out to locate resistant genes through the wheat genome by the use of wide genome screenings with SNP, STS and SSR markers. Khan et al. (2005) reported SSR markers linked to stem rust, Sr22 located on chromosome 7A, although cfa2019 and cfa2123 are loosely linked to Sr22 they can be used for screening of presence of this gene; on the other hand, Tsilio and coworkers (2007) reported Xgwm47 as linked marker to *Sr*9a gene, while Wu et al. (2009) found three markers for the identification of *Sr*40 gene. Börner et al. (2000) mapped a major gene that provides resistance to stripe rust in wheat, they found on 3BS linked to wheat microsatellite WMS493 (around 20.5-21.5 cM, depending of the filial generation).

Major genes are easy to identify under field conditions, provide high degree of resistance due to a hypersensitive response from plant to the pathogen (De Wit, 1992), in this sense, farmers can directly see the benefits resistance of crops without pustules or spores in their crop; the reason is that major genes give great effect upon phenotype, commonly are race-specific, can be combined with different resistant genes for wide spectrum of defense against races (Lowe et al. 2011). Disadvantage of these genes is that, the pathogen evolve in order to solve

the barrier placed by the plant, usually this kind of genes can be used for 5 years before the pathogen can colonize the tissue (Eagles et al. 2008).

1.1 Causal agent of stem rust

Puccinia graminis Pers. f sp. *tritici* Eriks and Henn (Pgt) is a fungus called stem rust or black rust, is one of the most important pathogen of wheat (Singh et al. 2011). It is an obligate pathogen, member of Basidiomycetes, family Pucciniaceae, the spores can be generated as basidiospores and aeciospores (formed on alternative host), urediospores and teloispores (formed in wheat).

In the alternative host (Barberry) the infection is carried out by basidiospores and two sexes are present in this pathogen. In the teliospores there are two nucleus which are divided in two in order to achieve a basidium (four-celled), each cell produces a sporidium (two+ and two – named + and – instead of male and female). This sporidium, is not able to infect barberry, so develops a haploid mycelium, which will develops pycnidium containing pycniospores. These pycnidiospores are insect-helped and moved for crossing with opposite hyphae and sexual reproduction takes place. Once formed the binucleate mycelium, the aecia is produced and filled with aeciopores. The aeciospores are wind-aided to reach cereals (wheat) or grasses. In these hosts, the mycelium will continue being binucleated in order to fusion in the teliospore. On the other hand, the urediniospores will be formed from asexual cycle in wheat crop (Figure 4; Roelf et al. 1992; Horst 2008).

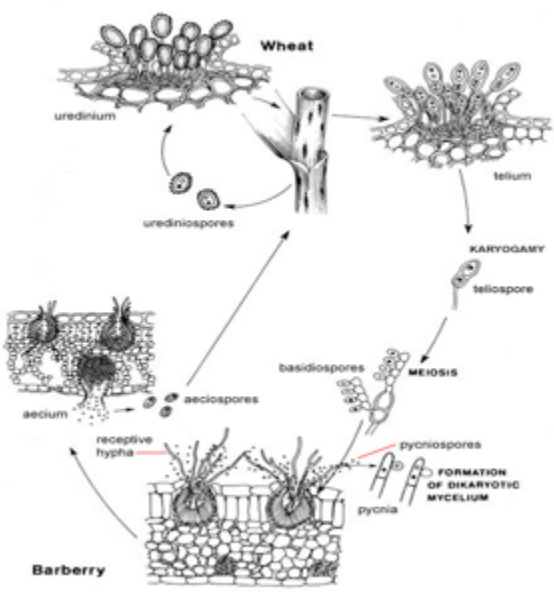

Figure 4 Stem rust cycle.

The disease severity is scored using a modified Cobb's scale (Peterson et al. 1948). This rust scoring estimates from no infection or resistant plants (R), moderately resistant (MR), moderately susceptible (MS) and susceptible (S) (Figure 5).

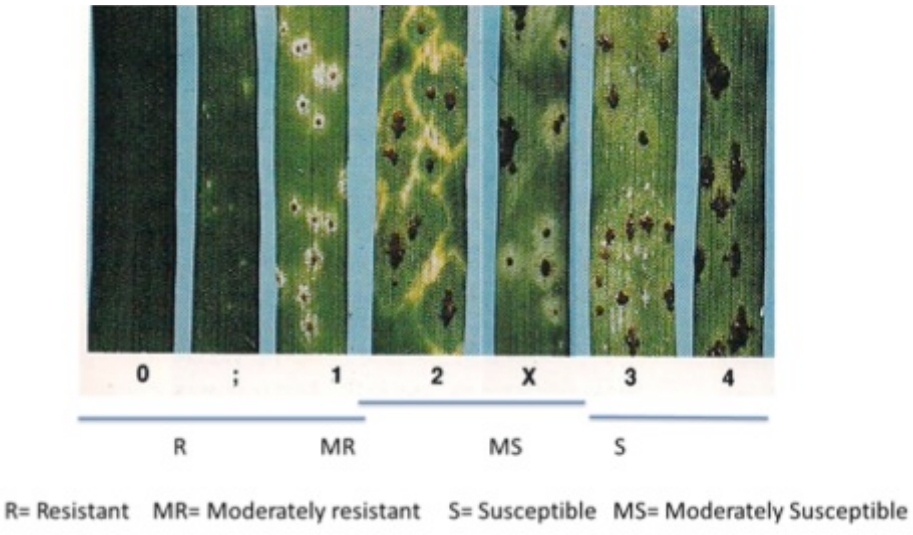

Figure 5. Infection types caused by stem rust.

1.2 Plant pathogen interaction

The plant protects itself against biotic factors using structural barriers and metabolites such as antibiotics, which stop pathogen development (Mert-Turk, 2002). Some metabolites are preformed and change to an active form when the pathogen attacks (Grayer and Kokubun, 2001). Additional metabolites against pathogens have been reports, such as phytoalexins, these are synthetized during plant pathogen interaction, have low molecular weight and its synthesis involves the utilization of new metabolic routes (Harborne, 1999).

In order to study the interaction between the plant and the pathogen, Jones and Dangl (2006) described this relationship as compatible or non-compatible (Figure 6). Once the pathogen is in contact with the plant cell, a germinal tube is produced, exerting pressure on stomata, epidermal cells or even wounds, thus forming haustoria. In these specialized structures, pathogens take nutrients and releases molecular effectors (virulence factors) such as pathogen-activated molecular patterns (PAMP). On the other side, if the plant has R proteins, pathogen´s effectors will be recognized otherwise and the colonization by pathogen will be successful. Two classes of PAMPs are identified: i)

transmembrane pattern recognition receptors (PRR´s) and ii) nucleotide binding (NB) leucine rich repeat (LRR), these last are effective against biotrophic pathogens. If the effector is indirectly recognized, the plant starts PAMP-tiggered immunity, and no colonization occurs, but if the effectors are direct recognized, the hypersensibility response is launched by the effector-triggered immunity (ETI). On the other hand, if pathogen releases effectors that inhibit the PRRs, then is called effector-triggered susceptibility (ETS). There is another way to avoid plant response: by natural selection. Races, which send effectors not recognizable by the plant, will have successful colonization. In wheat, sequences coding for NBS-LRR (CIN14) has been described, those likely acts during resistance to leaf rust (Zhang et al., 2011).

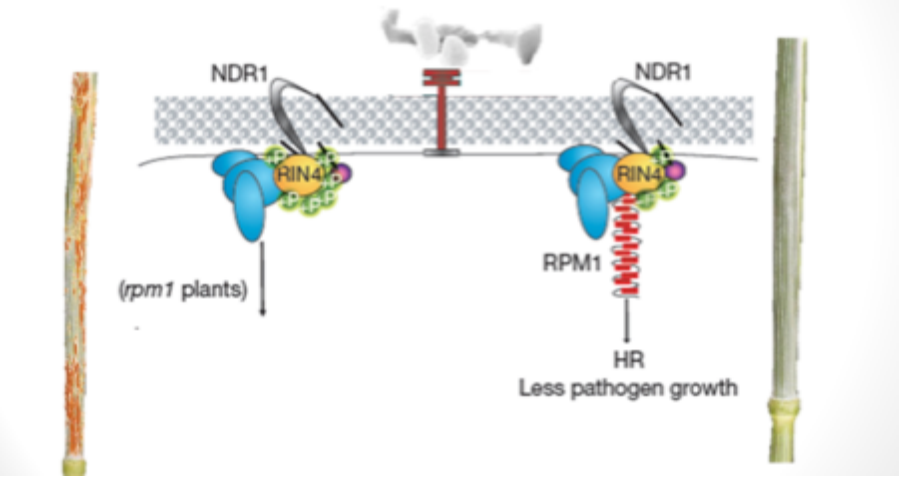

Figure 6. Responses of susceptible and resistant plant during plant-pathogen interaction

Sukhwinder Singh and Ravi Singh's research work is underway to identify and incorporate more durable resistance in high yield wheat varieties at CIMMYT and many other breeding programs. CIMMYT is involved in the characterization of wheat varieties with APR or race specific resistance for rusts. The proposed study will further strengthen the research to examine wheat diseases (rusts) including Ug99 at multi-environment level on different wheat mapping populations and to know what the effect of resistant region or gene is.

Pathogen resistance is a quantitative trait, there are at least 50 genes reported for stem rust resistance (Table 1). These genes can be classified as minor genes, which have a light effect upon phenotype, although if they are present with more minor genes, can provide a substantial improvement to the phenotype. This pyramiding of minor genes, give more durable resistance against pathogens. Usually, the resistance given by several minor genes is sown at adult stage, and receives the name of Adult Plant Resistance (APR). The main advantages of APR are i) non-specific, which means can act against wide spectrum of races target of the breeding and ii) is durable (Ayliffe et al. 2008).

1.3 Molecular markers to identify genotypic differences

Genetic marker is defined as specific landmark in the genome whose expression at phenotypic level is usually easy to identify between individual or cell that contains the genetic trait; on the other hand, discontinuous variants between genotypes occurring simultaneously are called genetic polymorphisms (Semagn et al., 2006).

Molecular markers are useful to study genotypic differences between individuals of a given population. Some molecular markers used are RFLP (restriction fragment length polymorphism), which consist in use the DNA and digest it with an 4,6 or 8 bp-cutter restriction enzyme. The digested products are resolved in agarose gels and blotted onto membranes for further hybridization (Southern blot) using a labeled probe. The type of polymorphism detected using this markers are: single base, insertion and deletion, the inheritance detected is co-dominant and has high reproducibility; however, the limitation using this markers are they need high quantity and quality of DNA, specific probes are required, time consuming laborious and expensive, labeled probes are required (Figure 7; Semagn et al. 2006; Collard et al. 2005).

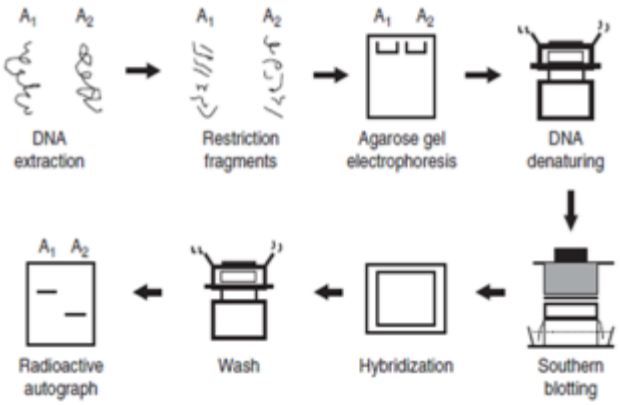

Figure 7. RFLP chart flow.

Amplified fragment length polymorphism (AFLP), is a technique in which amplification is carried out from digested genomic DNA. Firstly, DNA is digested with restriction enzymes, usually 4-6 bp, adaptors of 18-20 bp of know sequence are added to DNA ends through ligation reaction by a DNA ligase. Then, PCR is set with primers in order to generate a subset of fragments of different sizes. The product amplified is observed by radio-labeling one of the primers followed by electrophoresis (Figure 8; Collard et al. 2005).

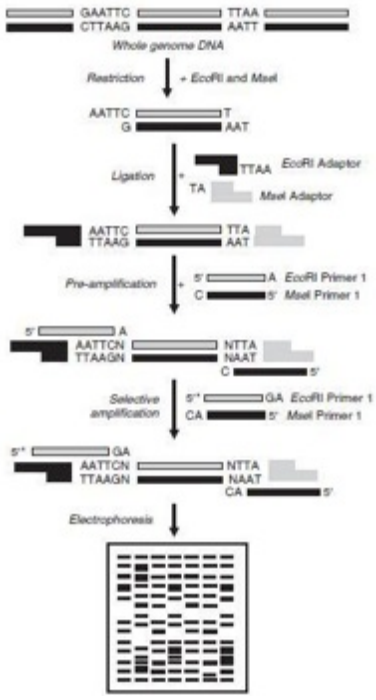

Figure 8. AFLP chart flow

Simple sequence repeat (SSR) markers use forward and reverse primers that anneal DNA, using PCR (polymerase chain reaction) technique. SSR markers reflect variation in the number of repeat units in a defined region of the genome (Figure 9, Varshney et al. 2004).

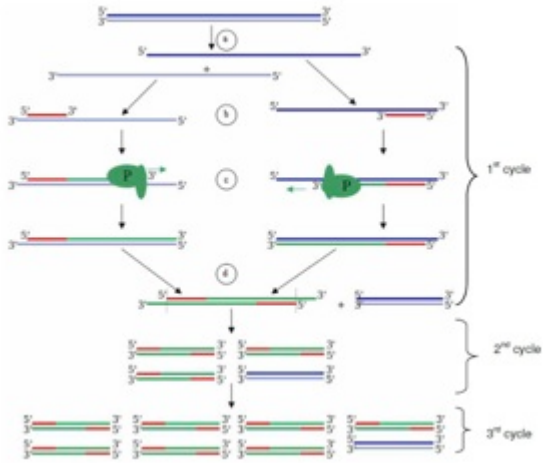

Figure 9. PCR using SSR and STS markers chartflow

Sequence tagged site (STS) markers, are designed from short regions of DNA whose exact sequence is not found in other individual. These kinds of markers usually are co-dominant and dominant, highly reproducible and technically simple to use (Semagn et al. 2006; Xu 2010).

1.4 Wheat genome complexity

Wheat plant is the result of genetic crosses from three different grasses, plants are diploid (2n=2X=14) tetraploid (2n=4X=28) and Hexaploid (2n=6X=42; Figure 10). Wheat bears one of the largest genomes in nature, about 16 million base pairs; 128 times larger than Arabidopsis and five times larger than *Homo sapiens*. Microsatellites represent from 80 to 90 %. Nullisomic varieties can be obtained; these plants maintain normal physiology, due likely to the buffer effect of several alleles, known as “a buffer effect”.

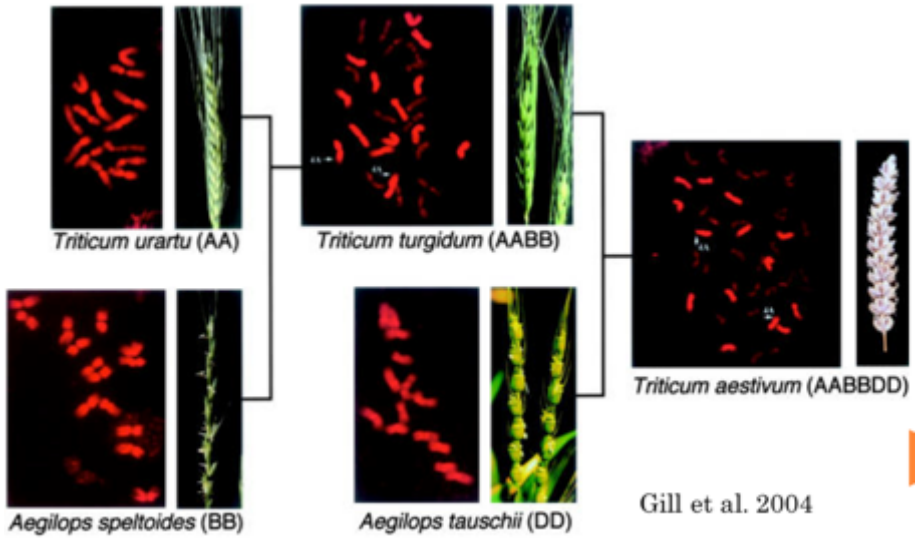

Figure 10 Hexaploid wheat

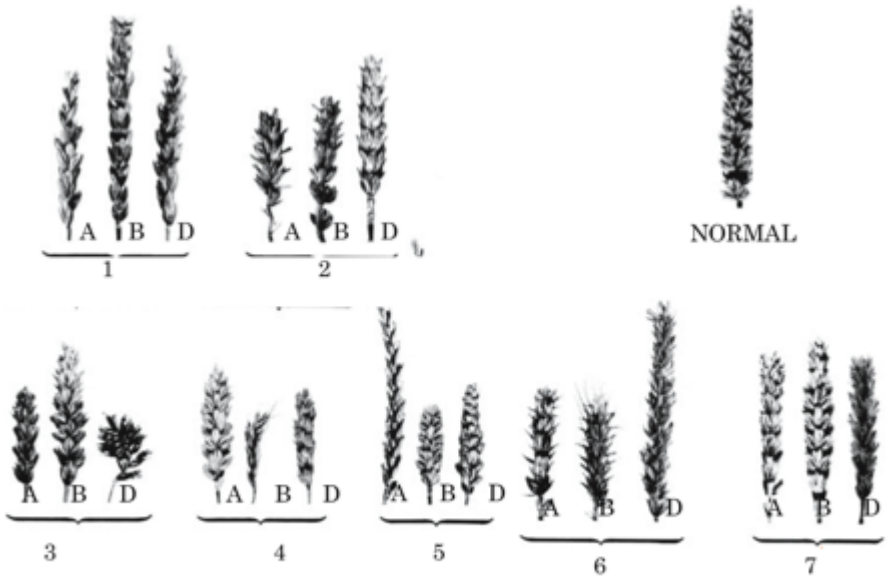

Figure 11 Nullisomic wheat lines

Nullisomy is a condition in which a cell or individual lacks both representatives of a pair of homologous chromosomes. Nullisomy is viable only in allopolyploids where the homologous chromosomes can compensate for the loss. The nullisomic compensation in allopolyploids can survive as nullisomic but it is a deleterious condition. If they are made tetrasomic for another homologous chromosome, their condition is ameliorated because of some degree of restoration of the genetic balance (Figure 11; Sears, 1953).

1.5 Justification

Despite of the identification of resistance genes in wheat, the pathogen Ug99 is an important fungal pest for which new wheat resistant varieties are needed. Genetic mapping of new resistant regions to wheat stem rust need to be discover to employ them in assisted selection of resistance varieties.

2 GENERAL OBJECTIVE

- Identification and characterization of rust resistance genomic regions in wheat using molecular tools.

2.1 Particular objectives

- To locate the major genes ruling resistance against stem rust including Ug99 strain in different mapping populations.

- Design of closer markers to the resistance regions.

3. MATERIALS AND METHODS

3.1 Phenotypic data

Phenotypic data were collected from recombinant inbred lines that carry on major genes, artificially infected with stem rust. Scored will include disease severity and infection type by using modified Cobb´s scale (Peterson et al. 1948).

Parental lines of these populations were screened with molecular markers in order to find polymorphism across the wheat genome. Once obtained polymorphic primers, bulked segregant analysis technique (BSA) was used to increase the number of screened plants. The use of susceptible and resistant bulks will allow finding differences between them for rapid identification of stem rust resistant region. After obtaining differences in bulks, susceptible and resistant lines were used to set the same primer(s) and make the statistical analysis and linkage map. More primers were tested on the chromosome region to identify those that better flank the resistance region.

3.2 Genotypic data

3.2.1 DNA extraction

CTAB method was used (CIMMYT, 2006). Briefly, the samples collected are placed at -80^{o}C for 8-12 hours. Then, samples are placed in to lyophilizer for 4 days. After that, 60 mg of tissue are added into a 2-ml eppendorf tube. Samples are grinded using a tissuelyzer for 3 min, then CTAB solution (1mL) is added and the tubes, which are then closed and shaken for 1.5 h at 65^{o}C. Then, 1 mL of a mix chloroform:octanol (24:1) is added and shaken for 15 min at room temperature. The tubes are centrifuged at 3750 rpm for 30 min. The supernatant is transferred into new tube, RNAase 1 mg/ml is added, the tubes are put in oven at 37^{o}C for 30 min, then cool isopropanol is added, the tubes are softly shaken and placed into -20^{o}C for 1 h. Then, the tubes are centrifuged at 3750 rpm for 30-40 min, the supernatant is discarded and ethanol (70%) is added, the tubes are

centrifuged for 15 min. The supernatant is discarded and the pellet is dried at room temperature (Figure 12). The DNA is suspended in 200µL of water, the DNA quantitation is made in Nanodrop-8000™. The dilutions are adjusted to 20 ng/µl.

3.2.2 Polymerase chain reaction (PCR)

The conditions for PCR were set using the follow reagents and volumes: buffer 5X (1.55µL), $MgCl_2$ 25mM (0.75µL), dNTP´s 100 mM (0.6µL) Primer 1µM (2µL) and Taq enzyme (0.4µL). The annealing temperature of each SSR was obtained from Grain Genes 2.0 wep page (http://wheat.pw.usda.gov/cgi-bin/graingenes/browse.cgi?class=marker). The PCR was set in 96-well microplates.

The PCR product was loaded on polyacrilamide gels (12%), and ran for 2.5-3 h at 300 volts. DNA is stained with a silver solution as follows: Polyacrylamide gel is incubated in fixative buffer (15 min, alcohol 10% and acetic acid 1%), 5-10 min in silver nitrate solution (2%) developer (10-15 min, 10% NaCl and 1% folmaldehide), and stopper 10 min (EDTA 60%) were used.

The banding pattern in the population is scored according to each parent, genotype parent 1 and genotype parent 2, a mix of two genotypes is heterozygous genotype.

Statistical analysis

Statistical analyses were done using different software as SAS for lineal and mixed models, R, to calculate variance between genotypes.

Use programs to construct the linkage map as Mapmaker which allows the fit of single-QTL models, QTL Cartographer provides facilities for the fit of multiple-QTL models.

R/qtl: open surce QTL mapping programs, includes many important diagnostics present in MapMaker, but allows the fit of multiple-QTL models.

4. RESULTS

Paper 1. Resistance to Ug99 stem rust in six bread wheat cultivars maps to chromosome 6DS

Abstract Over 80% of wheat area worldwide is currently grown to varieties that are susceptible to Ug99 race group of stem rust fungus. Wheat lines Niini, Tinkio, Coni, Pfunye, Blouk and Ripper were resistant to Ug99 at the seedling and adult plant stages. We mapped stem rust resistance in populations derived from crosses of a susceptible parent with each of the resistant lines. The segregation of resistance in each population indicated the presence of a single gene. The resistance gene in Niini mapped to short arm of chromosome 6D and was flanked by SSR markers *Xcfd49* and *Xbarc183* at distances of 3.9 cM and 8.4 cM proximal and distal, respectively. The chromosome location of this resistance was validated in three other populations PBW343/Coni, PBW343/Tinkio and Cacuke/Pfunye. Resistance initially postulated to be conferred by *SrTmp* in Blouk and Ripper was also linked to *Xcfd49* and *Xbarc183* on 6DS, however mapped proximal to *Xbarc183* at a similar position to previously mapped genes *Sr42* and *SrCad.* Based on the variation in diagnostic marker alleles it is possible that Niini and Pfunye may carry different resistance genes/alleles. Further studies are needed to determine the allelic relationships between various genes located on chromosome arm 6DS. Our results provide valuable molecular marker and genetic information for developing Ug99 resistant wheat varieties in diverse germplasm.

Introduction

Stem rust (SR), also known as black rust, caused by *Puccinia graminis* f. sp. *tritici* (*Pgt*), is an important disease of wheat. Severe yield losses due to stem rust epidemics were reported in Europe, Asia, Australia, and the U.S. in the 20th century (Leonard and Szabo 2005; Nagarajan and Joshi 1975; Roelfs 1978). Stem rust was controlled successfully by growing resistant semi-dwarf spring wheat varieties during the "Green Revolution" and subsequently (Singh et al. 2008). The slow rusting, adult plant resistance (APR) gene *Sr2*, transferred to hexaploid wheat 'Hope' and 'H44-24' from tetraploid emmer wheat 'Yaroslav' by McFadden (1930), when combined with other unknown APR genes has likely conferred durable resistance to stem rust (McIntosh, 1988; Rajaram et al. 1988; Singh et al. 2011b).

The appearance in Uganda of race TTKSK of the SR fungus, commonly known as Ug99, and its evolution and spread outside eastern Africa was recognized as a serious threat to wheat production because of the susceptibility of numerous important varieties grown worldwide (Singh et al. 2011a; Sharma et al. 2013). This race possessed a unique virulence combination, including virulence to *Sr31*, *Sr38*, and for a number of resistance genes used widely by breeding programs worldwide (Pretorius et al. 2000; Jin and Singh 2006). About 80-90% of wheat varieties and other germplasm was found to be susceptible to Ug99 (Jin and Singh 2006; Fetch 2007; Singh et al. 2008). Variants of race TTKSK possess additional virulence to resistance genes *Sr24* and *Sr36* (Jin et al. 2008, 2009). These two genes were widely used in Australia, South America and in winter

wheat varieties of the USA. (Jin et al. 2009). Race TTKSK, or its variants, are also now known to occur in Kenya, Ethiopia, Sudan, Yemen, Iran, Tanzania, Zimbabwe, and South Africa (Nazari et al. 2009; Pretorius et al. 2010; Singh et al. 2011a; Sharma et al. 2013).

Ug99 resistant varieties must be developed and grown for economic and environment friendly disease control. Significant efforts were made by the Borlaug Global Rust Initiative (BGRI) to counter the challenges posed by Ug99. Several effective resistance genes were identified and can be utilized in breeding (Jin et al. 2007; Singh et al. 2008, 2011a & b). Most of these genes have been introgressed from wild relatives of wheat and some possess genetic linkage to undesirable traits (Singh et al. 2008). To achieve long-lasting resistance, combinations of multiple effective genes need to be deployed in varieties (Singh et al. 2008). Identification of molecular markers closely linked to resistance genes could enable incorporating multiple resistance genes in breeding materials. Molecular markers are available for genes transferred from alien wheat relatives or related species (Mago et al. 2002, 2005 & 2011; Sambasivam et al. 2008; Tsilo et al. 2008; Qi et al. 2011; Simons et al. 2011), and also three genes native to bread wheat (Hiebert et al. 2010; Hiebert et al. 2011; Rouse et al. 2012).

Field and greenhouse screenings of the International Maize and Wheat Improvement Center (CIMMYT) and United States wheat germplasm with Ug99 identified some lines that appeared to possess uncharacterized resistance genes (Jin and Singh 2006; Njau et al. 2010; Rouse et al. 2011). Wheat lines ‘Niini#1’, ‘Tinkio#1’ and ‘Coni#1’ were identified by Njau et al. (2010) as possessing seedling and field resistance to the Ug99 race group derived from a Chinese

resistance source (Njau et al. 2010). It was hypothesized that Blouk#1 possessed *SrTmp*, a stem rust gene derived from 'Triumph 64' (McVey and Hamilton 1985), based on seedling infection type and pedigree. CIMMYT line Pfunye#1 was also identified to possess seedling and field resistance. Jin and Singh (2006) suggested that many United States hard red winter wheat varieties (HRWW) resistant to Ug99 possessed *SrTmp* based on seedling infection type, race specificity, and pedigree. We hypothesized that HRWW variety Ripper (Haley et al. 2007) also possessed *SrTmp* based on seedling infection type, race specificity, and pedigree. The above Ug99 resistance sources identified in CIMMYT germplasm are being utilized by the bread wheat improvement program. Several advanced lines that possess a combination of adult plant and race specific resistance derived from different sources, such as Niini#1, Coni#1, Tinkio#1, Blouk#1 and 'Pfunye#1', were already developed and distributed worldwide (Singh et al. 2011b). Some varieties carrying race-specific resistance to Ug99 were released in different countries. 'Gambo' in Ethiopia and 'Koshan 09' in Afghanistan possess Ug99 resistance derived from 'Babax/Lr42/Babax', the resistant parent of Blouk#1 (Njau et al. 2010). Similarly, 'Shanghai 7' derived Ug99 resistant varieties 'Digalu' and 'Morvarid' were released in Ethiopia and Iran, respectively. Digalu is now the predominant bread wheat variety in Ethiopia with over 0.5 million hectares.

The objectives of present study were: (1) to determine the genetic basis of resistance to Ug99 in six bread wheats, (2) to identify the chromosomal location(s) of resistance gene(s), and (3) to identify molecular markers linked to resistance genes for marker-assisted selection in breeding programs.

Material and methods

Plant materials

Five of the six Ug99 resistant parents, Niini#1, Pfunye#1, Blouk#1, Tinkio#1 and Coni#1 included in the study were spring wheat derived from crosses and selections made at CIMMYT (Table 1). For simplicity in this manuscript, hereafter the selection number will be omitted from the line name. The sixth Ug99 resistant parent was the US hard red winter wheat (HRWW) variety Ripper (Haley et al. 2007). The Ug99 susceptible parents used in developing mapping populations with CIMMYT lines were 'Cacuke#1' or 'PBW343' (Table 1). Susceptible parent 'Bill Brown' (Haley et al. 2008) was used in developing mapping population with Ripper.

Cacucke was crossed with Niini and Pfunye and 148 F_5 Recombinant inbreed lines (RILs) were developed for each of the cross as described by Singh et al. (2013). PBW343 was crossed with Tinkio, Coni and Blouk and 148, 190 and 142 F_5 RILs, respectively were included in the study. A population of 139 $F_{2:3}$ families, obtained by harvesting individual F_2 plants, from the cross Ripper with Bill Brown was used in mapping.

Field evaluation for stem rust resistance

The parents and four RIL populations, Cacuke/Niini, Cacuke/Pfunye, PBW343/Tinkio and PBW343/Coni, were phenotyped for SR infection responses in field trials at the Kenya Agricultural Research Institute (KARI) research station in

Njoro, Kenya, during two growing seasons (off-season: December 2009 to April 2010 and main-season: June to October 2010). Approximately 4 g seed (expected 60-70 plants) of each line was planted in 0.7 m long paired-row plots with 0.3 m row spacing and 0.3 m wide pathways between plots. To enhance the inoculum multiplication in the field, spreader rows, consisting of a mixture of Ug99 susceptible lines, were planted as border around the experimental area. The spreaders were also planted as hill on one side of the plots in the middle of 0.3 m pathways to ensure the uniform disease development and spread within the field. A light-weight mineral oil suspension of freshly collected urediniospores of Pgt race TTKST, the *Sr24* virulent variant of Ug99, was sprayed on the spreaders as described in Njau et al. (2013). Disease severity and infection response was recorded when the plants reached flowering to soft dough stages. The susceptible parent Cacuke displayed about 60-80% rust severity in both seasons. The infection responses are based on *P. graminis* f. sp. *tritici* uredinia size and shape (Roelfs et al. 1992) where R= resistant, MR=moderately resistant, MS=moderately susceptible, and S=susceptible. Plants observed with a mixture of infection responses were described by listing all infection responses, such as MR-MS. We further classified RIL families as resistant if all plants displayed low infection responses of R, MR, or MR-MS. RIL families were considered susceptible if all plants displayed MS to S infection responses and segregating (Seg) if plants segregated for resistant and susceptible infection responses. We also determined the disease severity using the modified Cobb Scale (Peterson et al. 1948) however these data were not useful in mapping of major-effect resistance. To avoid complication in linkage map construction, the RILs with Seg and ambiguous

responses were removed from the analyses. The final disease scores for each RIL were given based on the consistent expression of resistance response across the seasons.

Seedling evaluation with Pgt race TTKSK

The parents and three populations (Cacuke/Pfunye, PBW343/Blouk and Bill Brown/Ripper) were assayed at the seedling stage with race TTKSK (isolate 04KEN156/04). For the PBW343/Blouk F_5 RIL population, 5 seeds of each family were planted. Whereas, for the Bill Brown/Ripper and Cacuke/Pfunye populations, 20 seeds of each family were sown. After the full emergence of the primary leaf, 7 to 10 days after planting, the seedlings were inoculated at a Biosafety Level 3 greenhouse facility in St. Paul, MN (Rouse et al. 2011). Fourteen days after inoculation each plant was rated for seedling infection type according to a 0 to 4 Scale where 2 or less corresponded to resistance and 3 or more corresponded to susceptibility following Stakman et al. (1962). Families were characterized as resistant, segregating, or susceptible. After preliminary mapping, all families that displayed allelic disassociation with linked markers were rephenotyped. For the PBW343/Blouk population, families identified as segregating in the first screening were also rephenotyped using 20 seedlings for each family.

Molecular marker analysis

Genomic DNA of the parents of four mapping populations (Cacuke/Niini, Cacuke/Pfunye, PBW343/Tinkio and PBW343/Coni) and the corresponding derived families was extracted using a hexadecyltrimethyl ammonium bromide (CTAB) method according to CIMMYT laboratory protocol (CIMMYT 2005). For these populations, bulked segregant analysis (BSA) was used to ascertain the chromosome location of resistance (Michelmore et al. 1991; Lowe et al. 2011). Genomic DNAs from the 15 homozygous resistant and 15 homozygous susceptible families were mixed in equivalent amounts to establish the resistant and susceptible bulks, respectively. For pooling, DNA of each of the susceptible and resistant family was diluted to 30 ng/µL and then equal amounts were mixed in the respective bulks. Three hundred and seventy two SSR markers (*Xwmc, Xgwm, Xbarc, Xcfd* and *Xcfa*) covering the whole genome of wheat, were screened on the parents and the resistant and susceptible bulks. The primer sequences and annealing temperatures for each SSR marker was obtained from GrainGenes web database (http://wheat.pw.usda.gov). The PCR reaction was performed in a volume of 10 µL reaction mix containing 1X of 5X green GoTaq® Flexi buffer, 2.5mM $MgCl_2$, 100 mM dNTPs, 1 pM of forward primer, 1 pM of reverse primer, 1 unit of GoTaq® DNA Polymerase and 100 ng of template DNA. PCR products were separated on 12% polyacrylamide (29:1) gel at 300V for 3h. After electrophoresis, gel was silver- stained for visualization of the bands (CIMMYT 2005).

Four Single Nucleotide Polymorphism (SNP) markers, *XBS00009514*, *XBS00010742*, *XBS00009806*, *XBS00021867,* previously mapped on chromosome 6DS (http://www.cerealsdb.uk.net) were also screened on parents. Marker *XBS00010742* was polymorphic among the parents of the four populations and was assayed on the RILs. For genotyping RILs with SNP markers, 100 ng DNA of each RIL was dried at 60°C for 1 h to avoid difference in DNA concentration between distinct samples. The technique used for SNP genotyping was based on the KASPar genotyping system as described on KBiosciences portal (http://www.kbioscience.co.uk/) The PCR program used was a touchdown with initial denaturation at 94°C for 15 min followed by 11 cycles of 94°C for 30 sec, 65°C for 1 min with 0.8^0C decrease per cycle, 72°C for 30 sec, followed by 26 cycles of 94°C for 30 sec, 57°C for 1 min, 72°C for 30 sec and last step of extension at 72°C for 5 min. The alleles were visualized with a Pherastar Plus plate reader equipment® (BMG labtech company) using 30% of gain and 5.7 focus.

For the PBW343/Blouk population, DNA was extracted from a leaf tissue bulk of 10 plants from each F_5 family according to previously described methods (Rouse et al. 2012). For the Bill Brown/Ripper population, DNA was extracted from the F_2 parent plant of each $F_{2:3}$ families. A total of 10 resistant and 10 susceptible families from each population were used to create resistant and susceptible bulks. The bulks and parents were screened for polymorphism with 23 SSR markers previously mapped to chromosome arm 6DS (Roder et al. 1998; Somers et al. 2004; Song et al. 2005). Identified polymorphic markers were assayed on the

entire populations. SSR genotyping was performed using an ABI 3730 utilizing M13-tagged fluorescent dyes as described previously (Rouse et al. 2012).

After finding that resistance in these populations mapped to chromosome arm 6DS (see Results) the DNA of the resistant and susceptible lines was genotyped with marker FSD_RSA (closely linked to *Sr42*) according to previously described methods (Ghazvini et al. 2012) in order to test if the resistant lines carried the allele of FSD_RSA linked to *Sr42*.

Statistical analyses and genetic mapping

Chi-squared (χ^2) test for goodness of fit was used to test for deviation of observed data from expected monogenic segregation ratios. For the Cacuke/Niini, Cacuke/Pfunye, PBW343/Tinkio and PBW343/Coni populations, genetic mapping was performed using MAPDisto program (Lorieux 2012) with LOD 3.0, algorithm SER, ripping SARF criterion, and Kosambi mapping function (Kosambi 1994). For the PBW343/Blouk and Bill Brown/Ripper populations, JoinMap software v4.0 (Stam 1993; Van Ooijen 2006) with an LOD of 5.0 and the Kosambi mapping function was used to create genetic maps.

Results

Phenotypic response and inheritance of SR resistance

Stem rust disease pressure was high during the crop season and field evaluation allowed discrimination of infection response between the parents and within the

mapping populations. The parents Cacuke and PBW343 showed susceptible infection responses, whereas Niini, Pfunye, Tinkio and Coni displayed resistant (R) to moderately resistant (MR) responses (Table 1). The infection response in each RIL population varied from R to moderately resistant-moderately susceptible (MR-MS) with a range of severities representing that the expression of the race-specific gene was influenced by the genetic background. The distribution of 70 homozygous resistant (R to MR-MS) and 67 homozygous susceptible (MS or S) RILs of Cacuke/Niini conformed to 1:1 segregation ratio expected for a single gene (Table 2). The Cacuke/Pfunye RILs also segregated for a single gene with a distribution of 60 homozygous resistant (R to MR) and 58 homozygous susceptible (MS to S) (Table 2). The seedling and field phenotypic data correlated well in this population excepting for four lines where seedling phenotypes were considered accurate due to the presence/absence of flanking markers linked to resistance genes. This is not unusual with the resistance gene as its expression is known to be influenced by the genetic background (Singh et al. 2011a). In the remaining 2 populations, the distribution of resistant and susceptible RILs conformed to a 1:1 ratio; 63 homozygous resistant and 43 homozygous susceptible in PBW343/Tinkio and 84 homozygous resistant and 106 homozygous susceptible in PBW343/Coni (Table 2). For both Blouk#1 and Ripper, seedling infection types to race TTKSK ranged from 2 to 2+ among replications. PBW343 and Bill Brown displayed seedling infection types of 3+. Resistant progenies in the PBW343/Blouk and Bill Brown/Ripper populations displayed infection types ranging from 2 to 2+. A total of 117 $F_{4:5}$ families phenotyped from PBW343/Blouk population segregated with 1:1 ratio expected for a single after excluding few segregating families (Table 2). From

phenotypic data available for 130 of the 139 Bill Brown/Ripper $F_{2:3}$ families, resistance to race TTKSK segregated 36 resistant: 69 segregating: 25 susceptible; in accordance to a 1:2:1 ratio expected for a single gene (Table 2).

Bulk segregant analysis and polymorphic marker identification

For the Cacuke/Niini, Cacuke/Pfunye, PBW343/Tinkio and PBW343/Coni populations, the number of markers screened varied by genome with 113, 162 and 97 SSR marker loci from the A, B and D genomes, respectively. Fifteen percent of the markers were polymorphic between parents. Only markers located on chromosome 6DS revealed linkage to Ug99 resistance. Thus, this chromosome region was directed for additional mapping in the four populations. The marker *Xcfd49* positioned on chromosome 6DS was polymorphic in all the populations producing an amplified fragment of 214 bp in Cacuke and PBW343 and a fragment of 202 bp in resistant lines Niini, Tinkio and Coni. Bill Brown produced a 210 bp fragment. In contrast Pfunye, and the Pfunye/cacuke resistant bulk produced a 160 bp band with the marker *Xcfd49,* similar to that produced by 'Norin40' which is reported to carry *Sr42* (Fig 1). This result was further confirmed on 10 resistant and 10 susceptible lines from Cacuke/Pfunye population. However, it is not clear if the Pfunye carries the gene *Sr42* as the marker FSD_RSA, closely linked to *Sr42* (Hiebert et al. 2011), failed to amplify the fragment in Pfunye (Fig 2) and the RILs. A 142 bp fragment was amplified for *Xcfd49* in Ripper and Blouk (excluding the 19 bp M13 tag). Marker *Xbarc183* was most closely linked to resistance in Ripper and Blouk. The alleles of both Ripper and Blouk were 151 bp (excluding M13), which is

different from Norin 40 and AC Cadillac (Hiebert et al. 2011). Both Bill Brown and PBW343 did not amplify a fragment for *Xbarc183*, and this marker was scored as a dominant marker in these populations. The other 6DS markers that showed polymorphism between resistant and susceptible bulks were *Xbarc173*, *Xcfd13*, *Xcfd42*, *Xcfd75*, *Xgdm132*, *Xgwm469*, *Xugwm61, Xwmc749* and BS00010742. Only *Xcfd49* and *Xbarc183* were amplified in all six resistant parents.

Genetic maps of markers on chromosome arm 6DS linked to stem rust resistance

Polymorphic markers from 6DS between parental lines were evaluated on susceptible and resistant lines of all the populations and linkage maps were constructed. Total map lengths in the populations Cacuke/Niini, Cacuke/Pfuney, PBW343/Tinkio, PBW343/Coni, PBW343/Blouk, and Bill Brown/Ripper were 55.3, 57.9, 50.2, 54.8, 38.0, and 33.3 cM, respectively. The markers *Xcfd49* and *Xbarc183* flanked the resistance gene in the maps of the Cacuke/Niini, Cacuke/Pfunye, PBW343/Tinkio, and PBW343/Coni populations (Fig 3). In the PBW343/Blouk and Bill Brown/Ripper populations, both *Xcfd49* and *Xbarc183* were distal to the mapped resistance gene. The genetic distance between closest distal and proximal markers to the resistance gene in the Cacuke/Niini population was 3.9 cM and 8.4 cM, respectively. In the Cacuke/Pfunye population, the distal marker *Xcfd49* was located 5.3 cM from the resistance gene and proximal marker *Xbarc183* was located 6.2 cM from the gene (Fig 3). In the PBW343/Tinkio population, the markers *Xcfd49* and *Xbarc183* were located at 6.6 and 27.5 cM from the gene, respectively (Fig 3). In the PBW343/Coni population, the resistance

gene was located 5.8 cM from the distal marker *Xcfd49* and at 2.6 cM from the proximal marker *Xbarc183*. The resistance loci in the PBW343/Blouk and Bill Brown/Ripper populations mapped 5.9 and 3.8 cM proximal to *Xbarc*183, respectively. These observations indicate that the resistance loci mapped in six different populations on chromosome arm 6DS.

Marker FSD_RSA alleles

Molecular marker FSD_RSA is reported to be closely linked to resistance gene *Sr42* in cultivar Norin 40 (Ghazvini et al. 2012) and *SrCad* in AC Cadillac (Hiebert et al. 2011). Resistant parents Niini, Coni, Pfunye and Tinkio and susceptible parents Cacuke and PBW343 were haplotyped for the presence of FSD_RSA marker. FSD_RSA produced a diagnostic band of 275 bp invariably in all resistant and susceptible parents except Pfunye (Fig 2). Experiments to amplify FSD_RSA in Norin 40, Ripper, Bill Brown, Blouk, and PBW343 at the USDA-ARS Cereal Disease Laboratory yielded a band observed closer to 300 bp compared to the expected 275 bp amplicon size (Fig. 5). In order to validate this difference, we used primers FS and RS described in Laroche et al. (2000) to amplify the 604 bp fragment, within which the 275 bp FSD_RSA fragment was reported to be included (Qiagen PCR cloning kit; sanger sequencing at University of Minnesota Biomedical Genomics Center). Sequencing of the 5' and 3' ends of the fragment from Ripper, Bill Brown, Blouk, and PBW343 identified that the sequence amplified by FSD_RSA was 292 bp and that there is a single nucleotide polymorphism (SNP) within the FSD primer region of these wheat lines. The FSD primer 5'-gttttatctttttatttc-3' corresponded to 5'-gttttatctttttattta-3' in the wheat lines. The

replacement of a cytosine with an adenine at the 3' end is consistent with the sequence of wheat variety 'Neepawa' that did not produce the FSD_RSA fragment (Laroche et al. 2000). In fact the FSD_RSA marker was designed to specifically amplify sequences with a cytosine at the 3' end. This suggests that though we amplified a 292 bp fragment for these lines, the fragment does not share the SNP characteristic of the bunt resistant wheat line 'BW553' for which the FSD_RSA marker was developed. We suggest that the Taq polymerase we used for the PCR assay (TaKaRa Ex Taq, Takara Bio Inc.) may be too aggressive for accurate genotyping of FSD_RSA.

Parental line contributing resistance to Niini

The resistant parent Niini was derived from a three-way cross utilizing parents 'Ningmai9558', 'Chilero' and 'Chuanmai18'. Genotyping of these parents showed that the resistance-linked allele of the marker *Xcfd49* was present in Ningmai9558 and absent in Chilero and Chuanmai18 (Fig 4). However Niini and Norin 40, donor of *Sr42*, had different sizes of bands of 202 and 160 bp, respectively (Fig 1).

Discussion

Niini, Pfunye, Tinkio, Coni, Blouk and Ripper wheat were resistant to Ug99 and are being used in breeding for rust resistance at CIMMYT and Colorado State University. Race-specific resistance in all six wheats was conditioned by a single gene. Based on results for different mapping populations the resistance gene was

located on chromosome 6DS, 3.9 to 12.5 cM proximal to SSR marker *Xcfd49* (Fig 3). *Xcfd49* is the terminal SSR marker on the genetic map of 6DS. Marker *Xbarc183* was located proximal or distal to stem resistance loci in four and two of the populations, respectively (Fig 3). Our data provide some evidence that the resistance locus in Niini, Coni, Tinkio and Pfunye could be different from the resistance locus in Blouk and Ripper based on marker order (Fig 3). Further experiments, such as allelism tests, will be necessary to confirm results from our mapping studies. Alternatively, the parents and resistant RILs need to be tested with an array of races of stem rust fungus that have diverse avirulence/virulence combinations including virulence for the mapped genes(s) on 6DS.

The phenotyping of moderately effective race-specific resistance is challenging in the field trials as the disease severity may not necessarily serve any demarcation point to distinguish between resistant and susceptible lines. The effect of moderately effective resistance genes on disease severity depends upon the genetic background (Singh et al. 2011b; Hiebert et al. 2011). Singh et al. (2011b) have demonstrated that genes *SrTmp* and *SrSha7* displayed a disease severity ranging from 5 to 60% and 1 to 30%, respectively, in CIMMYT derived advanced wheat breeding materials tested in Njoro, Kenya in 2010. Similarly, *SrCad* has been shown to display a disease severity of 10-30% depending upon the presence or absence of adult plant resistance gene *Lr34* in the background (Hiebert et al. 2011). In our study, we also observed a wide range of disease severity with incompatible reactions in the RILs. So, we used the discrete classification of RILs into resistant and susceptible classes based on host infection response to map the single genes in the populations evaluated in field trials.

Chromosome 6D has been reported to be a gene rich region of the wheat genome containing resistance to powdery mildew (Ma et al. 2011), leaf rust (Mebrate 2008), common bunt (Laroche et al. 2000) and stem rust (Ghazvini et al. 2012; Hiebert et al. 2011). Four *Sr* genes were previously mapped to chromosome 6D. These are *Sr5*, *Sr29*, *Sr42* (McIntosh et al. 1995; Ghazvini et al. 2012) and *SrCad* (Hiebert et al. 2011). Of these, *Sr29* is reported to be located on the long arm of chromosome 6D. The remaining three genes, *Sr5, Sr42* and *SrCad*, were mapped to the short arm of chromosome 6D (McIntosh et al. 1995; Hiebert et al. 2011; Ghazvini et al. 2012). The *Sr5* gene, however, does not confer resistance to Ug99 (Jin et al. 2009) and both *Sr42* and *SrCad* confer resistance to Ug99 and produce a low seedling infection type similar to the resistant lines. Further, comparison of the maps in the present study with the published map of *Sr42* and *SrCad* (Ghazvini et al. 2012) reveals high similarity in the marker order and genetic distance between linked SSRs (Fig. 3). We therefore conclude that we cannot rule out the possibility that the resistance loci reported in this study are the same as *Sr42* or *SrCad*.

To evaluate further the relationship between the mapped *Sr* gene(s) in the present study and *Sr42* and *SrCad*, we used a PCR based diagnostic marker FSD_RSA which is the closest marker to both *SrCad* (Hiebert et al. 2011) and *Sr42* (Ghazvini et al. 2012). FSD_RSA was developed as a marker for the common bunt resistance gene *Bt10*, which is closely linked to *SrCad* (Hiebert et al. 2011). Ghazvini et al. (2012) reported that *Sr42* (in Norin40) co-segregated with FSD_RSA that mapped 1.5 cM from *SrCad* (Hiebert et al. 2011). It has also been hypothesised that *Sr42* and *SrCad* represent the same allele or different alleles of

the same locus (Ghazvini et al. 2012). We evaluated the presence and absence of FSD_RSA in six resistant (Niini, Pfunye, Blouk, Ripper, Tinkio and Coni) and the three susceptible (Cacuke, PBW343, and Bill Brown) parents. Excepting Pfunye, the other three resistant and two susceptible parents possessed the diagnostic band of ~275 bp (Fig 2). This result was further confirmed by evaluating FSD_RSA on the Cacuke/Pfunye RIL population. This indicates that resistance derived from Pfunye could be different from *Sr42/SrCad* and also from resistance derived from Niini, Coni and Tinkio. Sequencing of the FSD_RSA region identified that this marker may not be robust in diverse germplasm as we identified Ripper, Bill Brown, Blouk, and PBW343 to amplify the fragment, but not possess the diagnostic SNP. Allelism tests will be required to determine if the resistance gene in Pfunye is different from resistance in other parents and *Sr42* or *SrCad* .

The closest SSR markers (*Xcfd49* and *Xbarc183*) identified in this study will expedite marker-assisted selection and stacking of resistance on 6DS in CIMMYT and Colorado State University wheat breeding germplasm. If further testing identifies more than one gene present on 6DS, recombinants could be selected to create a desirable linkage block of two or more disease resistance genes that would be easy to track and select in wheat breeding populations. When desirable genes are linked in coupling, as is the case for *SrCad* and *Bt10* (Hiebert et al. 2011), breeding populations can be easily enriched with the linked genes.

Based on linked marker alleles, map location, pedigree information, and field infection response, it is likely that resistance in Niini, Coni and Tinkio represents the same gene. The Chinese wheat lines Ningmai9558, Ningmai9415.16 (Ningmai50) and Ningmai9415 (Ningmai7) could share the

common resistance gene identified in Niini, Tinkio and Coni, respectively. On the other hand, it is also possible that Pfunye possesses a distinct gene based on the diagnostic marker allele and pedigree. Because Blouk and Ripper mapped to the same location as *Sr42*, and displayed seedling infection types similar to *Sr42*, it is possible that Blouk, Ripper, Norin 40 and AC Cadillac share a common resistance gene. Since Blouk and Ripper were postulated to possess *SrTmp,* our data indicate that *SrTmp* may be the same gene as *Sr42* and *SrCad*. Studies examining the stem rust resistance in the *SrTmp* donor, 'Triumph64', and allelism tests among these resistance sources will be needed to sort out the relationships between *SrTmp*, *SrCad*, *Sr42* and the resistance in six lines included in this study.

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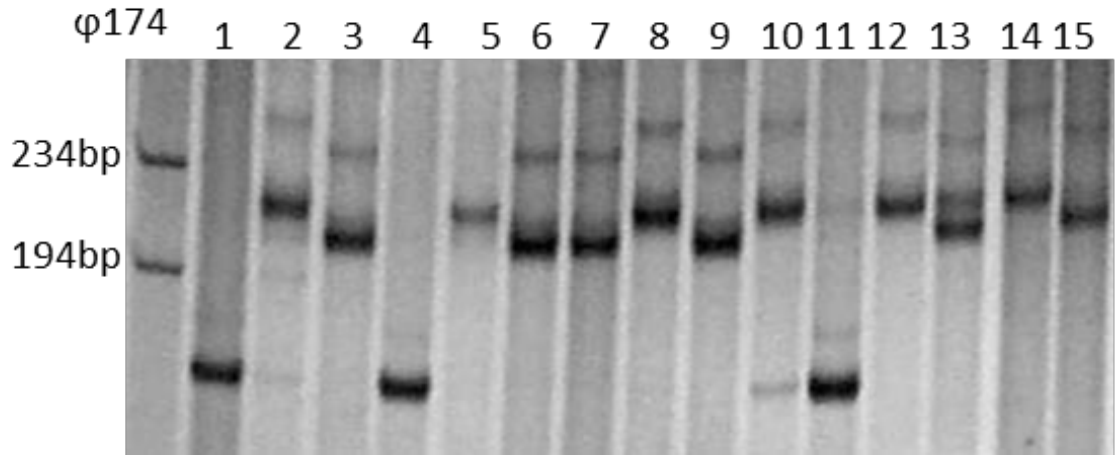

Figure 12. Fig. 1. Molecular polymorphism between susceptible and resistant parents and F_5 resistant and susceptible bulks detected with SSR marker Xcfd49.

The first column is the size marker (φX174 digested with the enzyme HaeIII). The numbers on the left indicate the molecular size in bp. The numbers on the top are DNA samples: 1=Norin40, 2=Cacuke, 3=Niini, 4= Pfunye, 5= PBW343, 6=Tinkio, 7=Coni, 8=Cacuke/Niini Susceptible bulk, 9= Cacuke/Niini Resistant bulk, 10= Cacuke/Pfunye susceptible bulk, 11= Cacuke/Pfunye resistant bulk, 12= PBW343/Tinkio susceptible bulk, 13=PBW343/Tinkio resistant bulk, 14= PBW343/Coni susceptible bulk, and 15=PBW343/Coni resistant bulk.

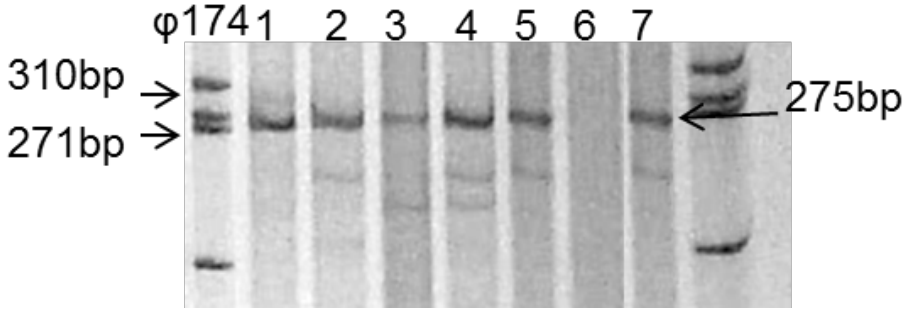

Figure 13. Fig 2. Amplification of DNA with the marker FSD_RSA on six wheat lines.

The arrow on the right shows the diagnostic band of 275 bp in Norin 40. The first column is lamda φX174 digested with the enzyme *HaeIII*. Numbers to the left indicate the size of ladder in bp. Numbers on top are: 1=Norin40, 2=Cacuke, 3=PBW343, 4=Niini, 5=Tinkio, 6= Pfunye, and 7=Coni.

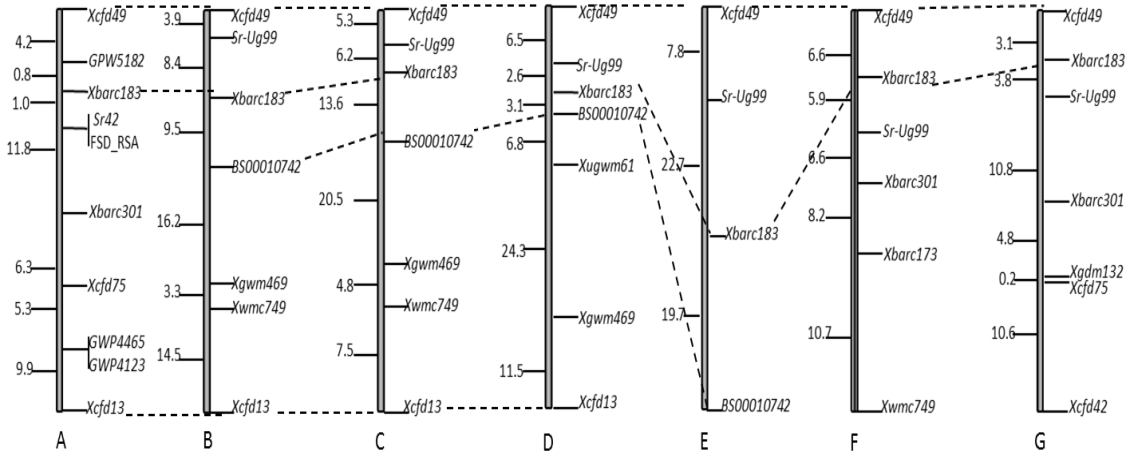

Figure 14. Fig 3**.** Comparison of genetic maps showing the positions of Sr genes on chromosome 6D short arm.

A) 6DS map from double haploid population LMPG/ Norin40 reconstructed from Ghazvini et al. (2012), B) map constructed using Cacuke/Niini derived RIL population, C) map constructed using Cacuke/ Pfunye#1 derived RIL population, D) map constructed using PBW343/Coni derived RIL population , E) map constructed using PBW343/Tinkio derived RIL population and F) map constructed using PBW343/Blouk derived RIL population, G) map constructed using Bill Brown/Ripper derived F3 population. Loci names are indicated on the right and map distances are shown on the left in CentiMorgans.

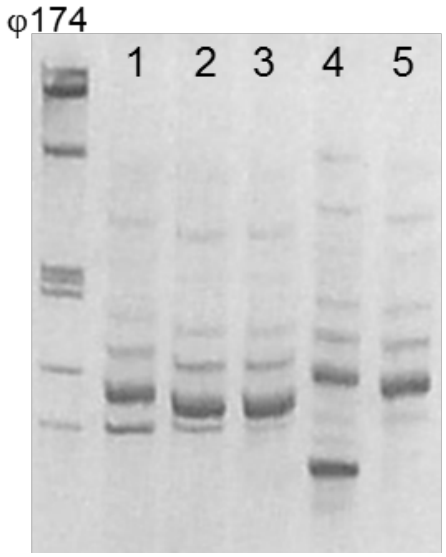

Figure 15. Fig 4. Amplification of DNA samples with the marker Xcfd49:

(from left to right) 1=Cacuke (susceptible to Ug99), 2= Niini (resistant to Ug99); and parents of Niini: 3= Ningmai9558, 4= Chilero and 5=Chuanmai18. The first column is lambda produced by the size marker (φX174 digested with the enzyme *HaeIII*).

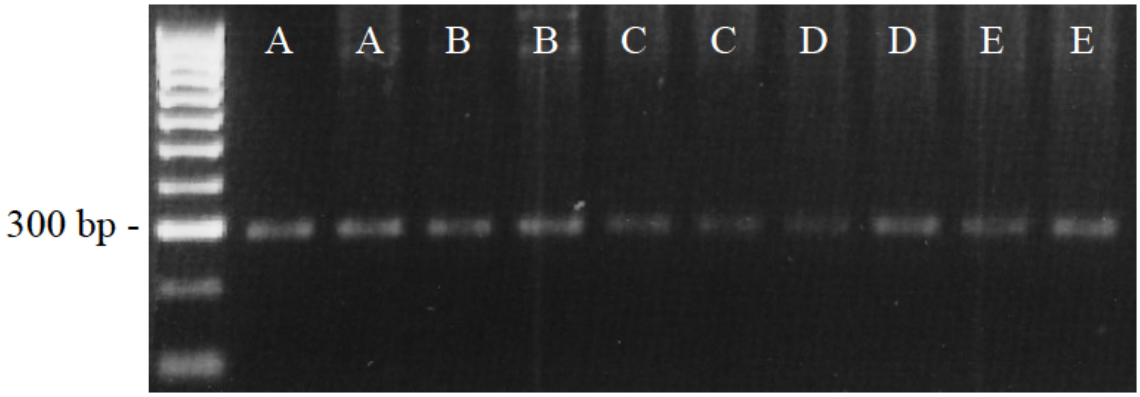

Figure 16. Fig. 5. Amplificaiton of marker FSD_RSA in lines Norin 40

(A), Ripper (B), Bill Brown (C), Blouk (D), and PBW343 (E) and separation on a 1% agarose gel. The 300 bp fragment of the ladder is indicated.

Table 1. Table 1. Parental lines used in mapping of resistance to Ug99 race group of stem rust fungus with their pedigrees and responses to Ug99 in seedlings and adult plants

Parents	Pedigree	GID[a]	Seedling reaction to Ug99	Field reaction to Ug99
Niini#1	Ningmai9558//Chilero/Chuanmai18	4902635	2	5R-15RMR
Phunye#1	Pfau/Milan/3/Skauz/KS94U215//Skauz	5534349	22+	5R-10R
Blouk#1	Babax/LR42//Babax*2/3/Kuruku	5398610	22+	10RMR-40MR
Tinkio#1	Ningmai9415.16//Shanghai#4/Chilero/3/Ningmai50	4942852	2+	5R-30RMR
Coni#1	Ningmai9415/3/Ures/Bow//Opata/4/Ningmai7	4911181	2+	5R-10RMR
Ripper	CO940606/TAM107R-2	-	22+	-
Cacuke#1	Canadian/Cunningham//Kennedy	5347441	3+	80S-100S
PBW343	Nord Deprez/VG9144//Kalyansona/Bluebird/3/Yaco/4/Veery#5	2430154	3+	60MSS-90S
Bill Brown	Yumar/Arlin	-	3+	-

[a] Germplasm identification number in CIMYYT database

Table 2. Table 2. Phenotypic distribution of lines in the six mapping populations based of stem rust infection response evaluated in field or greenhouse trials

Population	Generation	Lines (No.)	Observed frequency[a]	Expected ratio[a]	χ^2 (1:1)	*P*-value
Cakuke/Niini	F_5	110	50: 60	1:1	0.91	0.34
Cakuke/Pfunye	F_5	116	57: 59	1:1	0.03	0.85
PBW343/Tinkio	F_5	106	43: 63	1:1	3.70	0.05
PBW343/Coni	F_5	190	106: 84	1:1	2.52	0.11
PBW343/Blouk	F_5	117	58:59	1:1	0.01	0.93
Bill Brown/Ripper	F_3	130	36:69:25	1:2:1	2.35	0.31

[a] Expected frequency and ratio are for resistant: susceptible in F_5 RILs and resistant: segregating: susceptible in F_3 families.

Paper 2. Molecular mapping and validation of *SrND643*: a new gene effective against stem rust race Ug99 in bread wheat

Abstract

We identified a new source of genetic resistance to race Ug99 of *Puccinia graminis* f. sp. *tritici*, in wheat (*Triticum aestivum* L.). Advanced wheat breeding line ND643/2*Weebil1 has been observed to carry a resistance gene effective to the Ug99 group of stem rust races and expressed at both seedling and adult growth stages, temporarily designated as *SrND643*. This study was conducted to map the genetic location of *SrND643*, and identify closely linked molecular markers to allow for selection of *SrND643* in breeding populations. To map the gene, 148 recombinant inbred lines (RILs), developed by crossing ND643/2*Weebil1 with susceptible line Cacuke, were evaluated for stem rust field reaction in Njoro, Kenya, during two growing seasons in 2010, and were genotyped with several DNA markers (DArT, SSR and SNP). Genetic linkage mapping identified that *SrND643* is located at the distal end of 4AL close to SSR markers *Xgwm350* (0.5 cM), *Xwmc219* (4.1 cM) and *Xwmc776* (2.9 cM). The race specificity of *SrND643* is different from *Sr7a* and *7b*, indicating that the resistance we identified is a new gene or allele. The flanking markers *Xgwm350* and *Xwmc219* were predictive of the presence of *SrND643* in advanced germplasm, thus validating our map location and suggesting that these markers could be useful in marker-assisted selection.

Introduction

Wheat stem rust (SR), caused by *Puccinia graminis* Pers. f. sp. *tritici* Eriks. & E. Henn, (*Pgt*) is one of the most devastating diseases of wheat (*Triticum aestivum* L.). Several historical stem rust epidemics have been recorded in different parts of the world, including India (Nagarajan and Joshi 1975) and North America (Roelfs, 1978) with estimated yield loss up to 20 and 55% respectively. Severe stem rust epidemics can cause 100% loss in individual fields when susceptible cultivars are grown (Saari and Prescott, 1985). The recent emergence of a new race of the

stem rust pathogen, commonly known as Ug99 named after the country (Uganda) and year of first description (1999), has threatened global wheat production because of its broad virulence on modern cultivars. Ug99 was characterized as race TTKSK based on the North American system of *Pgt* race nomenclature (Roelfs and Martens, 1988; Jin et al. 2008). Race TTKSK, has been evolving across years defeating additional SR genes present in currently grown cultivars, such as *Sr24* and *Sr36* (Jin et al. 2008, 2009). The Ug99 group of races has spread to wide geographical regions including most of the countries in the Eastern African highlands, Zimbabwe, South Africa, Sudan, Yemen and Iran (Singh et al. 2011b).

As more than 90% of wheat cultivars grown worldwide are susceptible to Ug99 (Singh et al. 2011b), incorporating genetic resistance into the new cultivars and their rapid dissemination to farmers can help reduce the risk of epidemics and associated large yield losses in vulnerable areas. The majority of the 58 characterized SR resistance genes, originated from *Triticum* and relative species (McIntosh et al. 2012), confer race-specific resistance that is effective at both the seedling and adult plant growth stages. The Ug99 group of races carries virulence to several SR genes, including *Sr31*, *Sr24*, *Sr36* and *Sr38* which are very common in wheat cultivars worldwide (Pretorius et al. 2000; Jin et al. 2007, 2009; Singh et al, 2011b). Using non-race-specific resistance effective only at the adult plant stage is a viable strategy of breeding wheat cultivars with durable resistance. However, multiple adult plant resistance genes are necessary to achieve adequate resistance and combining multiple genes in high yielding advanced lines is often a difficult task. For example, *Sr2* is one of the most studied and widely distributed adult plant resistance genes (Sunderwirth and Roelfs, 1980; Singh et al. 2008). However, it does not provide enough protection to the crop as it reaches a disease severity of 60-70% when present alone during high disease pressure (Singh et al. 2008). It has been suggested that to achieve the near immune response against rust, at least 4 to 5 adult plant resistance genes need to be combined into a single cultivar (Singh et al. 2000). It has been observed that high level of rust resistance can be achieved when a moderately effective race-specific gene is combined with

adult plant resistance genes (Basnet et al. 2013b; Singh et al. 2011a, 2011b). To accelerate the effort of developing new cultivars which carry genetic resistance to the virulent stem rust race Ug99, identification of new sources of resistance and their molecular characterization is necessary. *SrCad* is one of the newly characterized race-specific genes present in Canadian wheat varieties AC Caddillac and Peace (Hiebert et al. 2010). Similar to *SrCad*, some CIMMYT (International Center for the Improvement of Maize and Wheat) lines have been found to carry moderately effective race-specific resistance which is mapped on chromosome arm 6DS (Lopez-Vera et al. 2013). Some other race specific genes which are newly postulated and being used in CIMMYT breeding program are *SrSha7* derived from Shanghai#7, *SrHuw234* derived from HUW234 and *SrND643* derived from ND643 (Singh et al. 2011b). Molecular mapping of these genes will help to identify the closely linked molecular markers and expedite breeding for rust resistance through marker-assisted selection.

SrND643 is a moderately effective stem rust resistance gene present in the CIMMYT spring wheat line 'ND643/2*Weebil1'. In the seedling stage, *SrND643* produces an infection type of 2 to 22^{+} against Ug99 race TTKSK on 0 to 4 scale (Stakman et al. 1962). In the adult plant stage the disease severity ranges from 10 to 40% with intermediate infection responses following Peterson et al. (1944) and Roelfs et al. (1992). CIMMYT's bread wheat improvement program has successfully developed several ND643-derived high yielding lines which carry *SrND643* and constitute important entries in different international nurseries and trials. It has been observed that the effectiveness of *SrND643* is dependent upon genetic background. Recently, the Kenya Agricultural Research Institute (KARI) has released two varieties of wheat 'Kenya Tai' and 'Kenya Sunbird' which were selected from CIMMYT advanced lines derived from ND643/2*Weebil1 cross, and are postulated to carry *SrND643*. The search for new sources of resistance and their molecular characterization will not only increase gene diversity but also help to expedite breeding to combat newly evolving virulent races of stem rust. The objectives of this study were: 1) to map the genetic location of *SrND643* using Cacuke/3/ND643/2*Weebil1 recombinant inbred line population, and 2) to identify

the molecular markers closely linked with *SrND643* and validate their usefulness in marker-assisted selection.

Materials and Methods

Plant Materials

A mapping population comprised of 148 $F_{4:5}$ RILs were developed by crossing resistant parent ND643/2*Weebil1 with susceptible line Cacuke. The North Dakota State University line ND643 was selected as a high protein line from three-way cross RL4352-1/*T. dicoccoides* FA-15-3//Len; where, RL4352-1 was a selection from Canadian cultivar Columbus for rust resistance (Khan et al. 1989) and Len was derived from the cross ND499/3/Justin/RL4205//Wisc261 (http://www.ars-grin.gov/). The *T. dicoccoides* accession was included in the cross to introgress a gene for high grain protein content. Similarly, Weebil1 and Cacuke1 were selected from cross Babax/Amadina//Babax and Canadian/Cunningham//Kennedy at CIMMYT. The mapping population from the cross Cacuke/3/ND643/2*Weebil1 was developed by using the single head descent method as described by Basnet et al. (2013a).

Evaluation of Ug99 resistance

Parents and RILs were evaluated for field reaction to stem rust race Ug99 at the Kenya Agricultural Research Institute (KARI) in Njoro, Kenya during off- and main-growing seasons in 2010. The off season rust screening nursery is planted during November and harvested in May, whereas the main season begins in June and ends in October. About 4 grams seed of each RIL and parents were plated in 0.7 m long paired-row plots with 0.3 m wide row and plot spacing. To initiate the infection and establish SR epidemics in the nursery, spreaders, which consisted of a mixture of Ug99 susceptible lines, were hill planted on one side of each plot in the middle of 0.3 m wide pathways. The spreaders were also planted along the border of the experimental field on 1 m wide plots. To create the uniform disease epidemics, the spreaders were inoculated with a field bulk collection of race TTKST (Jin et al., 2008), 6 to 8 weeks after planting using the method described

by Njau et al. (2012). The infection responses on each RIL and parent were recorded at post-flowering when the susceptible parent displayed moderately susceptible to susceptible infection response with 80% disease severity. The infection data were recorded based on the shape and size of the *Pgt* uredinia on the stem (Roelfs et al. 1992) where R = resistant, MR = moderately resistant, MR-MS or M = moderately resistant to moderately susceptible, MS = moderately susceptible, and S = susceptible. To further mendelize infection responses as a monogenic trait, the RIL families were classified as resistant when they displayed low infection responses of R, MR or M. Whereas, they were classified as susceptible if they displayed high infection responses of MS or S. The families with both resistant and susceptible plants were classified as segregating. The percent disease severity (DS) was also recorded following the modified Cobb Scale (Peterson et al. 1944) in the main-season.

Molecular marker analysis and genetic mapping

To identify the chromosomal location of the gene present in the Cacuke/3/ND643/2*Weebill population, both selective genotyping and bulk segregant analysis techniques were used. For selective genotyping, a total of 46 entries, consisting of parents and twenty two resistant and susceptible lines each were genotyped with diversity arrays technology (DArT) markers at Triticarte Pty Ltd., Australia (Akbari et al. 2006). Similarly, resistant and susceptible bulks were prepared by mixing equal amount of DNA samples from 10 resistant and 10 susceptible lines respectively. Then, targeted chromosome specific SSR and SNP markers were screened for polymorphism between parents and their respective bulks. To construct the final genetic linkage map, all the RILs were genotyped with polymorphic markers. Linkage mapping was carried out with Inclusive Composite Interval Mapping (ICIM) software (Li et al. 2008). The recombination frequency was converted to map distance with the Kosambi mapping function (Kosambi 1944). Similarly, the linkage map was ordered and rippled with nearest neighbor two-opt (nnTwoOpt) and sum of adjacent recombination fraction (SARF)

algorithms respectively (Li et al. 2008). The graphical representation of the linkage map was constructed with MapChart 2.2 (Voorrips 2002).

Validation of flanking markers for marker assisted selection

Two SSR markers (*Xwmc219* and *Xgwm350*) which flanked the resistant gene *SrND643* were used to haplotype 53 wheat lines with ND643 in their pedigrees selected from the 3rd, 4th, 7th and M8th stem rust resistance screening nurseries (SRRSN), the M47th international bread wheat screening nursery (IBWSN) and the M25th hard red wheat screening nursery (HRWSN) of CIMMYT (Table 1). The seedling reactions to race TTKSK were recorded at the Kenya Agricultural Research Institute, Njoro, Kenya (3rd and 4th SRRSN) and at the USDA-ARS Cereal Disease laboratory (7th and M8th SRRSN). The seedling evaluation of these advanced lines was completed as described by Jin et al. (2007). The seedling infection type (IT) data were recorded on a 0 to 4 scale following Stakman et al. (1962), where ITs 0, 1, 2 or their combinations were considered incompatible reactions i.e., the host was resistant, whereas, ITs 3 and 4 were considered compatible reactions i.e., the host was susceptible to the race being tested. Unfortunately, the seedling data for M47th IBWSN and M25th HRWSN were not available. For the 53 wheat lines, stem rust infection response and severity data were obtained from the international stem rust nurseries evaluated in the 2008 off-season in Kenya (3rd and 4th SRRSN), the 2013 off-season in Ethiopia (7th SRRSN) and the 2013 off-season in Kenya (M8th SRRSN, M47th IBWSN and M25th HRWSN). The marker haplotype and stem rust phenotype in each line were compared to evaluate the usefulness of the markers to select for the resistant gene.

Results

Disease evaluation and inheritance of resistance

Substantial and uniform disease pressure was observed in the field nursery during both main- and off-seasons in 2010. The susceptible parent Cacuke displayed susceptible infection responses of MS to S and at least 80% disease severity

across the seasons. The resistant parent ND643/2*Weebill showed M and MR infection responses during main- and off-season, respectively, and a disease severities from 5 to 15%. A wide range of infection responses (MR to S) were observed in the RILs. Similarly, the disease severity ranged from 5 to 80% among the RILs. Most of the RILs consistently produced the same type of infection responses across the two seasons. A greater number of segregating types was observed during the off-season. The RILs were classified as resistant or susceptible based on a consistent infection response during both seasons. To avoid the risk of misclassification, the lines with ambiguous or inconsistent infection responses across two experiments (25 RILs) were discarded from the analysis. Similarly, in order to meet the requirement to perform the linkage mapping in RILs, segregating lines (13 RILs) were also discarded. Based on the total 110 lines used in the final analysis, 52 susceptible and 58 resistant types, conformed to mono-genic inheritance of resistance with an expected 1:1 ratio (χ^2 = 0.33, P = 0.57).

Genetic mapping of resistance

Selective genotyping on 46 entries identified a total of 629 polymorphic DArT markers which were distributed among all the 21 chromosomes of bread wheat. Among them, at least 12 markers from chromosome 4A showed high linkage with stem rust resistance (recombination frequency, r = 0.02 to 0.15). For further analysis, the most closely associated marker, *wPt-7590* (r = 0.02), was selected to design PCR primers. The DNA sequence of *wPt-7580* was obtained from http://www.diversityarrays.com/sequences.html, and the forward (5´-CGTCCAATGTTTGCTCAGAA-3´) and reverse (5´-GCAACTACGGGGGTAATTGTT-3´) primers were designed by using the primer-BLAST program available at http://blast.ncbi.nlm.nih.gov. Similarly, 48 SSR markers which were previously mapped on 4A (Somers et al. 2004; Sourdille et al. 2004; Song et al. 2005; Pillard et al. 2003) were used to survey polymorphim between parents and their bulks. The primer sequences and annealing temperatures for all the SSR markers were obtained from the GrainGenes

database (http://wheat.pw.usda.gov). Similarly, 4 SNP markers (*BS00016097*, *BS00013985*, *BS00020741* and *BS00018740*) reported on chromosome 4A were also screened for polymorphism (http://www.lgcgenomics.com/). Among the markers screened, 7 SSR (*Xbarc78*, *Xgwm160*, *Xwmc722*, *Xwmc497*, *Xwmc776*, *Xgwm350* and *Xwmc219*), and 1 SNP (*BS00016097*) markers showed distinct polymorphism between parents and their respective bulks. For final genetic map construction, polymorphic SSR, SNP and DArT-PCR markers were used to genotype the whole mapping population of 148 RILs.

Before linkage mapping, the segregation distortion of all the markers was analyzed using Chi-square tests. In the analysis, all the 9 markers conformed to a 1:1 segregation ratio of parental alleles (Chi-square probability, P = 0.09 to 0.80). The partial genetic map of 4A was constructed with a total genetic distance of 31.2 cM, where the stem rust resistance locus, *SrND643*, was mapped between markers *Xwms350* and *Xwmc219* at a distance of 0.5 and 4.1 cM respectively (Fig. 1). The markers *Xbarc78*, *Xgwm160*, *wPt-7590*, *Xgwm350* and *Xwmc219* have been placed on the most distal deletion bin (4AL4-0.8-1.0) of 4AL in the publicly available physical-genetic map of wheat (Sourdille et al. 2004; Gadaleta et al. 2009; http://www.cerealsdb.uk.net). In our partial map, *Xbarc78* and *Xwmc219* represent the two most proximal and distal markers respectively. This confirms that *SrND643* is located at the distal end of the long arm of chromosome 4A. The marker order in the 4AL partial genetic map is consistent with most of the previously published maps including the microsatellite consensus map of Somers et al. (2004). The flanking markers *Xgwm350* and *Xwmc219* produced 148 and 136bp PCR fragments, respectively, associated with *SrND643* in ND643/2*Weebil1.

Effect of SrND643 on disease severity

To analyze the effect of *SrND643* on disease severity, all RILs were reclassified into resistant (+*SrND643*) and susceptible (-*SrND643*) groups based on the flanking markers *Xgwm350* and *Xwmc219*. The disease severity on +*SrND643*

lines ranged from 5 to 40% (mean = 12%), whereas it ranged from 10 to 90% (mean = 38%) in -*SrND643* lines (Fig. 2). The mean difference between two groups was highly significant (*t*-value = 9.09, *P* <0.0001) indicating that *SrND643* is associated with disease severity as well. In all the cases, +*SrND643* lines always displayed MR or M type of infection responses in the field. On the other hand, -*SrND643* lines showed a wide range of disease severity with continuous and approximately normal distribution (Fig. 2). Irrespective of disease severity, -*SrND643* RILs always displayed compatible infection responses (MS, MSS or S) indicating that genes modifying disease severity apart from *SrND643* are present in the population.

Validation of flanking markers for marker assisted selection

To assess the effectiveness of marker assisted selection for *SrND643*, a total of 53 CIMMYT advanced lines derived from ND643 were haplotyped with flanking markers *Xgwm350* and *Xwmc219* (Table 1). Out of 53 lines studied, 46 lines were postulated to have *SrND643* based on the field and seedling disease data (Table 1). In all of the 46 lines, *Xgwm350* and *Xwmc219* amplified 148 and 136bp fragments, respectively, predicting the presence of *SrND643*. The ITs in most of the SrND643 postulated lines ranged from 2^- to 22^+ except some lines which produced IT of 23^- or 2^+3^-, whereas, the disease severity ranged from 5 to 40% with infection responses ranging from R to M. Similarly, in all the 7 lines which were not postulated to carry *SrND643*, flanking markers did not amplify the *SrND643* associated allele fragments. ITs in these lines were 3, 3^+ or 4 except one line, ND643/2*WAXWING from the 4^{th} SRRSN, which showed an intermediate IT of 2^+3^-. Similarly, the disease severity in these lines ranged from 10 to 70% with infection responses of MS to S, except one, ND643/2*WBLL1// 2*KACHU from $M47^{th}$ IBWSN, which was found to have few plants with M responses as well (Table 1). Overall, these two flanking markers were found to be highly predictable for the presence of *SrND643* and all lines with the *SrND643* associated markers were resistant to Ug99 in seedling and field assays.

Discussion

Using selective genotyping and bulk segregant analysis methods, a new gene, *SrND643*, effective against the Ug99 group of *Puccinia graminis* f. sp. *tritici* races, was mapped on the long arm of chromosome 4A. As the resistant line ND643/2*Weebil1 has been a very common parent in the CIMMYT bread wheat improvement program since its development in 2007, *SrND643* is expected to have a significant presence in the recently developed Ug99 resistant advanced lines. Though this gene is sometimes moderately effective, the resistance has been observed to be significantly enhanced depending on genetic background. As gene *SrND643* has been mapped to within 0.5 cM from its closest marker *Xwmc219*, marker assisted selection or backcrossing can be effectively carried out to transfer this gene into new germplasm.

To this date, only one SR resistance gene, *Sr7*, has been reported on chromosome 4A using Chinese spring monosomics (Knott and Anderson, 1956; Knott, 1958). Similarly, *Sr7* has been observed to be genetically independent of the centromere, thus, it was suggested to be located on 4AL (RA McIntosh, unpublished 1973 cited in McIntosh et al. 1995). *Sr7* was originally identified in several Kenyan wheat varieties, such as Kenya farmer, Kenya 117A and Kenya Governor (Knott and Anderson, 1956; Knott, 1958). Later on, *Sr7* was transferred to Marquis and other non-Kenyan lines from Kenya 117A (Green et al. 1960). Loegering and Sears (1966) reported a different allele, *Sr7b*, in variety Hope. Though *Sr7b* is commonly present in Australian, European, North American and CIMMYT wheat germplasm, it has not been deliberately selected as a source of stem rust resistance (McIntosh et al. 1995). As *Sr7* has not been genetically mapped with molecular markers, it is very difficult to compare the location of *Sr7* with the location of *SrND643*. However, in contrast to *SrND643*, which displays an intermediate IT of 2 to 22^{+} to race TTKSK, *Sr7a* and *Sr7b* have been observed to produce high ITs of 3^{+} and 4, respectively, to race TTKSK (Jin et al. 2007; 2008). Similarly, *SrND643* flanking markers *Xgwm350* and *Xwmc219* amplified 139 and 218bp PCR fragments on ISr7b-Ra (*Sr7b* differential genotype). Similarly,

Xgwm350 amplified 139bp, whereas *Xwmc219* did not amplified any fragments (null allele) on Kenya Governor/10*MQ//8*LMPG (*Sr7a* differential genotype). Both the SSR alleles on *Sr7a* and *Sr7b* were different from those amplified on ND643 (148bp for *Xgwm350* and 136bp for *Xwmc219*). Moreover, the pedigree of ND643 does not appear to have any source of *Sr7*. Thus, we believe that *SrND643* is a novel gene or allele mapped to chromosome arm 4AL. In association studies on CIMMYT wheat germplasm, Crossa et al. (2007) and Yu et al. (2011, 2012) have reported that some closely linked DArT markers (*wPt-4487*, *wPt-7807*, *wPt-3795*, *wPt5749*, *wPt-5857*, *wPt-3349*) on 4AL are associated with stem rust resistance. As the markers *wPt-7807*, *wPt-3795*, *wPt5749* and *wPt-3349* have been placed on the same bin (4AL4-0.8-1.0) as *SrND643* in the physical bin map (http://www.cerealsdb.uk.net), it is possible that the resistance loci reported in the above association studies might represent the same gene as *SrND643*. Because our 4AL partial map does not have any common markers with the above reports, the exact relationship of *SrND643* with previous studies cannot be confirmed at this time.

In this study, RILs carrying *SrND643* were observed to have disease severities ranging from 5 to 40% depending upon the genetic background (Fig. 2). However, these lines still displayed the incompatible reaction to *Pgt* with infection responses of R, MR or M. On the other hand, in the absence of *SrND643*, the disease severity in the RILs ranged from 10 to 90% with compatible infection responses of MS to S. This observation indicates that disease severity as such may not be useful to classify the lines into resistant or susceptible categories for a segregating major-effect (?) gene. Similar observation were reported by Singh et al. (2011a) where CIMMYT advanced lines with major (??) genes *Sha7* and *SrTmp* displayed 1 to 30% and 5 to 60% of disease severities, respectively, accompanied by R or MR infection responses.

The markers *Xgwm350* and *Xwmc219* were validated for their usefulness for selecting the *SrND643* gene in the ND643-derived CIMMYT lines. The advanced lines were developed by a selected bulk approach where segregating

populations went through at least one season of selection for leaf and yellow rust in Mexico, and two seasons of selection for stem rust in Kenya. Lines were derived by selecting the single plants in the F_5 or F_6 generation, and were further evaluated for disease, yield and quality traits in subsequent breeding cycles. About 1500 advanced lines which passed the specific requirements of yield, quality and disease resistance criteria were considered as the candidates for different types of international bread wheat nurseries including IBWSN, HRWSN and SRRSN of CIMMYT. Since ND643 was identified to carry resistance effective against Ug99, it has been continuously used as a potential parent in crossing blocks of the bread wheat improvement program at CIMMYT. As *SrND643* is a moderately effective gene, its postulation based on field data often becomes a difficult task in the advanced breeding lines. With the availability of newly developed markers, *Xgwm350* and *Xwmc219*, screening and transfer of *SrND643* in bread wheat will be easier and more efficient in the future.

In conclusion, *SrND643* represents a new gene or allele for Ug99 resistance in common wheat. Being a moderately effective gene effective at the seedling and adult plant stages, *SrND643* can be deployed into new cultivars in conjunction with other all-stage resistance genes or genes effective only at the adult plant stage. Newly developed markers, *Xgwm350* and *Xwmc219*, are very reliable to screen and select for *SrND643* via marker assisted selection.

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Table 3. Table 1 Phenotype based SrND643 postulation and corresponding haplotypes of flanking markers in CIMMYT germplasm

SN	CIMMYT Nursery[a]	Entry No	GID[b]	Pedigree	SR field[c] response	Reaction[d] to TTKSK	Postulated *SrND643*	*Xwmc219* (136bp)	*Xgwm350* (148bp)
1	3rdSRRSN	6086	5535274	ND643/2*WBLL1	5 RMR	2^{-}	+	+	+
2	3rdSRRSN	6087	5535275	ND643/2*WBLL1	5 RMR	2	+	+	+
3	3rdSRRSN	6088	5535276	ND643/2*WBLL1	5 R	2	+	+	+
4	3rdSRRSN	6089	5535277	ND643/2*WBLL1	5 R	22^{+}	+	+	+
5	3rdSRRSN	6090	5535280	ND643/2*WAXWING	30 S	4	-	-	-
6	3rdSRRSN	6092	5535287	ND643/2*WAXWING	15 MS	3	-	-	-
7	3rdSRRSN	6093	5535312	ND643//2*PRL/2*PASTOR	20 S	3	-	-	-
8	4thSRRSN	6035	5535271	ND643/2*WBLL1	5 M	2	+	+	+
9	4thSRRSN	6036	5535285	ND643/2*WAXWING	30 S	$2^{+}3^{-}$	-	-	-
10	4thSRRSN	6038	5535313	ND643//2*PRL/2*PASTOR	5 RMR	2	+	+	+
11	4thSRRSN	6039	5535350	ND643/2*WBLL1	5 RMR	22^{+}	+	+	+
12	4thSRRSN	6041	5535351	ND643/2*WBLL1	5 RMR	2^{+}	+	+	+
13	4thSRRSN	6042	5535357	ND643/2*WBLL1	5 RMR	2^{+}	+	+	+
14	7thSRRSN	6110	6415386	CHIBIA//PRLII/CM65531/3/FISCAL/4/ND643/2*WBLL1	5 M	2	+	+	+
15	7thSRRSN	6115	6417213	BECARD//ND643/2*WBLL1	15 M	2	+	+	+
16	7thSRRSN	6141	6416382	ND643/2*WBLL1//ATTILA*2/PBW65/3/MUNAL	20 MR	$2^{+}3$	+	+	+
17	7thSRRSN	6142	6417455	ND643/2*WBLL1//ATTILA*2/PBW65/3/MUNAL	30 MR	$2^{+}3$	+	+	+
18	7thSRRSN	6143	6416477	ND643/2*WBLL1/3/KIRITATI//PRL/2*PASTOR/4/KRT// PBW65/2*SERI.1B	5 RMR	2^{+}	+	+	+
19	7thSRRSN	6144	6416507	ND643/2*WBLL1//2*BAJ #1	5 RMR	2^{+}	+	+	+
20	7thSRRSN	6145	6416509	ND643/2*WBLL1//2*BAJ #1	5 RMR	$2^{+}3$	+	+	+
21	7thSRRSN	6146	6417467	ND643/2*WBLL1//2*BAJ #1	10 RMR	2^{+}	+	+	+
22	7thSRRSN	6147	6416530	ND643/2*TRCH//BECARD/3/BECARD	70 S	3^{+}	-	-	-
23	7thSRRSN	6148	6417471	ND643//2*ATTILA*2/PASTOR/3/WBLL1*2/KURUKU/4/WBLL1*2/ BRLG	30 MR	2	+	+	+
24	7thSRRSN	6149	6417472	ND643//2*ATTILA*2/PASTOR/3/WBLL1*2/KURUKU/4/WBLL1*2/ BRLG	30 MR	2^{+}	+	+	+
25	M-8SRRSN	42	6681796	ND643/2*WBLL1/4/WHEAR/KUKUNA/3/C80.1/3*BATAVIA//2*WBLL1	5 RMR	2	+	+	+
26	M-8SRRSN	43	6683550	ND643/2*WBLL1/4/WHEAR/KUKUNA/3/C80.1/3*BATAVIA//2*WBLL1	5 RMR	$2^{-}/22^{+}$	+	+	+
27	M-8SRRSN	44	6683560	ND643/2*WBLL1//KACHU	10 M	22^{+}	+	+	+
28	M-8SRRSN	198	6680756	ND643/2*WBLL1//BECARD	5 M	2	+	+	+

Table 1 (Continued)

SN	CIMMYT Nursery[a]	Entry No	GID[b]	Pedigree	SR field[c] response	Reaction[d] to TTKSK	Postulated SrND643	*Xwmc219* (136bp)	*Xgwm350* (148bp)
31	M-8SRRSN	297	6681200	ND643/2*WBLL1/4/CHIBIA//PRLII/CM65531/3/SKAUZ/BAV92/5/BECARD	10 M	2	+	+	+
32	M-8SRRSN	396	6685270	ND643/2*WBLL1//HEILO	5 M	22^{+}	+	+	+
33	M-8SRRSN	397	6685271	ND643/2*WBLL1//HEILO	10 M	2	+	+	+
34	M-8SRRSN	429	6684608	ND643/2*WBLL1//BECARD	15 MR	2	+	+	+
35	M-8SRRSN	446	6684742	ND643/2*WBLL1/4/CHIBIA//PRLII/CM65531/3/SKAUZ/BAV92/5/BECARD	5 MR	2	+	+	+
36	M-8SRRSN	447	6684751	ND643/2*WBLL1/4/CHIBIA//PRLII/CM65531/3/SKAUZ/BAV92/5/BECARD	10 RMR	23^{-}	+	+	+
37	M-8SRRSN	448	6684770	ND643/2*WBLL1/3/KIRITATI//2*PRL/2*PASTOR/4/BECARD	10 M	$2^{+}3^{-}$	+	+	+
38	M25HRWSN	1015	6570095	ND643/2*WBLL1//KACHU	10 M	NA	+	+	+
39	M25HRWSN	1017	6684812	ND643/2*WBLL1//KACHU	5 M	NA	+	+	+
40	M25HRWSN	1018	6684970	ND643/2*WBLL1//KACHU	10 MR	NA	+	+	+
41	M25HRWSN	1021	6684981	ND643/2*WBLL1/3/KIRITATI//2*PRL/2*PASTOR	5 MR	NA	+	+	+
42	M25HRWSN	1076	6569660	ND643/2*WBLL1/3/BERKUT//PBW343*2/KUKUNA	5 M	NA	+	+	+
43	M25HRWSN	1077	6569788	ND643/2*WAXWING//SAAR/2*WAXWING	5 R	NA	+	+	+
44	M25HRWSN	1121	6684735	ND643/2*WBLL1//2*KACHU	5 M	NA	+	+	+
45	M25HRWSN	1123	6684748	ND643/2*WBLL1/4/CHIBIA//PRLII/CM65531/3/SKAUZ/BAV92/5/BECARD	10 M	NA	+	+	+
46	M25HRWSN	1125	6684759	ND643/2*WBLL1/4/CHIBIA//PRLII/CM65531/3/SKAUZ/BAV92/5/BECARD	10 M	NA	+	+	+
47	M25HRWSN	1126	6684761	ND643/2*WBLL1/4/CHIBIA//PRLII/CM65531/3/SKAUZ/BAV92/5/BECARD	10 M	NA	+	+	+
48	M25HRWSN	1127	6684767	ND643/2*WBLL1/3/KIRITATI//2*PRL/2*PASTOR/4/BECARD	10 M	NA	+	+	+
49	M25HRWSN	1129	6684771	ND643/2*WBLL1/3/KIRITATI//2*PRL/2*PASTOR/4/BECARD	10 M	NA	+	+	+

50	M47IBWSN	91	6681793	ND643/2*WBLL1/4/WHEAR/KUKUNA/3/C80.1/3*BATAVIA//2*WBLL1	10 MR	NA	**+**	**+**	**+**
51	M47IBWSN	92	6681794	ND643/2*WBLL1/4/WHEAR/KUKUNA/3/C80.1/3*BATAVIA//2*WBLL1	5 MR	NA	**+**	**+**	**+**
52	M47IBWSN	650	6679711	ND643/2*WBLL1//2*KACHU	40 S, 30 M	NA	**-**	**-**	**-**
53	M47IBWSN	652	6681187	ND643/2*WBLL1//2*KACHU	10 MSS	NA	**-**	**-**	**-**

[a] IBWSN, International bread wheat screening nursery; HRWSN, Hard red wheat screening nursery; SRRSN, Stem rust resistance screening nursery, M represents the Mexicali block

[b] Germplasm identification number, a unique identifier of CIMMYT germplasm

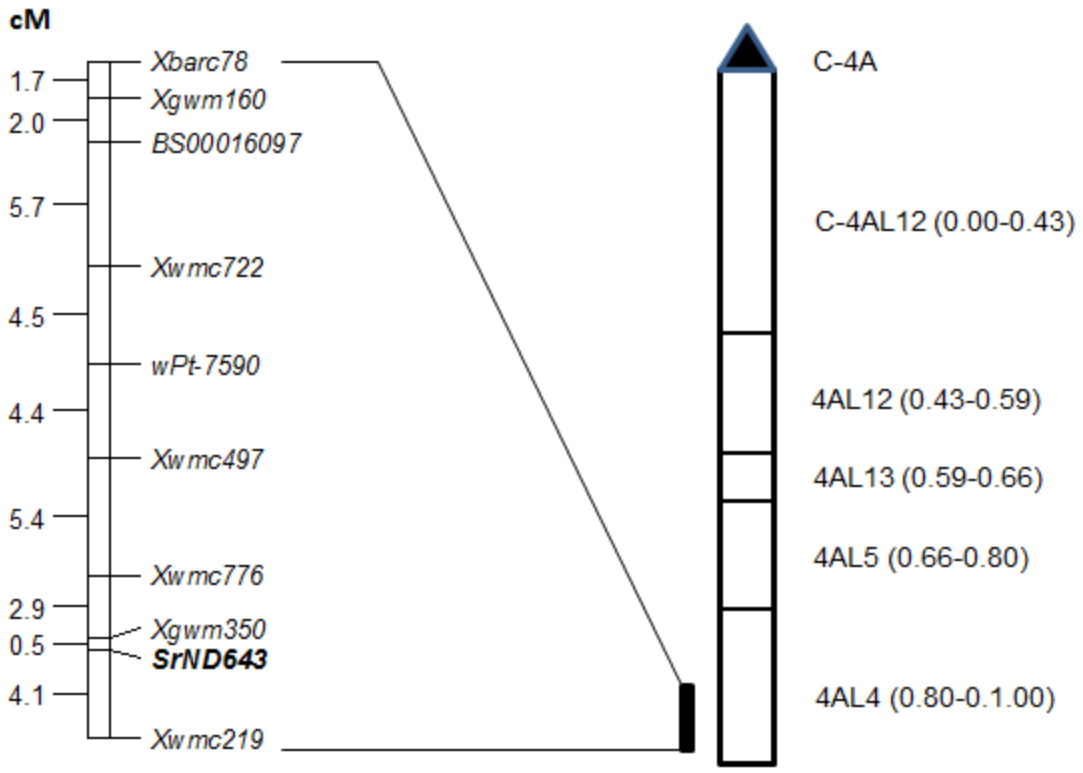

Figure 17. Figure 1. Genetic position of SrND643

on a partial linkage map of 4AL with corresponding physical bin map. The genetic distances between the markers are given in centi-morgans.

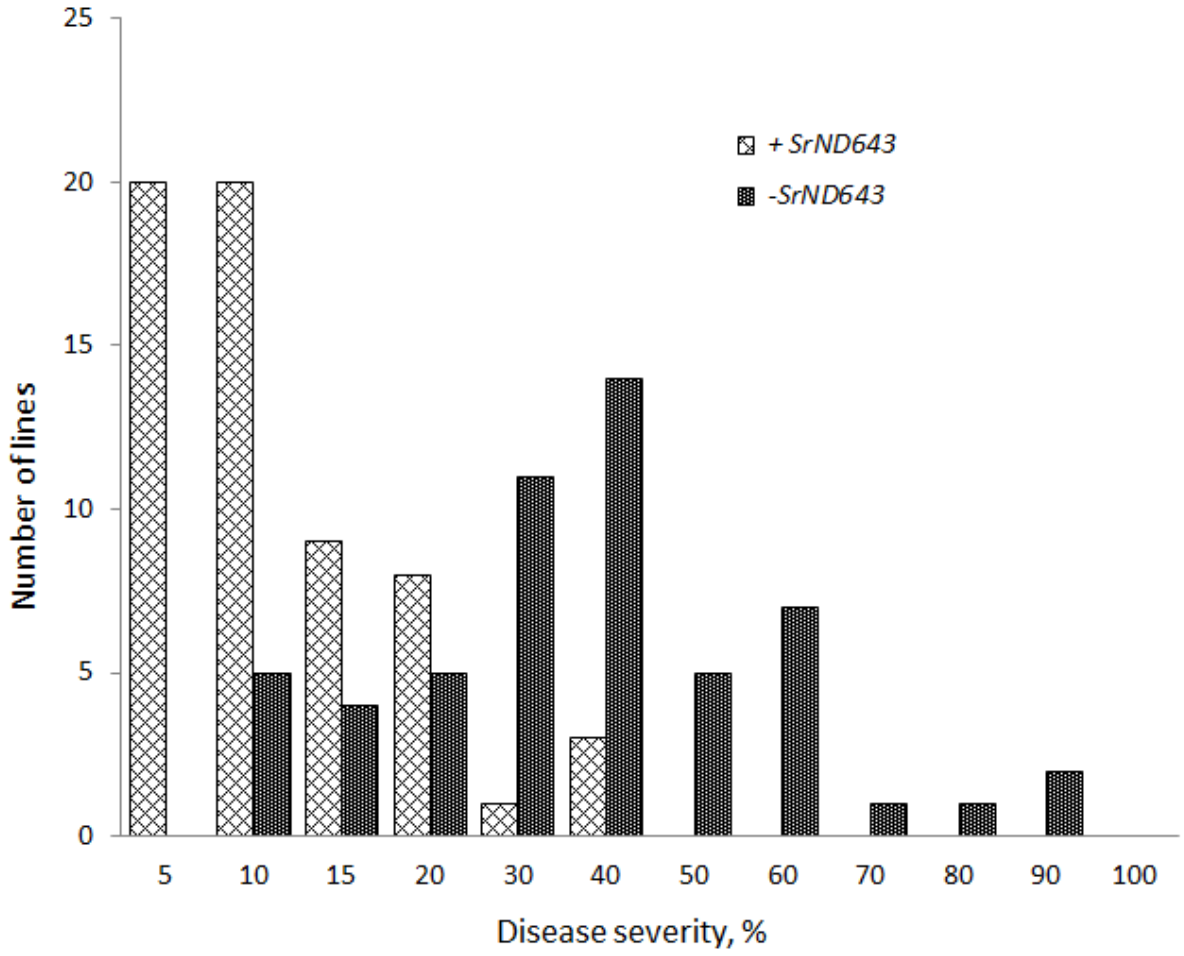

Figure 18. Figure 2. Distribution of disease severity among Cacuke/3/ND643/2*Weebil derived RILs with (+) or without (-) *SrND643.* Data were collected in the 2010 main-season at Njoro, Kenya.

Paper 3. Characterization of two qualitative trait loci against stem rust-Ug99 located in chromosome 2B of wheat.

Introduction

Wheat is one of the most important cereal crop in the world, it provides adequate quantity of carbohydrates and calories (55% and 20%, respectively) for human diet. As many crops, wheat is affected by diseases that decreased its productivity. Rust are cataloged among important disease for wheat production. A long of the wheat history, epidemics caused by *Puccinia graminis* Pers f. sp. *tritici* Eriks. & E. Henn (Pgt) have been reported, occurred in countries such as Australia, Canada and the USA (Roelfs 1978) causing devastating yield losses (Nagarajan and Joshi 1975; Joshi et al. 1986). Since the 1970´s, the stem rust control have been carried out with resistant semi-dwarf spring wheat varieties (Singh et al. 2011b).

The wheat stem rust fungus Ug99 (*Puccinia graminis* Pers. f. sp. *tritici* Eriks. & E. Henn.) (Pgt) is one of the disease that threaten wheat production and thereby the food security (Singh et al. 2011a). The new race of stem rust pathogen (TTKSK), commonly known as Ug99 recorded for the first time in Uganda, and its further evolution and spread outside eastern Africa is a challenge for maintain the wheat production (Roelfs and Martens 1988; Jin et al. 2008).

These new races confers pathogenicity to race specific *Sr* genes, widely distributed in varieties planted, its estimated 80-90% of wheat plants are susceptible to variants of Ug99, causing losses of 70% of yield (Shiferaw and Hoffman, 2012; Jin and Singh 2006; Fetch 2007; Singh et al. 2008; Waynera 2009). Since the report of Ug99, other races (TTKST, TTTK, TTKSF, TTKSP, PTKSK, PTKST) infecting crops carrying major genes this race and related races have been detected in Kenya, Ethiopia, Sudan, Yemen, Iran, Tanzania, Zimbabwe, and South Africa (Wanyera et al. 2006; Nazari et al. 2009; Pretorius et al. 2010; Singh et al. 2011b). The report of Pgt in Iran in 2007 is a concern for its spread in Asia (Nazari et al. 2009), and has increased the threat for an epidemic in wheat growing countries with susceptible varieties to Ug99 on all continents. Not only affecting Sr genes from wheat, but also Sr genes introduced from relatives such as Sr38 (Pretorius et al. 2000; Jin et al.

2007) or Sr24 and Sr36 (Jin et al. 2008, 2009), even a combined virulence to Sr31 and Sr24 (Pretorius et al. 2010). Wheat breeding for disease resistance is one of the economic and environmental friendly way for grain producers to ensure productivity.

Near 50 stem rust resistance genes have been designed to date (McIntosh et al. 2008), some of them conferring race-specific resistance can be overcome easily by new stem rust races (Jin et al. 2007; Singh et al. 2011a).

The discovery of new genes is important to pyramid with race non-specific genes and achieve durable resistance; to save time improving wheat varieties for rust, the molecular markers approach provides an opportunity to discover resistant genes and combine them for pyramiding (William et al. 2007). Molecular tools can help to discriminate between new or different sources of resistance effective to Ug99 races and offers alternative methods for germplasm selection, which facilitates effective pyramiding of resistance in the absence of the pathogens (Krattinger et al. 2009).
Around 28 race specific resistant genes across wheat genome have been reported effectives to Ug99; most of them transferred from related species (Singh et al 2011b). The chromosome 2B of wheat is one of the most containing stem rust resistant genes, and the tests against Ug99 indicated that there are effectives (Sr39, Sr9, Sr40, Sr47, Sr28, SrWeb and Sr36) and non-effectives (Sr10, Sr20, Sr19, Sr23, and Sr16). Although there are several gene effectives for Ug99, the search for new alleles is important to maintain the risk of disease low.

One source of resistance to Pgt is provided by the gene Sr-Huw234, this gene was first scored in the 2nd stem rust resistant screening nursery in 2007, is an moderately effective gene (Singh et al. 2011b; Yu et al. 2011). On the other hand, the resistance provided by the gene Sr-Yanac was also, first scored in the 2nd stem rust resistant screening nursery in 2007 under the temporally name of “Sr-Synthetic” (www.globalrust.org/traction/permanlink/screening20).
These genes come from CIMMYT material and now properly named as the donor of resistance, the line Huwha is the donor for SrHuwha previously Sr-Huw234, and the line Yaye is the donor of SrYaye before named Sr-Yanac. This last variety has the

same pedigree and selection history than Hidase (synonym ETBW5795) released in Ethiopia in 2012.

The objective of this work was characterize the resistant region to stem rust Ug99 in two recombinant inbreed line populations. We hypothesize that according to reaction type tested in stem rust resistance screening nurseries the parental resistant lines Yaye#1 and Huwha could have different resistant gene.

Materials and Methods

Plant Materials

Two mapping populations comprised the crossing of susceptible line Cacuke and the stem rust resistant lines HUWHA and Yaye#1 hereafter called only Yaye, for simplicity. A total of 148 and 192 $F_{4:5}$ RILs were developed, using the mentioned parents, respectively.

Field evaluation of stem rust resistance

Parents and the two RIL populations were phenotyped for their infection response to stem rust race Ug99 in field trials at the Kenya Agricultural Research Institute (KARI) in Njoro, Kenya. F3:4 RILs derived from Cacuke/Yaye were evaluated during off-growing season in 2011 and the $F_{4:5}$ RILs derived from the cross Cacuke/Huwha were evaluated in Off-growing Season 2011 and Main-growing season 2012.

The off season rust screening nursery was planted during November and harvested in May, and the main season was started in June and finalized in October. Four grams of seed of each RIL and parent were plated in 0.7 m long paired-row plots with 0.3 m wide row and plot spacing. A mixture of susceptible lines was hill planted and used as spreaders for initiating and guarantee the infection of stem rust. A light-weight mineral oil suspension of freshly collected urediniospores of *Puccinia graminis tritici* race TTKST, was sprayed on the spreaders as described by Njau et al. (2013).

Severity and infection responses were recorded when the plants achieved adult and soft dough stage, when the susceptible line Cacuke showed moderately susceptible to susceptible infection response. The infection type was evaluated based on size and shape of the uredinia according to Roelfs et al. (1992) scale, where R=resistant, MR= moderately resistant, MS= moderately susceptible, and S=susceptible. Plants observed with a mixture of infection responses were recorded by the predominant infection type and subsequently by the next low infection type. The RIL families were classified as resistant when they displayed low infection responses of R, MR or M, and susceptible if they displayed infection responses of MS or S. The families with both resistant and susceptible plants were classified as segregating. The percent disease severity was recorded following the modified Cobb Scale (Peterson et al. 1948). To avoid complication in linkage map construction, the segregating RILs were removed from analyses. Final disease scores for each RIL were given based on the consistent expression of resistance response across the seasons.

Seedling evaluation with the race TTKSK of stem rust

The parents and two $F_{4:5}$ RILs were assayed with the race TTKSK (isolate 04KEN156/04) at the seedling stage. Five seeds of each family were planted. After 7 or 10 days of planting, the seedlings were inoculated when the primary leaf was fully emerged. Fourteen days after inoculation each plant was scored according to infection type displayed, using the Stakman et al. (1962) scale from 0-4, where 2 or less are registered resistant and 3 or more as susceptible.

Molecular marker analysis

Genomic DNA of Cacuke, Yaye, Huwha and the corresponding derived families was extracted using the CTAB method according to CIMMYT laboratory protocol (CIMMYT 2005). To identify the chromosomal location of the gene present in the Cacuke/Yaye and Cacuke/HUW234 populations, both selective genotyping and bulk segregant analysis techniques were used. For selective genotyping, a total of 46 entries, consisting of parents and twenty two resistant and susceptible lines each population were genotyped with diversity arrays technology (DArT) markers at

Triticarte Pty Ltd., Australia (Akbari et al. 2006). Similarly, resistant and susceptible bulks were prepared by mixing equal amount of DNA from 10 resistant and 10 susceptible lines respectively (Michelmore et al. 1991; Lowe et al. 2011). Then, targeted chromosome specific SSR markers were screened for polymorphism between parents and their respective bulks. The annealing temperature and the sequence of each SSR marker was obtained from the GrainGenes database (http://wheat.pw.usda.gov). To construct the final genetic linkage map, all the RILs were genotyped with polymorphic markers. Linkage mapping was carried out with Inclusive Composite Interval Mapping (ICIM) software (Li et al. 2007). The recombination frequency was converted to map distance with the Kosambi mapping function (Kosambi 1944). Similarly, the linkage map was ordered and rippled with seriation (SER) and sum of adjacent recombination fraction (SARF) algorithms respectively (Li et al. 2007). The graphical representation of the linkage map was constructed with GGT software (Van Berloo, 2008).

Infection type of SrYaye and SrHuwha in previous stem rust resistance screening nurseries

Infection type of *SrYaye* and *SrHuwha* was registered since the 2nd stem rust resistance screening nursery (SRRSN). Using the races TTKSK, TTKST, TTTSK and TRTT, we used this information to compare with the previously reported resistant genes against Ug99 such as *Sr39, Sr40, SrAes7, Sr28, Sr36, Sr47* and *SrWeb* located on the chromosome 2B.

Results

Field evaluation of stem rust resistance

Uniform disease pressure in the field was present during both crop seasons main season 2011 for Cacuke/Yaye population and Off-season 2011 and Main season 2012 for Cacuke/HUWHA population. Parental lines Cacuke and Yaye sprayed a disease severity of 30 and 1 %, respectively. The reaction type was moderately susceptible-susceptible for Cacuke and moderately resistant- resistant for Yaye. The

derived population ranged from 1 up to 70% for stem rust severity in the condition tested of the main cycle of 2011.

For the survey of disease severity in the Cacuke and Huwha derived population, Cacuke showed form 70-90% of susceptibility and Huwha from 15-50% being moderately resistant at adult plant stage. The progeny mainly ranged between 10 to 90% of disease severity.

In both populations, there were several segregating types in each field experiment. In order to avoid the misclassification, lines with inconsistent or ambiguous infection responses across each experiment were discarded from the analysis in each population.

Seedling evaluation with the race TTKSK of stem rust

Hundred forty eight plants in CCK/Huwha derived lines were evaluated against stem rust race TTKSK, 39 showed segregation, 61 were scored as susceptible and 50 were resistant. Based on 101 lines used in the analysis, the chi square fitted to a 1:1 ratio ($\chi 2 = 2.2$, $P = 0.14$). For the Lines derived from Cacuke/Yaye, the susceptible lines scored were 86 and resistant 96, conforming the ratio 1:1 ($\chi 2 = 0.5$, $P = 0.46$).

Molecular marker analysis

For marker analysis, the susceptible and resistant lines of each population were first valuated using DArT technology. For Cacuke/Yaye, 233 DArT markers were polymorphic and formed 21 groups. The trait for stem rust was linked with 10 markers belonged to the chromosome 2B. Once the chromosomal location was knew, 20 SSR markers were set in the parental lines Cacuke, Yaye and susceptible and resistant bulks. Polymorphism between parents was found in four markers, *Xwmc154, Xbarc200, Xwmc770* and *Xgwm148,* according to Sourdille et al. (2004), located in

the short arm of the chromosome 2B. To construct the molecular map, these four markers were set in all lines. The segregation distortion of these markers followed to a 1:1 segregation ratio of parental alleles (Chi-square probability, *P*= 0.30 to 1.44). The map was constructed with a total genetic distance of 23.04 cM, the trait locus for the resistance to Ug99 was located between the markers *Xbarc200* and *Xwmc770* at a distance of 4.44 and 7.61, respectively (Fig 1). The markers *Xwmc154*, *Xbarc200 and Xwmc770* have been placed on the deletion bin 2BS1-0.53 of 2BS in the previous consensus physical-genetic map of wheat (Sourdille et al. 2004, Marone et al. 2012). These results indicate that the qualitative trait *SrYaye* is located between in the distal and proximal bin in the short arm of the chrosome 2B. The marker order is consistent with previous reported by Sourdille et al. (2004). The flanking markers produced a band of around 151 and 163 bp, respectively, associated with *SrYaye* in Yaye. $F_{4:5}$ Lines scored with the band present for *SrYaye* deployed a disease severity ranging from 1 to 30, and lines with missing allele ranged from 15 to 80. The average of severity disease for resistant and susceptible lines were 6.9 and 51.0 %, respectively, and were statistically different (α=0.05) between them.

For Cacuke/Huwha population, 466 DArT markers were polymorphic between parents, forming 46 linkage groups. Among them, the qualitative trait for resistance to Ug99 was grouped with three markers belonged to chromosome 2B. Once knew the chromosomal location Using the DArT technology, 67 SSR markers were used for construct the genetic map, 43% were polymorphic between parents and 11% were polymorphic between susceptible and resistant bulks. Ten markers located on 2B chromosome were grouped using F4:5 lines giving a map with total length of 108.3 cM almost half of them belonging to short arm; however, only five SSR marker*s* were taken to construct the final map, because the *SrHuwha* trait was linked with those markers in the long arm of this chromosome (Figure 2).

Infection type of SrYaye and SrHuwha in previous stem rust resistance screening nurseries

Resistance to stem rust group of Ug99 fungus by the genes *SrYaye* and *SrHuwha* was registered in 2007 in the 3rdSRRSN. The infection type was clearly easy to differentiate between the resistance given by these two genes, from fleck to 2 for *SrYaye* and 2 to 2+ for *SrHuwha* according to Stakman et al. (1962) scale and depending the race (Table 5). Other genes located on the chromosome 2B effective against Ug99 are *Sr39, Sr40* and *SrAes7* in the short arm, and *Sr28, Sr36, Sr47* and *SrWeb* in the long arm. The search indicated that these two genes mapped in this work, shows different reaction type than those reported previously.

Discussion

Around 14 resistant genes against stem rust have been reported in the chromosome 2B (McIntosh et al 2011). However, the races derived from Ug99 are capable to defeat almost 50% of them (Singh et al. 2006). More recently, quantitative trait loci are being reported linked to stem rust resistance in both arms of chromosome 2B (Singh et al 2013b, Bansal et al. 2013). In the sort arm, molecular DArT markers have been useful for finding QTLs against rust, such as those reported by Li et al. (2013) for yellow rust and Singh et al. (2013b) for stem rust, both in short arm. We found linkage of 10 DArT markers with the resistant trait to stem rust, one of them shared linkage with previous reported by Singh et al (2013), the marker wPt-2430 was linked 86 cM far away from the QTL for stem rust in PBW343/Muu population, while 42 cM from *SrYaye* in this work. Singh et al (2013a) also found a DArT marker (wPt-1064) groped with resistance but 2 to 6 cM, while in this work, this marker was located at cM away and based on the SSR markers is more likely their QTL for stem rust be different from the location of *SrYaye*. Other markers grouped were wPt-9423 and wPt-8072, these were previously reported by Bansal et al (2013), grouped with a QTL for stem rust, these markers where located around 52 cM from the QTL, while in

this work these markers were 17 cM away from Sr-Yaye. In addition, Singh et al (data not published) found a QTL in PBW343/Diniza in the 2BS chromosome, linked with the marker *Xgwm148* in Main crop season, in this work, the marker *Xgwm148* was located at more than 10 cM of the resistant qualitative trait. Sort arm of chromosome 2B has resistant genes to Ug99 already named, the genes *Sr36* introgressed from *Triticum timopheevii* and *Sr39* and *SrAes7* introgressed from *Aegilops speltoides* (Purnhauser et al. 2011; Gold et al 1999; Klindworth et al. 2012), and *Sr40* from *Triticum araraticum*. The gene *Sr39* is mapped with the markers Sr39#22, Sr39#50 and BE500705 (Mago et al. 2009) the first one for resistant genotype and two remaining for susceptible genotype, while *Sr7Aes* and *Sr40* linked with the markers *Xbarc200* among others. We tested the marker Sr39#22 in control lines carrying the gene Sr39 (RWG1 and RWG4 lines), Cacuke, Yaye and susceptible and resistant bulks, we did not find the diagnostic band in Cacuke, yaye and bulks but in controls (picture not shown). The only SSR marker for mapping *Sr40* and *SrAes7* shared with our results was Xbarc200; the gene *Sr40* is linked with the markers *Xgwm344*, *Xwmc661* and *Xwmc474*, which were not polymorphic between Cacuke and Yaye. The gene *SrAes7* according to Kindworth et al. (2012), could be related with the gene *Sr39*, we tested the marker Sr39#22r for resistant genotypes (Mago et al. 2009) confronting with lines carrying the gene Sr39 (RWG1 and RWG4), no band was found in cacuke or Yaye whilst in the line containing Sr39 a band around 800 bp was present.

On the other hand, the SrHuwha was located on the long arm of the chromosome 2B, we obtained a linkage group of two DArT markers along with the resistant gene SrHuwha (map not shown). The marker wPt-1394 and wPt-9736 were located at 11 and 3 cM respectively, corresponding to chromosome 2B. We use SSR markers for obtain the location across the chromosome. Microsatellite Xwmc332 and Xgwm388 flanked the gene on Cacuke/Huwha population. Chromosome 2BL carries Sr9, Sr16, Sr28 (Rouse et al 2012), Sr40 (Wu et al 2009) and Sr47 (Klindworth et al. 2012). All alleles of Sr9, and Sr16 are ineffective against TTKSK. Sr28 mapped on 2BL (Hiebert

et al. 2010) remains effective and reported on different mapping population (Bansal et al. 2012; Rouse et al. 2012), this gene share the marker *Xwmc332* with the resistance region found in this work, however, more markers linked to Sr28 gene were not linked in this work. Wu et al (2009) mapped the gene Sr40 on the long arm, the SSR markers flanking the gene were *Xwmc474* and *Xwmc661* in two populations, there were no linkage with these markers and the markers in the map of *SrHuwha*, an allelism test is required.

The infection type scored for the resistance region on 2BS and 2BL is shown in the Table 5. SrYaye and SrHuwha have different reaction type between them such as the genes effective against TTKSK. In the sort arm, SrYaye reaction type has been scored from fleck to 2, different from Sr39, 40, SrAes7. With the races TTKST and TTTSK could not show differences but with the race TRTTF the genes show infection type of 2 with small pustules while SrYaye although also the infection type is 2, the score registered has been with large pustules (+).

On the other hand, SrHuwha with the race TTKSK showed an infection type of 2 with large pustules, and this reaction is different from the genes Sr28, Sr36, Sr47 and SrWeb. SrWeb displayed a similar reaction type for the race TTKSK; however, the large pustules in *SrHuwha* and different infections types with the races TTKST and TRTTF shows differences between the resistances provided by each of these two genes (Table 5). It might possible, under these results, the major genes in the resistant material Yaye and Huwha are different from the mayor genes located on the chromosome 2B; However, an allelism test should be carry out in order to provide an accurate determination.

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Table 4. Table 1. Parental lines and field/greenhouse reaction of Cacuke, Huwha and Yaye.

Parents	Pedigree	GID[a]	Seedling reaction to Ug99	Field reaction to Ug99
Cacuke#1	Canadian/Cunningham//Kennedy	5347441	3+	80S-100S
HUWHA	HUW234+LR34/PRINIA//KRONSTAD F2004	5552006	22+	15MR
YAYE#1	YANAC/3/PRL/SARA//TSI/VEE#5/4/CROC_1/AE.SQUARROSA (224)//OPATA	5343322	-	1RMR

[a] Germplasm identification number in CIMYYT database

Table 5. Table 2. Reaction type of lines carrying resistance genes to stem rust located on chromosome 2B short and long arm with four races of Ug99 lineage.

		Race (IT)			
	Gene	**TTKSK**	**TTKST**	**TTTSK**	**TRTTF**
Short arm	*Sr39*	22-	22-	22-	22-
	Sr40	22-	22-	22-	22-
	SrAes7	2-	2-	2-	2-
	Sr36	0;	34	34	34
	SrYaye	**;12**	**2-**	**2**	**2+**
Long arm	*Sr28*	0;	34	0;	34
	Sr47	;	2-	;	2+
	SrWeb	2	34	2	34
	SrHuwha	**22+**	**2**	**;2-**	**2**

IT: infection type. Reaction type registered in the 3rdSRRSN for the genes *SrYaye* and *SrHuwha*

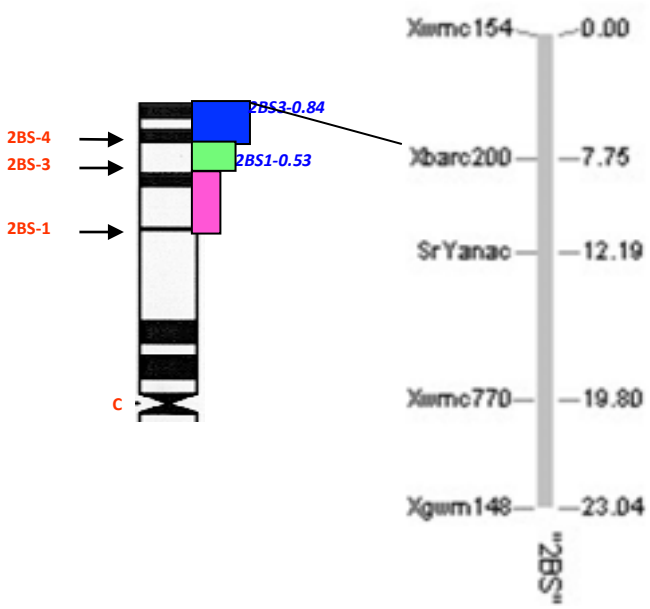

Figure 19. Figure 1. Genetic position of SrYaye

on a partial linkage map of 2BS with corresponding physical bin map. The genetic distances between markes are given in centi-morgans

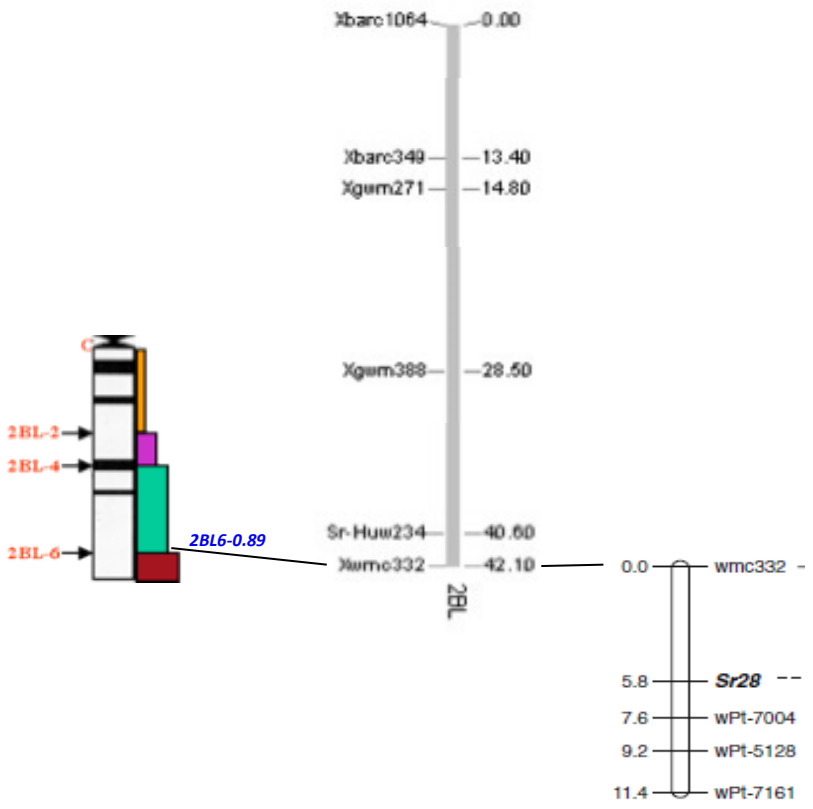

Figure 20. Figure 2. Comparision of bin map (left)

generated by Sourdille et al. (2004) and the genetic position of *SrHuwha* and map to the right was developed by Rouse et al. (2012). The genetic distances between markes are given in centi-morgans.

6. GLOBAL CONCLUSIONS

Genes located on chromosome 6DS similar to Niini could be different from Pfunye or *Sr42*

Infection type tests must be run to evaluate differential response between lines carrying the Sr gene in chromosome 6DS

More SNP´s markers should be run in lines carrying *SrND643* in order to reduce the distance between markers

SNP markers must be run in population Cacuke and Huwha and Yaye

Allelism tests should be carried out to differentiate between knowledge genes and new genes in the chromosomes 6DS, 4AL and 2B short and long arm

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7. APPENDIX

Publication and congress

Lopez-Vera EE, Nelson S, Singh RP, Basnet BR, Haley SD, Bhavani S, Huerta-Espino J, Xoconostle-Cazares BG, Ruiz-Medrano R, Rouse MN, Singh S (2014) Resistance to stem rust Ug99 in six bread wheat cultivars maps to chromosome 6DS. Theor Appl Genet, 127: 231-239.

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