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ORIGINAL PAPER

Molecular mapping of resistance genes to tan spot [*Pyrenophora tritici-repentis* race 1] in synthetic wheat lines

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Abstract Synthetic wheat lines ($2n = 6x = 42$, AABBDD), which are amphiploids developed from the hybrid between tetraploid wheat (*Triticum turgidum* L., $2n = 4x = 28$, AABB) and *Aegilops tauschii* Coss. ($2n = 2x = 14$, DD), are important sources of resistance against tan spot of wheat caused by *Pyrenophora tritici-repentis*. In the present study, inheritance, allelism and genetic linkage analysis in synthetic wheat lines have been carried out. Segregation analysis of the phenotypic and molecular data in $F_{2:3}$ populations of CS/XX41, CS/XX45, and CS/XX110 has revealed a 1:2:1 segregation ratio indicating that resistance of tan spot in these synthetic lines is controlled by a single gene. Allelism tests detected no segregation for susceptibility among F_1 and F_2 plants derived from intercrosses of the resistance lines XX41, XX45 and XX110 indicating that the genes are either allelic or tightly linked. Linkage analysis using SSR markers showed that all the three genes: *tsn3a* in XX41, *Tsn3b* in XX45 and *tsn3c* in XX110 are clustered in the region around *Xgwm2a*, located on the short arm of chromosome 3D. The linked markers and genetic relationship of these genes will greatly facilitate their use in wheat breeding and deployment of cultivars resistant to tan spot.

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Introduction

Tan spot, caused by the fungus *Pyrenophora tritici-repentis* (Died.) Drechs., anamorph *Drechslera tritici-repentis* (Died.) Shoem, is one of the major foliar diseases of wheat spreading worldwide at an increasing rate, and can cause a yield loss of up to 50% in susceptible wheat cultivars (Hosford 1982; Rees et al. 1988; Riede et al. 1996). According to Duveiller et al. (2005), 20–30% yield loss of wheat was frequently recorded in farmers' fields. Adoption of new farm management practices such as minimum or zero tillage, banning of stubble burning, and intensive wheat after wheat cultivation systems have contributed to the fast spread of the pathogen *Pyrenophora tritici-repentis* (Wolf and Hoffmann 1993; Tekauz et al. 2004).

The development and use of resistant cultivars is regarded as the most cost effective, socially feasible and ecologically safe means of controlling tan spot. Because of the co-evolution of the host and pathogen, however, the deployment of individual resistance genes leads to the emergence of new virulent pathogen mutants. Hence, identification of new resistance sources and pyramiding of more resistance genes in a cultivar are of paramount importance for effective and better genetic control. However, selection of genotypes with such gene combinations via classical genetics and breeding methods is very time consuming and even may be impossible due to the lack of pathogen isolates with specific virulence genes.

On the other hand, the development of molecular markers that are closely associated with the respective resistance genes would enable to pyramidize genes of interest effectively and successfully (Gupta et al. 1999; Huang et al. 2000). Among the different molecular

markers developed to date, restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), amplified length polymorphisms (AFLPs) and micro satellites also called simple sequence repeats (SSRs) have been used for gene mapping in wheat. The use of RFLP (Chao et al. 1989; Kam-Morgan et al. 1989) and RAPD (Devos and Gale 1992) for wheat gene mapping, however, is very limited due to their very low level of frequency and polymorhism which may be associated with the polyploid nature, high proportion of repetitive DNA, large genome size and recent origin of hexaploid wheat (Gupta et al. 1999). On the other hand, AFLPs and SSRs have been used extensively for wheat gene mapping (Huang et al. 2000; Hartl et al. 1999; Singrün et al. 2004; Schmolke et al. 2005; Mohler et al. 2005). In particular, SSR loci are employed much more frequently than other markers due to their advantages of higher level of polymorphism, known map location, accuracy, repeatability and PCR-based amplification (Röder et al. 1998; Gupta et al. 1999; Huang et al. 2004).

To date, unlike powdery mildew and rust resistance genes of wheat, only very few sources of tan spot resistance genes are identified and mapped (Faris et al. 1996, 1997; Friesen and Faris 2004; Cheong et al. 2004). Recently, Tadesse et al. (2006a) through monosomic analysis have identified tan spot resistance genes on chromosome 3D in three synthetic wheat lines (XX41, XX45 and XX110) which are amphiploids ($2n = 6x = 42$, AABBDD) developed from the hybrid between tetraploid wheat (*Triticum turgidum* L., $2n = 4x = 28$, AABB) and *Aegilops tauschii* Coss. ($2n = 2x = 14$, DD), the progenitor of wheat D genome. The objectives of the present study were to study tan spot inheritance, allelism among the resistant synthetic wheat lines, and genetic linkage in order to identify molecular markers which could be useful for marker assisted selection (MAS).

Materials and methods

Plant material

Synthetic wheat genotypes: XX41 (a hybrid between *Langdon durum* and *Ae. tauschii*, CI 00017), XX45 (*Langdon durum*/*Ae. tauschii*, RL 5565) and XX110 (*T. dicoccum*, A38/*Ae. tauschii*, CI 33) were developed at the Institute of Plant Breeding, Technical University of Munich, Germany. About 60–66 F_2 derived F_3 families ($F_{2:3}$) originating from crosses between the susceptible cultivar Chinese Spring (CS) and each of the three synthetic lines, XX41, XX45 (highly resistant) and XX110 (moderately resistant) were used to study modes of inheritance and linkage to SSR markers. Crosses among the resistant synthetic lines were made in all possible combinations and their F_1 and F_2 lines were used to study allelism.

Tan spot evaluation

Individual lines of each $F_{2:3}$ families were planted along with the parents at a rate of about ten seeds per row in two rows per pot using pots of 13 cm diameter containing peat moss. The pots were arranged on a box (40 pots/box) and placed on a bench in the greenhouse at a temperature of 20–23°C with 16 h photoperiod. Water was supplied by capillary action via holes in the base of the pots. After 2 weeks, the second leaf from each plant was cut and bulked per family and used for DNA extraction, while the first leaf of each line was inoculated using the most virulent race 1 *Ptr* isolate ASC1b. Inoculum production followed the method of Lamari and Bernier (1989) in a medium prepared from 150 ml V8 juice, 10 g potato dextrose agar (PDA), 3 g $CaCo_3$, 10 g Bacto agar and 850 ml distilled water. Conidia were harvested and diluted approximately to 3,000 spores ml^{-1}. Plants were inoculated using a hand sprayer until runoff and placed into a 2 m × 1.5 m × 1 m portable plastic tent which was further covered by a black plastic sheet to ensure complete darkness for 24 h at a relative humidity of 100% as explained in Tadesse et al. (2006a). The plants were then transferred into a growth chamber at a temperature of 22°C and photoperiod of 12 h for about 7 days. Disease readings were taken on the 7th day post-inoculation using the 1–5 rating scale developed by Lamari and Bernier (1989).

Microsatellite analysis

Genomic DNA was extracted from the second leaf of 2 weeks old seedlings of each lines of the F_3 families and the parental lines using the cetyltrimethyl amonium bromide (CTAB) method as described by Saghai-Maroof et al. (1984). A total of 12 SSR markers from wheat chromosome 3D was screened for polymorphism (Table 1) following the procedure of Huang et al. (2000).

PCR reactions were performed in a PE 9600 thermal cycler in a total volume of 20 µl containing 2 µl of 10 × PCR buffer (50 mmol of KCl, 10 mmol of Tris–HCl, 1.5 mmol of $MgCl_2$, pH 8.3), 2.5 mM of each dNTPs, 2.5 mM of each labelled and unlabelled primer, 1 U Taq DNA polymerase (Qiagen) and 100 ng template DNA. The PCR was programmed at

Table 1 Description of SSR markers tested

Locus	Anealing temperature (°C)	SSR motif	Chromosome arm
Xgwm2	50	$(CA)_{18}$	3DS
Xgwm52	55	$(GT)_4AT$ $(AT)_{20}$	3DL
Xgwm161	60	$(CT)_{15}$	3DS
Xgwm314	55	$(CT)_{25}$	3DL
Xgwm3	55	$(CA)_{18}$	3DL
Xgwm497	55	$(GT)_{29imp}$	3DL
Xgwm645	55	$(GT)_{28}$	3DL
Xbarc1040	55	$(ATCT)_8$	3DS
Xwmc366	55	$(CA)_{12}$	3DL
Xbarc42	55	$(TTA)_{12}$	3DL
Xbarc52	55	$(ATCT)_5$	3DL
Xgwm114	55	$(GA)_{53}$	3DS

an initial denaturation step of 3 min at 95°C followed by 35 cycles of 1 min denaturation at 95°C, annealing at 50, 55 or 60°C (depending on the primer) for 1 min, initial extension at 72°C for 1 min and final extension at 72°C for 15 min. The PCR product was checked along with the molecular weight standard λ*Hind*III and a non-template control by running on 1.5% agrose gel containing 5 μg/μl of ethidium bromide for about 30 min at 5 v/cm. Depending on the intensity of the bands, PCR products were diluted with double distilled water at 1:3 or 1:4 ratio. The samples were mixed with 0.15 μl GenScan-500 TAMRA internal size standard (PE Biosystems) and 0.85 μl formamide dye (98% formamide, 0.01% dextran blue), denatured at 95°C for 2 min and chilled on ice.

Samples were loaded on 5% denaturing polyacrylamide gel (Long Ranger TM, FMC Bioproducts) in 1 × TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3). Electrophoresis was carried out in an ABI Prism™ 377 DNA sequencer (Applied Biosystems) at 1,200V for 1.5 h. ABI collection software version 1.1 was used for raw data collection. Microsatellite fragments were analysed using GENSCAN™ analysis software version 2.1.

Linkage analysis

Linkage between SSR markers and the *tsn3* loci was established with MAPMAKER/EXP, version 3.0b using a LOD value of 3.0 and a maximum distance of 50 cM (Lander et al. 1987). The Kosambi function was applied to convert recombination fractions into map distances (Kosambi 1944), and linkage maps were drawn using the Mapchart software (Voorrips 2002).

Results

Inheritance of tan spot resistance

According to Tadesse et al. (2006a), the F_2 populations of CS/XX41 and CS/XX110 crosses segregated into 1 resistant:3 susceptible (1:3) ratio *to Ptr* race 1 isolate ASC1b, indicating that resistance in XX41 and XX110 is controlled by a single recessive gene. The CS/XX45 F_2 population, on the other hand, segregated into 3 resistant:1 susceptible (3:1) ratio indicating that resistance is controlled by a single dominant gene. The heterozygous (Aa) F_2 plants are susceptible in CS/XX41 and CS/XX110 populations, but resistant in CS/XX45 population. In the present study, inoculation of $F_{2:3}$ seedlings (F_2 derived F_3 families) of each of the three populations (CS/XX41, CS/XX45 and CS/XX110) with the same *Ptr* race 1 isolate ASC1b has resulted in a segregation ratio of 1:2:1 indicating that tan spot resistance in these synthetic lines is controlled by a single gene. The recessive genes in XX41 and XX110 are named as *tsn3a* and *tsn3c*, respectively, while the dominant gene in XX45 is named *Tsn3b*. This is in line with the recommendation in Tadesse et al. (2006a) with a little modification in the naming of the genes

As indicated in Table 2, for the recessive gene *tsn3a*, the CS/XX41 population segregated into 14 AA (homozygous susceptible), 31Aa (heterozygous susceptible) and 17 aa (homozygous resistant), a satisfactory fit for segregation at a single locus ($\chi^2_{1:2:1} = 0.29$, $P = 0.865$ at 2 df). Similarly, CS/XX110 population segregated into 12 AA (homozygous susceptible), 37 Aa (heterozygous susceptible) and 11 aa (homozygous resistant) ($\chi^2_{1:2:1} = 1.71$, $P = 0.425$ at 2 df) for the recessive *tsn3c* gene. On the other hand, the dominant gene, *Tsn3b*, in CS/XX45 population segregated into 12 AA (homozygous resistant), 40 Aa (heterozygous resistant) and 14 aa (homozygous susceptible) ($\chi^2_{1:2:1} = 3.07$, $P = 0.215$ at 2 df). The respective flanking markers in each of the populations, except *Xgwm2b* in population CS/XX45, have also shown a satisfactory fit for a 1:2:1 segregation ratio (Table 2). *Xgwm2b* was dominant in this population, and showed a satisfactory fit for a 1:3 segeregation ratio ($\chi^2_{1:3} = 3.73$, $P = 0.053$ at 1 df).

Allelism studies

As indicated in Table 3, all F_1 and F_2 progenies of the crosses between the resistant synthetic lines in all possible combinations were resistant to the race 1 *Ptr* isolate ASC1b. The lack of segregation into susceptible plants both in the F_1 and F_2 crosses among the three resistant lines indicated that the three genes are very tightly

Table 2 Genotypes inferred from seedling reactions of $F_{2:3}$ families and the corresponding alleles at SSR loci for the CS/XX41, CS/XX45 and CS/XX110 populations

Population	R* gene/ flanking markers	F_2 genotypes					
CS/XX41		AA	Aa	aa	Total	χ^2 (1:2:1, df 2)	P
	Xbarc42	8	30	24	62	8.23	0.016
	tsn3a	14	31	17	62	0.29	0.865
	Xgwm2a	13	36	13	62	1.61	0.447
CS/XX45	*Xgwm2a*	13	40	12	65	3.49	0.175
	Tsn3b	12	40	14	66	3.07	0.215
	Xgwm2b	23	0	42	65	3.73 ($\chi^2$1:3, df 1)	0.053
CS/XX110	Xgwm2a	8	35	17	60	4.35	0.114
	tsn3c	12	37	11	60	1.71	0.425
	Xgwm341	20	22	18	60	4.39	0.111

**R* refers to resistance genes (*tsn3a*, *Tsn3b*, *tsn3c*); AA = homozygous susceptible in CS/XX41 & CS/XX110 populations but homozygous resistant in CS/XX45 population; Aa = segregating; aa = homozygous resistant in CS/XX41 & CS/XX110 populations but homozygous susceptible in CS/XX45 population; df = degrees of freedom

Table 3 Response of F_1 and F_2 populations for resistance to *Ptr* isolate ASC1b in resistant x resistant synthetic wheat crosses

Crosses	Number of F_1 plants		Number of F_2 plants	
	Resistant	Susceptible	Resistant	Susceptible
XX41/XX45	8	0	150	0
XX41/XX110	10	0	200	0
XX45/XX110	8	0	150	0

linked.. The recessive genes, *tsn3a* and *tsn3c*, can also be allelic genes. However, they can be differentiated from one another in their mode of inheritance and differential reactions to differential isolates (Tadesse et al. 2006a). Phenotypically, the recessive genes *tsn3a* (aa) and tsn3c (aa), are highly resistant and moderately resistant to *Ptr* race 1 isolate ASC1b, respectively. The dominant gene *Tsn3b* (AA) showed a highly resistant response.

Linkage analysis and genetic map

A total of 12 SSR markers located on chromosome 3D (Somers et al. 2004; Röder et al. 1998) was screened for polymorphism (Table 1), and 6, 7 and 9 of these markers were found to be polymorphic for CS/XX41, CS/XX45 and CS/XX110 populations, respectively. The SSR locus *Xgwm2* was classified into *Xgwm2a* and *Xgwm2b* since it showed two distinctly different bands in CS and the three resistant synthetic lines. *Xgwm2a* has amplified 126 bp in CS, and 120 bp in XX41, XX45 and XX110 lines. *Xgwm2b*, on the other hand, has amplified 258 bp marker allele in CS, and 256 bp marker allele in XX41 and XX110, but was not amplified in XX45. *Xbarc1040*, *Xgwm2a*, *Xbarc42*, *Xgwm52*, *Xgwm341*, *Xgwm114* were polyorphic in CS/XX41 population. All these markers plus *Xgwm2b* were also found to be polymorphic in CS/XX45 population. *Xbarc1040*, although it was polymorphic in all the three populations, it was linked only in CS/XX110 population. Markers which were polymorhic in CS/XX41 and CS/XX45, except *Xgwm114*, were also polymorphic and linked in CS/XX110. Electropherograms showing the variation between the parents, and some selected homozygous and heterozygous lines of CS/XX41, CS/XX45 and CS/XX110 populations for some selected markers are indicated in Fig 1a, b, and c, respectively.

As shown in Fig. 2, all the three genes: *tsn3a* in CS/XX41, *Tsn3b* in CS/XX45 and *tsn3c* in CS/XX110 were clustered in a region around *Xgwm2a*, which showed 120 bp marker allele in CS, and 126 bp in XX41, XX45 and XX110. *Xbarc42*, *Xgwm2b* and *Xgwm341* were the other flanking markers for *tsn3a*, *tsn3b* and *tsn3c*, respectively. *Xgwm2a* was the closest marker to *Tsn3b* and *tsn3c* at genetic distances of 14.4 and 9.5 cM, respectively. *Xbarc42* with a linkage distance of 11 cM was the closest marker to *tsn3a*.

Maps for CS/XX41 and CS/XX45 differed in the order of SSR loci *Xbarc42*, *Xgwm52* and *Xgwm341*. Furthermore, *Xgwm2b* was not polymorphic in CS/XX41. Maps for CS/XX45 and CS/XX110 varied in order of markers *Xgwm2b* and *Xgwm341*. *Xgwm2b* was dominant in CS/XX45 showing only the 258 bp from CS, but co-dominant in CS/XX110 amplifying marker alleles of 258 and 256 bp in CS and XX110, respectively. Furthermore, markers *Xgwm161*, *Xbarc52*, *Xbarc1040* were not linked in CS/XX45 and CS/XX41.

Discussion

Synthetic wheat genotypes ($2n = 6x = 42$, AABBDD) which are amphiploids developed from the hybrid

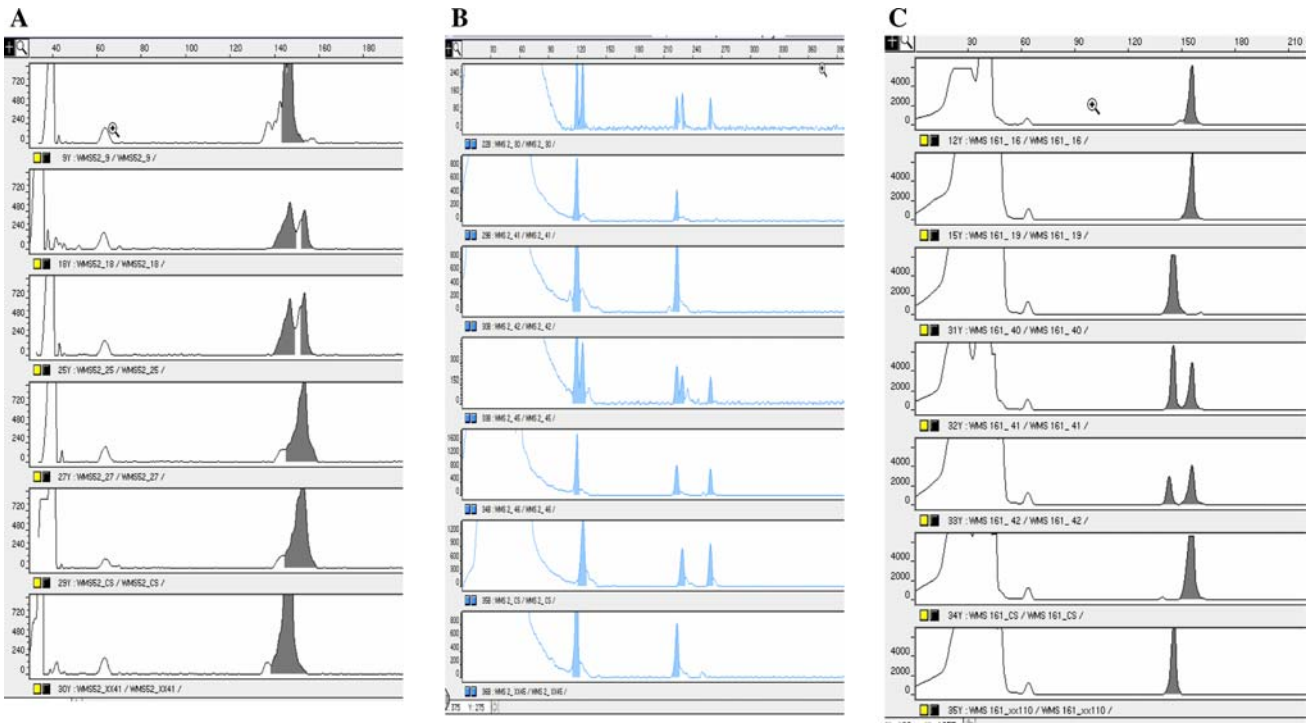

Fig. 1 Electropherograms showing polymorphism in: CS, XX41 and some selected lines of CS/XX41 population using *Xgwm52* (**a**); CS, XX45, and some selected lines of CS/XX45 population using *Xgwm2a* (**b**); CS, XX110 and some selected lines of CS/XX110 population using *Xgwm161* (**c**)

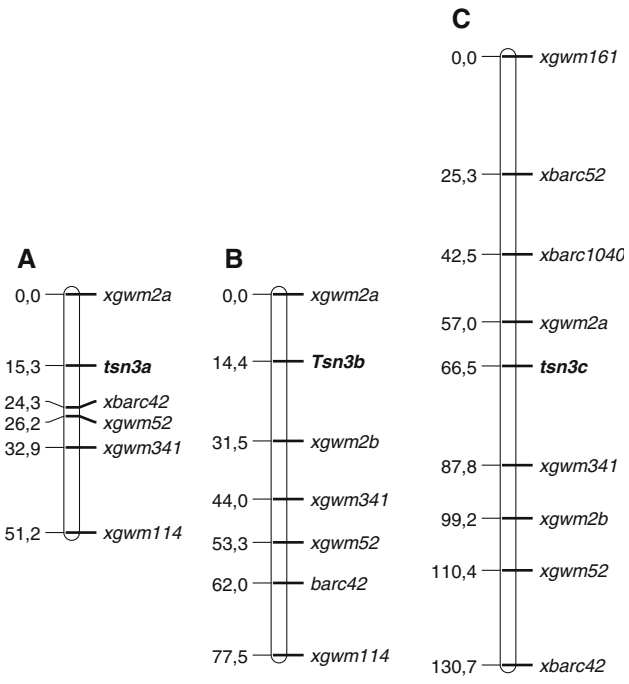

Fig. 2 Microsatellite linkage maps showing *tsn3* genes on chromosome 3D in the synthetic populations: CS/XX41 (**a**), CS/XX45 (**b**) and CS/XX110 (**c**). Locus names and map distances (*cM*) are indicated on the right and left sides of the maps, respectively

between tetraploid wheat (*Triticum turgidum* L., $2n = 4x = 28$, AABB) and *Aegilops tauschii* Coss. ($2n = 2x = 14$, DD) have a large degree of genetic variation for resistance to different wheat diseases in general, and to tan spot of wheat in particular (Siedler et al. 1994; Xu et al. 2004; Tadesse et al. 2006a). The source of resistance in the synthetic lines XX41, XX45 and XX10 was identified to be the *Aegilops tauschii* parents (Tadesse et al. 2006a).

In the present study, inheritance, allelism and genetic maps of tan spot resistance genes in synthetic wheat lines were investigated. Inheritance reports for tan spot resistance ranged from qualitative to quantitative. Some researchers (Nagle et al. 1982; Elias et al. 1989; Faris et al. 1997; Effertz et al. 2002) reported quantitative inheritance, while others (Lee and Gough 1984; Lamari and Bernier 1991; Gamba and Lamari 1998; Lamari et al. 2003; Singh and Hughes 2005) have reported the inheritance of tan spot to be qualitative, controlled by single major recessive genes. More recently, Tadesse et al. (2006a, b) have also reported qualitative inheritance from the F_2 segregation analyses of the disomic Chinese Spring/synthetic populations and CS/Salamouni crosses. The current study using the $F_{2:3}$ CS/synthetic lines has also confirmed qualitative inheritance of tan spot resistance in synthetic wheat lines.

The lack of segregation in the F_1 and F_2 populations for the *Ptr* isolate ASC1b in the crosses between the

resistant synthetic lines indicated all the three genes (*tsn3a*, *Tsn3b* and *tsn3c*) belong to the same resistant gene cluster. The recessive genes *tsn3a* and *tsn3c* can also be allelic while the dominant gene *Tsn3b* is a tightly linked gene. Singh and Hughes (2005) have also reported allelism among winter wheat cultivars for tan spot resistance using *Ptr* race 1isolate. Resistance genes occurring as a single gene with one or more alleles encoding different resistance specificities have been reported in many crops. In wheat, a total of ten different resistance specificities (*Pm3a* to *Pm3j*) against powdery mildew has been reported at the *Pm3* locus on the short arm of chromosome 1A (Zeller and Hsam 1998; Hsam et al. 1998). Allelic/linked genes have been also reported for wheat leaf rust resistance (Singh et al. 2004), resistance to Russian wheat aphid (Miller et al. 2001; Liu et al. 2005) and resistance to flax rust (Ellis et al. 1997).

Linkage analysis has also shown that the genes in all the three populations are located in the vicinity of *Xgwm2a* (Fig. 2). Maps for CS/XX41 and CS/XX45 differed in the order of SSR loci *Xbarc42*, *Xgwm52* and *Xgwm341* which is probably due to a single inversion. Furthermore, *Xgwm2b* was not polymorphic in CS/XX41. More number of markers were polymorphic and linked in CS/XX110 than in CS/XX41 and CS/XX45 populations. The difference in the order of *Xgwm2b* and *Xgwm341* for the CS/XX45 and CS/XX110 maps may be due to the variation in the informativeness of the marker in these two populations. The order of markers *Xbarc42*, *Xgwm52*, *Xgwm341* in the present genetic map for CS/XX41 was in line with the consensus map (Somers et al. 2004). However, their position was inverted in the maps for CS/XX45 and CS/XX110 populations. Such variations in the location of markers between the genetic and consensus maps were also reported recently by Wang et al. (2006).

In general, this variation in the order of some markers among the maps in CS/XX41, CS/XX45 and CS/XX110 may be due to the low number of F_3 lines tested, the difference in populations, and the position of crossovers along chromosomes within the progeny lines (Somers et al. 2004). The relatively wide gap between some of the markers can also be associated to the low number of SSR markers available on the D genome as compared to the A and B genomes of wheat.

This is the first report of mapping allelic/linked genes for tan spot resistance in the D genome of wheat using SSR markers. However, SSR markers were used to map allelic genes to powdery mildew of wheat indicating that they are ideal for comparative mapping of alleles at the same gene locus in different mapping populations (Singrün et al. 2004; Huang et al. 2004).

Most of the tan spot resistant genes reported to date were located in the B genome of hexaploid wheat. Faris et al. (1996) mapped the resistant locus *tsn-1* on the long arm of 5B using restriction fragment length polymorphism (RFLP) markers. The *tsn-1* gene is recently fine mapped and markers which are important for cloning of this gene are identified (Haen et al. 2004). A major QTL designated as *tsc*2, which is located on the short arm of chromosome 2B, was reported by Friesen and Faris (2004). Cheong et al. (2004) have also identifed a major QTL on 5BL, which actually is expected to be the same as *tsn-1*, in the Australian cultivar Brookton. More recently, Faris and Friesen (2005) have identified QTL on chromosome arms 1BS and 3BL in cultivar BR34 using *Ptr* races 1–3 and 5 indicating presence of race- nonspecific tan spot resistance. Recently, Singh et al. (2006) have identified *tsn2* on the long arm of chromosome 3B using race 3 *Ptr* isolate in tetraploid wheat, which actually may be the same gene reported as QTL on 3BL by Faris and Friesen (2005). There are few reports of tan spot resistance in the A genome of wheat. A major QTL on the short arm of chromosome 1A (*QTsc.ndsu-1A*), and a minor QTL on the long arm of chromosome 4A were reported in W-7984/Opata85 population (Faris et al. 1997). Recently, Tadesse et al. (2006b) have located the *tsn4* gene in the spring wheat cultivar Salamouni on chromosome 3A.

In conclusion, the absence of susceptible plants both in the F_1 and F_2 lines of the different intercrosses among the resistant parental lines in the allelism tests, and linkage analysis using SSR markers showed that all the three genes: *tsn3a* in XX41, *Tsn3b* in XX45 and *tsn3c* in XX110 are closely linked genes clustered in the vicinity of *Xgwm2a*, located on the short arm of chromosome 3D. The linked markers and genetic relationship of these genes will greatly facilitate their use in wheat breeding and deployment of tan spot resistant cultivars. As the currently available SSR markers in the D genome of wheat are limited, it is advisable to carry out fine mapping in the future when more markers are developed on wheat chromosome 3D in order to effectively delimit the genomic region containing the *tsn3* genes for cloning purpose.

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