Chromosomal Location of a Gene Conditioning Insensitivity in Wheat to a Necrosis-Inducing Culture Filtrate from *Pyrenophora tritici-repentis*

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ABSTRACT

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Previous research indicates that infection by the tan spot fungus (*Pyrenophora tritici-repentis*) produces two genetically distinct symptoms in wheat (*Triticum aestivum*): tan necrosis and extensive chlorosis. Necrosis-inducing isolates of *P. tritici-repentis* release a host-selective toxin in culture that produces a reaction highly associated with the induction of tan necrosis in susceptible wheat genotypes. The objectives of this research were to determine the number of genes conditioning insensitivity to a necrosis-inducing culture filtrate in a population of wheat F_3 families, and to map the insensitivity gene(s) using restriction fragment length polymorphisms (RFLPs). The population consisted of 58 F_3 families derived from the cross of a resistant synthetic hexaploid, W-7976, with the susceptible cultivar 'Kulm.' At least 16 individuals from each F_3 family were infiltrated with culture filtrate from the *P. tritici-repentis* isolate 86-124 and were scored as insensitive or sensitive. Low-copy DNA clones that hybridized to group 5 wheat chromosomes were used to detect RFLPs associated with insensitivity. The families segregated in a ratio of 15:29:14 homozygous insensitive/segregating/homozygous sensitive, suggesting that a single nuclear gene was responsible for conditioning insensitivity to the pathogenic factor(s) in the culture filtrate. RFLPs were detected that flanked the locus conferring insensitivity at distances of 5.7 and 16.5 cM. Aneuploid analysis indicated that this gene resided on the long arm of chromosome 5B. We proposed the symbol *tsn1* to designate this gene.

Additional keywords: host-pathogen interactions, molecular markers, resistance, yellow leaf spot.

Tan spot, caused by *Pyrenophora tritici-repentis* (Died.) Drechs., occurs worldwide and is an economically important disease in major wheat (*Triticum aestivum* L.) production areas including Australia and the Great Plains of the United States and Canada (6,28,32). Isolates of *P. tritici-repentis* differ in aggressiveness (6). Susceptibility of wheat to the fungus is manifested by the development of tan necrosis and/or extensive chlorosis. Isolates of the fungus are currently grouped into five races based on their virulence pattern and ability to induce tan necrosis and extensive chlorosis (nec+ chl+, race 1), tan necrosis only (nec+ chl–, race 2), extensive chlorosis only (nec– chl+, races 3 and 5), or avirulence (nec– chl–, race 4) (14,16,17).

Tan necrosis-inducing isolates of *P. tritici-repentis* release a host-selective necrosis-inducing toxin in culture (14,29,31). Sensitivity to the toxin was found to be highly correlated with the induction of tan necrosis in the host, and insensitivity to the toxin and resistance to tan necrosis caused by the fungus are controlled by a common gene, or closely linked genes (14,29). Ballance et al. (4) determined that a toxin produced by the isolate 86-124 was a protein with a molecular mass of 13.9 kDa and designated it the Ptr necrosis toxin. They reported that purified toxin at a concentration of 0.2 nM produced necrosis in susceptible cultivars. Tomás et al. (30) isolated a toxin from the isolate Pt-1c and reported it to be a protein of 14.7 kDa (designated Ptr toxin) with an average minimum active concentration of 90 nM. Tuori et al. (31) reported that multiple toxins are produced by the *P. tritici-repentis* isolate Pt-1c. The major necrosis-inducing protein (designated ToxA) was described as a 13.2-kDa heat-stable protein that produced necrosis in susceptible wheat cultivars at an average minimum concentration of 60 nM. Isolates of race 5 have been collected from Algeria that produce a host-selective chlorosis-inducing toxin in culture (22). Insensitivity to the toxin and resistance to the fungus were found to be highly correlated, and controlled by a single recessive gene in the host.

Lamari et al. (16) suggested that the symptoms of tan necrosis, produced by nec+ isolates, and extensive chlorosis, produced by chl+ isolates, were genetically distinct. Lamari and Bernier (15) studied segregation ratios of F_2 and F_3 populations and determined that a single recessive gene was responsible for conditioning resistance to tan necrosis, and a single dominant gene was responsible for controlling resistance to extensive chlorosis, with the possibility of minor gene action occurring in some populations. We also found that independent factors controlled resistance to extensive chlorosis and tan necrosis (J. D. Faris, J. A. Anderson, L. J. Francl, and J. G. Jordahl, *unpublished data*). 'Chinese Spring' is a unique resistant source in that a number of series of chromosome substitution lines are available. R. G. Rees and G. J. Platz (*unpublished data*) evaluated disomic substitution series of 'Kenya Farmer,' 'Timstein,' and 'Thatcher' into 'Chinese Spring' as juvenile plants. In each series, chromosomes 1A and 5B appeared to contribute most of the resistance to conidial inoculations of *P. tritici-repentis*.

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Molecular markers such as restriction fragment length polymorphisms (RFLPs) can be used to tag genes of interest and aid in their efficient transfer from otherwise undesirable genotypes. These RFLPs have been used to map genes regulating many important qualitatively and quantitatively inherited traits in wheat (2,3,10,12,19,20,25).

The objectives of this research were to determine the number of genes conditioning insensitivity of wheat to a necrosis-inducing culture filtrate produced by *P. tritici-repentis* in a population of F_3 families, and map the insensitivity gene(s) using RFLPs.

MATERIALS AND METHODS

Plant materials. The original population consisted of 60 F_3 families derived from the cross of W-7976 × 'Kulm.' Thirty of the 60 families were derived from the reciprocal cross and each F_3 population was derived from a single F_1 plant. W-7976 is a synthetic hexaploid wheat with the pedigree Cando/R143//mexi 'S'/3/*Triticum tauschii* (C122) and was provided by A. Mujeeb-Kazi, CIMMYT. This synthetic wheat was determined to be highly resistant to tan spot at the seedling stage, as well as the adult stage (24). 'Kulm' is a tan spot-susceptible hard red spring wheat cultivar developed at North Dakota State University and was provided by R. Frohberg. The resistant cultivar 'Erik' and the susceptible line ND495 were used as checks.

Culture filtrate production. Culture filtrate produced by the *P. tritici-repentis* isolate 86-124 was used to test F_3 families. This isolate was previously characterized as a producer of the necrosis toxin (nec+) and a noninducer of extensive chlorosis (chl–) (14). The fungus was cultured on V8–potato-dextrose agar (PDA) to produce mycelial plugs (7). Ten plugs were transferred to 250-ml Erlenmeyer flasks containing 50 ml of Fries medium amended with 0.1% yeast extract (medium 66 in Dhingra and Sinclair [7]) and incubated at 20°C in total darkness without agitation for 3 weeks. The cultures were then filtered through Whatman No. 1 paper, passed through a 0.45-µm Millipore membrane (Millipore Corp., Bedford, MA), adjusted to pH 6.5 with 1 N NaOH, and stored at 4°C.

Infiltration of culture filtrate. Parents, checks, and at least 16 F_3 plants of each of the 60 families (except for one family from which only 10 plants were available) were infiltrated with the necrosis-inducing filtrate. Plants were grown in plastic cones containing Fison sunshine blend #1 (Fison Horticulture, Vancouver, BC) in the greenhouse at an average temperature of 21°C with a 16-h photoperiod. The middle of the youngest, fully expanded leaf at the third leaf stage was infiltrated by injecting 15 to 25 µl of full-strength filtrate using a 1-ml syringe with the needle removed. The boundaries of the infiltration sites were marked with a nontoxic felt pen before water-soaking disappeared. Inoculated leaves were evaluated 3 days after infiltration and rated as insensitive or sensitive.

Population analysis. F_3 families were classified as being homozygous insensitive, segregating, or homozygous sensitive for reaction to the filtrate. Chi-square analysis was used to test for fit to a 1:2:1 ratio among families. By evaluating 16 individuals of each family, we were confident at the 0.01 level of probability that our classification was correct. Families that were classified as being homozygous based on the 16 individuals were further investigated using 16 additional F_3 individuals. This increased our confidence of detecting all segregating families to the 0.0001 level of probability. Because family size was small, segregating F_3 families were subjected to a binomial test for fit to a 1:3 ratio within the family. Lack of fit to tested ratios was indicated when $P < 0.05$.

RFLP analysis. An equal amount of leaf tissue from each of the 16 F_3 plants of each family was collected for DNA extraction. DNA isolation, restriction digests, electrophoresis, Southern blotting, hybridization, and membrane washing procedures were executed as described by Riede and Anderson (23).

A total of 62 low-copy RFLP clones were selected from barley cDNA, barley genomic DNA, oat cDNA, and wheat genomic DNA libraries. Clones with their specific prefix and the providing individuals were as follows: CDO (oat cDNA), BCD (barley cDNA), and WG (wheat genomic DNA) by M. Sorrells; ABC (barley cDNA) and ABG (barley genomic DNA) by A. Kleinhofs; KSU (*T. tauschii* cDNA and genomic DNA) by B. Gill; and MWG (barley genomic DNA) by A. Graner. The clones were described by the following authors: BCD, CDO, and WG by Heun et al. (11), KSU by Gill et al. (8), MWG by Graner et al. (9), and ABC and ABG by Kleinhofs et al. (13). Clones that hybridized to group 5 wheat chromosomes were selected based on genetic linkage maps developed by Nelson et al. (21). Markers were selected at approximately 20-cM intervals along chromosomes 5A, 5B, and 5D of the previously developed map to construct group 5 skeleton maps in this population. Detection of linkage between RFLP loci and phenotypic reactions directed our efforts to select and map additional clones in the corresponding chromosome region.

Bulked DNA of F_3 families was scored as being one of the two parental types or as heterozygous for segregating RFLP bands. Because the copy number of each parental allele at a given locus varied within each F_3 family, the detection of RFLP patterns consisting of both parental states was scored as heterozygous. Linkage relationships were evaluated with MAPMAKER (18), using a minimum $\log_{10}$ odds likelihood (LOD) of 3.00 and a maximum theta equal to 0.30.

Nullisomic-tetrasomic (NT) lines of 'Chinese Spring' wheat (26) (complete except for 2A and 4B) and ditelosomics (DT) of

Fig. 1. Leaves of W-7976 and 'Kulm' 3 days after infiltration with culture filtrate produced by the *Pyrenophora tritici-repentis* isolate 86-124.

'Chinese Spring' (27) (complete except for 2AL, 4AS, 5AS, 2BS, 4BL, 5BS, 5DS, and 7DL) were used to locate RFLP markers to chromosome arms, similar to the method used by Anderson et al. (1). Clones identifying restriction fragments linked to the insensitivity gene were hybridized to NT and DT stocks, and resulting autoradiographs of the probings were visually scored to identify fragments absent in any of the stocks. If a fragment was absent in a particular NT stock, we inferred its location on the chromosome in the nullisomic condition. Concomitant presence of a double-dose fragment in the stocks tetrasomic for a particular chromosome was used as additional evidence for the proper localization of fragments. In the analysis of DTs, a fragment absent in a stock indicated its presence on the opposing arm of that chromosome. In those cases in which a complete DT set was not available, the assignment of restriction fragments to chromosome arms was inferred based upon the presence of the fragment in the DT stock available.

RESULTS

Infiltration analysis. Infiltration of the culture filtrate into wheat leaves produced insensitive or sensitive reactions. Compatible reactions on susceptible genotypes could be detected after 24 h, and after 3 days were clearly evident (Fig. 1). Reactions of insensitive and sensitive genotypes closely resembled reactions of W-7976 and 'Kulm,' respectively, and no intermediate reactions were observed in the progeny.

Fifty-eight of the 60 families were used in the chi-square analysis because one family exhibited nonparental fragments in the RFLP analysis, indicating the possibility of contamination or an outcross. A second excluded family showed a segregation ratio of 13:3 (insensitive/sensitive) and was subsequently evaluated on 16 additional individuals. All 16 individuals were insensitive, indicating that this family may actually be homozygous. The three sensitive plants detected in this family may have resulted from an outcross in the previous generation.

The families segregated in a pooled ratio of 15:29:14 for homozygous insensitive, segregating, and homozygous sensitive, respectively (Table 1). The heterogeneity chi-square test for the cross and the reciprocal cross was nonsignificant, indicating that no reciprocal effects existed. This suggested that a single nuclear gene was responsible for controlling reaction to the pathogenic factor(s) in culture filtrate from the isolate 86-124.

Segregation ratios within families ranged from 8:8 to 2:14, insensitive/sensitive, suggesting the insensitivity gene was recessive (Table 1). Only two families segregated 8:8 and did not fit the binomial test for fit to 1:3 ($P < 0.05$). But, the chi-square test for heterogeneity among segregating families was nonsignificant, indicating families were homogeneous.

TABLE 1. Segregation ratios among and within F_3 families derived from the cross and the reciprocal cross of W-7976 × 'Kulm' after infiltration with a necrosis-inducing culture filtrate from the *Pyrenophora tritici-repentis* isolate 86-124

Cross	Ratios among families[a]	Probability[b]	Ratios within families[c]	No. of families	Probability[d]
W-7976/'Kulm'	4:18:6	$0.50 > P > 0.25$	8:8	1	$P = 0.04$
			7:9	2	$P = 0.14$
			6:10	2	$P = 0.39$
			5:11	4	$P = 0.77$
			4:12	5	$P = 1.00$
			3:13	1	$P = 0.77$
			2:14	3	$P = 0.39$
'Kulm'/W-7976	11:11:8	$0.50 > P > 0.25$	8:8	1	$P = 0.04$
			7:9	1	$P = 0.14$
			5:11	3	$P = 0.77$
			4:12	3	$P = 1.00$
			3:13	1	$P = 0.77$
			2:14	1	$P = 0.39$
			1:9[e]	1	$P = 0.32$
Deviation X^2	15:29:14	$0.95 > P > 0.90$	131:327	29	$0.10 > P > 0.05$
Heterogeneity X^2					$0.50 > P > 0.25$

[a] Homozygous insensitive/segregating/homozygous sensitive.
[b] Chi-square probability of expected fit to a 1:2:1 ratio.
[c] Insensitive/sensitive.
[d] Binomial probability of expected fit to a 1:3 ratio.
[e] Only 10 plants available for analysis.

Mapping and linkage analysis. Forty-five of 62 clones exhibited polymorphism and were subsequently hybridized with membranes containing DNA from the bulked F_3 families (Fig. 2). Fifty-seven of the 60 families were used for RFLP analysis, because one sample was lost during DNA preparation and two families were eliminated for reasons explained previously. Sixty-six RFLP loci were obtained from the 45 clones hybridized, and formed five linkage groups. Fragments detected by *Xbcd1030* and *Xwg583* flanked the locus conferring insensitivity at distances of 5.7 and 16.5 cM, respectively (Fig. 3). Hybridization of clones WG583, MWG914, BCD450, CDO584, and BCD1421 onto NT stocks of 'Chinese Spring' resulted in the absence of fragments linked to the resistance locus in N5BT5D stocks, and double-dose fragments in N5AT5B and N5DT5B stocks. Because all fragments linked to the resistance locus were present in DT 5AL, 5BL, and 5DL (DT 5AS, 5BS, and 5DS stocks were unavailable), it was concluded that the fragments detected by these clones and linked to this insensitivity gene resided on the long arm of chromosome 5B.

The relative order of RFLP markers on the long arm of chromosome 5B in this population was identical to the map developed by Nelson et al. (21) using recombinant inbred lines derived from the cross of a different synthetic hexaploid with a cultivated hard red spring wheat.

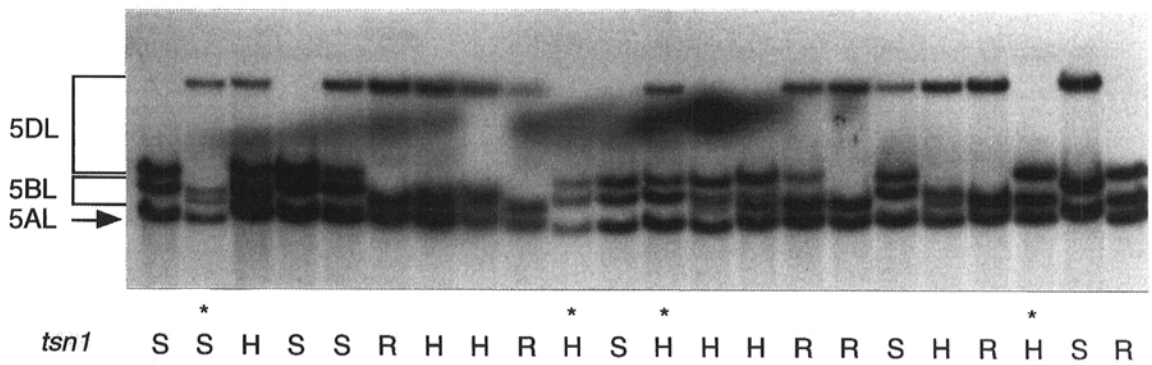

Fig. 2. Autoradiograph of clone BCD450 hybridized to genomic DNA of F_3 families derived from W-7976 × 'Kulm.' The two bands of highest molecular mass are allelic and map to 5DL. The band of lowest molecular mass is monomorphic and maps to 5AL. The two middle bands, which are allelic, are linked to *tsn1* and map to 5BL. Phenotypic scores of F_3 families are indicated: S = homozygous susceptible family, H = segregating family, and R = homozygous resistant family. Asterisks indicate recombination between *Xbcd450* and *tsn1*.

DISCUSSION

Our research was consistent with previous reports that concluded insensitivity to tan necrosis is controlled by a single recessive nuclear gene in the host. This research also agreed with studies done by R. G. Rees and G. J. Platz (*unpublished data*) in which they determined that chromosome 5B contained a factor(s) contributing to tan spot resistance. We proposed the symbol *tsn1* to designate the gene on 5BL of W-7976 that controlled insensitivity to culture filtrate produced by the *P. tritici-repentis* isolate 86-124.

Previous research suggests that *P. tritici-repentis* produces multiple toxins including one major toxin (ToxA) responsible for producing tan necrosis (31). Culture filtrate was used for infiltration in this study, therefore all toxins were presumed present in the inoculum. Segregation ratios and the fact that reactions were clearly either sensitive or insensitive suggested that either a single recessive gene alone controlled insensitivity to all toxins produced by 86-124, or phenotypes of reactions to minor toxins may have been masked by the major toxin. But, research has produced evidence suggesting that isolate 86-124 produces only one toxin (4). Furthermore, an array of diverse genotypes, including the parents and checks used in this study, were tested for reaction to culture filtrate and pure toxin and produced identical results, suggesting that one toxic protein was produced by the isolate 86-124 (24). More studies are needed to determine if other genes are involved in controlling reactions to other necrosis-inducing toxins produced by other isolates.

The insensitivity gene identified in W-7976 was responsible for conditioning resistance to the toxin at the seedling stage; however, it is not known whether this gene also conferred resistance at the adult stage or if other factors were involved. This synthetic hexaploid was found to be insensitive to the culture filtrate and necrosis toxin of 21 isolates (L. J. Francl, J. G. Jordahl, and S. W. Meinhardt, *unpublished data*) and resistant to conidia of three isolates (J. D. Faris, J. A. Anderson, L. J. Francl, and J. G. Jordahl, *unpublished data*). The high level of tan spot resistance possessed by W-7976 has been previously reported and insensitivity to culture filtrate and purified necrosis toxin has been identified in other hexaploid wheats as well (24). The insensitivity gene identified in W-7976 was undoubtedly derived from one of its durum progenitors, and it is likely that this gene exists in other tan necrosis resistant genotypes. Accessions of *T. tauschii* have also been determined to be insensitive to the toxin (5). Because *T. tauschii* is the D genome progenitor of common bread wheat, it is likely that some hexaploid wheat genotypes possess a gene(s) on the D genome conferring insensitivity to toxins. As more isolates and races of *P. tritici-repentis* are identified, it is probable that other resistance genes will be identified.

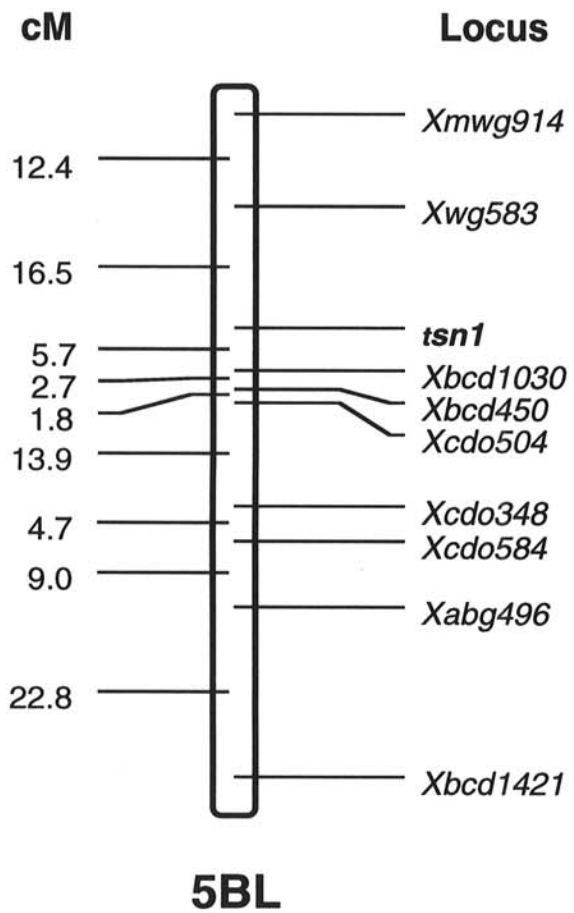

Fig. 3. Linkage map of the long arm of chromosome 5B in the population derived from W-7976 × 'Kulm.' Centimorgan distances were obtained using the Kosambi mapping function. RFLP loci are indicated with the prefix X.

Knowledge of the location of the necrosis resistance gene and the identification of RFLP markers linked to the gene could facilitate a marker-assisted selection scheme and aid breeders in making selections for resistant genotypes, while avoiding the masking effects of environmental inconsistencies that frequently create selection difficulties. However, this may not be necessary in this particular situation. Inoculations using culture filtrate or necrosis toxin can be made at the seedling stage without destroying the plant, and unambiguous results can be obtained 3 to 5 days after inoculation. Furthermore, since this insensitivity gene is recessive, heterozygous genotypes can be eliminated on a phenotypic basis. Therefore, direct infiltration of the culture filtrate or necrosis toxin may be a more efficient method of selection for this gene as compared with a marker-assisted selection scheme. Though markers linked to this insensitivity gene may be of little value for marker-assisted selection, they may be quite useful in evaluating other resistant germ plasms to determine if a common gene is responsible for controlling insensitivity to the toxin or if different gene(s) are involved. These markers may also prove useful in map-based cloning experiments.

Tan necrosis produced by *P. tritici-repentis* is a significant virulence component of tan spot. But, selection for resistance to tan necrosis alone will not necessarily lead to tan spot-resistant cultivars. The ability to produce extensive chlorosis on susceptible wheat genotypes is a major destructive property of many tan spot isolates (15,16). Using a different population, we have observed a diverse range of symptoms even though both parents and the entire population was insensitive to the necrosis-inducing filtrate produced by the isolate 86-124 (J. D. Faris, J. A. Anderson, L. J. Francl, and J. G. Jordahl, *unpublished data*). Further research has been done to determine the number and location of genes involved in conditioning resistance to chlorosis-inducing factors of *P. tritici-repentis* and will be reported elsewhere.

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