Genetic Analysis of Sensitivity to a *Pyrenophora tritici-repentis* Necrosis-Inducing Toxin in Durum and Common Wheat

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ABSTRACT

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The fungus *Pyrenophora tritici-repentis* produces a toxin (Ptr ToxA) that causes rapid cell necrosis in sensitive wheat genotypes. A single recessive gene (*tsn1*) on chromosome 5BL in common wheat confers insensitivity to this toxin. Our objectives were to analyze the allelic relationships of genotypes that have shown insensitivity to a *P. tritici-repentis* necrosis-inducing toxin, map the gene for insensitivity to the necrosis-inducing factor produced by *P. tritici-repentis* in a durum wheat population, and determine the reaction to *P. tritici-repentis* of aneuploid genotypes that do not contain the gene. Greenhouse-grown plants of seven populations from crosses of insensitive genotypes; an F_2 population of durum wheat; and 'Chinese Spring' aneuploid, substitution, and deletion lines were infiltrated with Ptr ToxA. All crosses involving insensitive genotypes failed to produce sensitive progeny, indicating that the same gene is present in these genotypes. The gene for insensitivity in the durum population was mapped to the same region on 5BL as in common wheat using restriction fragment length polymorphism markers. 'Chinese Spring', its homoeologous group 5 nullisomic-tetrasomic stocks, and 5BL deletion lines were insensitive to the toxin. Substitution of a 5B chromosome from sensitive genotypes into 'Chinese Spring' resulted in sensitivity. Therefore, insensitivity is not conferred by a gene product per se, but rather conferred by absence of a gene for sensitivity.

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

Tan spot, a foliar leaf-spotting disease incited by *Pyrenophora tritici-repentis*, causes losses in grain yield of wheat (*Triticum* species) in many regions worldwide (11,19,30). Symptoms of tan spot typically include large brown necrotic lesions surrounded by a chlorotic halo, with a small black point in the center of the lesion. In some genotypes, an extensive chlorosis spreads throughout the entire leaf. These lesions can coalesce, resulting in the death of the entire leaf (9,11).

P. tritici-repentis produces several toxins that mimic the tan necrosis or extensive chlorosis symptoms in susceptible wheat lines (1,3,4,10,18,27,28). Two types of necrosis-inducing toxins have been described. Ptr ToxA (a necrosis-inducing toxin produced by *P. tritici-repentis*) is a low-molecular-weight protein (1,27,28), and the others are a group of spirocyclic lactams (10). The necrosis-inducing protein has been well characterized. The amino acid composition and sequence of Ptr ToxA have been determined, and the gene encoding the Ptr ToxA polypeptide has been cloned and sequenced (2,5,31). Two chlorosis-inducing toxins have been reported; however, the chemical characteristics of these toxins have not been described to date (3,18).

Two major genes in wheat are responsible for resistance to tan spot (13,14). One dominant gene provides resistance to a chlorosis-inducing factor (15). A quantitative trait locus (QTL) designated *QTsc.ndsu-1A*, which explains 35% of the variation in chlorosis induction in one recombinant inbred population, is located on the short arm of chromosome 1A (8). Four minor QTL and one epistatic interaction also were identified in the restriction fragment length polymorphism (RFLP) mapping of chlorosis induction and, in combination with *QTsc.ndsu-1A*, explained 49% of the variation in this population. A second gene conditions insensitivity to necrosis induction by *P. tritici-repentis* and is recessive (7,15,26). This gene was mapped to the long arm of chromosome 5B and has been designated *tsn1* (7). The fact that a gene was located to the same chromosome from a different resistance source (26) is an indication that these genes may be allelic. The resistance to necrosis induction shows absolute correlation with insensitivity to Ptr ToxA (15).

The excellent aneuploid stocks of wheat allow for the localization of genes to specific chromosomes (23,24) and, with the recent availability of deletion stocks (6), to subarm locations. The employment of deletion stocks for the physical mapping of genes provides knowledge regarding gene density and frequency of recombination in the chromosomal segment harboring the gene and can lead to the identification of additional closely linked markers, which is an important initial step for positional cloning of the gene. Furthermore, the testing of genetic materials that are missing specific chromosome segments can give insight into gene expression and host-pathogen interactions, because this represents a null condition that is not readily found in nature.

Although there is a broad range of genotypic response to infection by *P. tritici-repentis* in wheat germ plasm (20,22), resistance to both chlorosis and necrosis appear necessary to provide high levels of resistance under field conditions (22). Due to the locations of the major resistance genes on the A and B genomes, the

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resistance genes found in common wheat may be present in durum wheat (*T. turgidum* var. *durum*) and, if not, could be introgressed from *T. aestivum*.

The objectives of this research were to (i) analyze the allelic relationships of genotypes that have shown insensitivity to a *P. tritici-repentis* necrosis-inducing toxin; (ii) map the gene for insensitivity to the necrosis-inducing factor produced by *P. tritici-repentis* in a durum wheat population; and (iii) determine the reaction to *P. tritici-repentis* of aneuploid genotypes that do not contain the gene.

MATERIALS AND METHODS

Plant materials. The common wheats 'BR 34', 'CEP 17', 'IA 807', 'IA 905', and 'Opata 85'; the durum wheats 'Altar 84', 'Ben', 'Medora', and 'D87450'; and the synthetic wheat 'W-7984' were tested for their response to Ptr ToxA. Additional checks included the toxin-sensitive common wheat line ND495, provided by R. Frohberg, North Dakota State University (NDSU), and the toxin-insensitive common wheat cultivar Erik. The foregoing genotypes except for 'Ben' were used as parents in the development of nine populations that also were tested for Ptr ToxA response. All populations except the International Triticeae Mapping Initiative (ITMI) population were developed at NDSU by selfing F_1 plants and advancing by single-seed descent (SSD). Development of the ITMI population was described by Van Deynze et al. (29).

Wheat aneuploid stocks and chromosome substitution lines in the cultivar Chinese Spring were maintained and provided by the Wheat Genetics Resource Center at Kansas State University and were used to investigate the gene responsible for reaction to Ptr ToxA. The homoeologous group 5 nullisomic-tetrasomic (NT) lines (N5AT5D, N5BT5D, and N5DT5B) (24); 12 chromosome deletion lines for the long arm of chromosome 5B (6); the cultivars Hope (25), Cheyenne (17), and Timstein (25); and their corresponding chromosome 5B disomic substitutions into 'Chinese Spring' were assayed for their reaction to Ptr ToxA. Based on the known position of the chromosome breakpoints in the 12 5BL deletion lines analyzed, 2 were expected to contain *tsn1* and 9 were not. The presence of the gene in the other deletion line could not be predicted because of the close proximity of the gene and deletion breakpoint. Our goal was to determine the location of *tsn1* on the chromosome 5B physical map by assigning it to a deletion interval. Normal 'Chinese Spring' and the common wheat cultivar Jagger were used as toxin-insensitive and -sensitive checks, respectively.

Ptr ToxA screening—Crosses among insensitive genotypes. Greenhouse-grown plants from the populations were evaluated for their reaction to infiltration with Ptr ToxA. Each experiment consisted of all genotypes in the population, parents, and one or more known sensitive and insensitive genotypes as checks. Plants were grown in greenhouse soil beds at 22 to 26°C. Toxin from *P. tritici-repentis* isolate 86-124 was purified by the method of Zhang et al. (32), and 50 to 100 µl of purified Ptr ToxA at a concentration of 10 µg/ml was infiltrated into the middle section of the second fully expanded leaf as described by Faris et al. (7). The *P. tritici-repentis* isolate 86-124 was originally provided by L. Lamari, University of Manitoba, and was the same isolate used by Faris et al. (7) and Stock et al. (26). One plant was used to represent each SSD line, and five to seven plants of each check were used in each experiment. After 3 days, plants were scored as either insensitive or sensitive based on whether necrotic tissue formed outside of the area wounded as a result of the infiltration procedure.

Durum wheat segregating population and mapping. A population of F_2 plants derived from the cross of 'Altar 84'/'Medora' and its reciprocal were infiltrated with Ptr ToxA by infusing the fourth leaf after it was fully expanded. Toxin preparation was as described earlier. Included in this screening were four plants each of 'Altar 84' and 'Medora'. Some of the F_3 families derived from sensitive F_2 individuals were evaluated for their response to Ptr ToxA infiltration. The F_3 plants and 24 plants each of 'Altar 84' and 'Medora' were grown in plastic cones (3.8 cm in diameter and 21 cm in length) filled with a peat moss/perlite (3:1) mix and grown at 22 to 26°C in a greenhouse. Our goal was to evaluate 24 F_3 in-

TABLE 1. Wheat parental lines, pedigrees, origins, and responses to infiltration of Ptr ToxA produced by *Pyrenophora tritici-repentis*

Genotype	Pedigree	Origin[a]	Response to necrosis toxin[b]
Durums			
'Altar 84'	Ruff/Free Gallipoli/2/Mexicali 75/3/Shwa	1	Insensitive
'D87450'	D7224/Crosby//Cando/3/Aust#820198/4/D7075/Edmore//Cando/Edmore	2	Insensitive
'Ben'	D65150/Lds//Rugby/3/Ward/4/Vic/5/Monroe	2	Sensitive
'Medora'	Ward/Macoun	3	Sensitive
Common wheats			
'BR 34'	Alv110/2*IAS 54/6/Tp/4/TzPP/Son64//Napo/3/Cno/5/PF6968	4	Insensitive
'CEP 17'	PEL72380/Art71//CEP 75336/3/Ald "S"/PF72707//PAT 19	5	Insensitive
'IA 807'	Kvz/K4500 L.A.4	6	Insensitive
'IA 905'	Inia66/A.dist.//Inia66/Gen	6	Insensitive
'ND 495' (susceptible check)	Justin*2/3/ND 259/Conley//ND 112	2	Sensitive
'Opata 85'	Bluejay/Jupateco F73	1	Insensitive
'Erik' (resistant check)	Kitt//Waldron/Era	7	Insensitive
Synthetic wheats			
'W-7984'	Altar 84/*T. tauschii* (CI18)	1	Insensitive

[a] Origin: 1 = CIMMYT, 2 = North Dakota State University, 3 = Agriculture and Agri-Food Canada, 4 = EMBRAPA, 5 = FUNDACEP/FECOTRIGO, 6 = IAPAR/CIMMYT, and 7 = Agri-Pro Biosciences .

[b] The responses of the common wheats and the synthetic wheat were identical to those reported in Riede et al. (22). The durums and 'Opata 85' were not evaluated in that study.

TABLE 2. Responses of progeny from crosses of durum and common wheats after infiltration of Ptr ToxA produced by *Pyrenophora tritici-repentis*

Cross no.	Pedigree	Generation	No. of progeny	No. of insensitive	No. of sensitive
CR93-98-3	BR 34/IA 905	F_5	113	113	0
X94A11-3	CEP 17/W-7984	F_4	70	70	0
X94A24-1	IA 807/BR 34	F_4	127	127	0
X95B20-A	D87450/Altar 84	F_2	165	165	0
X95B18-A	Altar 84/D87450	F_2	181	181	0
ITMI population	W-7984/Opata 85	F_8	114	114	0
X95A112-B(3)	W-7984/Opata 85//D87450	F_2	20	20	0

dividuals per F_2 family. This number would give a 0.001 probability of being able to distinguish between a 3:1 sensitive/insensitive ratio (segregating family) and a 4:0 sensitive/insensitive ratio (nonsegregating sensitive family). Due to lack of seed, less than 24 F_3 individuals were available for some families. Only those with at least 12 individuals evaluated ($P < 0.05$ of distinguishing between the two ratios) were included in the analyses. Chi-square analysis tested goodness-of-fit for segregation among F_2 individuals (expected 3:1 sensitive/insensitive ratio), F_3 families from sensitive F_2 individuals (expected 1:2 nonsegregating sensitive/segregating ratio), and segregation of progeny within segregating F_3 families (expected 3:1 sensitive/insensitive ratio). DNA marker data were obtained from 77 of 96 F_2 individuals, and these were classified as to their homozygosity or heterozygosity by screening F_2 individuals and F_3 families.

The RFLP marker most closely linked to *tsn1*, *Xbcd1030* (7), was mapped in the 'Altar'/'Medora' population. A single leaf from 96 (48 from each of the two reciprocals) lines from this F_2 population was harvested for DNA extraction and Southern hybridization with clone BCD1030, according to the protocol of Riede and Anderson (21). Due to poor quality DNA of some genotypes, only 77 of the 96 individuals were genotyped at this RFLP locus. The necrosis toxin screening results and *Xbcd1030* RFLP scores of the F_2 individuals were analyzed for linkage using the Macintosh version of the computer program MAPMAKER (16).

'Chinese Spring' aneuploids, deletion lines, and substitution lines. The 'Chinese Spring' aneuploids, deletion lines, substitution lines, and checks were grown in pots containing a 1:1 mixture of pasteurized soil and vermiculite in a greenhouse at 22 to 26°C. At least two plants of each stock, including the checks 'Chinese Spring' and 'Jagger', were inoculated on three different dates in the case of the deletion lines, and on two different dates in the case of the nullisomic-tetrasomic stocks and substitution lines. Screening and analysis of reaction to Ptr ToxA was as described for the crosses among insensitive genotypes.

RESULTS

The checks 'Erik' and 'ND495' were insensitive and sensitive, respectively, in all experiments in which they were included. In all experiments, the parents of the populations responded as indicated in Table 1. All crosses involving insensitive parents failed to produce any sensitive progeny (Table 2). The lack of sensitive progeny indicated that at least one gene conferring insensitivity was shared between the two parents used to develop these populations. Because no sensitive progeny were found among their crosses, the durum genotypes 'Altar 84' and 'D87450' share the same resistance gene with the hexaploid genotypes 'Opata 85' and 'CEP 17' and the synthetic hexaploid 'W-7984'.

To investigate the insensitivity to Ptr ToxA in durum wheat, we mapped the gene using a marker known to be closely linked with the *tsn1* locus. The F_2 lines from the cross of 'Altar 84'/'Medora' and its reciprocal segregated in a manner consistent with a 3:1 sensitive/insensitive ratio, as expected for the case of a single recessive gene (Table 3). Progeny from a portion of the sensitive F_2 individuals were evaluated as F_3 seedlings to completely classify F_2 individuals as homozygous sensitive or heterozygous (Table 3). The F_3 families segregated in the expected 1:2 nonsegregating sensitive/segregating ratio. The RFLP marker *Xbcd1030* was mapped on 77 F_2 individuals from the 'Altar 84'/'Medora' cross and its reciprocal. The only DNA fragment segregating from an *Eco*RV digest segregated in a 1:2:1 homozygous 'Altar 84'/heterozygous/homozygous 'Medora' ratio (data not shown). This restriction fragment mapped 3.7 cM from a gene conditioning insensitivity to Ptr ToxA. This was a result of recombination events in 2 of the 77 progeny analyzed.

The three 'Chinese Spring' NT lines as well as all of the chromosome 5BL deletion lines were completely insensitive to Ptr ToxA, and reactions to infiltration did not differ from that of euploid 'Chinese Spring' (Table 4). These results were consistent in all replications of the experiments. The collapse of cells within the infiltration site on the sensitive check 'Jagger' was visible 12 h after infiltration and, after 3 days, developed into complete necrosis.

To determine if 'Chinese Spring' could be rendered sensitive to the toxin by the substitution of a pair of 5B chromosomes from a

TABLE 4. Responses to infiltration of Ptr ToxA produced by *Pyrenophora tritici-repentis* in 'Chinese Spring' aneuploid and substitution lines and in checks

Genotype[a]	Response to necrosis toxin
'Jagger'	Sensitive
'Chinese Spring'	Insensitive
'Chinese Spring' N5AT5D	Insensitive
'Chinese Spring' N5BT5D	Insensitive
'Chinese Spring' N5DT5B	Insensitive
'Chinese Spring' 5BL deletion lines[b]	
5BL-6	Insensitive
5BL-2	Insensitive
5BL-15	Insensitive
5BL-7	Insensitive
5BL-8	Insensitive
5BL-3	Insensitive
5BL-1	Insensitive
5BL-11	Insensitive
5BL-14	Insensitive
5BL-9	Insensitive
5BL-16	Insensitive
5BL-13	Insensitive
'Timstein'	Sensitive
'Cheyenne'	Sensitive
'Hope'	Sensitive
'Chinese Spring' – 'Timstein' 5B substitution	Sensitive
'Chinese Spring' – 'Cheyenne' 5B substitution	Sensitive
'Chinese Spring' – 'Hope' 5B substitution	Sensitive

[a] All genotypes maintained at the Wheat Genetics Resource Center, Kansas State University, Manhattan 66506.

[b] 'Chinese Spring' 5BL deletion lines are listed in order of most proximal to most distal deletions.

TABLE 3. Segregation of response to infiltration of Ptr ToxA in parents, F_2 individuals, and F_3 families derived from sensitive F_2 individuals from the cross 'Altar 84'/'Medora' and the reciprocal

Genotype/family	Generation	No.	No. of sensitive	No. of segregating[a]	No. of insensitive	P ($>\chi^2$)[b]
'Altar 84'	Parent	24	0	N/A	24	N/A
'Medora'	Parent	24	24	N/A	0	N/A
'Altar 84'/'Medora'	F_2	105	78	N/A	27	0.86
'Medora'/'Altar 84'	F_2	99	78	N/A	21	0.38
'Altar 84'/'Medora'	F_3[c]	27	9	18 (296:105)	0	1.00 (0.58)
'Medora'/'Altar 84'	F_3[c]	24	11	13 (227:85)	0	0.19 (0.36)

[a] Ratio in parentheses is total number of sensitive/insensitive individuals within the segregating families. N/A = not applicable.

[b] F_2 populations were tested to a 3:1 sensitive/insensitive ratio. F_3 populations were tested to a 1:2 nonsegregating sensitive/segregating ratio. In parentheses is the chi-square test to a 3:1 sensitive/insensitive fit of individuals within the segregating families. N/A = not applicable.

[c] From sensitive F_2 individuals only.

sensitive genotype for the native 5B chromosomes of 'Chinese Spring', we tested 'Hope', 'Cheyenne', and 'Timstein' and their corresponding chromosome 5B disomic substitutions into 'Chinese Spring'. The three cultivars were all sensitive to Ptr ToxA as were the corresponding chromosome 5B disomic substitution lines (Table 4).

DISCUSSION

All evidence reported to date suggests that the presence of a single gene in durum and common wheat confers sensitivity to Ptr ToxA. Insensitivity to Ptr ToxA was conditioned by a single recessive gene in several genetic backgrounds (7,15,26), and we found no sensitive genotypes from crosses among insensitive common and durum wheats. The lack of sensitive individuals in crosses among and within insensitive durums and common wheat indicates that the same gene can provide insensitivity at both ploidy levels.

The RFLP marker *Xbcd1030* that was linked to the *tsn1* locus from the synthetic hexaploid 'W-7976' at a distance of 5.7 cM (7) was mapped in our durum population at a distance of 3.7 cM. The proximity of this gene in a synthetic hexaploid and durum to *Xbcd1030* indicated that this was the same genomic region. Either selecting for the DNA marker *Xbcd1030* or screening with Ptr ToxA can increase the efficiency of introgressing *tsn1* to eliminate sensitive germ plasm.

Using aneuploid, deletion, and substitution lines of 'Chinese Spring', a genotype insensitive to Ptr ToxA, we have shown that deletion of the chromosome segment carrying the gene responsible for its insensitivity does not alter its response to the toxin. However, 'Chinese Spring' was rendered susceptible to the toxin after substitution of chromosome 5B from a sensitive genotype. This has also been shown by Stock et al. (26). The insensitivity to Ptr ToxA of the N5BT5D stock and all of the chromosome 5BL deletion lines suggests that insensitivity to the toxin is not conferred by the expression of a recessive gene for insensitivity, but rather the lack of a gene for sensitivity. These results provide conclusive evidence that the gene controlling the reaction to Ptr ToxA lies on chromosome 5B and that no factors on chromosomes other than 5B are involved. Because the allele at the *tsn1* locus that confers insensitivity in euploid genotypes behaves as a heritable unit allelic to the gene resulting in sensitivity, we refer to it as a gene in this manuscript. However, the dominant allele and its designation, *Tsn1*, should take precedence over the recessive allele when discussing Ptr ToxA-sensitive genotypes.

Our results with the N5BT5D stock are not consistent with those of Stock et al. (26) using nullisomic 5B plants. They reported that 7 out of 100 progeny obtained by selfing a single 'Chinese Spring' monosomic 5B plant were sensitive to *P. tritici-repentis* toxin from the same isolate used in these investigations. All 7 plants were nullisomic, while of the 12 insensitive progeny analyzed, 9 were monosomic and 3 were disomic. While there is no clear explanation for this discrepancy, it should be noted that their apparent recovery rate of nullisomic 5B plants from a monosomic 5B plant of 7/100 is about sevenfold higher than observed in previous work (23). Based on our results using N5BT5D and the 5BL deletion lines, the nullisomic 5B plants should have been insensitive to the toxin. The aneuploid stocks used in our investigations have been tested with more than 100 RFLP probes specific for group 5 chromosomes. None of these probings have caused us to question the authenticity of our stocks.

It is our hypothesis that, in order to manifest necrosis, Ptr ToxA requires interaction with the product of the gene conferring sensitivity in the host. Kwon et al. (12) examined the role of wheat metabolism in the host-pathogen interaction and indicated that active transcription, active translation, and functional host H^+-ATPase were required for toxin activity. The absence of the sensitivity gene would result in the absence of a receptor or binding target for Ptr ToxA, leading to a disruption of the signaling cascade required for toxin activity and ultimately to insensitivity of the host.

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