Identification of a Chlorosis-Inducing Toxin from *Pyrenophora tritici-repentis* and the Chromosomal Location of an Insensitivity Locus in Wheat

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ABSTRACT

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Culture filtrate from *Pyrenophora tritici-repentis* race 1 isolate 78-62 contained a genotype-specific toxin which elicited extensive chlorosis on sensitive wheat genotypes. This toxin was partially purified using gel filtration, ion exchange, and reversed-phase chromatography. The chlorosis toxin was found to be a polar, nonionic, low-molecular-weight molecule. Wheat genotypes infiltrated with crude culture filtrate and partially purified chlorosis toxin exhibited the same chlorotic symptoms seen with conidial inoculations of isolate 78-62. All tested wheat genotypes that exhibited extensive chlorosis to the toxin also exhibited

extensive chlorosis to conidial inoculations, and all wheat genotypes insensitive to the toxin did not exhibit extensive chlorosis to conidial inoculations. The recombinant inbred population derived from the cross W-7984 × Opata 85 segregated for chlorosis induction from infiltration with partially purified chlorosis toxin from isolate 78-62. The locus identified by the marker *XGli1*, associated with resistance to conidial inoculations from race 1 isolates Pti2 and 78-62 and race 3 isolate D308, also was associated with insensitivity to infiltration of crude culture filtrate and partially purified chlorosis toxin. The marker *XGli1*, located on the short arm of chromosome 1A, is linked to the insensitivity locus within 5.7 cM. We propose that this chlorosis toxin be designated Ptr ToxC.

Additional keywords: host selective toxin, resistance, restriction fragment length polymorphism, tan spot.

The fungus *Pyrenophora tritici-repentis* (Died.) Drechs. anamorph *Drechslera tritici-repentis* (Died.) Shoem. is the causal agent of the foliar disease tan spot on wheat (27). Tan spot is an economically important disease in the major wheat-producing areas of the world, including the Great Plains region of the United States and Canada (12,24,33). The symptoms caused by *P. tritici-repentis* on susceptible wheat cultivars can be small dark-brown infection sites with the development of necrotic lesions with or without chlorotic halos, necrotic lesions with extensive chlorosis throughout the infected leaf, or extensive chlorosis without necrotic lesions (13,14). Isolates of *P. tritici-repentis* have been grouped into five races based on their ability to induce tan necrosis, extensive chlorosis, both symptoms, or neither symptom on wheat differentials (15,16). Race 1 isolates can induce both tan necrosis and extensive chlorosis in susceptible wheat genotypes. The race 2 isolates can only induce necrosis, race 3 isolates can only induce extensive chlorosis, and race 4 isolates do not induce either symptom in genotypes susceptible to other races and are considered avirulent. Race 5 isolates are similar in response to race 3 isolates, but are differentiated by the ability to induce chlorosis in cv. Katepwa and not in the line 6B365.

It has been reported that resistance to tan necrosis and extensive chlorosis are controlled by independent loci (15). Resistance to tan necrosis is controlled by a single recessive gene, *tsn1*, which has been mapped to the long arm of chromosome 5B (9,28).

Inheritance of resistance to extensive chlorosis in seedlings was quantitative, and a single, major quantitative trait loci (QTL), *QTsc.ndsu-1A*, explained 35% of the variation for resistance and was mapped to the short arm of chromosome 1A (10). Inheritance of resistance to extensive chlorosis in adult plants also was quantitative and *QTsc.ndsu-1A* was responsible for the major effect (8), explaining 26% of that variation. *QTsc.ndsu-1A* also was associated with seedling resistance to two race 1 isolates and one race 3 isolate in a recombinant inbred population derived from the cross W-7976 × Trenton. This QTL explained 47, 58, and 64% of the variation for resistance to isolates Pti2, 78-62, and D308, respectively (8).

P. tritici-repentis produces several toxins that mimic the tan necrosis or extensive chlorosis symptoms in susceptible wheat lines (1,3,11,23,29–31,35). Two types of necrosis-inducing toxins have been described. Ptr ToxA is a low-molecular-weight protein (1,30,31,35) and the others are a group of spirocyclic lactams (11). The amino acid composition and sequence of Ptr ToxA have been determined and the gene encoding this protein has been cloned and sequenced (2,5,34). The spirocyclic lactams consist of six structurally similar triticones that have been characterized by X-ray diffraction (11). Two chlorosis-inducing toxins have been reported (3,23). One of these toxins has been characterized with host-specificity being established. This host-specific toxin (Ptr ToxB) was purified from the race 5 isolate Alg. 3-24 and was determined to be a proteinaceous molecule with a molecular mass of 6.61 kDa (29).

An isolate of *P. tritici-repentis* (78-62) was identified that produced a toxic compound in culture, which mimicked the extensive chlorosis symptom associated with conidia inoculations of wheat cv. 6B365. The previously identified Ptr ToxB (16) does not

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induce chlorosis in wheat cv. 6B365, suggesting that isolate 78-62 secretes a previously uncharacterized chlorosis toxin. The objectives of this study were to purify the chlorosis-inducing toxin from the *P. tritici-repentis* race 1 isolate 78-62, establish host specificity, and to determine the number and location of a gene or genes conditioning insensitivity to this toxin in seedlings of a wheat recombinant inbred population.

MATERIALS AND METHODS

Plant materials. The wheat plants used for restriction fragment length polymorphism (RFLP) mapping and QTL analysis in this experiment were a recombinant inbred (RI) population consisting of 114 F_7 -derived single-seed-descent lines from the cross of W-7984 (a synthetic hexaploid wheat derived from the cross of the durum wheat Altar 84 and *Triticum tauschii* (Coss.) Schmal.) and Opata 85, a hard red spring wheat from The International Maize and Wheat Improvement Center, Mexico (Centro Internacional para Mejoramiento de Maiz y Trigo, CIMMYT). This population was provided by M. E. Sorrells, Cornell University, Ithaca, NY. The synthetic hexaploid parent of this population showed high levels of resistance to tan spot in greenhouse studies (25). Seedlings of this population segregated for the chlorosis symptom induced by *P. tritici-repentis* race 1, but were resistant to necrosis induction from conidia inoculations and necrosis toxin infiltrations (10). Checks in the mapping experiment included both parents, the resistant cv. Erik and the susceptible line 6B365. Wheat plants used for the bioassay to isolate the chlorosis toxin included the sensitive check 6B365 and the insensitive cv. Erik. Wheat differentials used to determine the race of *P. tritici-repentis* isolate 78-62 were 6B365, Erik, ND495, Glenlea, W-7976, W-7984, Katepwa, Salamouni, Opata 85, Kulm, and Trenton.

Conidial inoculation and rating. The *P. tritici-repentis* isolate 78-62 was used in this study. Isolate 78-62 from the American Type Culture Center (ATCC 44142) produced identical reactions in wheat differentials when compared with the isolate stored at NDSU (data not shown). Dried mycelial plugs of 78-62 were stored at -20°C and used for inoculum preparation throughout the study. Inoculum was produced as previously reported by Faris et al. (10). The conidia then were used directly, or they could be stored at -20°C and used up to 1 month later with no noticeable decrease in infection rate.

Wheat differentials were grown in plastic cones containing a mixture of peat moss/perlite (3:1) in the greenhouse at an average temperature of 22°C with a photoperiod of 16 h and a light intensity of $900\ \mu\text{mol photons m}^{-2}\text{s}^{-1}$ at plant height. Fourteen seeds of each differential were planted, and seven plants were selected based on similarity of growth stage. This was done to minimize age effects of inoculated leaves, because it had been noted that younger leaves tend to be more resistant than older leaves (6). *P. tritici-repentis* isolate 78-62 was inoculated on all plants in a completely randomized design.

Seedlings were inoculated with a concentration of 3,000 conidia per ml when the third leaf was fully emerged. The conidial solution was kept on ice and continually stirred to minimize germination before being sprayed on the seedlings. Plants were sprayed to run off with the conidial solution using a pressurized atomizer. The seedlings were allowed to dry for 30 min and then were misted using an ultrasonic humidifier in a mist chamber at 20°C in total darkness for 24 h. The plants then were placed in a growth chamber at 20°C and subirrigated to avoid further leaf wetting. The third leaf was rated 7 days after inoculation for the presence or absence of extensive chlorosis.

Crude culture filtrate production. Culture filtrate produced by isolate 78-62 was used as the source of chlorosis toxin. The fungus was cultured on V8-potato dextrose agar (PDA) (150 ml of V8 juice, 10 g of PDA, 3 g of CaCO_3 , 10 g of agar, and 850 ml of distilled water) to produce mycelial plugs. Approximately 10 my-

celial plugs (5 mm in diameter) were transferred to a 100-ml Erlenmeyer flask containing 50 ml of potato dextrose broth (PDB) and cultured on a wrist-action shaker at room temperature for 5 days or until the plugs formed mycelial balls. The cultures were blended under sterile conditions and transferred with a syringe in 2-ml aliquots to 50 ml of Fries medium amended with 0.1% yeast extract (7) in 250-ml Erlenmeyer flasks. The cultures were incubated at 20°C in total darkness without agitation for 28 days.

Crude culture filtrate was separated from mycelial fragments by filtration through a Whatman No. 1 filter (Whatman International, Ltd., Maidstone, England) under vacuum. The crude filtrate was frozen (-20°C) overnight, thawed, and centrifuged for 20 min at $34,000 \times g$. The supernatant was collected and filtered through a $0.45\text{-}\mu\text{m}$ Whatman nylon filter to ensure that no fungal cells were in the filtrate. Filtrate was diluted 10-fold with 20 mM sodium acetate, pH 4.8, and chromatographed at a flow rate of 2 ml/min on an SP-Sepharose fast-flow cation exchange column (16 by 75 mm). The flow through was collected and used for all experiments described as using crude culture filtrate.

Partial purification of the chlorosis toxin. Crude culture filtrate was prepared in 0.5- to 1-liter batches from 78-62 cultures. The pH stability of chlorosis activity from crude culture filtrate was tested at pH values of 2, 3, 4, 5, 6, 7, 8, 9, and 10 using sodium phosphate (2, 3, 7, and 8), sodium citrate (3, 5, and 6), sodium acetate (4 and 5), MES (6), Mops (7), sodium borate (9), Hepes (8), bistrispropane (9), and Caps (10) buffers. Crude culture filtrate (1 ml) was diluted to 5 ml with each of the buffers (20 mM) at each of the indicated pH values. The diluted filtrate and buffer controls were infiltrated into 6B365 (sensitive check) and Erik (insensitive check) and scored for extensive chlorosis 5 days after infiltration. Temperature stability was checked by placing 100- μl samples of crude culture filtrate in water baths at 20, 40, 60, 80, and 100°C for 10 min. The samples were immediately placed on ice until infiltration into Erik and 6B365.

Organic extractions were attempted using *n*-butanol, cyclohexane, benzyl alcohol, 1-hexanol, and chloroform. Crude culture filtrate (50 ml) was diluted to 100 ml with each of the organic solvents and shaken vigorously to mix the phases. The phases were allowed to separate and were collected using a separatory funnel. The organic phase was diluted to its azeotrope with dH_2O and concentrated to 1 ml by vacuum distillation. The sample was again diluted to 20 ml with dH_2O and infiltrated, as well as the aqueous phase, into the checks.

Chromatography. Chromatography strategies used to separate the chlorosis toxin included size exclusion (low pressure [FPLC] and high pressure liquid chromatography [HPLC]), low pressure ion exchange (cation and anion), and HPLC reversed-phase (C18 and pyrene). The following chromatography instruments and columns were used in these experiments: Pharmacia FPLC (Pharmacia, Biotechnology Inc., Uppsala, Sweden), Beckman 126 HPLC with a Beckman 210A injector and a 166 UV detector (Beckman Instruments, Inc., San Ramon, CA), Pharmacia Hi Load 16/60 Superdex HR-75 gel filtration column, Rainin Microsorb-MV C18 reversed-phase column (Rainin Instrument Co. Inc., Woburn, MA) and a Cosmocil 5PYE Waters (4.6 by 150 mm) reversed-phase pyrene column (Phenomenex, Torrence, CA).

Toxin purification protocol. Crude culture filtrate was concentrated from 500 ml to 50 ml by rotovaping at 38°C with 5% butanol added to prevent foaming. The sample was loaded in 5-ml aliquots onto the Superdex HR-75 gel filtration column (16/60) (equilibrated with 20 mM sodium acetate, 150 mM NaCl, pH 4.8) and chromatographed at a flow rate of 1 ml/min. The eluent profile was measured spectrophotometrically at 280 nm and the active fractions were collected and combined between runs. The sample was diluted 10-fold with dH_2O and chromatographed at a flow rate of 2 ml/min on a DEAE sephadex column (16 mm by 15 cm) equilibrated with 20 mM sodium acetate, pH 4.8. The flow through was collected and concentrated by rotovaping at 38°C to

approximately 10 ml. The 10-ml sample was further concentrated by vacuum distillation to a volume of 4 ml.

The concentrated sample was chromatographed by HPLC in 500- μ l aliquots and eluted on a Rainin Microsorb-MV C18 reversed-phase column with a 20-min linear gradient from 1 to 18% acetonitrile and 20 mM sodium phosphate, pH 6.8, at a flow rate of 1 ml/min and measured spectrophotometrically at 280 nm. The active fractions from the C18 column were combined and concentrated to 500 μ l by vacuum distillation. The entire concentrated sample was chromatographed by HPLC and eluted on a Cosmocil 5PYE Waters reversed-phase pyrene column with a 25-min linear gradient from 1.2 to 16.2% acetonitrile and 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The eluent profile was measured spectrophotometrically at 215 nm and collected in 0.5-ml fractions. The active fractions from the pyrene column were combined and diluted 1:20, 1:50, 1:100, and 1:500 with dH₂O and infiltrated into the checks. An ultraviolet/visible (UV/VIS) spectral analysis was conducted on active fractions from the pyrene column using a Beckman DU-600 spectrophotometer.

Biochemical tests of chlorosis toxin. Partially purified chlorosis toxin from the C18 reversed-phase column was checked for the presence of primary and secondary amines, carbohydrate, and protein using the ninhydrin test for amino groups, the phenol-sulfuric acid method for detecting carbohydrates, and the micro-bicinchoninic acid (BCA) protein assay (Pierce Chemical Co., Rockford, IL) for determining protein concentration.

Partially purified chlorosis toxin (10 μ l) was diluted to 500 μ l with dH₂O, after which 25 μ l of 0.1% ninhydrin in 95% ethanol was added to the diluted chlorosis toxin sample. The sample combined with ninhydrin was placed in a 100°C water bath for 10 min and checked for color development. Carbohydrate determination was made using 10 μ l of partially purified chlorosis toxin diluted to 500 μ l with dH₂O, then adding to the sample 500 μ l of 5% aqueous phenol and 2.5 ml of concentrated sulfuric acid. The solution was mixed and allowed to sit at room temperature (21 to 23°C) for 20 min. The control used was 500 μ l of dH₂O with the phenol and sulfuric acid added. Both samples were read at 470 nm for color development.

Protein concentration was determined using the Pierce micro-BCA assay. A standard curve was prepared using bovine serum albumin (BSA) at 1 mg/ml to make protein standards at concentrations of 1, 10, and 100 μ g/ml. The sample was the same as in the previous experiments, and the control was 500 μ l of dH₂O. The micro-BCA assay was performed as per the instructions from Pierce, and the absorbence of the standards and sample was read spectrophotometrically at 562 nm with the zero set to the control.

Infiltration and chlorosis rating. Crude culture filtrate was checked for chlorosis activity by diluting 1:2 with sterile distilled water (for a total 1:20 dilution of the original fungal culture filtrate) and infiltrating approximately 10 μ l into the insensitive check (Erik) and the sensitive check (6B365). The boundaries of the infiltration area were marked with a felt pen before water soaking disappeared. Infiltrated leaves were checked for the presence of extensive chlorosis 5 days after infiltration. Plants were scored as either sensitive or insensitive based on the presence or absence of extensive chlorosis beyond the infiltration zone and on a scale from 0 to 4 (0 indicating no chlorosis, 1 to 3 indicating varying degrees of chlorotic intensity within the infiltration zone, and 4 indicating extensive chlorosis beyond the infiltration zone). Although modeled after the 1-to-5 scale of Lamari and Bernier (13), this scale deals exclusively with the chlorosis symptom.

Crude culture filtrate infiltration. Two runs with three replications of each RI line, parents, and checks were grown in the greenhouse at an average temperature of 22°C with a photoperiod of 16 h and a light intensity of 900 μ mol of photons m⁻²s⁻¹. In the first run, plants were grown in soilbeds with a 10-cm spacing between plants and 30-cm spacing between rows. Ten seeds of

each RI line were pregerminated in petri dishes and three of these were planted into the greenhouse beds. The second run was set up as in the first run, except for growing plants in tree pots (10 by 10 by 20 cm) containing a mixture of peat moss/perlite (3:1).

Approximately 10 μ l of crude culture filtrate was infiltrated into the youngest, most fully expanded leaf at the three-leaf stage of each RI line, parents, and checks using a 1-ml syringe with the needle removed. Seedlings were rated for chlorosis using the 0-to-4 scale described earlier and analysis of variance (ANOVA) was conducted to identify significant variation among lines. Variances were tested for homogeneity between runs. RI lines that exhibited both sensitive and insensitive reactions to crude culture filtrate were eliminated from the analysis based on the possibility that such lines may be heterogeneous.

Partially purified chlorosis toxin infiltration. The RI lines, parents, and checks were grown in plastic cones containing a mixture of peat moss/perlite (3:1) in the greenhouse at an average temperature of 22°C with a photoperiod of 16 h and a light intensity of 750 μ mol of photons m⁻²s⁻¹ at plant height. Fourteen seeds of each RI line were planted and six plants were selected based on similarity of growth stage for infiltration. Approximately 10 μ l of partially purified chlorosis toxin (diluted 50-fold with dH₂O) was infiltrated into the youngest, most fully expanded leaf at the three-leaf stage. Chlorosis ratings were as described for the crude culture filtrate.

RFLP analysis. Chlorosis scores were used to associate putative QTL with insensitivity to crude culture filtrate and partially purified chlorosis toxin produced by isolate 78-62 in the W-7984 $\times$ Opata 85 RI population. An extensive RFLP map which exists for this population and utilizes 541 markers selected to give non-redundant, complete genome coverage with an average distance of 7 cM between markers was used to identify associations between extensive chlorosis and RFLP loci (18,20–22,32). Linkage relationships were determined using the computer program Mapmaker V2.0 (17) with a minimum log likelihood ratio (LOD) of 3.0 and a maximum theta equal to 0.30.

The computer program QGene V2.25b (19) was used to execute interval regression by a flanking-marker regression technique to infer the best position for QTL between marker loci. Missing data points existed; therefore, the “complete missing data” feature was used to estimate QTL from the flanking markers of the nearest nonmissing data points. All the loci with significant main effects were tested against all the other markers on the map to detect significant ($P < 0.01$) interactions. Markers showing significant main effects and interactions were used to identify the best multiple regression model and markers that failed to retain significance in the full model were eliminated. The coefficient of determination (R^2) is the proportion of the total phenotypic variance explained by the markers in the multiple regression model. The type I error threshold of $P = 0.01$ was used for all QTL statistical analyses. The chance of declaring a nonsignificant QTL as significant (type I error) is enhanced in this experiment because of the large number of RFLP–tan spot associations evaluated in the W-7984 $\times$ Opata 85RI population. The higher probability of committing this type of error is tolerated to reduce the chance of declaring a significant QTL as nonsignificant (type II error).

RESULTS

Toxin purification. *P. tritici-repentis* isolate 78-62 culture filtrate ranged in color from light amber to dark red-brown depending on the batch grown. Chlorosis activity also varied depending on the batch. There was no relationship apparent between culture filtrate color and chlorosis activity. At no time was chlorosis seen in any of the wheat genotypes infiltrated with either full-strength or diluted (1:20) Fries medium alone. No difference was seen in chlorosis induction with either conidial inoculation or crude culture filtrate infiltration (Table 1).

Crude culture filtrate was checked for chlorosis-inducing stability at nine pH values using 15 buffering systems. Chlorosis activity remained stable at all pH values tested, pH 2 to 10. No reduction in chlorosis activity was seen after heating crude culture filtrate to any temperature tested, 20 to 100°C. All organic extractions resulted in chlorosis activity remaining in the aqueous phase. Anion and cation exchange columns were used at seven different pH values to establish the ionic characteristics of the chlorosis toxin and chlorosis activity was associated with the flow through of every column (data not shown). Dialysis of crude culture filtrate using 3,500 molecular weight cutoff dialysis tubing resulted in both the interior and exterior volumes causing chlorosis when infiltrated into 6B365.

Chlorosis toxin activity eluted from the Superdex HR-75 gel filtration column in a volume of 10 to 15 ml per 0.5- to 1-liter batch of crude culture filtrate. This resulted in no net concentration of the toxin; however, it removed over half the material that absorbed light at 280 nm, including Ptr ToxA. The DEAE sephadex column removed a large portion of the colored material from the flow through. Chlorosis activity eluted from the HPLC C18 column at 13% acetonitrile in a volume of 1 ml. The final separation by reversed-phase HPLC using a pyrene column resulted in chlorosis activity eluting in one 0.5-ml fraction at 12.6% acetonitrile (Fig. 1). A spectral analysis of the active fraction from the pyrene column resulted in no absorbance above 215 nm. All dilutions of partially purified chlorosis toxin (1:20, 1:50, 1:100, and 1:500) exhibited chlorotic activity when infiltrated into 6B365.

Biochemical tests of the chlorosis toxin were performed on the active fractions from the C18 reversed-phase column. The ninhydrin test resulted in no color development. The Pierce micro-BCA assay indicated protein at less than 5 µg/ml in the sample. Results of the phenol sulfuric acid method for the determination of carbohydrates indicated approximately 5 µg of carbohydrate in the sample.

Chlorosis screening and RFLP analysis. Chlorosis score means of checks and parents to crude culture filtrate and partially purified chlorosis toxin from isolate 78-62 are presented in Table 1. Reaction of differential lines to conidial inoculations indicated that 78-62 was a race 1 isolate. Although not apparent from the scale used, genotypes sensitive to crude culture filtrate exhibited

varying degrees of chlorosis intensity within the infiltration site with or without extensive chlorosis beyond the infiltration zone. Extensive chlorosis was mottled, while chlorosis seen inside the infiltration zone was not mottled, but solid. Insensitive genotypes exhibited no chlorosis in the leaf. Variances among runs were homogeneous for chlorosis scores, which allowed the means to be pooled. ANOVA indicated highly significant differences among the RI lines from the W-7984 × Opatá 85 cross. Genotypes sensitive to partially purified chlorosis toxin exhibited extensive chlorosis. All chlorosis seen was extensive and mottled. None of the RI lines exhibited chlorosis limited to the infiltration zone. Insensitive genotypes exhibited no chlorosis in the leaf. ANOVA indicated highly significant differences among the RI lines.

Chlorosis score means of the RI lines to crude culture filtrate segregated in a ratio of 60:46, insensitive/sensitive, and fit the binomial test for fit to 1:1 ($0.25 > P > 0.1$) when the Opatá 85 parental score was used as the base for sensitivity. A histogram of chlorosis score means was produced which resembled bimodal distribution with transgressive segregation for sensitivity (Fig. 2), suggesting more than one gene conditions insensitivity to crude culture filtrate from *P. tritici-repentis* isolate 78-62 in this population. Eight RI lines infiltrated with crude culture filtrate exhibited chlorosis scores ranging from insensitive to sensitive and were not included in the analysis.

Chlorosis score means were regressed on the RFLP marker data using the computer program QGene to identify loci significantly associated ($P = 0.01$) with insensitivity to crude culture filtrate of isolate 78-62. Two regions were significantly associated with insensitivity at the $P = 0.01$ level of probability. Chromosomal locations, identification of contributing parent, and multiple regression model are presented in Table 2. The main effects of *XG11* and *Xcdo395* explained 55.6% of the variation for insensitivity to crude culture filtrate in seedlings of the RI population. The locus identified by the marker *XG11* on the short arm of chromosome 1A explained the largest portion of variation for insensitivity to crude culture filtrate in seedlings at 49.4% (Table 2).

RI lines infiltrated with partially purified chlorosis toxin showed variation in intensity of extensive chlorosis seen, but only were analyzed for presence or absence of extensive chlorosis. The insensitivity locus was mapped to chromosome 1AS and is linked to

TABLE 1. Reaction of 12 wheat genotypes to inoculation with conidia and means of extensive chlorosis scores after infiltration with crude culture filtrate and partially purified chlorosis toxin from *Pyrenophora tritici-repentis* isolate 78-62

Wheat genotype	Conidial inoculations		Toxin infiltrations ^w	
	Chlorosis ^x	Necrosis ^y	CCF	PPT
6B365	+	—	4.0 a	4.0 a
Kulm	+	+	4.0 a	4.0 a
Trenton	+	+	4.0 a	4.0 a
W-7984	—	—	0.0 c	0.0 b
W-7976	—	—	0.0 c	0.0 b
Erik	—	—	0.0 c	0.0 b
ND495	— ^z	+	0.0 c	0.0 b
Salamouni	—	—	0.0 c	0.0 b
Glenlea	—	+	0.0 c	0.0 b
Katepwa	—	+	0.0 c	0.0 b
Opatá 85	— ^z	—	2.0 b	0.0 b
Altar 84	—	—	0.0 c	0.0 b

^w Chlorosis score means from infiltration of crude culture filtrate (CCF) and partially purified toxin (PPT) rated on a scale from 0 to 4, where 0 = no chlorosis, 1 to 3 = varying degrees of chlorosis within the infiltration zone, and 4 = extensive chlorosis throughout the leaf. Means within the same column followed by the same letter are not significantly different at $P = 0.05$.

^x Presence of extensive chlorosis is indicated by + and absence is indicated by —.

^y Presence of necrosis is indicated with + and absence is indicated by —.

^z Chlorotic halo was seen surrounding the infection site, but extensive chlorosis throughout.

Fig. 1. High performance liquid chromatography of the active fraction from the C-18 column using a pyrene reversed-phase column. Ptr ToxC eluted at 19 min in 12.6% acetonitrile at a flow rate of 1 ml/min, with buffer A composed of 5% acetonitrile and 95% water plus 0.1% trifluoroacetic acid, and buffer B composed of 95% acetonitrile and 5% water plus 0.1% trifluoroacetic acid, and was measured spectrophotometrically at 215 nm. The dashed line indicates the acetonitrile gradient.

the RFLP marker *XGli1* at 5.7 cM (Fig. 3). Seven RI lines infiltrated with partially purified chlorosis toxin exhibited both sensitive and insensitive reactions and were not included in the analysis. RI lines showing sensitivity and insensitivity to crude culture filtrate and partially purified chlorosis toxin may be heterogeneous at a major insensitivity locus. An interval map was generated showing association of the chlorosis symptom from Pti2 conidial inoculations of seedling and adult plants, crude culture filtrate from isolate 78-62, and Ptr ToxC from isolate 78-62 with markers on chromosome 1A of wheat (Fig. 4). In each case, the major effect is associated with the marker *XGli1*.

DISCUSSION

A molecule that is to be designated a toxin needs to meet several essential criteria. Scheffer (26) stated that the molecule has to be produced by the pathogen, it has to cause obvious damage to the host, and it needs to be established with confidence that the molecule is involved in disease development. The toxic compound or compounds in the culture filtrate of isolate 78-62 met these requirements. Moreover, wheat lines reacted in a genotype-specific manner. Infiltration of crude culture filtrate and partially purified chlorosis toxin into sensitive wheat genotypes produced a chlorotic symptom similar to that seen with conidia inoculations on the same susceptible genotypes. Wheat genotypes that were resistant to conidia inoculations also were insensitive to crude culture filtrate and partially purified chlorosis toxin infiltrations. Furthermore, the locus associated with resistance to conidia of 78-62 is the same or closely linked to the locus associated with insensitivity to partially purified chlorosis toxin. In accordance

Fig. 2. Histogram of chlorosis score means of 107 seedling recombinant inbred (RI) lines derived from the cross W-7984 x Opata 85 after infiltration with crude culture filtrate of *Pyrenophora tritici-repentis* isolate 78-62.

with the precedence for naming toxins produced by *P. tritici-repentis* set at the Third International Tan Spot Workshop (4), we propose Ptr ToxC to designate the toxin responsible for extensive chlorosis seen in this study.

P. tritici-repentis isolate 78-62 was the only isolate of 21 tested that produced culture filtrate capable of inducing chlorosis in sensitive wheat genotypes (J. Jordahl, unpublished data). Other race 1 and race 3 isolates may lack the toxin altogether or may not have produced sufficient quantities of Ptr ToxC in culture to cause chlorosis in sensitive wheat genotypes. Isolate 78-62 produces a necrosis toxin (Ptr ToxA) in culture (35). Effective screening of wheat genotypes for chlorosis sensitivity to culture filtrate of isolate 78-62 required the elimination of Ptr ToxA from the culture filtrate. This was done to prevent the masking of chlorosis by the necrosis symptom and to reduce the possibility of interactions between the toxins. However, the chlorotic symptom produced by conidia from race 1 isolate Pti2 and race 3 isolate D308 were very similar to that produced by isolate 78-62. Previous work has established that the locus *QTsc.ndsu-1A*, detected by the RFLP marker *XGli1*, was associated with resistance to conidial inoculations of Pti2, D308, and 78-62 (8). This is the same locus that

Fig. 3. Genetic linkage map of the short arm of chromosome 1A indicating linkage of insensitivity to Ptr ToxC of *Pyrenophora tritici-repentis* isolate 78-62 to the restriction fragment length polymorphism marker *XGli1*.

TABLE 2. *P* values and coefficients of determination of two unlinked markers significant at the *P* = 0.01 level of probability and multiple regression model of the significant markers associated with insensitivity to chlorosis following infiltration of the W-7984 x Opata 85 recombinant inbred population with crude culture filtrate of *Pyrenophora tritici-repentis* isolate 78-62

Variables	Chromosome	Resistance source	<i>P</i>	<i>R</i> ²	df	Mean square	<i>F</i> value	<i>P</i> value	Coefficient	Standard error	<i>T</i> (2-tail)
RFLP marker ^y											
<i>XGli1</i>	1AS	W-7984	<0.0001	0.494	...	...	...	...	...	...	...
<i>Xcdo395</i>	3A	Opata 85	<0.0001	0.063	...	...	...	...	...	...	...
Source of variation											
Model ^z	...	...	...	...	2	51.179	61.59	<0.0001	...	...	...
Error	...	...	...	...	98	0.831	...	...	...	...	...
Factor											
Intercept	...	...	...	...	...	...	...	<0.0001	1.832	0.090	20.430
<i>XGli1</i>	...	...	...	...	...	...	...	<0.0001	0.927	0.090	10.339
<i>Xcdo395</i>	...	...	...	...	...	...	...	0.0002	0.346	0.089	3.873

^y RFLP = restriction fragment length polymorphism.
^z Model adjusted *R*² = 0.556.

has been shown to be associated with insensitivity to crude culture filtrate and Ptr ToxC produced by 78-62 (Fig. 4).

Ptr ToxC was found to be a nonionic, polar, low-molecular-weight molecule. These physical properties made concentration of Ptr ToxC from large quantities of culture filtrate difficult. The nonionic nature of Ptr ToxC prevented ion exchange chromatography from being used to concentrate the toxin. Likewise, the polar nature of the toxin prevented organic extraction of the toxin from culture filtrate. Lyophilization of crude culture filtrate concentrated the sample, but it was found that activity was lost when the sample was resuspended. This left distillation as the only alternative for concentrating Ptr ToxC. Ptr ToxC was a low-molecular-weight molecule; therefore, desalting could not be accomplished by dialysis. A protocol of concentration by distillation followed by desalting on a gel filtration column seemed the only option available. The gel filtration column desalted the sample and removed all high-molecular-weight compounds from the sample. The DEAE column was used in a reverse chromatography step to remove most of the colored compounds, which were found to adhere very tightly to column materials and lens surfaces in UV/VIS detectors.

The reversed-phase C18 column interacted with Ptr ToxC, and the hydrophobic interactions between the C18 column and Ptr ToxC allowed concentration of the toxin within limits of column saturation and injection loop size. Colored material not removed from the sample resulted in saturation of the UV/VIS detector when large amounts of material were loaded onto the column. Ptr ToxC eluted in a volume of 1 ml, which separated it from most of the other material in the sample. The pyrene column utilized the slightly different hydrophobic interactions of π -bonding to retain Ptr ToxC on the column. Initially, the bioassay indicated two 0.5-ml active fractions that eluted at 9.6 and 12.6% acetonitrile, but when large amounts of material were run, these two peaks would run together. It was subsequently found that Ptr ToxC was apparently undergoing chemical reaction, which shifted the amount of acetonitrile required to release Ptr ToxC from the column (S. Meinhardt, unpublished data).

The limited biochemical tests performed with the partially purified toxin did little to classify the toxin into a chemical group. The micro-BCA assay indicated that peptide bonds were present in the sample and the ninhydrin assay indicated that no free primary or secondary amines were present in the sample. The

phenol-sulfuric acid assay for carbohydrates indicated that reducing compounds were present in the sample. These results are not conclusive because both the micro-BCA and phenol-sulfuric acid assays were at the limits of their detection errors.

Two other chlorosis-inducing toxins have been reported in the literature, Ptr ToxB from race 5 isolates (23,29) and a low-molecular-weight toxin from the Oklahoma isolate OKD4 (3). Ptr ToxC and Ptr ToxB are clearly different toxins. Ptr ToxB induces chlorosis on the wheat differential Katepwa and does not induce chlorosis on 6B365. Ptr ToxC induces chlorosis on the differential 6B365 and does not induce chlorosis on Katepwa. Brown and Hunger (3) reported toxin recovery in the organic phase of a butanol extraction. Ptr ToxC was recovered in the aqueous phase of all organic extractions tested, including *n*-butanol. Although toxin from OKD4 was similar in size to Ptr ToxC, host-pathogen interactions cannot be compared due to the lack of information regarding symptoms induced by OKD4 and reaction types of host differentials.

During the isolation of Ptr ToxC, the phenotype of solid chlorosis seen inside the infiltration zone was lost. Opat 85 was sensitive to this type of chlorosis but was insensitive to Ptr ToxC. The phenotype was eliminated during the purification protocol; this may indicate another chlorosis toxin was being produced by 78-62. Isolate 78-62 produces at least two toxins (Ptr ToxA and Ptr ToxC) and multiple toxin production by isolates of *P. tritici-repentis* has been suggested by Tuori et al. (31).

The sensitivity or insensitivity response to Ptr ToxC in the W-7984 $\times$ Opat 85 RI population was interesting because both parents appeared insensitive to the toxin, whereas the RI progeny segregated for insensitivity. There are several possible explanations for this phenomena. One possibility is that a suppressor system may be involved. Another explanation may be that the source of Opat 85 used in these experiments is different than the Opat 85 parent used for the development of the original population of RI lines, resulting in a different genetic background. The gene or tightly linked group of genes at the locus marked by *XGll1* may encode a product that is responsible for insensitivity to Ptr ToxC. There were varying degrees of chlorotic intensity seen in the progeny of the W-7984 $\times$ Opat 85 cross. This chlorotic intensity appeared consistent within RI lines and varied among RI lines, but a suitable scale needs to be developed to score chlorotic intensity before the genetics of these phenotypes can be uncovered.

Although Opat 85 contributed a locus on chromosome 3A, detected by the RFLP marker *Xcdo395*, which was associated with a portion of the insensitivity to crude culture filtrate, this locus was not associated with insensitivity to Ptr ToxC. This could represent partial insensitivity to a factor that was purified out of the crude culture filtrate or it could be an artifact due to the high level of Type I error tolerated in this experiment.

Further research is needed to purify fully Ptr ToxC, establish its physical characteristics, and determine the role of Ptr ToxC in pathogenesis. The means by which insensitivity to Ptr ToxC is conditioned and whether other chlorosis-inducing toxins are involved in the host-pathogen system also need to be determined. The *P. tritici-repentis* isolate 78-62 represents a model system from which to approach these questions, because this isolate produces multiple toxins.

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Fig. 4. Chromosome 1A interval map indicating the major effect from *XGll1* associated with resistance in recombinant inbred lines derived from the cross W-7984 $\times$ Opat 85 to extensive chlorosis caused by *Pyrenophora tritici-repentis* in seedlings from isolate Pt2, in adults from isolate Pt2, in seedlings from crude culture filtrate produced by isolate 78-62, and in seedlings from Ptr ToxC produced by isolate 78-62. Pt2 seedling data provided by J. Faris.

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