Advances in the Characterization of the *Pyrenophora tritici-repentis*–Wheat Interaction

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ABSTRACT

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Tan spot of wheat, caused by the fungus *Pyrenophora tritici-repentis*, is a destructive disease found in wheat-growing regions worldwide that can lead to serious yield losses. Changes in cultural practices have led to an increase in the severity and incidence of tan spot. Following infection, compatible races of the fungus elicit two distinct symptoms in differential wheat lines: tan necrosis and (extensive) chlorosis. Tan necrosis has been clearly demonstrated by several groups to result from the action of a protein toxin, Ptr ToxA. Wheat sensitivity to this toxin is conditioned by a single dominant gene. The chlorosis response may be more complex

and appears to involve at least two other toxins, Ptr ToxB and Ptr ToxC, produced by different races of the fungus. Distinct genes apparently condition the reaction of wheat lines to each of these chlorosis-inducing toxins. This review concentrates on significant advances that have occurred during the past decade in the characterization of this disease interaction, ranging from the epidemiology and management of tan spot to molecular host-parasite interactions. Particular emphasis is placed on work describing fungal race differentiation, production of toxins and their importance in pathogenicity, and the genetics and physiology of host response to infection.

Additional keywords: chlorosis toxin, fungal toxin, host-pathogen interactions, host-selective toxin, necrosis toxin.

Pyrenophora tritici-repentis (Died.) Drechs. (synonym *P. trichostoma* (Fr.) Fuckel), anamorph *Drechslera tritici-repentis* (Died.) Shoemaker (synonym *Helminthosporium tritici-repentis* Died.), is a homothallic ascomycete that is responsible for the foliar disease tan spot (synonym yellow leaf spot) of wheat (*Triticum aestivum* L.) and durum wheat (*T. turgidum* L. var. *durum*) (45,51,74). Much of the earlier work on the *P. tritici-repentis*–wheat interaction concentrated on epidemiological aspects of this disease. An increase in the economical significance and distribution of tan spot has recently fostered an increased awareness and interest among researchers. As such, the past decade has shown significant advances in understanding the molecular aspects of this host-pathogen interaction including toxin purification and biochemistry, the significance of toxins to disease development, and the genetics of the host response. We attempt here to provide the reader with a history of the system and an overall scope of the research developments.

DISEASE OCCURRENCE AND SEVERITY

Tan spot of wheat has been identified in major wheat-growing areas throughout the world and is a disease of economic importance. The disease was first detected in the United States in New York in 1940 and in Kansas in 1947 and has increased in incidence and severity to the present (7,42,78,81,92,104). By the late 1970s, the incidence of tan spot increased in Oklahoma and the southern plains of the United States (47). The disease was identified in Canada in 1939, but the first serious outbreak occurred in 1974 (19, 98). Yield losses attributed to this disease have ranged from 3 to 50% in the central plains of the United States and Canada (44,84, 97). Tan spot was reported as the fastest-spreading disease in the Southern Cone region of South America (50), and serious losses have been reported in Argentina, Brazil, and Paraguay (2,49). It is

a major leaf disease in the northern wheat-growing areas of Australia (69). In 1986, tan spot was seen for the first time in Central Asia and soon after was considered to be one of the main wheat diseases, based on surveys of cereal crops (80). The disease was first reported in Mexico in 1982 (37). Due to the wide-ranging spread of the disease and potential destruction, it is of concern to CIMMYT (International Maize and Wheat Improvement Center, Mexico, D.F., Mexico), as evidenced by a special workshop, “*Helminthosporium* Blights of Wheat: Spot Blotch and Tan Spot,” held at CIMMYT to strengthen research partnerships directed at reducing yield losses and at development of potential strategies for disease control (25).

Increases in disease incidence have been attributed to changes in cultural practices (82) including shifts from conventional tillage to conservation- and zero-tillage practices (3,22,55,96,104), shorter rotations and continuous wheat cultivation (36,81,86,96), and the culture of highly susceptible cultivars (22,85). In addition, the change from stubble burning to its retention is believed to be the cause of the increased incidence in Australia and other areas of the world (10,60,84,85). Interestingly, areas that have reported a decline in leaf and stem rusts due to the release of resistant germ plasm are now experiencing an increase in the predominance of tan spot (23,35). Foliar fungicides can be effective in the control of the disease, but in some areas, costs are prohibitive (59,81,99).

PATHOGEN DESCRIPTION

The fungus propagates both asexually by conidia and sexually by ascospores. Conidiophores are erect and simple with a swollen base and give rise to conidia that are subhyaline, cylindrical, typically four- to seven-septate, and multinucleate. The basal cell of the conidium is conically tapered and diagnostic. Pseudothecia are black and can be beaked; asci are typically eight-spored. Ascospores are brown, three-septate transversely, multinucleate, and range from oval to globose. The asci contain slight septal constrictions, and median cells may exhibit a longitudinal septation (27,40,91,105).

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DISEASE CYCLE

The fungus is a diurnal sporulator (35,72,74) and conidia are disseminated by wind (88,106). Pseudothecia of *P. tritici-repentis* develop on infected wheat residue and mature over fall and winter, giving rise to ascospores that are forcibly discharged during wet periods the following spring (41,74,86). Ascospores are generally thought to be the source of primary inoculum (40,51,81,84,96), followed by repeated cycles of conidial production on diseased leaf tissue (45,83,88,106). In contrast, studies by Krupinsky (54) indicate conidia may play a primary role in the initiation of a tan spot epidemic in spring wheat in the northern Great Plains. Further studies indicate that ascospores may serve to disperse *P. tritici-repentis* over short distances, while long-distance dispersal by wind may be limited to conidia (88). Tan spot severity has been correlated with the levels of infected wheat residue in the field (76,86, 96,104,106). *P. tritici-repentis* is known to infect other gramineous species present in major wheat-growing areas (53,74,91). As such, overwintering of the pathogen on these grasses may provide additional inoculum for spread of the disease. Moisture (30,42,44,77, 79,81,95), temperature (44,70,95), light (48), plant age or leaf position (20,43,81,84,90), host genotype (21,59,60,87,97), and virulence of the isolate (37,52,60,71,84) are variables reported to contribute to the amount of inoculum produced and the onset and severity of the disease, either together or separately. An increase in nitrogen fertilizer and the proportion of nitrogen utilized as ammonium was shown to reduce lesion development in tan spot (46). Later studies by Bockus and Davis (9), however, suggest that the apparent reduction of disease from N fertilizers is due to a delay in leaf senescence and not due to a direct effect on tan spot. The conflicting results of the two studies could be due to different rates and forms of N used; differences in soil type; differences in the wheat cultivars tested (9); or all of the above. Infected kernels, called red smudge (12), cause kernel discoloration; can affect seedling emergence, seedling vigor, yield, and grain quality; and may provide inoculum for epidemics and dispersal of pathogenic strains to new geographic areas (8,12,31,32,89,103).

SYMPTOM DEVELOPMENT AND PATHOTYPE/RACE DESIGNATIONS

The fungus penetrates the epidermis directly and grows intercellularly through the leaf mesophyll (24,66,68). Typical disease symptoms were originally described as yellow to tan-brown elliptical necrotic lesions with a brown to black point at the center frequently surrounded by a chlorotic border, often spreading to cover the entire leaf (40,42). The two symptoms (necrosis and chlorosis) were considered to be part of the tan spot syndrome, and a number of rating systems were adopted to describe host reactions to *P. tritici-repentis*. The work of Lamari and Bernier (60,62) demonstrated that specific interactions between certain isolates of the fungus and wheat genotypes resulted in the differential development of tan necrosis and chlorosis (including extensive chlorosis). Recognition of independent symptoms (necrosis and chlorosis) on various wheat genotypes provided the basis of a disease system of classification. To categorize host reactions to *P. tritici-repentis*, Lamari and Bernier (59) suggested a rating system based on lesion type (37,40). Under this system, symptoms range from small brown to black spots (resistant) to symptoms consisting of coalescing chlorotic or tan necrotic areas with or without the brown to black centers (highly susceptible) (59,60). Based on the ability of an isolate to induce necrosis and chlorosis on a set of wheat differentials, Lamari and Bernier (60) further suggested a disease system of classification that placed isolates into four pathotypes. They identified isolates that cause necrosis and chlorosis (nec^+chl^+ , pathotype 1), necrosis only (nec^+chl^- , pathotype 2), chlorosis only (nec^-chl^+ , pathotype 3), and those causing neither phenotype (nec^-chl^- , pathotype 4, avirulent). The majority of *P. tritici-repentis* isolates identified be-

long to pathotype 1 (60). The development of necrosis and chlorosis was found to be dependent upon a combination of the host genotype and the isolate of *P. tritici-repentis* tested. Subsequent to the proposed pathotype system, Lamari et al. (65) reported chlorosis-inducing isolates of *P. tritici-repentis* from Algeria that could be distinguished from the original isolates of pathotype 3 by differential wheat response. The Algerian isolates are considered members of pathotype 3 (nec^-chl^+) in that they are able to induce chlorosis, but in genotypes previously shown to be resistant to other isolates of pathotype 3. As such, a race designation system was suggested by Lamari et al. (65) that consisted of five races: races 1 to 4 represent the isolates previously designated pathotypes 1 to 4, respectively, and race 5 represents isolates with the genotype-specific chlorosis-inducing activity of the Algerian isolates. The pathotype 3 designation (nec^-chl^+) given to the Algerian isolates and, in fact, to other members of pathotype 3 can be misleading. This confusion lies in the fact that these isolates are also capable of inducing necrosis in certain susceptible durum wheat cultivars (such as 'Coulter' and 'Sceptre'). Similarly, isolates of race 3 have also been shown to be capable of inducing necrosis in 'Coulter' (60,65). This differential response of necrosis or chlorosis could possibly be attributed to different manifestations of the same recognition event in the different host genotypes. Additional studies are necessary to further resolve these apparent discrepancies.

TOXIN IDENTIFICATION AND PURIFICATION

The involvement of a toxin(s) in pathogenesis was suggested by both the symptomology of tan spot (40) and the method of fungal colonization of the host (60,66,68). Tomás and Bockus (100) provided the initial evidence that a cultivar-specific toxic compound produced by *P. tritici-repentis* was involved in the development of tan spot of wheat. Further studies by Lamari et al. (61) supported this view. The involvement of a host-specific toxin (HST) in tan spot of wheat was supported by the following. (i) All wheat cultivars or near-isogenic lines that developed typical tan spot necrosis upon inoculation with the fungus were also sensitive to the toxin (61,100). (ii) Resistant lines of wheat were insensitive to the toxin (61,100). (iii) All *P. tritici-repentis* isolates that caused tan spot symptoms also produced toxin in vitro (61,100). (iv) All isolates lacking the ability to cause tan spot necrosis did not produce toxin in vitro (61). (v) Development of necrotic symptoms, sensitivity to the toxin, and disease susceptibility all appear to be controlled by the same dominant gene (61).

Necrosis-inducing toxin(s)

The major necrosis-inducing toxin from culture filtrates of *P. tritici-repentis* was initially purified by two groups (4,101) and later by two other groups (102,107) employing slightly different procedures. Although each report described a polypeptide of approximately the same molecular weight, amino acid composition, and sensitivity to chemical reduction, discrepancies regarding the heat stability and specific activity of the toxin exist. Ballance et al. (4) reported that the toxin was a 13.9-kDa protein (designated Ptr necrosis toxin) with an average minimum active concentration of 0.2 nM. Tomas et al. (101) described the toxin as a 14.7-kDa protein (designated Ptr toxin) with an average minimum active concentration of 90 nM. Tuori et al. (102) described the toxin as a 13.2-kDa protein (designated ToxA) with an average minimum active concentration of 60 nM. The lower specific activity reported by Ballance et al. (4) could be attributed to differences in fungal isolates or wheat cultivars used as compared with Tomas et al. (101) and Tuori et al. (102). Differences in molecular weight and heat stability can be attributed to the different methods of characterization; e.g., electrophoretic mobility (4,101) versus the more accurate, mass spectroscopy (102). Although these apparent differences led researchers to speculate that different toxins were described, it now seems more likely that all groups described the same toxin

(described below). Zhang et al. (107), working with the same isolate (86-124) as Ballance and coworkers (4), reported the primary amino acid sequence of the toxin with the exception of the extreme N-terminus and determined both experimentally, by circular dichroism spectroscopy, and theoretically, by computer-aided secondary structure predictions, that the protein's secondary structure is comprised mostly of β -sheets.

The necrosis-inducing HST is produced by the fungus both in culture (4,101,102,107) and in planta (58). The 13.2-kDa ToxA protein was shown to be causal to tan necrosis by bioassays of the products of indirect immunoprecipitations (102) and of the protein eluted from polyacrylamide gels (101,102). In addition, incubation of the toxin with antisera raised against the protein eliminated toxic activity (58).

Other necrosis-inducing toxic components from culture filtrates of the fungus also have been described. Tuori et al. (102) isolated anionic (AI) and cationic (CI) protein fractions that elicited cultivar-specific necrosis. Both toxic components were essentially free of ToxA, as determined by native gel electrophoresis and immunological analysis with mouse anti-ToxA antisera. Similarly, Meinhardt and Zhang (73) isolated a protein fraction distinct from Ptr necrosis toxin that induced necrosis; however, this fraction reacted with antisera raised against Ptr necrosis toxin and was thus described as being a possible folding precursor of the toxin. The relation of the major necrosis-inducing toxin (Ptr toxin, Ptr-necrosis toxin, and ToxA) to the other necrosis-inducing toxic components (AI and CI) observed in culture filtrates of certain isolates of *P. tritici-repentis* (102) is currently unknown. A possibility that has not yet been ruled out is that the same gene could condition multiple toxins. Potential alternate processing of the necrosis-inducing toxin could result in proteins that are distinct, both chemically and immunologically, yet retain an affinity for the same receptor and, therefore, elicit the same necrotic response. We are currently investigating whether the ToxA protein undergoes alternate proteolytic cleavage that would yield chemically differentiable yet biologically active peptides (R. P. Tuori and L. M. Ciuffetti, *unpublished data*).

Chlorosis-inducing toxin(s)

Isolates of races 1 and 3 have been reported to induce extensive chlorosis in particular wheat lines (60,62). A constituent from cell-free culture filtrates capable of inducing similar chlorosis has recently been identified from race 1 isolates and efforts are underway to purify this putative HST (S. W. Meinhardt, *personal communication*). Additionally, a distinct chlorosis-inducing toxin that is also cultivar specific was identified and partially characterized from culture filtrates of race 5 isolates and was initially described as being between 3.5 and 10 kDa and heat labile (75). Subsequently, an approximately 5-kDa protein, designated Ptr chlorosis toxin, was partially purified (94).

Another low-molecular-weight, chlorosis-inducing compound (also called Ptr toxin) from cultures of *P. tritici-repentis* has been reported (11). Based on its predicted molecular weight of between 800 and 1,800 Da and its lack of host specificity (it affects barley), it was presumed to be a different molecule than the Ptr chlorosis toxin reported by Orolaza et al. (75). Two other groups have reported on phytotoxic components isolated from *P. tritici-repentis* (1,38). It is apparent that isolates of *P. tritici-repentis* are capable of producing multiple toxins, and different isolates of the fungus likely make different combinations of toxins.

CLONING AND CHARACTERIZATION OF THE NECROSIS-INDUCING TOXIN GENE

Purification of the proteinaceous necrosis-inducing toxin(s) from *P. tritici-repentis* led to the isolation of a gene responsible for toxin production. Two groups independently cloned a cDNA for the major necrosis-inducing toxin. Ciuffetti et al. (15,17,18) re-

ported the molecular cloning of a 917-base pair (bp) cDNA identified from an expression library with antibody to purified ToxA. The identity of this cDNA (designated *ToxA*) was confirmed by comparison of the predicted open reading frame to amino acid sequence determined from the purified toxin. Ballance et al. (5,6) independently reported the cloning and analyses of a 900-bp cDNA (designated *PtrNEC*) encoding the Ptr necrosis toxin. Interestingly, the sequences of the two cDNAs differ in only a single base that does not change the coding sequence, although the genes were obtained from geographically separated isolates. Indirect immunoprecipitations of in vitro translation products indicated that the toxin transcript is relatively abundant and the protein is initially synthesized as an approximately 20-kDa precursor. Sequence analysis of the cDNA clones indicated that *ToxA* encodes a 19.7-kDa preproprotein. The precursor is composed of a signal peptide necessary for secretion, followed by a small domain at the N-terminus comprising an anionic peptide not present in the mature toxin, and finally a larger domain at the C-terminus comprising the mature, cationic toxin (17). Originally, two potential proteolytic cleavage sites were proposed; between Arg-60 and Gln-61 or between Gln-61 and Gly-62 (17). However, we now favor the proteolytic cleavage site following Arg-60 yielding a pyroglutamate residue as the N-terminus of the mature toxin (18). The C-terminal domain very likely does not undergo any significant posttranslational modifications, other than the cyclization of the N-terminal glutamine residue to form a pyroglutamate residue (R. P. Tuori and L. M. Ciuffetti, *unpublished data*). A genomic clone of the *ToxA* gene was isolated along with its endogenous promoter, and expression was shown to be sufficient for pathogenicity (16,17). When transformed into a non-toxin-producing, nonpathogenic isolate of the fungus, the gene conferred upon this isolate toxin-producing ability and pathogenicity (16,17). To date, no striking similarity to other genes or proteins has been detected in database searches.

In isolates analyzed thus far, both the *ToxA* gene and the *PtrNEC* gene appear to be a single copy gene. The gene is apparently absent from non-toxin-producing (those that do not induce races 1- and 2-type necrosis) isolates of *P. tritici-repentis* as demonstrated by polymerase chain reaction and Southern blot analyses (5,16,17). Additionally, the *PtrNEC* gene was shown to be absent from isolates of races 3 and 5, both considered pathotype 3 (5). We have separated the chromosomes in selected *ToxA*⁺ and *ToxA*⁻ isolates of *P. tritici-repentis* by pulsed-field gel electrophoresis. Our results indicate that the *ToxA* gene appears to be localized to a single chromosome in all *ToxA*⁺ isolates tested thus far. Additionally, chromosomal polymorphisms exist among the *ToxA*⁺ isolates and between *ToxA*⁺ and *ToxA*⁻ isolates (A. Lichter and L. M. Ciuffetti, *unpublished data*).

STANDARDIZATION OF TOXIN NOMENCLATURE

Researchers working on the *P. tritici-repentis*-wheat interaction have proposed a nomenclature for the HSTs and the genes responsible for their production (14). The need for standardization arose from the fact that there are multiple names in the literature for the same toxin (i.e., Ptr necrosis toxin, Ptr toxin, and ToxA) and gene (i.e., *ToxA* and *PtrNEC*), and, apparently, multiple toxins produced by *P. tritici-repentis*. The following designations were adopted: (i) Ptr ToxA = Ptr necrosis toxin, Ptr toxin, and ToxA; (ii) Ptr ToxB = Ptr chlorosis toxin from race 5; and (iii) Ptr ToxC = Ptr chlorosis toxin from race 1 (and potentially from race 3), etc. The genes will be named in accordance with their respective toxins, and as such, *ToxA* = *ToxA* and *PtrNEC*. The gene (locus) for Ptr ToxB, when identified, would be *ToxB*, etc. (14).

GENETICS OF RESISTANCE TO TAN SPOT

The most economical and environmentally safe method for the control of tan spot is durable genetic resistance. Identification of

the number of loci conditioning resistance and their dominance relationships and linkages is the first step in developing a successful breeding strategy. Determination of resistance and susceptibility relationships of wheat to isolates of *P. tritici-repentis* is not a simple task, given that there are apparently several HSTs produced by this fungus. The genetics of sensitivity to the toxins produced by *P. tritici-repentis* is apparently conditioned by different loci that express either dominant or recessive alleles (62,64,67). Genetic data for sensitivity to Ptr ToxA (synonyms Ptr necrosis toxin, Ptr toxin, and ToxA) clearly indicates a role in pathogenicity (17). Segregation analysis of progeny from crosses between cultivars resistant and susceptible to tan necrosis (and sensitivity to Ptr ToxA) indicates that a single dominant gene confers sensitivity to the toxin and susceptibility to toxin-producing isolates of the fungus (61,62, 97). The single recessive gene, designated *tsn1*, conferring insensitivity to Ptr ToxA and resistance to necrosis-inducing isolates of the fungus was mapped to the long arm of wheat chromosome 5B (28,93). Resistance to infection by some isolates of *P. tritici-repentis* has also been described as polygenic (13,26,67); however, it is not known what toxins, if any, were produced by the isolates tested.

Distinct genes appear to condition the reaction of wheat lines to chlorosis-inducing isolates of races 1 and 3 (62) and to chlorosis-inducing isolates of race 5 (75). Lamari and Bernier (62) reported that resistance to races 1 and 3 is attributed to a single dominant gene with incomplete dominance observed with some crosses, suggesting the possibility of minor gene(s) action. In contrast, susceptibility to race 5 and sensitivity to Ptr ToxB (synonym Ptr chlorosis toxin) (94) were suggested to be conditioned by a single dominant gene (75). Recently, Faris et al. (29), using conidial inoculations, reported quantitatively controlled resistance to races 1 and 3. A quantitative trait locus (QTL) was identified and demonstrated to be responsible for 35% of the chlorosis-inducing variation, with four minor QTL contributing to the variation seen in the recombinant population tested. The major QTL was mapped to the short arm of chromosome 1A (29).

RECEPTOR AND SITE- AND MODE-OF-ACTION STUDIES

As stated above, genetic analyses indicate that sensitivity of wheat to Ptr ToxA and susceptibility to the fungus is dominant and controlled by a single gene. These results suggest that a single gene product directs the differential response of wheat to this toxin. Data explaining the physiological and molecular toxicity of Ptr ToxA in planta is lacking. However, there are a number of laboratories actively working on the molecular characterization of the site- and mode-of-action of this HST.

One hypothesis is that sensitivity to Ptr ToxA results from a dominant allele encoding a gene product involved in the toxin-recognition process. Purification of Ptr ToxA and cloning of the *ToxA* gene (synonym *PtrNEC*) provide the tools to investigate this hypothesis. One approach in progress in our laboratory is the use of the yeast two-hybrid assay (33,34) to identify interactions in vivo between the Ptr ToxA protein and proteins from toxin-sensitive wheat. A clear advantage to this approach is that not only does it have the potential to identify wheat proteins that interact with Ptr ToxA, but facilitates the cloning of the genes that encode these proteins. We have identified and sequenced a number of wheat cDNA clones whose products appear to interact with Ptr ToxA (39). Further analyses of these clones should provide insights into relationships of these putative Ptr ToxA-interacting proteins to one another and possibly to the mode-of-action of this toxin.

Kwon et al. (56,57) demonstrated that Ptr ToxA (synonym Ptr necrosis toxin and Ptr toxin) elicited electrolyte leakage in susceptible wheat genotypes. Their data indicate that inhibitors such as vanadate (an inhibitor of H⁺-ATPases) reduce the amount of toxin-induced electrolyte leakage. However, this protection decreased with time and did not prevent the development of necrosis (57).

Cycloheximide (inhibitor of protein synthesis) was also shown to protect against toxin-induced electrolyte leakage. Interestingly, cycloheximide prevented necrosis development when the inhibitor was coinfiltrated with the toxin (57). The electrolyte-leakage bioassay was used to investigate the effect of temperature on toxin action. Susceptible wheat seedlings failed to develop necrosis or to leak electrolytes when incubated at 4°C prior to infiltration of the toxin. The effect of cold treatment on toxin action was reversible (57). Kwon and coworkers (57) suggest that these results support the requirement of active host metabolism and putative signaling mechanisms for toxin action in sensitive wheat cultivars. Previous studies have also shown that the development of necrosis is temperature sensitive and necrosis fails to develop at temperatures ≥30°C (56,63).

CONCLUDING REMARKS

Significant progress has been made in understanding the epidemiology and management of tan spot, with particular emphasis on the effectiveness of fungicides for disease management practices, cultural practices affecting incidence and severity, and the identification of sources of resistance for use in breeding programs. Current efforts to understand the molecular aspects of the *P. tritici-repentis*-wheat interaction could be exploited to identify and develop genetically resistant wheat cultivars. Further advances in the purification of the toxins, cloning of their respective genes, and the identification and characterization of the site- and mode-of-action of the toxins involved in this plant-microbe interaction will likely be forthcoming. It is anticipated that continued molecular characterization will be exploited for the control of this wide-ranging and potentially serious disease.

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