PURIFICATION AND MODE OF ACTION OF PTR

(*PYRENOPHORA TRITICI-REPENTIS*) CHLOROSIS TOXIN

By

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University

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MASTER OF SCIENCE

Stephen E. Strelkov 1997 (c)

Dedicated to the memory of my grandmother, Elsa C. Davi.

ACKNOWLEDGMENTS

FOREWORD

This thesis is written in manuscript style. A general introduction and a review of the literature precedes the two manuscripts presented. Each manuscript consists of an abstract, introduction, materials and methods, results, and a discussion. The manuscripts are followed by a general discussion, a list of literature cited, and an appendix.

ABSTRACT

Pyrenophora tritici-repentis differentially induces tan necrosis and extensive chlorosis in its hexaploid wheat host. A chlorosis inducing host-specific toxin, termed the Ptr chlorosis toxin, has been identified from race 5 of *P. tritici-repentis*. Ptr chlorosis toxin was purified from the culture filtrates of race 5 isolates, and the physiological development of chlorosis was investigated. Partial purification was performed by 25-80% ammonium sulfate precipitation and passage through a CM-S C25 cation exchange column. Final purification was performed on fast performance liquid chromatography (FPLC), using a MonoS 5/5 cation exchanger, followed by size fractionation on a Superose 12 HR 10/30 column. Purity was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the molecular weight of the toxin determined to be 6.61 kDa by mass spectrometry. The physiological development of chlorosis in sensitive wheat was investigated using partially purified toxin. Treatment with the toxin had no effect on the greening of etiolated tissue, suggesting that chlorosis results from chlorophyll degradation, rather than inhibition of chlorophyll synthesis. Development of chlorosis was light-dependent, suggesting that it may be a consequence of photochemical bleaching. To test for the involvement of active oxygen (AO) species in photobleaching, toxin-treated tissue was floated in solutions of various AO scavengers. The compound p-benzoquinone, which quenches singlet oxygen and triplet chlorophyll, prevented the development of chlorosis, suggesting that AO species are involved in chlorophyll degradation. High performance liquid chromatography (HPLC) chlorophyll degradation profiles were also consistent with photooxidation. Decreases in carotenoid levels were smaller than and concurrent with the declines in chlorophyll, indicating that toxin-

induced chlorosis was not the result of a carotenoid deficiency. It appears that Ptr chlorosis toxin, directly or indirectly, inhibits photosynthesis, leading to chlorophyll photodestruction as illuminated thylakoid membranes become unable to dissipate excitation energy normally used in photosynthesis.

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1. INTRODUCTION

Tan spot, caused by the fungus *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph *Drechslera tritici-repentis* (Died.) Shoem.), is an important foliar disease of wheat (*Triticum aestivum* L.) worldwide. While its occurrence as an endemic disease of wheat has been known in many regions since the 1940s (Rees et al., 1982), there has been an increased incidence of this disease in recent years, as a consequence of the shift towards stubble retention by farmers (Rees, 1982). This in turn has elevated the economic significance of tan spot, as severe epidemics may result in yield losses of up to 50% (Rees et al., 1982). Tan spot consists of two distinct symptoms: tan necrosis and extensive chlorosis (Lamari and Bernier, 1989a). Host reaction to the pathogen is qualitative in nature (Lamari and Bernier, 1989a), as is the variation for virulence in the pathogen (Lamari and Bernier, 1989b). Isolates of *Pyrenophora tritici-repentis* can be grouped into four pathotypes based on their ability to cause necrosis and/or chlorosis on differential wheat cultivars (Lamari and Bernier, 1989b). This symptom based classification system allows for the description of a maximum of four pathotypes; pathotype 1 (P1) causes both necrosis and chlorosis (nec+chl+), pathotype 2 (P2) causes only necrosis (nec+chl-), pathotype 3 (P3) causes only chlorosis (nec-chl+), and pathotype 4 (P4) is avirulent and produces neither symptom (nec-chl-).

The discovery of Algerian fungal isolates possessing a virulence pattern different from those reported in North America has served to illustrate the limitations of the pathotype system in describing isolates of *P. tritici-repentis* (Lamari et al., 1995). Isolates collected in Algeria belong to pathotype 3, in that they can induce chlorosis but not necrosis on hexaploid

differential wheat cultivars. However, while North American pathotype 3 isolates are virulent on wheat cv. 6B365 and avirulent on cv. Katepwa, the Algerian isolates are virulent on cv. Katepwa and avirulent on cv. 6B365 (Lamari et al., 1995). Hence, a race designation has been proposed (Lamari et al., 1995) to classify isolates of *P. tritici-repentis* based on their virulence on differential wheat lines. This race-based system is a refinement of the symptom based classification system, since the number of races which it can describe is limited only by the size and effectiveness of the differential set. Under the race classification system, the isolates within the original pathotypes 1 to 4 have been re-classified as belonging to races 1 to 4, while the new Algerian chlorosis inducing isolates have been classified as members of race 5 (Lamari et al., 1995).

A host-specific toxin, termed the Ptr necrosis toxin, has been shown to be responsible for the development of the necrotic symptom in susceptible wheat lines (Lamari and Bernier, 1989c). This toxin has been purified to homogeneity from the culture filtrates of necrosis-inducing isolates, and has been characterized by several independent research groups (Ballance et al., 1989; Tomas et al., 1990; Tuori et al., 1995; Zhang et al., 1997). Ptr necrosis toxin is a 14kDa protein, with a very high level of toxic activity; bioassays have shown that it can induce necrosis in susceptible wheat plants within three days after infiltration at a concentration of only 2.4 ng/ml (Ballance et al., 1989). This toxin shows the same host-specificity as the fungal isolates from which it is obtained, and has been recognized as an important factor in the development of tan spot (Tomas and Bockus, 1987; Ballance et al., 1989; Lamari and Bernier, 1991). The encoding gene for Ptr necrosis toxin has been recently cloned (Ballance et al., 1996; Ciuffeti et al., 1997).

A second host-specific toxin, capable of inducing chlorosis in various wheat cultivars, has been isolated from race 5 culture filtrates of *P. tritici-repentis* (Orolaza et al., 1995). This toxin, which has been termed the Ptr chlorosis toxin, appears to be a small, hydrophilic protein, stable to exposure to organics (Orolaza et al., 1995; N.P. Orolaza, *unpublished data*). Like Ptr necrosis toxin, Ptr chlorosis toxin possesses a host specificity identical to that of the isolates from which it has been obtained. This and other evidence strongly suggest that Ptr chlorosis toxin is responsible for the development of extensive chlorosis in susceptible hosts (Orolaza et al., 1995).

The objectives of the present investigation were to (1) develop an effective method to purify Ptr chlorosis toxin, and to (2) elucidate its mode of action.

2. LITERATURE REVIEW

2.1. Tan Spot of Wheat

Tan spot of wheat is caused by the fungal pathogen *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph *Drechslera tritici-repentis*), a homothallic ascomycete. This disease, also known as yellow spot, generally begins as tan-brown flecks on the leaves of affected plants. These flecks expand into brown coloured, oval shaped spots surrounded by a yellow margin. As the disease progresses, these spots can coalesce, producing large areas of dead and chlorotic tissue. Tan spot has been identified throughout the major wheat growing regions of the world, including Canada, Australia, and the United States (Hosford, 1982; Wiese, 1987). While its occurrence as an endemic disease of wheat has been known since the 1940s (Rees et al., 1982), it is only in recent decades that tan spot has become an important constraint to wheat production (Rees and Platz, 1992). Yield losses can be very high; Rees et al. (1982) reported losses of up to 50% in severe epidemics. However, losses of 5 to 10% are not uncommon (Hosford, 1982), particularly since most wheat cultivars are susceptible to tan spot (Martens et al., 1988).

The increased incidence of tan spot in recent years can be largely attributed to the widespread adoption of conservation and zero tillage techniques by farmers. Tan spot is caused by a stubble-borne pathogen and hence, reduced tillage practices allow for longer survival of the pathogen on crop residues on the soil surface, leading to an increase in inoculum levels. Changes in cultivar genotypes may have also played a role in the increased importance of tan spot; many of the semi-dwarf wheat varieties introduced after 1960 have a high susceptibility to this disease (Rees and Platz, 1992). Compounding the problem is the

fact that *Pyrenophora tritici-repentis* has the widest host range of any member of the genus *Pyrenophora* (Shoemaker, 1962). *P. tritici-repentis* has been identified on many species of grasses, as well as on several cereal species, such as triticale (*Triticum secale*) and rye (*Secale cereale*) (Sissons, 1996). This large host range allows *P. tritici-repentis* to overwinter on a large number of grasses, which could provide inoculum to start tan spot epidemics in successive wheat crops (Krupinsky, 1986).

The tan spot syndrome is associated with two distinct symptoms, tan necrosis and/or extensive chlorosis. The development of each symptom is the result of specific interactions between individual isolates of *P. tritici-repentis* and wheat genotypes (Lamari and Bernier, 1989a). Some wheat cultivars develop both symptoms when challenged with appropriate isolates of the fungus, while others selectively develop necrosis or chlorosis (Lamari and Bernier, 1989a; Lamari et al., 1991). Isolates of *P. tritici-repentis* have been classified into four pathotypes based on their ability to induce necrosis and chlorosis (pathotype 1), necrosis only (pathotype 2), chlorosis only (pathotype 3), or neither symptom (pathotype 4) in differential hexaploid wheats (Lamari and Bernier, 1989b). The recent identification of Algerian pathotype 3 isolates that can induce chlorosis in wheat genotypes previously known to be resistant to all isolates of this pathotype has led to the adoption of a race classification system (Lamari et al., 1995). In this system, isolates of *P. tritici-repentis* are classified into races based on their virulence on wheat differential genotypes. The number of races which can be described is limited only by the size and effectiveness of the differential set. This is in contrast with the pathotype classification system, which can describe a maximum of only four pathotypes (Lamari et al., 1995). Currently, five races have been identified. Races 1 to 4

correspond to the original pathotypes 1 to 4, and race 5 is the recently identified Algerian race. Isolates belonging to race 5 can also be classified as being of pathotype 3, since they induce only chlorosis in hexaploid wheats.

To date, three host-specific toxins have been identified from the culture filtrates of *P. tritici-repentis* (Ballance et al., 1989; Orolaza et al., 1995; Meinhardt et al., 1997). The best characterized of these is Ptr necrosis toxin, which has been shown to be responsible for the development of the necrotic symptom in susceptible wheat lines (Lamari and Bernier, 1989c). Ptr necrosis toxin was purified from the culture filtrates of necrosis inducing *P. tritici-repentis* isolates by several independent research groups (Ballance et al., 1989; Tomas et al., 1990; Tuori et al., 1995; Zhang et al., 1997), and its encoding gene recently cloned (Ballance et al., 1996; Ciuffeti et al., 1997). The clone (PtrNEC) encodes a 19.7 kDa protein precursor of Ptr necrosis toxin, which is a water soluble protein with a molecular mass of 14 kDa and a very high level of toxic activity. Although Ptr necrosis toxin has been recognized as an important factor in the development of tan spot (Tomas and Bockus, 1987; Ballance et al., 1989; Lamari and Bernier, 1991), its mode of action has not yet been elucidated. There is evidence to suggest, however, that the toxin may trigger a programmed cell death in sensitive wheat (Kwon and Rasmussen, 1997).

The two other host-specific toxins identified from the culture filtrates of *P. tritici-repentis* are both associated with the development of the chlorotic symptom. One of these toxins, which has not yet been named, has been detected in the culture filtrates of a race 1 isolate (Meinhardt et al., 1997). This toxin is a very polar, non-ionic molecule with a molecular weight of less than 2 kDa. The other chlorosis-inducing toxin, termed Ptr chlorosis

toxin, has been identified in the culture filtrates, spore germination fluids, and intercellular washing fluids from race 5 of *P. tritici-repentis* (Orolaza et al., 1995). Ptr chlorosis toxin appears to be a small, hydrophilic protein, stable to exposure to organics (Orolaza et al., 1995; N.P. Orolaza, *unpublished data*). Several lines of evidence, including the fact that Ptr chlorosis toxin possesses a host specificity identical to that of the isolates from which it was obtained, suggest that this toxin is a pathogenicity factor (Orolaza et al., 1995). Although the amount of Ptr chlorosis toxin needed to produce chlorosis is unknown, the toxin seems to have a relatively high activity (Orolaza et al., 1995). Furthermore, the development of chlorosis in toxin-treated tissue is strictly light dependent (N.P. Orolaza, *unpublished data*). However, nothing is known or has been published with respect to the mode of action of Ptr chlorosis toxin, the primary focus of the present investigation.

2.2. Phytotoxins

Many bacterial and fungal plant pathogens produce chemical compounds which are toxic to plants. These substances are generally referred to as "phytotoxins" (Strobel, 1982; Graniti, 1991). Although there is no one definition on what exactly constitutes a phytotoxin, many of the definitions that have been suggested are quite similar. For instance, Strobel (1982) used the term "phytotoxins" to refer to "compounds produced by parasites that are toxic to plants and play some role in symptom expression." Durbin (1991) provided a more inclusive definition of phytotoxins, stating that they are "compounds synthesized by the pathogen during pathogenesis that are deleterious to the host." Either of these definitions would suffice for our discussion of phytotoxins. However, a compound should be judged to be a phytotoxin based on its function, and not on its structure or chemical characteristics. As

a group, the phytotoxins currently recognized have no common features; they vary dramatically both in size and structure (Strobel, 1982; Strobel et al., 1991; Graniti, 1991). Most known phytotoxins have low molecular weights and are not antigenic (Scheffer and Livingston, 1984; Graniti, 1991). However, some phytotoxins, such as Ptr necrosis toxin, are relatively large and antigenic (Ballance et al., 1989).

Phytotoxins can either be host-specific (host-selective), or non-specific (non-selective). Host-specific toxins affect only plants of a genotype susceptible to the pathogen (Graniti, 1991). Furthermore, the virulence of pathogenic strains varies with their ability to produce the toxin (Goodman et al., 1986). In addition, host-specific toxins are able to produce, in susceptible hosts, the symptoms of the natural infection at low or physiological concentrations (Goodman et al., 1986; Graniti, 1991). Non-specific phytotoxins, on the other hand, do not reproduce the patterns of resistance and susceptibility of the host to the pathogen (Graniti, 1991). Plants of a genotype which may be resistant to a particular toxin-producing pathogen may be sensitive to the isolated toxin. All known bacterial phytotoxins as well as a majority of fungal phytotoxins are non-specific (Graniti, 1991). Nevertheless, the impact of both groups of toxins on economically important crops can be very serious.

Many plant pathogenic bacteria and fungi produce phytotoxins. The mode of action of a few of these toxins is relatively well understood, and for a somewhat greater number of toxins, there is some understanding as to what organelle and/or metabolic process is affected. However, for the majority of phytotoxins, very little is known regarding their mechanism of action (Durbin, 1991). This is unfortunate, since a knowledge of the mode of action of a toxin can be useful in better understanding the nature of host-pathogen interactions, both in

general and within specific systems. Nevertheless, in recent years, more research has been devoted to elucidating the mechanisms responsible for the toxicity of these compounds (Ballio, 1991). Most of the visible and physiological changes caused by phytotoxins appear to be secondary to prime, or initial biochemical lesions somewhere in the metabolic machinery of the plant (Scheffer and Livingston, 1984). This is particularly true with respect to symptoms such as necrosis or chlorosis. These symptoms require several days to develop, which implies that they are secondary effects of a primary molecular event (Daly, 1981). Nevertheless, plant tissue or cell responses to toxins can provide some clues as to their molecular mode of action.

Ptr chlorosis toxin causes the development of chlorosis in sensitive wheat genotypes. A wide variety of other phytotoxins are also known to produce the same symptom, and the modes of action of several non-specific chlorosis inducing toxins have been at least partially elucidated. The roles that various herbicides have in producing chlorosis are also relatively well understood. Therefore, it is useful to consider what is known regarding the mechanisms of action of some of these compounds, in order to examine Ptr chlorosis toxin within a broader context.

2.2.1. Phaseolotoxin (Halotoxin)

Phaseolotoxin (halotoxin) is a non-specific toxin produced by the bacterial pathogen *Pseudomonas syringae* pv. *phaseolicola* (*Pseudomonas phaseolicola*), causal agent of haloblight disease of French bean (*Phaseolus vulgaris* L.). Phaseolotoxin can induce all the major symptoms of haloblight, including the development of chlorotic haloes on leaves, and the accumulation of certain amino acids, especially ornithine (Smith and Rubery, 1982; Turner

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and Mitchell, 1985). This toxin consists of sulfamyl phosphate linked to a tripeptide of ornithine, alanine, and homoarginine (Mitchell, 1976). Most of the work on the mode of action of phaseolotoxin has focused on the accumulation of ornithine in affected tissues (Smith and Rubery, 1982). The accumulation of ornithine is believed to result from the specific inhibition of ornithine carbamyltransferase (OCT), which catalyzes the formation of citrulline from ornithine and carbamoyl phosphate (Patil et al., 1972). This is the first reaction in the only biosynthetic pathway to arginine in plants. However, the manner in which the toxin produces the other important symptom, chlorosis, is a point of some contention. While it has been shown that phaseolotoxin inhibits chlorophyll synthesis and does not cause its degradation (Patil et al., 1972; Smith and Rubery, 1982; Turner and Mitchell, 1985), there are two major hypotheses as to how this inhibition occurs.

The first hypothesis stipulates that there is a direct link between OCT-inhibition and the development of chlorotic haloes (Patil et al., 1972; Turner and Mitchell, 1985). In this scenario, the toxin causes chlorosis by blocking the accumulation of protein through the primary inhibition of arginine synthesis at the OCT stage (Turner and Mitchell, 1985). Hence, according to Turner and Mitchell (1985), the development of chlorosis is principally the result of arginine starvation in affected tissues. In support of this hypothesis, these workers note that when arginine is applied to chlorotic tissue, chlorophyll begins to accumulate and chlorosis is reversed (Turner and Mitchell, 1985). A reduction in net protein synthesis could also mean a reduction in chlorophyll *b* synthesis, because a large proportion of chlorophyll *b* exists in association with a chloroplast membrane polypeptide, and the synthesis of this peptide is apparently metabolically linked to the synthesis of chlorophyll *b* (Turner and

Mitchell, 1985; Wettstein et al., 1995). Thus, if net protein synthesis was reduced, chlorophyll

b synthesis would also decline. According to Turner and Mitchell (1985), this reduction in

chlorophyll *b* synthesis could account for at least part of the chlorosis caused by

phaseolotoxin.

The second hypothesis as to how the inhibition of chlorophyll synthesis occurs was

formulated by Smith and Rubery (1982). They suggested that phaseolotoxin interferes

directly with an early process common to the synthesis of chlorophyll and of different lipids.

Smith and Rubery (1982) found that there was an inhibition of 5-aminolevulinic acid (5-ALA)

production in affected leaf tissue. 5-ALA is a chlorophyll precursor, two molecules of which

are condensed to make porphobilinogen early in the chlorophyll biosynthetic pathway

(Wettstein et al., 1995). Therefore, Smith and Rubery (1982) suggested a causal relationship

between the inhibition of 5-ALA production and chlorophyll synthesis in this system.

Furthermore, they found that ^{14}C-acetate incorporation into lipid was inhibited in toxin treated

tissue, without affecting the distribution of the label within the fatty acid spectrum (Smith and

Rubery, 1982). Thus, Smith and Rubery (1982) concluded that phaseolotoxin interferes at

some early stage in fatty acid biosynthesis. They stated that this could account for the

inhibition of chlorophyll synthesis, and therefore chlorosis. In contrast to Turner and Mitchell

(1985), these researchers found it difficult to establish a direct connection between the

inhibition of OCT and the development of chlorosis.

While it is hard to evaluate which hypothesis has the most merit, it should be noted

that Turner and Mitchell (1985) used pure toxin, whereas Smith and Rubery (1982) used only

partially purified toxin. Therefore, the chemical nature of their toxin preparation is uncertain.

In light of this fact, it is possible that the results of Smith and Rubery (1982) are an artefact of some other compound present in their toxin preparation.

2.2.2. Tabtoxin

Tabtoxin is a non-specific toxin produced by *Pseudomonas syringae* pv. *tabaci*, causal agent of wildfire disease of tobacco (*Nicotiana tabacum* L.) (Turner, 1981; Turner, 1988). Wildfire disease is characterized by distinct chlorotic haloes that develop around the site of infections. These symptoms are due to the action of tabtoxin, a peptide containing two residues, tabtoxinine β-lactam linked to either threonine or serine. Plant enzymes process the toxin by hydrolyzing it to yield tabtoxinine β-lactam (Turner, 1981). Turner (1981) found that the earliest detectable change in tabtoxin-treated tissue is the loss of glutamine synthetase activity. This is followed by a measurable increase in ammonia, which is a substrate for this enzyme, and a reduction in the incorporation of ammonia-N into protein amino acids (Turner, 1981; Turner, 1988). The ammonia that accumulates in tabtoxin-treated tissue is produced largely from the photorespiratory cycle, and treatments that inhibit this cycle inhibit ammonia accumulation and also prevent development of chlorosis (Turner, 1986; Turner, 1988).

These facts suggest that additional major physiological changes may occur during the development of chlorosis (Turner, 1988). Turner (1988) showed that photosynthesis is inhibited in tabtoxin treated tissue, but that this is not a result of a lower chlorophyll content. In addition, tabtoxin does not appear to have a direct effect on photosynthesis, because photosynthetic inhibition has not been observed in tissues maintained in darkness following treatment with tabtoxin, even though glutamine synthetase is inactivated (Turner, 1988). Turner (1988) suggests that the light-dependent inhibition of photosynthesis is a consequence

of the inhibition of the photorespiratory nitrogen cycle at glutamine synthetase. If this is the case, then the most probable chain of events leading to the development of chlorosis begins with the inactivation of glutamine synthetase by tabtoxin, resulting in a lesion in the photorespiratory nitrogen cycle, which is expressed only in illuminated tissue (Turner, 1981; Turner, 1988). Photosynthesis may then be inhibited as a result of either the reduced return of photorespiratory cycle intermediates to the Calvin cycle, or of the uncoupling of photophosphorylation by ammonia (Turner, 1988). This could result in an inability of the illuminated thylakoid membranes to dissipate the excitation energy that would normally be used in photosynthesis, leading to the photodestruction of the chlorophylls and the subsequent development of chlorosis (Turner, 1988). Therefore, it appears that the inhibition of photosynthesis is important in the development of symptoms caused by tabtoxin (Turner, 1988).

2.2.3. Coronatine

Coronatine is a non-specific, chlorosis-inducing toxin produced by several pathovars of the phytopathogenic bacterium *Pseudomonas syringae*. It was first isolated from the culture fluids of *P. syringae* pv. *atropurpurea*, which causes chocolate spot disease of *Lolium multiflorum*. However, it has since been found in cultures of *P. syringae* pv. *tomato* and *P. syringae* pv. *glycinea*, causal agents of bacterial speck of tomato (*Lycopersicon esculentum* Mill.) and bacterial blight of soybean (*Glycine max* L.), respectively (Kenyon and Turner, 1990; Durbin, 1991). All three of these diseases produce a symptom complex that is characterized by a diffuse chlorotic halo surrounding the site of infection.

The development of chlorosis in coronatine treated tissue has been shown to result from chlorophyll degradation (Kenyon and Turner, 1990). However, the fact that pigment loss and inhibition of photosynthesis occur simultaneously after treatment with this toxin, along with other evidence, suggests that photochemical bleaching of chlorophyll is not involved (Kenyon and Turner, 1990). Rather, studies with coronatine seem to suggest that the chlorotic symptom is the result of an alteration in growth regulator metabolism of affected tissues (Kenyon and Turner, 1990). For instance, it has been found that coronatine treated leaves retain a level of physiological competence not usually associated with the pathological state of tissues affected by other toxins. Furthermore, changes observed in photosynthesis and protein synthesis occur relatively slowly after treatment with coronatine, and are therefore unlikely to be the primary consequences of the treatment (Kenyon and Turner, 1990). Other studies have focused on the ability of coronatine to cause hypertrophy of potato tuber disks and open stomata of Italian ryegrass (Durbin, 1991). Results obtained from these studies also seem to support the hypothesis that coronatine may affect phytohormone activities in treated tissues (Durbin, 1991).

2.2.4. Cercosporin

Cercosporin is a non-specific toxin produced by members of the genus *Cercospora*, a group of fungal pathogens which cause damaging leaf spot diseases on a wide range of crops (Daub and Hangarter, 1983; Daub and Payne, 1989). Lesions on affected leaves first appear as small chlorotic spots, which soon enlarge and turn necrotic (Howard et al., 1994). Cercosporin is unique among known phytotoxins in that it is a proven photosensitizer; photosensitizers are compounds that absorb light to form a long-lived, electronically excited

state (triplet state) which can then react with molecular oxygen to produce compounds that are toxic to living cells (Daub and Hangarter, 1983; Ballio, 1991).

Cercosporin, when activated by light in the presence of O_2, rapidly kills plant cells (Daub and Hangarter, 1983; Daub and Payne, 1989; Ballio, 1991). Results obtained from studies with cercosporin suggest that this toxin produces its toxic effects by reacting with O_2 to produce the active oxygen (AO) species singlet oxygen ($^1O_2$) and superoxide (O_2^-) (Daub and Hangarter, 1983). Proteins, membranes, and lipids are all vulnerable to AO attack (Sutherland, 1991; Tzeng and DeVay, 1993). Hence, the oxygen species formed in the reaction between cercosporin and O_2 cause peroxidation of plant membrane lipids (Daub and Hangarter, 1983; Ballio, 1991). This leads to changes in membrane structure and fluidity, and eventually to cell death (Daub and Hangarter, 1983; Ballio, 1991). As would be expected, cercosporin shows generalized toxicity to many organisms, including all plants tested so far, as well as to animal and bacterial cells.

Interestingly, *Cercospora* species producing this toxin are unaffected by it. It has been suggested that carotenoids, which are effective quenchers of $^1O_2$, may play a role in resistance of these fungi to cercosporin, in conjunction with other mechanisms (Daub and Payne, 1989). If this were the case, the distribution of carotenoids within cells would be important in determining how effective they are at providing protection against the effects of cercosporin. In plants, carotenoids and other quenchers of AO are confined to the chloroplasts. However, since the effects of cercosporin are generalized throughout the cell, plant carotenoids are unable to protect the plasmalemma against damage (Daub and Payne, 1989). Nevertheless, recent studies seem to indicate that carotenoids have no role in resistance of *Cercospora*

species to cercosporin and other photosensitizers. Ehrenshaft et al. (1995) found that carotenoid-minus *Cercospora nicatianae* mutants were no more sensitive to cercosporin than the wild-type strains from which they were derived. Thus, it appears that *Cercospora* species have a distinct and highly effective mechanism for photosensitizer resistance which does not involve carotenoids (Ehrenshaft et al., 1995).

2.2.5. Tentoxin

Tentoxin is a non-specific toxin produced by the plant pathogenic fungus *Alternaria alternata* (= *tenuis*), which induces chlorosis in many higher plants (Bocker and Novacky, 1981). Early studies indicated that chlorosis develops only when tentoxin, a cyclic tetrapeptide, is applied to tissue prior to or during the initiation of greening in developing seedlings (Daly, 1981). Hence, the toxin causes chlorosis by interfering with chlorophyll synthesis rather than by causing its degradation (Daly, 1981). Steele et al. (1976) provided strong evidence that the site of tentoxin action is the coupling factor 1 (CF_1) domain of chloroplast ATPase, and it is now widely accepted that this is the binding site for tentoxin (Bocher and Novacky, 1981; Ballio, 1991). More recent studies indicate that the β-subunit of CF_1 in particular may be the target of tentoxin (Ballio, 1991). The α-β-subunit complex of CF_1 is involved in the photophosphorylation activity of CF_1. Thus, the binding of tentoxin to the β-subunit results in an inhibition of the normal catalytic function of CF_1, leading to an inhibition of light-driven protein and RNA synthesis in isolated chloroplasts. These effects result in the chloroplast specific ultrastructural changes and chlorosis caused by tentoxin (Strobel, 1982; Ballio, 1991).

The possibility of a second site for tentoxin action has arisen from experiments with stomata (Durbin et al., 1973; Daly, 1981; Ballio, 1991). It has been reported that tentoxin causes a rapid closure of broad bean (*Vicia faba* L.) stomates and a marked inhibition of potassium uptake by guard cells (Durbin et al., 1973). Furthermore, tentoxin has been observed to counteract fusicoccin-induced stomatal opening in the dark (Bocher and Novacky, 1981). These experiments suggest a possible direct interaction between tentoxin and the plasmalemma (Bocher and Novacky, 1981; Ballio, 1981). Although negative evidence for an interaction of tentoxin with the cell membrane has been produced (Bocher and Novacky, 1981), the idea of a second site of action has failed to garner convincing support (Ballio, 1991). The results of another study on the effects of tentoxin on stomatal movement in broad bean seem to favour the inhibition of photophosphorylation as the primary cause of stomatal closure (Dahse et al., 1988). Hence, it would appear that stomatal closure is a secondary consequence of tentoxin binding to the CF_1 domain of chloroplast ATPase (Dahse et al., 1988; Ballio, 1991).

2.3. Herbicides

Herbicides can be broadly defined as xenobiotics used in weed control (Boger and Sandmann, 1989). These compounds, although not phytotoxins, may act in a manner similar to certain phytotoxins. There are many different modes of herbicidal action (Boger and Sandmann, 1989), but we shall restrict our discussion to those herbicides which cause chlorosis in affected tissues. Herbicides can induce chlorosis via several modes of action. Many herbicides interfere with electron transport, thereby inhibiting photosynthetic electron flow (Salisbury and Ross, 1992). This leads to an inability of illuminated thylakoid

membranes to dissipate the excitation energy normally used in photosynthesis, and to the formation of toxic radical species (Kunert and Dodge, 1989). The result is the development of chlorosis in affected tissues and eventually their death (Kunert and Dodge, 1989). Herbicides can disrupt electron transport at several points along the electron transport chain (Sandmann and Boger, 1982; Salisbury and Ross, 1992). For instance, several urea derivatives, such as DCMU (3-(3,4-dichlorophenyl)-1,1-dimethyl-urea), block electron transport by replacing the electron carrier Q_B, which is a specialized plastoquinone on the D1 polypeptide of photosystem II (PSII) (Salisbury and Ross, 1992). Other herbicides, specifically the bipyridylium salts, accept electrons at the reducing site of PSI. Bipyridylium radicals are then formed, which in turn give rise to active oxygen species. These AO species initiate the breakdown of membrane components by a peroxidative process (Sandmann and Boger, 1982).

Certain herbicides act by inhibiting chloroplast ATPase, thereby preventing or stalling ATP formation by photophosphorylation (Boger, 1989). For example, certain p-nitrophenylethers exert a strong influence on the CF_1 domain of ATPase, presumably through their binding to the α- and β- subunits of CF_1. Chlorosis would result from a lack of chlorophyll-forming enzymes, which cannot be produced because of a lack of ATP (Boger, 1989). Hence, some ATPase inhibitors act in a manner similar to tentoxin, which was discussed earlier. Another effective target for herbicides is the carotenoid biosynthetic pathway in photosynthetic membranes (Burns et al., 1971; Sandmann and Boger, 1982; Sandmann and Boger, 1989). As was discussed briefly with respect to cercosporin, carotenoids serve to protect the photosynthetic apparatus from destruction by singlet oxygen,

which can be produced under conditions in which triplet-state chlorophyll transfers its energy to molecular oxygen (Lawlor, 1993). Furthermore, carotenoids also quench triplet chlorophyll. Hence, if carotenoid levels in the chloroplast decrease as a result of herbicide action, their protection is no longer available to the photosynthetic apparatus (Lawlor, 1993). Many herbicides are known to affect carotenoid synthesis (Sandmann and Boger, 1982; Sandmann and Boger, 1989). For instance, CPTA [2-(4-chlorophenylthio)-triethylamine HCl] inhibits the enzyme lycopene cyclase. This enzyme catalyzes the conversion of lycopene to β-carotene (Lawlor, 1993). Therefore, after treatment with CPTA, β-carotene levels decrease, resulting in photobleaching of the affected tissue (Sandmann and Boger, 1989).

3. ISOLATION OF A HOST-SPECIFIC CHLOROSIS-INDUCING TOXIN FROM RACE 5 OF *PYRENOPHORA TRITICI-REPENTIS*

3.1. Abstract

Pyrenophora tritici-repentis, causal agent of tan spot of wheat, differentially induces tan necrosis and/or chlorosis in hexaploid wheat lines. A chlorosis inducing host-specific toxin, termed the Ptr chlorosis toxin, has been identified from race 5 of *P. tritici-repentis*. Preliminary characterization of the toxin suggested that it is a small hydrophilic protein. Ptr chlorosis toxin was purified from the culture filtrates of race of 5 isolates. Partial purification was performed by 25-80% ammonium sulfate precipitation and passage through a CM-S C25 cation exchange column. Final purification was performed on fast performance liquid chromatography (FPLC), using a MonoS 5/5 cation exchanger, followed by size fractionation on a Superose 12 HR 10/30 column. Purity was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the molecular weight of the toxin determined to be 6.61 kDa by mass spectrometry. The toxin was found to be heat stable, maintaining full toxic activity even after 1 hour at 55°C.

3.2. Introduction

Tan spot, caused by *Pyrenophora tritici-repentis* (Died.) Drechs., anamorph *Drechslera tritici-repentis* (Died.) Shoem., is an important foliar disease of wheat worldwide. The incidence of this disease has been increasing in recent years, as a consequence of the shift by farmers towards soil conservation practices, such as minimum and zero-tillage (Hosford, 1982). Tan spot consists of two distinct symptoms: tan necrosis and extensive chlorosis (Lamari and Bernier, 1989a; Lamari and Bernier, 1989b). Five races of the fungus have been described so far, producing necrosis and/or chlorosis on differential hexaploid wheat lines (Lamari et al., 1995). Race 1 isolates are capable of producing both necrosis and chlorosis on susceptible wheat, race 2 isolates can only produce the necrotic symptom, race 3 and race 5 isolates both cause chlorosis, but on different cultivars, and race 4 isolates are avirulent, producing neither symptom (Lamari et al., 1995).

The necrotic symptom has been shown to be associated with a host-specific toxin, termed the Ptr necrosis toxin (Lamari and Bernier, 1989c). Ptr necrosis toxin, a 14 kDa protein, has been characterized by several independent research groups and its encoding gene has been recently cloned (Ballance et al., 1996; Ciuffeti et al., 1997). This toxin exhibits the same host-specificity as the isolates from which it is obtained, and has been recognized as an important factor in the development of the tan spot disease (Tomas and Bockus, 1987; Ballance et al., 1989; Lamari and Bernier, 1989c).

A second host-specific toxin, termed the Ptr chlorosis toxin, was identified from race 5 isolates of *P. tritici-repentis* (Orolaza et al, 1995). Preliminary characterization of Ptr chlorosis toxin suggested that this toxin was a small hydrophilic protein (N.P. Orolaza and

S.E. Strelkov, *unpublished data*). It stained as a protein on sodium dodecyl sulfate polyacrylamide gels, and bound to chromatography columns in a manner consistent with a protein. The toxin also appears to be a pathogenicity factor (Orolaza et al., 1995). Although partial results on the purification of Ptr chlorosis toxin were presented previously (Strelkov et al., 1997), the present work fully describes a procedure developed to purify the toxin from the culture filtrate of *P. tritici-repentis* race 5 isolates.

3.3. Materials and Methods

Isolates and fungal cultures. The race 5 isolate, Alg. 3-24, of *P. tritici-repentis* used in this investigation is a laboratory stock that was previously collected from Algeria (Lamari et al., 1995). Cultures of this race 5 isolate were grown on V8 potato-dextrose agar (Lamari and Bernier, 1989a) until they were 4 to 5 cm in diameter. Five plugs (1 cm in diameter) were cut from each colony and transferred to 1 liter Roux bottles, each containing 250 ml of Fries medium amended with 0.1% yeast extract (Dhingra and Sinclair, 1985). The cultures were incubated without agitation at 20°C for 21 days. The filtrates were removed from the mycelial mat by filtering through Whatman No. 1 filter paper and 0.45 μm cellulose nitrate filters (Sartorious GmbH, Gottingen, Germany). Culture filtrates were freeze-dried and stored at -20°C until processed.

Plant materials and bioassays. Wheat cultivars Katepwa (sensitive to Ptr chlorosis toxin) and Glenlea (insensitive to Ptr chlorosis toxin) were used throughout the study. The seedlings were grown in plastic pots (12 cm in diameter) filled with a 1:1 soil/peat mix, and planted at a rate of 5 to 6 seeds per pot. The plants were maintained in a growth room at 22/18°C (day/night) with a 16 hour photoperiod at a light intensity of 250 μE/m²s. Seedlings

were watered and fertilized as required. Bioassays were conducted by infiltrating seedlings at the 2 to 4 leaf stage with ca. 20 µl of sample using a Hagborg device (Hagborg, 1970; Fig. 1).

Toxin purification. The procedure followed in the purification of the toxin is outlined in Fig. 2. Approximately 2 liters of culture filtrate were freeze-dried to give ca. 20 g of dry-matter. Freeze-dried culture filtrate was re-suspended in 40 ml of 20 mM sodium acetate pH 4.6 buffer, containing 100 µM phenylmethylsulfonyl fluoride (PMSF). The re-suspended culture filtrate was centrifuged (Beckman model J2-21 centrifuge) at 17400 X g for 10 minutes and the supernatant retained. Sequential salt fractionation of the protein in the supernatant was conducted with ammonium sulfate in two steps (see Appendix). Material precipitating out at 0 to 25 % salt saturation was discarded, and that precipitating out at 25 to 80 % saturation was retained. The precipitate was re-dissolved in 30 ml of 20 mM sodium acetate pH 4.6 buffer, containing 100 µM PMSF, and dialyzed against the same buffer. The dialyzed sample was loaded onto a CM-Sephadex C25 column (19 X 2.5 cm) equilibrated with 20 mM sodium acetate pH 4.6 buffer containing 200 µM PMSF. The column was washed with seven bed volumes of equilibration buffer, and the bound fraction was eluted with a 0 to 400 mM NaCl gradient (250 ml total volume) in equilibration buffer at a flow rate of 30 ml/hour. The eluate was collected in 8 ml fractions while monitored at 280 nm. Toxic activity of the fractions was assessed following a 1/5 dilution with water by the bioassay procedure described above. Toxic fractions were pooled, concentrated by freeze-drying, and dialyzed against 20 mM sodium acetate pH 4.6 buffer. The dialysate was loaded onto a Fast Performance Liquid Chromatography (FPLC) MonoS 5/5 column (Pharmacia, Baie d'Urfe,
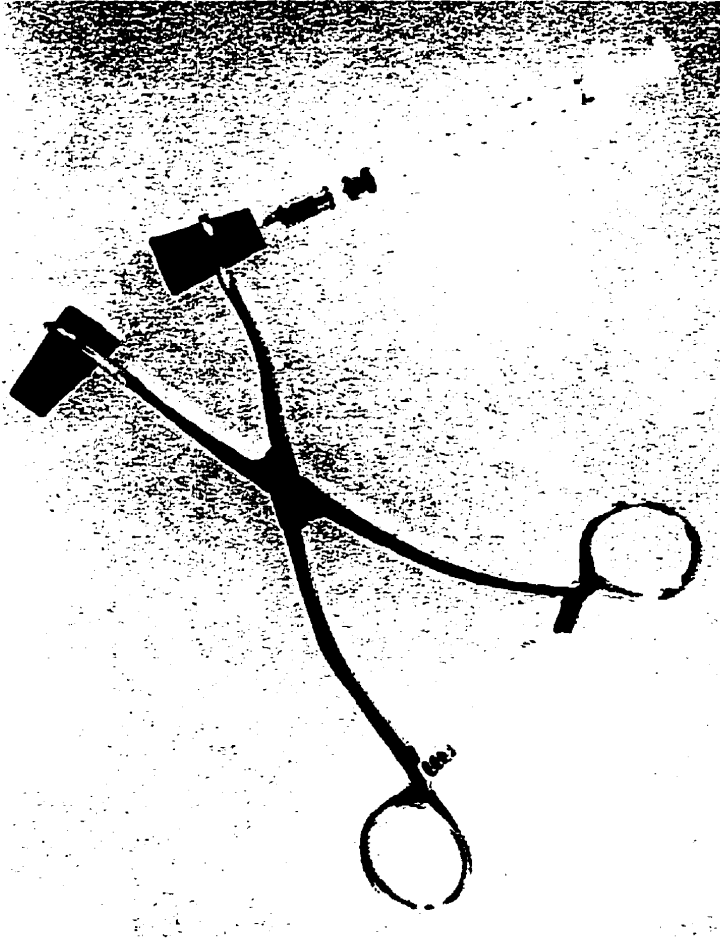
Fig. 1. Hagborg device used to infiltrate solutions into leaves.

Concentrated culture filtrate from race 5 isolate of *P. tritici-repentis*

⇓

Re-dissolved in 20 mM sodium acetate (pH 4.6) buffer and centrifuged

⇓

Supernatant

⇓

Ammonium sulfate fractionation of supernatant

⇓

25-80 % ammonium sulfate precipitate re-dissolved in buffer and dialyzed

⇓

CM-Sephadex C25 cation exchanger

⇓

Toxic fractions pooled, concentrated and dialyzed

⇓

FPLC MonoS 5/5 cation exchanger

⇓

Toxic fractions pooled and concentrated

⇓

FPLC Superose 12 HR 10/30 gel filtration column

⇓

Toxic fractions pooled, concentrated and desalted with EconoPac 10DG column
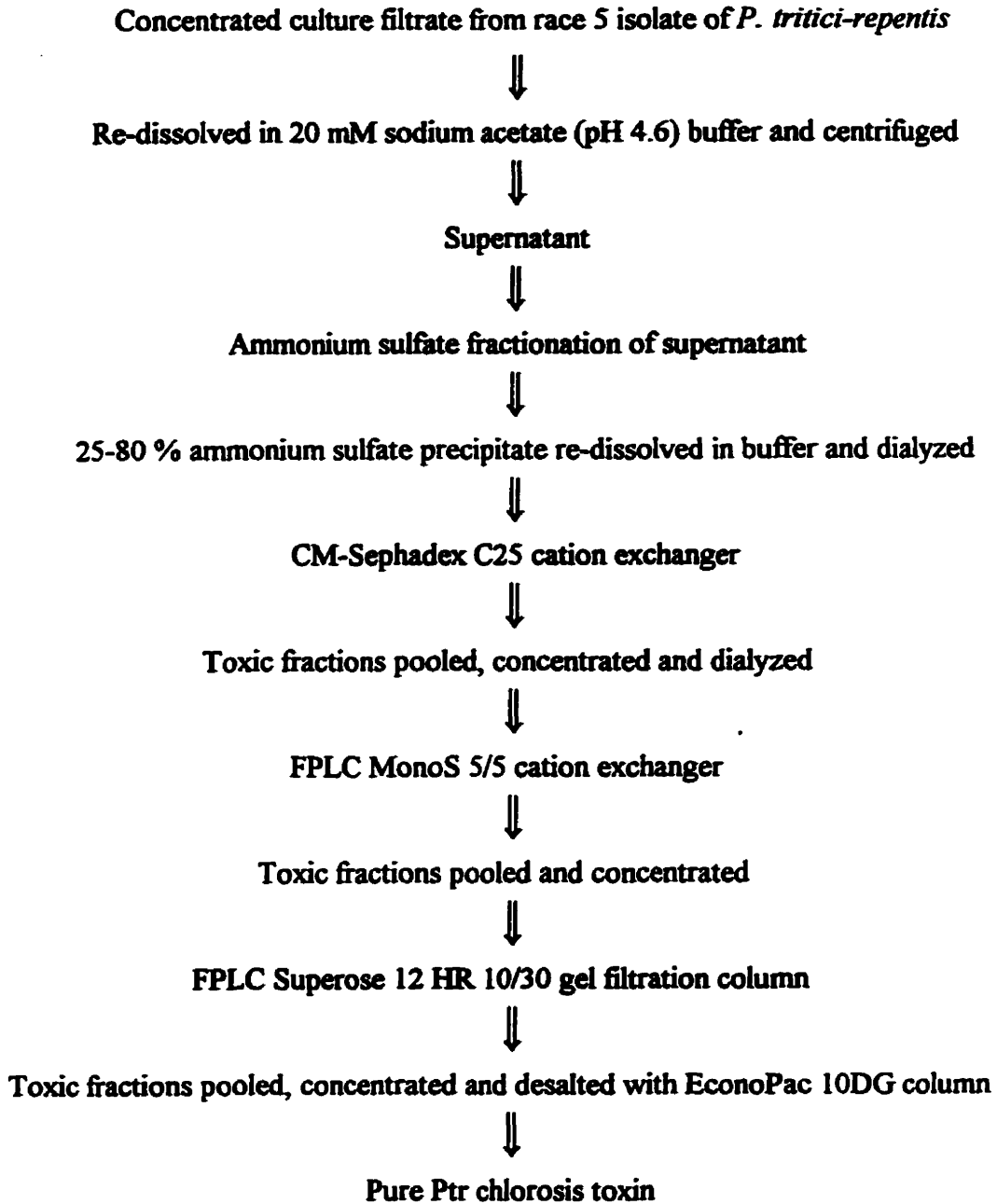
⇓

Pure Ptr chlorosis toxin

Fig. 2. Schematic of the Ptr chlorosis toxin purification procedure. See text for details.

Quebec; 1 column volume = 1 ml) equilibrated with 20 mM sodium acetate, pH 4.6. The

bound proteins were resolved with a linear gradient of 0 to 400 mM NaCl (in equilibration

buffer) over 25 column volumes at 0.5 ml/min. Toxic fractions were again pooled, freeze-

dried and fractionated based on size on a FPLC Superose 12 HR 10/30 column (Pharmacia,

1 column volume = 24 ml) equilibrated with 20 mM sodium acetate, pH 4.6, and eluted with

the same buffer at 0.5 ml/min. Fractions containing toxic activity were pooled, desalted on

an Econo-Pac 10DG column (Bio-Rad Canada Ltd., Mississauga, Ontario), and concentrated

by freeze-drying.

Protein estimation. The protein concentration of the various solutions obtained

during the purification procedure was calculated spectrophotometrically. The optical density

of the solutions was measured at 280 nm in a 10 mm cell with a spectrophotometer (Hewlett

Packard model 8452A), and the value obtained was used to calculate the protein

concentration.

Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was run under

denaturing conditions with sodium dodecyl sulfate (SDS). This was carried out using the

buffer system of Fling and Gregerson (1986) at pH 8.5 with a 10 % separating gel and 5 %

stacking gel. Samples of the toxin and protein molecular weight markers (Bio-Rad Canada

Ltd., Mississauga, Ontario) were prepared in sample buffer (10 µl) consisting of 0.2 M Tris-

HCl, pH 8.8, 0.5 M sucrose, 0.01% bromophenol blue, and 10 mM EDTA. To this were

added 2.5 µl of 10% SDS and 1 µl of 0.25 M dithiothreitol (DTT). Samples were heated for

5 minutes at 100^{0}C and cooled on ice. After cooling, 2.5 µl of 0.5 M iodoacetamide were

added and the samples allowed to stand for 15 minutes before loading onto a 0.75 mm thick

mini-gel. After running (1 h at 100 V, constant current) the mini-gel was fixed for 15 minutes in methanol-acetic acid-water (5:1:5), stained with Coomasie Brilliant Blue (0.05 % in fix solution) for 1 hour, and destained in methanol-acetic acid-water (2:0.8:7.2).

Molecular weight determination. The molecular weight of Ptr chlorosis toxin was determined using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS). Spectra were obtained from samples deposited onto non-porous ether-type polyurethane (PU) membranes (McComb et al., 1997). This work was conducted by Helene Perrault and Mark McComb of the Chemistry Department, University of Manitoba, Winnipeg, Canada.

Thermal stability. The heat stability of Ptr chlorosis toxin was determined by incubating 500 μl samples of toxin (0.45 μg/ml) at 30, 40, 55, 80 and 100^{0}C for 30 and 60 minute periods. After heat treatment the samples were allowed to cool and then bioassayed for toxic activity.

3.4. Results

Purification. The electrophoretic pattern of the proteins precipitated at 25 to 80% ammonium sulfate saturation is illustrated in Fig. 3. Toxic activity was associated with this fraction. Material precipitating out before 25% saturation possessed no toxic activity and neither did the 80% supernatant. After the 25 to 80% precipitate was re-dissolved and dialyzed, it was loaded onto a CM-S C25 column, and a large unbound fraction of UV-absorbing material was washed through. Toxic activity was eluted at ca. 200 mM NaCl. Fig. 4 shows the elution profile from the CM-S C25 column, and Fig. 5 shows the electrophoretic pattern of the proteins in the toxic fractions. After the toxic fractions were pooled,
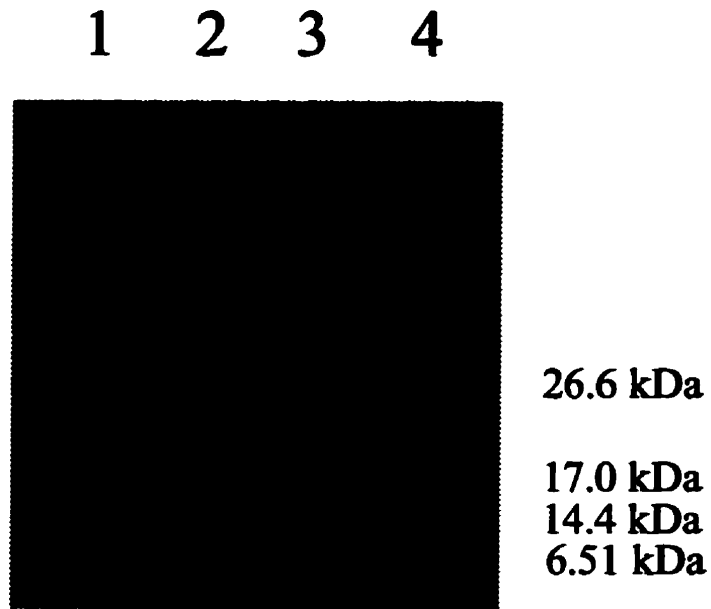
Fig. 3. SDS-polyacrylamide gel electrophoresis showing the serial dilutions of the 25 to 80% ammonium sulfate precipitate; lane 1 contained 61 μg of protein, lane 2 contained 30 μg of protein, and lane 3 contained 12 μg of protein. Lane 4 contained 10 μg of molecular weight markers.
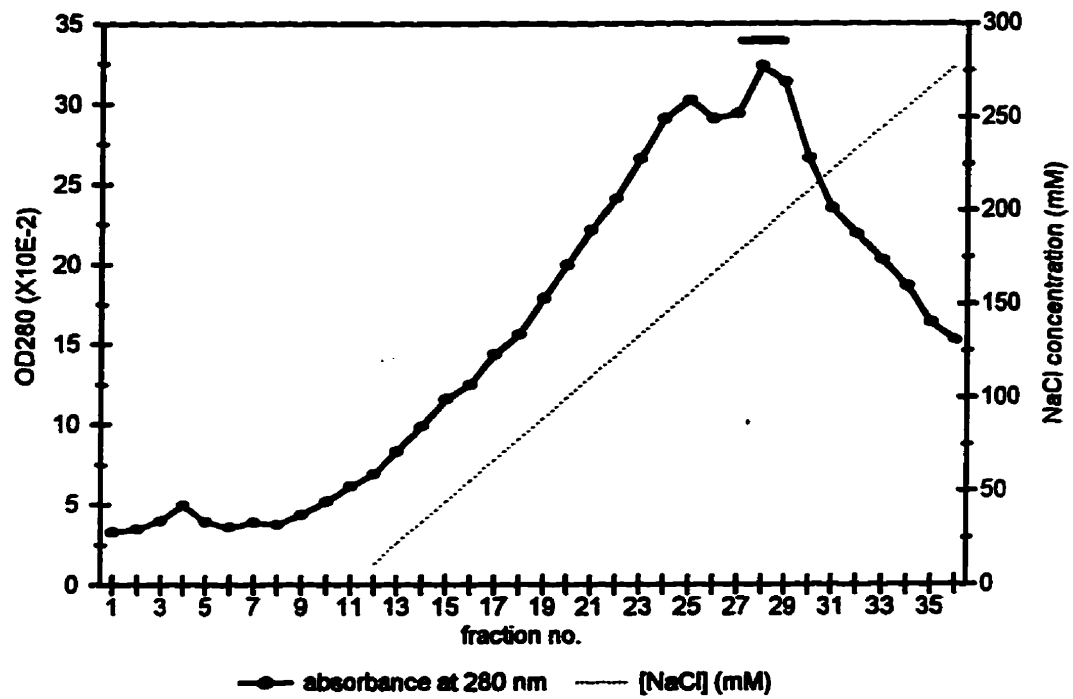
Fig. 4. Elution profile of proteins bound to CM-S C25 cation exchange column (8 ml/fraction). The solid bar indicates where the toxic fractions were eluted.

1 2 3 4 5

26.6 kDa

17.0 kDa
14.4 kDa
6.51 kDa

⇐ Toxin
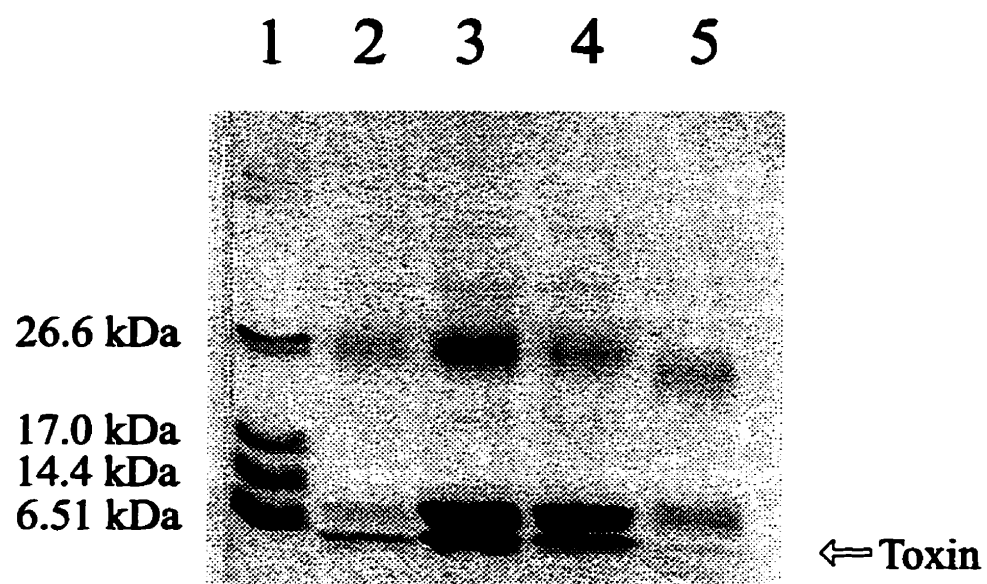
Fig. 5. SDS-polyacrylamide gel electrophoresis containing toxic fractions from CM-S ion exchange chromatography. Lane 1 contains 10 μg of molecular weight markers, lanes 2 to 5 are the toxic fractions. Lanes 2, 3, 4 and 5 were loaded with 20 μl of sample.

concentrated and desalted, the sample was loaded on the FPLC MonoS 5/5 column, and fractionated by gradient elution as described earlier into several peaks absorbing at 280 nm. The elution profile is illustrated in Fig. 6. Toxic activity was associated with only one of these peaks, which was in close proximity to a peak corresponding to a 26 kDa contaminant (as determined by SDS-PAGE; Fig. 7). Ptr chlorosis toxin was purified to homogeneity by concentrating and running the near-pure toxic fractions obtained from the MonoS column through an FPLC Superose 12 column. Fig. 8 contains the elution profile from the Superose 12 column. The toxin, corresponding to a single UV-280 absorbing peak, was separated from all remaining contaminants. Ptr chlorosis toxin was desalted on a desalting column and concentrated for further analysis. Since earlier work had indicated that Ptr chlorosis toxin was proteinaceous in nature, the relative purity of the toxin was examined through electrophoresis and protein staining. SDS-PAGE of the final concentrate of the toxin revealed only one band stainable with Coomasie blue (Fig. 9). Fig. 10 shows the symptoms produced by the purified toxin on sensitive (Katepwa) and insensitive (Glenlea) wheat leaves. The protein concentration during the various purification steps is shown in Table 1.

Molecular weight determination. The molecular weight of Ptr chlorosis toxin was determined to be 6612.3 Da +/- 7 (0.1% error). The MALDI-TOF mass spectrum of the toxin is shown in Fig. 11.

Thermal stability. The thermal stability of the toxic activity was determined after several heat-time treatments. Activity was fully present after heating for 60 minutes at 30,
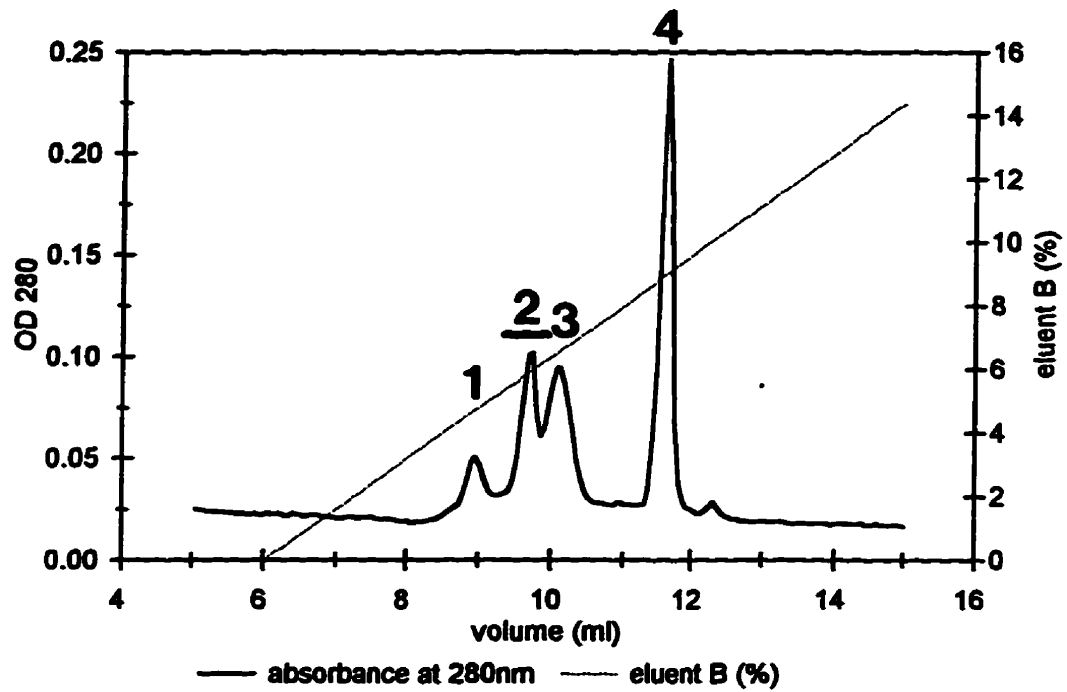
Fig. 6. Elution profile of toxic fraction on MonoS 5/5 FPLC column. The solid bar indicates fractions containing toxic activity. Eluent B was 1 M NaCl.

1   2   3   4   5

Toxin ⟹

26.6 kDa

17.0 kDa
14.4 kDa
6.51 kDa
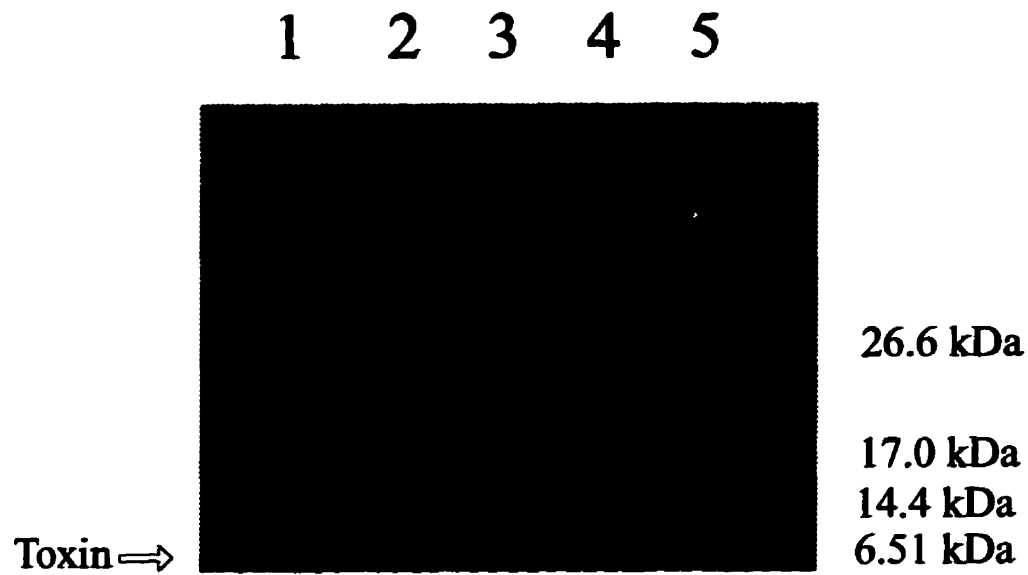
Fig. 7. SDS-polyacrylamide gel electrophoresis of the peaks from the MonoS FPLC column. Lanes 1 to 4 corresponded to peaks 1 to 4, respectively, in Fig. 6. Fractions in lanes 1 and 4 possessed no toxic activity. Lanes 2 and 3 corresponded to toxic fractions and contained the toxin band. Lane 5 contained 10 μg of molecular weight markers. Lanes 1 to 4 were loaded with 20 μl samples..
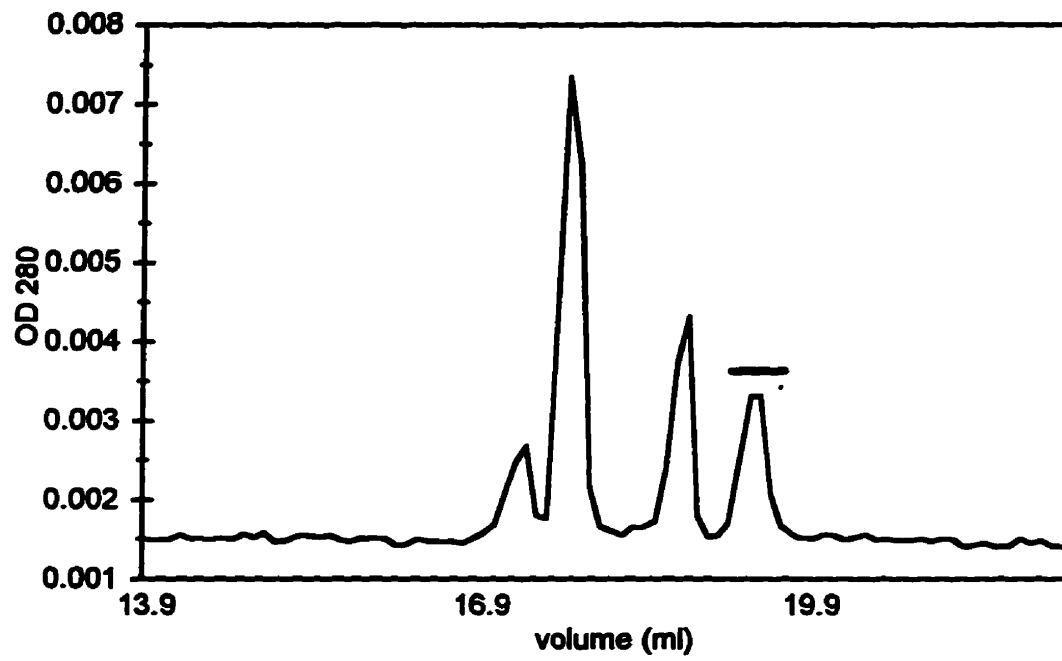
Fig. 8. Elution profile of MonoS pooled peaks 2 and 3 (Fig. 6) on Superose 12 FPLC column, with the location of Ptr chlorosis toxin indicated with the solid bar.
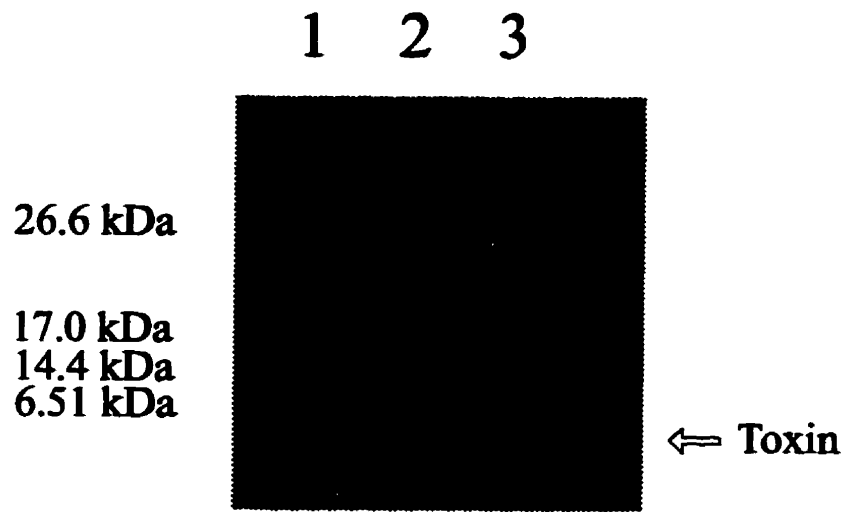
Fig. 9. SDS-polyacrylamide gel electrophoresis of purified Ptr chlorosis toxin. Lane 1 contains 10 ug of molecular weight markers, lane 2 is empty, and lane 3 contains 20 µl (0.1 µg) of the purified toxin.

Fig. 10. Toxin-sensitive Katepwa leaves (left) and toxin-insensitive Glenlea leaves (right), 72 hours after infiltration with purified Ptr chlorosis toxin at a concentration of 0.45 µg/ml.

TABLE 1: Protein content at various steps of the purification procedure, as calculated from the optical densities at 280 nm.

Step	Volume (ml)	Total Protein Concentration (μg/ml)	Total Protein (μg)
Concentrated culture filtrate	60	3.04×10^3	182×10^3
25-80% $(NH_4)_2SO_4$ ppt.[1]	30	2.72×10^3	81.5×10^3
CM-S C25 toxic fractions	24	260	6.24×10^3
MonoS 5/5 toxic fractions	2.2	76.0	167
Superose 12 HR 10/30 toxic fractions	1.6	2.80	4.48

[1]ppt.: precipitate
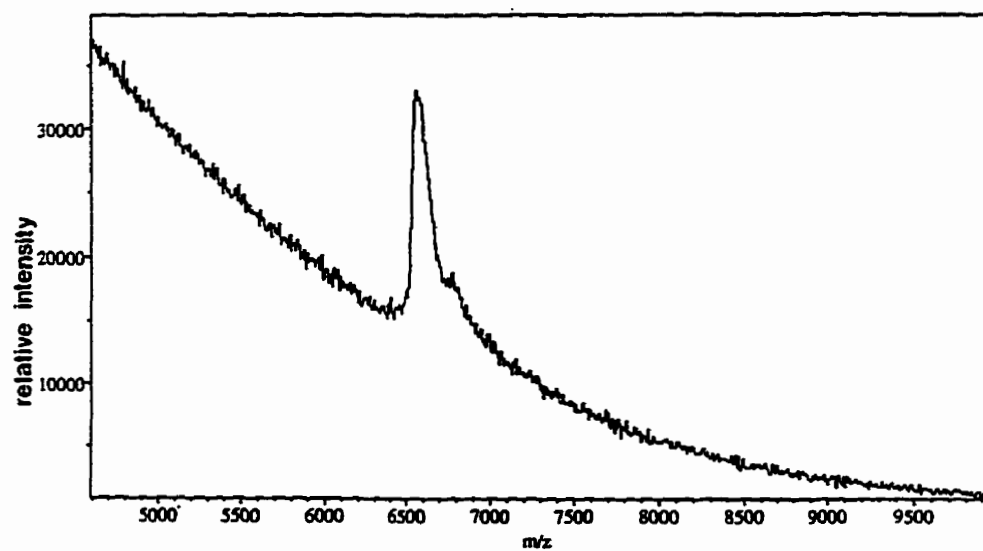
Fig. 11. MALDI-TOF mass spectrum of Ptr chlorosis toxin obtained using a PU membrane. The peak corresponds to a molecular weight of 6.61 kDa.

40, and 55°C. Slightly weaker activity was still present after 30 minutes at 80°C, and a low level of chlorosis was detectable after 60 minutes at 80°C. A very low level of chlorosis was still detectable after 30 and 60 minutes of heating at 100°C.

3.5. Discussion

Earlier work on Ptr chlorosis toxin provided evidence that this is a hydrophilic molecule, stable to exposure to organics (Orolaza et al., 1995). It was also found to be proteinaceous in nature (N.P. Orolaza and S.E. Strelkov, *unpublished data*). The toxin stained as a protein on SDS-polyacrylamide gels and bound to chromatography columns in a manner consistent with a protein. The present investigation confirmed that Ptr chlorosis toxin behaves in a manner consistent with a small protein; the toxin was purified to homogeneity using protein purification techniques. The fact that Ptr chlorosis toxin bound to cation exchange columns indicates that it has a net positive charge and is presumably a basic protein.

The protein estimation technique which was used probably resulted in an underestimation of the toxin concentration. The intensity of the toxin bands obtained when purified Ptr chlorosis toxin was run on SDS-polyacrylamide gels did not correlate well with the theoretical amounts of toxin that were loaded; the bands were stronger than expected. This could be due to the fact that we were basing our calculations on the absorbance of the protein solutions at 280 nm, the absorption maximum of tryptophan (Haurowitz, 1963). Since Ptr chlorosis toxin is only 6.61 kDa in size (ca. 52 amino acids), it may not contain tryptophan residues.

Most other known host-selective toxins are low molecular weight compounds and are not antigenic. Nevertheless, there are exceptions, most notably with toxins produced by *P.tritici-repentis*; Ptr necrosis toxin is a protein with a molecular weight of 14 kDa (Ballance et al., 1989; Tomas et al., 1990; Tuori et al., 1995; Zhang et al., 1997). Ptr chlorosis toxin, as a small protein, is therefore also unique. This raises the possibility that it may be binding a toxic constituent, and may not actually be the toxic principle. However, during ion exchange chromatography the polypeptide and toxic activity always co-migrated. Furthermore, the final purification step involving FPLC gel filtration provided no evidence for the existence of a lower molecular weight toxic constituent.

It appears that Ptr chlorosis toxin is heat stable. There was no reduction in toxic activity after heating for 1 hour at 55°C, conditions which resulted in the irreversible denaturation of Ptr necrosis toxin (Ballance et al., 1989). However, even after 1 hour at 100°C some toxic activity was retained. Since we did not have a way to monitor the loss of structure of the toxin with heating, it is not clear whether this thermal stability was due to an inability of the heat to disrupt the active site, or if the small nature of the protein made it difficult to denature. It is also possible that the toxin renatured after cooling. Further analyses of Ptr chlorosis toxin will be necessary to conclusively show the protein nature of this molecule and to determine its structure.

4. INDUCED CHLOROPHYLL DEGRADATION BY A CHLOROSIS TOXIN FROM PYRENOPHORA TRITICI-REPENTIS

4.1. Abstract

Pyrenophora tritici-repentis, causal agent of tan spot of wheat, can differentially induce tan necrosis and extensive chlorosis in hexaploid wheat lines. A chlorosis inducing host-specific toxin, termed the Ptr chlorosis toxin, was isolated from a race 5 isolate of *P. tritici-repentis*. The physiological development of chlorosis in sensitive wheat was investigated using partially purified toxin. Treatment with the toxin had no effect on the greening of etiolated tissue, suggesting that chlorosis results from chlorophyll degradation, rather than inhibition of chlorophyll synthesis. Development of chlorosis was light-dependent, indicating that it may be a consequence of photochemical bleaching. To test for the involvement of active oxygen (AO) species in photobleaching, toxin-treated tissue was floated in solutions of various AO scavengers. The compound p-benzoquinone, which quenches singlet oxygen and triplet chlorophyll, prevented the development of chlorosis, suggesting that AO species are involved in chlorophyll degradation. High performance liquid chromatography (HPLC) chlorophyll degradation profiles were also consistent with photooxidation. Decreases in carotenoid levels were smaller than and concurrent with the declines in chlorophyll, suggesting that toxin-induced chlorosis was not the result of a carotenoid deficiency. It appears that Ptr chlorosis toxin, directly or indirectly, inhibits photosynthesis, leading to chlorophyll photodestruction as illuminated thylakoid membranes become unable to dissipate excitation energy.

4.2. Introduction

Tan spot is an important foliar disease of wheat caused by the fungal pathogen *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph: *Drechslera tritici-repentis* (Died.) Shoem.). Disease development can be associated with two distinct symptoms: tan necrosis and/or extensive chlorosis (Lamari and Bernier, 1989a; Lamari and Bernier, 1989b). The necrotic symptom is caused by the action of a host-specific toxin, termed the Ptr necrosis toxin (Lamari and Bernier, 1989c). While the mode of action of Ptr necrosis toxin has not yet been elucidated, there is evidence to suggest that the toxin may trigger a programmed cell death in sensitive wheat lines (Kwon and Rasmussen, 1997).

A second host-specific toxin, termed the Ptr chlorosis toxin, has been shown to be associated with the development of the chlorotic symptom in tan spot of wheat (Orolaza et al., 1995). This toxin, which is produced by race 5 isolates of *P. tritici-repentis*, appears to be a small hydrophilic protein, stable to exposure to organics (Orolaza et al., 1995; Strelkov et al., *in preparation*). Ptr chlorosis toxin is a pathogenicity factor (Orolaza et al., 1995). However, little is known about the mechanism of chlorosis induction in sensitive tissue. Preliminary investigations revealed that the development of chlorosis was light-dependent; chlorosis does not develop under darkness, and is much weaker under reduced light conditions (N.P. Orolaza, *unpublished data*). Furthermore, development of the chlorotic symptom was independent of tissue age. These observations suggested that the development of chlorosis was a consequence of photochemical bleaching. The objective of this study was to elucidate the mode of action of Ptr chlorosis toxin. Preliminary results were presented earlier (Strelkov et al., 1997).

4.3. Materials and Methods

Terminology. The terms "sensitivity" and "insensitivity" are used to describe the host reaction to the toxin, while "susceptibility" and "resistance" are used to refer to the host reaction to *P. tritici-repentis*.

Plant materials and bioassays. Wheat cultivars, Katepwa (sensitive to Ptr chlorosis toxin) and Glenlea (insensitive to Ptr chlorosis toxin), were used throughout this study. Unless otherwise stated, the seedlings were grown in plastic pots, 12 cm in diameter, filled with a 1:1 soil/peat mix. Plants were seeded at a rate of 5 to 6 seeds per pot, and maintained in a growth room at $22/18^0C$ (day/night) with a 16 hour photoperiod, at a light intensity of 250 $\mu E\ m^{-2}s^{-1}$. The plants were watered and fertilized as required. Bioassays were conducted by infiltrating seedlings at the 2 to 4 leaf stage with ca. 20 μl of water or toxin using a Hagborg device (Hagborg, 1970). All experiments were repeated three times, and treatments were replicated three times in each run. Results presented are from a typical run.

Production of culture filtrates. A race 5 isolate (Alg. 3-24) of *P. tritici-repentis* was used throughout this study. Cultures of the fungus were grown on V8 potato-dextrose agar (Lamari and Bernier, 1989a) until they were 4 to 5 cm in diameter. Five plugs, 1 cm in diameter, were cut from each colony and transferred to Roux bottles containing 250 ml of Fries medium, amended with 0.1% yeast extract (Dhingra and Sinclair, 1985). The cultures were incubated without shaking at 20^0C for 21 days. Culture filtrates were removed from the mycelial mat by filtering through Whatman No. 1 filter paper and 0.45 μm cellulose nitrate filters (Sartorious GmbH, Gottingen, Germany).

Partial purification of the toxin. A partially purified toxin mixture (N.P. Orolaza, *unpublished data*) was used in this study. Approximately 500 ml of culture filtrate were freeze-dried to yield ca. 5 g dry-matter. Freeze-dried culture filtrate was re-suspended in 20 ml of 20 mM sodium acetate pH 4.6 buffer and centrifuged (Beckman model J2-21 centrifuge) at 17400 X g for 10 minutes at 4^0C. The supernatant was extracted twice with an equal volume of ethyl acetate. Traces of ethyl acetate were removed from the supernatant with a stream of nitrogen and the toxic activity was precipitated with ammonium sulphate (0-80% saturation). The precipitate was resuspended in 20 mM sodium acetate (pH 4.6) buffer and desalted on a 10 ml Bio-Gel 10 DG column, pre-equilibrated with 20 mM sodium acetate, pH 4.6, and eluted with the same buffer. The pooled sample was loaded onto a CM-Sephadex C-25 cation exchange column (2.5 X 19 cm), equilibrated with 20 mM sodium acetate pH 4.6 buffer. The column was washed with 5 bed volumes of equilibration buffer, and the bound fraction was eluted with a sodium chloride gradient (250 ml of 0 to 150 mM followed by 250 ml of 500 mM) at a flow rate of 30 ml/hour. The eluate was monitored at 280 nm while 8 ml fractions were collected. Toxic activity of the fractions was assessed following dilution with water by the bioassay procedure described above. Toxic fractions were pooled and concentrated by acetone (90% v/v) precipitation. The resulting stock toxin solution had a greatly enriched level of Ptr chlorosis toxin, although a number of contaminants were still present (Fig. 12).

Estimation of chlorophyll content. Four leaf segments (0.5 to 1.0 cm in length, total weight ca. 40 mg), randomly chosen from each treatment, were cut, weighed, and frozen in liquid nitrogen. They were then ground with a mortar and pestle and extracted three times
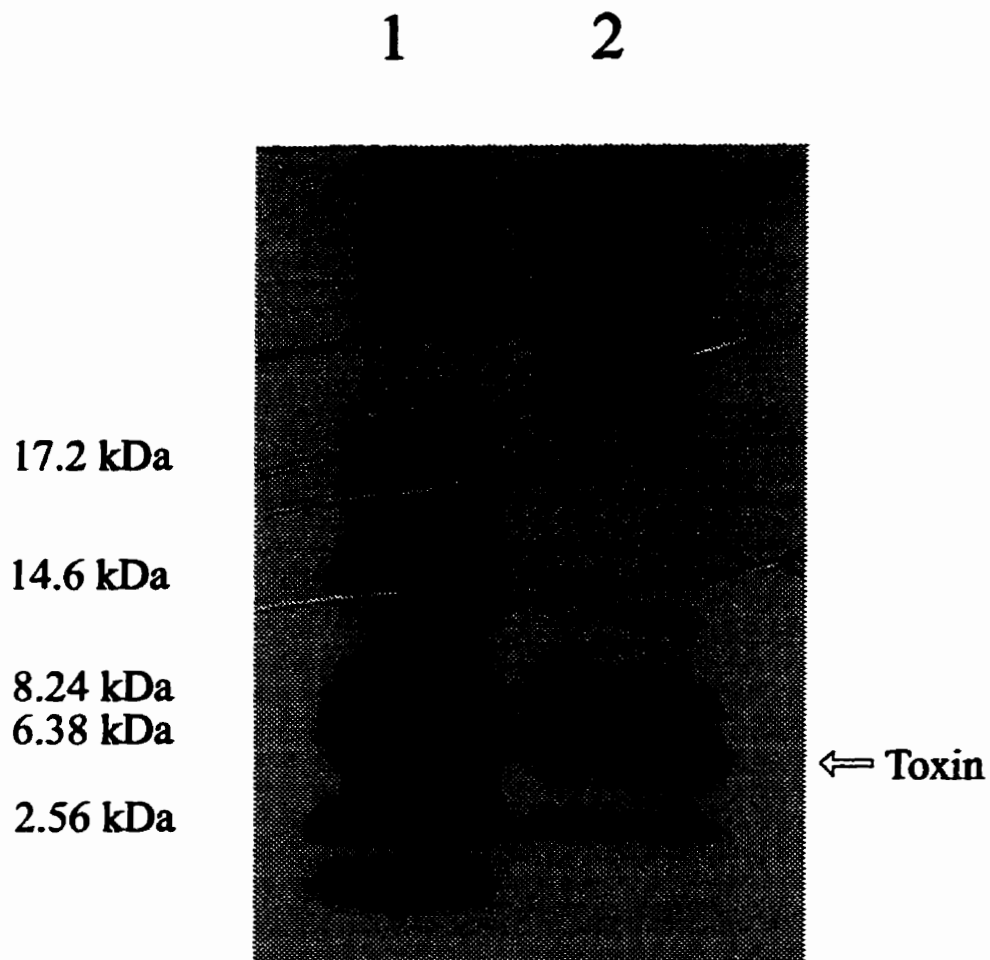
Fig. 12. SDS-polyacrylamide gel electrophoresis of the partially purified toxin that was used throughout the mode of action studies. Lane 1 contains 10 µg of molecular weight markers, and lane 2 contains 10 µg total protein of the partially purified stock toxin solution. It was infiltrated into leaves at a concentration of 1 µg total protein/ml.

with 80% acetone (v/v) in microcentrifuge tubes. The final extracts had a volume based on 10 mg (fresh weight) plant material extracted per 1 ml of 80% acetone (Witham et al, 1971), and were kept in the dark prior to measurement. The optical density of the extracts was read at 645 and 663 nm in a 10 mm cell with a spectrophotometer (Hewlett Packard model 8452A). The amount of chlorophyll present was then calculated as milligrams of chlorophyll per gram of leaf tissue extracted using the equations of Arnon (1949).

Estimation of carotenoid content. Estimation of carotenoid content was conducted in a manner identical to that described above for chlorophylls, except that the optical density of the extracts was measured at 470, 647 and 663 nm. The equations used to calculate the amount of carotenes and xanthophylls present in the extracts were as listed in Lichtenthaler (1987), for pigments in 80% acetone (v/v).

Effect of toxin on pigment content in light-grown seedlings. Sensitive and insensitive wheat seedlings were infiltrated with either Ptr chlorosis toxin or with water using the Hagborg device and the same procedure described for the bioassay. Chlorophyll levels were determined at the time of infiltration, and then every 24 hours up to 120 hours in leaf sections that had been infiltrated.

Effect of a dark period on development of chlorosis. Leaves of sensitive and insensitive wheat seedlings were infiltrated with either partially purified Ptr chlorosis toxin or with water. Three leaves were infiltrated on each seedling, and three seedlings were used for every treatment in each repitition of the experiment. The plants were then moved into the dark for varying periods of time (0, 24, 48, 72 and 96 hours), after which they were transferred back into the light. Plants were assessed daily for development of the chlorotic

symptom at the infiltrated areas, and were rated as (+) or (-) for the presence and absence of chlorosis, respectively.

Effect of toxin on chlorophyll accumulation in etiolated seedlings exposed to light. Sensitive and insensitive wheat seedlings were germinated and grown in 5 X 5 cm jiffy pots (Poly Pak, Jiffy Products (N.P.) Ltd., Shippagan, Canada) for one week under complete darkness. They were then transferred to a growth chamber with a 16 hour photoperiod, at which time they were infiltrated with either Ptr chlorosis toxin or water. Chlorophyll levels were measured as indicated above at 0, 5, 10, 24, 48, 72, and 96 hours after infiltration.

Effect of active oxygen scavengers on development of chlorosis. This study was adapted from Kar and Feierabend (1984). Leaves of sensitive and insensitive seedlings were infiltrated with either Ptr chlorosis toxin or water. Twenty-four hours after infiltration, the treated leaf segments were cut into 1 cm strips, and placed in petri plates (8.5 cm in diameter) containing 25 ml each of solutions of various active oxygen (AO) scavengers. The treatments were distilled water, 10 mM 1,4-diazobicyclo(2,2,2)octane, 1 mM p-benzoquinone, 5 mM formate (pH 7), and 5 mM ascorbate (pH 7) (Sigma, Oakville, Canada). One set of plates was placed under complete darkness, the second under a 16 hour photoperiod. The temperature was $22^0C/18^0C$ (day/night) for plates under light, and $22^0C/18^0C$ (16 h/8 h) for plates in the dark. After 48 hours in the petri plates (72 hours after infiltration), the leaf segments were collected and their chlorophyll content determined.

HPLC chlorophyll degradation profiles. Pigments were extracted and prepared for analysis with high performance liquid chromatography (HPLC) using a method modified from Johnson-Flanagan and Singh (1993). Leaf segments (0.5 to 1 cm long) were cut, weighed,

and placed in microcentrifuge tubes for freezing and storage. The leaf tissue in the microcentrifuge tubes was frozen in liquid nitrogen and ground to a fine powder with a pestle. Pigments were extracted twice with ice cold 80% acetone, so that the final volume was based on 10 mg tissue per 1 ml of 80% acetone. Microcentrifuge tubes containing the ground tissue and the acetone were centrifuged at 10000 X g. Half the volume of the resulting supernatant was removed and dried under nitrogen in the dark. Pigments were then resuspended in 100 μl methylene chloride for analysis with HPLC. The injection volume was 15 μl.

Pigments were separated using a method developed by Johnson-Flanagan and Thiagarajah (1990). An 8 mm X 100 mm Radial Pak reverse phase column (Waters 85721) was used with a Spectra Physics HPLC system consisting of a SP8800 ternary pump, a Spectra 200 UV-VIS variable wavelength detector, and an SP 4270 integrator. The solvent system consisted of methanol/water (4:1) (solvent A) and ethyl acetate (solvent B). Chromatographs were developed in a linear gradient from 75%:25% A:B (v/v) to 50%:50% A:B (v/v) over 50 minutes, at a flow rate of 2 ml/minute. The pigments were detected at 660 nm, with a bandwidth of 8 nm. Chlorophyll a and b, pheophytin a and b, chlorophyllide a and b, and pheophorbide a and b were identified by comparing their retention times and absorption spectra with those of authentic standards. Chlorophyll a and b standards were purchased from Sigma (Oakville, Canada). Pheophytin a and b standards were obtained by acidification of the chlorophyll a and b standards (Johnson-Flanagan and Thiagarajah, 1990). Chlorophyllides were prepared from their respective chlorophylls by treatment with citrus chlorophyllase. Pheophorbides a and b were prepared by the acidification of their respective chlorophyllides (Johnson-Flanagan and Thiagarajah, 1990).

4.4. Results

Effect of toxin on pigment content in light-grown seedlings. Chlorophyll *a* levels

in the sensitive Katepwa seedlings treated with water, as well as in the insensitive Glenlea

seedlings treated with either water or toxin, remained relatively constant throughout the time-

course of the study, in the range of 2-3 mg/g tissue (Fig. 13). However, the chlorophyll *a*

level in Katepwa seedlings treated with toxin began to decrease after 48 hours post-

infiltration. Thus, while during the first 48 hours of the time-course the chlorophyll *a* level

had remained constant at approximately 2 mg/g tissue for this treatment, by 120 hours it had

decreased to 1.13 mg/g tissue. The same pattern was observed with respect to chlorophyll

b levels. The level of this pigment remained constant (at ca. 0.80 to 1.0 mg (chl. b)/g tissue)

throughout the time-course for all treatments except for the toxin-treated Katepwa seedlings

(Fig. 14). Katepwa exhibited a large decrease in the chlorophyll *b* level beginning at 48 hours

post-infiltration; the chlorophyll *b* level declined from nearly 0.80 mg/g tissue to only 0.43

mg/g tissue at 120 hours. Similarly, the total chlorophyll (chlorophyll *a* and *b*) levels only

decreased in the toxin-treated Katepwa seedlings (Fig. 15). The initial total chlorophyll level

in this treatment was 2.72 mg/g tissue, but it begun to markedly decrease after 48 hours post-

infiltration, to a value of only 1.56 mg/g tissue at 120 hours. Total chlorophyll levels in the

other treatments remained constant throughout the study. The ratio of chlorophyll *a* to *b* was

similar for all treatments, including the Katepwa + toxin treatment, at all points during the

time-course of the study (results not shown). Differences in carotenoid content were within

experimental error in all treatments for 48 hours after infiltration. It was at 72 hours that the

difference between the toxin-treated and water-treated sensitive tissues became significant

Fig. 13. Chlorophyll *a* content over time after infiltration of seedlings with Ptr chlorosis toxin or water. The treatments were cv. Katepwa + toxin (●), Katepwa + water (■), Glenlea + toxin (▲), and Glenlea + water (▼). Error bars indicate the standard deviation.

Fig. 14. Chlorophyll *b* content over time after infiltration of seedlings with Ptr chlorosis toxin or water. The treatments were cv. Katepwa + toxin (●), Katepwa + water (■), Glenlea + toxin (▲), and Glenlea + water (▼). Error bars indicate the standard deviation.
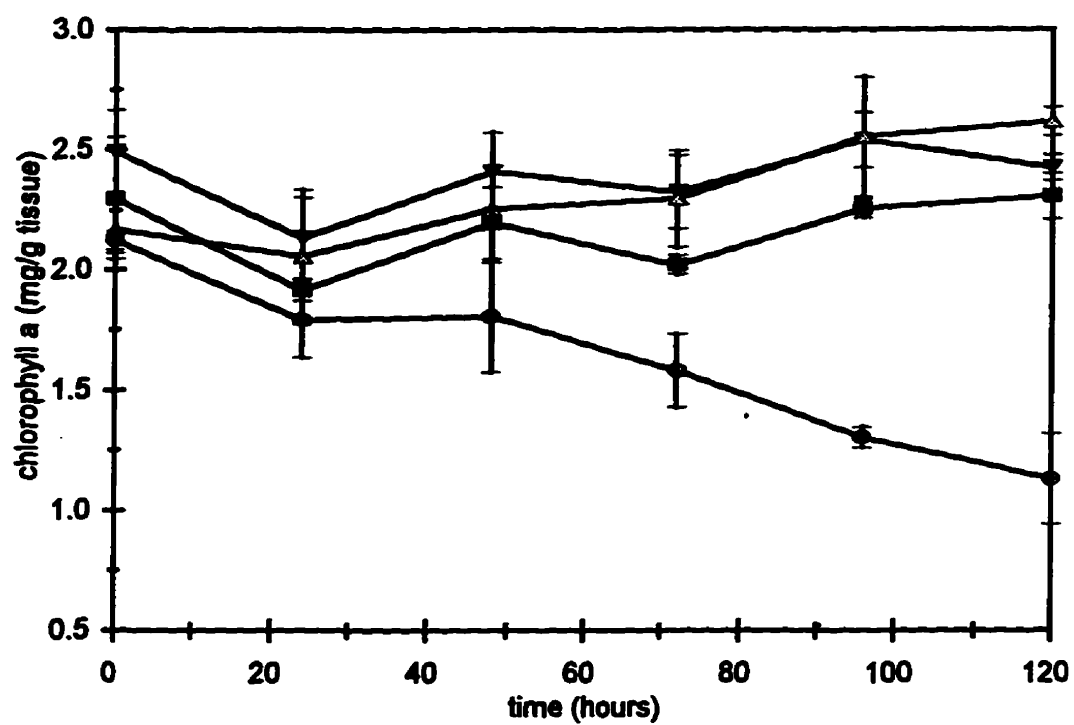
Fig. 15. Total chlorophyll content over time after infiltration of seedlings with Ptr chlorosis toxin or water. The treatments were cv. Katepwa + toxin (●), Katepwa + water (■), Glenlea + toxin (▲), and Glenlea + water (▼). Error bars indicate the standard deviation.
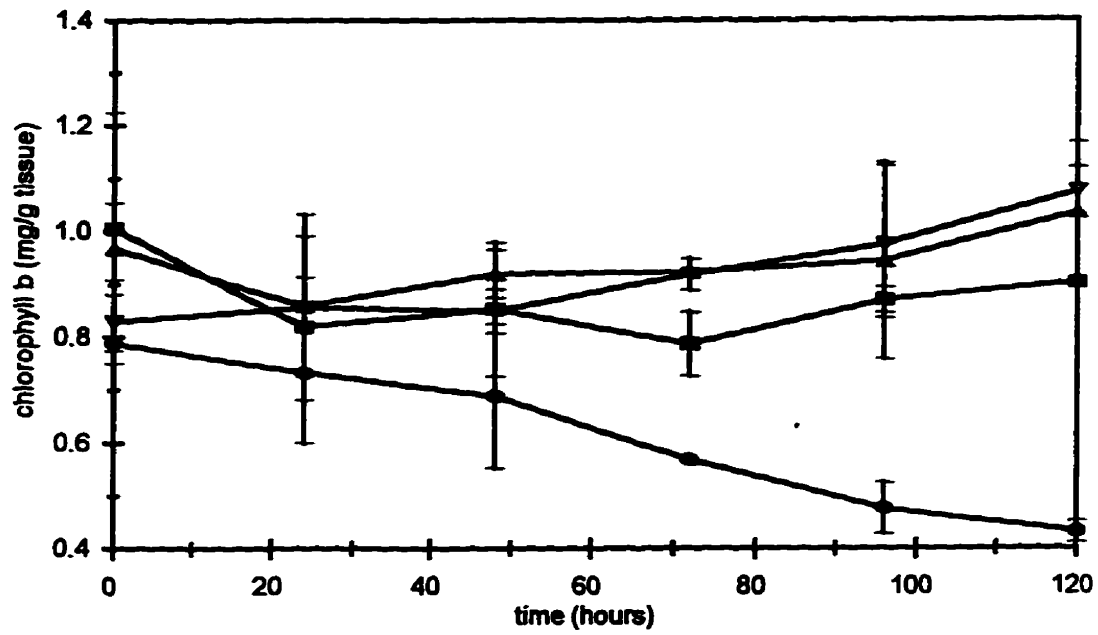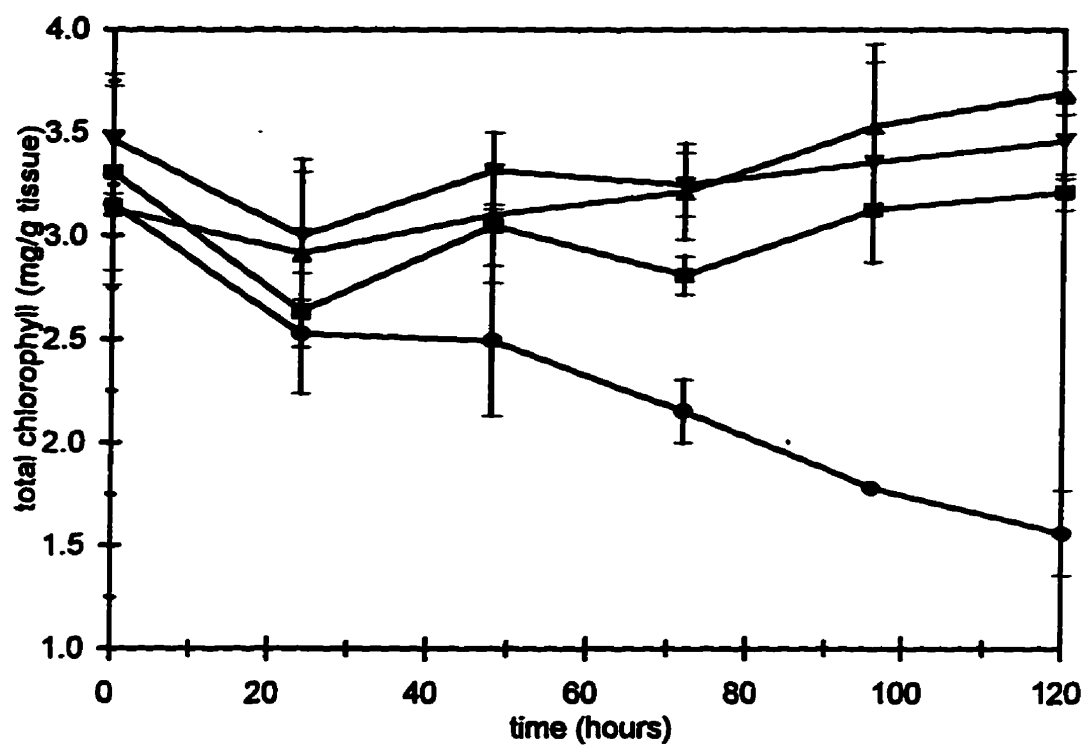
(Fig. 16). This difference was never as large as the one observed in chlorophyll levels. For instance, at 72 hours post-infiltration, the carotenoid level in the water-treated Katepwa controls was only 17% higher than the level in the toxin-treated Katepwa seedlings (0.454 vs. 0.387 mg carotenoid/g tissue).

Effect of dark period on development of chlorosis. When toxin-treated Katepwa seedlings were subjected to a dark period of 24 to 72 hours, the onset of chlorosis from the time of infiltration was delayed by a period of time similar in length to the dark period (Table 2). Once seedlings were transferred back into the light, chlorosis developed after 48 hours. Measurements were not carried out after 72 hours in the dark, as all treatments, including water-treated controls, became chlorotic.

Effect of toxin on chlorophyll accumulation in etiolated seedlings exposed to light. During the first 48 hours of the time-course of this study, chlorophyll levels appeared to increase at a similar rate in all the treatments (Fig. 17). However, the chlorophyll level in the toxin-treated Katepwa seedlings declined during the period 48 to 72 hours after infiltration with the toxin. In all of the remaining treatments, chlorophyll levels continued to increase during this period. The trend persisted between 72 and 96 hours, with the Katepwa + toxin treatment continuing to exhibit a decline in chlorophyll, and the control treatments continuing to show an increase in chlorophyll levels, approaching those observed in control leaves (Fig. 15).

Effect of active oxygen scavengers on development of chlorosis. The effect of the different AO scavengers on the development of chlorosis varied. When plates were kept in the dark, chlorophyll content was similar in all treatments. When plates were kept in the light,
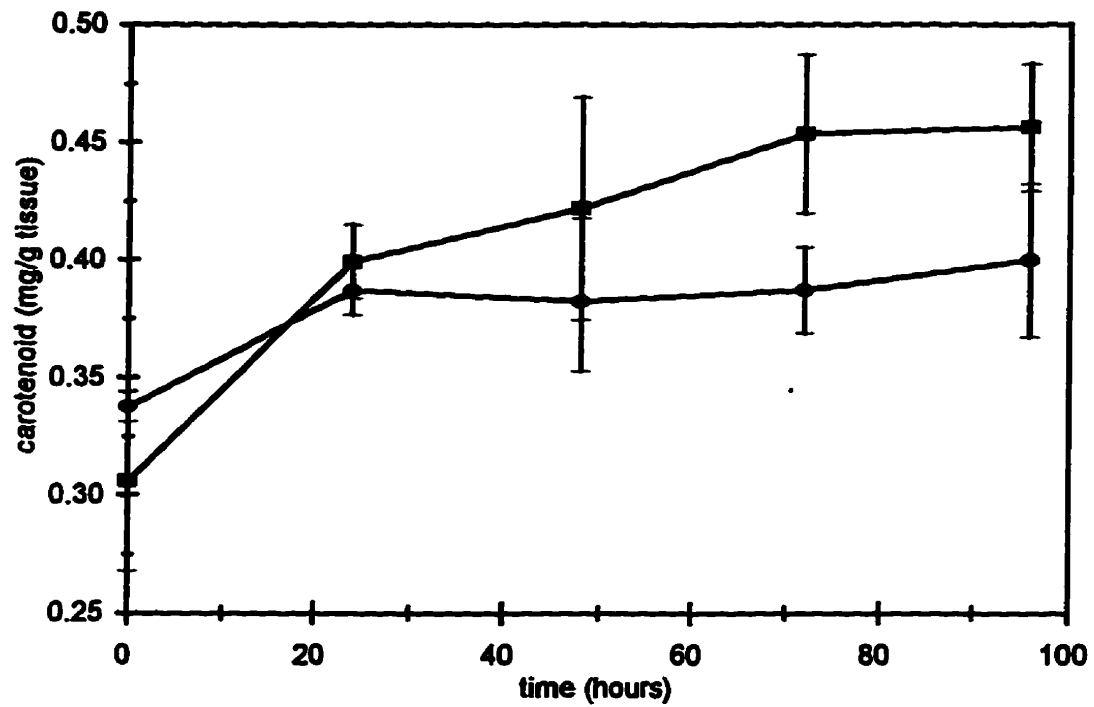
Fig. 16. Carotenoid content over time after infiltration of cv. Katepwa seedlings with Ptr chlorosis toxin (●) or water (■). Error bars indicate the standard deviation.

TABLE 2: The effect of dark periods on the development of chlorosis following infiltration of Katepwa seedlings with Ptr chlorosis toxin. Presence and absence of chlorosis are indicated as + and -, respectively.

	Infiltration	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Not placed in dark	light	-	-	+	+	+	+
24 hours in dark	dark	light	-	-	+	+	+
48 hours in dark	dark	dark	light	-	-	+	+
72 hours in dark	dark	dark	dark	light	-	-	+
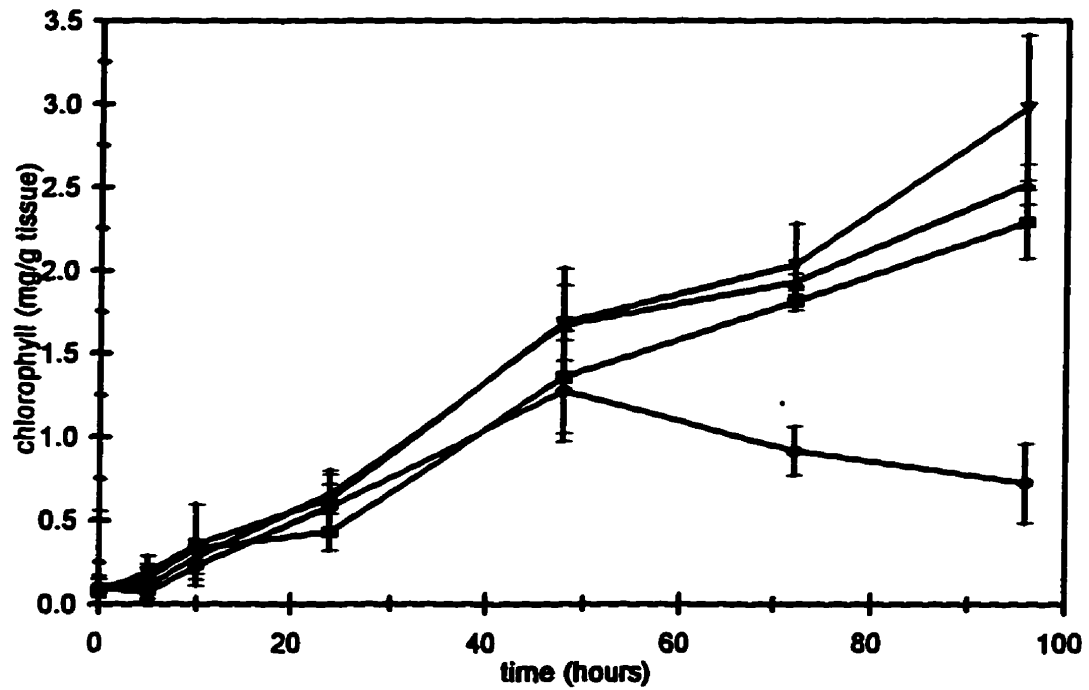
Fig. 17. Chlorophyll content in etiolated seedlings over time after transfer into light. The treatments were cv. Katepwa + toxin (●), Katepwa + water (■), Glenlea + toxin (▲), and Glenlea + water (▼). Error bars indicate the standard deviation.

chlorophyll content was similar in all the insensitive Glenlea tissue treatments. However, chlorophyll content in toxin-infiltrated Katepwa tissue was significantly lower than in water-infiltrated Katepwa tissue when both were floated in distilled water (Fig. 18). The same differential results occurred when toxin-infiltrated and water-infiltrated Katepwa tissue was floated in 10 mM 1,4-diazobicyclo(2,2,2)octane (a scavenger of singlet oxygen), in 5 mM formate (a hydroxyl radical scavenger), and in 5 mM ascorbate (a scavenger of superoxide). These scavengers, at least at the concentrations used, did not inhibit development of chlorosis in toxin-treated Katepwa tissue. However, chlorophyll content in toxin-infiltrated Katepwa tissue floated in 1 mM p-benzoquinone, a quencher of both singlet oxygen and triplet chlorophyll, was comparable to that of water-infiltrated controls floated in the same solution. Decreases in chlorophyll content in toxin-infiltrated Katepwa tissue were relative to the chlorophyll level in H_2O or toxin-insensitive controls. The chlorophyll levels of the control treatments were lower in this study than in studies in which leaves remained on the seedlings throughout the time-course.

HPLC chlorophyll degradation profiles. No differences were observed at any time in the chlorophyll degradation profiles between sensitive tissue in which chlorosis was developing, and control tissues in which chlorosis was not developing (Fig. 19). The major peaks identified in chromatograms of all treatments at all time points consisted of a chlorophyll *a* peak, and a smaller chlorophyll *b* peak. In a few of the sampled control tissues, there was a very small peak corresponding to pheophytin *a*. Chlorophyll derivatives, including pheophytin *a* and *b*, chlorophyllide *a* and *b*, and pheophorbide *a* and *b*, were generally not detected.
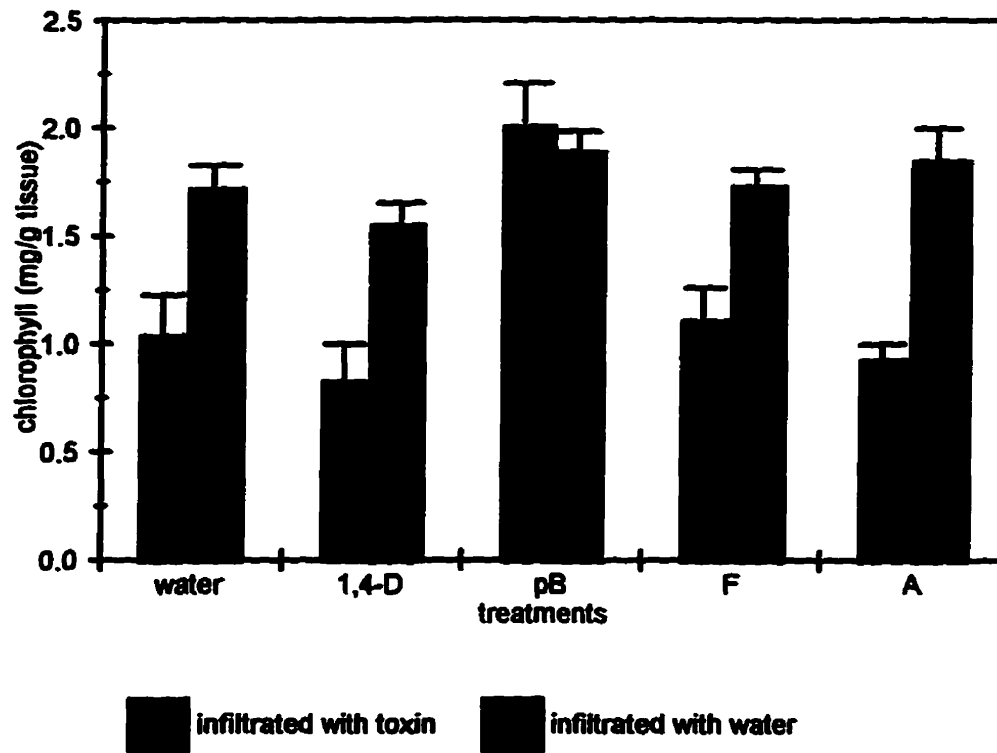
Fig. 18. The effect of various oxygen scavengers on the development of chlorosis. Toxin-treated Katepwa leaf segments were floated in distilled water (water), 10 mM 1,4-diazobicyclo(2,2,2)octane (1,4-D), 1 mM p-benzoquinone (pB), 5 mM formate pH 7 (F), or 5 mM ascorbate pH 7 (A). Error bars indicate the standard deviation.

Chl. A

Chl. B

28.19

21.31

26.97

29.62
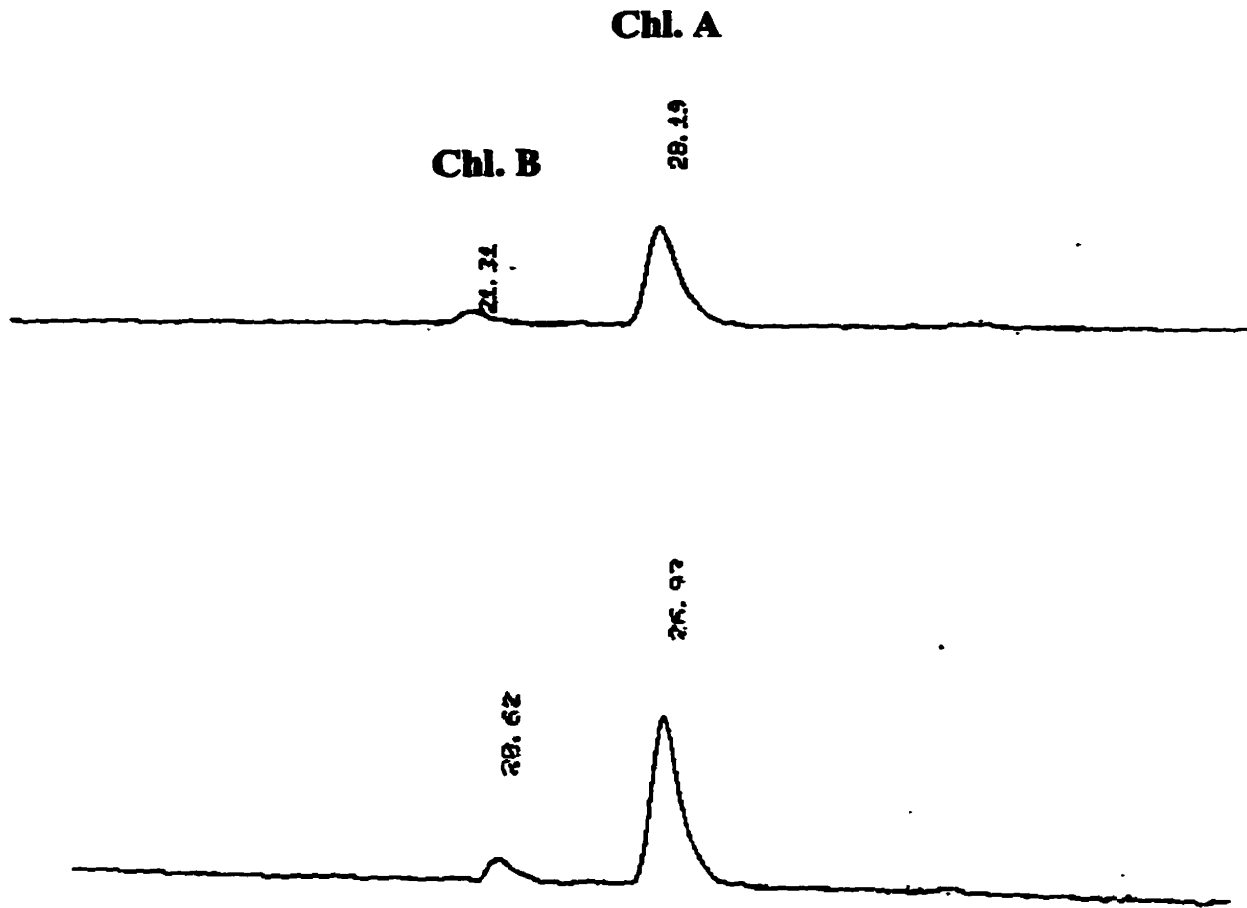
Fig. 19. HPLC chlorophyll degradation profiles of Katepwa tissue 72 hours after infiltration with Ptr chlorosis toxin (top) and water (bottom).

4.5. Discussion

The results obtained in this study provided some clues as to the mode of action of Ptr chlorosis toxin. The fact that the development of toxin-induced chlorosis is independent of tissue age (N.P. Orolaza, *unpublished data*) suggests that the toxin may promote chlorophyll degradation, rather than inhibit the synthesis of chlorophyll or associated chloroplast proteins. Chlorophyll synthesis is normally a major process in young, expanding leaves and decreases as plants age (Hendry et al., 1987). Therefore, if chlorophyll synthesis was being inhibited, greater symptom development would be expected in younger leaves. The fact that the toxin did not interfere, for the first 48 hours, with the accumulation of chlorophyll in etiolated seedlings exposed to light strongly suggests that chlorophyll synthesis was not being affected (Fig. 17). Although it could be argued that inhibition of chlorophyll synthesis might not begin until 48 hours after infiltration (and hence the lapse in time before symptom development), this scenario is unlikely. If this were the case, we would have expected a leveling off or a slight decrease in chlorophyll levels in the greening seedlings, rather than the sharp decline which we consistently observed.

Several lines of evidence suggest the involvement of photochemical bleaching in the degradation of chlorophyll: i) toxin-induced chlorosis is strictly light-dependent, and ii) when green seedlings were infiltrated with the toxin and subjected to dark periods of 24 to 72 hours, and then returned to the light, the onset of chlorosis was consistently delayed by a period of time similar in length to the dark period (Table 2). Further evidence for photochemical bleaching was also obtained from the study with AO scavengers. Photochemical bleaching involves the formation and action of AO species (Lawlor, 1993),

which cause the photooxidation of the chlorophyll molecules. Thus, it would be expected that scavengers of AO could be useful in preventing chlorosis. Three of the AO scavengers tested had no detectable effect on the development of the toxin-induced chlorotic symptom. However, the compound p-benzoquinone was highly effective at preventing chlorosis (Fig. 18). Other researchers have also observed that p-benzoquinone can inhibit chlorophyll photobleaching. For example, Feierabend and Winkelhusener (1982) found that p-benzoquinone effectively prevented photooxidation of chlorophyll in herbicide-treated rye leaf segments. This compound is a scavenger of singlet oxygen, and has been shown to reduce the half-life of the triplet state of chlorophyll (Fujimori and Livingston, 1957). This latter effect of p-benzoquinone could be the most important in preventing the development of chlorosis in the tan spot system: the reduction in the half-life of the energetic triplet chlorophyll molecules makes it less likely that they will react with molecular oxygen and produce AO species. It is also possible that by directly quenching AO species, p-benzoquinone prevents the photooxidation of chlorophyll. It is unclear why the three other AO scavengers used failed to have any effect. These compounds, 1,4-diazobicyclo(2,2,2)octane, formate and ascorbate, are scavengers of singlet oxygen, hydroxyl radical and superoxide, respectively, all of which have been implicated in the photodynamic decomposition of chlorophyll (Harbour and Bolton, 1978; Ginkel and Raison, 1980; Hoober, 1984). It is possible that these scavengers were ineffective at the concentrations used, or that they were not taken up in sufficient quantities by the tissue segments. Nevertheless, the fact that p-benzoquinone prevented the development of chlorosis provides strong evidence for the involvement of AO species in the production of this symptom.

The HPLC chlorophyll degradation profiles obtained (Fig. 19) are consistent with photooxidation of chlorophyll molecules into low molecular weight compounds. Hendry et al. (1987) identified two patterns of chlorophyll degradation, termed type I and type II. Type I involves the hydrolytic removal of the phytol moiety and magnesium from chlorophyll, resulting in the formation of pheophytins, chlorophyllides, and pheophorbides, compounds easily detectable by HPLC. Type II degradation involves the breakdown of the macrocyclic ring structure and subsequent degradation into smaller carbon/nitrogen fragments. Type II degradation occurs in the presence of oxygen and light (Ziegler and Schanderl, 1969), and it has been argued that degradation of the macrocyclic ring into carbon/nitrogen fragments occurs following attack by AO species (Dupont and Siegenthaler, 1986). In our studies with HPLC, we attempted to monitor type I degradation products in toxin-treated and non-treated, sensitive and insensitive tissue, but failed to record their presence (Fig. 19), suggesting that chlorophyll was being photooxidized into low molecular weight colourless compounds. The small pheophytin a peak which was observed in a few of the control tissues was most likely attributable to sample degradation prior to running through the HPLC. Although our failure to detect type I degradation products is not definite proof of their absence, the HPLC chlorophyll degradation profiles are consistent with photooxidation, particularly when considered together with our other results.

Carotenoids play an important role in the dissipation of the excess energy of chlorophyll molecules and in the detoxification of AO species (Hoober, 1984; Lawlor, 1993). We examined the possibility that a carotenoid deficiency was responsible for the development of chlorosis by measuring carotenoid levels in various treatments. Although we did observe

a difference in carotenoid levels between toxin-treated sensitive and insensitive tissue, this difference was concurrent with and of much lower magnitude than that of chlorophyll, suggesting that it was a reflection of the fact that AO-mediated damage was becoming more widespread. Whether other protective systems, such as the various antioxidant enzymes found in plants, are being affected remains unclear and will be the focus of future studies. Based on our current results, however, it appears that Ptr chlorosis toxin, directly or indirectly, inhibits photosynthesis, leading to chlorophyll degradation as illuminated thylakoid membranes become unable to dissipate excitation energy.

5. GENERAL DISCUSSION

The studies into the mode of action of Ptr chlorosis toxin were conducted prior to the development of an effective method to purify the toxin. Therefore, they were carried out using only partially purified toxin. However, given the fact that Ptr chlorosis toxin selectively induces chlorosis in sensitive wheat cultivars, it is unlikely that the physiological events associated with that chlorosis were the result of the action of some other compound present in the toxin mixture. Furthermore, the contaminants, when isolated from the toxin, did not produce chlorosis. Nevertheless, the fact that we were not using pure Ptr chlorosis toxin in the mode of action studies made it difficult to relate the development of physiological effects with a particular quantity of toxin. The partially purified toxin used in all the mode of action studies came from the same stock; the toxin concentration, although unknown, was therefore the same in all the studies.

The results obtained from studies on the mode of action strongly suggested that toxin-induced chlorosis develops as a result of the photooxidation of chlorophyll molecules. However, it is worth noting that we did not observe a decline in the chlorophyll a to b ratio. This appears, at least initially, to be at odds with some of the literature, where a decline in the chlorophyll a to b ratio is taken to be a characteristic of photochemical destruction (Turner, 1988). This conclusion by some researchers stems from the results of various studies that have found that chlorophyll b bleaches at a much slower rate than chlorophyll a (Sauer and Calvin, 1962; Thomas and Nijhuis, 1968; Carpentier et al., 1986). However, a brief examination of some of these studies may explain why we observed what appeared to be photooxidation of chlorophylls, without a decline in the chlorophyll a to b ratios.

Thomas and Nijhuis (1968) studied photobleaching of various chlorophyll-protein complexes *in vivo* at high light intensities with isolated chloroplasts. They found that chlorophyll *b* initially bleached more slowly than chlorophyll *a*, resulting in a decline in chlorophyll *a* to *b* ratios. However, in their 20 minute time-course experiments, they observed an approximately linear degradation rate for chlorophyll *a*, but an almost-exponential rate for chlorophyll *b*. Hence, over a sufficiently long period, the decline in the chlorophyll *a* to *b* ratio would be reversed. Other studies that reported a decline in chlorophyll *a* to *b* ratios have also used very short time courses. For instance, Miller and Carpentier (1991) saw a decline in chlorophyll *a* to *b* ratios during photobleaching in thylakoid membranes, but their time-courses were only 40 minutes long. Therefore, over a period of 72 hours (*ie*. the time it takes for toxin-induced chlorosis to develop in the tan spot system), a decline in chlorophyll *a* to *b* ratios would not be detected, as it would have occurred only over a short period of time. Furthermore, the issue of whether chlorophylls *a* and *b* bleach at different rates is itself controversial. Jen and MacKinney (1970) observed that while for most chemical reactions the rate for chlorophyll *a* is substantially higher than for chlorophyll *b*, no such difference existed with respect to photochemical bleaching rates. Thus, our finding that chlorophyll *a* to *b* ratios did not decline during toxin-induced photooxidation does not appear to be a paradox.

Some of the strongest evidence for the involvement of AO species in the development of toxin-induced chlorosis came from the studies with AO scavengers. The compound p-benzoquinone, a quencher of triplet chlorophyll and singlet oxygen, was highly effective at

preventing chlorosis (Fig. 18). However, it is unclear why the remaining compounds tested failed to have a measurable effect. Ascorbate, formate and 1,4-diazobicyclo(2,2,2)octane are known scavengers of superoxide, hydroxyl radical and singlet oxygen, respectively, all of which have been implicated in the photodynamic decomposition of chlorophyll (Harbour and Bolton, 1978; Ginkel and Raison, 1980; Hoober, 1984). It is possible that these scavengers were ineffective at the concentrations used, or that they were not taken up in sufficient quantities by the tissue segments. This second alternative could be a drawback of using intact leaf tissue segments; in an *in vivo* system, uncertainties exist as to whether the substances were sufficiently taken up by the tissue and whether they reached the chloroplast membranes. Nevertheless, an *in vivo* system was still preferable to an *in vitro* system, such as one consisting of isolated chloroplasts. The *in vitro* system does not truly reflect the *in vivo* situation, since some photoprotective mechanisms are lost during the isolation of chloroplasts (Feierabend and Winkelhusener, 1982).

Although our results strongly suggested that toxin-induced chlorosis is a direct consequence of chlorophyll photooxidation, it is not clear whether this photooxidation is a result of an inhibition of photosynthesis, or whether it is due to a failure in the normal photoprotective mechanisms of affected plants. If Ptr chlorosis toxin is inhibiting photosynthesis, directly or indirectly, then chlorosis could result from the photodestruction of chlorophylls, as illuminated thylakoid membranes would become unable to dissipate the excitation energy that would normally be used in photosynthetic processes. Given this scenario, we would have to determine at which point photosynthesis is being inhibited. It is possible that the toxin or a processed, lower molecular weight toxic compound derived from

it, could block photosynthetic electron flow in either photosystem (PS) I or II. However, it is also possible that the inhibition of photosynthesis could be more indirect. For instance, suppression of photosynthetic carbon metabolism could lead to a lack of $NADP^+$, as NADPH would not be used up in the carbon reduction reactions. Hence, reduced ferredoxin from PSI, having no $NADP^+$ to reduce, could react with molecular oxygen, resulting in the formation of AO species.

Alternately, if it is the photoprotective mechanisms of the plant which are being disrupted, then the possible sequence of events leading to the development of chlorosis would be quite different. Carotenoids play an important role in the dissipation of the excess energy of chlorophyll molecules and in the detoxification of AO species. Although carotenoid deficiencies have been shown to be responsible for the development of chlorosis in many herbicide systems (Burns et al., 1971; Sandmann and Boger, 1989), it seems unlikely that a carotenoid deficiency led to chlorosis in the wheat-Ptr chlorosis toxin interaction. In our studies, the decline in carotenoids was concurrent with and smaller than that of chlorophylls, suggesting that it was simply a reflection of the fact that AO-mediated damage was becoming more widespread (Fig. 16). Nevertheless, this is a point which requires further study. It is also possible that the activities of one or more of the various antioxidant enzymes found in plants, such as superoxide dismutase, ascorbate peroxidase, or catalase, are being affected. This would lead to an inability by the plant cells to detoxify AO species, which would then be available to cause widespread damage.

This investigation served to lay the groundwork for further research. Although we have established that Ptr chlorosis toxin caused degradation of chlorophyll through a

photooxidative process, many questions remain unanswered. It will be necessary to conduct more studies to clearly ascertain whether it is photosynthesis that is being affected, or whether the plant photoprotective mechanisms are being compromised. Once this is resolved, we will be able to focus our attention on the exact nature of the primary event(s) leading to the development of chlorosis.

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7. APPENDIX

Ammonium sulfate concentration is usually expressed as percent saturation (relative to full saturation at a given temperature). The equation used in this investigation to calculate grams of ammonium sulfate (solid) to be added to 1 liter of a solution at 20^0C to take it from S_1 % saturation to S_2 % saturation was:

$$g = 533(S_2-S_1)/100-0.3S_2$$

(Scopes, 1982)