Localization of Ptr ToxA Produced by *Pyrenophora tritici-repentis* Reveals Protein Import into Wheat Mesophyll Cells

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The plant pathogenic fungus *Pyrenophora tritici-repentis* secretes host-selective toxins (HSTs) that function as pathogenicity factors. Unlike most HSTs that are products of enzymatic pathways, at least two toxins produced by *P. tritici-repentis* are proteins and, thus, products of single genes. Sensitivity to these toxins in the host is conferred by a single gene for each toxin. To study the site of action of Ptr ToxA (ToxA), toxin-sensitive and -insensitive wheat (*Triticum aestivum*) cultivars were treated with ToxA followed by proteinase K. ToxA was resistant to protease, but only in sensitive leaves, suggesting that ToxA is either protected from the protease by association with a receptor or internalized. Immunolocalization and green fluorescent protein tagged ToxA localization demonstrate that ToxA is internalized in sensitive wheat cultivars only. Once internalized, ToxA localizes to cytoplasmic compartments and to chloroplasts. Intracellular expression of ToxA by biolistic bombardment into both toxin-sensitive and -insensitive cells results in cell death, suggesting that the ToxA internal site of action is present in both cell types. However, because ToxA is internalized only in sensitive cultivars, toxin sensitivity, and therefore the ToxA sensitivity gene, are most likely related to protein import. The results of this study show that the ToxA protein is capable of crossing the plant plasma membrane from the apoplastic space to the interior of the plant cell in the absence of a pathogen.

INTRODUCTION

The outcome of an interaction between a plant pathogen and its host is often governed by the expression of a single dominant gene in the pathogen and a single dominant gene in the host. In classical gene-for-gene interactions, the expression of an avirulence (*Avr*) gene in the pathogen and the corresponding resistance (*R*) gene in the host results in the initiation of the defense response (reviewed in Flor, 1971; Dangl and Jones, 2001). In this type of gene-for-gene interaction, defense is associated with the localized cell death of the host called the hypersensitive response, which may play a role in marginalizing the pathogen (reviewed in Heath, 2000). There are many examples of classical gene-for-gene interactions where Avr proteins produced by bacteria, fungi, oomycetes, or viruses are detected by a corresponding R protein in their hosts (reviewed in Bonas and Lahaye, 2002). In these interactions, avirulence determinants can be described as agents of incompatibility. However, some plant pathogenic fungi produce agents of compatibility, namely host-selective toxins (HSTs) (reviewed in Walton, 1996; Wolpert et al., 2002). The interaction of some HST-producing pathogens with

their hosts also follows a gene-for-gene model where toxin production by the pathogen and toxin sensitivity in the host are each conferred by a single dominant gene. In these interactions, the toxins, like avirulence gene products, affect the localized cell death of the host but with the inverse effect of disease susceptibility. An emerging model for the study of this inverse form of gene-for-gene interaction is the *Pyrenophora tritici-repentis*/wheat (*Triticum aestivum*) pathosystem (Lamari et al., 2002; Wolpert et al., 2002; Strelkov and Lamari, 2003).

Cell death–inducing proteins produced by plant pathogens have been shown to act both externally and internally. Perhaps the greatest understanding of extracellular-acting, cell death–inducing proteins comes from the study of the *Cladosporium fulvum*/tomato (*Lycopersicon esculentum*) interaction (reviewed in Joosten and de Wit, 1999). For example, Avr4 and Avr9 produced by *C. fulvum* are recognized at the surface of cells expressing the cognate *R* genes *Cf-4* and *Cf-9*, respectively. *Cf-4* and *Cf-9* encode R proteins that contain an extracellular leucine-rich repeat region, a transmembrane domain, and a short cytoplasmic tail (reviewed in Kruijt et al., 2005). Many hypersensitive response–inducing Avr proteins have also been shown to act intracellularly. Avr proteins produced by plant pathogenic bacteria typically act within plant cells and are introduced into the plant cell via a type III secretion system (reviewed in Alfano and Collmer, 2004). This system is also utilized by animal pathogens to introduce their effector proteins (reviewed in Buttner and Bonas, 2003; He et al., 2004). The oomycete *Hyaloperonospora parasitica* and fungus *Magnaporthe grisea*, as well as other oomycetes and hemibiotrophic/biotrophic fungi, also produce Avr proteins that act intracellularly to induce cell death (Jia et al.,

2000; Allen et al., 2004; Dodds et al., 2004; Armstrong et al., 2005). The exact mechanism of delivery of these proteins is unclear but is thought to require the intimate contact of the pathogen and plant cell membranes. Viral Avr proteins are produced within infected host cells after the virus has already gained entry (reviewed in Culver, 1996). To date, no proteins have been shown to independently cross plant plasma membranes from the apoplast.

P. tritici-repentis is a necrotrophic fungus and the causal agent of tan spot of wheat. The ability of *P. tritici-repentis* to cause disease is correlated with the production of several HSTs. Different races produce different toxins, or combinations of toxins, that act as pathogenicity/virulence factors and define host range (reviewed in De Wolf et al., 1998; Ciuffetti and Tuori, 1999; Strelkov and Lamari, 2003). The HSTs produced by *P. tritici-repentis* are both proteinaceous and nonproteinaceous. For instance, Ptr ToxA (ToxA) and ToxB are proteins (Ballance et al., 1989; Tomas et al., 1990; Tuori et al., 1995; Ciuffetti et al., 1998; Strelkov et al., 1999; Martinez et al., 2001), whereas ToxC appears to be a polar, nonionic, low-molecular-weight molecule (Effertz et al., 2002). Other toxins have been identified but not fully characterized (Tuori et al., 1995; Ciuffetti et al., 2002; Meinhardt et al., 2002a). It has been shown that sensitivity of the host to ToxA, ToxB, and ToxC is conferred by a single gene for each toxin (Faris et al., 1996; Stock et al., 1996; Effertz et al., 1998, 2002; Gamba et al., 1998; Anderson et al., 1999; Friesen and Faris, 2004) and that host susceptibility to pathogenic races that produce these toxins cosegregates with toxin sensitivity (Gamba et al., 1998). Although the genetic nature of the patho-system is well understood, the underlying mechanisms governing host sensitivity to a particular toxin are not.

ToxA is the most studied toxin in the *P. tritici-repentis* patho-system. ToxA was the first HST isolated that was shown to be a protein (Ballance et al., 1989; Tomas et al., 1990; Tuori et al., 1995) and the product of a single gene (Ballance et al., 1996; Ciuffetti et al., 1997). Transformation of a nonpathogenic isolate with the *ToxA* gene is sufficient to render that isolate pathogenic on ToxA-sensitive wheat lines (Ciuffetti et al., 1997). The mature toxin as produced in culture is 13.2 kD and contains an N-terminal pyroglutamate (Tuori et al., 1995, 2000); however, the *ToxA* gene encodes a pre-pro-protein that contains a signal sequence to target the protein to the secretory system (Ballance et al., 1996; Ciuffetti et al., 1997) and a pro-sequence (N-domain) that is necessary for proper folding and is removed prior to secretion (Tuori et al., 2000) of the mature toxin, ToxA (C-domain). Introduction of the mature toxin into the apoplastic space of a sensitive plant results in a necrotic response similar to the disease symptoms induced by ToxA-producing isolates (Ballance et al., 1989; Tomas et al., 1990; Tuori et al., 1995). Therefore, the ToxA protein is capable of inducing cell death in the absence of pathogen. The sequence of ToxA is completely unique in that, to date, there are no similar protein sequences found in any database. While the lack of similarity makes it difficult to hypothesize how this protein might function, it also implies a completely novel mechanism of cell death induction.

One clue guiding current mechanistic hypotheses is that the amino acid sequence of ToxA has an Arg-Gly-Asp (RGD) tripep-tide (Ballance et al., 1996; Ciuffetti et al., 1997; Zhang et al., 1997)

in a stretch of 10 amino acids (Manning et al., 2004) that is 60% identical to the RGD loop of vitronectin (Suzuki et al., 1985), a protein present in the extracellular matrix of animals. Interestingly, site-directed mutagenesis of the vitronectin-like region of ToxA has shown that 9 of the 10 amino acids (Manning et al., 2004), including the RGD residues, are necessary for ToxA function (Meinhardt et al., 2002b; Manning et al., 2004). Vitronectin relays environmental cues to cells via RGD-mediated interactions with integrin receptors with or without the internalization of the receptor and its ligand (Cherny et al., 1993; Memmo and McKeown-Longo, 1998; Hynes, 2002). Internalization of receptor has been coopted as a mechanism for pathogen uptake by animal cells (Marjomaki et al., 2002). Although no integrin homologs have been identified in plants, RGD-containing peptides can perturb the interaction between the cell wall and plasma membrane of plant cells (Canut et al., 1998; Mellersh and Heath, 2001), suggesting that RGD/integrin-like interactions occur in plants. Recently, it has been shown that an RGD-containing protein from the oomycete *Phytophthora infestans* binds with high affinity to an 80-kD protein in purified plasma membrane vesicles of *Arabidopsis thaliana* (Senchou et al., 2004). Because protein import from the extracellular space across plant plasma membranes (in the absence of pathogen) is unprecedented, the most recent hypothesis is that ToxA exerts its effects externally by either (1) disrupting cell-to-cell communication or (2) triggering receptor-mediated events that ultimately lead to cell death (Meinhardt et al., 2002b; Manning et al., 2004).

We initiated this study to determine where ToxA exerts its effects to induce cell death of its target cell. We provide evidence that, contrary to the expectation that ToxA acts extracellularly, ToxA traverses the plasma membrane and localizes to the cytoplasm and chloroplasts in sensitive but not insensitive wheat mesophyll cells. In addition, intracellular expression of ToxA through the introduction of the ToxA coding sequence leads to cell death in both ToxA-sensitive and -insensitive genotypes. ToxA traverses from the extracellular space through plant cell membranes and, as such, provides a unique vehicle to study protein internalization from the apoplast into mesophyll cells and the induction of plant cell death.

RESULTS

ToxA Is Protected from Protease Degradation in Sensitive but Not Insensitive Wheat

We developed a simple approach to determine whether ToxA remains extracellular following introduction into the apoplast of the leaf; if ToxA acts extracellularly, it should be available for digestion by an extracellular protease. Therefore, sensitive and insensitive wheat cultivars were treated with heterologously expressed N-terminally His-tagged ToxA (His-ToxA) (Tuori et al., 2000) for 2 h, followed by treatment with proteinase K (PK). ToxA followed by PK treatment (ToxA/PK) of sensitive leaves results in necrosis similar to ToxA treatment without protease (Figure 1A), indicating that 2 h of exposure to extracellular ToxA is sufficient to induce cell death. As expected, no symptoms of cell death are detected in ToxA-treated, insensitive
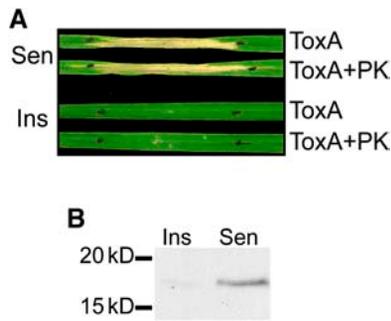
Figure 1. ToxA Is Protected from PK Degradation in ToxA-Sensitive Leaves.

(A) PK treatment of leaves 2 h after ToxA treatment does not reduce ToxA-induced necrosis. Leaves of sensitive (Sen) and insensitive (Ins) wheat cultivars infiltrated with ToxA alone (ToxA) or ToxA followed by proteinase K (ToxA+PK) were harvested 2 d after infiltration. Black dots on leaves define the treatment area.
(B) ToxA can be isolated from sensitive, but not insensitive, wheat cultivars treated with ToxA/PK. The immunoblot was probed with anti-ToxA antibody. Sizes of proteins in the molecular mass markers are noted on the left.

leaves (Figure 1A). PK treatment alone has no effect on either sensitive or insensitive cultivars (data not shown).

To determine if ToxA was degraded with proteinase treatment, nickel beads were used to retrieve His-ToxA from whole cell lysates of ToxA/PK-treated leaves. Protein gel blot analysis of eluates from the nickel beads using an anti-ToxA antibody indicates that after PK treatment, intact ToxA is still present in total lysates of sensitive leaves but nearly undetectable in insensitive leaves (Figure 1B). This indicates that ToxA is protected from the extracellular protease only in sensitive leaves. The size of His-ToxA detected on the protein gel blot is identical to the size of toxin used for treatment; therefore, ToxA does not appear to be processed in planta. Similar results were obtained with native ToxA (data not shown). The protection of ToxA from degradation in sensitive leaves suggests that it is either internalized or tightly held by a receptor and thus is inaccessible to the protease.

Immunolocalization of ToxA

To determine if ToxA is internalized in sensitive cells, both sensitive and insensitive wheat were treated with either water or native ToxA for 4 h and thin sections prepared for immuno-localization (Figure 2). Sections were stained with anti-ToxA antibody followed by an Alexa 488–tagged secondary antibody and visualized using confocal microscopy to detect both chloroplast autofluorescence (magenta) and ToxA-labeling (green). Very little label is detected in either water-treated insensitive (Figure 2A) or sensitive (Figure 2B) sections or in ToxA-treated insensitive sections (Figure 2C). However, extensive labeling in ToxA-treated sensitive wheat (Figure 2D) indicates the presence of ToxA in the cytoplasm and on the inside of the plasma membrane, occasionally in large aggregates (Figure 2D, closed arrowheads). ToxA is also found associated with chloroplasts (Figure 2D, open arrowheads). The green fluorescence in meso-

phyll cell membranes (Figures 2A to 2D), epidermal cell membranes (Figures 2A, 2B, and 2D), and vascular tissue (Figures 2B and 2C) is due to autofluorescence seen in both treated and untreated tissue and not due to toxin treatment. Plasma membranes appear intact in both ToxA-treated sensitive and insensitive wheat (Figures 2C and 2D); therefore, ToxA does not rupture the plant cell membrane for internalization. A similar pattern of localization is observed with two additional, distinct ToxA-specific antibodies (data not shown). Thus, ToxA protease protection in sensitive wheat occurs via internalization rather than receptor binding.

Visualization of ToxA Internalization via Green Fluorescent Protein–ToxA Fusion Treatment of Sensitive and Insensitive Wheat

To further confirm the sites of intracellular ToxA accumulation in vivo, a green fluorescent protein–ToxA fusion protein (GFP-ToxA) was utilized. Because N-terminal tagging of ToxA with a His tag does not alter ToxA activity (Tuori et al., 2000) and ToxA is not processed in planta (Figure 1B), GFP was fused
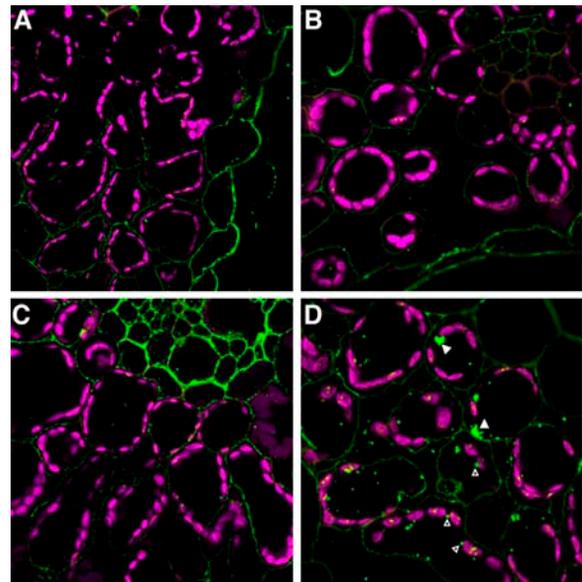

Figure 2. Immunolocalization of ToxA in ToxA-Sensitive and -Insensitive Wheat.

(A) to **(D)** Confocal imaging of thin sections of fixed/methacrylate-embedded leaf tissue immunostained with anti-ToxA antibody and Alexa-488–tagged secondary antibody. Chloroplast autofluorescence (magenta) and Alexa-488 fluorescence (green) images are merged. Green autofluorescence of mesophyll cell membrane (**[A]** to **[D]**), vascular tissue (**[B]** and **[C]**), and epidermal tissue (**[A]**, **[B]**, and **[D]**) is present in both treated and untreated tissue.
(A) Water-treated insensitive leaves.
(B) Water-treated sensitive leaves.
(C) ToxA-treated insensitive leaves.
(D) ToxA-treated sensitive leaves. Closed arrowheads indicate ToxA-specific signal present in aggregates. Open arrowheads indicate ToxA-specific signal associated with chloroplasts.

N-terminally to ToxA. The fusion protein of ~55 kD was expressed and purified from *Escherichia coli*. As a control, and to ensure that the GFP domain of the fusion protein does not induce symptoms in wheat, an unfused version of GFP was also heterologously expressed. GFP-ToxA is a valid tool for the study of ToxA function because GFP-ToxA infiltration into ToxA-sensitive wheat leaves (Figure 3A, fourth leaf) induces similar levels of necrosis as native ToxA (Figure 3A, first leaf), whereas GFP alone does not induce necrosis (Figure 3A, second leaf) or alter the amount of necrosis induced by ToxA when the two proteins are coinfiltrated (ToxA + GFP) (Figure 3A, third leaf). As expected, necrotic symptoms do not develop in ToxA-insensitive wheat with treatment by any of these proteins (data not shown).

Cotreatment for 7 h with ToxA + GFP in both sensitive and insensitive leaves, or treatment with GFP-ToxA in insensitive leaves, results in a haze of fluorescence (Figures 3B to 3D), consistent with GFP remaining in the apoplastic space. However, in GFP-ToxA–treated sensitive wheat leaves, in addition to a slight haze of fluorescence, fluorescence is also present inside mesophyll cells (Figure 3E). Fluorescence within these cells localizes to both the cytoplasm and chloroplasts (Figures 3F and 3G). The unperturbed cell morphologies and the observation that GFP does not enter cells in the ToxA + GFP coinfiltration control (Figure 3D) indicate that ToxA enters the cell without disruption of the membrane. Additionally, the mechanism of ToxA entry into the cell is robust enough to carry a GFP fusion tag along. GFP-ToxA persists inside of sensitive mesophyll cells for several hours, and then rapid cell death ensues (data not shown).

For higher-resolution localization, GFP-ToxA–treated leaves were fixed and viewed with a confocal microscope to detect chloroplast autofluorescence (magenta) and GFP (green) (Figures 3H to 3M). Mesophyll cells in both GFP-ToxA–treated insensitive (Figure 3H) and sensitive leaves (Figure 3K) are intact. The chloroplasts are also intact, although some chloroplasts in the GFP-ToxA–treated sensitive cells appear more elliptical than rounded (Figure 3K). GFP fluorescence is not visible in GFP-ToxA–treated insensitive leaves (Figure 3I); however, it is apparent throughout treated sensitive cells (Figure 3L). The GFP-ToxA fluorescence in sensitive cells is concentrated in discrete regions of the cytoplasm (Figures 3L and 3M, cell labeled "a") and associated with the chloroplasts (Figure 3M, arrows), confirming immunolocalization (Figure 2D) and in vivo observations (Figures 3E to 3G). Again, membrane integrity and cell structure do not appear to be altered by the import of ToxA into sensitive cells at this stage, as cellular morphology looks normal and unperturbed (Figure 3M).

ToxA Activity Is Light Dependant

Demonstration of the localization of ToxA to chloroplasts raised the possibility that light-induced chloroplast function is necessary for toxin activity. To test this, sensitive wheat leaves were infiltrated with native ToxA or GFP-ToxA fusion protein, and a region of the infiltration zone was covered to exclude light (Figure 4). Light exclusion virtually eliminates toxin-induced tissue collapse and necrosis, while necrosis produced by ToxA and GFP-ToxA in the unexcluded leaf regions confirms that ToxA

is fully active. Therefore, both ToxA and GFP-ToxA require light in order to induce necrosis.

ToxA Expression by Plant Cells Leads to Cell Death

ToxA entry into sensitive plant cells does not establish that ToxA internalization is required for its toxicity; it could be that signals initiated by extracellular ToxA are the cause of cell death. If internalization of ToxA is required and sufficient to induce necrosis, then expression of ToxA by sensitive wheat cells should result in cell death. Biolistic bombardment, a technique that has been used to demonstrate that some cell death–inducing proteins produced by plant pathogens have an internal site of action (Leister et al., 1996; Jia et al., 2000; Allen et al., 2004; Dodds et al., 2004), was used to cotransform both sensitive and insensitive wheat cultivars with ToxA (minus the signal sequence) and β-glucuronidase (GUS) expression plasmids. In this assay, if cells die in response to expression of ToxA, fewer cells will show GUS activity than cells that have been cotransformed with GUS and a control plasmid.

ToxA coexpression with GUS in both sensitive and insensitive wheat results in an ~50% decrease in the number of GUS-expressing cells compared with controls (Figure 5), which is within the range expected if ToxA has an internal site of action (Leister et al., 1996). The similar response of sensitive and insensitive cells to internal expression of ToxA illustrates not only that ToxA internalization is necessary and sufficient for toxicity but also that the ability of a cell to internalize toxin is the factor that distinguishes sensitivity from insensitivity.

DISCUSSION

ToxA Internalization into Sensitive Wheat Cultivars

ToxA has been shown to traverse the plant plasma membrane from the apoplastic space into the interior of a plant cell. Protection of ToxA from PK (Figure 1), the intracellular detection of ToxA by immunolocalization (Figure 2), and the direct visualization of a functional GFP-ToxA fusion protein in the cytoplasm and in association with chloroplasts (Figure 3) all show that ToxA is internalized into toxin-sensitive cells.

In planta protection from PK degradation provided an indication that ToxA is internalized and only in sensitive wheat cultivars (Figure 1). Such studies demonstrate that ToxA protection from PK occurs within a 2-h incubation period and suggests that ToxA is rapidly taken into the cell. In addition, protected ToxA is not proteolytically processed in planta; therefore, N-terminal tagging of ToxA with a fluorescent tag for localization studies was possible. ToxA protein contains all of the information required for internalization, and the internalization process is sufficiently robust to still function despite increasing the size of ToxA from a 13.2-kD, single-structural domain protein (Tuori et al., 1995; Sarma et al., 2005) to an ~55-kD, two-domain protein (GFP-ToxA).

In immunolocalization studies (Figure 2), ToxA is clearly visible in the cytoplasm of sensitive cells 4 h after toxin treatment. Some ToxA is present in aggregates that are occasionally associated with the interior of the plasma membrane. Aggregation of ToxA
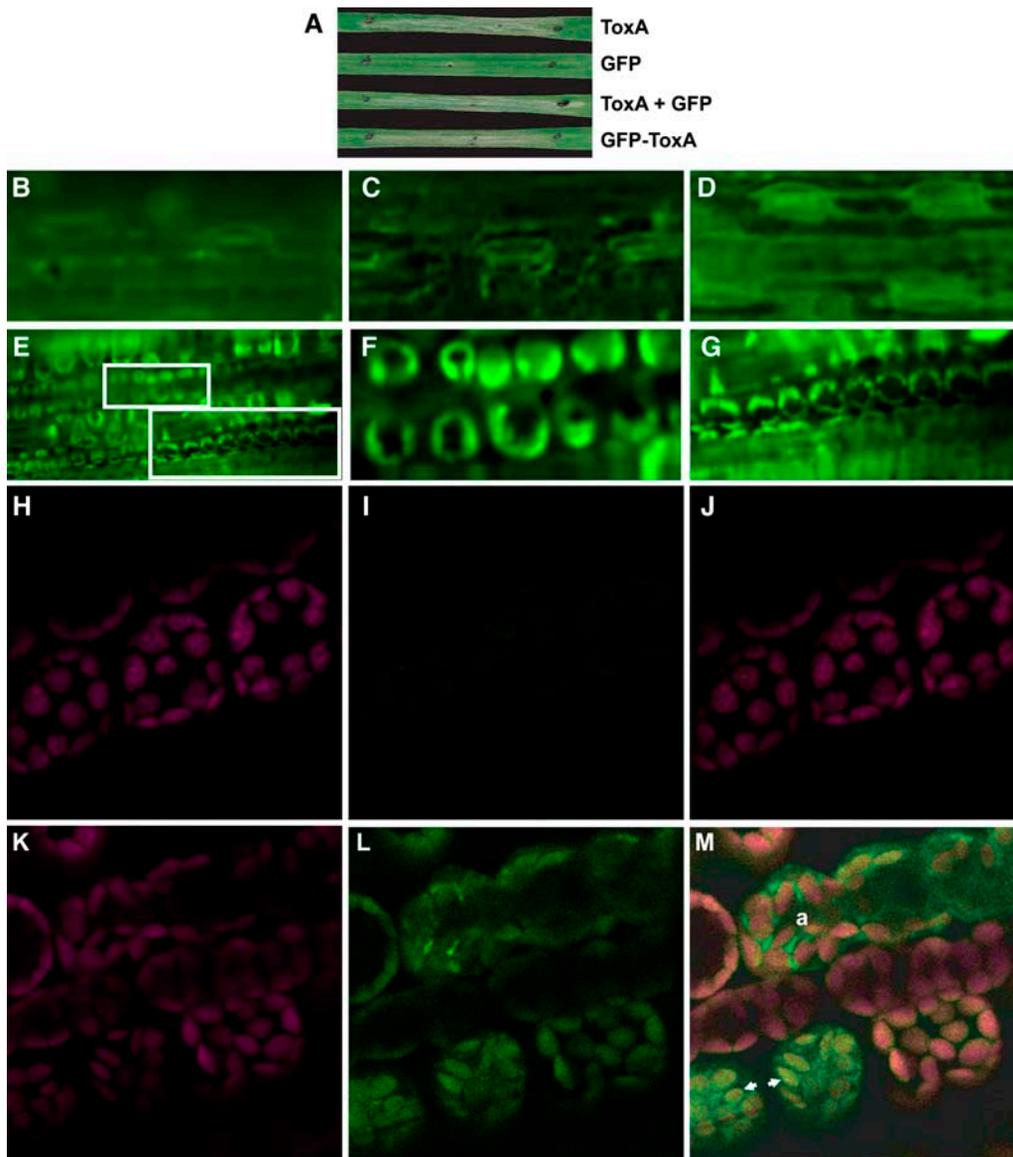
Figure 3. Localization of GFP-ToxA Fusion Protein in ToxA-Sensitive and -Insensitive Wheat.

(A) GFP-ToxA fusion protein induces symptoms comparable to native ToxA. Leaves were treated with proteins indicated on the right and harvested 24 h after infiltration. Black dots on leaves define the treatment area.

(B) to **(G)** Epifluorescence microscopy on live, whole leaf tissue of ToxA-sensitive and -insensitive wheat treated with ToxA + GFP (nonfused) or GFP-ToxA fusion protein.

(B) Insensitive leaves treated with ToxA + GFP.

(C) Insensitive leaves treated with GFP-ToxA.

(D) Sensitive leaves treated with ToxA + GFP.

(E) Sensitive leaves treated with GFP-ToxA.

(F) and **(G)** Enlargements of boxed sections in **(E)**.

(H) to **(M)** Confocal imaging of paraformaldehyde-fixed, ToxA-insensitive (**[H]** to **[J]**) and ToxA-sensitive (**[K]** to **[M]**) leaves treated with GFP-ToxA. Chloroplast autofluorescence is shown in **(H)** and **(K)**, GFP fluorescence is shown in **(I)** and **(L)**, and merged autofluorescence and GFP fluorescence is shown in **(J)** and **(M)**. Note GFP fluorescence in sensitive leaves is compartmentalized (e.g., cell marked ''a'') and associated with chloroplasts (arrows).

Figure 4. ToxA Activity Is Light Dependent.

Sensitive leaves were treated with 5 μM ToxA or GFP-ToxA fusion protein and covered with a light-exclusion clip. Black dots on leaves define the treatment area. The solid line below the leaves corresponds to the area covered. Leaves were harvested and photographed 48 h after treatment.

suggests that it is compartmentalized. Localization studies performed using confocal microscopy (Figure 3) confirm compartmentalization of GFP-ToxA fusion protein. As discussed below, these observations, together with other properties of ToxA, are consistent with ToxA being internalized via receptor-mediated endocytosis (RME). RME in plants is not well understood; however, proteins of the plant plasma membrane as well as cell wall components have been shown to be recycled via endocytosis, and endocytosis of nonprotein elicitors has been demonstrated (Horn et al., 1989; Holstein, 2002; Romanenko et al., 2002; Samaj et al., 2004). Characteristics of RME include the requirement for a specific receptor, temperature and energy dependence, competition with free ligand, and saturation of uptake (Horn et al., 1989). While a specific receptor for ToxA has not yet been found, there appears to be a high affinity binding site present on sensitive cells. Tuori et al. (1995) infiltrated increasing amounts of purified ToxA into sensitive wheat cultivars and found that at low concentrations, only cells near the infiltration site showed necrosis. As the concentration of ToxA infiltrated was gradually increased, the area that displayed necrosis also increased, spreading outward from the infiltration point, until eventually the entire treatment zone was affected. If ToxA did not interact with a high affinity receptor, we would expect that treatment with low amounts of toxin would result in diffuse and/or delayed cell death throughout the entire zone of infiltration, as opposed to concentrated necrotic symptoms near the point of treatment. ToxA activity is also temperature dependent (Kwon et al., 1998). Though energy dependence for ToxA activity has not been directly addressed, cell death caused by ToxA treatment requires light (Figure 4) and host metabolism (Kwon et al., 1998). ToxA activity can be reduced in a dose-dependent manner by coinfiltration of RGD-containing peptides (Meinhardt et al., 2002b; Manning et al., 2004) or by a nonfunctional mutant-ToxA protein containing an Asp-to-Glu mutation in the RGD motif (Manning et al., 2004). This suggests that yet another criterion for RME, competition with free ligand, is met. Uptake of ToxA has not been previously demonstrated; therefore, the saturation of uptake of ToxA has not been established. However, the tools necessary to address this question (i.e., GFP-ToxA fusion protein [Figure 3] and His-ToxA; Tuori et al., 2000) are now available. That ToxA-induced necrosis shares characteristics required for RME warrants further investigation into RME as the mechanism of ToxA internalization.

We have shown that ToxA behaves differently in sensitive and insensitive wheat cultivars, and this difference appears to lie in the ability of ToxA to traverse the cell membrane. Because only sensitive plants internalize toxin (Figures 1 to 3), it is likely that the

single host gene that conditions toxin sensitivity (Faris et al., 1996; Stock et al., 1996; Gamba et al., 1998) controls toxin uptake. As shown by biolistic bombardment, intracellular expression of ToxA causes cell death in both ToxA-sensitive and -insensitive genotypes (Figure 5). This strongly suggests a common intracellular site of action in both sensitive and insensitive cells, providing further support that the difference between sensitivity and insensitivity is the ability to internalize toxin.

It is through the cell wall–plasma membrane interface that plant cells perceive and respond to their extracellular environment (reviewed in Baluska et al., 2003). Several studies have shown that RGD motifs are important for linking cell walls to plasma membranes (Canut et al., 1998; Mellersh and Heath, 2001). It is also known that ToxA mutants in the vitronectin-like RGD motif are no longer active (Meinhardt et al., 2002b; Manning et al., 2004). The crystal structure of ToxA (see companion article, Sarma et al., 2005) demonstrates that the vitronectin-like sequence is at the surface of the protein on a solvent-exposed loop and very accessible for protein–protein interactions. Thus, it is plausible that ToxA internalization relies on recognition of the RGD motif by a plant protein receptor. In fact, although plant genome sequencing has not revealed integrin homologs, immunologically related proteins exist (Faik et al., 1998; Kiba et al., 1998; Laboure et al., 1999; Laval et al., 1999; Nagpal and Quatrano, 1999; Swatzell et al., 1999; Sun et al., 2000; Baluska et al., 2003) and may provide candidates for mediating ToxA internalization. Deciphering the recognition mechanism of ToxA at the plasma membrane should help to clarify host susceptibility/resistance mechanisms and provide insight into the importance of the cell wall–plasma membrane–cytoskeleton continuum in host defense.

Localization of ToxA to the Chloroplast

Immunolocalization and GFP-ToxA fusion protein localization indicate that ToxA interacts with the chloroplast (Figures 2 and 3)
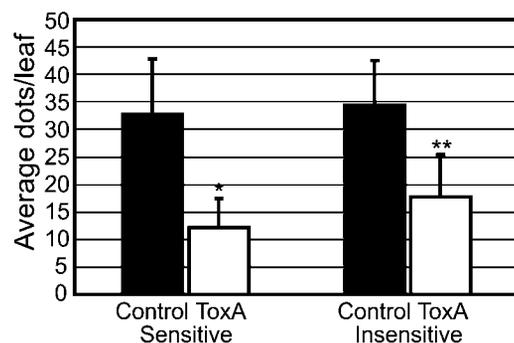

Figure 5. ToxA Expression in Planta in Both ToxA-Sensitive and -Insensitive Wheat Cultivars Leads to Cell Death.

Average GUS expression (dots/per leaf) for cobombardment of 1 μg each of pBS/35S:GUS (control) or 35S:ToxA/35S:GUS (ToxA). Graph represents the average of four independent experiments. The error bars represent standard error. *P* values were calculated by Student's paired, one-tailed *t* test. One asterisk indicates P < 0.05, and two asterisks indicates P < 0.01. GUS expression was decreased by 63 and 48% in sensitive and insensitive leaves, respectively.

in sensitive wheat. A yeast two-hybrid library screen of a ToxA-sensitive wheat cultivar with ToxA as the bait reveals that ToxA interacts with a chloroplast- and stromule-localized protein (V.A. Manning, L.K. Hardison, and L.M. Ciuffetti, unpublished data) The ToxA protein does not contain a recognizable transit peptide for targeting to the chloroplast (reviewed in Bruce, 2001). Recently, chloroplast proteins that do not contain these N-terminal targeting peptides have been found (Miras et al., 2002; Nada and Soll, 2004); therefore, it is possible that the ToxA protein contains information for chloroplast targeting that has not yet been described.

The significance of ToxA chloroplast localization is not yet known; however, the loss of chloroplast integrity after ToxA treatment has been established. Chloroplast morphology is affected in ToxA-treated, ToxA-sensitive wheat (Figure 3), and thylakoid structure is disrupted (Freeman et al., 1995). The *Arabidopsis* homolog of the chloroplast protein with which ToxA interacts has been shown to be required for thylakoid formation (Wang et al., 2004), suggesting that the interaction of ToxA with this protein may disrupt thylakoids. Future studies to determine the effect of ToxA on chloroplast structure and function should provide insights into the mechanism of ToxA-induced cell death.

For the work described here, several valuable tools were developed. In the first tool developed, the PK sensitivity assay, proteins present in the apoplastic space should be degraded, whereas internalized proteins should be protected. While protection from protease degradation is not conclusive of internalization, it provides a good initial test. The ToxA molecule itself provides a powerful tool. ToxA is the first protein shown to contain all of the structural information necessary for internalization and targeting to the chloroplasts of sensitive wheat. Furthermore, ToxA can be modified at the N terminus and still be imported into a cell. This allowed for the development of another tool, GFP-ToxA fusion protein; GFP-ToxA behaves like native ToxA and is easily visualized by microscopy. The study of ToxA function will not only provide insight into understanding the *P. tritici-repentis*–wheat interaction but also has the potential to contribute to our understanding of plasma membrane–cell wall interactions, protein import, and organellar targeting of proteins in plant cells.

METHODS

Methods for plant growth, maintenance, and toxin infiltration have been published elsewhere (Manning et al., 2004). The ToxA-sensitive and -insensitive wheat cultivars used in this study were Katepwa and Auburn, respectively.

PK Treatment of ToxA-Infiltrated Leaves

Six sensitive and insensitive leaves were treated with 0.1 mg/mL of His-ToxA (Tuori et al., 2000) and returned to the growth chamber for 2 h. The same leaf area was subsequently treated with 200 μg/mL of PK (Fisher Scientific) made fresh and returned to the growth chamber for 4 h. To determine the optimal time for PK treatment post-ToxA infiltration, a time course of toxin treatment followed by PK treatment was performed (data not shown). The 2-h time point was selected because PK treatment

2 h post-ToxA treatment results in ToxA-induced necrosis in PK-treated samples that is equivalent to ToxA-induced necrosis without PK treatment. In vitro digestion of ToxA by PK is complete within 5 min of protease treatment (data not shown). The treatment area was infiltrated with deionized distilled water, and a 3-cm zone was removed, cut in half, and centrifuged at 4000*g* for 10 min to remove intercellular wash fluid. Leaves were ground in 4.5 mL of immunoprecipitation extraction buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, and 1% [v/v] Triton X-100) (Boyes et al., 1998) + 1 mM phenylmethylsulfonylfluoride (Sigma-Aldrich) and filtered through 200 μm Nitex. Fifty-microliter Ni-NTA beads (Qiagen) were added to the lysate and incubated 1 h, 4°C, with rotation. Beads were washed three times with 1 mL of TBS-T (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween-20), eluted with 500 mM imidazole (Sigma-Aldrich), boiled 3 min, and run on a 12% SDS-PAGE gel as described by Fling and Gregerson (1986). Proteins were blotted onto nitrocellulose (Osmonics) and blocked overnight in TBS-T/3% nonfat dry milk. Anti-ToxA antibody was used at a 1:10,000 dilution and anti-rabbit-horseradish peroxidase antibody (Sigma-Aldrich) at 1:80,000. Horseradish peroxidase was detected with the SuperSignal West Dura Extended Duration Substrate (Pierce).

Cloning of Fusion Proteins

Standard cloning techniques were used to construct the fusion protein vectors (Sambrook et al., 1989). The N- and C-domains of the cDNA of *ToxA* were subcloned into the multiple cloning site of pET43.1 (Novagen) to create an in-frame *Nus-ToxA* fusion protein expression vector, pCVM43. The *Nus* coding region was removed by digestion with *NdeI* and *SpeI* and replaced by *gfp* amplified by PCR from pCT74 (Lorang et al., 2001) with primers GFP-F1 (5′-GAATATAGcatatgGTAACCAAG-GGC-3′) and GFP-R2 (5′-CCactagtCTTGTACAGCTCGTCCAT-3′) to create the GFP-ToxA fusion protein plasmid, pCM20. The nucleotides shown in lowercase letters are the *NdeI* and *SpeI* restriction sites added to facilitate cloning. The GFP expression construct, pCVM36, was created by removal of the *ToxA* coding region from pCM20.

Protein Production and Purification

For expression in *Escherichia coli*, plasmids were transformed into Origami cells (Novagen). Overnight cultures were diluted at 1:100 and grown for 4.5 h at 37°C in Luria-Bertani media (10 g tryptone, 5 g NaCl, and 5 g yeast extract). Cultures were cooled to room temperature, induced with a 1:50 dilution of the Inducer (Molecula), and grown overnight with shaking at room temperature. Cells were lysed with BugBuster and Benzonase (Novagen). GFP-ToxA (pCM20) was purified from inclusion bodies using the His-Bind purification kit (Novagen) under denaturing conditions as recommended by the manufacturer. The purified protein was refolded using urea step dialysis as previously described (Tuori et al., 2000). GFP (pCVM36) was purified from supernatants using the same kit under nondenaturing conditions following the manufacturer's recommendations. Both proteins were dialyzed against Epure water (Barnstead International). Protein concentration was determined with the Detergent Compatible protein assay kit (Bio-Rad) with BSA as the standard.

Native ToxA was isolated as previously described (Tuori et al., 1995).

In Vivo Detection of GFP Proteins

Sensitive and insensitive leaves were treated with 5 μM GFP-ToxA, GFP alone, native ToxA alone, or 5 μM each of GFP plus native ToxA. Leaves were detached and placed in a humidity chamber in the growth chamber for 6 h, after which leaves were infiltrated with 100 mM MOPS, pH 7.2. Fluorescence on live tissue was observed in at least three independent

samples using a Leica DMBR microscope fitted with an Endow GFP band-pass emission filter (Chroma). For confocal imaging, leaves were fixed by vacuum infiltration of 4% paraformaldehyde in fixation solution (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 4 mM $MgCl_2$, pH 6.9, 0.1% Triton X-100, 2% glycerol, and 5% dimethylsulfoxide) for 15 min, left overnight at 4°C, and washed three times with PBS (150 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, and 3.3 mM KH_2PO_4, pH 7.5). Confocal imaging was performed on two independent samples on a Zeiss LSM 510 Meta microscope using a ×40 water objective.

Immunolocalization

Sensitive and insensitive leaves were treated with water or 5 μM native ToxA for 4 h. Leaves were fixed by vacuum infiltration for 30 min in 4% paraformaldehyde/0.1 M phosphate buffer, pH 7.2, and incubated overnight at 4°C in fixative. Leaves were washed in 0.1 M phosphate buffer as above and then step-dehydrated in acetone at 4°C. Leaves were then embedded in Technovit 7100 (Energy Beam Sciences) + hardener for >12 h, and 4-μm-thin sections were layered onto slides. Sections were blocked for 30 min at room temperature with blocking solution (0.1% Tween-20, 1.5% glycine, 2% BSA, and 3% goat serum in PBS). After blocking, slides were rinsed with PBS wash buffer (0.25% Tween-20 and 0.8% BSA in PBS) followed by PBS. Sections were incubated for 90 min at 37°C in a humidity chamber with anti-ToxA antiserum diluted 1:5 in PBS, washed two times, 10 min each, in high salt PBS (350 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 3.3 mM KH_2PO_4, pH 7.5, 0.25% Tween-20, and 0.1% BSA), and rinsed with PBS wash buffer followed by PBS. Sections were further incubated with 1:500 Alexa-488 in PBS for 1 h at 37°C in a humidity chamber, washed two times, 10 min each with PBS, rinsed with running distilled water for 15 min, then allowed to dry and cover slipped with Cytoseal 60 (Stephens Scientific).

Biolistics

The ToxA expression plasmid pCVM63 was constructed by removing the *uidA* gene from pBI505 (Warkentin et al., 1992) and replacing it with the N- and C-domains of *ToxA*. One microgram each of pBluescript/pBI505 or pCVM63/pBI505 was used to coat 1.6-μM gold beads following the manufacturer's instructions (Bio-Rad). Two different preparations of plasmid were used to control for the possibility of an effect of the plasmid preparation on expression. Beads were spotted onto 1100-p.s.i. rupture disks and fitted into the PDS-1000/He system (Bio-Rad) 9 cm above the target. Ten- to twelve-day-old leaves were cut to 7-cm lengths, sterilized 30 s in 10% bleach, and rinsed two times with sterile water. Eight to ten leaves were placed adaxial side up onto prewet (sterile water) filter paper and aligned so that maximum surface area could be bombarded. Leaves were bombarded in a vacuum of 27 inches Hg and removed to a new sterile Petri dish onto prewet filter paper, and dishes were parafilmed and placed at 25°C with constant light. After 48 h, leaves were stained for GUS expression (Rueb and Hensgens, 1989). The average number of dots per leaf was calculated for each experiment by counting the number of dots per bombardment and dividing by the number of leaves bombarded. Four independent experimental averages were used to calculate average dots/ leaf and standard error.

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Localization of Ptr ToxA Produced by *Pyrenophora tritici-repentis* Reveals Protein Import into Wheat Mesophyll Cells

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