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Short communication

Requirement of host signaling mechanisms for the action of Ptr ToxA in wheat

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Abstract

Ptr ToxA, the host-selective toxin produced by *Pyrenophora tritici-repentis*, is genetically associated with the development of tan spot disease of wheat. The toxin was shown previously to cause a programmed cell death in the host that requires *de novo* mRNA and protein synthesis. In the present study, inhibitors of plant signaling mechanisms protected wheat leaves from toxin action, as determined by electrolyte leakage bioassays, when applied to leaves with toxin. Okadaic acid, calyculin A and phenylarsine oxide, all inhibitors of protein phosphatase activity, reduced toxin-induced electrolyte leakage by more than 90%. Inorganic calcium channel blockers ($LaCl_3$ and $CoCl_2$) reduced toxin-induced electrolyte leakage by 78–95%, depending on inhibitor and time of measurement. By comparison, about 50% protection was achieved by the application of the protein kinase inhibitors staurosporine and K-252A. Nonetheless, the reduction in toxin-induced electrolyte leakage by protein kinase inhibitors was reproduced in multiple trials and was statistically significant. The data indicate that host signaling mechanisms, including calcium fluxes and a protein phosphorylation cascade, are required for the Ptr ToxA-induced cell death in wheat. Our current model holds that the signaling events occur between toxin perception by the cell and the toxin-directed gene expression in the host associated with cell death. As an alternative, the toxin-induced mRNA synthesis required for cell death may be for protein phosphatase and/or protein kinase genes. Additional work is required to resolve these possibilities.

Races 1 and 2 of *Pyrenophora tritici-repentis*, causal agent of tan spot of wheat (*Triticum aestivum*), produce Ptr ToxA, a host-selective toxin associated with disease development (Ciuffetti et al., 1998). Host perception of Ptr ToxA, apparently mediated through receptor binding (Meinhardt et al., 2002), results in a programmed cell death that requires the *de novo* synthesis of host mRNA and protein (Kwon et al., 1998). Our model for toxin action predicts the need for host signaling mechanisms, such as calcium influx and protein phosphorylation and dephosphorylation events, to bridge the gap between toxin perception and gene expression during toxin action (Kwon et al., 1998). The current work uses inhibitors of signaling mechanisms to test this hypothesis.

Ptr ToxA, a 13.2 kDa protein, was obtained from strain 86–124 (Zhang et al., 1997). The electrolyte leakage bioassay of Kwon *et al.* (1998) and seedlings of the toxin-sensitive wheat line ND495 were used throughout. Electrolyte leakage has been directly associated with toxin sensitivity (Friesen et al., 2002). The loss of host plasmalemma function associated with electrolyte leakage is thought to occur late in the process of toxin action and be a consequence rather than a cause of cell death. The bioassay has been useful for other mechanistic studies for Ptr ToxA (Meinhardt et al., 2002; Kwon et al., 1998) and currently is the only published quantitative bioassay for Ptr ToxA.

Each treatment used 10 seedlings divided into two replicates of five seedlings. The second leaf of each seedling was infiltrated with purified Ptr ToxA ($10\,\mu g\,ml^{-1}$), inhibitor only (each obtained from Sigma Corp., St. Louis, MO, USA), toxin and inhibitor together or water. Infiltrated seedlings were incubated

for 8 h at room temperature (21–23 °C). Leaf sections (2.5 cm) were obtained from the infiltrated region of the second leaf (Kwon et al., 1998) and placed in distilled water (15 ml) to begin a period during which electrolytes collected in the ambient solution. The conductivity of the water was determined at the beginning of the leaching period (time point zero in the data set) and periodically thereafter. Data are the mean and SD of the two replicates. Percent inhibition was determined by the formula: $[1-(C_{\text{toxin+inhibitor}}-C_{\text{Water}})/(C_{\text{Toxin}}-C_{\text{Water}})]\times 100$ where C = the conductivity value at the time point in question. Significant differences between treatments was determined by one way ANOVA and LSD mean separation tests. Data are from a single representative experiment that was repeated at least two additional times with similar results.

The inorganic calcium channel blocker $LaCl_3$ (5 μM) reduced the level of electrolyte leakage when co-infiltrated with toxin (Figure 1). Complete protection was observed 1 h into the leaching period, but the level of protection gradually decreased to 82% after 6 h. Electrolyte leakage from leaves exposed to toxin plus $LaCl_3$ was significantly less than from leaves that received toxin only at each time point. The gradual loss of protection from toxin action offered by the one-time application of other biochemical inhibitors has been observed previously (Kwon et al., 1998; Meinhardt et al., 2002). Another calcium channel blocker, $CoCl_2$ (0.5 M) gave 78% protection after 6 h of leaching (data not shown).

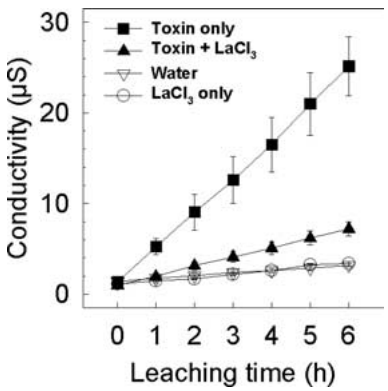

Figure 1. Time course of electrolyte leakage from wheat leaves infiltrated with water, $LaCl_3$ (5 μM), Ptr ToxA (10 μg ml^{-1}), or toxin and $LaCl_3$ together. After infiltration with experimental solutions and incubation for 8 h, leaf sections were placed in distilled water and the conductivity of the ambient solution was measured (Leaching time = 0) and at hourly intervals thereafter. Data are average and SD of two replicates.

Nearly complete protection was observed with calyculin A (1 μM) and okadaic acid (2 μM), both inhibitors of protein phosphatases (Table 1). In another experiment, the phosphatase inhibitor phenylarsine oxide (1 μM) gave 93% protection under identical conditions (data not shown). By comparison, only 49% protection was achieved by the co-application of the protein kinase inhibitor staurosporine (1 μM) (Figure 2). Although this level of protection was less than observed with calcium channel blockers and inhibitors of protein phosphatase, the results were reproduced in multiple trials and differences between treatments were statistically significant (Figure 2). K-252a (5 μM), another protein kinase inhibitor, also

Table 1. Inhibition of toxin-induced electrolyte leakage by the co-infiltration of the protein phosphatase inhibitors okadaic acid (0.2 μM) and calyculin A (1 μM). Wheat leaves were exposed to experimental solutions for 8 h after infiltration. Leaching time was 4 h. Conductivity values followed by a different letter were significantly different ($P = 0.05$)

Treatment	Conductivity (μS)
Water	6.0 ± 0.5 a
Okadaic acid	6.2 ± 0.5 a
Calyculin A	6.8 ± 0.6 a
Okadaic acid + toxin	6.8 ± 1.0 a
Calyculin A + toxin	6.8 ± 1.0 a
Toxin only	22.9 ± 2.7 b

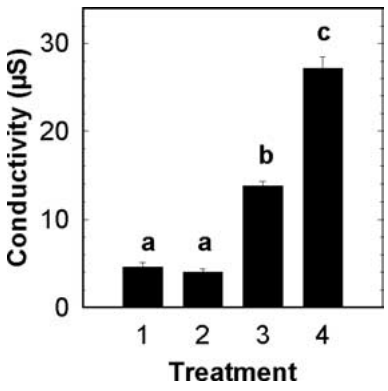

Figure 2. Effect of the protein kinase inhibitor staurosporine on toxin-induced electrolyte leakage. Data are mean and SD conductivity values for wheat leaves infiltrated with water (treatment 1), 1 μM staurosporine (treatment 2), staurosporine + Ptr ToxA (10 μg ml^{-1}) together (treatment 3) or toxin only (treatment 4). Exposure to experimental solutions was for 8 h and the leaching period was 4 h. Different letters above the bars indicate significant differences ($P = 0.05$).

gave statistically-significant protection in the 40–60% range in multiple, independent experiments (data not shown).

Experiments that use metabolic inhibitors always need to be viewed with caution because of the potential for secondary, nonspecific effects. However, taken together, the data presented here indicate the requirement for calcium signaling and activity of host protein kinase and phosphatase for cell death of wheat in response to Ptr ToxA. It seems likely the host signaling occurs between toxin perception and gene expression associated with toxin action (Kwon et al., 1998), although there are no direct data to support this conclusion. It is also possible that gene expression involved in toxin action may actually direct the *de novo* synthesis of a protein phosphatase or kinase that is itself part of the signal transduction machinery for cell death.

The inorganic calcium channel blockers (i.e., $CoCl_2$ and $LaCl_3$) and inhibitors of protein phosphatase 2A (i.e., okadaic acid, calyculin A and phenylarsine oxide) gave nearly complete protection from Ptr ToxA. This suggests that calcium and protein phosphatase activity may have early or critical roles in toxin action. Amino acids 140–142 of Ptr ToxA form an RGD tripeptide motif postulated to interact with the host receptor (Meinhardt et al., 2002). In animal systems, protein binding to receptors known as integrins occurs to through the RGD motif. The signal for integrin binding, which can regulate programmed cell death, involves calcium fluxes and protein phosphatase as an early step (Giancotti and Ruoslahti 1999). The wheat receptor for Ptr ToxA is not known but the data presented here and elsewhere (Meinhardt et al., 2002) suggest possible similarities to integrin binding in animals.

The data suggest a multi-component signaling cascade in the host associated with the action of Ptr ToxA. The order of events in this putative signaling sequence currently are not known, but testable hypotheses are clear. For example, calcium influx is known to be a secondary messenger that activates various processes in plants, including protein kinases (Hunter, 1995). Staurosporine and K-252A, the two protein kinase inhibitors tested, affect calcium-dependent protein kinases. Thus, calcium influx may precede and activate protein kinase activity. Also, the identity of specific protein kinases and phosphatase and proteins affected by the activities are not known. All these questions, along with the search for the toxin receptor, should be the focus of future research.

Acknowledgements

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