Tsn1*-Mediated Host Responses to ToxA from *Pyrenophora tritici-repentis

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Submitted 20 April 2009. Accepted 19 May 2009.

The toxin sensitivity gene *Tsn1* interacts with Ptr ToxA (ToxA), a host-selective toxin produced by the necrotrophic fungus *Pyrenophora tritici-repentis*. The molecular mechanisms associated with cell death in sensitive wheat cultivars following ToxA application are not well understood. To address this question, we used the Affymetrix GeneChip Wheat Genome Array to compare gene expression in a sensitive wheat cultivar possessing the *Tsn1* gene with the insensitive wheat cv. Nec103, which lacks the *Tsn1* gene. This analysis was performed at early timepoints after infiltration with ToxA (e.g., 0.5 to 12 h postinfiltration [hpi]); at this time, ToxA is known to internalize into mesophyll cells without visible cell death symptoms. Gene expression also was monitored at later timepoints (24 to 48 hpi), when ToxA causes extensive damage in cellular compartments and visible cell death. At both early and late timepoints, numerous defense-related genes were induced (2- to 197-fold increases) and included genes involved in the phenylpropanoid pathway, lignification, and the production of reactive oxygen species (ROS). Furthermore, a subset of host genes functioning in signal transduction, metabolism, and as transcription factors was induced as a consequence of the *Tsn1*-ToxA interaction. Nine genes known to be involved in the host defense response and signaling pathways were selected for analysis by quantitative real-time polymerase chain reaction, and the expression profiles of these genes confirmed the results obtained in microarray experiments. Histochemical analyses of a sensitive wheat cultivar showed that H₂O₂ was present in leaves undergoing cell death, indicating that ROS signaling is a major event involved in ToxA-mediated cell death. The results suggest that recognition of ToxA via *Tsn1* triggers transcriptional reprogramming events similar to those reported for avirulence-resistance gene interactions, and that host-derived genes play an important role in the modulation of susceptibility to *P. tritici-repentis*.

Tan spot, caused by *Pyrenophora tritici-repentis* (anamorph: *Drechslera tritici-repentis* (Died) Shoemaker), is a devastating disease in wheat (*Triticum aestivum* L.). When *P. tritici-repentis* infects susceptible wheat lines, necrotic or chlorotic symp-

toms are induced due to the production of host-selective toxins (Lamari and Bernier 1989; Strelkov et al. 1999; Tomàs et al. 1990; Tuori et al. 1995). Eight pathogenic races of *P. tritici-repentis* have been reported based on their interaction with a set of differential wheat cultivars (Lamari and Bernier 1989; Strelkov and Lamari 2003). Race 1 of *P. tritici-repentis* is predominant worldwide (Adhikari et al. 2008) and produces both Ptr ToxA and Ptr ToxC (Ciuffetti and Tuori 1999; Effertz et al. 2002; Strelkov and Lamari 2003; Zhang et al. 1997).

Ptr ToxA (ToxA) (Ciuffetti et al. 1998) is a 13.2-kDa protein (Ballance et al. 1996; Ciuffetti et al. 1997) that induces cell death in sensitive wheat cultivars (Kwon et al. 1998; Manning et al. 2004; Meinhardt et al. 2002). It has been suggested that *P. tritici-repentis* acquired the ToxA gene from *Stagonospora nodorum* (Friesen et al. 2006), which causes leaf blotch in wheat. In wheat tissue exposed to ToxA, rapid damage to the plasmalemma was detected by transmission electron microscopy (Freeman et al. 1995), and exposure to ToxA for ≥4 h enhanced electrolyte leakage in a sensitive wheat cultivar (Kwon et al. 1998). Recent studies indicate that ToxA shares similarity with the mammalian protein vitronectin (Manning et al. 2004; Suzuki et al. 1985). Furthermore, the vitronectin-like sequence of ToxA (Manning et al. 2004) contains a three amino acid arginyl-glycyl-aspartic (RGD) cell-attachment motif that is necessary for toxin-induced cell death in sensitive wheat (Manning et al. 2004; Meinhardt et al. 2002). ToxA is internalized into the mesophyll cells and localizes to the chloroplast of sensitive but not resistant lines of wheat (Manning and Ciuffetti 2005; Manning et al. 2008). In addition, ToxA interacts with a chloroplast protein, designated ToxA-binding protein 1 (ToxABP1), suggesting that the chloroplast is a possible target for ToxA (Manning and Ciuffetti 2007; Sarma et al. 2005). Pharmacological investigations indicated that calcium signaling and protein phosphorylation were necessary for ToxA action (Meinhardt et al. 2002; Rasmussen et al. 2004). Sensitivity to ToxA is controlled by a single locus designated *Tsn1* on chromosome 5BL in wheat (Anderson et al. 1999; Faris et al. 1996; Lamari and Bernier 1989). Map-based cloning of *Tsn1* is in progress, and one of the candidate genes encodes a nucleotide-binding site with a leucine-rich repeat (NBS-LRR) protein (Faris et al. 2008). The interaction between *Tsn1* in wheat (Anderson et al. 1999; Faris et al. 1996; Lamari and Bernier 1989) and ToxA in the fungus (Ciuffetti et al. 1997) follows an inverse gene-for-gene model (Lamari et al. 2002; Wolpert et al. 2002).

Gene expression studies have significantly advanced our understanding of the host defense responses that occur in plant-

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*The e-Xtra logo stands for “electronic extra” and indicates that five supplementary tables are published online.

pathogen interactions. Microarray analysis has provided global gene expression profiling and identified genes of interest in both compatible and incompatible plant–pathogen interactions. The Affymetrix GeneChip Wheat Genome Array has been used to monitor gene expression profiling during the infection of wheat by *Puccinia tritici* (Bolton et al. 2008; Hulbert et al. 2007) and *P. striiformis* (Coram et al. 2007). Although knowledge of the genetics of resistance and molecular mapping has progressed by the identification and characterization of ToxA (Tomás et al. 1990; Tuori et al. 1995; Zhang et al. 1997), information regarding the molecular processes altered during the *Tsn1*–ToxA interaction is limited.

In a classical gene-for-gene model (Flor 1971), resistance (*R*) genes directly or indirectly interact with pathogen effectors (avirulence [*Avr*] genes) (Abramovitch et al. 2006; Hammond-Kosack and Jones 1996; Martin et al. 2003). Activation of defense responses in *R*-gene-mediated resistance is coordinated by transcriptional reprogramming (Eulgem 2005). Such defense responses include a localized form of plant programmed cell death (PCD), changes in plasma membrane integrity (e.g., influx of cellular Ca^{2+} and H^{+} and efflux of K^{+} and Cl^{-}), production of reactive oxygen species (ROS), alternation of gene expression, and activation of mitogen-activated protein kinases (MAPK) (Abramovitch et al. 2006; Lamb and Dixon 1997; Martin et al. 2003). We hypothesize that recognition of ToxA via *Tsn1* may activate important genes involved in both the host defense response and signaling pathways. This hypothesis was addressed in the present study by using the Affymetrix GeneChip Wheat Genome Array to investigate cellular and molecular processes elicited in response to ToxA in the sensitive wheat cv. Kulm (with *Tsn1*) and in the insensitive wheat cv. Nec103 (without *Tsn1*). To confirm the transcriptome results, quantitative real-time polymerase chain reaction (qPCR) was used to compare the expression patterns of selected defense response and signal transduction genes in the compatible *Tsn1*–ToxA interaction. In addition, histochemical procedures were performed to detect cell death and H_2O_2 production in ToxA-sensitive and -insensitive wheat. To our knowledge, this study is the first to use global gene expression profiling to examine *Tsn1*-mediated host responses to ToxA.

RESULTS

Induction of cell death in sensitive wheat in response to ToxA.

A visible water-soaked region was observed in the leaves of the sensitive wheat cv. Kulm exposed to ToxA at 12 h postinfiltration (hpi) (data not shown). Symptoms expanded within the ToxA-infiltrated areas, and cell death was clearly visible by 48 hpi (Fig. 1A). In contrast, no cell death was observed in the insensitive wheat cv. Nec103, even at 48 hpi (Fig. 1B). As expected, plants inoculated with water (mock

Fig. 1. Phenotype of ToxA-sensitive and -insensitive wheat cultivars. **A**, Cell death induced by ToxA in the sensitive wheat cv. Kulm (with *Tsn1*) and **B**, absence of cell death in the ToxA-insensitive wheat cv. Nec103 (without *Tsn1*); an arrow indicates where the toxin was infiltrated. Two-week-old seedlings were infiltrated with ToxA (10 $\mu\text{g}/\text{ml}$) from *Pyrenophora tritici-repentis* and photographed at 48 h postinfiltration.

inoculated) showed no cell death at any of the timepoints (data not shown).

Identification and classification of differentially expressed genes in sensitive wheat in response to ToxA.

To identify significantly expressed genes during the *Tsn1*–ToxA interaction, we used two selection criteria: i) genes considered biologically significant had to exhibit at least a twofold change in expression, and ii) the probability (*P*) value had to be ≤ 0.05 for specific pairwise comparison of treatments. Using these criteria, we predicted a false detection rate $< 1\%$ for each timepoint. At 0.5 h after ToxA infiltration, 178 genes were upregulated and 74 were downregulated; similarly, at 4 hpi, 304 genes were upregulated and 78 were downregulated (Fig. 2). At 12 hpi, the ratio changed, with 238 genes induced and 433 genes repressed. At 24 hpi, 385 genes were upregulated and 6 genes were downregulated; and, at 48 hpi, 1,101 genes were upregulated and 78 were downregulated (Fig. 2).

Cluster analysis showed two patterns of differentially expressed genes (Fig. 3). Those genes with significantly altered mitochondrial (m)RNA levels from 0.5 to 12 hpi were categorized as early-response genes (Fig. 3A, B, and C), whereas those genes induced between 24 and 48 hpi were considered late-response genes (Fig. 3D and E). To identify *Tsn1*-mediated molecular responses to ToxA, we used the Munich Information Center for Protein Sequences Gene Ontology (MIPS GO) descriptions (MIPS *Arabidopsis thaliana* database) to annotate proteins encoded by differentially expressed genes and classified these into 16 GO biological processes (Fig. 4). At all timepoints, approximately 60% of the up- and down-regulated genes had unknown functions. Nearly 17% of the genes were annotated as transcribing proteins with unknown function. Mean overall timepoints in the categories of plant defense response, metabolism, energy, and signal transduction were 5.6, 5.3, 3.0, and 2.3%, respectively (Fig. 4). The remaining categories were poorly represented.

Fig. 2. Differential gene expression in the sensitive wheat cv. Kulm compared with the insensitive wheat cv. Nec103 after infiltration with ToxA (10 $\mu\text{g}/\text{ml}$) from *Pyrenophora tritici-repentis*. Total RNA was extracted from leaves at 0.5, 4, 12, 24, and 48 h postinfiltration and used for microarray analysis. Genes were considered differentially expressed if their fold change was ≥ 2 with $P \leq 0.05$. Cross-hatched bars represent the number of genes where transcription was significantly increased and solid bars indicate a decrease in RNA abundance.

Induction of defense response and signal transduction genes during the compatible *Tsn1*–ToxA interaction.

A large number of genes modulated by ToxA were classified as defense related (Table 1). Approximately 154 upregulated and 36 downregulated genes are involved in defense at multiple timepoints after ToxA infiltration; these included genes encoding pathogenesis-related (PR) proteins, ROS-related enzymes (e.g., glutathione transferase, glutathione S-transferase, lipoxy-

Fig. 3. Cluster analysis showing differentially expressed genes in the toxin-sensitive wheat cv. Kulm compared with the insensitive wheat cv. Nec103 at 0.5, 4, 12, 24, and 48 h after infiltration with ToxA (10 µg/ml) from *Pyrenophora tritici-repentis*. The discriminators $\log_{10} (P \leq 0.05)$ and log twofold change are indicated by horizontal and vertical green lines, respectively.

genase, and peroxidase), cell-wall-modifying enzymes (chitinase and β -1,3-endoglucanase) (Table 1), and stress-related genes (germin-like protein, heat shock protein, osmotin, proline-rich protein, and thaumatin-like protein) (Supplementary Table S2). Interestingly, several genes involved in the phenylpropanoid pathway were induced, including chalcone synthase (*CHS*), lipoxigenase (*LOX*), and phenylalanine ammonia lyase (*PAL*). Genes repressed at early timepoints were amine oxidase (Ta.6420.2.S1_a_at), glutathione synthase (Ta.14856.1.S1_at), and peroxidases (Ta.27461.1.S1_a_at and Ta.12587.1.S1_at).

A significant number of genes encoding protein kinases (e.g., casein kinases, receptor-like protein kinases, and serine threonine kinases) and phosphatases also were induced at both early and late timepoints (Table 2). An ortholog (Ta.30053.1.S1_at) of Pto kinase was activated at 48 hpi, and genes encoding calcium-binding proteins and interactors (e.g., calmodulin and calmodulin-transporting ATPase) were upregulated at later timepoints. One of the signaling proteins, calmodulin (Ta.1018.2S1_x_at), was constitutively expressed in this study (Table 2). Several genes encoding proteins in the receptor kinase protein family were downregulated at early timepoints.

Activation of genes related to metabolism, energy, transcription factors, and cellular transporters during the compatible *Tsn1*–ToxA interaction.

Among genes related to energy and chloroplast biogenesis, 71 were upregulated and 26 were downregulated across all timepoints (Supplementary Table S3). Genes encoding chloroplast functions such as ribulosebiphosphate carboxylase (Ta.2752.1.S1_x_at) and mitochondrial precursors (e.g., Ta.7715.1.S1_at) were downregulated at 12 hpi. Among transcription factors, 24 upregulated and 5 downregulated genes were identified across all timepoints (Supplementary Table S4). The two major transcription factors identified in this study were Myb and zinc finger proteins. Seven ABC transporter genes were upregulated at the later stages of cell death (Supplementary Table S5).

Expression of defense response and signaling genes using qPCR analysis.

We used qPCR analysis to validate the expression of six genes associated with the plant defense response, including chitinase (*CHI*), β -1,3-glucanase (*PR-2*), glutathione transferase (*GST*), *PAL*, peroxidase (*PER*), and NADPH oxidase (*NAD*). Three genes from established signal transduction pathways also were analyzed by qPCR, including calmodulin (*CaM*), receptor-like kinase (*RLK*), and serine threonine protein kinase (*PK*). The expression of each gene was examined after the accumulation of mRNAs in a toxin-sensitive and a toxin-insensitive wheat cultivar at 0, 4, 8, 12, 14, and 48 h after ToxA application. The relative fold changes in the wheat cultivars were compared with a mock inoculation (H_2O) at each timepoint. Among the six genes from the defense response category, *CHI* displayed the highest induction in the ToxA-sensitive wheat cv. Kulm at 12 hpi (Fig. 5A). An increase in the expression of *PR-2* was first observed at 8 hpi (threefold) in Kulm but did not show maximal induction (sevenfold) until 48 hpi (Fig. 5B). Although there was a twofold induction of *GST* in Kulm at 4 hpi, induction was substantially higher (sevenfold) at 48 hpi (Fig. 5C). *PAL* was induced across all timepoints in cv. Kulm, with maximal expression at 48 hpi (Fig. 5D). *PAL* also was induced (approximately fourfold) in the ToxA-insensitive Nec103 at 48 hpi (Fig. 5D). The *PER* gene was induced at 8 and 12 hpi (16-fold) in the ToxA-sensitive cv. Kulm; a slight induction of *PER* (two to threefold) also was observed at 24 hpi in Nec103 (Fig. 5E). Maximum induction (13-fold) of

NAD occurred in Kulm at 48 hpi (Fig. 5F), confirming that *NAD* was expressed during the later stages of cell death.

The signaling gene *CaM* was differentially induced in cv. Kulm at 4, 12, 24, and 48 hpi, with the highest induction (18-fold) at 48 hpi (Fig. 5G). *RLK* was the most highly expressed gene in Kulm (50-fold increase at 48 hpi) and was not induced in the ToxA-insensitive Nec103 (Fig. 5H). The highest level of *PK* induction was observed in the ToxA-sensitive Kulm at 48 hpi, with little to no induction in the ToxA-insensitive Nec103 (Fig. 5I). Overall, gene expression patterns determined by qPCR analysis were consistent and correlated with microarray analysis ($r = 0.914$, data not shown).

Measurement of cell death using Evans blue.

The survival of plant cells can be examined by staining tissue sections in a solution of Evans blue (Baker and Mock 1994). Viable cells exclude Evans blue at the plasma membrane and remain unstained, whereas dead cells are unable to exclude Evans blue and stain deep blue (Baker and Mock

1994). Previous investigations reported that a rapid loss of host cell membrane integrity occurs during cell death and can be monitored via electrolyte leakage assays (Meinhardt et al. 2002; Rasmussen et al. 2004). Thus, the relationship between cell death and dye uptake was monitored in the *Tsn1*-ToxA interaction using a spectrophotometer and by microscopic examination at early (12 hpi) and late (48 hpi) stages after ToxA infiltration. At 12 hpi, Evans blue uptake was higher in the ToxA-sensitive Kulm compared with the insensitive cv. Nec103 (Fig. 6A). Cell death measured by microscopy was higher in cv. Kulm compared with Nec103 (Fig. 6B). At 48 hpi, the estimated cell death by both methods (Evans blue uptake and microscopic evaluation) was more correlated in sensitive wheat than insensitive wheat and was significantly ($P \leq 0.01$) different between two wheat cultivars (Fig. 6A and B).

Detection of H_2O_2 using 3,3'-diaminobenzidine.

Staining with 3,3'-diaminobenzidine (DAB) is commonly used to detect H_2O_2 production during plant-pathogen interac-

Fig. 4. Pie charts showing classification of functional category genes that were differentially expressed at 0.5, 4, 12, 24, and 48 h after infiltration of ToxA (10 µg/ml) from *Pyrenophora tritici-repentis*. Each gene was categorized to a functional class according to annotations assigned by MIPS (MIPS *Arabidopsis thaliana* Database) as described previously (Keon et al. 2007). Values represent the percentage of differentially expressed genes (fold-change values > 2.0) after infiltration of the toxin-sensitive wheat cv. Kulm compared with the insensitive wheat line Nec103. Values <0.5% in some functional categories are not shown.

tions (Thordal-Christensen et al. 1997). Wheat segments infiltrated with water (control) or ToxA were collected from both sensitive and insensitive wheat cultivars at 4, 12, 24, and 48 hpi. H₂O₂ was not detected in leaf segments of Kulm or Nec103 when infiltrated with water (Fig. 7A through H). Similarly, no difference in H₂O₂ production in mesophyll cells was observed between sensitive and insensitive wheat cultivars at 4 hpi (Fig. 7I and M). DAB staining occurred in mesophyll cells undergoing ToxA-elicited cell death in the sensitive wheat cv. Kulm at 12 hpi (Fig. 7J). Extensive staining was detected at the stomatal aperture or near the periphery of guard cells in Kulm treated with ToxA (Fig. 7K), indicating the effect of DAB polymerization. ToxA-induced cell death became necrotic in Kulm, particularly at 48 hpi (Fig. 7L). In contrast, H₂O₂ was not detected in the ToxA-insensitive wheat cv. Nec103 (Fig. 7M through P), which agrees with the absence of cell death. These results suggest a strong correlation between H₂O₂ production and the ability of ToxA to induce cell death in the ToxA-sensitive Kulm.

DISCUSSION

A combination of Affymetrix GeneChip Wheat Genome Array, qPCR assays, and histochemical methods was used to determine *Tsn1*-mediated host responses to ToxA. Aims of the present study were to determine what genes are induced in response to ToxA, and to compare ToxA-induced cell death in

wheat to other plant-pathogen interactions. We show that the fungal virulence effector ToxA can induce cell death by altering host gene expression and cell physiology. Our data provide a conceptual framework for understanding the role of ToxA in modulating *Tsn1*-mediated defense and fungal pathogenesis. To our knowledge, induction of host cell death by ToxA follows mechanisms similar to *Avr-R* gene model systems.

Many of the genes induced as a consequence of the *Tsn1*-ToxA interaction allowed us to make comparisons with other compatible interactions (Restrepo et al. 2005; Thilmony et al. 2006). The numbers of differentially expressed genes were related to proteins and enzymes associated with defense, signal transduction, secondary metabolism, energy, transcription factors, and ATP-binding cassette (ABC) transporters. Our microarray analysis identified the expression patterns of several hundred genes in the *Tsn1*-ToxA interaction, and one of the challenges was how to utilize these large data sets. Thus, we chose two major functional gene categories that are modulated in the *Tsn1*-ToxA interaction (e.g., defense and signal transduction) (Tables 1 and 2).

In this study, Affymetrix GeneChip Wheat genome arrays identified numerous genes that were differentially expressed in the *Tsn1*-ToxA interaction, including genes encoding PR proteins associated with secondary metabolism and cell wall modifications (e.g., *PR-1*, *PR-2*, *PR-5*, and *PR10*) and the phenylpropanoid pathway (e.g., *CHS*, *LOX*, and *PAL*). The expression patterns of selected genes were confirmed in an independent

Table 1. Differentially expressed representative genes involved in plant defense response revealed by microarray analysis of a toxin-sensitive wheat cultivar versus an insensitive wheat cultivar infiltrated with ToxA from *Pyrenophora tritici-repentis*

Probe set ^a	Putative function	Time (h)	Fold change ^b	P value
Ta.14946.1.S1_at	Basic chitinase	48	4.1	1.64E-05
Ta.26983.1.A1_at	Basic chitinase	12	0.3	0.041
Ta.26983.1.A1_at	Basic chitinase	48	3.2	0.001
Ta.30501.1.S1_at	Basic chitinase	24	3.2	0.029
		48	5.5	0.001
Ta.2784.1.A1_at	Chitinase 1 /F	24	3	0.044
		48	4.7	0.009
Ta.21342.1.S1_x_at	Chitinase 3	24	13	0.010
		48	15	6.72E-05
Ta.221.1.S1_at	Chitinase II precursor	48	14.9	7.45E-06
Ta.1830.2.S1_x_at	Glutathione S-transferase	48	2.4	0.041
Ta.3118.1.S1_at	Glutathione S-transferase	24	4.2	0.014
		48	2	0.001
Ta.25377.1.S1_at	Glutathione S-transferase	48	4.9	0.001
Ta.3118.1.S1_at	Glutathione S-transferase	24	4.2	0.014
Ta.25838.1.A1_at	Glutathione S-transferase	12	5.6	0.046
Ta.14856.1.S1_at	Glutathione synthase	0.5	0.4	0.039
Ta.28354.1.S1_at	Glutathione transferase	48	2	0.001
Ta.3679.1.S1_x_at	Glutathione transferase	24	7	0.008
		48	4	0.006
Ta.278.1.S1_at	Pathogenesis-related protein 1	24	18.1	0.008
		48	50.1	5.01E-06
Ta.278.1.S1_x_at	Pathogenesis-related protein 1	24	4	0.002
		48	45.2	1.01E-05
Ta.8304.1.S1_a_at	Pathogenesis-related protein 1	24	10.9	0.010
Ta.30739.2.S1_at	Pathogenesis-related protein	48	40	2.20E-05
Ta.14010.3.S1_x_at	Peroxidase ATP19a	4	5.1	0.041
Ta.24015.1.A1_at	Peroxidase ATP23a	12	0.5	0.017
		48	3.7	0.015
Ta.12587.1.S1_at	Peroxidase ATP2a	0.5	0.2	0.021
Ta.21307.2.S1_x_at	Peroxidase ATP4a	4	4.8	0.035
		48	2.2	0.012
Ta.29496.1.S1_x_at	Peroxidase ATP4a	48	5.2	0.001
Ta.30028.1.S1_x_at	Peroxidase ATP4a	48	2.2	0.007
Ta.5235.1.S1_x_at	Peroxidase ATP4a	48	7.7	0.005
Ta.29603.1.A1_at	Peroxidase N	48	8.6	0.029

(continued on next page)

^a Blank indicates that probe induced more than one time point.

^b Genes were considered to be differentially expressed if their fold change was >2 with $P \leq 0.05$.

biological experiment by qPCR analysis and found to be highly correlated ($r = 0.914$) between microarray gene expression and qPCR gene expression. A previous study (Bolton et al. 2008) has also shown a high concordance between these two analyses. Consistent with our microarray analysis, most of these genes were induced as early as 4 hpi in sensitive wheat cv. Kulm infiltrated with ToxA. Local and systemic accumulation of PR proteins such as *PR-1*, *PR-2*, and *PR-5* along with activation of the phenylpropanoid pathway have been implicated in plants exhibiting a hypersensitive response to fungal and bacterial pathogens (Balaji et al. 2008; Martin et al. 2003; Venisse et al. 2002). In addition, several comparative studies have shown marked differences in the temporal and spatial expression patterns of defense genes in compatible and incompatible plant-pathogen interactions (Joosten and de Wit 1989; Venisse et al. 2002). Genes encoding *CHS*, cinnamoyl-CoA reductases, *LOX*, *PAL*, and cytochrome P450 hydroxylases, which are involved in the phenylpropanoid pathway and electron transport, were induced in the *Tsn1*-ToxA interaction in the present study (Fig. 8). These enzymes are important in the production of secondary metabolites that are necessary for lignin biosynthesis during cell wall thickening (Hahlbrock and Scheel 1989). In addition, defense-related secondary metabolites were previously shown to be generated from the hydroxylation of phenylalanine from cytochrome P450 hydroxylases (La Camera et al. 2004). In soybean, temporal changes in the expression of genes in the phenylpropanoid pathway were ob-

served in both compatible and incompatible interactions (Zabala et al. 2006). In our study, numerous cytochrome P450 hydroxylases and ABC transporters were induced in the *Tsn1*-ToxA interaction. These results may indicate a role for these enzymes and transporters in producing defense-related secondary metabolites, and for transporting and utilizing these metabolites during the necrotrophic fungal lifestyle and pathogenesis.

Several lines of evidence suggest that ROS play important roles in the activation of plant defense via the reinforcement of cell walls (Bradley et al. 1992), the regulation of defense genes (Levine et al. 1994), and the production of toxic free radicals (Brisson et al. 1994). The production of ROS during plant-pathogen interactions is one of the earliest cellular responses (Lamb and Dixon 1997). Potential enzyme sources for ROS include plasma membrane-located NADPH oxidase (Desikan et al. 2001), cell-wall-bound peroxidases, and amine oxidase (Desikan et al. 2001; Lamb and Dixon 1997). In this study, several anionic peroxidases, cationic peroxidases, and an NADPH oxidase were induced in the *Tsn1*-ToxA interaction. These membrane-bound enzymes may be major sources for ROS production, thus leading to cell death in ToxA-sensitive wheat. Several ROS-related enzymes, including germins, glutathione S-transferases, and a set of ROS marker genes (encoding heat shock protein, dirigent protein, HSP90-like protein, proline-rich protein, thaumatin-like protein, and osmotin), also were induced in sensitive wheat cultivars across all timepoints. Comparison of these results with previous findings (Dowd et

Table 1. (continued from preceding page)

Probe set ^a	Putative function	Time (h)	Fold change ^b	P value
Ta.20985.1.A1_at	Peroxidase	12	9.1	0.038
Ta.12472.1.S1_x_at	Peroxidase	4	2.2	0.046
Ta.13307.3.S1_at	Peroxidase	48	2.7	0.013
Ta.18560.1.S1_s_at	Peroxidase	48	3.5	0.006
Ta.21505.1.S1_at	Peroxidase	48	2.2	0.005
Ta.22564.1.S1_at	Peroxidase	24	5.3	0.016
		48	15.8	0.003
Ta.22564.2.S1_a_at	Peroxidase	48	197.3	0.018
Ta.22564.2.S1_x_at	Peroxidase	48	81.4	0.003
Ta.22593.1.A1_at	Peroxidase	48	2.5	0.002
Ta.22593.2.S1_x_at	Peroxidase	4	4.6	0.050
		48	2.6	0.013
Ta.24710.1.S1_at	Peroxidase	48	8.3	0.009
Ta.26230.1.S1_at	Peroxidase	48	2.7	0.001
Ta.26230.1.S1_x_at	Peroxidase	48	5.5	0.002
Ta.26230.2.S1_at	Peroxidase	48	15.8	0.007
Ta.27461.1.S1_a_at	Peroxidase	0.5	0.4	0.032
Ta.5406.1.S1_at	Peroxidase	12	0.5	0.024
Ta.7816.1.A1_at	Peroxidase	12	0.18	0.032
Ta.82.1.S1_at	Peroxidase	24	4.2	0.039
		48	11.2	0.001
Ta.962.1.A1_at	Peroxidase	48	3.3	0.003
Ta.28182.1.A1_x_at	Phenylalanine ammonia lyase	48	3.2	0.012
Ta.7022.1.S1_at	Phenylalanine ammonia lyase	48	3.1	0.007
Ta.7022.1.S1_s_at	Phenylalanine ammonia lyase	48	3.3	0.005
Ta.7022.1.S1_x_at	Phenylalanine ammonia lyase	48	3.1	0.005
Ta.7022.2.S1_at	Phenylalanine ammonia lyase	48	2.7	0.046
Ta.7022.2.S1_x_at	Phenylalanine ammonia lyase	48	2.3	0.048
Ta.9220.1.S1_a_at	Phenylalanine ammonia lyase	24	3.7	0.026
		48	7.9	0.001
Ta.21208.1.S1_at	Putative superoxide generating NADPH oxidase	4	4.2	0.041
Ta.21028.3.S1_at	Putative superoxide-generating NADPH oxidase	0.5	2.3	0.003
Ta.10.1.S1_a_at	β -1,3;1,4-glucanase	4	5.7	0.032
Ta.10.1.S1_x_at	β -1,3;1,4-glucanase	4	6.7	0.021
Ta.8584.1.S1_at	β -1,3-glucanase class I precursor	24	7	0.030
		48	19.6	0.001
Ta.223.1.S1_at	β -1,3-glucanase precursor	12	0.3	0.011
		48	7.3	0.001
Ta.225.1.S1_at	β -1,3-glucanase precursor	48	32.6	0.001
Ta.22562.1.S1_at	β -1,3-glucanase	48	2.3	0.006
Ta.21354.1.A1_at	β -1,3-glucanase-like protein	48	14.2	0.001

al. 2004; Moy et al. 2004; Shimizu et al. 2007) suggests commonalities with respect to elevated expression of host defense response genes during compatible interactions. Our results indicate that the cell death associated with ROS in the presence of ToxA plays an important role in developing necrotic symptoms in sensitive wheat. Furthermore, a common mechanism is likely to exist for *Tsn1*-mediated susceptibility and *R*-gene-mediated plant immunity in response to plant pathogens (Hammond-Kosack and Jones 1996; Martin et al. 2003).

The most striking evidence for the involvement of ROS in cell death in the present study was based on the quantitative measurement of dye uptake (estimated by a spectrophotometer) and H₂O₂ production detected by DAB staining. Dark-brown regions were visible after DAB staining in ToxA-treated

sensitive wheat between 12 and 48 hpi (Fig. 7J, K, and L). However, H₂O₂ did not accumulate in the ToxA-insensitive wheat Nec103 even at 48 hpi (Fig. 7P). Our histochemical analyses show that ROS is a major signal leading to the development of cell death in ToxA-sensitive wheat, and may be a pivotal step in the development of necrosis in wheat infected by *Pyrenophora tritici-repentis*. Photosystem II is regarded as a major source of the large, secondary oxidative burst in hypersensitive response-associated resistance (Lamb and Dixon 1997). As reported previously, ToxA acts on the chloroplasts of sensitive wheat (Manning and Ciuffetti 2007). A notable change that we observed in the present study was the downregulation of several chloroplast-related genes such as chloroplast ribulosebiphosphate carboxylase and ribosomal proteins, and

Table 2. Differentially expressed representative genes involved in signal transduction revealed by microarray analysis of a toxin-sensitive wheat cultivar versus an insensitive wheat cultivar infiltrated with ToxA from *Pyrenophora tritici-repentis*

Probe set ^a	Putative function	Time (h)	Fold change ^b	P value
Ta.1018.1.S1_x_at	Calmodulin	48	2.9	0.001
Ta.1018.2.S1_x_at	Calmodulin 7	0.5	9.5	0.001
		4	8.3	0.001
		12	3.8	0.031
		24	4.5	0.016
		48	8.3	2.16E-05
Ta.7730.2.S1_a_at	Calmodulin-binding protein homolog	48	2.3	2.35E-07
Ta.7730.1.A1_at	Calmodulin-binding protein homolog	48	2.7	0.093
Ta.23713.1.A1_at	Protein kinase	48	8.2	0.043
Ta.15958.1.3.S1_at	Protein kinase	48	2.3	0.008
Ta.1674.1.S1_x_at	Protein kinase	48	7.2	0.001
Ta.1674.2.S1_s_at	Protein kinase	48	6	0.001
Ta.13013.2.S1_x_at	Protein kinase	48	3.6	0.001
Ta.17696.1.A1_at	Protein kinase	24	4.2	0.039
Ta.7752.1.S1_x_at	Protein kinase	24	2.2	0.006
Ta.8400.2.S1_at	Protein kinase	12	8	0.432
Ta.23679.1.S1_at	Protein phosphatase 2C-like protein	48	3.5	9.70E-05
Ta.27767.1.S1_at	Protein phosphatase 2C	48	7.2	0.041
Ta.23713.1.A1_x_at	Putative protein kinase	24	9.2	0.043
Ta.10207.1.S1_at	Putative protein phosphatase 2C	48	2	0.015
Ta.6517.1.S1_x_at	Protein phosphatase 2C	4	2.2	0.039
		24	4.2	0.03
Ta.30053.1.S1_at	Pto kinase interactor	48	2.4	0.032
Ta.22697.2.A1_at	Putative casein kinase II	12	0.4	0.429
Ta.22269.1.A1_a_at	Putative casein kinase	0.5	6.1	0.018
		24	5.1	0.002
Ta.22269.1.A1_at	Putative casein kinase	0.5	97.5	0.027
		4	38.5	0.004
		48	90	0.002
Ta.12127.1.A1_at	Receptor-like protein kinase	48	3.6	0.017
Ta.29629.1.S1_s_at	Putative receptor-like protein kinase	12	0.5	0.030
Ta.29629.2.S1_a_at	Putative receptor-like protein kinase	0.5	0.4	0.031
Ta.5331.1.A1_x_at	Receptor protein kinase-like protein	48	2.1	0.001
Ta.24562.1.A1_at	Receptor-like protein kinase	0.5	5.2	0.011
		24	4.8	0.041
		48	9	0.027
Ta.5331.1.A1_a_at	Receptor protein kinase-like protein	48	2.4	0.001
Ta.8590.1.S1_s_at	Receptor protein kinase-like protein	48	2.1	0.002
Ta.8590.2.S1_a_at	Receptor protein kinase-like protein	48	2.4	0.002
Ta.8590.2.S1_at	Receptor protein kinase-like protein	48	2.3	0.045
Ta.12007.2.S1_at	Receptor serine threonine protein kinase	48	2.3	0.002
Ta.25397.1.S1_at	Receptor-like kinase	48	3.7	0.002
Ta.941.1.A1_at	Receptor-like protein kinase - like protein	4	136.6	0.026
Ta.3929.2.A1_a_at	Receptor-like protein kinase	48	3.8	0.046
Ta.18480.2.S1_at	Receptor-like protein kinase	12	0.1	0.002
Ta.22666.1.S1_at	Receptor-like protein kinase	12	0.2	0.482
		48	3.3	0.001
Ta.5161.2.S1_x_at	Receptor-like protein kinase	0.5	0.5	0.012
Ta.14561.3.S1_at	Receptor-protein kinase-like protein	24	11.9	0.045
Ta.28502.2.S1_at	Serine threonine kinase	12	13.3	0.037
Ta.5041.2.S1_a_at	Serine threonine kinase	0.5	0.5	0.044
Ta.12156.1.A1_at	Serine threonine protein kinase	0.5	3.4	0.029
Ta.4106.1.A1_at	Serine threonine-specific protein kinase	48	2.6	0.001

^a Blank indicates that probe induced more than one time point.

^b Genes were considered as differentially expressed if their fold change was >2 with *P* ≤ 0.05.

transcription factor genes (e.g., translation elongation factors), in response to ToxA. Thus, it is possible that the inhibition of photosystem activity in the chloroplast due to ToxA may lead to high levels of ROS that can impact cellular homeostasis (Lamb and Dixon 1997; Manning et al. 2009). We hypothesize that the photosystem of toxin-sensitive chloroplasts could be the origin of ROS in the *Tsn1*–ToxA interaction.

Evidence exists that the calcium signaling and protein phosphorylation cascades may be necessary for ToxA-induced cell death (Rasmussen et al. 2004). The majority of signaling transcripts accumulated during cell death in the present study were attributed to protein kinases (e.g., casein kinases, protein kinases, receptor-like kinases, and serine threonine kinases) and phosphatases. The products of these genes could play an important role in signal transduction after ToxA is perceived by the host receptor. Similarly, two MAPK (TaMPK3 and TaMPK6) were induced differentially during compatible interactions between wheat and *Mycosphaerella graminicola* (Rudd et al. 2008). Interestingly, the calmodulin gene (Ta.1018.2.S1_x_at) was expressed constitutively in response to ToxA. The expression of genes involved in calmodulin and

NADPH oxidase suggests a possible role in cross talk between calcium and ROS signaling in ToxA-mediated cell death.

In the current study, we assumed that the ToxA-insensitive wheat cv. Nec103 lacks a receptor for ToxA; consequently, ToxA would not be recognized in the insensitive cultivar. In support of this hypothesis, H₂O₂ production and cell death were absent in ToxA-treated Nec103. Although most of the defense-related genes monitored by qPCR (Fig. 5A through F) were downregulated in insensitive Nec103, a few genes (e.g., *PAL* and *PER*) showed some induction in this cultivar. It has been shown that defense response genes are closely associated with quantitative trait loci or *R* genes in several cereal diseases (Faris et al. 1999, Liu et al. 2004). Future studies designed to investigate gene expression in incompatible Nec103–ToxA interaction are warranted.

Conclusion.

To gain insight into the molecular processes in the *Tsn1*–ToxA interaction, we used microarray analysis to identify differentially expressed genes in ToxA-sensitive and -insensitive wheat cultivars. Host gene expression remained relatively low

Fig. 5. Validation of differentially expressed representative genes using real-time polymerase chain reaction (qPCR) assays. The expression of nine genes was investigated in the ToxA-sensitive wheat cv. Kulm and compared with insensitive mutant wheat line Nec103 after infiltration with ToxA (10 µg/ml) from *Pyrenophora tritici-repentis*. The relative expression of each gene was calculated relative to the H₂O-infiltrated control from 0 to 48 h postinfiltration. Genes analyzed by qPCR assays include **A**, chitinase (*CHI*); **B**, β-1-3-glucanase (*PR-2*); **C**, glutathione transferase (*GST*); **D**, phenylalanine ammonia lyase (*PAL*); **E**, peroxidase (*PER*); **F**, NADPH oxidase (*NAD*); **G**, calmodulin (*CaM*); **H**, receptor kinase (*RLK*), and **I**, serine threonine protein kinase (*PK*). Error bars represent standard deviations of the measurements for three replications at each timepoint.

during the early timepoints prior to cell death, with a substantial increase in gene induction at 24 and 48 hpi when ToxA triggered extensive cell death. Our study supports the hypothesis that chloroplasts may be the origin of ROS production in the *Tsn1*-ToxA interaction. We propose that recognition of ToxA by its cognate host receptor occurs in ToxA-sensitive wheat but not in insensitive wheat. Specific signaling pathways appear to be activated due to up- and downregulation of transcription factors such as the Myb and zinc finger proteins (Fig. 8), suggesting complex positive and negative modulation of *Tsn1*-mediated metabolic pathways. These pathways activate protein phosphorylation, alter Ca^{2+} levels, and modulate enzymes involved in the production of ROS and phenylpropanoid biosynthesis (Fig. 8). This transcriptional reprogramming results in the induction and repression of genes involved in the plant defense response, cell wall modification, photosynthesis, and chloroplast biogenesis. We conclude that similar host responses are activated during the recognition of avirulence gene products (effectors) and elicitor-like proteins (ToxA) during the *R*-gene-mediated resistance to biotrophic fungi and susceptibility to necrotrophic fungi, respectively. This study generated an extensive list of potential candidate

genes that can regulate the successful establishment of infection in sensitive wheat plants. Functional analysis of candidate genes will advance our understanding of the molecular processes of plant susceptibility to *P. tritici-repentis*.

MATERIALS AND METHODS

Selection of wheat cultivars.

The hard red spring wheat cv. Kulm, sensitive to ToxA and susceptible to *P. tritici-repentis* (Friesen et al. 2003), was selected as the toxin-sensitive cultivar. The wheat line Nec103, which was previously developed by mutagenesis (Friesen et al. 2002), was used as a toxin-insensitive cultivar. To identify genes in the *Tsn1*-ToxA interaction and validate gene expression patterns, several precautions were adopted to reduce biological variation. First, all experiments were conducted in an environmental growth chamber to provide controlled conditions for all timepoints. Next, fully expanded secondary leaves of 2-week-old wheat seedlings were infiltrated with ToxA, which was purified from *P. tritici-repentis* as described below. Unless otherwise stated, two independent experiments were conducted on different days to represent two biological replicates. Each experiment was carried out three times to represent three technical replicates. Infiltration with sterile distilled H_2O was included in each experiment to represent a mock-inoculated control.

RNA isolation and experimental design.

P. tritici-repentis number 86-124, an isolate of race 2, produces Ptr ToxA (Strelkov and Lamari 2003) and was obtained from Dr. L. Lamari, University of Manitoba, Canada, and maintained at the Department of Plant Pathology, North Dakota State University. ToxA was purified and characterized from *P. tritici-repentis* isolate number 86-124 as described previously (Zhang et al. 1997). To identify host genes triggered by ToxA, seedling bioassays were performed, and a 10- μl aliquot of purified ToxA (10 $\mu\text{g}/\text{ml}$) was infiltrated into the second leaf of each seedling using a 1-ml needleless syringe (Kwon et al. 1998). The infiltrated area was marked with a nontoxic permanent marker. Immediately after toxin infiltration, plants were maintained in growth chambers at a temperature regime of 21 and 18°C (light and darkness, respectively) and a 16-h photoperiod. To compare visual reactions, wheat cvs. Glenlea (sensitive to ToxA) and Salamouni (insensitive to ToxA) were included as controls. The entire experiment was conducted using a factorial split-plot design with three replications, where wheat cultivars and timepoints were represented as the main plot and subplot, respectively (Gomez and Gomez 1984). Each plastic cone was 3.8 cm in diameter and 20 cm long (Stuewe and Sons, Inc. Tangent, OR, U.S.A.), contained three seedlings per cone, and was regarded as a sampling unit, and two independent experiments were conducted.

After ToxA infiltration, total RNA was purified from the ToxA-sensitive wheat cv. Kulm and the insensitive wheat cv. Nec103 using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.). Total RNA was extracted from three seedlings per treatment and, following extraction, total RNAs were pooled to represent one biological replicate. For microarray analyses, leaf samples from both ToxA-sensitive and ToxA-insensitive wheat cultivars were collected at 0.5, 4, 12, 24, and 48 hpi. Prior to microarray analysis, RNA integrity was evaluated using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, U.S.A.). RNA preparation, purification, and labeling were performed as described previously (Hulbert et al. 2007). Approximately 5 μg of total RNA was used for first-strand cDNA and second-strand cDNA synthesis; cRNA synthesis and cRNA labeling were conducted as recom-

Fig. 6. Detection of Evans blue uptake in sensitive wheat cv. Kulm and insensitive mutant wheat line Nec103 after infiltration with ToxA (10 $\mu\text{g}/\text{ml}$) from *Pyrenophora tritici-repentis* at 12 and 48 h postinfiltration. For both experiments, leaf segments (5 cm long) were collected from the two wheat cultivars (a total of 10 from two biological replicates). Cell death values were obtained by monitoring the retention of Evans blue by **A**, spectrophotometry or **B**, microscopic examination. Error bars represent standard deviations of the measurements for three replications at each timepoint.

mended by the manufacturer (Affymetrix, Inc., Santa Clara, CA, U.S.A.). cDNA (15 µg) was used for hybridization.

Gene expression profiling during the *Tsn1*-ToxA interaction.

The Affymetrix GeneChip Wheat Genome Arrays contain 61,127 probe sets representing 55,052 transcripts, which cover all 21 wheat chromosomes (Affymetrix). Each probe set contained 11 matched and 11 mismatched 25-mer oligonucleotides and was regarded as a transcript (Hulbert et al. 2007). The standard protocol used for microarray analysis was developed by Affymetrix and optimized by the Kansas State University Integrated Genomics Facility, Manhattan. Data analysis was performed using Genespring software (Agilent Technologies) as described previously (Hulbert et al. 2007). Analysis of variance was performed with three biological replicates to determine the effect of ToxA in a sensitive wheat cultivar versus an insensitive wheat cultivar. Differential gene expression in response to ToxA was classified as either qualitative or quantitative, depending on transcript detection patterns (present or absent) or transcript abundance differences, respectively. Fold changes were calculated as sensitive wheat cultivar values divided by insensitive wheat cultivar values. A gene was re-

garded as differentially expressed if the fold change was >2.0 and P was ≤ 0.05 . Differentially expressed genes (up- and downregulated) were classified according to functional categories using the MIPS *Arabidopsis thaliana* Database as described previously (Keon et al. 2007).

qPCR analysis.

Planting of wheat seed was performed as described above. Leaf tissues of both sensitive and insensitive wheat cultivars were infiltrated with ToxA as described previously (Kwon et al. 1998). Two-week-old seedlings were spray inoculated and transferred to a mist chamber (100% relative humidity) to provide continuous leaf wetness for 24 h. Next, the plants were transferred into a growth chamber and maintained at 21 and 18°C (light and darkness, respectively) with a 16-h photoperiod. Leaf tissues from both wheat cultivars were collected at 0, 4, 8, 12, 24, and 48 hpi. A mock inoculation consisting of infiltrated water was used as a control. The entire experiment was conducted using a factorial split-split-plot design with three replications, where wheat cultivars, ToxA infiltration or pathogen inoculation, and timepoints were represented as main plot, subplot, and sub-subplot, respectively (Gomez and Gomez 1984).

Fig. 7. Detection of H_2O_2 production and cell death in the ToxA-sensitive wheat cv. Kulm and the insensitive wheat line Nec103 infiltrated with H_2O and ToxA (10 µg/ml) from *Pyrenophora tritici-repentis*. For each experiment, leaf segments (5 cm long) were collected from the two wheat cultivars (a total of 10 segments from two biological replicates). Leaf tissues were stained with 3,3'-diaminobenzidine (DAB), and H_2O_2 was assessed using a digital stereomicroscope. A through H, Upper panels show leaves infiltrated with H_2O and I through P, lower panels show leaves infiltrated with ToxA. Kulm infiltrated with water at A, 4 h postinfiltration (hpi); B, 12 hpi; C, 24 hpi; and D, 48 hpi. Nec103 infiltrated with water at E, 4 hpi; F, 12 hpi; G, 24 hpi; and H, 48 hpi. Kulm infiltrated with ToxA at I, 4 hpi; J, 12 hpi; K, 24 hpi; and L, 48 hpi. Nec103 infiltrated with ToxA at M, 4 hpi; N, 12 hpi; O, 24 hpi; and P, 48 hpi.

For qPCR analysis, total RNA and cDNA were prepared as described previously (Adhikari et al. 2007). Briefly, 100 mg of ground leaf powder was transferred into sterile 1.5-ml centrifuge tubes, and total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies). Random hexamers were used as the first-strand cDNA primer (Promega Corporation, Madison, WI, U.S.A.). The cDNA concentration of each sample was quantified using a NanoDrop spectrophotometer (Model ND-1000; Thermo Scientific Inc., Waltham, MA, U.S.A.). Six genes involved in either the plant defense response or ROS production were analyzed for expression and included *CHI* (Ta.21342.1.S1_x_at), *PR-2* (Ta10.1.S1_a_at), *GST* (Ta.3679.1.S1_x_at), *PER* (Ta.82.1.S1_at), *PAL* (Ta.9220.1.S1_a_at), and *NAD* (Ta.21208.1.S1_at). Three genes involved in signal transduction were also monitored and included *CaM* (Ta.1018.2.S1_x_at), *RLK* (Ta.24562.1.A1_at), and *PK* (Ta.28502.2.S1_at). Analysis of these genes was confirmed by qPCR at 0, 4, 8, 12, 24, and 48 hpi with ToxA. The 18S ribosomal RNA gene from wheat was used as an internal control (Adhikari et al. 2007). cDNA was prepared from the ToxA-sensitive Kulm infiltrated with ToxA at 48 hpi; this cDNA preparation was serially diluted and 10-fold dilutions (100 to 0.01 ng/μl) were used to generate a standard curve for each gene. Primer pairs for qPCR analyses (Supplementary Table S1) were selected using the same criteria as described previously (Adhikari et al. 2007). Each qPCR reaction (10 μl) contained 1× SYBR Green PCR Master Mix (Applied Biosystems, Inc., Foster City, CA, U.S.A.), 10 ng of cDNA template per reaction, and 4 to 7 μM each forward and reverse gene-specific primer. All qPCR were performed using the Stratagene Mx3005P System (Agilent Technologies). The protocol used for PCR reactions was as

follows: preincubation at 25°C for 2 min; denaturation at 95°C for 10 min; and 45 cycles at 95°C for 30 s and annealing at 55°C for 1 min; and extension at 72°C for 30 min/cycle. Immediately after the final PCR cycle, a melt-curve analysis was performed to determine the specificity of the primers. The reaction was incubated at 95°C for 1 min, annealed at 55°C for 30 s, and then gradually increased to 95°C for 30 s. Each reaction was run in triplicate. qPCR data were analyzed by the relative standard curve method as described previously (Adhikari et al. 2007). Gene expression in Kulm and Nec103 was expressed as fold changes relative to mock-inoculated tissue. Standard deviations were calculated to find differences ($P \leq 0.05$) between fold changes in the ToxA-sensitive and -insensitive wheat cultivars. Correlation analysis between qPCR gene expression and GeneChip gene expression was performed to validate the results of this study, as described previously (Bolton et al. 2008). Nine genes analyzed by qPCR analysis were selected and used for comparison. R^2 and r values were calculated from these data.

Measurement of cell death using Evans blue.

In separate experiments, the second leaves of 2-week-old seedlings of Kulm and Nec103 were infiltrated with ToxA and water. Leaf samples were collected at 4, 12, 24, and 48 hpi. To measure changes in absorbance values, leaf segments (2.5 cm long) were excised (a total of 10 from two biological replicates) and placed in 2-ml centrifuge tubes. The leaf segments were submerged for 20 min in 1 ml of Evans blue solution (0.5%, wt/vol) (MP Biomedicals, Solon, OH, U.S.A.) and homogenized using plastic pestles. Excess dye was removed with deionized water. The homogenates were centrifuged at 14,000 ×

Fig. 8. Hypothetical model showing classes of genes and cellular processes altered during the susceptible response triggered by ToxA from *Pyrenophora tritici-repentis*. Blue arrows indicate upregulated genes ($\geq$ twofold change, $P \leq 0.05$) and pink arrows represent downregulated genes from the different functional categories described in this investigation. Enzymes involved in the phenylpropanoid pathway include chalcone synthase (*CHS*), lipoxigenase (*LOX*), and phenylalanine ammonia lyase (*PAL*).

g for 5 min, transferred to 1% (wt/vol) sodium dodecyl sulfate, and incubated for 10 min at 37°C. The absorbance values of dye in the supernatant were quantified by spectrophotometry (Thermo Electron Corp., Vantaa, Finland) at 600 nm. Water was used as a control. To measure cell death, leaf sections (2.5 cm long) were excised (a total of 10 from two biological replicates), prepared without homogenization, and stained with Evans blue solution as described above. The leaf segments were examined using a Nikon Sclipse-E600 stereo microscope (Fryer Company, Inc., Bloomington, MN, U.S.A.). Dark blue staining of cells indicated cell death. The percentages of cell death values (scale of 0 to 100%) were recorded (Baker and Mock 1994), and relative cell death values were calculated and level of significance was determined at $P < 0.01$.

Detection of H₂O₂ using DAB.

The second leaves of 2-week-old seedlings of Kulm and Nec103 were infiltrated with ToxA or water (control). Leaf samples were collected at 4, 12, 24, and 48 h hpi. Approximately 2.5-cm segments (a total of 10 from two biological replicates) from each treatment were stained using the DAB Liquid Substrate System (Sigma-Aldrich, Inc., St. Louis) as described by the manufacturer. Tissues were destained by boiling them in 90% ethanol alcohol for 5 min and rinsing in 50% ethanol, then examined for the presence of a reddish-brown stain using a stereomicroscope equipped with a digital camera (Lecia Microsystems Heidelberg GmbH, Mannheim, Germany). Photographs were taken for each treatment.

ACKNOWLEDGMENTS

We thank N. Wang for analyzing the microarray data, Melvin Bolton for correlation analysis between the microarray data and qPCR data, and Scott Payne for photography. This work was supported in part by the National Research Initiative of the United States Department of Agriculture–Cooperative State Research Education and Extension Service (06-35319-17442), the North Dakota–National Science Foundation program to stimulate competitive research (grant 044-7679, ND-NSF EPSCoR), and the North Dakota State University Agricultural Experiment Station.

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