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Functional Characterization of ToxA and Molecular Identification of its Intracellular Targeting Protein in Wheat

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Abstract: The fungus *Pyrenophora tritici-repentis* causes tan spot disease in wheat. Typical symptoms include tan-colored necrotic lesions surrounding small infection sites. The necrosis is induced by the host-selective proteinaceous toxin, ToxA. The ability of ToxA to be internalized into plant cells is required for the toxin to cause cell death. Using yeast two-hybrid (Y2H) analyses, we detected the oligomerization of ToxA and we identified amino acid E145 and D149 residues that were critical for this ToxA-ToxA oligomerization. However, a G141A mutation in the RGD motif, which is important for necrosis, still can form the oligomer. We have developed a transient assay system to provide direct evidence that intracellular expression of ToxA causes cell death in wheat. By screening a cDNA library, we identified wheat plastocyanin as a host target protein of ToxA. Plastocyanin is a component of the electron transport chain of photosynthesis. Virus-induced gene silencing of wheat plastocyanin produced a similar phenotype of ToxA-induced cell death. In Y2H analyses, the mutant G141A does not interact with plastocyanin, but the E145R and D149K mutants do interact with plastocyanin. However, all G141, E145 and D149 residues are required for ToxA-induced necrotic symptom *in planta*, which indicates that both oligomerization and plastocyanin association are important for ToxA activity.

Key words: Ptr ToxA, plastocyanin, virus-induced gene silencing (VIGS), virus-mediated transient assay

INTRODUCTION

In many host-pathogen interactions, host sensitivity to a toxin is usually associated with disease susceptibility (Markham and Hille, 2001; Walton and Panaccione, 1993). The necrotrophic fungus *Pyrenophora tritici-repentis*, a world-wide threat in wheat growing areas, causes tan spot disease in bread wheat (*Triticum aestivum* L.) and durum wheat (*T. turgidum* L. var. *durum*). The typical phenotype of tan-colored and necrotic lesion is due to the action of a host-selective toxin (HST), ToxA (Tomas *et al.*, 1990; Lamari *et al.*, 1995), which is the first proteinaceous HST cloned from fungi (Ballance *et al.*, 1996; Ciuffetti *et al.*, 1997; Zhang *et al.*, 1997). ToxA-induced cell death is distinct from gene-for-gene mediated hypersensitive response (Tai, 2007). Based on the virulence of isolates on specific wheat genotypes, eight races of *P. tritici-repentis* have been identified (Lamari *et al.*, 2003). A ToxA homolog was also identified in *Stagonospora nodorum* (Friesen *et al.*, 2006), the causal agent of wheat *Stagonospora nodorum* blotch disease. A single dominant gene, *Tsn1*, which confers sensitivity to ToxA infiltration, was mapped to wheat chromosome 5BL (Faris *et al.*, 1996; Stock *et al.*, 1996; Lu *et al.*, 2006). Other HSTs have been demonstrated to act through ligand-receptor relationships, such as victorin produced by *Cochliobolus victoriae* to oats (*Avena sativa* L.) (Wolpert and Macko, 1989) and Hmt toxin produced by *C. heterostrophus* race T to corn (*Zea mays* L.)

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(Braun *et al.*, 1990). It was proposed that the ability of ToxA to be internalized into plant cells determines the host sensitivity to this toxin (Manning and Ciuffetti, 2005; Tai and Bragg, 2007).

The ToxA cDNA encodes a pre-pro-protein that contains a signal peptide (residues 1 to 22) for targeting to the secretory compartment (Ballance *et al.*, 1996; Ciuffetti *et al.*, 1997). The pro-protein contains a second N-terminal signal sequence (residues 23 to 60) that is removed to generate the mature protein (residues 61 to 178) prior to secretion into culture medium (Tuori *et al.*, 2000). However, pre-pro-protein and pro-protein ToxA produced from *E. coli* still cause cell death when infiltrated to sensitive wheat (Ballance *et al.*, 1996; Tuori *et al.*, 2000; Meinhardt *et al.*, 2002), indicating that post-translational modifications in mature ToxA protein secreted by the fungus are not required for cell death *in planta* (Tuori *et al.*, 2000). Transient assays using particle bombardment into wheat leaves showed ToxA-dependent but non-host specific cell death (Manning and Ciuffetti, 2005). Additionally, using a *Barley stripe mosaic virus* (BSMV)-mediated gene expression system, direct evidence demonstrating cell death following the intracellular production of ToxA in wheat was obtained (Tai and Bragg, 2007). Infection of mesophyll tissues and epidermal cells (Haupt *et al.*, 2001) makes the BSMV-mediated expression system useful in mimicking the action of ToxA effects during the fungal infection.

Originally, based on ToxA infiltration into wheat leaves, wheat cultivars were classified as ToxA sensitive or insensitive (Ciuffetti *et al.*, 1997). However, ToxA produced intracellularly in “insensitive” cultivars, either by particle bombardment (Manning and Ciuffetti, 2005) or by BSMV-mediated transient expression (Tai and Bragg, 2007), also resulted in cell death, indicating that a ToxA target exists in all wheat cultivars tested, regardless of sensitivity to ToxA infiltration. Here we present the identification and characterization of a ToxA target protein by screening a wheat cDNA library using the yeast two-hybrid system. Additionally, virus-induced gene silencing (VIGS) was used to verify the role of the ToxA target protein in ToxA-induced necrotic cell death in wheat.

MATERIALS AND METHODS

Plant Materials

Wheat plants used in this study include the ToxA-sensitive Langdon (LDN) and the ToxA-insensitive LDN/J00M-935, LDN/J03M2411, ND495/633, Chinese Spring (CS) and Auburn (provided by USDA National Small Grains Research Facility, Patterson *et al.*, 1982). LDN/J00M-935 is derived from an EMS-induced mutation in the LDN genetic background. LDN/J03M2411 and ND495/633 are fast neutron-induced mutations in LDN and ND495 genetic backgrounds, respectively. Both wheat and *Nicotiana benthamiana* plants were kept in a growth chamber with fluorescent lamps at 23°C with 10/14 h light/dark cycle for two to three weeks until the seedlings reached the two-leaf stage. Photographs of cell death induced by ToxA infiltration in wheat were taken three days post-infiltration. Photographs of *N. benthamiana* leaves were taken after 5 days of agroinfiltration. BSMV-mediated ToxA systemic cell death was recorded 14 to 21 days post-infection.

Yeast Two-hybrid Analyses and cDNA Library Screening

The MATCHMAKER GAL4 Two-Hybrid System 3 (BD Clontech Laboratories, Inc., Mountain View, CA, USA) was used to test the formation of ToxA dimer and to screen a wheat cDNA library. The protocols were according to the manufacturer’s instructions. Wheat LDN leaves were infiltrated with ToxA for 2, 4, 8, 12 and 24 h. Total RNA was isolated with TRI-ZOL reagent (Invitrogen, Carlsbad, CA, USA). A pool of total RNAs from these different harvests was used as a template to synthesize a cDNA library. Yeast mating procedure was performed using Y187 strain carrying the bait ToxA and AH109 strain containing the prey library. About 20 million clones were screened on SD plates in the presence of X-Gal but without leucine, tryptophan, adenine and histidine (SD/X-Gal/-L-

W-A-H). The prey plasmids of candidate clones were isolated and transformed into *E. coli*. The amplified prey plasmids were isolated from bacteria and then retransformed back to AH109 cells for mating with Y187 cells carrying either negative control (p53) or ToxA to confirm the positive interactions. All constructs were confirmed by DNA sequencing. All protein-protein interactions were repeated at least three times.

Western Blots

Protein isolation from yeast cells and the Western blots were performed according to the yeast protocols handbook (Clontech). Protein samples were electrophoresed through a 4 to 15% gradient SDS-PAGE (Bio-Rad, Hercules, CA). Protein was electrotransferred to Immun-Blot PVDF membrane (Bio-Rad). Immunodetection was performed with 0.5 $\mu\text{g mL}^{-1}$ anti-Gal4 BD monoclonal antibody (Clontech) and then the membrane was incubated with 1:5,000 dilution of goat-anti-mouse IgG HRP-conjugated secondary antibody (Bio-Rad). The blot was visualized with ECL Western blotting detection reagents (GE Healthcare-Amersham, Piscataway, NJ). The procedures for ToxA antibody were described as previously (Meinhardt *et al.*, 2002).

BSMV-mediated Virus-induced Gene Silencing (VIGS)

VIGS was performed as described previously (Tai *et al.*, 2005). The genome of BSMV strain ND18 is comprised of three RNAs, designated α , β and γ (Bragg and Jackson, 2004). We used modified β and γ components of the ND18 strain to facilitate insertion of a PCR product and to increase efficiency of VIGS. The BSMV RNA β mutant 'B7' was derived from wild-type RNA β in which the expression of coat protein was disrupted by a mutation in the start codon, thereby enhancing gene silencing (Holzberg *et al.*, 2002). The start codon of the γ b open-reading frame was modified to create a *Bam*HI site for insertion of cloned DNA fragments (γ - γ bBamHI), while also blocking expression of the VIGS suppressor γ b (Bragg and Jackson, 2004). Each construct for VIGS was tested in five wheat plants.

BSMV-mediated ToxA Expression in Planta

The sequence of the mature ToxA protein has flanking *Bam*HI and *Hpa*I restriction sites and was used for cloning into BSMV γ - γ bBamHI vector (Tai *et al.*, 2005), which replaced most of γ b gene. According to the NCBI accession number U79662 of *ToxA*, the PCR product of gene sequence encoding the mature polypeptide, with the created ATG start codon after the *Bam*HI site in the forward primer and an *Hpa*I site before the stop codon in the reverse primer (Table 1), was used to replace the fragment between *Bam*HI and *Hpa*I sites in the γ - γ bBamHI vector DNA (12). Each construct was tested in at least 10 plants.

Site-directed Mutagenesis of ToxA

The point mutations of ToxA E145R and D149K were created by sequential PCR steps (Cormack, 1991). The primers used to create point mutations and to amplify the full-length sequence

Table 1: The primers used for amplification of normal and mutant ToxA. Restriction enzyme sites for *Bam*HI, *Eco*RI and *Hpa*I are in bold and italic. The created start codon (ATG) and codons (AGG and AAG) for point-mutations E145R and D149K are underlined

Primers	Sequences
ToxA_F184BH	5'- <i>GGATCC</i> ATGGGAAGCTGCATGTCAATCACA-3'
ToxA_F184RI	5'- <i>GAATTC</i> CGGAAGCTGCATGTCAATCACAATC-3'
ToxA_R537BH	5'- <i>GGATCC</i> CTAATTTTCTAGCTGCATTCTCCA-3'
ToxA_537HpaI_R	5'- <i>GTTAACT</i> AATTTTCTAGCTGCATTCT-3'
toxa_e145R_F	5'-ACGTTTATAGGCTTTTGGTGATTACGCT-3'
toxa_e145R_R	5'-ACCAAAAAGCCTATAAACGTCCTCCCGCA-3'
toxa_d149K_F	5'-TTTTGGTAAGTACGCTTTAATTCAAGGT-3'
toxa_d149K_R	5'-TAAAGCGTACTTACCAAAAAGCTCATAAACGT-3'

of mature ToxA are shown in Table 1. The pair of F184BH / 537HpaI_R primers was used to clone the mature ToxA into BSMV γ - γ BamHI vector. The primer pair F184RI / R537BH was used to clone the mature ToxA into yeast two-hybrid bait pGBKT7 and prey pGADT7 vectors. These constructs carrying point mutations were confirmed by DNA sequencing.

Agrobacterium-mediated Transient Assays in *Nicotiana benthamiana*

The cDNAs were each inserted into the *Agrobacterium tumefaciens* binary vector pCB302-3 (Xiang *et al.*, 1999) mediated by Gateway cloning vector pDONR207 (Invitrogen). These constructs were then electroporated into *A. tumefaciens* strain EHA105. The transformed cells were selected on LB plates containing 50 $\mu\text{g mL}^{-1}$ kanamycin. In preparation for infiltration of *N. benthamiana*, overnight cultures of the transformed cells were pelleted, washed and resuspended in infiltration media (10 mM morpholineethanesulfonic acid (MES) pH 5.6, 10 mM MgCl_2 and 150 μM acetosyringone).

The OD_{600} was adjusted to 2.0. Infiltrations were performed by infusion of the bacterial suspension from a 1 mL syringe into the leaves of 6 to 8-week-old *N. benthamiana* plants. The infiltrated plants were kept in a growth chamber for 2 to 7 days and assessed daily for the appearance of cell death. The results of agroinfiltration for each construct were repeatable for at least three times.

RESULTS

ToxA Protein-protein Interactions in the Yeast Two-hybrid System

Because ToxA is a protein, we used the yeast two-hybrid (Y2H) system to investigate the ligand-receptor hypothesis. We first characterized the interaction properties of ToxA in this Y2H system. The mature ToxA fragment was cloned as bait and prey. Neither bait nor prey interacted with either of the empty vectors, nor did they interact with the control proteins, p53 and LTA (Fig. 1A). The interaction between p53 and LTA served as a positive control. In this assay, ToxA showed the formation of homodimer (Fig. 1A). Interestingly, the mutant G141A still could interact with wild type ToxA and itself (Fig. 1A), suggesting that the glycine residue within the RGD motif (the region containing the amino acids of arginine, glycine and aspartate) was not required for dimer formation and that the G141A mutation is unlikely to significantly change the protein conformation needed for dimer formation. Based on the ToxA crystal structure (Sarma *et al.*, 2005), we hypothesize that the RGD motif is required for binding to its target protein, since this motif protrudes from the crystal oligomer structure. From the crystal structure, we also predict the region spanning residues E145 to D149 might be involved in the dimer formation. In contrast to wild type ToxA and mutant G141A, neither mutant E145R nor D149K formed homodimers in Y2H analyses (Fig. 1B). The presence of protein for each construct was verified by Western blots (Fig. 1C). The greater mobility of E145R bait might be due to a change in protein conformation or phosphorylation status, as was observed in the E145A mutation (Manning *et al.*, 2004). The results of protein-protein interactions in Y2H analyses indicate that ToxA requires at least two domains for proper function --- the RGD motif for target recognition and the region of residues E145 to D149 for inter-molecular interactions of the ToxA oligomer.

BSMV-mediated ToxA Expression in Wheat

In the study of ToxA-induced cell death, particle bombardment resulted in a high frequency of cell death that was easily confused with the ToxA-induced cell death phenotype. To avoid this problem, a novel virus-mediated assay for transient and systemic expression of ToxA in wheat was employed. We used BSMV as a vector to express mature ToxA protein and ToxA-derived mutants in different wheat cultivars. Transient assays were used to directly express ToxA inside the cells and therefore avoid any problems with ToxA uptake. After confirming that BSMV-mediated ToxA expression occurred in the sensitive wheat cultivar LDN (Fig. 2), we tested the ToxA insensitive wheat plants. BSMV-mediated systemic expression of mature ToxA resulted in cell death in leaves of all wheat tested (Fig. 2), regardless of wheat sensitivity to ToxA infiltration.

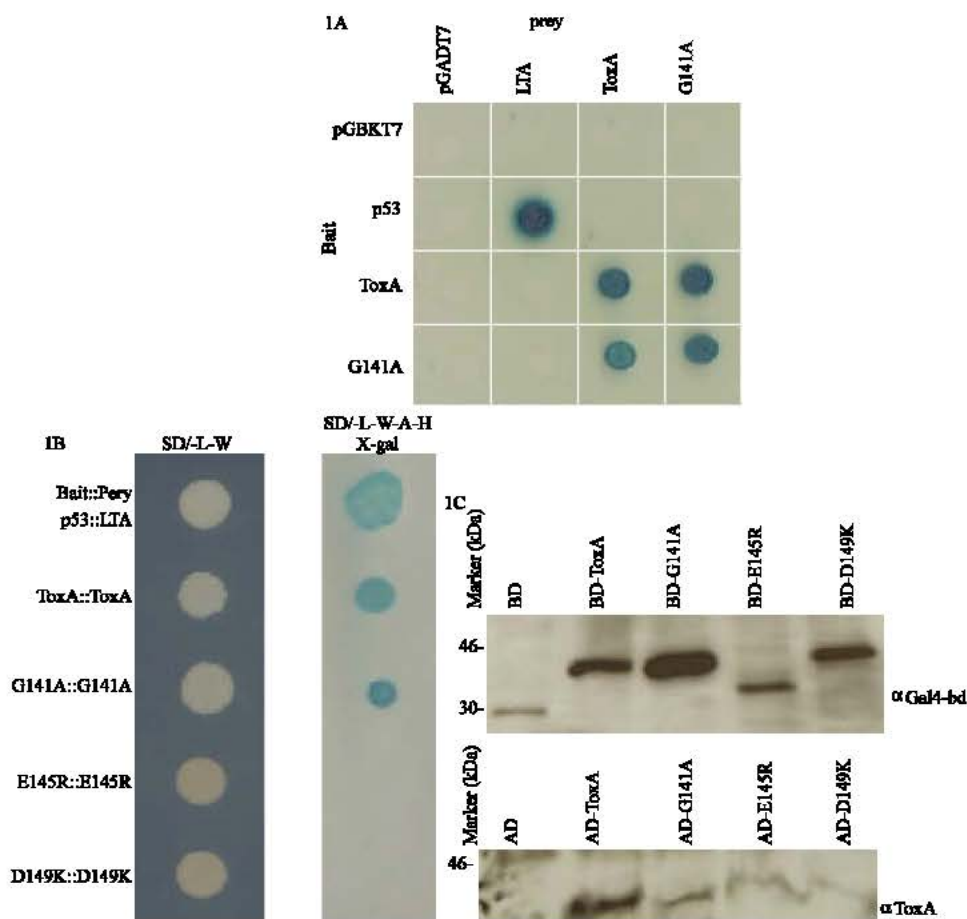


Fig. 1: Protein-protein interactions of ToxA oligomers in yeast two-hybrid analyses. (A) and (B), pGBKT7 and pGADT7 are empty vectors of bait and prey, respectively. The interaction between p53 and LTA serves as a positive control. Yeast cells carrying both bait and prey plasmids were selected on SD medium without leucine and tryptophan. Yeast cells can survive under the selection of SD without leucine, tryptophan, adenine and histidine only when the bait and prey proteins interact with each other. These protein interactions were also detected using X-Gal to measure the expression of β -galactosidase. © Western blot of ToxA fusion proteins isolated from yeast cells. The approximate sizes of GAL4-BD and the mature ToxA are 30 kDa and 12 kDa, respectively. The blot was probed with anti-Gal4-BD monoclonal antibody. For prey constructs, the proteins were isolated from the yeast cells and ToxA antibody was used

In BSMV-mediated expression assays, ToxA derivatives containing the point mutation G141A, E145R, or D149K did not cause cell death (Fig. 3). The ToxA G141A derivative has been shown to be inactive in protein infiltration assays (Meinhardt *et al.*, 2002; Manning *et al.*, 2004). Current results show that intracellular expression of the G141A mutant also failed to induce necrosis in wheat and furthermore our results indicate that this failure is due to the loss of interaction with an intracellular target. However, this does not exclude the possibility that G141A may not be internalized in protein infiltration assays. The failure of mutants ToxA E145R and ToxA D149K to induce cell death demonstrated that the interaction of ToxA to form an oligomer is critical for its biological activity

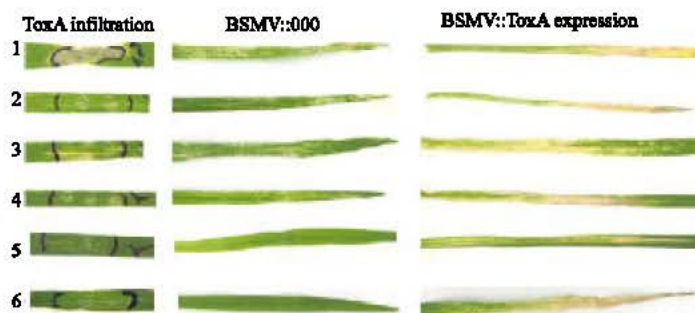


Fig. 2: BSMV-mediated *ToxA* expression in plants. Numbers 1 through 6 indicate the wheat: LDN, LDN/J00M935, ND495/633, LDN/J03M-2411, CS and Auburn, respectively. Only LDN showed sensitivity to *ToxA* protein infiltration. The other cultivars were *ToxA* insensitive. BSMV::000 represents the empty vector and BSMV::*ToxA* represents virus carrying the *ToxA* gene. All tested wheat cultivars showed cell death resulting from the virus-mediated *ToxA* expression



Fig. 3: BSMV-mediated gene expression of *ToxA* and mutants in the leaves of LDN wheat. The BSMV constructs were infected in the second leaf of wheat and the phenotypes were recorded from the third leaf in 21 days post-infection

leading to cell death. The *ToxA* E145A mutant had been previously shown to have lost cell death activity (Manning *et al.*, 2004). Consistent with this, we observed that the E145R mutant did not cause cell death in BSMV-mediated transient assays (Fig. 3) and in addition, we demonstrated that an additional mutant, D149K, failed to induce cell death (Fig. 3). The contribution of the D149 residue to the *ToxA* necrosis phenotype has not been previously characterized. Both of these residues were shown to be required for *ToxA* self interactions and the failure of these mutants to induce cell death indicate that *ToxA* oligomer formation is critical for the biological activity leading to cell death. The absence of cell death induced by the mutants G141A, E145R and D149K was not due to the failure of gene expression. The *ToxA* protein was not detectable in Western blots of extracts from infected leaves due to high levels of non-specific cross-reactivity of the *ToxA* polyclonal antibody in samples prepared from plant extracts (data not shown). However, the expression of *ToxA* in wheat was verified by reverse transcription PCR (Tai and Bragg, 2007).

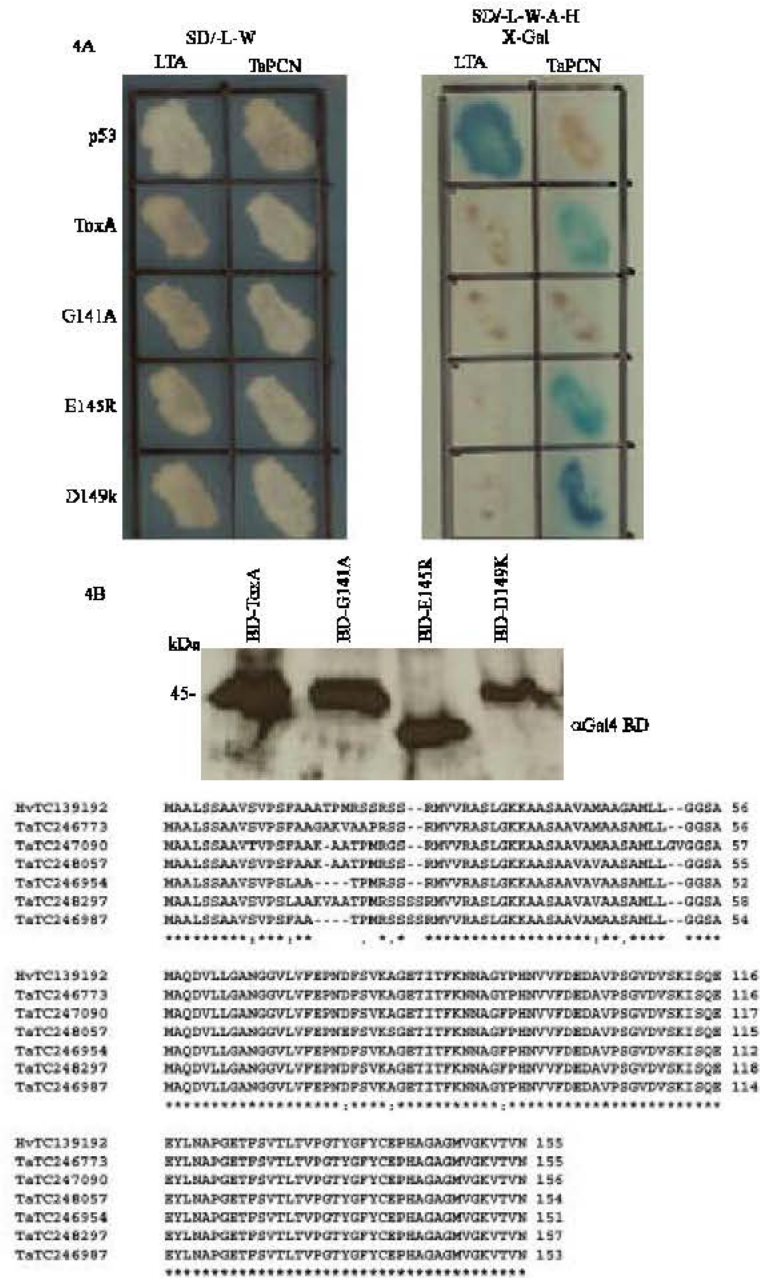


Fig. 4: Protein-protein interactions between TaPCN and ToxA or mutants of ToxA in yeast two-hybrid analyses. (A) G141A was unable to interact with TaPCN, whereas wild-type ToxA and mutants of E145R and D149K all demonstrated interactions with TaPCN in this system. (B) Western blot of proteins isolated from the yeast two-hybrid cells. (C) Amino acid sequence alignment of barley and wheat plastocyanin (HvPCN and TaPCN, respectively). The star symbol under the sequence stands for the conserved amino acid in the alignment. Sequences were obtained from the DFCI database and the accession numbers were shown in the figure

Identification of the ToxA Target Protein in Wheat

To identify the intracellular host target protein of ToxA, we constructed a cDNA library for Y2H screening. The cDNA library was made from a pooled total RNA of 2, 4, 8, 12 and 24 h treatments of ToxA infiltration in LDN wheat leaves. After screening approximately 20 million yeast colonies, 17 clones were identified to be ToxA interactors (Fig. 4A). All 17 clones encode plastocyanin. Here, we designate the wheat plastocyanin as TaPCN and the homolog of *Nicotiana benthamiana* as NbPCN, according to The DFCI (<http://compbio.dfci.harvard.edu/tgi/plant.html>) *Nicotiana benthamiana* Gene Index (NbGI) TC7014. The G141A mutant that failed to elicit necrosis in protein infiltration and intracellular expression assays also lost the ability to interact with TaPCN, but the E145R and D149K mutants were still able to interact with TaPCN (Fig. 4A). The presence of the G141A protein was confirmed by Western blot (Fig. 4B) and therefore, the negative result of TaPCN::G141A interaction is not due to the lack of G141A expression. The ToxA::TaPCN interaction was further confirmed *in vivo* by vector swap between bait and prey constructs (data not shown). These data further support the hypothesis that ToxA contains two independent domains for protein-protein interactions. A summary for the functions of these three critical amino acid residues of ToxA was presented in Table 2.

Plastocyanin is a nuclear encoded chloroplast protein involved in electron transfer in photosynthesis (Kato, 2003; De Rienzo *et al.*, 2004). In wheat, *TaPCN* is a small gene family. The amino acid sequences of TaPCN family members are varied in the N-terminal signal peptide (residues 1 to 60), but the mature polypeptides of TaPCN are highly conserved (96 out of 99 amino acids identical, Fig. 4C). Despite the similarity between the amino acid sequences of mature NbPCN and TaPCN (74% identity), ToxA did not interact with NbPCN (Fig. 5A and B). The predicted secondary structures (Fig. 5C, DNASIS MAX Version 2.5, MiraiBio Inc.) of mature NbPCN and TaPCN may explain the different protein affinities for ToxA. Therefore, the Y2H interaction of ToxA is specific to TaPCN.

Manning and Ciuffetti (2005) proposed that protein internalization was the major factor determining ToxA sensitivity. In addition to wheat and barley with 98% identity at amino acid level of mature plastocyanin (Tai and Bragg, 2007), we would like to test ToxA response across a broad spectrum of plant species (from monocots to dicots). The mature ToxA was expressed under the control of 35S promoter in the binary vector pGD (Goodin *et al.*, 2002) designed for *Agrobacterium*-mediated transient protein expression (agroinfiltration). Using agroinfiltration assays, we can easily express ToxA in *N. benthamiana*, a well-established model plant for agroinfiltration. As a positive control of cell death, agroinfiltration of a constitutively active kinase (LeMEK2*) led to cell death in *N. benthamiana* leaves (Fig. 6A) (Chang *et al.*, 2002). In contrast, agroinfiltration of ToxA did not result in cell death and this was consistent with results observed when the ToxA protein was directly infiltrated into *N. benthamiana* leaves (Fig. 6A). We also constructed a ToxA-GFP fusion protein to monitor the localization of the fusion protein (Fig. 6B). Since ToxA did not bind to NbPCN, most ToxA-GFP was localized near the plasmamembrane but not in the chloroplast as in wheat. Collectively, the Y2H and *in planta* analyses indicate that the ToxA interaction with plastocyanin is specific to monocot wheat and barley but not the dicot *N. benthamiana*.

BSMV-induced Gene Silencing of TaPCN

Under normal light conditions, the plastocyanin knockout mutant in *Arabidopsis* is lethal (Weigel *et al.*, 2003) and establishing stable transgenic wheat cultivars is tedious and time-consuming

Table 2: Summary of three critical amino acid residues of ToxA

ToxA	Dimer	<i>In vivo</i> function	Bind to TaPCN
Wild type	+	+	+
G141A	+	-	-
E145R	-	-	+
D149K	-	-	+

+: Functional; -: Loss of function

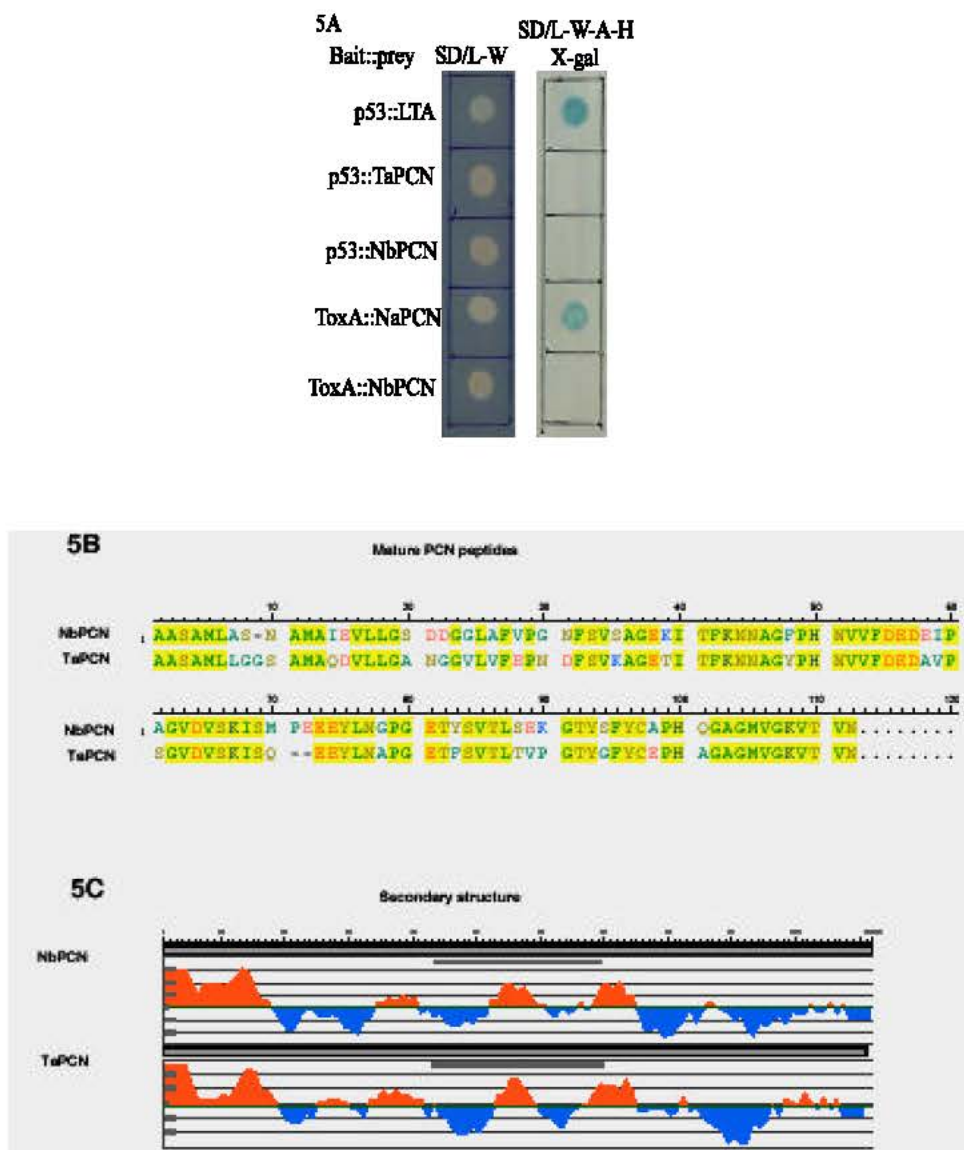


Fig. 5: Protein-protein interactions between ToxA and wheat or *N. benthamiana* plastocyanin. (A) Interaction of ToxA to TaPCN and NbPCN in yeast two-hybrid analyses. (B) Amino acid alignment of mature polypeptides of NbPCN and TaPCN. © Predicted secondary structures of mature NbPCN and TaPCN

(Shewry and Jones, 2005; Bhalla, 2006). Although not yet as commonly used as in dicots, a virus induced gene silencing (VIGS) system has been recently established to facilitate convenient knockdown of genes of interest in wheat (Scofield *et al.*, 2005; Tai *et al.*, 2005). Compared to wheat transformation, the advantage of VIGS is that it is localized and will not kill the whole plant and takes only a few weeks to generate results. Silencing of TaPCN using VIGS was not lethal to whole plants. However, silencing of TaPCN resulted in cell death in the silenced areas of the leaves; this phenotype was similar

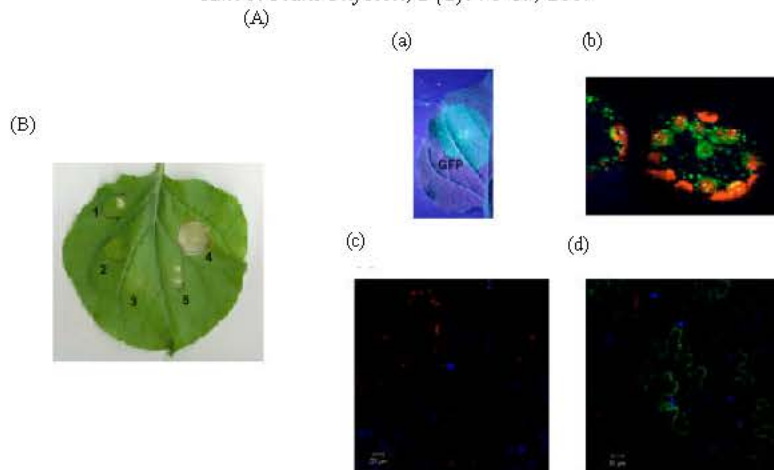


Fig. 6: ToxA effects on tobacco leaves. (A) Numbers 1 to 4 illustrate agroinfiltration assays. *Agrobacterium* contained pGD plasmids encoding the following proteins (counter-clockwise from upper-left): 1: empty vector, 2 and 3: two independent clones of ToxA, 4: infiltration of ToxA with the purified protein from *E. coli*. Area of number 5 illustrates a constitutively active form of protein kinase LeMEK2*, a positive control for cell death. (B) LSCM images of agroinfiltrated *N. benthamiana* leaves expressing GFP (a) or ToxA-GFP (b and d). (a) The leaf was agroinfiltrated with GFP construct and the image was taken under UV light. (b) Guard cells expressing ToxA-GFP. © Negative control of empty vector pGD only. (d) Epidermal cells expressing ToxA-GFP. Green is GFP fluorescence. Red is chlorophyll autofluorescence and blue is DAPI staining of nuclei



Fig. 7: Phenotypes of BSMV-mediated ToxA expression and VIGS of TaPCN

to that of ToxA following systemic expression using the BSMV-mediated expression (Fig. 7) and during infiltration of ToxA protein in sensitive cultivars (Fig. 2). No cell death was in plants infected with empty vector (Fig. 7) or when other genes were silenced, such as TaRar1, TaSGT1, TaSHP90 (Scofield *et al.*, 2005; Y.-S. Tai unpublished results), TaEDS1, TaCoi1 and TaEIN2 (Y.-S. Tai unpublished results).

DISCUSSION

The major goal of this study was to understand the molecular mechanism (s) of ToxA-induced cell death in wheat. Like other host-specific toxins (Wolpert *et al.*, 2002), the function of ToxA is usually associated with tan spot disease (Ciuffetti *et al.*, 1997). Analysis of ToxA action can provide insight into the complexity of host-pathogen interactions. In this study, we have examined the role of individual domains of ToxA in relation to oligomerization and target binding. Using site-directed

mutagenesis, we demonstrate that ToxA contains at least two domains required for function: one for binding to its target protein and the other for the formation of a homo-oligomer. The interaction of ToxA and its host target protein provides clues how a proteinaceous toxin causes cell death and therefore can act as a virulence factor for this necrotrophic pathogen.

Analyses of circular dichroism spectroscopy (Zhang *et al.*, 1997) indicated that the ToxA was mostly beta sheet and turns with a small amount of α and this is in agreement with the crystal structure (Sarma *et al.*, 2005). The RGD motif is located in a loop region protruding out from the crystal oligomers ([http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?form=6 and db=t and Dopt=s and uid=34730](http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?form=6&db=t&Dopt=s&uid=34730)). There are intra-molecular hydrogen bonds between E145 and Q133 as well as salt bridges creating an inter-molecular network that include a stretch of residues from V143 to L146 in β_6 sheet (Sarma *et al.*, 2005). The point mutation E145A was previously shown to abrogate cell death activity (Manning *et al.*, 2004). Although it has been shown that the mutated protein is stably expressed and the importance of E145 for ToxA activity is obvious, it was not clear whether the loss of function in E145A mutation is due to the disruption of oligomer formation or due to the loss of binding activity to host target protein. In contrast, the role of residue D149 has not been addressed in the literature. Using Y2H analysis and our novel transient assays, we showed the correlations of ToxA protein-protein interactions and cell death activity. We also identified TaPCN as the ToxA target protein. *Tsn1* is located in chromosome 5BL and was proposed to determine the ToxA sensitivity in protein infiltration assays (Lu *et al.*, 2006). *Tsn1* may be a membrane protein and therefore is not easily detected by the Y2H system that we used. *Tsn1* may play a role similar to components involved in endocytosis (Ayscough, 2005) or autophagy (Edinger and Thompson, 2004; Liu *et al.*, 2005). The function of *Tsn1* may be required for the internalization of ToxA and therefore *Tsn1* would be important in determining the ToxA sensitivity of different wheat cultivars. Functional redundancy of plastocyanin gene family members may explain why only *Tsn1* but not plastocyanin was identified in mutation screening (Faris *et al.*, 1996; Stock *et al.*, 1996).

Another ToxA-binding protein reported in the NCBI GenBank is called TaThf1 (AY377991). Since no published paper about the TaThf1 relevant to ToxA action, it remains unclear about its biological function. However, we could not detect the interaction between ToxA and TaThf1 in our Y2H system (data not shown). This might be due to the uses of different methods for detecting protein-protein interactions (Parrish *et al.*, 2006). We also tried to silence the TaThf1 by VIGS, but there no cell death was observed in the infected plants (data not shown). This might be due to the typical property of RNA silencing with a wide range of effects on gene expression (Waterhouse and Helliwell, 2003). Both TaPCN and TaThf1 are compartmentalized to the chloroplast. Further investigation is needed to determine their roles in ToxA-induced cell death.

The light-dependent cell death that correlated with ToxA effects (S. Meinhardt, unpublished data; Manning and Ciuffetti, 2005) might stem from the interruption of electron flow by the binding of ToxA to plastocyanin. Interaction of plastocyanin with ToxA could affect both protein binding to components of the photosystem (e.g., Cyt. b_6/f) and electron transfer properties. Correct and timely interaction of specific molecules is pivotal to the electron transfer processes. The blocking of electron flow may generate Reactive Oxygen Species (ROS) leading to cell death (van Breusegem and Dat, 2006). The signal transduction networks activated by ROS may explain the phenomenon of ToxA-induced cell electrolyte leakage, the low-temperature effect and the cycloheximide effect (Kwon *et al.*, 1998).

In conclusion, we have shown that ToxA activity is dependent on both oligomerization and plastocyanin binding. Future work will pursue protein-protein interactions *in planta*, using coimmunoprecipitation with different tags. However, wheat transformation is a great limitation. We intend to apply methods of bimolecular fluorescence complementation (BiFC, Shyu *et al.*, 2006; Walter *et al.*, 2004) or tandem affinity purification (TAP, Rubio *et al.*, 2005) adapted to our BSMV-mediated transient expression in wheat.

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