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Dual Applications of a Virus Vector for Studies of Wheat-Fungal Interactions

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Abstract: Obtaining stable transgenic plants is still a highly challenging barrier for analyses of gene function in wheat. Unlike the situation with dicots and other model organisms, RNA silencing and transient assays of gene expression are not well-established in wheat. We previously developed and reported a Virus-induced Gene Silencing (VIGS) system in wheat and now we present here a transient expression system for assays in wheat. To demonstrate the utility of this transient assay system, we have applied it to the study of ToxA, a fungal toxin important in tan spot disease of wheat.

Key words: BSMV, transient assay, ToxA, wheat, barley

INTRODUCTION

The necrotrophic fungus *Pyrenophora tritici-repentis* (*Ptr*), a world-wide threat in wheat-growing areas, causes tan spot disease in wheat. Typical disease symptoms include tan-colored, diamond-shaped necrotic lesions surrounding small, dark-brown infection sites. Wheat diseases caused by fungal pathogens are often associated with the secretion of fungal toxins (Walton and Panaccione, 1993) and the necrotic symptoms induced by *Ptr* are associated with the production of a host-selective toxin (HST), ToxA (Tomas *et al*., 1990; Lamari *et al*., 1995). ToxA is the first proteinaceous toxin among the HSTs to be cloned from fungi and ToxA protein infiltration alone causes necrotic cell death in sensitive wheat cultivars (Ballance *et al*., 1996; Ciuffetti *et al*., 1997). Host sensitivity to ToxA is proposed to be mediated by the wheat *Tsn1* locus (Faris *et al*., 1996; Stock *et al*., 1996; Lu *et al*., 2006). Although it has been proposed that Tsn1 mediates internalization of ToxA, experiments also indicate that there is an intracellular ToxA target (Manning and Ciuffetti, 2005; Tai *et al*., unpublished data). After intracellular ToxA expression via particle bombardment (Manning and Ciuffetti, 2005) or virus-mediated expression (this report), the ToxA-induced necrotic symptoms were no longer restricted to sensitive host.

The generation of stable transgenic plants and induction of RNA silencing are two commonly used approaches to verify gene function *in planta*. However, the recalcitrance of wheat to transformation remains a highly challenging barrier (Shewry and Jones, 2005; Bhalla, 2006). A transient expression assay, such as particle bombardment, affords an alternative methodology (Manning and Ciuffetti, 2005). However, in the study of ToxA-induced cell death, particle bombardment causes a high frequency of cell death that is easily confused with the ToxA-induced cell death phenotype. In order to circumvent this confusion associated with particle bombardment, we used a novel virus-mediated transient assay for systemic expression of ToxA in wheat and barley.

MATERIALS AND METHODS

Plant materials: Durum wheat (*Triticum turgidum* L. subsp. *durum*) cultivar Langdon (LND) and barley (*Hordeum vulgare* L.) cultivar Black Hulless plants were used in this study. LDN wheat is susceptible to *Ptr* and sensitive to ToxA, but barley is not a host of *Ptr*. Plants were kept in a growth chamber at 23°C with a 10/14 h light/dark cycle for two to three weeks until the seedlings reached the two-leaf stage. Photos of cell death were taken at 21 days post-inoculation (dpi).

BSMV-mediated ToxA expression *in planta*: *BamHI* and *Hpa*I restriction sites were engineered to flank the sequence encoding the mature ToxA protein and were used for cloning into the BSMV γ-γb*BamHI* vector (Tai *et al*., 2005), replacing most of γb gene. The *ToxA* gene sequence encoding the mature polypeptide (based on the NCBI accession number U79662) was amplified by PCR using the following primers: The forward primer introduced a *BamHI* site directly before the ATG start codon (5'-*GGATCC* ATGGGAAGCTGCATGTC AATCACA-3') and the reverse primer encoded an *Hpa*I

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site following the stop codon (5'-*GTTAAC* TAATTTTCTAGCTGCATTCT-3'). The procedure for inoculating the RNAs in wheat and barley was described in Tai *et al*. (2005). Each construct was tested in 10 plants.

Reverse transcription PCR: In addition to observing the necrotic cell death symptoms in plants, ToxA RNA levels were assayed to evaluate gene expression by reverse transcription PCR (RT-PCR). Total RNA from systemically-infected leaves was isolated at 21 dpi, using the TriZOL reagent per manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from 1 μg of total RNA using BD PowerScript Reverse Transcriptase (SMART II RACE kit, BD Biosciences Clontech, Palo Alto, CA). Using the synthesized cDNAs as templates, PCR was performed with the FastStar High Fidelity PCR System kit (Roche Applied Science, Indianapolis, IN, USA). As a control to confirm the cDNA templates and to compare the levels of PCR products, actin was amplified using the following primer pair: forward 5'-GAGGTCCTCTT CCAGCCATCCAT-3' and reverse 5'-TTGGATATCCA CATCTGTTGGAAAGT-3'. According to the NCBI sequence (Accession # AY663391), a 300-bp PCR product of actin gene is predicted. To amplify the ToxA sequence the same ToxA primers described above were used to generate a 354-bp product.

RESULTS AND DISCUSSION

Recent studies have demonstrated that *Barley Stripe Mosaic Virus* (BSMV) can be used as a vector for virus-induced gene silencing (VIGS) in wheat (Tai *et al*., 2005; Scofield *et al*., 2005) and barley (Holzberg *et al*., 2002). The genome of the BSMV strain ND18 is comprised of three RNAs, designated α,-β and γ (Tai *et al*., 2005) and the RNAγ component has been modified to express foreign genes of interest. The *ToxA* sequence was used to replace the fragment between *BamHI* and *Hpa*I sites in the γ-γb*BamHI* vector DNA (Tai *et al*., 2005) to generate the γ-ToxA construct. The size of PCR product is 354 bp, which is smaller than the replaced fragment (747 bp) of γb. However, this insertion of *ToxA* does not significantly distort the size of the viral genome.

To test the systemic expression of ToxA *in planta*, the BSMV α, β and γ-ToxA RNAs were prepared by *in vitro* transcription and inoculated to wheat and barley leaves. The ToxA-induced cell death phenotype was visible and became prominent by 21 dpi (Fig. 1A). 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. However, the expression of *ToxA* in wheat was verified by RT-PCR. Compared to the actin control, the expression of *ToxA* was

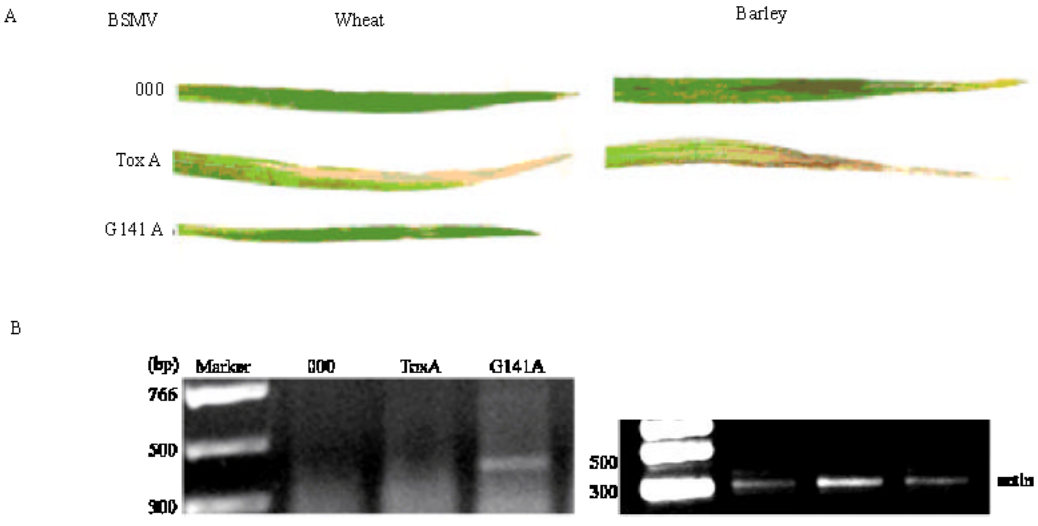

Fig. 1: ToxA-induced necrotic cell death in wheat and barley. (A) The BSMV empty vector (000) and BSMV carrying an insert of wild type ToxA or mutant ToxA-G141A were inoculated on the second leaves of two-leaf-age wheat and barley. The pictures of systemic (third) leaves were taken at 21 dpi. (B) Reverse transcription PCR using the total RNA isolated from the wheat leaves inoculated with BSMV::000, BSMV::ToxA and BSMV::ToxA-G141A, respectively. The primer sequences for ToxA and actin are described in the text

detectable in leaves infected by the loss-of-function mutant ToxA-G141A but not in the leaves inoculated with the empty vector or the wild type ToxA (Fig. 1B). The intracellular expression of the ToxA-G141A mutant in wheat did not cause cell death (Fig. 1A) and this result is consistent with observations that ToxA-G141A protein infiltrations fail to cause necrosis in a sensitive wheat cultivar (Meinhardt *et al*., 2002). One possible explanation for the very low levels of wild type ToxA detected in wheat is that the total RNA was isolated from tissue that was highly necrotic due to the presence of the toxin and the small portion of live, surrounding cells were not infected by the virus.

Since the intracellular host target of ToxA (TaPCN, Tai *et al*., unpublished) is non-host specific in wheat, we further tested whether ToxA could induce cell death in barley. We have identified the sequence of TaPCN homolog in the barley genome that is highly similar at the amino acid level (94% identity for the full-length coding region and 98% identity for the mature protein) to the wheat ToxA interactor (Tai *et al*., unpublished data). Barley was inoculated with the BSMV α, β and γ-ToxA infectious transcripts to mediate expression of ToxA and infected tissue showed necrotic cell death similar to the ToxA phenotype observed in wheat (Fig. 1A).

To our knowledge, this is the first published report of the successful application of BSMV-mediated gene expression for the study of fungal pathogens in wheat, although it has been a decade ago using *Potato Virus X* (PVX) as a vector to express tomato or bacterium genes in dicots (Rommens *et al*., 1995; Tobias *et al*., 1999). This BSMV-mediated transient assay system provides a powerful tool and has excellent potential for genome-wide application for fungal functional genomics. In combination with the approaches of proteomics and bioinformatics, we and colleagues are currently using this system to identify candidate toxin genes in a major pathogen of wheat and barley, *S. nodorum*, that secretes proteinaceous toxins including a ToxA homolog (Friesen *et al*., 2006) and for which genomic sequence is available in a public database (http://www.broad.mit.edu/annotation/genome/stagonospora-nodorum/Home.html).

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