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Analysis of small RNA silencing in *Zymoseptoria tritici* - wheat interactions

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1. Cross-kingdom gene silencing

The ascomycete fungus *Zymoseptoria tritici* is the causative agent of Septoria tritici blotch (STB) disease and is a major threat to wheat production globally (Dean *et al.*, 2012). *Z. tritici* is a hemibiotrophic foliar pathogen, which invades leaf tissue through natural openings such as stomata. *Z. tritici* remains exclusively apoplastic through its infection cycle, which is characterized by a long symptomless infection phase (10-14 days), followed by the transition to necrotrophy (Kema *et al.*, 1996).

Recent observations suggest that natural cross-kingdom gene silencing (RNAi) can occur in some plant-fungal interactions (Weiberg *et al.*, 2013; Wang *et al.*, 2016). These developments prompted us to ask whether this phenomenon was involved in the colonization of wheat by *Z. tritici*, an exclusively apoplastically-dwelling pathogen. The existence of fungal sRNA in *Z. tritici* is to date unexplored. Here we report our findings to characterize the sRNA populations of this fungal species during wheat leaf infection, predict putative wheat transcripts that may be subject to cross-kingdom RNAi and biochemically validate such interactions.

2. sRNA discovery in *Z. tritici*

The genome of *Z. tritici* isolate IPO323 is predicted to encode a single Dicer (*ZtDCL - Mycgr3G47983*) and two Argonaute (*ZtAGO1 - Mycgr3G38035*, *ZtAGO2 - Mycgr3G10621*) ribonucleases. This provided indirect evidence that a system for sRNA biogenesis may exist in *Z. tritici*. We therefore sequenced sRNA preparations from in vitro cultured fungus, and from infected susceptible wheat plants (cv. Bobwhite) over a 21-day timecourse (Fig.1).

In planta sample	BWZt4 (4 dpi)	BWZt9 (9 dpi)	BWZt13 (13 dpi)	BWZt21 (21 dpi)	
Number of sRNA loci	192	277	320	348	Total = 389 loci

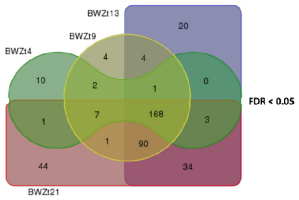

Figure 1. Predicted *Z. tritici* sRNA loci active during different timepoints of wheat infection. Numbers of sRNA loci upregulated at 4, 9, 13, and 21 days post inoculation (dpi) are shown in the upper panel. Venn diagram showing numbers of infection stage-specific sRNA loci as well as those shared between consecutive infection timepoints is presented in the lower panel.

3. Transcriptional profiling of *Z. tritici* sRNAs and putative wheat targets

We identified 262 fungal sRNAs computationally predicted to target 737 wheat transcripts. It was expected that wheat transcripts successfully targeted by fungal sRNAs would display downregulation during some or all stages of infection. We prioritized ten wheat mRNAs (Table 1), predicted to be targeted by four *Z. tritici* sRNAs (by mRNA cleavage) for investigation based on expression profile (Fig.2) and role in pathogen defense.

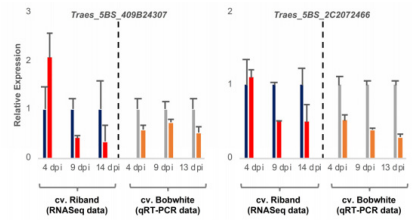

Figure 2. Expression profiling of candidate wheat target mRNAs Left panels show RNAseq data (re-analysis of Rudd et al. 2015) for candidate genes for mock (blue) and *Z. tritici* IPO323 (red) treatments. Right panels show qRT-PCR analysis of duplicate sample set, mock (grey) and *Z. tritici* IPO323 (orange).

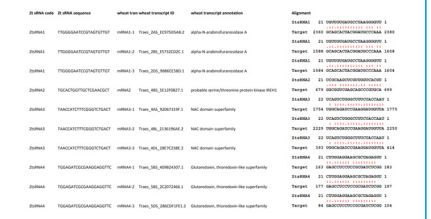

Table 1. *Z. tritici* mature sRNAs selected for detailed study and their respective wheat mRNA targets.

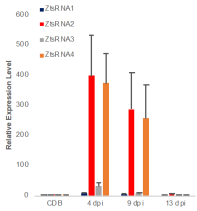

Figure 3. Expression profiling of *Z. tritici* sRNAs by stem-loop qRT-PCR. Relative expression of four sRNAs (ZtsRNA1 - ZtsRNA4) from the fungus grown in vitro (CDB) or in planta (leaf tissue at 4, 9, and 13 dpi).

4. *Z. tritici* RNAi mutants are fully pathogenic

To examine the role of the fungal RNAi pathway in the interaction between wheat and *Z. tritici*, we produced several fungal mutants deficient in key RNAi components (Fig.4). These experiments demonstrated that the *ZtDCL*, *ZtAGO1*, and *ZtAGO2* genes are dispensable for virulence.

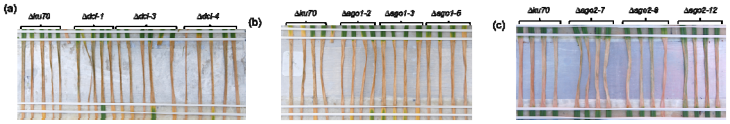

Figure 4. *Z. tritici* IPO323 strain Δ*ku70* and its derivatives deficient in the RNAi pathway (a) Δ*dcl*, (b) Δ*ago1* or (c) Δ*ago2* were inoculated onto wheat cv. Bobwhite. Fungal inoculations were done using suspension of conidiospores at 1 x 10^6 mL^{-1} and the inoculated leaves were photographed at 16 dpi.

5. Dicer-independent sRNA production in *Z. tritici*

We carried out wheat infection time courses with *Z. tritici* IPO323 Δ*ku70* (control) and the Δ*dcl* mutant followed by fungal sRNA expression analysis (Figs.5,6). Expression of some fungal sRNAs was maintained in the Δ*dcl* mutant indicating DCL-independent sRNA biogenesis in *Z. tritici* (Fig.5). Expression analysis of a wheat mRNA target for the DCL-dependent ZtsRNA2 showed no difference between Δ*ku70* (control) and Δ*dcl* mutant infections (Fig.6), indicating that this mRNA is unlikely to be a genuine sRNA target.

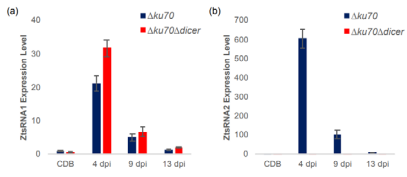

Figure 5. Expression profiling of two fungal sRNAs in an RNAi-deficient *Z. tritici* mutant (Δ*ku70* Δ*dicer*).

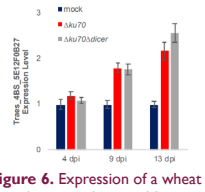

Figure 6. Expression of a wheat mRNA target of ZtsRNA2 in same samples used in Fig.5.

6. Host-induced gene silencing (HIGS) and in vitro dsRNA uptake are ineffective against *Z. tritici*

To test whether plant-derived sRNAs could translocate and induce RNAi in *Z. tritici* during infection, we used a plant RNA virus-based vector as an RNA silencing inducer in a procedure known as HIGS (Fig.7). In parallel, we investigated whether external application of dsRNAs targeting essential for life fungal genes could restrict *Z. tritici* growth in vitro (Fig.8). To directly assess the uptake of dsRNA by *Z. tritici*, we co-incubated germinating conidiospores with fluorescently-labelled short or long dsRNA (Fig.9).

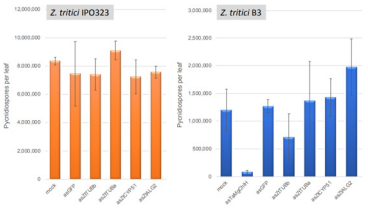

Figure 7. *Z. tritici* (strains IPO323 and B3) pycnidiospore production in wheat cv. Riband plants pre-treated with various BSMV-HIGS and BSMV-VIGS constructs.

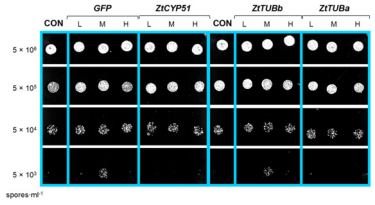

Figure 8. In vitro silencing using *Z. tritici*-specific dsRNAs generated in vitro. *Z. tritici* IPO323 conidiospore suspensions at four different concentrations were incubated overnight with 12.5 ng (L), 125 ng (M) or 1250 ng (H) of dsRNA specific for different *Z. tritici* mRNAs before growth on YPD agar for 4 days at 17°C. CON is untreated *Z. tritici* conidiospores.

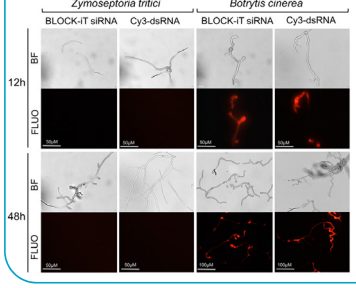

Figure 9. Assessment of dsRNA uptake in *Z. tritici* and *B. cinerea*. Uptake of fluorescently labelled short dsRNA (Alexa Fluor Red BLOCK-iT siRNA) or 250-long long Cy3-labelled dsRNA against GFP was monitored at 12 h and 48 h of co-incubation. Images were captured with bright field (BF) and fluorescent (FLUO) absorption 555 nm, emission 565 nm) settings. *B. cinerea* was used as a positive control for dsRNA uptake.

Conclusions

- *Z. tritici* encodes sRNAs that are transcriptionally-induced during infection but we could not validate targeting of wheat mRNAs
- RNAi pathway is dispensable for fungal virulence
- Some *Z. tritici* sRNAs are generated by an unknown Dicer-independent mechanism
- Unlikely *Z. tritici* can absorb functional sRNA from host plants or environment
- RNAi likely to be less effective at control of *Z. tritici* than for some other fungal pathogens

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