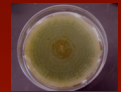


Understanding the Link between a Natural Antisense Transcript and the Regulation of Aflatoxin Biosynthesis



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Background

The full impact of RNA molecules on gene expression is only now becoming clear. In addition to microRNAs and siRNAs, other types of naturally occurring antisense transcripts (NATs) can impact gene expression and have been observed in many different eukaryotic organisms. NATs can be defined as RNA containing sequences that are complementary to other endogenous RNAs. With more and more genomes being sequenced, it is now possible to use bioinformatic analyses to estimate the number of NATs in an organism. Only a fraction of these NATs have been studied in detail to assign function. Modes of action of NATs include transcriptional interference, RNA masking, antisense induced methylation, and ds-RNA dependent mechanisms. We identified a NAT in *Aspergillus flavus* that overlaps *afIR*, the transcriptional regulator of the aflatoxin (AF) biosynthesis pathway.

Does *afIRas* really exist?

Several years ago, the *afIRas* transcript was identified in a cDNA library constructed from tissue grown in media conducive for AF production. The *afIRas* transcript is complementary to the 5' region of *afIR* and its promoter (Fig 1).

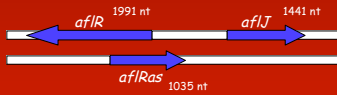


Figure 1 – Genomic Arrangement of *afIR* and *afIRas*. *afIRas* is transcribed from the complementary strand of *afIR* in the opposite direction. The region of overlap between *afIR* and *afIRas* occurs at the 5' end of each transcript. The *afIRas* transcript extends past the transcriptional start site of *afIR* and into its promoter region.

To verify that *afIRas* transcripts were present in cultures actively producing AF, we used quantitative RT-PCR (Fig 2).

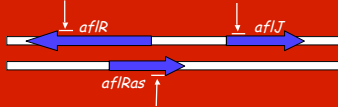
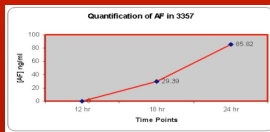


Figure 2 – Targets for q RT-PCR Analysis. Primers were designed to amplify amplicons of approximately 70bp. The primers that were used to distinguish *afIR* and *afIRas* amplify 3' regions that are not included in the area of overlap.

The RNA used for the q RT-PCR analysis was extracted from a wild type strain of *A. flavus* (3357). A mother culture was inoculated with spores and used to seed daughter cultures. The daughter cultures were harvested at 12hrs, 16hrs, and 24hrs (Fig 3) and liquid medium was analyzed for AF.

Figure 3 – HPLC Analysis of AF for Tissue Samples in YES medium used for q RT-PCR.



q RT-PCR was used to look at transcript levels of *afIR*, *afIRas*, and *afIJ* at the three time points. *afIJ* was used to make sure that *afIRas* is separate from this transcript. At the 12hr time point (before aflatoxin production), *afIR* and *afIRas* are equal. At the 18hr time point in which AF production has started, *afIRas* begins to decrease in relation to *afIR*. When AF is accumulating at higher levels in the 24hr time point, there is 2 fold decrease of *afIRas* compared to *afIR*.

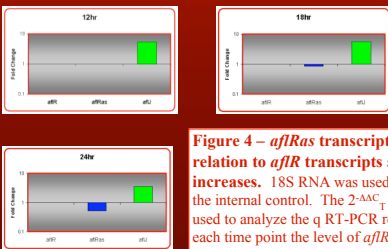


Figure 4 – *afIRas* transcripts decrease in relation to *afIR* transcripts as toxin increases. 18S RNA was used in this study as the internal control. The $2^{-\Delta\Delta C_T}$ method was used to analyze the q RT-PCR results and at each time point the level of *afIR* was set to one for comparison.

What happens if you over-express *afIRas*?

Because *afIR* and *afIRas* have overlapping transcripts, gene replacement cannot be used to study *afIRas* without also modifying *afIR*. Since a knock out is problematic, we decided to over-express *afIRas* to see if this had an effect on *afIR* and toxin levels. An *afIRas* over-expression construct was built and deemed pGAP40 (Fig 5).

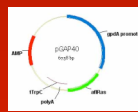


Figure 5 – *afIRas* Over-expression Construct (pGAP40). This construct contains the *gpdA* promoter that constitutively expresses *afIRas*. The full-length transcript of *afIRas* isolated from the cDNA library was used with an additional 19 bases at the 5' end and 6 bases at the 3' end.

A *pyr* mutant strain of *A. flavus* (3357-5) was used for a cotransformation with pGAP40 and pBSK-pyr that contains *pyr-4* to complement the mutation as a selection. Ninety-seven transformants were recovered on medium lacking uracil. In order to determine which of these transformants contained the pGAP40 construct, genomic DNA from each transformant was used in a PCR screen. The PCR results showed that twenty-nine transformants contained both the pBSK-pyr and pGAP40 plasmids (Fig 6).

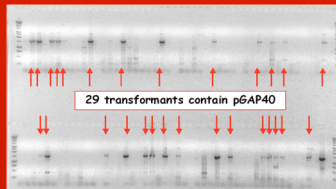


Figure 6 – PCR Screen of Putative *afIRas* Over-expression Transformants. Genomic DNA was extracted from each transformant and used for the PCR screen. Primers that are specific to the over-expression construct were used to amplify a 1.5kb product.

Spore suspensions for each over-expression transformant were used to inoculate liquid cultures of PDB. While YES and PDB media are both conducive for AF production, PDB supports higher levels of AF, therefore we used PDB for this experiment. After 24 hrs, tissue was collected by filtration and liquid media was quantified for aflatoxin using HPLC analysis (Fig 7).

Sample	Aflatoxin, ng/ml in PDB		
	Parent	Total	
3357-5	2478	AS 338	1100
AS 326	1100	AS 325	6436
AS 328	1100	AS 343	4548
AS 329	26	AS 342	7349
AS 342	2675	AS 340	3117
AS 388	2675	AS 339	3438
AS 351	984	AS 346	3483
AS 381	984	AS 380	112
AS 387	420	AS 382	411
AS 376	709	AS 378	2929
AS 384	709	AS 393	2668
AS 374	816	AS 385	2568
AS 395	311	AS 373	7485
AS 371	76	AS 368	2636
AS 338-1	675	AS 329	173

Figure 7 – HPLC Analysis of AF in *afIRas* Over-expression Transformants in PDB medium.

A subset of the *afIRas* over-expression transformants was selected to use for q RT-PCR analysis. This subset had varying aflatoxin quantities ranging from 0 to 11,648 ng/ml as compared to the 3357-5 parental strain that had 2478 ng/ml in order to get examples from the full range. q RT-PCR was used to determine the levels of *afIR* and *afIRas* in the over-expression transformants. Transformants as344, as252, and as328 show over-expression of *afIRas* whereas as349 and as335 contained less *afIRas* than the parental strain. There does not seem to be any correlation between *afIRas*, *afIR*, and toxin levels.

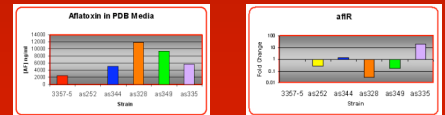
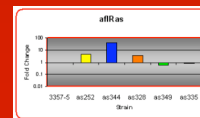


Figure 8 – *afIRas* over-expression Transformants q RT-PCR results. 18S RNA was used in this study as the internal control. The $2^{-\Delta\Delta C_T}$ method was used to analyze the q RT-PCR results and the *afIRas* over-expression transformants were compared to 3357-5 (parental strain).



Conclusions

- In relation to *afIR*, *afIRas* accumulation decreases as AF levels increase in conducive media
- Some *afIRas* over-expression transformants accumulate more AF when compared to the parental strain while some accumulate less
- Further studies are needed to determine how over-expression of *afIRas* affects *afIR* and toxin production

Future Directions

- Is *afIRas* conserved in other species of *Aspergillus*?
- Does *afIRas* encode a protein?
- Time course of relative levels under conducive and nonconductive conditions for AF

References

Chen J, et al., Over 20% of human transcripts might form sense-antisense pairs. *Nucleic Acids Res.* (2004), 48:12-20.
 Kiyonawa, H, et al., Antisense transcripts with FANTOM2 clone set and their implications for gene regulation. *Genome Res.* (2003), 13:24-34.
 Lavorgna, G, et al., In search of antisense. *Trends Biochem Sci.* (2004) 88-94.
 Livak, K.J. and T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻ $\Delta\Delta C_T$ Method. *Methods.* (2001) 402-8.
 Lofas, B.J, et al., The genome of the basidiomycetous yeast and human pathogen *Cryptococcus neoformans*. *Science.* (2005) 1211-4.
 Mira, S, et al., Annotation of the *Drosophila melanogaster* euchromatic genome: a systematic review. *Genome Biol.* (2002) 83.
 Osato, N, et al., Antisense transcripts with rice full-length cDNAs. *Genome Biol.* (2003), R5.
 Woloshuk, C.P, et al., Molecular characterization of *afIR*, a regulatory locus for aflatoxin biosynthesis. *Appl Environ Microbiol.* (1994) 2486-14.