Characterizing MADS-box transcription factor-mediated regulation of fumonisin biosynthesis in *Fusarium verticillioides* with computational subnetwork module analysis

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Abstract

Fusarium verticillioides **is an important maize pathogen, leading to Fumonisin B1 (FB1) in infested kernels. MADS-box transcription factors (TF) were found to modulate polyketide synthase (PKS) gene expression and FB1 production in F. verticillioides. With next-generation sequencing of F. verticillioides wild type and MADS-box TF mutant cultures, we used a suite of computational network-based tools, e.g. partial correlation, log-likelihood ratio matrix and seed-and-extend approach, to perform a system-oriented transcriptome analysis to predict downstream genetic subnetwork modules associated with FB1 production. Our aim was to identify and characterize system-level changes across correlated genes rather than simply focusing on individual gene expression. The resulting subnetwork modules are predicted to contain hub genes, which likely play a crucial regulatory role within functional modules. Two predicted subnetwork modules were analyzed in silico, and five putative hub genes were subjected to functional characterization. Deletion of RAS GTPase (FvRSR1) and methyltransferase (FvEFM3) led to a significant reduction in FB1 levels. A comparative qPCR was performed to investigate the impact of FvRsr1 and FvEfm3 on the expression of neighboring genes in two subnetworks, which did show significant changes. Collectively, these results support our prediction that FvRSR1 and FvEFM3 serve as a key hub gene in each subnetwork and regulate FB1 biosynthesis.**

Introduction to *Fusarium* stalk and kernel rot in maize

- *Fusarium verticillioides* is an economically important, fungal pathogen causing stalk and ear rot in maize
- Worldwide distribution
- A heterothallic, ascomycete (*Gibberella moniliformis*)
- Produces fumonisin B_1 (FB_1) linked to esophageal cancer in humans and leukoencephalomalacia in horses

Background: MADS-box TFs

- The MADS-box TFs are involved in the co-regulation of genes, signal transduction and developmental functions
- Have a highly conserved motif across a wide range of eukaryotes
- First four proteins identified -- *MCM1*, *AGAMOUS*, *DERCIENS*, and *SRF1*
- Knockout mutants were constructed to analyze the role of *Mads1* and *Mads2* in secondary metabolism and sexual reproduction in *F. verticillioides*
- *Mads1* produced significantly less FB_1 than the WT
- PKS gene expression levels decreased as time passed, with 14 of the PKS transcripts in *Mads1* at least half of that of the WT, 10-dpi

Identification of MADS-Box TF regulatory pathways

- Wild type and *Mads1*
  - Myro medium
  - Initial inoculum
    - 7-day old, 150 rpm
  - Biological replicates
    - 0.3 g of mycelia as inoculum
    - Harvested 5- and 7-dpi
  - RNA extraction
  - Sequencing
    - 5 samples/time point/ strain

Method

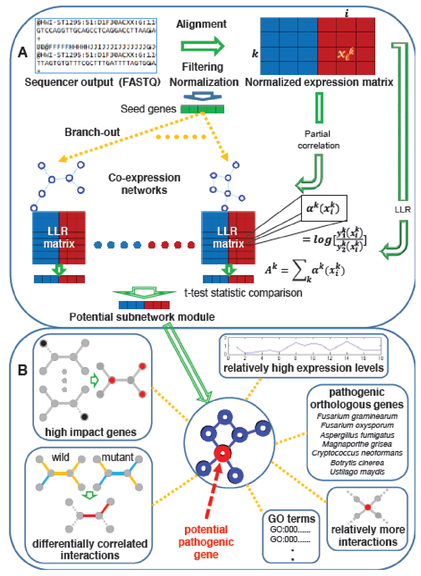
Expected outcomes

- PKS genes and other secondary metabolite genes
- Vegetative development genes
- Other genes and signaling pathways under MADS-box TF regulation

Our Subnetwork Prediction Workflow

- Preprocessing
  - alignment
  - filtering
  - normalization
- Seed-and-Extend Approach
  - computationally efficient technique
  - activity from log-likelihood ratio (LLR)
  - t-test statistic comparison

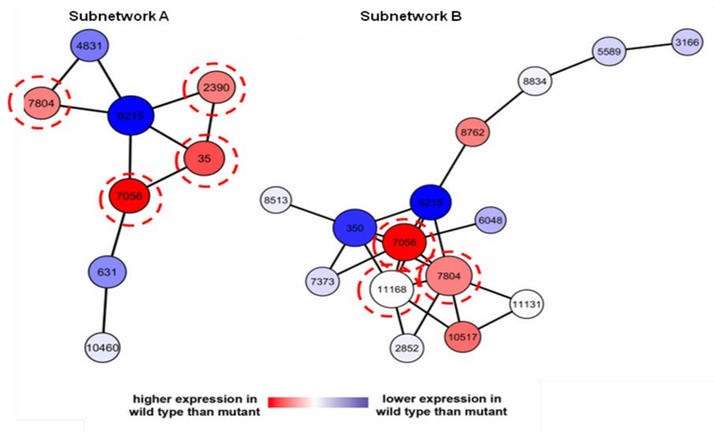
Seed genes: strongly differentially expressed in the two phenotypes at the last time point



Aims

- **Identification of putative hub genes with *in silico* analysis**
- **Gene-deletion by homologous combination**
- **Functional characterization**
  - **Growth and development**
  - **FB_1 production**
- **Putative hub impact on network robustness and transcriptional directionality**

Putative hub genes



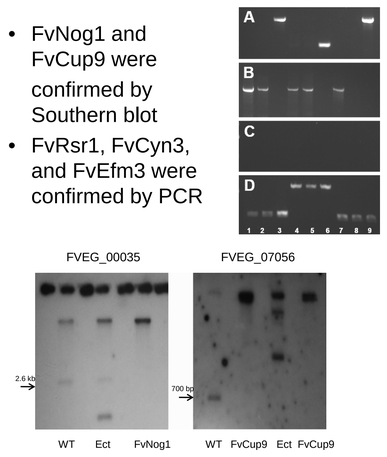
In silico analysis

Gene	Protein domain prediction	# of Paralogs	Yeast Homolog	Known Function
FVEG_00035	50S Ribosome-binding GTPase		NOG1	Required for 60s ribosome sub-unit biogenesis in yeast
FVEG_02300	RAS GTPase	1 copy	RSR1, RAS2, RAS1, GSP1, GSP2	GTP-binding protein of the Ras superfamily, required for bud site selection, morphological changes in response to mating pheromone, and efficient cell fusion
FVEG_07056	Homeobox KN Domain	4 copies	CUP9, TOS8	Homeodomain-containing transcriptional repressor, regulates expression of PTR2, which encodes a major peptide transporter, imported peptides activate ubiquitin-dependent proteolysis, resulting in degradation of Cup9p and de-repression of PTR2 transcription, protein abundance increases in response to DNA replication stress
FVEG_07804	Cyanase_C (cyanate lyase)	1 copy	None	Unknown

Gene	Protein domain prediction	# of Paralogs	Yeast Homolog	Known Function
FVEG_11168	Methyltransferase	1 copy	EFM3, NNT1, YNL024C	S-adenosylmethionine-dependent methyltransferase, seven-beta-strand lysine methyltransferase which trimethylates translation elongation factor EF2 (Eft1p and Eft2p)
FVEG_07804	Cyanase_C (cyanate lyase)	1 copy	None	Unknown
FVEG_07056	Homeobox KN Domain	4 copies	CUP9, TOS8	Homeodomain-containing transcriptional repressor, regulates expression of PTR2, which encodes a major peptide transporter, imported peptides activate ubiquitin-dependent proteolysis, resulting in degradation of Cup9p and de-repression of PTR2 transcription, protein abundance increases in response to DNA replication stress

Gene-deletion Confirmation

- FvNog1 and FvCup9 were confirmed by Southern blot
- FvRsr1, FvCyn3, and FvEfm3 were confirmed by PCR

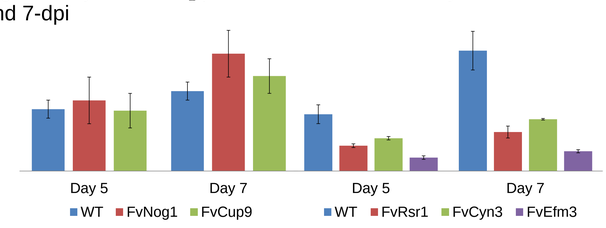


Growth analysis

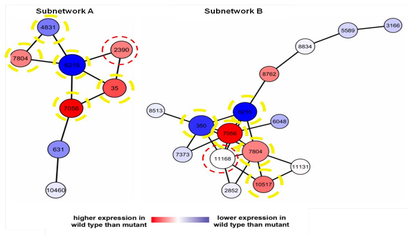
- WT and gene-deletion mutants were analyzed for growth on PDA, V8, and DL (solid) media
- Significant difference:
  - FvNog1 and FvCup9 on PDA and DL, respectively
- No difference between WT and FvRsr1, FvCyn3, FvEfm3
- Increased conidia production in FvRsr1 (V8)
- Pigment altered in DL (all mutants)

FB_1 production

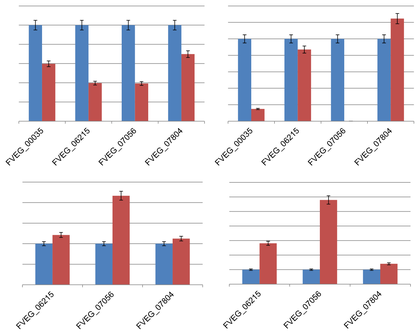
- Significantly **higher** FB_1 production in FvNog1 vs WT at 7-dpi
- Significantly **lower** FB_1 production in FvRsr1, FvCyn3, and FvEfm3 at 5- and 7-dpi



Impact of hub genes on neighboring genes



Relative Expression in WT vs mutants (FvRsr1 & FvEfm3)



- Expression of neighboring genes were altered in FvRsr1 when compared to WT (5- and 7-dpi)
- Two neighboring genes (FVEG_06215 and FVEG_07056) were significantly upregulated in FvEfm3 when compared to WT

Summary

- Network-based gene association analysis was used to predict two subnetworks downstream of *MADS1* important for FB1 regulation
- Five genes were selected for knockout and functional characterization
- All five gene-deletion mutants affected secondary metabolism
- FvRsr1 and FvEfm3 mutations resulted in a significant FB_1 reduction
- Subnetwork gene expression was altered in FvRsr1 and FvEfm3
- *FvRSR1* – RAS GTPase and *FvEFM3* – methyltransferase have a directional impact on transcription in neighboring genes
- *FvRSR1* and *FvEFM3* likely play hub role in predicted subnetworks

Literature cited

Ortiz, C.S., and Shim, W.-B. 2013. The role of MADS-box transcription factors in secondary metabolism and sexual development in the maize pathogen Fusarium verticillioides. Microbiology 159:2259-2268.

Kim, M., Zhang, H., Woloshuk, C., Shim, W.-B., and Yoon, B.-J. 2015b. Computational identification of genetic subnetwork modules associated with maize defense response to Fusarium verticillioides. BMC Bioinformatics 16:S12.

Acknowledgements

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