

A Transgenic Approach to Alfalfa Crown Rot Control

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Alfalfa Crown Rot

- Pervasive disease, occurs to some extent in all alfalfa stands
- Significant cause of stand decline
- Predispose to winter kill; effects might not be evident until next season
- Repeated wounding, pathogen entrance



Disease Characteristics

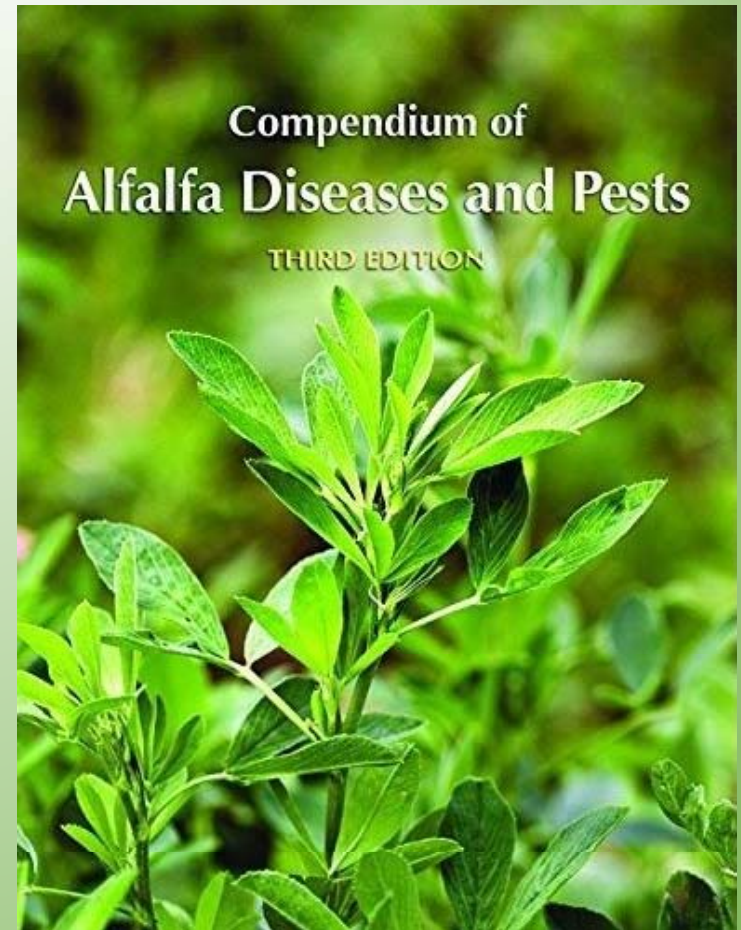
- Chronic Disease: occurs over the scale of months/years
- Brown, necrotic areas restricted to the crown or root cortex
- Asymmetric plant growth
- Development of secondary crown branches



ent.iastate.edu/imagegal/plantpath/alfalfa/1303.80crownrot.html; Samac et al., 2011

Disease Complex

- Cannot be ascribed to the activity of a single pathogen
- *Fusarium oxysporum* f. sp. *medicaginis*, *F. solani*, *F. roseum*, *Phoma medicaginis*, *Rhizoctonia solani*, *Colletotrichum trifolii*, *Pythium* spp.
- *Pseudomonas syringae* pv. *syringae*
Clavibacter michiganensis subsp. *insidiosus*
- Differ substantially by geographic location



Current Control Methods

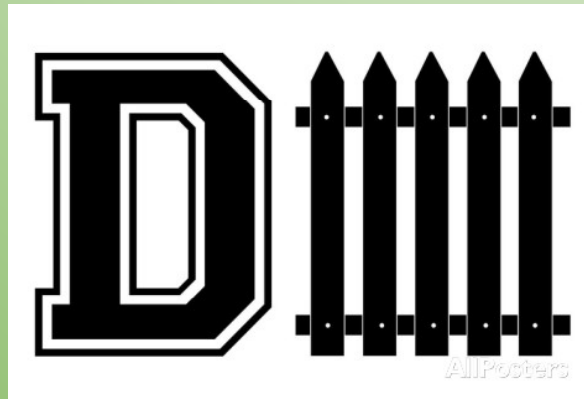
- Breeding efforts to increase quantitative resistance have made only minor progress (Miller-Garvin and Viands, 1994).
- Chemical control does not have the necessary persistence
- Prevention using cultural practices: use adapted cultivars and maintain proper cutting schedule
- Need for development of innovative methods to manage crown rot, to increase alfalfa persistence and yields.



<http://ucanr.edu/blogs/blogcore/postdetail.cfm?postnum=17810>

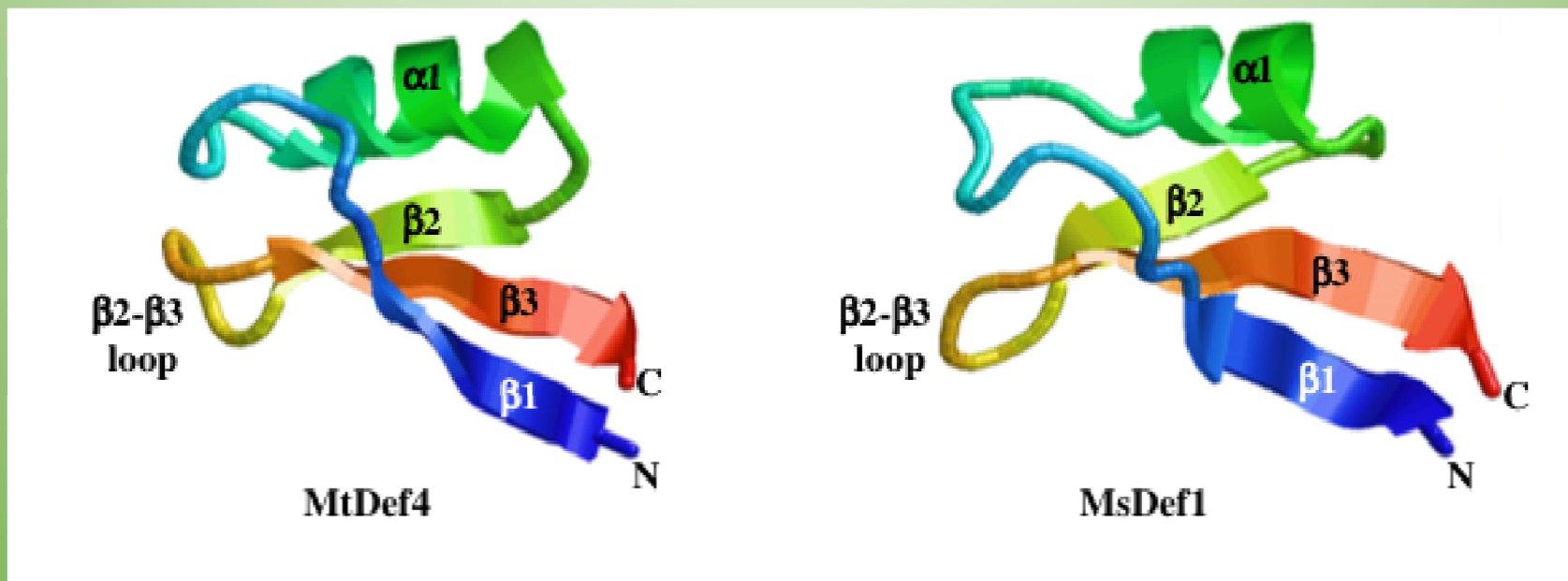
Candidate Transgene: Plant Defensin

- Small cationic, cysteine-rich antimicrobial peptides (45-54 aa)
- First line of defense against pathogen invasive in plants, vertebrates, invertebrates, and fungi
- Plant defensins are primarily antifungal

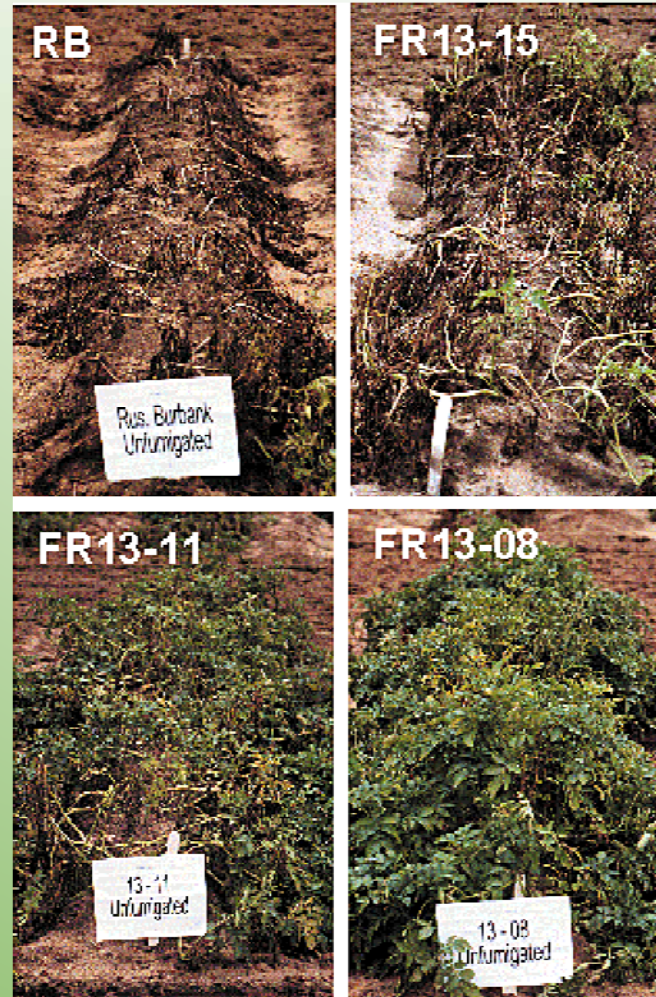


Plant Defensin Structure/Function

- Cluster of positive charges attracted to negatively charged pathogen membranes
- Create pores in membranes leading to cell death



Successful Applications in other Crops



Activity Against Crown Rot Pathogens?

- *In vitro* Fungal Assay
- Microplate study that uses absorbance to quantify fungal growth
- Measure change in absorbance (growth) and see what defensin concentration limits fungal growth by 50% (IC_{50})

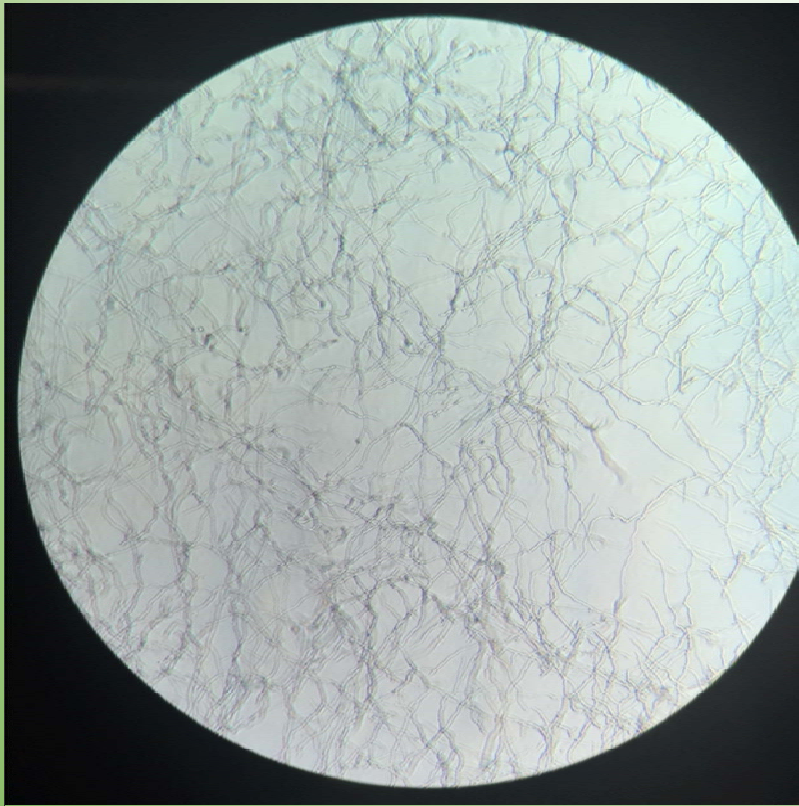


Synthetic plant defensins

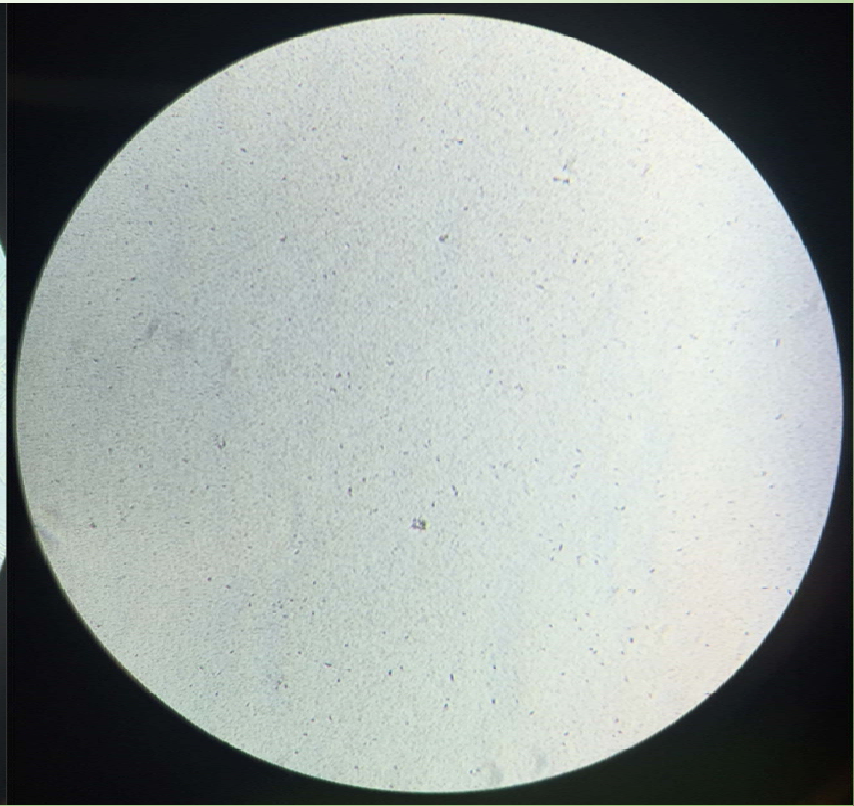
Defensin	Amino Acid Sequence
MsDef1	GRCRDDFRCWCTKRC
MtDef4	GRCRGFRRRCFCTTHC
MtDef5	GACHRQGFGFACFCYKKC
RsAFP-2	GSCNYVFPAHKCICYFP
So-D2	GDCKGIRRRCMCSKPL

γ -core motif: $\text{GXCX}_{3-9}\text{C}$

Fusarium oxysporum f. sp. *medicaginis*
(Fusarium Wilt)

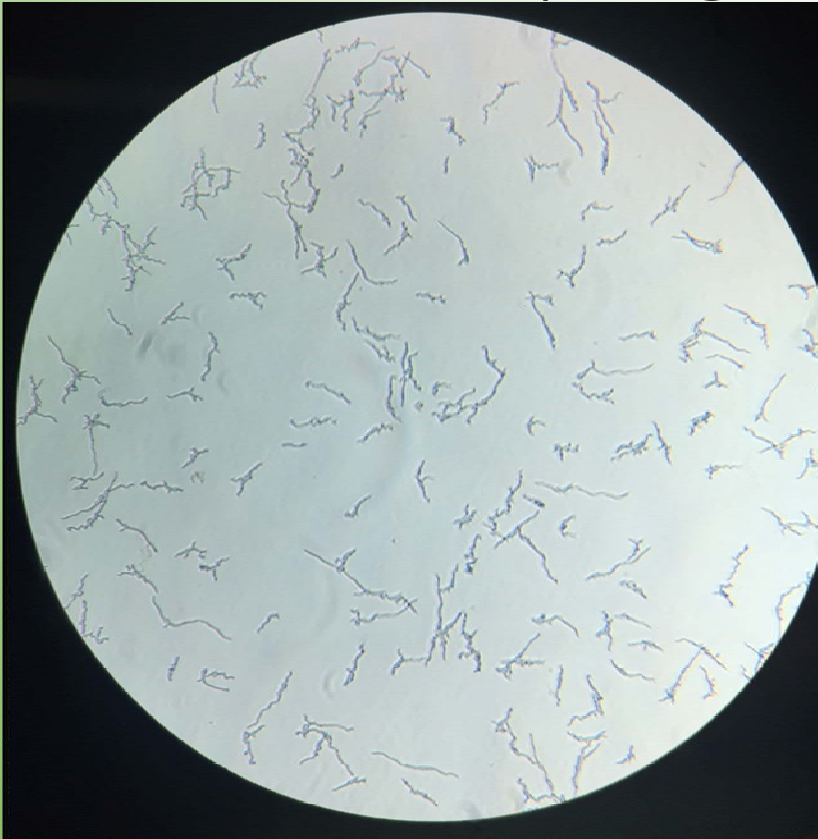


Control

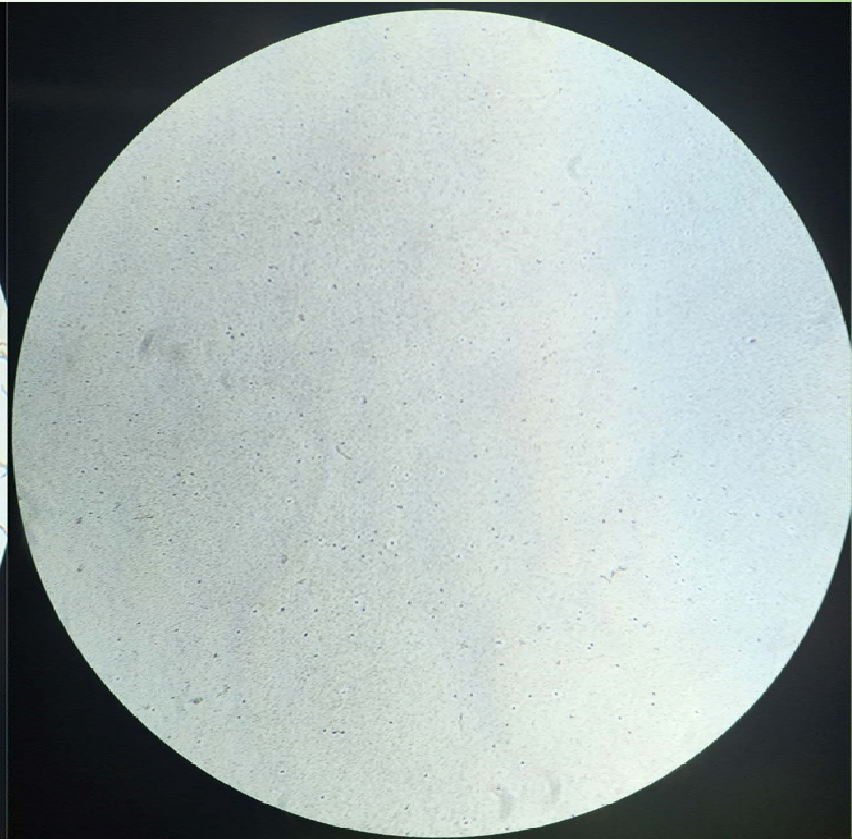


MtDef4 at 30 µg/mL

Phoma medicaginis
(Spring Black Stem)



Control



MtDef4 at 30 µg/mL

Defensin	<i>Fusarium oxysporum</i> f. sp. <i>medicaginis</i>		<i>Phoma medicaginis</i>		<i>Colletotrichum trifolii</i>		<i>Aphanomyces euteiches</i>		<i>Fusarium solani</i>	<i>Fusarium tricinctum</i>	<i>Fusarium redolens</i>	<i>Fusarium incarnatum</i>
	Strain 1	Strain 2	Strain 1	Strain 2	Strain 1	Strain 2	Race 1	Race 2				
Core MsDef1	NA	NA	12.7	14.8	NA	NA	NA	NA	NA	NA	NA	NA
Core MtDef4	7.1	6.9	7.3	5.3	NA	NA	NA	NA	6.0	14.7	NA	NA
Core MtDef5	NA	NA	19.5	8.5	NA	NA	NA	NA	4.1	NA	NA	NA
Core RsAFP2	NA	NA	NA	NA	NA	NA	NA	NA	NA	5.3	NA	NA
Core So-D2	33.1	NA	6.4	6.1	NA	NA	NA	NA	13.8	NA	NA	NA
MtDef4	0.7	1.9	0.3	2.6	NA	NA	NA	NA	-	-	-	-
MtDef5	0.8	1.3	1.5	1.6	NA	NA	NA	NA	-	-	-	-

Antibacterial Activity?

- Incubate defensins with bacterial solutions for 3 hours
- Serial dilute solutions and plate
- Measure colony-forming units to calculate IC_{50}



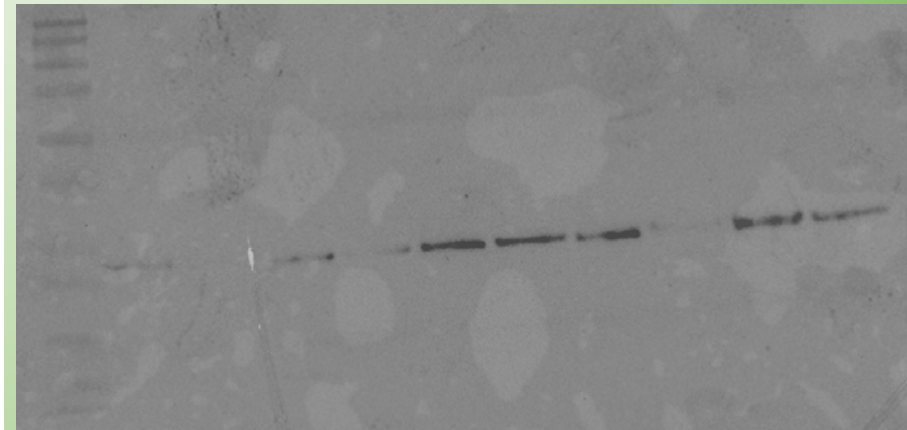
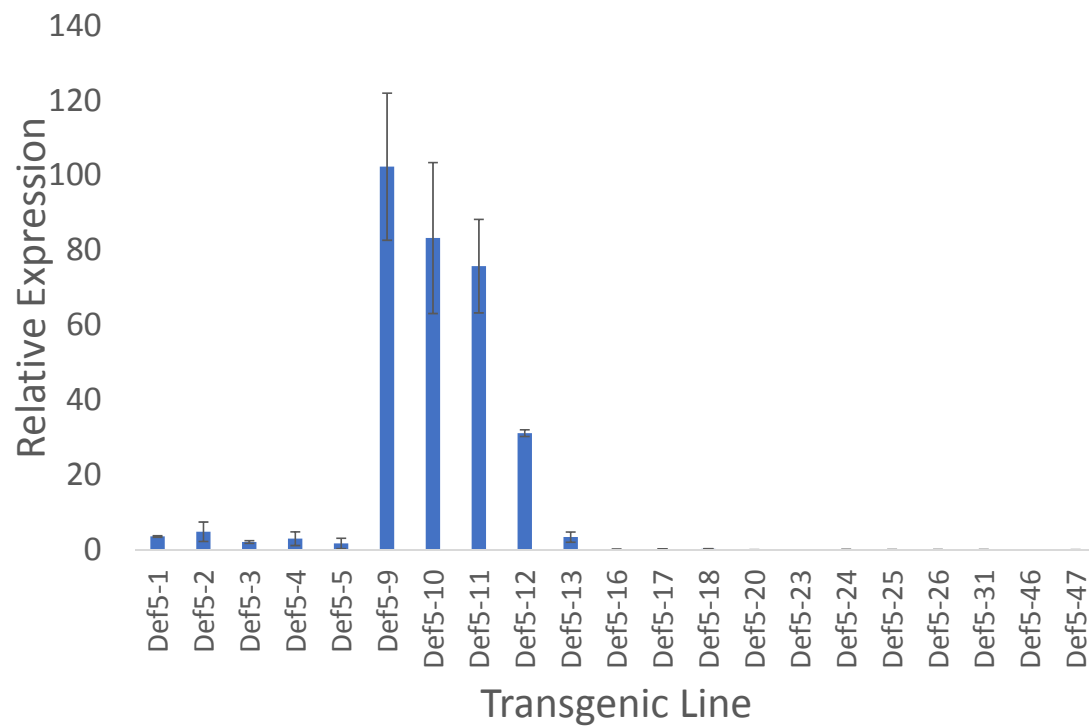
Defensin	<i>Xanthomonas alfalfae</i> subsp. <i>alfalfae</i>	<i>Pseudomonas syringae</i> pv. <i>syringae</i>	<i>Clavibacter michiganensis</i> subsp. <i>insidiosus</i>					
Core MtDef4	11.4	3.4	-					
			Defensin	<i>Serratia marcescens</i>	<i>Enterobacter aerogenes</i>	<i>Enterococcus casseliflavus</i>	<i>Pseudomonas aeruginosa</i>	
Core MtDef5	NA	4.5	Core MtDef4	8.4	2.3	NA	2.7	
Core So-D2	19.3	25.9	Core MtDef5	6.0	2.8	NA	11.8	
Core MsDef1	7.9	8.8						
MtDef4	0.6	0.4	0.1					
MtDef5	NA	0.1	NA					

Generation of Transgenic Alfalfa Expressing MtDef5

- *Agrobacterium*-mediated transformation
- MtDef5 constitutively expressed under control of CsVMV promoter
- 21 verified Transgenic Lines

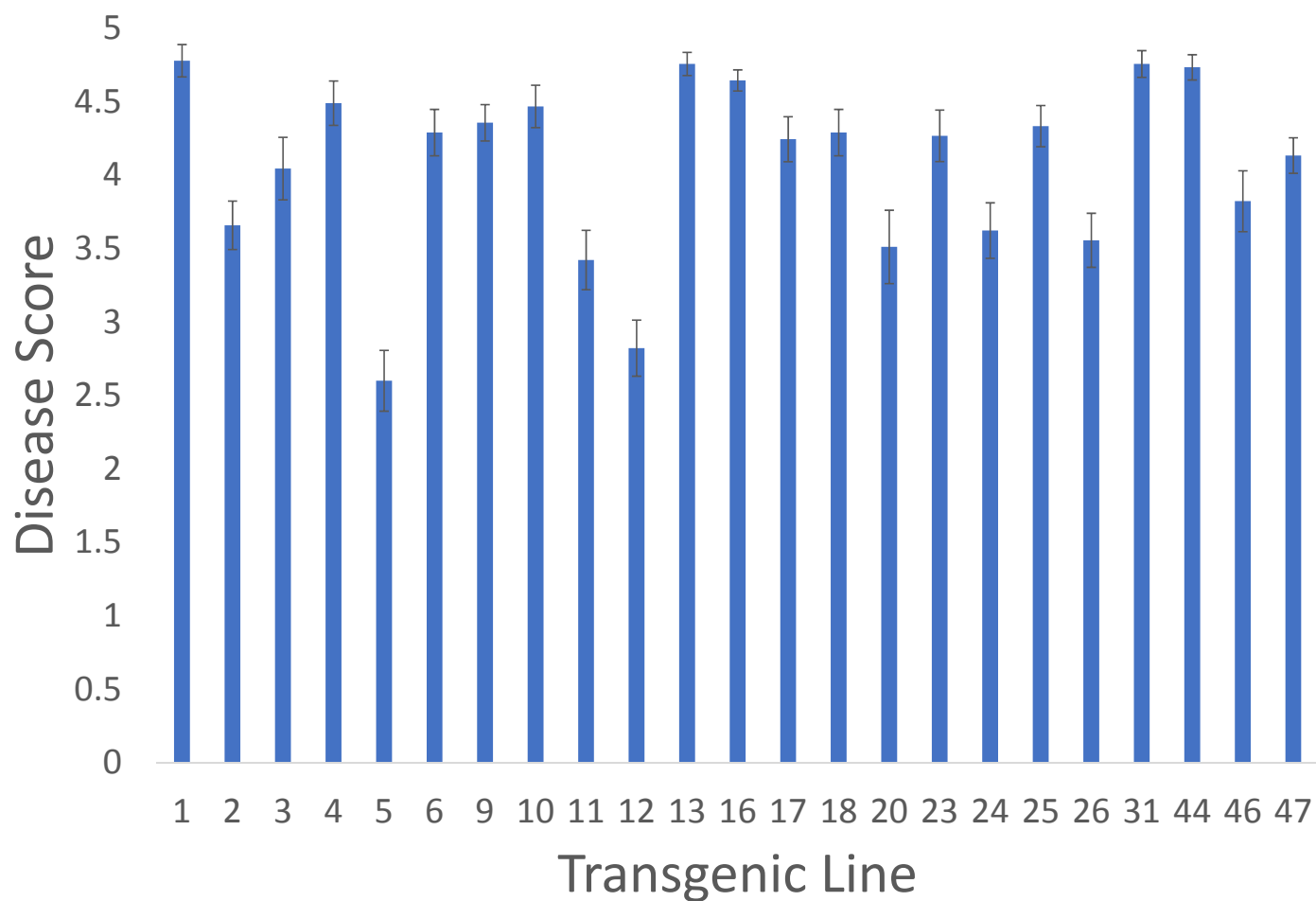


Transgene Expression



Western Blot using polyclonal anti-MtDef5 antibody

Disease Bioassays



Bioassay: *Phoma medicaginis*



Control

MtDef5-12

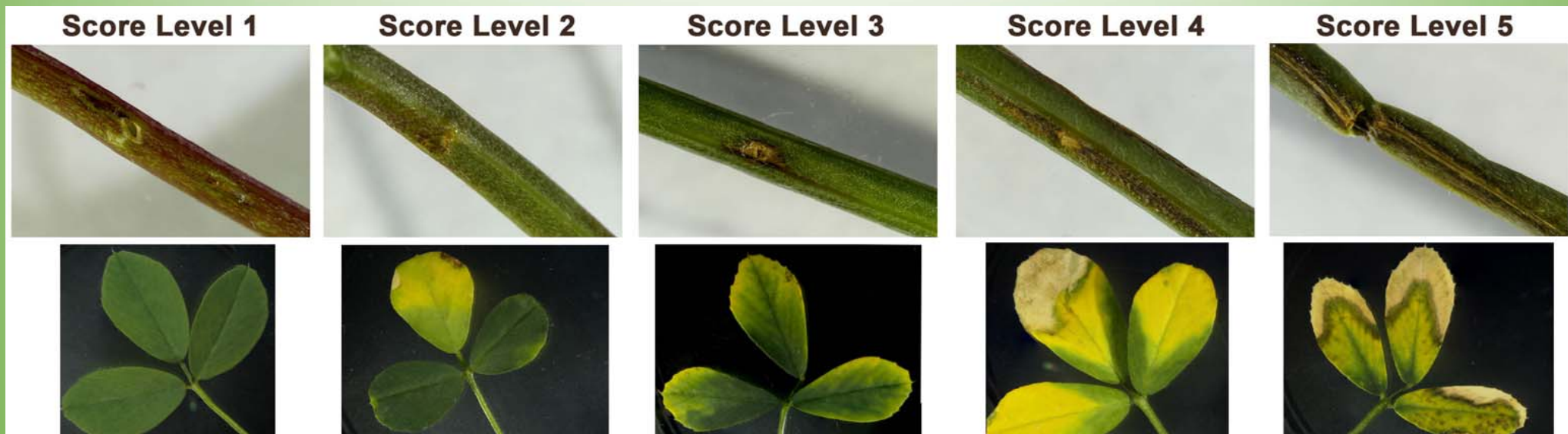
Bioassay: *Colletotrichum trifolii*
(Anthracnose)



Control

MtDef5-12

Bioassay: *Pseudomonas syringae* pv. *syringae* (Bacterial Stem Blight)



MtDef5-9: 2.8, 2.2

MtDef5-12: 2.8, 2.2

Control: 4.4, 2.4

Summary of Preliminary Results: Transgenic Alfalfa

- Transgenic lines with greater levels of MtDef5 expression corresponded to increased crown rot pathogen resistance.
- *In vitro* testing an indicator of antimicrobial activity, but additional activity present
- Disease resistance is broad spectrum. Line MtDef5-12 shows resistance against all three pathogens tested.

Conclusions

- Plant defensins have extensive and strong antimicrobial activity against both bacterial and fungal alfalfa crown rot pathogens
- Transgenic expression of defensins could be utilized to implement an eco-friendly, protein-based strategy that provides alfalfa with enhanced resistance against crown rot
- New strategy for disease resistance breeding?

Thank You!

- My Advisor: Debby Samac
- My Lab Teachers: Mindy Dornbusch and Sue Miller
- My Collaborators at Donald Danforth Plant Science Center: Dilip Shah and Siva Velivelli
- Plugging Myself: Looking for a Post-doc position for Summer/Fall 2019

