Plant defensins inhibit growth of pathogens in the alfalfa crown rot disease complex

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Abstract

Alfalfa crown rot is a disease complex that severely limits alfalfa stand density and productivity in all alfalfa-producing areas. Currently, there are no viable methods of control. Plant defensins are small cationic antimicrobial peptides with a conserved signature of cysteines. Defensins have a γ-core motif, a cluster of positively charged residues, which is essential for antimicrobial activity. The γ-core motifs of five synthetic defensins were tested for antimicrobial activity against the pathogens in the alfalfa crown rot disease complex. Using a microplate reader assay, the amount of defensin needed to inhibit growth of pathogen strains by 50% (IC_{50}) was calculated. The γ-core motif of MtDef4 was shown to be the most effective peptide with IC_{50} values of 5.3 µM against *Phoma medicaginis* and 6.9 µM against *Fusarium oxysporum* f.sp. *medicaginis*. In addition, MtDef4 had activity against *Pseudomonas syringae* pv. *syringae* but not the oomycete *Aphanomyces euteiches* in *in vitro* assays.

Introduction

- Crown rot occurs to some extent in every alfalfa stand that is over one year old.
- Resistance has not been identified for developing crown rot resistant cultivars.
- Defensins are cationic peptides that have broad antimicrobial activity.
- They constitute one of the first lines of defense against pathogen invasion in plants.
- Defensins can cause pore formation and permeabilize pathogen plasma membranes.

Objectives

- Evaluate the functionality of truncated defensins (just the γ-core region) as antimicrobial peptides.
- Discover the inhibitory concentrations of the defensins against alfalfa crown rot disease complex pathogens.
- Reveal the defensin with the greatest potential as an effective transgene in alfalfa.

Materials and Methods

Microplate Assay

A microplate reader assay was used to monitor fungal growth inhibition as previously described (1).

- Spore suspensions were filtered, and densities were determined microscopically.
- In a 96-well microplate, each well contained half strength potato dextrose broth, approximately 2000 spores, and concentrations of defensin peptide up to 30 µg/mL in a total volume of 100 µL.
- After 48 h at 25 °C in the dark, absorbance of the wells was measured at 595 nm to quantify the inhibition of fungal growth.
- From these values, the amount of defensin needed to inhibit growth of the pathogens strains by 50% (IC_{50}) was calculated.

Disk and MIC Bacterial Assays

- Defensins were spotted on filter paper disks at various concentrations.
- The filter paper disks were placed on a bacterial lawn and grown overnight.
- Zones of inhibition surrounding disks indicate antibacterial activity.

An agar dilution method was used to determine, the minimal inhibitory concentration (MIC), the lowest concentration of the defensin that inhibits the visible growth of the bacterium (2).

- Pure cultures of bacteria were grown overnight in a shaker at 225 rpm at 25°C.
- Bacterial suspensions were adjusted to 10^8 cfu/ml.
- 200 µL of the bacteria were treated with the defensins and incubated for 3 h at 30°C.
- Bacterial suspensions were serially diluted and plated out in triplicate on King's B media. Colony-forming units per milliliter were measured after 2 d of incubation at 30°C.

Results

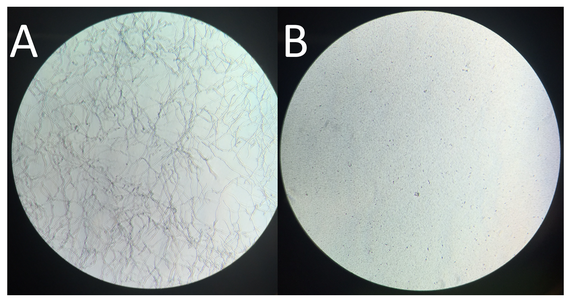

Figure 1. Spores of *Fusarium oxysporum* of were grown for 24 h at 25°C in culture medium in the absence (A) or presence (B) of 30 µg/ml of MtDef4

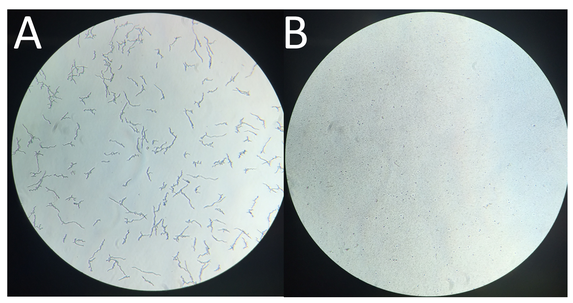

Figure 2. Spores of *Phoma medicaginis* were grown for 24 h at 25°C in culture medium in the absence (A) or presence (B) of 30 µg/ml of MtDef4

Table 1. Amino acid sequences of γ-core region (bold) and C-terminal region (italics) of defensins tested *in vitro*.

Defensin	Amino Acid Sequence
MsDef1	**GRCRDDFRC***WCTKRC*
MtDef4	**GRCRGFRRRC***FCTTHC*
MtDef5	**GACHRQGFGFAC***FCYKKC*
So-D2	**GDCKGIRRRC***MCSKPL*

Synthetic γ-core motifs of four defensins (Table 1) were tested *in vitro* against pathogens associated with the alfalfa crown rot disease complex. MtDef4 visibly inhibited fungal growth (Fig. 1 and 2). MtDef4 also displayed visible inhibition of bacterial growth with zones of inhibition increasing in diameter as defensin concentration increases (Fig. 3). Other defensin also displayed antimicrobial activity at a micromolar level. No defensin, even at the highest concentrations, was able to inhibit *Aphanomyces euteiches* or *Colletotrichum trifolii*.

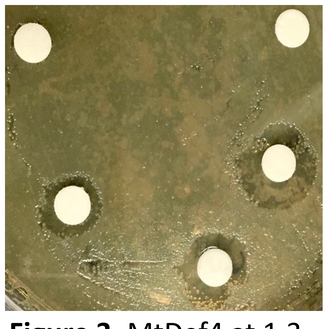

Figure 3. MtDef4 at 1.3, 5.1, 10.2, 15.3, and 20.41 µM on lawn of *Pseudomonas syringae* pv. *syringae*

Table 2. Activity of γ-core defensin peptides against alfalfa pathogens. For fungal and oomycete pathogens the IC_{50} (µM) is reported. For bacterial pathogens the minimum inhibitory concentration is reported.

Defensin	*Fusarium oxysporum*	*Phoma medicaginis*	*Colletotrichum trifolii*	*Aphanomyces euteiches*	*Pseudomonas syringae*
MsDef1	> 30	12.753	> 30	> 30	
MtDef4	6.972	5.354	> 30	> 30	< 5
MtDef5	> 30	8.516	> 30	> 30	< 5
So-D2	33.144	6.137	> 30	> 30	

Conclusion

- The γ-core regions of the defensins displayed antimicrobial activity.
- Against several pathogens in the alfalfa crown rot disease complex, the defensins showed activity at micromolar levels.
- MtDef4 consistently had the greatest activity of the tested defensins.
- These results indicate that transgenic expression of plant defensins in alfalfa has the potential to lead to improved crown rot resistance.

References

1. Broekaert, W., Terras, F., Cammue, B., and Vanderleyden, J. 1990. An automated quantitative assay for fungal growth inhibition. FEMS Microbiol Lett 69: 55–59.
2. Kim, M., Chen, Y., Xi, J., Waters, C., Chen, R., and Wang, D. 2015. An antimicrobial peptide essential for bacterial survival in the nitrogen-fixing symbiosis. Proc. Natl. Acad. Sci. USA 112:15238-15243.