Plant Defensins: An Innovative Approach to Control Alfalfa Crown Rot

A dissertation submitted to the faculty of the University of Minnesota

by Andrew Edward Sathoff

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Dedication

I dedicate this dissertation to my wife, Holly. Without her love, humor, furry shadow (Butters), and support, graduate school would have been much more difficult to complete.

Abstract

Plant defensins are antimicrobial host defense peptides expressed in all higher plants. These peptides inhibit the growth of a broad range of fungi and bacteria. Crown rot is a disease complex that reduces alfalfa (*Medicago sativa*) stand density and causes substantial losses in productivity in all alfalfa-growing areas. Currently, there are no effective methods of disease control. To evaluate plant defensins as a potential control for alfalfa crown rot, defensins were screened for antimicrobial activity. MtDef5, a defensin from *Medicago truncatula*, displayed high activity against both bacterial and fungal crown rot pathogens *in vitro*. *Agrobacterium*-mediated transformation was used to create transgenic lines of alfalfa (genotype Regen SY27x) constitutively expressing MtDef5. Disease bioassays demonstrated increased resistance against fungal crown rot pathogens in the transgenic lines expressing MtDef5. The transgenic lines with greater levels of MtDef5 expression corresponded to increased disease resistance. Transgenic expression of defensins could be utilized to implement an eco-friendly, protein-based strategy that could provide alfalfa with enhanced resistance against crown rot and corresponding gains in alfalfa yield.

Defensin antibacterial mode of action (MOA) is well characterized in invertebrate and vertebrate systems, but the MOA of most antibacterial plant defensins has not been investigated. Mini-Tn*5*-*lux* mutant strains of *Pseudomonas aeruginosa* with Tn insertions disrupting outer membrane protective modifications were assessed for sensitivity against plant defensin peptides. These transcriptional *lux* reporter strains were also evaluated for *lux* gene expression in response to sublethal plant defensin exposure. A defensin from *M. truncatula*, MtDef4, induced dose-dependent gene expression of the aminoarabinose

modification of LPS and surface polycation spermidine production operons. This indicates that MtDef4 damages the outer membrane akin to polymyxin B, which stimulates antimicrobial peptide resistance mechanisms similar to plant defensins. A plant pathogen, *Pseudomonas syringae* pv. *syringae* was modified through transposon mutagenesis to create mutants that are resistant to *in vitro* MtDef4 treatments. The transposon insertion site on defensin resistant bacterial mutants was sequenced, and modifications of ribosomal genes were identified to contribute to enhanced resistance to defensin treatments. Therefore, the MtDef4 antibacterial mode of action may also involve inhibition of translation.

Genetic modification of alfalfa for the introduction of novel traits requires promoters for controlling gene expression. Constitutively active promoters impose a great energy load on the plants and a strong selective pressure on the pathogens. *M. truncatula* promoter regions of pathogenesis-related (PR) genes, PR5 and PR10, were identified as being highly up-regulated during the initial stages of infection by root and foliar pathogens. These promoters, along with the alfalfa homolog for PR10, were cloned into plant transformation vectors ahead of the β-glucuronidase (*gus*) gene. *Agrobacterium*-mediated transformation was used to create transgenic lines of alfalfa. Quantitative PCR assays were utilized to evaluate pathogen-induced GUS expression. Consistently, the MtPR10 promoter had greater fold amplifications and greater activity than the MsPR10 and MtPR5 promoters. The MtPR10 promoter is functional in alfalfa for expression of transgenes and up-regulates genes after infection by a wide range of alfalfa pathogens.

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Chapter 1: Antibacterial activity of plant defensins*

Introduction. Plants produce a wide array of antimicrobial peptides (AMPs) constitutively or in response to pathogen infection, abiotic stresses, and mechanical injury, including damage from insects and herbivores. Plant AMPs are classified based on their structure and presence of disulfide bonds into the main classes of cyclotides, defensins, hevein-like proteins, lipid transfer proteins, snakins, thionins, and vicilin-like proteins (Goyal and Mattoo 2014). The plant defensins are among the best characterized of the AMPs with an abundance of representatives from diverse plant species (Tam et al. 2015). The term plant defensin was coined due to structural and functional homology of the plant peptides to insect and human defensins (Broekaert et al. 1995). Plant defensins are cationic, with a basic isoelectric point, and are identified by an invariant tetradisulfide array. Although they share little amino acid sequence identity, these peptides share a conserved 3-D structure consisting of one α-helix and three antiparallel β-strands that are connected by four disulfide bonds, which forms a distinctive cysteine-stabilized αβ (CSαβ) motif. Defensins from plants, fungi, and invertebrates share the CSαβ motif, which defines the *cis*-defensin superfamily, distinct from the *trans*-defensin superfamily, consisting of vertebrate α-, β-, θ-, and invertebrate big defensins (Shafee et al. 2016). Proteins in the two defensin superfamilies likely arose from extensive convergent evolution (Shafee et al. 2016). Plant defensins possess an additional conserved sequence, a γ-core motif, defined as $GXCX_{3-9}C$, which is required for their antifungal activity

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(Sagaram et al. 2011). Plant defensins have been shown to be promiscuous peptides, meaning that a single defensin peptide can have multiple functions (van der Weerden and Anderson 2013). For example, in addition to antimicrobial activity, defensins with diverse functions, such as amylase activity, conferring zinc tolerance, and involvement in pollen tube elongation have been identified (Carvalho and Gomes 2009; Franco 2011). Though plant defensins share a common tertiary structure, there is extensive variation in amino acid sequences and length of peptides. This sequence diversity corresponds to variation in antimicrobial activity.

Numerous recent review papers have summarized the extensive antifungal activity of plant defensins and their mode of action against fungi, but these reviews do not acknowledge antibacterial activity, or only cite an example without going into detail (Cools et al. 2017; De Coninck et al. 2013; Lacerda et al. 2014; Montesinos 2007; Parisi et al. 2018; Rautenbach et al. 2016; Vriens et al. 2014). The lack of information on the antibacterial activity of plant defensins is surprising because plant defensins were originally classified as γ-thionins, a subclass of thionins, which have long been known to have antibacterial activity. Purothionins extracted from wheat (*Triticum aestivum*) display extensive antibacterial activity against both Gram-positive and Gram-negative strains of phytopathogenic bacteria (de Caleya et al. 1972). Additionally, human and invertebrate defensins have potent antibacterial activity (Ageitos et al. 2017), and the mode of action (MOA) of these defensins against bacterial pathogens is well characterized (Ageitos et al. 2017; Guilhelmelli et al. 2013). Vertebrate defensins rely on electrostatic interactions with the anionic lipid components of bacterial membranes, which leads to rapid membrane permeabilization through pore formation (Morgera et al. 2008), which is a

MOA for many other AMPs. For example, HNP-1, the most studied human α-defensin, has an antibacterial MOA similar to several other vertebrate defensins which occurs via HNP-1 dimerization, then electrostatic interaction of dimers with the bacterial membrane in which β-sheet dimers span the membrane forming a pore, with higher order oligomers of HNP-1 forming upon dimers when the defensin is in high concentration (Zhang et al. 2010). Another well-studied antibacterial human defensin, human β-defensin-3 (HBD3), has been shown to inhibit bacterial cell wall biosynthesis by interacting with lipid II components, which allows for HBD3 to have widespread activity against both Gram-positive and Gram-negative bacteria (Sass et al. 2008).

Frequently, plant defensins display antifungal activity and lack antibacterial activity. For example, Lc-Def from lentil (*Lens culinaris*), showed antifungal activity, but failed to show activity against either Gram-positive (*Clavibacter michiganensis*) or Gram-negative (*Agrobacterium tumefaciens* and *Pseudomonas syringae*) bacterial plant pathogens (Shenkarev et al. 2014). However, it is likely that many defensins have not been tested for antibacterial activity or the testing method used may have erroneously led to the conclusion that the defensin lacked antibacterial activity. The focus on antifungal activity may reflect the relative importance of fungal pathogens opposed to bacterial pathogens in plant pathogenesis compared to human and animal disease. Because of their potent antifungal activity, plant defensins are being exploited in agricultural biotechnological applications to generate disease resistant crops (Gao et al. 2000; Gaspar et al. 2014; Sharma et al. 2017).

Though fungal plant pathogens may be more prevalent, plant pathogenic bacteria have great economic significance and influence over food security (Mansfield et al.

2012). *Ralstonia solanacearum* has a broad host range, infecting over 200 plant species, including important food crops such as potato, banana, and tomato. From potato alone, the yearly worldwide losses are estimated to be $1 billion US dollars (Elphinstone 2005). During the monsoon season in India and Southeast Asia, outbreaks of bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* reduce the yield of rice, a staple crop, by up to one half (Mew et al. 1993). There are a limited number of management strategies for controlling bacterial diseases of plants. Although genetic resistance to bacterial diseases can be found in many crops, it is often only effective against a single or small number of pathotypes (races), which would require "stacking" of numerous resistance genes, and this genetic resistance can be overcome through mutagenesis in the pathogen. Antibiotic usage on crops is expensive and contributes to the generation of antibacterial-resistant bacterial populations. Transgenic expression of antibacterial plant defensins would reduce producer application of costly antibiotics and is an eco-friendly method of disease control that may be difficult to overcome, depending on the specific MOA.

Plant defensin nomenclature. The inconsistent naming of plant peptides with a defensin structure may have resulted in the loss of information on the defensins with antibacterial activity. Until recently, the term thionin was used synonymously with plant defensin in the small grains literature. For example, a cold acclimation-induced, antibacterial plant peptide isolated from winter wheat (*Triticum aestivum*) crown tissue, Tad1, was referred to as being both a thionin and a plant defensin throughout its characterization (Koike et al. 2002). A thionin from potato (*Solanum tuberosum*) tubers, Pth-St1, which was named before the term plant defensin was developed, was shown to display broad-spectrum activity against fungi, Gram-positive, and Gram-negative bacteria

(Moreno et al. 1994). Cp-thionin II from cowpea (*Vigna unguiculata*) displays antibacterial activity against both Gram-positive and Gram-negative pathogens (Franco et al. 2006). Thionins and defensins are currently considered to be two separate classes of AMPs, but the naming conventions do not reflect this change of classification.

The following peptides have the structure of plant defensins but were given a wide variety of names using no particular system of nomenclature. ZmESR-6, isolated from kernels of *Zea mays*, shows antimicrobial activity against both fungi and bacteria (Baladin et al. 2005), with greater efficacy against Gram-positive bacteria compared to Gram-negative bacteria. Fabatin was isolated from broad bean (*Vicia faba*) seeds and displays activity against Gram-positive and Gram-negative bacteria but not fungi (Zhang and Lewis 1997). Fujimura et al. (2003; 2004) identified peptides from tulip (*Tulipa gesneriana*; Tu-AMP1 and Tu-AMP2) and buckwheat (*Fagopyrum esculentum*; Fa-AMP1 and Fa-AMP2) that have both antibacterial and antifungal activity. These cases highlight the need for a consistent nomenclature system for plant defensins. Renaming these previously discovered plant defensins would make them more accessible to the research community. Following the current naming conventions, a two-letter genus/species designation should be followed by a defensin indication (Def) and number. Renaming of plant defensins has already been done in a few cases. For example, the plant defensin alfAFP from alfalfa (*Medicago sativa*) was renamed, MsDef1 (Spelbrink et al. 2004). Suggested new names for previously characterized peptides are listed in Table 1.

Evaluating antibacterial activity. The antibacterial activity of a plant defensin can be described by different parameters determined by several distinct methods of testing. The selected experimental approach can obscure validation of plant defensin

antibacterial activity. Frequently, *in vitro* assays are designed to find the minimal inhibitory concentration (MIC) of a defensin against the bacterium of interest. The MIC is defined as the concentration of an antimicrobial agent at which no bacterial growth is detected. MIC determination uses optical density to quantify bacterial growth and is often achieved through serial microdilution of agents in microplates with a defined number of bacterial cells (Wiegand et al. 2008). Slowing bacterial growth may result in a delayed bacterial infection, and that can be a desirable trait in crop production. Plant defensins may alter the kinetics of bacterial growth, which could be missed because MIC assays are influenced by inoculum concentration and cannot distinguish between bacteriostatic and bactericidal mechanisms. Additionally, MIC tests could lead to false negatives regarding the activity of a plant defensin if insufficient peptide is used to completely inhibit growth. To fully capture inhibitory activity of a defensin, including partial suppression of bacterial growth, a more appropriate method of evaluating antibacterial activity would be to measure the IC_{50} value, the concentration of defensin peptide that reduces bacterial growth by half. Colony-forming unit-based assays are frequently utilized to determine IC_{50} values. Throughout this review, the antibacterial inhibitory concentrations will be carefully noted as an IC_{50} or MIC parameter (Table 1).

Antibacterial plant defensins. Many of the first plant defensin peptides characterized were isolated from crude seed extracts. The defensins from the seeds of horticultural species, horse chestnut (*Aesculus hippocastanum*; Ah-AMP1), butterfly pea (*Clitoria ternatea*; Ct-AMP1), and *Dahlia merckii* (Dm-AMP1, Dm-AMP2), were evaluated against four Gram-positive bacterial species and two Gram-negative bacterial species causing diseases in humans. Ah-AMP1, Ct-AMP1, and Dm-AMP1 inhibit

Bacillus subtilis but not *Escherichia coli*, *Micrococcus luteus*, *Proteus vulgaris*, *Staphylococcus aureus*, or *Streptococcus faecalis*. Additionally, the concentration of the peptides needed for 50% growth inhibition (IC_{50}) for inhibition of *B. subtilis* was relatively high ranging from 15 to 150 μg/mL (Osborn et al. 1995). This limited antibacterial activity in this initial, fundamental study may have dissuaded other researchers from further antibacterial testing during the characterization of newly discovered plant defensins.

Spinach defensin (So-D2) is the most commonly referenced plant defensin with antibacterial activity. So-D2 and So-D7 were isolated from spinach (*Spinacia oleracea*) leaves and display antibacterial activity against *Clavibacter sepedonicus* and *Ralstonia solanacearum*, Gram-positive and Gram-negative plant pathogenic bacteria, respectively (Segura et al. 1998). These defensins have potent activity against the bacterial isolates tested with IC_{50} values ranging from 0.1-2 μM. Also, So-D2 and So-D7 display activity against the fungus, *Fusarium solani*, with IC_{50} values of 9 and 11 μM, respectively. The citrus industry has utilized these defensins to generate transgenic sweet orange and grapefruit trees through *Agrobacterium*-mediated transformation in hopes of combatting economically important bacterial diseases. Transgenic citrus trees constitutively expressing So-D2 and So-D7 in combination were shown to be highly resistant to citrus greening caused by the bacterial pathogens *Candidatus* Liberibacter spp. and to citrus canker caused by *Xanthomonas axonopodis* pv. *citri* (Mirkov and Gonzalez-Ramos 2014). Simultaneously expressing both spinach defensins resulted in significantly greater disease control than expression of an individual defensin. Disease resistance for citrus greening was determined by both grafting and psyllid inoculation greenhouse trials, and

citrus canker resistance was assessed using a detached leaf assay. Additionally, *Citrus tristeza* virus (CTV) has been modified into a remarkably stable, viral vector to transfect citrus and provide long-term expression of spinach defensins (Kress 2015). In greenhouse studies, inoculation of citrus plants with the CTV-based vector imparted resistance to both citrus greening and citrus canker. The CTV vector can be utilized as a microbial pesticide in the field, which is currently a disease control method approved by the United States Environmental Protection Agency. Synthetic AMPs, with structures similar to plant defensins, have also been utilized to control citrus greening and citrus canker (Hao et al. 2017).

Antibacterial activity was identified in OsDef7 and OsDef8, defensins from rice (*Oryza sativa* L. ssp. *japonica*) (Tantong et al. 2016). In contrast to the conventional method of testing activity of crude protein extracts, these defensins were first identified through an *in silico* co-expression network analysis of genes encoding defensin and defensin-like peptides. Using RiceArrayNet (Lee et al. 2009) with data collected from 183 rice microarrays, the putative defensins were found to be co-expressed with pathogen-responsive genes. Additionally, the analytical software Genevestigator (Hruz et al. 2008) was used to analyze the expression levels of candidate rice defensins in response to seven rice pathogens. OsDef7 and OsDef8 display potent antibacterial activity *in vitro* against *Xanthomonas oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, and *Erwinia carotovora* subsp. *atroseptica* but do not show activity against Gram-positive bacteria and are weakly active against phytopathogenic fungi (Tantong et al. 2016). With IC_{50} values as low as 0.6 μg/mL, OsDef8 demonstrates greater antibacterial activity than OsDef7. When these defensins were transiently expressed in *Nicotiana benthamiana*

leaves, increased resistance was shown against *Xanthomonas campestris* pv. *glycines* (Weerawanich et al. 2018).

MtDef5, a bi-domain defensin peptide containing two defensin domains, isolated from the model legume *Medicago truncatula* displays antibacterial activity along with previously characterized extensive antifungal activity (Islam et al. 2017; Velivelli et al. 2018). MtDef5 was able to inhibit the Gram-negative pathogen, *Xanthomonas campestris* pv. *campestris*, at a MIC value of 6 μM, but MtDef5 failed to inhibit the Gram-positive pathogen, *Clavibacter nebraskensis*. This lack of antibacterial activity against *C. nebraskensis* was suggested to be a result of limited defensin binding to the thick, outer layer of peptidoglycan present in the cell walls of Gram-positive bacteria (Velivelli et al. 2018).

Many more plant defensins have been discovered through genome analyses than through protein isolation and characterization (Maroti et al. 2015). However, because the specificity of antimicrobial activity is unique to each peptide, the activity of putative defensins needs to be tested experimentally. But, there are some features that antibacterial plant defensin appear to share, which may allow for the prediction of antibacterial activity. J1-1, OsDef7, OsDef8, Tu-AMP2, and MtDef5 are among the few plant defensins that can form oligomeric structures, most commonly dimers, and they also have antibacterial activity (Fujimura et al. 2003; Guillen-Chable et al. 2017; Islam et al. 2017; Tantong et al. 2016). For mammalian defensins, the ability to form dimers is related to their antibacterial activity (Schibli et al. 2002). Even though all plant defensins that form dimers are not antibacterial, the ability to form dimers may be an indicator of antibacterial activity. Phylogenetic studies of plant defensins have demonstrated that the

highest sequence homologies are between plant defensins that share the same biological activities (Zhu et al. 2005). Fabatin-2 and Cp-thionin II show structural and sequence homology to the well-characterized human antibacterial defensin, HBD3 (Kraszewska et al. 2016). This may allow for the identification of conserved residues necessary for antibacterial activity. Also, buckwheat defensins, Fa-AMPs, are particularly rich in glycine residues, which suggests a connection with the glycine-rich peptide family that is known to display activity against Gram-negative bacteria (Fujimura et al. 2003). Therefore, an abundance of glycine residues may be correlated with antibacterial activity. Additionally, studies could be performed that explore plant defensin bacterial lipid binding ability as an indicator of antibacterial activity. Lipid binding has been used to predict the antibacterial activity of defensins from fungi, mollusks, and arthropods (Schneider et al. 2010).

Defensin-like peptides. Plant genomes encode a large repertoire of defensin-like (DEFL) peptides with a conserved pattern of cysteine residues but with a highly variable mature peptide sequence (Mergaert et al. 2003; Silverstein et al. 2007). Based on sequence diversity, over 100 subgroups of DEFLs have been identified (Silverstein et al. 2007). The functions of most members of this large gene family are largely unknown, although roles in plant development and plant-microbe interactions have been identified (Tesfaye et al. 2013). DEFLs that share secondary structural and functional homologies with plant defensins are the nodule-specific cysteine-rich peptides (NCRs) (Maroti et al. 2015). Also, similar to plant defensins, cationic NCRs (pI > 9) have antimicrobial activity while neutral and anionic peptides are inactive (Mikuláss et al. 2016). But, opposed to the eight cysteines found in plant defensins, NCRs have a conserved motif of four to six

cysteine residues (Stonoha-Arther and Wang 2018). In the inverted repeat-lacking clade (IRLC) of legumes, NCRs are produced extensively in root nodules, and several NCRs have been shown to regulate interactions with rhizobial microsymbionts. In *M. truncatula*, NCR peptides are expressed exclusively in the nodules and are proposed to trigger terminal bacteroid differentiation of *Sinorhizobium meliloti*. Bacteroids are characterized by arrested cell division, cell elongation, DNA multiplication, and a partially permeabilized cell membrane, which appears to improve the efficiency of symbiosis (Van de Velde et al. 2010). Additionally, NCR peptides may eliminate rhizobial strains that use host resources to accumulate carbon resources but do not provide fixed nitrogen. Some NCR peptides have been shown to function in the elimination of infecting but unadapted rhizobial strains and to determine compatibility between the host and endosymbiont (Wang et al. 2017; Yang et al. 2017). *In vitro*, synthetic NCRs display broad-spectrum activity against many bacteria, including rhizobia, as well as fungi (Mergaert 2018). Two particularly well-studied *M. truncatula* NCRs, NCR247 and NCR335, display extensive antibacterial activity against both Gram-positive and Gram-negative bacteria although their spectra of activity were only partially overlapping, with NCR335 inhibiting a greater number of bacteria (Tiricz et al. 2013). *Clavibacter michiganensis* and *Xanthomonas campestris* were particularly sensitive to both NCRs since bacterial growth was completely eliminated with peptide treatments of 50 μg/mL (Tiricz et al. 2013).

Antibacterial mode of action. In contrast to vertebrate defensins, the mode of action of most antibacterial plant defensins has not been investigated. Recently, Velivelli et al. (2018) examined the antibacterial activity of MtDef5 from *M. truncatula*. Through

site-directed mutagenesis, the cationic amino acid residues found in the γ-core motif of MtDef5 were discovered to be essential for antibacterial activity. These residues were previously shown to be critical for antifungal activity (Islam et al. 2017). *In vitro* treatment of *X. campestris* pv. *campestris* with MtDef5 causes morphological changes making the normally rod-shaped bacteria cells spherical or dumbbell-shaped, with associated loss of cell viability. MtDef5 was shown to permeabilize the bacterial plasma membrane and translocate into the cells of *X. campestris* pv. *campestris* (Velivelli et al. 2018). *In vitro*, MtDef5 binds to DNA, suggesting that the peptides may also inhibit DNA replication or gene transcription. VaD1, an antibacterial defensin isolated from azuki bean (*Vigna angularis*), was found to inhibit *in vitro* protein synthesis in a cell-free system derived from wheat germ (Chen et al. 2005). Additional studies are needed for understanding the modes of action of plant defensins against different species of bacteria.

Valuable insights into mechanisms of antibacterial activity can be gained from investigations into the MOA of NCRs. Synthetic NCRs were shown to induce membrane permeabilization and alter bacterial cell morphology causing death of *S. meliloti* cells with MICs as low as 5 μM (Van de Velde et al. 2010). NCR247 and NCR335 cause cell envelope damage to both the outer and inner bacterial membranes, resulting in the loss of membrane potential, which is proposed to be the primary reason for their antibacterial activity (Mikulass et al. 2016). However, the two NCRs interact differently with bacterial membranes though both appear to target the lipid matrix (Nagy et al. 2015). The transcriptome of *S. meliloti* cells treated with NCR247 and NCR335 was found to be similar to that of *Staphylococcus aureus* treated with the membrane-depolarizing agent carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP) (Tiricz et al. 2013). CCCP

interferes with membrane potential and the proton gradient as well as inhibiting protein transport (Park et al. 1997). NCR247 binds to several intracellular targets to facilitate bacteroid formation. NCR247 binding to FtsZ, required for bacterial septum formation, inhibits cell division, binding to GroEL, a chaperone, was found to amplify NCR247-mediated processes, and binding to ribosomes was shown to inhibit translation (Farkas et al. 2014). Additionally, NCR247 treatments were found to block bacterial cell division through reducing the expression of the cell cycle regulator, *ctrA* (Penterman et al. 2014).

NCRs can be viewed as specialized, antibacterial peptides and can provide insights for understanding potential resistance mechanisms to antibacterial plant defensins. In the NCR-rhizobial system, there is a co-evolutionary relationship and molecular arms race that mimics the interaction between pathogen effectors and the plant immune system. For example, responding to the antibacterial activity of NCRs, some *S. meliloti* strains express a metallopeptidase, a host range restriction peptidase (HrrP) that degrades NCRs (Price et al. 2015). Through endosymbiont modifications to protect against host NCRs, we can infer potential host resistance mechanisms to antibacterial plant defensins.

BacA, a peptide transporter in *S. meliloti*, is required for bacterial survival in the presence of NCRs (Haag et al. 2011). The BacA knockout mutant is characterized by an altered distribution of the fatty acids in the lipopolysaccharide membrane (Ferguson et al. 2002). BacA is proposed to promote the uptake of NCRs and redirect them away from the bacterial membrane to limit membrane damage. This suggests that bacterial pathogens with a BacA homolog may not be susceptible to the antibacterial activity of NCRs and likely, some plant defensins. NCR treatment induces the σ^{32}-stress factor RpoH1, a heat-

shock protein transcription factor, in *S. meliloti*, and *rpoH1* mutant nodules phenocopy the nodules from *bacA* mutants (Mitsui et al. 2004; Penterman et al. 2014). This suggests that RpoH1-regulated stress response may be required for bacterial survival since *S. meliloti* knockout mutants quickly die in the presence of NCRs (Penterman et al. 2014). Plant defensins may elicit this same stress response in bacteria and defeating this response may be necessary for antibacterial activity. Extracellular polysaccharides (EPS) and lipopolysaccharide membrane coatings affect the affinity of NCRs to rhizobial membranes (Mergaert 2018). Bacterial strains that have an EPS with few negatively charged succinate groups display increased susceptibility to NCRs compared to strains with greater amounts of succinate groups, which implies that this membrane modification shields against the antibacterial activity of NCR peptides (Simsek et al. 2007; Wang et al. 2018). Therefore, plant defensins may display less antibacterial activity against bacteria with membrane modifications that interfere with defensin binding. Similarly, bacterial pathogens of vertebrates have been shown to produce capsular polysaccharides to shield the membranes from defensins, inactivate or degrade the defensin peptide, and actively expel defensins from cells (Koprivniak and Peschel 2011). Our current understanding of the bacterial targets of defensins/NCRs and bacterial host responses to defensins/NCRs is diagramed in Figure 1.

Plant defensins in human and animal health. With the rising rates of antibiotic resistance in clinical bacterial strains, there is a renewed interest in alternative therapeutic compounds. Plant defensins are attractive candidates for antibiotic development. Pathogens are predicted to rarely develop resistant phenotypes because defensins specifically target the bacterial plasma membrane, do not interact with specific receptors,

and/or likely have multiple targets. Thus, plant defensins may be an untapped reservoir for antibiotic and drug development for human and animal health.

When evaluated against human bacterial pathogens, several plant defensins had potent antibacterial activity. PaDef, a defensin from avocado (*Persea americana* var. *drymifolia*), displays antibacterial activity against *E. coli* and *S. aureus* but failed to show antifungal activity against *Candida albicans* (Guzmán-Rodríguez et al. 2013). Since the synthesis of plant defensins can be difficult in prokaryotic expression systems due to improper post-translational modifications, PaDef was expressed in the bovine endothelial cell line BE-E6E7, and the bacteria were evaluated in conditioned media containing the secreted PaDef at concentrations up to 100 μg/mL (Guzmán-Rodríguez et al. 2013). A defensin isolated from bell pepper (*Capsicum annuum*), J1-1, exhibits antibacterial activity against *Pseudomonas aeruginosa* with a MIC of 250 μg/mL (Guillen-Chable et al. 2017). This interaction may be facilitated by the ability of J1-1 to bind to phosphoinositides (PIs) and phosphatidic acid (PA) *in vitro*. Also, limenin, a defensin from lima bean (*Phaseolus limensis*), displays broad antibacterial activity against numerous human-associated bacterial species (Wong and Ng 2006).

Against mammalian cancer cells, plant defensins have demonstrated specificity and cytotoxicity. PaDef is cytotoxic against K562 chronic myeloid leukemia cells with an IC_{50} value of 18.65 μM (Flores-Alvarez et al. 2018). Surprisingly, PaDef was shown not to cause membrane permeation against K562 cells, which indicates that cytotoxicity is not related to cell membrane damage. In the breast cancer cell line MCF-7, PaDef was shown to induce apoptosis with an IC_{50} value of 27.23 μM (Guzmán-Rodríguez et al. 2016). Also, NaD1 a defensin from sweet tobacco (*Nicotiana alata*) had an IC_{50} value of

10 μM against leukemia cells (Poon et al. 2014). The cytotoxic mechanism of NaD1 against mammalian melanoma and leukemia cells at subacute concentrations was shown to be membranolytic rather than apoptotic, which implies that NaD1 and PaDef have different modes of action against cancer cells (Baxter et al. 2017).

Conclusions and prospects. Plant defensins appear to be an untapped reservoir for developing plants with greater resistance to bacterial diseases as well as for combatting human and animal bacterial pathogens. Due to the possible misconception that plant defensins are solely antifungal and rarely display antibacterial activity, evaluation of defensins against bacterial pathogens may have been neglected in the past and a reevaluation of previously characterized defensins for antibacterial activity is warranted. Expression of antibacterial defensins in genetically modified plants could lead to broad-spectrum disease resistance to provide a new tactic for combating economically important plant pathogens. The resistance conferred is likely to be highly durable and difficult to overcome because plant defensins presumably target integral, conserved bacterial membrane components. Improving resistance to phytobacterial pathogens would contribute to substantial gains in crop yields. Additionally, these peptides have potential as applied plant protectants because the structure of defensins is compact and heat-stable, which suggests high environmental stability.

***Medicago* defensin antifungal MOA.** *M. truncatula* and *M. sativa* defensins are among some of the best characterized plant defensins. Studies investigating the antifungal MOA of MsDef1, MtDef4, and MtDef5 have been performed (Islam et al. 2017; Sagaram et al. 2011; Spelbrink et al. 2004). MOA investigations frequently start by uncovering defensin binding preferences for specific fungal membrane components. MsDef1 binds

specifically to glucosylceramide (GlcCer) residues, which are sphingolipids found in fungal cell walls (Spelbrink et al. 2004). MtDef4 is a phospholipid-binding defensin and targets phosphatic acid (PA) in fungal cell membranes (Sagaram et al. 2013). A strain of *F. graminearum* was modified to be defective in GlcCer production, and it acquired resistance to MsDef1 but not to MtDef4 (Ramamoorthy et al. 2007a). When the γ-core region of MsDef1 was replaced with the γ-core of MtDef4, the antifungal MOA and lipid binding were altered making MsDef1 behave in the manner of MtDef4 (Sagaram et al. 2011). Also, in MtDef5, the γ-core region is required for dimerization and lipid binding. MtDef5 binds to a number of different phospholipids but displays a strong preference for phosphatidylinositol 3-phosphates (PI3P), PI4P, and PI5P (Islam et al. 2017). Without proper lipid binding, these defensins have reduced antifungal activity. These findings support the existence of a "phospholipid code" that identifies target membranes for defensin-mediated attack (Baxter et al. 2017b).

Despite numerous studies exploring plant defensin antifungal MOAs, their complex behavior is still not yet fully understood. MOAs differ between plant defensins interacting with the same fungal pathogen. MsDef1 induces prolific hyperbranching of the hyphae in *F. graminearum*, and MtDef4 reduces *F. graminearum* growth without causing significant morphological changes (Ramamoorthy et al. 2007b). Additionally, MsDef1 remains on the outside of *F. graminearum* cells while MtDef4 is internalized and accumulates in the cytoplasm (Sagaram et al. 2013). Interfering with calcium signaling and increasing the calcium uptake appear to be crucial to the antifungal MOA of MsDef1. MsDef1 blocks mammalian L-type Ca^{2+} channels, and in *Neurospora crassa*, MsDef1 perturbs Ca^{2+} homeostasis through binding to GlcCer (Munoz et al. 2014; Spelbrink et al.

2004). Surprisingly, closely related fungal species interact with a particular defensin in different ways. MtDef4 was found to have different MOAs against two ascomycete fungi, *N. crassa* and *F. graminearum*; membrane permeabilization is required for antifungal activity against *F. graminearum*, but it is not required against *N. crassa* (El-Mounadi et al. 2016). The authors suggested that these findings were due to different molecular compositions of cell wall and plasma membranes in these two fungi, which restricted MtDef4 entry. MtDef5 was shown to be internalized and rapidly permeabilize membranes of both *N. crassa* and *F. graminearum*, but MtDef5 uses spatially distinct routes of entry into these fungi (Islam et al. 2017).

Transgenic expression of *Medicago* defensins. Numerous crop plants have been engineered for resistance to fungal pathogens using *Medicago* defensins. In an influential, early study, MsDef1 was expressed in potato (Gao et al. 2000). The transgenic potatoes demonstrated increased resistance to *Verticillium dahliae* in both greenhouse and field trials. Also, MsDef1 when expressed in tomato confers resistance to *F. oxysporum* f. sp. *lycopersici* (Abdallah et al. 2010). Pleiotropic effects that reduced yield traits are frequently observed with constitutive defensin expression. For example, tuber size was reduced when MsDef1 was constitutively expressed in potato (Gao et al. 2000). Additionally, MsDef1 seed treatments were demonstrated to inhibit root growth in germinating *Arabidopsis* seeds but not on germinating *M. truncatula* seeds (Allen et al. 2008). Therefore, MtDef4 became a desirable plant defensin to constitutively express because it has limited pleiotropic effects. In transgenic wheat, MtDef4 provided increased resistance to *Puccinia triticina* without significantly reducing yield (Kaur et al. 2017).

Both MsDef1 and MtDef4 provided *Aspergillus flavus* resistance and greatly reduce the levels of aflatoxins in transgenic peanut (Sharma et al. 2017).

Introduction to Dissertation Research

Plant defensins have the capability of inhibiting bacterial, oomycete, and fungal pathogen growth (Parisi et al. 2018). A single defensin peptide can have antimicrobial activity against a diverse scope of pathogens (Franco 2011). But, plant defensin antimicrobial activity has not been determined against alfalfa pathogens. Alfalfa crown rot is a disease complex including numerous bacterial, oomycete, and fungal alfalfa pathogens (Rhodes 2015). Currently, there are limited management strategies for this complex alfalfa disease. Out of a suite of plant defensin peptides, I hypothesized that at least one would demonstrate antimicrobial activity against multiple crown rot pathogens. The first research objective of my dissertation was to evaluate the antimicrobial activity of plant defensin peptides against alfalfa crown rot pathogens *in vitro*. Chapter two contains my first objective and was published in *Phytopathology* (Sathoff et. al 2019).

In numerous crop plants, the transgenic expression of a plant defensin has led to increased disease resistance, but notably, this has not been done not in alfalfa (Lacerda et al. 2014). Innovative approaches are needed to manage alfalfa crown rot because there are currently no chemical treatments or resistant cultivars marketed for crown rot control. Considering that the *in vitro* testing demonstrated that *M. truncatula* plant defensins have antimicrobial activity against crown rot pathogens, I hypothesized that MtDef5 would display antimicrobial activity against crown rot pathogens *in planta*. My second research objective was to transgenically express MtDef5 in alfalfa and evaluate resistance of the plants to fungal and bacterial members of the crown rot complex.

Little is known about the antibacterial MOA of plant defensins. Defensin sequence data alone are not sufficient to predict antimicrobial activity, and the various bacterial pathogens that have been identified as being sensitive to plant defensin treatments do not form a pattern to aid in the prediction of antibacterial activity (Sathoff and Samac 2019). Knowing the MOA will allow for the predication of plant defensin activity against bacterial pathogens and will reduce the amount of laborious *in vitro* testing. In concert with the extensive knowledge on the MOAs of antifungal defensins, I hypothesized that certain bacterial membrane residues or modifications will determine plant defensin antibacterial activity. My third research objective was to identify bacterial membrane modifications and resistance mechanisms that influence the antibacterial activity of plant defensin peptides.

When constitutively expressed, defense gene expression can lead to undesirable pleiotropic effects that reduce yield. Transgenic potato that constitutive expressed MsDef1 had increased disease resistance but also had smaller tubers and reduced yields compared to the non-transgenic control (Kaur et al. 2011). In order for transgene expression to be optimized, expression needs to be spatially and temporally controlled. Pathogen-inducible promoters will restrict transgene expression in the absence of disease and therefore, will eliminate the deleterious effects of constitutive expression. I hypothesized that *M. truncatula* PR genes shown to be upregulated during the infections by root and foliar alfalfa pathogens will contain pathogen-induced promoters that can be utilized for transgene expression in alfalfa. My fourth and final research objective was to characterize gene promoters from *M. sativa* and *M. truncatula* for pathogen-induced activity.

TABLE 1 Comprehensive list of all known plant defensins with antibacterial activity[x]

Peptide	Source	Suggested Defensin Nomenclature	Sensitive Bacteria	IC50[y]	MIC[z]
Ah-AMP1 (Osborn et al. 1995)	*Aesculus hippocastanum*	AhDef1	*Bacillus subtilis*	100 µg/mL	
Cp-thionin II (Franco et al. 2006)	*Vigna unguiculata*	VuDef2	*Pseudomonas syringae* *Staphylococcus aureus* *Escherichia coli*		42 µg/mL 128 µg/mL 64 µg/mL
Ct-AMP1 (Osborn et al. 1995)	*Clitoria ternatea*	CtDef1	*Bacillus subtilis*	15 µg/mL	
Dm-AMP1 (Osborn et al. 1995)	*Dahlia merckii*	DmDef1	*Bacillus subtilis*	150 µg/mL	
Fa-AMP1 (Fujimura et al. 2004)	*Fagopyrum esculentum*	FeDef1	*Erwinia carotovora* subsp. *carotovora* *Agrobacterium tumefaciens* *Clavibacter michiganensis* *Curtobacterium flaccumfaciens* pv. *oortii*	11 µg/mL 24 µg/mL 14 µg/mL 13 µg/mL	
Fa-AMP2 (Fujimura et al. 2004)	*Fagopyrum esculentum*	FeDef2	*Erwinia carotovora* subsp. *carotovora* *Agrobacterium tumefaciens* *Clavibacter michiganensis* *Curtobacterium flaccumfaciens* pv. *oortii*	15 µg/mL 17 µg/mL 17 µg/mL 15 µg/mL	
Fabatin (Zhang and Lewis 1997)	*Vicia faba*	VfDef1	*Escherichia coli* *Enterococcus hirae* *Pseudomonas aeruginosa*	14.53 µg/mL 28.75 µg/mL 0.8 µg/mL	
J1-1 (Guillen-Chable et al. 2017)	*Capsicum annuum*	CaDef1	*Pseudomonas aeruginosa*		250 µg/mL
Limenin (Wong and Ng 2006)	*Phaseolus limensis*	PlDef1	*Mycobacterium phlei* *Proteus vulgaris* *Bacillus megaterium* *Bacillus subtilis*	96 µM 81 µM 102 µM 112 µM	

MtDef5 (Velivelli et al. 2018)	*Medicago truncatula*	-	*Xanthomonas campestris* pv. *campestris*		6 µM
OsDef7 (Tantong et al. 2016)	*Oryza sativa*	-	*Xanthomonas oryzae* pv. *oryzae* *X. oryzae* pv. *oryzicola* *Erwinia carotovora* subsp. *atroseptica*		3.9 µg/mL 3.9 µg/mL 63 µg/mL
OsDef8 (Tantong et al. 2016)	*Oryza sativa*	-	*Xanthomonas oryzae* pv. *oryzae* *X. oryzae* pv. *oryzicola* *Erwinia carotovora* subsp. *atroseptica*		3.9 µg/mL 0.6 µg/mL 63 µg/mL
Pth-St1 (Moreno et al. 1994)	*Solanum tuberosum*	StDef1	*Pseudomonas solanacearum* *Clavibacter sepedonicus*	2 µM 0.3 µM	
So-D2 (Segura et al. 1998)	*Spinacia oleracea*	SoDef2	*Clavibacter sepedonicus* *Ralstonia solanacearum*	1 µM 2 µM	
So-D7 (Segura et al. 1998)	*Spinacia oleracea*	SoDef7	*Clavibacter sepedonicus* *Ralstonia solanacearum*	0.1 µM 1 µM	
Tad1 (Koike et al. 2002)	*Triticum aestivum*	TaDef1	*Pseudomonas cichorii*	25 µg/mL	
Tu-AMP1 (Fujimura et al. 2003)	*Tulipa gesneriana*	TgDef1	*Erwinia carotovora* subsp. *carotovora* *Agrobacterium tumefaciens* *Clavibacter michiganensis* *Curtobacterium flaccumfaciens* pv. *oortii*	11 µg/mL 15 µg/mL 14 µg/mL 13 µg/mL	
Tu-AMP2 (Fujimura et al. 2003)	*Tulipa gesneriana*	TgDef2	*Erwinia carotovora* subsp. *carotovora* *Agrobacterium tumefaciens* *Clavibacter michiganensis* *Curtobacterium flaccumfaciens* pv. *oortii*	15 µg/mL 17 µg/mL 17 µg/mL 15 µg/mL	

VaD1 (Chen et al. 2005)	*Vigna angularis*	VaDef1	*Staphylococcus epidermis*	36.6 µg/mL
			Xanthomonas campestris pv. *vesicatoria*	40.8 µg/mL
			Salmonella typhimurium	143.4 µg/mL
ZmESR-6 (Balandin et al. 2005)	*Zea mays*	ZmDef6	*Clavibacter sepedonicus*	0.2 µM
			Xanthomonas campestris	15 µM
			Sinorhizobium meliloti	5 µM

[x]The IC_{50} or MIC values are listed for each defensin depending on which assay was used for the initial characterization.
[y]The IC_{50} is the concentration of defensin peptide that reduces bacterial growth by half.
[z]The MIC is the concentration of defensin peptide at which no bacterial growth is detected.

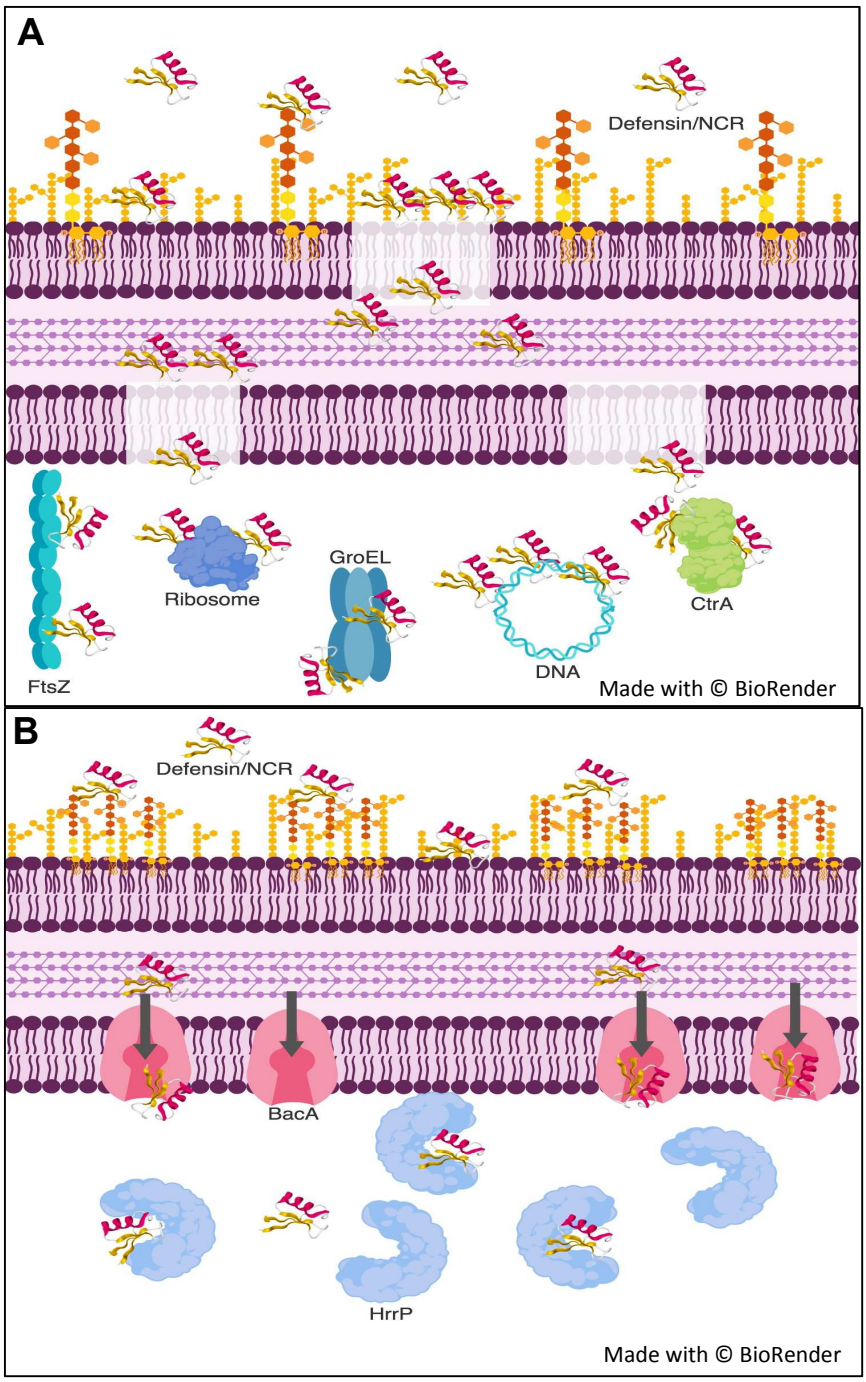

Fig. 1 Defensins and NCRs interfere with bacterial physiology. The membrane system for a typical Gram-negative bacterium illustrates the bacterial targets of defensins/NCRs **A**, and the bacterial host responses to defensins/NCRs **B**. The structure of a typical defensin peptide is shown by the colored ribbon structures.

Chapter 2: Plant defensin peptides have antifungal and antibacterial activity against human and plant pathogens*

INTRODUCTION

Peptides and small proteins with antimicrobial activity have been identified in a wide array of organisms (Dias and Franco 2015). Because they are found in vertebrates, invertebrates, plants, and fungi, they may constitute an ancient, conserved line of defense against pathogen invasion that predates the divergence in eukaryotes (Carvalho and Gomes 2009). Plant defensins are cysteine-rich, cationic antimicrobial peptides of 45-54 amino acid residues. These peptides have a highly conserved three-dimensional structure consisting of one α-helix and three antiparallel β-strands that are connected by four disulfide bonds forming a cysteine-stabilized αβ (CSαβ) motif (de Coninck et al. 2013; Francisco and Georgina 2017; Lay and Anderson 2005; Vriens et al. 2014). The structure of each plant defensin has a functionally important γ-core motif $GXCX_{3-9}C$ which alone can confer antimicrobial activity (Sagaram et al. 2011; Yount and Yeaman 2004). Despite their structural uniformity, plant defensins exhibit very low sequence similarity outside the eight distinctively conserved cysteines (Thomma et al. 2002; van der Weerden and Anderson 2013). This divergence in primary sequences may account for different biological functions attributed to plant defensins including antifungal and antibacterial activity, pollen tube guidance, and roles in plant development (Carvalho and Gomes 2009).

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Many plant defensins exhibit potent activity *in vitro* inhibiting the growth of fungi and oomycetes at micromolar concentrations. However, they differ considerably in the spectrum of organisms inhibited and modes of action (MOA). The initial studies aimed at revealing MOA of plant defensins identified interactions with fungal-specific membrane components (Thevissen et al. 1997; 2000; 2004). Defensins were shown to permeabilize fungal plasma membranes, induce Ca^{2+} influx, and disrupt a Ca^{2+} gradient essential for polar growth of hyphal tips (Thevissen et al. 1996; 1997; 1999). Some defensins bind with high affinity to specific sphingolipids present in the fungal cell wall and/or plasma membrane of their target fungi (Aerts et al. 2008; Thevissen et al. 2005; 2007).

During the last few years, several antifungal plant defensins including Psd1 from *Pisum sativum*, NaD1 from *Nicotiana alata*, TPP3 from *Solanum lycopersicum*, NsD7 from *N. suaveolens*, and MtDef4 and MtDef5 from *Medicago truncatula* have been shown to gain entry into fungal cells and interact with bioactive plasma membrane resident phospholipids, cause membrane permeabilization, and induce fungal cell death (Aerts et al. 2008; Lobo et al. 2007; Parisi et al. 2018; Thevissen et al. 2004). It has been proposed that these peptides have multiple targets in fungal cells. Thus, in addition to disrupting the plasma membrane, these peptides likely bind to intracellular targets, induce production of reactive oxygen species, and inhibit cell division.

Although several plant defensins have been extensively studied for their antifungal activity, fewer defensins with antibacterial activity have been reported (van der Weerden and Anderson 2013). For example, Cp-thionin II from cowpea (Franco et al. 2006), DmAMP1 from *Dahlia merckii*, CtAMP1 from *Clitoria ternatea*, AhAMP1 from *Aesculus hippocastanum* (Osborn et al. 1995), ZmESR-6 from maize (Balandin et al. 2005), fabatin

from broad been (Zhang and Lewis 1997), and So-D2 and So-D7 from spinach (Segura et al. 1998) have been reported to exhibit antibacterial activity against a range of Gram-positive and Gram-negative bacterial pathogens. Among antibacterial defensins, So-D2 and So-D7 from spinach have been demonstrated to confer resistance to Asiatic citrus canker and Huanglongbing caused by *Xanthomonas citri* spp. *citri* and *Candidatus* Liberibacter sp., respectively, in transgenic citrus (Stover et al. 2013). The MOA of antibacterial plant defensins have yet to be deciphered in detail.

Alfalfa (*Medicago sativa*), a perennial plant in the legume family, is among the most valuable crops in the United States with a direct value of over $8 billion annually. Alfalfa production is essential for sustaining the dairy industry which used an estimated 14 million tons of alfalfa in 2016. Plant pathogens and nematodes that infect alfalfa account for an estimated $400 million in economic losses annually (Leath et al. 1988). Crown rot, caused by a complex of soil microbes, is one of the most important alfalfa diseases across the United States. The organisms causing crown rot can differ substantially by geographic location. Some of the most common pathogenic fungal species are *Phoma medicaginis, Rhizoctonia solani, Fusarium oxysporum, F. roseum,* and *F. solani* (Turner and Van Alfen 1983; Uddin and Knous 1991; Wilcoxson et al. 1977). Bacteria (*Clavibacter insidiosus* and *Pseudomonas* species) and oomycetes (*Pythium* species) are also associated with the disease complex. Another economically important alfalfa disease, Aphanomyces root rot caused by *Aphanomyces euteiches* often accompanies crown rot in soil with poor drainage. Crown rot occurs to some extent in every alfalfa stand that is over one year old and is the major source of stand decline and yield loss.

Breeding for resistance has been successfully employed to manage several alfalfa diseases, but resistance has not been identified for developing crown rot resistant cultivars. Fungicides with the required persistent root and crown activity are not available. Lack of cultural and chemical management practices for this disease severely limits alfalfa production. Thus, there is an immediate need for development of innovative methods to manage crown rot for enhanced alfalfa persistence and yields.

We have recently reported the antibacterial activity of a bi-domain defensin, MtDef5, from *M. truncatula*, a model plant species closely related to alfalfa, against the Gram-negative bacterial pathogen *Xanthomonas campestris*. MtDef5 permeabilizes the plasma membrane and translocates into the cells of this bacterial pathogen. *In vitro*, it also binds to DNA (Velivelli et al. 2018). In this study, we identified plant defensin peptides that inhibit the *in vitro* growth of alfalfa crown rot pathogens. We also discovered that they inhibit the growth of several human bacterial pathogens. Synthetic γ-core motifs were used to initially screen for activity against numerous fungal and bacterial pathogens. The γ-core motifs from *M. truncatula* had the greatest antagonistic biological activity against the evaluated pathogens. However, the corresponding full-length defensins displayed enhanced activity compared to the γ-core motifs. These results not only indicate that transgenic expression of plant defensins in alfalfa has the potential to provide improved resistance to crown rot disease in alfalfa, but also that plant defensins and short peptides derived from them may be a valuable resource for the development of therapeutic compounds with novel modes of action to combat human pathogens.

MATERIALS AND METHODS

Pathogen cultures and growth media. All fungal pathogen strains were isolated from infected alfalfa plants collected in Minnesota from commercial production fields and deposited in the University of Minnesota Mycological Culture Collection. The fungal strains, *Fusarium oxysporum* f. sp. *medicaginis* 7F-3, *F. oxysporum* f. sp. *medicaginis* 31F-3, *F. solani*, *F. tricinctum*, *F. incarnatum*, *F. redolens*, *Colletotrichum trifolii* WS-5, *C. trifolii* FG-1, *Phoma medicaginis* STC, and *P. medicaginis* WS-2 were grown on potato dextrose agar (Difco, Sparks, MD) at 25°C. After one to two weeks of culture growth, conidia were harvested by washing the plates with sterile water. The spore suspensions were filtered and spore densities were determined microscopically using a hemocytometer.

Aphanomyces euteiches MF-1 (race 1) and *A. euteiches* MER4 (race 2) were grown on corn meal agar (Difco, Sparks, MD) at 25°C for one week. Agar disks (7 mm diameter) from the margin of the *A. euteiches* colonized medium were cultured in liquid peptone glucose (PG) medium containing 20 g peptone and 5 g glucose per liter at 20°C for 24 h. To trigger zoospore production, PG medium was removed and agar disks were washed with sterile spring water at 0, 1, 2, 4, and 6 hours after PG media removal by resuspending in 100 mL of sterile spring water. The final resuspension had just enough volume to immerse the disks in ~15 mL of sterile spring water. Zoospores were harvested 18 h after the final resuspension. Spore densities were determined microscopically using a hemocytometer.

From glycerol stocks, the bacterial strains, *Pseudomonas syringae* pv. *syringae* ALF3, *Xanthomonas alfalfae* subsp. *alfalfae* F3, *E. coli* DH5α, *Sinorhizobium meliloti* 102F51, *Clavibacter insidiosus* R1-3, and *C. insidiosus* R1-1 were cultured on nutrient

broth yeast extract (NBY) agar at 30°C. After two days of growth, the bacterial cells were harvested by flooding the plates with sterile water. The ATCC reference strains of human pathogenic bacteria, *Pseudomonas aeruginosa* PAO1, *Serratia marcescens*, *Enterobacter aerogenes*, and *Enterococcus casseliflavus* were obtained from Dr. Foster-Hartnett at the University of Minnesota and were cultured on LB agar (Difco, Sparks, MD) at 37°C. After one day of growth, the bacterial cells were harvested by flooding the plates with sterile water.

Defensin peptide synthesis. The γ-core motif peptides derived from plant defensins, MsDef1, MtDef4, MtDef5, RsAFP-2, and So-D2 (Gao et al., 2000; Islam et al., 2017; Sagaram et al., 2011; Segura et al., 1998; Terras et al., 1992) (Table 2) were chemically synthesized and purified by HPLC (LifeTein, Somerset, NJ).

Full-length clones encoding MtDef4 and MtDef5 were expressed in *Pichia pastoris*, and the peptides were purified as previously described (Islam et al. 2017; Spelbrink et al. 2004). Defensins were lyophilized and re-suspended in nuclease-free water. The concentration of each defensin was determined by NanoDrop spectrophotometry. Approximately 3 mg of purified protein was collected from one liter of *P. pastoris* culture expressing the defensin.

***In vitro* defensin antifungal activity determination.** A microplate reader assay adapted from Broekaert et al. (1990) using absorption as a measure for fungal growth was utilized to monitor growth inhibition by the γ-core motif peptides and full-length defensins. Flat clear bottom 96 well microplates (Corning, Corning, NY) were used with each well containing half-strength potato dextrose broth (Difco, Sparks, MD), approximately 2,000 spores, and a defensin peptide at concentrations of 0, 5, 10, 15, or

30 μg/mL in a total volume of 100 μl. Samples were assayed in triplicate. The microplates were shaken on an orbital shaker and spores were allowed to sediment for 30 minutes before absorbance was measured. The absorbance of the wells was measured at 595 nm on a Synergy H1 microplate reader (BioTek, Winooski, VT). Further absorbance measurements were carried out after 24-h and 48-h incubation periods at 25 °C. To quantify fungal growth, the initial absorbance measurement was subtracted from the final absorbance measurement at 48 h. The changes in absorbance were averaged across the three replications and a dose response curve was created by performing a regression using Microsoft Excel 2016. The amount of defensin needed to inhibit growth of the fungal pathogens strains by 50% (IC_{50}) was calculated from dose response curves as previously described (Terras et al. 1992). This assay was repeated three times for each fungal pathogen. The IC_{50} values are presented as mean ± standard error from the three experiments.

Antibacterial activity screen. Cell suspensions were diluted with sterile water to an OD_{600} of 0.1. Plates of NBY were spread with 100 μL of bacteria to create a bacterial lawn. The plates were dried for 10 minutes before placing sterile filter paper disks containing 30, 10, 5, or 0 μg defensin peptide onto plates. Each bacterial lawn had 12 filter paper disks, three disks of each defensin concentration, and this experiment was repeated three times on separate NBY plates. The bacterial plates were incubated for one or two days at 25 °C. If zones of bacterial growth inhibition were observed, the defensin was considered to have antibacterial activity. The diameter of zones of inhibition was measured, and the average area of the zones of inhibition was determined using the formula, area = πr^2.

***In vitro* defensin antibacterial activity determination.** To quantify antibacterial activity, a spread-plate assay was used for both full-length defensins and γ-core defensin peptides and was repeated three times for each bacterial pathogen. As was done in our initial antibacterial screen, bacterial lawns of *P. syringae* pv. *syringae* and *X. alfalfae* subsp. *alfalfae* were grown on NBY plates for two days. The human pathogens, *S. marcescens, E. aerogenes*, and *P. aeruginosa*, were grown on LB plates for one day. The alfalfa bacterial wilt pathogen, *C. insidiosus* was grown on NBY for one week. The plates were flooded with sterile water, and bacteria were harvested by rubbing with a sterile rubber policeman. Cultures were diluted with sterile water to an OD_{600} of 0.1. In microcentrifuge tubes, 200 µL of bacteria were incubated at 30 °C with shaking for 3 h with various concentrations of a defensin peptide (0, 2.5, 5, 10, 15, or 30 µg/mL). After the peptide treatment, 10-fold serial dilutions were made, and 100 µL were plated in triplicate onto NBY plates. Colony-forming units (CFUs) were counted for *P. syringae* pv. *syringae* and *X. alfalfae* subsp. *alfalfae* after incubation for two days at 30 °C, for *S. marcescens, E. aerogenes*, *E. casseliflavus*, and *P. aeruginosa* incubated one day at 37 °C, and for *C. insidiosus* incubated for seven days at 25 °C. Regression of the average CFUs were across experimental replications versus the defensin concentration was used to create a dose response curve using Microsoft Excel 2016. From these curves, the amount of defensin needed to inhibit growth of bacterial strains by 50% (IC_{50}) was calculated. The IC_{50} values are presented as mean ± standard error from three experiments.

RESULTS

Antifungal activity. The γ-core motif peptides derived from MsDef1, MtDef4, MtDef5, RsAFP-2, and So-D2 (Table 2) demonstrated antifungal activity at micromolar concentrations (Table 3). Overall, the γ-core motif of MtDef4 exhibited a wider spectrum of antifungal activity than the corresponding motif from other defensins tested. In particular, MtDef4 inhibited the growth of *P. medicaginis* and *F. solani* with IC_{50} values of 5.3-7.3 and 6.0 μM, respectively. This peptide inhibited spore germination as well as germ tube elongation and mycelial growth of both fungi but did not result in morphological changes of spores or hyphae (Fig. 2). The γ-core motif of MtDef5 also inhibited the growth of *P. medicaginis* but had enhanced inhibition of *F. solani*, with an IC_{50} value of 4.1 μM, compared to the core motif of MtDef4. None of the γ-core motif peptides demonstrated activity against the oomycete pathogen *Aphanomyces euteiches*, the fungal pathogen *Colletotrichum trifolii*, or surprisingly, against either *Fusarium redolens* or *Fusarium incarnatum*.

Because the γ-core motifs of MtDef4 and MtDef5 showed the greatest antifungal activity against alfalfa crown rot fungal pathogens, the corresponding full-length defensin peptides were evaluated for activity. Full-length MtDef4 and MtDef5 had greater inhibitory activity than their corresponding γ-core peptides against *P. medicaginis* and *F. oxysporum* f. sp. *medicaginis* with IC_{50} values as low as 300 and 700 nM, respectively. The full-length defensins also caused inhibition of spore germination and mycelial growth (Fig. 3). Like the γ-core motifs, the full-length defensins also failed to inhibit the growth of *A. euteiches* and *C. trifolii* (Table 3). These results indicate that the γ-core motif peptides may be used to predict the relative antifungal activity of the corresponding

full-length defensins though additional comparisons need to be performed to see if this trend holds for other defensins.

Antibacterial activity. Considering that plant defensins rarely display antibacterial activity, a somewhat qualitative screen for biological activity was first utilized. The γ-core defensin peptides were initially screened for antibacterial activity on a bacterial lawn by measuring zones of inhibition resulting from defensin peptides spotted onto filter paper disks. The γ-core defensin peptides inhibited the growth of *E. coli*, *Pseudomonas syringae* pv. *syringae*, *Sinorhizobium meliloti*, or *Xanthomonas alfalfae* subsp. *alfalfae* to varying degrees (Fig. 4). The γ-core peptide from MtDef4 displayed the greatest antibacterial activity of the γ-core motif peptides tested. However, neither MtDef4 nor MtDef5 γ-core motifs inhibited the growth of the beneficial, nitrogen-fixing microsymbiont *S. meliloti*. Overall, the γ-core peptides displayed the greatest growth inhibition against *E. coli* using the filter paper disk assay.

Using a more quantitative spread-plate assay, the antibacterial activity of the γ-core motifs from MsDef1, MtDef4, MtDef5, and So-D2 against the bacterial plant pathogens were evaluated and found to exhibit antibacterial activity at micromolar concentrations. The MtDef4 and MtDef5 γ-core motifs inhibited the growth of *P. syringae* pv. *syringae* with IC_{50} values of 3.4 and 4.5 μM, respectively (Table 4). Notably, the MtDef4 γ-core motif peptide but not the MtDef5 γ-core peptide displayed antibacterial activity against *X. alfalfae* subsp. *alfalfae*.

The full-length defensins, MtDef4 and MtDef5, were also characterized for antibacterial activity using the spread-plate method. In addition to the previously tested

Gram-negative bacteria, antibacterial activity against a Gram-positive alfalfa bacterial wilt bacterium, *Clavibacter insidiosus*, was evaluated. The antibacterial activity of full-length MtDef4 and MtDef5 was enhanced as compared to their corresponding γ-core motifs. MtDef4 and MtDef5 had IC_{50} values at nanomolar concentrations. In accordance with the γ-core motif results, MtDef4 and MtDef5 exhibited high activity against *P. syringae* pv. *syringae* with IC_{50} values of 400 and 100 nM, respectively (Table 4). MtDef4 was most active against *C. insidiosus* with an IC_{50} value of 100 nM. Again, MtDef5 displayed no antibacterial activity against *X. alfalfae* subsp. *alfalfae*, which further supports the predictive capacity of the γ-core motif peptides. The broad antibacterial activity of MtDef4 and MtDef5 against plant bacterial pathogens led us to conduct antibacterial tests against human pathogens using the spread-plate assay. MtDef4 and MtDef5 γ-core peptides displayed low IC_{50} values against the majority of human bacterial pathogens tested, with *Enterobacter aerogenes* being the most sensitive to both MtDef4 and MtDef5 (Table 5). No antibacterial activity was seen against the Gram-positive bacterium *Enterococcus casseliflavus*.

DISCUSSION

Plant defensins are well known to have activity against plant fungal pathogens, inhibiting *in vitro* growth as well as reducing damage from fungal diseases when expressed in heterologous plant hosts. We tested plant defensin peptides against diverse pathogens and found that they displayed broad inhibitory activity against plant fungal pathogens causing alfalfa crown rot disease along with, remarkably, both human and plant bacterial pathogens. In addition to displaying extensive antifungal activity, MtDef4 had strong and broad-spectrum antibacterial activity with nanomolar IC_{50} values against both gram-

positive and gram-negative bacteria (Table 4). There are few reports that cite antibacterial activity of plant defensins because traditionally they have been regarded to possess solely antifungal activity (Fujimura et al. 2003; Guillen-Chable et al. 2017; Segura et al. 1998; Velivelli et al. 2018). Spinach defensin (So-D2) displayed high activity against both *Clavibacter michiganensis* subsp. *sepedonicus* and *Ralstonia solanacearum*, which corresponds to gram-positive and gram-negative bacteria, but So-D2 displayed limited activity against fungal pathogens (Segura et al. 1998). MtDef4, MtDef5, and MsDef1 are noteworthy defensins because they exhibit potency against gram-positive, gram-negative, and fungal pathogens. Our report demonstrates that extensively studied plant defensins can have high antibacterial activity against human and plant pathogens, which was previously overlooked. This suggests that other well-characterized plant defensins should be retested for antibacterial activity.

Of the fungi tested, none of the plant defensins inhibited *C. trifolii*, *F. redolens,* or *F. incarnatum* (Table 3). Defensin antimicrobial specificity is commonly observed. For example, RsAFP2 demonstrated extensive antifungal activity but displayed no activity against either *Sclerotinia sclerotiorum* or *Rhizoctonia solani* (Terras et al. 1992). This pathogen specificity could occur due to diverse modes of action, resulting from the rich diversity of the primary amino acid sequences of plant defensins. RsAFP-1 and RsAFP-2 differ from each other by only two amino acids in the primary structure but exhibit a striking difference in their antimicrobial activity (Terras et al. 1992). The antifungal modes of action of MsDef1, MtDef4, MtDef5, and RsAFP-2 all differ, but their molecular modes of action all involve interactions with fungal membrane components (Cools et al. 2017;

Islam et al. 2017; Parisi et al. 2018). These resistant fungal pathogens could have structural differences in their membranes that inhibit defensin recognition.

The *M. truncatula* defensins that we tested against alfalfa crown rot pathogens and human bacterial pathogens are among the well-characterized plant defensins and have potent activity against other plant pathogens (Muñoz et al. 2014; Sagaram et al. 2011; 2013). When expressed in Arabidopsis and tomato, they give strong protection against virulent fungal pathogens and protect the plants from disease (Abdallah et al. 2010; Kaur et al. 2012; Sharma et al. 2017). But, the broad antibacterial activity of these defensins had not been previously characterized. In contrast to the antifungal modes of action, there is no proposed antibacterial mode of action for any plant defensin. Both the full-length MtDef5 and its γ-core motif failed to inhibit the growth of *X. alfalfae* subsp. *alfalfae* (Table 4), which was surprising because MtDef5 was previously shown to be active against *X. campestris* (Velivelli et al. 2018). The resistance of *X. alfalfae* subsp. *alfalfae* to MtDef5 may be due to the presence of a homolog of MtDef5 in alfalfa (Scc4a34_1890, 78.8% sequence identity). *X. alfalfae* subsp. *alfalfae*, a common alfalfa pathogen, could have become resistant to the MtDef5 homolog in alfalfa. Therefore, *X. alfalfae* subsp. *alfalfae* would be considered an adapted pathogen that has overcome the antibacterial activity of MtDef5. Plant defensins may be an excellent source for antibiotic development because human bacterial pathogens would be considered non-adapted to plant derived defensins.

Currently, there are limited control and management strategies for the alfalfa crown rot disease complex. Alfalfa crown rot is ubiquitous and leads to stand decline, which brings on financial losses to the growers. This disease complex poses a complex

problem. Pathogens gain entry into the crown through cut stems and mechanical damage to the root and crown that occur during the multiple foliage harvests throughout the year. Chemical control does not have the necessary persistence because pathogens decay the crowns during a period of months or years, predisposing it to winterkill and eventually killing the plant. Breeding efforts to increase quantitative resistance have made only minor progress (Miller-Garvin and Viands 1994). This report establishes plant defensins as potential agents for enhancing resistance to alfalfa crown rot, and possibly other diseases, through genetic modification.

Obtaining functional defensin peptides through heterologous expression can be a fastidious process. Amino acid substitutions, improper folding, and incorrect disulfide bridge formation all inhibit the biological activity of plant defensins (Vriens et al. 2014). Eukaryotic expression systems, like the often-utilized *Pichia pastoris* expression system, can create constructs with the proper structure, disulfide bonds, and post-translational modifications, but the experimental setup can be difficult. Specialized *E. coli* based bacterial expression systems can generate copious amounts of defensin peptides, but these peptides have low biological activity due to problematic structural integrity (Lacerda et al. 2014). We have shown that truncated defensin peptides containing the γ-core motif can be chemically synthesized and may mimic the relative antimicrobial activity of the full-length defensins (Table 3 and 4). This warrants the further investigation of the predictive capabilities of γ-core motif defensin peptides from species other than *M. truncatula*. In combination with the described microplate and spread-plate methods, γ-core motif peptides could be used to quickly screen defensins for antimicrobial activity, which would greatly simplify and expedite defensin bioassays.

In this report, we characterized the *in vitro* antifungal and antibacterial activity of plant defensins against alfalfa crown rot pathogens and human bacterial pathogens. Full-length defensins were shown to have antimicrobial activity against both fungal and bacterial pathogens at nanomolar concentrations. These experiments show the previously overlooked high biological activity of plant defensins against bacterial pathogens. Additionally, these results indicate that the γ-core motif peptide may be used to predict the relative biological activity of the full-length defensin. Specifically, MtDef4 and MtDef5 were identified as ideal candidates for transgenic expression in alfalfa due to their broad-spectrum and strong antimicrobial activity. Transgenic expression of these defensins could be utilized to implement an eco-friendly, protein-based strategy that could provide alfalfa with enhanced resistance against crown rot and growers with the corresponding gains in yield.

TABLE 2 Amino acid sequences of γ-core motif (bold) and C-terminal region (italics) of plant defensins tested *in vitro*

Defensin	**Amino Acid Sequence**
MsDef1	**GRCRDDFRC***WCTKRC*
MtDef4	**GRCRGFRRRC***FCTTHC*
MtDef5	**GACHRQGFGFAC***FCYKKC*
RsAFP-2	**GSCNYVFPAHKC***ICYFP*
So-D2	**GDCKGIRRRC***MCSKPL*

TABLE 3 Activity of the γ-core motif defensin peptide constructs and full-length defensin peptides against fungal and oomycete alfalfa crown rot pathogens[z]

Defensin	*Fusarium oxysporum* f. sp. *medicaginis*		*Phoma medicaginis*		*Colletotrichum trifolii*		*Aphanomyces euteiches*		*Fusarium solani*	*Fusarium tricinctum*	*Fusarium redolens*	*Fusarium incarnatum*
	7F-3	31F-3	STC	WS-2	FG-1	WS-5	Race 1	Race 2				
Core MsDef1	NA	NA	12.7 ± 1.1	14.8 ± 1.0	NA	NA	NA	NA	NA	NA	NA	NA
Core MtDef4	7.1 ± 0.8	6.9 ± 0.8	7.3 ± 0.7	5.3 ± 0.7	NA	NA	NA	NA	6.0 ± 1.0	14.7 ± 1.3	NA	NA
Core MtDef5	NA	NA	19.5 ± 1.2	8.5 ± 1.0	NA	NA	NA	NA	4.1 ± 0.5	NA	NA	NA
Core RsAFP2	NA	NA	NA	NA	NA	NA	NA	NA	NA	5.3 ± 0.5	NA	NA
Core So-D2	33.1 ± 1.9	NA	6.4 ± 0.6	6.1 ± 0.6	NA	NA	NA	NA	13.8 ± 0.9	NA	NA	NA
MtDef4	0.7 ± 0.1	1.9 ± 0.1	0.3 ± 0.1	2.6 ± 0.1	NA	NA	NA	NA	ND	ND	ND	ND
MtDef5	0.8 ± 0.1	1.3 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	NA	NA	NA	NA	ND	ND	ND	ND

[z]The mean IC_{50} (μM) values are reported ± SE of three independent experiments (n=3). NA indicates that the defensins at a concentration of 30 μg/mL showed no biological activity against the pathogens. ND indicates no data.

TABLE 4 Activity of the γ-core motif defensin peptides and full-length defensin peptides against bacterial alfalfa crown rot pathogens[z]

Defensin	***Xanthomonas alfalfae* subsp. *alfalfae***	***Pseudomonas syringae* pv. *syringae***	***Clavibacter insidiosus***
Core MtDef4	11.4 ± 0.2	3.4 ± 0.4	ND
Core MtDef5	NA	4.5 ± 0.5	ND
Core So-D2	19.3 ± 2.2	25.9 ± 1.2	ND
Core MsDef1	7.9 ± 0.7	8.8 ± 1.1	ND
MtDef4	0.6 ± 0.04	0.4 ± 0.05	0.1 ± 0.01
MtDef5	NA	0.1 ± 0.01	NA

[z] The mean IC_{50} (μM) values are reported ± SE of three independent experiments (n=3). NA indicates that the defensins at a concentration of 30 μg/mL showed no biological activity against the pathogens. ND indicates no data.

TABLE 5 Activity of the γ-core motif defensin peptides against human bacterial pathogens[z]

Defensin	***Serratia marcescens***	***Enterobacter aerogenes***	***Enterococcus casseliflavus***	***Pseudomonas aeruginosa***
Core MtDef4	8.4 ± 1.0	2.3 ± 0.3	NA	2.7 ± 0.1
Core MtDef5	6.0 ± 0.7	2.8 ± 0.3	NA	11.8 ± 1.4

[z]The mean IC_{50} (μM) values are reported ± SE of three independent experiments (n=3). NA indicates that the defensins at a concentration of 30 μg/mL showed no biological activity against the pathogens.

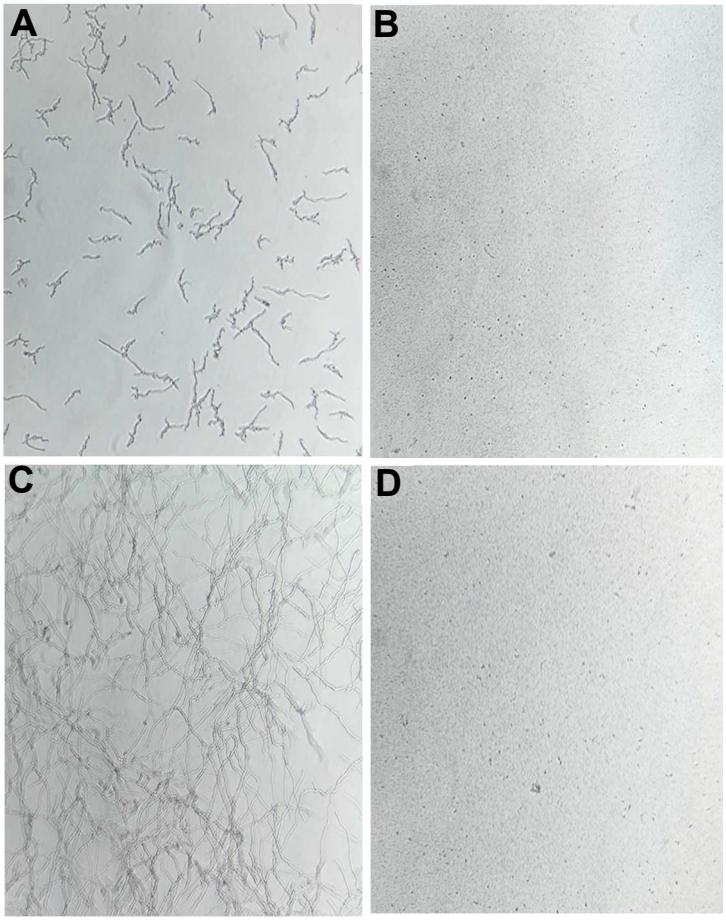


Fig. 2 The γ-core motif peptide from MtDef4 inhibited growth of alfalfa fungal crown rot pathogens *in vitro*. Spores of *Phoma medicaginis* were grown for 24 h at 25°C in potato dextrose broth (PDB) culture medium in **A,** the absence or **B,** presence of 30 μg/mL of γ-core MtDef4. Spores of *Fusarium oxysporum* f. sp. *medicaginis* were grown for 24 h at 25°C in PDB culture medium in **C,** the absence or **D,** presence of 30 μg/mL of γ-core MtDef4.

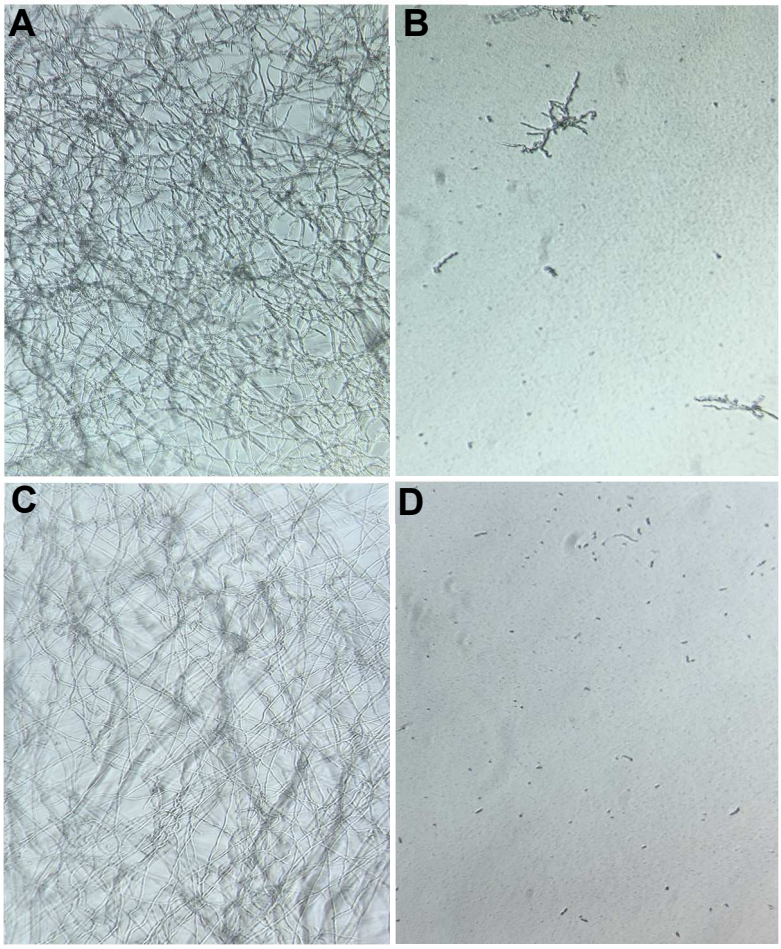


Fig. 3 Full-length MtDef4 inhibited growth of alfalfa fungal crown rot pathogens *in vitro*. Spores of *Phoma medicaginis* were grown for 48 h at 25°C in potato dextrose broth (PDB) culture medium in **A,** the absence or **B,** presence of 30 µg/mL of full-length MtDef4. Spores of *Fusarium oxysporum* f. sp. *medicaginis* were grown for 48 h at 25°C in PDB culture medium in **C,** the absence or **D,** presence of 30 µg/mL of full-length MtDef4.

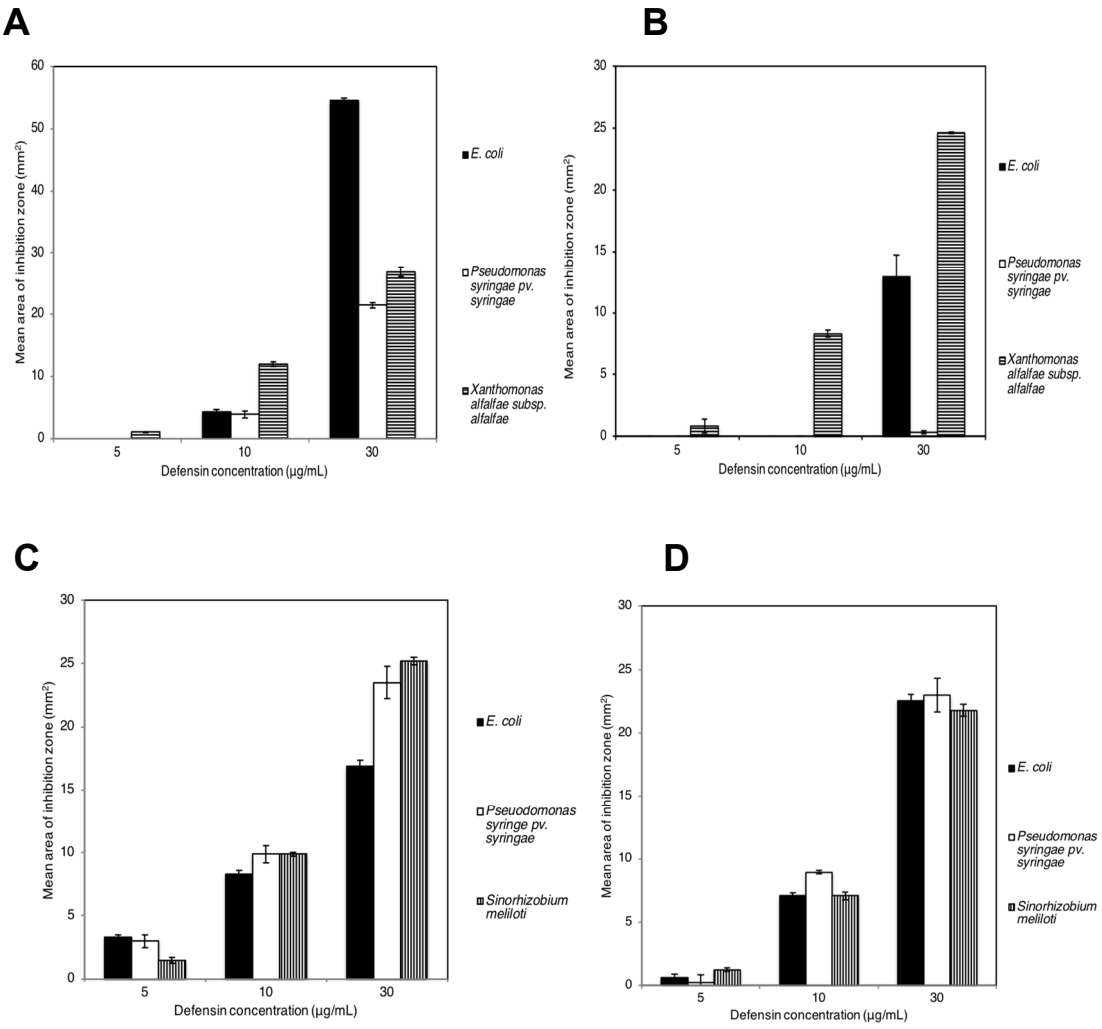


Fig. 4 Antibacterial activity of plant defensin γ-core motif peptides. On bacterial lawns of *E. coli*, *P. syringae* pv. *syringae*, *S. meliloti*, or *X. alfalfae* subsp. *alfalfae*, the area of the zone of inhibition was measured around blank filter paper disks spotted with varying concentrations of the γ-core defensin peptides. The γ-core defensin peptides tested were **A,** MtDef4, **B,** MtDef5, **C,** MsDef1, and **D,** RsAFP2. Bars represent means and error bars indicate standard error (n=9).

Chapter 3: Transgenic expression of a plant defensin in alfalfa (*Medicago sativa*) confers increased resistance to alfalfa crown rot pathogens.

INTRODUCTION

Alfalfa (*Medicago sativa*) is the third most valuable field crop in the United States with a value of $9.9 billion per year estimated by the USDA (https://quickstats.nass.usda.gov). Crown rot is an economically important, chronic alfalfa disease found wherever alfalfa is grown. The disease is characterized by a dark, dry-rot of crown and root tissues, the loss of crown buds, and asymmetric plant growth accompanied by the formation of secondary crowns. Infections initially develop from pathogen entrance into freshly cut stems damaged from harvest, and the lesions expand into the crown tissue (Gossen 1994). Through wounds from insect feeding, grazing animals, machinery, or winter injury, root tissue may also be colonized by crown rotting pathogens (Richard et al. 1980; Wilcoxson et al. 1977). Crown rot reduces alfalfa stand density and persistence with increases in both disease incidence and severity as the stand ages. Profitable alfalfa production depends on maintaining adequate forage harvests for at least 3 years before incurring the cost of stand reestablishment (Kalb et al. 1994). Additionally, decreases in stand density may not be recognized until the following growing season because infected alfalfa stands experience increases in winterkill due to the deteriorated taproot and crown tissues, which store nutrients required for winter survival.

The versatility of crown rot, demonstrated through worldwide distribution and persistence across multiple growing seasons, may result from the diversity of organisms involved in the disease etiology (Rodriguez and Leath 1992). Crown rot is caused by a complex of microbes that varies based on geographic location. The most commonly isolated pathogenic fungal species include *Fusarium oxysporum*, *F. solani*, *F. acuminatum*, *F. avenaceum*, *F. tricinctum, Phoma medicaginis*, *Rhizoctonia solani*, and *Colletotrichum trifolii* (Richard et al. 1980; Turner and Van Alfen 1983; Uddin and Knous 1991; Wilcoxson et al. 1977). The bacterial component of the disease includes *Pseudomonas syringae* pv. *syringae*, *P. viridiflava*, and *P. marginalis* pv. *alfalfae* (Heydari et al. 2014; Lukezic et al. 1983; Turner and Van Alfen 1983).

The pathogens in the crown rot disease complex may only cause crown rots under specific, stress-induced conditions. Damages incurred from feeding of the three-cornered alfalfa leafhopper (*Spissistilus festinus*), potato leafhopper (*Empoasca fabae*), and clover root curculio (*Sitona hispidula*) larvae increase the severity of crown rot (Ariss et al. 2007; Kalb et al. 1994; Moellenbeck et al. 1992). For example, *F. oxysporum* extensively colonizes alfalfa root and crown tissues with relatively minor effects until an additional stress, such as potato leafhopper damage, triggers the disease (Ariss et al. 2007). Currently, crown rot management is concentrated on preventative measures that reduce plant stresses caused by low soil pH, grazing animals or wheel traffic that damages the crowns, and other diseases. Also, frequent harvesting and harvesting too late in the season were shown to increase crown rot severity (Koch and Knox-Davies 1989).

Crown rot damage can be reduced by choosing and planting alfalfa varieties that have resistance to multiple diseases. But, little progress has been made in breeding for

crown rot resistance over the past 45 years with the last reported attempt made in 1994 (Barnes et al. 1990; Miller-Garvin and Viands 1994; Richard et al. 1980; Salter et al. 1994; Wilcoxson et al. 1977). There are currently no cultivars available with documented crown rot resistance. Long-lasting, broad spectrum fungicides with enduring root and crown activity are not available. Incorporation of green manure treatments failed to reduce crown rot (Samac et al. 2013). Therefore, innovative approaches are needed for alfalfa crown rot management.

Plant defensins are small, cysteine-rich pathogenesis-related (PR) proteins found throughout a wide array of plant species (Lacerda et al. 2014; van Loon et al. 2006). Though plant defensins have a highly conserved three-dimensional structure, they are diverse in their amino acid sequences (Lay and Anderson 2005). This sequence diversity likely leads to divergent biological functions and pathogen specificity (Carvalho and Gomes 2009). Several cationic plant defensins inhibit fungal, oomycete, and bacterial growth at micromolar concentrations, and this antimicrobial activity corresponds to disease resistance in transgenic plants (Kaur et al. 2011).

Defensins from *Medicago* species have been transgenically expressed in numerous crop plants leading to enhanced disease resistance. An alfalfa defensin, MsDef1, when expressed in potato generates increased resistance to *Verticillium dahliae*, and in tomato, MsDef1 expression confers resistance to *Fusarium oxysporum* f. sp. *lycopersici* (Abdallah et al. 2011; Gao et al. 2000). MtDef4, a defensin from *Medicago truncatula*, was recently shown to provide *Aspergillus flavus* resistance in transgenic peanut (Sharma et al. 2018). Also, when transgenically expressed in wheat, MtDef4 provides substantial resistance to *Puccinia triticina* (Kaur et al. 2017).

MtDef5 is a unique defensin from *M. truncatula* because it has two defensin domains (MtDef5A and MtDef5B) linked by a 7-amino acid peptide and is the first characterized antimicrobial bi-domain defensin (Islam et al. 2017). We previously demonstrated that MtDef5 inhibits the growth of several bacterial and fungal alfalfa crown rot pathogens *in vitro* (Sathoff et al. 2019). The objective of this current investigation was to determine if MtDef5 provides resistance to crown rot when expressed in alfalfa. Transgenic alfalfa lines expressing MtDef5A were evaluated for resistance to several fungal and bacterial crown rot pathogens. The results indicate that this protein reduces the overall disease symptoms resulting from multiple crown rot pathogens, which indicates that overexpression of a single plant defensin can lead to broad-spectrum disease resistance.

MATERIALS AND METHODS

Construction of plant transformation vector for MtDef5 expression. A previous search of the *M. truncatula* GeneIndex (MtGI 4.0) revealed a bi-domain plant defensin gene identified as Tentative Consensus 87273 (TC87273) at the genomic locus (*MTR8g012775*) (Hanks et al. 2005). The signal peptide and the first defensin domain of TC87273 (MtDef5A) were PCR amplified from pDsRed:MtDef5 using the primers listed in Table 6 (Islam et al. 2017). The PCR product was ligated into pGEM-T Easy (Promega, Madison, WI) and transformed into *E. coli* strain DH5α. Plasmids were purified using a QIAprep spin miniprep kit (Qiagen, Valencia, CA) and digested with *EcoR*I and *Xba*I to remove the MtDef5 gene insert. The insert was isolated via electrophoresis and extracted from the gel using a QIAquick gel extraction kit (Qiagen). The binary plant transformation vector pILTAB381 (Verdaguer et al. 1996) was digested

with *EcoR*I and *Xba*I to remove the *GUS* gene, the products separated on a 1% agarose gel, and the digested pILTAB381 vector was extracted from the gel using a QIAquick gel extraction kit (Qiagen). To create the plant transformation vector pILTAB::MtDef5, MtDef5A was ligated into the binary vector behind the *Cassava vein mosaic virus* (CsVMV) promoter (Fig. 5). Ligation reactions contained Promega 2X Rapid Ligation Buffer and T4 DNA Ligase. The plant transformation vector was used to transform *E. coli* strain DH5α. Plasmids were purified using a QIAprep spin miniprep kit (Qiagen), and positive clones were verified by Sanger DNA sequencing. Plasmids from verified clones were used to transform *Agrobacterium tumefaciens* LBA4404 by electroporation. Transformants were selected on Luria-Bertani (LB) agar (Difco, Sparks, MD) plates with 50 mg/L kanamycin and 25 mg/L rifampicin.

Plant transformation. Alfalfa (genotype Regen SY27x) was transformed using *Agrobacterium*-mediated transformation as described previously (Samac and Austin-Phillips 2006). Briefly, alfalfa leaflets were surface sterilized with 70% ethanol and 10% household bleach, rinsed in sterile water, leaf margins were removed, and leaflets cut into 0.5 cm pieces. Explants were inoculated with *A. tumefaciens* LBA4404 containing the plant transformation vector pILTAB::MtDef5 and co-cultured for 7 days. Bacteria were removed by rinsing explants in sterile distilled water and by culturing on regeneration medium containing the antibiotic ticarcillin (500 mg/L). Transformed callus was selected using kanamycin (25 mg/L), and somatic embryogenesis was induced by culture of callus on medium lacking plant growth regulators. As mature embryos arose, they were transferred to a fresh medium for shoot development and finally to a medium lacking

kanamycin to promote further shoot and root development. The transgenic plants were confirmed by genomic DNA extraction and PCR for the *nptII* and *MtDef5* genes.

Molecular analysis of transgenic plants. A Western blot was performed to identify plants expressing MtDef5. Total protein extracts were prepared by homogenizing 500 mg of fully expanded leaf tissue of an empty vector line lacking the transgene and transgenic alfalfa lines into 1 mL of chilled protein extraction buffer (100 mM 2-[N-morpholino]-ethane sulfonic acid (MES), 100 mM sucrose, 5% 2-mercaptoethanol, and 15% ethylene glycol at pH 6.8). The extract was centrifuged at 10,000 g for 15 min, and the supernatant was used to determine the protein concentration using the Bradford assay kit (Bio-Rad, Hercules, CA). Equal amounts (10 µg) of protein samples were separated on 8–16% Criterion TGX Stain-Free™ precast SDS-PAGE gels (Bio-Rad) and electroblotted onto a 0.45 µm nitrocellulose membrane (Bio-Rad). Membranes were blocked in 30 mL of 5% nonfat dry milk in PBS with 0.05% Tween 20 for 1 h at room temperature with gentle agitation. The membranes were incubated for 1 h with the Rabbit anti-MtDef5 derived primary antibody (1 µg/ml) (GenScript, Piscataway, NJ), followed by incubation for 1 h with the Goat anti-Rabbit IgG horseradish peroxidase conjugate secondary antibody (Bio-Rad). SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fischer, Carlsbad, CA) was used for the development of the blot, and images were taken using a ChemiDoc Imaging System (Bio-Rad). Purified MtDef5 protein (100 ng) was used as a positive control.

Reverse-transcriptase quantitative PCR (RT-qPCR) was performed to measure MtDef5 transcript accumulation in transgenic plants. RNA was isolated from detached leaves using the RNeasy Plant Mini kit (Qiagen). Concentration and purity of the samples

were examined with a NanoDrop spectrophotometer (Thermo Fischer). The first strand of cDNA for each sample was made from 1 μg total RNA using an iScript cDNA Synthesis Kit (Bio-Rad). qPCR primers were designed with Primer3 (Untergasser et al. 2012) (Table 6). qPCR was performed using the iTaq Universal SYBR Green Supermix (Bio-Rad) in 25 μL reactions containing 4 pmol of each MtDef5 forward and reverse primer and 5 μL of template cDNA. Samples from three biological repetitions were run in triplicate on an Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fischer) following the manufacturer's recommendations. The PCR conditions were as follows: 2 min of denaturation at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 58 °C, followed by steps for melting curve generation (15 s at 95 °C, 1 min at 60 °C, 30 s at 95 °C, 15 s at 60 °C). The 7500 Fast Real-Time PCR software (Thermo Fischer) was used for data collection. Melting curves showed that only one transcript was amplified demonstrating that the primers were specific. Relative transcript accumulation for each sample was obtained using the $2^{-\Delta Ct}$ method (Schmittgen and Livak 2008) using the C_t value of the alfalfa f-actin gene (JQ028730.1) for sample normalization.

Pathogen cultures and growth media. All fungal pathogen strains were isolated from infected alfalfa plants collected in Minnesota from commercial production fields and deposited in the University of Minnesota Mycological Culture Collection. The fungal strains, *Colletotrichum trifolii* WS-5, *C. trifolii* 2SP2, *C. trifolii* SM, and *Phoma medicaginis* STC were grown on potato dextrose agar (Difco) at 25 °C. After two weeks of culture growth, conidia were harvested by washing the plates with sterile water. The spore suspensions were filtered and spore densities were determined microscopically

using a hemocytometer. From a glycerol stock, the bacterial strain, *Pseudomonas syringae* pv. *syringae* ALF3 was cultured on nutrient broth yeast extract (NBY) agar at 30 °C. After two days of growth, cells were harvested in sterile distilled water, and cultures were diluted with sterile water to an OD_{600} of 0.1.

Plant Material. Selected primary transformants were propagated clonally by stem cuttings and grown in the greenhouse. Primary transformants were used due to severe inbreeding depression in alfalfa when self-pollinated. Plants were grown in a soil:sand mixture (1:1, v/v), one plant per cone-tainer (Stuewe & Sons, Tangent OR; 7 cm width, 35 cm depth). Plant material from each replicate was combined, frozen in liquid nitrogen, and stored at -80 °C until assayed.

Disease resistance analysis. A detached leaf assay was used to evaluate plants for resistance to crown rot pathogens. Fully expanded trifoliates from the top two to three nodes of transgenic alfalfa plants, pooled from multiple plants in each line, were removed and placed in 100 x 15 mm Petri plates lined with moist filter paper. Each leaflet was inoculated with 5 µL drops of a conidial suspension of either *P. medicaginis* PSTC, *C. trifolii* 2SP2, or *C. trifolii* WS5 at a concentration of 10^6 spores/mL with 50 ppm Tween 20. Mock-inoculated leaves received 5 µL drops of sterile water with 50 ppm Tween 20. Each plant line was evaluated in triplicate plates that contained five trifoliates. Leaflets were scored 7 days after inoculation with *P. medicaginis* and 10 days after inoculation with *C. trifolii*. The disease scale from Garcia et al. (2014) was utilized: 1:Healthy leaflet, 2:Countable injuries, 3:Uncountable injuries, 4:Chlorosis, 5:Completely damaged.

Transgenic alfalfa plants, three replicates for each line, were placed in a mist chamber and sprayed with fungal inoculum, either *P. medicaginis* PSTC or *C. trifolii* SM

adjusted to a concentration of 10^6 spores/mL with 50 ppm Tween 20 until run-off. Mock-inoculated plants were sprayed with sterile water with 50 ppm Tween 20. After 48 hours in a dark mist chamber with 100% humidity, the plants were returned to the greenhouse for 8-10 days. Then, disease severity was scored visually using the rating scales from the standardized tests to characterize alfalfa cultivars (https://www.naaic.org/stdtests). On the rating scale for *P. medicaginis*, classes 1 and 2 are considered resistant; 1:Healthy, symptom-free top growth, 2:Small (<2 mm), brown or black lesions with no defoliation, 3:Larger (2 to 3 mm), discrete lesions with no chlorosis or defoliation, 4:Large (>3 mm) lesions with no defoliation, 5:Lesions >3 mm with pycnidia and dead leaves or defoliation. On the rating scale for *C. trifolii*, 1:Healthy, symptom-free top growth, 2:Minor wilting with no formation of shepherd's crooks (wilting and death of upper portion of stems), 3:Single shepherd's crook with no foliar damage or chlorosis, 4:Multiple shepherd's crooks with chlorosis and defoliation. This experiment was repeated three times for each of the fungal pathogens evaluated. On disease score data, statistical analyses including ANOVA Tukey HSD test ($P < 0.05$) and multiple pairwise T tests with a Bonferroni correction ($P < 0.05$) were performed in R (https://www.r-project.org).

A stem injection assay was used to evaluate plants for resistance to *P. syringae* pv. *syringae*. Transgenic plants were inoculated after 14 days of regrowth. The stem was wounded at a single site using a 22-gauge needle and swabbed with bacterial inoculum adjusted to an $OD_{600} = 0.1$. Control plants were inoculated with water. Five replicate plants were assayed for each line, and this experiment was repeated three times. At 10 days after inoculation symptoms were scored using the following scale for stems; 1:Stem

with no visible damage; 2:Hypersensitive response; 3:Lesion at site of inoculation; 4:Lesion extending from site of inoculation; 5:Stem collapse. Foliar symptoms were scored as 1:No symptoms; 2:Marginal leaf necrosis; 3:Yellowing of leaf at inoculated node; 4:Systemic yellowing and/or necrosis; 5:Dead plant. After scoring symptoms, the inoculated internode was removed, cut into 1 mm sections, and placed in 1 mL sterile water. Serial dilutions were made and plated on King's B medium to determine colony forming units per internode. On disease score data, statistical analyses including ANOVA Tukey HSD test ($P < 0.05$) and multiple pairwise T tests with a Bonferroni correction ($P < 0.05$) were performed in R (https://www.r-project.org).

RESULTS

Generation of transgenic alfalfa. *Agrobacterium*-mediated transformation of alfalfa was performed using the pILTAB::MtDef5 vector (Fig. 5). For constitutive expression, *MtDef5* was placed under the control of the CsVMV promoter, which was previously shown to drive high-level transgene expression in alfalfa (Samac et al. 2004). Integration of *MtDef5* into the genome was confirmed through PCR using *MtDef5* and *nptII* gene specific primers (Table 6). Overall, 21 different alfalfa lines were verified to contain *MtDef5*. Transgenic alfalfa plants with constitutive *MtDef5* expression formed root nodules and displayed no toxic effects or obvious phenotypic changes when compared with untransformed plants.

Expression analysis of *MtDef5* in transgenic alfalfa. RT-qPCR was used to confirm transcription of *MtDef5* in the transgenic alfalfa lines. Transgenic lines Def5-9, Def5-10, Def5-11, and Def5-12 displayed 102-, 83-, 76-, and 31-fold expression of *MtDef5* mRNA in leaves relative to the untransformed control (Fig. 6). Leaves of

transgenic alfalfa lines were analyzed by Western blot using an anti-MtDef5 antibody to detect the presence of MtDef5. Transformed plants show expression of MtDef5 protein, which was not detectable in the empty vector control line (Fig. 7). MtDef5A formed dimers with the major band forming at ~ 11 kDa. Oligomer formation was previously observed in a MtDef5 Western blot analysis (Islam et al. 2017).

Antifungal bioassays for transgenic alfalfa. As a first step to investigate the antifungal activity of the *MtDef5*-expressing transgenic alfalfa, a detached leaf assay was performed. Detached leaflets of transgenic alfalfa were inoculated with a spore solution of *P. medicaginis* or *C. trifolii* and incubated in moist chambers for either 7 or 10 days, respectively. An empty vector line of alfalfa lacking the transgene was also inoculated as a control. Disease severity was estimated by visually scoring disease symptoms using the scale developed by Garcia et al. (2014). While the degree of fungal resistance varied among transgenic lines, generally, the lines with higher levels of MtDef5 protein expression displayed enhanced resistance. Against both *C. trifolii* 2SP2 and *P. medicaginis*, Def5-12 showed a highly resistant phenotype having disease scores of leaflets significantly lower compared to leaflets from the empty vector line, which were completely damaged (Fig. 8 and 9). Lines Def5-5, Def5-11 and Def-12 exhibited the highest levels of resistance to *P. medicaginis*, which were significantly more resistant than the empty vector line (Fig. 8). The lines of transgenic alfalfa were assessed against two different strains of *C. trifolii*, and strain specificity was observed. Line Def5-12 displayed the highest level of resistance against *C. trifolii* 2SP2, a race 1 isolate, and lines Def5-46, Def5-9, and Def5-23 all displayed significant levels of resistance against *C. trifolii* WS-5, a newly described race 5 isolate that is highly aggressive (Fig. 9). This

observed strain specificity could be caused by pathogens of the same species having different membrane compositions, effectors, or transporters that function as intrinsic resistance mechanisms.

Whole transgenic alfalfa plants were spray inoculated with either *P. medicaginis* PSTC or *C. trifolii* SM spores at a concentration of $1x10^6$ spores/mL until runoff. *P. medicaginis* rating was performed in accordance with the standard tests utilized to characterize alfalfa cultivars. Generally, transgenic lines with higher levels of MtDef5 protein expression (Def5-9, Def5-10, and Def5-23) demonstrated significantly increased resistance against *P. medicaginis* compared to the empty vector line that lacks MtDef5 expression (Fig. 10). Significant levels of *C. trifolii* resistance were observed in lines Def5-5 and Def5-12 (Fig. 11). Plants with increased resistance were easily identified because their stems did not form the distinctive shepherd's crook symptom, which is where the upper portion of the stem wilts and dies, and the susceptible plants possessed numerous shepherd's crooks.

Antibacterial bioassay for transgenic alfalfa. Transgenic lines of alfalfa were inoculated with *P. syringae* pv. *syringae* using a stem injection. Resistant plants displayed a necrotic response at the injection site without the systemic water soaking and collapse at the injection site observed in the susceptible plants. Though lines Def5-2 and Def5-12 displayed the greatest level of stem resistance, they did not display statistically significant levels of resistance compared to the empty vector line (Fig. 12). Also, in all transgenic lines evaluated, levels of leaf resistance failed to differ from the empty vector line. Additionally, CFUs in the inoculated internode were not significantly reduced in any of the transgenic lines.

DISCUSSION

The plant defensin MtDef5 provides strong *in vitro* antimicrobial activity against both bacterial and fungal alfalfa crown rot pathogens (Sathoff et al. 2019). In this study, we show that expression of *MtDef5* in transgenic alfalfa increases broad-spectrum resistance to crown rot pathogens. Crown pathogens were tested individually against the transgenic lines of alfalfa because synergism among crown rot pathogens has been shown not to lead to enhanced disease (Miller-Garvin and Viands 1994; Turner and Van Alfen 1983). In particular, transgenic alfalfa lines Def5-12 and Def5-9 in some cases displayed enhanced resistance compared to the empty vector line lacking the transgene (Fig. 8 and 9). Greenhouse assays are conducted under highly conducive conditions for infection to occur, thus resistance under field conditions, where less conducive conditions occur, may be expected to be higher than that observed in greenhouse assays. Though a range of *MtDef5* expression levels were observed, which may be due to transgene copy number or position effects, we did discover an imperfect correlation between the level of *MtDef5* RNA transcription and disease resistance. Alfalfa lines showing higher degrees of resistance, such as Def5-9 and Def5-12, have increased levels of *MtDef5* mRNA transcription (Fig. 6). MtDef5 protein expression in the transgenic lines may be a better indicator of disease resistance. Line Def5-23 had high levels of protein expression observed on the Western blot analysis, increased *in planta* resistance to *C. trifolii*, but had low relative transcription levels of *MtDef5* (Fig. 6, 7, and 11). Overexpression of *MtDef5* likely reduces the lag phase for the activation of disease resistance mechanisms and protects alfalfa from sudden pathogen attacks (Kaur et al. 2011). We believe that this is

the first report of alfalfa being engineered to transgenically express a plant defensin and the first time that the MtDef5 defensin has been expressed in a transgenic plant.

Detached leaf and whole plant assays in transgenic alfalfa revealed enhanced resistance to *P. medicaginis* (Fig. 8 and 10). *P. medicaginis* may be one of the most destructive pathogens in the alfalfa crown rot disease complex because it was previously identified as the crown rot pathogen responsible for the greatest disease severity (Perez 1983). Additionally, limited genetic resistance to *P. medicaginis* is found in the USDA core alfalfa collection, and resistant varieties are not available commercially (Castell-Miller et al. 2007). When alfalfa harvests were delayed until the flowering stage, foliar alfalfa diseases, including *P. medicaginis*, caused moderate to severe (32 to 64%) leaf loss in both conventional and reduced lignin alfalfa varieties (Samac et al. 2018). This defoliation may be due to the strong induction of the tricarboxylic acid cycle pathways by *P. medicaginis* in the later stages of infection (Fan et al. 2018). In addition to reducing forage quality, this defoliation of infected leaves serves as a source of inoculum for crown rot infections. We have demonstrated that *MtDef5* expression can reduce foliar disease, which will likely reduce crown rot.

Previously completed *in vitro* tests of MtDef5, indicated that the full-length and γ-core defensin peptides were not active against *C. trifolii* (Sathoff et al. 2019). But in this study, both the detached leaf assays and whole-plant assays demonstrated that transgenic alfalfa expressing MtDef5 have significantly increased resistance to *C. trifolii* (Fig. 9 and 11). This same phenomenon was previously seen during the *in vitro* testing of So-D2, a defensin from spinach (*Spinacia oleracea*). When So-D2 was evaluated against the citrus canker pathogen, *Xanthomonas axonopodis* pv. *citri*, no antibacterial activity

was observed at concentrations up to 30 μM (Stover et al. 2013). But, transgenically expressed So-D2 displays potent activity against *X. axonopodis* pv. *citri* and '*Candidatus* Liberibacter spp.' in both greenhouse and field trials of the transgenic citrus (Mirkov and Gonzalez-Ramos 2014). Therefore, *in vitro* testing of plant defensins can provide some guidance on transgene selection, but the test results should be considered with caution because good candidates can be missed due to false negatives.

In addition to crown rot, the anthracnose disease of alfalfa caused by *C. trifolii* can lead to seedling mortality, foliar disease, and stem death. The management of anthracnose through the deployment of resistant cultivars has recently become more challenging due to arrival of new pathotypes of *C. trifolii* (Mackie et al. 2003). Along with the newly discovered race 5, three other physiological races have been described, and most modern alfalfa cultivars only have resistance to race 1 (O'Neill 1996; Samac et al. 2014). Race 5 causes latent infections and late symptom development compared to other races of anthracnose, which allows it to escape the standard anthracnose resistance screening assays (Rodgers et al. 2019). Several MtDef5 expressing transgenic lines displayed increased resistance against *C. trifolii* WS-5, a race 5 isolate, in detached leaf assays (Fig. 9). Glyphosate treatments in glyphosate-tolerant alfalfa cultivars were previously shown to control alfalfa rust (*Uromyces striatus*), another alfalfa foliar disease, but limited protection was observed against both *C. trifolii* and *P. medicaginis* (Samac and Foster-Hartnett 2012). Fungicides are available for controlling foliar disease in alfalfa, but they must be applied before symptoms occur and do not consistently have a positive economic return based on increased yield or quality (Samac et al. 2013).

Transgenic alfalfa expressing MtDef5 has the potential to provide a generic resistance against various pathogens and is likely to reduce the usage of chemical pesticides.

Under the greenhouse conditions used, we observed no morphological differences in foliar, stem, and root tissues between transgenic and non-transgenic alfalfa lines. But, it is possible that overexpression of *MtDef5* over a certain threshold confers yield loss, which was observed when potato constitutively expressed MsDef1 (Gao et al. 2000). Therefore, for commercial utilization of this technology, using a pathogen-induced or tissue-specific promoter to limit defensin expression would be beneficial. Recently, root-specific expression of a pepper defensin, J1-1, enhanced resistance in tobacco to *Phytophthora parasitic* var. *nicotianae* (Lee et al. 2018). Numerous *Medicago* promoters are well characterized and could potentially be used for pathogen-induced or tissue-specific expression of plant defensins.

The crown rot disease complex is a product of interactions among environmental and biological factors. Changing environmental conditions may distort the balance in a disease complex, which would lead to new disease outbreaks. Increased atmospheric CO_2 levels have recently been determined to drive increased susceptibility towards biotrophic foliar diseases, increased resistance against necrotrophic foliar disease, and similar susceptibility towards root pathogens in Arabidopsis (Zhou et al. 2019). Therefore, biotrophic and hemibiotrophic foliar pathogens such as *P. syringae* pv. *syringae* and *C. trifolii* may become relatively more important in the alfalfa crown rot disease complex. Also, climate change causes more extreme weather events and alters freeze-thaw cycles (Urakawa et al. 2014). Crown rot resistance is typically positively correlated with increased winter hardiness since alfalfa plants with rotten crowns and roots are less able

to harden and accumulate soluble sugars needed to survive the winter (Castonguay et al. 1995; Richard et al. 1982). Low-set, broad crowns of winter-hardy alfalfa cultivars consistently have the least crown rot and winter injury (Wilcoxson et al. 1977). Previous breeding programs directed at improving winter hardiness may have indirectly selected cultivars for increased crown rot resistance. Therefore, the *MtDef5* expressing transgenic alfalfa (Regen SY27x genotype) should be crossed with a winter hardy elite line of alfalfa before being evaluated for crown rot resistance in field trials.

Increasing broad-spectrum horizontal resistance through conventional breeding has previously been a strategy proposed to combat alfalfa crown rot (Richard et al. 1980). But, little progress has been made, and there are currently no commercial cultivars available with noted crown rot resistance. Expression in alfalfa of a single transgene, *MtDef5*, was shown to provide increased resistance to a group of crown rot pathogens, but field testing with transgenic alfalfa derived from seed is needed for further crown rot analysis since there has not been a greenhouse assay developed for crown rot screening. Potentially, plant defensin transgenes could be stacked in alfalfa, which could increase disease resistance above the level achieved with a single transgene. This enhanced resistance due to plant defensin stacking was previously observed when So-D2 and So-D7 were expressed in tandem in citrus (Mirkov and Gonzalez-Ramos 2014). A new strategy for plant disease resistance breeding may be screening plant defensins for *in vitro* activity against economically important pathogens and transgenically incorporating several defensins into the crop of interest.

TABLE 6 Primers used in MtDef5 cloning and quantitative RT-PCR assay.

Annotation	**Forward primer**	**Reverse primer**
MtDef5 cloning	GCATCGGAATTCTTAGCACTTCTTATAGC AAAAAC	GGTAGCTCTAGAATGACCAGTTCAG CATCTAAG
MtDef5 qPCR	ACATCAGAGGTGGAGGCAAA	CAGGGTTTTGGTTTTGCTTGT
f-actin qPCR	CCACATGCCATCCTTCGTTT	TGTCACGAACAATTTCCCG

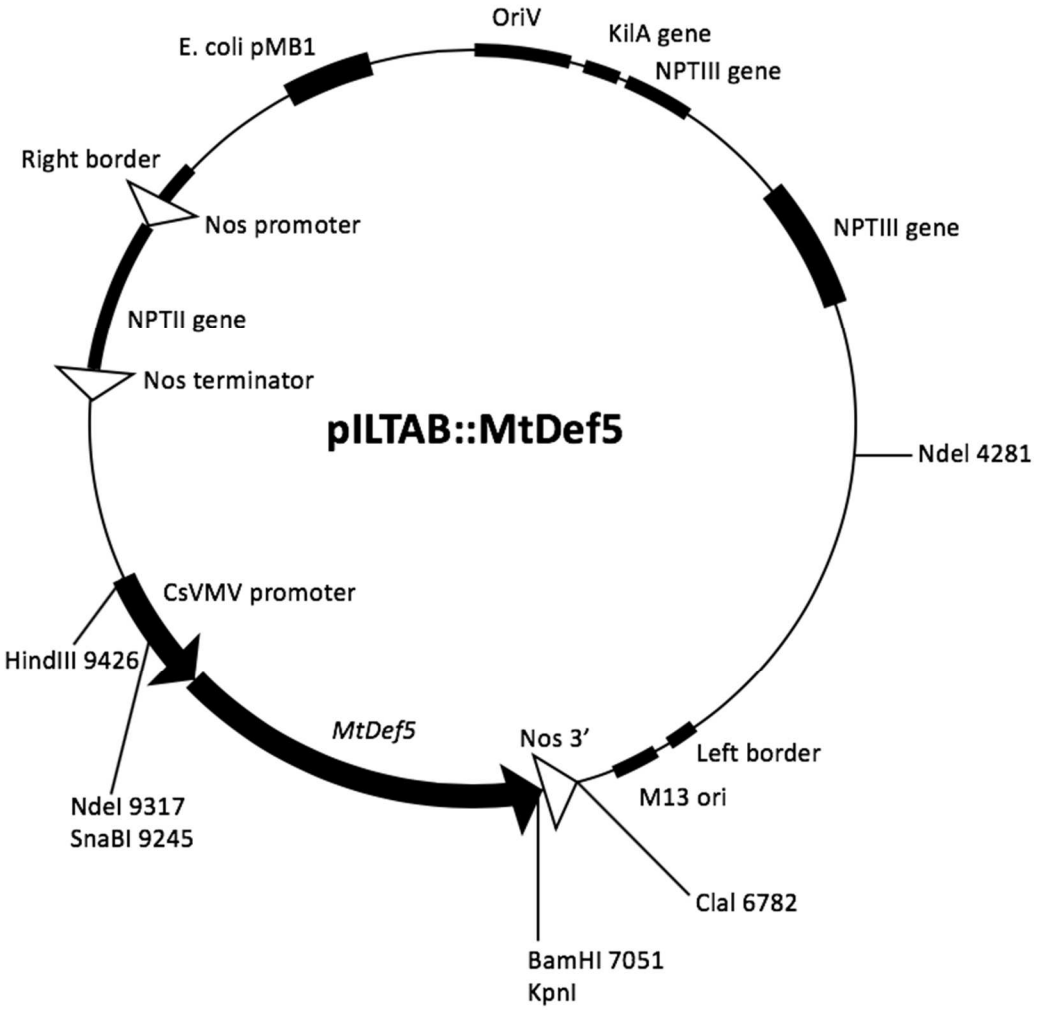


Fig. 5 The plant vector pILTAB::MtDef5 used for alfalfa transformation. The binary plant transformation vector pILTAB381 (Verdaguer et al. 1996) was modified to express MtDef5 under the control of the constitutive *Cassava vein mosaic virus* (CsVMV) promoter.

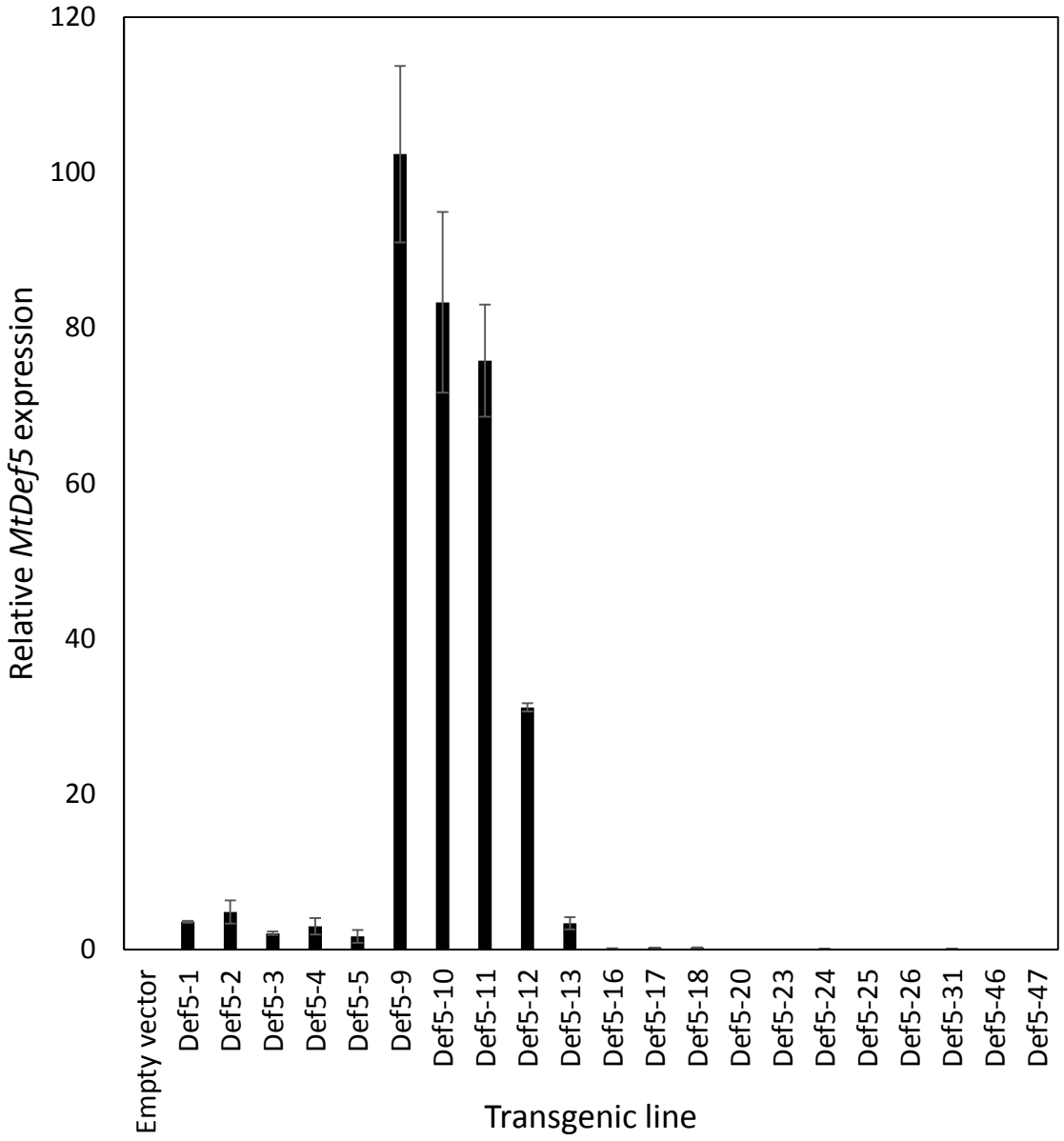


Fig. 6 Quantitative RT-PCR of *MtDef5* in the leaf tissue of transgenic alfalfa lines. The relative transcription levels of *MtDef5* were normalized to the transcription of alfalfa f-actin gene (JQ028730.1). Error bars represent the SE from three different biological replications.

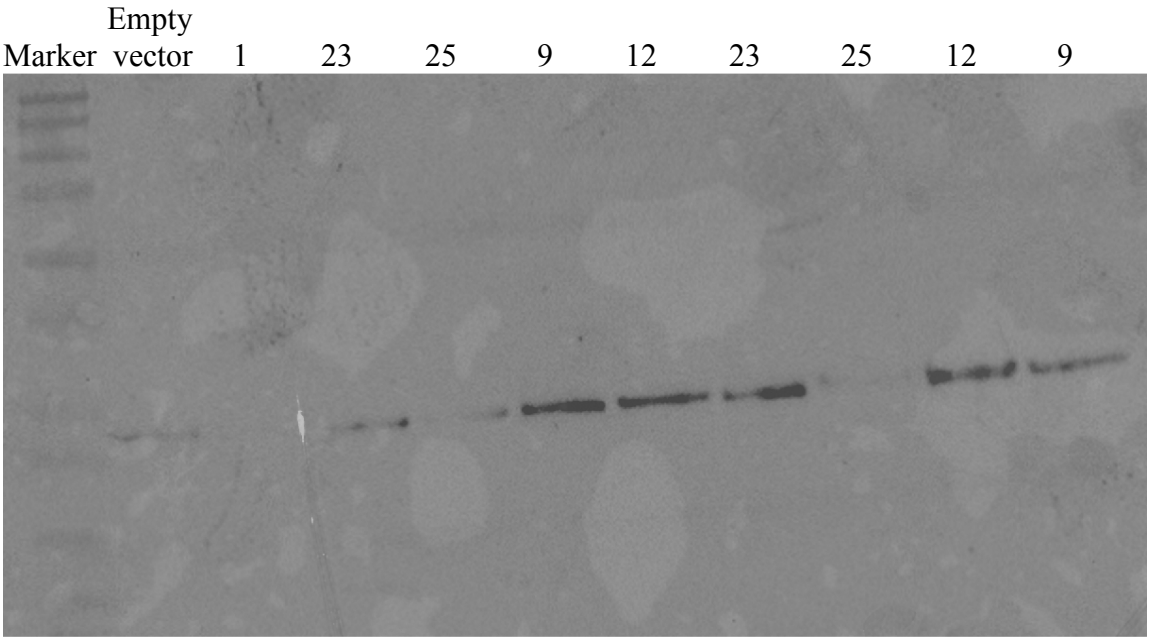


Fig. 7 Western blot analysis of protein extracts from transgenic alfalfa. Representative Western blot using proteins extracted from transgenic lines of alfalfa and the empty vector line. The affinity purified MtDef5-derived polyclonal antibody recognizes MtDef5 protein extracted from transgenic lines of alfalfa. Bands are present at ~11 kDa, which indicates MtDef5A dimer formation.

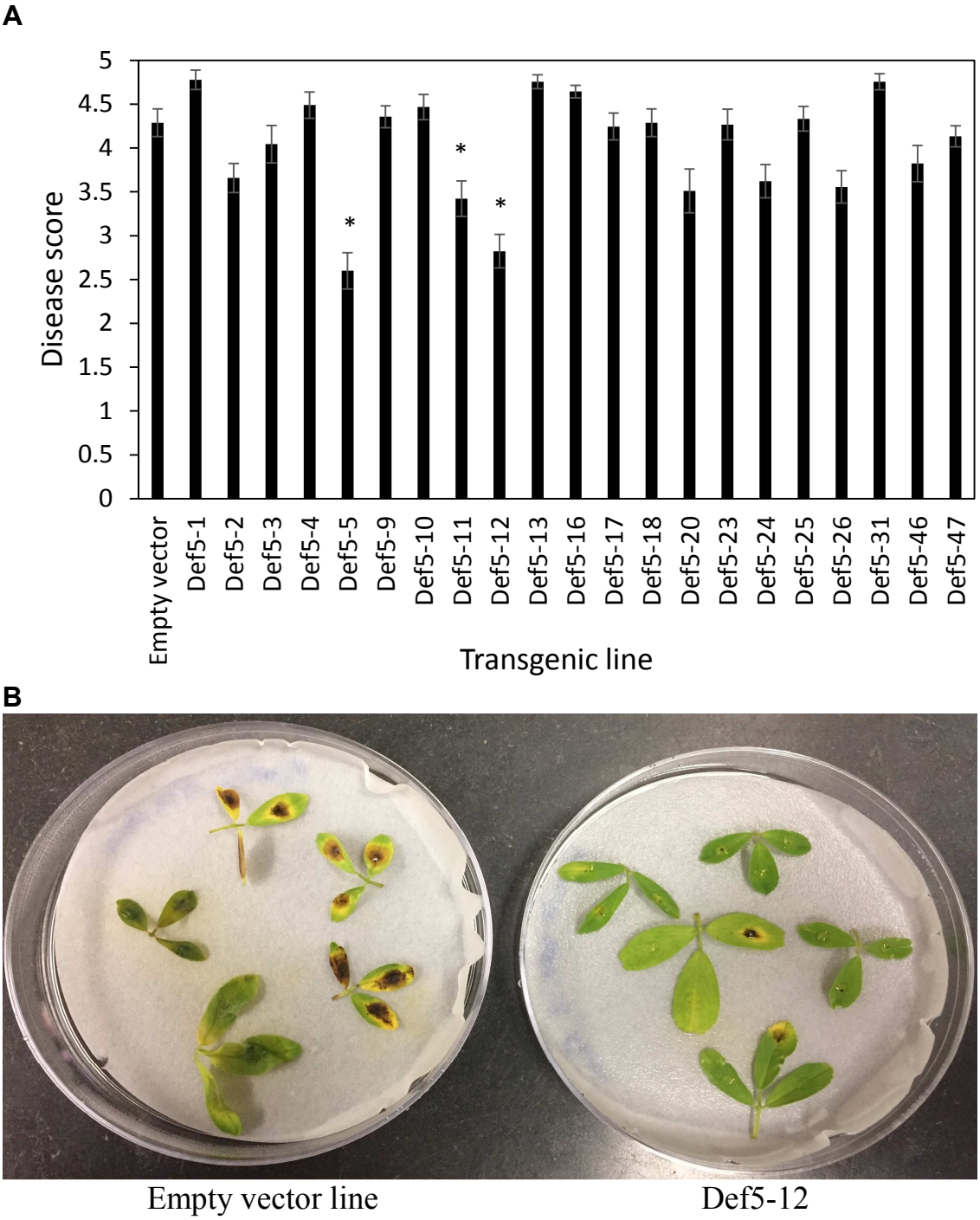
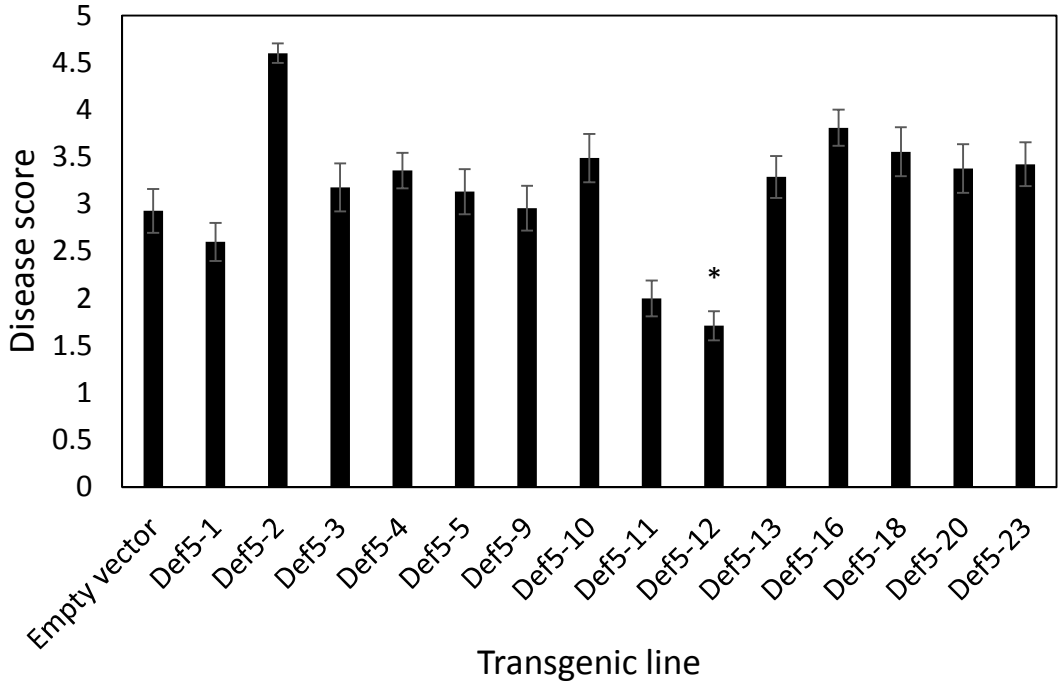


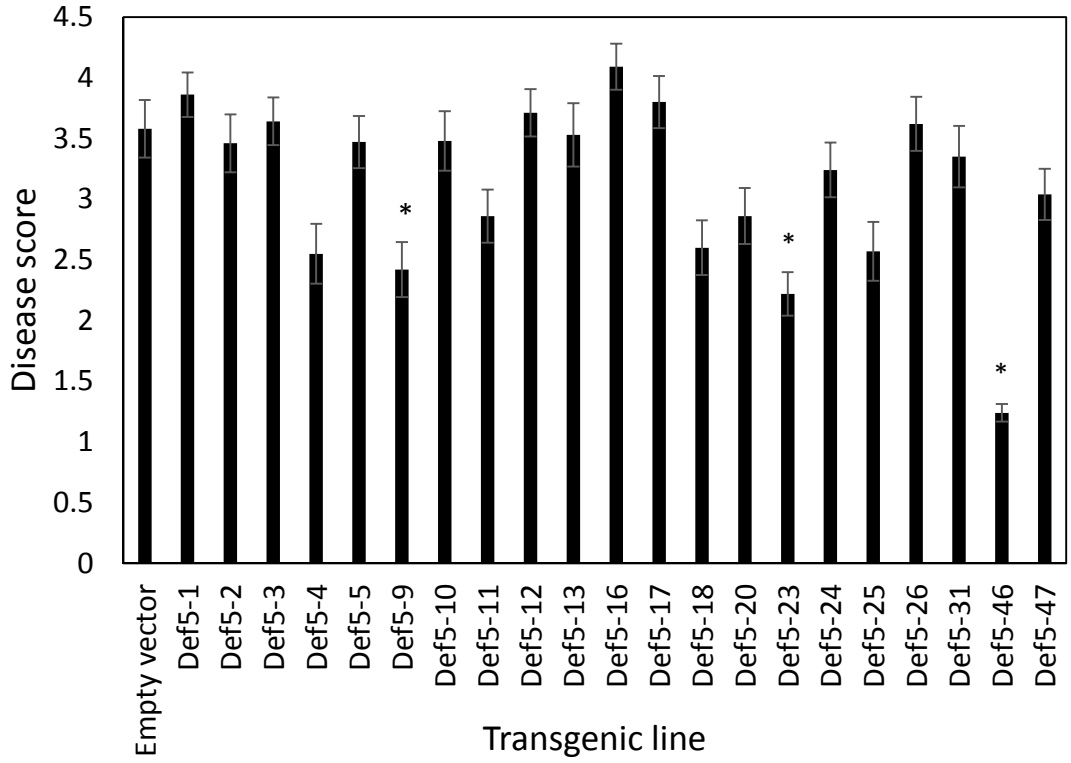
Fig. 8 *In vitro* antifungal activity of *MtDef5* expressing transgenic alfalfa against *P. medicaginis*. **(A)** Disease phenotype scores of transgenic alfalfa lines infected with *P. medicaginis*. Scored on a 1-5 scale where 1:Healthy leaflet, 2:Countable injuries,

3:Uncountable injuries, 4:Chlorosis, 5:Completely damaged per Garcia et al. (2014). * indicates significant difference in disease scores compared to the empty vector line (ANOVA Tukey HSD test; $P < 0.05$). **(B)** Representative *P. medicaginis* detached leaf assay with empty vector line lacking the transgene and transgenic alfalfa (line Def5-12) inoculated leaflets.

A


Disease score
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Empty vector
Def5-1
Def5-2
Def5-3
Def5-4
Def5-5
Def5-9
Def5-10
Def5-11
Def5-12
Def5-13
Def5-16
Def5-18
Def5-20
Def5-23
Transgenic line

B


Disease score
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
*
Empty vector
Def5-1
Def5-2
Def5-3
Def5-4
Def5-5
Def5-9
Def5-10
Def5-11
Def5-12
Def5-13
Def5-16
Def5-17
Def5-18
Def5-20
Def5-23
Def5-24
Def5-25
Def5-26
Def5-31
Def5-46
Def5-47
Transgenic line

C

Empty vector line Def5-12

Fig. 9 *In vitro* antifungal activity of *MtDef5* expressing transgenic alfalfa against two different strains of *C. trifolii*. Disease phenotype scores of transgenic alfalfa lines infected with *C. trifolii* **(A)** 2SP2 or **(B)** WS-5. Scored on a 1-5 scale where 1:Healthy leaflet, 2:Countable injuries, 3:Uncountable injuries, 4:Chlorosis, 5:Completely damaged per Garcia et al. (2014). * indicates significant difference in disease scores compared to the empty vector line (ANOVA Tukey HSD test; $P < 0.05$). **(C)** Representative *C. trifolii* 2SP2 detached leaf assay with empty vector line lacking the transgene and transgenic alfalfa (line Def5-12) inoculated leaflets.

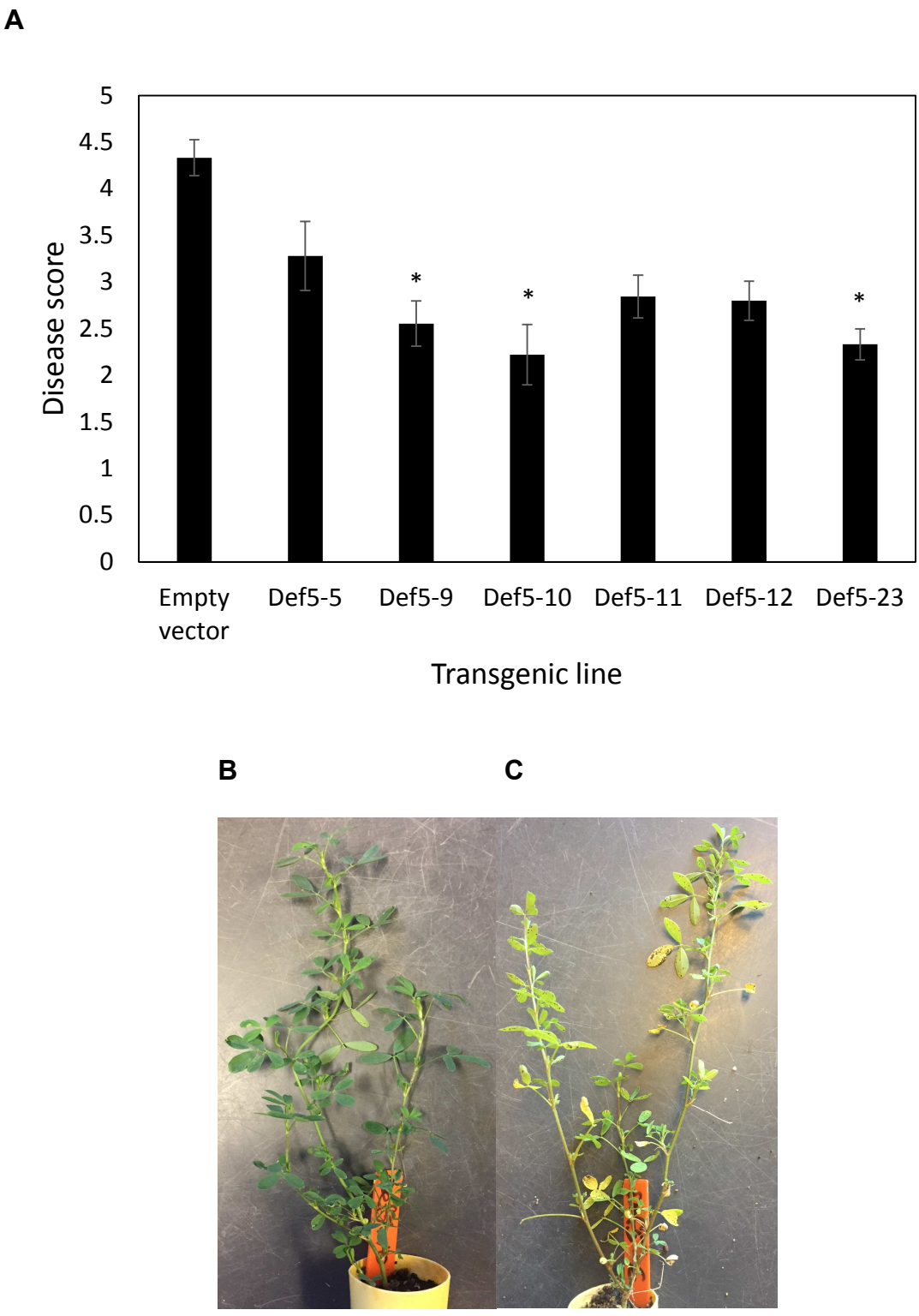


Fig. 10 *In planta* antifungal activity of *MtDef5* expressing transgenic alfalfa against *Phoma medicaginis*. **(A)** Disease phenotype scores of transgenic alfalfa lines infected

with *P. medicaginis*. Scored on a 1-5 scale where 1:Healthy, symptom-free top growth, 2:Small (<2 mm), brown or black lesions with no defoliation, 3:Larger (2 to 3 mm), discrete lesions with no chlorosis or defoliation, 4:Large (>3 mm) lesions with no defoliation, 5:Lesions >3 mm with fruiting and dead leaves or defoliation. * indicates significant difference in disease scores compared to the empty vector line (ANOVA Tukey HSD test; $P < 0.05$). **(B)** Line Def5-10 displays increased resistance to *P. medicaginis* compared to the empty vector line lacking the transgene **(C)**

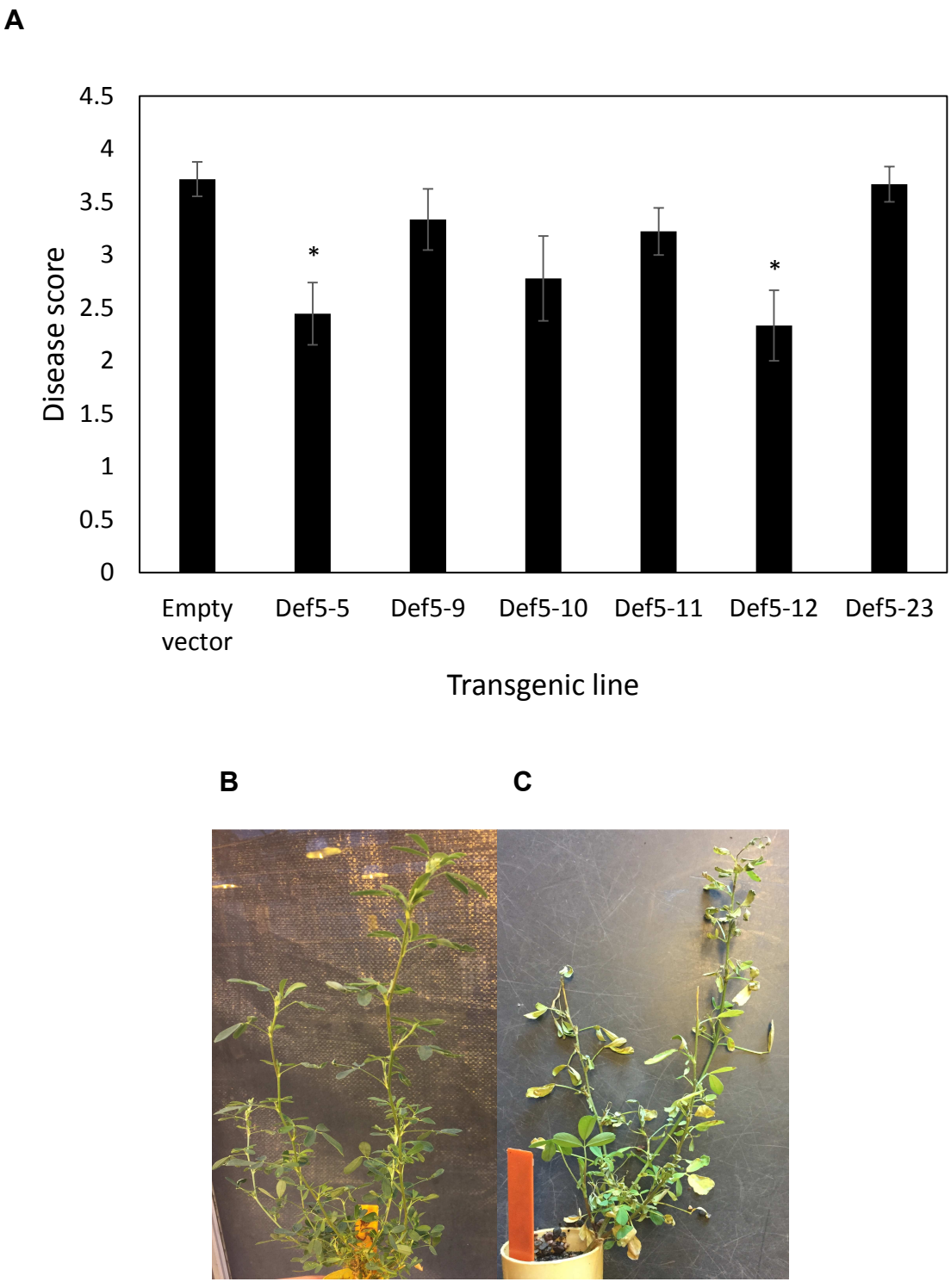
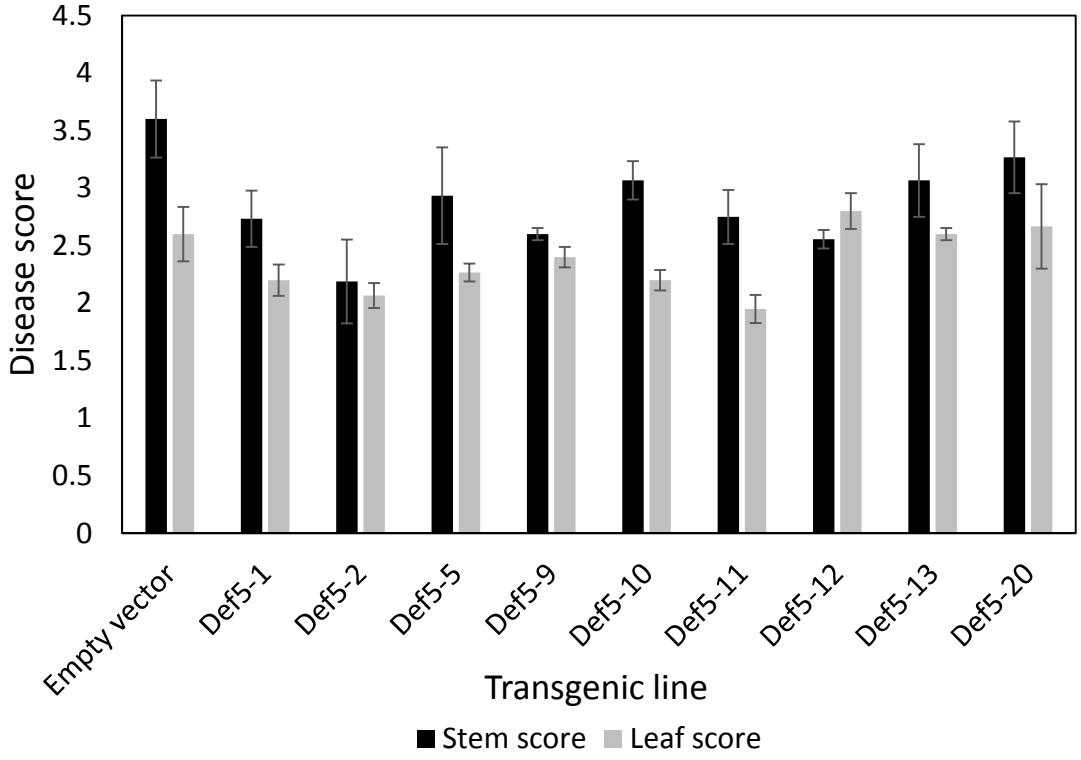


Fig. 11 *In planta* antifungal activity of *MtDef5* expressing transgenic alfalfa against *Colletotrichum trifolii*. **(A)** Disease phenotype scores of transgenic alfalfa lines infected

with *C. trifolii*. Scored on a 1-4 scale where 1:Healthy, symptom-free top growth, 2:Minor wilting with no formation of shepherd's crooks, 3:Single shepherd's crook with no foliar damage or chlorosis, 4:Multiple shepherd's crooks with chlorosis and defoliation. * indicates significant difference in disease scores compared to the empty vector line (ANOVA Tukey HSD test; $P < 0.05$) **(B)** Line Def5-5 displays increased resistance to *C. trifolii* compared to the empty vector line lacking the transgene **(C)**

A


4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Disease score
Empty vector
Def5-1
Def5-2
Def5-5
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Def5-10
Def5-11
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Def5-13
Def5-20
Transgenic line
Stem score
Leaf score

B

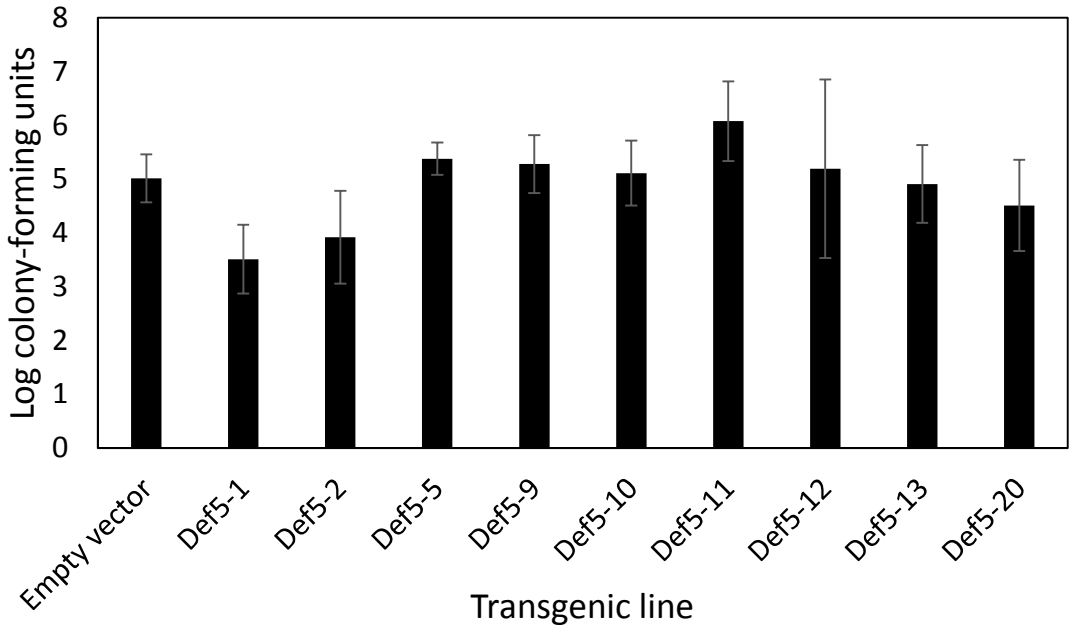

8
7
6
5
4
3
2
1
0
Log colony-forming units
Empty vector
Def5-1
Def5-2
Def5-5
Def5-9
Def5-10
Def5-11
Def5-12
Def5-13
Def5-20
Transgenic line

Fig. 12 *In planta* antibacterial activity of *MtDef5* expressing transgenic alfalfa against *P. syringae* pv. *syringae*. **(A)** Disease phenotype scores of transgenic alfalfa lines infected with *P. syringae* pv. *syringae*. Scored on a 1-5 scale. Stem symptoms were scored as; 1:Stem with no visible damage; 2:Hypersensitive response; 3:Lesion at site of inoculation; 4:Lesion extending from site of inoculation; 5:Stem collapse. Foliar symptoms were scored as 1:No symptoms; 2:Marginal leaf necrosis; 3:Yellowing of leaf at inoculated node; 4:Systemic yellowing and/or necrosis; 5:Dead plant. **(B)** Sections of the *P. syringae* pv. *syringae* inoculated internode were placed in 1 mL of sterile water, and serial dilutions were made and plated on King's B medium to determine colony-forming units per internode of each inoculated transgenic line. * indicates significant difference in disease scores compared to the empty vector line (ANOVA Tukey HSD test; $P < 0.05$).

Chapter 4: Plant defensin antibacterial mode of action against *Pseudomonas* species

INTRODUCTION

Plants produce a suite of antimicrobial peptides (AMPs) to defend against the extensive array of potential pathogens encountered in their environment. Plant AMPs are classified based on their structure and presence of disulfide bonds (Goyal and Mattoo 2014). With an abundance of representatives from diverse plant species, plant defensins are among the most widespread and best characterized plant AMPs (Tam et al. 2015). Plant defensins are cationic, cysteine-rich antimicrobial peptides that usually contain four disulfide bonds. They have a conserved three-dimensional structure, a cysteine-stabilized $\alpha\beta$ (CS$\alpha\beta$) motif, with a concentration of positively charged amino acid residues on the β2- β3 loop, which is classified as the γ-core motif ($GXCX_{3-9}C$). The γ-core motif alone has been shown to impart antimicrobial activity and mimic the activity of the corresponding full-length defensin (Sathoff et al. 2019). Plant defensins are promiscuous peptides, which means that a single peptide can have multiple distinct functions (Franco 2011). Along with having antimicrobial activity, plant defensins are controls for plant development, contribute to zinc tolerance, and act as inhibitors of digestive enzymes (van der Weerden and Anderson 2013). In crop plants, the transgenic expression of plant defensins has been used to engineer fungal and oomycete disease resistant plants. When MsDef1, a defensin from alfalfa (*Medicago sativa*), was expressed in potato, field-grown potatoes displayed resistance to *Verticillium dahliae* (Gao et al. 2000). NaD1, a defensin from sweet tobacco (*Nicotiana alata*), provided transgenic cotton with resistance to

Fusarium oxysporum f. sp. *vasinfectum* and *V. dahliae* throughout three years of field trials (Gaspar et al. 2014).

Though considered to be primarily antifungal, plant defensins have been shown to demonstrate antibacterial activity against both plant and vertebrate bacterial pathogens (reviewed by Sathoff and Samac 2019). Spinach defensin (So-D2) is the most frequently cited plant defensin with antibacterial activity, and transgenic sweet orange and grapefruit trees expressing So-D2 exhibited increased resistance to the bacterial diseases, citrus greening and citrus canker, caused by *Candidatus* Liberibacter spp. and *Xanthomonas axonopodis* pv. *citri,* respectively (Mirkov and Gonzalez-Ramos 2014). Plant defensins also display *in vitro* antibacterial activity against human pathogens. For instance, J1-1, a defensin from bell pepper (*Capsicum annum*) has a minimum inhibitory concentration (MIC) value of 250 μg/mL against *Pseudomonas aeruginosa* (Guillén-Chable et al. 2017). Also, PaDef, a defensin from avocado (*Persea americana* var. *drymifolia*), displays antibacterial activity against *Staphylococcus aureus* (Guzmán-Rodríguez et al. 2013). Therefore, plant defensins not only appear to be a resource for improving plant immunity to bacterial diseases but also for combatting human and animal bacterial pathogens.

A major obstacle blocking the widespread usage of plant defensins as antibacterial compounds is that their antibacterial mode of action (MOA) is poorly characterized (Sathoff and Samac 2019) although their MOA against fungal pathogens is well-described (Cools et al. 2017; Lacerda et al. 2014; Parisi et al. 2019). Recently, the antibacterial activity of a defensin from *Medicago truncatula*, MtDef5, was characterized (Velivelli et al. 2018). MtDef5 is a bi-domain defensin with two defensin domains

(MtDef5A and MtDef5B) connected by a 7-amino acid linker peptide. The cationic amino acid residues found in both γ-core motifs of MtDef5 were mutated and discovered to be essential for antibacterial activity, which were the same residues previously found to be essential for antifungal activity (Islam et al. 2017). Additionally, MtDef5 was shown to permeabilize the plasma membrane of *Xanthomonas campestris* pv. *campestris*, a gram-negative bacterial plant pathogen, but not the gram-positive plant pathogen *Clavibacter michiganensis* subsp. *nebraskensis* (Velivelli et al. 2018). The MtDef5 peptide binds to DNA indicating that it may kill bacterial cells by inhibiting DNA synthesis or transcription.

The MOA of human and invertebrate defensins against bacterial pathogens is well characterized (Ageitos et al. 2017; Guilhelmelli et al. 2013). Vertebrate defensins interact with the negatively charged lipopolysaccharide (LPS) in the bacterial outer membrane, which leads to rapid membrane permeabilization through pore formation (Morgera et al. 2008). For example, HNP-1, the most studied human α-defensin, has an antibacterial MOA typical of many AMPs. HNP-1 dimerization occurs, and the electrostatic interaction of dimers with the bacterial membrane causes β-sheet dimers to span the membrane forming a pore, with higher order oligomers of HNP-1 forming upon dimers when the defensin is in high concentration (Zhang et al. 2010). Another well-studied antibacterial human defensin, human β-defensin-3 (HBD3), has been shown to inhibit bacterial cell wall biosynthesis by interacting with lipid II components, which allows for HBD3 to have widespread activity against both gram-positive and gram-negative bacteria (Sass et al. 2008).

In response to the electrostatic interactions between cationic AMPs and negatively charged bacterial membranes, gram-positive and gram-negative bacteria have demonstrated the ability to modify their membrane surfaces (Anaya-Lopez et al. 2013). In *P. aeruginosa* and many other gram-negative bacteria, the PhoPQ/PmrAB systems control various genes required for resistance to AMPs (McPhee et al. 2003). The *pmr* operon (*PA3552-PA3559*) is controlled by both PhoPQ and PmrAB and is required for the addition of aminoarabinose to mask the phosphates of lipid A in *P. aeruginosa* (Mulcahy et al. 2008). Upstream of PmrAB, the spermidine synthesis genes *PA4773* (*speD*) and *PA4774* (*speE*) in *P. aeruginosa* are required for production of this polycation on the outer surface of the bacterial membrane (Johnson et al. 2012). These surface modifications protect bacteria from cationic AMPs through masking of the negative surface charges, which limits AMP binding to bacterial membranes (Johnson et al. 2012; Mulcahy et al. 2008). The mini-Tn*5*-*luxCDABE* mutant library in *P. aeruginosa* has been used extensively to identify antimicrobial peptide MOAs and bacterial resistance mechanisms (Lewenza et al. 2005).

Pseudomonas syringae pv. *syringae* is a bacterial plant pathogen that causes bacterial stem blight of alfalfa, which is an economically important disease with widespread distribution in the Western United States (Nemchinov et al. 2017). Currently, there are no effective means to control bacterial stem blight of alfalfa. *P. syringae* pv. *syringae* strain ALF3 has a draft genome sequence (Harrison et al. 2016) and was shown to be sensitive to *M. truncatula* defensins, MtDef5 and MtDef4, with IC_{50} values of 0.1 and 0.4 μM, respectively (Sathoff et al. 2019). Additionally, MtDef4 displays activity against *Xanthomonas alfalfae* subsp. *alfalfae* and the gram-positive bacterium

Clavibacter insidiosus, while MtDef5 displays no activity against these pathogens (Sathoff et al. 2019). There is insufficient knowledge to explain this observed specificity of plant defensin antibacterial activity. Generating tools to explore plant defensin MOA against bacterial plant pathogens is necessary for evaluating the risk of bacterial evolution towards defensin resistance and for the development of plant defensins into a spray-on peptide-based biological pesticide or transgenic expression of defensins for plant protection. Furthermore, knowing the antibacterial MOA of plant defensins allows for prediction of antibacterial activity without extensive *in vitro* testing.

In this study, we investigated plant defensin MOA against plant and vertebrate bacterial pathogens belonging to the genus *Pseudomonas*. Characterized *P. aeruginosa* *lux*-reporter strains with mutations in genes involved with cationic antimicrobial peptide resistance mechanisms were screened for sensitivity to γ-core motif plant defensin peptides. We discovered that plant defensin γ-core motif peptides exhibit potent activity against *P. aeruginosa* with the membrane modification mutants displaying increased sensitivity compared to the wild type. Exploiting the transcriptional *lux* reporter feature of these mutant strains (*PA3553::lux* and *PA4774::lux*), we found that MtDef4 induces the expression of both resistance determinants, indicating that MtDef4 likely acts on the *P. aeruginosa* outer membrane. Transposon insertion libraries of *P. syringae* pv. *syringae* were generated and screened for plant defensin resistance. Slow-growing resistant bacterial mutants were identified, transposon insertion sites were sequenced, and interrupted genes annotated as 16S and 23S ribosomal rRNA genes were found to be involved with plant defensin resistance. This suggests that MtDef4 may also function as a protein synthesis inhibitor.

MATERIALS AND METHODS

Bacterial strains and growth media. All bacterial strains utilized in this study are listed in Table 7. *Pseudomonas aeruginosa* strains were obtained from Dr. Lewenza at the University of Calgary. The *P. aeruginosa lux*-reporter strains have inactivated lipopolysaccharide (LPS) modification genes, which are bacterial genes involved in the resistance to cationic antimicrobial peptides. *PA4774::lux* has an interrupted outer membrane surface spermidine synthesis gene. *PA3553::lux* has an interrupted lipid A modification gene, which is responsible for the addition of aminoarabinose to lipid A. When the *lux*-reporter bacteria produce bioluminescence, they act as a real-time reporter for the induction of the inactivated gene (Lewenza et al. 2005). PAO1 was used as the wild type strain of *P. aeruginosa*. The *P. aeruginosa* strains were cultured on Luria-Bertani (LB) agar (Difco, Sparks, MD) at 37 °C. From a glycerol stock, the sequenced bacterial strain, *Pseudomonas syringae* pv. *syringae* ALF3, originally isolated from an infected alfalfa plant near Cheyenne, WY, was cultured on nutrient broth yeast extract (NBY) agar at 30 °C (Harrison et al. 2016). ALF3 was used as the wild type strain of *P. syringae* pv. *syringae*.

Plant defensin peptide synthesis. The γ-core motif peptides derived from plant defensins, MtDef4, MtDef5A, and So-D2 (Islam et al. 2017; Sagaram et al. 2011; Segura et al. 1998) (Table 8) were chemically synthesized and purified by HPLC (LifeTein, Somerset, NJ). Lyophilized defensin peptides were rehydrated in sterile water prior to each assay.

Determination of plant defensin antibacterial activity against *Pseudomonas aeruginosa*. To quantify defensin antibacterial activity, a spread-plate assay was used as

previously described (Sathoff et al. 2019). This assay was repeated three times for each strain of *P. aeruginosa*. Lawns of *P. aeruginosa* were grown on acidic LB (pH adjusted to 5.5 with HCl) plates for 15 h at 37 °C, conditions which induce antimicrobial peptide resistance mechanisms (Wilton et al. 2016). The plates were flooded with sterile water to harvest the bacteria. Cultures were diluted with sterile water to an OD_{600} of 0.1. In microcentrifuge tubes, 200 µL of bacteria were incubated at 37 °C with shaking for 3 h with various concentrations of a γ-core motif defensin peptide (0, 2.5, 5, 10, 20, or 30 µg/mL). After the defensin peptide treatment, 10-fold serial dilutions were made, and 100 µL were plated in triplicate onto LB plates. Colony forming units (CFUs) were counted for *P. aeruginosa* after incubation for 24 h at 37 °C. Regression of the average CFUs across experimental replications versus the defensin peptide concentration was used to create a dose response curve using Microsoft Excel 2016. From these dose response curves, the IC_{50} value, the amount of γ-core motif defensin peptide needed to inhibit the growth of bacterial strains by 50%, was calculated. The IC_{50} values are presented as mean ± standard error from the three repeated experiments.

***Lux*-reporter gene expression assay.** *Lux*-reporter gene expression assays, adapted from Mulcahy et al. (2008), were performed in a high-throughput manner using 96-well microplates. Bacterial cultures were grown overnight in acidic LB broth adjusted to a pH of 5.5. Overnight cultures were diluted by 1000 into LB broth, and 150 µL of culture medium with γ-core motif defensin peptide added at a sublethal concentration (0, 5, 15, or 30 µg/mL) was added to flat clear bottom 96-well microplates (Corning, Corning, NY) and overlaid with 50 µL of mineral oil to prevent evaporation. As a

positive control, the antibiotic, polymyxin B, which is known to cause high gene induction of the *lux*-reporter strains, was added at a sublethal concentration of 0.5 μg/mL. Samples were assayed in triplicate. Microplate cultures were incubated at 37 °C for 18 h in a Synergy H1 microplate reader (BioTek, Winooski, VT) with optical density (600 nm) and luminescence (counts per second [CPS]) readings taken every 20 min throughout the incubation period. Gene expression values were normalized to growth (CPS/OD_{600}).

***Pseudomonas syringae* pv. *syringae* transposon mutagenesis.** An EZ-Tn*5* <R6K*γori*/KAN-2> Tnp Transposome Kit (Lucigen, Middleton, WI) was used to generate mutants of *Pseudomonas syringae* pv. *syringae* strain ALF3 through random transposon insertions. The transposome was transformed into the ALF3 strain using the *P. syringae* pv. *syringae* electroporation protocol previously described by Scholz-Schroeder (2001). The transformed bacteria were plated onto NBY agar plates with 50 mg/L kanamycin and incubated at 25 °C for 48 h. Colonies were pooled by flooding the plates with sterile water. Bacterial cultures were diluted with sterile water to an OD_{600} of 0.1. In microcentrifuge tubes, the MtDef4 γ-core motif peptide at 80 μg/mL, approximately 10 times the IC_{50} concentration, was added to 200 μL of the transformed bacteria, and the cultures were incubated at 25 °C with shaking for 3 h. After the defensin treatment, 10-fold serial dilutions were made and 100 μL were plated in triplicate onto NBY plus kanamycin plates. Plates were grown at 25 °C overnight. Single colonies were selected, restreaked on NBY plus kanamycin plates, grown overnight at 25 °C, and the defensin treatment was repeated. From the recovered *P. syringae* pv. *syringae* mutants resistant to the MtDef4 γ-core motif peptide, genomic DNA was extracted and digested

with *EcoR*I (NEB, Ipswich, MA). The DNA was self-ligated using T4 DNA ligase (NEB). Electrocompetent TransforMax EC100D *pir*-116 *E. coli* (Lucigen) were electroporated with 2 μL of the ligation mix. The transformed *E. coli* were plated on LB agar plus 50 mg/L kanamycin and grown overnight at 37 °C. Plasmid DNA was extracted using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The plasmid DNA was Sanger sequenced on both sides of the transposon insertion at the University of Minnesota Genomics Center using the supplied primers from the Tnp Transposome kit, KAN-2 FP-1 (5'-ACCTACAACAAAGCTCTCATCAACC -3') and R6KAN-2 RP-1 (5'-CTACCCTGTGGAACACCTACATCT-3'). The resulting DNA sequences near the transposon insertion were validated using Sequencer (Gene Codes Corporation, Ann Arbor, MI). Nucleotide BLAST searches using the Pseudomonas Genome Database (Winsor et al. 2016) were performed on the DNA sequences near the transposon insertion site to identify the locations in the ALF3 genome of the insertions and the corresponding interrupted genes with annotations.

RESULTS

Plant defensin derived inhibition of *P. aeruginosa* growth. The antibacterial activity of γ-core motif peptides from MtDef4, MtDef5A, and So-D2 were evaluated against wild-type and antimicrobial peptide sensitive mutants of *P. aeruginosa*. The *P. aeruginosa lux*-reporter strains had inactivated LPS modification genes, either an interrupted outer membrane surface spermidine synthesis gene (*PA4774*) or an interrupted lipid A aminoarabinose modification gene (*PA3553*). These mutants are incapable of producing outer membrane surface modifications used for protection against cationic antimicrobial peptide treatments (Lewenza 2013; Lewenza et al. 2005; Wilton et

al. 2016). Using a spread-plate assay, the γ-core motif peptides exhibited antibacterial activity at micromolar concentrations. Against *P. aeruginosa* PAO1, the γ-core peptides inhibited bacterial growth with MtDef4 displaying the greatest activity corresponding to an IC_{50} value of 4.2 μM (Table 9). The *lux*-reporter *P. aeruginosa* strains had the expected increase in sensitivity towards both MtDef4 and So-D2 peptides compared to the wild type strain (Table 9). Overall, MtDef5 displayed the least antibacterial activity of the evaluated γ-core motif defensin peptides with the highest recorded IC_{50} value of 14.6 μM against *PA4774::lux*. In contrast, MtDef4 was the most potent against *PA4774::lux* with an IC_{50} value of 1.7 μM.

***Lux* gene expression assay.** Expression of the *lux* gene in the modified *P. aeruginosa* reporter strains has been previously shown to be induced by a Mg^{2+} limiting environment, acidic pH, the presence of extracellular DNA, or the presence of antimicrobial peptides at a sublethal concentration (Lewenza 2013; McPhee et al. 2003; Wilton et al. 2016). Expression of *lux* in the reporter strains was confirmed to be induced by an acidic pH of 5.5 in a LB broth medium (data not shown). The *lux*-reporter strains of *P. aeruginosa* were grown overnight in acidic LB broth, diluted with LB broth, treated with plant defensin γ-core motif peptides, and monitored for bioluminescence in a microplate reader, where bioluminescence would indicate the induction of the inactivated bacterial membrane modification genes. Therefore, if the γ-core peptides cause bacterial membrane stress, the *lux*-reporter will be induced and bioluminescence will be recorded. In response to MtDef4 treatment at sublethal concentrations, *lux* expression was greatly induced in a concentration dependent manner in *PA4774::lux* (Fig. 13). The level of induction from a treatment of 30 μg/mL of MtDef4 was greater than the induction caused

by the antibiotic positive control, polymyxin B. Additionally, *PA3553::lux* expression was induced by MtDef4 at levels near those achieved by polymyxin B. MtDef5 and So-D2 failed to induce *lux* expression at levels near or greater than the antibiotic control in all mutant strains evaluated (Fig. 14). But, during the first 3 hours after defensin treatment, the level of induced *lux* expression caused by all plant defensin treatments is greater than the antibiotic control, which indicates different kinetics and possibly MOAs between plant defensins and polymyxin B (Fig. 13 and 14).

***P. syringae* pv. *syringae* transposon mutagenesis.** The prior application of the mini-Tn*5*-*luxCDABE* mutant library in *P. aeruginosa* lead us to develop transposon-insertion mutant screen of a bacterial plant pathogen, *P. syringae* pv. *syringae* strain ALF3. The γ-core motif peptide of MtDef4 was previously shown to have an IC_{50} value of 3.4 μM against *P. syringae* pv. *syringae* (Sathoff et al. 2019), so the transposon-insertion mutants were screened for resistance at 40 μM MtDef4 (10 x IC_{50}). Three slow-growing, MtDef4 resistant *P. syringae* pv. *syringae* mutants were recovered following two repetitions of the γ-core defensin peptide treatment. Genomic DNA was extracted, digested with *Eco*RI, and self-ligated with T4 DNA ligase to generate plasmids that were transformed into *E. coli*. Plasmid DNA surrounding the transposon insertion sites was sequenced for two mutants. Although sequencing was attempted from several clones of the third mutant, no sequence was obtained suggesting that the mutation was not due to a transposon insertion. The resulting sequence data from the two MtDef4 insensitive mutants (*ALF3::Tn5-1* and *ALF3::Tn5-2*) were characterized using BLAST analyses. The transposon insertion sites for both *ALF3::Tn5-1* and *ALF3::Tn5-2* were found to be located on scaffold 32544_1.1 of the ALF3 genome assembly and are 3,824 base pairs

apart. The mutated gene (RS24240) in *ALF3::Tn5-1* is annotated as a 16S ribosomal RNA gene, and the mutated gene (RS24220) in *ALF3::Tn5-2* is annotated as a 23S ribosomal RNA gene (Table 10).

DISCUSSION

Plant defensins are able to kill a broad spectrum of gram-positive and gram-negative bacteria, and therefore, they are valuable candidates for generating a new class of antibiotics to treat multidrug-resistant bacteria. Full-length defensin peptides have IC_{50} values approximately ten-fold lower than those from the corresponding γ-core motif peptides (Sathoff et al. 2019), which indicates that full-length defensins may have a more nuanced MOA where another undiscovered motif may be acting in synergy with the γ-core. A major drawback of peptide-based antibiotics is that they are much more expensive to produce than conventional antibiotics, so to reduce cost, the size of the peptide should be minimized (Hilpert et al. 2005). Truncated plant defensins (γ-core motif peptides) were assessed to evaluate a more realistic peptide-based antibacterial treatment. The plant defensin γ-core motif peptides demonstrated potent activity against *P. aeruginosa* (Table 9).

Gram-negative bacteria contain an outer membrane composed of LPS in the outer leaflet. Divalent inorganic cations (Mg^{2+} and Ca^{2+}) stabilize the outer leaflet by binding neighboring LPS molecules, and the displacement of these cations results in membrane destabilization and bacterial cell death (Hancock 1984). Polycation spermidine production and aminoarabinose-modification of lipid A contribute to reduce outer membrane permeability and therefore, the entrance of cationic AMPs (Johnson et al. 2012; Moskowitz et al. 2004). Random mini-Tn*5* transposon mutagenesis has been

performed on *P. aeruginosa* PAO1, and the sites flanking the insertion have been sequenced and mapped, which has allowed for the characterization of outer membrane modification mutants (Lewenza et al. 2005). These *P. aeruginosa* membrane modification mutants have increased sensitivity to MtDef4 and So-D2 γ-core motif peptides with IC_{50} values reduced by 2-4 fold compared to PAO1 (Table 9). This suggests that these plant defensins may have a MOA that involves pore creation in which the displacement of divalent metal cations causes destabilization of the LPS and disruption of membrane integrity. When evaluated against the MtDef5A γ-core motif peptide, *PA3553::lux* shows a limited increase in susceptibility and *PA4774::lux* has increased resistance. This lack of greatly enhanced susceptibility implies that MtDef5 does not directly act on the bacterial outer membrane and may have an intracellular target considering that MtDef5 does not induce gene expression of the reporters. MtDef5 was previously shown to be internalized in *X. campestris* pv. *campestris* (Velivelli et al. 2018). Also, MtDef5 demonstrates no activity towards gram-positive pathogens, *C. insidiosus* and *C. michiganensis* subsp. *nebraskensis*, while MtDef4 had high antibacterial activity against *C. insidiosus* (Sathoff et al. 2019; Velivelli et al. 2018). This could be due to the inability of MtDef5 to enter the bacterial cell through the thick outer layer of peptidoglycan present in the cell wall of gram-positive cells and interact with its intracellular target. These results suggest differing MOAs between MtDef5 and the other plant defensins evaluated.

The *P. aeruginosa* mini-Tn*5-luxCDABE* mutants contain a promoterless luciferase gene cassette, which serves as a sensitive, real-time reporter of gene expression for the inactivated gene (Lewenza et al. 2005). Highly induced expression of the *lux* gene

following plant defensin treatments at sublethal concentrations signals that the defensin peptide acts on the bacterial membrane, similar to other known antimicrobial peptides (McPhee et al. 2003). MtDef4 γ-core motif peptide treatments cause a strong concentration-dependent induction of *lux* in the *P. aeruginosa* mutant, *PA4774::lux* (Fig. 13). The *PA4774::lux* mutant is deficient in production of outer membrane spermidine, a polyamine, which serves as a substitute for inorganic cations that bind to and stabilize LPS in the outer membrane (Hancock and Wong 1984). Antimicrobial peptides compete with cations for binding to LPS, but spermidine protects the outer membrane by ensuring that the negative surface charges are masked (Johnson et al. 2012). High concentrations of exogenous spermidine (20mM) have been demonstrated to increase the resistance of *P. aeruginosa* to cationic peptides (Kwon and Lu 2006). Therefore, bacteria with high production levels of spermidine or other polyamines may be more resistant to plant defensin treatments.

The emergence of multidrug-resistant (MDR) gram-negative bacterial isolates has led to the renewed usage of both polymyxin B and colistin (polymyxin E) as therapeutic agents (Nation and Li 2009). Polymyxins have a polycationic ring that binds to the LPS on the outer bacterial membrane and competitively displaces Ca^{2+} and Mg^{2+} leading to membrane destabilization and increased permeability (Zavascki et al. 2007). With the increased prevalence of polymyxin treatments, polymyxin-resistant *P. aeruginosa* isolates have been reported worldwide (Lee et al. 2011; Wang et al. 2006). Throughout our study, a polymyxin B treatment was used as positive control against *P. aeruginosa*. In all *lux*-reporter assays, the plant defensin treatments displayed rapid levels of *lux* induction, and *lux*-expression was induced faster with plant defensin treatments

compared to polymyxin B treatments (Fig. 13). These different induction dynamics in the *lux*-reporter assays suggest that plant defensins and polymyxin B have different MOAs on the outer membrane. Therapeutic compounds with novel MOAs are needed to treat MDR bacterial isolates, and plant defensins may be an untapped reservoir.

The transposon insertion mutants of *P. syringae* pv. *syringae*, *ALF3::Tn5-1* and *ALF3::Tn5-2*, had increased resistance to MtDef4 γ-core motif peptide treatments, which may be due to changes in ribosome structure (Table 10). The ribosome is a common target for antibacterial compounds because binding to bacterial ribosomes causes disruption of translation (Tenson and Mankin 2006). For example, aminoglycoside antibiotics target 16S rRNA in the small ribosomal subunit and tylosin targets 23S rRNA (Hansen et al. 2002; Ogle et al. 2001). Target site mutations are a frequently utilized bacterial resistance mechanism. To gain resistance to several antibiotics, *Mycobacterium tuberculosis* acquired mutations in multiple regions of the *rrs* gene, which encodes 16S rRNA (Nguyen et al. 2018). But, the multiplicity of rRNA genes in most bacterial species slows the development of this type of resistance (Cundliffe 1990). Also, the antifungal MOAs of MtDef4 against *Fusarium graminearum* and *Neurospora crassa* requires γ-core motif mediated entry into fungal cells, which implies that MtDef4 has an intracellular target (Sagaram et al. 2013).

Our results suggest that the antibacterial MOA of the MtDef4 γ-core motif peptide involves ribosomal targeting, and the transposon insertions in *P. syringae* pv. *syringae* rRNA encoding genes were target site mutations leading to increased MtDef4 resistance. Furthermore, spermidine interacts closely with RNA because in *E. coli* cells spermidine exists predominantly as a polyamine-RNA complex (Igarashi and Kashiwagi

2010). Polyamines play crucial roles as modulators of RNA structure and can induce changes in RNA structure in context-dependent manner (Lightfoot and Hall 2014). Polyamine binding to 23S rRNA on the central loop region of domain V, a site where several antibiotics are known to bind, caused structural alterations, which is suggested to restrict spiramycin binding to the ribosome (Petropoulos et al. 2004). In addition to having decreased outer membrane spermidine content, *PA4774::lux* may also have a reduction of intracellular spermidine. Both spermidine and MtDef4 may normally interact with 23 and 16S rRNA, but in *PA4774::lux*, this intracellular spermidine-based protection does not occur, which leads to increased susceptibility to MtDef4. The *P. syringae* pv. *syringae* transposon insertion mutants may also disrupt the interaction between MtDef4 and rRNA, which would explain the observed resistance to MtDef4. Additionally, the antibacterial MOA of MtDef4 against different *Pseudomonas* species may not be conserved or multiple MOAs could be utilized. The AMP melittin, the main component of European honeybee (*Apis mellifera*) venom, killed bacterial cells of the plant pathogen *Xanthomonas oryzae* pv. *oryzae* using multiple MOAs including membrane permeabilization, inhibition of protein synthesis, and DNA/RNA binding (Shi et al. 2016). Also, the antifungal MOA of MtDef4 is not conserved between ascomycete fungi, *N. crassa* and *F. graminearum* (El-Mounadi et al. 2016).

In this report, we gain insights into the antibacterial MOA of plant defensins against two pseudomonads, *P. aeruginosa* and *P. syringae* pv. *syringae*. In *P. aeruginosa*, we propose that MtDef4 and So-D2 interact with the bacterial outer membrane and possibly create pores leading to bacterial cell death. MtDef5 appears to have a different antibacterial MOA where outer membrane binding is not as vital and,

therefore, may have an intracellular target. This hypothesis is consistent with the reported antibacterial MOA of MtDef5 against *X. campestris* pv. *campestris* in which DNA binding by MtDef5 likely interferes with DNA synthesis and transcription (Vellivelli et al. 2018). Additionally, plant defensins seem to have a different MOA than polymyxin B. The *P. syringae* pv. *syringae* mutational analysis suggests that MtDef4 may also target the ribosome and interfere with bacterial translation. Resistance mechanisms that bacteria use to combat MtDef4 and other plant defensins may include increased cell membrane thickness through outer membrane spermidine synthesis or target site mutations. Plant defensin γ-core motif peptides can be utilized for the development of treatments against both plant and human bacterial pathogens and for furthering knowledge of mechanisms of antimicrobial resistance.

TABLE 7 Bacterial strains used in this study

Strain or Mutant	**Description**	**Reference**
PAO1	Wild-type *Pseudomonas aeruginosa*	Stover et al. 2000
PA3553::lux	Transposon mutants and transcriptional fusion, homolog to *pmr* gene (*pmrF*) responsible for the addition of aminoarabinose to lipid A	Lewenza et al. 2005
PA4774::lux	Transposon mutant and transcriptional fusion, homolog to *speE* gene responsible for spermidine synthesis	Lewenza et al. 2005
ALF3	Wild-type *Pseudomonas syringae* pv. *syringae*	Harrison et al. 2016
ALF3::Tn5-1	ALF3 with random transposon insertion conferring MtDef4 insensitivity, Mu_4-1	This paper
ALF3::Tn5-2	ALF3 with random transposon insertion conferring MtDef4 insensitivity, Mu_5-1	This paper

TABLE 8 Amino acid sequences of γ-core motif (bold) and C-terminal region (italics) of plant defensins tested *in vitro*

Plant Species	**Defensin**	**Amino Acid Sequence**
Medicago truncatula	MtDef4	**GRCRGFRRRC***FCTTHC*
M. truncatula	MtDef5A	**GACHRQGFGFAC***FCY KKC*
Spinacia oleracea	So-D2	**GDCKGIRRRC***MCSKPL*

TABLE 9 Activity of the γ-core motif defensin peptides against *Pseudomonas aeruginosa* strains[z]

***Pseudomonas aeruginosa* strains**	**MtDef4 core**	**MtDef5A core**	**So-D2 core**
PAO1	4.2 ± 0.4	11.8 ± 1.4	11.6 ± 0.6
PA3553:lux	2.7 ± 0.3	8.5 ± 0.8	3.0 ± 0.3
PA4774:lux	1.7 ± 0.2	14.6 ± 1.0	5.2 ± 0.5

[z]The mean IC_{50} (μM) values are reported ± SE of three independent experiments (n=3).

TABLE 10 BLASTn results from the Pseudomonas Genome Database identifying the transposon insertion site in the *Pseudomonas syringae* pv. *syringae* ALF3 Tn*5* mutant strains.

***Pseudomonas syringae* pv. *syringae* ALF3 mutant strain**	**Transposon insertion site**	**Interrupted Gene**
ALF3::Tn5-1	1346 in scaffold 32544_1.1	16S ribosomal RNA gene (RS24240)
ALF3::Tn5-2	5170 in scaffold 32544_1.1	23S ribosomal RNA gene (RS24220)

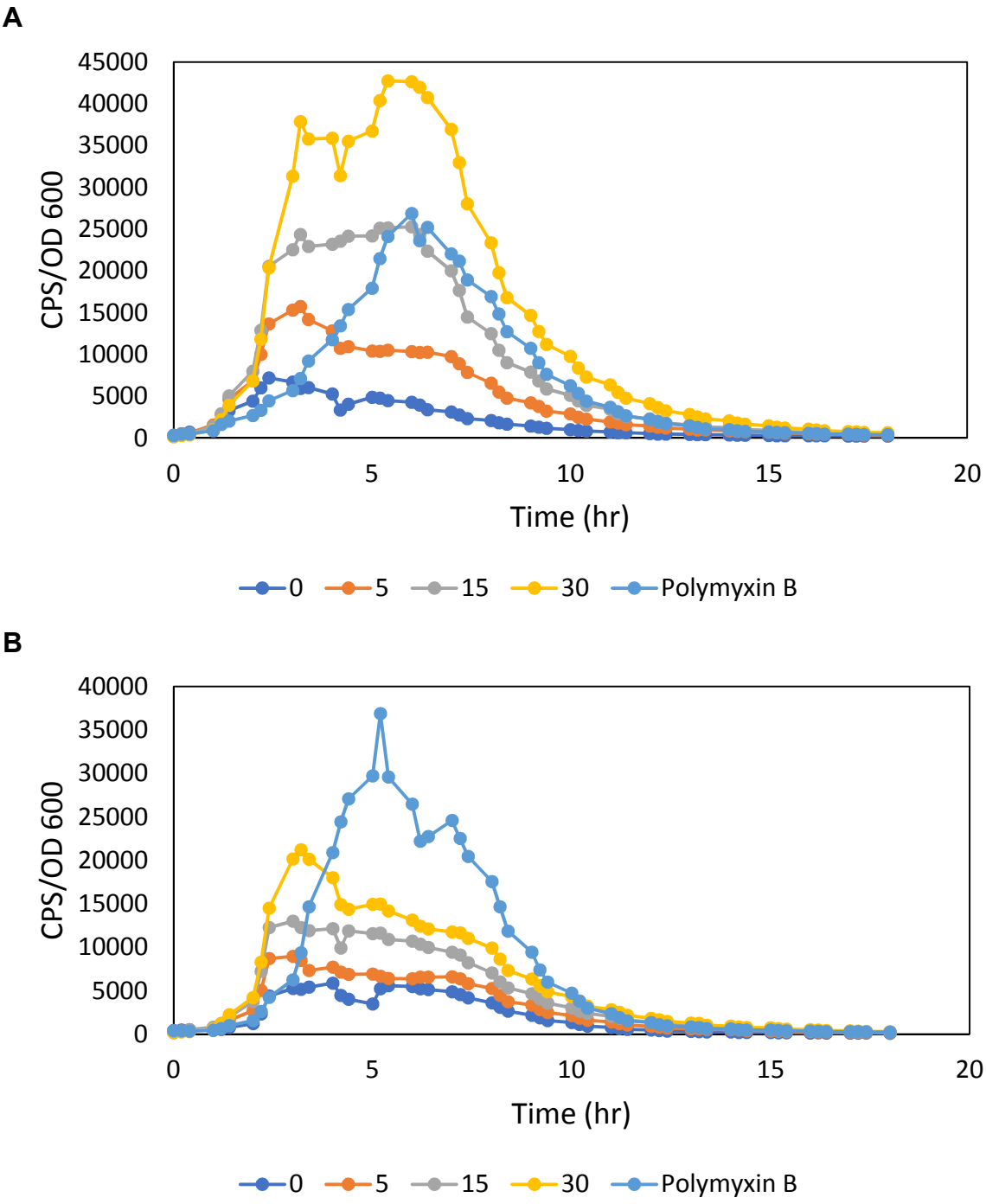


Fig. 13 MtDef4 γ-core motif peptide induces *PA4774* and early *PA3553* gene expression. Effects of MtDef4 γ-core peptide at sub-MIC concentrations of 0, 5, 15, or 30 µg/mL or polymyxin B at 0.5 µg/mL on the expression of the *PA4774::lux* **(A)** and *PA3553::lux* **(B)** transcriptional fusion in planktonic cultures in LB broth. Gene expression was

normalized for growth and CPS/OD600 values for the average of the triplicates are presented. Each growth experiment was performed three times and representative curves are shown.

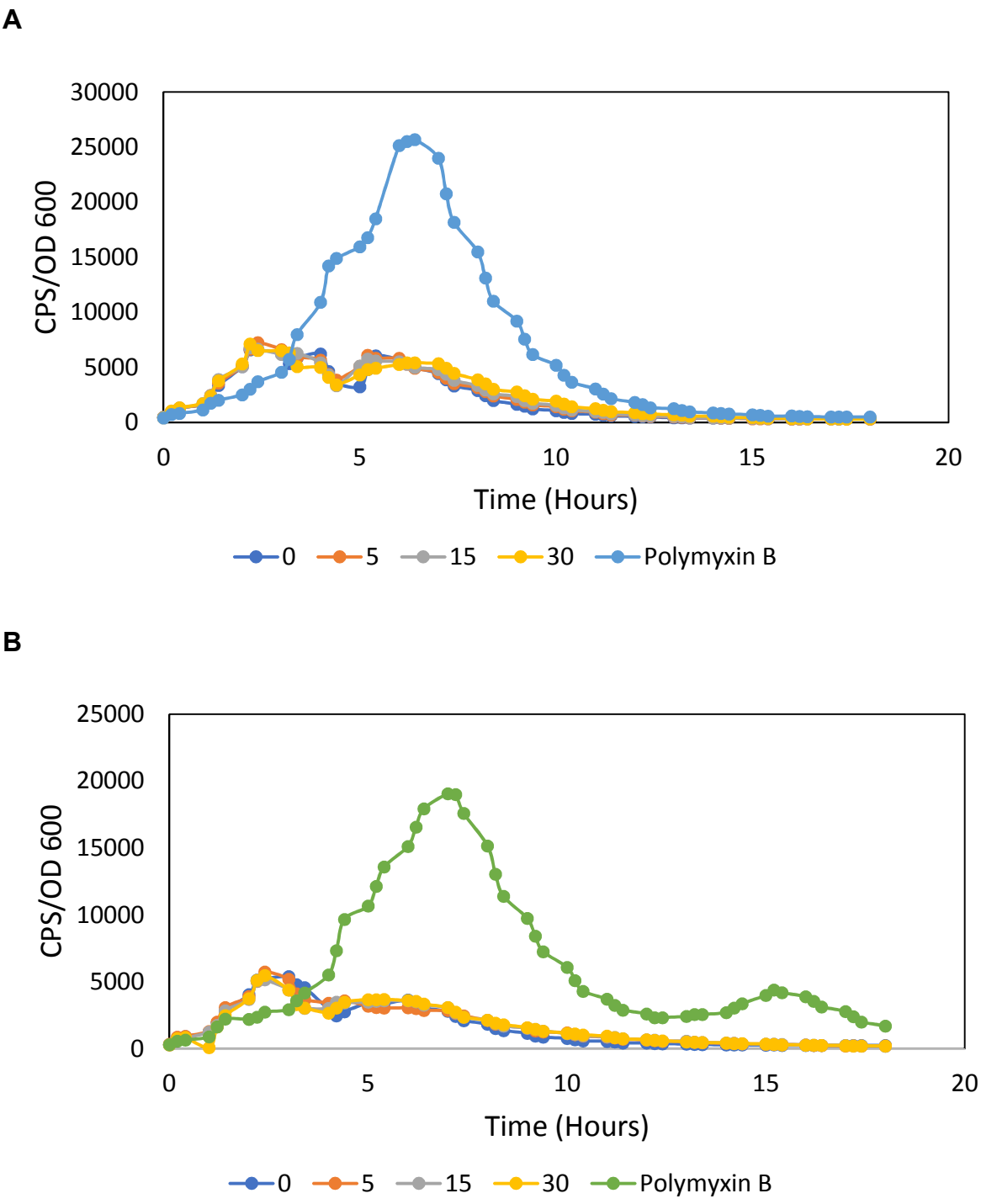


Fig. 14 So-D2 and MtDef5 γ-core motif peptides fail to induce *PA4774* gene expression. Effects of So-D2 γ-core peptide **(A)** and MtDef5 γ-core peptide **(B)** at sub-MIC concentrations of 0, 5, 15, or 30 µg/mL or polymyxin B at 0.5 µg/mL on the expression

of the *PA4774::lux* transcriptional fusion in planktonic cultures in LB broth. Gene expression was normalized for growth and CPS/OD600 values for the average of the triplicates are presented. Each growth experiment was performed three times and representative curves are shown.

Chapter 5: Functional analysis of *Medicago*-derived pathogen-induced gene promoters

INTRODUCTION

Genetic modification of crop plants to introduce novel traits requires promoters for initiating and regulating gene expression. These promoters need to be well-characterized in order to achieve predictable and desirable transgene expression. Characterizing promoters requires analyzing both their temporal and spatial expression patterns. Currently, there is a need for tissue-specific expression systems to deliver transgene products more efficiently in plant cells under attack by plant pathogens to achieve enhanced disease control. The ideal promoter for expression of genes to protect plant cells would be responsive to multiple types of pathogens. The cauliflower mosaic virus (CaMV) 35S promoter is the most widely used promoter for improving disease resistance and leads to constitutive expression of the transgene of interest (Odell et al. 1985). The CaMV 35S promoter generates strong gene expression in most plant cells with transcripts accumulating to high levels, which in some instances has been observed to lead to poor quality plants with reduced size or altered morphology (Chen et al. 2003). This may be due in part to defense reactions being activated by the over expression of the transgene in uninfected plant cells. Furthermore, constitutive transgene expression places a strong selective pressure on the pathogens for mutations that can overcome the engineered resistance. Also, the use of the CaMV 35S promoter for expression of multiple genes may increase the chances of transcriptional inactivation due to homology-dependent gene silencing (Matzke and Matzke 1995). The characterization of promoters

that are induced upon pathogen invasion is needed for engineering plants with efficient and effective disease resistance.

Medicago species are a source for novel promoters regulating gene expression in a tissue-specific manner or in response to environmental stimuli, and many of these promoters have been described through spatial and temporal gene expression studies. In the model legume, *Medicago truncatula*, a sieve element occlusion gene promoter, MtSEO-F1, generates tissue-specific gene expression in the immature sieve elements in developing phloem tissue (Bucsenez et al. 2012). Additionally, a nodulin-induced promoter, MtEBNOD12, from *M. truncatula* was characterized, and symbiosis-specific gene expression was induced in root tissue after the addition of *Sinorhizobium meliloti* nodulation factors (Chabaud et al. 1996). The MtHP promoter from *M. truncatula* was fused to a β-glucuronidase (GUS) gene and was transformed into white clover (*Trifolium repens*) where it displayed strong constitutive expression in leaf, petiole, root, and flower tissues (Xiao et al. 2005). Higher transgene expression was observed using the MtHP promoter compared to the CaMV 35S promoter, and fragments of the MtHP promoter, as small as 107 bp, could still lead to a moderate level of expression (Xiao et al. 2005). In alfalfa (*Medicago sativa*), the promoter from *hi7* displays harvest-induced and abscisic acid-induced activity in both leaf vascular and mesophyll tissues (Zhang et al. 2011). An alfalfa zinc finger protein promoter, *MsZPP*, was characterized, and transcription levels in the roots, stem vascular tissues, floral reproductive organs, and leaves were found to be stimulated by darkness, indoleacetic acid, and methyl jasmonate (MeJA) (Li et al. 2012). The promoter from the alfalfa gene encoding the plastid form of aspartate aminotransferase (AAT2) exhibits high levels of expression in *Sinorhizobium*-infected

cells of root nodules in alfalfa (Yoshioka et al. 1999). In contrast to other types of environmental stimuli, there is very little data available regarding *Medicago* promoter activity in response to pathogen infection.

Pathogenesis-related (PR) proteins were discovered to primarily accumulate in plants in response to pathogen infection (van Loon and van Strien 1999). PR proteins are currently separated into 17 distinct classes (van Loon et al. 2006). PR5 and PR10 genes are often among the most highly upregulated PR protein genes in response to infection by a wide range of pathogens, and the promoters from a number of these genes have been characterized. PR5 proteins, also called thaumatin-like proteins, are typically expressed constitutively in roots with upregulation in leaves occurring after pathogen infection, treatment with salicylic acid, jasmonic acid, or ethylene, and after wounding or cold stress (Velazhahan et al. 1999). In peach, PR5 gene expression was shown to be induced by MeJA and *Xanthomonas campestris* pv. *pruni* (Sherif et al. 2012). Pathogen-induced PR10 gene expression by a wide variety of pathogens including fungi, oomycetes, bacteria, and viruses has been established in numerous plant species. For example, *Magnaporthe grisea* on rice (McGee et al. 2001), *Phytophthora infestans* on potato (Matton and Brisson 1989), *Pseudomonas syringae* pv. *pisi* and *Xanthomonas campestris* pv. *alfalfae* on alfalfa (Borsics and Lados 2002), and *Tobacco mosaic virus* on *Capsicum annuum* (Park et al. 2004) have all led to the induction of PR10 gene expression. PR10 genes are often detected in multi-gene families. In western white pine (*Pinus monticola*), multiple members of the PR10 gene family are differentially expressed upon pathogen infection (Liu et al. 2003). In alfalfa, individual PR10 genes have been shown to have

significantly different patterns of expression dependent on a structural difference, the number of β-bulges, found in each protein structure (Bahramnejad et al. 2010).

M. truncatula PR genes, PR5 and PR10, were previously identified as being highly up-regulated during the initial stages of infection by root and foliar pathogens (Samac et al. 2011). In this work, we describe the isolation of the promoter sequences from three different *Medicago* PR genes and the design of plant transformation vectors linking these promoter sequences with the GUS reporter gene. The vectors were used for alfalfa transformation, and transgene expression was evaluated qualitatively through GUS histochemical assays and quantitatively through RT-qPCR. These analyses indicated strong transgene expression in response to pathogen attack and limited expression under disease-free conditions. Additionally, putative transcription regulator elements (REs) responsible for the pathogen-induced promoter activity were identified in the *Medicago* promoters. These promoters provide a means to engineer localized and pathogen-regulated disease resistance in transgenic alfalfa and other transgenic crops.

MATERIALS AND METHODS

Isolation of promoter regions, binary vector construction, and plant transformation. The *Medicago truncatula* EST TC113538 encoding a thaumatin-like protein (TLP) in the PR5 class of pathogenesis-related proteins was used in a BLAST search to identify the corresponding genome sequence Medtr1g062590.1 in Mt4.0 (http://jcvi.org/medicago/index.php), and the putative promoter region from 1 kbp upstream of the transcription start site and the 162 bp of leader sequence to the initiating ATG were retrieved. Similarly, *M. truncatula* EST TC192586 encoding a PR10 gene was used to identify Medtr2g035150.1, and the putative promoter region sequence (1201 bp)

was retrieved. The *Medicago sativa* genome was searched for a MtPR10 promoter homolog, and the putative promoter from *MsPR10-1* (AJ311049.1) a gene previously characterized by Breda et al. (1996) was identified. The promoter sequence of PR10 from alfalfa was named MsPR10, the *M. truncatula* PR10 promoter was named MtPR10, and the PR5 promoter from *M. truncatula* was named MtPR5. For each promoter sequence, PCR primers were designed with a *Bgl*II site immediately preceding the initiating ATG and a *Hin*dIII site at -1,000 bp (Table 11). PCR amplification was conducted using 250 µg *M. truncatula* A17 or *M. sativa* Regen SY27x genomic DNA, 50 pmol forward and reverse primers, and 2X GoTaq Master Mix (Promega, Madison, WI) in a 25 µl reaction for 30 cycles consisting of 94 °C for 30 sec, 55 °C for 1 min, and 72 °C for 1 min. Reactions were gel purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), ligated into pGEM-T Easy (Promega), and used to transform *E. coli* strain JM109 following the manufacturer's instructions. Plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen), and 0.5 µg DNA was digested with *Hin*dIII and *Bgl*II. The MtPR5 and MtPR10 promoter fragments were gel purified and ligated into the binary transformation vector pBI101.2 (Jefferson et al. 1987) digested with *Hin*dII and *BamH*I to create plant transformation vectors pBI:MtPR5 and pBI:MtPR10 with the promoters in transcriptional fusion with the β-glucuronidase gene, *gusA*. The MsPR10 promoter fragment was gel purified and ligated into the binary plant transformation vector pILTAB381 (Verdaguer et al. 1996) digested with *Xba*I and *Hin*dIII to created plant transformation vector pILTAB:MsPR10 with the promoter upstream of *gusA*. Ligation reactions contained Promega 2X Rapid Ligation Buffer and T4 DNA ligase. The vector was used to transform *E. coli* strain JM109. The promoter sequences were verified in

each vector by DNA sequencing. Verified clones were used to transform *Agrobacterium tumefaciens* LBA4404 by electroporation. Transformants were selected on Luria-Bertani (LB) agar (Difco, Sparks, MD) plates with 50 mg/L kanamycin and 25 mg/L rifampicin.

The alfalfa genotype (Regen SY27x) was transformed by co-cultivating leaf explants with *A. tumefaciens* LBA4404 containing the transformation vectors as described previously (Samac and Austin-Phillips 2006). Transformed callus cells and somatic embryos were selected using kanamycin (25 mg/L). Transgenic plants were identified by PCR amplification of genomic DNA using primers targeting the *nptII* gene and *gusA* with the MtPR5, MtPR10, or MsPR10 promoter, as described previously (Saruul et al. 2002).

Plant Material. Selected primary transformants were propagated clonally by stem cuttings and grown in the greenhouse. Primary transformants were used due to severe inbreeding depression in alfalfa when plants are self-pollinated. Plants were grown in a soil:sand mixture (1:1, v/v), one plant per cone-tainer (Stuewe & Sons, Tangent OR; 7 cm width, 35 cm depth). For RNA extraction, plant material from each replicate was combined, frozen in liquid nitrogen, and stored at -80 °C until assayed.

Pathogen cultures and growth media. Fungal pathogen strains were isolated from infected alfalfa plants obtained in Minnesota from commercial production fields and are deposited in the University of Minnesota Mycological Culture Collection. The fungal strains, *Colletotrichum trifolii* WS-5, *C. trifolii* FG-1, *Phoma medicaginis* STC, and *P. medicaginis* WS-2 were grown on potato dextrose agar (Difco) at 25 °C. After two weeks of culture growth, conidia were harvested by washing the plates with sterile water. The

spore suspensions were filtered, and spore densities were determined microscopically using a hemocytometer.

From a glycerol stock, the bacterial strain, *Pseudomonas syringae* pv. *syringae* ALF3, was cultured on nutrient broth yeast extract (NBY) agar at 30 °C. After one day of growth, the bacterial cells were harvested by flooding the plates with sterile water. Cultures were diluted with sterile water to an OD_{600} of 0.1.

Histochemical localization of GUS expression. Samples of transgenic alfalfa stems, leaves, petioles, and roots with nodules were cut into large pieces and placed in 24-well microplates. A GUS staining solution containing 1 mg/mL 5-bromo-4-chloro-3-indoyl β-D-glucuronic acid sodium salt (X-GlcA; Research Products International, Mt. Prospect, IL) (Jefferson et al. 1987) was added (25 mL per plate), which was enough to cover the samples, and vacuum infiltration was used to increase stain penetration into plant tissues. Plates were sealed with Parafilm and placed at 37 °C for 24 hours. The stain was removed, the samples were washed twice with distilled water, and 70% ethanol was added to remove pigments in order to better visualize the staining.

GUS staining was also performed on infected detached leaves from each transgenic line and a non-transformed Regen SY27x control line. Young leaves were removed from top three nodes of alfalfa plants, and five trifoliates from the same transgenic line were placed in 100 x 15 mm Petri plates lined with moist filter paper, then inoculated. For fungal pathogens, *Colletotrichum trifolii* or *Phoma medicaginis*, each leaflet was inoculated with a 5 µl spore suspension at a concentration of $1x10^6$ conidia/mL with 50 ppm Tween 20. For inoculations with a bacterial pathogen, *Pseudomonas syringae* pv. *syringae*, each leaflet received 5 µl droplet of a bacterial

suspension at an OD_{600} of 0.1 with 50 ppm Tween 20. Droplets were placed on wounds created by pressing a pipette tip on the leaf tissue. Control leaves were mock-inoculated with water. Plates were incubated at 25 °C for 24 h, 48 h, 72 h, or 120 h. Leaves were then cut and stained with GUS staining solution as previously described.

Expression analysis using reverse-transcriptase quantitative PCR (RT-qPCR). Detached leaves of transgenic alfalfa were inoculated as previously described with *C. trifolii*, *P. medicaginis*, or *P. syringae* pv. *syringae* and harvested 48 h after inoculation. Control leaves were mock-inoculated with water. RNA was isolated from detached leaves using the RNeasy Plant Mini kit (Qiagen). Concentration and purity of the RNA samples were tested with a NanoDrop spectrophotometer (Thermo Fischer, Carlsbad, CA). The first strand of cDNA for each sample was made from 1 µg total RNA using an iScript cDNA Synthesis Kit (BioRad, Hercules, CA). GUS and promoter-specific qPCR primers were designed using Primer Express (Thermo Fischer) (Table 11). qPCR was performed using the iTaq Universal SYBR Green Supermix (BioRad) in 25 µL reactions containing 4 pmol of each forward and reverse primer and 5 µL of template cDNA. Samples from three biological repetitions from each line were run in triplicate on a 7500 Fast Real-Time PCR System (Thermo Fischer) following the manufacturer's recommendations. The PCR conditions were as follows: 2 min of denaturation at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 58 °C, followed by steps for melting curve generation (15 s at 95 °C, 1 min at 60 °C, 30 s at 95 °C, 15 s at 60 °C). The 7500 Fast Real-Time software (Thermo Fischer) was used for data collection. Melting curves showed that only one transcript was measured demonstrating that the primers were specific for transcripts of each isoform. Relative transcript accumulation for each sample

was obtained using the comparative C_t method (Schmittgen and Livak 2008) using the C_t value of the alfalfa f-actin gene (JQ028730.1) for sample normalization.

***In silico* sequence analysis.** *Nsite* (Shahmuradov and Solovyev 2015) was used to identify regulatory elements (REs), which facilitate transcription factor binding on promoters, on the MtPR5, MtPR10, and MsPR10 promoter DNA sequences. The *Nsite* analysis performed searches for statistically non-random motifs of known REs using the RegSite dataset, a plant-specific RE dataset. Both single and composite REs were identified with the statistical significance of each hit being reported. Also, EMBOSS Needle pairwise sequence alignments (Li et al. 2015) were used to compare MtPR10 and MsPR10 DNA sequence similarity for both promoter and coding sequences.

RESULTS

Genetic transformation of alfalfa. The predicted promoter regions from *M. sativa* PR10 and *M. truncatula* PR10 and PR5 were PCR amplified to generate approximately 1,000 bp segments of each putative promoter. The fragments were cloned and sequenced before creating promoter::GUS constructs. Promoter sequences were submitted to GenBank under the accession numbers MK618665, MK618666, and MK618667 for the MsPR10, MtPR10, and MtPR5 promoters, respectively. Plant transformation vectors (pBI:MtPR10, pBI:MtPR5, and pILTAB:MsPR10) were confirmed to contain the anticipated promoter sequences and were used to transform alfalfa (cultivar Regen SY27x) through *Agrobacterium*-mediated transformation. A total of 20 lines containing MtPR10::GUS, 14 lines containing MtPR5::GUS, and 41 lines containing MsPR10::GUS were confirmed to be transgenic by PCR amplification of both the GUS gene and *nptII* selectable marker gene.

GUS expression patterns. GUS expression varied among the transgenic lines, which is commonly seen and attributed to positional effects of the inserted transgene (Peach and Velton 1991). Of the 34 transgenic lines with *M. truncatula* promoters, GUS expression was visualized in 11 different lines of uninoculated plants, and 25 out of 41 transgenic lines with the MsPR10 promoter had detected GUS expression in uninoculated plants. In uninoculated plants, GUS staining was primarily observed in the root vascular tissues in the transgenic lines containing the MtPR10, MtPR5, and MsPR10 promoters (Fig. 15). Staining appeared to be intensified near regions of developing nodules (Fig. 15G). Limited GUS expression was detected in the leaf tissue compared to the petiole and root tissues. The MsPR10 lines had the greatest intensity of staining with several lines displaying staining in the petiole, root cortex and vascular tissues, root tip, and stem vascular tissue. Pathogen inoculation of detached leaves was shown to induce GUS expression near the sites of infection in transgenic plants (Fig. 16). Following *P. medicaginis* inoculation, GUS expression was greatly enhanced throughout the leaf vascular tissue in the MtPR10::GUS, MtPR5::GUS, and MsPR10::GUS transgenic lines (Fig. 16). *C. trifolii* inoculations also induced foliar GUS expression in lines containing the MtPR5 and MtPR10 promoters (data not shown). Following inoculation with a bacterial pathogen, *P. syringae* pv. *syringae*, GUS staining was concentrated near centers of infection in leaves from the MtPR5::GUS and MtPR10::GUS transgenic lines (Fig. 16). There appeared to be more diffuse staining in transgenic alfalfa following inoculation with fungal pathogens compared to bacterial pathogens. Also, foliar GUS staining first emerged 48 hours after inoculation and increased in intensity over time. GUS expression was not observed in plant tissues of the non-transformed control line,

although staining of *P. medicaginis* mycelium was seen at 120 h post-inoculation (Fig. 16).

Relative gene expression in response to pathogen infection. Transgenic alfalfa plants with observed GUS expression after pathogen inoculation were further investigated through RT-qPCR analyses to measure GUS transcript accumulation after inoculation with either *P. medicaginis* strain PSTC or *P. syringae* pv. *syringae* ALF3. The expression of the GUS gene was normalized by the alfalfa reference gene, f-actin, and compared relative to the GUS transcript accumulation in mock-inoculated leaves. The MtPR10 promoter resulted in greater levels of fungal pathogen-induced GUS transcription than either the MtPR5 and MsPR10 promoters at 48 hours post-inoculation (Fig. 17). Relative GUS expression in plants with the MtPR10::GUS construct ranged from a low of 1.4-fold to 359-fold up-regulation in line MtPR10-12 when infected with *P. medicaginis* (Fig. 17). Whereas, for the MsPR10::GUS transgenic lines, the highest up-regulation in GUS gene transcripts was 78-fold in line MsPR10-7 when infected with *P. medicaginis*. GUS expression in plants with the MtPR5 promoter inoculated with *P. medicaginis* ranged from 1.2-fold to 22-fold up-regulation. In response to bacterial pathogen infection, transcript up-regulation was limited, which agrees with the results from the GUS histochemical staining. The MtPR5-45 line demonstrated the greatest level of GUS transcript up-regulation with an increase of 8-fold upon *P. syringae* pv. *syringae* infection (Fig. 18).

Since the *M. truncatula* promoters displayed substantial pathogen-induced expression in response to a fungal pathogen, they were further evaluated using another strain of *P. medicaginis* and two highly aggressive race 5 strains of *Colletotrichum*

trifolii. In response to inoculation with *C. trifolii* FG-1, a race 5 isolate, MtPR5 promoters had high levels of induced GUS expression with relative increases in expression of 100-fold in line MtPR5-44 (Fig. 19). MtPR10-12 again displayed high levels of induction against *P. medicaginis* WS-2 with an increase in GUS expression of 189-fold (Fig. 19). But, strain specificity was identified in both the *P. medicaginis* and *C. trifolii* inoculation trials. Line MtPR10-33 had a 6-fold increase in GUS expression when inoculated with *P. medicaginis* strain PSTC and a 423-fold increase in GUS expression when inoculated with *P. medicaginis* strain WS-2 (Fig. 17 and 19).

Expression of the MsPR10 and MsPR5 genes were also measured in response to pathogen infection and compared to mock-inoculated plants. In response to infection with *P. medicaginis* WS-2, PR10 expression ranged from 2-fold to 316-fold up-regulation, and PR5 expression was increased by 3-fold to 60-fold (Fig. 20). When inoculated with *P. syringae* pv. *syringae*, expression averaged around a 120-fold increase although line MtPR10-52 had a large increase in PR10 expression of 5,288-fold (Fig. 21). PR5 gene expression had relatively small increases of 9-fold in line MtPR5-13 after *P. syringae* pv. *syringae* inoculation and an increase of 5-fold in line MtPR5-13 inoculated with *C. trifolii* WS-5 (Fig. 21).

***In silico* sequence analysis.** To identify potential transcription regulatory elements (REs), the promoter DNA sequences were analyzed utilizing the program *Nsite* (Shahmuradov and Solovyev 2015). *Nsite* predicts both single and composite REs in query sequences using a large plant-specific RE database and estimates the statistical significance of each identified RE. Nonrandom motifs (a level of homology between known RE and motif of ≥90%; the statistical significance of 95%) of 15 known REs were

predicted for MtPR10, 14 REs were predicted for MtPR5, and 28 REs were predicted for MsPR10. Selected statistically significant, nonrandom REs identified in the *Medicago* promoters are given in Tables 12, 13, and 14. Several pathogen responsive REs were present throughout the regulatory sequence. The binding sites for WRKY (SA signaling) and ERF (JA/ET signaling) transcription factors were found in both plus and minus DNA-strands of the promoters. In the MsPR10 promoter, a RE from an *Arabidopsis* PR1 gene was identified. Additionally, a putative TATA box was present in both the MtPR10 and MsPR10 promoters. Diagrams of the pathogen-inducible elements in the MtPR5, MtPR10, and MsPR10 promoters are shown in Figure 22.

Given that the MtPR10 and MsPR10 promoters come from similar species, *M. truncatula* and *M. sativa*, and both promote PR10 gene expression, a pairwise sequence alignment was performed. An EMBOSS Needle alignment (Li et al 2015) was used to compare the promoter and coding DNA sequences for MtPR10 and MsPR10. The nucleotide sequences of MtPR10 and MsPR10 promoters revealed 45.6% identity, and the PR10 coding sequences had 47.5% identity. In a previous phylogenetic analysis, the *M. truncatula* and *M. sativa* PR10 proteins were closely clustered together and grouped in the same subfamily based on amino acid similarity (Liu and Ekramoddoullah 2004).

DISCUSSION

Alfalfa is the most widely grown forage legume throughout the world and is the third most valuable row crop in the United States with a production value of $9.9 billion for 2018 as estimated by the USDA (https://quickstats.nass.usda.gov). Through genetic engineering, transgenic alfalfa has been developed and commercialized for glyphosate herbicide tolerance and for reduced lignin, which increases forage digestibility.

Constitutive expression of transgenes in alfalfa can be achieved using the viral promoters CaMV 35S, cassava vein mosaic virus promoter, or sugarcane bacilliform badnavirus promoter (Samac et al. 2004). When engineering plants for disease resistance, localized pathogen-induced gene expression is preferable due to limiting both the energy load on the plant and the selective pressure on the pathogen. Additionally, public disapproval towards the usage of genes from unrelated species encourages the isolation and characterization of genetic elements from the species of interest (Lassen et al. 2002). Discovery of pathogen-induced promoters lags behind identification of the genes for disease resistance (Gurr and Rushton 2005). Therefore, the development of *Medicago* genetic elements for tissue-specific, pathogen-induced expression to efficiently deliver the transgene product to plant cells under attack is essential.

Alfalfa diseases reduce forage quality and yields causing financial losses for the growers. The pathogens utilized in this study are a diverse group of economically important alfalfa fungal and bacterial pathogens. *P. medicaginis* is the most destructive foliar alfalfa pathogen causing spring black stem and leaf spot disease. During cooler weather, *P. medicaginis* greatly reduces forage quality, especially from the first spring harvest (Castell-Miller 2015). Losses from the first cutting are particularly economically damaging because the first harvest typically contains the best forage quality and is the highest yielding. *P. syringae* pv. *syringae* is a bacterial pathogen that causes bacterial stem blight of alfalfa in which diseased plants are stunted with spindly stems that can be easily broken (Gray and Hollingsworth 2015). The bacterium typically penetrates host stems at sites of frost injury, so with global climate change, the geographic range and economic impact of bacterial stem blight may increase (Nemchinov et al. 2017). Though

most alfalfa has resistance to race 1 of *C. trifolii*, we used a newly identified race 5 isolated (WS-5) that is very aggressive against most alfalfa cultivars. *C. trifolii* can infect alfalfa root and crown tissues contributing to crown rot. Crown rot reduces alfalfa stand persistence and density, which often requires growers to incur the cost of replanting the stand (Kalb et al. 1994). Also, these alfalfa pathogens have different lifestyles. *P. medicaginis* is a necrotroph, *C. trifolii* is a hemibiotroph, and *P. syringae* pv. *syringae* is a biotroph. The *Medicago* promoters analyzed in this experiment were all responsive to this varied suite of significant alfalfa pathogens.

In this study, promoters from three *Medicago* PR genes were isolated and used to drive GUS expression. Transgenic alfalfa plants expressing the GUS reporter gene using the promoters MsPR10, MtPR10, or MtPR5 were qualitatively and quantitatively assayed for GUS transcriptional activity. The MtPR5, MtPR10, and MsPR10 promoters were functional in alfalfa and reflected the expression patterns seen for the respective genes. In some instances, promoters from one species may not be expressed similarly when transgenically expressed in another species (Mithra et al. 2017). For example, the *Arabidopsis* class III chitinase promoter leads to enhanced expression in root tissue in *Arabidopsis*, but when the promoter::GUS construct is expressed in alfalfa, GUS activity is limited to vascular tissue (Samac and Temple 2004). In a previous study, the relative expression of PR5 and PR10 genes in *M. truncatula* was analyzed by RT-qPCR, and foliage-enhanced expression was observed for *PR5*, while root-enhanced expression was observed for *PR10* (Samac et al. 2011). This same spatial pattern of expression was observed during the GUS staining of uninoculated transgenic alfalfa. The MtPR10 and MsPR10 promoters primarily led to expression in root tissue (Fig. 15). The transgenic

lines of alfalfa displayed pathogen-induced expression visualized through greatly enhanced GUS staining of the leaf vascular tissue, especially after inoculation with a fungal pathogen (Fig. 16). This characteristic pathogen-induced expression pattern was previously seen with *MsPR10-1*, the alfalfa gene used for isolation of the MsPR10 promoter, after inoculation with *Pseudomonas syringae* pv. *pisi*, an incompatible pathogen (Breda et al. 1996). A characterized *M. truncatula* PR10 promoter, MtHP, displayed different patterns of expression than MtPR10 with constitutive GUS histochemical staining without pathogen induction or tissue specificity (Xiao et al. 2005), which could be due to the genetic background of the alfalfa lines used.

The MtPR10 promoter exhibited greater pathogen-induced activity than the MsPR10 promoter in RT-qPCR assays (Fig. 17). But, when inoculated with *P. syringae* pv. *syringae*, the MtPR5 promoter had higher levels of activation than MtPR10 promoter (Fig. 18). The differential expression of highly similar PR genes provides an opportunity for plants to produce protein isoforms that are most selected evolutionarily in response to environmental stresses (Liu et al. 2005). Although smaller relative increases in GUS expression throughout the leaf were observed in response to inoculation with the bacterial pathogen compared to fungal pathogens (Fig. 18), the level of induction near the bacterial infection site may be relatively high based on GUS staining (Fig. 16). Similarly, after inoculation with *Xanthomonas campestris* pv. *alfalfae*, MsPR10.1A and MsPR10.1B showed a moderate increase in GUS expression of 1.1 and 1.6-fold, respectively (Bahramnejad et al. 2010). GUS expression varied for MtPR10::GUS and MtPR5::GUS lines after inoculation (Fig. 17 and 19). Variability from plant to plant in GUS expression from tissue specific promoters has been observed previously in alfalfa (Pathirana et al.

1997; Trepp et al. 1999).

The analysis of REs in the studied promoter sequences identified motifs responsible for precise transcription initiation, such as the TATA box (Butler and Kadonaga 2002). Several defense response REs were present throughout the *Medicago* promoters. W-boxes, *cis*-acting elements that are recognized by WRKY transcription factors, were identified in each of the *Medicago* promoters (Fig. 22). Clustering of W-boxes within pathogen-controlled promoters is frequently observed, but a single W-box can be sufficient for pathogen inducibility (Eulgem et al. 2000). For example, PR10a, a pathogen-responsive promoter from rice, has a single W-box that was essential for induction (Hwang et al. 2008). Furthermore, a single type of *cis*-acting element can confer pathogen-induced expression, which was demonstrated with synthetic plant promoters (Rushton et al. 2002). MYB motifs involved in the defense response were identified in the MtPR10 and MtPR5 promoters. In the parsley PAL1 promoter, the MYB binding sites were discovered to be the sites of fungal elicitor-inducible DNA-protein interactions (Lois et al. 1989). Also, two MYB binding sites were found to be essential for *Phytophthora sojae*-induced expression in the soybean promoter *GmaPPO12* (Chai et al. 2013). Additionally, the MtPR5 promoter contains a GBF3 binding site thought to confer drought and other abiotic stress tolerance (Ramegowda et al. 2017).

Promoters with a full spectrum of activities need to be available for the research community, so expression systems can be finely modulated. Localized pathogen inducibility of promoters would allow for the expression of proteins that may be detrimental when expressed ubiquitously in the entire plant. Promoters that are weakly induced upon pathogen infection could be advantageous because defensive signaling

molecules are potent and expensive for the plant to produce. Having a larger toolbox of characterized promoters will make transgenic plants more efficient and effective. The MtPR10, MtPR5, and MsPR10 promoters are functional in alfalfa for expression of transgenes and up-regulate gene expression after infection by a range of different alfalfa pathogens. These promoters potentially could be used for the transgenic expression of antimicrobial peptides or other avirulence factors.

TABLE 11 Primers used in *Medicago* promoter cloning and real-time PCR assays[1]

Annotation	**Forward primer**	**Reverse primer**
Cloning MtPR10	GGGagatctGATGGTATAATGTATTAA	GGGaagcttCTTAGAACACTTGTTAAT
Cloning MtPR5	GGGagatctATTCTTGTTATTGTTTTA	GGGaagcttTGTATAAGTGAAGGAGAG
Cloning MsPR10	GCTCAGtctagaGATGATATAATACTAATGTGTG	GTGACCaagcttGGACGGATCTACAGTCAC
qPCR f-actin	CCACATGCCATCCTTCGTTT	TGTCACGAACAATTTCCCG
qPCR GUS	CAGTTCGCCGATGACGATATTCG	GCCCTGATGCTCCATCACTTCC
qPCR MsPR10	GCCGGAACCATCAAGAAACT	AAGCCAACACCTCCAACAA
qPCR MsPR5	GGACCTGGAGCAACGAACTC	GCAACTTGACCAGATGCACAA

[1]Primers are in the 5’ to 3’ direction and restriction sites are in lower case letters

TABLE 12 Motifs of known REs predicted by the *Nsite* program in the [-1201:-1] region upstream of the translation start of the PR10 gene (Medtr2g035150.1) in *Medicago truncatula.*

Name, Accession Number[1] and Binding Factor of known RE	**Organism and Gene**	**Positions of identified RE motifs[2]**	**Sequence of identified RE motifs[3]**
CE1 (RSP00058); Unknown nuclear factor	*Hordeum vulgare*: HVA22	-770:-762	TtCCACCGG
NIT2 BS III (RSP01202), NIT2	*Chlorella vulgaris*: NR	-666:-659	CCAAAGGT
EM1 (CArG box 1) (RSP01209), MADS box proteins	*Lepidium africanum*: LaCRC	-124:-115	CTTTTTTGG
TATA box (RSP01301), TBF	*Triticum aestivum*: Amy2/54	-91:-82	CTATAAATAC
E6.3 (RSP01974), Unknown nuclear factor	*Oryza sativa*: SAG39	-700:-693	CTTGACCA
Fp30/VI (RSP02187), Unknown nuclear factor	*Glycine max*: CHS8	-288:-268	aAAGaAGAAAAAAAAAAat AGT
WRKY28 BS (m2.8) (RSP02309), WRKY28	*Arabidopsis thaliana*: ICS1	-702:-691	CgCTTGACCaAT
DOF2 domain 2 (RSP02710), DOF2	*Arabidopsis thaliana*: AmidP (At4g34880)	-767:-757	gTGGAAAGGAG
MYB46 BS2 (PAL1) (RSP02752), MYB46	*Arabidopsis thaliana*: PAL1	-671:-664	CACCAACC

[1]In Regsite Plant Database. [2]Positions are given relative to the ATG start codon. [3]Lower-case letters indicate mismatches.

TABLE 13 Motifs of known REs predicted by the *Nsite* program in the [-1162:-1] region upstream of the translation start of the PR5 gene (Medtr1g062590.1) in *Medicago truncatula.*

Name, Accession Number[1] and Binding Factor of known RE	**Organism and Gene**	**Positions of identified RE motifs[2]**	**Sequence of identified RE motifs[3]**
56/59 box (RSP00039); GT-1 related transcription factors	*Lycopersicon esculentum*: LAT56; LAT59	-648:-638	TGAATTGTGA
C-rich R (RSP00601), Unknown nuclear factor	*Lycopersicon esculentum*: rbcS2	-393:-385	tCCCACCAA
CT-LB (RSP00653), Unknown nuclear factor	*Spinacia oleracea*: petH	-1150:-1140	TTCTCTCTCCT
AtSIRKp (WRKY11) BS1 (RSP01739), WRKY11	*Arabidopsis thaliana*: SIRK/FRK1 (At2g19190)	-127:-115	TTATTGACTgAAT
Aux28B2 (RSP00969), STGA1	*Glycine max*: GmAux28	-900:-891	TTGACGACAA
-190 half G-box (core) (RSP00683), GBF3	*Arabidopsis thaliana*: Adh	-1085:-1076	GCaAAGTGGA
Box-L3 (RSP01081), DcMYB1	*Daucus carota*: DcPAL1	-94:-83	TtCAACCcTCCA
RY (RSP02234), LEC2	*Arabidopsis thaliana*: Oleosin	-819:-811	TGCATGATG
GA-6 (RSP00865), BPC1	*Arabidopsis thaliana*: STK	-1149:-1141	GGAGAGAGA

[1]In Regsite Plant Database. [2]Positions are given relative to the ATG start codon. [3]Lower-case letters indicate mismatches.

TABLE 14 Motifs of known REs predicted by the *Nsite* program in the [-1524:-1] region upstream of the translation start of the MsPR10 gene (AJ311049.1) in *Medicago sativa.*

Name, Accession Number[1] and Binding Factor of known RE	**Organism and Gene**	**Positions of identified RE motifs[2]**	**Sequence of identified RE motifs[3]**
ERRE (RSP00030); EREBP	*Nicotiana sylvestris*: OLP	-716:-710	AGCCGCC
B3 (RSP00915), Opaque-2	*Zea mays*: b-32	-160:-151	GATGATGTGT
Box VI (RSP00037), 3AF1	*Pisum sativum*: rbcS-3A	-84:-64	AAATtGATAAATAAAAtatTT
GCC box (RSP02365), ERF1	*Arabidopsis thaliana*: GSTF7	-716:-710	AGCCGCC
GbWRKY1 BS1 (RSP02874), GbWRKY1	*Gossypium hirsutum*: GhJAZ1	-823:-812	TAAcGTCAAAGA
LS10 (RSP02162), DOF factors	*Arabidopsis thaliana*: PR-1	-887:-872	TTCTTCAGGtCaTTTg
TATA box (RSP01301), TBF	*Triticum aestivum*: Amy2/54	-94:-85	CTATAAATAC
GCC box (RSP01523), StEREBP1	*Solanum tuberosum*: Synthetic oligonucleotides	-716:-710	AGCCGCC
Motif III (RSP00063), unknown nuclear factor	*Oryza sativa*: rab16B	-1510:-1501	GCCGCGTGaC

[1]In Regsite Plant Database. [2] Positions are given relative to the ATG start codon. [3]Lower-case letters indicate mismatches.

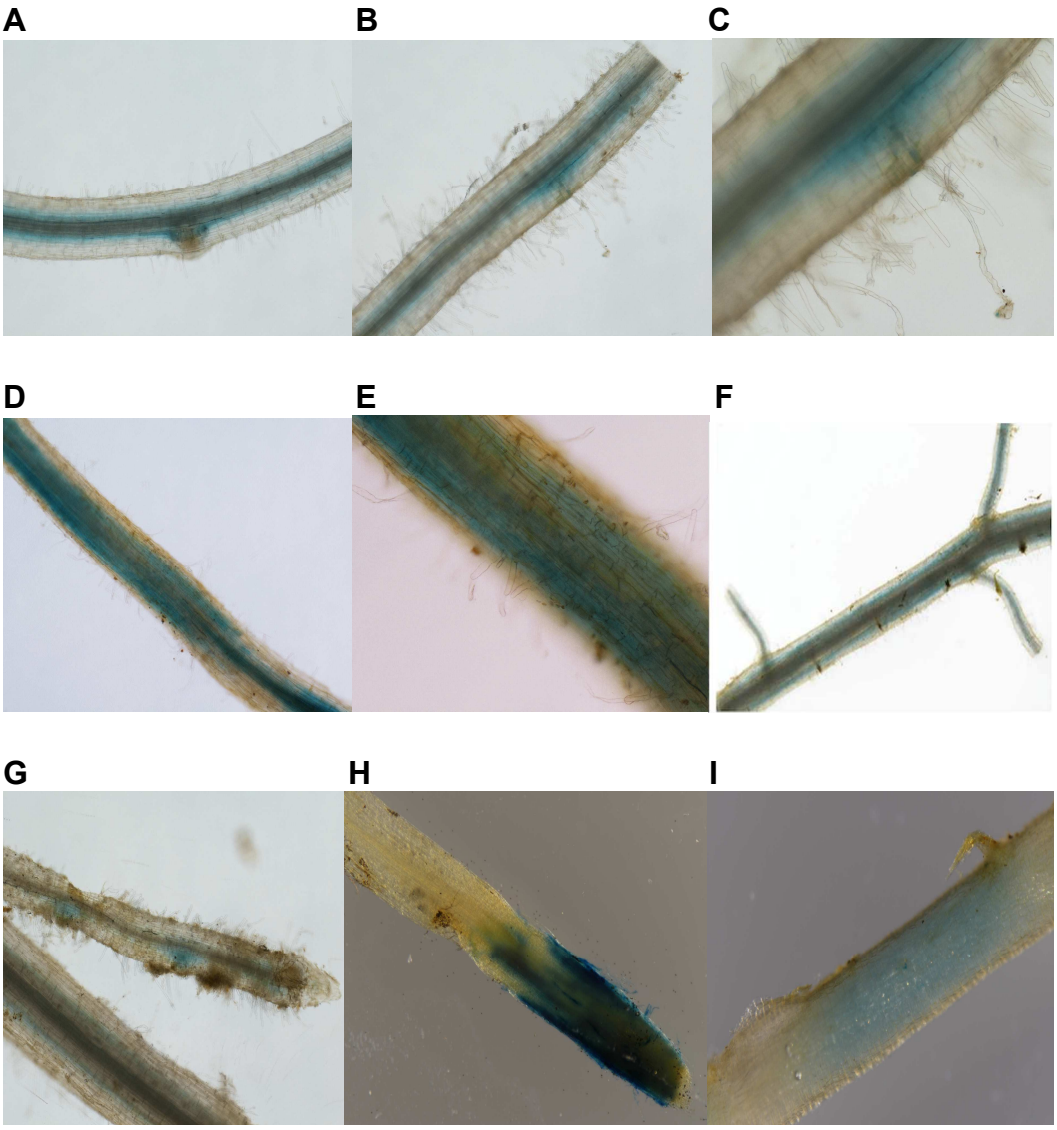


Fig. 15 Expression of GUS gene fusions in the roots of transgenic alfalfa plants. Expression pattern in root sections of the GUS gene regulated by the MtPR10 promoter (line 12, **A-C**; line 19, **D** and **E**; line 25, **F**), the MtPR5 promoter (line 44, **G**), and the MsPR10 promoter (line 12, **H**; line 35, **I**).

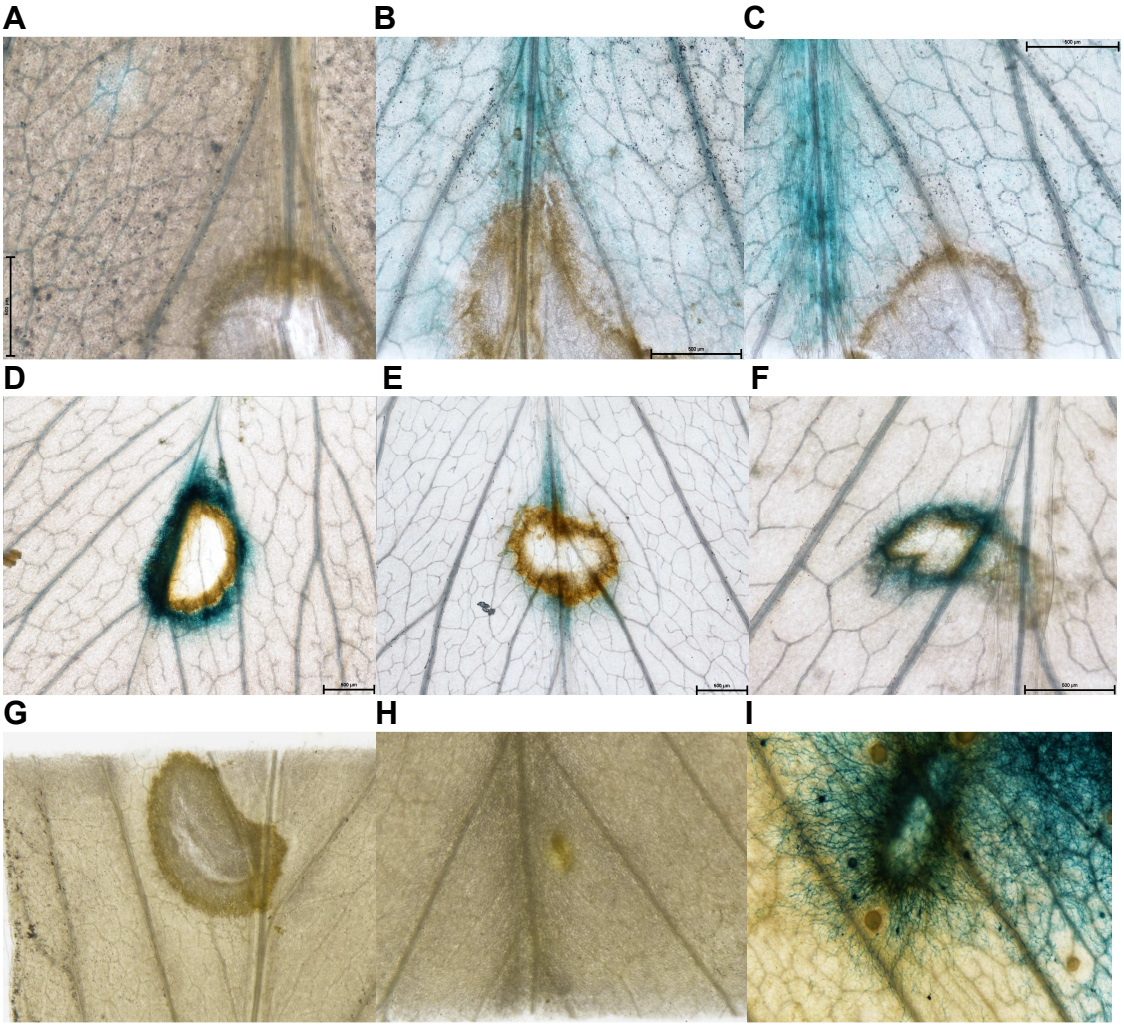


Fig. 16 Histochemical localization of GUS activity after pathogen inoculation of transgenic alfalfa. Leaves of transgenic alfalfa with the MsPR10::GUS construct (line 14) were infected with *Phoma medicaginis* PSTC and staining was done at 48 **A** and 72 **B, C** hours after inoculation. Leaves of transgenic alfalfa with the MtPR10::GUS construct (line 19) were infected with *Pseudomonas syringae* pv. *syringae* ALF3 and staining was done at 72 **D, E, F** hours after inoculation. Leaves from a non-transformed line were stained 72 hours after inoculation with *P. syringae* pv. *syringae* ALF3 **G** and *P. medicaginis* **H**. 120 hours after inoculation with *P. medicaginis*, pathogen staining was observed on the non-transformed line **I**.

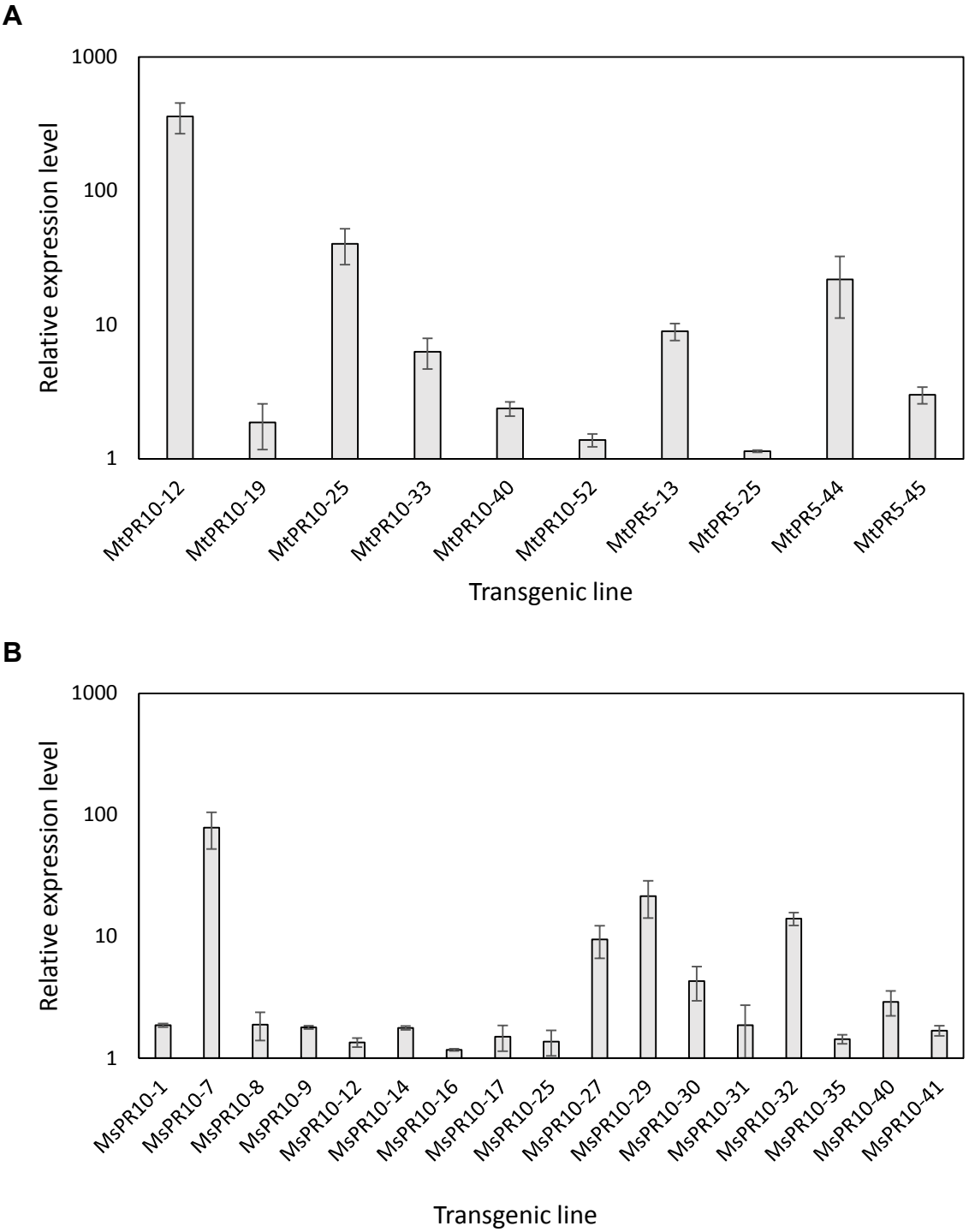
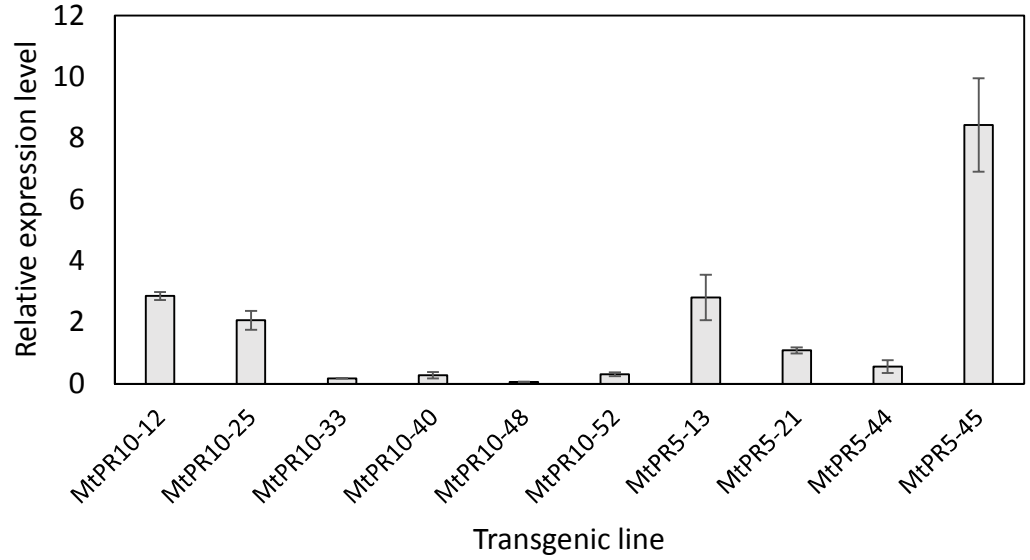


Fig. 17 Quantitative RT-PCR analysis indicates fungal pathogen-induced GUS gene expression in transgenic alfalfa plants containing the MtPR10::GUS or MtPR5::GUS constructs **A** or the MsPR10::GUS construct **B**. Total RNA was extracted from leaves

infected with *Phoma medicaginis* (PSTC). The relative transcription level of *GUS* was normalized to the transcription of alfalfa f-actin gene (JQ028730.1) and to transcription level of *GUS* in mock-inoculated leaves. Bars represent means and error bars indicate the standard error ($n = 9$).

A



B

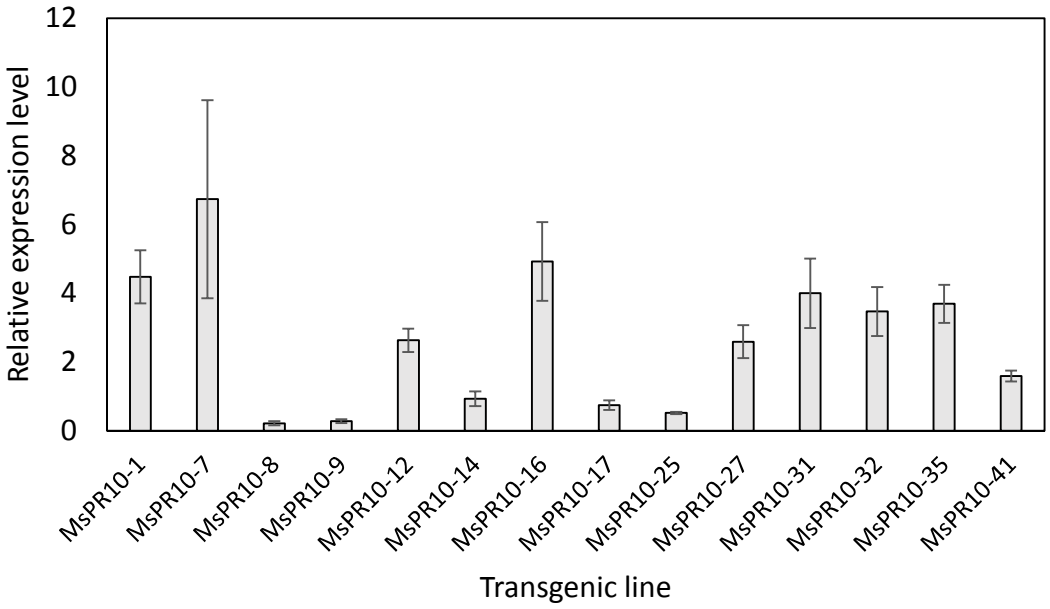


Fig. 18 Quantitative RT-PCR analysis indicates bacterial pathogen-induced GUS gene expression in transgenic alfalfa plants containing the MtPR10::GUS or MtPR5::GUS constructs **A** or the MsPR10::GUS construct **B**. Total RNA was extracted from leaves infected with *Pseudomonas syringae* pv. *syringae* (ALF3). The relative transcription level of *GUS* was normalized to the transcription of alfalfa f-actin gene (JQ028730.1) and

to transcription level of *GUS* in mock-inoculated leaves. Bars represent means and error bars indicate the standard error ($n = 9$).

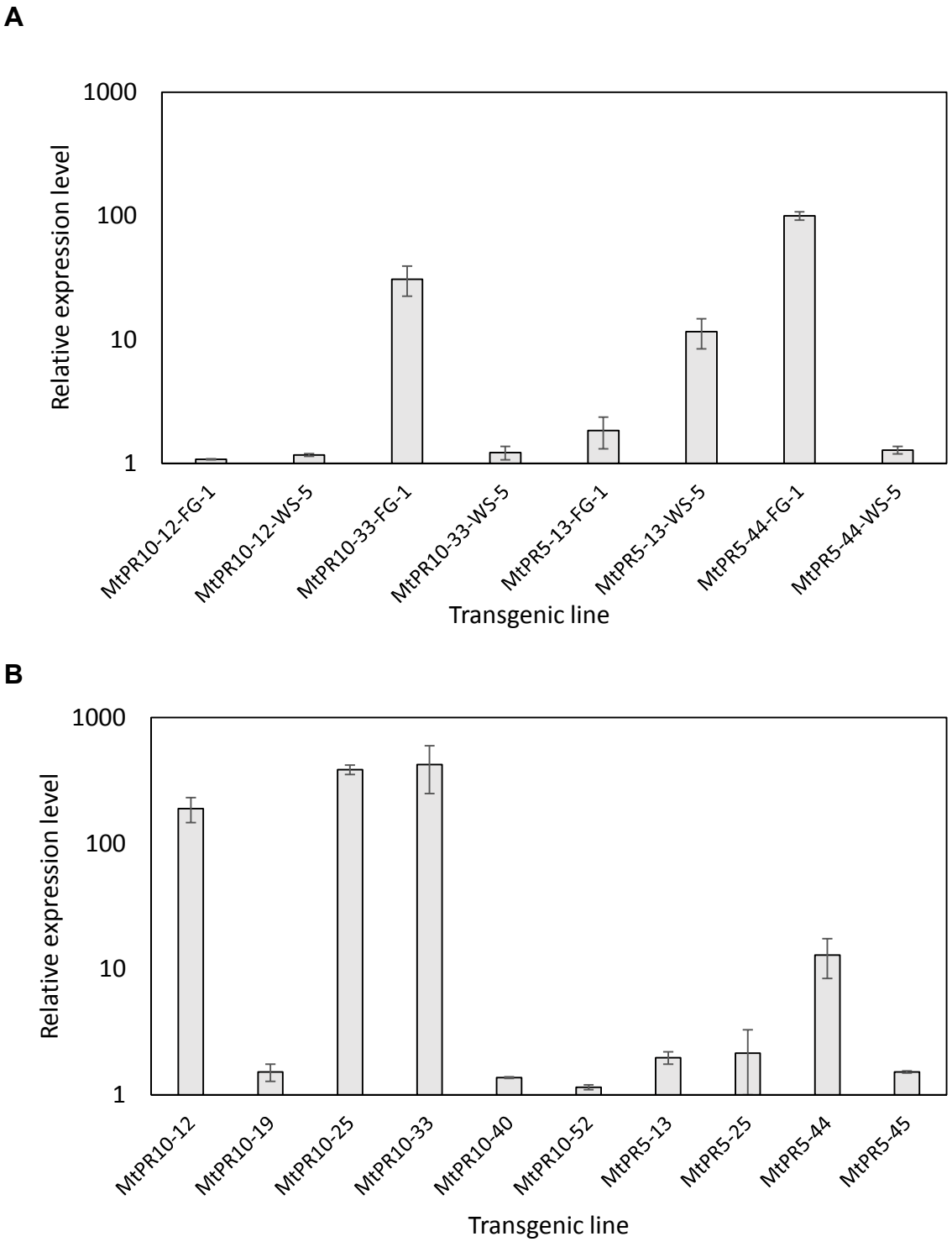


Fig. 19 Quantitative RT-PCR analysis indicates fungal pathogen-induced GUS gene expression in transgenic alfalfa plants containing the MtPR10::GUS or MtPR5::GUS

constructs. Total RNA was extracted from leaves infected with two strains of *Colletotrichum trifolii* (FG-1, WS-5) **A** and *Phoma medicaginis* (WS-2) **B**. The relative transcription level of *GUS* was normalized to the transcription of alfalfa f-actin gene (JQ028730.1) and to transcription level of *GUS* in mock-inoculated leaves. Bars represent means and error bars indicate the standard error ($n = 9$).

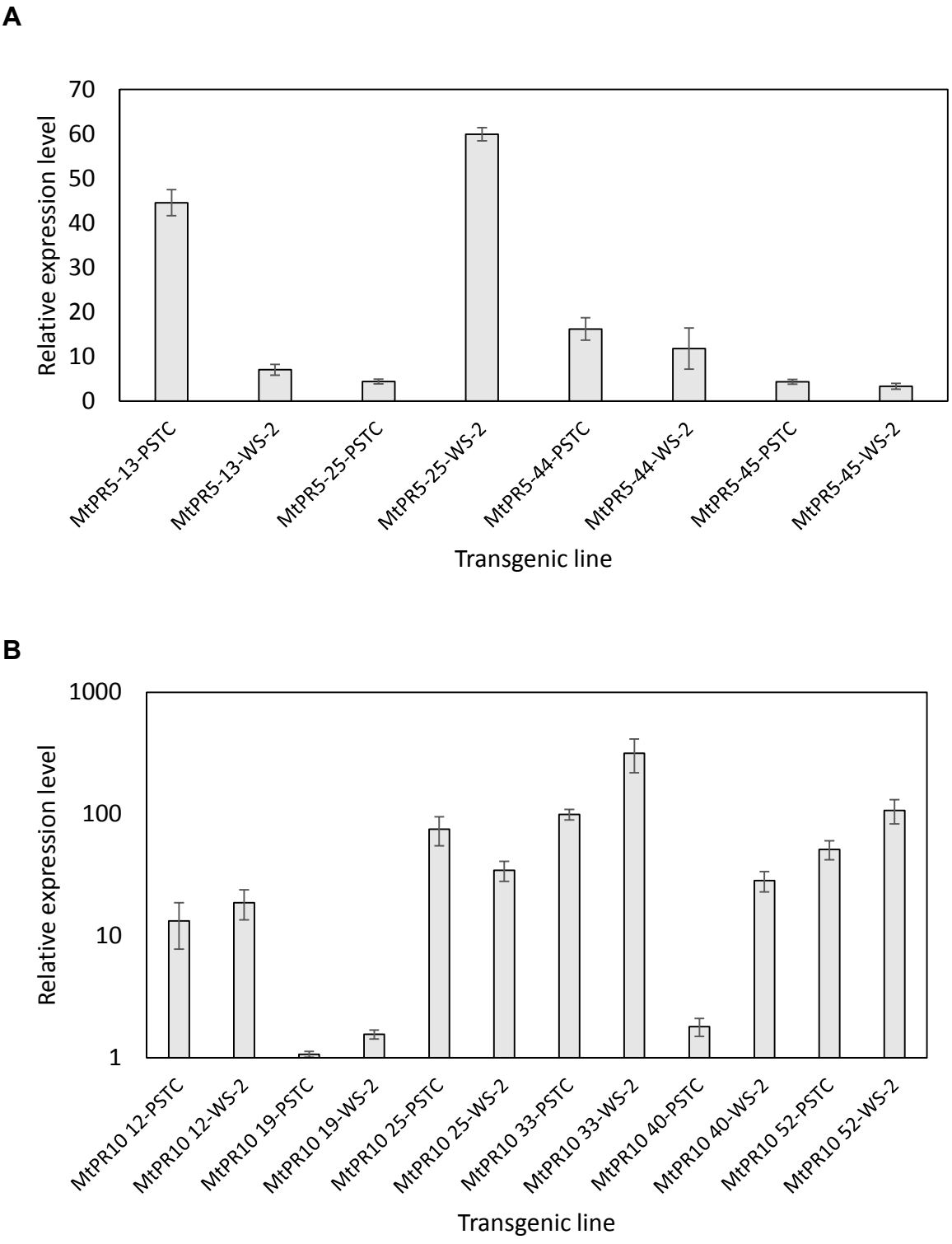


Fig. 20 Quantitative RT-PCR analysis indicates *Phoma medicaginis*-induced PR5 and PR10 gene expression in transgenic alfalfa plants containing the MtPR10::GUS or

MtPR5::GUS constructs. Total RNA was extracted from MtPR10::GUS and MtPR5::GUS lines infected with two strains of *Phoma medicaginis* (PSTC, WS-2). The relative transcription levels of *PR5* **A** and *PR10* **B** were normalized to the transcription of alfalfa f-actin gene (JQ028730.1) and to transcription levels of *PR5* and *PR10* in mock-inoculated leaves. Bars represent means and error bars indicate the standard error ($n = 9$).

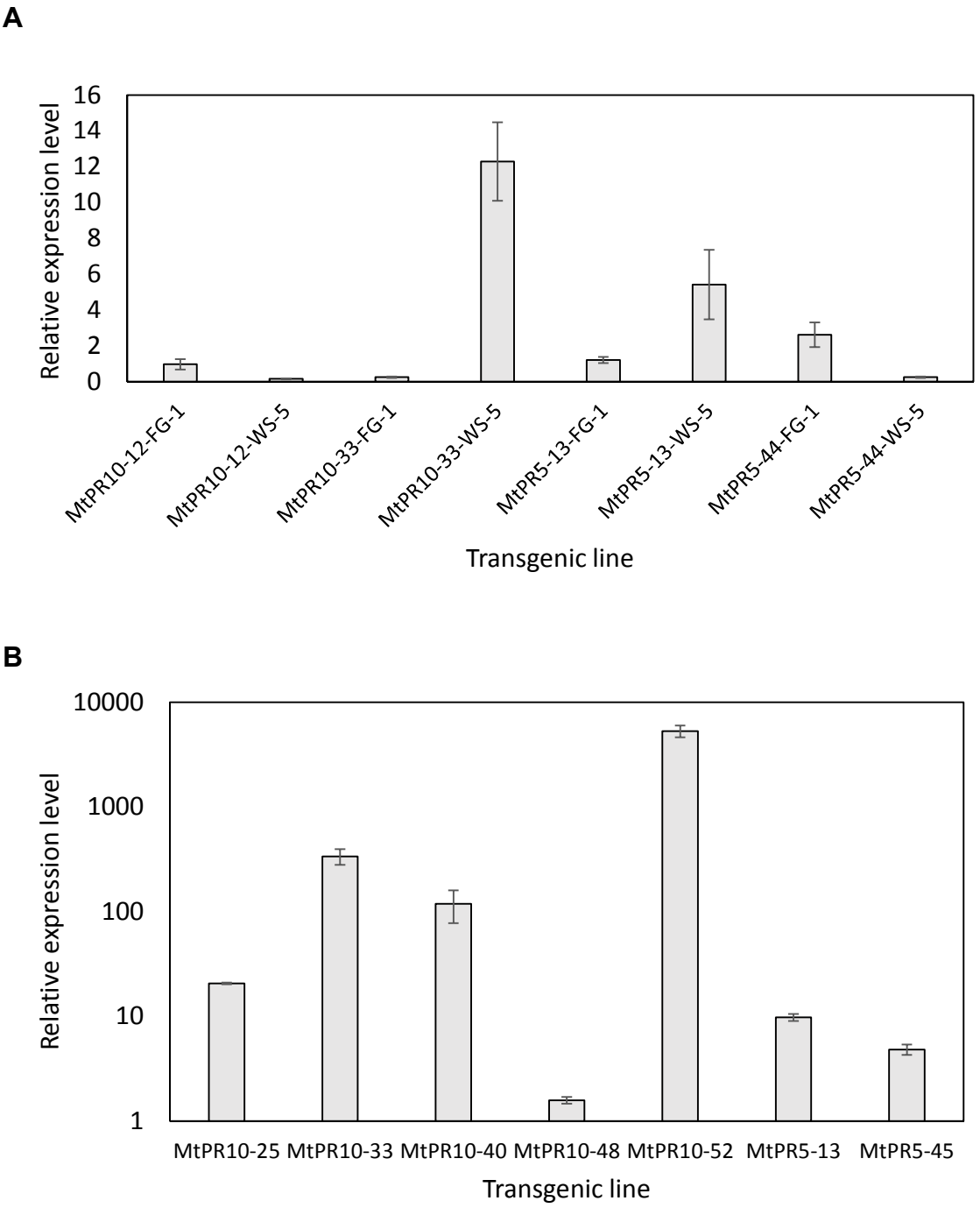


Fig. 21 Quantitative RT-PCR analysis indicates *Colletotrichum trifolii* and *Pseudomonas syringae* pv. *syringae*-induced PR5 and PR10 gene expression in transgenic alfalfa plants containing the MtPR10::GUS or MtPR5::GUS constructs. Total RNA was extracted from leaves infected with two strains of *Colletotrichum trifolii* (FG-1, WS-5) **A** and

Pseudomonas syringae pv. *syringae* (ALF3) **B**. The relative transcription levels of *PR5* and *PR10* were normalized to the transcription of alfalfa f-actin gene (JQ028730.1) and to transcription levels of *PR5* and *PR10* in mock-inoculated leaves. Bars represent means and error bars indicate the standard error ($n = 9$).

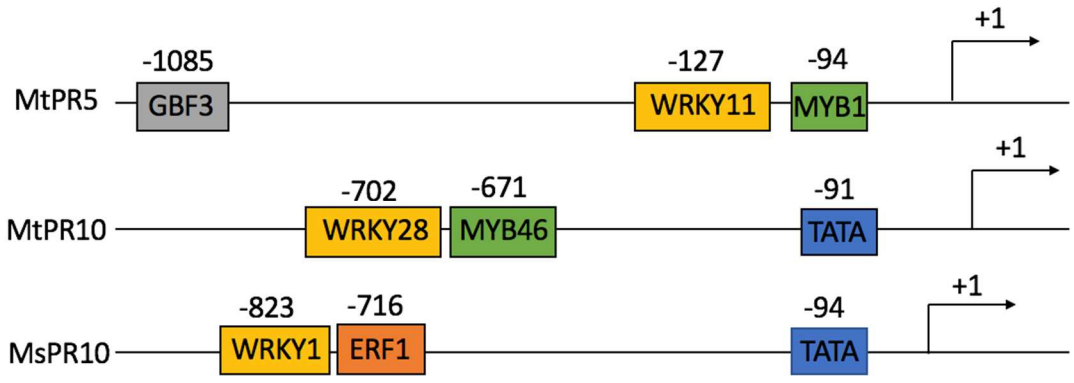


Fig. 22 Promoters from MtPR5, MtPR10, and MsPR10 are diagramed with pathogen inducible transcription regulator elements (REs) predicted by the *Nsite* program. REs positions are given relative to the ATG start codon.

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