Mol Breeding (2020) 40:66
https://doi.org/10.1007/s11032-020-01144-6

Functional analysis of *Medicago*-derived pathogen-induced gene promoters for usage in transgenic alfalfa

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Received: 28 January 2020 / Accepted: 15 June 2020

Abstract Three gene promoters from the model legume species *Medicago truncatula* and *Medicago sativa* (alfalfa) pathogenesis-related (PR) proteins (MtPR5, MtPR10, and MsPR10) were isolated and investigated using in silico and in situ approaches. For the functional analysis of these promoters, plant transformation vectors linking promoter sequences with the β-glucuronidase (GUS) reporter gene were constructed and utilized to generate transgenic alfalfa. Histochemical GUS staining established that the PR5 and both PR10 promoters were functional in alfalfa and induce GUS expression primarily in the root tissue. When transgenic alfalfa leaves were inoculated with a diverse set of alfalfa pathogens, either *Phoma medicaginis*, *Colletotrichum trifolii*, or *Pseudomonas syringae* pv. *syringae*, the promoters were responsive to induction by both bacterial and fungal pathogens. Gene expression analysis also indicated that all three PR promoters were pathogen-induced and upregulate transgene expression. Additionally, several putative transcription regulator elements (REs) responsible for the observed pathogen-induced promoter activity were predicted in each of the promoter sequences. These characterized *Medicago* PR promoters may be a unique and valuable tool for pathogen-induced transgenic expression of antimicrobial proteins or other genes enhancing disease resistance in alfalfa.

Keywords Alfalfa · β-glucuronidase · Pathogenesis-related protein · Promoter · Pathogen-induced

Introduction

Alfalfa (*Medicago sativa*) is the most widely grown forage legume throughout the world and is the third most valuable row crop in the USA 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 cauliflower mosaic virus (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

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11032-020-01144-6) contains supplementary material, which is available to authorized users.

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Published online: 26 June 2020

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 or other closely related species (Lassen et al. 2002). Discovery of pathogen-induced promoters lags behind identification of genes increasing 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. Also, these alfalfa pathogens have different lifestyles. *Phoma medicaginis* is a necrotroph, *Colletotrichum trifolii* is a hemibiotroph, and *Pseudomonas syringae* pv. *syringae* is a biotroph. *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 assessed a newly identified race 5 isolate (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).

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. Promoters from the model legume species, *Medicago truncatula*, are frequently characterized for usage in alfalfa because there still is no genome sequence for tetraploid alfalfa. A *M. truncatula* 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 with 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 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 formed by gene duplication events. 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 upregulated during the initial stages of infection by diverse 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.

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. After observing strong pathogen-induced activity in the *M. truncatula* PR10 promoter, 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 − 1000 bp (Supplementary Table 1). 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 s, 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 create 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 2 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 1 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 (top, from third internode), leaves (fresh, entire trifoliate harvested), 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 h. The GUS staining solution 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 × 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 1×10^6 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

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) (Supplementary Table 1). 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 of three biological repetitions from each separate line with every biological repetition having three technical repetitions 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 (Szucs et al. 2006).

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 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 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 Velten, 1991). Of the 34 transgenic lines with *M. truncatula* promoters, GUS expression was visualized in four lines of plants containing the MtPR5 promoter and seven lines containing the MtPR10 promoter. In the MsPR10 promoter plants, 25 out of 41 transgenic lines had detected GUS expression. 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. 1). Staining appeared to be intensified near regions of developing nodules (Fig. 1g). Limited GUS expression was detected in the leaf tissue compared with 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. 2). 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. 2). *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. 2). There appeared to be more diffuse staining in transgenic alfalfa following inoculation with fungal pathogens compared with bacterial pathogens. Also, foliar GUS staining was first visible 48 h after inoculation with both fungal and bacterial pathogens 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. 2), which is commonly observed due to background glucuronidase activity (Schoenbeck et al. 1999).

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. Specialized qPCR primers were generated (Supplementary Table 1), and the expression of the GUS gene was normalized by the alfalfa reference gene, f-actin. The MtPR10 promoter resulted in greater levels of fungal pathogen-induced GUS transcription than either the MtPR5 or MsPR10 promoters at 48 h post-inoculation (Fig. 3). Relative GUS expression in plants with the MtPR10::GUS construct ranged from a low of 1.4-fold to 359-fold upregulation in line MtPR10-12 when infected with *P. medicaginis* (Fig. 3). Whereas, for the MsPR10::GUS transgenic lines, the highest upregulation 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 upregulation. In response to bacterial pathogen infection, transcript upregulation was limited, which agrees with the results from the GUS histochemical staining. The MtPR5-45 line demonstrated the greatest level of GUS transcript upregulation with an increase of 8-fold upon *P. syringae* pv. *syringae* infection (Fig. 4).

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 (Supplementary Fig. 1). MtPR10-12 again displayed high levels of induction against *P. medicaginis* WS-2 with an increase in GUS expression of 189-fold (Supplementary Fig. 1). But, strain specificity was identified in both the *P. medicaginis* and *C. trifolii* inoculation trials. Line MtPR10-33 had a sixfold 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. 3 and Supplementary Fig. 1).

To support the previous GUS expression analysis, the expression of the alfalfa PR10 and PR5 genes themselves was also measured in response to pathogen infection and compared with mock-inoculated plants. In response to infection with *P. medicaginis* WS-2, PR10 expression ranged from twofold to 316-fold upregulation, and PR5 expression was increased by threefold to 60-fold (Supplementary Fig. 2). 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 5288-fold (Supplementary Fig. 3). PR5 gene expression had relatively small increases of ninefold in line MtPR5-13 after *P. syringae* pv. *syringae* inoculation and an increase of fivefold in line MtPR5-13 inoculated with *C. trifolii* WS-5 (Supplementary Fig. 3).

In silico sequence analysis

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 the PR10 genes from *M. truncatula* and alfalfa. The nucleotide sequences of MtPR10 and MsPR10 promoters revealed 45.6% identity, and the PR10 coding sequences had 88.9% 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).

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. Non-random 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, non-random REs identified in the *Medicago* promoters are given in Supplementary Tables 2, 3, and 4. 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

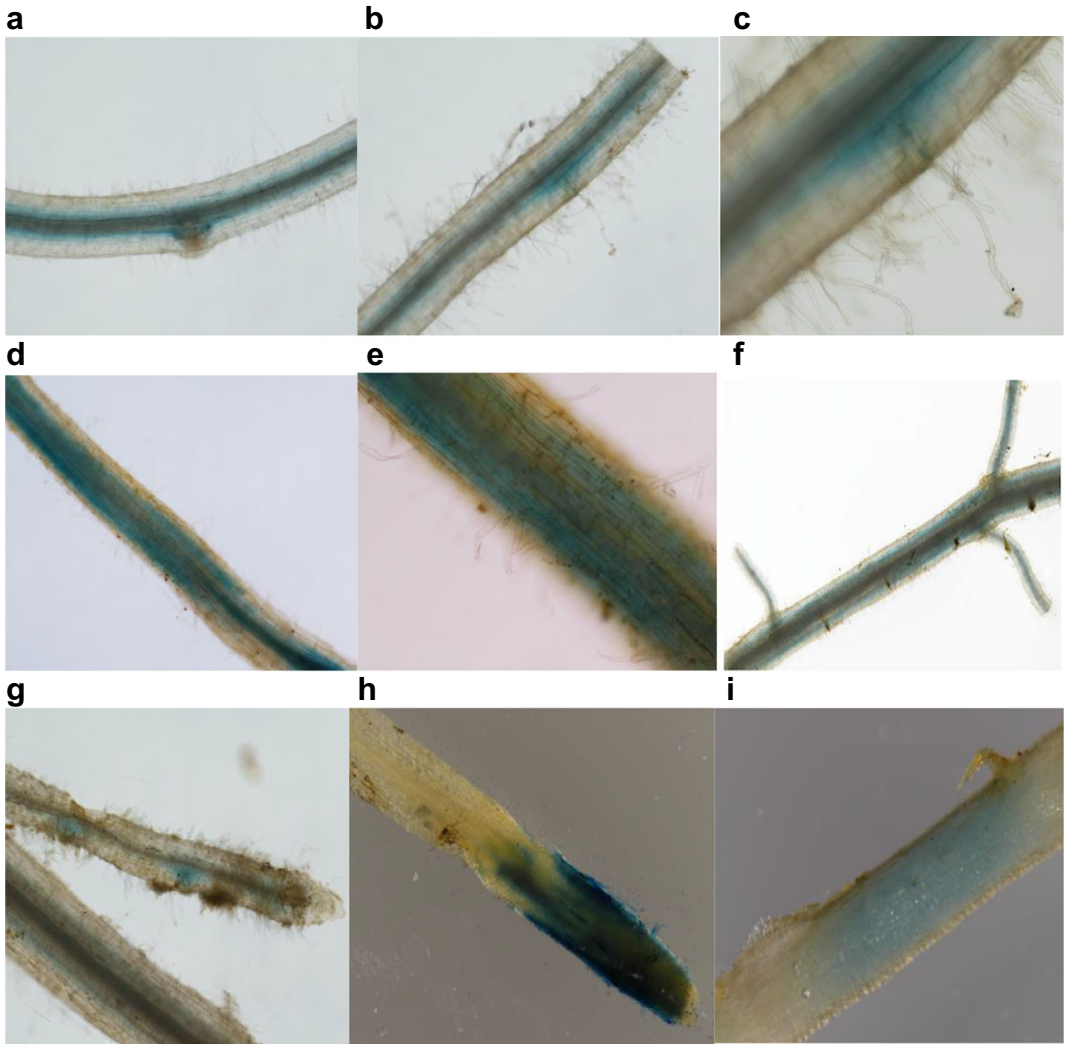

Fig. 1 Expression of GUS gene fusions in the roots of transgenic alfalfa plants. Expression pattern in root sections of the GUS gene regulated by the MtPR10 promoter (line 12, **a**–**c**; line 19, **d** and **e**; line 25, **f**), the MtPR5 promoter (line 44, **g**), and the MsPR10 promoter (line 12, **h**; line 35, **i**)

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 Fig. 5.

Discussion

Genetic modification of crop plants to introduce localized and pathogen-regulated disease resistance traits requires promoters for initiating and regulating gene expression. 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 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). Constitutive transgene expression places a strong selective pressure on the pathogens for mutations that can overcome the engineered resistance. The *Medicago* promoters analyzed in this experiment were all responsive to this varied suite of significant

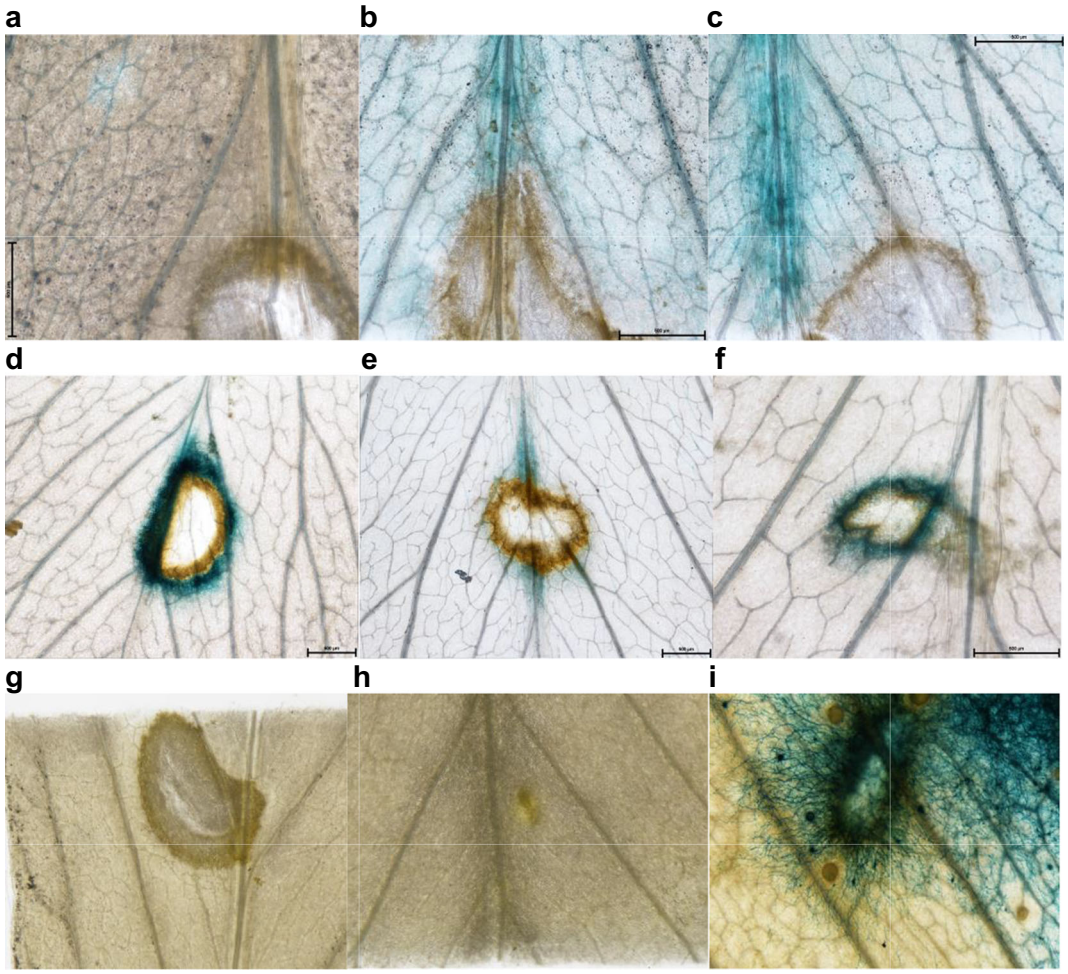

Fig. 2 Histochemical localization of GUS activity after pathogen inoculation of transgenic alfalfa. Leaves of transgenic alfalfa with the MsPR10::GUS construct (line 14) were infected with *Phoma medicaginis* PSTC, and staining was done at 48 (**a**) and 72 (**b**, **c**) hours after inoculation. Leaves 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 h after inoculation with *P. syringae* pv. *syringae* ALF3 (**g**) and *P. medicaginis* (**h**). A total of 120 h after inoculation with *P. medicaginis*, pathogen staining was observed on the non-transformed line (**i**)

alfalfa pathogens and could be used to engineer efficient and effective disease resistance in alfalfa.

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 expressed ectopically 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. 1). The

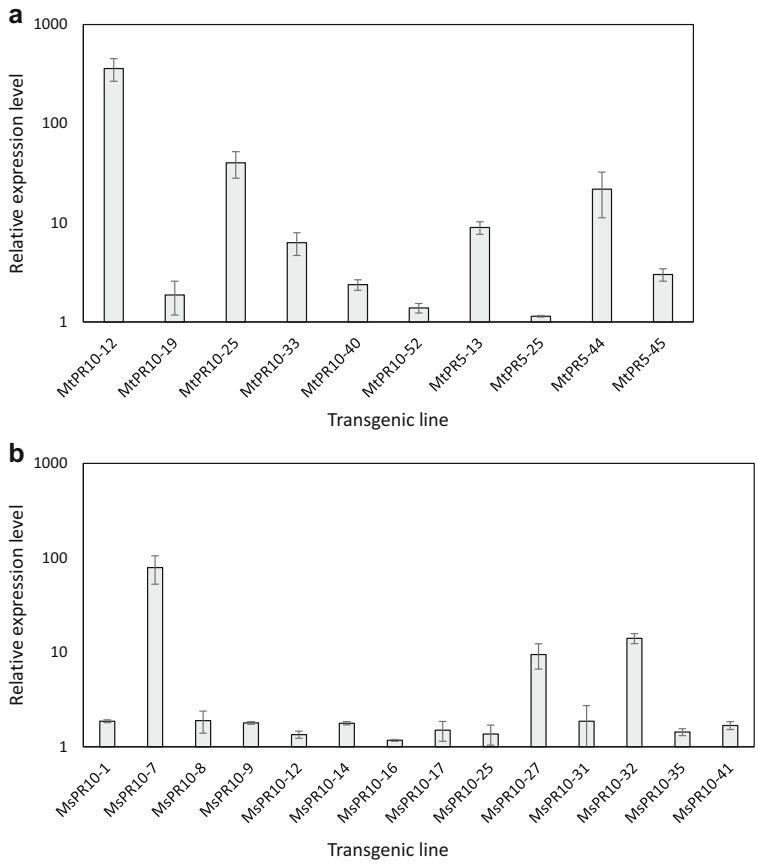

Fig. 3 Quantitative RT-PCR analysis indicates fungal pathogen-induced GUS gene expression in transgenic alfalfa plants containing the MtPR10::GUS or MtPR5::GUS constructs (**a**) or the MsPR10::GUS construct (**b**). Total RNA was extracted from leaves infected with *Phoma medicaginis* (PSTC). The relative 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 ($n = 9$)

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. 2). 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. 3). But, when inoculated with *P. syringae* pv. *syringae*, the MtPR5 promoter had higher levels of activation than MtPR10 promoter (Fig. 4). The differential expression of distinct 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 with fungal pathogens (Fig. 4), the level of induction near the bacterial infection site may be relatively high based on GUS staining (Fig. 2). 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. 3 and Supplementary Fig. 1). 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 predicted 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,

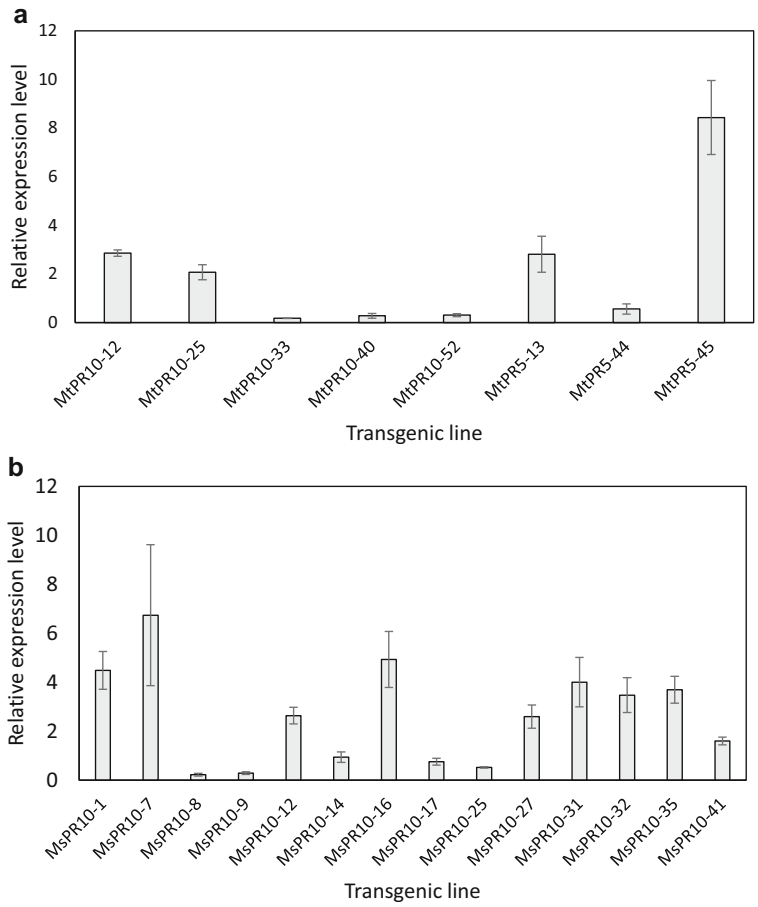

Fig. 4 Quantitative RT-PCR analysis indicates bacterial pathogen-induced GUS gene expression in transgenic alfalfa plants containing the MtPR10::GUS or MtPR5::GUS constructs (**a**) or the MsPR10::GUS construct (**b**). Total RNA was extracted from leaves infected with *Pseudomonas syringae* pv. *syringae* (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 ($n = 9$)

cis-acting elements that are recognized by WRKY transcription factors, were identified in each of the *Medicago* promoters (Fig. 5). 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).

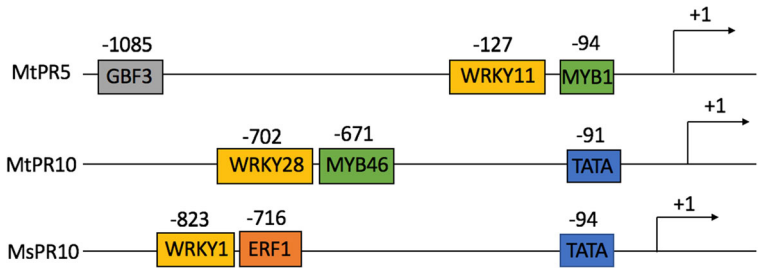

Fig. 5 Promoters from MtPR5, MtPR10, and MsPR10 are diagramed with pathogen-inducible transcription regulator elements (REs) predicted by the *Nsite* program. RE positions are given relative to the ATG start codon

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 upregulate 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 avirulence factors in alfalfa or other crop plants.

Acknowledgments We thank Bruna Bucciarelli for the photography of plant tissues. This paper is a joint contribution from the Plant Science Research Unit, USDA-ARS, and the Minnesota Agricultural Experiment Station.

Author contributions AES and DAS conceived and the designed the research. AES, MRD, and SSM conducted the experiments. AES and DAS wrote the manuscript. All authors read and approved the manuscript.

Funding information The work was supported by funding by the USDA-ARS project 5062-12210-003D and the Torske Klubben Fellowship.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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