

A comparison of two class 10 pathogenesis-related genes from alfalfa and their activation by multiple stresses and stress-related signaling molecules

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Abstract A collection of 29 pathogenesis-related 10 (PR10) genes of *Medicago sativa* and *Medicago truncatula* showed that they were almost all obtained from cDNA libraries of tissues undergoing abiotic or biotic stresses. The predicted proteins could be divided into two subclasses, PR10.1 and PR10.2, but in silico predicted models of their three-dimensional structures revealed that they could be further divided based on size of the hydrophobic internal cavity and number of β -bulges. A comparison of the expression of two highly similar *M. sativa* subclass PR10.1 genes, *MsPR10.1A* and *MsPR10.1B*, predicted to have similar sized hydrophobic internal cavities, but a different number of β -bulges revealed differences in their expression patterns. *MsPR10.1A* was induced faster than *MsPR10.1B* by ABA, ethylene, and *X. campestris* pv. *alfalfae*, but slower than *MsPR10.1B* by harvesting and wounding. Unlike *MsPR10.1A*, *MsPR10.1B* expression was induced in non-harvested tissues following harvesting, but was not induced by heat treatment. Histochemical observations of

Nicotiana benthamiana transformed with 657 bp of the *MsPR10.1A* promoter fused to the β -glucuronidase (GUS) gene showed that GUS expression was wound-inducible in leaves, which was consistent with *MsPR10.1A* expression in alfalfa leaves. GUS expression in stems and leaves was mostly in vascular tissue. The *MsPR10.1A* promoter may be valuable in controlling the expression in vascular tissues and disease resistance.

Keywords PR10 · Alfalfa · Promoter · Harvesting

Introduction

Pathogenesis-related (PR) proteins were originally discovered to accumulate in plants that are infected by pathogens, but were later found to accumulate in plants in response to a variety of biotic and abiotic stresses (Datta and Muthukrishnan 1999). Van Loon and Van Strien (1999) recognized a number of PR proteins that are induced by pathogens in tissues that normally do not contain the protein. One of these is the PR10 family, in which the type member, parsley PR1, has been shown to have ribonuclease activity (Liu and Ekramoddoullah 2006).

Induction of PR10 gene expression has been demonstrated in a wide variety of plant species following infection by pathogens, including *Phytophthora megasperma* f.sp. *glycinea* on parsley (Somssich et al. 1986), *Phytophthora infestans* on potato (Matton and Brisson 1989), TMV on *Capsicum annuum* (Park et al. 2004), *Cronartium ribicola* on *Pinus monticola* (Liu et al. 2003), *Cochliobolus heterostrophus* and *Colletotrichum sublineolum* on sorghum (Lo et al. 1999), *Pseudomonas syringae* pv. *pisi* on *Vitis vinifera* (Robert et al. 2001), *Magnaporthe grisea* and *Acidovorax avenae* on rice (McGee et al. 2001), and

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P. syringae pv. *pisii* on alfalfa (Borsics and Lados 2002). However, PR10 genes have also been shown to be induced by various abiotic stresses, such as treatment with NaCl (Borsics and Lados 2002), heat, cold, PEG (Borsics and Lados 2002), UV irradiation (Rakwal et al. 1999), and ozone (Agrawal et al. 2002). Expression of some PR10 genes are also upregulated following treatment with plant hormones and signaling molecules, such as jasmonic acid (JA) (Rakwal et al. 1999; McGee et al. 2001; Borsics and Lados 2002), salicylic acid (SA) (McGee et al. 2001), ethylene (Poupart et al. 2003), abscisic acid (ABA) (Borsics and Lados 2002), and kinetin (Rakwal et al. 1999).

PR10 genes are found in multi-gene families. For example, nine different yellow lupin (*Lupinus luteus*) PR10 genes have been described that can be divided into two classes, namely *LIPR-10.1* and *LIPR-10.2*, based on their predicted AA sequences as well as certain features of their three-dimensional structures (Pasternak et al. 2005). Considerable variation in expression patterns could be found between the different PR10 genes in yellow lupin. For example, *LIPR10.1B* was constitutively expressed in the petioles and leaves, while *LIPR10.1A* was induced in these organs in response to infection with the pathogenic bacteria, *Pseudomonas syringae* or during senescence of leaves (Sikorski et al. 1999). These differences would be consistent with variation in the promoter sequences of different members of the PR10 genes family.

A *Medicago sativa* PR10 gene, designated *MsPR10.1A*, was isolated from cDNA clones generated following subtractive hybridization between cDNA from harvested tissue and non-harvested alfalfa tissues that had high homology to PR10 genes from other plants in the Fabaceae, such as those from yellow lupin (Zhang 2004). A genomic clone of *MsPR10.1A* was also obtained containing a total of 657 bp upstream of the start codon (Zhang 2004). Although the promoter of this gene may be useful to direct genes for harvest-inducible expression, the ability of PR10 genes to be affected by many different biotic and abiotic stimuli raises questions about its specificity. To further investigate the diversity and regulation of PR10 genes, its sequence, putative three-dimensional protein structure, and expression was compared to that of a previously described PR10 gene, namely *PPRG2* (Borsics and Lados 2002), hereafter referred to as *MsPR10.1B*. Expression of the two genes was measured in alfalfa following harvesting, wounding, heat, ABA, ethylene, and pathogen treatments. In addition, a 657-bp portion of the promoter region of *MsPR10.1A* was fused to a GUS reporter gene, and the construct transferred to *Nicotiana benthamiana* to determine the tissue-specificity conferred by this regulatory region.

Materials and methods

Biological materials

Plants of *M. sativa* cv. Gala were grown from seeds kindly provided by Northrup King Canada in Sunshine LA4 Mix (Sun Gro Horticulture Canada Ltd, Vancouver, BC, Canada) at 21°C with a photoperiod of 12 h at 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Treatments were applied at 8 weeks when plants were approximately 10 cm in height. *N. benthamiana* was grown from seed under the same conditions. *Xanthomonas campestris* pv. *alfalfae* strain X61 was kindly provided by Dr. Diane Cuppels, Agriculture and Agri-Food Canada, London, ON, Canada.

Sequence comparisons

A search of the NCBI EST and NR databases (<http://blast.ncbi.nlm.nih.gov>) delimited by “*Medicago*” and the *Medicago truncatula* Gene Index Release 10.0 (<http://compbio.dfci.harvard.edu>) accessed June 2010 using BLASTX with *MsPR10.1A* as a query identified PR10 sequences from *M. sativa* and *M. truncatula* (Tables 1, 2). In addition, PR10 sequences of *L. luteus* previously analyzed for their three-dimensional structure by Pasternak et al. (2005) were included (Tables 1, 2). These sequences were aligned with CLUSTALW (Chenna et al. 2003) using default parameters, and then subjected to dendrogram and bootstrap analysis using MEGA 2.1 (Kumar et al. 2001). The dendrogram was generated using the distance methods procedure, UPGMA (Sneath and Sokal 1973).

The putative three-dimensional structures of the predicted PR10 sequences from *M. sativa* and *M. truncatula* (Tables 1, 2) were generated with Swiss-Model (<http://swissmodel.expasy.org/>) using Automated Mode, and the protein models of the *L. luteus* PR10 proteins, LIPR10.1A, LIPR10.1B, LIPR10.2A, and LIPR10.2B, were obtained from the Protein Data Bank (1ICX, 1IFV, 1XDF, and 3E85, respectively) in PDB format. The PDB file from Swiss-Model was viewed using PyMOL version 1.2r1 (San Carlos, CA, USA) (<http://www.pymol.org/>), and the angle in the C-terminal helix $\alpha 3$ resulting in a severe distortion of its central region (i.e., kink) was determined using the ANGLES command based on the amino acids (labeled: start, midpoint, and end) that formed the distortion. The PDB file from Swiss-Model was also submitted to PDBsum (<http://www.ebi.ac.uk/pdbsum/>), which uses ProMotif to determine the amino acids that caused the distortion of the β -strands resulting in the β -bulges. In PyMOL, a filling model was created with the SHOW AS SURFACE command, and the locations of the midpoint of the kink in the helix $\alpha 3$, as well as the β -bulges were marked with the

Table 1 Subclass I PR10 predicted amino acid sequences of *Medicago sativa*, *M. truncatula*, *Lupinus luteus*, and some of their characteristics

GenBank or gene index accession number ^a	Source	MW (kDa)	pI	Angle in helix $\alpha 3$ (°)	Largest internal cavity with opening (Å ³)	Number of β -bulges	EST frequency (%) ^b
CAA56298 ^c	<i>L. luteus</i>	16.859	5.19	101.5	1,528	7	–
CAA56299 ^c	<i>L. luteus</i>	16.654	5.35	109.7	1,216	7	–
AAD55099 ^c	<i>L. luteus</i>	16.750	5.08	109.9	1,375	8	–
CAC86467 ^d	<i>M. sativa</i>	16.591	5.80	110.4	1,150	8	–
CO512791	<i>M. sativa</i>	16.513	4.98	110.5	1,162	8	0.0075
CO512055	<i>M. sativa</i>	16.515	4.98	110.4	1,162	8	0.0075
CO512118	<i>M. sativa</i>	16.515	4.98	110.4	1,162	8	0.0150
ADB44209 ^d	<i>M. sativa</i>	16.636	5.09	102.0	1,188	7	–
CO514574	<i>M. sativa</i>	16.501	4.98	110.5	1,261	8	0.0075
TC119223	<i>M. truncatula</i>	16.574	4.98	110.4	1,159	8	0.0060
TC119143	<i>M. truncatula</i>	16.526	4.95	110.4	1,204	8	0.0056
TC120627	<i>M. truncatula</i>	16.606	4.95	110.4	1,289	8	0.0067
TC124784	<i>M. truncatula</i>	16.606	4.95	110.4	1,289	8	0.0048
TC122461	<i>M. truncatula</i>	16.607	4.95	102.0	1,346	7	0.0677

^a Accession numbers preceded by TC are from the *Medicago truncatula* Gene Index Release 10.0 (<http://compbio.dfci.harvard.edu>). Other accession numbers are from NCBI EST and NR databases (<http://blast.ncbi.nlm.nih.gov>)

^b For the *M. truncatula* ESTs, the percent EST frequency is based on 268,712 ESTs in the *M. truncatula* Gene Index. For the *M. sativa* ESTs, the percent EST frequency is based on 13,358 *M. sativa* ESTs in NCBI EST_others database

^c CAA56298, CAA56299, and AAD55099 refer to LIPR10.1A, LIPR10.1B, and LIPR10.1C, respectively

^d ADB44209 and CAC86467 refer to MsPR10.1A and MSPR10.1B, respectively

COLOR command. In PyMOL, the display of the surface form of the protein was modified to show only the cavity and pockets by selecting Setting > Surface > Cavities and Pockets (Culled). The surface was set to 30% transparency by the command set transparency to 0.3. The volume of the cavities with predicted entrances were determined with CASTp (<http://sts.bioengr.uic.edu/castp>) and viewed through PyMOL.

Hydrophobicity was determined in PyMOL using the “color_h” script obtained through PyMOL Wiki’s Script Library (<http://pymolwiki.org>). The code for the “color_h” script was saved in a text file (colorh.py), and then the script was used with the File > Run command in PyMOL. The script used amino acid hydropathicity values (Eisenberg et al. 1984) to color the hydrophobic residues and was modified so that more intense shades of orange represented more hydrophobic amino acids and more intense shades of green represented more polar amino acids. The images were saved as a PNG file with raytracing. A gray scale version of the image was created in Photoshop CS5 (Adobe, San Jose, CA) with orange converted to black, green converted to white and gray for intermediate values.

To determine the number of ESTs and their source cDNA libraries, *M. sativa* PR10 sequences were identified using the AA sequence as a query in tBLASTn against the NCBI EST and NR databases. The sequence was also used as a query against the *M. truncatula* Plant Gene Index

Release 9.0 to identify *M. truncatula* PR10 tentative consensus sequences and the identity of their ESTs. Sequences with an e-value less than $1.1e^{-29}$ were selected. The source libraries were determined from the GenBank accession description.

Harvesting, wounding, and heat treatments

For the harvesting treatment, aerial portions of six *M. sativa* plants were excised 4 cm above the crown, placed in aluminum foil, and incubated at 21°C for 0, 0.5, 3, 6, 12, and 24 h for RNA extraction. The remaining 4 cm of non-harvested plant tissue was excised from the same plants at the same times and incubated in the same manner.

Alfalfa leaves were wounded by making 4–5 parallel incisions with a razor blade lengthwise on attached leaves. The wounded leaves were harvested at 0, 0.5, 3, 6, 12, or 24 h for RNA extraction.

For the heat treatment, alfalfa plants grown at 21°C were moved to a growth chamber at 38°C for 0, 0.5, 3, 6, 12, or 24 h, and leaves were then harvested for RNA extraction.

ABA and ethylene treatments

A 100- μ M solution of ABA (\pm cis–trans–abscisic acid, Sigma A-1012) or distilled water as a control was sprayed on alfalfa plants until run-off, and leaf samples were

Table 2 Subclass II PR10 predicted amino acid sequences of *Medicago sativa*, *M. truncatula*, *Lupinus luteus*, and some of their characteristics

GenBank or gene index accession number ^a	Source	MW	pI	Angle in helix $\alpha 3^{\circ}$ (°)	Largest internal cavity with opening (\AA^3)	Number of β -bulges	EST frequency (%) ^b
AAF77633 ^c	<i>L. luteus</i>	16.904	4.95	58.0	208	6	–
AAF77634 ^c	<i>L. luteus</i>	16.888	4.81	84.3	2,182	7	–
CAA67375	<i>M. sativa</i>	16.644	4.59	57.7	195	6	–
CAC37690	<i>M. sativa</i>	16.656	4.59	57.7	195	6	–
CO512778	<i>M. sativa</i>	16.631	4.59	57.6	200	6	0.0075
AAB58315	<i>M. sativa</i>	16.628	4.59	57.5	201	6	–
CAC37691	<i>M. sativa</i>	16.829	4.84	83.2	2,221	7	–
TC147457	<i>M. truncatula</i>	16.615	4.59	56.7	202	7	0.0037
TC147835	<i>M. truncatula</i>	16.656	4.59	57.7	202	6	0.0026
TC147958	<i>M. truncatula</i>	16.656	4.59	57.7	202	6	0.0063
TC148514	<i>M. truncatula</i>	16.656	4.59	57.7	202	6	0.0662
TC152152	<i>M. truncatula</i>	16.656	4.59	57.7	202	6	0.0141
TC152425	<i>M. truncatula</i>	16.656	4.59	57.7	202	6	0.0030
TC161990	<i>M. truncatula</i>	16.656	4.59	57.6	202	6	0.0960
TC152734	<i>M. truncatula</i>	16.781	4.88	83.2	2,172	7	0.0123
TC155212	<i>M. truncatula</i>	16.757	4.88	83.2	2,174	6	0.0089
TC120093	<i>M. truncatula</i>	16.891	4.62	83.2	2,220	7	0.0238
TC169494	<i>M. truncatula</i>	16.712	4.83	83.2	2,255	7	0.0007
TC146207	<i>M. truncatula</i>	16.814	4.76	83.2	2,266	7	0.0089
TC118987	<i>M. truncatula</i>	16.749	4.73	83.1	2,299	7	0.0443

^a Accession numbers preceded by TC are from the *Medicago truncatula* Gene Index Release 10.0 (<http://compbio.dfci.harvard.edu>). Other accession numbers are from NCBI EST and NR databases (<http://blast.ncbi.nlm.nih.gov>)

^b For the *M. truncatula* ESTs, the percent EST frequency is based on 268,712 ESTs in the *M. truncatula* Gene Index. For the *M. sativa* ESTs, the percent EST frequency is based on 13,358 *M. sativa* ESTs in NCBI EST_others database

^c AAF77633 and AAF77634 refer to LIPR10.2A and LIPR10.2B, respectively

collected at 0, 0.5, 3, 6, 12, or 24 h for RNA extraction. Seedlings were also exposed to ABA by placing surface-sterilized seeds for 6 days on filter paper moistened with sterile dH₂O at 21°C, which was then replaced with filter paper moistened with 100 μ M ABA. After 0, 0.5, 3, 6, 12, or 24 h, four seedlings were collected for RNA extraction.

For the ethylene treatment, 10 mM Ethrel[®] (Bayer CropScience, Toronto, ON, Canada) in distilled water was sprayed until run-off onto alfalfa plants in a 18.5-L plastic container lined with a plastic bag, following which the bag was sealed tightly. Control plants were sprayed with distilled water and similarly sealed. At 0, 0.5, 3, 6, 12, or 24 h, leaves were harvested for RNA extraction.

Pathogen treatment

Cultures of *X. campestris* pv. *alfalfae* were grown in YDA medium (10 g/L glucose, 5 g/L yeast extract, and 5 g/L peptone (Difco, Detroit, MI, USA) at 30°C overnight, collected by centrifugation and resuspended in 10 mM MgCl₂ at a final concentration of 10⁷ cells/mL. Approximately 30 μ L of this bacterial suspension was infiltrated

into the abaxial surface of two fully developed leaves using a needleless syringe. Control leaves were similarly infiltrated with 10 mM MgCl₂. The infiltrated leaves were harvested at 0, 3, 6, 12, 24, and 48 HPI for RNA extraction.

Relative RT-PCR

After each treatment the plant material was immediately frozen in liquid nitrogen and stored at –70°C for RNA extraction following the method of Chen et al. (2000). Single-stranded cDNA was synthesized using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Invitrogen, Burlington, ON, Canada) and oligo (dT) primer with total RNA following the manufacturer's instructions.

To ensure specificity in the RT-PCR, the nt sequences of the PR10 genes from alfalfa were compared to find unique regions for gene-specific primers for *MsPR10.1A* (GU290205) and *MSPR10.1B* (AJ320476). Primers, HI7F1, 5'-TCTTGTCATATTGATCCTTG-3', and HI7Re1, 5'-AAA TAGCACTCAAACCTCAC-3', were designed to amplify 673 bp of *MsPR10.1A*. Primers, MSPR10.1BF1, 5'-GCA TATTAATCTTTTTTGTC-3', and MSPR10.1BRe1, 5'-A

CACCACTCAAACCGG-3', were designed to amplify 643 bp of *MsPR10.1B*. For a constitutive internal control for relative RT-PCR, primers MSC27F, 5'-GAATGGAATG TTGTGGGAGG-3', and MSCRe, 5'-ATTTGGTTGCTC CCTCAATG-3' were designed to amplify 300 bp of the alfalfa gene, *Msc27* (X63872), which encodes a protein with homology to a human tumor protein (TCTP) (Pay et al. 1992). This gene has been used similarly for RT-PCR analyses of alfalfa genes in several studies (Allison et al. 1993; Chinchilla et al. 2003; Rodriguez-Llorente et al. 2004).

For relative RT-PCR, 1.5 μ L cDNA, 0.25 μ L *Taq* polymerase (Invitrogen), 10 \times *Taq* polymerase buffer, 0.5 mM dNTPs, 1.5 mM MgCl₂, 0.1 μ M of MSC27F/ MSCRe, and 1 μ M of each primer for the gene of interest (*MsPR10.1A* or *MsPR10.1B*) were combined, and the final volume was adjusted to 20 μ L with distilled water. PCR conditions were 94°C for 3 min, 30 cycles of 94°C for 30 s, 53°C for 1 min, and 72°C for 1 min, with a final extension period of 10 min at 72°C. The RT-PCR products were separated in 1% TAE agarose gels containing 0.5 μ g/ml ethidium bromide. For gel band quantification, the RT-PCR products were separated as described above. Gel images were saved as tiff files and imported into NIH Image (Scion Corporation, Frederick, MD, USA). The band intensities were determined for both the gene of interest and *Msc27* in each lane, and relative expression was calculated from the ratio of the band intensity to that of *Msc27* (Dean et al. 2002). To determine that the number of amplification cycles was not too great for quantification, cDNA samples were also amplified with three fewer cycles, and the bands again quantified. The results with the different number of cycles were compared to demonstrate that the expression patterns were not significantly different. To confirm the identity of the RT-PCR product of *MsPR10.1A* and *MsPR10.1B*, the band obtained using cDNA from harvested tissue after 12 h incubation was excised from the gel, purified using the QIAquick PCR Purification Kit (QIAGEN, Mississauga, ON, Canada), and the purified product was sequenced at Laboratory Services Division, University of Guelph, Guelph, ON, Canada.

Cloning and sequence analysis of the *MsPR10.1A* promoter

Genomic DNA of *M. sativa* cv. Gala was extracted as described by Edwards et al. (1991). A 657-bp fragment of the *MsPR10.1A* promoter (GU290205) was amplified from genomic DNA using a forward primer 5'-TATTTAA GCTTCGGGCTGGTACTAAAG-3' with a *Hind*III restriction site at the 5' end, and a reverse primer 5'-TTTT CTAGAATATCAAGATAACAAGGATC-3' with a *Xba*I restriction site at the 5' end. The reverse primer was designed to match the +20 position in the 5' UTR of the gene as primer

extension data indicated that this was the transcription initiation site (Zhang 2004).

The promoter fragment was excised from pBluescript with *Hind*III and *Xba*I, and transcriptionally fused to the GUS gene in pBI101 (Datla et al. 1992), digested with the same enzymes. The resulting plasmid and pBI121.1 (Clontech, Palo Alto, CA, USA), a related vector containing the GUS gene controlled by the constitutive 35S promoter (35S-GUS), were transferred to *Agrobacterium tumefaciens* strain C58C1 and subsequently to *N. benthamiana* by the leaf disk co-cultivation method (Horsch et al. 1985). When regenerating plantlets developed roots, they were transferred to Sunshine LA4 Mix and grown under lights as described above.

Putative transgenic plants were tested for the presence of the fusion transgene by PCR using genomic DNA, extracted as described previously, and primers anchored in the promoter, HI7PF_{Forw}, 5'-GGGCTGGTACTAAAGTA TTAC-3', and within the GUS gene, GUS_{fusre}, 5'-GAT TTCACGGGTTGGGG-3'. The presence of the GUS transgene in the 35S-GUS plants was verified by PCR with primers GUS 156F, 5'-CGATCAGTTCGCCGATG-3', and GUS676R, 5'-CCCGCTAGTGCCTTGTC-3'. For the histochemical GUS assay, leaves and stems from plants at the eighth true-leaf stage were stained and cleared of chlorophyll according to the method of Jefferson (1987). The leaves of the transgenic plants and non-transgenic controls were wounded as described for alfalfa, and remained attached for 24 h after wounding before excision and extraction of RNA.

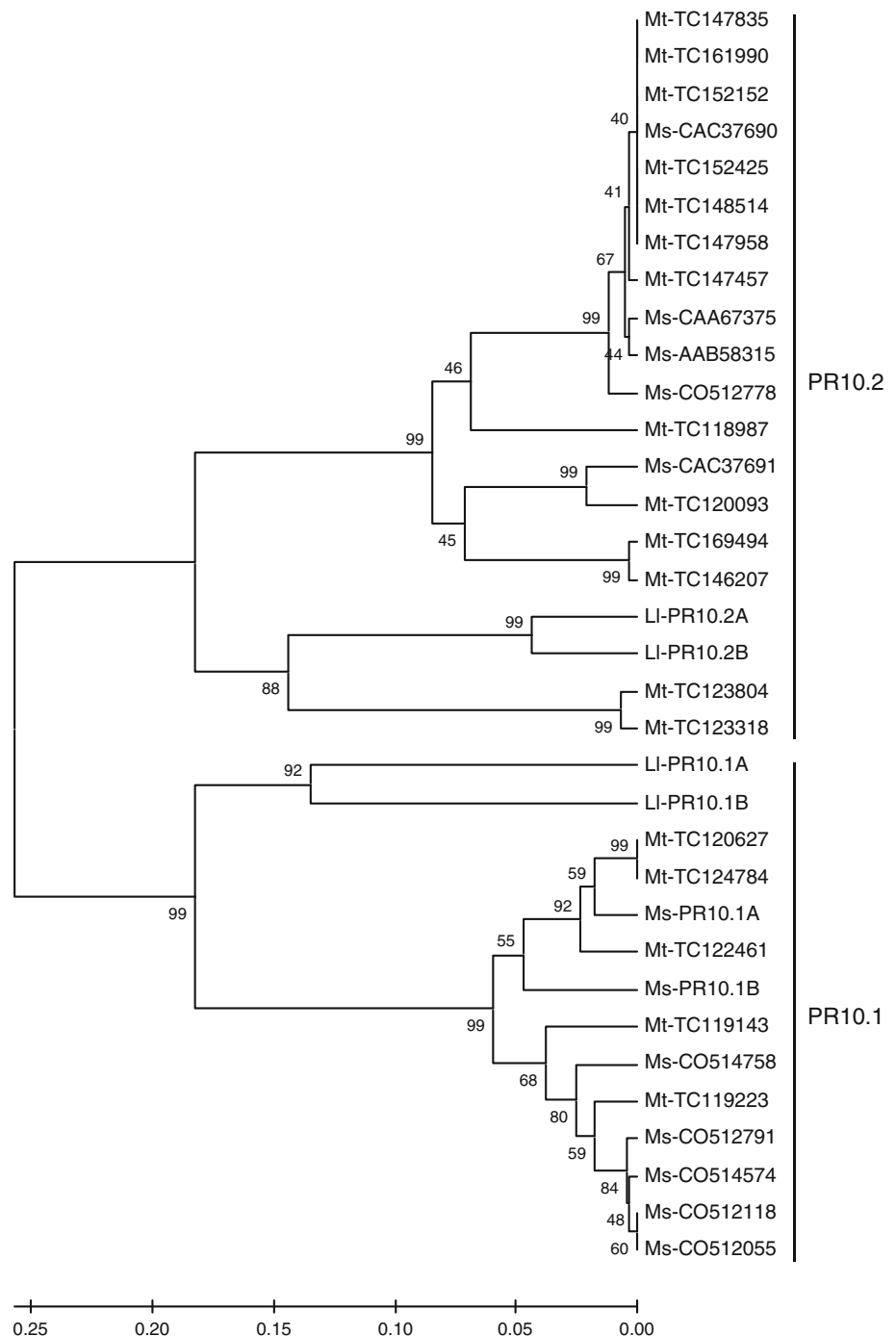
Results

Sequence comparison and putative structural features of *MsPR10.1A* compared to other *M. sativa* and *M. truncatula* predicted PR10 proteins

A comparison was made of 11 putative PR10 genes of *M. sativa* and 18 putative PR10 genes of *M. truncatula* (Tables 1, 2). The proteins ranged in molecular weight from 16.5 to 16.9 kDa. They were all relatively acidic proteins with *pI* ranging from 4.6 to 5.8. All the predicted proteins, except for CAC86467, possess a glycine-rich motif (G46-T52), E101, E148, and Y150, that is presumed to function as a nucleotide-binding site involved in the RNase activity of some PR10 proteins (Liu and Ekramoddoullah 2006). For CAC86467, there is a Lys rather than Glu at position 148.

A dendrogram of the *M. sativa* and *M. truncatula* predicted PR10 proteins revealed two clusters, and the *L. luteus* sequences, LIPR10.1A, LIPR10.1B, LIPR10.1C, LIPR10.2A, and LIPR10.2B, could be included in those

Fig. 1 Phylogenetic analysis of PR10 amino acid sequences from *Medicago sativa* (Ms–), *M. truncatula* (Mt–), and *Lupinus luteus* (Ll–). Multiple alignment was done using CLUSTALX, and the tree was constructed by the UPGMA method. The tree was drawn using MEGA4. The accession numbers are listed in Table 1. Subclass I (PR10.1) and subclass II (PR10.2) are indicated to the right of the tree



clusters (Fig. 1). Pasternak et al. (2005) had shown that the *L. luteus* sequences were divided into subclass I (LIPR10.1) and II (LIPR10.2). Sequences from *M. sativa* and *M. truncatula* were intermixed in the tree indicating a close relationship between the PR10 sequences of the two species. However, the *L. luteus* sequences clustered together in each subclass. In subclass I, MsPR10.1A clustered with two *M. truncatula* sequences, TC120627 and TC124784, but with low bootstrap support.

In order to determine how variation in the primary sequence might relate to variation in certain features of the putative three-dimensional structures, in silico models of the structures of the 29 *M. sativa* and *M. truncatula* PR10 sequences were made based on sequences that had been verified by crystallography in the Protein Data Bank (<http://www.rcsb.org>) and modeled using the automated mode at Swiss-Model, which requires relatively high target-template identity (<http://swissmodel.expasy.org>) (Tables 1,

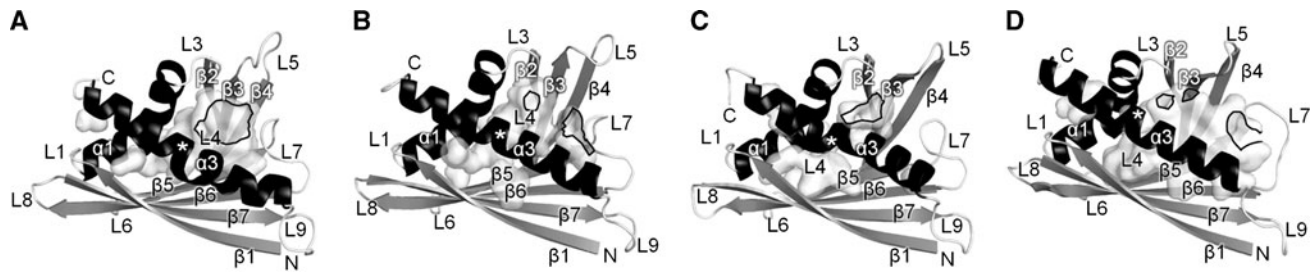


Fig. 2 Variance in the $\alpha 3$ helix and β -bulges in the three-dimensional structure of the predicted proteins of *MsPR10.1A* (a, e), *MsPR10.1B* (b, f), TC161990 (c, g), and TC118987 (d, h). The loops, β -strands, and α -helices are numbered as per LIPR-10.2B (Fernandes et al. 2009), except that there is no label for $\alpha 2$ and L2 as they were not

present in *MsPR10.1A*, *MsPR10.1B*, TC161990, and TC118987. The opening to the internal cavity is outlined in *black*. The *star* shows the location in $\alpha 3$, where there is either no kink (a, b), a kink pointing inwards (c) or a kink pointing outwards (d)

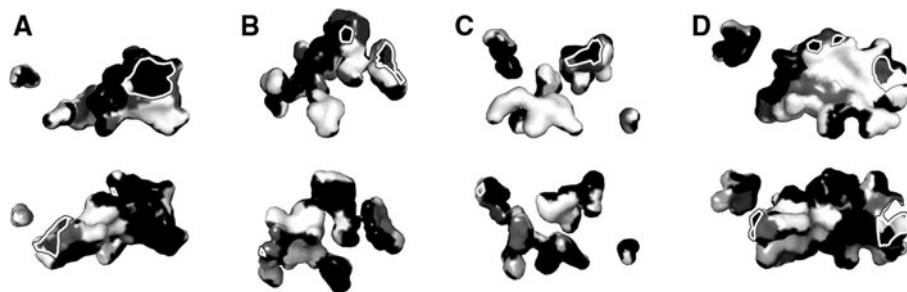


Fig. 3 Hydrophobicity in the predicted three-dimensional structure of the internal cavities of *MsPR10.1A* (a), *MsPR10.1B* (b), TC161990 (c), and TC118987 (d). The *front view* is the side with the hydrophobic internal cavity as shown in Fig. 2, and the *back view*

the reverse side. The intensity of *white* indicates the greater polarity of the residues, while the intensity of *black* indicates greater hydrophobicity of the residues. Entrances to the cavity are outlined in *white*

2). Features of the putative three-dimensional structures and hydrophobic internal cavities are shown in Figs. 2 and 3 for four examples of those sequences, *MsPR10.1A*, *MsPR10.1B*, TC131880, and TC118987.

Like other PR10 proteins, the PR10 proteins of *M. sativa* and *M. truncatula* have seven antiparallel β -strands forming a β -sheet with more variable regions linking them to two α -helices (Fig. 2). The helices and β -sheet surround a large cavity that has one or more openings (Figs. 2, 3). Pasternak et al. (2005) noted that one structural difference between the LIPR10.1 and LIPR10.2 proteins is the pattern of β -bulges, which distort the β -sheet to give it a curved shape. Seven β -bulges were found in LIPR10.1A and LIPR10.1B, and six were found in LIPR10.2A. For the *M. sativa* and *M. truncatula* PR10s, all the sequences in subclass I had seven or eight β -bulges, whereas all the sequences in subclass II had six or seven β -bulges (Tables 1, 2). The seven β -bulges matched those of Pasternak et al. (2005) and were common between all the sequences, but some subclass I sequences had an additional β -bulge, while some subclass II sequences were missing one β -bulge. Therefore, the number of predicted β -bulges does not appear to be a universal marker distinguishing subclasses I and II in *M. sativa* and *M. truncatula* PR10s.

The C-terminal helix $\alpha 3$ is the most divergent structural feature of PR10 proteins (Fernandes et al. 2008). Pasternak et al. (2005) noted that the C-terminal helix $\alpha 3$ of the subclass I proteins, LIPR10.1A or LIPR10.1B, had angles of 101.5°, and 109.7°, respectively, at Leu141. All of the subclass I PR10 proteins from *M. sativa* and *M. truncatula* had similar angles (102.0°–110.5°) at the corresponding location in the helix $\alpha 3$, Leu143, and had a similar sized internal cavity (Table 1; Fig. 2; Supplemental Fig. 1).

The C-terminal helix $\alpha 3$ of LIPR10.2A has a severe distortion in its central region, which creates a kink of 58.0° at Phe142 resulting in a reduction in the central internal cavity (Pasternak et al. 2005). For the PR10 proteins in subclass II of *M. sativa* and *M. truncatula*, all had a kink at residue 142 or 143 (Table 2; Fig. 2). However, the subclass II proteins of *M. sativa* and *M. truncatula* could be divided into two groups based on the direction of the kink. One group was similar to LIPR10.2A with a predicted kink of 56.7°–57.7° inwards to the internal cavity at Leu143 resulting in a drastic reduction in the predicted internal cavity. For those proteins, the volume of the largest predicted internal cavity with an opening ranged from 195 to 202 Å³ (Table 2; Fig. 3; Supplemental Fig. 2). A second group had a predicted kink of 83.1°–83.2° outwards from

the internal cavity at Gly142 resulting in a relatively large predicted internal cavity, which ranged in volume from 2,172 to 2,299 Å³ (Table 2; Fig. 3; Supplemental Fig. 3). The outward kink was also observed for LIPR10.2B, which had a kink of 84.3° projecting away from the cavity at Ala141, and Fernandes et al. (2008) noted that LIPR10.2B had an internal cavity with an unusually large volume. The predicted volumes of the *M. sativa* and *M. truncatula* PR10 proteins should be considered estimates that are relative to each other rather than absolute values.

The internal cavity of PR10 proteins is described as being surrounded by hydrophobic residues but with a few polar residues, and both can be involved in ligand binding (e.g., Fernandes et al. 2008). For *MsPR10.1A*, *MsPR10.1B*, TC131880, and TC118987, the walls of the internal cavity of are primarily hydrophobic, but do have some hydrophilic regions (Fig. 3). In general, the walls of subclass I proteins of *M. sativa* and *M. truncatula* are primarily hydrophobic with hydrophilic regions being mostly in side chambers (Fig. 3a, b; Supplemental Fig. 1). However, subclass II proteins of *M. sativa* and *M. truncatula* appear to have a higher percentage of the cavity wall with hydrophilic regions, typically with part of one side of the cavity being hydrophobic and part of the other side being slightly to strongly hydrophilic (Fig. 3c, d; Supplemental Figs. 2, 3). Because of its larger cavity, this is more obvious for the subclass II proteins with an outward-pointing kink in the C-terminal helix $\alpha 3$ (Fig. 3c). Similar patterns were observed for the hydrophobic and hydrophilic regions walls of the internal cavities of LIPR10.1A, LIPR10.1B, LIPR10.2A, and LIPR10.2B in the Protein Data Bank, whose structures have been verified by crystallography (data not shown).

The direction of the kink in the helix $\alpha 3$ generally correlated with the number of β -bulges, with proteins with an inward kink having six β -bulges and proteins having an outward kink having seven β -bulges. The exceptions are TC147457, which had an inward kink and seven β -bulges, and TC155212, which had an outward kink and six β -bulges. The division of the sequences based on the number of β -bulges or kink angle in the C-terminal helix $\alpha 3$ did not correlate with the clusters observed within the subclasses in the tree (Fig. 1). Although the primary sequence of *MsPR10.1A* was more similar to that of *MsPR10.1B* among the *M. sativa* subclass I predicted proteins (Fig. 1), *MsPR10.1A* had seven β -bulges, whereas *MsPR10.1B* had eight β -bulges. They also differed in the shape of the internal cavities (Fig. 3).

Abundance of ESTs for PR10 genes of *M. sativa* and *M. truncatula*

A total of 1,121 ESTs for PR10 genes were identified from the 259,642 ESTs in the *M. truncatula* Gene Index with most of these (855 ESTs) belonging to subclass II sequences

(Table 1). Among the subclass I genes, the abundance of TC122461, which is the only *M. truncatula* sequence in subclass I with no putative kink in the helix $\alpha 3$ and seven β -bulges, was by far the most abundant comprising 74% of the subclass I ESTs. The next subclass I sequences in abundance of ESTs were approximately ten times lower. ESTs of TC122461 were most common in roots, but they were also found in leaves, flowers, and other tissues (Supplemental Table 1). It appeared that TC122561 was expressed under a variety of abiotic stresses, including drought and phosphate starvation. However, it was also expressed in many biotic associations, such as leaves infected with Alfalfa Mosaic Virus or *Colletotrichum trifolii* and roots infected with *Phytophthora medicaginis*, *Aphanomyces euteiches*, *Sinorhizobium meliloti*, *Glomus versiforme*, or *Meloidogyne incognita*. Although harvesting or wounding were not among the treatments for the cDNA libraries of *M. truncatula*, it appears that expression of TC122461 also occurred during stresses that result in cell death, like elicitors and necrotrophic pathogens. *MsPR10.1A* is the closest homolog of TC122461 in *M. sativa* (Supplemental Table 1).

Among the subclass II genes, 70% of the ESTs were for proteins with a putative kink inwards in the helix $\alpha 3$ and six β -bulges, but this was dominated by just one gene, TC131880, which included 87% of the ESTs of the “inward kink” group (Table 1). The next most abundant subclass II gene was TC117333, but it had about five times fewer ESTs. TC117333 had a putative kink outwards and seven β -bulges. TC131880 appeared to be expressed under similar conditions as TC117333 with many of the ESTs from cDNA libraries for root-associated abiotic stress and biotic interactions (Supplemental Table 2). One difference of TC131880 from all the other *M. truncatula* PR10 sequences was that it was much more expressed in irradiated seedlings and roots infected with the mycorrhizal fungus, *Glomus intraradices*. However, in general, there was no obvious distinguishing feature between the EST library sources for subclass I versus subclass II proteins in terms of tissue types, abiotic or biotic stresses.

The EST abundance indicates that expression can occur under a variety of conditions, and expression levels of members of the subclass I and II sequences can differ considerably, even between even closely related PR10 genes. A direct comparison was made between the patterns of expression of two subclass I genes, *MsPR10.1A* and *MsPR10.1B*, following treatments with various abiotic stresses, signaling molecules and pathogen attack.

Expression of *MsPR10.1A* and *MsPR10.1B* following harvesting, wounding, and heat

Expression of *MsPR10.1A* significantly increased in harvested tissues (stems, petioles and leaves) by 3 h after

harvesting, peaking at 12 h, and then declining slightly by 24 h (Fig. 4a). In contrast, expression of *MsPR10.1B* increased much more rapidly than *MsPR10.1A* with a large increase by 0.5 h after harvesting, peaking at 3 h, and then remaining relatively constant. In the above-ground, non-harvested tissue (stems), expression of *MsPR10.1A* was much lower than in the harvested tissue (Fig. 4b). Another difference from *MsPR10.1A* is that *MsPR10.1B* expression was significantly induced in the non-harvested tissues by 0.5 h and remained high until 12 h, decreasing by 24 h to pre-treatment levels.

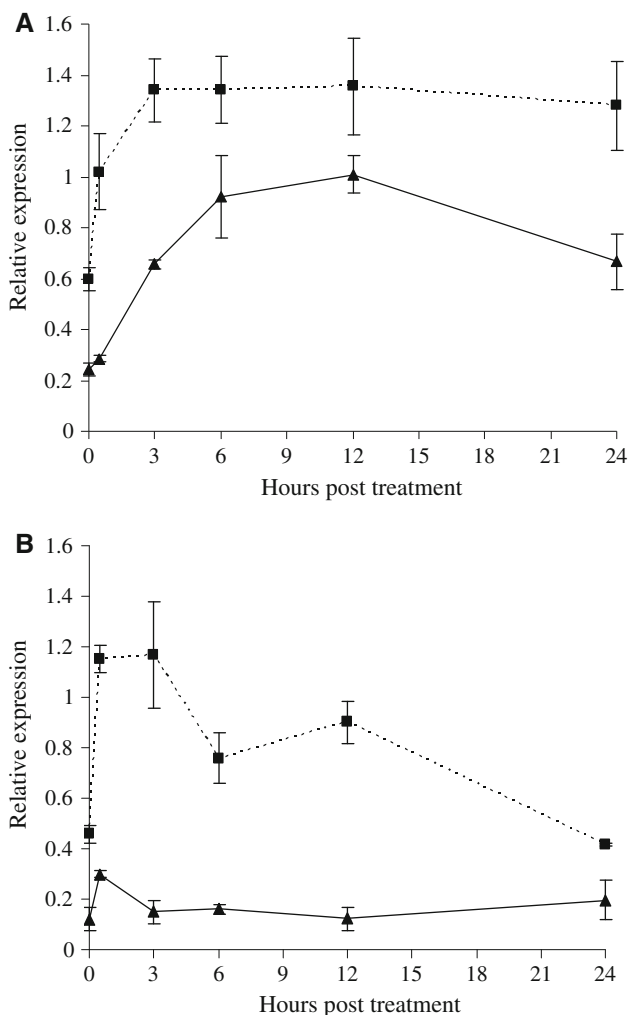


Fig. 4 Relative RT-PCR of *MsPR10.1A* and *MsPR10.1B* in alfalfa tissues following harvesting treatment. **a** Expression of *MsPR10.1A* and *MsPR10.1B* in harvested tissues. **b** Expression of *MsPR10.1A* and *MsPR10.1B* in remaining non-harvested portions of plants. The levels of *MsPR10.1A* and *MsPR10.1B* mRNA were determined relative to the expression of constitutive internal control *Msc27*. Means are plotted with *standard error bars* based on three replications (*MsPR10.1A* filled triangle with *solid line* and *MsPR10.1B* filled square with *dotted line*)

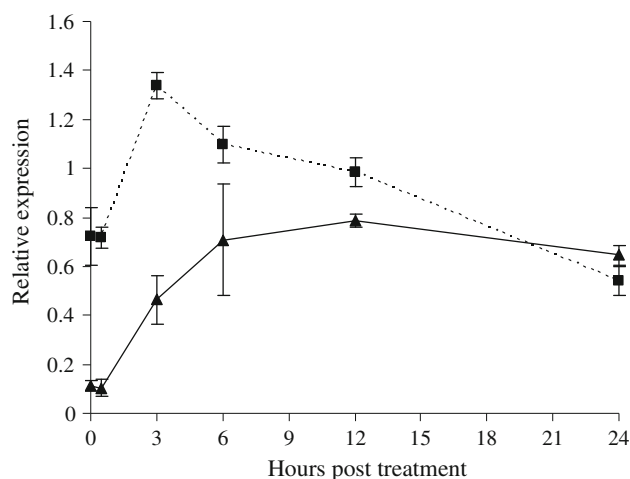


Fig. 5 Relative RT-PCR of *MsPR10.1A* and *MsPR10.1B* in alfalfa leaves following wounding. The levels of *MsPR10.1A* and *MsPR10.1B* mRNA were determined relative to the expression of constitutive internal control *Msc27*. Means are shown with *standard error bars* based on three replications (*MsPR10.1A* filled triangle with *solid line* and *MsPR10.1B* filled square with *dotted line*)

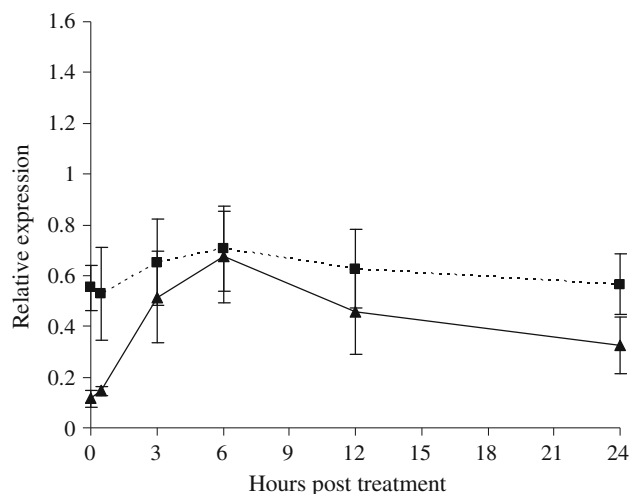


Fig. 6 Relative RT-PCR of *MsPR10.1A* and *MsPR10.1B* in heat-treated leaves. The levels of *MsPR10.1A* and *MsPR10.1B* mRNA were determined relative to the expression of constitutive internal control *Msc27*. Means are shown with *standard error bars* based on three replications (*MsPR10.1A* filled triangle with *solid line* and *MsPR10.1B* filled square with *dotted line*)

Expression of *MsPR10.1A* in wounded leaves was similar to that in harvested tissue with a significant increase at 3 h (Fig. 5). Expression of *MsPR10.1B* also showed a significant increase at 3 h, but then a gradual decrease to 24 h.

The heat treatment significantly elevated expression levels of *MsPR10.1A*, which peaked at 6 h, and then remained unchanged to 24 h. In contrast, *MsPR10.1B* showed a slight but not significant induction by heat (Fig. 6).

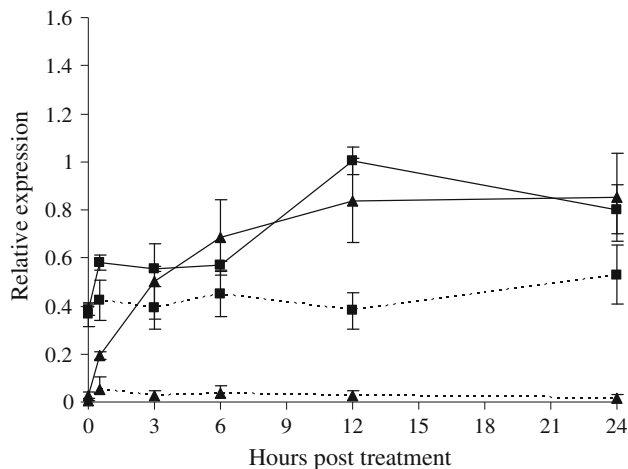


Fig. 7 Relative RT-PCR of *MsPR10.1A* and *MsPR10.1B* in alfalfa leaves following ethylene treatment using 10 mM Ethrel. Control plants were sprayed with distilled water as with Ethrel. The quantities of *MsPR10.1A* and *MSPR10.1B* mRNA levels were determined relative to the expression of constitutive internal control *Msc27*. Means are shown with *standard error bars* based on three replications (*MsPR10.1A* ethylene filled triangle with solid line, *MsPR10.1A* water filled triangle with dotted line, *MsPR10.1B* ethylene filled square with solid line, and *MsPR10.1B* water filled square with dotted line)

Expression of *MsPR10.1A* and *MsPR10.1B* following ethylene and ABA

MsPR10.1A was significantly induced by 0.5 h after the ethylene treatment and continued to increase until 12 h, while expression levels in water-sprayed control leaves remained low and did not change significantly (Fig. 7). By 12 h, expression was approximately 16 times greater than that of the water control. The expression of *MsPR10.1B* also significantly increased after 0.5 h, but then remained unchanged until a second increase by 12 h after ethylene treatment when it was approximately twice that of the water control.

In plants sprayed with ABA, the expression level of *MsPR10.1B* was significantly increased by 0.5 h, whereas the response by *MsPR10.1A* was slower (Fig. 8a). Expression of both genes reached a maximum at 24 h. In germinating seeds transferred to medium containing ABA, both *MsPR10.1A* and *MsPR10.1B* mRNA levels increased, but in this case, *MsPR10.1A* expression was higher at 0.5 h (Fig. 8b). Expression of *MsPR10.1A* significantly declined at 24 h.

Expression of *MsPR10.1A* and *MsPR10.1B* following inoculation with *Xanthomonas campestris* pv. *alfalfae*

Inoculation of alfalfa leaves with the compatible pathogenic bacterium, *X. campestris* pv. *alfalfae*, resulted in a

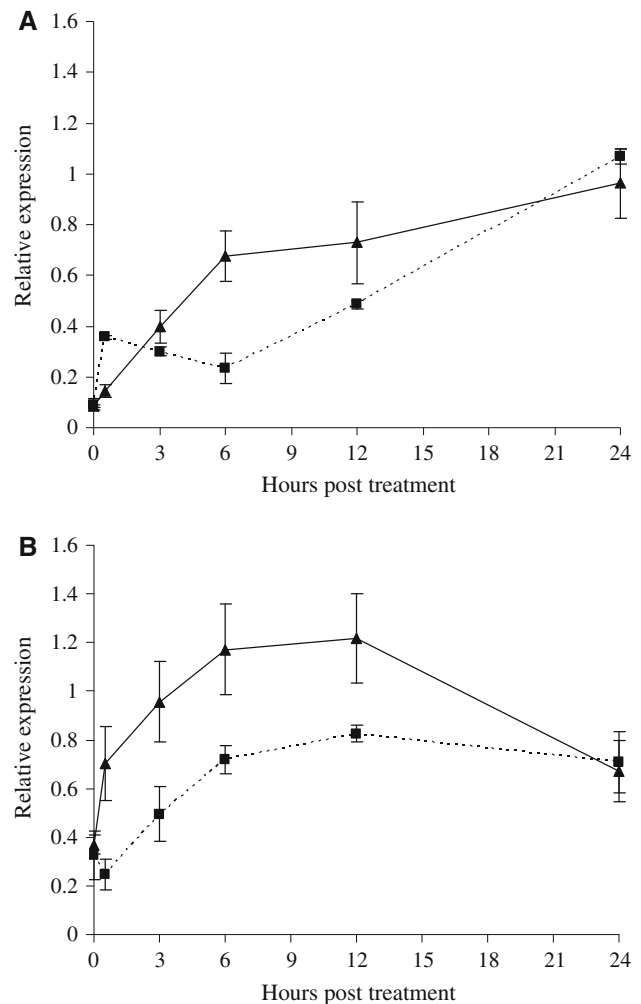


Fig. 8 Relative RT-PCR of *MsPR10.1A* and *MsPR10.1B* in alfalfa after ABA treatments. **a** Expression of *MsPR10.1A* and *MsPR10.1B* in leaves following ABA spray. **b** Expression of *MsPR10.1A* and *MsPR10.1B* in alfalfa seedlings following transfer to media containing ABA. The levels of *MsPR10.1A* and *MsPR10.1B* mRNA were determined relative to the expression of constitutive internal control *Msc27*. Means are shown with *standard error bars* based on three replications (*MsPR10.1A* filled triangle with solid line and *MSPR10.1B* filled square with dotted line)

much greater elevation of *MsPR10.1A* expression at 3 h than that of *MsPR10.1B* (Fig. 9). *MsPR10.1A* expression peaked at 12 h, but *MsPR10.1B* expression continued to increase to 48 h. Thus, it appears that *MsPR10.1B* expression progressively increased with the continuing growth of the bacteria in the leaves, while expression of *MsPR10.1A* increased mostly due to the early stages of infection. As the development of water-soaked lesions was observed at approximately 96 h, both *MsPR10.1A* and *MsPR10.1B* expression showed a rapid response to the pathogen beginning well before symptoms appeared. In the $MgCl_2$ -treated control plants, there was a marginal induction of both *MsPR10.1A* and *MsPR10.1B* by 3 h, perhaps

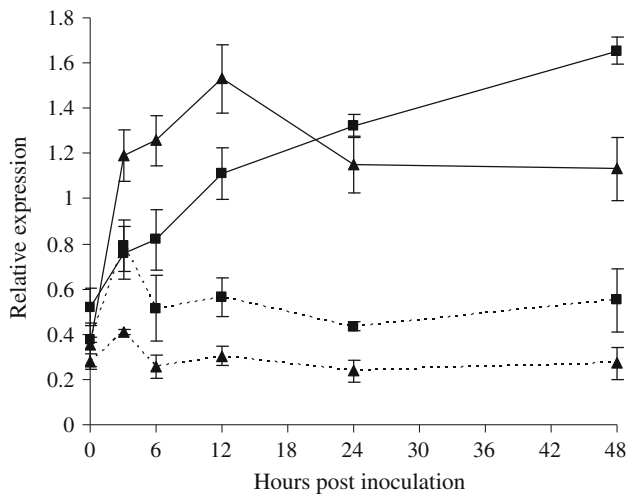


Fig. 9 Relative RT-PCR of *MsPR10.1A* and *MsPR10.1B* in alfalfa leaves inoculated with *Xanthomonas campestris* pv. *alfalfae*. Control plants were injected with 10 mM MgCl₂. The levels of *MsPR10.1A* and *MsPR10.1B* mRNA were determined relative to the expression of constitutive internal control *Msc27*. Means are shown with standard error bars based on three replications (*MsPR10.1A* *Xanthomonas campestris* pv. *alfalfae* filled triangle with solid line, *MsPR10.1A* MgCl₂ filled triangle with dotted line, *MsPR10.1B* *Xanthomonas campestris* pv. *alfalfa* filled square with solid line, and *MsPR10.1B* MgCl₂ filled square with dotted line)

due to the effects of infiltration, but transcript levels quickly returned to pre-inoculation levels and remained constant throughout the remainder of the experiment.

Analysis of transgenic *N. benthamiana* plants containing the *MsPR10.1A-GUS* fusion

According to primer extension data (Zhang 2004), transcription of *MsPR10.1A* begins at G in the sequence, ATTGATCCT. The PLACE database (<http://www.dna.affrc.go.jp/PLACE/>) of plant *cis*-acting regulatory elements identified a TATA box at –36 bp upstream of that start site. Within the first 621 bp upstream of the TATA box, a total of 67 *cis*-acting regulatory elements were identified using the PLACE database. Among those were seven *cis*-elements for ABA response, ten for dehydration, one each for wounding and heat shock, as well as three WRKY elements, and seven associated with various defense responses. However, no *cis*-elements related to ethylene responses were identified.

A total of 35 kanamycin-resistant plants were regenerated that contained the 657 bp *MsPR10.1A* promoter-*GUS* fusion construct based on PCR detection of the *MsPR10.1A* promoter and *GUS* gene. As controls, an additional four kanamycin-resistant plants containing a 35S-*GUS* construct were regenerated, and verified by PCR detection of a fragment spanning the 35S promoter and *GUS* gene. All

regenerated plants appeared morphologically normal compared to non-transgenic plants. All the transgenic lines were self-pollinated, and the resulting seeds were germinated on media containing kanamycin. Histochemical staining of the *MsPR10.1A-GUS* construct lines showed that line 16 exhibited the highest *GUS* activity, and line 16 was subsequently used for comparison with the non-transgenic control along with line 2 expressing the 35S-*GUS* construct at the highest level.

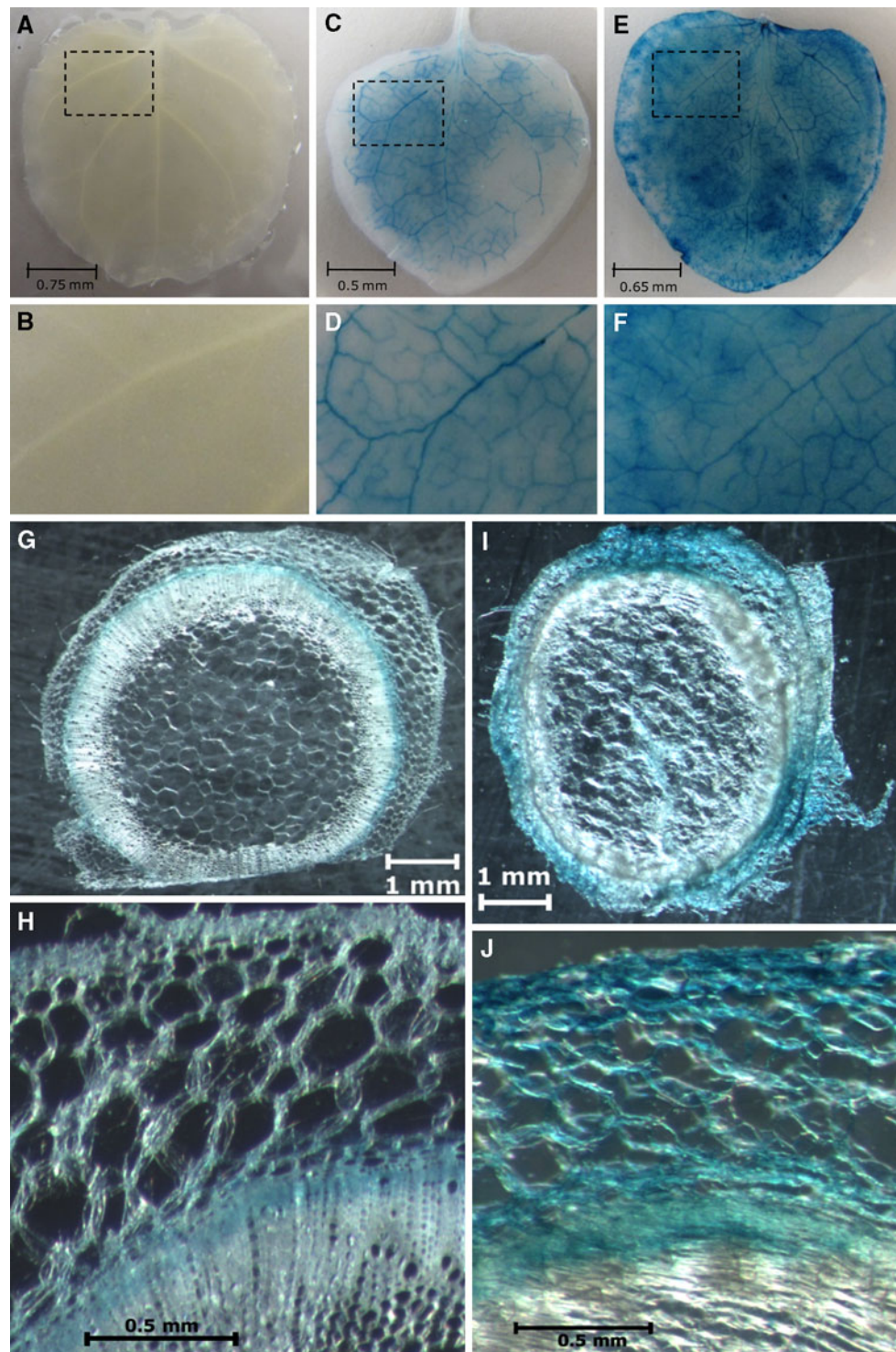
No blue staining for *GUS* activity was evident in any tissue of the non-transgenic control plants (Fig. 10a, b). Leaves containing *MsPR10.1A-GUS* displayed staining that was restricted largely to the veins (Fig. 10c, d). By comparison, leaves from plants containing 35S-*GUS* also showed strong *GUS* activity in the veins together with other tissues (Fig. 10e, f). More specifically, the promoter of *MsPR10.1A* appears to direct expression to the phloem tissues as cross-sections of the stem from the *MsPR10.1A-GUS* plants revealed a restricted ring of expression correlating with the phloem tissue (Fig. 10g, h), whereas the staining in the 35S-*GUS* plants was much more widespread throughout the stem (Fig. 10i, j).

Wounding of *MsPR10.1A-GUS* leaves also resulted in *GUS* gene expression predominantly in veins (Fig. 11a), whereas control 35S-*GUS* leaves did not show *GUS* activity (Fig. 11b).

Discussion

For both *M. sativa* and *M. truncatula*, two subclasses of PR10 AA sequences were observed. Thus, it appears that subclasses are common among members of the Fabaceae as this was also observed for pea and yellow lupin (Tewari et al. 2003; Pasternak et al. 2005). In silico analysis of the predicted structures of the proteins modeled on similar sequences in the Protein Data Bank showed that a key structural difference between subclasses I and II is a kink in the AA chain resulting in a deformation of the helix α 3 in all members of subclass II. This predicted kink either reduced or increased the size of the internal cavity compared to the subclass I proteins, and Pasternak et al. (2005) proposed that changes in the shape and size of the internal cavity provided a way to regulate the specificity of PR10 proteins toward their ligands and thus their biological role. Both *MsPR10.1A* and *MsPR10.1B* belong to subclass I and are quite similar in their AA sequence. They have a similar sized internal cavity, but they differ in the predicted shape of the internal cavity as well as the number of β -bulges. The significance of these differences is not clear, but could reflect their roles in the plant. It is important to note that all the three-dimensional structures of the PR10 sequences in this study are speculative structures as they are based on

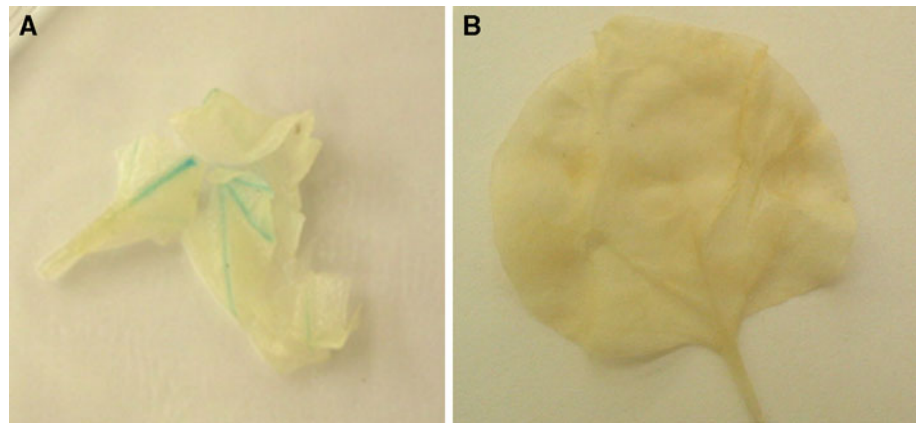
Fig. 10 Expression of GUS gene fusions in transgenic *N. benthamiana* plants. Expression pattern in leaves from non-transgenic control plant (a) with enlarged view (b), transgenic plants containing the *MsPR10.1A* promoter-GUS construct (c) with enlarged view (d), and transgenic plants containing the 35S promoter-GUS construct (e) with enlarged view (f). Expression pattern in stem sections of the GUS gene regulated by the *MsPR10.1A* promoter (g) with enlarged view (h), and the 35S promoter (i) with enlarged view (j)



predictions using similar sequences that had been determined by crystallography. The purpose of comparing PR10 sequences was not to determine *in vivo* protein conformation, but to examine potential changes to the protein structures due to primary sequence variation within this gene family.

PR10 proteins are proposed to have a wide range of possible roles in plants (Liu and Ekramoddoullah 2006). They have direct antiviral activity, as was observed against TMV by CaPR10 in *C. annuum* (Park et al. 2004), or direct antibacterial and antifungal activities, as was observed against *A. tumefaciens*, *Phytophthora cinnamomi*, *Fusarium*

Fig. 11 Histochemical localization of GUS activity after wounding of transgenic *N. benthamiana*. *N. benthamiana* transgenic plant with *MsPR10.1A::GUS* construct were wounded by making 4–5 parallel incisions with a razor blade lengthwise on attached leaves (a) or were not wounded *N. benthamiana* (b). Transgenic plant with *MsPR10.1A::GUS* construct as control



oxysporum, *Rhizoctonia solani*, and *Nectria hematococcus* by ocatin in *Oxalis tuberosa* (Flores et al. 2002). However, no activity was observed for LIPR10.2B against three different plant pathogenic fungi (Fernandes et al. 2009). PR10 proteins may also have a role in dehydration stress acting as cryoprotectants, perhaps due to their structural similarity to dehydrin (Liu and Ekramoddoullah 2006). However, most of the evidence is that PR10 primarily functions in ligand binding in stress response signaling. This includes binding with cytokinins, fatty acids, flavonoids, and brassinosteroids (Fujimoto et al. 1998; Mogensen et al. 2002; Markovic-Housley et al. 2003). For example, both natural and synthetic cytokinins were observed to bind to the relatively large internal cavity of LIPR10.2B, and it was proposed that PR10 proteins show general rather than specific ligand binding with both the PR10 and ligand showing mutual conformational adjustments permitting transport of different hydrophobic ligands from the cytosol to their receptors (Fernandes et al. 2008, 2009). On the other hand, some PR10 proteins are reported to have enzymatic activities, such as *HpHyp-1* from *Hypericum perforatum* that encodes a PR10 enzyme for synthesis of hypericin (Bais et al. 2003). However, Michalska et al. (2010) were unable to show that the Hyp-1 protein had enzymatic activity although it did bind polyethylene glycol molecules to the internal cavity, and they suggested that Hyp-1 may bind hypericin for storage or transport supporting the idea that the biological activity of PR10 proteins is ligand binding. The ability of PR10 proteins to make conformational adjustments may help explain why there is not a wider range of putative structures observed among the full-length predicted PR10 protein structures in *Medicago* spp. and determine if there is a relationship between structural features, like the deformation of $\alpha 3$ helix and number of β -bulges, to particular biological activities.

An examination of the collection of *M. truncatula* ESTs for PR10 proteins indicates that there are a fairly large

number of PR10 genes, but it is mostly a single gene in subclass I, TC122461, and subclass II, TC118987, that are predominately expressed. However, for all the PR10 genes, ESTs related to cDNA libraries based on microbial infections and different abiotic stresses were common with considerable overlap between the PR10 genes in terms of treatments and tissues from which the sequences were obtained.

The expression pattern of *MsPR10.1A* showed a slower response than did *MsPR10.1B* in harvested tissue. Harvesting constitutes a unique combination of stresses, including wounding, dehydration, and separation from nutrient supply, which results in changes in metabolism and protein content (Davies and King 1993). However, Ferullo et al. (1996) demonstrated that alfalfa plants synthesize proteins that are specific to harvesting and not synthesized by wounding or water stress treatments alone. This would not include *MsPR10.1A* and *MsPR10.1B* as their expression increased for both wounding and harvesting.

Although both genes responded to harvesting, *MsPR10.1A* mRNA levels were only induced transiently in non-harvested tissue and were much lower than those reached in harvested tissue. However, the expression pattern of *MsPR10.1B* was fairly similar in both harvested and non-harvested tissue. This indicates that *MsPR10.1B* expression may be affected by some sort of signal generated by harvesting that spreads throughout the plant, whereas *MsPR10.1A* expression is not. This may be an advantage for using the *MsPR10.1A* promoter to limit expression to only harvested tissues and restrict expression in the remaining non-harvested plant tissues.

In wounded leaves of *M. sativa*, expression of *MsPR10.1A* showed a more gradual increase than that of *MsPR10.1B*. Reynolds (1999) proposed that proteins expressed early after wounding are important for sealing wound tissue and activating defensive compounds, while proteins expressed late after wounding are important for recovering from the wound. It may be, therefore, that *MsPR10.1B* is more important for such early responses

compared to *MsPR10.1A*. Many other genes for PR10 proteins are induced by wounding, such as *AoPRI* in asparagus (Warner et al. 1993), *PR10a* in potato (Constabel and Brisson 1995), *PR10a* and *PR10c* in birch (Poupard et al. 1998), *PmPR10-1.13* and *PmPR10-1.14* in western white pine (Liu et al. 2005), and *MsPR10.1B* in alfalfa (Borsics and Lados 2002). The frequent association of PR10 gene expression with wounding may be due to a role in defense against microorganisms that infect through wounds (Reynolds 1999).

In contrast, expression of *MsPR10.1A* was much more responsive to ethylene compared to the control than was expression of *MsPR10.1B* compared to the control. Signal transduction after wounding goes through an ethylene and/or JA-dependent pathway (Thaler et al. 2004), and increased expression of PR10 genes, such as *OsPR10a* from rice (Jwa et al. 2006) and *Pg1* from ginseng (Yasnetskaya et al. 2003), following ethylene treatment has been observed (Jwa et al. 2006). While expression of both genes was induced by ethylene, the faster response of *MsPR10.1B* expression to harvesting and wounding cannot be explained by a faster response to ethylene, and thus other factors may be involved.

These two genes also differed in their expression response to heat, with *MsPR10.1A* showing strong early induction while *MsPR10.1B* remaining unchanged. However, increased expression of *MsPR10.1B* was observed when alfalfa plants were treated at 42°C for 3 h (Borsics and Lados 2002). The failure of this study to observe increased expression of *MsPR10.1B* may be due to the use of 38°C rather than 42°C or perhaps due to a difference in cultivars as *M. sativa* cv. Gala was used in these experiments, while *M. sativa* cv. Verko was used by Borsics and Lados (2002).

Like the response to ethylene, expression of *MsPR10.1A* was more responsive than *MsPR10.1B* to ABA. ABA is a plant hormone that regulates processes such as embryo maturation, seed development and germination, cell division and elongation, stomatal opening, root development, floral transition, and tolerance to abiotic and biotic stress (Finkelstein et al. 2002). In addition to induction of *MsPR10.1A* and *MsPR10.1B*, ABA treatment is reported to induce a PR-10 gene of *Lilium longiflorum* (Wang et al. 1999). This may be related to an increase in ABA during drought and salt stresses. In *Arabidopsis*, knock-out mutants with defects in ABA biosynthesis have impaired drought tolerance, which shows the importance of ABA to this stress (Iuchi et al. 2001). As dehydration is a major stress in harvested tissue, some of the harvest inducibility of *MsPR10.1A* and *MsPR10.1B* could be related to ABA.

Expression of *MsPR10.1A* and *MsPR10.1B* significantly increased after alfalfa leaves were inoculated with *X. campestris* pv. *alfalfae*, a compatible pathogen in alfalfa, which causes water-soaked lesions leading to necrosis

(Esnault et al. 1993). However, expression of *MsPR10.1A* responded much faster than *MsPR10.1B*, which was previously shown to be induced by *X. campestris* pv. *alfalfae* (Borsics and Lados 2002). In contrast, the alfalfa PR10 gene, AAB41557, was not affected by *X. campestris* pv. *alfalfae* inoculation (Esnault et al. 1993). Most examples of PR10 genes being induced following bacterial inoculations are for incompatible bacteria inoculations, such as a PR10 gene in *Vitis vinifera* that was induced by *Pseudomonas syringae* pv. *pisi* (Robert et al. 2001), LIPR10.1A in yellow lupin that was induced by *Pseudomonas syringae* (Sikorski et al. 1999), AAB41557 in alfalfa that was induced by *P. syringae* pv. *pisi* (Esnault et al. 1993), and *MsPR10.1B* in alfalfa that was induced by *P. syringae* pv. *pisi* (Borsics and Lados 2002).

Transgenic *N. benthamiana* plants containing the *MsPR10.1A-GUS* fusion showed some GUS activity in mesophyll tissues but predominantly in the veins of leaves and stems. This is similar to the promoter of the PR10 gene, YPR-10, from *G. max* that was fused to the GUS reporter gene, transformed into *Nicotiana tabacum* and showed GUS activity in vascular tissues (Walter et al. 1996). Breda et al. (1996) demonstrated by in situ hybridization that a subclass II PR 10 gene *MsPR10-1* (CAC37690) from alfalfa was expressed in vascular bundles and mesophyll tissues adjacent and distant from the inoculation site on the same leaf following inoculation with *P. syringae* pv. *pisi*.

Localization of expression of other defense-related genes to vascular tissue has been reported. For example, in *Capsicum annuum*, mRNA of the defensin gene, *CADEF1*, was localized to the phloem in leaves treated with SA, MeJA, and ABA (Do et al. 2004). Expression of the *C. annuum* transcription factor gene, *CARAVI*, was localized to the phloem of leaves during infection by *Xanthomonas campestris* pv. *vesicatoria* and ethylene treatment (Sohn et al. 2006). Moreover, transcripts of a *C. annuum* gene encoding a protein related to acquired disease resistance, *CASAR82A*, were localized to phloem and epidermal cells of leaves and stems infected by *Colletotrichum coccodes* and *Phytophthora capsici*, or treated with SA (Lee and Hwang 2003).

The many similarities in the expression patterns of *MsPR10.1A* and *MsPR10.1B* to treatments described in this work imply that they may have similar functions related to stress responses. However, the two related genes have some significant differences in expression, such as their speed of response to wounding and harvesting or their ability to respond to heat or in tissues remaining after harvesting. Those may indicate important differences in terms of plant signaling as well as their potential biotechnology applications for using the promoters of PR10 genes for directing transgene expression.

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