GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Molecular cloning and characterization of two novel *NAC* genes from *Mikania micrantha* (Asteraceae)

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Genet. Mol. Res. 11 (4): 4383-4401 (2012)
Received March 4, 2012
Accepted June 22, 2012
Published September 19, 2012
DOI http://dx.doi.org/10.4238/2012.September.19.3

ABSTRACT. NAC proteins, which are plant-specific transcription factors, have been identified to play important roles in plant response to stresses and in plant development. The full-length cDNAs that encode 2 putative NAC proteins, designated as *MmATAF1* and *MmNAP*, respectively, were cloned from *Mikania micrantha* by rapid amplification of cDNA ends. The full-length cDNAs of *MmATAF1* and *MmNAP* were 1329 and 1072 bp, respectively, and they encoded deduced proteins of 260- and 278-amino acid residues, respectively. The proteins MmATAF1 and MmNAP had a calculated molecular mass of 29.81 and 32.55 kDa and a theoretical isoelectric point of 7.08 and 9.00, respectively. Nucleotide sequence data indicated that both *MmATAF1* and *MmNAP* contained 2 introns and 3 exons and that they shared a conserved genomic organization. Multiple sequence alignments showed that MmATAF1 showed high sequence identity with

ATAF1 of *Arabidopsis thaliana* (61%) and that MmNAP showed high sequence identity with NAP of *A. thaliana* (67%) and CitNAC of *Citrus sinensis* Osbeck (62%). Phylogenetic analysis showed that the predicted MmATAF1 and MmNAP proteins were classified into the ATAF and NAP subgroups, respectively. Transient expression analysis of onion epidermal cells indicated nuclear localization of both MmATAF1-GFP and MmNAP-GFP fusion proteins. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis indicated that *MmATAF1* was expressed in all the tissues tested, but in varying abundance, while *MmNAP* was specifically expressed in stems, petioles, shoots, and leaves, but not in roots. The transcript levels of *MmATAF1* and *MmNAP* in shoots and in infected stems were induced and strengthened by wounding, exogenous $ZnSO_4$, abscisic acid, salicylic acid, and *Cuscuta campestris* infection on the basis of semi-quantitative RT-PCR and real-time PCR analyses, respectively. Collectively, these results indicated that *MmATAF1* and *MmNAP*, besides having roles in *M. micrantha* adaptation to *C. campestris* infection and abiotic stresses, also integrated signals derived from both *C. campestris* infection and abiotic stresses.

Key words: *Mikania micrantha*; *NAC* genes; Sequence characterization; Transcript expression

INTRODUCTION

Plants have evolved gene expression and regulation mechanisms to survive under a variety of abiotic and biotic stresses, such as drought, cold, high salinity, insect feeding, and pathogen and virus infections. Changes in gene expression are often regulated by transcription factors that bind to specific cis-elements of target gene promoters and then lead to activation or suppression of the target genes. In recent years, a novel class of NAC proteins with a highly conserved NAC domain (for NAM, ATAF1/2, CUC2) (Aida et al., 1997) was identified. The proteins were recognized as transcription factors (Fujita et al., 2004; Tran et al., 2004) that participated in the formation of embryos (Duval et al., 2002), leaf senescence (Guo and Gan, 2006; Uauy et al., 2006), flower organ development (Sablowski and Meyerowitz, 1998), fruit development and senescence (Liu et al., 2009), cell cycle control (Kim et al., 2006), seed development (Meng et al., 2007), secondary-wall formation (Mitsuda et al., 2007; Mitsuda and Ohme-Takagi, 2008), hormone signaling (Fujita et al., 2004; Jensen et al., 2008), responses to pathogens and insect feeding (Delessert et al., 2005; Jensen et al., 2007; Lin et al., 2007), as well as responses to different abiotic stresses (Kim et al., 2007; Lu et al., 2007).

Mikania micrantha, a perennial vine belonging to the Asteraceae family and native to Central and South America (Wirjahar, 1976; Holm et al., 1977), has been listed as one of the 100 worst invasive alien species in the world (Lowe et al., 2001). In recent decades, *M. micrantha* has caused severe damage to many ecosystems in Southern China (Zhang et al., 2004). A previous study has shown that *Cuscuta campestris* can suppress the growth of *M. micrantha* (Parker, 1972). *C. campestris* (field dodder) belongs to the Con-

volvulaceae family and is a rootless and leafless plant that lives as a holoparasite on host plants (Dawson et al., 1994). Recently, the effects of *C. campestris* on *M. micrantha* have been studied with respect to biomass, physiology, ecology, and genes expressed at early post-penetration stages. For example, pot trials showed that *C. campestris* significantly reduced the total biomass, changed the biomass allocation patterns, completely inhibited flowering, and reduced photosynthesis and growth of the parasitized *M. micrantha* plants (Shen et al., 2005, 2007); field trials also showed that *C. campestris* significantly reduced the biomass of *M. micrantha* (Lian et al., 2006). Furthermore, 303 presumably upregulated expressed sequence tags were identified from *M. micrantha* as being induced by the early stages of *C. campestris* infection (Li et al., 2009). However, possible roles for *NAC* genes in *M. micrantha* have not yet been extensively explored.

In a previous study, we obtained 2 cDNA fragments, EY274758 (519 bp) and EY274879 (282 bp, containing 5'-untranslated region, 5'-UTR) from a suppression subtractive hybridization cDNA library of *M. micrantha* stems infected by *C. campestris* (Li et al., 2009). These 2 cDNA fragments were homologous to the *Arabidopsis thaliana ATAF1* (Lu et al., 2007) and *NAP* (Guo and Gan, 2006) genes and were designated as *MmATAF1* and *MmNAP*, respectively. In this study, as the first step to illustrating the functions of NAC proteins in *M. micrantha*, the full-length cDNAs of *MmATAF1* and *MmNAP* genes and the genomic DNA of their coding regions were cloned and characterized, and the corresponding putative proteins were analyzed using various bioinformatics tools and methods. Both MmATAF1-GFP (green fluorescence protein) and MmNAP-GFP fusion proteins were localized in the nucleus of onion epidermal cells. Transcript levels of the *MmATAF1* and *MmNAP* genes in different tissues and in non-infected shoots treated with different abiotic stress factors were tested using semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Finally, expression patterns of the 2 *NAC* genes in *M. micrantha* stems during *C. campestris* infection were studied at different time points by using real-time PCR. To the best of our knowledge, this study is the first to report the isolation and characterization of these 2 genes in *M. micrantha*.

MATERIAL AND METHODS

Plant materials, growth conditions, and treatments

Whole *M. micrantha* H.B.K. plants were collected from a population in Zhuhai, Guangdong Province, China. Two-node segments, similar in size, were planted into pots (18 cm in height, 22 cm in upper diameter, and 15 cm in lower diameter), which were filled with a mixture of humus soil, perlite, and sand (3:1:1 by volume). Plants were maintained under greenhouse conditions for 2 months (25° ± 1°C; 75 ± 10% relative humidity; 300 µmoL photons$\cdot m^{-2} \cdot s^{-1}$ light intensity with a 16-h light/8-h dark photoperiod). A bamboo cane (about 1.5 m in length) was placed vertically in each pot, on which *M. micrantha* climbed. Healthy, 2-month-old *M. micrantha* plants were used for treatments and nucleic acid extraction.

Different tissue samples from roots, stems, leaves, petioles, and shoots of the 2-month-old *M. micrantha* plants were collected for analysis of *MmATAF1* and *MmNAP* gene expression by semi-quantitative RT-PCR, as described below.

For chemical stress and plant hormone treatments, the *M. micrantha* plants were sprayed with a solution containing the indicated concentration of chemicals: 20 mM $ZnSO_4$, 100 µM abscisic acid, and 2 mM salicylic acid. Mechanical wounding was performed by injuring shoots (8 cm in length) with needles. In all cases, 15 *M. micrantha* plants were treated with each stress. Three control shoots and 3 shoots from each treatment were collected after 6, 24, 48, and 96 h to detect *MmATAF1* and *MmNAP* gene expression by semi-quantitative RT-PCR.

For infection of *M. micrantha* plants, *C. campestris* shoots (about 20 cm in length) were coiled around the green stems (80 cm in height) of *M. micrantha* plants, and this time point was considered as the initial time of infection. Non-infected and infected plants were watered with sterile distilled water once a day and grown as described above. Three infected stem parts close to the parasite *C. campestris* attachment site (0.5 cm) and similar non-infected stems of *M. micrantha* plants were harvested at 0, 2, 4, 7, and 14 days post-infection (dpi). These samples were used to assess *MmATAF1* and *MmNAP* gene expression by using real-time PCR, as described below.

Isolation of total RNA

Total RNA was extracted using the method previously described (Ding et al., 2008). The quality of RNA was determined by agarose gel electrophoresis, and the amount of RNA was quantified spectrophotometrically (Nanodrop ND-2000C, Thermo, USA). The RNA samples were stored at -80°C until use.

Cloning of the full-length cDNA of the target genes *MmATAF1* and *MmNAP*

First-strand and second-strand cDNA was synthesized from 1.0 µg total RNA extracted from infected *M. micrantha* stems at 7 dpi by using the SMART™ PCR cDNA Synthesis Kit (Clontech, USA), according to manufacturer instructions. To obtain full-length cDNAs of *MmATAF1* and *MmNAP*, 5'- and 3'-RACE were performed using the SMART™ RACE cDNA Amplification Kit (Clontech). On the basis of the sequences of EY274758 and EY274879, gene-specific primers (GSP) were designed for *MmATAF1* and *MmNAP* (*MmATAF1* 5'-RACE-GSP, *MmATAF1* 3'-RACE-GSP, *MmNAP* 3'-RACE-GSP; Table 1) and synthesized (Invitrogen, China).

Aimed at each target gene, 3'-RACE-GSP (*MmATAF1* 3'-RACE-GSP, *MmNAP* 3'-RACE-GSP) and 3'-RACE universal primer (3'-RACE-P provided by the RACE kit; Table 1) were used with first-strand cDNA at 7 dpi as a template for the first amplification. For the second amplification, 3'-RACE-GSP and 3'-RACE-P were used with the templates from the previous PCR products. In a similar manner, 5'-RACE-GSP (*MmATAF1* 5'-RACE-GSP, *MmNAP* 5'-RACE-GSP) and 5'-RACE universal primer UPM (UPM, NUP provided by the RACE kit; Table 1) were performed using second-strand cDNA at 7 dpi as a template for the first PCR. For the second PCR, 5'-RACE-GSP and NUP were used with the templates from the first PCR products. Amplification was performed in a 25-µL reaction mixture, which contained 12.5 µL 2X GC buffer, 4.0 µL 2.5 mM of each dNTP Mix, 0.25 µL 5 U/µL *LA* Taq DNA polymerase (TaKaRa, China), 1.0 µL of each gene-specific primer and 10 µM of each universal primer, 1.0 µL template, and 5.25 µL sterile water. PCRs were performed in a PTC-200 thermocycler (MJ Research, USA) with the following amplification protocol: 4

min at 94°C, followed by 31 cycles of 30 s at 94°C, 30 s at 60°C for 5'-RACE (30 s at 55°C for 3'-RACE), 1 min at 72°C, and a final step of 10 min at 72°C.

Table 1. Primers used in molecular cloning and expression patterns.

Primer codes	Sequences (5'→3')
MmATAF1 5'-RACE-GSP	CCAACGCCTTCTTAATCCC
UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT CTAATACGACTCACTATAGGGC
NUP	AAGCAGTGGTATCAACGCAGAGT
MmATAF1 3'-RACE-GSP	GTCCTGAACAATCGGTCCAAAGT
3'-RACE-P	AAGCAGTGGTATCAACGCAGAGTACT($_{30}$)N$_{-1}$N (N = A, C, G, T; N$_{-1}$ = A, G, C)
MmNAP 3'-RACE-GSP	TCCTTGGGACTTGCCTGAGAA
MmATAF1-F1	GAGCTCATGAATTGTGGCGATTTGGA, underlined added *Sac*I site
MmATAF1-R1	CTCGAGCATCTGAAACGGCTTGGACG, underlined added *Xho*I site
MmNAP-F1	GGATCCATGGTGGGAAGAGATAACT, underlined added *Bam*HI site
MmNAP-R1	GTCGACCTGAAATTGAAGCATTGGATTA, underlined added *Sal*I site
MmATAF1-F2	AGATCTATGAATTGTGGCGATTTGGAGTTAC, underlined added *Bgl*II site
MmATAF1-R2	ACTAGTCATCTGAAACGGCTTGGACG, underlined added *Spe*I site
MmNAP-F2	AGATCTATGGTGGGAAGAGATAACT, underlined added *Bgl*II site
MmNAP-R2	ACTAGTCTGAAATTGAAGCATTGGATTA, underlined added *Spe*I site
MmATAF1-F3	TGGCGATTTGGAGTTACCG
MmATAF1-R3	CAAAGCCATACCAGGAAGC
MmNAP-F3	CAGGGACAGACAAAGCAATC
MmNAP-R3	AACCCAGTCATCCAGCCTCA
Mmactin F1	CGGTCTTTCCCAGTATTGTA
Mmactin R1	GTTTAGTGGTGCCTCGGTGA
MmATAF1-F4	GTCCTGAACAATCGGTCCAAA
MmATAF1-R4	TTGTAACGGGGAGAGCTGAAA
MmNAP-F4	GGCTGGATGACTGGGTTTTATG
MmNAP-R4	CCTGGGGTGTTTTGGGTATTT
Mmactin F2	AGGCGGGATTTGCTGGT
Mmactin R2	TACCTCTTTTGGACTGGGCTTC

By comparing and aligning the nucleotide sequences from EY274758, EY274879, 5'- and 3'-RACE, the entire coding regions of *MmATAF1* and *MmNAP* were deduced and amplified with 2 pairs of gene-specific primers (*MmATAF1*-F1 and *MmATAF1*-R1, *MmNAP*-F1 and *MmNAP*-R1; Table 1). The first-strand cDNA at 7 dpi was used as a PCR template. PCRs were performed by denaturing the cDNA at 94°C for 4 min, 31 cycles of at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 10 min.

PCR products were detected on 1.0% agarose gels stained with ethidium bromide, purified using the QIAquick gel extraction kit (Qiagen, Germany), cloned into the pGEM-T Easy vector (Promega, USA), and finally transformed into *Escherichia coli* strain DH5α (Dingguo, China) competent cells. Three positive clones for each PCR product were sequenced (Invitrogen).

Isolation of *MmATAF1* and *MmNAP* genes from *M. micrantha* genomic DNA

Genomic DNA was extracted from the shoots of 2-month-old *M. micrantha* plants by using the modified CTAB protocol, as previously described (Li et al., 2007). Two pairs of gene-specific primers (*MmATAF1*-F1 and *MmATAF1*-R1, *MmNAP*-F1 and *MmNAP*-R1; Table 1) were used for amplification of *MmATAF1* and *MmNAP* genes from genomic DNA, respectively. PCR volume was 25 µL, which contained 12.5 µL 2X GC buffer, 4.0 µL 2.5 mM of each dNTP Mix, 0.25 µL 5 U/µL *LA* Taq DNA polymerase (TaKaRa), 1.0 µL 10 µM of each gene-

specific primer, 1.0 µL 100 ng/µL genomic DNA, and 5.25 µL sterile water. Reactions were performed with a pre-denaturation step at 94°C for 5 min, followed by 33 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 1.5 min, and a final extension at 72°C for 7 min. The obtained PCR fragments were gel-purified, sub-cloned, and sequenced as described above.

Bioinformatics analysis

The nucleic acid sequences of the target genes (*MmATAF1* and *MmNAP*) and corresponding amino acid sequences of the proteins (MmATAF1 and MmNAP) were calculated and analyzed with bioinformatics tools. Sequence data were analyzed online at the NCBI by using the BLAST (Altschul et al., 1997) and Expasy websites (http://www.expasy.org). The open reading frame (ORF) was predicted using ORF Finder. Isoelectric point (pI) and molecular mass were predicted with the Compute pI/Mw tool. Genomic sequence structure was analyzed using InterProScan. Multiple sequence alignments of the amino acid sequences of MmATAF1, MmNAP, and NACs from other plant species were performed using ClustalX version 1.81 (Thompson et al., 1997). The phylogenetic tree was constructed by the neighbor-joining method with 1000 bootstrap replicates by using MEGA version 3 (Kumar et al., 2004).

Subcellular localization analysis of transiently expressed fusion proteins

Localization of the MmATAF1 and MmNAP proteins was assessed by transient expression of MmATAF1-GFP and MmNAP-GFP fusion proteins in onion epidermal cells. The coding regions of the target genes were amplified with 2 pairs of gene-specific primers (*MmATAF1*-F2 and *MmATAF1*-R2, stop codon deleted; *MmNAP*-F2 and *MmNAP*-R2, stop codon deleted; Table 1). The resulting PCR product was cloned by the TA-ends method (pGEM-T Easy Vector Kit, Promega), excised with the restriction enzymes *Bgl*II and *Spe*I, eluted from 1.0% agarose gel (QIAquick Gel Extraction Kit, Qiagen), and inserted into the pCAMBIA1302 (CAMBIA, Canberra, Australia) plasmid containing the *CaMV35S* promoter and the *GFP* gene. Likewise, the 3'-end of the *MmATAF1* or *MmNAP* cDNA was ligated to the 5'-end of the *GFP* of pCAMBIA1302, resulting in the gene-protein fusion *MmATAF1-GFP* or *MmNAP-GFP*. The construct was under the control of a single *CaMV35S* promoter. An NOS terminus is located at the 3'-end of the *GFP* sequence.

Gold particles (1 µm) were loaded with plasmid DNA, as described by the manufacturer (BioRad). The bombardment was performed using a particle gun (PDS-1000/He biolistic particle delivery system, BioRad) with 900 psi helium pressure on onion epidermal cells. The bombarded onion cells were incubated on Murashige and Skoog (MS) agar plates for 1-2 days at 25°C in the dark and then analyzed using a fluorescence microscope.

Semi-quantitative RT-PCR analysis of *MmATAF1* and *MmNAP* expressions

To analyze transcript levels of *MmATAF1* and *MmNAP* in different tissues, the roots, stems, leaves, petioles, and shoots of 2-month-old *M. micrantha* plants were collected for RNA extraction.

To investigate the expression profile of induced *MmATAF1* and *MmNAP*, 3 shoots of each treatment, including 20 mM $ZnSO_4$, 100 µM abscisic acid, 2 mM salicylic acid,

and mechanical wounding treatments, were collected at 6, 24, 48, and 96 h. Total RNA was isolated from the treated and non-treated shoots, as already described.

Prior to cDNA synthesis, all RNA samples were treated with RNase-free DNaseI (TaKaRa) to remove DNA contamination. First-strand cDNA of each RNA sample (1.0 µg) was synthesized using the Prime-Script™ Reagent Kit (TaKaRa), according to the manufacturer protocol. PCR volume was 25 µL, which contained 2.0 µL first-strand cDNA (10X dilution) and 0.2 µM of each gene-specific primer (*MmATAF1*-F3 and *MmATAF1*-R3, *MmNAP*-F3 and *MmNAP*-R3; Table 1). The amplified fragments of *MmATAF1* and *MmNAP* were 166 and 185 bp in length, respectively. PCR was performed under the following conditions: 3 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, 45 s at 72°C, and a final extension at 72°C for 5 min. The *M. micrantha* actin (*Mmactin*) gene (GenBank accession No. EY456955) was amplified using gene-specific primers (*Mmactin*-F1 and *Mmactin*-R1; Table 1) as an internal control. The amplified fragment of *Mmactin* was 248 bp in length. PCR for *Mmactin* was similar to the PCR for *MmATAF1*, but with 30 cycles. Seven microliters of the reaction mixture was analyzed on a 1.0% (w/v) agarose gel in 1X TAE buffer and then photographed for expression analysis. Each RT-PCR was performed in triplicate.

Real-time PCR analysis of *MmATAF1* and *MmNAP* expressions

Real-time PCR was used to analyze the expression patterns of *MmATAF1* and *MmNAP* genes in *M. micrantha* stems during *C. campestris* infection. Total RNA was isolated from stems of infected *M. micrantha* at 0, 2, 4, 7, and 14 dpi. First-strand cDNA was synthesized according to the method described for semi-quantitative RT-PCR.

Primers used for real-time PCR were as follows: *MmATAF1*-F4 and *MmATAF1*-R4, *MmNAP*-F4 and *MmNAP*-R4, *Mmactin*-F2, and *Mmactin*-R2 (Table 1). Real-time PCR was performed with the SYBR green kit (TaKaRa) in a final volume of 25 µL, which contained 0.2 µM of each primer, 12.5 µL 2X SYBR Premix, 2 µL first-strand cDNA (10X dilution), and distilled water. PCRs were performed in optical reaction strip tubes by using an iCycler iQ5 apparatus (BioRad). The cycling conditions were as follows: 1 min at 95°C, followed by 40 cycles of 10 s at 95°C, 15 s at 59°C, 15 s at 72°C, and data acquisition at 81°C. At the end of the amplification experiment, a melting curve was established (between 55° and 95°C by steps of 0.5°C) to ensure that the signal corresponded to a single-PCR product. Quantification of the target gene was assessed using relative standard curves. Each RT-PCR was conducted in triplicate. In order to obtain the relative expression ratio, the target gene transcript levels (*MmATAF1* or *MmNAP*) were normalized to the average amount of *Mmactin* transcripts. Normalized values of 3 replicated experiments were used to calculate average relative expression ratios (*MmATAF1*/*Mmactin* and *MmNAP*/*Mmactin*).

RESULTS

Cloning of the full-length cDNA of the target genes *MmATAF1* and *MmNAP*

By using the methods described above, the full-length cDNAs of *MmATAF1* and *MmNAP* were obtained. First, the cDNA sequence of *MmATAF1* was 1329 bp in length, containing a 780-bp ORF encoding 260 amino acids, a 5'-UTR 82 bp upstream from the start

codon ATG, and a 464 bp 3'-UTR incidental 30 bp poly(A) tail from the stop codon TAA, as shown in Figure 1. Second, the full-length cDNA sequence of *MmNAP* was 1072 bp, in which there was a 834-bp ORF that encoded a 278-amino acid protein, a 52-bp 5'-UTR and a 183-bp 3'-UTR with a putative polyadenylation signal AATAA at a position 98 bp downstream from the stop codon TGA (Figure 2). The cDNA sequences of *MmATAF1* and *MmNAP* have been submitted to GenBank under the accession Nos. EU296447 and EU296448, respectively.

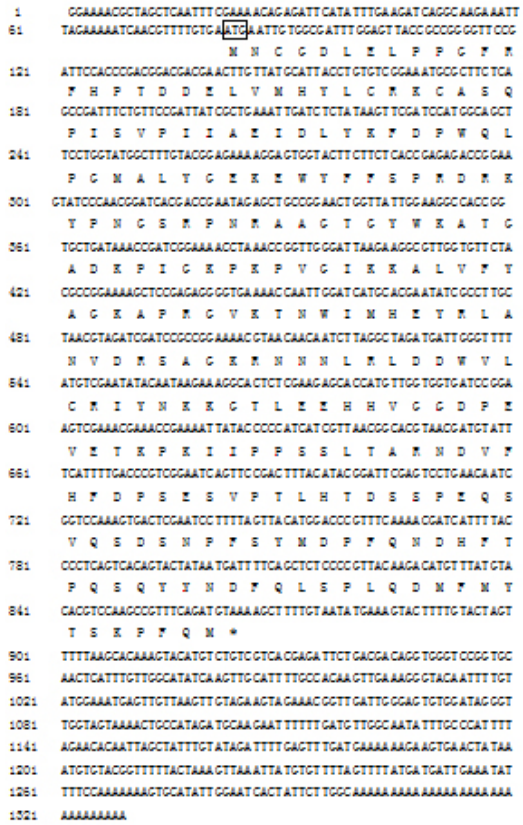

Figure 1. Full-length cDNA sequence and deduced amino acid sequence of *MmATAF1*. The start codon (ATG) is boxed and the stop codon (TAA) is marked with an asterisk.

Analysis of the genomic sequences of *MmATAF1* and *MmNAP*

The genomic DNA sequences of *MmATAF1* and *MmNAP* were 1001 and 997 bp in length from start codon to stop codon, respectively. They were submitted to GenBank under the accession Nos. FJ872509 and FJ872510, respectively. Comparisons of the cDNA sequences and the corresponding genomic sequences revealed that both genes contained 3 exons and 2 introns (Figure 3). The first 2 exons encoded the conserved *N*-terminal region (NAC domain), whereas the last one encoded the highly divergent C-terminal domain (see below). This specific genomic organization was shared by the 2 *MmNAC* genes. Moreover, the sites of the 2 introns were obviously conserved (Figure 3; Duval et al., 2002; Meng et al., 2007).

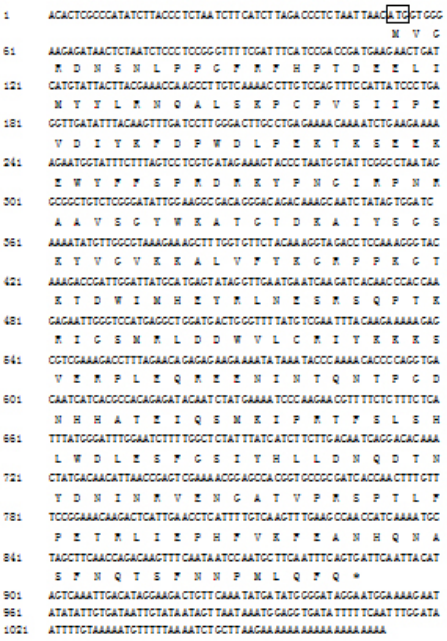

Figure 2. Full-length cDNA sequence and deduced amino acid sequence of *MmNAP*. The start codon (ATG) is boxed and the stop codon (TGA) is marked with an asterisk.

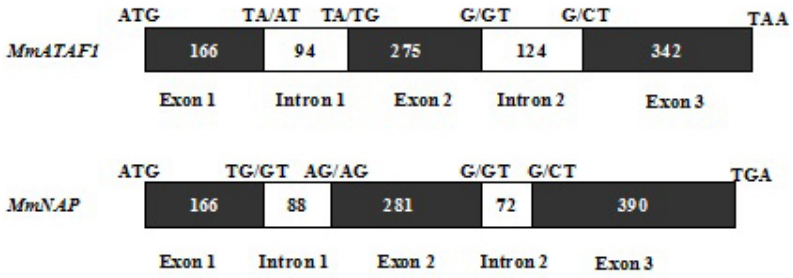

Figure 3. Genomic structure of *MmATAF1* and *MmNAP* genes. Black boxes indicate exons and open boxes represent introns. Exons and introns are drawn schematically to indicate their relative positions and sizes.

Analysis of the deduced amino acid sequences of the *MmATAF1* and *MmNAP* genes

The proteins coded by *MmATAF1* and *MmNAP* had a calculated molecular mass of 29.81 and 32.55 kDa, respectively, and a theoretical pI of 7.08 and 9.00, respectively. BLASTP comparison of the deduced amino acid sequence of MmATAF1 with that of other NAC proteins revealed that MmATAF1 had 61% sequence identity (Expect = 6e-91) with *A. thaliana* ATAF1 (Lu et al., 2007) and 59% sequence identity (Expect = 3e-82) with ATAF2 (Delessert et al., 2005). MmNAP had 67% sequence identity (Expect = 3e-83) with AtNAP (Guo and Gan, 2006) and 62% sequence identity (Expect = 2e-83) with CitNAC (Liu et al., 2009).

InterPro inquiry found that both MmATAF1 and MmNAP contained an InterPro NAM domain (IPR003441), which ranged between amino acid sites 8 to 133 and 9 to 135, respec-

tively (Figure 4). The NAC domains of the predicted MmATAF1 and MmNAP proteins were further compared with other NAC proteins, including *A. thaliana* ATAF1 (NP_171677), ATAF2 (NP_680161), AtNAM (NP_175696), AtNAP (NP_564966), CUC1 (NP_188135), NST1 (NP_182200), and *Citrus sinensis* CitNAC (ABM67699) by using ClustalX. Results showed that MmATAF1 and MmNAP had high levels of sequence identity with other NAC proteins in the N-terminal region and could be divided into 5 sub-domains (A-E) (Figure 4), according to Ooka et al. (2003).

Phylogenetic analysis

To predict the possible function(s) of MmATAF1 and MmNAP, a phylogenetic tree was constructed using MEGA. Phylogenetic analysis showed that NAC proteins could be classified into several subgroups on the basis of similarities in NAC domains (Figure 4; Ooka et al., 2003). For ease of comparison, only major subgroups were shown in Figure 5. MmATAF1 was classified into the ATAF subgroup, which was composed of the ATAF1, ATAF2, AtNAC1, and AtNAC19 proteins. MmNAP was classified into the NAP subgroup, which included CitNAC and AtNAP.

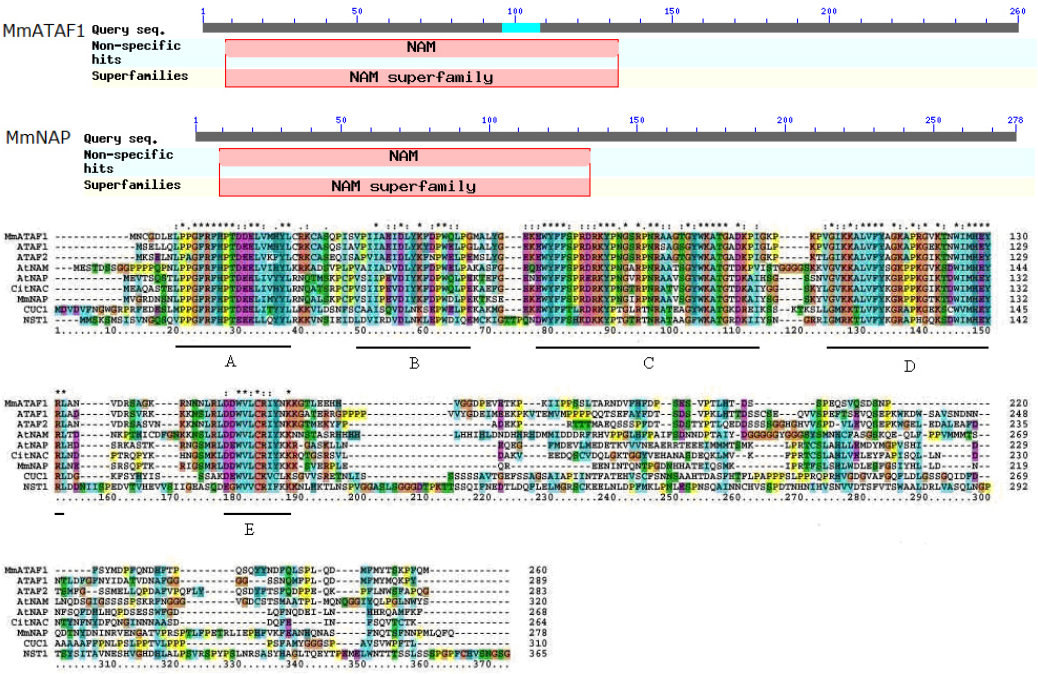

Figure 4. Multiple alignment of the deduced amino acid sequences of *MmATAF1* and *MmNAP* with other reported NAC proteins. Amino acid sequences were aligned as follows: ATAF1 (*Arabidopsis thaliana*, NP_171677), ATAF2 (*A. thaliana*, NP_680161), AtNAM (*A. thaliana*, NP_175696), AtNAP (*A. thaliana*, NP_564966), CUC1 (*A. thaliana*, NP_188135), NST1 (*A. thaliana*, NP_182200), CitNAC (*Citrus sinensis*, ABM67699), MmATAF1, and MmNAP. The numbers on the right side show the amino acid positions in sequence. The five sub-domains (A-E) are indicated by lines at the bottom. '-', '*', ':' and '.' indicate gaps, identical amino acid residues, conserved substitutions, and semi-conserved substitutions in all sequences used in the alignment, respectively.

Subcellular localization of MmATAF1 and MmNAP

In order to investigate the subcellular localization of proteins MmATAF1 and MmNAP, 2 plasmids, p35S-MmATAF1-GFP and p35S-MmNAP-GFP, were constructed; the expression of the fusion gene *MmATAF1-GFP* or *MmNAP-GFP* was driven by the 35S promoter of CaMV. The plasmid containing the fusion gene and vector control DNA were introduced into onion epidermal cells by bombardment. Cells expressing GFP alone displayed only diffuse cytoplasmic and nuclear staining (Figure 6). In contrast, both MmATAF1-GFP and MmNAP-GFP localized exclusively to the nuclei of transformed cells (Figure 6). These results indicated that both MmATAF1 and MmNAP are nuclear proteins.

Tissue-specific and abiotic stress-induced expression of the *MmATAF1* and *MmNAP* genes

To elucidate the tissue-specific expression of the *MmATAF1* and *MmNAP* genes, semi-quantitative RT-PCR was performed (Figure 7A). The *Mmactin* gene, an internal control for constitutive expression, was expressed in all tissues examined. Each *MmNAC* gene showed a unique expression pattern. *MmATAF1* was expressed strongly in roots, stems, petioles, and shoots, but weakly in leaves. *MmNAP* was constitutively expressed in stems, petioles, shoots, and leaves, but it was almost undetectable in roots. The different expression patterns of *MmATAF1* and *MmNAP* suggested that their products probably played functionally diverse roles during the process of *M. micrantha* growth and development.

The effects of diverse abiotic stresses on the expression of *MmATAF1* and *MmNAP* genes in *M. micrantha* shoots were analyzed by semi-quantitative RT-PCR. Both *MmATAF1* and *MmNAP* genes were significantly induced by mechanical wounding (Figure 7B). Transcript levels of *MmATAF1* rapidly increased and reached a peak at 6 h after wounding treatment, then declined gradually (Figure 7B). The transcript levels of *MmNAP* began to increase at 6 h post-treatment, then gradually increased and reached a peak at 96 h (Figure 7B). The *MmATAF1* and *MmNAP* genes were also affected differently by $ZnSO_4$ treatment (Figure 7C). The *MmATAF1* transcript levels showed a slight decline at 6 h after $ZnSO_4$ treatment, but they were strongly induced and reached a maximum at 24 h post-treatment (Figure 7C). Transcript levels of *MmNAP* rapidly increased and reached a peak at 6-24 h, then declined gradually thereafter (Figure 7C). Notably, the transcript levels of *MmATAF1* were rapidly induced within 6 h after abscisic acid treatment and reached a maximum at 24 h (Figure 7D). In contrast, the transcript levels of *MmNAP* slightly decreased within 6-24 h after abscisic acid treatment and then significantly increased within 48-96 h (Figure 7D). With salicylic acid treatment, *MmATAF1* transcript levels slightly decreased within 6 h, then gradually increased and reached a maximum at 96 h (Figure 7E). Transcript levels of *MmNAP* were markedly increased at 24 h after salicylic acid treatment and reached a maximum at 96 h (Figure 7E).

C. campestris infection induced expression of the *MmATAF1* and *MmNAP* genes

To confirm the expression profiles of *MmATAF1* and *MmNAP* genes in the stems of *M. micrantha* during *C. campestris* infection, relative real-time RT-PCR was performed (Figure 8). Compared with the expression levels of the *MmATAF1* gene in non-infected *M.*

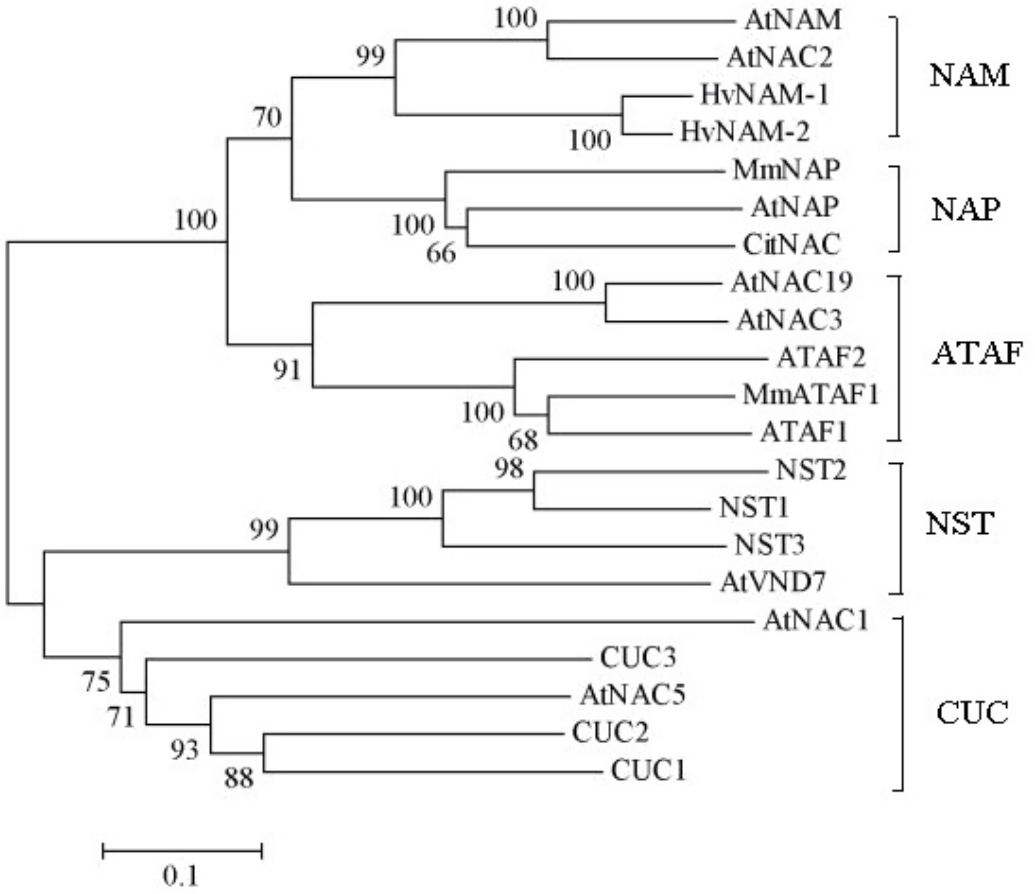

Figure 5. Phylogenetic analysis of plant NAC proteins. The tree was constructed with MmATAF1, MmNAP and the sequences from NCBI as follows: ATAF1 (*Arabidopsis thaliana*, NP_171677), ATAF2 (*A. thaliana*, NP_680161), AtNAM (*A. thaliana*, NP_175696), AtNAP (*A. thaliana*, NP_564966), AtNAC1 (*A. thaliana*, NP_175997), AtNAC2 (*A. thaliana*, NP_188170), AtNAC3 (*A. thaliana*, NP_188169), AtNAC5 (*A. thaliana*, NP_200951), AtNAC19 (*A. thaliana*, NP_175697), CUC1 (*A. thaliana*, NP_188135), CUC2 (*A. thaliana*, NP_200206), CUC3 (*A. thaliana*, NP_177768), NST1 (*A. thaliana*, NP_182200), NST2 (*A. thaliana*, NP_191750), NST3 (*A. thaliana*, NP_174554), AtVND7 (*A. thaliana*, NP_177338), CitNAC (*Citrus sinensis*, ABM67699), HvNAM-1 (*Hordeum vulgare* subsp *vulgare*, ABI94357), and HvNAM-2 (*H. vulgare* subsp *vulgare*, ABI94358). Numbers on the branches represent bootstrap support for 1000 replicates. The scale bar represents a genetic distance of 0.1.

micrantha plants, transcript levels of the *MmATAF1* gene were slightly decreased at 2 to 4 days after *C. campestris* infection, but significantly increased and achieved a maximum at 7 days (4-fold greater than the control levels) (Figure 8). *C. campestris* infection remarkably increased *MmNAP* transcript levels within 2 days (3-fold greater than the control levels) and reached a maximum after 14 days (294-fold greater than the control levels) (Figure 8). These results showed that both *MmATAF1* and *MmNAP* genes were induced in the stems of infected *M. micrantha* plants, and they may play important roles in the stems of *M. micrantha* during *C. campestris* infection.

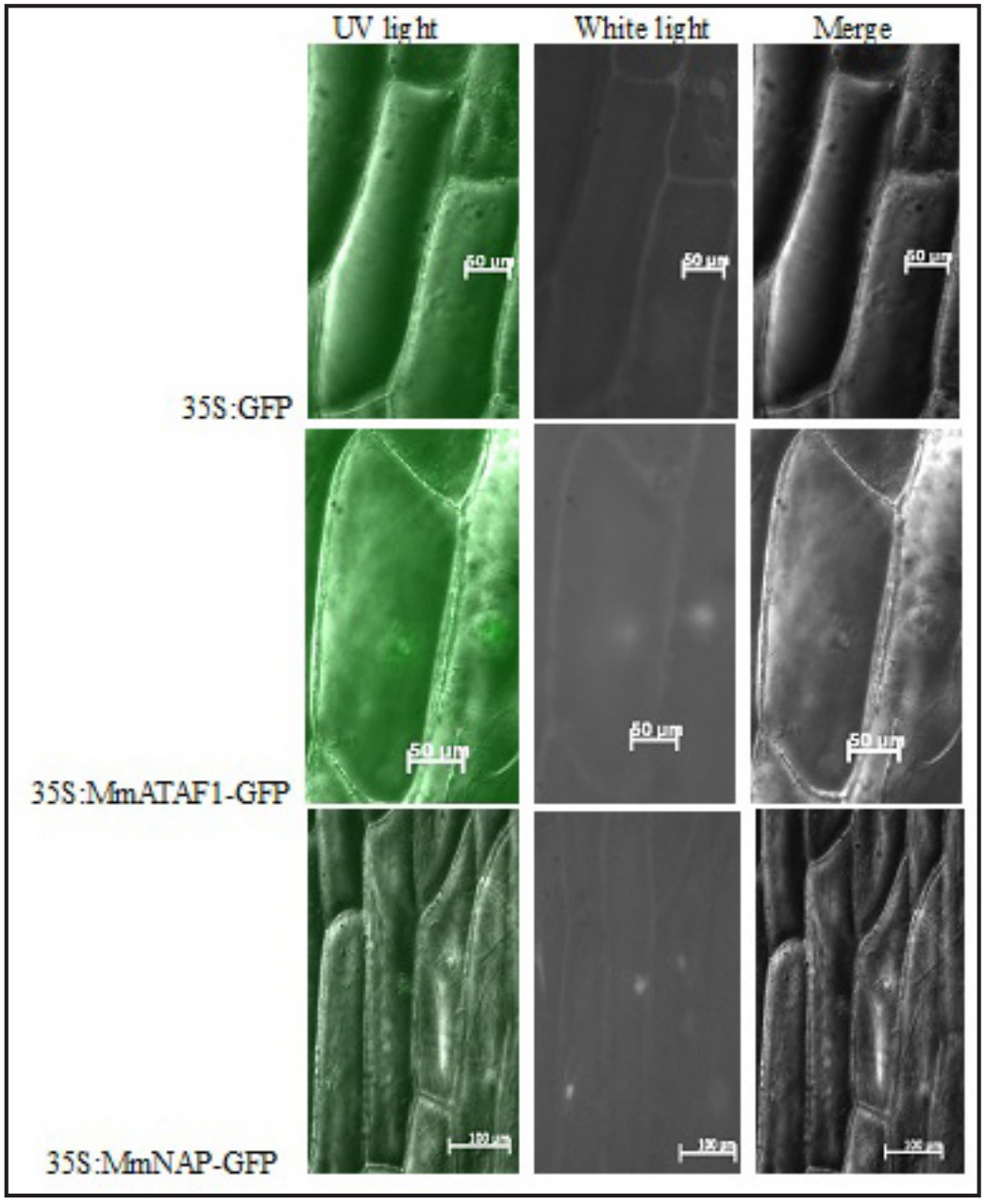

Figure 6. Subcellular localizations of the MmATAF1-GFP and MmNAP-GFP proteins. The plasmid expressing the fusion gene and vector control p35S-GFP were transformed into onion epidermal cells by bombardment. The fluorescence images (left column) of the GFP control (top row), fusion protein MmATAF1-GFP (middle row) and MmNAP-GFP (bottom row) under UV light (left column) or white light (middle column) are shown. The merged images are shown in the right column.

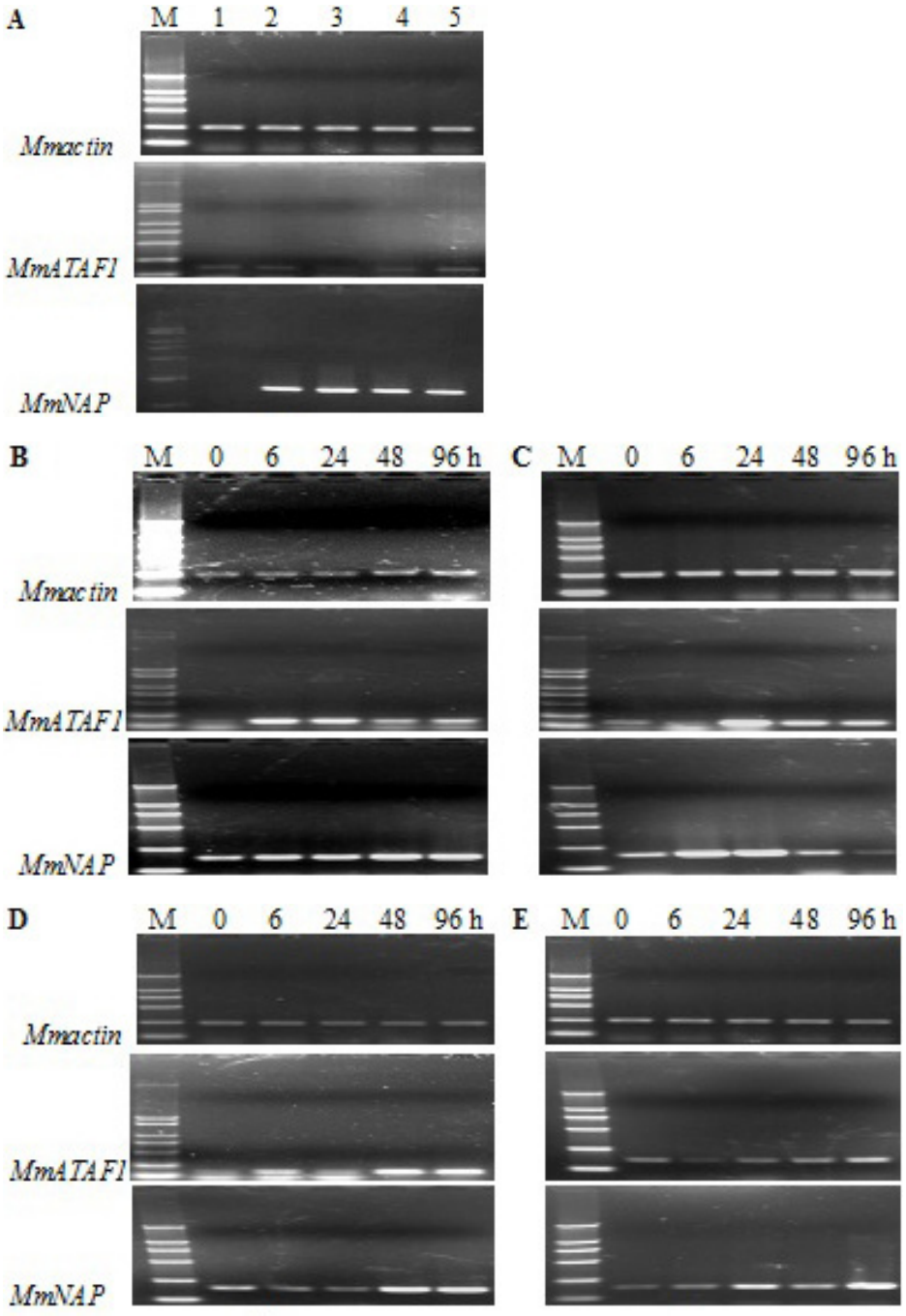

Figure 7. Semi-quantitative RT-PCR analysis of transcript levels for *MmATAF1* and *MmNAP*. *Lane M* = molecular mass marker. **A.** *Lanes 1* to *5* = roots, stems, leaves, petioles, and shoots, respectively; **B.** Wounding; **C.** 20 mM $ZnSO_4$; **D.** 100 µM abscisic acid; **E.** 2 mM salicylic acid. *Mmactin* was used as an internal control.

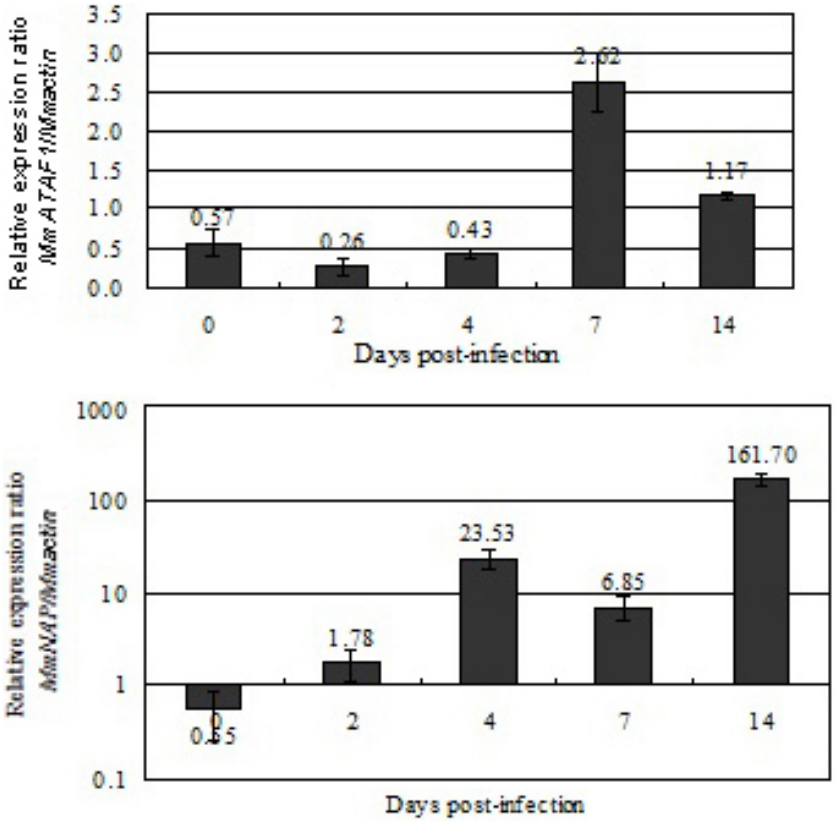

Figure 8. Expression analyses of *MmATAF1* and *MmNAP* in stems of *Cuscuta campestris*-infected *Mikania micrantha* at different time points. Error bars represent the variation among three independent replications.

DISCUSSION

The reported NAC members of this family have been characterized mainly in *Arabidopsis*, rice, wheat, soybean, and *Citrus* (e.g., Delessert et al., 2005; Ohnishi et al., 2005; Guo and Gan, 2006; Uauy et al., 2006; Lu et al., 2007; Meng et al., 2007; Nakashima et al., 2007; Liu et al., 2009), but the characterization of *M. micrantha NAC* genes still remains unknown. In the present study, we cloned and characterized 2 full-length cDNAs of *NAC* genes from the stems of *M. micrantha* (Figures 1 and 2), and they were named *MmATAF1* and *MmNAP*, respectively. In comparison with the sequences of other *NAC* genes, both *MmATAF1* and *MmNAP* contain introns. Although intron sequences varied in different *NAC* genes, the sites of these introns seem to be conserved. Introns in *MmATAF1* were 94 and 124 bp in length, whereas introns in *MmNAP* were shorter (88 and 72 bp) (Figure 3). MmATAF1 and MmNAP had highly conserved amino acid sequences at their N-terminals (Figure 4). The regions of highest amino acid identity were the 5 typical NAC sub-domains (Figure 4). Transient assay using onion epidermal cells showed that both fusion proteins MmATAF1-GFP and MmNAP-GFP were exclusively expressed within the nuclei (Figure 6). This provides direct evidence

that MmATAF1 and MmNAP can be properly targeted to the nucleus of plant cells. Phylogenetic analysis showed that MmATAF1 was closely related to the proteins of the ATAF subgroup, and MmNAP was closely related to the proteins of the NAP subgroup (Figure 5). Thus, we concluded that MmATAF1 is a putative ATAF subgroup protein and that MmNAP is a putative NAP subgroup protein.

Our data indicate that the expression of *MmATAF1* and *MmNAP* genes is tissue-specific in 2-month-old plants. Semi-quantitative RT-PCR analysis showed that higher levels of expression of *MmATAF1* transcripts were observed in root, stem, petiole, and shoot tissues, whereas lower expression levels were detected in leaves (Figure 7A). *MmNAP* had high and steady expression levels in the stem, leaf, petiole, and shoot, and almost no expression in the root (Figure 7A). Previous studies revealed that different *NAC* genes are involved in development and senescence, suggesting a role in specific developmental processes and organ senescence (Sablowski and Meyerowitz, 1998; Duval et al., 2002; Uauy et al., 2006; Guo and Gan, 2006; Kim et al., 2006; Meng et al., 2007; Mitsuda et al., 2007; Mitsuda and Ohme-Takagi, 2008; Liu et al., 2009). In soybean, for example, *GmNAC1* was predominantly expressed in roots and floral buds; *GmNAC2* was expressed strongly in leaves, stems, and developing seeds but only weakly in roots, floral buds, and pods (Meng et al., 2007). In *A. thaliana*, *ATAF2* was mainly expressed in roots, cotyledons, and leaves (Delessert et al., 2005). In rice, transcripts of *OsNAC19* were abundant in roots, relatively scarce in culms and blade sheaths, and very rare in leaves (Lin et al., 2007). These results indicate variable expression of NAC transcripts in various tissues across diverse plant species.

Previous studies showed that plant *NAC* genes were implicated in responses to abiotic and biotic stimuli (e.g., Delessert et al., 2005; Jensen et al., 2007; Kim et al., 2007; Lin et al., 2007; Lu et al., 2007). Our analysis of the expression of *MmATAF1* and *MmNAP* genes in shoots showed that they were also induced by mechanical wounding, $ZnSO_4$, abscisic acid, and salicylic acid treatments with variable expression levels (Figure 7B-E). Furthermore, *MmATAF1* and *MmNAP* were remarkably upregulated in stems of *M. micrantha* 2 weeks after *C. campestris* infection (Figure 8). These findings revealed that *MmATAF1* and *MmNAP* may contribute to both abiotic and parasitic plant stress responses.

In the current study, the response of *MmATAF1* to wounding was very rapid and strong (Figure 7B). Transcripts of *MmNAP* in shoots showed increased levels at 6 h and reached a maximum at 96 h after wounding (Figure 7B). These results agreed with the results reported previously for members of the NAC family. For example, *ATAF2* was highly and rapidly induced at the wound site in *A. thaliana* (Delessert et al., 2005), and *OsNAC6* was strongly induced by wounding in rice (Ohnishi et al., 2005). The effect of the heavy metal Zn on accumulation of *MmATAF1* and *MmNAP* transcripts in shoots was also tested. Our analysis indicated that transcript levels of *MmATAF1* were remarkably increased at 24 h post-treatment, while *MmNAP* transcript levels rapidly increased and reached a peak at 6 h, and then declined gradually thereafter (Figure 7C). These findings revealed that *NAC* genes were induced not only by salt, drought, and cold stresses (Hegedus et al., 2003; He et al., 2005; Lu et al., 2007; Nakashima et al., 2007), but also by heavy-metal stress.

In this study, we showed that the *MmATAF1* and *MmNAP* transcript levels in *M. micrantha* shoots were affected by abscisic acid and salicylic acid treatments (Figure 7D and E). Exogenous abscisic acid resulted in an early and notable increase in levels of *MmATAF1* transcripts (Figure 7D). The expression of *MmATAF1* was more strongly induced by abscisic acid

than by salicylic acid (Figure 7D and E). In this context, it is interesting that *ATAF1* was also induced by 100-μM abscisic acid in *A. thaliana* (Lu et al., 2007). However, these findings were different from *ATAF2* expression in *A. thaliana*. Transcripts of *ATAF2* in *A. thaliana* leaves were induced by the application of MeJA and salicylic acid, whereas abscisic acid treatment did not have any effect (Delessert et al., 2005).

Interestingly, previous reports had shown that *AtNAP* played an important role in leaf senescence in *Arabidopsis* (Guo and Gan, 2006), and *CitNAC*, which was closely related to *AtNAP*, was involved in fruit development and senescence (Liu et al., 2009). Here, we showed that both abscisic acid and salicylic acid treatments induced obvious accumulation of *MmNAP* transcripts at 4 days after treatments (Figure 7D and E). Additionally, transcript levels of *MmNAP* were remarkably upregulated in infected stems of *M. micrantha* at early and later infection stages (Figure 8). Therefore, we can presume that *MmNAP*, in addition to having roles in leaf senescence in infected *M. micrantha* plants, may be involved in the abscisic acid and salicylic acid signal pathways derived from biotic and abiotic stresses.

In summary, 2 full-length *NAC* cDNA clones, *MmATAF1* and *MmNAP* from *M. micrantha,* were isolated and characterized. The proteins encoded by *MmATAF1* and *MmNAP* share a conserved NAM domain with the corresponding proteins from other plants. Subcellular localization revealed that both MmATAF1 and MmNAP are nuclear proteins. Gene expression data indicate that *MmATAF1* is expressed in various tissues but at varying levels, and *MmNAP* is constitutively expressed in stems, leaves, petioles, and shoots, but not in roots. Increased accumulation of *MmATAF1* and *MmNAP* transcripts was found in response to mechanical wounding and exogenous $ZnSO_4$, abscisic acid, and salicylic acid. Furthermore, levels of *MmATAF1* transcripts were low in the early stages of *C. campestris* infection, whereas significantly higher levels were detected at later infection stages. Remarkably increased accumulation of *MmNAP* transcripts was measured at early and later infection stages. Our results point to the possibility that signal transduction pathways related to abscisic acid and salicylic acid regulate expression of *MmATAF1* and *MmNAP* under biotic and abiotic stresses. This establishes the foundation for further research on these 2 *M. micrantha NAC* genes. Future studies are required to elucidate the biological functions of *MmATAF1* and *MmNAP* in *M. micrantha* by using transgenic plants.

ACKNOWLEDGMENTS

Research supported by the Key Project Foundation of the Education Ministry (#704037) and the Guangdong Academy of Agricultural Sciences Fund (#201019).

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