

TWO METALLOTHIONEIN GENE FAMILY MEMBERS IN BUCKWHEAT: EXPRESSION ANALYSIS IN FLOODING STRESS USING REAL TIME RT-PCR TECHNOLOGY

DRAGANA B. MAJIĆ, JELENA T. SAMARDŽIĆ, MIRA Đ. MILISAVLJEVIĆ,
A. M. KRSTIĆ, and VESNA R. MAKSIMOVIĆ

Institute of Molecular Genetics and Genetic Engineering, 11010 Belgrade, Serbia.

Abstract — Metallothioneins (MTs) are an extensive and diverse family of small cysteine-rich proteins with metal-binding ability that are involved in metal homeostasis and detoxification. Two cDNA clones of the MT3 type, differing in 3' UTRs, were isolated from the developing buckwheat seed cDNA library. Following sequence analyses, expression profiles during flooding stress were monitored by Real Time RT PCR technology.

Key words: Buckwheat, flooding, metallothionein, Real Time RT-PCR

UDC 633.12:577.218:575

INTRODUCTION

Metallothioneins (MTs) are an extensive and diverse family of small cysteine-rich proteins found throughout the animal and plant kingdoms, as well as in some prokaryotes. MTs can sequester several metal ions, primarily Cu^{++} and Zn^{++} under normal physiological conditions, and also more toxic metals (like Cd^{++} or Hg^{++}) following exposure (Kagi and Vallee, 1960). Their metal-binding ability suggests that MTs are involved in metal homeostasis and detoxification, but their definite physiological function is still a matter of debate (Fisher et al., 1998; Palmiter, 1998).

The available scarce data on plant metallothioneins were mainly obtained in gene expression studies. Plant MT genes have been found to be expressed in all plant organs - leaves, seeds, roots, fruits, and are also induced by various abiotic and biotic factors, such as metal ions and hormones (Cobbet and Goldsbrough, 2002). Plant MTs have been suggested to play important roles in maintaining the homeostasis of essential transition metals, detoxification of toxic metals, and protection against oxidative stress (Palmiter, 1998, Coyle et al., 2002; Wong et al., 2004, Cobbet and Goldsbrough, 2002). Moreover, the diverse

expression patterns of MT genes suggest that MT-like protein variants differing in sequence may also have different functions in specific tissues (Cobbet and Goldsbrough, 2002). A number of investigations demonstrated that these proteins could have distinct but overlapping functions in the multigene family in a single species (Guo et al., 2003).

The main approach in investigations of gene expression regulation in general is analysis of promoter activity at the transcriptional level. However, the increasing evidence that post-transcriptional mechanisms are critical in gene regulation leads to the conclusion that study of gene expression should include consideration of RNA-based gene regulation involving the 5'- and 3'- untranslated regions (UTRs) (Hesketh et al., 1996, 2002). Given that, there is considerable scope for regulatory motifs within 3'-UTRs. In different animal and plant mRNAs, 3'-UTRs have been reported to be involved in regulation of mRNA stability, translation, and localization (Hesketh and Villette, 2002). There are some results on animal MTs which indicate that the association of MT mRNA with the cytoskeleton around the nucleus, depending upon its 3'-UTRs, is essential for efficient shuttling of the protein into the nucleus during the G_1 to S phase transition (Chabannon et al., 2004).

Buckwheat (*Fagopyrum esculentum* Moench) cDNA clone pBM 290 (GenBank accession number AF056203) coding for a metallothionein-like protein was isolated from the developing buckwheat seed cDNA library (Brkljačić et al., 1999). The deduced amino acid sequence showed the highest homology with the MT3-like protein from *A. thaliana* (Murphy et al., 1997). Expression analysis showed that buckwheat MT3 mRNAs are discernible in buckwheat root and leaf tissue and throughout seed development in normal conditions. Since metal ions and oxidative stress conditions influenced the MT3 expression levels in both leaf and seed (Brkljačić et al., 2004), there was great interest in identifying new MT3 family members in order to elucidate their possible involvement in plant protection during particular environmental stress conditions.

A common disaster that greatly reduces the survival and yield of many commercially important plant species is flooding, with consequent hypoxic stress. Since a protective role of MT in hypoxia has been shown for mammalian MTs (Li et al., 2004), we examined whether expression of buckwheat MT3 transcripts changes under the influence of flooding stress.

MATERIAL AND METHODS

cDNA synthesis

cDNA synthesis using poly-T primer (5'-TTCTAGAATTCAGCGGCCGC(T)₃₀-N₋₁N-3', where N₋₁ is G, A or C, N is G, A, C or T-3') and adaptor primer AP1 (5'-CCATCCTAATACGACTCACTATAGG GC-3') was carried out according to Chenchik et al. (1996) with some modifications as described by Samardžić et al. (2004).

Non-radioactive Southern blot analysis

Previously synthesized cDNA clones were digested by *EcoRI* endonuclease, and the obtained inserts were purified on agarose gels using the Wizard DNA Gel Purification Kit (Promega) and run on new agarose gel for Southern blot. After electrophoresis, cDNA was depurinated in 3.25% HCl for 15 min and denatured in 1.5 M NaCl and 0.5 M NaOH

for 30 min. Neutralization was performed twice in a neutralizing buffer (1.5 M NaCl; 0.5 M Tris-HCl, pH 7.2; 0.0001 M EDTA, pH 8.0) for 15 min. cDNA was then blotted on the Pall Biodyne® A Membrane (Pall Corporation) in 10xSSC buffer (1.5 M NaCl; 0.15 M Na-citrate) overnight. cDNA was fixed on the membrane by baking at 80°C for 2 h.

Hybridization was performed overnight in a hybridization buffer (5xSSC buffer; 0.1% N-laurylsarcosine; 0.02% SDS; and 1% casein) at 65°C, using as a probe pBM290 labeled by the BioPrime® DNA Labeling System (Invitrogen). After washing the membrane in SSC buffers of decreasing ionic strengths, followed by incubation in 3% BSA solution at 65°C for 1 h and incubation with streptavidine-alkaline phosphatase at room temperature for 15 min, the membrane was incubated with the BCIP-dye NBT (BRL) at room temperature for 15 min. The reaction was stopped after visualization of signals by placing the membrane in Tris-EDTA buffer (20 mM Tris, pH 7.5; 0.5 mM EDTA).

DNA Sequencing

Both strands of DNA were sequenced by the Sanger method using an Automated Laser Fluorescence DNA sequencer.

Data Analysis (Computer-Assisted Analysis)

Protein sequences were compared using the BLASTP search program and exploring all of the available sequence databases at the www.ncbi.nlm.nih.gov web server. Sequence analysis was done using the ExpASY program package (www.expasy.org). Sequence alignment was performed with the ClustalW program.

Plant material and flooding treatment

Three- to four-week old buckwheat (*Fagopyrum esculentum* Moench) plants grown in a greenhouse were used for RNA isolation and flooding stress experiments. Prior to submergence, control leaves were taken. The same plants were then submerged completely in water, and flooded leaves were taken at various time points.

Transcript quantification by real-time PCR

Total RNA was extracted from leaves of *wt* buckwheat plants before and after flooding stress using the RNeasy Plant Mini Kit (Qiagen, Germany). To eliminate residual genomic DNA, the samples were treated with RNase-free DNaseI (Amersham Pharmacia). 1 µg of RNA was transcribed into first-strand cDNA using M⁻MuLV Reverse Transcriptase (Biolabs) with random hexamers (Applied Biosystems) as primers in final concentration of 5-µM. Real-Time PCR was performed using the SYBR-Green master mix (Applied Biosystems) in the Applied Biosystems 7500 Real-Time PCR System. Primers annealing to both MT3 transcripts were used: FeMT3-F 5'-gacatcgttgagaccgacaactttg-3' and FeMT3-150-R 5'-tgtccacatagttggaataaagggtgactg-3'. Relative abundance of MT3 transcripts was calculated and normalized with respect to histone *H3* (endogenous control) mRNA according to the method of Muller et al. (2002). A serial two-fold dilution of cDNA derived from control leaves was used as a standard curve to calculate amplification efficiency for *FeMT3* and histone *H3* primers. PCR efficiencies were 1.85 and 1.92 for the *FeMT3* and *H3* genes, respectively. Each reaction was performed in triplicate, and the specificity of amplification products was confirmed by melting curve analysis.

RESULTS AND DISCUSSION

Identification of MT3 cDNA clones by Southern hybridization

In order to identify new MT3 family members from buckwheat, previously isolated partial MT3 cDNA clone pBM 290, lacking the end of the coding region and 3'-UTR, was used as a probe for hybridization with cDNAs present in the developing buckwheat seed cDNA library. Twelve clones were selected and sequenced. The obtained results were analyzed using computer software.

Sequence analyses of MT3 clones

After the performed analyses, only two clones were proven to contain MT sequences. They were named pFeMT3.5 (312 bp) and pFeMT3.10 (479 bp) and submitted to the Blast Gene Bank with acces-

sion numbers [DQ681063](#) and [DQ681064](#). Both of the clones included a 189-bp-long coding region with STOP signal and a 61-bp one that belonged to the 5'-UTR. The clones shared identical 62-bp-long 3'-UTR with extension of 167 bp in clone pFeMT3.10 (Fig. 1A). This was evidence for the presence of at least two MT3 gene family members in the buckwheat genome. In view of mind the importance of 3'-UTR sequences in messenger RNA stability and localization (Hesketh and Vilette, 2002), the detected differences could have an important impact on various functions of FeMT3 family members in buckwheat.

Both of the sequences encoded a 62-aa-long polypeptide which has the typical structure of plant MT-like proteins with two cysteine-rich terminal domains separated by a cysteine-free central domain. On the basis of the number and arrangements of cysteine residues in a specific and conserved pattern in two domains at the N- and C- terminals, clones pFeMT3.5 and pFeMT3.10 were confirmed to belong to type 3 of plant MTs (Cobbett and Goldsborough, 2002). Sequence comparison between the two identified clones showed 95% identity on the protein level. Differences consisted of two amino acid changes in the spacer (central) region of the protein (Fig. 1B). One of them, Ile-Thr, could have influence on conformation and function of the spacer region, which is presumed to be involved in protein-protein and DNA-protein interactions (Domenech et al., 2006). Crystallographic as well as functional analyses of transgenics in those two MT protein variants should reveal potential biological relevance of these "minor" changes.

FeMT3 expression analysis during flooding stress

FeMT3 transcripts have previously been detected in green and senescent leaf, root, and developing seed. Influences of different stress conditions, such as oxidative and heavy metal stress, on the level of *FeMT3* expression were noticed as well (Berkljaić et al., 2004). In order to examine changes in expression of two isolated MT3 transcripts in flooding stress, a quantitative Real Time RT-PCR technology was employed.

A

```

FeMT3.5   GCAAATCACTTCTTACTTCTTACATACAAGAACCTCTTTGTAACCTCAAGCTCATTAAC 60
FeMT3.10  GCAAATCACTTCTTACTTCTTACATGCAAGAACCTCTTTGTAACCTCAAGCTCATTAAC 60
*****

FeMT3.5   CATGTCGTCCAACCTGCGGAAGCTGCGATTGCTCTGACAAGAGCCAGTGTGGGAAGAGGG 120
FeMT3.10  CATGTCGTCCAACCTGCGGAAGCTGCGATTGCTCTGACAAGAGCCAGTGTGGGAAGAGGG 120
*****

FeMT3.5   ATACACCTTTGACATCGTTGAGATCGACAACCTTTGCCGACACCATGGTGATGAACGCCTC 180
FeMT3.10  ATACACCTTTGACATCGTTGAGACCGACAACCTTTGTTGACACCATGGTGATGAACGCCTC 180
*****

FeMT3.5   TGAGAACGATGGCAAGTGCAAAATGCGGAAGCAGCTGCGGGTGCACCAACTGCTCATGCGG 240
FeMT3.10  TGAGAACGATGGCAAGTGCAAAATGCGGAAGCAGCTGCGGGTGCACCAACTGCTCATGCGG 240
*****

FeMT3.5   TTGCCATTGAGCAGTCAACCTTTATTCCAACCTATGTGGACAAAAATGGGGTCTGTGTGAA 300
FeMT3.10  TTGCCATTGAGCAGTCAACCTTTATTCCAACCTATGTGGACAAAAATGGGGTCTGTGTGAA 300
*****

FeMT3.5   ACTAATGATGTTTCTA----- 316
FeMT3.10  ACTAATGATAGGTCCTTGCTAAAT AAGAAGCGGTGTTTCGCCCTTTGTGTCTTTGTGT 360
*****   ***

FeMT3.5   ----- 420
FeMT3.10  TGTGTCTGTTTCTGTGTTTGTTCAGTTCGTTTCATAGAAGCCATGCCCATGGCTTCCC 420

FeMT3.5   ----- 480
FeMT3.10  TTCCCCCTTCTCTGTGTATGTTTCACATAATTTATTGGACTAATGATGTTTCTATTGTACA 480

```

B

```

FeMT3.5   MSSNCGSCDCSDKSQCGKKGYTFDIVEIDNFA DTMVMNASENDGKCKCGSSCGCTNCSGCH 62
FeMT3.10  MSSNCGSCDCSDKSQCGKKGYTFDIVETDNFVDTMVMNASENDGKCKCGSSCGCTNCSGCH 62
*****

```

Fig. 1. Sequence alignment among pFeMT3.5 and pFeMT3.10 clones and their predicted amino acid sequences. Sequence alignment was performed with the aid of ClustalW software. A. Nucleotide sequences. Sequences given in not-bolded letters are the 5'-UTRs, bolded sequences represent coding regions and bases in italic letters are 3'-UTRs. B. Protein sequences. Stars indicate identical nucleotides and amino acids.

The level of *FeMT3* expression in control leaves was compared to that detected in leaves of plants completely submerged in water for different time periods. In contrast to mammalian MT gene transcripts, which increase in tissues under hypoxia during graft transplantation (Li et al., 2004), we observed a significant decrease in *FeMT3* expression after 2 days of flooding stress. No significant change

was detected after 3 hours (Fig. 2). The same was observed after 2 hours and 1 day under the same stress conditions (data not shown).

The obtained results should be discussed in the light of complexity of the plant response to different stress conditions and that of the defined stress *per se*. One of the most prominent consequences of

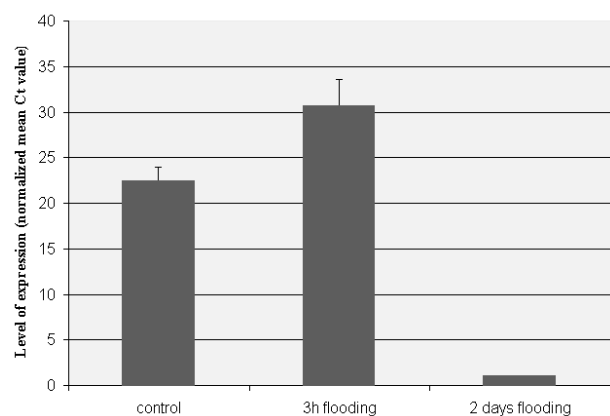


Fig. 2. Analysis of the relative expression level of FeMT3 during flooding by Real Time RT-PCR technology. All values were normalized with respect to the level of endogenous control H3 histone gene expression.

flooding is oxygen deprivation, since gas diffusion is extremely limited in water compared to air. Different plant species have developed different levels of tolerance and different ways to overcome flooding, including biochemical as well as morphological changes. Transient increase in reactive oxygen species (ROS) concentration is detected shortly after submergence and has a role in hypoxia signaling pathways (Vo es en ek et al., 2006). Still, the overall oxidative status is not expected to be elevated, contrary to mammalian hypoxic tissues experiencing oxidative stress due to nonspecific inflammation in graft transplantation (Li et al., 2004). Consequently, the need for MTs in plant vs. mammalian tissues during hypoxia is not the same. The most severe damage to the plant is incurred during oxidative stress upon its re-exposure to air. In some hypoxia-tolerant species, antioxidative enzymes like superoxide dismutase (SOD) are upregulated gradually during submergence, preparing the plant for the postanoxic phase (Monk et al., 1987.). However, FeMT3 expression was not increased in the same manner, but rather decreased. Further investigation will show if FeMT3 genes are upregulated after re-exposure to air, in the period of severe oxidative stress, thus when needed the most.

The existence of two or more highly homologous genes belonging to the same gene family in one species is relevant both from the point of view

of molecular evolution and for studies of different mechanisms governing regulation of gene expression. As mentioned above, various plant MT gene family members may have different expression profiles, indicating their distinct but overlapping functions. Our future work will be focused on elucidation and comparison of particular roles of the two MT3 transcripts by Real Time RT-PCR methodology as the most sophisticated tool in expression analyses of sequence-similar genes.

Abbreviations used

MT) metallothionein; ROS) reactive oxygen species; SOD) superoxide dismutase; UTR) untranslated region.

Acknowledgments — This work was supported by the Ministry of Science of the Republic of Serbia (Grant 143017).

REFERENCES

- Brkljacic, J. M., Maksimovic, V. R., Radovic, S. R., and A. P. Savic (1999). Isolation of metallothionein-like cDNA clone from buckwheat. *J. Plant Physiol.* **154**, 802-804.
- Brkljacic, J. M., Samardzic, J. T., Timotijevic, G. S., and V. R. Maksimovic (2004). Expression analysis of buckwheat (*Fagopyrum esculentum* Moench) metallothionein-like gene (MT3) under different stress and physiological conditions. *J. Plant Physiol.* **161** (6), 741-746.
- Chabanon, H., Nury, D., Mickleburgh, I., Burtle, B., and H. Hesketh (2004). Characterization of the cis-acting element directing perinuclear localization of the metallothionein-1 mRNA. *Biochem. Soc. Trans.* **32**, 702-704.
- Chenchik, A., Diachenko, L., Moqadam, F., Tarabykin, V., Lukyanov, S., and P. D. Siebert (1996). Full-length cDNA cloning and determination of mRNA 5' and 3' ends by amplification of adaptor-ligated cDNA. *Biotechniques* **21** (3), 526-534.
- Cobbett, C., and P. Goldsbrough (2002). Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annu. Rev. Plant Biol.* **53**, 159-182.
- Coyle, P., Philcox, J. C., Carey, L. C., and A. M. Roife (2002). Metallothionein: the multipurpose protein. *Cell Mol. Life Sci.* **59**, 627-647.
- Domenech, J., Mir, G., Huguet, G., Capdevila, M., Molinas, M., and S. Atrian (2006). Plant metallothionein domains: functional insight into physiological metal binding and protein folding. *Biochimie* **88**, 583-593.
- Fischer, E. H., and E. W. Davie (1998). Recent excitement

- regarding metallothionein. *Proc. Natl. Acad. Sci. USA* **95**, 3333-3334.
- Guo, W. J., Bundithya, W., and P. B. Goldsbrough (2003). Characterization of the *Arabidopsis* metallothionein gene family: tissue-specific expression and induction during senescence and in response to copper. *New Phytol.* **59**, 369-381.
- Hesketh, J. E. (1996). Sorting of mRNAs in the cytoplasm: mRNA localization and cytoskeleton. *J. Exp. Cell Res.* **225**, 219-236.
- Hesketh, J. E., and S. Vilette (2002). Intracellular trafficking of micronutrients: from gene regulation to nutrient requirements. *Proc. Nutr. Soc.* **61** (4), 405-414.
- Kagi and Vallee (1960). Metallothionein: a cadmium- and zinc-containing protein from equine renal cortex. *J. Biol. Chem.* **235**, 3460-3465.
- Li, X., Chen, H., and N. P. Epstein (2004). Metallothionein protects islets from hypoxia and extends islet graft survival by scavenging most kinds of reactive oxygen species. *J. Biol. Chem.* **279** (1), 765-771.
- Monk, S. L., Fagerstedt, V. K., and M. M. R. Crawford (1987). Superoxide dismutase as an anaerobic polypeptide - a key factor in recovery from oxygen deprivation in *Iris pseudacorus*? *Plant Physiol.* **85**, 1016-1020.
- Muller, P. Y., Janovjak, H., Miserez, A. R., and Z. Dobbie (2002). Processing of gene expression data generated by quantitative Real Time RT-PCR. *Biotechniques* **32**, 1372-1374, 1376, 1378-1379.
- Murphy, A., Zhou, J., Goldsbrough, P.B., and L. Taiz (1997). Purification and immunological identification of metallothioneins 1 and 2 from *Arabidopsis thaliana*. *Plant Physiol.* **113** (4), 1293-1301.
- Palmiter, R. D. (1998). The elusive function of metallothioneins. *Proc. Natl. Acad. Sci. USA* **95**, 8428-8430.
- Samardžić, J., Milisavljević, M., Brkljačić, J., Konstantinović, M., and V. Maksimović (2004) Characterization and evolutionary relationship of methionine-rich legumin-like protein from buckwheat. *Plant Physiol. Biochem.* **2** (42): 157-163.
- Voesenek, L. A. C. J., Colmer, T. D., Pierik, R., Millenaar, F. F., and A. J. M. Peeters (2006). How plants cope with complete submergence. *New Phytol.* **170**, 213-226.
- Wong, H. L., Sakamoto, T., Kawasaki, T., Umemura, K., and K. Shimamoto (2004). Down-regulation of metallothionein, a reactive oxygen scavenger, by the small GTPase OsRac1 in rice. *Plant Physiol.* **135**, 1447-1456.

АНАЛИЗА СТРУКТУРЕ ДВА ТРАНСКРИПТА ГЕНА ЗА МЕТАЛОТИОНЕИН ХЕЉДЕ И ИСПИТИВАЊЕ ЊИХОВЕ ЕКСПРЕСИЈЕ ТОКОМ ХИПОКСИЈЕ КОРИШЋЕЊЕМ ТЕХНОЛОГИЈЕ REAL TIME RT-PCR

ДРАГАНА Б. МАЈИЋ, ЈЕЛЕНА Т. САМАРЏИЋ, МИРА Ћ. МИЛИСАВЉЕВИЋ,
А. М. КРСТИЋ И ВЕСНА Р. МАКСИМОВИЋ

Институт за молекуларну генетику и генетичко инжењерство, 11010 Београд, Србија

Металотионеини (МТ) припадају великој групи протеина мале молекулске тежине богатих цистеином, изражене способности за везивање јона метала, укључених у процесе одржавања хомеостазе металних јона и детоксификације од тешких метала. У раду је анализирана структура

два транскрипта гена за МТ типа 3 пореклом из семена хељде у развићу. Разлике су нађене пре свега у оквиру 3'-UTR секвенци. Након анализа секвенци урађена је анализа експресије током хипоксије коришћењем технологије Real Time RT-PCR.