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Three *Brassica rapa* metallothionein genes are differentially regulated under various stress conditions

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Abstract The expression profiles of three *Brassica rapa* metallothionein genes (*BrMT 1–3*) were determined in 7-day-old seedlings exposed to various exogenous factors including plant hormones, heavy metals and abiotic stresses. *BrMT1*, *BrMT2*, and *BrMT3* were representatives of *MT* gene type 1, type 2, and type 3, respectively, according to their cysteine alignment. *BrMT2* showed a relatively higher basal expression level compared to *BrMT1* and *BrMT3* under normal conditions. The *BrMT1* transcript was markedly increased by various factors including ethephon, polyethylene glycol and hydrogen peroxide, with no down-regulation evident. On the contrary, *BrMT2* expression was down-regulated by abscisic acid, salicylic acid, and methyl jasmonate. Heavy metals did not increase *BrMT2* expression. *BrMT3* expression was only marginally and non-significantly up- and down-regulated by the stress conditions tested. Promoter regions of *BrMT1* and *BrMT2* display different *cis*-acting elements supporting the different responses of both genes against various stresses. The results demonstrate the differential regulation of *BrMT1–3* by various plant exogenous factors, and indicate the utility of the *BrMT1* promoter as a multiple stress inducible promoter.

Keywords Metallothioneins · Phytoremediation · *Brassica rapa* · Heavy metals · ROS · Promoter

Abbreviations

Br	*Brassica rapa*
MT	Metallothionein
PC	Phytochelatins
PEG	Polyethylene glycol
ROS	Reactive oxygen species
SA	Salicylic acid
ABA	Abscisic acid
MeJA	Methyl jasmonate
ET	Ethephon
H_2O_2	Hydrogen peroxide
UBQ	Ubiquitin

Introduction

Contamination of water and soil by heavy metals degrades the environment, causes direct health hazards to animals and human beings, and decreases crop productivity [1]. A variety of engineering technologies have been explored to develop remediation strategies for heavy metal contaminated sites. One approach of note is the cost-effective and environmentally friendly technology of phytoremediation, in which plants actively sequester heavy metals. Successful phytoremediation requires plants that have a short lifetime, contaminant hyperaccumulation capacity and high biomass. Heavy metal ions play essential roles in several biological process in plants, but excess amounts

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inhibit plant growth due to root damage, decreased chlorophyll content, and leaf chlorosis [2, 3].

To overcome heavy metal stress, plants produce metal chelating proteins such as phytochelatins (PCs) and metallothioneins (MTs) [4]. PCs are enzymatically synthesized low molecular weight peptides having the general structure (γ-Glu–Cys)n-Gly, where $n = 2$–11. Formation of a complex between the PC and a metal ion is followed by internal transport and sequestration of the ion in a vacuole as a detoxification mechanism [5].

MTs are low molecular weight, cysteine-rich, metal binding proteins widely distributed in living organisms and involved in the detoxification of heavy metals and in the homeostasis of intracellular metal ions [6]. The cysteines of MTs are responsible for metal binding via mercaptide bonds [7]. Plant MTs constitute a separate family that is subdivided into four types of MTs based on cysteine residue distribution [4, 8]. Because MT proteins were first isolated as cadmium-binding species in horse kidney [9], earlier studies of plant MTs focused on their role in maintaining metal homeostasis in cells and in mediating the response to metal toxicity [8]. However, more recent findings have proposed extended roles of plant MTs in development, fruit ripening, senescence, and defense against oxidative stress. Recent findings show that the expression of the *Brassica juncea MT2* gene in *Arabidopsis* increases copper and cadmium tolerance, but inhibits root elongation [10]. Two *MT*-like genes are up-regulated during natural leaf senescence in sweet potato [11]. Cotton *MT3* increases plant tolerance to oxidative stresses caused by NaCl, polyethylene glycol (PEG), and low temperature by scavenging reactive oxygen species (ROS) [12].

Plant MTs are thought to be primarily involved in cellular ion homeostasis. While it is likely that plant PCs and MTs both participate in heavy metal detoxification, their distinctive roles have not been clearly demonstrated. *Brassica* species are considered one of the most important models for the study of metal accumulation in plants due to their high biomass production, short life span, and increased acquisition and accumulation of heavy metals such as Cd, Cu, Ni, Zn, Pb and Se [13–16]. In a study that determined the tolerance index of three *Brassica* species for contaminated soils in the mediterranean region of Spain, *B. juncea*, *B. carinata* and *B. oleracea* displayed decreased tolerance to heavy metals such as Zn and Pb [17]. *B. juncea* accumulated high levels of Cd in both hydroponic and soil cultures, but its accumulation was less effective than that of other crops such as maize, rice, and sugar beet at low concentrations of Cd in the soil [18].

Although heavy metal tolerance in *Brassica* species has been well studied, the mechanisms that contribute to the tolerance of *Brassica* families to heavy metals remain unclear. Various plant MTs have been well characterized, but the in vivo functions of each MT gene type have not been elucidated. The aim of the present study was to investigate the responses of individual *Brassica* MTs to various exogenous factors through the exposure of *B. rapa* seedlings to hormones, heavy metals and abiotic stresses, and the assessment of their effect on three *B. rapa* MT (*BrMT*) genes.

Materials and methods

Plant material and growth conditions

Seeds of *B. rapa* L. ssp. *Pekinensis*, inbred line "Chiifu", were germinated on plates of 1/2 Murashige and Skoog [19] with 1.5% sucrose in a growth chamber (22°C, 16 h photoperiod) for 7 days, and the seedlings were used for experiments.

Stress treatment

Seven-day-old *B. rapa* seedlings were used in the assessment of the responses to all stress treatments. For the evaluation of responses to hormone treatment, seedlings were treated with 200 μM salicylic acid (SA), 10 μM abscisic acid (ABA), 50 μM methyl jasmonate (MeJA), and 0.3% ethephon (ET). For heavy metal toxicity, seedlings were exposed to 300 μM each of $CuSO_4$, $FeSO_4$, $ZnSO_4$, and $MnSO_4$. For abiotic stress assessment, seedlings were exposed to 100 μM methyl viologen, 10 mM hydrogen peroxide (H_2O_2), 300 mM NaCl, and 25% PEG. Cold tolerance was assessed through exposure to 4°C. Samples were collected at 3, 6, 12, 24, and 48 h after treatment, frozen in liquid nitrogen, and stored at −80°C until analyzed.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) analysis

Recovered frozen plant tissue was ground in liquid nitrogen with a mortar and pestle, and total RNA was extracted with the Easy-Spin™ total RNA extraction kit (iNtRON Biotechnology, Korea) according to the manufacturer's instructions. Prior to RT-PCR, RNA samples were treated with DNase I for 60 min to digest contaminating genomic DNA. First-strand cDNA synthesis was performed in a 20 μl reaction mixture containing 1 μg RNA, 0.5 μg oligo dT, 40 μM each of the four dNTPs, 2 μl 10× buffer, 200 units of M-MLV reverse transcriptase, and 20 units of RNase inhibitor (Fermentas, Canada). The reaction mixture was incubated at 42°C for 60 min and stopped by heating at 65°C for 10 min. To determine the expression patterns of genes, semiquantitative PCR using gene-specific primers was

performed using the ubiquitin transcript level as internal standard. *BrMT1* (TA5472_3711), *BrMT2* (TA5525_3711), and *BrMT3* (DN192421) sequences were obtained from the website TIGR Plant Transcript Assemblies (http://plantta.jcvi.org/index.shtml). The primers used were: *BrMT1* sense primer, 5′-GTGGATGTGGTTCCGGTTG-3′ and *BrMT1* antisense primer, 5′-GCTGTCCCCACAGCTACAGT-3′; *BrMT*2 sense primer, 5′-CAAGTGTG ATGGCTGCAAA G-3′ and *BrMT*2 antisense primer, 5′-TACAAGTGCAAGG GTTGTCG-3′; *BrMT*3 sense primer, 5′-GGAAACTGC GACTGTTCTGA-3′ and *BrMT*3 antisense primer, 5′-T AGAGCCACACTTGCATTGG-3′; and *Br ubiquitin* (UBQ) sense primer, 5′-ATTCGTGAAGACGCTGACG-3′ and *BrUBQ* antisense primer, 5′-GGCCACACT TCTTCT TCCTG-3′. PCR reaction mixtures included the sense and antisense primers and single-stranded cDNA as template. The thermal cycling conditions were 94°C for 30 s, 56–58°C for 30 s, and 72°C for 30 s for 25 cycles. PCR products were separated by 2% agarose gel electrophoresis and detected under ultraviolet illumination. The densitometry data for band intensities in different sets of experiments was generated by analyzing the gel images on the Image J program (Version 1.33, USA http://rsb.info.nih.gov/ij/).

Cis-acting elements analysis

The genomic sequences containing the regulatory regions of *BrMT1* and *BrMT2* genes were obtained from GenBank (http://www.ncbi.nlm.nih.gov/nucgss). The potential *cis*-acting elements in the 3 kb upstream regions of the genes were identified by using PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html), PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and DNA MOTIF Search (http://www.genome.jp/tools/motif/).

Results and discussion

Responses to various hormones

Several reports have described the responses of plant MTs to plant hormones. The expression levels of *OsMT1*, *OsMT2b*, and *GhMT3* were increased by ABA treatment [12, 20, 21]. The expression of *GhMT3* and *OsMT2b* was also up-regulated by exposure to ethylene and GA, respectively [12, 21]. In contrast, *OsMT2b* was reported to be down-regulated by cytokinin [21].

In the present study, 7-day-old *B. rapa* seedlings were treated with four plant hormones, four heavy metals and five abiotic stresses. The genomic and physiological responses of the seedlings to each stress treatment were assessed 3, 6, 12, 24 and 48 h after exposure. The basal expression levels of three *BrMT* genes (*BrMT1–3*) were determined using

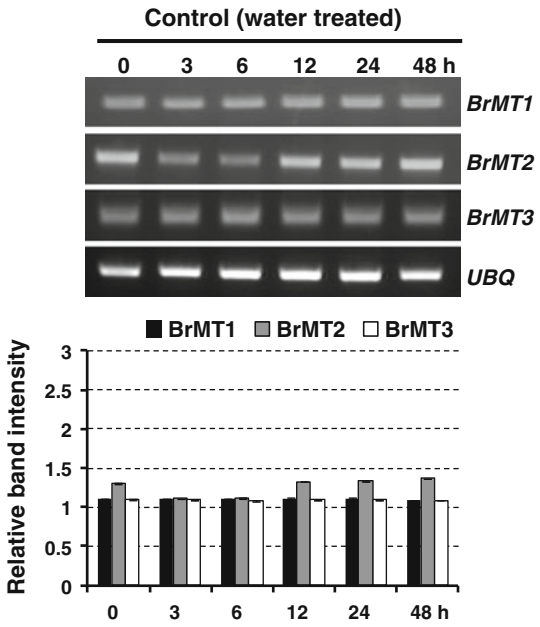

Fig. 1 Comparison of *BrMT1*, *BrMT2*, and *BrMT3* temporal expression in water-treated, 7-day-old *B. rapa* seedlings. Total RNAs were isolated from the seedlings and analyzed by RT-PCR, using gene-specific primers. Ubiquitin mRNA was amplified to confirm that a constant amount of total mRNA was used and ubiquitin was equally expressed in all samples

RT-PCR in water-treated samples. *BrMT2* expression was higher than that of *BrMT1* and *BrMT3* throughout the 48 h experimental period (Fig. 1). *BrMT2* expression was slightly reduced at 3 and 6 h (Fig. 1). RT-PCR was also used to evaluate the effects of ABA, ET, SA, and MeJA (Fig. 2). In ABA-treated seedlings, *BrMT1* transcript levels increased after 3 h and remained elevated until 48 h, while *BrMT2* and *BrMT3* expression was down-regulated. In ET-treated seedlings, *BrMT1* expression was increased at 3 h, decreased to basal levels from 6 to 12 h, and increased again from 24 to 48 h. The expression patterns of *BrMT2* and *BrMT3* were different, showing an elevation at 3 h, followed by a decrease by 6 h to a level that remained constant through the 48 h period. In SA-treated samples, the amount of total RNA recovered at 48 h was insufficient for analysis due to pronounced cell death, and RT-PCR analyses were therefore performed up to 24 h. *BrMT1* expression gradually increased from 6 to 24 h after treatment. *BrMT2* and *BrMT*3 expression, on the other hand, were either down-regulated or unchanged in response to SA. In MeJA- and ET-treated seedlings, *BrMT*1 expression was induced from 12 to 24 h, whereas *BrMT2* gene expression was significantly decreased from 3 to 12 h, recovered to basal level at 24 h, and decreased again by 48 h. *BrMT3* expression was decreased at 3 h but gradually increased thereafter. The overall response patterns of the three *BrMT1–3* genes showed similarities in the responses

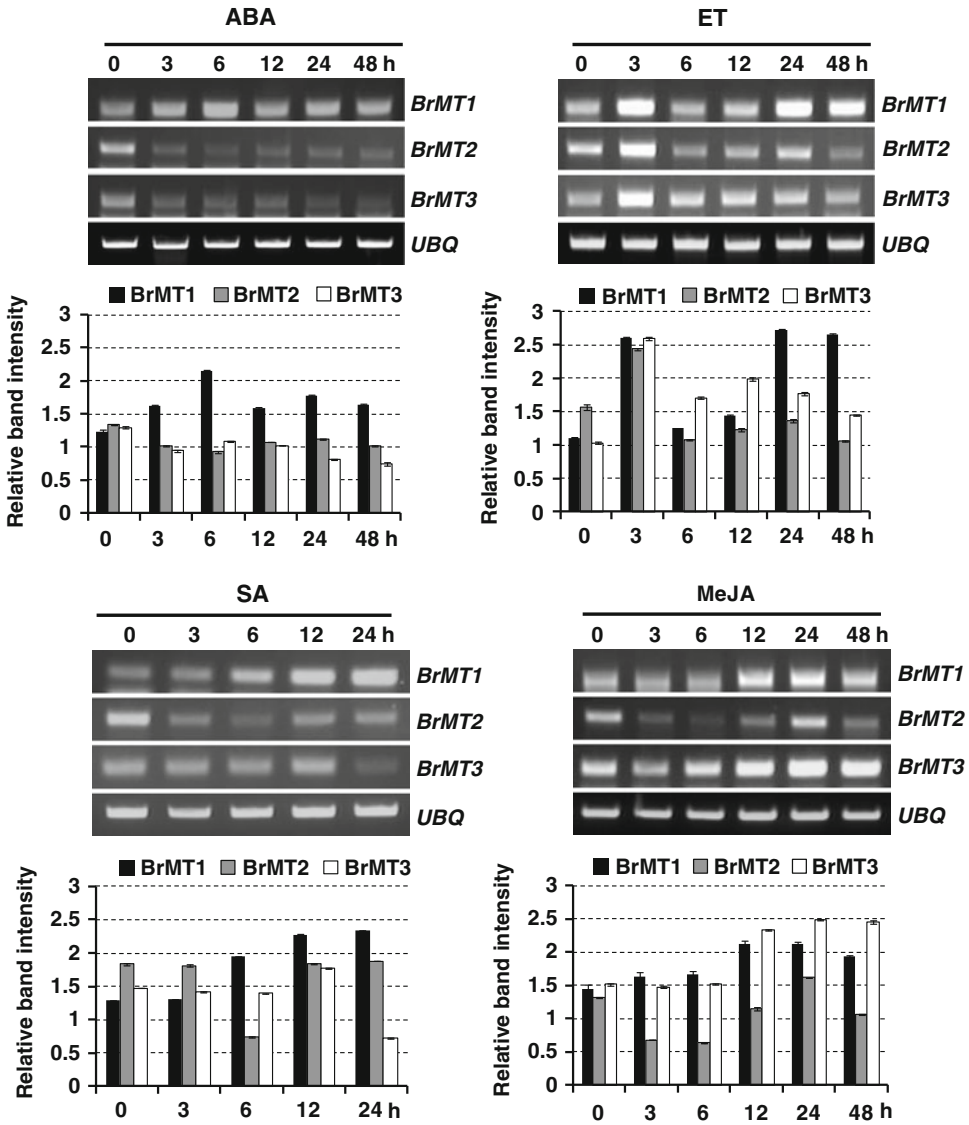

Fig. 2 Effect of ABA, ET, SA and MeJA on expression levels of *BrMT1*, *BrMT2* and *BrMT3*. Total RNA was isolated from the seedlings and analyzed by RT-PCR using gene-specific primers. Ubiquitin mRNA was amplified to confirm that a constant amount of total mRNA was used and ubiquitin was equally expressed in all samples

to ET and MeJA treatments, while *BrMT1* expression was markedly different in ABA- and SA-treated seedlings.

Responses to various metals

Figure 3 summarizes the RT-PCR results showing the changes in *BrMT1–3* gene expression in seedlings during exposure to various metals. In Fe-treated seedlings, *BrMT1* and *BrMT2* expression was not appreciably induced, while elevated expression of *BrMT3* was apparent at 6 and 24 h. In Cu-treated seedlings, *BrMT1* expression was increased at 3 h and it remained at the increased level thereafter, while *BrMT2* expression was down-regulated; no appreciable changes were apparent in the expression of *BrMT3*. In Zn-treated seedlings, *BrMT1* expression was slightly induced at 12 and 48 h, while *BrMT2* expression was marginally down-regulated and *BrMT3* expression showed no changes. In Mn-treated seedlings, *BrMT1* and *BrMT3* gene expression were induced until 12 h and thereafter declined gradually to the basal level. *BrMT2* expression did not change until 12 h and then was gradually down-regulated (Fig. 3).

The observed heavy metal-induced down-regulation was unexpected, given that many plant MT genes are up-regulated by heavy metals. For example, *OsMT1* transcription can be strongly induced by Zn treatment, although not by Mn, Fe and Cu [20]. Meanwhile, *OsMT2b* expression can be strongly increased by Fe, Mn and Zn [22]. Cu and Zn also enhance *GhMT3* expression [12]. Comparison of the expression of type 1 MT genes in *B. rapa* and *Oryza sativa* revealed that Zn could increase both *BrMT1* and *OsMT1* expression, while Cu and Mn increased only *BrMT1* expression. Thus, similar to the observations for hormone-treated seedlings, *B. rapa MTs* are differentially induced by heavy metals.

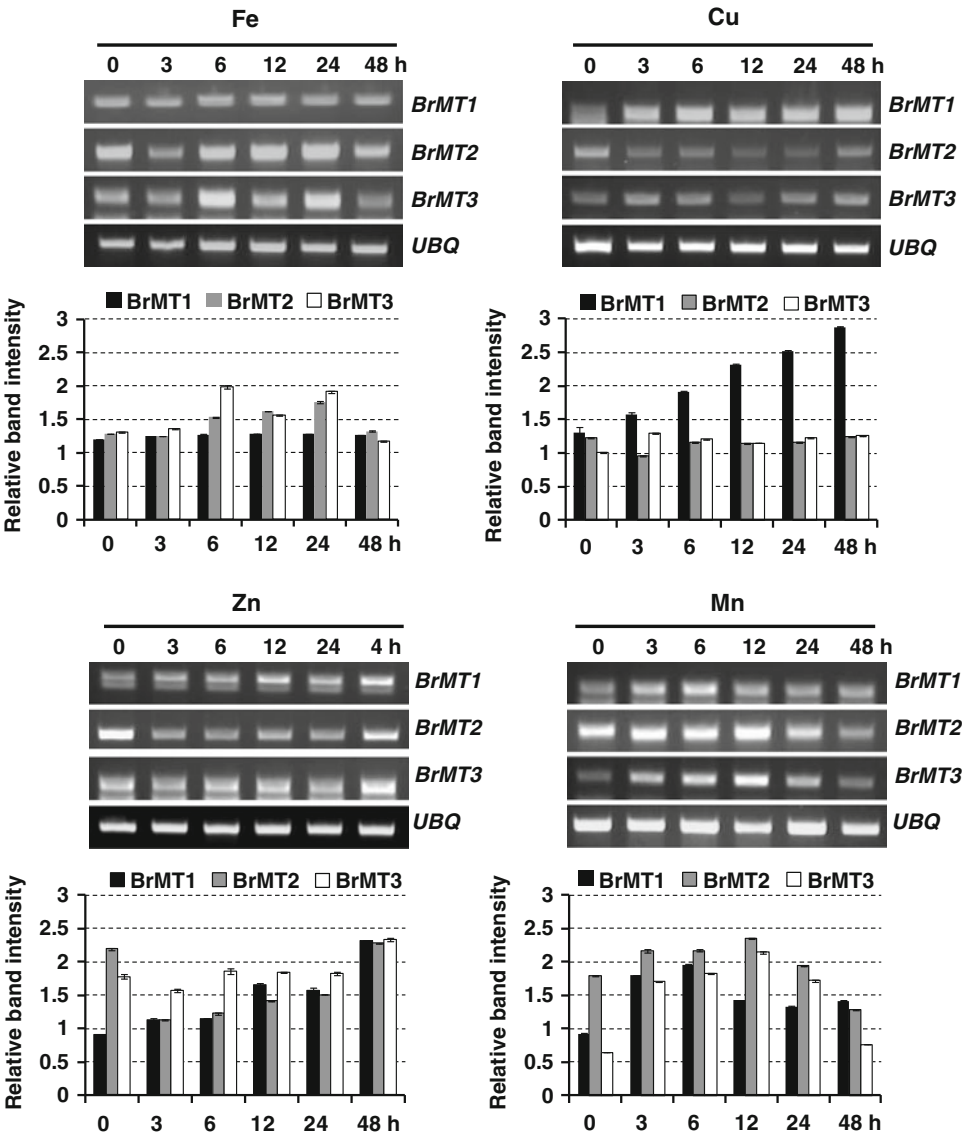

Fig. 3 Effect of Fe, Cu, Zn and Mn on expression levels of *BrMT1*, *BrMT2* and *BrMT3*. Total RNA was isolated from the seedlings and analyzed by RT-PCR using gene-specific primers. Ubiquitin mRNA was amplified to confirm that a constant amount of total mRNA was used and ubiquitin was equally expressed in all samples

Response to abiotic stresses

The RT-PCR results of the different *BrMT* responses to various abiotic stresses are summarized in Fig. 4. The RNA of PEG-treated seedlings was degraded by 48 h, therefore 48 h samples were excluded from the analysis. *BrMT1* expression significantly increased at 6, 12 and 24 h in PEG-treated seedlings, while this treatment did not result in distinct changes in the levels of *BrMT2* and *BrMT3*. In NaCl-treated seedlings, expression of all three *BrMT* genes reached a maximum level at 3 h. Thereafter, the expression levels of *BrMT2* and *BrMT3* gradually decreased, but *BrMT1* levels remained high until 48 h. In seedlings treated with H_2O_2 and methyl viologen (MV, a ROS generating non-selective herbicide), the expression patterns of the *BrMT*s were different. *BrMT1–3* displayed the same expression pattern in response to H_2O_2 treatment, showing induction at 3 and 6 h, a decrease in expression at 12 h, and another increase at 24 and 48 h. However, in seedlings treated with methyl viologen, only *BrMT1* was marginally induced at 48 h, while *BrMT2* and *BrMT3* expression were down-regulated at 24 and 48 h. *BrMT1* and *BrMT3* were also induced by cold treatment, showing a gradual up-regulation of expression from 3 to 24 h and a slight down-regulation at 48 h. However, expression of *BrMT2* was unaffected by cold. A recent report showed that *GhMT3* is induced by cold and *GhMT3* overexpressing tobacco plants are resistant to cold treatment [12]. Research concerning plant MTs has focused mainly on the elucidation of the function of these proteins. Shared common functions include metal chelation, regulation of ion homeostasis, and quenching of ROS under various stress conditions.

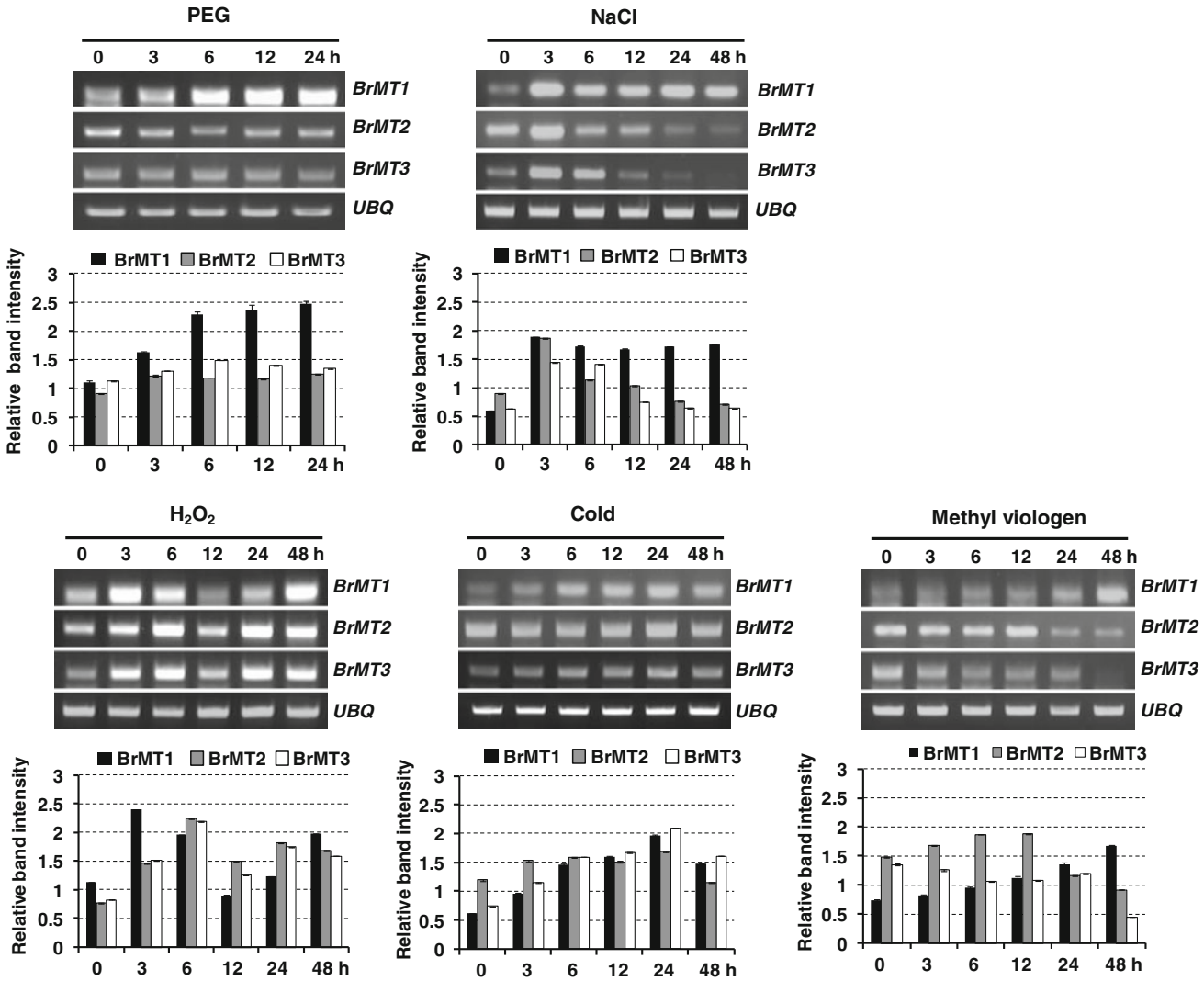

Fig. 4 Effect of PEG, NaCl, H_2O_2, cold and methyl viologen on the expression levels of *BrMT1*, *BrMT2*, and *BrMT3*. Total RNA was isolated from the seedlings and analyzed by RT-PCR, using gene-specific primers. Ubiquitin mRNA was amplified to confirm that a constant amount of total mRNA was used and ubiquitin was equally expressed in all samples

However, there is no unequivocal experimental evidence to compare and clarify the physiological roles of each MT gene in plants. The expression of individual MTs is spatially and temporally controlled in plants. Even under the same conditions, the strength of stimuli such as the concentration of heavy metals and exposure time affects the gene expression of individual MTs. Therefore, several approaches have been used to investigate the regulation of plant MTs, among them the use of functional promoter analysis with the β-glucuronidase reporter gene in several transgenic tobacco plants to assess tissue specific distribution [23–25] and in *Arabidopsis* to test for responses against biotic and abiotic stresses [26–29]. These analyses demonstrated that plant MT genes induced during senescence are also induced by oxidative stresses [28], indicating that ROS is a common signal in the control of MT gene expression. However, the present data suggests that the expression of three MTs from *B. rapa* does not show a direct temporal correlation with exposure to stress conditions. For example, SA and Cu treatments induced *BrMT1* expression, but suppressed *BrMT2* expression. *BrMT1*, which is a type 1 MT gene, was strongly induced by exposure to various plant hormones, metals and abiotic stresses, while *BrMT2* and *BrMT3* were not induced or down-regulated under the same stress conditions. This result suggests that the promoter region of *BrMT1* could be of value for the development of a multiple stress-inducible promoter. The present results support the existence of subtle differences between paralogs in response to the same abiotic stresses, providing clues to the prevalence of isozymes in plants.

In silico analysis of *cis*-acting elements in the 5′ regulatory regions of *BrMT1* and *BrMT2*

There have been a few reports on the presence of putative metal-responsive elements in plant *MT* promoters including Douglas-fir *PmMT* and *ricMT* genes [27, 30]. However, detailed functional analysis to identify the core *cis*-acting element for metal response in plants has not yet been performed. To figure out the different responses of three *BrMTs* under various stress treatments, we investigated the promoter regions of three *BrMT* genes. The sequences of 5′ regulatory regions were available only for *BrMT1* and *BrMT2* from the public GenBank (Supplement 1). The *cis*-acting sequence analysis of the obtained 3 kb upstream region detected the putative TATA box and CAAT box for both of the genes. A total of 13 and 7 gene specific *cis*-acting elements were detected from the 3 kb upstream regions of *BrMT1* and *BrMT2*, respectively, (Table 1). Most of the *BrMT1* gene specific *cis*-acting elements were stress responsive ones such as ABA, MeJA and wounding while *BrMT2*'s were auxin or gibberellins responsive elements. Only one low temperature responsive element (LTR, CCGAAA) was detected at −764 bp of *BrMT2*, which is not consistent with the result in Fig. 4. This is probably because of the different strength and/or exposure time of cold treatment. Ethylene responsive element was identified from both genes in different elements, GCC-box for *BrMT1* and ERE for *BrMT2*. In addition, the elements involved in K^+ influx channel were detected only in the regulatory region of *BrMT1* displaying clear induction of *BrMT1* by several metal treatments.

Although we could not get the promoter regions of *BrMT3*, there are several reports that promoters of type 3 MT genes have *cis*-acting elements responsible for plant hormone and metal ion homeostasis such as ARE and MRE motives [31, 32].

Taken all together, comparison of promoter regions of *BrMT1* and *BrMT2* provides strong support for the differential expression patterns of the *BrMT* genes by exogenous stresses obtained in this study.

Table 1 Potential *cis*-acting regulatory elements identified in the 5′ regulatory sequences of the *BrMT1* and *BrMT2* genes

Cis-acting elements	Sequence	Position		Function
		BrMT1	*BrMT2*	
A-box	CCGTCC		−725	Sugar repression
ABRE	TACGTG	−946		Abscisic acid responsive element
	CACGTG	−1,699		
	CGCACGTGTC	−1,968		
ARE	TGGTTT	−1,275	−833	Antioxidant responsive element
AuxRR-core	GGTCCAT		−118	Auxin responsive element
			−1,059	
CAAT-box	CAAAT	−174	−42	Common *cis*-acting element in promoter and enhancer regions
CAT-box	GCCACT	−1,690	−2,795	*Cis*-acting regulatory element related to meristem expression
CCGTCC-box	CCGTCC		−725	*Cis*-acting regulatory element related to meristem specific activation
CGTCA-motif	CGTCA	−1,650		MeJA-responsive element
ERE	ATTTCAAA		−2,364	Ethylene-responsive element
G-box	CACGTT	−502	−1,417	Light responsive element
	TACGTG	−1,927		
	GCCACGTGGA	−946		
	TCACACGTGGC	−1,701		
	GACATGTGGT	−1,969		
GARE-motif	AAACAGA	−2,274	−185	Gibberellin-responsive element
GCC-box	GCCGCC	−2,696		Ethylene-responsive element
HSE	AAAAAATTTC	−357	−2,068	Heat stress responsive element
			−2,383	
LTR	CCGAAA		−764	Low-temperature responsive element
MBS	TAACTG	−2,707	−508	MYB binding site involved in drought-inducibility
	CAACTG	−2,348	−1,477	
		−1,157	−213	
		−369		

Table 1 continued

Cis-acting elements	Sequence	Position		Function
		BrMT1	*BrMT2*	
Skn-1_motif	GTCAT	−1,628	−1,728	*Cis*-acting regulatory element required for endosperm expression
SURE	GAGAC	−1,761	−109	Sulfur-responsive element
		−2,119	−1,616	
			−1,636	
			−2,975	
TAAAG motif	TAAAG	−2,016		*Cis*-acting element involved in K^+ influx channel
		−531		
		−912		
		−744		
TATA-box	ATATAA	−284	−67	Core promoter element around -30 of transcription start
TATC-box	TATCCCA		−800	Gibberellin-responsive element
TC-rich repeats	ATTTTCTCCA	−1,222	−206	Defense and stress responsive element
	ATTCTCTAAC	−629	−1,380	
	ATTCTCTAAC	−676	−909	
	ATTTTCTTCA			
TCA-element	CCATCTTTTT	−414	−1,011	Salicylic acid-responsive element
T/G-box	AACGTG	−1,926	−1,736	Jasmonic acid-responsive element
		−501		
TGA-element	AACGAC	−1,021		Auxin-responsive element
TGACG-motif	TGACG	−423		MeJA-responsive element
W box	TGACY	−1,205		Involved in activation of ERF3 gene by wounding
WUN-motif	TCATTACGAA	−218		Wound-responsive element
Circadian	CAANNNNATC	−2,634	−1,576	*Cis*-acting regulatory element involved in circadian control
			−899	

Conclusion

To investigate responses of individual *BrMTs*, *B. rapa* seedlings were exposed to various exogenous stress factors. The response of three *BrMT* genes and their differential expression were assessed. In summary, *BrMT1* showed a relatively lower basal level of expression and was strongly up-regulated by all the tested stressors. On the contrary, the expression of *BrMT*2, which was the highest of the three *B. rapa* genes in untreated seedlings, was unchanged or down-regulated in response to the tested stressors. Interestingly, *BrMT2* transcription was not increased by heavy metal treatment and *BrMT3* transcription was increased, albeit insignificantly relative to *BrMT1*, by ET, Fe, and NaCl. Comparative promoter region analysis for both *BrMT1* and *BrMT2* clearly supports that regulatory regions of *BrMT1* and *BrMT2* contain different *cis*-acting elements and more of abiotic stress responsive elements were found in that of *BrMT1*. The collective results demonstrate the differential regulation of the *BrMT* genes by abiotic stressors.

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