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PLANT METALLOTHIONEINS: PUTATIVE FUNCTIONS IDENTIFIED BY PROMOTER ANALYSIS *IN SILICO*

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Metallothioneins are low-molecular-weight proteins capable of covalently binding heavy metal ions due to the presence of many cysteine residues in their sequences. We analyzed the predicted amino acid sequences of 19 metallothionein (7 from *Arabidopsis thaliana* and 12 from *Oryza sativa*) and their promoter sequences *in silico* in order to determine the potential regulatory *cis*-elements present in the promoters of metallothionein genes, from which it is possible to determine the putative functions of these genes. The PlantCARE and PLACE databases provided information about the putative regulatory elements in the metallothionein promoters. Metal response element sequences were found in the promoters of eleven *O. sativa* and two *Arabidopsis* metallothionein genes. Copper response elements were identified in both model plants, usually in many copies, particularly in *O. sativa*. Both the high cysteine content and the presence of metal response motifs in the promoters support the suggestion that metallothioneins play a key role in metal detoxification. The most common putative element in the analyzed promoters was CIRCADIAN, which was present in five *A. thaliana* and eight *O. sativa* sequences. The methyl jasmonate response sequence, root-specific expression element and drought response element were found only in *O. sativa* metallothioneins. Light and low temperature response elements, biotic and abiotic stress elements, an abscisic acid-responsive element and an ethylene-responsive element occur in selected metallothionein promoters of both species. A few promoters have putative organ- and cell-specific regulatory elements. The presence of many different motifs in the promoters of the *Arabidopsis* and *O. sativa* genes implies that metallothioneins are general stress response proteins with many important functions in plants, including regulation of their normal development and adaptation to changing environmental conditions.

Key words: Plant metallothioneins, promoter, *Oryza sativa*, *Arabidopsis thaliana*.

INTRODUCTION

The promoters of genes transcribed by RNA polymerase II are located upstream of transcription start sites. Promoters are responsible for controlling the timing, location and efficiency of gene expression. The specificity and strength of a promoter is determined mainly by the regulatory motifs present in its sequence. Some promoters ensure constitutive expression of the genes they control, while others respond to environmental or endogenous factors.

Metallothioneins (MTs) are small proteins; their molecular weight is relatively low, ranging from 4 to 8 kDa (Cobbett and Goldsbrough, 2002; Koszucka and Dąbrowska, 2006). They are also characterized by high cysteine residue (Cys) content; these cysteines are arranged in characteristic motifs. Depending on the number and arrangement of cysteine residues, four types of plant MTs are distinguished (Freisinger, 2008). The presence of a large number of sulfhydryl groups enables MTs to participate in coordinated binding of heavy metal ions. MTs are essential in heavy metal detoxification processes; they maintain tolerance to stress generated by increased concentrations of metals (Hassinen et al., 2009; Hrynkiewicz et al., 2012; Mierek-Adamska et al., 2009). The amount of *Nicotiana tabacum MT2* mRNA doubles in the presence of copper ions (Choi et al., 1996). Expression of plant *MT* genes rescued Cu^{2+} tolerance in a yeast mutant lacking endogenous MT (Zhou and Goldsbrough, 1994; Ma et al., 2003).

Other stresses influencing plant MT expression are light (Dunaeva and Adamska, 2001), drought (Kohler et al., 2004; Yang et al., 2009), low temperature (Xue et al., 2009) and oxidative stress (Navabpour et al., 2003; Lü et al., 2007). Light- and darkness-induced expression of MTs was observed

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in *Arabidopsis* and *Ipomoea batatas* respectively (Dunaeva and Adamska, 2001; Chen et al., 2003). An analysis of a root EST database showed that the *PtdMT1a* and *PtdMT1b* genes of *Populus* hybrids were repressed 2–3-fold under drought stress which slightly elevated the transcript level of *PtdMT2b* (Kohler et al., 2004). Studies on yeast suggest that MTs not only bind heavy metals but may also be involved in protection against oxidative stress (Xue et al., 2009). Both pathogen attack and wounding induce suberization, during which reactive oxygen species (ROS) are generated (Lamb and Dixon, 1997; Razem and Bernards, 2002) and both can also induce *MT* gene expression in *Nicotiana glutinosa* (Choi et al., 1996) and *Arabidopsis* (Butt et al., 1998; Reymond et al., 2000). The presence of microorganisms in substrate can alter the expression level of metallothionein (Dąbrowska et al., 2011, 2012; Hrynkiewicz et al., 2012).

The level of MT expression is also influenced by phytohormones. In *Gossypium hirsutum* the *GhMT3* transcript is up-regulated by abscisic acid and ethylene (Xue et al., 2009). The level of *Musa acuminata* MT3 increases during fruit ripening, peaking at the moment of ethylene biosynthesis (Clendennen and May, 1997). Steffens and Sauter (2009) showed that the *O. sativa* metallothionein gene (*OsMT2*) was down-regulated by ethylene (and H_2O_2) in epidermal cells undergoing cell death. The expression of type 1 and type 2 *MT* genes decreased in *Populus trichokarpa* × *deltoides* roots treated with auxin (Kohler et al., 2004).

Plant MTs are organ-specific (Ahmadi et al., 2003; Fukuzawa et al., 2004; Dąbrowska et al., 2012a). *MT1* transcripts have been identified in *Mimulus guttatus*, *Pisum sativum* and *Zea mays* roots (de Miranda et al., 1990; Evans et al., 1990; de Framond, 1991). In *Arabidopsis*, *MT2* transcripts are found mainly in aboveground organs (Zhou and Goldsbrough, 1994; García-Hernández et al., 1998). High levels of *MT3* transcripts occur in ripening fruits of *Malus domestica*, *Elaeis guineensis*, *Actinidia deliciosa* and *Vitis vinifera* (Ledger and Gardner, 1994; Reid and Ross, 1997; Davies and Robinson, 2000; Abdullah et al., 2002). *MT* type 4 presents the most specific expression pattern: it is limited to seeds and germinating pollen (Kawashima et al., 1992; White and Rivin, 1995; Guo et al., 2003; Mierek-Adamska et al., 2012). MTs play an important role in seed and root development (Yuan et al., 2008), suberization (Guo et al., 2003), pollen germination (Guyon et al., 2000) and embryogenesis (Reynolds and Crawford, 1996; Chattai et al., 1997). Yuan et al. (2008) showed that *OsMT2b* is expressed in the developing root and embryo and that silencing *OsMT2b* by RNAi causes serious defects in plant growth. During *Triticum aestivum* embryogenesis, an abundant level of *MT4* mRNA gradually decreased (Kawashima et al., 1992).

Plant MTs are not as well studied as animal MTs. Genomic sequencing and Southern blot analysis revealed that plant metallothionein genes form multimember families (Giritch et al., 1998; Liu et al., 2002). Few plant *MT* gene promoters have been described to date. Previously studied plant MTs include those from *Lycopersicon esculentum* (Whitelaw et al., 1997), *Pisum sativum* (Fordham-Skelton et al., 1997), *Pseudotsuga menziesii* (Chatthai et al., 2004), *Citrus unshiu* (Endo et al., 2007), *Phaseolus vulgaris* (Qi et al., 2007), *Elaeis guineensis* (Omidvar et al., 2010) and *O. sativa* (Dong et al., 2010).

The objective of this study was to identify the potential regulatory elements in the metallothionein promoter sequences of *A. thaliana* and *O. sativa* genes through *in silico* analysis, to enable prediction of the functions of the encoded proteins.

MATERIAL AND METHODS

IDENTIFICATION OF GENE SEQUENCES

Gene sequences encoding metallothioneins in *A. thaliana* (*AtMT*) and *O. sativa* (*OsMT*) were found by searches of the NCBI/Gene database (www.ncbi.nlm.nih.gov/gene) using the keyword phrases "*Arabidopsis thaliana* metallothionein" and "*Oryza sativa* metallothionein". Promoter sequences of the analyzed genes at least 1000 bp long were also derived from this database. The one exception is the *O. sativa OsMT 2B* promoter sequence; due to the nearby location of another gene in the same orientation, only 860 bp of this promoter sequence was used for analysis. The cDNA sequences of the analyzed metallothioneins came from the NCBI database of nucleotide sequences (www.ncbi.nlm.nih.gov/nuccore). Putative amino acid sequences were obtained by translating the cDNA sequences using Translate software from the Expasy server (www.expasy.org).

IDENTIFICATION OF REGULATORY MOTIFS

Regulatory motifs in promoter sequences were identified using the PLACE (http://www.dna.affrc.go.jp/PLACE/) (Higo et al., 1999) and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al., 2002) databases.

To identify metal response elements (MREs) in MT promoter sequences of *Arabidopsis* and *O. sativa*, we used the following sequences as well as their reverse and complementary sequences: 5'-TCGA/GCNC-3' (Dixon et al., 1996), 5'-TGCAGGC-3' and 5'-ATTCAAA(N)nAAACTTA-3' (Qi et al., 2007;

TABLE 1. Analysis of translated *MT* sequences of *Arabidopsis thaliana* and *Oryza sativa*

Gene	NCBI number	Chromosome localization	Amino acid number	Cysteine residue number	Cysteine content (%)
AtMT 1A	NM_100633	1	46	6+6	26
AtMT 1B	NM_001037008.2	5	45	6+4	22
AtMT 1C	NM_100634	1	46	6+6	26
AtMT 2A	NM_111773	3	82	8+6	17
AtMT 2B	NM_120316	5	78	8+6	18
AtMT 3	NM_112401	3	70	4+6	14
AtMT EC	NM_201791	2	85	5+5+6	19
OsMT 1A	NM_001056317.1	3	73	6+6	16
OsMT 1B	NM_001075076.1	11	75	6+6	16
OsMT 1C	NM_ 001073595.1	12	79	6+6	15
OsMT 1D	NM_001073598.1	12	80	6+6	15
OsMT 1E	NM_001073613.1	12	79	6+6	15
OsMT 1F	NM_ 001073615.1	12	79	6+6	15
OsMT 2A	NM_001048563.1	1	83	8+6	17
OsMT 2B	NM_001052087.1	1	81	8+6	17
OsMT 2C	NM_001060982.1	5	83	8+6	17
OsMT 3A	NM_ 001048854.1	1	63	4+6	16
OsMT 3B	NM_001073597.1	12	80	4+6	13
OsMT 4A	NM_001071727.1	10	88	6+6+5	19

Ren and Zhao, 2009), 5'-GAGAGCA-3' and 5'-TGCAACC3-' (Dong et al., 2010).

The 5'GTAC3' sequence was used to identify the copper response element (CuRE) (Quinn and Merchant, 1995; Quinn et al., 2000).

RESULTS AND DISCUSSION

GENERAL DESCRIPTION OF METALLOTHIONEINS IN *A. THALIANA* AND *O. SATIVA*

Seven sequences encoding proper MTs from *A. thaliana* (*AtMT 1A, 1B, 1C, 2A, 2B, 3, EC*) and twelve from *O. sativa* (*OsMT 1A, 1B, 1C, 1D, 1E, 1F, 2A, 2B, 2C, 3A, 3B, 4A*) were found in the NCBI/Gene database. The database search gave us even more *O. sativa* sequences, but we did not use them for further analyses as their predicted amino acid sequences lacked the characteristic cysteine-rich motifs.

The length of the predicted MT amino acid sequences ranged from 45 to 85 aa in *Arabidopsis* and from 63 to 88 aa *O. sativa*. The sequences included different quantities of conserved cysteine residues; for example, 10 in *AtMT 3*, *OsMT 3A* and *OsMT 3B*; 16 found in *AtMT* E_C and 17 in *OsMT 4A* (Tab. 1). Other plant MTs have sequences 45–87 aa long and 10–17 Cys residues (Freisinger, 2008). Both the arrangement and number of cysteine residues in the analyzed MTs are characteristic of plants and allow the MTs to be classified into four types (Cobbett and Goldsbrough, 2002; Hassinen et al., 2011; Freisinger, 2011). The MTs contain 2 (type 1–3) or 3 (type 4/E_C) domains (Tab. 1) separated by variable spacers. Each domain includes 4–8 Cys residues (Tab. 1) in characteristic clusters: Cys-X-Cys in type 1; Cys-Cys, Cys-X-Cys and Cys-X-X-Cys in type 2; Cys-X-Cys in type 3; and in type 4 only some Cys are clustered in Cys-X-Cys motifs.

Current knowledge of the metal-binding properties of plant MTs was reviewed by Hassinen et al. (2011) and Freisinger (2011).

IDENTIFICATION OF PUTATIVE *CIS* ELEMENTS CONNECTED WITH THE PLANT RESPONSE TO HEAVY METALS

Metal response elements with the core sequence 5'-TGCRCNCG-3' (where R = purine and N = any base) were initially found in promoters of animal genes regulated by metals (Stuart et al., 1985). Other MRE motifs have been described in plants: TGCAGGC and ATTCAAA(N)nAAACTTA (Qi et al., 2007), GAGAGCA and TGCAACC (Dong et al., 2010). Our analyses identified MRE-like sequences in the promoter regions of all *O. sativa MT* genes and five of the seven *A. thaliana MT* genes (Tab. 2, 3).

TABLE 2. Putative MRE and CuRE sequences in *MT* promoters of *Arabidopsis thaliana*

Gene	MRE motif sequence	MRE localization	CuRE motifs	CuRE localization
AtMT1A	AAACTTA	-1514/-1508, -136/-130	2	-1793/-1789, -1600/-1596
AtMT 1B	GCCTGCA	-639/-633	2	-980/-976, -409/-405
	TGCAACC	-102/-96		
	ATTCAAA....AAATCTTA	-428/-422...-369/-362		
AtMT 1C	AAACTTA	-1513/-1507, -135/-129	2	-1792/-1788, -1599/-1595
AtMT 2A	–	–	3	-225/-221, -211/-207, -145/-141
AtMT 2B	TGCTCTC	-269/-263	1	-808/-804
	ATTCAAA	-899/-893		
	TAAGTTT	-8/-2		
AtMT 3	TAAGTTT...TTGAAT	-1636/-1630...-1575/-1570	6	-2098/-2094, -2089/-2085, -1828/-1824, -1354/-1350, -887/-883, -393/-389
	ATTCAA...AAAGTTA	-536/-530...-489/-483		
AtMT EC	–	–	1	-1295/1291

MRE-like motifs (5'-TGCACACC-3' and 5'-TACGCGCG-3') in plant *MT* genes were first found in the $PsMT_A$ promoter from *Pisum sativum* (Evans et al., 1990) and the $LeMT_B$ gene from *Lycopersicon esculentum* (Whitelaw et al., 1997). Experimental studies confirmed a Cu-induced increase in the expression of a GUS reporter gene directed by the $PsMT_A$ promoter in roots of transgenic *Arabidopsis* plants (Fordham-Skelton et al., 1997), but the functionality of its MRE was not studied. Giritch et al. (1998) showed that the $LeMT_B$ gene is induced by heavy metal treatments, especially treatment with zinc ions. In a study of the promoter sequence of the *Fagopyrum esculentum FeMT3* gene, Nikolić et al. (2010) showed the presence of four MRE elements. Analyses of type 3 metallothionein gene promoters in *Populus alba* (*MT3b*) (Bereta et al., 2009), *Elaeis guineensis* (*MT3-B*) (Siti Nor Akumar et al., 2002) and *Porteresia coarctata* (*PcMT3*) (Usha et al., 2011) revealed the presence of MREs. Usha et al. (2009) described the sequences of three *Prosopis juliflora* MTs that do not contain the MRE motif within the promoter region. In the *MT3* gene of *Citrus unshiu* the MRE sequence does not occur either, and the expression of this gene does not change under the influence of heavy metal ions (Endo et al., 2007). Our analysis also showed that MRE-like sequences are not always present in MT promoters (Tab. 2). Zhou et al. (2006), who searched only for the canonical MRE sequence, reported its presence in six of eleven analyzed *O. sativa* MT promoters. The latest study by Dong et al. (2010) identified MRE motifs in the promoter of an additional *O. sativa* gene, *OsMT-1-4b*, the cDNA of which is identical to our *OsMT 1D*, but our search of the NCBI database sequences yielded a different promoter sequence of the gene than the one they amplified and analyzed. A version of the bipartite MRE motif was previously found in the promoter of the *OsMT2b* gene of *O. sativa* var. *indica* (Ren and Zhao, 2009). The *OsMT2b* cDNA and promoter sequence is nearly identical to our *OsMT 2C* sequences.

Another motif related to the plant response to copper is the CuRE *cis*-element, which has the conserved sequence 5'-GTAC-3', originally identified in the green alga *Chlamydomonas reinhardtii* (Quinn and Merchant, 1995; Quinn et al., 2000) and monocotyledonous plants (Dong et al., 2010). Research by Quinn et al. (2003) revealed that this element is also connected to the plant response to nickel. The CuRE *cis*-element is present in all analyzed promoters of *A. thaliana* MTs (Tab. 2). The *AtMT 3* promoter contains the largest number of copies (6) of the 5'-GTAC-3' motif; one copy of CuRE was identified in the promoters of *AtMT 2B* and *AtMT* E_C. The promoter sequences of most *O. sativa* MT genes contain the CuRE motif in many copies (Tab. 3), with up to 13 copies in the case of *OsMT 4A*. Only the *OsMT 3A* promoter does not contain the regulatory element. Bratiè et al. (2009) described the presence of two CuRE elements at the -485/-482 and -451/-448 positions of the *F. esculentum FeMT3* promoter. Deletion of the region containing both CuREs and other putative elements diminished the reporter gene activity in transgenic plants subjected to simultaneous hypoxia, different metal ions and osmotic stresses. Expression of *FeMT3* was previously recorded in leaves, induced by copper ions (Brkljaèić et al., 2004). Nikolić et al. (2010) demonstrated the protective role of the FeMT3 protein during the exposure of transgenic yeast and plants to heavy metals. Work by Omidvar et al. (2010) demonstrated the presence of the CuRE motif in the

TABLE 3. Putative MRE and CuRE sequences in *MT* promoters of *Oryza sativa*

Gene	MRE motif sequence	MRE localization	CuRE motifs	CuRE localization
OsMT 1A	TGCAGGC	-1890/-1884	12	-1914/-1910, -1584/-1580, -1367/-1363, 1341/-1337, -1249/-1245, -1182/-1178, -1174/-1170, -734/-730, -707/-703, -634/-630, -357/-353, -319/-315
	TGCGCCC	-1017/-1011		
	GCCTGCA	-774/-768		
OsMT 1B	TGCGCTC	-36/-30	8	-1824/-1820, -1622/-1618, -1196/-1192, -1138/-1134, -822/-818, -776/-772, -725/-721, -625/-621
	AAACTTA	-664/-658		
OsMT 1C	TGCAACC	-2966/-2960	7	-2491/-2487, -2159/-2155, -1629/-1625, -1069/-1065, -871/-867, -585/-581, -118/-114
	GAGCGCA	-2586/-2580, -1853/-1847		
	TGCAACC	-271/-265		
	TGCACCC	-175/169		
	ATTCAAA	-808/-802, -495/-489		
	TTTGAAT	-876/-870, -792/-786, -595/-589, -341/-335		
OsMT1D	TGCAACC	-1489/-1483, -502/-496	8	-1579/-1575, -1226/-1222, -1121/-1117, -1037/-1033, -827/-823, -809/-805, -260/-256, -223/-219
	TGCACGC	-493/-487		
	ATTCAAA...AAACTTG	-993/-987...-905/-899		
	ATTCAAA...ATTCAAA	-993/-987...-770/-764		
	ATTCAA...TTTGAAT	-770/-764...-568/-562		
	TTTGAAT	-1423/-1417, -1065/-1059		
OsMT 1E	TGCAGGC	-1661/-1655, -1454/-1448	10	-1594/-1590, -1495/-1491, -1386/-1382, -1353/-1349, -1116/-1112, -749/-745, 428/-424, -309/-305, -276/-272, -247/-243
	GAGAGCA	-1087/-1081		
	TTTGAACT..TTTGAAT	-1174/-1167..-1136/-1130		
	TTTGAAT	-929/-923		
	AAACTGA...TTTGAAT	-552/-546...-488/-482		
OsMT 1F	TGCAACC	-440/-434	7	-1125/-1121, -1098/-1094, -1081/-1077, -678/-674, -386/-382, -326/-322, -44/-40
	TGCACCC	-198/-192		
	TTTGAAT...AAACTTA	-763/-757...-594/-588		
OsMT 2A	ATTCAAA	-498/-492	6	-2242/-2238, -2121/-2117, -705/-701, -639/-635, -411/-407, -158/-154
OsMT 2B	GGTTGCA	-388/-382	4	-1556/-1552, -1406/-1402, -1328/-1324, -876/-872
	TGCGCGC	-231/225		
OsMT 2C	TGCAGGC	-1643/-1637	5	-1665/-1661, -1403/1399, -805/-801, -180/-176, -56/-52
	TGCTCTC	-45/-39		
	ATTCAAA...TTTGAAT	-1094/-1088...-882/-876		
OsMT 3A	TGCTCTC	-1184/-1178	-	-
	TTTGAAT...TAAGTTT	-1599/-1593..-1155/-1149		
OsMT 3B	TGCTCTC	-1926/-1920, -1281/-1275	7	-1498/-1494, -1041/-1037, -1014/-1010, -997/-993, -670/-666, -600/-596, -119/-115
	GAGAGCA	-1453/-1447		
	TGCAACC	-282/-276		
	TGCACCC	-186/-180		
	TAAGTTT...AAACTTA	-917/-911...-820/-814		
	TAAGTTT...TTTGAAT	-917/-911...-841/-835, -648/-642...-595/-589		
	ATTCAAA	-702/-696		
OsMT 4A	GCGCGCA	-1565/-1557	13	-2528/-2524, -1959/-1955, -1920/-1916, -1790/-1786, -1780/-1776, -1770/-1766, -1764/-1760, -1755/-1751, -1542/-1538, -675/-671, -394/-390, -121/-117, -88/-84
	GAGAGCA	-355/-349		

E. guineensis MT promoter sequence and strong induction of this gene in response to Cu^{2+} and ABA treatment. Usha et al. (2009) found CuRE within the promoter region of *Prosopis juliflora MT1*.

Numerous reports confirm the involvement of plant MTs in the homeostasis and detoxification of heavy metal ions (Cobbett and Goldsbrough, 2002; Hassinen et al., 2011). However, the absence of MRE and CuRE motifs in some *MT* promoters and the metal-independent expression of some plant MTs suggest that they might be involved in other processes.

IDENTIFICATION OF ADDITIONAL PUTATIVE REGULATORY ELEMENTS

Using the PlantCARE and PLACE databases we found many other regulatory elements in each of the analyzed promoter sequences, many more in the PLACE database than in the PlantCARE database (data not presented). Table 4 gives the elements in common to both databases.

Eight types of putative regulatory elements were found in the MT promoter sequences of *Arabidopsis*. The largest number of regulatory motif sequences (4) were identified in the promoters of *AtMT 1A*, *AtMT 1B* and $AtMTE_C$. Only one was found in *AtMT 3*. CIRCADIAN motifs are most frequent in the promoters of *Arabidopsis* MT genes, with the exception of *AtMT 1B* and *AtMT 2B*. In *O. sativa* metallothionein gene promoters we found 13 different regulatory elements. The largest number of motifs common to both databases (6 elements – ERE, TGACG, CCGTCC-box, as1, G-box and CIRCADIAN) were in *OsMT 1F*, and the fewest in the *OsMT 1D* and *OsMT 2C* promoters (2 elements). Both the CIRCADIAN and 5'-TGACG-3' motifs were present in eight of the twelve *O. sativa* sequences (Tab. 4).

In both *A. thaliana* and *O. sativa* MT promoters the following motifs occur: W-box, CIRCADIAN, ERE, LTRE, CCGTCC-box, ABRE and G-box. *O. sativa* MT gene promoters contained other regulatory elements not recorded in *Arabidopsis*: GT1, TCT, MBS, O_2 site, as1, and the methyl jasmonate response element. The RY motif associated with regulation of seed development was found only in *Arabidopsis AtMT 1A* (Tab. 4).

AtMT and *OsMT* promoters – hypothetical elements of response to other abiotic stresses

Light

The G-box motif was present in the promoter of two *Arabidopsis* (*AtMT 1B* and *1C*) and three *O. sativa* (*OsMT 1F*, *2A* and *2B*) MT genes (Tab. 4). The G-box element plays a key role in the plant response to light. The G-box element was already identified in the promoter of the gene encoding type 2 metallothionein in *L. esculentum* (Whitelaw et al., 1997). The G-box motif was also found in the promoters of the oil palm genes *MT3-A* and *MT3-B* (Omidvar et al., 2010). Our analyses revealed the presence of the GT1 (*OsMT 1E, 3A*) and TCT (*OsMT 4A*) elements in some promoters; these elements are also associated with the response to light (Tab. 4). In other work we assessed the influence of light on plant metallothionein expression by assaying the transcript levels of *Pharbitis nil MT1* under different light conditions; *MT1* transcript levels were high in plants growing under continuous light but even higher in plants induced to flower by 16 h darkness (Dąbrowska et al., 2010). Increased expression of *MTs* has been induced in *Arabidopsis* by intense light (Dunaeva and Adamska, 2001) and in *Ipomoea batatas* by darkness (Chen et al., 2003), suggesting that light-response elements in *MT* gene promoters may be functional.

Low temperature

The LTRE motif, which is involved in the response to cooling, is contained in the promoters of *AtMT 1A*-C and *OsMT 1B*, *1E*, 2C and *3B* genes (Tab. 4). Xue et al. (2009) used low temperature to induce expression of metallothionein *GhMT3*a in *Gossypium hirsutum* seedlings. Zhu et al. (2009) showed that *MT 2a* in *Arabidopsis* participates in maintaining the balance of ROS during oxidative stress, which is triggered by many factors and especially low temperature.

Drought

Our search demonstrated that the *OsMT 1A*, *2B* and *C* promoters contain the MBS motif with the 5'TAACTG3' sequence; this motif is involved in the response to drought (Tab. 4). Increased tolerance to drought was observed in transgenic *O. sativa* overexpressing *OsMT 1A* (Yang et al., 2009). Xue et al. (2009) demonstrated induction of type 3 metallothionein in *G. hirsutum* in response to drought; overexpression of this *MT* increased tolerance to drought through reduction of the hydrogen peroxide level. Brosche et al. (2005) observed a high level of *MT* expression in trees growing in dry areas. Berta et al. (2009) found that transcription of *MT3* in leaves and the cambial zone of *Populus alba* depended on changes in water status., and suggested the involvement of MT in protection of plant cells during dry seasons.

Cis elements governing cell- and organ-specific expression are also present in the MT promoters

We identified the CCGTCC-box, which is associated with regulation of meristematic cell activity, in the promoters of the $AtMTE_C$, *OsMT 1F*, *OsMT 2A* and

TABLE 4. Selected putative regulatory elements identified in *MT* gene promoter sequences in *Arabidopsis thaliana* and *Oryza sativa*

Motif	Consensus sequence (source)	Function	Occurence
ABRE	TACGTGTC, ACGTGGC, CACGTG (Ezcurra et al., 1999)	response to abscisic acid	*AtMT 1B, AtMT 2B, AtMT EC OsMT 1B, OsMT 2A, OsMT 4A*
ERE	ATTTCAAA (Yang et al., 1998)	response to ethylene	*AtMT 1A, OsMT1F*
TGACG	TGACG (Penninckx et al., 1998)	response to methyl jasmonate	*OsMT 1A, OsMT 1B, OsMT 1C, OsMT 1F, OsMT 2B, OsMT 3A, OsMT 3B, OsMT 4A,*
W-box	TTGACC (Ulker and Somssich, 2004)	response to fungi elicitors	*AtMT 1B, AtMT 2A, AtMT 2B, AtMT EC OsMT 2B*
CCGTCC-box	CCGTCC (Meshi et al., 2000, Silvente et al., 2008)	regulation of meristematic cell activity	*AtMT E OsMT 1F, OsMT 2A, OsMT 2B*
RY element	CATGCATG (Reidt et al., 2000)	regulation of seed development	*AtMT 1A*
as1	TGACGTCA (Lam et al., 1989)	root-specific expression	*OsMT 1A, OsMT 1C, OsMT 1D, OsMT 1F, OsMT 3B*
O_2 site	GATGACATGG (Vincentz et al., 1997)	regulation of metabolism level	*OsMT 1A*
LTRE	CCGAAA (Fenga et al., 2009)	response to low temperature	*AtMT 1A, AtMT 1B, AtMT 1, OsMT 1B, OsMT 1E, OsMT 2C, OsMT 3B*
MBS	TAACTG (Urao et al., 1993)	response to drought	*OsMT 1A, OsMT 2B, OsMT 2C*
G-box	TGACGTTGG, CACGTG, CACGTGG, TGACGTGG (Whitelaw et al., 1997)	response to light	*AtMT 1B, AtMT 1C, OsMT 1F, OsMT 2A, OsMT 2B*
GT1	GGTTAA, GGTTAAT (Argüello-Astroga and Herrere-Estrella, 1996)	response to light	*OsMT 1E, OsMT 3A*
TCT	TCTTAC (Hiratsuka and Chua, 1997)	response to light	*OsMT 4A*
CIRCADIAN	CAANNNNATC (Piechulla et al., 1998)	regulation by circadian clock	*AtMT 1A, AtMT 1C, AtMT 2A, AtMT 3, AtMT EC, OsMT 1A, OsMT 1C, OsMT 1D, OsMT 1E, OsMT 1F, OsMT 2B, OsMT 2C, OsMT 3A*

OsMT 2B. We also identified the O_2 site, known to control metabolism level, in *OsMT 1A*. The RY element, responsible for regulation of seed and development, was identified in *AtMT 1A*, and the as1 motif specifying root expression was found in *OsMT 1A, 1C, 1D, 1F* and *3B* (Tab. 4). Using polyclonal antibodies, Hassinen et al. (2009) detected a high level of MT2 protein in the epidermis of *Thlaspi caerulescens* roots and root hairs. Van de Mortel et al. (2006) showed that root expression of *MT2A* and *MT2B* in *T. caerulescens*, a metallophyte, is much higher than in *A. thaliana*. Expression of the rice *OsMT* 2B gene in the developing root and embryo of germinating seeds (Yuan et al., 2008) suggests that the as1 motif may be functional. Accumulation of *MT* mRNAs in intensively dividing tissues suggests a role for MTs in cell division (Mir et al., 2004).

The most frequent regulatory elements of MT genes are responsive to general stress signaling

The signaling pathways induced by many different types of stress are interrelated in plants (reviewed in Yanhui et al., 2006; Maksymiec et al., 2007; Hirayama and Shinozaki, 2010). Phytohormones and ROS are common signaling molecules in the stress response. Analysis of genes induced by abscisic acid (ABA) enabled us to identify the conserved promoter motif ABRE. The biological functions of ABA include control of seed development and germination (Brkljaèić et al., 2004). In vegetative tissues, ABA controls the response of plants to drought, salinity and low temperature. The ABRE motif is an 8–10-nucleotide sequence with the 5'-ACGT-3' core sequence. The requirement of the

presence of this element for induction of gene expression by ABA has been confirmed experimentally (Giraudat et al., 1994). The ABRE motif is recognized by proteins from the bZIP family with the leucine zipper motif (Busk and Pages, 1998). The core of the ABRE motif is also present in many different regulatory motifs connected with the response to other factors such as white light, UV, auxins and jasmonates. We found the ABRE element in the promoters of three *O. sativa MTs* (*OsMT 1B, OsMT 2A, OsMT 4A*) and three *Arabidopsis MTs* (*AtMT 1B*, *AtMT 2B*, *AtMTE*$_C$) genes (Tab. 4). Previously it has been found in MT gene promoters in *T. aestivum* (Giritch et al., 1998), *Hordeum vulgare* (Ozturk et al., 2002), *P. juliflora* (Usha et al., 2009), *E. guineensis* (Omidvar et al., 2010) and *P. coarctata* (Usha et al., 2011). It has also been shown that exposing plants to ABA induces MT expression in *G. hirsutum* (Xue et al., 2009) and *Thellungiella halophila* (Hobo et al., 1999) and that it induces rice *OsMT2A* and *OsMT4A* genes (Zhou et al., 2006).

The ERE ethylene response element (Quan et al., 2008) occurs in the promoters of genes associated with organ senescence and biotic stress defense. In our study this element was identified in the promoters of *OsMT1 F* and *AtMT1 A* (Tab. 4). The ERE motif has been found in the promoters of *P. juliflora PjMT1* and *PjMT2* (Usha et al., 2009), *P. coarctata PcMT3* (Usha et al., 2011), *E. guineensis* metallothionein (Omidvar et al., 2010) and *L. esculentum LeMTb* (Whitelaw et al., 1997). Expression of the latter gene is higher in mature leaves than in young leaves (Giritch et al., 1998). Coupe et al. (1995) observed MT transcript accumulation in *Sambucus nigra* L. during ethylene-promoted abscission. Steffens and Sauter (2009), however, showed that the gene encoding type 2 metallothionein in *O. sativa* is down-regulated by hydrogen peroxide and ethylene in epidermal cells directed to apoptosis.

Motifs with the consensus sequence 5'-TGACG-3' and 5'-CCACGTCACCG-3' present in the eight *O. sativa MT* promoters (Tab. 4) are known to determine the response to jasmonates. Jasmonic acid (JA) and its methyl ester (MeJA) induce plant defense reactions to fungi and bacteria (Wang and Wu, 2005). *In silico* analysis of the 1.15 kb promoter region of the *Casuarina glauca MT1* gene revealed the presence of three 5'-TGACG-3' motifs (Oberlello et al., 2007). Omidvar et al. (2010) showed the presence of the MeJa-responsive element in the *E. guineensis MT* promoter sequence.

The W-box regulatory sequence associated with the response of plants to fungal elicitors contains the conserved sequence 5'-TTGACC-3'. In *N. tabacum*, Chen and Chen (2000) described the ability of the WRKY transcription factor to bind the W-box and examined its relation to the plant response to pathogen attack. The immediate consequences of recognition of an elicitor by a plant cell are sudden reactions starting with the production of reactive forms of oxygen and followed by activation of many defense pathways induced by jasmonic acid, salicylic acid and ethylene (Berrocal-Lobo and Molina, 2008). Our search revealed the presence of a W-box motif in five metallothionein promoters, including four in *Arabidopsis* (*AtMT 1B*, *2A, 2B* and *E*$_C$) and *O. sativa OsMT 2B* (Tab. 4). Metallothionein expression is induced in *Nicotiana glutinosa* L. by tobacco mosaic virus infection (Choi et al., 1996) and in *Arabidopsis* by mechanical injury and insect attack (Reymond et al., 2000). It seems that the presence of the W-box in promoters enables plant MTs to be involved not only in the response to pathogen attack. The WRKY proteins are a very large family (e.g., 55 differentially regulated genes in *Cucumis sativus*) and are involved not only in biotic stress responses but also in abiotic stress responses, developmental processes, and phytohormone-mediated signal transduction (Ling et al., 2011).

The CIRCADIAN regulatory element with the consensus sequence 5'-CAANNNNATC-3' was first described in the regulatory region of *L. esculentum Lhc* (*light-harvesting complex*) genes. The presence of the motif was shown to be necessary for rhythmic changes in gene expression (Piechulla et al., 1998). Many processes in plants are subject to cyclic regulation. Elongation of the hypocotyl in *Arabidopsis* is controlled by the circadian clock immediately after germination (Dawson-Day and Millar, 1999). We found the CIRCADIAN element in the promoters of many *O. sativa* and *Arabidopsis* MTs, with the exception of *AtMT 1B* and *2B*, and *OsMT 1B, 2A*, *3B* and *4A* (Tab. 4). To date there are no reports suggesting that plant MTs are regulated by the circadian clock. The metallothionein of *Pharbitis nil* did not reveal any regulation by the endogenous rhythm (Dąbrowska et al., 2010), but *MT* expression in the fungus *Neurospora crassa* was shown to be subject to rhythmic changes (Bell-Pedersen et al., 1996). Transcriptome analysis revealed that nearly 70% of *Arabidopsis* genes controlled by the circadian clock are also regulated by abiotic stresses (Kreps et al., 2002) and that the plant circadian clock is interconnected in both ABA and non-ABA stress responses (Sanchez et al., 2011). This suggests a role for the putative CIRCADIAN elements in *MT* gene regulation.

The MBS motif, described above as a potential drought response element, binds MYB factors encoded by a gene superfamily with nearly 200 members in the *O. sativa* and *Arabidopsis* genomes. Plant MYBs are involved in a long list of processes and stress responses (reviewed by Yanhui et al., 2006).

Our analyses of promoter sequences indicate that plant MTs may have many important functions. The metallothionein genes respond not only to heavy metals but to many biotic and abiotic stress factors, so their gene products should be considered general stress proteins. Hassinen et al. (2011) discussed the role of MTs in ROS scavenging. The ABRE, ERE, LTRE and MRE-like motifs found in *Arabidopsis* and MT promoters have also been reported in the promoters of Cd-regulated rice *miR* genes with target genes that encode transcription factors and metabolic proteins controlling plant development and the stress response (Ding et al., 2011). Our results are in accord with the literature and add to the current understanding of plant MT function. The presence, in the promoters of many of the MT genes, of regulatory sequences associated with the response of plants to jasmonates, abscisic acid and fungal elicitors, and with activation of meristematic cells, indicates potential involvement of MTs in many processes enabling the proper growth and development of plants and adaptation to changing environmental conditions.

In silico analyses of the promoter sequences of genes encoding metallothioneins provide a platform for learning more about the functions of MTs in higher plants and represent a direction for future research.

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