Characterization and Expression of DNA Sequences Encoding Putative Type-II Metallothioneins in the Seagrass *Posidonia oceanica*[1]

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Posidonia oceanica is a marine phanerogam, largely widespread in the Mediterranean sea, representing an important food substrate for many marine organisms. A progressive reduction of *P. oceanica* meadows has been reported, due to anthropogenic coastal activity. Studying mechanisms by which this species responds to environmental stresses, three DNA sequences putatively encoding metallothioneins (MTs) have been isolated, by PCR. Two sequences, *Pomt2a* (accession no. AJ249603) and *Pomt2b* (accession no. AJ249602), show high similarities with genes encoding type-II MTs and are interrupted by two and one intron, respectively. The third sequence, *Pomt2c* (accession no. AJ249604), is supposed to be a pseudogene, originated by retrotranscription of the *Pomt2b* mRNA. These sequences belong to a multigene family with at least five members. Northern hybridizations indicated that MT transcripts accumulation is constitutive and seasonally regulated. MT encoding RNAs increase after rhyzome harvesting and (at a lesser extent) after 15 d of cultivation in an aquarium. As for animal MTs, transcripts accumulation is observed also after exposure to trace metals such as copper and cadmium. In the case of copper, the effect depends on concentration. Finally, taking into consideration the great interest in studying the biogeochemical cycle of mercury in the Mediterranean basin and since *P. oceanica* is commonly considered a bioindicator of this metal, the effect of mercury treatments on the accumulation of MT transcripts has been analyzed: in only a few experiments a small increase in the level of transcripts was recorded, suggesting that MTs are not key elements in the mercury accumulation by this species.

Seagrasses of the genus *Posidonia* are marine phanerogams of the Potamogetonaceae family. They grow along coastal waters of the Mediterranean basin and Australia and form dense infralittoral populations that frame the so-called *Posidonia* meadow ecosystem (Boudouresque and Meinesz, 1982). In particular, *Posidonia oceanica* (L.) Delile is largely spread in the Mediterranean basin, where large prairies of this plant represent a dynamic substrate that exceeds the area of the sediment surface several times over and allows settlement of epiphyte organisms. *P. oceanica* prairies extend from a few meters up to 30 to 40 m in depth and occupy an area of approximately 50,000 km^2 (Maserti et al., 1991).

Because of its high productivity, *P. oceanica* represents an important food substrate for many marine organisms (Novak, 1982; Pirc, 1985). The productivity is different in the various Mediterranean areas due to the sensitivity of the plants to changing local and temporal factors of its environment (Giaccone et al., 1981). During recent years in many areas of the Mediterranean sea, the progressive reduction of *P. oceanica* meadows due to coastal activity has been reported (Peres, 1984; Pergent-Martini and Pergent, 1990). This reduction was also related to the widespread vegetative propagation in *Posidonia* prairies (Loques et al., 1990; Buia and Mazzella, 1991; Capiomont et al., 1996; Procaccini and Mazzella, 1998). The resulting genetic uniformity should not allow plants to face environmental changes.

Like other seagrasses, i.e. *Zostera* and *Cymodocea*, *Posidonia* plays a major role in maintaining marine environments. However, basic understanding of seagrass molecular physiology is still limited (Cavallini et al., 1995; Fukuhara et al., 1996), in particular with regards to molecular mechanisms involved in environmental adaptation that could appear peculiar to these plant species compared to terrestrial phanerogams.

It is known that *P. oceanica* may absorb and accumulate metals from sediments in its organs and tissues (Maserti et al., 1988; Ferrara et al., 1989; Rainbow and Phillips, 1993; Warnau et al., 1996; Schlacher-Hoenlinger and Schlacher, 1998) determining metal bioavailability in the marine ecosystem. In particular, since *P. oceanica* can live on mercury-rich sites, it has been proposed to be a bioindicator of this metal (Maserti et al., 1988; Pergent-Martini, 1998).

Trace metal accumulation in plants may induce the production of small peptides, the phytochelatins, and/or 7,000- to 10,000-kD peptides, metallothioneins (MTs), whose involvements in the response of

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plants to trace metals is still controversial (Robinson et al., 1993; Zenk, 1996).

MTs are defined as low-M_r Cys-rich proteins that bind heavy metals. They are widely distributed in eukaryotic and prokaryotic species (Robinson et al., 1993). In higher plants, MT proteins have been purified and sequenced only in Arabidopsis (Murphy et al., 1997). In most cases, however, the presence of proteins that are enriched in Cys and bind metals has been largely inferred from DNA sequences, so that the term MT-like is preferred if protein-metal characterization is not established (Hsieh et al., 1996). Plants may carry three major classes of MT proteins, classes I, II, and III, which are distinguishable on the basis of their sequences (Robinson et al., 1993; Murphy et al., 1997). Class-I MT-like proteins are characterized by two Cys-rich domains separated by a central Cys-free spacer; class-II MTs are represented by the wheat Ec protein, lacking the internal spacer (Kawashima et al., 1992); class-III MTs include diverse glutathione-derived peptides as phytochelatins (Zenk, 1996). Class-I MT-like proteins are further distinguished as type I or type II, based on their Cys spacing; in both types there are six Cys residues in each of the two domains, arranged as CXC motifs (C stands for Cys and X for variable amino acids), but in the amino terminus of type-II MT-like proteins, two additional Cys are present in tandem and one of the CXC motifs exists as CXXC (Robinson et al., 1993). Moreover, in recent years other MT-like coding sequences have been isolated showing different Cys distribution, mainly at the amino terminus, for which the term type-III MT-like proteins has been proposed (Reid and Ross, 1997).

In animals and fungi, MTs have been shown to play a role in the detoxification of heavy metals (Robinson et al., 1993), although their exact function is not completely understood. In plants a correlation has been reported between MT RNA levels and naturally observed differences in the tolerance to heavy metals in Arabidopsis ecotypes, suggesting a role in metal homeostasis (Murphy and Taiz, 1995; Murphy et al., 1997).

The effect of metals on the expression of MTs varies with the plant species, tissue, and MT type. For example, in Arabidopsis (Zhou and Goldsbrough, 1994; Murphy and Taiz, 1995), wheat (Snowden et al., 1995), pea (Fordham-Skelton et al., 1997) and rice (Hsieh et al., 1995), transcription of MTs was enhanced by certain metals. By contrast, in certain species (De Miranda et al., 1990; Kawashima et al., 1991; Okumura et al., 1991), MT mRNA levels were decreased by copper treatment, whereas in other (Lane et al., 1987; Choi et al., 1996; Foley et al., 1997), MT expression was not affected by metals. As reported for animals (Robinson et al., 1993), a number of other stimuli, including abscisic acid, heat shock, cold shock, wounding, viral infection, senescence, fruit ripening, salinity, and Suc starvation, have been shown to affect the accumulation of MT transcripts in plants (Foley and Singh, 1994; Hsieh et al., 1995; Snowden et al., 1995; Choi et al., 1996; Buchanan-Wollaston and Ainsworth, 1997; Foley et al., 1997; Butt et al., 1998; Woodhead et al., 1998; Nam et al., 1999).

In the terrestrial environment, plants uptake trace metals mainly by the root system, whereas leaves can absorb metals only if associated to the particulate matter in the atmosphere or in volatile forms. By contrast, marine phanerogams may uptake trace metals by both roots and leaves, thus providing a valuable tool to study cellular mechanisms of response to the presence of trace metals in the environment. During a study on the mechanisms by which *P. oceanica* responds to trace metals, three DNA sequences putatively coding MTs were isolated by PCR using primers homologous to genes coding other plant MTs. In this paper the genetic and functional characterization of these sequences are reported.

RESULTS

Isolation of DNA Sequences Putatively Encoding Type-II MTs

Three genomic sequences putatively encoding MTs were isolated by PCR amplification, using primers homologous to genes encoding other plant MTs. These sequences are named *Pomt2a*, *Pomt2b*, and *Pomt2c* and are 747, 364, and 226 bp long, respectively. The deduced proteins of the three sequences were analyzed by the BLAST program at National Center for Biotechnology Information (Bethesda, MD) (Altschul et al., 1990) and showed high similarities to putative plant MTs.

After comparison to other MT sequences and using the program FEX at Baylor College of Medicine (Houston), it was inferred that *Pomt2a* should contain two introns, from bases 65 to 499 and from bases 576 to 660. *Pomt2b* should contain only one intron, from bases 143 to 279. The second intron of *Pomt2a* is located at the same site of the unique *Pomt2b* intron, but it is shorter than the unique *Pomt2b* intron (84 versus 136 bp).

The deduced amino acid sequences of *Pomt2a* and *Pomt2b* contain the Cys-rich regions typical of plant class-I type-II MTs, including the presence of a CXXC motif at the amino terminus (see the introduction). The alignment of *Pomt2a* and *Pomt2b* deduced proteins to other eight plant type-II MTs is reported in Figure 1. Only minor differences are observed between *Pomt2a* and *Pomt2b* with regard to their deduced amino acid sequence in the Cys domains, in particular three conservative substitutions in the 5′ domain and one conservative substitution in the 3′ domain, respectively. More differences are found in the internal spacer: 16 amino acids were substituted, nine of which were conservative and seven non-conservative.

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Pomt2a                          CGSGCNCGNGCGGCKMYPDL
Pomt2b                          CGSGCTCGSGCSGCKMYPDL
GI3900980,E.crassipes -MSCCGGNCGCGSGCKCGNGCSGCKMYPDM
GI3334260,M.acuminata -MSCSGGNCGCGSSCSCGSGCGGCRMLTDL
GI2497903,O.sativa    -MSCCGGNCGCGSGCQCGSGCGGCKMYPEM
GI1171035,A.chinensis -MSCCGGKCGCGSSCSCGSGCGGCGMYPDL
GI3334261,M.domestica MSSCCGGKCGCGSVCSCGSGCNGCGMAPDL
GI3901014,F.sylvatica -MSCCGGNCGCGTGCKCGSGCGGCKAYPDL
GI2815246,C.arietinum -MSCCGGNCGCGSSCKCGSGCGGCKMYPDM
GI2497901,N.glutinosa -MSCCGGNCGCGSGCN---GCGGCKMYPDL

Pomt2a                -LEMGSTSETLILGVAPEKKIFGGFEMA--
Pomt2b                -IEKCTSTETLIMGVASEKRSYDGFEMV--
E.crassipes           -EEKTVTAQTMIMGIAAEKGHFEDFE----
M.acuminata           GEERSSTSQTMIMGVAPQKGHFEELET---
O.sativa              A-EEVTTTQTVIMGVAPSKGHAEGLEAGAA
A.chinensis           SYSEMTTTETLIVGVAPQKTYFEGSEMG--
M.domestica           SYMEGSTTETLVMGVAPQKSHMEASEMG--
F.sylvatica           SYTEKTTTETLIVGVAPQKAHSEGSEMG--
C.arietinum           SYTEQTTSETLVMGVASGKTQFEGAEMG--
N.glutinosa           SYNESTTTETLVLGVGHEKTSFGTMEMGES

Pomt2a                GAS--EN-GCKCGSN
Pomt2b                AGT--EN-GCKCGSS
E.crassipes           -AAGSENEGCKCGSNCTCNPCNC-K
M.acuminata           -AAGSDN-GCKCGSNCTCDPCNC-K
O.sativa              AGAGAEN-GCKCGDNCTCNPCNCGK
A.chinensis           VAA--EN-GCKCGSDCKCDPCTC-K
M.domestica           VAA--EN-GCKCGDNCTCNPCNC-K
F.sylvatica           VGA--ENGGCKCGSNCTCDPCNC-K
C.arietinum           FGA--ENDGCKCGSNCTCNPCTC-K
N.glutinosa           PAA--EN-GCKCGSECKCNPCACSK
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Figure 1. Sequence alignment of *Pomt2a* and *Pomt2b* deduced proteins to other type-II MTs (for which GenBank code and species name is reported). Sequences were aligned by the CLUSTAL program. Conserved amino acids are reported in bold. Dashes are included for optimal alignment. Target sequences of *Pomt2a* and *Pomt2b* are not included in the comparison.

The third sequence, *Pomt2c*, varies from the others for one base deletion at base 62 and some base substitutions and for the absence of the DNA sequences corresponding to the introns of *Pomt2a* and *Pomt2b*. The deletion at base 62 determines a frame shift and the presence of a stop codon in the RNA. For this reason, and because of the absence of introns, *Pomt2c* can probably be considered as a pseudogene that originated by reverse transcription of the mRNA produced by other MT sequences. In particular it is conceivable that *Pomt2c* derived from reverse transcription of a mRNA encoded by *Pomt2b*, because only five base substitutions are observed between *Pomt2c* and the coding regions of *Pomt2b*, versus the 41 base substitutions between *Pomt2c* and those of *Pomt2a*.

The sequence comparison between *Pomt2a*-b and other 46 plant type-II MTs produced a single most parsimonious tree, reported in Figure 2.

Genomic Organization of the Isolated MT Coding Sequences

Southern hybridizations were performed to estimate the copy number of each gene. Southern-blot experiments were then made using DNAs restricted with enzymes that do not recognize sites within any of the three MT-like coding genomic sequences (Fig. 3).

It may be observed that *Pomt2b* and *Pomt2c* show the same restriction pattern (four *Eco*RI, four *Bam*HI, and two *Msp*I bands), indicating the presence of at least four members of these two sequences in the *P. oceanica* genome, altogether. By contrast, *Pomt2a* shows only one band, suggesting that this may be a single-copy gene. On the whole, at least five MT genes should be present in *P. oceanica* genome.

Steady-State Level of MT mRNAs in Leaf Tissues of *P. oceanica* in Vivo

The accumulation of MT-like transcripts was analyzed in vivo by northern-blot experiments and hybridization with *Pomt2b* probe. The minor differences in the coding regions between *Pomt2a* and *Pomt2b* did not distinguish *Pomt2a* or *Pomt2b* mRNA sequences in northern blots. In Figure 4, the steady-state levels of MT transcripts in basal and central portions of leaves collected in March or June of 1999 and frozen in liquid nitrogen immediately after explantations are reported.

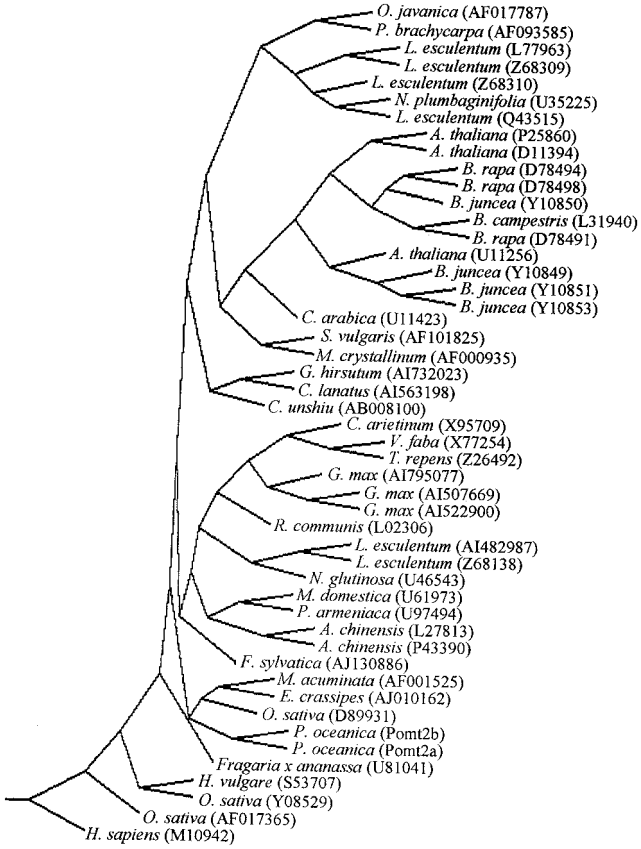

Figure 2. Strict consensus of 100 most parsimonious trees of plant type-II MTs, obtained using PROTPARS software (PHYLIP package version 3.572). An MT coding sequence of *Homo sapiens* (M10942) was used as the outgroup. Bootstrap support values (%) are shown at the forks. For each sequence the GenBank code is reported.

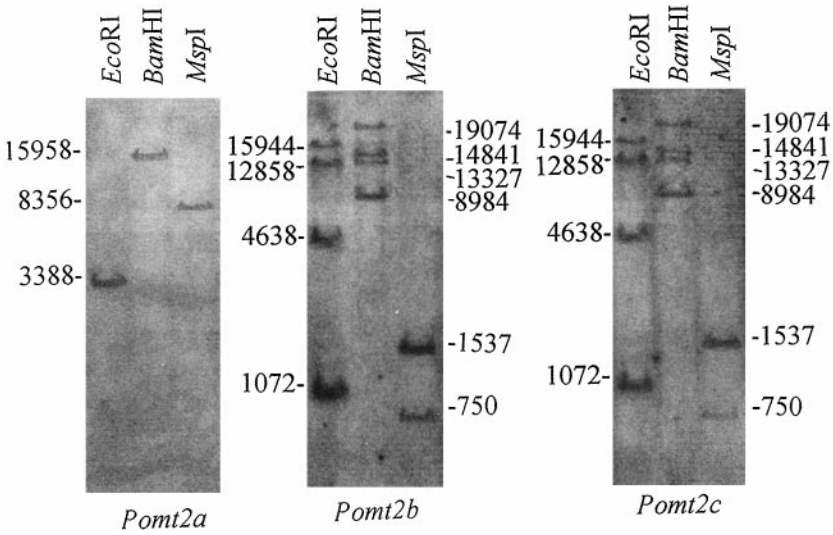

Figure 3. Southern blots of *P. oceanica* DNA, digested with enzymes that do not recognize sites within isolated putative MT coding sequences, hybridized with digoxygenin-labeled *Pomt2a*, *Pomt2b*, or *Pomt2c*. Molecular masses of bands are reported in bp.

Very low metal concentrations were measured in the seawater where seagrasses were collected at least for copper, cadmium, and mercury (Table I). Total (i.e. including both bioavailable and chelated) metal concentrations were about 10^{-2} μM for copper, 10^{-3} μM for cadmium, and 10^{-4} μM for mercury; these concentrations are much lower than those commonly used in experiments involving metal treatments for MT induction in plants. In this sense, the accumulation of MT transcripts in vivo should be considered as constitutive.

However, differences were observed between leaf portions: younger leaf portions accumulated more MT transcripts than differentiated ones in March, whereas in June, more transcripts were found in the differentiated portions than in the basal ones (Fig. 4, top).

The copper, cadmium, and mercury content of leaf tissues collected in March and June of 1999 is reported in Table I. No relationship may be found between metal concentrations and MT transcripts accumulation in the different leaf portions and time periods.

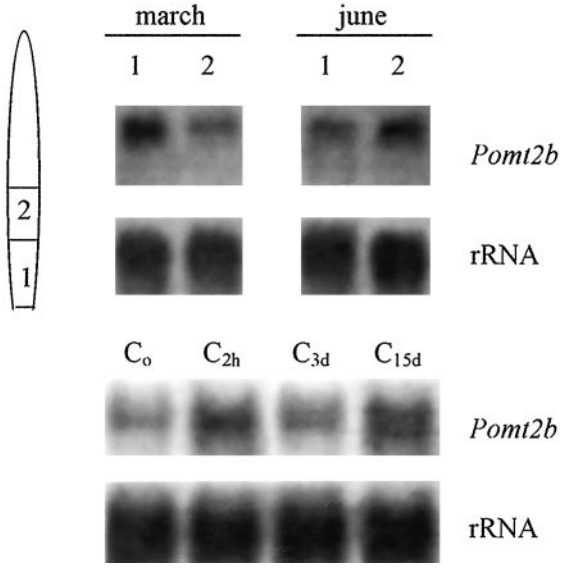

Figure 4. Northern blots of RNAs isolated from: different regions of leaves of *P. oceanica* (1, basal; 2, central; a schematic representation of leaf is reported) collected from Antignano meadows in different months and immediately frozen in liquid nitrogen (top) or isolated from the basal region of leaves of *P. oceanica* collected in March, immediately frozen in liquid nitrogen (C_o) or frozen after 2 h (C_{2h}), 3 d (C_{3d}), or 15 d (C_{15d}) in an aquarium, hybridized with digoxygenin-labeled *Pomt2b* probe (bottom). Ten micrograms of total RNA per sample was loaded. A *Phaseolus coccineus* 25S rDNA fragment was used as a control probe (rRNA).

Steady-State Level of MT mRNAs in Leaf Tissues of *P. oceanica* in the Aquarium

The steady-state level of MT transcripts in the basal leaf portions of plants acclimated in the aquarium was studied by northern blot and hybridization with labeled *Pomt2b* as probe (Fig. 4, bottom). An increase in the amount of MT transcripts was observed after 2 h from shoot explantation, with a subsequent decrease in shoots acclimated for 3 d in the aquarium and a slight increase after 15 d in the aquarium.

Trace-Metal-Induced Accumulation of MT Transcripts in Leaf Tissues of Plants Cultivated in the Aquarium

To study metal induction of MT transcripts accumulation, experiments were performed on shoots of *P. oceanica* after 1 d of acclimation in aquarium. After this time, seawater was substituted and $CuCl_2$, $Cd(NO_3)_2$, or $Hg(NO_3)_2$ was added at final concentrations of 10 μM, 10 μM, and 10^{-2} μM, respectively. After 2 d, total RNA was isolated from basal leaf portions, of which the copper, cadmium, and mercury concentrations are reported in Table II. Experi-

Table I. *Copper, cadmium, and mercury concentrations in seawater and in plant tissues in vivo*

Cu_{2+}, Cd_{2+}, and Hg_{2+} concentrations measured in the seawater where plants were collected (μM) and in young (1) or differentiated (2) portion of *P. oceanica* leaves (μg/g dry wt; see Fig. 4) of plants collected in March and June of 1999 at the same sampling site. For each metal determination in plant tissues, values are not significantly different at the 5% level with Tukey's ranking test.

	Copper (Seawater 10^{-2})		Cadmium (Seawater 10^{-3})		Mercury (Seawater 10^{-4})	
Sampling time	1	2	1	2	1	2
March	20.7	17.5	2.3	3.5	0.3	0.7
June	21.6	13.4	2.8	2.3	0.2	0.2

ments of northern blot and hybridization with labeled *Pomt2b* probe (Fig. 5) indicate that MT transcripts accumulation is increased by copper and cadmium, whereas no apparent effect is observed after mercury treatment.

In other experiments, plants were treated with different (1 and 10 μM) $CuCl_2$ concentrations. Northern-blot experiments (Fig. 5) show that the increase in steady-state level of MT transcripts depends on the copper concentration, at least in the range of concentrations used in the experiments undertaken here. The increase of copper concentration in leaves treated with 1 μM $CuCl_2$ is very small, hence $CuCl_2$ concentrations lower than 1 μM were not used. On the other hand, treatments with $CuCl_2$ concentrations higher than 10 μM was fatal to the plants in the aquarium after 1 d.

Finally, since *P. oceanica* is known to be mercury tolerant and a bioindicator of environmental mercury contamination, shoots have been treated with different mercury concentrations [10^{-2}, 1, and 5 μM $Hg(NO_3)_2$] for 2 and 14 d. Only a small (and not regularly observed) increase of MT transcripts was found in treated plants for every mercury concentration and treatment duration tested. In Figure 5, northern blot of RNA of leaves of plants treated with 5 μM $Hg(NO_3)_2$ hybridized with labeled *Pomt2b* probe is reported. The results obtained in the other mercury treatments are similar and are not shown.

DISCUSSION

Physiological studies on marine phanerogams are very rare, probably due to difficulties on collecting and cultivating plants, though fundamental for the great importance in maintaining the marine ecosystem. In particular, studies on the response of these plants to environmental stimuli can be useful to establish adequate strategies of protection of marine environment.

P. oceanica is commonly studied as a trace-metal bioaccumulator (see the introduction), but no information on molecular mechanisms of metal tolerance is currently available. On the other hand, such molecular mechanisms are yet to be completely established for every plant species. It is known, for example, that genes coding for putative MTs are present in plant genomes, but their role in stress response is still controversial (Zenk, 1996).

In our experiments, three DNA sequences had been isolated whose deduced proteins show strong similarities with deduced class-I type-II MTs of other plant species (Fig. 1). Two of them, *Pomt2a* and *Pomt2b*, are probably different genes, as shown by the different hybridization-labeling pattern in Southern-blot experiments (Fig. 3). Both *Pomt2a* and *Pomt2b* are related to type-II MTs of other monocotyledons, as shown by phylogenetic analysis (Fig. 2).

It is interesting that the third sequence, *Pomt2c*, is very similar to *Pomt2b* except for the absence of the intron and some point mutations, of which one (in position 62) determines the presence of a stop codon. These characteristics support the hypothesis that this sequence is a pseudogene derived from retrotranscription of a *Pomt2b* mRNA. A similar case was described in human genome (Hamer, 1986), where five MT pseudogenes were isolated in addition to six functional genes. The origin of *Pomt2c* from a transcribed *Pomt2b* sequence is also indicated by the results of Southern-blot and hybridization experi-

Table II. *Copper, cadmium, and mercury concentrations in metal-treated plants*

Cu_{2+}, Cd_{2+} and Hg_{2+} levels (μg/g dry wt) measured in *P. oceanica* leaves from plants experienced, at laboratory conditions, with different metals and metals concentrations, independently. For each metal, means followed by the same letter are not significantly different at the 5% level with Tukey's ranking test.

Treatment Time	Copper		Cadmium	Mercury		
	1 μM	10 μM	10 μM	10^{-2} μM	1 μM	5 μM
2 d (Control)	18.0a	15.7a	3.3a	0.4a	0.2a	0.3a
2 d (Treated)	27.9b	80.2c	302.9b	40.8b	252.0c	400.8d
15 d (Control)	–	–	–	0.4a	0.5a	0.2a
15 d (Treated)	–	–	–	50.3b	290.0c	635.8e

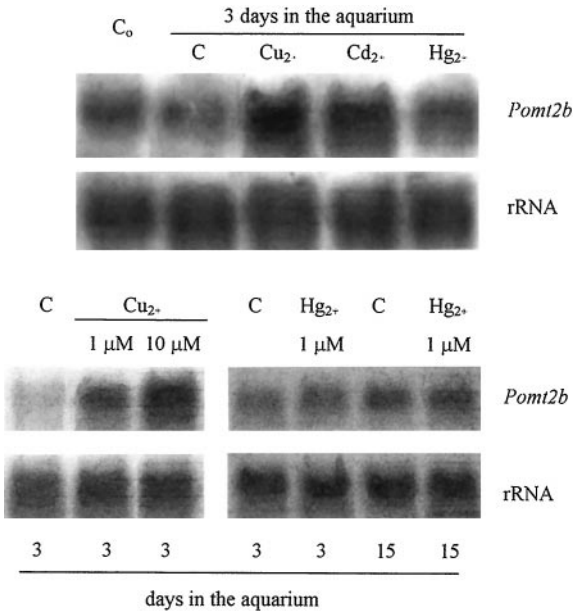

Figure 5. Northern blots of RNAs isolated from the basal region of leaves of *P. oceanica* collected in June from Antignano meadows. Top, Immediately frozen in liquid nitrogen (C_o) or frozen after 3 d in an aquarium supplemented (for the last 2 d) with 10 μM CuCl2 (Cu_{2+}), 10 μM $Cd(NO_3)_2$ (Cd_{2+}), 1 μM $Hg(NO_3)_2$ (Hg_{2+}) or with no trace metal added (C); bottom left, frozen after 3 d in an aquarium supplemented with no trace metal (C) or treated (for the last 2 d) with 1 μM or 10 μM CuCl2; bottom right, frozen after 3 or 15 d in an aquarium supplemented with no trace metal (C) or treated (for the last 2 or 14 d, respectively) with 1 μM $Hg(NO_3)_2$. Digoxygenin-labeled *Pomt2b* was used as probe. Ten micrograms of total RNA per sample was loaded. A *P. coccineus* 25S rDNA fragment was used as a control probe (rRNA).

ments: the same pattern is obtained using the two sequences as probes (Fig. 3). However, only analysis of sequences flanking *Pomt2c* in the genome of *P. oceanica* may definitely clarify the nature of this sequence.

Other considerations may derive from the results of Southern-blot experiments. For example, the single band observed for *Pomt2a* indicates that this is a single-copy gene; moreover, unless two different allele hybridize on fragments of the same length, the single band should indicate that these plants are probably homozygous, supporting the theory that, besides through vegetative propagation, these plants reproduce (even rarely) autogamously, as shown by microsatellite analyses on different populations (Procaccini and Mazzella, 1998).

According to this hypothesis, since *Pomt2b* or *Pomt2c* show four hybridization bands, it should be conceivable that at least four of these genes are present in *Posidonia* genome, with other pseudogenes than *Pomt2c* possibly contributing to this number. Obviously, it is also possible that these plants are homozygous only for *Pomt2a* gene, and heterozygous for the other MT genes, that consequently would be in number lower than four.

The expression of the isolated sequences was studied by northern-blot and hybridization experiments. No specificity could be established in expression pattern between *Pomt2a* and *Pomt2b* in northern blots, because of the high homology between coding regions of these sequences. We assume that hybridization signals using labeled *Pomt2b* as probe are due to *Pomt2b* mRNAs; however, it is also possible that those signals were partly due to *Pomt2a* mRNAs. Experiments of quantitative RT-PCR (using specific primers) are being planned to establish if different genes are expressed in response to different stimuli or metals, as observed for MT genes of Arabidopsis (Murphy and Taiz, 1995).

MT mRNAs were found in leaves collected from plants in the sea and immediately frozen (Fig. 4, above). Actually, the concentration of copper, cadmium, and mercury in the seawater where seagrasses were collected was very low (Table I), i.e. much lower than metal concentrations commonly used to induce gene expression in other plants. On the other hand, total metal concentrations in the water is similar to those measured in other sites of the Tyrrhenian Sea: hence, it is conceivable that MT genes are expressed constitutively, as reported for Arabidopsis roots (Murphy et al., 1997). Alternatively, it is possible that metals induce MT gene expression in *P. oceanica* at thresholds lower than those established for other plant species. Moreover, it is also possible that MT mRNA accumulation can be induced by metals different from those studied in the experiments described here.

The steady-state level of MT mRNA in young and differentiated portions of the leaf and in different time periods was also analyzed (Fig. 4, top). Differences were observed between leaves collected in March or in June, in particular related to the leaf portion analyzed. Younger tissues showed higher MT mRNA levels than older ones in March; whereas in June, a somehow opposite result was obtained. Copper, cadmium, and mercury concentrations in the water did not vary between March and June. Concerning the leaf portions from which RNAs were isolated, no significant differences were observed in copper, cadmium, and mercury concentrations (Table I), indicating that variations found in the steady-state MT mRNA level between young and differentiated leaf portions are not related to metal translocations in the plant.

Different stimuli are known to determine high steady-state levels of MT mRNAs in other plant species (see the introduction). For example, tissue wounding is known to induce MT mRNA increase in *Nicotiana* spp. (Choi et al., 1996). MT mRNA levels were studied in leaves from plants after 2 h from harvesting, after 3 and 15 d in the aquarium without adding metals (Fig. 4, bottom). Leaves of plants 2 h after sampling show an increase of MT mRNA levels, probably induced by wounding. After 3 d, MT

mRNA signal is similar to those recorded at time 0, i.e. MT mRNA accumulation reduces with acclimation to aquarium conditions. A further increase in MT mRNA levels is found in leaves from plants cultivated for 15 d in the aquarium. This is probably because of the stress imposed on plants by prolonged time periods in the aquarium, which indicates the necessity of studying *P. oceanica* physiology only in the first days after harvesting.

Among the trace metals that can induce the expression of MT genes, the effects of copper, cadmium and mercury were studied. Concerning copper and cadmium (Fig. 5), an increase of MT mRNA levels after 2-d treatments with 10 μM copper or cadmium was noted, confirming that these metals can induce MT gene expression, as already reported for other plant species (for example, see Murphy and Taiz, 1995). It is worth noting that an MT gene product (with similarities to plant MT sequences) has recently been isolated from a marine microalga *Fucus vesicolosus*; this gene is induced by copper and its encoded protein binds cadmium and copper (Morris et al., 1999).

In particular the effect of copper was studied using different $CuCl_2$ concentrations (1 and 10 μM). Even at a concentration of 1 μM, copper induces a strong increase of MT mRNA levels in leaves of *P. oceanica* (Fig. 5); even relatively small increases of copper uptake (Table II) may determine an increase in MT mRNAs. It is possible that the stimulus inducing mRNA accumulation is not (or it is not only) the increase of total metal concentration, but possibly a variation in copper cellular compartmentalization following copper treatment. Such a variation has been assumed to result also after stresses as senescence and Suc deficiency (Fordham-Skelton et al., 1997).

In other experiments, the effect of mercury on the level of MT transcripts was analyzed (Fig. 5). Although in human tissues mercury is known to induce MT synthesis (Palmiter, 1994), studies on MT induction by this metal in plants do not exist.

Different mercury concentrations for 2 or 14 d in the aquarium were used. A slight increase in the steady-state level of MT transcripts was recorded in only a few experiments (independently of concentration and treatment time) indicating that MTs are not (or are only slightly) involved in the high-mercury tolerance shown by *P. oceanica*.

In conclusion, the experiments reported in this paper show that putative MT genes are present in *P. oceanica* genome, where they form a multigene family. Our data show that MT transcripts are present constitutively in the plant and are increased by different stimuli, supporting the hypothesis that MTs participate to processes of metal homeostasis and possibly tolerance. Moreover, MT induction seems to be metal-specific. MT mRNAs are increased by copper and cadmium, but not by mercury.

To further elucidate these aspects, we plan to isolate flanking sequences of these genes to verify the presence of metal-responsive elements in the promoters, as described for other MT genes, both in animals (Culotta and Hamer, 1989) and in plants (Whitelaw et al., 1997). Moreover, other experiments are programmed to isolate type-I MT genes from *Posidonia* genome.

Such studies will help to clarify the role of these genes in metal homeostasis and to isolate *P. oceanica* genotypes particularly tolerant to stress, to be used in experiments of recovery of *P. oceanica* populations in the Mediterranean Sea.

MATERIALS AND METHODS

Plant Materials

Posidonia oceanica (L.) Delile shoots were collected in different periods by scuba diving at between 2 and 5 m depth in the *P. oceanica* meadow of Antignano (Leighorn, Italy). Excess sediment was removed by washing the plants gently in seawater, before placing them in plastic bags filled with seawater. Some plants were frozen in liquid nitrogen immediately after explantation for RNA analyses and metals determinations.

Shoots were then acclimated to laboratory conditions (closed circuit aquaria, constantly aerated; 16.5°C ± 0.5°C; light/dark cycle, 16 h/8 h; six shoots per aquarium containing 12 L of natural seawater, collected in the same place) for 24 h before starting metal treatments.

Metal Treatments

Seawater culture medium was spiked with $CuCl_2$, $Cd(NO_3)_2$, or $Hg(NO_3)_2$ solutions to reach total concentrations of 1 or 10 μM copper; 10 μM cadmium; and 10^{-2}, 1, or 5 μM mercury. Metal treatment duration was 2 d for plants coping with $CuCl_2$ and $Cd(NO_3)_2$ and 2 and 14 d for those experiencing $Hg(NO_3)_2$. During mercury prolonged treatments, seawater in the aquarium was substituted every 2 d.

Analyses were performed on two leaf portions: the basal region (undifferentiated and light-green) and the intermediate region (differentiated and green). The leaf tip was not used because of the presence of epiphytes.

Metal Content Determination

Samples Preparation

Leaf portions were rinsed carefully in seawater and dried until constant weight in an electric oven at 40°C. One hundred milligrams of each dried sample was mineralized by a 7-mL solution (5:2) of $H(NO_3)$:H_2O_2 in a microwave oven (Milestone MSL 1200, Bergamo, Italy) for 25 min. The mineralized samples were diluted 1:100 with Milli-Q (Millipore, Bedford, MA) and then analyzed for metal determination.

Copper and Cadmium Determination in Plants

The copper and cadmium content of each leaf sample was independently determined by an atomic absorption spectrometer (1100B, Perkin Elmer, Foster City, CA) equipped with a graphite furnace (HGA 700, Perkin Elmer) and fitted with autosampler. Pro-analysis grade reagents (Merck) were used in every case. Standards were prepared by serial dilution of commercially available stock solutions within the linear range of respective metals.

Copper and Cadmium Determination in Water

The copper and cadmium content in seawater was measured by differential pulse anodic stripping voltammetry using a MetroHom (Zurich) 646 VA processor and 647 electrode system with hanging mercury drop electrode. Due to the very low background levels in seawater the metals concentrations were estimated about 10^{-2} μM for copper and 10^{-3} μM for cadmium.

Mercury Determination in Plants

The mercury content of each leaf sample was determined independently by cold vapor atomic absorption spectrometry with a detection limit of 8 ng, and was the mean of three replicates. The typical contribution of the blank was 1 to 2 ng of mercury. Standards were prepared with serial dilution of commercially available stock solutions within the linear range of the metal.

Mercury Determination in Water

A 400-mL seawater sample was acidified with 400 μL of a solution of mercury-free potassium dichromate (0.4% [w/v] $K_2Cr_20_7$ in 9 M H_2SO_4) and then photooxidized for 15 min using a UV immersion lamp (90 W). The mercury was reduced with 10 mL of mercury-free tin chloride solution (10% $SnCl_2$ in 4 M H_2SO_4) as described elsewhere (Seritti et al., 1980). Mercury was measured by cold vapor atomic absorption spectometry after preconcentration on a gold absorber (Seritti et al., 1980).

Isolation of Genomic DNA

DNA was purified according to the method devised by Doyle and Doyle (1989) with modifications. Leaf portions were homogenized in liquid nitrogen in a mortar and lysed in hexadecyl-trimethyl ammonium bromide (CTAB) isolation buffer (3% [w/v] CTAB [Sigma], 1.4 M NaCl, 0.2% [v/v] 2-mercaptoethanol, 20 mM EDTA, and 100 mM Tris-HCl, pH 8.0), and 1 mL/g tissue, at 60°C. Samples were incubated at 60°C for 30 min with occasional gentle swirling and then extracted once with chloroform:isoamyl alcohol (24:1, v/v). After centrifugation (5,000 rpm) at room temperature, nucleic acids were precipitated from the aqueous phase by adding 0.67 volume of cold isopropanol and then spooled with a glass hook, washed in 76% (v/v) ethanol and 10 mM ammonium acetate for 1 to 2 h, allowed to dry briefly, and resuspended in water.

For further purification, solid CsCl and ethidium bromide were added to the nucleic acids solution up to final concentrations of about 0.8 mg/mL (refraction index of 1.3890–1.3895) and 200 μg/mL, respectively. The solution was centrifuged at 44,000 rpm in an ultracentrifuge (L5–65, Beckman Instruments, Fullerton, CA) for 30 h at 15°C using the model 65 Ti rotor, and the DNA band, which was visualized under long-wave UV illumination, was collected. Ethidium bromide was then removed by gentle inversion of the solution with *n*-butanol and dialyzed against water at 4°C for 3 h. Finally, DNA was ethanol-precipitated and resuspended in the appropriate buffer.

Isolation of Genomic Sequences Coding Putative Type-II MTs

DNA sequences homologous to MT coding genes were isolated by PCR on *P. oceanica* genomic DNA. PCR was performed using two degenerated oligonucleotides based on the published DNA sequences encoding MTs in other plant species: 5′-ATGTCTTGCTGYGGAGGAARCTGT-3′ (sense) and 5′-ACAAKYGCARGGGTYACASK TGCA-3′ (antisense). Sequences were amplified using 100 ng of genomic DNA as a template; thermocycling was performed at 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s, for 30 cycles, using *Taq*-DNA polymerase (Promega, Madison, WI).

The amplified fragments were cloned into a pGEM-T Easy plasmid vector (Promega). The cloned fragments were sequenced by the dideoxy-chain termination method (Sanger et al., 1977) using the PRISM dye terminator cycle sequencing kit (Perkin-Elmer) according to the manufacturer's instructions; sequences were analyzed using the Sequencing Analysis 2.1.2 and the Sequencer 3.0 analysis programs (Perkin-Elmer Applied Biosystems, Foster City, CA).

Southern Analysis

Ten micrograms of genomic DNA was digested with the restriction endonucleases *Eco*RI, *Bam*HI, and *Msp*I in a 5-fold excess according to the instructions of the suppliers (Boehringer, Mannheim, Germany). Complete digestion was checked by including unmethylated bacteriophage λ-DNA, which, when digested with *Eco*RI plus *Hin*dIII (DNA molecular size marker III, Boehringer), was also used as a fragment size marker. Southern blotting of digested DNAs was performed according to standard protocols (Sambrook et al., 1989) and hybridizations were made with digoxigenin-labeled riboprobes transcribed from *Pomt2a*, *Pomt2b*, or *Pomt2c* clones using the DIG RNA labeling kit SP6/T7 (Boehringer).

Hybridizations were performed using the labeled RNA probes under high-stringency conditions, at 50°C in 50% (v/v) formamide, 5× SSC (1× SSC is 0.15 M NaCl and 0.015 M trisodium citrate, pH 7.0), 2% (w/v) blocking reagent (Boehringer), 0.02% (w/v) SDS, 0.1% (w/v) sodium lau-

royl sarcosinate. Filters were washed twice in 2× SSC, 0.1% (w/v) SDS for 15 min at room temperature, once in 1× SSC, 0.1% (w/v) SDS for 30 min at 68°C, and once in 0.3× SSC, 0.1% (w/v) SDS for 30 min at 68°C.

RNA Isolation and Northern-Blot Analysis

Total RNA extraction was performed by the same CTAB method used for DNA isolation, except that higher volumes extraction buffer were used in this case to increase the RNA yield. RNA was solubilized in diethylpirocarbonate-treated water. RNA integrity was assayed on agarose gels, before northern blotting.

Total RNAs (10 μg) were separated by 1% (w/v) formaldehyde agarose gel electrophoresis and blotted onto positively charged nylon membranes (Boehringer). The integrity and the equal amount of RNA loading were confirmed by ethidium bromide staining and subsequent densitometric image analysis and hybridization with a *Phaseolus coccineus* rDNA probe. The ribosomal probe was the *Eco*RI-*Sau*3A 25S-rDNA fragment of clone pPH1 (Maggini et al., 1992), subcloned in pBluescript II SK+ (Stratagene, La Jolla, CA).

The *Pomt2b* DNA sequence (or the *P. coccineus* rDNA) was digoxygenin-labeled using the DIG RNA labeling kit SP6/T7 (Boehringer). Hybridizations and subsequent washes were performed under high-stringency conditions, as described for Southern analysis, except for hybridization temperature (68°C). Northern blots were repeated three times per experiment. When hybridization signals seemed over saturation, experiments were repeated loading reduced amounts of RNA.

DNA Sequence Analysis

Intron delimitation within genomic sequences was made by comparing other putative MT-like genomic sequences and confirmed by the use of the program FEX (Baylor College of Medicine).

Database searches were conducted using the program BLAST (National Center for Biotechnology Information). Alignment of deduced amino acid sequences of *Pomt2a* and *Pomt2b* to other 46 type-II MT sequences was obtained using the program CLUSTALW (GenomeNet, Kyoto).

Maximum parsimony analysis was performed using the PHYLIP program package version 3.572 (Felsenstein, 1989): using the SEQBOOT program, 100 versions of the original alignment were generated. Trees were constructed using the PROTPARS and NEIGHBOR programs. A strict consensus tree was generated from the available trees using the CONSENSE program.

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