A plant type 2 metallothionein (MT) from cork tissue responds to oxidative stress

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Abstract
Expression of plant metallothionein genes has been reported in a variety of senescing tissues, such as leaves and stems, ripening fruits, and wounded tissues, and has been proposed to function in both metal chaperoning and scavenging of reactive oxygen species. In this work, it is shown that MT is also associated with suberization, after identifying a gene actively transcribed in Quercus suber cork cells as a novel MT. This cDNA, isolated from a phellem cDNA library, encodes a MT that belongs to type 2 plant MTs (QsMT). Expression of the QsMT cDNA in E. coli grown in media supplemented with Zn, Cd, or Cu has yielded recombinant QsMT. Characterization of the respective metal aggregates agrees well with a copper-related biological role, consistent with the capacity of QsMT to restore copper tolerance to a MT-deficient, copper-sensitive yeast mutant. Furthermore, in situ hybridization results demonstrate that RNA expression of QsMT is mainly observed under conditions related to oxidative stress, either endogenous, as found in cork or in actively proliferating tissues, or exogenous, for example, in response to H2O2 or paraquat treatments. The putative role of QsMT in oxidative stress, both as a free radical scavenger via its sulphydryl groups or as a copper chelator is discussed.

Key words: In situ hybridization, metal co-ordination, metallothionein, oxidative stress, senescent plant tissue, Quercus suber.

Introduction
Metallothioneins (MTs) are low molecular weight (4–8 kDa), cysteine-rich proteins with the ability to co-ordinate metal atoms, which are widely distributed among the animal and plant kingdom. Although classically these extremely heterogeneous polypeptides were grouped into three classes (Kági and Nordberg, 1979; Fowler et al., 1987), current classification, based on taxonomic relationships, considers 15 MT families, with plant MTs being placed into Family 15 (Kojima et al., 1999; Binz and Kági, 1999; http://www.unizh.ch/~mtpage/classif.html). Plant MTs have been further organized into four types, according to the distribution of cysteine residues in the amino- and carboxy-terminal regions (Robinson et al., 1993; Cobbett and Goldsbrough, 2002). The Arabidopsis MT gene family, which includes representatives of the four above-mentioned MT types, has been described by Zhou and Goldsbrough (1995) and Guo et al. (2003). In spite of this information about the primary sequence of plant MTs, relatively little is known about the structure, metal co-ordination, and in vivo function of plant MTs, especially in comparison with the state of knowledge about animal MTs and phytochelatins (PCs), which are the enzymatically synthesized peptides identified as the main cadmium detoxification agents in plants and some fungi (Rauser, 1999).

Because plant MTs efficiently bind metals (Kille et al., 1991) and some MT genes are positively regulated by metals, MTs are thought to be involved in cellular metal homeostasis and tolerance mechanisms (Cobbett and Goldsbrough, 2002). Moreover, an increasing number of observations suggest a role for plant MTs in senescing tissues, since expression of MT genes has been shown to be
induced in senescing leaves and stems (Buchanan-Wollaston, 1994; Yu et al., 1998; Miller et al., 1999; Chen et al., 2003), in ripening fruits (Davies and Robinson, 2000), in tapetum cells (Charbonnel-Campaa et al., 2000), and in wounded tissues (Choi et al., 1996; Butt et al., 1998). Senescence is a developmental process characterized by the programmed loss of metabolic function that ultimately leads to cell death. Before being shed from the plant, senescent leaves and tissues actively redistribute nutrients, including Zn and Cu ions, to other parts of the plant (Robert et al., 1996). Therefore, MTs may be involved in chaperoning metals released by catabolism (Buchanan-Wollaston, 1994; Butt et al., 1998) and/or in scavenging the reactive oxygen species generated during the complex senescence program (Chubatsu and Meneghini, 1993; Hussain et al., 1996). Cork (phellem) is a senescent tissue that replaces the epidermis in woody plants and acts as a barrier to radiation, water loss, and the entry of pathogens. Cork is composed of dead cells, which, during their maturation process, accumulate large amounts of suberin in their walls. Due to the phenoxy radicals generated by suberin synthesis and deposition (Whetten and Sederoff, 1995; Razem and Bernard, 2003), cork cells are subjected to high oxidative stress (Pla et al., 1998). In cork, proteins that function as metal chelators may be required to translocate metal ions and/or to protect cells from the toxic effects of oxidative radicals.

The cloning of a type 2 MT (QsMT) isolated from a Quercus suber cork cDNA library is reported here. The metal binding properties of the recombinant protein expressed in E. coli was investigated. Furthermore, the capacity of QsMT to protect against Cu toxicity is demonstrated by functional complementation of a Cu-sensitive yeast mutant. Moreover, because cork is an oxidatively stressed tissue, QsMT expression was also examined in other tissues that are subjected to oxidative stress by their nature or by the effects of treatments that stimulate oxidative stress. This work contributes to a better understanding of plant MTs and is the basis for further analyses of the structure/function relationship in these proteins.

Materials and methods

Isolation of QsMT

A cDNA library was made using RNA from Q. suber cork (phellem) tissue (Pla et al., 1998). The DNA sequence of one cDNA was identified as encoding a MT protein. Genomic DNA was extracted from young shoots using the NucleoSpin Plant XL kit (Macherey-Nagel), and used as the template for PCR with oligonucleotides complementary to the 5′ and 3′ ends of the QsMT coding region (underlined in Fig. 1A of the Results). The PCR product was purified and sequenced using the ABI PRISM dye terminator-cycle sequencing ready reaction kit (Perkin Elmer) using an Applied Biosystems ABI PRISM 310 automatic sequencer.

Cloning of QsMT in pGEX-4T2

The coding region of QsMT was amplified by PCR, using synthetic anchored primers (forward, 5′-CTCTGGATCCATGT-CTTGCTGGAGGA; reverse, 5′-CTCTGAATTCTGCATTCAATTTGCAAGG) that contained, respectively, BamHI and EcoRI restriction sites (underlined). After digestion, the QsMT coding region was subcloned into the same sites of the vector pGEX-4T2 (Amersham Pharmacia Biotech), downstream from the glutathione-S-transferase (GST) open reading frame. The recombinant plasmid, pGEX-QsMT, was confirmed by sequencing and transferred into the protease defective strain E. coli BL21.

Expression and purification of recombinant QsMT

To produce the QsMT recombinant protein, 3.0 l of fresh LB medium were inoculated with 300 ml of an overnight culture of E. coli BL21 carrying pGEX-QsMT. Induction with isopropyl β-D-thiogalactopyranoside (IPTG) was performed at OD600=0.8, and cultures were grown for a further 3 h in the presence of either 300 μM ZnCl2, 300 μM CdCl2, or 500 μM CuSO4. Cells were harvested by centrifugation (Servall RC5C, 15 min at 9600 g) and resuspended in PBSx1. Cells were lysed by sonication (Branson Sonifier 250, 0.6 Hz) in the presence of 0.5% β-mercaptoethanol to prevent protein oxidation. From this step onwards, all procedures were carried out in argon-saturated buffers. After sonication, cellular debris was pelleted by centrifugation (20 min at 20 000 g) and the fusion protein GST-QsMT isolated from the supernatant by affinity chromatography using Glutathione-Sepharose 4B (Amersham Pharmacia). QsMT was purified from the fusion protein by thrombin cleavage and batch-affinity chromatography. Several rounds of protein concentration were performed using a Centriprep Microcon 3 (Amicon). QsMT was finally purified by FPLC using a Superdex75 column (Pharmacia), equilibrated with 50 mM TRIS-HCl, pH 7.0. Selected fractions were confirmed by 15% SDS-PAGE and kept at −70 °C until further use.

Analysis and characterization of the recombinant QsMT

Depending on the metal ions supplemented in the bacterial cultures, three recombinant metal–QsMT aggregates were obtained. The S, Zn, Cd, and Cu content was analysed in the three metal–QsMT preparations by Inductively Coupled Plasma Optic Emission Spectroscopy (ICP-OES). A Polyscan 61E (Thermo Jarrell Ash) spectropolarimeter was used, measuring S at 182.040 nm, Zn at 213.856 nm, Cd at 228.802 nm, and Cu at 324.803 nm. Samples were prepared as previously described (Bongers et al., 1988) and treated with 1% (v/v) HNO3 1 h for 25 °C to digest the protein. When required, further acidification was carried out by the addition of HCl (1 M final concentration). Two independent methods were used to estimate protein concentration. The first method was based on S content and assumed that all S atoms in the sample were contributed by the QsMT polypeptide, that is 17 S atoms per mol of QsMT (14 from Cys and 3 from Met residues). The second method used amino acid analysis data to determine protein concentration. After hydrolysis in 6 M HCl for 22 h at 110 °C, samples were run on an Alpha Plus Amino acid Autoanalyzer (Pharmacia LKB Biotechnology). Serine, lysine, and glycine contents were used to extrapolate the concentration of QsMT in the sample.

ESI-MS (Electrospray Ionization-Mass Spectrometry)

ESI-MS was performed on a Fisons Platform II instrument (VG Biotech) controlled by the MassLynx Software and calibrated with horse-heart myoglobin (0.1 mg ml−1). 20 μl of the sample were injected through a PEEK column (1 m × 0.168 mm i.d.) at 20 μl min−1 under the following conditions: source temperature: 120 °C; capillary-counterelectrode voltage, 3.5 kV; lens-counterelectrode voltage, 1.5 kV; cone potential, 60 V. A m/z range from 850 to 2284 was scanned at 2 s scan−1 with an interscan delay of 0.2 s. The liquid carrier was a 20:80 (v/v) mixture of acetonitrile and 5 mM ammonium acetate, pH 7. To analyse apo-QsMT, the protein sample was acidified with HCl to pH 1.5 to dissociate metals, and mass
spectrometry measurements were carried out as described for the holo-forms, except that the liquid carrier was a 5:95 (v/v) mixture of methanol and ammonium/formate ammonia at pH 2.5. In all cases, molecular masses were calculated as described by Fabris et al. (1996).

Yeast functional complementation assays

Two copper-sensitive Saccharomyces cerevisiae strains were used: DTY3 (MATa, leu2-3, 112his3D1, trp1-1, ura3-50, gal1 CUP1S) and DTY4 (the same with cup1::URA3), referred to hereafter as cup1S and cup1D, respectively (Longo et al., 1996). The yeast vector p424-QsMT was constructed as follows: the QsMT coding region was excised from pGEX-QsMT by digestion with BamHI/PstI and ligated into the yeast expression vector p424 (Mumberg et al., 1995) under the transcriptional control of the yeast GPD (glyceraldehyde-3-phosphate dehydrogenase) promoter. The p424 vector also contains the CYC1 (cytochrome c oxidase) terminator, the 2μ replication origin and the TRP1 tryptophan marker. Vector p424 and the construct p424-QsMT were introduced into cup1D cells using the lithium acetate procedure (Stearns et al., 1991) and transformed cells were selected by their capacity to grow in complete synthetic medium (SC), lacking Trp and Ura (SC–Trp–Ura).

For the functional complementation experiments, cultures of cup1D yeast cells carrying either p424 or p424-QsMT were grown in SC–Trp–Ura medium at 30°C and 220 rpm, to OD600=0.5. Three 10-fold dilutions were performed, and 3 ml of each dilution were spotted on SC plates and on SC supplemented with 75 μM CuSO4 plates. Plates were incubated for 3 d at 30°C and photographed. The parental cup1S yeast strain was grown in SC medium and used as a positive control.

Fig. 1. Nucleotide and deduced amino acid sequences of QsMT (EMBL accession number AJ577299). (A) The ORF (indicated in uppercase) encodes a putative protein of 77 amino acids including 14 cysteines distributed in two Cys-rich regions. Two introns interrupt the coding region at the points signalled by arrowheads. Oligonucleotides used for QsMT gene amplification are boxed. (B) DNA sequence of QsMT introns 1 and 2 (see arrowheads in A). A region of approximately 500 bp of the intron 1 (marked by parallel lines) remains unsequenced.
SC–Trp–Ura medium were inoculated with mid-log precultures of \textit{cup1} \textsuperscript{A} p424-QsMT or \textit{cup1} \textsuperscript{B} p424 cells to attain a starting optical density of 0.02 at 600 nm. Cells were grown at 30 °C and 220 rpm and CuSO\textsubscript{4} was added 5 h after inoculation to a final concentration of 75 µM. As a positive control, \textit{cup1} \textsuperscript{A} p424 cells were grown in SC–Trp–Ura medium without the addition of CuSO\textsubscript{4}. The optical densities of the cultures were measured at 2–3 h intervals for 26 h.

**Plant material**

Cork oak (\textit{Quercus suber} L.) radicle tips and plantlets were obtained by germination of acorns in water-imbibed peat for 1 month. Standard growth conditions were 22 °C at 70% humidity in a light/dark cycle of 16/8 h. As a source of somatic embryos, a cork oak recurrent embryogenic line maintained on a medium free of plant-growth regulators was used (Puigderrajols \textit{et al.}, 1996). Macronutrients were those from SH medium (Schenk and Hildebrandt, 1972), and micro-nutrients and vitamins were from MS medium (Murashige and Skoog, 1962), including 3% (w/v) sucrose. The culture was solidified with 0.6% agar and the pH adjusted to 5.7. The cultures were incubated in a growth chamber at 25 °C and a 16 h photoperiod (50 µmol m\textsuperscript{-2} s\textsuperscript{-1}) was provided by cool-white plus Grolux fluorescent lamps.

**Stress treatment**

Stress treatment was performed using somatic embryos at the translucent immature stage as a model system (Puigderrajols \textit{et al.}, 1996). Translucent embryos (5–7 mm in length) were isolated, subcultured on agar medium in agar baby-food jars for 5 d, and then transferred to liquid medium and grown at 25 °C on a rotary shaker (100 rpm). Oxidative stress treatments were imposed by incubating the embryos in culture medium supplemented with either 0.5% (v/v) H\textsubscript{2}O\textsubscript{2} or 5 µM paraquat (Sigma) for 3 h. Heat stress was performed at 42 °C for 3 h. Controls were maintained at 25 °C. Immediately after all treatments, the tissue samples were fixed and processed.

**In situ hybridizations**

Formalin-fixed plant material was dehydrated in an ethanol series and embedded in paraffin. Serial sections 10 µm were fixed onto poly-t-lysine coated slides overnight at 42 °C. Digoxigenin-labelled sense and antisense RNA probes were transcribed from the \textit{QsMT} cDNA using T3 and T7 polymerases (Stratagene) and DIG-RNA-Labelling-Mix (Roche). The probes contained both the coding and non-coding regions of the cDNA. In \textit{in situ} hybridizations were performed as previously described (Pla \textit{et al.}, 1998). Hybridization was performed overnight at 55 °C; hybridized probes were detected with the anti-digoxigenin alkaline-phosphatase conjugate antibody (anti-DIG-AP, Roche) and colour substrates were Nitro Blue Tetroazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Controls were hybridized with sense probes.

**Results**

**\textit{QsMT} cDNA isolation and characterization**

A cDNA library prepared with RNA isolated from living cork tissue from a 1-year-old cork oak sprouts was sequenced at random. Clone sequences were identified using an EMBL DataBank BLAST search. One of the cDNAs showed high similarity to the metallothionein-like family and was, therefore, referred to as \textit{QsMT}, from \textit{Quercus suber} metallothionein (EMBL accession number AJ277599). This clone is 643 bp long and contains an open reading frame of 234 bp, encoding a 77 amino acid polypeptide with a theoretical molecular mass of 7672.5 Da. Based on its sequence (Fig. 1A), the putative encoded protein was identified as a type 2 metallothionein. Typical of plant type 2 MTs, \textit{QsMT} contains 14 cysteine residues occurring as C-X-C, C-C and C-X-X-C motifs in the N-terminal, and of C-X-C in the C-terminal region. The cysteine-rich regions are separated by a 39 amino acid spacer devoid of cysteines. The genomic sequence includes two introns (Fig. 1B), interrupting the coding region (Fig. 1A, arrowheads).

Sequence similarity trees for plant MTs were constructed using the nucleotide and amino acid sequences retrieved by a BLAST search (E-value cutoff of 0.028) in EMBL DataBank aligned by Clustal W (1.75). In both the nucleotide (not shown) and protein trees (Fig. 2), maximum identity was to a type 2 MT from European beech (\textit{Fagus sylvatica}) (93% for the nucleotide and 92% for the amino acid sequence). Cork oak and beech MT sequences appear close to the rose family and to avocado and kiwi tree species. Most of the sequences retrieved using \textit{QsMT} as a query were type 2 MTs, only a small cluster of nine sequences, curiously located close to \textit{QsMT}, were type 1 MTs. Broadly, tree phyletic lineages correspond with taxonomic units, except for the type 1 MTs cluster, that appears more related to type 2 MTs than to other type 1 MT sequences (data not shown). No other type 1 or type 3 and 4 sequences were retrieved by this BLAST search.

**Metal binding properties**

The homogeneous metal–\textit{QsMT} preparations, purified from \textit{E. coli} grown in Cu, Zn, or Cd-enriched culture medium, had final protein concentrations between 0.24×10\textsuperscript{-4} M and 0.93×10\textsuperscript{-3} M. Acidification of the metal aggregates to pH 1.5 yielded the \textit{QsMT} apoform, with a molecular mass of 7816.4±0.7 Da. This is in good agreement with the value predicted from the polypeptide sequence (7816.8 Da) for recombinant \textit{QsMT}, which includes two residues from the expression vector. These results confirm both the identity and integrity of the recombinant protein. Metal–\textit{QsMT} stoichiometry was first measured using the same approach previously reported for animal MTs (Capdevila \textit{et al.}, 1997). Thus, assuming that all S content was contributed by Cys and Met amino acids (17 S per mol of \textit{QsMT}), the total sulphur ICP-OES measurement was used to deduce \textit{QsMT} concentration and the metal measurements were related to this value. These data were then analysed in relation to the molecular species detected by ESI-MS (Table 1, columns a and d). As there was disagreement between the results obtained for Cd–\textit{QsMT} with ICP-OES and ESI-MS, the protein concentration was also calculated through amino acid analysis as an alternative method (Table 1, column c). The ICP-OES analyses were repeated with prior strong acidification of the samples in order to remove other ligands that would be volatilized under these conditions (Table 1, column b).
In Cu-supplemented medium, the expression of QsMT yielded metal aggregates that not only contained Cu(I) but also Zn(II), in a global ratio ranging from $\text{Zn}_{1.5}\text{Cu}_{4.2}$ to $\text{Zn}_{1.7}\text{Cu}_{5.5}$ per protein (Table 1). As ESI-MS cannot discriminate between Cu and Zn, the only conclusion is that a major aggregate, $\text{M}_8\text{-QsMT}$, and two minor aggregates, $\text{M}_9\text{-QsMT}$ and $\text{M}_4\text{-QsMT}$, are formed. In Zn-enriched medium, ESI-MS results revealed a major Zn$_4$-QsMT and two minor Zn$_5$-QsMT and Zn$_3$-QsMT aggregates. Since ICP-OES data indicated an average ratio of 2.7 Zn per QsMT molecule, the stoichiometry of the zinc-containing samples was in better agreement with stoichiometries derived from the amino acid analyses, which yielded a ratio of 3.5 per protein molecule (Table 1). Finally, in Cd-supplemented medium, the global Cd:QsMT ratio was unexpectedly low, amounting to 2.5 Cd(II) per protein, when calculated according to the conventional ICP-OES methodology. Moreover, ESI-MS detected two main species, whose molecular masses could not be matched with those of QsMT aggregates containing only cadmium. This led to the assumption that putative Cd$_6$-QsMT and Cd$_7$-QsMT species included other elements. One possibility was the presence of zinc ions in the aggregates, but the ICP-OES analysis revealed a total absence of this metal. Moreover, stoichiometry based on protein concentration estimated by amino acid analysis yielded 6.5 Cd(II) per protein, clearly

Fig. 2. Protein distance tree constructed with the plant MT sequences retrieved by a BLAST search in the EMBL database, using QsMT as a query and an E-value cutoff of 0.028. Alignment was performed using Clustal W and the phylogenetic tree was constructed using the MEGA software, version 2.1 (Kumar et al., 2001). Nearly all sequences retrieved were type 2 MTs, except for a small cluster of type 1 MTs (bordered). QsMT is indicated by an arrow.
shown in Fig. 3B, p424-QsMT was confirmed in liquid culture assays. As shown in Fig. 3A, 75 mM CuSO₄ inhibited the growth of CUP1 cells, whereas the same cells carrying QsMT, CUP1 cells, were transformed with protection against copper toxicity, Cu-sensitive yeast cells medium containing 75 mM CuSO₄. The Cu-tolerance threshold is reduced to 10⁻³ M CuSO₄, whereas the growth of Cu₃-QsMT·[X] species is due to the different masses of all the possible Zn and Cu contents summing the total metal atoms of the metallopeptide. ‘X’ denotes an additional ligand with a calculated MW of 159.6±1.4.

Expression of MT mRNA in cork oak tissues

In situ hybridization using a DIG-labelled QsMT riboprobe was performed on cork oak plantlets and germinating acorns in order to localize QsMT mRNA expression. For this purpose, stem and root tissue from cork oak plantlets grown in non-stress conditions, as well as from embryo radicle tips 8 d after germination, were processed and analysed. In mature stem and root tissue, the QsMT riboprobe hybridized with a set of specific cells. In stem sections, the strongest hybridization signal was observed in lignified cortical fibres, but QsMT RNA was also detected in vascular tissue (Fig. 4A). In root cross-sections, the QsMT probe hybridized mainly with endodermal and vascular cylinder cells (Fig. 4C). In radicle tip tissue, the highest signal was seen in the region of root initials and a somewhat weaker signal in the inner layers of the root cap (Fig. 4E–H).

To investigate the expression of QsMT in response to oxidative stress, cork oak somatic embryos were used as a model system for oxidative and temperature stress treatments, as previously described (Puigderrajols et al., 1996). Translucent immature embryos (5–7 mm in length) were transferred into a liquid medium and subjected to either oxidative (H₂O₂ 0.5% (v/v) or paraquat 5 μM, 3 h at 25 °C) or temperature stress treatments (42 °C, 3 h), while control embryos were maintained at 25 °C. When embryo sections were hybridized with the QsMT riboprobe, they generally showed a weak reaction with some tissue-specificity that depended on the stress (Fig. 5B–I). Specificity was observed in the proliferating mass at the periphery of the

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<th>ICP-OES³</th>
<th>Amino acid analysis⁴</th>
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<td>Protein concentration (M)</td>
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Table 1. Protein concentrations and metal (Zn, Cd or Cu) to protein ratios of the recombinant metal–QsMT samples

All the data correspond to at least three independent expression experiments, and two measures of each parameter.

Matching the ESI-MS results. These values were confirmed by ICP-OES measures of strongly acidified samples; it was therefore concluded that Cd–QsMT aggregates consist of a mixture of Cd₅- and Cd₆-QsMT species containing an additional acid-labile ligand.

Functional complementation in yeast copper-sensitive strains

To test whether the expression of QsMT could provide protection against copper toxicity, Cu-sensitive yeast cells were transformed with QsMT. In Saccharomyces cerevisiae, CUP1 encodes a Cu-thionein that is induced by and binds to Cu. Most laboratory yeast strains contain multiple copies of this gene, but the cup1S strain harbours a single copy of this MT gene and its growth is inhibited at 300 μM CuSO₄. The Cu-tolerance threshold is reduced to 75 μM CuSO₄ in cup1A strain (cup1::URA3), which has no functional copies of CUP1 (Hamer et al., 1985). cup1A cells were transformed with p424-QsMT and the growth in solid and liquid medium containing copper was analysed. As shown in Fig. 3A, 75 mM CuSO₄ inhibited the growth of p424 cup1A cells, whereas the same cells carrying p424-QsMT were able to grow at a similar rate to parental cup1S cells. The restoration of copper tolerance by expression of QsMT was confirmed in liquid culture assays. As shown in Fig. 3B, p424-QsMT cup1A cells grew well in medium containing 75 μM CuSO₄, whereas the growth of p424 cup1A cells was inhibited. However, p424 cup1A cells showed normal growth when cultured in a Cu-free medium.
root cap and in the shoot and root apical meristems. The proliferating mass is an actively dividing tissue that originates from the outer layers of the root cap and gives rise to secondary embryogenesis. In all these regions, the level of hybridization signal increased after heat stress compared with control embryos (Fig. 5B, C). However, the strongest hybridization signal was observed after oxidative stress (Fig. 5D, E) with no significant differences between the H$_2$O$_2$ and paraquat treatments. In cross-sections of the radicle tip (Fig. 5J), the proliferating mass and root initials were strongly labelled, and a weak signal was also seen in the inner layers of the root cap, as was the case in zygotic embryos. A detailed examination of cotyledon sections showed labelling of tracheary elements (Fig. 5H, I), that increased after stress.

**Discussion**

The results presented here describe the characterization of a new cork oak type 2 plant MT that binds metal ions in a manner that is analogous to the well characterized animal MTs, provides metal tolerance to copper-sensitive yeast strains, and is positively regulated by oxidative stress.

Research on plant MT structure and function has been impaired by the difficulties encountered in purifying these proteins from native sources, mainly due to the inherent instability of cysteine-rich polypeptides in the presence of oxygen. Few MTs have been recovered from plant tissues, and even fewer as intact, non-cleaved metal aggregates (Cobbett and Goldsbrough, 2002). Heterologous expression strategies were supposed to overcome these difficulties, but initial reports showed that pea MT synthesized in E. coli as a native protein was also susceptible to significant proteolysis (Kille et al., 1991). Alternative strategies to produce plant MTs as fusion proteins generated intact proteins that retained their metal binding and antigenic features (Tommey et al., 1991; Murphy et al., 1997, respectively). Unfortunately, cleavage and recovery of the MT portion was not attempted and thus characterization of the corresponding metal aggregates was not reported. Nevertheless, the GST-based expression system has been applied to several animal MT forms allowing the synthesis of large amounts of homogeneous metal-MT preparations. Analysis of these complexes by spectroscopic and spectrometric techniques has yielded unprecedented information about MT aggregate stoichiometric and folding behaviour (Capdevila et al., 1997). It was shown that the application of this procedure to QsMT consistently renders a full length protein, free of proteolytic cleavage. Sufficient quantities of Zn-, Cd- and Cu-QsMT aggregates were then synthesized in order to determine the metal-chelating capacity and to characterize the basic features of the metal–QsMT clusters.

**QsMT and metal co-ordination**

Zn-supplemented cultures yielded a major Zn$_4$–QsMT and two minor Zn$_3$–QsMT and Zn$_5$–QsMT species in good agreement with other literature data on type 1 and type 2 plant MTs (Tommey et al., 1991). These results suggest a poorer capacity for plant MTs to bind Zn (Cys/Zn ratio of 3.5) compared with animal MTs (2.57 Cys/Zn ratio for the canonical mammalian Zn$_7$-MT1). Copper-supplemented cultures yielded a major mixture of aggregates containing both zinc and copper. The presence of zinc ions in metal-MT aggregates has been described for several animal MTs when synthesized in copper-supplemented media (Bofill et al., 2001; Valls et al., 2001), and has been thought to be a structural requirement to maintain stability in biological environments. Thus, for Zn and Cu, analysis of the QsMT co-ordination features are largely in agreement with those reported for animal MTs. This was not the case for cadmium co-ordination. When synthesized in Cd-supplemented medium, QsMT shows a binding capacity of 6–7 Cd(II) ions, similar to that reported for *Pisum sativum* type 1 MT.
(PsMTA) (5.6–6.1 Cd(II), Kille et al., 1991). This stoichiometry was obtained when the amount of protein was determined by amino acid analyses, or by ICP-OES provided that the samples had previously been strongly acidified, which indicates the presence of acid-labile ligands in Cd–QsMT. The participation of sulphide ions in Cd(II)-cadystin and Cd(II)-phytochelatin aggregates, giving rise to the Cd(II)-crystallites, is well reported in the literature (Hayashi et al., 1991; Mutoh and Hayashi, 1991; Robinson et al., 1993). Thus, it is reasonable to hypothesize that plant MTs may also include this kind of ligand when co-ordinating cadmium, probably due to the considerably bulkier size of this metal compared with zinc or copper. It should be noted that the different methods of estimating metal stoichiometry have produced very consistent results for the stoichiometry of Zn– and Cu–QsMT aggregates. Consideration of the additional ligands as sulphide ions is not only consistent with its acid lability, but also mainly with the fact that this provides a sensible explanation of the distortion of conventional ICP-OES stoichiometry calculations, because they invalidate the assumption that all the sulphur in the sample comes from the MT polypeptide. Although participation of inorganic, non acid-labile ligands, such as ammonia and chloride, has been claimed in animal MT aggregates (Domenech et al., 2003), this is the first time that the participation of sulphide ions is foreseen. Further research is currently being developed to identify these ligands directly, and to evaluate their impact on the structure and function of MTs.

In general, the literature suggests that plant type 1 and type 2 MTs have a biological function related to copper homeostasis, based on copper-induction of MT genes (Murphy et al., 1997), increased copper accumulation in transgenic Arabidopsis hosting a pea MT gene (Evans et al., 1992), and because the highest affinity of certain plant MTs is for Cu (Tomme et al., 1991; Morris et al., 1999). These results support this hypothesis rather than a role related with divalent metals. Copper co-ordination follows a conventional pattern, with the formation of aggregates with a high metal content that are stable in the absence of additional ligands. Furthermore, the results presented here prove that QsMT confers increased tolerance for Cu to sensitive yeast cells, thus replacing the physiological role of CUP1, the canonical copper-thionein, as previously reported for Arabidopsis type 1 and type 2 MTs (Zhou and Goldsbrough, 1994). The Zn content in the Zn–QsMT aggregates is low considering the number of thiolate ligands that 14 cysteines could provide for tetrahedral co-ordination of Zn(II). In fact, among plant MTs only the wheat Ec-MT has been isolated as a native zinc-containing polypeptide (Lane et al., 1987). Moreover, QsMT is expressed in Cd-enriched media only as Cd(II) aggregates stabilized by additional ligands, which, together with the poor inducibility of plant MT genes by cadmium reported in the literature (Cobbett and Goldsbrough, 2002) agrees with a secondary role for plant MTs in cadmium detoxification.

QsMT and oxidative stress
The expression of QsMT was analysed by in situ hybridization using a riboprobe that includes 5' and 3' UTR regions of QsMT under high stringency conditions in order

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Fig. 4. In situ hybridization of cork oak plantlets and germinating acorns with QsMT riboprobe. (A, B) Stem longitudinal sections. Note the intense hybridization signal in fibres and a weaker signal in phloem cells. (B) A negative control hybridized with the sense riboprobe. (C, D) Plantlet root in cross-sections. MT RNA is detected in the endodermis and cells of the vascular cylinder. (D) A negative control with the sense probe. (E–G) Transverse sections of zygotic embryo radicle tip. (E) A negative control with the sense probe. (H) Detail of (G) showing MT expression in root initials. c, cortex; e, endodermis; f, fibres; ph, phloem; rc, root cap; ri, root initials; vc, vascular cylinder. Bar 100 μm.
to minimize cross-reaction. These results show that QsMT mRNA is expressed in cortical fibres, vascular cells (tracheary and phloem elements), endodermal cells, and in specific regions of meristems, and that gene expression is enhanced by stress. Like cork cells, fibres, tracheary elements, and endodermal cells synthesize aromatic polymer species during cell wall lignification or suberization. A number of other reports indicate a correlation between the presence of a lignified or suberized cell wall and elevated expression of MT genes (Garcia-Hernandez et al., 1998; Guo et al., 2003; Nakazono et al., 2003). Due to the oxidative coupling of polyphenolic components through a peroxidase/H2O2 free radical formation, cells that synthesize aromatic polymer species, such as lignin and suberin, are subjected to high oxidative redox status (Ros-Barcelo, 1998; Wheten and Sederoff, 1995; Razem and Bernards, 2003). Previous experiments in the authors’ laboratory using a 3,3′-diaminobenzidine assay (as described in Sigma Tablet test Set) demonstrated high endogenous oxidative stress in cells with lignified and suberized cell walls (Pla et al., 1998). Reactive oxygen species (ROS) are also generated in association with suberization in response to wounding (Razem and Bernards, 2002) and to pathogen attack (Kuzniak et al., 1999), conditions that are also known to induce the expression of MT genes (Choi et al., 1996; Butt et al., 1998). Moreover, MTs have been associated with organ senescence (Buchanan-Wollaston, 1994; Guo et al., 2003), a process also related to oxidative stress. Increased production of ROS is characteristic of the degenerative process in senescence, and plants respond to this situation with enhanced expression of senescence-related genes, including MTs (Navapour et al., 2003). The relationship between MT gene expression and oxidative stress is corroborated by the experiments in somatic embryos. Although heat treatment resulted in some increase in expression, the most consistent enhancement was obtained in embryos treated with either H2O2 or paraquat.

Interestingly, it was found that MT gene expression was observed in tissues where cell division occurs. QsMT mRNA was conspicuous in meristem initials and the proliferating mass that gives rise to secondary embryogenesis,

Fig. 5. In situ hybridization of stressed and non-stressed cork oak somatic embryos with QsMT riboprobe. (A) Translucent somatic embryo (5–7 mm long) surrounded by proliferating mass, embryos at this stage were isolated and used for stress treatment and in situ hybridization analysis. (B–E) Nearly median longitudinal sections of control and stressed embryos: (B) control tissue (25 °C); (C) heat-stressed (42 °C); (D) oxidatively stressed (paraquat); (E) oxidatively stressed (H2O2). The hybridization signal is greatest in the proliferating mass and shoot apical meristem of oxidatively stressed embryos. (F, G) Details of (C) (25 °C) and (E) (H2O2), respectively, showing the shoot apical meristem. (H, I) Details of cotyledonal xylem vessels (H) 25 °C and (I) paraquat. (J) Transverse section at radicle tip level of an oxidatively stressed embryo (H2O2); note the intense signal in the root initials and proliferating mass. co, cotyledon; pm, proliferating mass; ri, root initials; sam, shoot apical meristem; xv, xylem vessels. (B–E) (H, I) bar 1 mm; (F, G) bar 100 μm; (H, I) bar 10 μm.
all these tissues consisting of undifferentiated, rapidly dividing cells undergoing complex morphogenetic processes (Puigderrajols et al., 1996, 2001). In these cells, MT function could be related to balancing the redox status rather than a general antioxidant function. It is widely accepted that ROS have a central signalling role in meristem regulation (Sanchez-Fernandez et al., 1997; Jiang et al., 2003; Vranova et al., 2002). In the root apical meristem of Arabidopsis, glutathione participates in the regulation of cell division by balancing the redox status via its sulphhydril groups (Sanchez-Fernandez et al., 1997; Vormoux et al., 2000). Thiol groups in MTs contribute to the pool of cellular thiols that can regulate cellular redox status and, in vitro, this can be modulated by metal chelation (Maret and Vallee, 1998; Chen and Maret, 2001). In animal cells, an increased MT expression has been reported in rapidly proliferating tissues (Coyle et al., 2002).

Taken together, these results support a role for plant MTs in maintaining the local redox balance either by sequestering copper and preventing potentially deleterious Fenton chemistry reactions (Zhang et al., 1999), or by directly scavenging deleterious oxygen radicals.

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References


A type 2 metallothionein from cork tissue


