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Metallothionein genes from the flowering plant *Mimulus guttatus*

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In response to excess metal, higher plants produce metal-binding peptides ($[\gamma EC]_nG$) whose biosynthesis is believed to be mediated by enzymes involved in glutathione (γECG) metabolism. In contrast, animals synthesize metallothioneins, gene-encoded low molecular weight cysteine-rich metal-binding proteins. In an investigation of copper-regulated genes in the copper-tolerant flowering plant *Mimulus guttatus*, we have isolated a series of cDNA clones identifying two genes which encode a protein with class I metallothionein domains. This represents the first description of a metallothionein gene in a flowering plant.

Class I metallothionein; Plant metallothionein; Metallothionein gene; Root-specific; Metal tolerance; Metal metabolism

1. INTRODUCTION

MTs are low molecular weight, cysteine-rich metal-binding proteins which have been divided into three classes [1]. Class I and class II MTs are gene-encoded polypeptides. Class I MTs possess arrangements of cysteine residues (as Cys-Cys and Cys-X-Cys clusters, where X is an amino acid other than Cys) which align with those of equine renal MT [1]. Class II MTs also contain Cys-X-Cys and Cys-Cys clusters, but these cannot be easily aligned with those of equine renal MT. Class III MTs are not synthesized on an mRNA template: their structure, $[\gamma EC]_nG$, is similar to glutathione from which they are synthesized by the enzyme γ-glutamylcysteinyl dipeptidyl transpeptidase [2]. Classes I and II, but not class III, MTs have been isolated from a wide variety of animals and certain fungi; whereas all 200 species of plants tested so far, together with a few fungal species, appear to contain only class III MTs [3–6]. There is only one description of a plant class II MT, the wheat Ec protein, which is based on a partial amino acid sequence [1,7]: since this is present in the early stage of germination of wheat, it could be a cysteine-rich storage protein which binds zinc. To date there have been no reports of a class I MT in any higher plant. Here, we describe for the first time the isolation of cDNA clones from a flowering plant which encode a protein containing class I MT domains.

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Abbreviations: MT, metallothionein; $[\gamma EC]_n$ G, poly(γ-glutamylcysteinyl) glycine or class III metallothionein; γECG, glutathione

2. MATERIALS AND METHODS

Copper-tolerant *M. guttatus* plants were grown for 21 days in 1/10th strength Long Ashton salt solution [8], metal was added either 24 h before harvest, or on day 0. Roots were harvested after an 8 h light period and RNA was isolated as described previously [9], except that the final RNA pellet was again resuspended in water, centrifuged and RNA was selectively precipitated with 0.5 vol 8 M LiCl. The pellet was resuspended in water and stored at $-70°C$.

A cDNA library in λ gt10 was constructed as described previously [10], using poly A^+ RNA isolated from roots treated with 10 μM $CuSO_4$ for 24 h before harvesting. The library was plated at a density of 300–500 plaques per 9 cm plate and duplicate plaque lifts were made of each plate using Colony/Plaque Screen (DuPont). The duplicate filters were divided into two groups: one set was probed with ^{32}P-labelled first-strand cDNA from poly A^+ RNA from roots grown identically to those used to make the library; the other set were probed with ^{32}P-labelled first-strand cDNA from poly A^+ RNA from roots grown in without copper-treatment. Hybridization, washing and autoradiography were carried out according to the manufacturer's instructions. λ Clones which showed a differential response, either induced or repressed, were picked, purified and retested.

RNA gels were run as described previously [11]. Northern transfer, hybridisation (42°C in 50% formamide) and washing (2 × SSC, 1% SDS, 65°C) were as recommended by the manufacturers of GeneScreen Plus (DuPont). The probe was insert cDNA from λ gt10 clone J87, labelled with ^{32}P as described previously [12].

For DNA sequencing, cDNA inserts were isolated from the λ clones by *Eco*RI digestion, subcloned into M13mp18 and sequenced by the dideoxynucleotide method of Sanger [13,14].

3. RESULTS AND DISCUSSION

In an investigation of copper-tolerant *M. guttatus* [8,15,16], a λ gt10 cDNA library made with poly A^+ RNA from the roots of plants exposed for 24 h to 10 μM $CuSO_4$ was differentially screened with first-strand cDNA prepared from the roots of copper-treated and untreated plants. 30 000 primary cDNA clones were screened yielding 40 clones which proved to be con-

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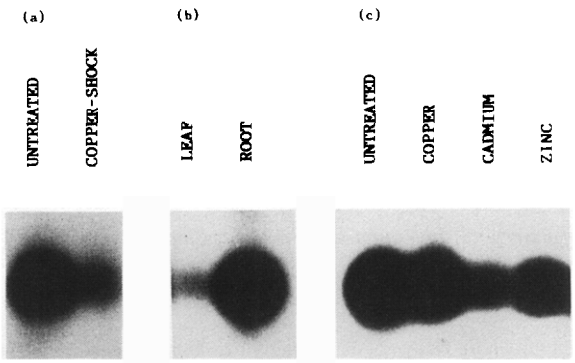

Fig.1. Northern transfer of total RNA from copper-tolerant *M. guttatus*: (a) 10 μg (per track) of total RNA from roots of plants either exposed to 10 μM $CuCO_4$ for 24 h (copper shock) or untreated; (b) total RNA from roots (6 μg) and leaves (12 μg) of plants without copper treatment; (c) 6 μg (per track) of total RNA from the roots of plants grown in the absence of metal, or continuously in the presence of 5 μM $CuSO_4$, 5 μM $CdSO_4$, 15 μM $ZnSO_4$.

sistently copper-regulated when rescreened with fresh probes. Half of these clones were copper-repressible and Northern transfer studies revealed that, with one exception, all had similar transcript sizes, 450–550 bases (fig.1a). Eighteen of these were found to cross-hybridise (data not shown) implying that they constitute a single class of message and that this was the major change in the mRNA population upon treatment of the plant roots with copper, since almost half of the 40 selected clones belong to this class. Despite its abundance in roots, the level of this transcript in leaf tissue was low (fig.1b), irrespective of copper treatment (data not shown). Furthermore, roots from plants initiated and grown continuously in copper appeared to possess similar levels of message to those in non-metal grown roots (fig.1c). The repression observed after 24 h or less therefore appears to be a copper shock effect. Roots of plants grown continuously in cadmium or zinc, however, had levels of message lower than in untreated plants (fig.1c).

Five of these copper-repressible cDNA clones (J39,49,55,73,87) have been sequenced (fig.2). The clones are representatives of two distinct sequences which differ by 3 bp in the open reading frame (one in the termination codon), and at 13 positions outside this coding region: none of these changes affect the predicted amino acid sequence. Minor differences in the length of the 5′ and 3′ ends demonstrate that four of the five cDNAs represent independently created clones. This indicates that the high frequency of these clones is not an artefact of amplification of the library, rather that this is genuinely an abundant message relative to other mRNA species even under the repressing conditions of copper shock.

Since all 5 cDNA clones fall into either of two classes, it would seem that there must be at least two copies of this sequence per genome. Because the plants from which these clones were isolated resulted from backcrossing tolerant to non-tolerant plants, we have yet to determine whether these sequences represent two alleles in a heterozygous plant, or two gene copies per haploid genome.

The cDNA sequence has a single open-reading frame encoding a putative 72 amino acid polypeptide (fig.2). When this sequence was submitted to computerized comparisons with the NBRF protein database (DNASTAR software), 19 of the top 23 matches found were MTs, the top 8 matches being from sources as diverse as *Neurospora crassa*, Chinese hamster, sea urchin and *Drosophila*. This is in contrast to the only other higher plant MT, the wheat E_c protein, which failed to reveal significant similarity to any sequence in the database [7]. The strong similarity of the *M. guttatus* sequence to class I and class II MTs is due to two domains of 14/15 amino acids, each of which contain 6 cysteine residues arranged exclusively as Cys-X-Cys clusters: similar domains are also present in a 75 amino acid open-reading frame in a cDNA isolated from *Pisum sativum* [Evans, I.M., Gatehouse, L.N., Gatehouse, J.A., Robinson, N.J. and Croy, R.R.D., unpublished]. This hypothetical *M. guttatus* protein also has other common features of MTs: low molecular weight (7348 Da); usually serine or lysine as the bridging amino acid in the Cys-X-Cys clusters; and an absence of aromatic amino acids in the MT domains. The major difference between this plant MT and the animal and fungal ones is the presence of an intervening sequence of amino acids between the two MT domains. This sequence has no significant homology to any region of any MT and contains aromatic amino acids. In all other MTs, cysteine residues are distributed throughout the sequence and the protein appears to fold either into one metal-binding domain, or two metal-binding domains with a very short amino acid bridge between them [17]. This is clearly not the case with the *M. guttatus* protein which has 39 amino acids between the two MT domains. Computer analysis predicts extensive MT-like folding for the two domains but a largely extended configuration for the intervening region. This implies that this plant MT does not function exactly as the animal and fungal MTs: either the spacer region between the domains is required, for example, to facilitate interaction with another macromolecule or subcellular component, or the protein is processed to remove the spacer region. There is some circumstantial evidence for the latter possibility. In experiments to purify class III MTs ($[\gamma EC]_nG$) from *M. guttatus*, we have also isolated a low molecular weight copper-binding protein which is not $[\gamma EC]_nG$ [8,15]. The amino acid composition and size of this polypeptide are remarkably similar to an average of the two MT domains as 20 amino acid peptides. This peptide fraction is currently being purified to check this possibility.

Animal MTs are thought to have roles in metal detoxification as well as in normal cellular homeostatis

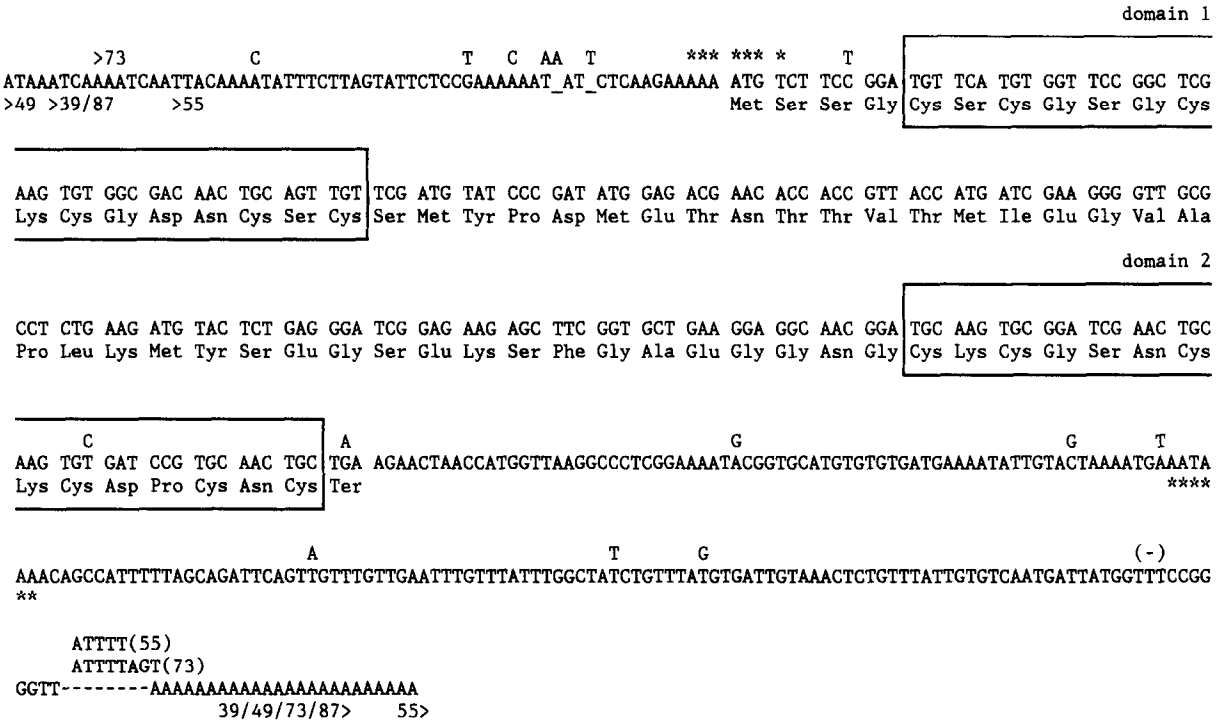

Fig.2. DNA sequence of the cDNA clones encoding the putative metallothionein. Clones J39, J49, and J87 are independently isolated clones which have identical cDNA sequences except that J49 is 4 bp longer at the 5′ end (main sequence). J55 and J73 are identical except for 3 bases immediately before the poly A tail, but differ from the J87 clones at 16 positions (base changes shown above the main sequence). The consensus eukaryote ribosome binding site sequence A/GNNATGG [19] and polyadenylation signal AATAAA [20] are also indicated (**).

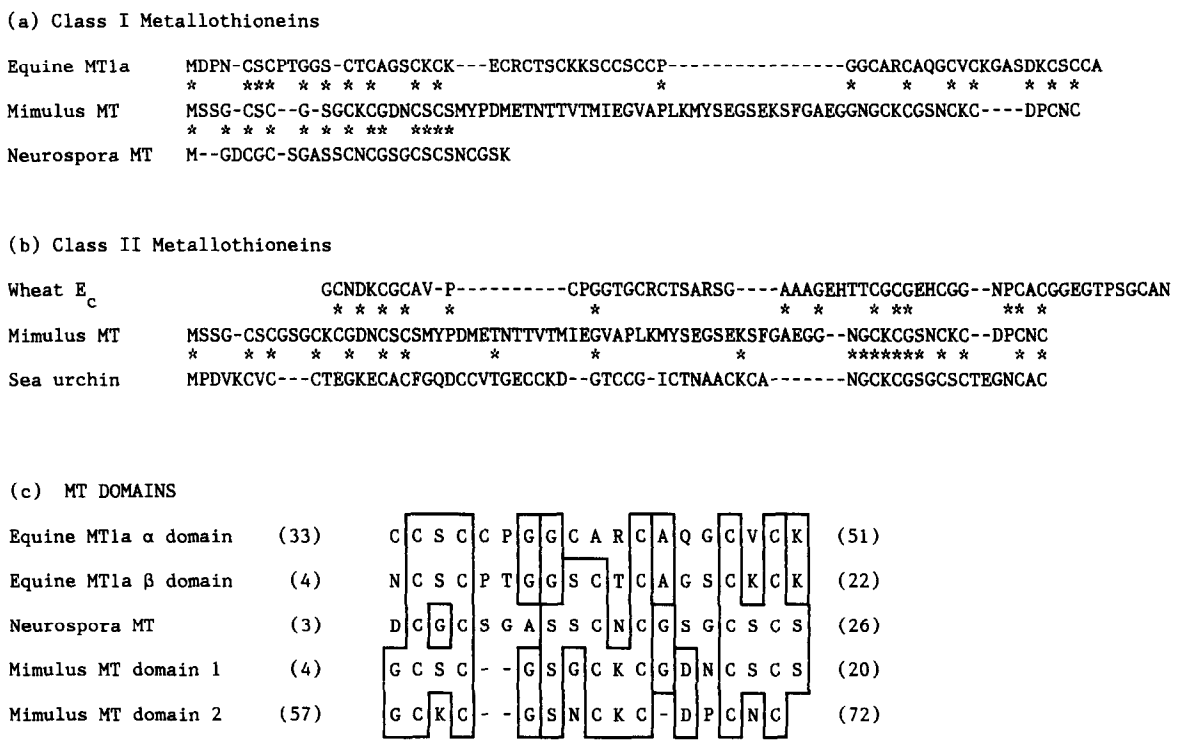

Fig.3. Amino acid sequence alignment of the putative *M. guttatus* MT with (a) class I and (b) class II metallothioneins [1]. In (c), domains 1 and 2 of the *M. guttatus* MT are compared with the α and β domains of equine MT and with the single domain of *N. crassa* MT. The inclusion of (–) in the sequence indicates a gap introduced for optimal alignment of the cysteine residues; (*) denotes amino acid homology; numbers in parentheses indicate the amino acid residue number within the protein. A comparison of the amino acid sequences of the *M. guttatus* MT domain 1 with *N. crassa* MT shows 50% identity (13/26 residues). On this basis, we conclude that this plant protein is a class I rather than class II MT, even though it has lower homology (18/61) to equine MT1a (whose primary structure is used to define class I MTs).

[1,18]. There are two striking differences between previously characterized MTs and the protein described here, the spacer region and its copper-shock repressibility. At present, the significance of these differences is unknown. Since $[\gamma EC]_nGs$ are the major metal-binding component in higher plants exposed to excess metal, perhaps this protein is exclusively involved in metal homeostatis. If so, this may explain both the structural and regulatory differences between this protein and animal MTs.

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