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A metallothionein-like gene from maize (*Zea mays*)

Cloning and characterization

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A differentially expressed maize gene has been cloned and sequenced. Transcriptional and translational start sites have been mapped and 2.5 kb of 5' flanking DNA were sequenced. The 8 kDa protein encoded by this gene shows striking similarity to the metallothionein-like proteins recently described in *Pisum sativum* [1] and *Mimulus guttatus* [2]. The maize MT-L gene message is very abundant in roots without exposure to high levels of metals, present at lower concentration in leaves and pith, and at very low concentration in seed.

Plant metallothionein; Metallothionein gene; 5' Flanking sequence; Differential expression; *Zea mays*

1. INTRODUCTION

Class I and class II MTs are low molecular weight, cysteine-rich, metal-binding proteins that have been identified throughout the animal kingdom and in some fungi [3]. Class I MTs display a typical arrangement of cysteine residues (different from class II MTs cysteine clusters) resembling that of equine MT [4], the first MT isolated [5]. MTs are encoded by genes inducible by high levels of metals [6] and are believed to play a role in the metabolism and detoxification of trace metals [3]. Plants exposed to excess metals accumulate [γEC]nG, metal-binding polypeptides also called phytochelatins (PCs) that belong to class III MTs [4,7]. PCs are not synthesized from an mRNA template but by the enzyme γ-glutamylcysteinyl dipeptidyl transpeptidase [8]. The synthesis of class I MTs by plant cells has long been a controversial subject [7,9,10] until Miranda et al. [2] and Evans et al. [1] reported the cloning of plant cDNAs and genes coding for class I MT-L proteins. While searching for organ-specific genes from maize, a similar gene was cloned. This report describes the characterization of this MT-L gene and its expression pattern in maize.

Abbreviations: MT, metallothionein; MT-L, metallothionein-like; PC, phytochelatin; [γEC]nG, poly(γ-glutamylcysteinyl)glycine = class III MT.

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2. MATERIALS AND METHODS

2.1. *cDNA library construction and differential screening*

Poly (A⁺)-RNA isolated from maize roots (*Zea mays*, CIBA-Geigy Seed inbred 211D) [11,12] was used for the synthesis of cDNA [13]. Oligo-dG-tailed cDNA was cloned into *Pst*I cut oligo-dC-tailed pUC9 (Pharmacia) and transformed into *E. coli* DH5α (BRL). Duplicate colony lifts on nitrocellulose filters were differentially screened for clones hybridizing to first strand cDNA probe from root but not to first strand cDNA probe from seed.

2.2. *Northern and Southern blotting*

Northern and Southern blot hybridizations were performed on nitrocellulose as described by Maniatis et al. [12] using random primed ^{32}P-labeled probes. Genomic DNA was isolated from corn leaves as

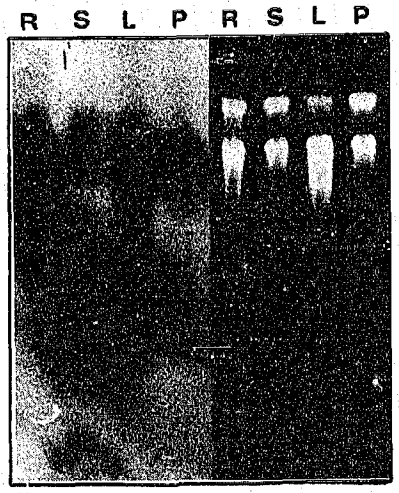

Fig. 1. Differential expression of the MT-L gene in maize. Left panel: 10 μg of root (R), seed (S), leaf (L) and pith (P) total RNA from maize were subjected to electrophoresis on a 1.2% denaturing formaldehyde gel. The RNA was blotted and probed with ^{32}P-labeled pCIB1325 cDNA insert. Right panel: picture of gel before Northern transfer (ethidium bromide was added to the RNA sample).

in Shure et al. [14] and genomic Southern analysis was done according to Klessig and Berry [15].

2.3. *Genomic library screening and DNA sequencing*

*Sau*3A partial genomic fragments of maize inbred 211D DNA were ligated into Lambda Dash/*Bam*HI (Stratagene). Nitrocellulose duplicate plaque lifts were screened with cDNA probes. Lambda phage DNA was extracted from plate lysates [12] and recombinant genomic clones were mapped directly in Lambda [16]. Genomic fragments of interest were subcloned into Bluescript vector for sequencing, which was performed using Sanger's dideoxynucleotide technique [17] as well as Maxam and Gilbert chemical sequencing [18].

2.4. *Primer extension*

Primer extension was done as described by Metraux et al. [19]. 30 μg of root total RNA were hybridized with 0.01 pmol of ^{32}P-end-labeled primer at 40°C for 4 h. Reverse transcriptase was then added and primer extension carried out at 37°C for 1 h. The extended fragments were subjected to electrophoresis on a 10% sequencing gel together with the sequencing reaction of the genomic clone.

3. RESULTS AND DISCUSSION

Differential screening of the maize root cDNA library led to the identification of pCIB1325, a cDNA clone of a transcript more abundant in roots than in seeds. This ~600 nt long mRNA is most abundant in roots, less abundant in green leaves and pith and much less abundant in kernels (Fig. 1). Six cDNA clones, ranging in length from 250 bp to 500 bp (truncated either on the

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-1020  GGCCCATAACAGCATCTCCTCCACCAGTTTATTGTAAGAATAAATTAAGTAGAGATATTT   -961
 -960  GTCGTCGGGCAGAAGAAACTTGGACAAGAAGAAGAAGCAAGCTAGGCCAATTTCTTGCCG   -901
 -900  GCAAGAGGAAGATAGTGGCCTCTAGTTTATATATCGGCGTGATGATGATGCTCCTAGCTA   -841
 -840  GAAATGAGAGAAGAAAAACGGACGCGTGTTTGGTGTGTGTCAATGGCGTCCATCCTTCCA   -781
 -780  TCAGATCAGAACGATGAAAAAGTCAAGCACGGCATGCATAGTATATGTATAGCTTGTTTT   -721
 -720  AGTGTGGCTTTGCTGAGACGAATGAAAGCAACGGCGGGCATATTTTTCAGTGGCTGTAGC   -661
 -660  TTTCAGGCTGAAAGAGACGTGGCATGCAATAATTCAGGGAATTCGTCAGCCAATTGAGGT   -601
 -600  AGCTAGTCAACTTGTACATTGGTGCGAGCAATTTTCCGCACTCAGGAGGGCTAGTTTGAG   -541
 -540  AGTCCAAAAACTATAGGAGATTAAAGAGGCTAAAATCCTCTCCTTATTTAATTTTAAATA   -481
 -480  AGTAGTGTATTTGTATTTTAACTCCTCCAACCCTTCCGATTTTATGGCTCTCAAACTAGC   -421
 -420  ATTCAGTCTAATGCATGCATGCTTGGCTAGAGGTCGTATGGGGTTGTTAATAGCATAGCT   -361
 -360  AGCTACAAGTTAACCGGGTCTTTTATATTTAATAAGGACAGGCAAAGTATTACTTACAAA   -301
 -300  TAAAGAATAAAGCTAGGACGAACTCGTGGATTATTACTAAATCGAAATGGACGTAATATT   -241
 -240  CCAGGCAAGAATAATTGTTCGATCAGGAGACAAGTGGGGCATTGGACCGGTTCTTGCAAG   -181
 -180  CAAGAGCCTATGGCGTGGTGACACGGCGCGTTGCCCATACATCATGCCTCCATCGATGAT   -121

 -120  CCATCCTCACTTGC**TATAAAAA**GAGGTGTCCATGGTGCTCAAGCT**CA**GC**CA**AGCAAATAA   -61

  -60  GACGACTTGTTTCATTGATTCTTCAAGAGATCGAGCTTCTTTTGCACCACAAGGTCGAGG   -1

    0  **ATGTCTTGCAGCTGCGGATCAAGCTGCGGCTGCGGCTCAAGCTGCAAGTGCG**GGTAATAT   59
       **MetSerCysSerCysGlySerSerCysGlyCysGlySerSerCysLysCysG**

   60  ATAATAATATATAAGTGCACCGTGCATGATTAATTTCTCCAGCCTTCTTCTTGTCTTGTC   119

  120  TAGTTAATTTCCCTTCTTTATTTATTTTTTCCATTGCAAAACAAACAAACAAAAAACAAA   179

  180  GTTAATCTGGATCGAGTAGTTCAATCCATTTGCGCGCTGTCCTTTTCA**GCAAGAAGTACC**   **239**
                                                      **lyLysLysTyrP**

  **240**  **CTGACCTGGAGGAGACGAGCACCGCCGCGCAGCCCACCGTCGTCCTCGGGGTGGCCCCGG**   **299**
           **roAspLeuGluGluThrSerThrAlaAlaGlnProThrValValLeuGlyValAlaProG**

  **300**  **AGAAGAAGGCCGCGCCCGAGTTCGTCGAGGCCGCGGCGGAGTCCGGCGAGGCCGCCCACG**   **359**
           **luLysLysAlaAlaProGluPheValGluAlaAlaAlaGluSerGlyGluAlaAlaHisG**

  **360**  **GCTGCAGCTGCGGTAGCGGCTGCAAGTGCGACCCCTGCAACTGCTGA**TCACATCGATCGA   419
           **lyCysSerCysGlySerGlyCysLysCysAspProCysAsnCys***

  420  CGACCATGGATATGATTATTATCTATCTAGCTTGTGGTGGTGGTTGAACAATAATAAGCG   479

  480  AGGCCGAGCTGGCTGCCATACATAGGTATTGTGTGGTGTGTGTGTGAGAGAGAGAGAAAC   539

  540  AGAGTTCTTCAGTTTGCTATCTCTCTCTGCATGTTTGGCGTCAGTCTTTGTGCTCATGTA   599

  600  CGTGTGTCTACATGCATGTTGGTTGATCCGATTGCGTCTGCTGTAACCATATATTAATTG   659

  660  GTCCACGATGATATGATTTGATACTATATATATATACTAAAACCGGACTTATTATAATAC   719

  720  TTGTAGTATATAAGTTTCTTACGCCCGCAATTGATCGATTCAGAACGAAGGAGTTCTAGC   779
  780  TAGCTAAAACATGCAGATTCAGAATATCAGATTTTACGACTACTGGAGGACAAGAATAT 838
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Fig. 2. Nucleotide sequence of the MT-L gene, including 1 kb of 5' flanking sequence and the predicted amino-acid sequence of its product. The extent of the cDNA sequence is indicated by two arrows below the gene sequence. The bases are numbered from the start of translation. The TATA box is in bold and underlined at position −105 as are the 4 transcriptional start sites at positions −75, −74, −71 and −70.

3' or 5' end or both), that hybridized to this transcript were isolated, mapped and sequenced. All overlapping sequences were identical.

A maize genomic library was screened using pCIB1325 as a probe. Two independent Lambda clones were mapped, subcloned into Bluescript and found to carry the same fragment of genomic DNA homologous to pCIB1325. One of these genomic subclones, pCIB1324, was sequenced and was 100% identical to the cDNA sequence except for a 176 bp long intron (Fig. 2). Genomic Southern analysis is shown in Fig. 3. The number and intensity of hybridizing fragments is consistent with the hypothesis that the most intense bands correspond to the isolated genomic clone and the fainter bands to another related gene in the maize genome that cross-hybridizes weakly to pCIB1324. Mung Bean nuclease mapping (data not shown) and primer extension (Fig. 4) gave consistent results in the mapping of the transcriptional start site of this gene and revealed four clustered start sites (Figs. 2, 4). About 35 bp upstream of these start sites is a TATA box (Fig. 2). The first ATG, presumed site of translation initiation, is found following 75 bp of 5' untranslated leader. None of the 6 cDNAs isolated from corn inbred 211D had a poly-A tail or a polyadenylation signal. A full-length cDNA, isolated from another corn inbred, had a poly-A tail and was used to identify the coding strand. The open reading frame of this gene codes for an 8 kDa protein (76 AA) shown in Fig. 2. The in vitro translation of hybrid-selected mRNA yielded an 8 kDa peptide (data not shown), supporting the identification of the open reading frame. Based on the full-length cDNA, the 3' untranslated region is 350 nt long.

The protein encoded by pCIB1325 was found to have significant homology with class I MTs after comparison

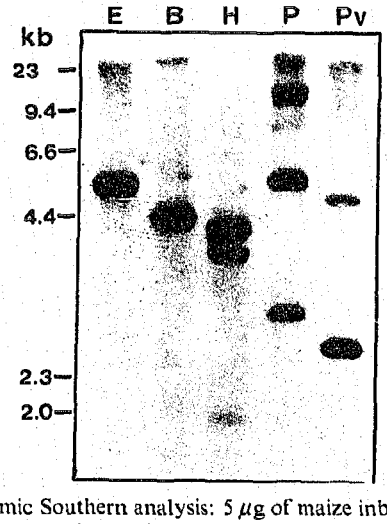

Fig. 3. Genomic Southern analysis: 5 μg of maize inbred 211D genomic DNA were restricted with *Eco*RI (E), *Bam*HI (B), *Hin*dIII (H), *Pst*I (P) and *Pvu*II (Pv), subjected to electrophoresis on a 0.7% agarose gel, blotted onto nitrocellulose and hybridized with pCIB1325 cDNA insert. Lambda DNA digested with *Hin*dIII was used as molecular weight markers.

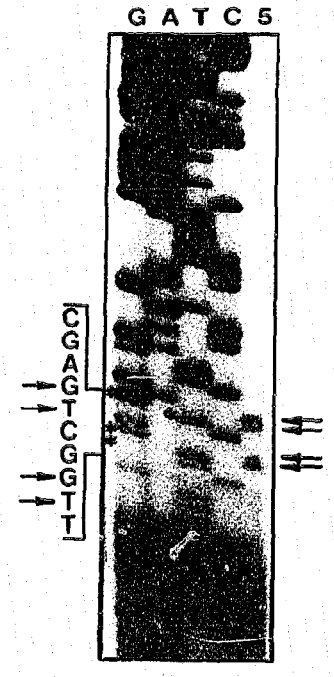

Fig. 4. Mapping of the maize MT-L gene transcriptional start using primer extension (see section 2). The extended DNA fragments were subjected to electrophoresis on a 10% sequencing gel (5). A sequencing reaction of the genomic subclone covering this region was run in lanes G,A,T,C. The arrows point to 4 closely spaced transcription start sites.

of its amino-acid sequence with that of the putative MT-L proteins recently described in pea [1] and in *Mimulus gutttatus* [2] (Fig. 5). At the nucleotide sequence level, the maize gene coding region shows 58% and 67% identity to the pea and *Mimulus guttatus* genes, respectively. The location of the intron is the same in the maize and pea genes, between codons 17 and 18. This gene was designated MT-L for metallothionein-like as opposed to MT because of lack of characterization of the MT-L gene product, in particular regarding its metal-binding properties. Metal regulatory elements (MREs) present in the 5' flanking region of the animal MT genes have been identified. 2.5 kb of 5' flanking region of the maize MT-L gene was sequenced (1 kb of this sequence is shown in Fig. 2) and searched for these MREs, but contrary to the pea gene [1], no such consensus sequence

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PEA       M.SGCGCGSSCNCGDSCKCNKRSSGLSYSEMETTETVIL..GV
CORN      M.S.CSCGSSCGCGSSCKCGKKYPDLEETSTAAQPTVVL..GV
MIMULUS   MSSGCSCGSGCKCGDNCSCSM.....YPDMETNTTVTMIEGV

PEA       GPAK...IQFEGAEMSAASEDG.GCKCGDNCTCDPCNCK
CORN      APEKKAAPEFVEAAAESGEAAH.GCSCGSGCKCDPCNC.
MIMULUS   APLK...MYSEGSEKSFGAEGGNGCKCGSNCKCDPCNC.
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Fig. 5. Amino-acid alignment of the maize MT-L protein with that of the pea [1] and *Mimulus guttatus* [2]. Vertical lines indicate matching amino acids between the maize gene and either one of the other 2 genes. The maize MT-L protein shows 46% amino-acid identity with the pea and *Mimulus guttatus* proteins. Most of the homology resides in the amino- and carboxy-termini domains that contain the Cys-X-Cys motifs typical of other MT proteins.

was found in the maize gene promoter. Furthermore, the maize MT-L gene is expressed constitutively at high level in roots without exposure to elevated concentrations of metals. Similar observations were made for the pea gene [1]. Whether metals regulate the expression of the maize MT-L gene is currently under investigation.

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