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Metallothioneins 1 and 2 Have Distinct but Overlapping Expression Patterns in Arabidopsis[1]

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The spatial and temporal expression patterns of metallothionein (MT) isoforms *MT1a* and *MT2a* were investigated in vegetative and reproductive tissues of untreated and copper-treated Arabidopsis by in situ hybridization and by northern blotting. In control plants, *MT1a* mRNA was localized in leaf trichomes and in the vascular tissue in leaves, roots, flowers, and germinating embryos. In copper-treated plants, *MT1a* expression was also observed in the leaf mesophyll and in vascular tissue of developing siliques and seeds. In contrast, *MT2a* was expressed primarily in the trichomes of both untreated and copper-treated plants. In copper-treated plants, *MT2a* mRNA was also expressed in siliques. Northern-hybridization studies performed on developing seedlings and leaves showed temporal variations of *MT1a* gene expression but not of *MT2a* expression. The possible implications of these findings for the cellular roles of MTs in plants are discussed.

MTs are defined as low-M_r, Cys-rich proteins that bind heavy metals. MTs are widely distributed in eukaryotic and prokaryotic organisms (for review, see Kagi, 1991; Robinson et al., 1993). In animals and fungi MTs have been shown to play a role in the detoxification of heavy metals, although their exact function is not completely understood. In plants a correlation has been observed between MT RNA levels and tolerance to heavy metals in different Arabidopsis ecotypes (Murphy and Taiz, 1995a), suggesting a role in metal homeostasis in plants.

In animals and yeast MT expression is regulated by metals (Robinson et al., 1993). In plants the effect of metals on the expression of MTs varies with the plant species, tissue, and MT type. In *Mimulus guttatus* (de Miranda, 1990), soybean (Kawashima, 1991), and barley (Okumura et al., 1991), MT mRNA levels were decreased by copper treatment, whereas in bean (Foley and Singh, 1994; Foley et al., 1997), wheat germ (Lane et al., 1987), and *Nicotiana glutinosa* (Choi et al., 1996), MT expression was not affected by metals. In Arabidopsis (Zhou and Goldsbrough, 1994, 1995; Murphy and Taiz, 1995a), wheat (Snowden and Gardner, 1993), pea (Evans et al., 1992), and rice (Hsieh et al., 1995), transcription of MTs was enhanced by certain metals only. As in animals (Robinson et al., 1993), a variety of other stimuli, including ABA, heat shock, cold shock, wounding, viral infection, senescence, salt stress, and Suc starvation, have been shown to influence expression of plant MTs (Buchanan-Wollaston, 1994, 1997; Foley and Singh, 1994; Hsieh et al., 1995; Murphy and Taiz, 1995a; Snowden et al., 1995; Choi et al., 1996; Foley et al., 1997).

In Arabidopsis, three MT gene families have been identified: *MT1*, *MT2*, and *MT3* (Zhou and Goldsbrough, 1994; Murphy et al., 1997), homologs of which have been identified in other species. The data available regarding the expression of MT genes from a variety of plant species indicate that each MT gene type exhibits characteristic temporal and tissue-specific expression patterns. Expression of most *MT1*-like sequences has been detected primarily in roots (de Miranda et al., 1990; de Framond, 1991; Evans et al., 1992; Zhou and Goldsbrough, 1994; Hsieh et al., 1995; Hudspeth et al., 1996) and senescent leaves (Kawashima et al., 1991; Buchanan-Wollaston, 1994, 1997; Hsieh et al., 1995; Foley et al., 1997). *MT2*-type transcripts have been detected primarily in leaves (Snowden and Gardner, 1993; Foley and Singh, 1994; Zhou and Goldsbrough, 1994, 1995; Coupe et al., 1995; Choi et al., 1996) and roots of mature plants (Zhou, 1994; Snowden et al., 1995; Murphy, 1996). *MT3*-like mRNAs have been detected in leaves (Murphy, 1996; Bundithya and Goldsbrough, 1997), fruits (Ledger and Gardner, 1994), and developing embryos (Dong and Dunstan, 1996).

In Arabidopsis each MT type appears to belong to a small gene family, the members of which appear to exhibit differential gene expression patterns. *MT1* consists of three isoforms, *MT1a, MT1b,* and *MT1c. MT1a* is constitutively expressed in seedlings and is induced by copper in excised leaves, whereas *MT1b* seems to be a pseudogene. *MT1c* is expressed in young and mature roots and in mature leaves and is not affected by copper treatment. The *MT2* gene family consists of *MT2a* and *MT2b*. They are both constitutively expressed in mature leaves, and only the *MT2a* gene is copper inducible in seedlings (Zhou and Goldsbrough, 1994; Murphy and Taiz, 1995a; Zhou and Goldsbrough, 1995; Murphy et al., 1997). Immunocytochemical studies have recently shown similar patterns of accumulation of the gene products (Murphy et al., 1997). All of these data suggest that each MT isoform may have specialized functions in different tissues. Some of the functions proposed for plant MTs include a role during development (Kawashima et al., 1992; Ledger and Gardner, 1994; Dong

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Abbreviations: MT, metallothionein; RT, reverse transcriptase.

and Dunstan, 1996), in senescence (Buchanan-Wollatson, 1994; Coupe et al., 1995; Hsieh et al., 1995), and in protection against oxidative stress (Choi et al., 1996).

To date, in situ-hybridization studies of metallothionein gene expression have been performed in just two plant species, bean and wheat, and only for *MT2* transcripts. In bean *MT2* expression was localized specifically in foliar trichomes and veins (Foley and Singh, 1994). In wheat the *MT2*-like *WALI 1* gene was specifically expressed in the apical meristem of roots (Snowden et al., 1995).

Additional information about the tissue-specific expression of MTs is needed to help clarify the biological function(s) of MT genes in plants. We focused our studies on Arabidopsis because its response to copper is well characterized, and because it is the only plant species in which more than one MT gene family has been identified. To further characterize the developmental regulation of MTs in Arabidopsis, we also performed northern-hybridization analysis on developing seedlings and aging leaves. Our results have confirmed that there are differences in the spatial localization of *MT1a* and *MT2a* expression in the tissues examined. *MT1a* was expressed in most vegetative and reproductive organs in vascular tissues and in trichomes, whereas *MT2a* was predominantly expressed in leaf trichomes. Moreover, developmental studies showed a very distinct pattern of expression for the two MT genes.

MATERIALS AND METHODS

Plant Material

Arabidopsis ecotype Wassilewskija (Ws) was used in all experiments. Plants were grown in a medium consisting of 1.1 g/L Murashige-Skoog basal salt mixture (Sigma) and 1 mM Mes, pH 4.8. For growth periods of less than 8 d, the seedlings were germinated using the vertical mesh transfer system (Murphy and Taiz, 1995a). For longer growth periods, the Aquamist hydroponic system (Pure Food Hydroponics, San Jose, CA) was used. For copper treatment, 40 μM $CuCl_2$ was added to the growth medium 36 h before harvesting.

Preparation of Probes

The coding and flanking untranslated regions of *MT1a* and *MT2a* cDNAs were amplified, using sequence-specific primers, by RT-PCR from total RNA prepared from 30 μM $CuCl_2$-treated seedlings of the Ws ecotype as previously described (Murphy and Taiz, 1995a). The *MT1a* RT-PCR product contained the complete translated region (135 residues) plus 24 nucleotides upstream of the start codon and 182 nucleotides of the 3′ untranslated region. The *MT2a* PCR product consisted of the complete translated region (236 bp) plus 12 nucleotides upstream of the start codon and 224 nucleotides of the 3′ untranslated region. Both sequences were cloned into the pZero-2 plasmid (Invitrogen, San Diego, CA) in the antisense orientation. The orientation of the inserts was verified by dye-termination dideoxy sequencing utilizing a DNA sequencer (ABI 310, Applied Biosystems) with M13 forward and reverse primers.

The clone encoding the 33-kD PSII-binding protein O from Arabidopsis (psbO) was a gift from Neil Hoffman (Carnegie Institution of Washington, Stanford, CA). The psbO cDNA was cloned into pGEM4 and contained the complete translated region flanked by 5′ and 3′ untranslated regions of 80 and 135 nucleotides, respectively.

The *MT1a* and *MT2a* cDNA sequences were amplified by PCR with M13 sequencing primers and digested with the appropriate restriction enzymes (*Sac*I for sense *MT1a*, *Xho*I for antisense *MT1a*, *Hin*dIII for sense *MT2*, and *Not*I for antisense *MT2*) to remove most of the remaining vector sequences. The *psbO* clone was linearized with *Bam*HI to produce a template of 386 nucleotides. The purified cDNA fragments were then used in the preparation of digoxigenin-labeled riboprobes by in vitro transcription. Sense and antisense strands were transcribed with SP6 or T7 polymerase according to the manufacturer's instructions (Boehringer Mannheim), except for the transcription buffer (5 mM each ribonucleotide triphosphate, 40 mM Tris-HCl, pH 8.0, 26 mM $MgCl_2$, 3 mM spermidine, 0.01% Triton X-100, and 10 mM DTT).

In Situ Hybridization

Plant tissues were fixed in 4% paraformaldehyde and 50 mM Pipes, pH 7.2, and washed twice for 15 min with the same buffer. Roots, leaves, germinating seeds, and siliques were fixed for 2 to 4 h at room temperature. Flowers were fixed overnight at 4°C. Roots were embedded in 0.6% agarose to facilitate further handling. After fixation, specimens were dehydrated in a graded ethyl alcohol series (15%, 30%, 50%, 70%, 85%, 95%, and 100%) and embedded in paraffin. Sections (8 μm thick) from paraffin-embedded material were mounted on glass slides coated with 3-aminopropyltriethoxysilane (Aldrich) in acetone. Paraffin was removed by incubating slides twice in xylene for 10 min. Section pretreatment and hybridization were performed according to the method of Lincoln et al. (1994) with some modifications. Slides were incubated with 2 μg/mL proteinase K (Boehringer Mannheim) for 30 min at 37°C. Hybridization was carried out overnight at 50°C in 50% formamide, 300 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5% dextran sulfate, 1% blocking reagent, 150 μg/mL tRNA, and about 500 ng/mL riboprobe. Slides were covered with HybriSlip (Research Products International, Mt. Prospect, IL) and sealed with rubber cement. RNase treatment was with 10 μg/mL RNase A (Boehringer Mannheim) for 30 min at 37°C. Detection of hybridized transcripts with antidigoxigenin antisera conjugated with alkaline phosphatase (1:500 dilution; Boehringer Mannheim) was performed according to the method of Coen et al. (1990). Slides were passed through an ethyl alcohol series and xylene before mounting in Eukit (Calibrated Instruments, Inc., Hawthorne, NY). The sections were observed using differential interference contrast optics on an Aristoplan microscope (Leitz, Wetzlar, Germany). Photographs were taken with Kodak Ektachrome 160T film using a Leitz Orthomat E camera.

Image Processing

Photographs were scanned by using a Sprintscan 35 (Polaroid, Inc., Cambridge, MA). For RNA blots, an Arcus II flatbed scanner (AGFA Division, Miles Inc., Ridgefield, NJ) was used. Images were processed using Photoshop, version 4.0. (Adobe, Mountain View, CA), and printed with an NP1600 printer (Codonics, Inc., Middleburg Heights, OH).

RT-PCR of Seedling Tissues

RNA isolation and limiting (22 cycle) quantitative RT-PCR of *MT1a* and *MT2a* expression was as described previously (Murphy and Taiz, 1995b). Primary leaves and apical buds, cotyledons, hypocotyls, and roots were excised from 200 seedlings with a razor blade and placed directly into liquid nitrogen before total RNA extraction. Results are summarized from two separate experiments.

RNA Isolation and Northern Blotting

RNA was isolated from liquid-nitrogen-ground plant tissues using Trizol reagent (GIBCO-BRL) following the instructions provided by the manufacturer. Total RNA was fractionated and transferred onto a nylon membrane (Nytran). Filters were hybridized with digoxigenin-labeled riboprobes and washed according to the method of Zhou and Goldsbrough (1994). Detection of transcripts with antidigoxigenin antisera coupled to alkaline phosphatase (Boehringer Mannheim) was carried out following the directions of the manufacturer. Each northern blotting experiment was repeated at least three times, and representative experiments are shown.

RESULTS

Expression of *MT1a* and *MT2a* Genes in Seedling Roots

All of the experiments with roots were carried out with 6-d-old seedlings. The results of in situ-hybridization analyses of *MT1a* and *MT2a* RNAs using antisense riboprobes are summarized in Figure 1, A to F. In the meristematic region of the root, the hybridization signal obtained for *MT1a* was low and in many cases difficult to distinguish from the background (Fig. 1A). However, significant amounts of *MT1a* transcript in the elongation and maturation regions of the roots was detected (Fig. 1B). Accumulation of *MT1a* transcript in these regions appeared to be higher in cells of the stele and cortex (Fig. 1B). In epidermal cells the hybridization signal obtained with *MT1a* probes was much less intense. Similar hybridization patterns were observed in roots from copper-treated seedlings (data not shown). No hybridization signals above background levels were detected in sections treated with the *MT1a* sense probe (Fig. 1C), indicating that the reactions observed with the antisense probe were specific.

The level of expression of *MT2a* in the meristematic region of the root was near the limit of our detection system (Fig. 1D). However, as in the case of *MT1a*, a strong hybridization signal was detected in the phloem, although the identity of the cells could not be determined (Fig. 1E). No significant *MT2a* transcript levels were found in other cells of the root. Surprisingly, when roots from plants that had been treated with excess copper were examined, we did not find any difference in the expression patterns of *MT2a* compared with untreated plants (data not shown). The absence of hybridization signal above background levels obtained with the *MT2a* sense probe (Fig. 1F) indicates that the signal detected with the *MT2a* antisense probe was specific.

To determine the relevance of these results to earlier findings (Murphy and Taiz, 1995b; Zhou and Goldsbrough, 1995), which showed copper-induced increases in *MT2* expression in Arabidopsis seedlings, *MT1a* and *MT2a* mRNA expression in primary leaves and apical buds, cotyledons, hypocotyls, and roots were quantitated by fluorometric assay of RT-PCR products. As shown in Table I, *MT2a* expression was specifically induced by copper in cotyledons and, to a lesser extent, in hypocotyls but not in the roots. This finding is consistent with the failure to detect *MT2a* expression in seedling roots by in situ hybridization, even in the presence of copper. Copper increased the level of *MT1a* expression only in primary leaves and apical buds.

Localization of *MT1a* and *MT2a* mRNAs in Arabidopsis Leaves

The youngest visible leaves of 2-week-old Arabidopsis plants were subjected to RNA in situ-hybridization analysis to determine which cells were responsible for the expression of *MT1a* and *MT2a* that had been observed previously by northern hybridization. Leaves from plants of the same age that had been treated with 40 μM $CuCl_2$ for 36 h were also examined. In leaves from control plants, *MT1a* expression was detected at high levels in trichomes and at lower levels in vascular bundles of minor veins (Fig. 1G). The hybridization pattern of *MT1a* in plants treated with excess copper was somewhat variable, but in all experiments *MT1a* was expressed at higher levels in copper-treated plants than in untreated ones (Fig. 1, H and I). Expression of *MT1a* was high in leaf trichomes of copper-treated plants, but since the hybridization signals were saturated in both control and treated trichomes, it was impossible to determine whether the amount of *MT1a* transcripts in trichomes of copper-treated plants was higher than in control plants. However, the stimulatory effect of copper on *MT1a* expression was discernible in other parts of the leaf. In some cases, hybridization signals were evenly distributed in all leaf tissues, including the rest of the epidermis and the mesophyll (Fig. 1H). In other cases, the signal was restricted in the mesophyll to vascular bundles but was much stronger than in control leaves (Fig. 1I). In the vascular bundles, the signal appeared to be localized in the phloem, possibly in the sieve elements (Fig. 1I, inset). No hybridization signal was observed in sections probed with the sense *MT1a* probe (Fig. 1M).

In the same leaves, *MT2a* was expressed at very high levels in trichomes of both control (Fig. 1J) and copper-

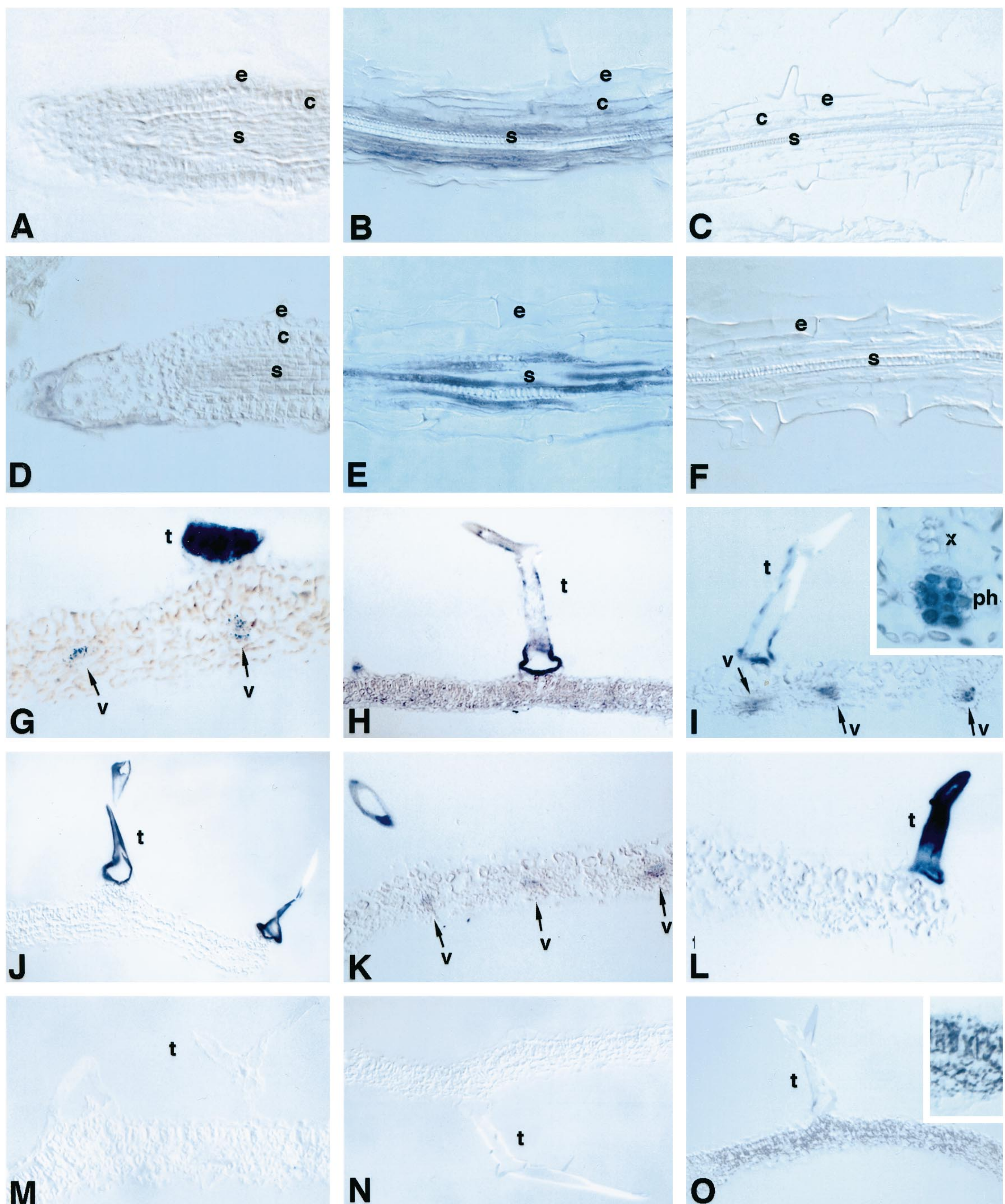

Figure 1. Expression of *MT1a* and *MT2a* in Arabidopsis roots and leaves. The hybridization signal ranged from purple to dark blue. A, B, G, H, and I, Sections probed with *MT1a* antisense; C and M, sections probed with *MT1a* sense; D, E, J, K, and L, sections probed with *MT2a* antisense; F and N, sections probed with antisense probe with MT2a sense; O, section probed with antisense probe for the chloroplast-specific transcript *psbO*. All roots sections are from 6-d-old seedlings grown without excess copper. A and D, Longitudinal sections. A and D, Root-tip sections. B, C, E, and F correspond to sections through the maturation region. All leaf sections are from the youngest leaves of 2-week-old untreated plants (G, J, M, and O) or plants treated with excess copper (H, I, K, and L). Inset in I corresponds to a higher magnification of one of the vascular bundles. e, Epidermis; c, cortex; s, stele; t, trichome; v, vascular bundle. Arrows, Locations of hybridization signal.

Table I. *RT-PCR quantitation of MT1a and MT2a mRNA expression in excised roots, hypocotyls, cotyledons, and primary leaves from 6.5-d-old Ws seedlings*

mRNA expression levels were quantitated fluorometrically after size verification on agarose gels (see "Materials and Methods") and are expressed as mean percentages ± SD of measured β-tubulin mRNA levels. High SD of primary leaf mRNA is the result of the difficulty in excising small (approximately 7% of total seedling fresh weight) primary leaves at this stage of development.

Plant Part	MT1a		MT2a	
	Control	40 μM $CuCl_2$	Control	40 μM $CuCl_2$
		%		
Root	62 ± 29	54 ± 24	75 ± 32	68 ± 22
Hypocotyl	31 ± 20	91 ± 35	27 ± 34	77 ± 26
Cotyledon	139 ± 20	142 ± 11	13 ± 27	182 ± 28
Primary leaves	20 ± 13	183 ± 47	130 ± 51	137 ± 43

treated (Fig. 1, K and L) plants. In control plants, trichomes were the only leaf cell type in which *MT2a* mRNA could be detected. In some leaf sections from copper-treated plants, low levels of expression were seen also in vascular bundles (Fig. 1K). In most cases, however, expression of *MT2a* in leaves from copper-treated plants remained restricted to trichomes exclusively (Fig. 1L). As before, the possibility of higher expression levels of *MT2a* in trichomes of copper-treated than in control plants cannot be ruled out because of color saturation.

In all cases, the *MT2a* antisense riboprobe hybridized more strongly with trichomes than the antisense *MT1a* probe, judging by the much shorter development times needed for the appearance of the *MT2a* signal (1 h versus more than 9 h for *MT1a*). This suggests that *MT2a* may be expressed at higher levels than *MT1a* in trichomes, although it could also be due to differences in the affinities of the probes for their respective sequences. However, the fact that the *MT1a* probe gave darker signals than the *MT2a* probe in other tissues (Fig. 1, H versus K) suggests that *MT2a* was expressed at higher levels than *MT1a* in trichomes. Hybridization reactions using the *MT2a* sense probe did not produce signals above background levels, indicating that the strong trichome staining is specific (Fig. 1N).

As a second, positive control, we also used a probe for a gene clone encoding psbO from Arabidopsis. As shown in Figure 1O, this probe hybridized with only chloroplast-containing mesophyll cells rather than the trichomes, further indicating that the hybridization patterns obtained with our MT antisense probes were specific.

Expression of *MT1* and *MT2* in Arabidopsis Flowers

To investigate the expression patterns of MT genes in Arabidopsis flowers, tissue sections from flowers at different stages of development were hybridized with *MT1a* and *MT2a* riboprobes. At floral stages 9 to 10 (Smythe et al., 1990), during which the ovule protrusions elongate, the petals are below the level of stamens and the anthers contain microspore mother cells or tetrads, the antisense *MT1a* probe hybridized to varying degrees with all tissues of the flower (Fig. 2A). *MT1a* expression was higher in the gynoecium, especially in the developing ovules, in anthers, in cells of the tapetum, and in tetrads. Hybridization with vascular tissues of the flower, especially in the stamens and the receptacle, was also detectable (Fig. 2A). No hybridization signal was observed in sections of flowers treated with the sense *MT1a* riboprobe (Fig. 2B).

In the next stages of flower development (11–12; after the stigmatic papillae appear, when the petals are level with the long stamens, and the integuments extend toward the apex of the nucellus), *MT1a* expression was still stronger in all tissues of the gynoecium, especially in the integuments and funiculus of developing ovules, in the placenta, and along some strands of vascular tissue (data not shown). In anthers the strong hybridization signal observed in the pollen sacs in stages 9 to 10 had disappeared. In mature flowers, *MT1a* was highly expressed in vascular strands of sepals and the receptacle, especially the provascular tissue connecting the receptacle with the sepals (Fig. 2C), in the nectaries (Fig. 2D), in the tissue surrounding the vascular strands of anthers (Fig. 2E), in the vascular tissues of stamens and petals (Fig. 2F), and in the cells of the stigmatic core (Fig. 2G).

In contrast to *MT1a*, *MT2a* was expressed at very low levels in the gynoecium and only at the early stages of ovule development (Fig. 2K). At later stages of development, *MT2a* mRNA levels were below the limits of detection in all floral tissues (Fig. 2, H–J). The only cells expressing *MT2a* in mature flowers were the trichomes (Fig. 2L).

No effects of copper on the expression of either *MT1a* or *MT2a* in flowers was observed (data not shown).

In Situ Hybridization of *MT1a* and *MT2a* in Germinating Seeds

We also studied the localization of *MT1a* and *MT2a* gene expression during the early stages of seedling development in the presence or absence of copper. A sample of the results obtained from copper-treated seedlings is shown in Figure 3, A to D. The antisense *MT1a* riboprobe hybridized with specific cells of the vascular tissue in the hypocotyl and cotyledons (Fig. 3A). In the hypocotyl these cells were tentatively identified as phloem sieve elements (Fig. 3B). No hybridization signal was detected in any other region of the hypocotyl or radical. The hybridization pattern of control plants was essentially the same as in copper-treated seedlings (data not shown). However, the

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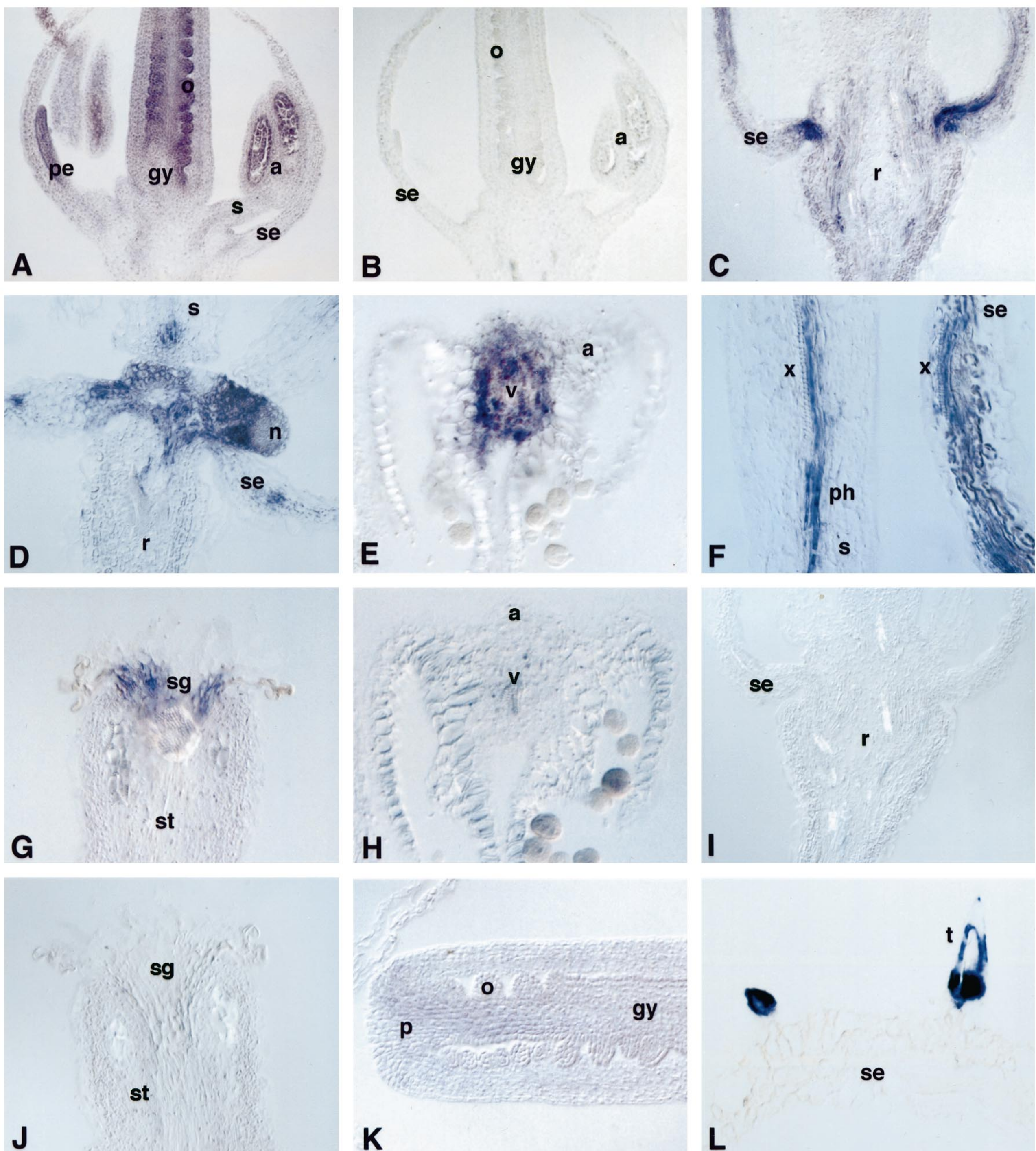

Figure 2. Localization of *MT1a* and *MT2a* in Arabidopsis flowers. A and C to G, Sections hybridized with *MT1a* antisense riboprobe; B, section probed with *MT1a* sense; H to L, sections probed with *MT2a* antisense. A, B, and K, Flowers at stage 8 to 9. C to J and L, Flowers at anthesis. All sections are from plants grown without excess copper. a, Anthers; gy, gynoecium; n, nectary; o, ovule; p, placenta; pe, petal; ph, phloem; r, receptacle; s, stamen; se, sepal; sg, stigma; st, style; t, trichome; v; vascular bundle; x, xylem.

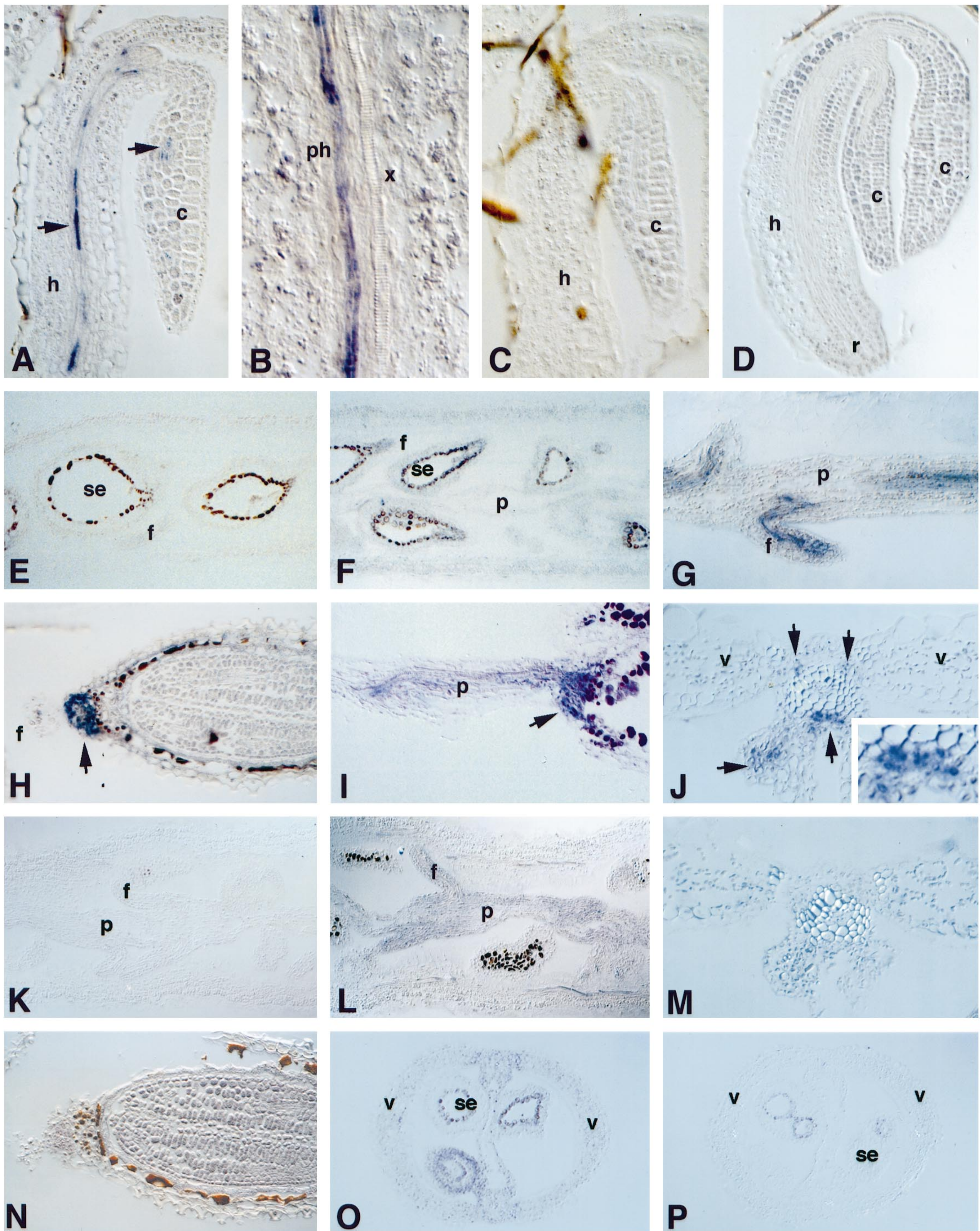

Figure 3. Expression of *MT1a* and *MT2a* in germinating seedlings and developing siliques. Sections were probed with: A, B, and E to J, *MT1a* antisense; C, *MT1a* sense; D and K to O, *MT2a* antisense; P, *MT2a* sense. A to D, F, G, I, J, L, M, and O were treated with excess copper; E, H, K, and N are untreated plants. c, Cotyledons; h, hypocotyl; f, funiculus; p, placenta; ph, phloem; se, seed; v, vascular tissue; x, xylem. Arrows indicate the location of the transcripts. Blue or purple stain corresponds to hybridization signals. The seed coat stains brown because of its normal pigmentation.

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number of seedlings that showed a hybridization signal was 3- to 4-fold higher in the copper-treated seeds than in the untreated controls. From these observations we inferred that *MT1a* is expressed at low levels in germinating seedlings in phloem tissue and that copper caused a slight induction in the expression of *MT1a* in the same cell type. The *MT1a* sense probe control failed to hybridize with any seed tissue (Fig. 3C). In contrast, expression of *MT2a* was not detected in any tissue in germinating seedlings, either with (Fig. 3D) or without (data not shown) copper treatment.

Expression of *MT1* and *MT2* in Siliques and Developing Seeds

In sections of siliques from control plants, we did not detect hybridization signals above background levels with either *MT1a* (Fig. 3E) or *MT2a* (Fig. 3K) antisense probes. In siliques from copper-treated plants, low levels of *MT1a* expression were detected in most tissues of the silique (Fig. 3F). Higher levels of *MT1a* expression were detected in vascular strands of the placenta, central septum, and funiculus (Fig. 3, G, I, and J). Cross-sections through the central septum showed *MT1a* hybridization with cells surrounding the xylem tissue, which appeared to be derived from the phloem (Fig. 3J). In developing seeds we found a strong hybridization signal with *MT1a* antisense probe in cells in the region where the funiculus attaches to the chalazal end of the seed (Fig. 3, H and I). The signal had a ring shape and surrounded the vascular bundle of the funiculus, in which tracheary elements were visible in the middle (Fig. 3H). In the longitudinal view the signal was localized to tissue that appeared as a continuation of the vascular strand of the funiculus, which is funnel-shaped inside the seed (Fig. 3I). This hybridization pattern was not apparent in very immature seeds, only in seeds in which the embryo occupied at least one-half of the seed volume. The same results were obtained in developing seeds from both untreated and copper-treated plants. In younger, copper-treated developing seeds, low levels of *MT1a* mRNA were observed in the integuments (Fig. 3F).

MT2a mRNA was not detected in siliques of untreated plants (Fig. 3K). In siliques from copper-treated plants, *MT2a* was localized at low levels in most tissues (Fig. 3, L, M, and O). *MT2a* expression was higher in the placenta and funiculus (Fig. 3L), but was evenly distributed in all cells rather than being predominant in vascular strands, as it was with *MT1a* expression. No significant levels of *MT2a* expression were observed in tissues of developing seeds at more mature stages, either untreated (Fig. 3N) or copper-treated (data not shown). Younger seeds from copper-treated plants showed low levels of *MT2a* expression in the integuments (Fig. 3O). No hybridization signals above background were obtained in siliques and developing seed tissues with *MT2a* sense control probe (Fig. 3P).

Northern-Hybridization Analysis of *MT1a* and *MT2a* mRNA in Developing Seedlings

To determine whether the expression of *MT1a* and/or *MT2a* was regulated during seedling development, and whether the effects of excess copper would vary with the developmental stage of the plants, total RNA was isolated from Arabidopsis seedlings grown for 5 to 8 d on vertical mesh transfer plates and then analyzed by northern blotting using the same riboprobes that were used for the in situ-hybridization studies. The results show that the amount of *MT1a* mRNA in the seedlings increased more than 10-fold from d 5 to 8 (Fig. 4A). The effect of copper on *MT1a* expression depended on the age of the seedlings. In the youngest (5 d old) seedlings, excess copper did not affect *MT1a* expression. In 6- and 7-d-old seedlings, excess copper caused an increase in *MT1a* RNA accumulation. In the 8-d-old seedlings, copper had either no effect or caused only a slight increase in *MT1a* expression.

In contrast to *MT1a*, the expression of *MT2a* in the absence of copper did not vary significantly during the stages of seedling development examined (Fig. 4B). Copper caused a slight (approximately 2-fold) increase in *MT2a* transcript level, but the effect was consistently detected only in 8-d-old seedlings (Fig. 4B). In all cases, the time required for the appearance of hybridization signals on the membranes probed with *MT2a* was about 3 to 5 times longer than with *MT1a*. Neither *MT1a* nor *MT2a* sense probes hybridized with any other RNA band on the filters (data not shown).

Expression of *MT1a* and *MT2a* in Senescing Leaves

We also investigated the developmental regulation of *MT1a* and *MT2a* expression in senescing leaves. Leaves of

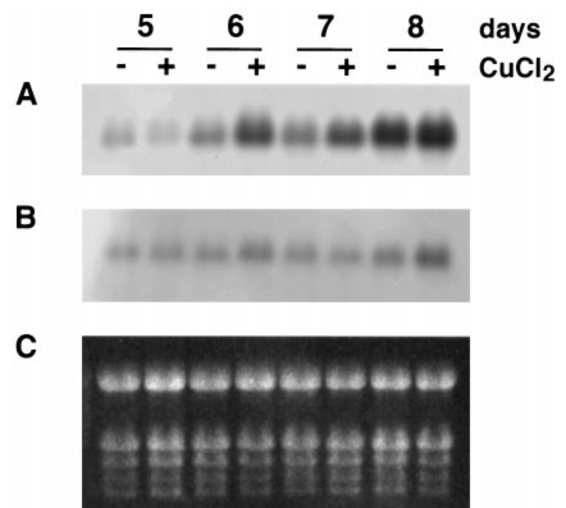

Figure 4. Northern analysis of MT1a and *MT2a* expression in developing Arabidopsis seedlings. Total RNA (10 mg) was extracted from seedlings germinated for 5, 6, 7, or 8 d in the absence (−) or presence (+) of 40 μM copper. RNA was separated in a formaldehyde agarose gel, blotted, and hybridized with digoxigenin-labeled RNA probes. A, Blot probed with *MT1a* antisense. B, Blot probed with *MT2a* antisense. C, Gel stained with ethidium bromide showing rRNA.

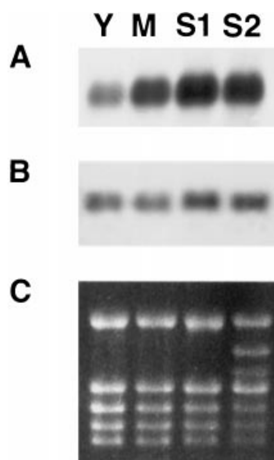

Figure 5. Northern hybridization of *MT1a* and *MT2a* in aging Arabidopsis leaves. Total RNA (8 μg) was isolated from young (Y), mature (M), mid-senescent (S1), and almost completely senescent (S2) leaves. RNA was processed as in Figure 4. Filters were hybridized with *MT1a* antisense (A) or *MT2a* antisense (B) riboprobes. C, Ethidium bromide-stained gel.

different ages (young, mature, and early- and late-senescent) were collected and their total RNA analyzed by northern blotting. As shown in Figure 5, the expression of *MT1a* increased by >2-fold from the young to the mature leaf stage and then increased further from the mature to the early-senescent stage (Fig. 5A).

In contrast to *MT1a*, the level *MT2a* mRNA did not vary significantly during the four stages of leaf maturation and senescence examined (Fig. 5B).

DISCUSSION

We have investigated the spatial distribution of *MT1a* and *MT2a* mRNAs by in situ hybridization in vegetative and reproductive tissues of Arabidopsis in the presence and absence of excess copper. Our in situ hybridization results indicate that the two MT genes have contrasting expression patterns, suggesting that they play distinct roles in metal ion homeostasis and development.

The riboprobes used in the in situ and northern hybridization experiments were synthesized using clones of *MT1a* and *MT2a* that contained the complete translated regions and most of the 3′ untranslated regions. The sequence identities between *MT1a* and *MT1c* and between *MT2a* and *MT2b* in these regions were 67% and 60%, respectively. These values are sufficiently low to expect isoform specificity of the probes under the high-stringency conditions used. In the case of *MT2a*, we were able to confirm by northern blotting that the antisense probe did not hybridize with sense transcripts prepared from the *MT2b* clone (data not shown). We were unable to obtain a cDNA clone of *MT1c* for parallel tests of the specificity of the *MT1a* probe. Although a recently deposited expressed sequence tag sequence has been identified, which appears to correspond to *MT1c* (expressed sequence tag clone no. 222N3T7, accession no. N38326), the abundance of this transcript is probably much lower than that of *MT1a* (P. Goldsbrough, personal communication). Thus, the two probes used in this study appear to be relatively isoform specific.

In young control roots, both *MT1a* and *MT2a* genes were expressed in the maturation zone of the root. However, compared with *MT1a*, the level of expression of *MT2a* was low, and its detection was not always possible.

Little or no *MT1a* or *MT2a* expression was detected in control root tips by in situ hybridization. This result is consistent with the finding that the pea *MT1*-like *PsMTa* gene promoter directed GUS expression in Arabidopsis to all tissues of the root except the apex (Fordham-Skelton et al., 1997). However, the wheat *MT2*-like *WALI1* gene was expressed predominantly in the root apical meristem (Snowden et al., 1995), and other expression studies of cotton using GUS fused to the *MT1* promoter showed the highest stain at the root tip (Hudspeth et al., 1996). These discrepancies may reflect the complexity of the expression regulation of the different MT genes, as well as the possible pitfalls associated with various methods of detection. For example, localization data obtained by reporter genes may not always reflect in vivo gene-expression pattern (Taylor, 1997).

Copper treatment failed to cause a significant increase in the expression of either *MT1a* or *MT2a* in growing tips and maturation zones of 6-d-old roots, as measured by in situ hybridization. This finding was unexpected, since previous northern blotting and RT-PCR studies with Arabidopsis seedlings had demonstrated an increase in the total *MT2* transcript level in the presence of copper (Zhou and Goldsbrough, 1994; Murphy and Taiz, 1995a). However, these studies were based on total mRNA extracted from whole seedlings. When we repeated our RT-PCR measurements of *MT1* and *MT2* mRNA using excised regions of the seedling, we were able to confirm that the copper-induced increase in *MT2* mRNA was restricted to the cotyledons and, to a lesser extent, the hypocotyl (Table I). This finding indicates that the previous correlation we observed between seedling copper tolerance (as measured by root growth) and *MT2* mRNA (Murphy and Taiz, 1995a) probably reflected *MT2* expression in the cotyledons rather than the root, suggesting that the cotyledon plays a key role in the copper tolerance of the seedling as a whole. Since *MT2a* expression is low in seedling roots, it does not appear to play a role in copper homeostasis in young roots. However, it is important to point out that the response of mature roots to copper appears to be different from that of seedling roots (Zhou, 1994; Snowden et al., 1995; Murphy et al., 1997).

Both *MT1a* and *MT2a* were expressed at very high levels in young leaf trichomes of both untreated and copper-treated plants. *MT2a* expression in trichomes appeared to be higher than that of *MT1a*. Perhaps because of the high levels of expression, which saturated the signal, it was not possible to detect any copper stimulation relative to the controls. In control plants low levels of *MT1a* expression in young leaves were also detected in minor veins, and copper stimulated the expression of *MT1a* in the vascular bundles, primarily in the phloem. In some cases, low levels were also detected in the mesophyll cells. In contrast, *MT2a* in leaves appeared to be expressed almost exclusively in

the trichomes. Occasionally, very low levels of *MT2a* transcript were observed in the mesophyll of copper-treated plants, primarily in the minor veins. Overall, these results are in good agreement with previous studies showing copper stimulation of *MT1*, but not *MT2*, in Arabidopsis leaves (Zhou and Goldsbrough, 1994; Murphy et al., 1997). In bean, *MT2* was also found to be specifically expressed in trichomes and to be absent from the mesophyll (Foley and Singh, 1994).

The expression patterns of *MT1a* and *MT2a* in leaves suggest possible functions for these proteins. The high levels of expression of *MT1a* and *MT2a* in trichomes are particularly striking and may indicate that trichomes play an important role in metal detoxification in leaves. In Indian mustard, for example, Cd accumulates preferentially in trichomes (Salt, 1995), and nickel accumulation has been demonstrated in the trichomes of *Alyssum lesbiacum* (Krämer et al., 1997). By analogy to the salt-secreting trichomes of halophytes, leaf trichomes may provide a pathway for secreting excess heavy metals outside the mesophyll. The volume of trichome cells is enormous compared with that of a typical mesophyll cell (Fig. 1H), allowing them to serve as large reservoirs for sequestering potentially toxic metal ions. Eventually, as the trichomes senesce, the metal ions would be deposited harmlessly onto the leaf surface.

Alternatively, the high expression levels of *MT1a* and *MT2a* in trichomes may reflect a high requirement for copper. Trichome cells are active in sulfur (Gotor et al., 1997), flavonoid (Charrier et al., 1996), and anthocyanin (Lloyd et al., 1994) metabolism. A number of genes involved in defense mechanisms, including polyphenol oxidase (Shahar et al., 1992; Thipyapong et al., 1997), peroxidase (Mohan et al., 1993), Phe ammonia-lyase (Prasad et al., 1995), and chalcone synthase (Sistrunk et al., 1994), are expressed in trichomes. In addition, trichomes have been shown to develop lignified cell walls (Wyatt et al., 1993). Many of the enzymes in the above pathways require copper for activity. Copper is a cofactor in at least two enzymes involved in lignin biosynthesis: polyphenol oxidase and diamine oxidase. *MT1a* could be involved in lignification processes in all of these tissues, whereas *MT2a* might act only in trichomes. The expression of MTs has been shown to increase after wounding (Snowden et al., 1995; Choi et al., 1996). Since rapid lignification is associated with wound healing, the increase in MTs following wounding might reflect an increase in copper demand. The function of MTs in these cells might be to facilitate the transfer of free copper ions to copper-requiring enzymes. Further studies are needed to determine whether copper accumulates in the trichomes of Arabidopsis.

The localization of *MT1a* expression in vascular bundles, especially the phloem, or to tissues that function in the transport of nutrients to the seed (placenta and funiculus), suggests that *MT1a* may play a role in metal-ion transport and/or vascular development. During leaf senescence micronutrients are remobilized and transported via the phloem to the growing regions of the plant. The elevated expression of *MT1a* observed by northern blotting in senescing leaves could be related to the remobilization of micronutrients, specifically copper and zinc. Other MTs have been shown to be transcriptionally activated during senescence (Buchanan-Wollaston, 1994; Ledger and Gardner, 1994; Coupe et al., 1995; Hsieh et al., 1995; Clendennen and May, 1997; Foley et al., 1997; Reid and Ross, 1997). Recently, two different MT-like cDNA clones were identified from senescing Arabidopsis leaves (Thomas and Villers, 1996). One of the MT clones followed a pattern of expression similar to the one we observed for *MT1a*, increasing at mid-senescence and decreasing thereafter.

The stigmatic core is another possible example of the role of MTs in micronutrient remobilization. The cells of the stigmatic core play an important role in pollen germination and growth, supplying the growing pollen tube with water and nutrient reserves. After the passage of the pollen tubes, these stigmatic cells senesce and die. *MT1a* could be involved in the remobilization of copper and other metals from those cells to the growing pollen tube. In general, *MT1a* was most highly expressed in either tissues with a high demand of copper or tissues involved in the transport or mobilization of nutrients.

MT1a, but not *MT2a*, was strongly expressed in flowers, particularly in the gynoecium and the anthers. Given the strong expression of *MT1a* in various parts of the flower, it may be significant that copper deficiencies typically affect flower formation and maturation much more than vegetative growth, and the ovary and anthers are particularly high in copper content (Märschner, 1995). Thus, *MT1a* may play an important role in flower development by facilitating the transfer and exchange of copper in those tissues with the highest copper requirement.

Finally, northern-hybridization analysis indicated that *MT1a*, but not *MT2*, expression is developmentally regulated in seedlings and leaves. The increasing expression in seedlings could be the reflection of active vascular differentiation in these tissues and/or an increasing demand of copper as the plant grows.

In conclusion, we have confirmed and extended previous observations that *MT1* and *MT2* are differentially expressed in plant tissues, although there are areas of overlapping expression as well. Of particular interest were the high expression levels of both MTs in leaf trichomes. Further investigations into the role of leaf trichomes in metal homeostasis are thus warranted.

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