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Immunolocalization of Mannitol Dehydrogenase in Celery Plants and Cells[1]

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Immunolocalization of mannitol dehydrogenase (MTD) in celery (*Apium graveolens* L.) suspension cells and plants showed that MTD is a cytoplasmic enzyme. MTD was found in the meristems of celery root apices, in young expanding leaves, in the vascular cambium, and in the phloem, including sieve-element/companion cell complexes, parenchyma, and in the exuding phloem sap of cut petioles. Suspension cells that were grown in medium with mannitol as the sole carbon source showed a high anti-MTD cross-reaction in the cytoplasm, whereas cells that were grown in sucrose-containing medium showed little or no cross-reaction. Gel-blot analysis of proteins from vascular and nonvascular tissues of mature celery petioles showed a strong anti-MTD sera cross-reactive band, corresponding to the 40-kD molecular mass of MTD in vascular extracts, but no cross-reactive bands in nonvascular extracts. The distribution pattern of MTD within celery plants and in cell cultures that were grown on different carbon sources is consistent with the hypothesis that the *Mtd* gene may be regulated by sugar repression. Additionally, a developmental component may regulate the distribution of MTD within celery plants.

MTD, an enzyme that catalyzes the oxidation of mannitol to Man (Stoop and Pharr, 1992), constitutes the initial reaction in the pathway by which mannitol enters the central metabolism in vascular plants (Pharr et al., 1995a, 1995b). MTD is distinctly different from M6PR, an enzyme that is localized almost exclusively in the cytosol of mature photosynthetic leaves (Everard et al., 1993), that catalyzes the central reaction of mannitol biosynthesis (Rumpho et al., 1983). Mannitol is an early photosynthetic product in celery (*Apium graveolens* L.; Davis et al., 1988; Keller and Matile, 1989; Davis and Loescher, 1990) and other species (Loescher and Everard, 1996) and makes up to 50% of the phloem-translocated carbohydrate in celery plants (Loescher et al., 1995).

Previous studies have focused on the role of mannitol as an important source of carbon for growth as well as its role in salt- and water-stress tolerance (Pharr et al., 1995a, 1995b). The regulation of MTD expression has been shown to be an important control point in determining whether mannitol is to be used in metabolism for the production of carbon skeletons for assimilation and energy or whether it will accumulate for use as an osmoprotectant (Pharr et al., 1995a). In celery carbohydrate-utilizing sinks such as growing root tips and young leaves contain relatively high MTD activity (Stoop and Pharr, 1994), whereas mature photosynthetic leaves, mature roots, and storage sinks, such as fleshy petioles and the storage organ (knob) of celeriac (Stoop and Pharr, 1992), contain little or no MTD activity. Under conditions of high salinity and/or osmostress, MTD is strongly down-regulated in celery sink tissues, and this results in decreased mannitol utilization by these tissues and a corresponding increase in mannitol accumulation throughout the plant. In contrast, *Mtd* is induced in cell cultures in response to salicyclic acid and has strong sequence homology to ELI3 pathogenesis-related proteins (Williamson et al., 1995). Thus, MTD may function in the resistance to an attack by plant pathogens (Stoop et al., 1996).

MTD has been purified to homogeneity (Stoop et al., 1995), and this antigen was used for the production of polyclonal antiserum. This MTD-specific antiserum was used here to examine the tissue- and cell-type localization in celery of this important enzyme of mannitol catabolism. It is anticipated that such studies will help to elucidate the metabolic role of MTD in whole plants and assist in identifying the physiological and metabolic mechanisms that modulate *Mtd* gene expression.

MATERIALS AND METHODS

Celery (*Apium graveolens* L. var *dulce* [Mill] Pers. cv Tall Golden Self-Blanching) seeds were grown in a greenhouse with a minimum day/night temperature of 24/18°C. Mature leaves were collected from plants 14 months after planting. Three-week to 3-month-old plants, cv Florida 638, were used as the source for the different developmental stages of leaves and roots. Celery suspension cultures were grown in Murashige-Skoog medium (Murashige and Skoog, 1962) that contained 180 mM mannitol or 90 mM Suc as the sole carbon source (Stoop and Pharr, 1993). Cells were subcultured every 14 d.

Antiserum Preparation and Specificity

Polyclonal anti-MTD serum was raised in rabbits by HPR (Denver, PA). Antiserum that was used in previous studies

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Abbreviations: AP, alkaline phosphatase; M6PR, Man-6-P reductase; MTD, NAD-dependent mannitol dehydrogenase.

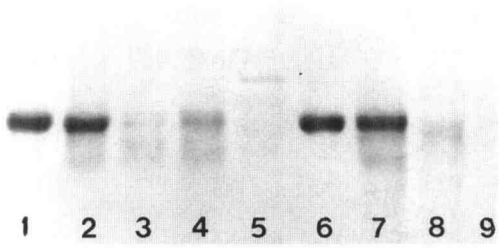

Figure 1. Immunoblot of celery proteins separated by denaturing SDS-PAGE and challenged with anti-MTD serum followed by visualization with AP. Lanes 1 and 6, 0.2 μg of purified MTD; lane 2, 20 μg of protein from mannitol-grown cells; lane 3, 20 μg of protein from Suc-grown cells; lane 4, 20 μg of protein from young, expanding leaf blades (second visible leaf within the central rosette); lane 5, 20 μg of protein from mature leaf blades; lane 7, 20 μg of protein from root tips; lane 8, 20 μg of protein from young petioles; and lane 9, 20 μg of protein from mature petioles. No cross-reaction was observed on identical blots challenged with preimmune serum.

(Stoop et al., 1995) was raised against gel-purified MTD (Enzyme Commission number unassigned). The antigen used here was highly purified MTD that was judged to be homogeneous by several criteria (Stoop et al., 1995). Specificities of the preimmune and immune sera were tested using western blotting of 0.2 μg of purified MTD and 20 μg of crude protein extracts of various celery tissues. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose, and immunodetection was conducted as described below. Crude, desalted protein extracts were prepared from mannitol- and Suc-grown celery suspension-cultured cells, mature leaf tissue, young expanding leaf tissue, root tips, expanding petioles, and mature petioles as described previously (Stoop et al., 1995).

Fixation, Embedding, and Sectioning

Plant materials (root tips and mature leaves) were fixed in 2.5% glutaraldehyde in 100 mM phosphate buffer, pH 7.2, for 6 h and dehydrated in a graded ethanol series. Fixed material was embedded in a "hard grade" London Resin White either by cold curing using an accelerator or by heat curing at 60°C for 24 h without an accelerator. Sections (1 μm), prepared using a JB-4A ultramicrotome (Sorvall), were mounted on ProbeOn Plus microscope slides (Fisher Scientific) for use in immunolocalization and light microscopy (Axiophot, Zeiss) with differential interference contrast optics.

Immunodetection of MTD

Immunolocalization of MTD was performed using both slide-mounted tissue sections and tissue print blots on nitrocellulose membranes (NitroBind, Schleicher & Schuell). Immunodetection was performed according to standard protocols using primary anti-MTD or preimmune sera followed by anti-IgG secondary antibodies that were conjugated with either AP or gold particles enhanced with silver.

Visualization using AP was performed by a modification of the ProtoBlot Western Blot AP System (Promega). Tissue print blots were prepared as described below and processed in Petri dishes using 20-mL solution volumes. Microscope slides were processed in 130-mm Petri dishes, and each slide was covered with 200-μL solution volumes. Sections and blots were blocked in 1% BSA for 30 min and challenged for 1 h with anti-MTD serum (1:100 or 1:1000

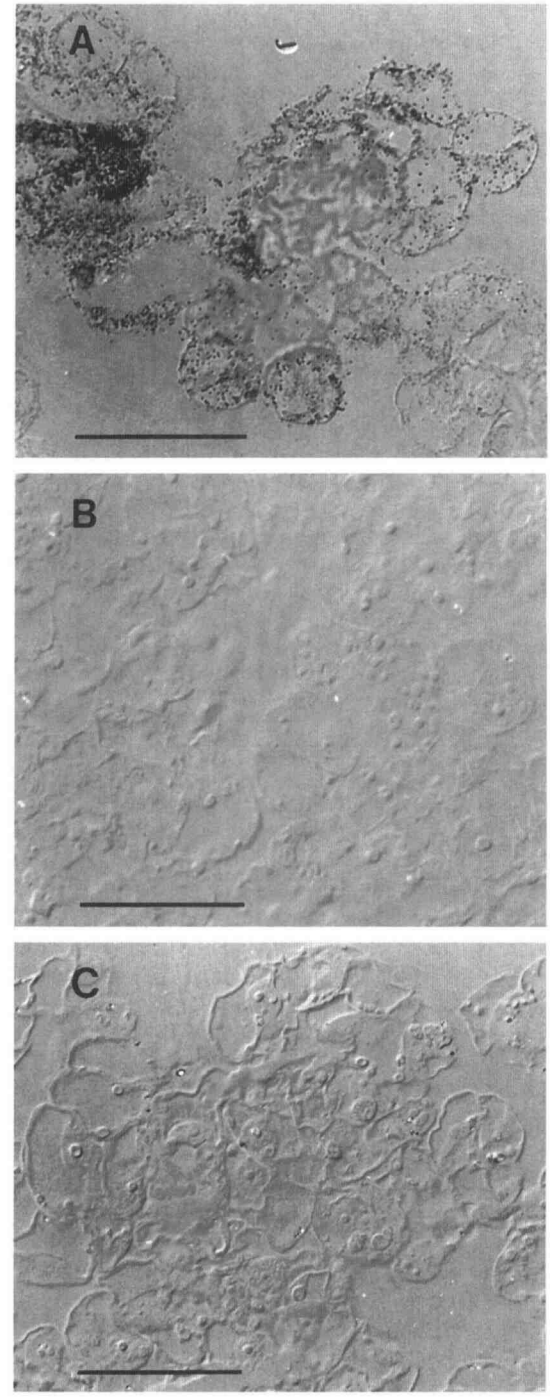

Figure 2. Localization of MTD in celery suspension cells. MTD antigens were visualized using silver-enhanced colloidal gold in 7-d-old mannitol-grown cells (A) or Suc-grown cells (C). No cross-reaction was observed in mannitol-grown cells treated with preimmune serum (B) or in Suc-grown cells (C). Bars = 50 μm.

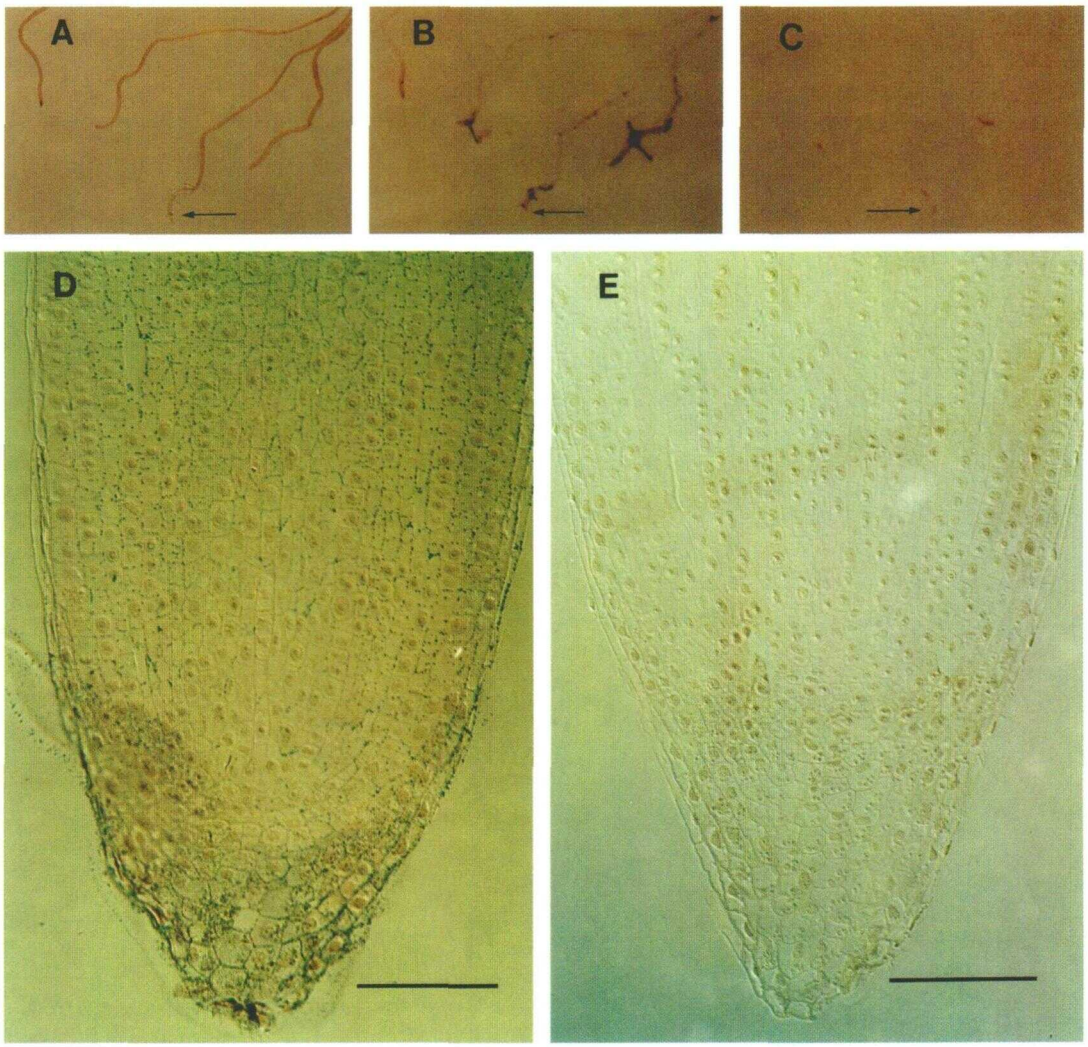

Figure 3. MTD localization in young celery roots. The presence of anti-MTD serum cross-reacting material was visualized using AP-conjugated secondary antibodies and appropriate color development in tissue blots of young roots (A–C), and in longitudinal sections of root tips (D and E). A, Roots in place on nitrocellulose after blotting. B, Tissue print showing anti-MTD serum cross-reaction at root tips (one is marked by arrow). C, Tissue print treated with preimmune serum (mirror image blot). D, Longitudinal section showing anti-MTD sera cross-reaction in cap cells and in the cytoplasm of root tip meristematic cells. Little or no cross-reaction is seen in the root initials (quiescent center). E, Serial section incubated with preimmune serum. Bars = 50 μm.

dilutions, respectively). Secondary antibodies (anti-rabbit IgG conjugated to AP, Promega) were applied to slides (1:500) or blots (1:5000) and the reaction was visualized according to the manufacturer's instructions. Color appeared within 5 min on blots and within 20 to 30 min on slide sections (in the dark). Slides were washed with distilled H_2O, dried, and sealed with Permount (Fisher Scientific). For tissue print blots of the mature leaf blade only, it was necessary to preheat the printed membrane at 80°C for 20 min to inactivate the endogenous AP.

Visualization using colloidal gold-protein complexes enhanced by silver was essentially as described in Sigma Technical Bulletin no. SE-1, except that slides were blocked with 1% BSA and volumes were adjusted to 200 μL of solution per slide.

Protein Gel-Blot Analyses

Nonvascular (control) and vascular tissues were isolated from the mature petioles of 14-month-old plants and from petioles procured from a local supplier. Nonvascular tissue was manually dissected from the adaxial (concave) side of the petioles with a curved spatula, and this exposed the vascular bundles, which were then isolated using fine tweezers. They were immediately frozen in liquid nitrogen and stored at −80°C until used for extraction. Tissues were ground in a chilled mortar and pestle using a tissue to buffer ratio of 1:4, and crude protein extracts were prepared as described previously (Stoop and Pharr, 1994).

Protein concentrations were determined spectrophotometrically by the Bradford (1976) method using BSA as a

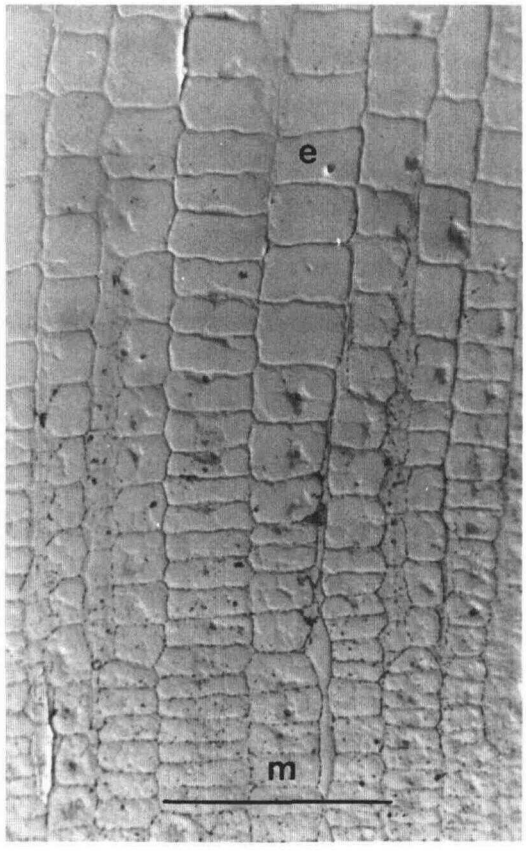

Figure 4. Localization of MTD in the root elongation zone. The presence of anti-MTD serum cross-reacting material was visualized using AP-conjugated secondary antibodies and appropriate color development in a longitudinal section at the beginning of the root elongation zone, 600 to 700 μm from the root tip. MTD cross-reacting material is localized in the cytoplasm of meristematic cells (m) but is absent in elongating cells (e). Bar = 50 μm.

standard. Proteins were separated by denaturing SDS-PAGE (Laemmli, 1970), with a final acrylamide concentration of 12% (w/v) separating gel and 4.5% (w/v) stacking gel. Proteins were blotted onto nitrocellulose using a Bio-Rad semidry transfer apparatus. Immunodetection of the antigen was carried out using anti-rabbit IgG-AP conjugate (Promega) secondary antibodies (1:5000) as described previously. Immune and preimmune sera were used at a dilution of 1:6000.

RESULTS

Specificity of the Anti-MTD Serum

Immunoblots of extracts from various celery tissues are shown in Figure 1. The antiserum cross-reacted most strongly with an antigen that co-migrated with purified MTD. The intensity of the cross-reaction that was observed for MTD in extracts from different tissues or cultured cells was proportional to the relative MTD activity previously reported for these tissues (Stoop and Pharr, 1994). The antiserum also cross-reacted, although more weakly, with proteins slightly larger and smaller than MTD that were not present in the purified antigen against which the antiserum was raised. No cross-reacting antigens were detected with preimmune serum (data not shown).

MTD Localization in Celery Suspension Cells

Cells that were grown on either mannitol or Suc as the sole carbon source were collected 7 d after subculture. Analysis of MTD activity in mannitol-grown cells resulted in a value of 11.1 μmol h^{-1} g^{-1} fresh weight, whereas MTD activity was not detected in extracts from Suc-grown cells; this is similar to previously reported results (Stoop and Pharr, 1993). Cross-sections of fixed, embedded cells were challenged with either anti-MTD serum or preimmune serum as described in "Materials and Methods." Correlation of the visualized anti-MTD serum cross-reacting material with measured MTD activity and MTD protein level (Fig. 1) provides further evidence that the specificity of the serum is suitable for immunodetection of MTD. High levels of the MTD antigen were seen in mannitol-grown cells using both AP color reaction and silver enhancement techniques, whereas little (with AP) or no (with silver enhancement) anti-MTD serum cross-reacting material was observed in Suc-grown cells (Fig. 2). Results of the silver-enhanced colloidal gold visualization process are shown. Immunodetection of cytosolic proteins typically produces discrete spots rather than general staining of the cytoplasm (Everard et al., 1993). Thus, although MTD is clearly not present in the vacuole, it is not possible at these magnifications to distinguish between cytosolic and cytoplasmic (organellar) localization.

MTD Localization in Roots

For tissue printing, young roots were placed between two nitrocellulose membranes and gently pressed between two glass plates. The roots were photographed in position on the blot (Fig. 3A) before removal: one membrane (below the roots) was treated with anti-MTD serum (Fig. 3B) and the other (above the roots, a mirror image of the lower blot) was treated with preimmune serum as a control (Fig. 3C). A strong anti-MTD serum cross-reaction (purple-blue) appeared at the root tips, and occasionally stained liquid with a strong anti-MTD serum cross-reaction was discharged 5 to 10 mm from the tip end when pressure was applied (Fig. 3B). Anti-MTD serum cross-reactive material was observed on the longitudinal sections in the meristematic region of the root tip (Fig. 3D). The reaction was strong in the cap cells and in the meristematic region and was localized in the cytoplasm; the meristem initials (the quiescent center), however, were less reactive (Fig. 3D). Little or no anti-MTD sera cross-reaction was observed on sections that had been treated with preimmune serum (Fig. 3E). An MTD cross-reaction was not detected above the meristematic zone, about 650 μm above the root cap (Fig. 4). Further above this region, however, cross-reactive material was observed in the sieve elements and in the companion cells of differentiated primary and secondary phloem (Fig. 5). Identical results were obtained using AP and silver enhancement techniques; however, AP detection proved to have less

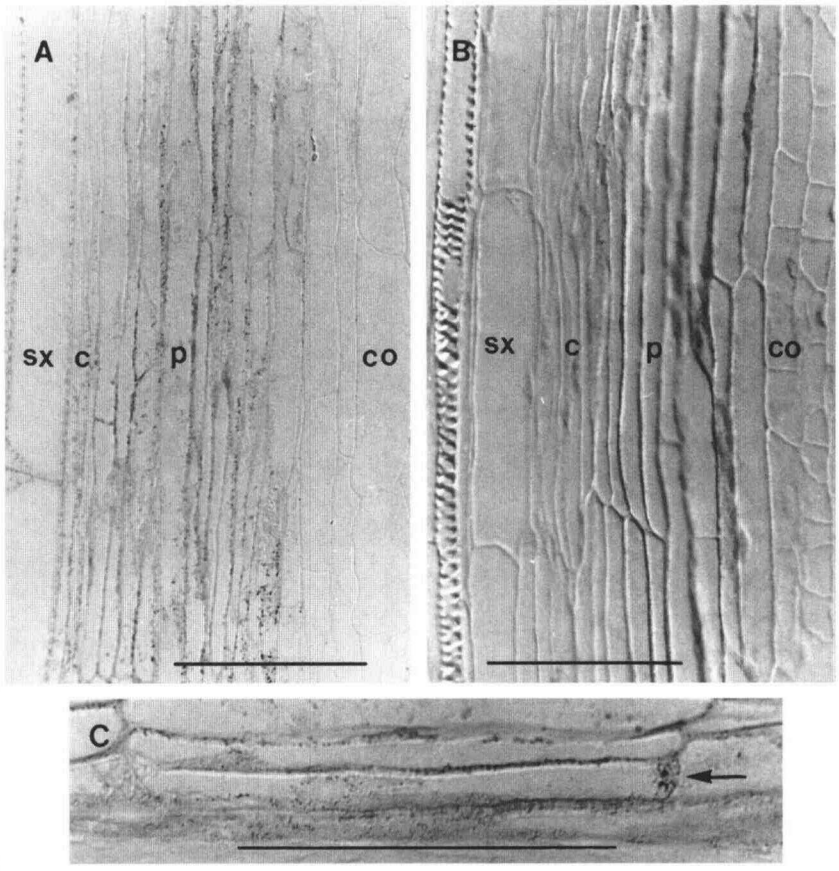

Figure 5. Localization of MTD in root vascular tissues of celery. Longitudinal sections in the differentiation zone, 2 cm from the root tip, incubated with anti-MTD serum (A) or preimmune serum (B), followed by visualization with AP. An anti-MTD sera cross-reaction appeared in the cambium zone (c) and phloem (p), including sieve elements, companion cells, and parenchyma, but not in the xylem (sx) and cortex (co). Arrow in C indicates labeling at the sieve plate of a sieve element. Bars = 50 μm.

background. Of all the plant tissues examined, the root tips showed the strongest anti-MTD serum cross-reaction.

MTD Localization in Petioles and Mature Leaves

Protein blots of young, expanding leaf blades and young petioles showed an anti-MTD serum cross-reaction (Fig. 1). Mature leaf blade and mature petioles, however, showed little or no anti-MTD serum cross-reaction (Figs. 1 and 6B) except in the vascular tissue (Fig. 6A).

MTD Identification in Vascular Tissue

Tissue print blots were prepared from cross-sections of 14-month-old petioles and leaf blades from greenhouse-grown plants as well as from plants procured from a local supplier. Blots were challenged with either anti-MTD serum or preimmune serum. Blots showed strong anti-MTD serum cross-reaction on the phloem and a weaker cross-reaction on the ground parenchyma (Figs. 6A and 7A). A much weaker nonspecific reaction was seen with preimmune sera (Fig. 7B). When 14-month-old petioles were cut at the base and immediately blotted onto nitrocellulose, the phloem sap exuded from the cut surface showed an intense anti-MTD serum cross-reaction (Fig. 8A).

Protein gel-blot analyses of crude extracts from isolated vascular tissues from 14-month-old petioles showed a strong anti-MTD serum cross-reaction with a band co-migrating with purified MTD (Fig. 8B). A much weaker band was seen in extracts from nonvascular tissue (Fig. 8B), and blots treated with preimmune serum showed no detectable cross-reaction (data not shown).

DISCUSSION

MTD is the central enzyme in celery plants that controls utilization of mannitol. This constitutes the first step whereby mannitol is committed to metabolic pathways for energy production and carbon assimilation (Stoop and Pharr, 1992). Previous studies found that extractable MTD activity was high in actively growing tissues, such as root tips and young expanding leaves, but was low or not detectable in mature photosynthetic leaves and mature petioles, the primary storage organ for mannitol in celery (Stoop and Pharr, 1992, 1994). Results from this study show that MTD protein localization parallels this pattern, being present in the cytoplasms of both root meristems and vascular cambium cells (Figs. 3 and 5). All of these cell types have high metabolic activity with consequent high energy and carbon demands. MTD was absent, however, in cells of the quiescent center (behind the root meristem, Fig. 3D), in root tips above the meristematic region (Fig. 4), and in mesophyll tissue of mature leaves (Figs. 6B), all places where carbon and energy demands are presumed to be substantially less. In contrast to the localization of MTD, M6PR, the central enzyme of mannitol biosynthesis, is found predominantly in the cytosol of mature leaf mesophyll cells (Everard et al., 1993). Thus, M6PR and MTD are

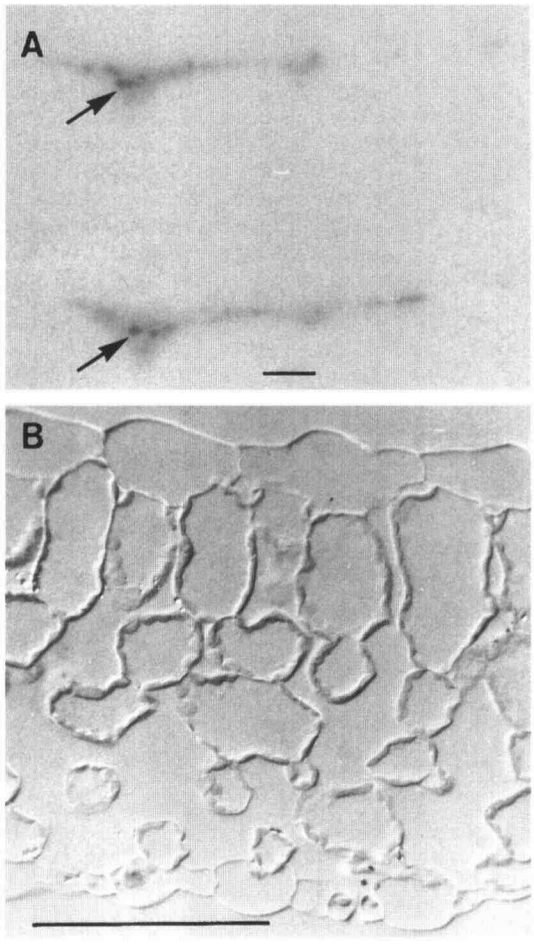

Figure 6. Cross-sections of mature celery leaf blades challenged with anti-MTD serum and visualized with AP-conjugated antibodies. A, Two consecutive tissue print blots of the same section showing an anti-MTD cross-reaction in the veins (arrows). B, Microscopic section with no anti-MTD cross-reaction in the mesophyll. No reaction was observed with preimmune serum. Bars = 1 mm (A) and 50 μm (B).

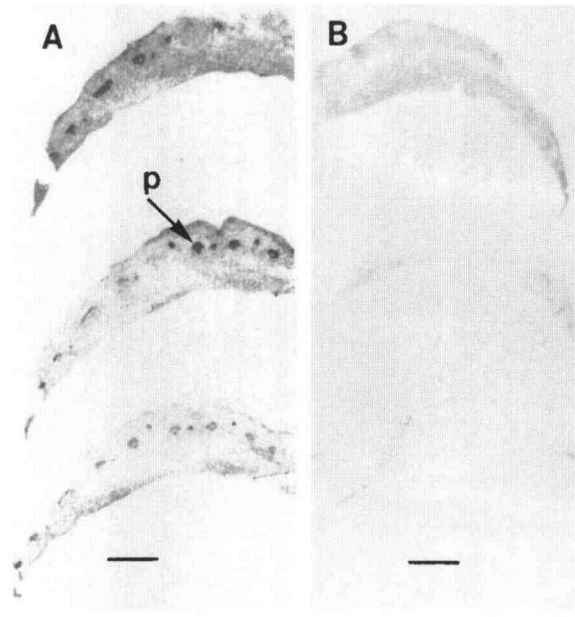

Figure 7. Localization of MTD in vascular tissue of mature celery petiole. Three consecutive tissue prints were made from a petiole cross-section of mature celery from a local supplier (top to bottom) and challenged with either anti-MTD serum (A) or preimmune serum (B), followed by visualization with AP. Bars = 5 mm. p, Phloem.

spatially separated in source and sink tissues of celery plants, supporting the concept that they serve distinctly separate metabolic functions in synthesis and catabolism, respectively.

MTD protein was found in sieve elements, companion cells, phloem parenchyma, and vascular cambium (Fig. 5), as well as in phloem and phloem exudate from the vascular tissue of mature petioles and blades (Figs. 6–8). The cross-reacting protein was confirmed by gel-blot analysis to co-migrate with purified MTD (Fig. 8B). As mentioned above, little or no MTD activity had previously been detected in extracts from mature petioles, perhaps because vascular tissues make up only a small portion of the petiole. There is growing evidence that there is active Suc breakdown in phloem companion cells and that the ATP required for H^+ pumping during phloem loading of Suc is generated within the phloem system (Geigenberger et al., 1993). In addition, immunolocalization studies (Nolte and Koch, 1993) have shown that Suc synthase is present in sieve element-companion cells of both importing and exporting organs but not in adjacent phloem cells. The presence of Suc synthase allows these cells to access translocated Suc to meet the high energy demands of loading, unloading, and retrieval of photoassimilates along the translocation pathway, as discussed elsewhere (Minchin and Grusak, 1988; Geigenberger et al., 1993; Nolte and Koch, 1993). This may

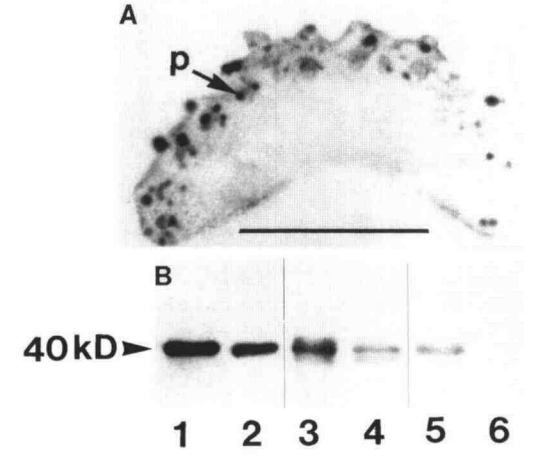

Figure 8. Tissue blot of a 14-month-old freshly cut petiole challenged with anti-MTD serum and visualized with AP-conjugated antibodies (A). Phloem sap (p) exuded from the cut surface showed an intense anti-MTD serum cross-reaction. Bar = 5 mm. B, Protein gel-blot analysis of vascular and nonvascular crude extracts from a 14-month-old petiole. Lanes 1 and 2, 1 and 0.5 μg of purified MTD. Lanes 3 and 4, 10 μg of protein of vascular and nonvascular tissues, respectively. Lanes 5 and 6, 5 μg of protein of vascular and nonvascular tissues, respectively. Blots treated with preimmune serum showed no cross-reactive bands (not shown).

be especially important for phloem cells, particularly the companion cell-sieve element complex, which generally do not contain significant hexose pools for ready metabolic use (Geigenberger et al., 1993). The energy demands of these cells is met through a strong glycolytic use of Suc involving Suc synthase rather than invertase (Geigenberger et al., 1993). Phloem-localized MTD in celery may serve an analogous function to that of Suc synthase in phloem by providing access to translocated mannitol for generating energy. Although little is known about phloem loading and unloading of mannitol, recent evidence suggests that a proton motive force is involved in its transport across plasma membrane vesicles that are isolated from celery phloem (Salmon et al., 1995).

The specific distribution of MTD in meristematic or rapidly growing cells, in which hexose pools are severely depleted, correlates with the hypothesis that these sugars repress *Mtd* expression (Pharr et al., 1995a). Celery cells cultured on Suc are known to contain substantial internal hexose pools but little MTD activity or *Mtd* transcript. In contrast, cells grown on mannitol have low internal hexose pools and high MTD activity and abundant *Mtd* transcript (Stoop and Pharr, 1993; Williamson et al., 1995). This correlates with the presence of abundant MTD protein in mannitol- but not Suc-grown cells that was observed in this study (Figs. 1 and 2). The localization of MTD in root tips, meristematic cells, and phloem cells is similar to the expression pattern of the sugar-repressed Suc synthase gene *Sh1* (Koch and Nolte, 1995) in other plants, although, unlike MTD, Suc synthase is apparently restricted to the companion cell-sieve element complex of the phloem.

Phloem-specific expression of *Sh1* has been demonstrated using transgenic tobacco transformed with a maize *Sh1* promoter-GUS fusion (Yang and Russell, 1990). In intact celery plants mobilization of stored mannitol pools from the leaves of plants transferred into the dark occurs only after sugars have been extensively mobilized (Fellman and Loescher, 1987). Likewise, during flowering the massive mobilization of mannitol storage pools throughout the plant to the growing reproductive structures occurs only after stored sugars are exhausted (Obaton, 1929). This hierarchical use of stored reserves, by which the high energy mannitol is mobilized only after sugars are exhausted, is consistent with the model of the sugar repression of *Mtd* expression (Pharr et al., 1995a). Thus, the apparent tissue- and cell-specific localization of MTD may be in part a reflection of sugar repression of *Mtd* expression (i.e. a response to tissue-/cell-type-specific energy demand as reflected by hexose pool size). However, mature celery leaf mesophyll expresses little or no MTD (Fig. 6B), and yet it contains barely detectable hexose pools (Stoop and Pharr, 1994). Thus, there also must be a developmental component to the expression of MTD in celery plants.

In addition to sugar repression, other layers of control of *Mtd* expression have been observed. Decreased MTD activity and protein were seen in the sink tissues of celery plants that were osmostressed with excess macronutrient salts. This causes an increased mannitol accumulation throughout the plant (Stoop and Pharr, 1994) where it functions as an osmoprotectant (Pharr et al., 1995a, 1995b; Stoop and Pharr, 1994). This loss of MTD is not associated with increased sugars in the sink tissues, nor are Suc synthase or invertase lost from these same tissues of stressed celery plants (Stoop and Pharr, 1994). Thus, it is clear that Suc synthase and MTD are differentially regulated by salinity stress. It has also been observed that salicylic acid, an inducer of pathogenesis-related proteins, induces *Mtd* in celery cells growing on Suc (Williamson et al., 1995), functionally overriding sugar repression of *Mtd* expression. Thus, there are regulatory elements that are not shared by *Mtd* and other sugar-regulated genes. The normal cell type and tissue distribution of MTD in celery plants that were grown in nonstressful environments have now been established; a more detailed study of MTD localization will assist in elucidating the regulation of MTD in various cell types and tissues in response to stress-related stimuli.

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