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Subcellular Localization of Celery Mannitol Dehydrogenase[1]

A Cytosolic Metabolic Enzyme in Nuclei

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Mannitol dehydrogenase (MTD) is the first enzyme in mannitol catabolism in celery (*Apium graveolens* L. var *dulce* [Mill] Pers. cv Florida 638). Mannitol is an important photoassimilate, as well as providing plants with resistance to salt and osmotic stress. Previous work has shown that expression of the celery *Mtd* gene is regulated by many factors, such as hexose sugars, salt and osmotic stress, and salicylic acid. Furthermore, MTD is present in cells of sink organs, phloem cells, and mannitol-grown suspension cultures. Immunogold localization and biochemical analyses presented here demonstrate that celery MTD is localized in the cytosol and nuclei. Although the cellular density of MTD varies among different cell types, densities of nuclear and cytosolic MTD in a given cell are approximately equal. Biochemical analyses of nuclear extracts from mannitol-grown cultured cells confirmed that the nuclear-localized MTD is enzymatically active. The function(s) of nuclear-localized MTD is unknown.

Celery (*Apium graveolens* L. var *dulce* [Mill] Pers. cv Florida 638) is one of many vascular plants that are capable of using mannitol as a photoassimilate and phloem-translocated carbohydrate. Advantages such as high carbon-exchange rates, equivalent to those of many C_4 plants (Loescher et al., 1995), and more efficient carbon use, especially in sink organs (Pharr et al., 1995b), have been inferred for plants that use mannitol. Additionally, mannitol functions as an osmoprotectant in these species. Mannitol accumulation in salt- and osmotically stressed plants and cells has been correlated with tolerance to salt and osmotic stress (Stoop et al., 1996, and refs. therein).

MTD is the first enzyme in mannitol catabolism (Pharr et al., 1995b). Cloning and sequencing a cDNA encoding celery MTD revealed that MTD is closely related to the ELI3 pathogenesis-related proteins of parsley (*Petroselinum crispum*) (Williamson et al., 1995) and *Arabidopsis thaliana*. The ELI3 protein of Arabidopsis, a plant that apparently does not possess a mannitol biosynthetic pathway, has been proposed to play a critical role in resistance to *Pseudomonas* spp. (Kiedrowski et al., 1992). The *Mtd* gene is related to *LeRse-1*, a gene from tomato (*Lycopersicon esculentum*), a species that apparently also does not possess a mannitol biosynthetic pathway. *LeRse-1* shows root-specific expression when the shoot is exposed to light (Lauter, 1996). It is unknown whether ELI3- and *LeRse-1*-encoded proteins have MTD enzyme activity. The presence of genes related to MTD in species that do not use mannitol implies that proteins encoded by these genes may have functions other than mannitol catabolism.

Celery MTD regulation is complex (Pharr et al., 1995b). First, MTD distribution is organ specific. Biochemical studies showed that celery MTD enzyme activity is high in root tip, young leaf, and petiole (Stoop and Pharr, 1992). We demonstrated by immunolocalization at the light microscope level that celery MTD is present in sink organs, consistent with the enzyme-activity distribution (Zamski et al., 1996). In addition, phloem cells and phloem exudate contain significant levels of MTD protein. Salt is another important factor that controls MTD expression. MTD activity in sink organs decreases dramatically when celery plants are exposed to high salinity (Stoop and Pharr, 1994). This leads to the accumulation of mannitol, an osmoprotectant. Furthermore, MTD expression in celery suspension-cultured cells is down-regulated by hexose sugars (Stoop and Pharr, 1993; Pharr et al., 1995a; Prata et al., 1997). Celery suspension-cultured cells express high levels of MTD when hexose sugars are depleted from the media (Prata et al., 1997). RNA-blot hybridization revealed that salt and hexoses affect the level of steady-state mRNA of the *Mtd* gene (Williamson et al., 1995; Prata et al., 1997). In addition to sugar and salt, *Mtd* gene expression is up-regulated by SA. The effect of SA overrides that of sugar repression (Williamson et al., 1995). The sensitivity to SA and the sequence similarity to ELI3 suggest that MTD may play a role in pathogen resistance (Stoop et al., 1996).

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Abbreviations: ADH, alcohol dehydrogenase; HMG, high-mobility group protein; M6PR, Man-6-P reductase; MTD, mannitol dehydrogenase; NLS, nuclear-localization signal; SA, salicylic acid.

In the present study we examined the subcellular distribution of MTD using immunogold labeling and subcellular fractionation. Electron micrographs clearly show that MTD is a cytosolic enzyme. In addition, nuclear localization of MTD was observed in all cells that express cytosolic MTD. An approximately equal density of MTD protein is present in the nucleus and cytosol. Immunoblot analyses and enzymatic assays using nuclear extracts of celery suspension-cultured cells confirmed that the nuclear antigen is enzymatically active MTD.

MATERIALS AND METHODS

Celery (*Apium graveolens* L. var *dulce* [Mill] Pers. cv Florida 638) plants were grown from seeds in a greenhouse with a minimum day/night temperature of 24/18°C. Three-week- to 3-month-old vegetative plants were used as the source for plant organs. Celery suspension cultures were maintained as described previously (Stoop and Pharr, 1993) and supplemented with either 180 mM mannitol or 90 mM Suc as the sole carbon source. Cells were subcultured every 7 d.

Antisera Preparation and Specificity

Polyclonal anti-MTD serum was raised against homogeneous MTD in rabbits by Covance Research Product, Inc. (Denver, PA). Its specificity was tested by immunoblot detection (Zamski et al., 1996). Anti-wheat (*Triticum aestivum* L.) HMGa (Spiker and Everett, 1987) was kindly provided by Dr. Steven Spiker (North Carolina State University, Raleigh).

Fixation, Embedding, and Sectioning

Plant materials (organs and cultured cells) were fixed in 2.5% (v/v) glutaraldehyde in 100 mM phosphate buffer, pH 7.2, for 6 h, and washed in the phosphate buffer overnight. Samples were then dehydrated in a graded ethanol series. Fixed material was embedded in a hard-grade London White Resin (London Resin Co., London, UK) by heat curing at 60°C for 18 h. Ultrathin sections (approximately 70–90 nm in thickness), prepared using a Nova ultramicrotome (LKB, Bromma, Sweden), were collected on 400-mesh nickel grids and used for immunogold labeling of MTD.

Immunogold Labeling of MTD

Sections on grids were treated in 50-μL drops on Parafilm as follows: blocking in 1% BSA in TBST buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% [v/v] Tween 20, pH 7.5) for 30 min, incubation with anti-MTD serum (1:100 in TBST) for 1 h, washing in TBST (3 × 5 min), and incubation with secondary antibodies (goat anti-rabbit IgG Polygold, 15 nm [Polysciences Inc., Warrington, PA], diluted 1:50 in TBST) for 1 h. The grids were then washed in TBST (3 × 5 min), stained in saturated uranyl acetate for 15 min, washed in running water, and examined in a transmission electron microscope (model 100S, JEOL).

Analyses of Gold Particles

Gold particles were counted in 15 to 36 fields (1 μm^2 each) of electron micrographs of cytosol or nuclei of at least five different cells representing each tissue or cell type. Electron micrographs for each tissue or cell type were chosen from two separate sample preparations using different plants or batches of cultured cells. Two to four blocks from each preparation were sectioned, and fields were selected randomly from sections of good quality derived from different blocks. For each cell type or tissue examined, the mean number of gold particles per unit area of cytosol was plotted against that of nuclei of the same cells. SE values were calculated to show the variation between fields.

Protein Gel-Blot Analyses

SDS-PAGE was performed according to Stoop et al. (1995). The fractionated proteins were electrotransferred to nitrocellulose membranes (NitroPure, Micron Separations Inc., Westborough, MA) using a transfer apparatus (Hoefer Scientific Instruments, San Francisco, CA) in transfer buffer (25 mM Tris, 192 mM Gly, 0.1% [w/v] SDS, pH 8.5) at 300 mA overnight. Immunoblot analyses were performed according to Saravitz and Siedow (1995) with minor modification. The nitrocellulose was treated as follows: blocking in a blocking solution (5% nonfat dry milk in TBST), washing in TBST (2 × 1 min, 15 min, 2 × 5 min), incubation with antiserum (1:10,000 for anti-MTD, 1:64,000 for anti-HMGa in blocking solution, preincubated 30 min), washing in TBST (2 × 1 min, 15 min, 3 × 5 min), incubation with secondary antibodies (goat anti-rabbit IgG-horseradish peroxidase, human/mouse adsorbed, [Southern Biotechnology Associates, Inc., Birmingham, AL], diluted 1:5000 in TBST), and washing in TBST (2 × 1 min, 15 min, 4 × 5 min). Chemiluminescent detection using SuperSignal substrate (Novagen, Milwaukee, WI) was performed according to the manufacturer's directions.

Nuclear Extract Preparation from Celery Suspension-Cultured Cells

Fifty grams (fresh weight) of 7-d-old celery suspension-cultured cells grown on mannitol as the sole carbon source was harvested and ground in liquid nitrogen. The nuclear-isolation protocol used was modified from that of Watson and Thompson (1986). The grinding, homogenization, and filtration procedures were performed in a cold room. Ground cells were homogenized in 300 mL of buffer B containing 1 M hexylene glycol, 10 mM Pipes (pH 6.8), 10 mM $MgCl_2$, 1 mM EDTA, 0.4 mM PMSF, and 4% (v/v) β-mercaptoethanol using a Polytron homogenizer (Brinkmann) at one-half of its maximum speed. Homogenates were filtered through a series of nylon meshes (500, 300, 100, and 50 μm). The filtered homogenates were washed once in buffer B, and separated at 200*g* at 4°C for 20 min on Percoll gradients (a step gradient of 15, 30% [v/v]) in buffer

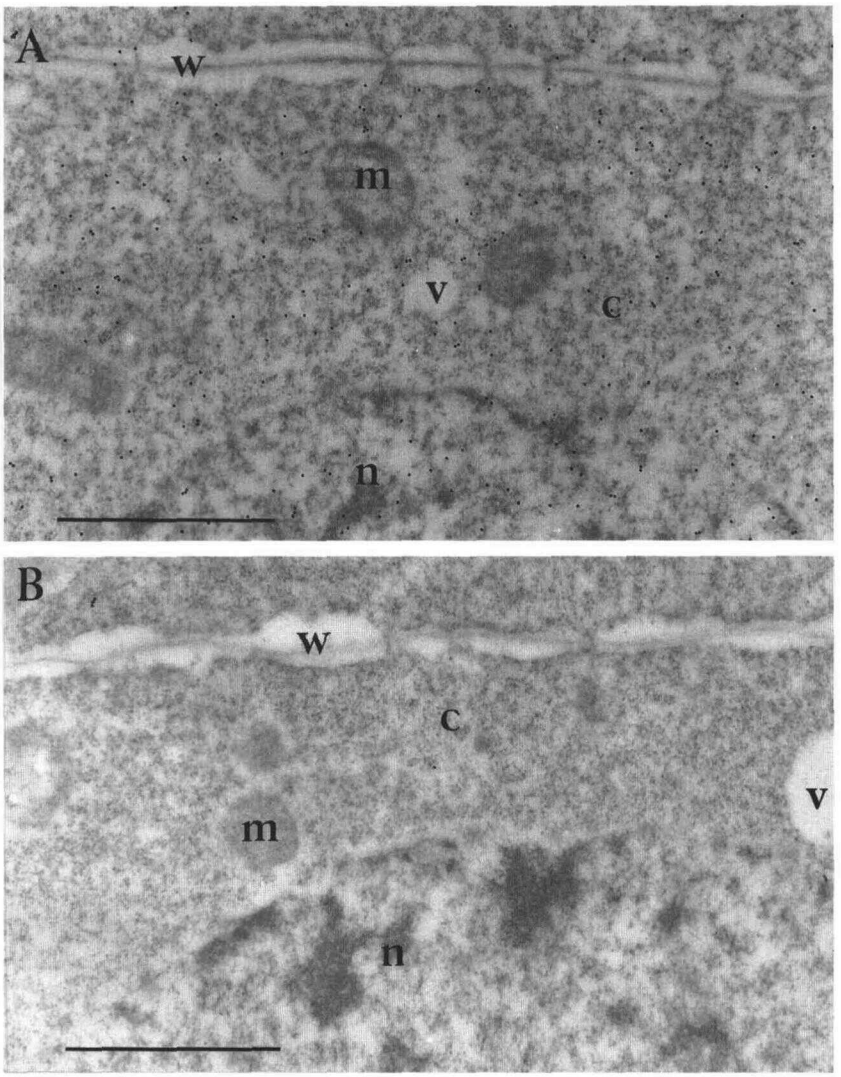

Figure 1. Immunogold labeling of celery root cells. A, Transmission electron microscopy of root meristematic cells labeled with anti-MTD serum and gold-conjugated goat anti-rabbit antibodies. B, Transmission electron microscopy of root meristematic cells treated with preimmune serum and gold-conjugated goat anti-rabbit antibodies. c, Cytosol; m, mitochondrion; n, nucleus; v, vacuole; and w, cell wall. Bars = 1 μm.

B. The pelleted nuclei were washed in buffer B. The nuclei were stored in buffer B in the presence of 20% (v/v) glycerol at −80°C.

Nuclear extract was prepared using a modification of the protocol of Green et al. (1987). Nuclei were thawed on ice and pelleted at 3,000*g* for 5 min, resuspended in 9 mL of nuclear lysis buffer (110 mM KCl, 15 mM Hepes/KOH [pH 7.6], 5 mM $MgCl_2$, 1 mM DTT, and 0.4 mM PMSF), and transferred to ultracentrifuge tubes. $(NH_4)_2SO_4$ was added to a final concentration of 0.4 M, and the tubes were shaken gently for 30 min at 4°C. Chromatin and particulate materials were removed by centrifugation at 147,000*g* in a vertical rotor (43,000 rpm, VTi80, Beckman) for 60 min at 4°C. Then, soluble proteins were precipitated from the supernatant by the addition of freshly ground $(NH_4)_2SO_4$ to 70% of saturation (a concentration at which all soluble MTD precipitates [Stoop et al., 1995]) with stirring for 30 min on ice. The pellet was recovered by centrifugation at 10,000*g* for 30 min, and resuspended in buffer containing 50 mM Mops (pH 7.5), 5 mM $MgCl_2$, 120 mM KCl, 5 mM DTT, 1 mM EDTA, 1% (v/v) Triton X-100, and 1 mM PMSF (Stoop et al., 1995). Insoluble materials were removed by centrifugation at 10,000*g* for 1 min. The nuclear extract was desalted into buffer containing 50 mM Mops (pH 7.5), 5 mM $MgCl_2$, and 1 mM DTT using a Sephadex G25–50 column (Stoop et al., 1995) before electrophoresis and enzyme activity assays.

Preparation of Crude Extracts from Celery Suspension-Cultured Cells

Cells were harvested and ground in liquid nitrogen. Whole-cell crude extracts for immunoblot detection and enzyme assays were isolated and desalted according to Stoop et al. (1995).

Enzyme Activity Assays

MTD activity assays of crude and nuclear extracts were performed spectrophotometrically according to Stoop et al. (1995). ADH assays of the extracts were identical to the MTD assays, except that 20 mM ethyl alcohol was used in place of 150 mM mannitol.

RESULTS

Cellular Distribution of MTD

MTD protein was present in root meristematic cells (Fig. 1A) and phloem cells (Fig. 2). Young leaf, petiole, and root cap cells also contained MTD protein (not shown). Mannitol-grown celery suspension-cultured cells contained high levels of MTD protein (Fig. 3A), whereas Suc-grown cells did not (Fig. 3B).

Subcellular Localization of MTD

Clearly, MTD is a cytosolic enzyme (Figs. 1–3). Very few gold particles were found in vacuoles, plastids, mitochondria, or cell walls. Furthermore, we observed that nuclei of MTD-expressing cells were labeled with gold particles (Figs. 1A and 3A). Only small numbers of gold particles were present in nucleoli (Fig. 3A). MTD was not present in the nucleus of cells that did not contain cytosolic MTD. For example, very few gold particles were found in the nuclei of Suc-grown cells (Fig. 3B). No gold particles were observed on the sections when preimmune serum was used in place of the anti-MTD serum (Fig. 1B).

Densities of Nuclear and Cytosolic MTD Are Similar

Table I and Figure 4 summarize the analyses of gold particle density. Numbers of 1-μm^2 fields and total numbers of gold particles counted from various electron micrographs are shown in Table I. Figure 4 shows the relationship between gold particle densities in the nucleus and cytosol. There was strong correlation between nuclear and cytosolic densities of MTD ($r^2 = 0.995$). As indicated by the slope of nearly 1.0 and the near-0 intercept of the regression, approximately equal densities of MTD existed within the two subcellular compartments. Very few gold particles were present in Suc-grown cells. Furthermore, large differences in the gold particle densities were observed between different cell types.

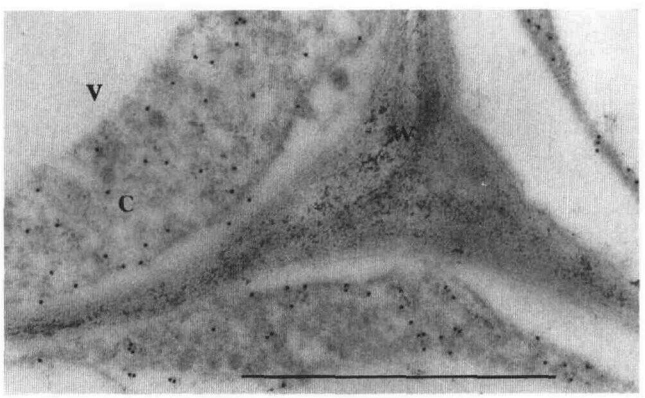

Figure 2. Transmission electron microscopy of celery phloem parenchyma cells labeled with anti-MTD serum and gold-conjugated goat anti-rabbit antibodies. c, Cytosol; v, vacuole; and w; cell wall. Bar = 1 μm.

Immunoblot Analysis of Crude and Nuclear Extracts from Celery Suspension-Cultured Cells

To determine whether the gold particles in nuclei were MTD or another antigen(s) that cross-reacted with anti-MTD, we prepared nuclear extracts from celery suspension-cultured cells. ADH enzyme activity in the crude and nuclear extracts was assayed to estimate the contamination levels of the nuclear extracts by soluble cytosolic proteins. Table II shows that the nuclear extracts contain no detectable ADH activity.

The extracted proteins were fractionated by PAGE and subjected to immunoblot analyses. To demonstrate nuclear enrichment of the extracts, one of the blots was incubated with anti-wheat HMGa. Wheat HMGa is an abundant chromosomal protein (Spiker, 1984) that binds to A/T-rich sequences (Pederson et al., 1991). Figure 5A shows that both nuclear and crude extracts from mannitol-grown cells contained a protein of the size of MTD (40 kD) that cross-reacted with anti-MTD. As a proportion of the total protein in each of the extracts, the concentration of the nuclear-localized MTD appears to be about one-fifth that of the soluble MTD in the crude extract. As expected, the nuclear-specific HMGa is highly enriched in the nuclear extracts (Fig. 5B).

Nuclear-Localized MTD Is Enzymatically Active

Based on the protein-blot analysis, it appears that gold particles in nuclei represented an antibody reaction with MTD. To investigate whether the nuclear-localized MTD is enzymatically active, MTD assay of the nuclear and crude extracts was performed. Assay results of two independent cell cultures are shown in Table II. Nuclear extracts of mannitol-grown cells exhibited approximately one-fifth the activity of the MTD in the crude extract, consistent with the results of the immunoblot analysis. These results demonstrate that celery cells containing cytosolic MTD also contain nuclear-localized MTD that is enzymatically active.

DISCUSSION

We used immunogold labeling and biochemical analyses to study the subcellular localization of MTD in celery organs and suspension-cultured cells. MTD is a cytosolic enzyme that is also present in the nuclei of the cells that express cytosolic MTD. Nuclear localization of MTD was confirmed by immunoblot analyses and MTD activity assay of nuclear extracts.

Cellular distribution of MTD protein was consistent with previous biochemical data (Stoop and Pharr, 1992) and immunolocalization at the light microscope level (Zamski et al., 1996). MTD is abundant in cells of sink organs, phloem, and mannitol-grown suspension culture. The differences in the gold-particle densities in different cell types seem to imply that different amounts of MTD are present in these cells. High levels of MTD protein in root meristematic cells and root cap cells are consistent with the biochemical data that demonstrated high-MTD activity in root tips

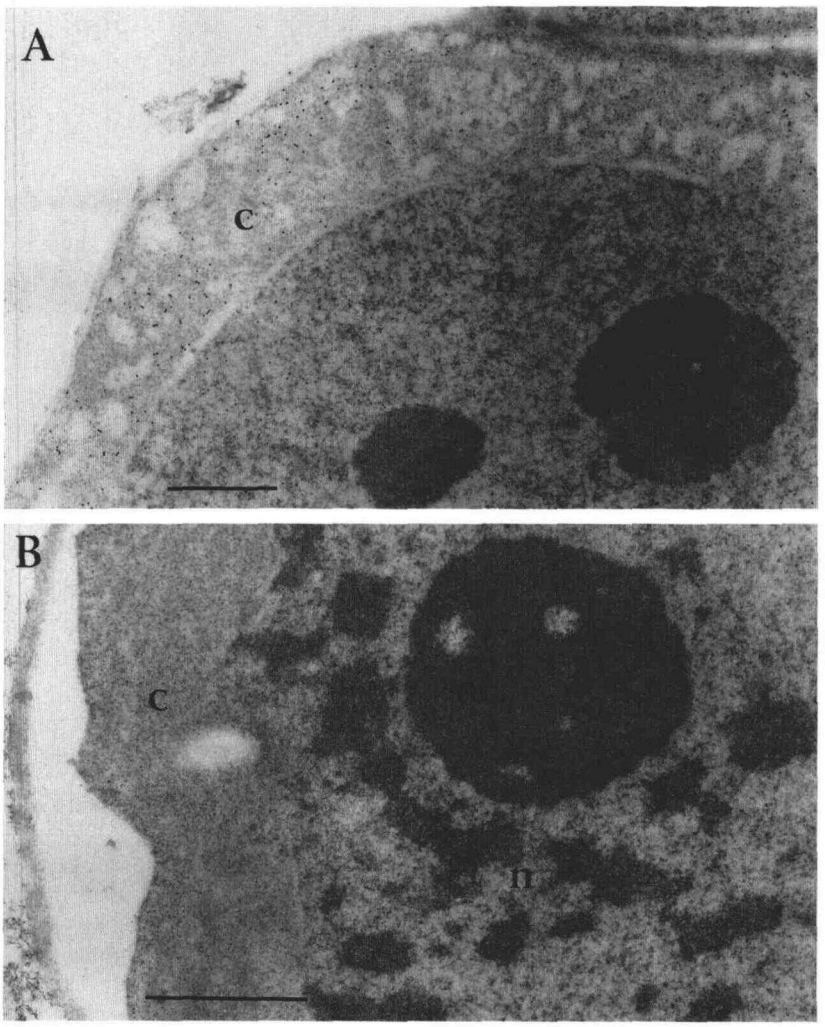

Figure 3. Immunogold labeling of celery suspension-cultured cells. A, Transmission electron microscopy of a 7-d-old mannitol-grown cell labeled with anti-MTD serum and gold-conjugated secondary antibody. B, Transmission electron microscopy of a 7-d-old Suc-grown cell labeled with anti-MTD serum and gold-conjugated secondary antibody. n, Nucleus; nu, nucleolus; and c, cytosol. Bars = 1 μm.

(Stoop and Pharr, 1992). Mannitol-grown cells, however, contain higher MTD activity than root tips (Stoop and Pharr, 1992), whereas gold-particle density of individual cells is lower than that of the root cells (Fig. 3). This is probably attributable to the fact that the root tips used for the biochemical study (3 cm from the tip) include many cell types that do not express MTD, such as the epidermis, cortex, and xylem (Zamski et al., 1996).

Nuclear localization of MTD protein was unexpected. To investigate whether the nuclear distribution of gold particles represents the presence of nuclear-localized MTD, or was a result of an artifact, we prepared nuclear and crude

Table I. *Analyses of gold particles*

The numbers of gold particles on electron micrographs were counted to analyze the density of gold particles in each cell type.

Cell Type	No. of 1-μm^2 Fields		Total No. of Gold Particles	Mean	SE
Root cap	Cytosol	15	226	15.07	0.71
	Nucleus	15	220	14.67	0.64
Root meristem	Cytosol	17	445	26.18	1.37
	Nucleus	20	568	28.40	1.52
Young leaf	Cytosol	15	108	7.20	0.54
	Nucleus	15	96	6.40	0.57
MT-grown[a]	Cytosol	36	265	7.36	0.58
	Nucleus	36	256	7.11	0.57
Suc-grown[b]	Cytosol	33	20	0.61	0.22
	Nucleus	30	20	0.67	0.18

[a] Mannitol-grown suspension-cultured cells. [b] Suc-grown cultured cells.

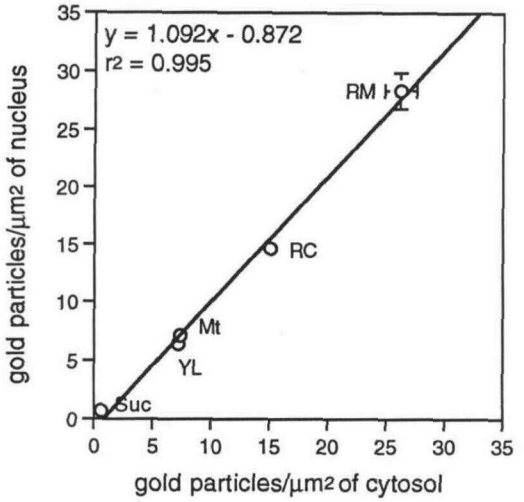

Figure 4. Correlation between the density of gold particles in the nucleus and cytosol of different cells and tissues of celery. Means of gold-particle density in cytosol are plotted against means of gold-particle density in nuclei for each cell and tissue type. Bars represent SE values. YL, Young leaf cells; RC, root cap cells; RM, root meristematic cells; Mt, mannitol-grown cultured cells; and Suc, Suc-grown cultured cells.

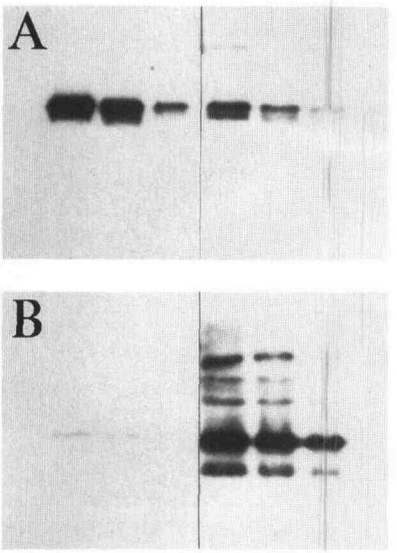

Figure 5. Immunoblot analyses of crude and nuclear extracts from 7-d-old celery cells. The amounts of 10, 5, and 1 µg (from left to right) of celery crude extracts (left three lanes) and celery nuclear extracts (right three lanes) were incubated with anti-MTD (A) and anti-HMGa (B) antisera. Specific cross-reaction was visualized using a chemiluminescent detection system.

extracts from mannitol-grown suspension-cultured cells of celery. We used ADH activity assays to determine the level of cytosolic contamination in the nuclear extracts, because ADH and MTD are both soluble cytosolic enzymes and are of similar size. The result of ADH assays of the extracts clearly showed that the nuclear extracts were free of cytosolic contamination (Table II). Immunoblot detection of the extracts revealed that the nuclear antigen is likely MTD (Fig. 5A). Furthermore, the nuclear-localized MTD is enzymatically active (Table II).

Although the densities of gold particles in nuclei and cytosol are similar (Fig. 4), the amount of nuclear-localized MTD protein detected on the immunoblot of nuclear extracts is much less compared with MTD protein in crude extracts. This may be attributable to the leakage of MTD, a 40-kD protein, from nuclei because of extensive washing during the nuclear-isolation procedure. Alternatively, MTD may simply represent a lesser portion of the total protein of the protein-dense nuclear compartment than of the cytosolic compartment.

Many nuclear proteins contain NLSs that allow them to pass through nuclear pores that exclude proteins larger than 40 to 60 kD (for review, see Silver, 1991; Raikhel, 1992; Carrington, 1995). MTD, a 40-kD protein, might simply diffuse through nuclear pores. Equal density of MTD between the two compartments implies that the nuclear and cytosolic MTD may be in "diffusional equilibrium." Alternatively, MTD may form a larger complex with other proteins that contain NLSs. Because the deduced amino acid sequence of MTD does not contain any known NLSs, only functional tests can provide answers to these questions.

Present biochemical data show that the nuclear-localized MTD is enzymatically active. Whether MTD has a catalytic function, another function, or no function within the nucleus needs to be investigated in the future. A number of metabolic enzymes are present in both the cytosol and the nuclei. Some of them have known nuclear functions. For instance, 3-phosphoglycerate kinase and annexin 2 monomer form a primer-recognition protein complex localized in the nuclei of mammalian cells (Kumble and Vishwanatha, 1991). The primer-recognition protein complex functions as an accessory protein for DNA polymerase α in the lagging strand. Cytosolic catabolic enzymes such as hexokinases in plants and yeasts and galactokinase in yeasts are involved in gene regulation (Gancedo, 1992; Ronne, 1995; Zenke et al., 1996; Jang et al., 1997). Galactokinase interacts directly with nuclear-localized proteins, and, therefore, at least some fraction of cellular galactokinase must be localized in the nucleus (Zenke et al., 1996). The catalytic activity of galactokinase is not required for its regulatory function. A model proposed by Jang and Sheen

Table II. *ADH- and MTD-specific activities of crude and nuclear extracts from celery suspension-cultured cells*

Crude and nuclear extracts were prepared from two batches of celery suspension-cultured cells grown in mannitol-containing medium.

Extract	Specific Activity	
	ADH	MTD
	$\mu mol\ h^{-1}\ mg^{-1}$ protein	
Crude		
Culture 1	11.28	0.65
Culture 2	11.14	0.78
Nuclear		
Culture 1	ND[a]	0.14
Culture 2	ND[a]	0.15

[a] ND, Not detectable (minimum detectable level was 0.03).

(1994) assumes nuclear localization of plant hexokinase. Glucokinase plays a critical role in Glc sensing in mammals (Grupe et al., 1995). Glucokinase is translocated to the cytoplasm from the nucleus of rat hepatocytes in response to a high concentration of Glc (Toyoda et al., 1994). Nuclear localization of celery M6PR, a mannitol biosynthetic enzyme, has also been reported (Everard et al., 1993). M6PR is expressed primarily in source leaves in which MTD is not expressed. It is unknown whether the nuclear M6PR has catalytic activity. Cofactor-independent phosphoglycerate mutase of lily is localized in the cytosol, plastids, and nuclei (Wang et al., 1996). Its nuclear functions are unknown.

MTD, likewise, may have multiple functions. Nuclear-localized MTD may play a role in gene regulation. The *Mtd* gene is regulated by many factors, and MTD protein could be involved in some of the regulatory processes of its own expression. MTD-related proteins in species that do not use mannitol may share such a function. Whereas the present report clearly demonstrates the nuclear localization of MTD, its possible functions need to be investigated in the future.

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