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Molecular Cloning of Mannose-6-Phosphate Reductase and Its Developmental Expression in Celery[1]

John D. Everard, Claudio Cantini, Rebecca Grumet, Julie Plummer, and Wayne H. Loescher*

Department of Horticulture, Michigan State University, East Lansing, Michigan 48824–1325 (J.D.E., R.G., W.H.L.); Istituto Propagazione Specie Legnose, Consiglio Nazionale delle Ricerche, Scandicci, Italy (C.C.); and Department of Horticulture, Faculty of Agricultural Science, University of Western Australia, Nedlands, Perth, Western Australia (J.P.)

Compared with other primary photosynthetic products (e.g. sucrose and starch), little is known about sugar alcohol metabolism, its regulation, and the manner in which it is integrated with other pathways. Mannose-6-phosphate reductase (M6PR) is a key enzyme that is involved in mannitol biosynthesis in celery (*Apium graveolens* L.). The M6PR gene was cloned from a leaf cDNA library, and clonal authenticity was established by assays of M6PR activity, western blots, and comparisons of the deduced amino acid sequence with a celery M6PR tryptic digestion product. Recombinant M6PR, purified from *Escherichia coli*, had specific activity, molecular mass, and kinetic characteristics indistinguishable from those of authentic celery M6PR. Sequence analyses showed M6PR to be a member of the aldo-keto reductase superfamily, which includes both animal and plant enzymes. The greatest sequence similarity was with aldose-6-phosphate reductase (EC 1.1.1.200), a key enzyme in sorbitol synthesis in Rosaceae. Developmental studies showed M6PR to be limited to green tissues and to be under tight transcriptional regulation during leaf initiation, expansion, and maturation. These data confirmed a close relationship between the development of photosynthetic capacity, mannitol synthesis, and M6PR activity.

Many higher plant species synthesize Suc and starch as the primary products of photosynthetic carbon assimilation. This is, however, not universal, and other species, including many of considerable economic importance, may partition between 15 and 60% of their newly assimilated carbon into acyclic sugar alcohols (e.g. sorbitol and mannitol). Sugar alcohols are rarely the exclusive products of carbon assimilation: all higher plant species that synthesize sugar alcohols as primary products also synthesize Suc and starch. Photosynthetic partitioning of carbon between these three products depends on the species (Flora and Madore, 1993; Loescher and Everard, 1996), stage of leaf development (Davis et al., 1988), and environmental factors (e.g. salinity [Everard et al., 1994; Loescher et al., 1995]). The influence of developmental and environmental factors on photoassimilate partitioning between sugar alcohols, Suc, and starch suggests that carbon partitioning into sugar alcohols is under strict metabolic control. This is consistent with what is known about nonsugar alcohol-producing species in which partitioning between Suc and starch is subject to diverse, complex, and coordinated regulatory mechanisms (Preiss and Sivak, 1996; Quick and Schaffer, 1996). There is, however, almost no equivalent information for sugar alcohol producers, and the mechanisms by which carbon partitioning into sugar alcohols is regulated and integrated with the Suc and starch biosynthetic pathways remain unknown.

Celery (*Apium graveolens* L.) is a particularly good species in which to study sugar alcohol metabolism. It is a mannitol producer and approximately 50% of newly assimilated carbon is partitioned into this compound in mature leaves. Both the anabolic (Rumpho et al., 1983; Loescher et al., 1992) and catabolic (Pharr et al., 1995) pathways have been described. The synthetic pathway appears to be present in other mannitol-synthesizing higher plant species (Loescher et al., 1992; Harloff and Wegmann, 1993; Simier et al., 1994) and comprises three unique enzymatic steps: (a) isomerization of Fru-6-P to Man-6-P by Man-6-P isomerase; (b) reduction of Man-6-P to mannitol-1-phosphate by M6PR; and (c) dephosphorylation of mannitol-1-phosphate to mannitol by mannitol-1-phosphate phosphatase. Radiotracer studies and kinetic analyses suggest that M6PR plays a regulatory role in this pathway. This enzyme has been purified and partially characterized (Loescher et al., 1992). Here we report on the cloning of cDNAs encoding M6PR, the partial purification and characterization of active M6PR isolated from transformed *Escherichia coli*, and a study to characterize the developmental regulation of M6PR during leaf development. The distribution of M6PR activity in the petioles, roots, and flowers of mature celery plants is also presented.

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* Corresponding author; e-mail loescher@msu.edu; fax 1–517–355–0249.

Abbreviations: A6PR, aldose-6-phosphate reductase; IPTG, isopropyl-β-D-thiogalactopyranoside; M6P, Man-6-phosphate; M6PR, Man-6-phosphate reductase.

MATERIALS AND METHODS

The mature celery (*Apium graveolens* L. cv Giant Pascal) plants used in this study were greenhouse-grown (Davis et al., 1988) and were approximately 6 months old.

RNA Isolation and Poly(A)$^+$ RNA Selection for Library Construction

Total RNA was extracted from approximately 10-g samples of the fifth and seventh leaves, according to Gilmour et al. (1988). Modifications included the addition of the polyphenol oxidase inhibitors cupferron (1 mM) and 2-mercaptobenzothiazole (1 μg/mL) to the extraction buffer immediately prior to use; the inclusion of three phenol:chloroform:isoamyl alcohol (25:24:1, v/v) partitioning steps on the aqueous phase, followed by three chloroform:isoamyl alcohol (48:2, v/v) partitionings to remove residual phenol; and a single LiCl precipitation followed by four ethanol precipitation steps. RNA yields averaged 500 ± 180 μg/g fresh weight. The absence of contaminating DNA was confirmed on an agarose gel after an RNase treatment.

Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography (Sambrook et al., 1989). Total RNA was loaded onto the column in a solution containing 0.12 M NaCl, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. Poly(A)$^+$ RNA was eluted from the column in the above buffer with the NaCl omitted.

cDNA Library Construction

A unidirectional cDNA expression library was constructed in a Uni-Zap XR vector (Stratagene) using a mixture of poly(A)$^+$ RNA from leaves 5 and 7 (2.5 μg of each). After packaging the phage library was amplified once before screening.

Library Screening

Two hundred thousand plaque-forming units were screened for M6PR expression at a density of 40,000 plaque-forming units per 140-mm Petri plate. Plates were incubated at 42°C until plaques were just visible. Nitrocellulose discs were soaked in IPTG (10 mM), air dried, and laid onto the surface of the plates. After 3.5 h at 37°C, the membranes were replaced and the plates incubated for a further 3 h. Both sets of membranes were screened using M6PR-specific antisera (Ried et al., 1992) at a dilution of 1:10,000 (see Everard et al., 1993). Over 100 plaques giving positive signals in the initial screening were recovered from the plates, and 10 of these were subjected to two additional rounds of screening. Twelve recombinant plaques that did not give a positive reaction with M6PR antisera were selected for use as non-M6PR control clones.

After selection through three rounds of screening, putative M6PR and non-M6PR control clones were in vivo excised to yield phagemid (plasmid) clones in *E. coli* strain SOLR. Although 10 individual putative M6PR clones were selected for further study, it is not certain that these represented 10 individual mRNAs isolated from the original population in the leaf material used. This is because the original library was amplified once before screening (see above).

Sequencing of M6PR Clones

Three putative M6PR clones were sequenced on both strands with a 373A sequencer (Applied Biosystems) using dye-primer and dye-terminator methodologies. Sequences were obtained using T3, T7, and 20-mer primers corresponding to internal sequences. The consensus sequence was derived by matching the two strands of each individual clone and by a comparison of the three independent clones.

Sequence analysis was performed using SeqEd (Applied Biosystems) and Lasergene (DNASTAR, Inc., Madison, WI). Sequence comparisons with other databases were performed through the National Center for Biotechnology Information via the BLAST server. Peptide comparisons were made through ExPASY-Prosite (expasy.hcuge.ch/sprot/prosite) and PRINTS (www.biochem.ucl.ac.uk/bsm/dbbrowser/PRINTS).

Clone Confirmation

Internal Peptide Sequencing

The amino acid sequence of native M6PR could not be determined, presumably because of a blocked N terminus. Purified M6PR (Loescher et al., 1992) was further purified by running approximately 200 μg on a preparative 10% polyacrylamide gel under denaturing conditions. The M6PR band, identified by staining with Coomassie blue R-250 for 2 min, was excised and electroeluted (Hunkapiller et al., 1983). The eluted protein was dried in vacuo and taken up in 80% ethanol. The precipitated protein was pelleted by centrifugation and dissolved in 0.1 M ammonium bicarbonate (pH 8.2) prior to digestion with trypsin at 37°C for 16 h. Trypsin was added at approximately 2% (w/w) at the start of the digestion and again after 8 h.

Digestion products were separated by reverse-phase chromatography on a 1 × 25 mm column (Applied Biosystems) eluted in a 90-min linear gradient of TFA (0.1% [v/v] in H_2O) and acetonitrile (acetonitrile:H_2O:TFA, 90:9.915:0.085, v/v), at a flow rate of 830 nL/s. Prominent peptides were collected, dried in vacuo, and subjected to amino acid sequencing on a 477A sequencer (Applied Biosystems).

Test for M6PR Activity in Putative Clones

Clones, three putative M6PR and one non-M6PR control, were tested for M6PR activity. Duplicate 10-mL cultures of each were grown in Luria broth + ampicillin (50 μg/mL). Once an average A_{600} of 0.5 had been attained, one culture of each pair was induced by the addition of IPTG to a final concentration of 10 mM. Noninduced control cultures had an equal volume of sterile water added. Cultures were maintained at 30°C for a further 3 h and were harvested at an average A_{600} of 1.29 ± 0.02. Cells were pelleted by centrifugation and washed twice by resuspending pellets in 5 mL of 0.1 M Tris, pH 7.5, 250 μM PMSF, with a

centrifugation step between each wash. After the final wash pelleted cells were maintained on ice. Just prior to disruption the cells were resuspended in 4 mL of the extraction buffer, which consisted of 0.1 M Tris-HCl, pH 7.5, 250 μM PMSF, 10 mM DTT, and 0.1% (v/v) Triton X-100. The suspensions were transferred to blood-dilution vials, and cells were ruptured by sonication at 4°C. Aliquots of the homogenate were centrifuged at 13,000*g* for 5 min at 4°C, and the supernatants were assayed for M6PR activity (Loescher et al., 1992). At least two different aliquot volumes from each extract were assayed to verify linearity. Supernatant aliquots containing between 100 and 200 μg of total protein were precipitated with 7 volumes of acetone. After standing overnight at −21°C, the samples were prepared for SDS-PAGE and western blotting (Everard et al., 1993). This experiment was performed twice with identical results.

Large-Scale Preparations and Purification

One-liter Luria broth/ampicillin cultures were initiated by the addition of 50 mL of an overnight culture of the M6PR clone D (initial A_{600}, approximately 0.15). At an A_{600} of approximately 0.25, IPTG was added to a final concentration of 1 mM and the culture was incubated at 30°C until A_{600} was between 0.8 and 1.0. A preliminary experiment had shown that M6PR specific activity began to level off at A_{600}>1.0. Cells were harvested by centrifugation and, after washing as described above, were frozen at −80°C. Prior to extraction, cells were thawed slowly on ice and ruptured by either sonication or decompression/shearing. Prior to sonication, cells were suspended in 20 mL of the extraction buffer (see above for composition) in a blood-dilution vial and sonicated for 5 min at full power. After centrifugation at 20,000*g* for 10 min, the supernatant was decanted and the pellet was resuspended in 10 mL of the extraction buffer and subjected to another round of sonication. This cycle was repeated three times. For decompression/shearing, cells suspended in 20 mL of the extraction buffer were passed through a French press (Aminco, Urbana, IL) three times followed by centrifugation at 20,000*g* for 20 min.

Subsequent purification steps were essentially as previously described (Loescher et al., 1992), except that the active fraction from the gel-filtration step here was split in two, and each fraction run separately on the reactive yellow 86 column (Sigma). The active fractions were pooled and either diluted by the addition of 0.5 volume of the column buffer or desalted using 30-kD cutoff centrifugal concentrating devices. This step was performed to dilute or remove NADPH. The sample was then loaded back onto the reactive yellow 86 column, and activity was eluted in a linear (100 mL) gradient between 0 and 0.2 mM NADPH in the column buffer. The purified M6PR was desalted and concentrated by ultrafiltration and was either used immediately for kinetic characterization or stored at −21°C after adding glycerol (1:1, v/v).

Kinetic parameters were determined at 30°C in 33 mM Tris-HCl buffer (pH 7.5) containing 3 mM DTT. Other assay details have previously been described (Loescher et al., 1992). M6P kinetics were determined at 12 concentrations of M6P ranging from 1 to 50 mM under saturating NADPH concentrations (200 μM). NADPH kinetics were determined at 9 NADPH concentrations ranging from 1 to 50 μM. M6P concentrations in these assays were 12 mM. Best line fits on double-reciprocal plots were calculated by linear regression: r^2 values were > 0.99 except for one recombinant NADPH determination, the r^2 value of which was 0.95. Kinetic determinations were repeated on at least two independent preparations of purified recombinant and authentic celery enzyme.

Developmental Study

Mature celery plants were reduced to their component parts by stripping the petioles from them. The leaf material was removed from the petioles, and material of a comparable developmental age from the two plants was pooled together and randomly divided into three subsamples (1 g for RNA extraction and 2 × 2 g batches for enzyme studies). Five-gram batches of petiole, root, and crown tissue were also harvested (see below). The material was wrapped in foil, frozen in liquid nitrogen, and stored at −80°C until further use. Three pairs of plants were harvested. Material was harvested from the crown (shoot meristem); leaves 1 through 9, 11, 13, 15, 17, and 19; petioles from leaves 3, 7, and 19; and the root. The root sample consisted of both root tips and more mature regions. Cotyledons were harvested from 21-d-old seedlings, prior to emergence of the first true leaf.

RNA Extraction

The RNA extraction protocol described above proved unsatisfactory for older leaf material, and the method of Chomczynski and Sacchi (1987), as modified by Puissant and Houdebine (1990), was adopted for the developmental study. Total RNA was quantified spectrophotometrically and stored frozen.

Northern Blotting

Ten micrograms of total RNA was separated on 1.5% agarose gels under denaturing (formaldehyde) conditions. RNA was capillary transferred (Sambrook et al., 1989) to Hybond-N nylon membranes (Amersham) overnight in 10× SSC and fixed to the membrane in a microwave oven (Angeletti et al., 1995). Membranes were prehybridized for 8 h at 60°C in 20 mL of 5× SSC (Sambrook et al., 1989), 63 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.2% (w/v) SDS, 2× Denhardt's reagent (Sambrook et al., 1989), 6% (w/v) PEG 6000, and 200 μg/mL denatured herring sperm DNA. Hybridization was performed overnight in 8 mL of the above buffer under the same conditions. Random priming was used to ^{32}P label a 1.3-kb *Eco*RI-*Kpn*I fragment of the M6PR clone. At the end of the hybridization period, membranes were rinsed briefly (2 × 1 min) in 2× SSC and 0.1% SDS and then washed for 20 min at room temperature in the same solution. Three further 20-min washes in 0.2× SSC and 0.1% SDS were performed at 60°C prior to image capture on film (Hyperfilm MP, Amersham) or a PhosphorImaging screen (Molecular Dynamics, Sunnyvale, CA). Im-

ages captured on the screen were analyzed using a PhosphorImaging densitometer (model 400B) and Image Quant software (Molecular Dynamics).

M6PR Activity and Characterization

Tissue samples were prepared and assayed for M6PR activity as described by Everard et al. (1994), except that Triton X-100, $MgCl_2$, and EDTA were omitted from the extraction buffer; the tissue-to-extraction-buffer ratio was 1:5 (w/v) for leaves and 1:2 (w/v) for the other plant parts. Assays were performed immediately after extraction and again after the extracts had been standing on ice for 12 h in a cold room. Samples for SDS-PAGE and western blotting were prepared as described above. Estimates of the quantity of M6PR protein in extracts were performed according to Everard et al. (1994) and by "quantitative" western blotting. In the latter technique 5 μg of total protein was separated by SDS-PAGE (12.5% acrylamide) and exhaustively transferred to PVDF membranes by electroblotting (60 V, 200 mA); membranes were changed four times over a 24-h period. Western blotting was performed as described by Everard et al. (1993) but using CDP Star (Boehringer Mannheim) as the substrate for alkaline phosphatase. Images were captured on x-ray film and quantified by densitometry (Everard et al., 1994). Experiments with purified M6PR showed a linear relationship between densitometry-determined band volume (arbitrary units) and the amount of protein loaded in the range of 1 μg to 25 ng. This was sufficient to cover the range of the protein contents that were present in the tissue extracts.

Protein and Chlorophyll Determinations

Protein content was determined by the method of Bradford (1976) using BSA as a standard. Chlorophyll was determined according to Wintermans and De Mots (1965).

RESULTS

Library Screening

Putative M6PR clones were identified based on western blotting with M6PR-specific antisera. Of the 200,000 plaque-forming units screened, an estimated 0.15 ± 0.04% gave a positive signal. Ten of these were subjected to two more rounds of screening, and three of these were further characterized and their authenticity as M6PR clones confirmed, as described below.

Expression of M6PR Protein and Enzyme Activity

Putative M6PR clones clearly expressed M6PR activity in an IPTG-dependent manner (Fig. 1A). Some M6P-dependent NADPH utilization was detected in extracts of the non-M6PR control clone, but this was very low and did not change with IPTG induction (Fig. 1A). The presence and absence of a polypeptide with an identical molecular mass to authentic celery M6PR correlated well with measured enzyme activities (Fig. 1B). This polypeptide reacted with M6PR-specific antisera (Fig. 1C). Trace amounts of the

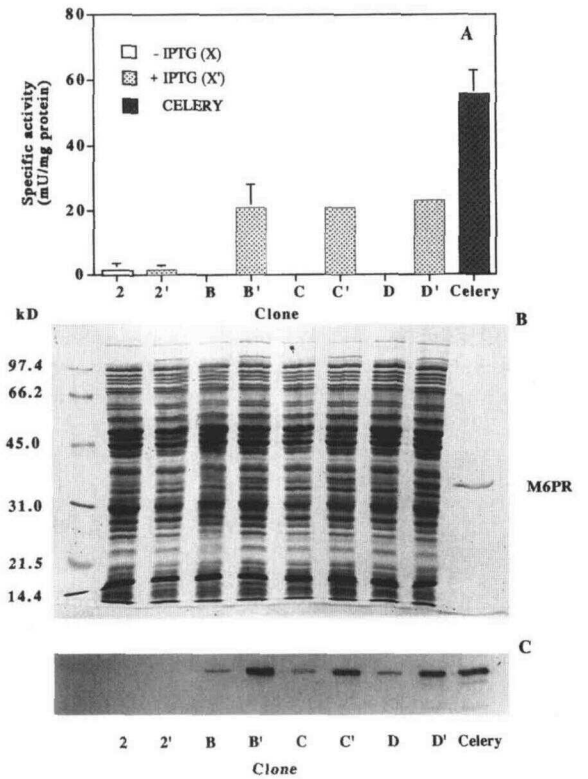

Figure 1. Verification of M6PR-specific clones. A, M6PR activities in sonicated extracts of one non-M6PR control (2) and three putative M6PR clones (B, C, and D) with and without IPTG induction. B, SDS gel of the extracts (100 μg/lane); the extreme right-hand lane contains 3 μg of authentic celery M6PR. C, A western blot. An SDS gel containing 5 μg of total protein per lane was blotted to a PVDF membrane and probed with M6PR-specific antisera; as in B the extreme right-hand lane contains authentic celery enzyme.

M6PR peptide were detected in noninduced cultures of the specific clones (Fig. 1C), but levels were below that detectable in the enzyme assay (Fig. 1A). No cross-reaction was observed between the M6PR-specific antibody and extracts of the non-M6PR control clone (Fig. 1C).

Sequencing M6PR Clones

EcoRI-KpnI restriction digestion of the three putative M6PR clones assayed above yielded inserts of approximately 1.3 kb. All three clones were sequenced on both strands. The consensus nucleic acid sequence (1207 bp; GenBank accession no. U83687) included a 930-bp open reading frame (stop codon included) (Fig. 2). Each clone differed in the length of the 5′ noncoding region and the poly(A) tail (data not shown), suggesting that they represented independent cloning events rather than duplications arising during library amplification. The predicted 309-amino acid polypeptide (Fig. 2A) had a calculated molecular mass of 35 kD. This value was identical to the previous SDS-PAGE-determined value for authentic celery M6PR (Loescher et al., 1992).

As a further confirmation of the authenticity of the putative clones, the deduced amino acid sequence contained a

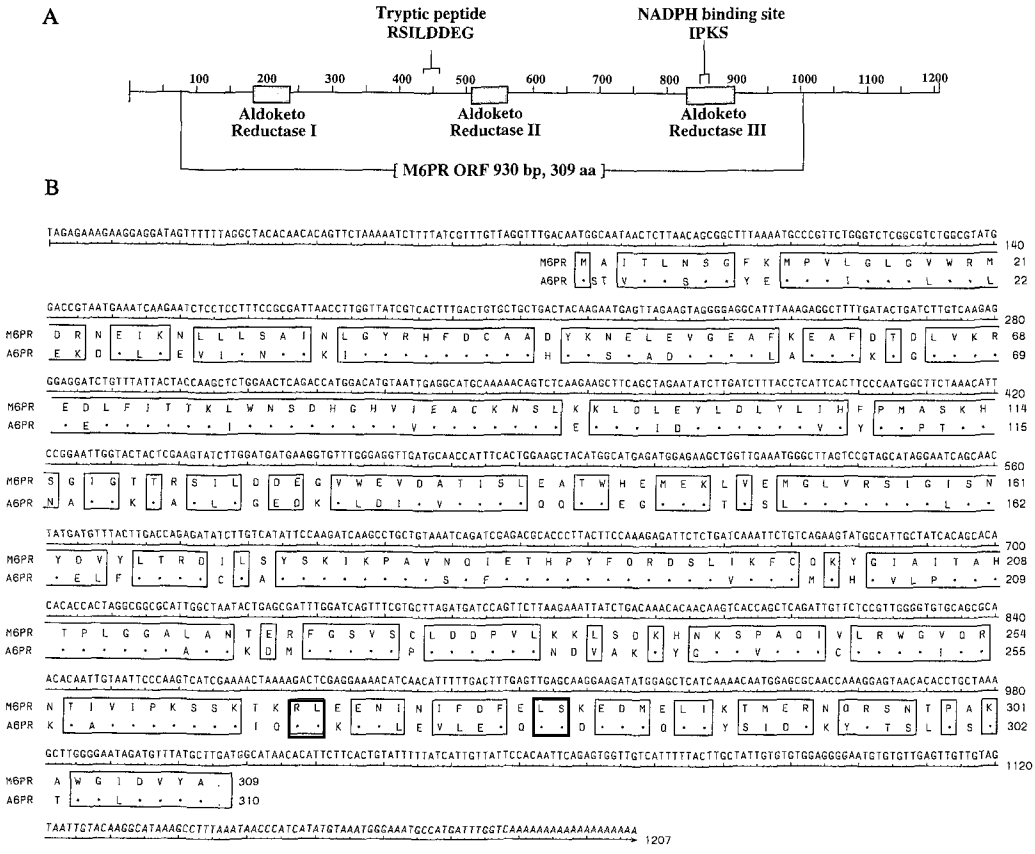

Figure 2. A, Schematic diagram showing the general structure of an M6PR cDNA clone. Features shown include the open reading frame (M6PR ORF), the position of a peptide obtained by trypsin digestion of authentic celery M6PR, and three conserved motifs typical of the aldo-keto reductase superfamily. Motif III contains the putative NADPH binding site IPKS. B, The nucleotide sequence of a celery M6PR cDNA clone (GenBank accession no. U83687) and the deduced amino acid sequence of M6PR. The open reading frame ran from base 78 through base 1007, including the stop codon ("."). The deduced amino acid sequence of A6PR from apple (EC 1.1.1.200; GenBank accession no. D11080) is also presented for a comparison. M6PR and A6PR were 64% identical at the amino acid level (conserved residues are indicated by asterisks) and showed 84% similarity when the functional relatedness of the residues was considered. Residues that are identical and functionally related have been enclosed within boxes. The classification used to group the amino acids was as follows: acidic (D and E), basic (H, K, and R), hydrophobic (A, F, I, L, M, P, V, and W), and polar (C, G, N, Q, S, T, and Y).

peptide (RSILDDEG) that was identical to an internal peptide obtained by sequencing a tryptic digestion product of purified celery M6PR (Fig. 2A).

Sequence Comparisons

Comparisons between the nucleic acid sequence of M6PR and those available through computer databases identified five sequences of plant origin having greater than 53% similarity. These included a 315-bp expressed sequence tag from Arabidopsis (accession number Z48383; 70% identity), 462- and 430-bp expressed sequence tags from rice (accession numbers D41273 [67% identity] and D48175 [73% identity], respectively), and an aldose reductase from barley (accession no. X57526 [Bartels et al., 1991], 53% identity). The only sequence (plant or animal) that showed homology across the complete open reading frame was NADP-dependent D-sorbitol-6-phosphate dehydrogenase (Kanayama et al., 1992) (also known as A6PR [EC 1.1.1.200], Negm and Loescher, 1981), a key enzyme in sorbitol biosynthesis in woody Rosaceae species. A direct comparison between the deduced amino acid sequences of M6PR and A6PR is given in Figure 2B. A6PR was 1 amino acid residue longer than M6PR (Fig. 2B) and had 66% nucleotide sequence identity (data not shown) and 64% amino acid identity (Fig. 2B). The similarity between the two proteins was 84% when amino acids with the same functional properties were considered (Fig. 2B).

Further database comparisons of the deduced amino acid sequence identified M6PR as a member of the aldo-keto reductase superfamily. A detailed description of the features and members of this group is given by Bohren et al. (1989). In brief, these proteins have molecular masses around 30 kD and are typified by the three conserved domains marked in Figure 2A. Domain three contains the motif IPKS, which is thought to be involved in pyridine nucleotide binding through the Lys (K) residue.

Purification and Characterization of Recombinant M6PR

Purification of M6PR from clone D typically resulted in a yield of approximately 16% and a 50- to 60-fold purification (data not shown). Purified recombinant enzyme had a specific activity of 3930 ± 830 milliunits/mg protein, which was similar to that of M6PR purified from celery (3760 milliunits/mg protein; Loescher et al., 1992). After purification a dominant polypeptide with a molecular mass identical to that of authentic celery M6PR accounted for 88 ± 4% of the protein present (data not shown). This polypeptide was the only one that cross-reacted with M6PR-specific antisera (data not shown).

Kinetic Characteristics of Recombinant M6PR

The kinetic characteristics of the purified celery leaf and recombinant enzymes were very similar, with no comparisons different by Student's t test (5% level). For leaf and recombinant M6PR, V_{max} values for M6P were 6.8 and 8.0 μmol mg^{-1} protein min^{-1}, respectively, and K_m values were 13.6 and 10.1 mM, respectively. For NADPH, V_{max} values were 3.7 and 6.0 μmol mg^{-1} protein min^{-1}, respectively, and K_m values were 2.1 and 6.2 μM, respectively.

M6PR Activity, Its Protein, and Its Message Levels in Developing Celery Leaves

The standard practice when reporting enzyme activities is to express data on a unit protein basis, or sometimes in the case of photosynthetic enzymes, a unit chlorophyll basis. When expressed in either fashion, M6PR activities rose from undetectable levels in the crown and young leaves, reached a plateau once full leaf expansion had been achieved, and remained almost constant throughout the remainder of the developmental series (Fig. 3). However, in a developmental study such as this in which the total protein and chlorophyll contents are also dependent on leaf age (Fig. 3, inset), a more appropriate method of expression is per unit fresh weight. Expression of M6PR activities on a fresh weight basis has the added advantage of providing data that are directly comparable to previously published in vivo developmental studies of photosynthetic gas exchange and ^{14}C partitioning (see "Discussion"). When the data were expressed on a fresh weight basis (Fig. 3), M6PR activity was clearly influenced by leaf development. Activity rose from undetectable levels in the crown and youngest leaves, peaked in the newly fully expanded leaves (leaves 5, 6, and 7), and declined in the more mature leaves. Activities in the two oldest leaves (17 and 19) were between 20 and 25% of the maximal values. Assaying the extracts for M6PR activity immediately after preparation and again after 12 h of storage on ice showed that the low activities in old leaves were not the result of inactivation during extraction from these older, senescing tissues. Regardless of leaf age, the activities after 12 h on ice were 95 ± 7% of the initial values (data not shown).

Figure 4 shows SDS-PAGE and western blot data for the extracts that were used to determine the above enzyme activities. In crown tissue, M6PR protein was below the detection level and only traces were present in leaf 2. With leaf 3, M6PR levels increased rapidly and started to plateau around leaf 6 (Fig. 4B). Northern analysis (Fig. 5) gave similar results. The transcript was below the detection levels in crown tissue and leaf 1, but increased sharply from leaf 2 and reached peak values in leaf 6; similar results were obtained with RNA extracted from three different sets of plants, including the subsamples taken from the leaves that were used for determination of the M6PR activities shown above (data not shown). From inspection and densitometry (data not shown) of the gel (Fig. 4A), western blot (Fig. 4B), and northern blot (Fig. 5), the M6PR-specific signal appeared to be maintained at almost constant levels throughout the developmental series, once leaves had reached full expansion (Figs. 4, A and B, and 5). However, because the total amount of protein (Fig. 3, inset) and RNA (Fig. 5, top) per gram fresh weight declined sharply in leaves older than leaf 7, we standardized the M6PR data on a fresh weight basis. In this form the data were directly comparable to the enzyme activities (Fig. 3) and to previously published studies (as outlined above). The data were then expressed relative to leaf 7 values so that the scale for the three parameters was the same. When expressed in this manner, a very close correlation was

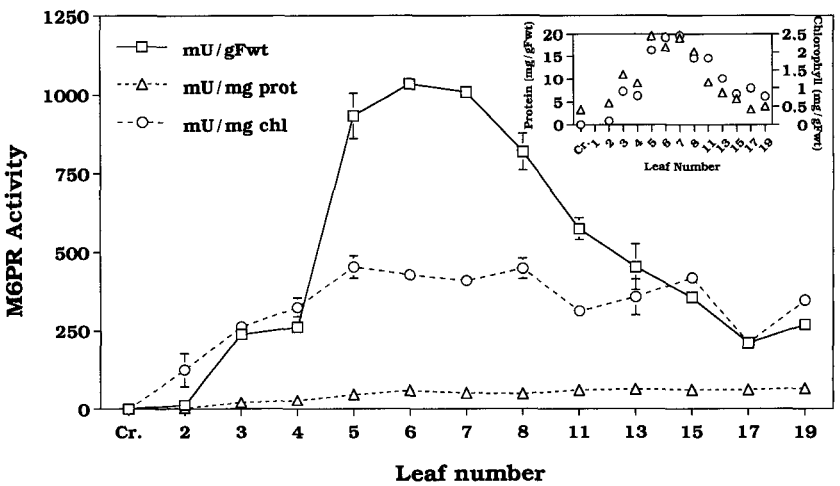

Figure 3. M6PR activity as a function of leaf age. The data have been expressed on a g fresh weight, mg protein, and mg chlorophyll basis. Values represent means ± SD of at least two determinations of activity on each extract; each individual determination used a different volume of tissue extract in the assay. Assays were repeated until the SD was <10% of the mean. One milliunit (mU) = 1 nmol NADPH oxidized per min. Error bars are shown where they were bigger than the symbols. Inset shows the protein (△) and chlorophyll (○) contents in the leaf material used to determine the enzyme activities. Similar results were obtained in at least four experiments using different plant material. Fwt, Fresh weight; prot, protein; chl, chlorophyll.

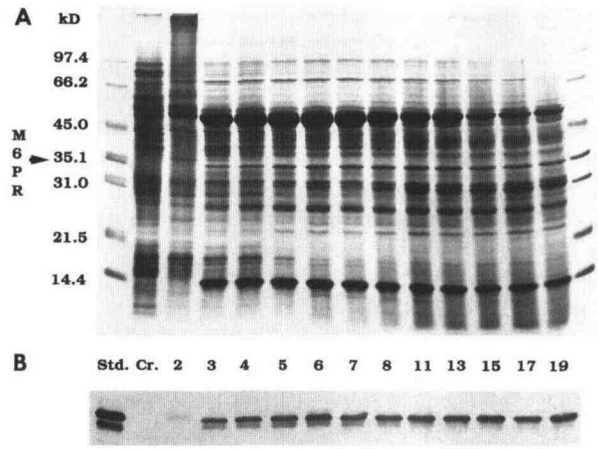

Figure 4. A, SDS-PAGE of extracts analyzed in Figure 3 (80 μg of protein per lane). The extreme right and left lanes contain Bio-Rad low-molecular-mass markers supplemented with authentic celery M6PR (2 μg); the position of M6PR is indicated on the extreme left margin. B, Western blot of the same extracts diluted to give 5 μg of total protein per lane. The extreme left lane (Std.) contained 140 ng of authentic celery M6PR. Cr., Crown.

observed between M6PR transcript, M6PR activities, and M6PR protein levels (Fig. 6). In young leaves, however, the increase in transcript preceded, by one leaf, the increases in M6PR protein and activity (Fig. 6).

M6PR Activities in Other Plant Tissues

M6PR enzyme activities were also determined in other plant tissues. There was no detectable activity in the roots and the petioles of the youngest leaves. Activity was detected in the oldest (leaf 19) petioles, but at approximately 15 milliunits/g fresh weight it represented <1.5% of the activity in leaf 7. Activity was also detected in 21-d-old

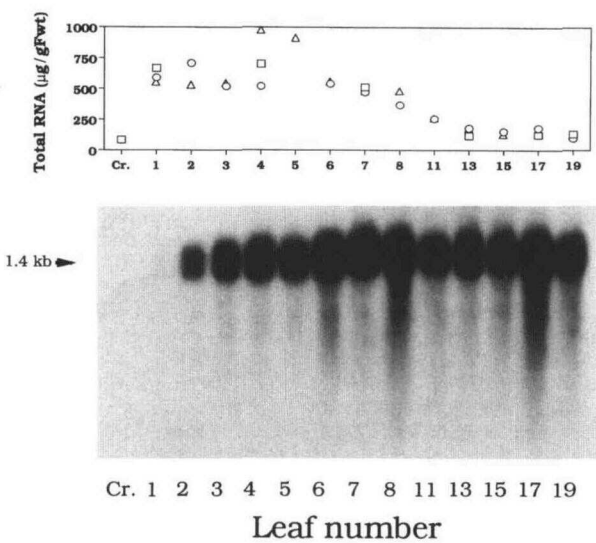

Figure 5. Northern analysis of total RNA (10 μg per lane) extracted from the crown and leaves of celery. The graph at the top shows the total RNA content of the various tissues. Data from three different preparations are shown for leaves 1, 4, 6, 15, 17, and 19. Cr., Crown.

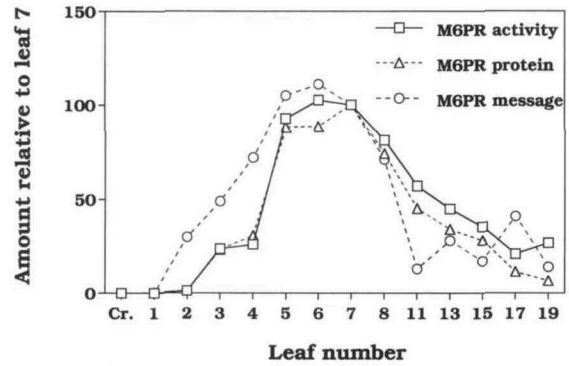

Figure 6. M6PR activity (milliunits/g fresh weight), M6PR protein (μg/g fresh weight), and M6PR transcript levels (PhosphorImager volume units/g fresh weight) as influenced by leaf age. All values are expressed as a percentage of that of the first fully expanded leaf (leaf 7). M6PR protein levels were determined by quantitative western blotting (see "Materials and Methods"). Similar data were obtained by densitometry of Coomassie blue-stained gels (data not shown).

cotyledons, but it was low in comparison to values measured in expanded true leaves: 50.0 ± 0.2 milliunits/g fresh weight for cotyledons versus 1008 ± 27 milliunits/g fresh weight for leaf 7. Immature flowers that were taken from secondary and tertiary umbels prior to anthesis displayed 6 ± 4 milliunits/g fresh weight (82 milliunits/mg chlorophyll) of activity. Mature flowers that were collected from primary whorls after anthesis but prior to desiccation, had activities of 36 ± 8 milliunits/g fresh weight (60 milliunits/mg chlorophyll).

DISCUSSION

In this paper we report the successful cloning of a full-length transcript encoding M6PR, a key enzyme in mannitol biosynthesis in celery. Clone authenticity was confirmed by several criteria. Only putative clones displayed M6PR activity when induced with IPTG. The activity correlated with the presence of a peptide of identical molecular mass to authentic celery M6PR. This peptide cross-reacted with M6PR-specific antisera. No consistent activity or immunoreactive peptide was observed in the non-M6PR control clones. The amino acid sequence of a tryptic digestion product from authentic celery M6PR had 100% identity to a peptide present within the deduced polypeptide of the clones.

Database comparisons showed M6PR to be a member of the aldo-keto reductase superfamily, which includes several animal enzymes (Bohren et al., 1989), including the extensively studied aldose reductases that have been implicated in complications associated with diabetes mellitus (Bohren et al., 1989; Vander Jagt et al., 1990; Borhani et al., 1992; Petrash et al., 1992). In plants the greatest degree of similarity between M6PR and any other enzyme was with NADP-dependent D-sorbitol-6-phosphate dehydrogenase (Kanayama et al., 1992) (also known as A6PR [Negm and Loescher, 1981]), a key enzyme in sorbitol biosynthesis in woody Rosaceae species. Four other plant sequences with nucleic acid sequence similarity came from species not

generally considered to be sugar alcohol synthesizers, i.e. Arabidopsis, rice, and barley. This finding, however, is consistent with the literature, since a homolog of mannitol dehydrogenase, a key enzyme in the mannitol catabolic pathway, has recently been discovered in Arabidopsis (Williamson et al., 1995). These findings may support Bieleski's admonition that the presence of sugar alcohols in any species should not be discounted until they are proven absent (Bieleski, 1982). The only other mannitol-metabolizing enzyme to have been cloned from plants is mannitol dehydrogenase, the first step in the mannitol catabolic pathway (Williamson et al., 1995). This clone, also from celery, showed <22% nucleic acid sequence and amino acid similarity to M6PR.

Purified clonal M6PR was indistinguishable, in terms of molecular mass, specific activity, and kinetic characteristics, from authentic celery M6PR. This appears to be a common feature of the aldo-keto reductase family. Enzymes cloned from both animal (Petrash et al., 1992) and plant (Kanayama et al., 1992) sources have been shown to be active when expressed in bacteria. This may be evidence that posttranslational modifications of these proteins are not required for activity or that any mechanisms that cause enzyme activation are present and universally effective in both eukaryotes and prokaryotes.

As mentioned in the introduction, there is little information on how sugar alcohol metabolism is regulated and integrated with Suc and starch assimilation in higher plants. The data here show that M6PR is under tight developmental control. M6PR activity is limited to photosynthetic tissues, with negligible activity in roots, petioles, and flowers. The close correlation between enzyme activity, transcript, and protein levels clearly indicates transcriptional regulation during leaf development. The pattern of M6PR expression in leaves closely mirrors photosynthetic assimilation (Fox et al., 1986) and the capacity to partition newly fixed carbon into mannitol (Davis et al., 1988; J.D. Everard, unpublished data). This further confirms a tight link between M6PR activity, carbon fixation, and mannitol synthesis.

From the literature on other enzymes involved in carbon partitioning (e.g. Walker and Huber [1989] for Suc phosphate synthase), it should be expected that there is developmental regulation of M6PR at the transcriptional level. However, in mature leaves it seems highly unlikely that a pathway that partitions up to 50% of newly fixed carbon should be regulated solely by transcriptional control. This is because very short-term (fractions of a second to minutes) changes in pathway flux are often required to maintain the balance in partitioning, which is essential for efficient carbon assimilation during the diurnal cycle (Geiger and Servaites, 1994). In the simplest case, the mannitol synthetic pathway could be regulated by carbon overflow from the Suc biosynthetic pathway, which shows tight control prior to Fru-6-P, the branch point for the two pathways (Rumpho et al., 1983). It seems more plausible, however, that the mannitol biosynthetic pathway is subjected to some form of posttranslational control. We have previously published evidence showing that extractable M6PR activity can vary independently of the amount of M6PR protein (Everard et al., 1994). This suggests that posttranslational regulation of M6PR may occur, but at present there is no evidence to suggest that such regulation is important in determining the flux of carbon into mannitol under normal, nonstressed conditions. Should further studies indicate that M6PR is indeed subject to posttranslational regulation, the ability to produce and purify a recombinant M6PR that is kinetically indistinguishable from the authentic enzyme should facilitate studies into the mechanisms involved.

Further investigations into the regulation of M6PR are currently underway. Such studies should provide insight into the roles of polyols in primary carbon metabolism. Given the increasing number of studies linking sugar alcohols to stress tolerance (Ahmad et al., 1979; Hirai, 1983; Ranney et al., 1991; Tarczynski et al., 1993; Everard et al., 1994; Stoop and Pharr, 1994; Pharr et al., 1995; Thomas et al., 1995; Tattini et al., 1996), we should also gain further insight into the mechanisms involved in the regulation of sugar alcohol levels in plants subjected to abiotic stress.

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