Abstract

BARB, ADAM WESLEY. Phosphomannose isomerase in *Nicotiana tabacum* L. NT1 and *Apium graveole*ns var. *dulce* L. cell suspension cultures. (Under the direction of Drs. D.M. Pharr and J.D. Williamson)

Phosphomannose isomerase (PMI) [E.C. 5.3.1.8] is a key enzyme required for Man metabolism in plants, and PMI activity is important in both mannitol metabolizing and mannitol non-metabolizing plants. In this manuscript, two studies are presented; one that describes PMI activity in a mannitol non-metabolizing plant, *Nicotiana tabacum* L. NT1 cell suspension cultures, and one that describes the partial purification of PMI from a mannitol metabolizing plant, *Apium graveolens* var. *dulce*.

Wild type cultures of NT1 cells grew slowly with Man as the sole carbohydrate source, doubling every 158 h, whereas NT1 cells doubled every 26 h when grown on Glc as the sole carbohydrate source. A mutant line of NT1 cells was selected from the wild type culture by repeated subculture into fresh Man medium, and after three months, exhibited a doubling time of 38 h on Man as the sole carbohydrate source. Additionally, this mutant culture exhibited a five-fold higher PMI activity level than the wild type culture from which it was selected. Interestingly, we noted that this mutant culture had a decreased growth rate on Glc, with a culture doubling time of 31 hours, and was found to have more than a 50% reduction in hexokinase (HK) [2.7.1.1] activity.

In the second study, *A. graveolens* cell suspension cultures were shown to have high levels of PMI activity (200 μmol hr^{-1} g^{-1} fresh weight) and provided an excellent starting material for the purification of PMI. PMI from Glc-grown *A. graveolens* cultures

was purified 23.5-fold with a 6% total recovery of PMI activity, using ammonium sulfate precipitation, gel filtration chromatography, and ion exchange chromatography. Low recovery of PMI activity was presumably because this activity was not stable. Although protease inhibitors in the extraction buffer resulted in the recovery of nearly twice the amount of nonspecific protein and PMI activity, neither protease inhibitors nor the addition of zinc to the enzyme solution aided in the maintenance or recovery of PMI activity in purification steps past ammonium sulfate precipitation. Additionally, different affinity chromatography resins did not bind PMI activity, but might still serve as a useful tool later in the purification of the enzyme.

Phosphomannose isomerase in *Nicotiana tabacum* L. NT1

and *Apium graveole*ns var. *dulce* L. cell suspension cultures

by

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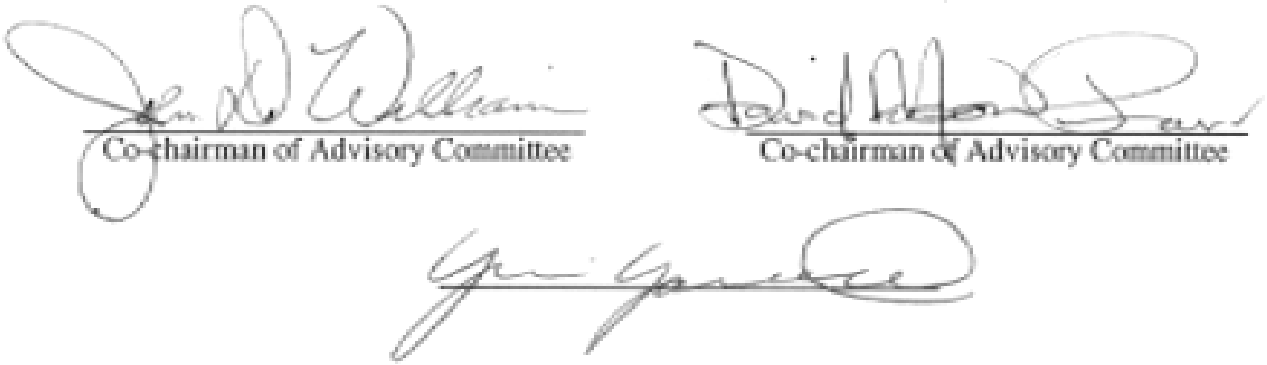

Biography

Adam Wesley Barb was born in Milwaukee, Wisconsin on August 14, 1978 to Stuart Clair and Paula Dye Barb. Adam's university education began in the Fall of 1996 at Purdue University (West Lafayette, Indiana), and he received a Bachelor of Science degree in Horticulture Science in May of 2000. While an undergraduate student at Purdue, Adam was persuaded to participate in research by Dr. Robert Joly. As a student, Adam took part in research to find and characterize T-DNA insert mutants in *Arabidopsis thaliana* plants that rendered the plants more sensitive to salinity and cold stress. While working closely with Dr. Hisashi Koiwa under the auspices of Dr. Ray Bressan and Dr. Mike Hasegawa, Adam decided to further his education and pursue a graduate degree in plant science. In August of 2000, Adam began his education as Master of Science student at the Department of Horticultural Science and as a member of the Plant Physiology Program at North Carolina State University.

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Abbreviations

A_{340}, absorbance at 340 nm

ATP, adenosine triphosphate

cDNA, complimentary deoxy ribonucleic acid

DEAE, diethyl amino ethylene

DNA, deoxy ribonucleic acid

E, einstein

EDTA, ethylenediaminetetraacetetate

FK, fructokinase

Fru, fructose

Glc, glucose

h, hour

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HK, hexokinase

Man, mannose

Min, minute

MOPS, 3-(N –morpholino)propanesulfonic acid

NAD, nicotinamide adenine dinucleotide, oxidized form

NADH, nicotinamide adenine dinucleotide, reduced form

P, phosphate

PCR, polymerase chain reaction

PMI, phosphomannose isomerase protein, mannose-6-phosphate isomerase protein

PMI, phosphomannose isomerase messenger RNA, cDNA or gene

RNA, ribonucleic acid

RT-PCR, reverse transcription polymerase chain reaction

s, second

Chapter 1

Phosphomannose Isomerase and Plant Carbohydrate Metabolism

Introduction

Phosphomannose isomerase (PMI) [E.C. 5.3.1.8] is a necessary enzyme of mannose metabolism in vascular plants, and catalyzes the interconversion of Man-6-P and Fru-6-P. In plants, the six carbon aldehyde Man is an intermediate in the pathway of mannitol metabolism (Stoop et al., 1996), may be a precursor to ascorbate synthesis (Wheeler et al., 1998), is a critical component of cell wall polymers (Olczak and Watorek, 2000), and is also a component of oligosaccharide modifications made to glycoproteins including cell membrane receptors (Strahl-Bolsinger et al., 1999).

PMI is present in many different organisms.

Proudfoot et al. (1994) described three different forms of PMI in plants, animals, fungi and bacteria. Type I PMI is a monomer with only PMI activity and is found in, but not limited to, *Homo sapiens* (Slein, 1950*), Saccharomyces cerevisiae* (Gracy and Noltmann, 1968), *Escherichia coli* (Miles and Guest, 1984), *Amophophallus konjac* (Murata, 1975a), a higher plant, and appears to be a ubiquitous enzyme among these four kingdoms. Type II PMI has been found only in bacteria and has both PMI and GDP-Man pyrophosphyrolase activity. Type II PMI has been described in, but is not limited to, *Acetobacter xylinum* (Griffen et al., 1997), *Azotobacter vinelandii* (Lloret et al., 1996) and the human pathogen *Pseudomonas aeruginosa* (Shinabarger et al., 1991) and functions by catalyzing two non-contiguous steps in the alginate (an exopolysaccharide) biosynthetic pathway. Type II PMI shares no obvious amino acid sequence similarity to Type I PMI. Type III PMI has only been found in *Rhizobium meliloti* and this enzyme has PMI activity but was not tested for GDP-Man pyrophosphorylase activity (Schmidt et

al., 1992). Type III PMI did not share any amino acid sequence similarity with either Type I or Type II PMI.

PMI is a critical component of mannitol metabolism.

Only Type I PMI has been found in higher plants. PMI in higher plants has been implicated as a critical enzyme in mannitol metabolism (Stoop et al., 1996), where it functions in both the synthesis and degradation of mannitol. More than 110 species of plants metabolize mannitol (Stoop et al, 1996), and plants that metabolize mannitol have been reported to be more drought and salinity tolerant (Everard et al., 1994, Pharr et al., 1995, Tarczynski et al., 1993) in addition to being more photosynthetically efficient than mannitol non-metabolizing C3 plants (Everard et al., 1993).

Currently, the only amino acid sequence information from higher plant PMI has been a result of predicted translation of a cloned PMI cDNA, and the only enzyme kinetic studies of plant PMI have been performed with PMI from *A. konjac* (Murata, 1975a, b).

PMI in *Apium graveolens* var. *dulce* L. and *Nicotiana tabacum* L.

PMI activity has been described in *A. graveolens*, a mannitol metabolizing plant, and PMI is an activity necessary for mannitol metabolism in this plant (Stoop and Pharr, 1992). Additionally, Stoop and Pharr (1993) reported that *A. graveolens* expressed very high PMI activity levels. Thus, with the intent of eventual description of the enzyme kinetic characteristics of this activity, we sought to purify PMI from *A. graveolens*.

Additionally, Fujiki et al. (200, 2001) demonstrated that PMI activity may be important in mannitol non-metabolizing plants, by showing that PMI is expressed in

response to carbohydrate starvation in *Arabidopsis thaliana* plants. We noted with interest that PMI activity had not been reported in *N. tabacum*, a mannitol non-metabolizing plant. However, it has been reported by Edwards and Walker (1983) that feeding Man to leaves of *N.tabacum* caused minimal disruptions of metabolism. Thus, we invesitgated the growth of *N.tabacum* NT1 cell suspension cultures on Man as the sole carbohydrate source.

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Chapter 2

Nicotiana tabacum culture selected for growth on Man has elevated phosphomannose isomerase activity

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ABSTRACT

Phosphomannose isomerase (PMI) [E.C. 5.3.1.8] is a key enzyme in Man metabolism. Many plant species express low levels of PMI, resulting in slow metabolism of this hexose. Assayed PMI activities in *Nicotiana tabacum* (NT1) heterotrophic cell suspension cultures were very low (<5 μmol hr^{-1} g^{-1} fresh weight). NT1 cultures provided with Man as the sole carbohydrate source grew very slowly, doubling every 158 h, compared to 26 h for NT1 cultures on Glc. A mutant cell line of NT1 was selected that exhibited a doubling time of 38 h on Man. This line of NT1 cells exhibited five-fold higher PMI enzyme activity than the parental line from which it was derived, along with an associated increase in PMI transcript. This increase presumably contributes to the mutant's increased ability to utilize Man for growth. The selected line continued to express high PMI activity when grown on Glc, suggesting a stable genetic change. However, the mutant line grew more slowly on Glc than the wild type and was found to have more than a 50% reduction in hexokinase (HK) [E.C. 2.7.1.1] activity. Fructokinase (FK) [E.C. 2.7.1.4] activity, however, was equal in the wild type and mutant cell lines, and the two cultures grew at equal rates with Fru as the sole carbohydrate source. Thus, the reduced growth rate of the mutant on Glc might be due to reduced capacity for *in situ* phosphorylation of Glc by HK.

Introduction

The enzyme phosphomannose isomerase (PMI) [E.C. 5.3.1.8] catalyzes the interconversion of Man-6-P and Fru-6-P, and is a necessary component of mannitol metabolism in vascular plants. Mannitol is a 6-carbon sugar alcohol that is found in over

110 species of plants (Stoop et al., 1996). PMI converts Fru-6-P to Man-6-P for synthesis of mannitol via Man-6-P reductase [E.C. 1.1.1.224] in photosynthetic leaves of *Apium graveolens* L. (Everard et al., 1993). PMI also plays a critical role in the utilization of mannitol in rapidly growing shoot and root tips of *A.graveolens* where mannitol is catabolized to Man via mannitol dehydrogenase [E.C. 1.1.1.255] (Stoop and Pharr 1992) producing Man. Man is then phosphorylated by hexokinase (HK)[E.C. 2.7.1.1] producing Man-6-P that is then converted to Fru-6-P via the reaction of PMI to enter central metabolism. Thus, PMI plays a critical role in the biosynthesis and catabolism of mannitol in mannitol translocating plants. In contrast, PMI is presumably often expressed at very low or undetectable levels in mannitol non-metabolizing plants, as is evident by the use of PMI and Man in a selectable marker system used in the production of transgenic plants (Bojsen et al., 1998, Joersbo et al, 1998). When Man is exogenously supplied to mannitol non-metabolizing plants, Man-6-P accumulates and phosphate sequestration can result in disruption of metabolism (Edwards and Walker, 1983). Sequestration of phosphate and the resulting depletion of phosphate pools may become extreme. A recent study reported that exogenously supplied Man resulted in induction of a DNA hydrolase and other apoptosis indicators in *Arabidopsis thaliana* and *Zea mays* cell cultures (Stein and Hansen, 1999).

Nevertheless, Fujiki et al. (2000, 2001) have demonstrated that conditional expression of Man metabolism (e.g. PMI) may be important in mannitol non-metabolizing plants. They found that *PMI* transcript levels are almost undetectable in photosynthesizing leaves of *A. thaliana* (Fujiki et al., 2000); but when removed from the light for more than 24 h, *PMI* transcript levels became detectable. Using Glc analogs they

demonstrated that *PMI* in *A. thaliana* responded to light like other HK-mediated, sugar-repressed genes (Fujiki et al., 2001). PMI is repressed under normal conditions in photosynthetic tissue. However, PMI is derepressed under starvation conditions (e.g. in the dark), suggesting that the *PMI* gene plays a role in a starvation-induced response. It is possible that Man could be liberated from cellular stores to relieve starvation, because Man is a component of cell wall polymers (Olczak and Watorek, 2000) as well as a component of oligosaccharide modifications made to glycoproteins including cell membrane receptors (Strahl-Bolsinger et al., 1999). During starvation, derepression of PMI might be necessary to avoid phosphate sequestration, and perhaps more importantly, would provide access to metabolic energy from the liberated Man.

Our interest in Man metabolism stems from our laboratory's interest in mannitol metabolism and the possibility of engineering it into mannitol non-metabolizing plants (see Stoop et al., 1996). PMI is a critical component for successful engineering of these pathways. We noted with interest that feeding Man to leaves of *Nicotiana tabacum* L. reportedly caused minimal disruptions of metabolism (Edwards and Walker, 1983) suggesting that *N.tabacum* might be capable of at least limited Man metabolism. Thus, we investigated the potential of a cell suspension culture of tobacco to grow on Man as the sole carbohydrate source. This in turn led to the isolation of a mutant cell line of tobacco with an acquired ability to grow vigorously on Man. Here we provide a characterization of this mutant cell line that exhibits increased PMI activity, increased PMI transcript, and reduced HK enzyme activity.

Results

PMI activity in NT1 cultures

When grown with Glc as the sole medium carbohydrate source, *N. tabacum* NT1 cell suspension cultures (An, 1985) had low levels of PMI activity, similar to those found in tobacco 'Burley 21' leaves (3.5 μmol h^{-1} g^{-1} fresh weight). As expected, NT1 cultures grew rapidly on medium containing Glc as the sole carbohydrate source, having a culture doubling time of 26 h. In contrast, cells exhibited extremely slow growth on Man as the sole carbohydrate source, with a doubling time of 158 h. Culture growth data from which doubling times were calculated are shown in Figure 1.

Selection of a mutant cell line

Presuming the slow growth on Man was likely due to the low PMI activity, we decided to determine if longer-term subculture on Man might result in selection of a line capable of more rapid growth on Man. NT1 cultures maintained on Man initially grew slowly, and PMI activity levels remained low. However, after continual two-week subculture over a three-month period, we noted that culture density at the end of each two-week growth cycle had increased over time and eventually stabilized. At the end of three months, growth on Man and PMI activity of this "mutant" culture were compared to wild type Glc-maintained NT1 cultures. With Man, the mutant culture exhibited a doubling time of 38 h as compared to 158 h for the wild type culture (Figure 1A and Table I), and the mutant cell line consistently expressed a higher PMI activity level than the wild type culture (Figure 1B). Additionally, PMI activity remained high in the mutant culture

when the cells were subcultured into medium containing Glc as the sole carbohydrate source, suggesting that the enhanced PMI expression of the mutant culture is stable in the absence of Man.

As discussed previously, plant tissues lacking adequate PMI can take up Man and convert it to Man-6-P as a dead end product of metabolism. This raised the question as to whether Man, taken up by the NT1 cultured cells, was being used as effectively for growth as was Glc. To assess this, we measured the depletion of sugars from the media (Fig. 2A) and correlated this with cell density in the cultures throughout the culture cycle (Fig. 2B) in all treatments. The relationship between media culture sugar concentration and cell density in the cultures was the same for Man and Glc grown cells. Thus, sugars were taken up by the cultures in proportion to their use for growth, and Man and Glc were used with apparent equal effectiveness. Additionally, these results show that media sugar did not become limiting to growth during the culture cycle (Figs. 1A and 2A).

Hexokinase activity in the mutant NT1 cell line

Interestingly, the mutant culture exhibited a small but consistent reduction in growth rate on Glc (Table I and Fig. 1A) when compared to wild type cultures. It seemed possible that this might be due to reduced HK in the mutant limiting the rate of Glc use. Further, we postulated that reduced HK might be the basis for increased PMI expression in the mutant, because recent work with *A. thaliana* showed that PMI expression was regulated through HK-mediated sugar repression (Fujiki et al., 2001). Consistent with this hypothesis, a reduction in HK expression in *A. thaliana*, achieved through antisense transformation, was shown to result in production of sugar hyposensitive phenotypes

wherein normally sugar regulated genes were less sugar repressed (Jang et al., 1997). To test this hypothesis, we assayed both soluble and detergent-extractable HK activities as well as fructokinase (FK)[E.C. 2.7.1.4] activity in both wild type and mutant NT1 cells. HK activity in both soluble and detergent extracted fractions in the mutant line was about 50% of wild type HK activity (Table II), while total FK activity was essentially the same. In wild type cells, FK activity was about 35% of the total HK activity. The slight apparent elevation of FK in wild type cultures (Table II) could be due to a small contribution of the higher HK activity in the wild type line. Neither HK nor FK activity was substantially affected by the growth medium carbohydrate source upon which the cells were grown.

Mutant and wild type growth on Fru

At this point, we needed to address the issue of whether the reduced HK itself or some more generalized mutation (e.g., in cytokinesis) was responsible for the reduced growth rate of the mutant culture on Glc. To do this we took advantage of the fact that while the mutant has lowered HK, the FK activity was essentially the same as the wild type. HK is thought to be primarily responsible for Glc and Man phosphorylation while Fru phosphorylation is catalyzed primarily by FK. In addition, the metabolism of Fru-6-P doesn't require PMI. If HK alone was responsible, then wild type and mutant cultures should grow at equal rates on Fru as the carbohydrate source. We observed while growing triplicate culture flasks in two independent experiments that the doubling times for wild type cultures and mutant cultures were indistinguishable in Fru as the sole carbohydrate source (Table I). Although growth rates on Fru were not as rapid as growth

of wild type cultures on Glc, the fact that cells grew at an equal rate on Fru suggests that the reduced growth of the mutant relative to the wild type on Glc may be due to the decreased HK (Table II). Further, the difference in growth rate in different hexoses might be influenced to some extent by rates of hexose phosphorylation by the different kinases.

PMI activity and carbohydrate starvation

Our data to this point suggests that reduced HK in the mutant culture might indirectly lead to derepression of PMI and thus increased growth rates on Man. To test this possibility, we next examined the extent to which PMI activity in wild type cultures responded to carbohydrate starvation. If, as is apparently the case in *A. thaliana*, PMI expression is regulated by HK-mediated sugar repression, carbohydrate starved cells might be expected to have higher (derepressed) PMI. However, we found that wild type cultures washed and starved of a carbohydrate source did not exhibit a substantial increase in PMI activity over cultures washed and incubated in Glc containing medium (Figure 3). PMI activity in the carbohydrate starved cells (4.5 μmol $h^{-1}g^{-1}$ fresh weight) was not sufficiently high to suggest that sugar repression was the basis of the difference in PMI expression between the wild type and mutant cell lines (maximum PMI activity in the mutant was 30.5 μmol $h^{-1}g^{-1}$ fresh weight) (Figs. 1B and 3).

Cloning of a partial NT1 *PMI* fragment.

A partial cDNA of NT1 *PMI* transcript was isolated using PCR for use as a molecular probe to determine relative levels of *PMI* transcript in mutant and wild type tissue.

Degenerate primers were designed (Rose, *et al*., 1998) from highly conserved amino acid sequence blocks aligned from four plant *PMI* sequences [one from *Cyamopsis tetragonoloba*, two from *A.thaliana* (*DIN9* and a proposed *PMI* homolog), and one putative *PMI* from *Oryza sativa*]. Reverse transcribed, single stranded cDNA prepared from RNA isolated from wild type NT1 cells was amplified using PCR and these degenerate primers. A single band was visualized on a ethidium-stained gel and was subsequently recovered, cloned, and sequenced. Predicted translation of the cDNA sequence revealed this 300 base pair fragment to have a 55% identity to *A.thaliana PMI* and high homology to other plant *PMI*s (Figure 4). The DNA sequence of the fragment did not contain a 30 bp sequence that is present in this region in *E.coli* and other microbial *PMI* transcripts.

PMI transcript levels in the mutant NT1 line.

To establish whether increased PMI activity was associated with increased *PMI* transcript, a blot of total RNA isolated from mutant and wild type cells was probed with the partial cDNA described above. Our results revealed that the PMI activity increase in the mutant line was paralleled by increased *PMI* transcript abundance relative to the wild type (Figure 5). Additionally, relative PCR was used to determine transcript abundance. *PMI* fragments were amplified from reverse transcribed, single stranded cDNA templates prepared from RNA of mutant and wild type tissues. PCR products from both mutant and wild type templates were compared to each other on a ethidium bromide stained agarose gel (data not shown), revealing that *PMI* transcript in the mutant was greater than *PMI* transcript in the wild type. An additional feature of the relative PCR procedure is

that RNA from NT1 cells was reverse transcribed using an oligo deoxy-thymidine primer. Therefore, only nucleic acid transcripts with a poly-adenosine tail would be amplified.

Discussion

We have shown that a mutant line of NT1 cells has stably acquired the ability to grow more vigorously on Man as the sole carbohydrate than the parent NT1 culture. Thus, the mutant line can be considered a gain of function mutant. This is only the second case known to us where a cell line was selected for growth on a carbohydrate source that is normally poorly utilized or toxic. Maretzki and Thom (1978) reported the selection of a line of sugarcane cells (*Saccharum sp.*) capable of growth on Gal, a hexose that is normally toxic to sugarcane cells in culture. UDP-Gal-4-epimerase activity was elevated 10-fold in the mutant sugarcane cells, and the capacity to grow on Gal was attributed to this increased activity. Studies of maize root tips using NMR (Loughman et al., 1989) demonstrated the toxic effects of high concentrations of Gal fed exogenously; poor metabolism of Gal resulted in accumulation of Gal-1-P and UDP-Gal with resultant sequestration of cytoplasmic phosphate. This is consistent with the fact that plant species that normally translocate and metabolize galactosyl- sugars grow well in culture on free galactose, because the enzymes of Gal metabolism are normally expressed at high levels in these species (Gross et al., 1981 and Gross and Pharr 1982).

By analogy, our mutant NT1 cells exhibit a five fold elevation of PMI activity relative to wild type cells, and increased PMI activity was associated with increased abundance of the *PMI* transcript, possibly indicating an increased rate of transcription of

the gene. Several related observations suggest that the increased PMI activity is likely to be the cause of the increased capacity of the mutant cells to grow on Man. For instance, species that normally express significant PMI activity exhibit minimal metabolic disruption when fed high concentrations of Man (Edwards and Walker, 1983). *A.graveolens*, a species that expresses extraordinarily high PMI activity, grows in culture on Man just as well as on Suc or Glc (Stoop and Pharr, 1993). Perhaps most convincing, the use of bacterial PMI as a selectable marker is the subject of a patent, wherein introduction of a single modified bacterial transgene encoding PMI into a Man sensitive plant will allow for growth of the transformant on Man (Bojsen et al., 1998). This technique has been successful with sugar beets (*Beta vulgaris* L.) (Joersbo et al., 1998), corn (*Z. mays* L.) and wheat (*Triticum aestivum* L.) (Wright et al., 2001). Our only reservation in concluding that the change in PMI in NT1 cells is solely responsible for the increased growth of the mutant cells is the fact that HK activity is also altered in the mutant. In plants sensitive to Man, phosphate sequestration may occur as a result of Man phosphorylation by HK at a rate faster than the ability of PMI to isomerize Man-6-P to Fru-6-P. Thus, down regulation of HK activity in concert with PMI up-regulation might be useful in achieving balance between phosphorylation of Man and isomerization of Man-6-P. In this regard, it may be important to note that the cells growing on Glc have multiple routes for utilization of Glc-6-P, such as oxidation by Glc-6-P dehydrogenase or conversion to Glc-1-P or Fru-6-P and subsequent metabolism. The only major outlet for Man-6-P is by conversion to Fru-6-P via PMI. Thus, one can speculate that cells are more tolerant of high rates of Glc phosphorylation than of Man phosphorylation.

Therefore, the down-regulation of HK in the mutant may actually serve a useful metabolic role with respect to its growth on Man.

In the mutant NT1 cells, it might be possible that the mutation is pleiotropic, affecting a group of genes including both PMI and HK by altering metabolite pools. It is well documented that perturbation of metabolite pools can markedly affect seemingly unrelated enzyme activities (Huber et al., 1989; Weiner et al., 1992). The regulation of PMI in the wild type NT1 culture has not been fully characterized, however one possibility can be ruled out. Unlike PMI regulation reported in *A. thaliana* (Fujiki et al., 2000), HK-mediated signaling of sugar repression of the PMI activity in NT1 cells seems to be minimal. This is based upon both a starvation where only little alteration in PMI enzyme activity was noted. Thus, our initial hypothesis that reduced HK in the mutant might be the cause of greater PMI activity seems untenable. However, our data do support the notion that the NT1 mutant's decreased growth rate on Glc might be due to reduced HK activity in the mutant cells. Reduction in HK might result in slower phosphorylation of Glc resulting in slower production of Glc-6-P for metabolism.

Because the Man adapted phenotype is associated with an alteration of at least two major enzymes of Man metabolism, it is tempting to consider interconnected regulation of the expression of the two enzymes. It is, of course, possible that we selected a mutant with two completely independent mutational events, one resulting in up-regulated PMI and the other in down-regulated HK. However, the relative rapidity with which the mutation arose makes this seem unlikely. Perhaps a more statistically likely possibility is the selection of a mutation resulting in loss of a single element in a pathway common to the regulation of both HK and PMI genes. A single mutation could lead to

the simultaneous, though apparently opposite, effects on the expression of both genes, and possibly many others. Regeneration of plants and classical genetic studies to distinguish between these possibilities is not feasible, because after almost two decades in culture (An, 1985), NT1 cells have lost the capacity to regenerate into whole plants. However, genomic approaches to investigate relative transcript abundance between mutant and wild type tissues could provide evidence for either cause and might reveal an as yet unknown interaction between the regulation of these two genes. A mutant cell culture that has altered metabolic enzyme activity has many uses. Isolation of a single mutant element with definite effects in control of key enzyme activities would be an important contribution to the understanding of carbohydrate metabolism. Somewhat less common is the isolation of a gain of function mutation, which may suggest the loss or reduced effectiveness of a regulatory cascade.

Finally, expression of adequate PMI activity is necessary if mannitol metabolism is to be engineered into plants using higher plant genes [mannitol dehydrogenase (Williamson et al., 1998), Man-6-P reductase (Everard et al., 1993), and a mannitol transporter (Noiraud et al., 2001)]. Mannitol has been shown to promote drought and salinity tolerance [Everard et al., (1994), Pharr et al., (1995), Tarczynski et al., (1993)], as well as being associated with enhanced photosynthetic capacity (Everard et al., 1993). Inadequate PMI activity levels can lead to severe metabolic shortcomings if Man is abundant within the plant cell. Whether selected for as an increased expression mutation or achieved through transformation, increased PMI expression will be necessary for efficient metabolism in plants engineered with genes of mannitol metabolism.

Materials and Methods

All chemicals and enzymes, except where otherwise noted, were obtained from Sigma. Upon request, all novel material described in this publication will be made available in a timely manner for non-commercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permission will be the responsibility of the requestor.

Plant Tissue Growth and Treatment

Suc grown *Nicotiana tabacum* L. (NT1) cell suspension cultures (An, 1985) were the generous gift of Dr. S. Spiker (North Carolina State University, Raleigh). Cell cultures were grown in 500 mL Erlenmeyer flasks at 25°C under alternating 12 h of fluorescent light followed by 12 h of darkness on a shaker table oscillating at 110 rpm. The cultures were grown at a light intensity of 5μE m^{-2} sec^{-1} in 100 mL of 1x MS salts + vitamins (Murashige and Skoog, 1962), 0.1 mg L^{-1} 2,4-D, 180 mM Glc or Man and initial pH adjusted to 5.6 with KOH. The mutant NT1 cultures were initiated by transferring inoculum from the Glc maintained cultures into 100 mL of Man containing media. A Man adapted line was selected for by repeated transfers into Man containing media, and referred to hereafter as mutant. This process was repeated for 11 culture cycles of approximately 14 d each, during which an obvious increased culture growth rate on Man was observed before the comparison experiments were conducted. Fresh leaf tissue for PMI activity assays was obtained from incubator grown *N. tabacum* 'Burley 21'.

Culture Treatments

Glc and Man cultures used in these experiments were inoculated from maintenance flasks that were transferred to fresh medium every 14 d. Experimental cultures were inoculated from 4 d old maintenance flasks that were washed three times with 100 mL of the appropriate experimental growth medium and resuspended in the growth medium. Washing was done to remove all residual carbohydrate from the previous culture cycle. Cultures were incubated in a final volume of 100 mL of the appropriate medium.

In order to monitor growth, cells were harvested from cultures in aliquots ranging from 20 mL (beginning of culture cycle) to 5 mL at timed intervals and placed into conical glass tubes. After the cells had settled, 1 mL of cleared medium was removed and frozen at –80°C for later medium carbohydrate analyses. The cells were vacuum filtered onto filter paper (Fisher, Cat. No 09-795A) and washed three times with one volume of distilled water. Cells were then weighed on an analytical balance, frozen in liquid nitrogen, and stored at –80°C until analysis. Relative growth rate was determined by fitting an exponential curve of the equation: Culture Density = (Culture Density at $time_0$)$e^{RGR*(Time(h))}$. This equation was fit to the culture density (g cells mL^{-1} culture) vs. time, excluding any late time points where the cultures did not exhibit log growth. Cell culture doubling time was determined by the following equation: Doubling Time = ln(2) RGR^{-1}.

Analysis of PMI Activity

Extracts for enzyme activity analyses were prepared from cells frozen in liquid nitrogen and stored at –80°C. Frozen cells were ground into a fine powder under liquid nitrogen

in a precooled mortar, at which point 4 volumes of ice cold extraction buffer (100 mM MOPS pH 7.5, 1 mM dithiothreitol, and 13% (w/v) Dowex-1) and 100 mg washed sea sand (to enhance cell lysis) was added and ground with a pestle as the mixture thawed. The resulting mixture was centrifuged at 10,000 rpm for 1 minute, and the supernatant was recovered and desalted via centrifugation using Sephadex^{TM} G50 resin equilibrated with 20 mM MOPS pH 7.5.

PMI activity was determined in a linked assay using 200 μL of the desalted enzyme extract that was added to 800 μL of assay buffer for a final concentration of 100 mM MOPS pH 7.5, 0.5 mM NAD^+, 5 mM Man-6-P and 10 units each of Phosphoglucose Isomerase (from *Saccharomyces cerevisiae*)(Sigma No P5381) and Glc-6-P Dehydrogenase (from *Lueconostoc mesenteroides*)(Sigma No G5885). A time course of change in A_{340} was measured with a spectrophotometer, and the linear slope was used to determine units of activity (μmol Fru-6-P h^{-1} g^{-1} fresh weight).

Analysis of Hexokinase and Fructokinase Activity

Cultures were treated as described above. Plant material grown on either Glc or Man for 72 h was ground into a fine powder under liquid nitrogen in a precooled mortar, at which point 4 volumes of ice cold soluble extraction buffer (20 mM HEPES pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA, 2.5 mM dithiothreitol, and 0.5 mM PMSF) and 100 mg washed sea sand were added and ground as the mixture thawed. The resulting mixture was centrifuged at 10,000 rpm for 1 minute, and the supernatant was recovered. The resultant pellet was extracted twice more with the same buffer, and the three extracts were combined (soluble fraction). Next, the pellet was extracted three times with the same

buffer, but now containing 1% (v/v) Triton X-100, and these three supernatants were pooled (detergent extracted fraction). Soluble and detergent pools were desalted using Sephadex^{TM} G50 resin equilibrated with 20 mM HEPES pH 8.0, 5 mM $MgCl_2$ and 1 mM dithiothreitol. HK and FK activity in soluble and detergent pools were assayed according to Yamamoto et al. (2000).

Medium Carbohydrate Determination

Frozen media (from growth experiments described above) were diluted with water to less than 100 μg sugar mL^{-1}. For Glc determination, 500 μL of diluted medium was mixed with 500 μL assay mix (200 mM HEPES/NaOH pH 8.0, 10 mM $MgCl_2$, 2 mM NAD^+, 2 mM ATP, 10 mM dithiothreitol, 4 units mL^{-1} Hexokinase, and 4 units mL^{-1} Glc-6-P Dehydrogenase) and incubated at room temperature for 30 minutes. Then, absorbency of the samples was read spectrophotometrically at 340 nm, with 500 μL of assay mix and 500 μL water subtracted from the sample A_{340}. These values were compared with a set of standards (0, 10, 20, 30, 40, and 50 μg Glc mL^{-1}).

The Man and Glc amount was determined by adding 500 μL of diluted sugar solution (with <50 μg Man + Glc) mixed with 500 μL assay mix (200mM HEPES/NaOH pH 8.0, 10 mM $MgCl_2$, 2 mM NAD^+, 2 mM ATP, 10 mM dithiothreitol, 4 units mL^{-1} Hexokinase, 4 units mL^{-1} Glc-6-P Dehydrogenase, and 4 units mL^{-1} Man-6-P Isomerase (from *Saccharomyces cerevisiae*)(Sigma No P5153) and 4 units phopshoglucose isomerase (from *Saccharomyces cerevisiae*)((Sigma No F2668) and incubated at room temperature for 30 minutes. Change in A_{340} was read spectrophotometrically, with 500 μL of assay mix and 500 μL water subtracted from the sample A_{340}, and compared to the

standard prepared earlier. The Glc amount determined previously was subtracted from this Man plus Glc amount to determine total Man.

Cloning of a partial *PMI* cDNA and RT-PCR

RNA was isolated using Tri-Reagent™ (Molecular Research Centers, Inc.) according to the manufacturer's protocol. cDNA was prepared from isolated RNA using SuperScript™ II reverse transcriptase (Gibco BRL) according to the manufacturer's instructions. An oligo deoxy-thymidine$_{21}$ primer and the 18SR primer (5'-TATTGGAGCTGGAATTACCGC) were used in the reverse transcription reaction to prepare cDNA copies of poly adenosine RNA and 18S rRNA, respectively. A partial NT1 *PMI* cDNA was amplified using degenerate primers F2 (5'-ATGCTGAATTTTGGATGGGNACNCA) and R2 (5'-CATAGCAAGAGCCATTTCTGGYTTRTGRTT). Primers were designed using the CODEHOP program (Rose, *et al*., 1998). The partial NT1 *PMI* cDNA PCR product was ligated into pGEM®-T Easy (Promega) and the cloned fragment (pPMI-1) was sequenced (MGIF, University of Georgia, Athens). The GenBank accession number of the sequenced fragment is AF469471.

Analysis of PMI Transcript Abundance

Total RNA was isolated as described above from 24 and 36 h Glc grown mutant and wild type cells and Man grown mutant cells. Total RNA was separated on a 1% (w/v) agarose/formaldehyde gel and transferred onto MagnaGraph™ membrane (Micron Separations, Inc.). A DIG labeled probe was prepared with DIG-dUTP (Roche) in a PCR

reaction as described above using the F2/R2 primer combination with pPMI-1 as the template. Blots were hybridized overnight and washed at high stringency (0.1x SSC and 0.1% SDS) (65°C). Hybridizing bands were visualized by using CDP-Star™ (Roche No 2041677), a chemiluminescent substrate, and autoradiography.

For relative, reverse-transcriptase PCR, reactions were mixed minus primers using separate cDNA preparations from both mutant and wild type RNA, and each preparation was split into halves. In one half, primers for amplification of *PMI* (F2 and R2) were added, and in the other half, primers for the amplification of 18S ribosomal cDNA (18SR and 18SF (5'-GACTGTGAAACTGCGAATGCG) were added. Complete reaction mixes were aliquoted and placed into a thermalcycler (MJ Research). PCR products were separated electrophoretically and identified on a ethidium bromide stained agarose gel.

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We would like to thank Dr. S. Spiker (NC State University) for the generous gift of the NT1 cultures, Dr. Wei Wen Guo for her technical expertise, Dr. Yuri Yamamoto for her discussions of culture treatments, Dr. Marilyn Ehrenshaft for her help with the cloning of NT1 PMI and Amanda White for her assistance in preparing culture media.

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Table I NT1 cell culture doubling times on three hexoses as sole carbon source[a].

hexose	wild type	mutant
	Doubling time (±SE)	
Glc	26.1 (0.3)	31.1 (1.1)
Man	157.8 (14.0)	38.0 (1.6)
Fru	33.6 (2.9)	36.7 (4.1)

[a] Glc and Man growth data from Figure 1. Fru data from a separate experiment discussed in Results. Additionally, cell growth rates were stable and consistent over time.

Table II Hexokinase activity in wild type and mutant NT1 suspension cultures growing for 72 hours on either Man or Glc.

	Soluble Hexokinase[a]	**Detergent Hexokinase[b]**	**Fructokinase[c]**
	Activity (μmol h^{-1} g^{-1} fresh weight) (±SE)		
Mut-Man	3.9 (0.1)	7.4 (0.7)	7.6 (0.2)
Mut-Glc	3.8 (0.5)	8.5 (0.7)	6.7 (0.4)
Wt-Man	9.0 (0.6)	15.4 (0.4)	9.9 (0.2)
Wt-Glc	10.6 (0.7)	16.8 (1.3)	8.3 (0.6)

[a] Soluble HK extracted from cell homogenate, and pellet reextracted two times with HEPES buffer

[b] Pellet from soluble HK is then extracted three times in a buffer containing HEPES fortified with 1% Triton X-100

[c] Fructokinase was assayed only in the soluble buffer explained in ([a]).

Figure Legends

Figure 1. A. Cell suspension culture density (g fresh weight mL^{-1} culture) of wild type and mutant NT1 cells on Man and Glc as the sole carbohydrate source. **B.** Enzyme activity (μmol Man-6-P h^{-1} g^{-1} fresh weight) of extracts from the cell suspension cultures. Data represent the mean values of three replications. Error bars represent one standard error of the mean.

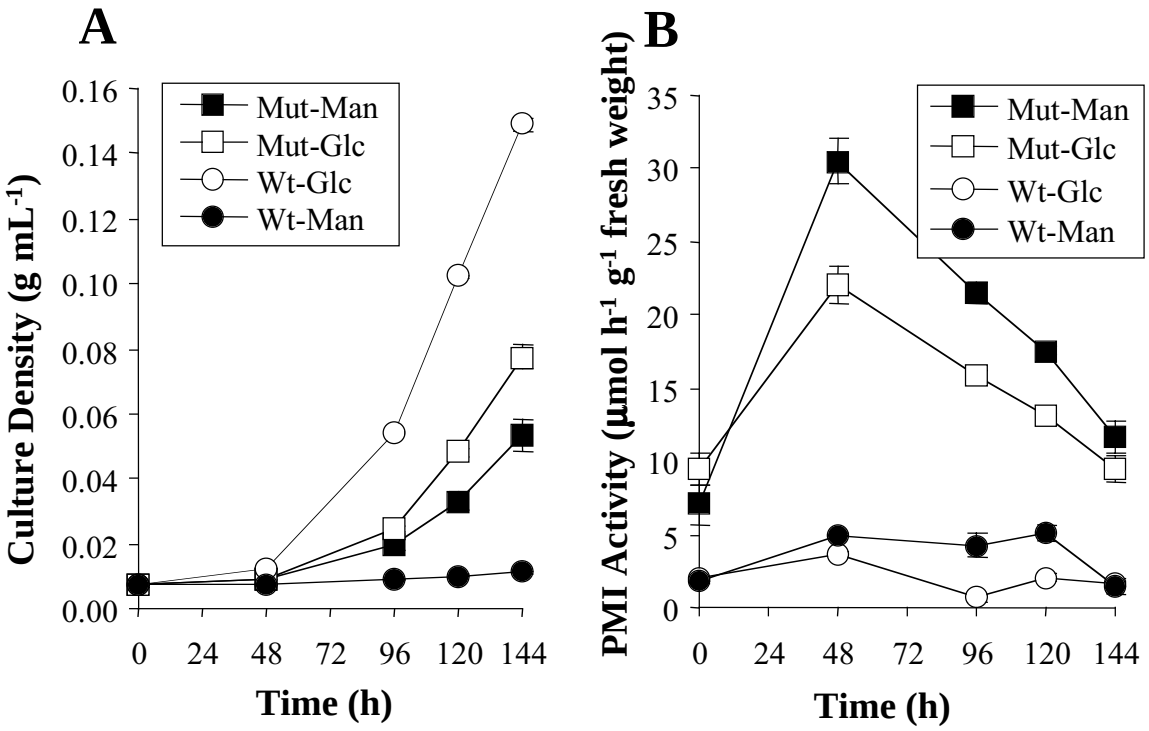
Figure 2. **A**. Hexose concentration in the growth medium sampled during the growth experiment shown in Figure 1. Each data point represents the mean value of three replications. Error bars represent one standard error of the mean. **B**. Correlation analysis of medium hexose concentration and culture density. Each point represents the hexose concentration and culture density of one culture at one time point. The experiment consisted of 12 cultures and 5 time points. The correlation has an R^2=0.95 and is described by the equation y=-134.5x + 33.7.

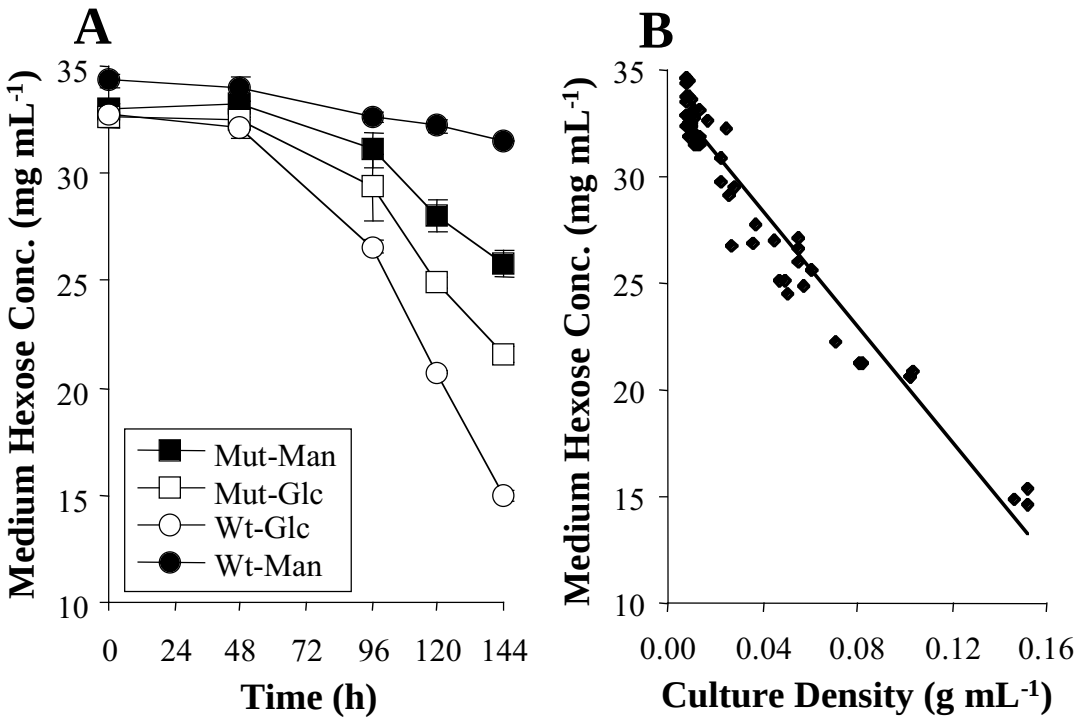
Figure 3. PMI enzyme activity (μmol Man-6-P h^{-1} g^{-1} fresh weight) of Glc grown and carbohydrate starved wild type NT1 cell suspension cultures. Cultures were washed free of media and resuspended in either Glc containing or carbohydrate free medium. Cultures were sampled and assayed for PMI activity.

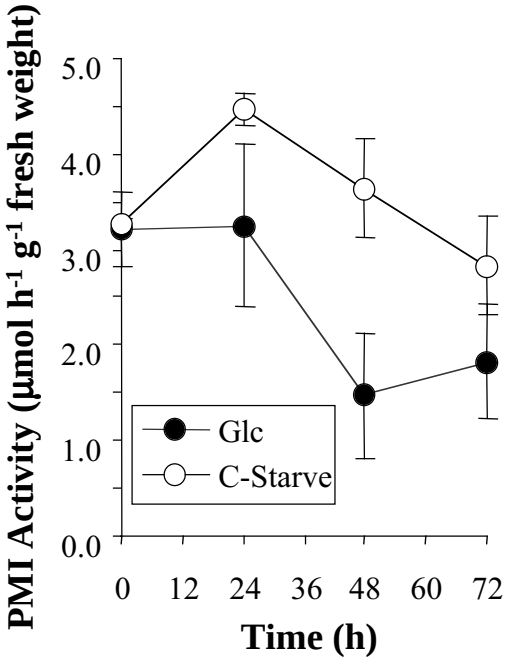
Figure 4. Amino acid alignment of PMI proteins and predicted proteins. A black background indicates identical amino acids; a gray background indicates similar amino

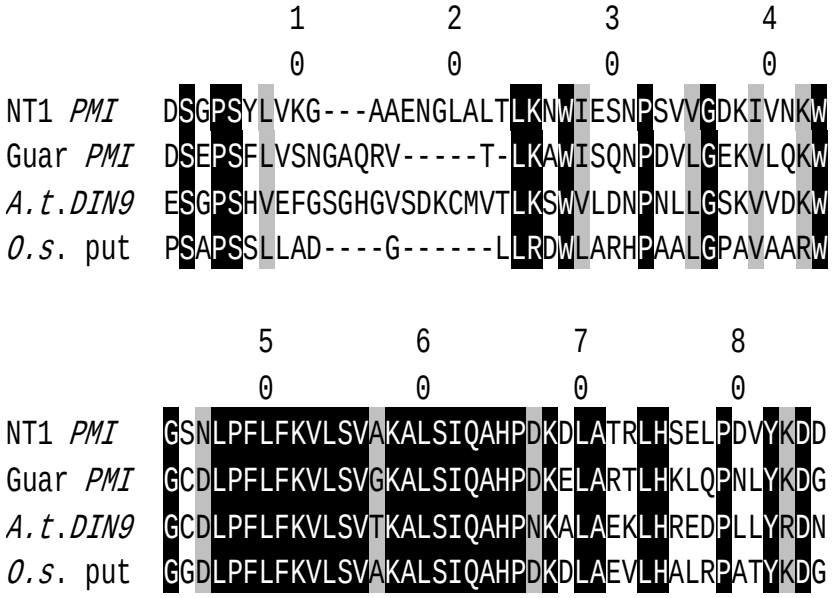
acids (neutral substitutions). The NT1 sequence was derived from the predicted translation of a partial cDNA sequence (with primers removed) attained using degenerate reverse transcription-PCR (*N.tabacum*, GenBank accession AF469471). Guar PMI (*C. tetragonoloba*, GenBank A63583), *A.thaliana* DIN9 (GenBank AC007152) and putative *O. sativa* PMI (GenBank AP002860) are shown for higher plant PMI comparisons. Identity is ~43% with an additional 14% similarity. Similarity groups used were A,G,S,T; I,V,L,M; F,Y,W; D,E,N,Q; and K,R,H.

Figure 5. Accumulation of the *PMI* gene transcript in *N. tabacum* NT1 cells. Blots were hybridized at high stringency with a DIG-labeled *PMI* probe (from NT1 *PMI*) and visualized with a chemiluminescent substrate. Gels were stained with ethidium bromide prior to transfer to assess RNA loading (lower panel). Migration of the 25S and 18S ribosomal RNAs is indicated. RNA from both mutant and wild type cells incubated in Glc is included as well as RNA from mutant cells incubated on Man. Similar results were observed with different RNA samples from different tissue in an independent blot.


A
Culture Density (g mL-1)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
24
48
72
96
120
144
Time (h)
Mut-Man
Mut-Glc
Wt-Glc
Wt-Man
B
PMI Activity (µmol h-1 g-1 fresh weight)
35
30
25
20
15
10
5
0
0
24
48
72
96
120
144
Time (h)
Mut-Man
Mut-Glc
Wt-Glc
Wt-Man


A
B
Medium Hexose Conc. (mg mL-1)
Time (h)
Culture Density (g mL-1)
Mut-Man
Mut-Glc
Wt-Glc
Wt-Man
35
30
25
20
15
10
0
24
48
72
96
120
144
0.00
0.04
0.08
0.12
0.16


PMI Activity (µmol h-1 g-1 fresh weight)
5.0
4.0
3.0
2.0
1.0
0.0
0
12
24
36
48
60
72
Time (h)
Glc
C-Starve


1 2 3 4
0 0 0 0
NT1 PMI DSGPSYLVKG---AAENGLALTLKNWIESNPSVVGDKIVNKW
Guar PMI DSEPSFLVSNGAQRV-----T-LKAWISQNPDVLGEKVLQKW
A.t.DIN9 ESGPSHVEFGSGHGVSDKCMVTLKSWVLDNPNLLGSKVVDKW
O.s. put PSAPSSLLAD----G------LLRDWLARHPAALGPAVAARW
5 6 7 8
0 0 0 0
NT1 PMI GSNLPFLFKVLSVAKALSIQAHPDKDLATRLHSELPDVYKDD
Guar PMI GCDLPFLFKVLSVGKALSIQAHPDKELARTLHKLQPNLYKDG
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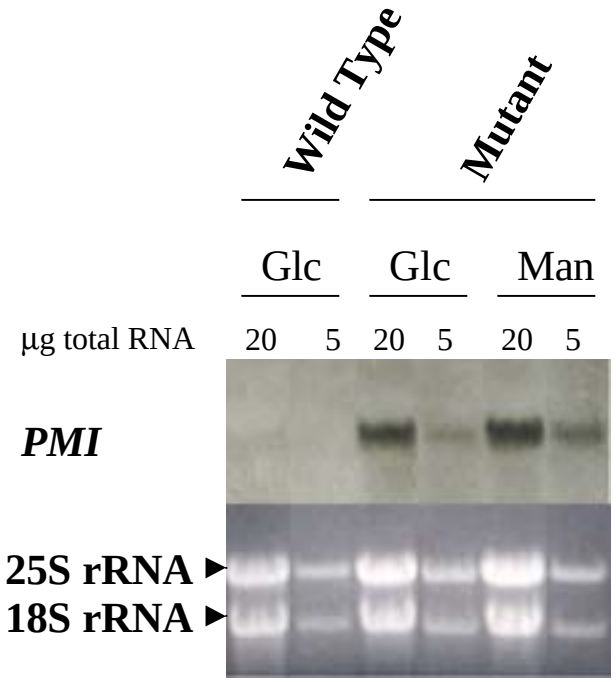
Wild Type
Mutant
Glc
Glc
Man
µg total RNA
20
5
20
5
20
5
PMI
25S rRNA
18S rRNA

Chapter 3

Partial purification of phosphomannose isomerase from *Apium graveolens* var. *dulce* L. cell suspension cultures

This chapter was prepared in the style of the journal Plant Physiology.

Abstract

Phosphomannose isomerase (PMI)[E.C. 5.3.1.8] is an essential enzyme required for the synthesis and degradation of mannitol in mannitol metabolizing plants. Cell suspension cultures of *Apium graveolens* var. *dulce* L., a mannitol metabolizing plant, expressed high levels of PMI activity (200 μmol hr^{-1} g^{-1}fresh weight). This high PMI activity *in A. graveolens* cultures was not influenced by different carbohydrate sources in the medium. PMI from Glc-grown *A. graveolens* cultures was purified 23.5-fold with a 6% total recovery of PMI activity using ammonium sulfate precipitation, gel filtration chromatography, and ion exchange chromatography. PMI activity was not stable during purification, and this was responsible for the low recovery. The addition of protease inhibitors to the extraction buffer resulted in more total protein and more PMI activity being recovered from the initial extract. However, protease inhibitors in the extraction buffer and addition of zinc to the enzyme solution did not aid in maintenance of PMI activity through subsequent steps of purification. Additionally, colored dye affinity columns and a Man-6-P affinity resin did not bind PMI activity, but might still serve as a useful tool later in purification of the enzyme, because at least two of the resins removed substantial protein.

Introduction

Mannitol is a six carbon sugar alcohol that is metabolized by more than 110 species of plants, including *Apium graveolens* L. (Stoop et al., 1996). Plants that metabolize mannitol have been reported to be more drought and salinity tolerant (Everard et al.,

1994, Pharr et al., 1995, Tarczynski et al., 1993) in addition to being more photosynthetically efficient than mannitol non-metabolizing C3 plants (Everard et al., 1993). Currently an effort is underway to describe the enzymes that are unique to mannitol metabolism and clone the genes encoding these proteins. To date, three of these unique enzymes have been characterized [mannitol dehydrogenase (Stoop and Pharr, 1992), Man-6-P reductase (Everard et al., 1993), and a mannitol transporter (Noiraud et al., 2001)]. One critical component of the mannitol catabolic and degradation pathways, phosphomannose isomerase (PMI)[E.C. 5.3.1.8], has been characterized from only a single higher plant species, *Amorphophallus konjac* C. Koch (Murata 1975 a, 1975b).

The enzyme PMI catalyzes the interconversion of Man-6-P and Fru-6-P, and is a necessary component of both mannose and mannitol metabolism in vascular plants. In addition to its role in mannitol metabolism, PMI from bacterial sources has been used as a selectable marker to aid in plant transformation (Bojsen et al., 1998, Joersbo et al., 1998, and Wright et al., 2001). While bacterial PMI has been reported to function adequately for use as a selectable marker, it could be that transformation using this form of PMI might not result in adequate catalytic capacity for the interconversion of Man-6-P and Fru-6-P in plants engineered to form mannitol at high rates. *A. graveolens* expresses very high PMI activity levels (Stoop and Pharr, 1993). While it is not known whether high PMI activity is due to a more active protein or higher transcript levels, PMI *from A. graveolens* may serve as a more useful tool for engineering mannitol metabolism pathways into plants.

In order to determine the molecular basis for high PMI activity in *A. graveolens*, purification of PMI activity will be necessary. Purification will permit accurate kinetic

characterization of the enzyme and could facilitate the isolation of a full length PMI cDNA clone which would be a useful tool in obtaining the full length gene from *A. graveolens*. Data from enzyme characterization combined with a PMI cDNA sequence ultimately will permit the determination of the molecular basis for high PMI activity and will be a useful tool in the engineering mannitol metabolism.

Our interest in PMI stems from our laboratory's interest in mannitol metabolism and the possibility of engineering it into mannitol non-metabolizing plants (see Stoop et al., 1996). As a primary step in the pursuit of a detailed molecular characterization of PMI activity, here we report the partial purification of the PMI activity from *A. graveolens*. Progress and problems associated with the purification of this enzyme are described.

Results

Treatment of the extract. In order to maximize the amount of PMI activity per g fresh tissue, PMI activity in *A. graveolens* cultures was tested in extracts from cultures grown on various carbohydrate sources. Cultures were grown for 2 weeks in a medium containing Glc as the sole carbohydrate source. At the end of this period, cultures were washed free of all Glc medium and resuspended in medium containing Glc, Man, mannitol or no carbohydrate source. After a two week incubation period, PMI was assayed in the cell homogenates from fresh tissue. PMI activity in *A. graveolens* cultures did not vary significantly in extracts from cells grown on the different carbohydrates, where PMI activity was 15 μmol Fru-6-P $hr^{-1}mg^{-1}$ protein (data not shown). Thus, all

tissue used for purification of PMI was grown on Glc, a relatively cheap and readily available carbohydrate source.

It was noted that upon freezing and storage (-80 °C) of the enzyme after extraction, minimal loss of specific activity occurred (<10% per freeze/thaw event). However, it became apparent that during early attempts with ammonium sulfate precipitation at 0°C, a significant loss of PMI activity occurred. In an attempt to minimize the activity loss, protease inhibitors were included in the extraction buffer. Table I. shows the result of addition of PMSF and Complete™ protease inhibitor tablets to the extraction buffer. Protease inhibitors in the extraction buffer did not result in a significant increase in PMI recovery after extraction and throughout ammonium sulfate precipitation. However, addition of the protease inhibitor PMSF and more dramatically the Complete™ tablets resulted in greater total recovery of PMI activity in the extract and a higher specific activity as compared to a protease inhibitor minus control. Therefore, in subsequent extractions of tissue for PMI purification, Complete™ tablets were added to the extraction buffer.

Purification of A. graveolens *PMI.* Increasing the concentration of an enzyme solution prior to chromatography is often useful so that a maximum amount of enzyme activity can be applied to a column in a minimum volume. We concentrated PMI five-fold using ammonium sulfate precipitation. Additionally, we used this technique to fractionate total protein from the crude cell homogenate, separating PMI activity from some cell homogenate protein (Figure 1). As a result, more than 90% of the PMI activity was present in the 60-80% ammonium sulfate fraction, whereas only 10% of the total cell

protein was present in this fraction. This resulted in a 8.5-fold purification of the enzyme.

As the next step in purification, different affinity resins were tested. Affinity chromatography, if successful, binds the chosen activity and few other proteins, resulting in the removal of non-target proteins and the recovery of a relatively pure activity. The 60-80% ammonium sulfate fraction was resuspended in extraction buffer, desalted by gel filtration, and applied to ten different affinity columns (Table II). None of the affinity media bound PMI activity and excluded a majority of the cellular proteins. This included an affinity medium with a Man-6-P ligand bound to agarose beads. However, some of the colored dye columns did result in a three-fold or slightly better increase in specific activity. Of note was the Cibacron Blue 3GA media, the use of which resulted in 3.3-fold purification, and, more than 100% of the activity applied was recovered. In this case, the PMI activity voided the column and the resin retained a significant amount of protein.

In lieu of a highly effective affinity resin, gel filtration chromatography was chosen as the next stage of purification to follow ammonium sulfate fractionation. Gel filtration chromatography is a logical next chromatography step, because the enzyme solution is desalted during the chromatography resulting in removal of small molecules (salts, carbohydrates, etc) that have persisted through ammonium sulfate precipitation. Additionally, this technique separates larger protein molecules based on molecular size, and allows for the removal of proteins that are significantly smaller or larger in size from the target activity. We were able to estimate an approximate size of PMI because the molecular size of PMI in a higher plant species (*A. konjac*) which was reported to be 45 kDa by Murata (1975a). The gel filtration resin Superdex^{TM} 75 was chosen because it has

a separation range between 3 and 70 kDa. A peak of enzyme activity containing >90% of the PMI activity from the column was recovered in six 5 mL fractions from the Superdex™ 75 media (Figure 2). Additionally, during the four separate uses of the Superdex™ 75 resin, the PMI activity peak consistently eluted from the column before a large peak of protein (fractions 40-44). Again, during each of the three independent runs with the Superdex™ 75 column, a 1.8-fold purification of the active enzyme and between 71-89% of the active enzyme was consistently recovered from this column.

As a subsequent step in purification of the PMI activity from *A. graveolens*, ion exchange chromatography was chosen. A tentacle-DEAE resin was used that binds proteins with a negatively charged ligand. With the resin we chose, the DEAE groups are bound at the end of a flexible chain, which is designed to permit the persistence of the native form of the enzyme. In theory, this precludes the protein from being pulled apart by fixed ion exchange groups binding on different sides. The first time this column was run, in seven 5 mL fractions only 16% of the total activity applied to the column was recovered in a single peak. We hypothesized that this poor recovery of enzyme activity might be due to the resin being 6 years old, and to test this hypothesis we began this step of enzyme purification again with new resin.

A second column using a new batch of tentacle DEAE resin was packed. However, a sharp peak of PMI activity was not eluted from the column with a liner KCl gradient (Figure 3). Additionally, using this column, PMI activity was spread over13 fractions, each of 5 mL. This suggests that the activity did not uniformly bind to or release from the ion exchange resin. Because of this trimodal profile, we cannot dismiss

the possibility that three forms of the enzyme exist, and perhaps only one of these forms was recovered with the previous run using the old column.

With the new column, 45% of the total activity applied to the column was recovered. Additionally, the center of the PMI peaks on both the old and the new columns centered at 300mM KCl when the linear KCl gradient was run. This was the same KCl concentration that Stoop and Pharr (1992) reported PMI from *Apium graveolens* var. *rapaceum* L. being eluted from the column.

In summary, through the three stages of purification of the PMI activity from *A. graveolens* cultures, a total purification of 23.5-fold was obtained with a yield of only 6% of the original activity from the crude extract (Table III). Included in these data is the loss of enzyme activity for each step, including the freezing and thawing cycle of the extract in addition to the chromatography. The total purification was completed in four separate days (extraction to ion exchange chromatography), with three freeze/thaw cycles included.

Discussion

Here we report the partial purification of PMI from a cell suspension culture of *A. graveolens* . This is the second case known to us where PMI has been partially purified from a higher plant species (Murata, 1975a). All of the published PMI protein sequences for higher plant species have come from the predicted translation of a cDNA.

While we were able to greatly improve the purity of PMI from total cell homogenate, problems afflicted the purification of PMI throughout the entire procedure. The greatest struggle was to maintain catalytic activity of the enzyme. Controlling the

loss of activity throughout the purification procedure could have a marked improvement on fold purification of the enzyme. Unfortunately, a small loss of activity was seen in every step of the procedure, including freezing and thawing of the protein solutions between purification steps. While only 10% of the activity was lost during each freezing and thawing event, three freeze/thaw cycles during the procedure presumably greatly reduced the amount of active enzyme. Additionally, it was noted that nearly 20% of the activity was lost during our experiments with gel filtration chromatography, and nearly 50% of the activity was lost during ion-exchange chromatography.

Surprisingly, addition of protease inhibitors to the initial extract did little to affect the stability of the enzyme in purification steps following extraction (Table I). We noted that the use of the protease inhibitor Complete™ tablets did increase the total protein content of the soluble tissue extract and also resulted in higher specific activity upon subsequent ammonium sulfate precipitation. Thus, we included the tablets in our extraction buffer. We can speculate that inclusion of protease inhibitors resulted in reduced nonspecific proteolysis during the extraction, but did not affect recovery of PMI activity after the extraction. This observation and hypothesis might lead us towards including a protease inhibitor as a component of column running buffers and resuspension solutions. In place of protease inhibitors, further concentration of the enzyme or inclusion of detergent or ethylene glycol in the elution buffer (Deutscher, 1990) has previously been shown to be effective in maintaining enzyme activity throughout purification.

It was reported by Murata (1975b) in *A. konjac* and by Gracy and Noltmann

(1968) in *Saccharomyces cerevisiae* that PMI was a zinc containing metalloenzyme, and that zinc was necessary for catalytic activity. However, we noted that zinc supplied to the enzyme solution during an activity assay did not result in the recovery of lost activity (data not shown). This led us to believe that the loss of zinc could not explain the loss of PMI activity throughout the purification procedure.

We also observed greater than 50% loss of activity during ion exchange chromatography. Initially, we hypothesized that the resin we were using could be ineffective, as a result of the resin having been stored at 4°C for more than 6 years. Replacement of the old with new resin did not have a great impact on stability of enzyme activity. Some of this loss might have been due to elution of broad peaks off the newly packed ion-exchange columns containing the new resin (Figure 3), where the tails on either side of the peak contained a significant portion of the active enzyme. It should be noted that the literature accompanying the DEAE resin states that some proteins do not interact favorably with the hydrophobic resin. We feel that this might be the reason for the great loss of activity during the use of these columns.

To this point, we believe that the partial purification of PMI has been encouraging. The initial steps of purification have been well defined and thoroughly tested. When the problem of enzyme stability is resolved, further purification with different columns will eventually result in the purification of PMI to homogeneity. Building upon the conditions we have defined, we feel that the use of one of the colored dye columns (specifically Cibacron Blue 3GA) would be a logical next step. While experiments with the colored dye columns (Table II) initially led us away from their use, the use of some resins did result in recovery of 100% of the activity. The content of

protein solution may have been changed to the degree that greater fold purification is possible using one of these resins.

Purification and characterization of a homogenous *A. graveolens* PMI protein would be a useful advancement in plant carbohydrate metabolism. Given the data of protein sequence and some enzymatic properties, it may then be possible to determine if *A. graveolens* expresses a uniquely high specific activity form of PMI. The addition of *A. graveolens* PMI to the suite of genes from *A. graveolens* necessary for engineering mannitol metabolism into plants [mannitol dehydrogenase (Williamson et al., 1998), Man-6-P reductase (Everard et al., 1993), and a mannitol transporter (Noiraud et al., 2001)] might be necessary for proper functioning of high--throughput mannitol metabolism. Additionally, isolation and characterization of PMI from *A. graveolens* would increase the understanding of mannitol metabolism in this plant, and would bring us closer to isolating and characterizing every unique step of mannitol metabolism in this plant that has been used as a model for mannitol metabolism.

Materials and Methods

Chemicals

All chemicals and enzymes, except where noted, were obtained from Sigma.
Upon request, all novel material described in this publication will be made available in a timely manner for non-commercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permission will be the responsibility of the requestor.

Materials

Celery (*Apium graveolens* var. *dulce*) cell suspension cultures were maintained at 22 °C under alternating 12 hours of fluorescent light followed by 12 hours of darkness in an incubator. Constant shaking at 100 rpm ensured aeration of the cultures. The cultures were grown in 500 mL Erlenmeyer flasks at a light intensity of 23 μE $m^{-2}sec^{-1}$ in 150 mL of sterile 1xMS salts +vitamins (Murashige and Skoog, 1962), 1.53 mg L^{-1} $KH_2 PO_4$, 0.25 mg L^{-1} 2, 4-D, 60 mg L^{-1} kinetin, 180 mM Glc and the pH was adjusted to 5.6. Cultures were transferred to fresh medium every 14 days. For growth of celery cultures on alternate substrates, Man, mannitol or no carbon source was substituted Glc in the medium. Cultures were washed free of the Glc medium and inoculated into fresh Glc, Man, and mannitol or carbon free medium.

Enzyme Activity

PMI activity was assayed by spectrophotometrically determining the changes in $NADH/NAD^+$ concentrations. Assays were carried out in 1mL volumes at 25 °C. PMI activity was assayed in 100 mM MOPS (pH 7. 5), 0.5 mM NAD+, 5 mM, Man-6-P, 10 units Glc-6-P dehydrogenase (*from Lueconostoc mesenteroides*, Sigma No G5885) and 10 units phosphoglucose isomerase (from *Saccharomyces cerevisiae*, Sigma No P5381). The linear slope of change in A_{340} vs. time was used to determine units of activity (μmol Fru-6-P hr^{-1}). The reaction was initiated with the addition of Man-6-P. Protein concentration of the extract was determined using the method of Bradford (1976).
For analysis of PMI activity in column fractions, a 96-well UVmax Kinetic microplate reader was used (Molecular Devices). The concentration of components of the PMI

assay was the same as spectrophotometic assays, but total assay volume was reduced to 300 μL. For the assay of PMI in the column fractions, 50 μL of the fraction was added to 250 μL of assay mix. The change in A_{340} per h for each fraction was estimated in triplicate in using the microplate reader. The mean of these three assays for each fraction is reported as "PMI Activity. "

Purification of PMI

Harvest. Cell suspension cultures were harvested after two weeks of growth by vacuum filtration onto filter paper (Fisher No 09-795A) and were subsequently washed three times with one volume of distilled water. The cells were then weighed on an analytical balance, frozen in liquid nitrogen, and stored at –80 °C until extraction.

Preparation of Crude Extract. Tissue was ground into a fine powder under liquid nitrogen in a precooled mortar, at which point 4 volumes of ice cold extraction buffer (20 mM MOPS (pH 7. 5), 1 mM DTT) and 100 mg washed sea sand (to enhance cell lysis) was added and ground with a pestle as the mixture thawed. The resulting mixture was centrifuged at 10,000 rpm for 1 min in a microfuge, and the supernatant was recovered. Tissue that was to be directly assayed was desalted via centrifugation using Sephadex™ G50 resin equilibrated with extraction buffer (see above).

Protease inhibitors. Protease inhibitors were added to the extraction buffer before adding extraction buffer to the frozen tissue. Extractions were prepared with no protease inhibitor, 1mM phenylmethylsulfonyl fluoride (PMSF) or the recommended

concentration of the Complete™, Mini EDTA-free, Protease Inhibitor Cocktail Tablets (Roche Molecular Biochemicals1 873 580) added to ice cold extraction buffer immediately before extraction.

Ammonium sulfate precipitation. Crude extract was fractionated using ammonium sulfate. Crude extract was stirred at 0°C and the temperature allowed to stabilize. An amount of crystalline ammonium sulfate was slowly added to bring the mixture up to 20% ammonium sulfate saturation (Englard and Seifter, 1990). This mixture was allowed to mix for at 0°C for 60 minutes, at which time the mixture was centrifuged at 20,000G for 10 min. The pellet was held at 0°C until resolubilized while the supernatant was returned to 0°C and stirred. This process was repeated, increasing the mixture by 20% saturation every iteration until 100% saturation was achieved. The recovered pellets were resolublized in extraction buffer (see above) by gentle agitation using a camel's hair brush. The resolubilized pellets were desalted via centrifugation using Sephadex™ G50 resin equilibrated with extraction buffer and assayed for PMI activity and protein content.

Chromatography. All enzyme solutions that were analyzed by chromatography were crude extracts that had been fractionated by ammonium sulfate precipitation (60-80% saturation fraction).

Affinity Chromatography. Two hundred μL of desalted ammonium sulfate precipitated extract (without protease inhibitors) was applied to each of nine Sigma Affinity Media Reactive Dye Ligand columns in the test kit (No RDL-9) and a Man-6-P affinity resin

(No M1795) after 3 column volumes of extraction buffer had been run through the columns. Two column volumes of buffer were run through after the extract was applied and the two washes were pooled. Then the columns were washed with two column volumes of 0.5 KCl in 20 mM MOPS (pH7. 5) and 1 mM DTT. The high salt washes were pooled. PMI activity and protein content was measured in both the pooled buffer wash fractions and the pooled 0.5 M KCl washed fractions.

Gel Filtration Chromatography. Crude extract was prepared as described above with protease inhibitors and then the 60-80% ammonium sulfate fraction of the crude extract was applied to a Superdex™ 75 prep grade column (Pharmacia Biotech No 17-1044-01). The column was 3.5 cm in diameter, 69 cm in height, and was run at a flow rate of 3 mL min^{-1}. The protein solution was run through the column in 20 mM MOPS (pH7. 5) and 100 mM NaCl buffer. Fractions of every 5 mL of elutant were collected using a fraction collector. Additionally, fractions were assayed on a plate reader to estimate relative PMI activity. Fractions containing 90% of the total PMI activity were pooled, and assayed for PMI activity and protein content.

Ion Exchange Chromatography. The pooled fractions containing PMI activity from the Superdex™ 75 column were applied to a Fractogel™ EMD-DEAE 650(M)(VWR Scientific No EM-116888) column. The column was washed with one column volume of 100 mM KCl in 20 mM MOPS (pH7. 5), and then a linear gradient was run between 100 mM KCl and 400 mM KCl in 20 mM MOPS (pH7. 5). The first (old column) was 2.5 cm in diameter, 32 cm in height, and was run with a flow rate of 10 mL min^{-1}. The

second column (with new resin) was 2.5 cm in diameter, 10 cm in height, and was run with a flow rate of 5 mL min^{-1}. Fractions of every 5 mL of elutant were collected using a fraction collector. Additionally, fractions were assayed on a plate reader to estimate relative PMI activity. Fractions containing 90% of the total PMI activity were pooled, and assayed for PMI activity and protein content.

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Table I. Effect of protease inhibitors on recovery of active PMI during extraction and ammonium sulfate precipitation.

	[Protein] ($\mu g\ \mu L^{-1}$)	Specific Activity	% Activity Recovered	Fold Purification
Extract-Control	1.5	18.9	100.0%	-
60-80% A.S.-Control	1.4	86.7	85.7%	4.6
Extract-PMSF	2.5	16.9	100.0%	-
60-80% A.S. PMSF	1.4	100.0	67.4%	5.9
Extract-Complete™	4.3	14.7	100.0%	-
60-80% A.S. Complete™	2.0	128.8	79.5%	8.5

Table II. Effectiveness of colored and Man-6-P columns on PMI purification. Crude extract was precipitated using ammonium sulfate, desalted by gel filtration, and then applied to the columns

		% Activity	% Protein	Fold Purification
Reactive Red 120	Buffer Wash	33.86%	29.31%	1.16
Agarose	0.5M KCl Wash	6.17%	56.36%	0.11
Reactive Yellow 3	Buffer Wash	57.30%	71.74%	0.80
Agarose	0.5M KCl Wash	3.05%	19.99%	0.15
Reactive Yellow 86	Buffer Wash	77.26%	23.85%	3.24
Agarose	0.5M KCl Wash	0.46%	40.36%	0.01
Reactive Green 86	Buffer Wash	49.46%	55.62%	0.89
Agarose	0.5M KCl Wash	4.58%	50.73%	0.09
Reactive Green 19	Buffer Wash	57.52%	42.77%	1.34
Agarose	0.5M KCl Wash	0.58%	29.19%	0.02
Reactive Blue 72	Buffer Wash	73.38%	38.96%	1.88
Agarose	0.5M KCl Wash	0.67%	34.69%	0.02
Cibacron Blue 3GA	Buffer Wash	109.27%	33.18%	3.29
Agarose Type 3000-Ct	0.5M KCl Wash	0.41%	32.05%	0.01
Reactive Blue 4	Buffer Wash	93.93%	28.36%	3.31
Agarose	0.5M KCl Wash	0.29%	29.66%	0.01
Reactive Brown	Buffer Wash	88.34%	38.60%	2.29
Agarose	0.5M KCl Wash	0.50%	31.72%	0.02
Man-6-P	Buffer Wash	107.95%	96.91%	1.11
Agarose	0.5M KCl Wash	0.00%	3.09%	0.00

Table III. Partial purification of PMI from celery (*Apium graveolens* var. *dulce* L.) suspension cultures grown on Glc.

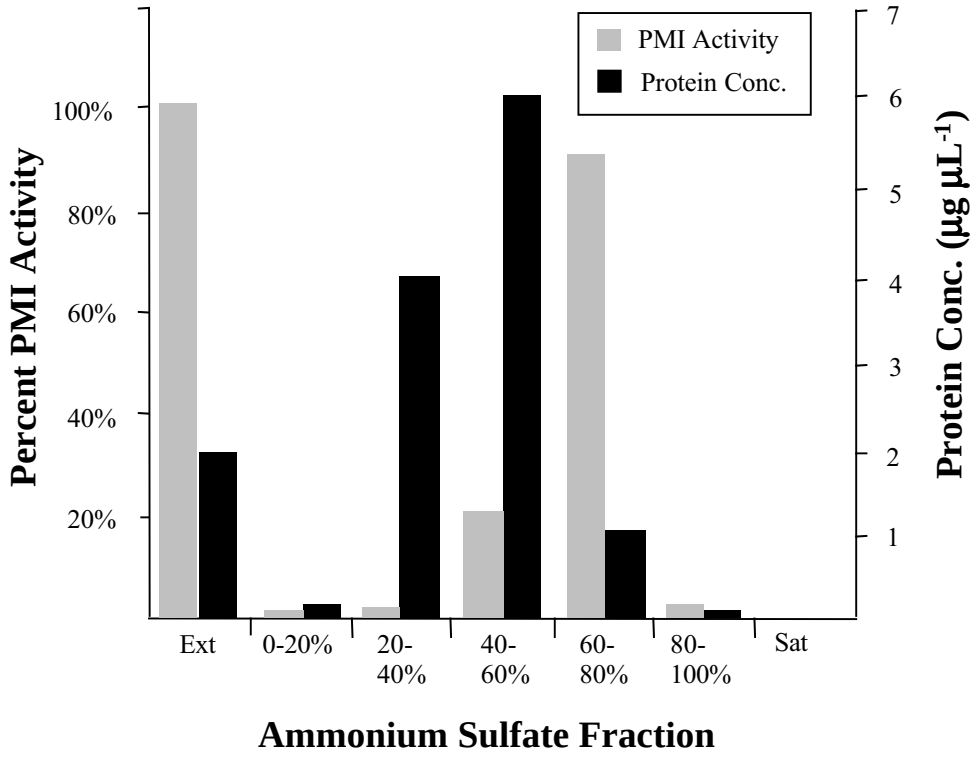
Fraction	Volume (mL)	Activity (μmol h^{-1})	Protein (mg)	Specific Activity (μmol mg^{-1} h^{-1})	Yield	Purification (fold)
Initial Extract	100	3300	214.48	15	100.0%	-
Ammonium Sulfate	20	2380	21.18	112	72.1%	7.5
Superdex™ 75	28.6	1056	5.20	203	32.0%	13.5
DEAE	40	199	0.56	352	6.0%	23.5

Figure Legends

Figure 1. Ammonium sulfate precipitation of total protein from *A.graveolens* var. *dulce* extraction. 110% of total PMI activity was recovered from the initial extract with 91% of the PMI activity being recovered in the 60-80% ammonium sulfate saturation fraction. This resulted in an 8.5-fold purification of the enzyme.

Figure 2. A typical Superdex^{TM} 75 column profile. Protein concentration was monitored by spectrophotometrically measuring A_{280}. PMI activity is expressed as the change in mOD min^{-1}. The path length of the reaction as assayed is unknown.

Figure 3. Column profile of the Fractogel^{TM} DEAE ion-exchange. Proteins were eluted with a linear KCl gradient. Protein concentration of the elutant was monitored by spectrophotometrically measuring A_{280}. PMI activity is expressed as the change in mOD min^{-1}. The path length of the reaction as assayed is unknown.


PMI Activity
Protein Conc.
Percent PMI Activity
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80%
60%
40%
20%
Protein Conc. (µg µL-1)
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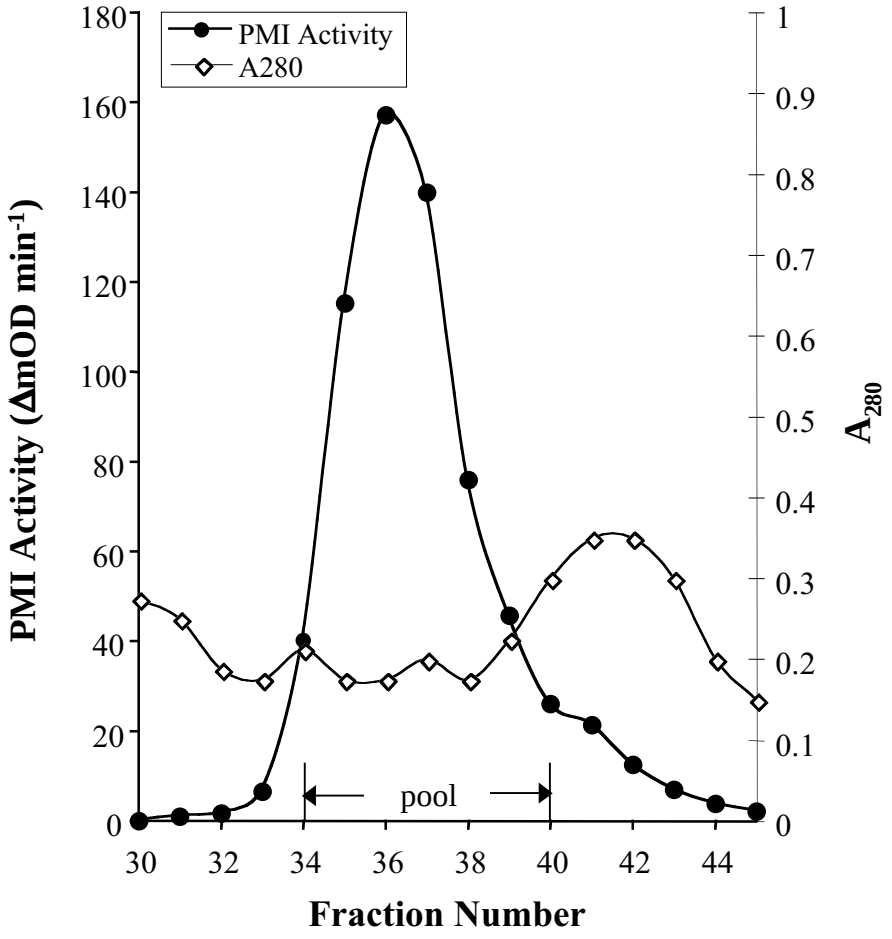
Ammonium Sulfate Fraction


PMI Activity
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PMI Activity (ΔmOD min-1)
A280
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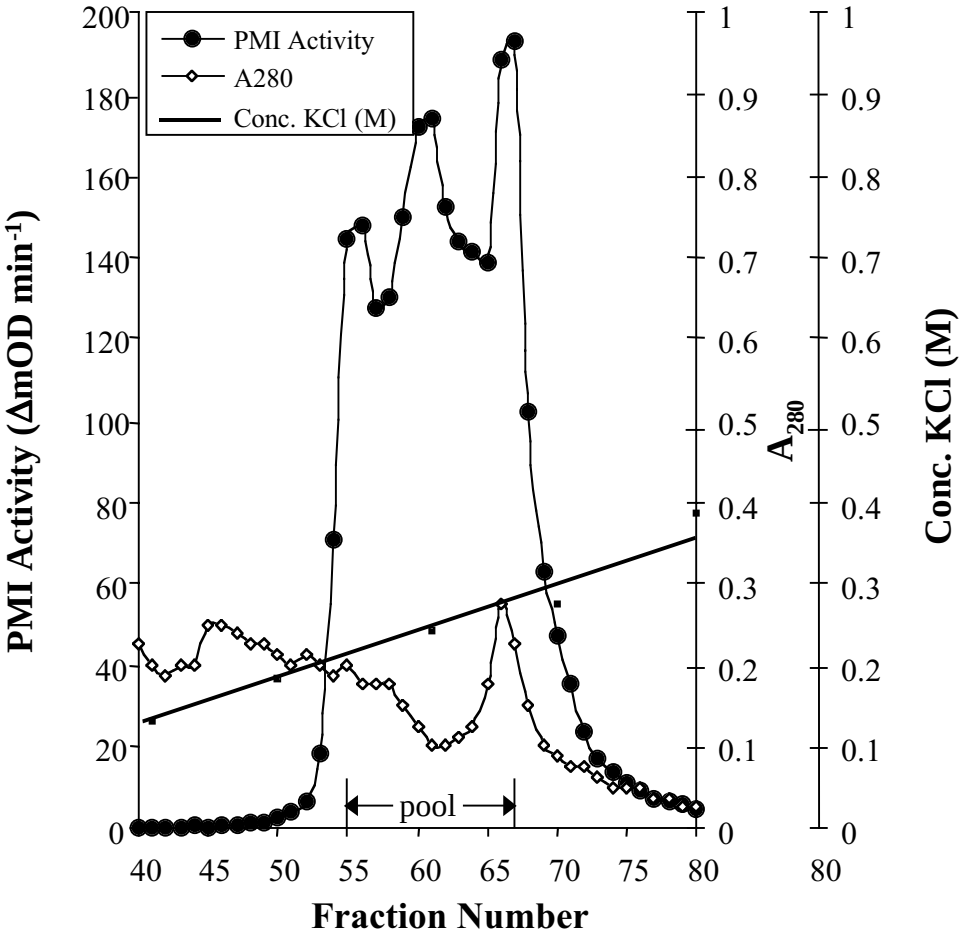
PMI Activity
A280
Conc. KCl (M)
PMI Activity (ΔmOD min-1)
A280
Conc. KCl (M)
Fraction Number
pool
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160
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Appendices

GenBank Entries

Included in this section are the nucleotide sequences of a partial PMI cDNA and a partial cDNA of 18S ribosomal RNA from *Nicotiana tabacum* as they were submitted to GenBank. Also included in these appendices are more detailed protocols and solution recipes for the procedures outlined in the Materials and Methods sections of Chapters 1 and 2. Both GenBank entries may be accessed using the world-wide web by typing the accession number into the search at the top of the National Center For Biotechnology Information web page (http://www.ncbi.nlm.nih.gov). The partial sequence for PMI will be available using this site beginning September 15, 2002. The partial sequence for 18S rRNA is currently available. A short description of the methods used to attain the 18S clone are included below. The description of the procedures used in attaining the PMI clone are described in Chapter 1.

While unplanned, the partial sequence for *Nicotiana tabacum* 18S RNA from NT1 cultures was recovered, and we felt it was prudent to submit this to the sequence database at NCBI. A primer set was designed that used 18S ribosomal RNA as a loading control for relative PCR. The primer 18SR (for sequence, see Material and Methods, Chapter 1) was included in the reverse transcription reaction to recover cDNA from ribosomal RNA. When the products of this reverse transcription reaction were combined with the PF1 primer (5'-AAGCAACCCAAGTGTTGTTGG; designed as an internal NT1 PMI primer) in a PCR reaction with no other primer, a 1042 bp band was recovered. This presumably used residual 18SR primer carried over from the reverse transcription reaction as the reverse primer in the PCR reaction. We did not expect that the PF1 primer would anneal to 18S RNA, but later sequence analysis revealed that this primer is highly

homologous to a inter-ribosomal spacer region on a transcribed, unprocessed pre-rRNA molecule. Two different clones of this fragment were sequenced in both directions, and the composite of these four sequence runs resulted in the 1042 bp sequence submitted to the database.

GenBank Entry for *Nicotiana tabacum* NT1 partial 18s rRNA clone

```
AY079155.   Nicotiana tabacum...[gi:19221239]
LOCUS       AY079155              1042 bp    DNA     linear   PLN 06-MAR-2002
DEFINITION  Nicotiana tabacum 18S ribosomal RNA gene, and internal
            transcribed spacer 1, partial sequence.
ACCESSION   AY079155
VERSION     AY079155.1  GI:19221239
KEYWORDS    .
SOURCE      common tobacco.
  ORGANISM  Nicotiana tabacum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta;
            Tracheophyta; Spermatophyta; Magnoliophyta;
            eudicotyledons; core eudicots; Asteridae; euasterids I;
            Solanales; Solanaceae; Nicotiana.
REFERENCE   1  (bases 1 to 1042)
  AUTHORS   Barb,A.W., Williamson,J.D. and Pharr,D.M.
  TITLE     Nicotiana tabacum partial 18s rRNA plus partial internal
            transcribed spacer sequence
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1042)
  AUTHORS   Barb,A.W., Williamson,J.D. and Pharr,D.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (15-FEB-2002) Horticultural Science, North Carolina
            State
            University, Box 7609 Kilgore Hall, Raleigh, NC 27695, USA
FEATURES             Location/Qualifiers
     source          1..1042
                     /organism="Nicotiana tabacum"
                     /db_xref="taxon:4097"
                     /cell_line="NT1"
     rRNA            complement(<1..577)
                     /product="18S ribosomal RNA"
     misc_RNA        complement(578..>1042)
                     /product="internal transcribed spacer 1"
BASE COUNT      273 a    285 c    243 g    241 t
ORIGIN
        1 GGCTGCTGGC ACCAGACTTG CCCTCCAATG GATCCTCGTT AAGAGGATTT AGATTGTACT
       61 CATTCCAATT ACCAGACTCA TAGAGCCCGG TATTGTTATT TATTGTCACT ACCTCCCCGT
      121 GTCAGGATTG GGTAATTTGC GCGCCTGCTG CCTTCCTTGG ATGTGGTAGC CGTTTCTCAG
      181 GCTCCCTCTC CGGAATCGAA CCCTAATTCT CCGTCACCCG TCACCACCAT GGTAGGCCAC
      241 TATCCTACCA TCGAAAGTTG ATAGGGCAGA AATTTGAATG ATGCGTCGCC GGCACGATGG
      301 CCGTGCGATC CGTCGAGTTA TCATGAATCA TCGCAGCAAC GGGCAGAGCC CGCGTCGACC
      361 TTTTATCTAA TAAATGCATC CCTTCCAGAA GTCGGGGTTT GTTGCACGTA TTAGCTCTAG
      421 AATTACTACG GTTATCCGAG TAGTAGATAC CATCAAACAA ACTATAACTG ATTTAATGAG
      481 CCATTCGCAG TTTCACAGTC TGAATTTGTT CATACTTACA CATGCATGGC TTAATCTTTG
      541 AGACAAGCAT ATGACTACTG GCAGGATCAA CCAGGTAGCA TTCCTTATTG ACGCCGGCAT
      601 CGCATGAGCA TGGCCGACCC AAAGGGCACC GCCAAGTCCA AGACGAGCAC GACCGTCATT
      661 CGTAAGGAGC ATTCTTTGGG CAAATAGGAG CCAATGAAGG CCCCATGCCC ATTGCGTTTA
      721 CCGAATCCGA GAGTCCGAGC ATACTAGTCA TGGACCAAGC CATCGCAAGG CAAAGCAAGG
      781 ACGACCTGGG ACACAACTAC AGCTTTTGGT TCACCCCGCA CGTCGAACGC GAGGGGCGAA
      841 AGGCAACCGA TTGCGCTTGA TAATGCCTTG GCATTAGGTA TGCAACACAG AAAACCGACA
      901 CCGAACAAGC CTAATTTGCG CTTGTGACAT GATTGAGATG GCATGCGGGC AAGGACGTCG
      961 CTGCTCAAGC AGGGATCCAA CCTAGCCACA CAAACCGAAC ACCACTCATG TGCCGCACGT
     1021 ACACTTGCCT CGCCGATACA AG
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GenBank Entry for *Nicotiana tabacum* NT1 partial PMI cDNA

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AF469471.   Nicotiana tabacum
LOCUS       AF469471                   243 bp    DNA     Release Date:9-15-02
DEFINITION  Nicotiana tabacum NT1 mannose-6-phosphate isomerase mRNA, partial
            cds.
ACCESSION   AF469471
VERSION     AF469471
KEYWORDS    .
SOURCE      common tobacco.
  ORGANISM  Nicotiana tabacum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta;
            Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons;
            core eudicots; Asteridae; euasterids I; Solanales; Solanaceae;
            Nicotiana.
REFERENCE   1  (bases 1 to 243)
  AUTHORS   Barb,A.W., Williamson,J.D. and Pharr,D.M.
  TITLE     Nicotiana tabacum culture selected for growth on Mannose has
            elevated phosphomannose isomerase activity
  JOURNAL   Plant Physiol (submitted)
REFERENCE   2  (bases 1 to 243)
  AUTHORS   Barb,A.W., Williamson,J.D. and Pharr,D.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-JAN-2002) Horticultural Science, North Carolina
            State University, Box 7609 Kilgore Hall, Raleigh, NC 27695, USA

FEATURES             Location/Qualifiers
     source          1..243
                     /organism="Nicotiana tabacum"
                     /db_xref="taxon:4097"
                     /cell_line="NT1"
     gene            <1..>243
                     /gene=”PMI” "
     cds             <1..>243
                     /gene=”PMI”
                     /note=”metabolic activity; E.C. 5.3.1.8”
                     /codon_start=1
                     /product=”mannose-6-phosphate isomerase, partial”
                     /translation=”DSGPSYLVKGAAENGLALTLKNWIESNPSVVGDKIVNKWG
                     SNLPFLFKVLSVAKALSIQAHPDKDLATRLHSELPDVYKDD”


BASE COUNT      62 a    47 c    62 g    72 t
ORIGIN
        1 GATTCTGGGC CTTCTTATCT TGTGAAAGGA GCAGCTGAGA ATGGATTGGC GTTGACATTG
       61 AAGAATTGGA TTGAAAGCAA CCCAAGTGTT GTTGGAGATA AGATTGTGAA CAAGTGGGGT
      121 TCCAACCTTC CTTTTCTCTT CAAGGTACTT TCTGTTGCAA AAGCTTTGTC CATACAGGCC
      181 CATCCAGACA AGGATTTGGC CACTCGTCTG CATAGTGAGC TCCCGGATGT TTATAAGGAT
      241 GAC

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Selected Protocols

Cell Preparation and Extraction

This was communicated by Dr. Wei Wen Guo from her recorded protocols in the laboratory of Dr. Mason Pharr and Dr. John Williamson (NC State University).

Cell Preparation / Freeze

- Prepare Büchner funnel aparatus
- Attach vacuum
- Wet Whatmann 1 circular filter paper
- From 75-150 mL culture, pour on cells
- Rinse with 150 mL dH_2O, repeat
- Prepare glass jars
- cut "X" in plastic lid
- label, add freezer tape
- Put washed cells in jar
- Pour ℓN_2 into jar, repeat until jar holds ℓN_2.
- Place jar in –80ºC.

Extraction Preparation

- Prepare Extraction Buffer.
- Prepare desalting column

  Be careful not to dry the columns by spinning the buffer out of the columns while packing, this is why only a 45 s spin is recommended.

  a. Setup clear (no filter) Büchner funnel apparatus w/o vacuum.

b. Pour pre swelled resin in (G-25). (Resin is prepared by autoclaving in H_2O for 20 min.)

c. Add ~150-200 mL dH_2O, suspend with rubber policeman.

d. Attach vacuum to remove wash.

e. Continue, wash with ~1 L.

f. Wash with 100 mL Equilibration Buffer (usually same as Extraction Buffer).

g. Resuspend occasionally over 10 min.

h. Add vacuum and remove Eq. Buffer.

i. Resuspend in a little Eq. Buffer.

j. Pour into columns.

k. Spin for 45-50 s at 500 G to remove Eq. Buffer.

Extraction

1. Weigh out 0.3 - 0.4 g. Record amount.
2. Grind into fine powder with ℓN_2.
3. Add 4x the weight of the tissue with Extraction Buffer. [ex- 0.400 g tissue→ 1.6 mL buffer]
4. Spin down extract in a microfuge tube for 1 min at <u>max speed</u>.
5. Load 700 µl of supernatant (extract) into previously prepared desalting column.
6. Centrifuge for 45-50 sec. (from start) at ~500 RPM. Keep sample on ice while assaying.
7. Store at –80°C.

Assay Enzyme Activity (Using Perkin Elmer Lambda Bio 20 Spectrophotomerter)

Warm up spectrophotometer 10 min before needed.

Computer→ double click on "Spectro."

Use "TD," time drive

Choose "T1 MTD"

Wave Length 340nm

Time 10 min

Time Interval 0.100 min.

AutoSave: ON

AutoPrint: ON

Set Sample # (choose 2x as many as you'll need, you can't return to a sample without stopping the method)

Press "START"

Insert blanks, proceed through method as directed.

PCR Analysis

All PCR analyses were completed using a MJ Research PTC 200 gradient thermal cycler. All of the protocols are developed from PCR methods communicated by Dr. Hisashi Koiwa (Purdue University). PCR primers were ordered from MWG Biotech (http://www.mwgbiotech.com). The goal of all homologous primer designs was to find a primer with a GC content that was between 40 and 50% and a length between 20 and 30 bp and contained at least one 3' G or C. The design of degenerate primers is described in Chapter 1 using CODEHOP (http://bioinformatics.weizmann.ac.il/blocks/help/CODEHOP/CODEHOP_program.html). It should be noted that only AmpliTaq Gold® (Applied Biosystems N808-0241) worked reliably with these primers.

Success or failure of a specific PCR reaction is usually based upon primer binding to the template molecule. Two sets of forward/reverse primers were designed for the amplification of a single product, and each tested against each other in a matrix design, across a 10°C gradient ranging from 52-62°C. Invariably, one or more of the primers will fail for an unknown reason. Additionally, it has been noted that $MgCl_2$ concentrations affect primer/template binding, but within the confines of the experiments reported in this manuscript, it was never necessary to change this parameter.

General PCR Reaction Mix (25µL), reagents added in this order to a PCR microfuge tube on ice. Total volume of all components (minus H_2O) is calculated, then the volume of H_2O is equal to 25 µL minus this calculated volume.

H_2O	to 25 µL final volume
PCR Buffer (minus $MgCl_2$)	
Usually supplied with *Taq* enzyme	to 10 % of final volume
$MgCl_2$	to 2.0 mM final concentration
Deoxy nucleotides	200 µM final concentration of each nucleotide
Forward Primer	to 0.5 µM final concentration
Reverse Primer	to 0.5 µM final concentration
Template	<1 µg in a volume less than 5 µL
Taq enzyme	0.25µL

General PCR program

1. 95°C	5 min
2. 94°C	20 s
3. 52-62°C (gradient)	20 s
4. 72°C	1 min 15 s / 1 kb
5. Go to Step 2	29 more times
6. 72°C	5 min
7. 4°C	indefinite hold.

PCR labeling of cloned pPMI-1

1. Same reaction concentrations as noted above were used as a control except the volume was increased to 50 µL.

2. In the DIG labeling reaction, the same 50 μL reaction mix described above was used, except, 1.75 μL of the Boehringer Mannheim (1 636 090) DIG PCR synthesis mix (10X) was added to the reaction mix with no decrease in any components.

After the PCR reaction, it is necesssary to test the incorporation of the DIG-dUTP into the DNA strand. To visualize the efficiency of DIG incorporation, 5 μL of unlabeled control DNA from the first reaction mix was run on an ethidium bromide stained agarose gel next to 5 μL from the second reaction mix. In a successful reaction, the DIG-labeled band will run about 10-15% larger than the unlabeled control. It is not necessary to further purify the PCR reaction before using it in a hydridization buffer for Northern analysis.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

This protocol is very similar to the instrctions provided with the reverse transcriptase enzyme (Super Script^{TM} II, Gibco BRL 18064-022). However, the amount of total RNA was repeatedly increased (5-10 µg) and no difference was observed. In order to verify the success of the protocol, 1µL of the final reaction mix was run on a 1% agarose gel. This would reveal a streak between about 200 bp and 1.5kb when visualized with ethidium bromide.

This procedure was adapted to be completed in a thermal cycler (PCR machine).

Reverse Transcription w/ SuperScript^{TM} II

20µl rxn volume, 1ng-5µg total RNA

Add to a PCR Microfuge Tube:

- 1µl Oligo dT (500 µg/mL)
- 1ng to 5µg total RNA
- 1µl 10 mM dNTP (10 mM each)
- ddH_2O to 13 µl

Heat to 65°C for 5 min and quickly chill on ice. Collect the contents of the tube by brief centrifugation and add:

- 4µl 5X First Strand Buffer
- 2µl 0.1M DTT

Mix Contents gently and incubate at 42°C for 2 min. Add:

- 1µl SuperScript II

Mix by pipetting up and down five or six times.

Incubate at 42°C for 50 min.

Heat at 70°C for 15 min.

Add 1µl RNase-H (10 mg/mL), incubate 37°C for 20 min.

Run a 1% agarose gel to test products.

RNA Northern Analysis

The RNA protocols are adapted from protocols generously provided by Dr. Marilyn Ehrenshaft (NC State University) for chemiluminescent visualization of the target message. In the detection of the message, here are some important points to note regarding this protocol:

- Preparation of the DIG-labeled DNA probe from cloned cDNA resulted in a much cleaner blot than a labeled probe from total reverse-transcribed cDNA.
- Adequate washing before and after the addition of the probe proved vital in the detection of presumably low copy number signals (Step 3, pre-hybridization and Step 5, high stringency washes.
- Fresh autoradiography film aided in the detection of a low signal message.
- Use FA buffer that is less than 1 month old and fresh loading buffer. If abnormalities in running the gel exist, replace the FA buffer.

Chemicals

DEPC ddH_2O 1 liter

1 l ddH_20

1 mL DEPC

Incubate for 24 h in fume hood with cap cracked. Occasionally, close cap tightly and invert bottle and mix thoroughly to coat inside.

Autoclave

1 mM EDTA 250 mL

mL 0.5M EDTA

DEPC ddH_2O to 250 mL

20x SSC 1 liter

Sodium Citrate 88.2 g

Sodium Chloride 175.3 g

pH to 7.0 with HCl or NaOH

ddH_2O to 1 liter

DEPC to 0.1%

Incubate for 24 h in fume hood with cap cracked. Occasionally, close cap tightly and invert bottle and mix thoroughly to coat inside.

Autoclave

Hybridization Buffer (or PerfectHyb Plus from Sigma) 250 mL

5x SSC 25 mL 20x SSC

0.1% (w/v) N-sarcosyl 1 mL 10%

0.02% (w/v) SDS 200 µl 10%

2% blocking reagent 2 g

DEPC ddH_2O to 250 mL

DO NOT AUTOCLAVE

Buffer 1 (Maleic Acid Buffer) 1 liter

11.6 g Maleic Acid (Caution, NOT Malic Acid)

8.8g NaCl

~40 pellets NaOH then base SLOWLY to pH 7.5

DEPC ddH_2O to 1 liter

DO NOT AUTOCLAVE

Buffer 2 (Blocking Buffer) Make Fresh

2% Blocking Reagent in Buffer 1

DO NOT AUTOCLAVE

Buffer 3 (Detection Buffer)	500 mL
0.1M Tris	6.05 g
0.1M NaCl	2.9 g

pH to 9.5 with HCl

DEPC ddH_2O to 500 mL

DO NOT AUTOCLAVE

Wash Buffer

Buffer 1 + 0.3% Tween-20 (v/v)

Anti-DIG AP Conjugate

(1 µl / 10 mL) DIG AP Conj in Buffer 2

10x FA	1 liter	500 mL
200mM MOPS	41.86g (Free Acid)	20.93
50mM NaAc	6.8g	3.4
10mM EDTA	20 mL 0.5M	10 mL 0.5M

pH to 7.0 w/ NaOH

DEPC to 0.1%

DO NOT AUTOCLAVE

FA / MOPS RNA Gel	100 mL
10 mL 10x FA	
1.0g Agarose	

90 mL DEPC ddH_2O

melt, cool to 65 C

add 0.5 ul 10 mg/mL EtBr

add 1.8 mL formaldehyde (37%)

mix well, pour

[can leave EtBr out, then stain membrane with 5 µg/100 mL in 1xFA, destain in 1xFA]

Running Buffer

50 mL 10xFA

10 mL formaldehyde

440 mL DEPC ddH_2O

RNA Loading Buffer (1µl buffer/ 4µl sample)

80µl 0.5M EDTA

720 µl formaldehyde

2 mL glycerol

3.084 mL deionized formamide

4 mL 10x FA

bromophenol blue and xylene cyanol (to taste)

DEPC ddH_2O to 10 mL

DO NOT AUTOCLAVE

Running RNA MOPS Gel

1. make Gel and pre-electrophorese at 65V for at least 30 min. Thaw sample, spin 2 min microfuge
2. Heat mixed samples to 65°C, centrifuge for 30 s in microfuge.
3. Wash wells thoroughly with running buffer.
4. Load 10-20 μg total RNA to each well and run at 50-60 V until bromophenol blue comes near end.
5. Record gel on UV light box
6. Wet membrane with H_2O, then 20X SSC.
7. Transfer gel to wick set up in the order listed below (a first through g) (remove all bubbles)
    a. 2 large filter paper (Whatmann 3M) into 20x SSC
    b. 1 filter paper gel size
    c. place gel, wells down
    d. MagnaGraph nylon membrane (Osmonics NJ0HY224F0)
    e. 2-3 filter papers gel size
    f. paper towel cut to gel size, stack 2-3" high
    g. weight, about 500 mg.
8. Leave at least 3 h
9. Tear apart set up. Take a picture of the membrane and gel on UV light box to check transfer.
10. Cross-link in a Stratalinker 1800 (Stratagene 400071) at 1200 μJoules

Detection of Message

1. Wet Dry membrane for 10 min in wash buffer (Maleic Acid Buffer + Tween 20).
2. Wet membrane in pre-hyb solution for 10 min before transferring to a hybridization tube in a roller oven.
3. Transfer wetted blots to hybridization tube and heat to 55-65°C. Add 15-20 mL pre-hyb solution for 2 h 30 min, ensuring that solution is covering the membrane. Incubate.
4. Boil PCR product (probe) for 5 min and add (1µl / mL) to pre-hyb solution in hybridization tube. Mix in bottom of tube so that undiluted probe does not contact membrane. Incubate for 12-24 hours.
5. Wash at 55-65C in (0.1-0.5x SSC, 0.2% SDS) in hybridization tube. Wash 2 times 15 min, then 1 time for 30 min. At this time turn on the 37°C incubator and move the CSPD to RT.
6. Move to Tupperware and shake in Wash Buffer RT twice for 2 min each.
7. Move to Blocking Solution (#2) and shake RT for 2 h. Can hold here for longer.
8. Move to Anti-DIG Solution and shake RT for 30 min.
9. Wash in Wash Buffer twice for 15 min.
10. Shake in Detection Buffer (#3) for 5 min.
11. Drop on CSPD to cover, cover with bag.
    a. Hold at RT for 5 min.
    b. Squeeze out excess and seal bag.

c. Incubate at 37°C to enhance detection for 10 min. At this point turn on autorad film developer.

12. Expose. For 1 to 16 h. Adjust as necessary.

Strip Membrane for Later Probing

1. Shake in Wash Buffer
2. Shake in boiling 0.5x SSC 0.2% SDS (Or any high stringency wash buffer)
3. Air dry between paper towels
4. Place between 2 3M blotting paper.
5. Wrap in aluminum foil or place in a plastic bag.

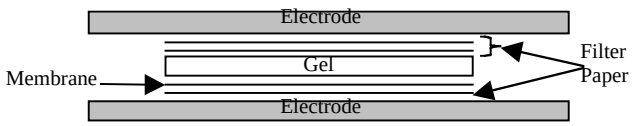
Alkali Labile Strip (not suitable for RNA)

1. 2x 20' wash in 0.2M NaOH + 0.1% SDS at 37°C
2. Rinse 2x SSC
3. Can be heat stripped or dried.

Western Protocol

This protocol is a hybrid of two protocols (the RNA Northern analysis (above), and the ECL™ (Amersham Pharmacia RPN 2106) Western blotting analysis system) to visualize protein amounts. As with the RNA Northern analysis, adequate washing of the blot before and after the addition of the antibody is essential.

1. Run 10% acrylamide Gel
    a. 150 V for 50 min
    b. Equilibrate Gel 15-30 min in Transfer Buffer
2. Transfer to Membrane (PVDF) using a Trans Blot® SD Semi-Dry Transfer Cell (BioRad 170-3940)
    a. Cut membrane and 3 filter paper sized to match equilibrated gel
    b. prewet membrane in 100% EtOH (PVDF in hydrophobic and will not adequately wet with H_2O)
    c. equilibrate in transfer buffer 10 min
    d. place filter paper, roll bubbles
    - place marked membrane (wells), roll out bubbles
    - place gel
    - place two filter papers, roll out bubbles
    - 12 V for 15 min. Current should be ~300-500 mA



3. Stain Membrane with Ponceau S stain to visualize transfer (not photographable).
    a. shake 5 min in Ponceau S Solution
    b. Wash with ddH_2O
    c. Reversible with 0.1M NaOH for 1 min.
4. Coomassie Blue Stain Gel
5. Shake gel in Coomassie Stain for 30-60 min
6. Wash 2x with ddH_2O
7. Add Destain and a foam tissue culture plug, shake until developed (1-2 h)
8. Preserve gel (dry, scan)
9. Block membrane in [5% blocking reagent or 1% BSA] in TBS-T for 2 h, shake, or over night at 2°C with no shaking.
10. Wash membrane in a 'large volume' of TBS-T briefly twice, then incubate for 15 min, then 2x 5 min.
11. Dilute primary antibody 1:10,000 in TBS-T. (It may be necessary to use a primary antibody concentration that is less that if the blot were visualized using AP)
12. Incubate membrane in the primary antibody solution for 1 h.
13. Wash membrane in a 'large volume' of TBS-T briefly twice, then incubate for 15 min, then 2x 5 min.
14. Dilute secondary antibody (1:10,000).
15. Incubate the membrane in the secondary antibody solution for 1 h.
16. Wash membrane in a 'large volume' of TBS-T briefly twice, then incubate for 15 min, then 2x 5 min.

17. Shake the membrane in detection buffer (RNA) for 5 min.
18. Drop on CSPD to cover, cover with bag.
19. Hold 5 min RT, squeeze out excess and seal bag, removing air bubbles.
20. Incubate 5-15 min 37°C to enhance detection.
21. Drop on CSPD to cover, cover with bag.
    a. Hold 5 min at RT, squeeze out excess and seal bag
    b. Incubate for 5-15 min at 37°C to enhance detection
22. Expose for 1 h. Adjust as necessary.