

Phosphomannose Isomerase, A Novel Selectable Plant Selection System: Mode of Action and Safety Assessment

Laura S. Privalle, Martha Wright, Janet Reed, Geneviève Hansen, John Dawson, Erik M. Dunder, Yin-Fu Chang, M. Luann Powell & Moez Meghji

SUMMARY

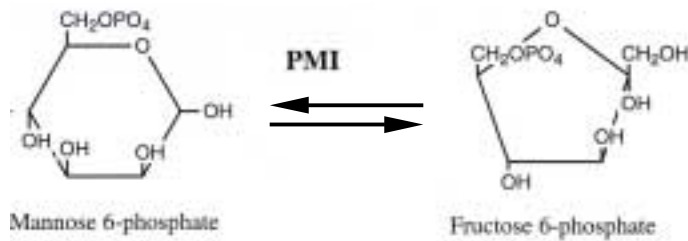
Phosphomannose isomerase (PMI), an enzyme not present in many plants, catalyzes the reversible interconversion of mannose 6-phosphate and fructose 6-phosphate. Plant cells lacking this enzyme are incapable of surviving on synthetic medium containing mannose. Plants derived from sugar beet and corn cells that had been genetically modified to express the *E. coli manA* gene encoding PMI were evaluated for biochemical changes in mannose-associated pathways. No detectable changes in glycoprotein profiles were detected in PMI-transformed plants as compared to non-transgenic controls. Purified PMI protein was readily digestible in a simulated gastric environment. Purified PMI protein demonstrated no adverse effects in an acute mouse oral toxicity study. The yield and nutritional composition of grain from PMI-transformed corn plants compared to their non-transformed isogenic counterparts were also determined and no statistically significant differences were found. These results indicate that PMI is an ideal selectable marker for plant transformation.

INTRODUCTION

Phosphomannose isomerase (PMI, EC 5.3.1.8) catalyzes the reversible interconversion of mannose 6-phosphate and fructose-6-phosphate (Figure 1A). PMI has utility as a selectable marker for transformation of many plant species (Bojsen *et al.*, 1994; Joersbo *et al.*, 1998) because expression of the *E. coli manA* gene encoding PMI allows the cells to utilize mannose as a carbon source and survive on media containing mannose. PMI is common in nature and found across kingdoms. However, PMI is less ubiquitous in the plant kingdom, having been reported to be present in soybeans but absent in many plants (Goldsworthy & Street, 1965; Lee & Matheson, 1984). The gene encoding this activity has been cloned from several bacteria and yeast species, as well as from humans (Miles & Guest, 1984; Darzins *et al.*, 1986; Shinaberge, *et al.*, 1991; Collins and Hackett, 1991; Schmidt *et al.*, 1992; Smith *et al.*, 1992; Smith *et al.*, 1993; Proudfoot *et al.*, 1994b). The enzyme has been purified and characterized from yeast, bacteria, pigs and humans (Proudfoot *et al.*, 1994a, b). Lack of the active enzyme in humans is associated with carbohydrate-deficient glycoprotein syndrome whose symptoms include hereditary fructose intolerance, galactosaemia, and hyperinsulinemic hypoglycemia (Keir *et al.*, 1999, deLonlay *et al.*, 1998).

Mannose and mannose derivatives are common constituents of living cells and are key components of intermediary metabolism (Figure 1B). Mannose is phosphorylated by hexokinase to mannose 6-phosphate and in the presence of PMI enters the glycolytic pathway after isomerization to fructose 6-phosphate. Mannose is also frequently the major sugar residue in the carbohydrate portion of glycoproteins. Recently mannose has been identified as a precursor for ascorbate synthesis (Wheeler *et al.*, 1998).

A.



B.

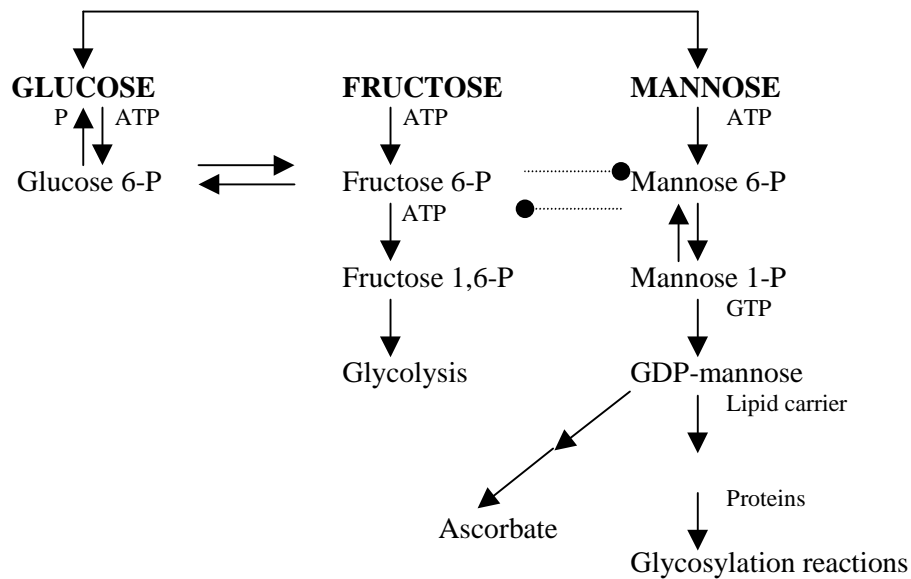


Figure 1. A. Reaction catalyzed by phosphomannose isomerase. B. Basic intermediary metabolism involving mannose in nonleguminous plant cells not transformed with PMI. The reaction catalyzed by PMI is indicated by the dashed lines and rounded arrows (.....●).

This presentation summarizes the plant species for which the PMI/mannose selection system have proven to be successful, reviews our current understanding about its mode of action and presents the safety assessment data that have been collected to date.

PLANT TRANSFORMATION

Plant cells genetically transformed to express PMI acquire a growth advantage (positive selection) on mannose-containing media. They are protected both from the negative impact of mannose derivatives (i.e. mannose 6-phosphate, see next section) and can utilize the mannose as a carbon source. Hence with the PMI gene, mannose is a useful selectable agent for the generation of transgenic plants (Hansen and Wright, 1999). This selection system was reported to increase sugarbeet transformation frequencies 10 fold compared to kanamycin selection (Joersbo *et al.*, 1998).

The table below summarizes the plant systems for which PMI and mannose have proved useful:

| Plant | Transformation Method | Target Tissue | Transformation Frequency* | Reference |
|-------------|-----------------------|-----------------------|---------------------------|-------------------------------|
| Maize | Biolistic | Callus | 50% (75%) | Wright <i>et al.</i> , 2000 |
| | Agrobacterium | Embryos | 30% (90%) | Negrotto <i>et al.</i> , 2000 |
| | Protoplasts | Protoplasts | - | Evans <i>et al.</i> , 1996 |
| Wheat | Biolistic | Callus | 25% (45%) | Wright <i>et al.</i> , 2000 |
| Sugar beet | Agrobacterium | Cotyledonary explants | 0.94% | Joersbo <i>et al.</i> , 1998 |
| Arabidopsis | Agrobacterium | Floral buds | - | Melanson <i>et al.</i> , 1999 |
| Rice | Agrobacterium | Embryos | 10 – 20% | Unpublished |
| Canola | Agrobacterium | Microspore embryos | 1% | Unpublished |
| Barley | Biolistic | Embryos | 3% (6%) | Unpublished |
| Tomato | Agrobacterium | Cotyledonary petioles | | Unpublished |

*Transformation frequency = the number of independent events obtained/number of targets for which transformation was attempted. Numbers between brackets indicate the maximum achieved.

MODE OF ACTION

The effect of mannose on plants was first described over 40 years ago as inhibiting respiration in wheat and tomato (Stenlid, 1954; Morgan & Street, 1959). This impact actually results from the accumulation of mannose 6-phosphate, which inhibits phosphoglucose isomerase, thus blocking glycolysis (Goldsworthy & Street, 1965). Other impacts include depletion of the pyrophosphate required for ATP production (Goldsworthy & Street, 1965; Herold & Lewis, 1977) and transcriptional repression of genes associated with photosynthesis and glyoxylate cycle (Jang & Sheen, 1994, 1997; Graham *et al.*, 1997).

Using traditional transformation protocols, plant cells are placed on culture media containing salts, hormones and a carbon source, usually sucrose. For the PMI/mannose selection system, plant tissues are cultured on a similar medium supplemented with either mannose as the unique source of carbon or with media containing both sucrose and mannose. While mannose has no direct adverse effect on plant cells, the subsequent selection is considered to be a consequence of its phosphorylation to mannose 6-phosphate by hexokinase. In the absence of PMI the mannose 6-phosphate accumulates and the cells stop growing. Stein & Hansen (1999) reported that mannose 6-phosphate itself induces apoptosis. The authors have identified a mannose 6-phosphate induced nuclease that is responsible for the development of the laddering of DNA, a characteristic of apoptosis.

SAFETY ASSESSMENT

Purified PMI protein, as well as whole plants derived from transformation events containing the gene and expressing the PMI protein, have been subjected to the event-independent portions of the typical safety assessment used in the registration process of transgenic plants. To assess mammalian safety, an allergenicity evaluation as well as an acute oral mouse toxicity test were conducted. The agronomic characteristics of plants from multiple independent corn transformation events have been monitored. The nutrient composition of grain from these events has been compared with that from their conventional counterparts.

Allergenicity Evaluation

Three approaches were taken to evaluate the allergenicity potential of PMI or plants that have been transformed to express this protein:

- 1) A search of public databases (e.g. Genbank, EMBL) was conducted. The database search revealed no significant homology of the *E. coli manA* gene product to any known toxin or allergen.
- 2) *In vitro* digestibility experiments were conducted to determine if PMI protein was readily digestible as conventional dietary protein. Allergenic food proteins are typically resistant to proteolytic degradation (Alpers, 1987; Taylor *et al.*, 1992; Franck-Oberaspach & Keller, 1997). The *in vitro* digestibility of PMI was monitored in both simulated mammalian gastric fluid containing pepsin (SGF) and simulated mammalian intestinal fluid containing pancreatin (SIF, U.S. Pharmacopoeia, 1990). These results are relevant to the overall safety assessment of PMI in transgenic plants, as the primary route of exposure, if any, to PMI would be dietary. PMI was rapidly degraded in SGF such that no intact PMI was detected upon immediate sampling of the reaction mixture (Figure 2A). PMI was also rapidly degraded in SIF and no intact PMI was detected after a 2 min incubation at 37°C (Figure 2B).

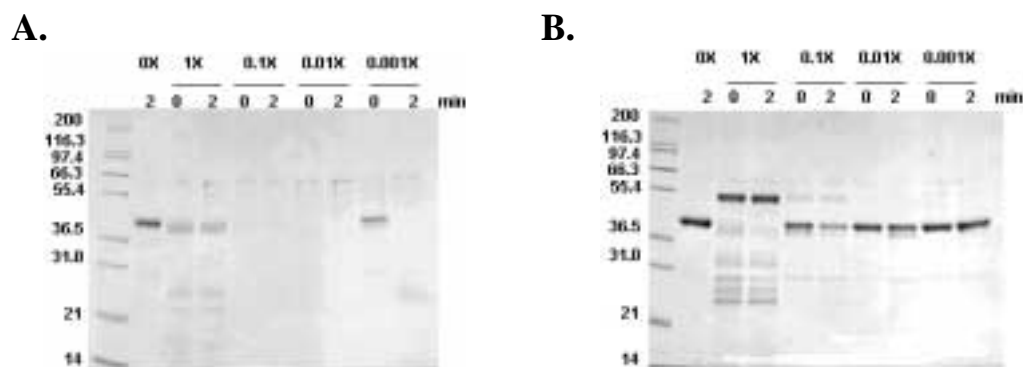
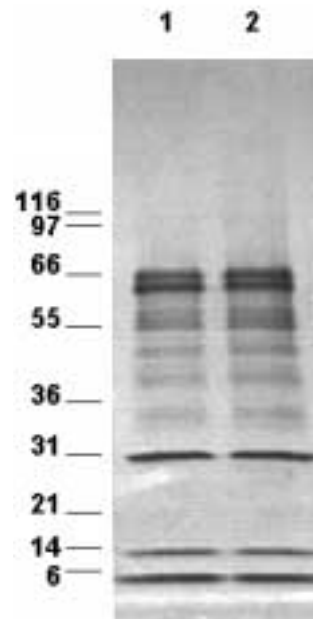


Figure 2. Digestion of PMI (approx. 45,000 mol. wt.) in SGF prepared at various concentrations of pepsin (approx. 40,000 mol. wt.) and SIF. Digestion reactions were prepared and PMI degradation was visualized via Coomassie blue staining. A. SGF was used at the standard concentration of pepsin (1X) and also prepared with 0.1X, 0.01X, and 0.001X the standard pepsin concentration. SGF prepared without pepsin was designated 0X. B. SIF was used at the standard concentration of pancreatin (1X) and also prepared with 0.1X, 0.01X, and 0.001X the standard pancreatin concentration. SIF prepared without pancreatin was designated 0X. Each lane represents a sample that initially contained approx. 6 µg of PMI. Molecular weight standards ($\times 10^{-3}$) are indicated.

These data indicate that PMI expressed in transgenic plants will likely be readily digested as conventional dietary protein under typical mammalian gastric conditions. Furthermore, in the unlikely event that PMI protein survives the gastric environment, it will rapidly be degraded in the intestines.

- 3) The third approach was to determine if the glycoprotein profiles differed in PMI transgenic maize and sugar beets from their negative segregants¹. This has relevance in the safety assessment of such transgenic plants as glycosylation is one common characteristic of known food allergens (Yunginger, 1991). The glycoproteins having affinity for Concanavalin A Sepharose were compared from transgenic plants expressing PMI with those from isogenic non-PMI-expressing plants. Concanavalin A binds mannose and glucose residues in the glyco portion of the glycoprotein. Samples obtained from plants from a representative dicot, sugar beet, and a representative monocot, maize, were used in this study. The results for maize are shown in Figure 3. No differences in the silver staining protein patterns of the glycoproteins isolated from the PMI-expressing and non-expressing plants were detected. Similar results were found for sugar beets (data not shown). This suggests that no unintended effects on the process of protein glycosylation would be expected to occur in PMI transgenic plants.

Figure 3. Silver stained 8- 16% polyacrylamide gel of proteins from PMI positive and negative segregants of maize which bound to a Concanavalin A-bound Sepharose column. Lane 1; PMI positive segregant, 1 μ g protein. Lane 2; PMI negative segregant, 1 μ g protein. Molecular weights ($\times 10^{-3}$) are indicated.



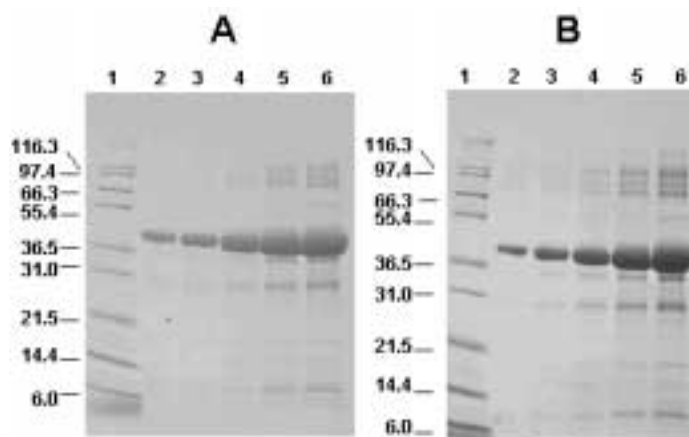
Toxicity Testing

PMI protein was purified from an *E. coli* over-expression system by differential ammonium sulfate precipitation followed by hydrophobic interaction chromatography. Following dialysis to remove salts, the resulting lyophilized protein preparation was determined to contain approx. 61% PMI by weight and was active in catalyzing the interconversion of mannose 6-phosphate to fructose 6-phosphate. PMI comprised approx. 95% of the total protein in this sample. An acute oral mouse toxicity study was conducted by an independent testing facility following standard protocols. The PMI sample was administered by gavage to 13 albino mice (6 females and 7 males) at a target dose of 5050 mg/kg body weight (3030 mg PMI/Kg body weight). A negative

¹Negative segregants are progeny that, through Mendelian segregation, have not inherited the transgenes, despite having a transgenic parent(s).

control group receiving only the dosing solution (carboxymethyl cellulose) was run concurrently, and mice in both groups were observed 14 days post-dosing. There were no clinical signs of toxicity, no effect on body weight gain in both groups and gross necropsy of all mice revealed no observable abnormalities. Based on the lack of adverse findings, the LD₅₀ was determined to be greater than 5050 mg/kg body weight (3030 mg PMI/Kg body weight). Re-analysis of the PMI sample following completion of the acute oral mouse toxicity study (approx. three months after the initial characterization) yielded comparable results for all parameters, including catalytic activity and purity, indicating that the sample was stable during this period (Figure 4).

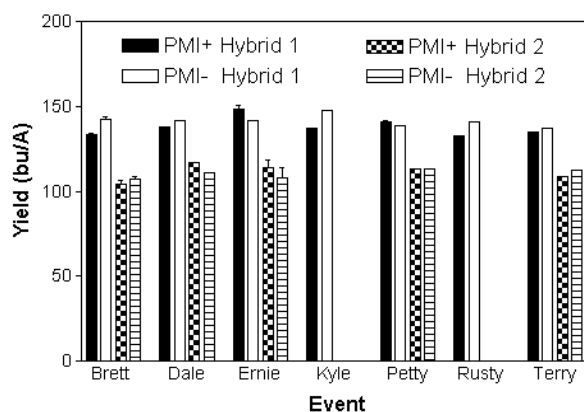
Figure 4. Molecular weight confirmation and purity determination of PMI. Coomassie blue-stained 14% polyacryl-amide SDS gel. PMI is approx. 45,000 mol. wt. A. Initial Analysis. B. Re-analysis. Lane 1, molecular weight markers ($\times 10^{-3}$); lanes 2 – 5 contain 1, 2, 5, 10, and 20 μg of protein, respectively.



Agronomic Characteristics

The agronomic characteristics of plants from seven independent transformation events expressing PMI were compared with their isogenic nontransgenic counterparts in field trials conducted in 1998. The parameters examined included moisture, stand, stalk quality, root lodging, green snap, plant height, ear height, incidence of Stewarts wilt, stay green, #plants emerge, dropped ears, gray leaf spot rating and yield. There were no statistical differences in any of these parameters between the transgenic and conventional plants. The yield data are shown in Figure 5 and are from 7 different PMI events in two different hybrid backgrounds. These studies indicate that expression of the PMI gene in maize plants from different events resulted in no adverse effect on growth or other agronomic characteristics.

Figure 5. Yield. Mean number of bushels per acre from two hybrid pairs from seven independent events are shown. For hybrid 1, PMI transgenic (■), nontransgenic (□); hybrid 2, PMI transgenic (▣) nontransgenic, (▤). Standard deviations are indicated.



Compositional Analysis

Grain from these 7 independent PMI transgenic maize events and their corresponding non-transgenic counterparts was analyzed for various nutritional components including proximates (moisture, ash, fiber, fat and protein), starch, β -carotene, and xanthophylls (Figure 6). No statistically significant differences were found between the PMI transgenic events and the PMI negative segregants. These findings indicate that events expressing the PMI gene are indistinguishable from their non-transformed counterparts for these measured parameters.

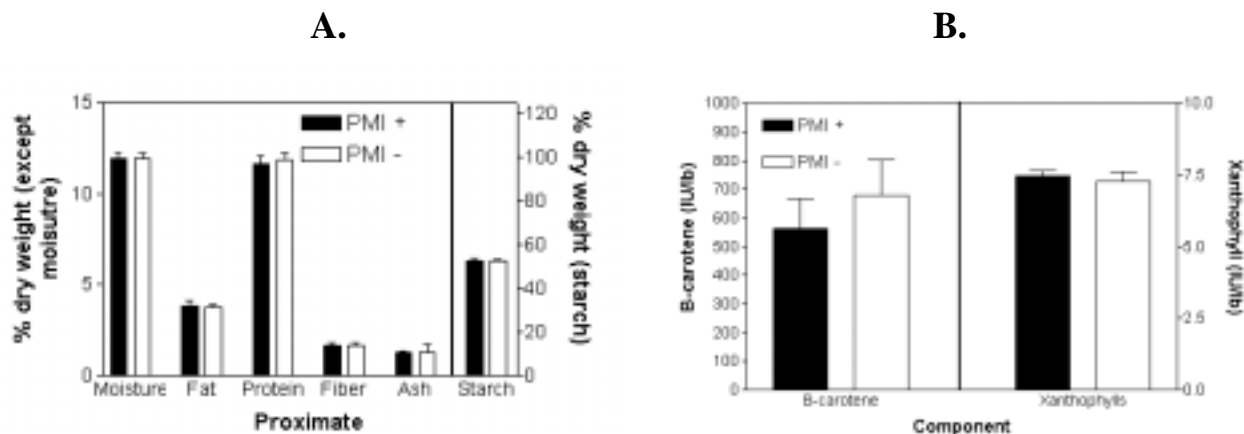


Figure 6. Compositional Analysis. A. Moisture, fat, protein, fiber, ash and starch levels in grain from PMI-transgenic plants (■) and isogenic nontransgenic conventional plants (□) are compared. B. Xanthophylls and β -carotene are compared. Standard deviations are indicated.

DISCUSSION

PMI and other potential enzymes in intermediary metabolism can be candidates as selectable markers for the plant transformation process, replacing more traditional antibiotic or herbicide selection systems. In this paper, *E. coli* PMI has been shown to be an effective selectable marker in multiple dicot and monocot crop species. PMI genes from other sources are also being examined. A similar positive selection system utilizing xylose isomerase, which catalyzes the reversible isomerization between D-xylose and D-xylulose, has been shown to be an effective selectable marker in tomato, potato and tobacco (Haldrup, *et al.*, 1998). Furthermore, the preliminary safety assessment of PMI found no adverse effects with respect to mammalian toxicity, allergenicity or unintended effects on the agronomic or nutritional composition of the plant events.

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