

Phosphomannose Isomerase, a Novel Plant Selection System

Potential Allergenicity Assessment

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ABSTRACT: 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. Thus PMI/mannose selection has utility in the identification of transformed plant cells. As part of the safety assessment transgenic plants undergo before commercialization, PMI has been evaluated for its potential allergenicity. Purified PMI protein was readily digestible in a simulated gastric environment. PMI has no sequence homology to known allergens, does not contain multiple disulfide bonds, and has no *N*-glycosylation consensus sequences. No detectable changes in glycoprotein profiles were detected in PMI-transformed plants as compared to nontransgenic controls. These results indicate that PMI lacks many of the attributes associated with known oral allergens.

KEYWORDS: allergenicity assessment; phosphomannose isomerase; transgenic plants; oral allergens

INTRODUCTION

One critical aspect of the safety assessment of transgenic crops is the allergenicity potential of the novel gene product. Approximately 1–2% of the adult population worldwide have true food allergies, defined as responding to oral allergens by the production of IgE resulting in the allergic response cascade. Unfortunately, much remains unknown about why certain proteins can cause these responses and why only certain predisposed individuals are affected. Therefore, the allergenicity potential of novel dietary proteins, includ-

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ing those that may be expressed in genetically enhanced crop plants, is difficult to assess. Primary considerations are centered on the source of the transgene—is it from a food source known to be allergenic? Many of the current products use genes from sources not normally part of the food chain, so paths of evaluation other than those used for foods are necessitated. General characteristics of known oral allergens are described below.

Oral allergens are proteins that may be 10,000–70,000 daltons in molecular mass. Multiple exceptions exist, and there are certainly many proteins of this size range that are not oral allergens. The Brazil nut allergen is less than 10,000 daltons, whereas the native peanut allergens are large polymers with molecular masses in the 200,000- to 300,000-dalton range.

- Many oral allergens are glycosylated. Glycosylation is common among oral allergens; however, many oral allergens are not glycosylated, and many glycosylated proteins are not oral allergens.
- Oral allergens may contain multiple disulfide bonds. Many allergenic proteins, such as lactoglobulin from milk, lose their ability to induce an allergenic response when their disulfide bonds have been reduced.¹ However, many allergens have no disulfide bonds.
- Oral allergens are frequently resistant to proteolytic digestion. In order to elicit the allergenic response, it is believed that the protein must survive the gastric system to be able to come into contact with the Peyer's patches in the intestine. Therefore, the rate of digestibility of the protein in simulated gastric fluid is examined because a highly digestible protein is less likely to elicit an allergenic response. We measure digestibility as one component of our safety evaluation for registration of the transgenic crop product. Separate laboratories have compared relative digestibility of known food allergens, and a whole range of rates of digestion exist, from not readily digested to rapidly digested.^{2,3}
- Oral allergens may share conserved amino acid epitopes. A computer search of allergen databases for sequence similarities at the 8–12 amino acid epitope size is conducted as part of the registration of any transgenic product.
- Some, but not all, oral allergens are heat stable. This parameter may simply be a consequence of multiple disulfide bonds.
- Oral allergens are generally present as a high percentage of the protein in the food source. In general, the allergenic protein ranges from 1 to 30% of the protein in the food supply. An exception is the major allergen of codfish, parvalbumin, which is found at lower quantities.⁴

In this chapter we describe the preliminary allergenicity evaluation of a novel plant selectable marker, phosphomannose isomerase (PMI). These re-

sults are part of the overall safety and environmental assessment evaluation of PMI that also includes an acute oral mouse toxicity test, agronomic and yield comparisons, and compositional analysis.

PHOSPHOMANNOSE ISOMERASE

Phosphomannose isomerase (PMI, EC 5.3.1.8) catalyzes the reversible interconversion of mannose-6-phosphate and fructose-6-phosphate (FIG. 1A). PMI has utility as a selectable marker for transformation of many plant species,^{5,6} because expression of the *E. coli manA* gene encoding PMI allows the

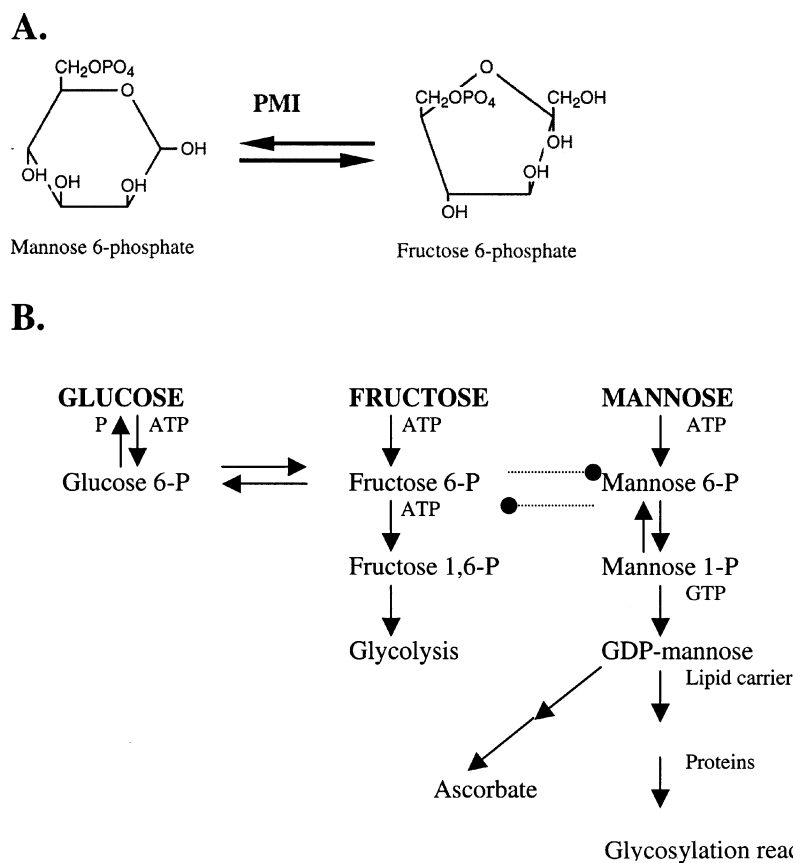


FIGURE 1. (A) Reaction catalyzed by phosphomannose isomerase. (B) Basic intermediary metabolism involving mannose in nonleguminous plant cells not transformed with PMI. The reaction catalyzed by PMI is indicated by the dotted lines and filled circles (.....●).

cells to use mannose as a carbon source and survive on media containing mannose. PMI is common in nature and is found across kingdoms. PMI is less ubiquitous in the plant kingdom, however, having been reported to be present in soybeans but absent in many plants.^{7,8} The gene encoding this activity has been cloned from several bacteria and yeast species, as well as from humans.^{9–16} The enzyme has been purified and characterized from yeast, bacteria, pigs, and humans.^{16,17} Lack of the active enzyme in humans is associated with carbohydrate-deficient glycoprotein syndrome, the symptoms of which include hereditary fructose intolerance, galactosemia, and hyperinsulinemic hypoglycemia.^{18,19}

Mannose and mannose derivatives are common constituents of living cells and are key components of intermediary metabolism (FIG. 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.²⁰

The effect of mannose on plants was first described over 40 years ago as inhibiting respiration in wheat and tomato.^{22,23} This impact actually results from the accumulation of mannose-6-phosphate, which inhibits phosphoglucose isomerase, thus blocking glycolysis.⁷ Other impacts include depletion of the pyrophosphate required for ATP production^{7,23} and transcriptional repression of genes associated with photosynthesis and glyoxylate cycle.^{24–26}

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 and Hansen²⁷ 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.

This chapter summarizes the allergenicity assessment of PMI that has been undertaken as part of the safety evaluation process necessary to achieve registration of a transgenic crop product containing this selectable marker.

SAFETY ASSESSMENT

Purified PMI protein and 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. Presented here is the allergenicity evaluation.

Allergenicity Evaluation

Several approaches have been 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) A search of an in-house allergen database constructed from public databases and containing over 900 sequences was conducted, looking for sequence identity of 8 amino acid stretches. This database search revealed no significant homology of the *E. coli manA* gene product to any known allergen.

(3) Examination of the amino acid sequence of PMI revealed no *N*-glycosylation consensus sequence. Consensus *N*-glycosylation sites have the sequence –NX(S or T)X–, where X can be any amino acid except proline, N is asparagine, S is serine, and T is threonine. *N*-glycosylation is the most common form of glycosylation; however, *O*-glycosylation can occur at any serine or threonine residue, of which, like most proteins, PMI contains many. PMI contains only two cysteines and therefore can contain at most one disulfide bridge.

(4) *In vitro* digestibility experiments were conducted to determine the rate and degree to which PMI protein is digested under conditions expected to be encountered in a typical monogastric stomach and small intestine. Allergenic food proteins are typically resistant to proteolytic degradation.^{28–30} The *in vitro* digestibility of PMI was monitored in both simulated mammalian gastric fluid containing pepsin (SGF) and simulated mammalian intestinal fluid containing pancreatin (SIF³¹). These results are relevant to the overall safety assessment of PMI in transgenic plants, because 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 (FIG. 2A). PMI was also rapidly degraded in SIF, and no intact PMI was detected after a 2-min incubation at 37°C (FIG. 2B). In order to demonstrate a time course of PMI degradation, the pepsin concentration in the SGF was reduced to 0.0001× the standard concentration in a separate experiment.

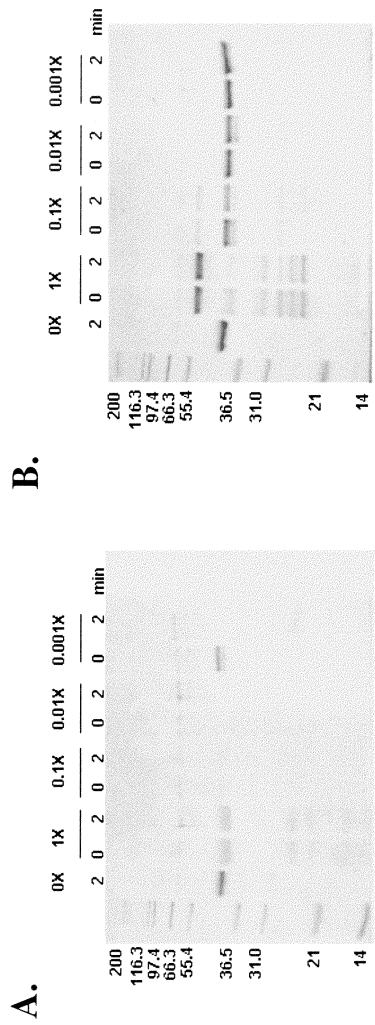


FIGURE 2. Digestion of PMI (approx. 45,000 MW) in SGF prepared at various concentrations of pepsin (approx. 40,000 MW) and SIF. Digestion reactions were prepared, and PMI degradation was visualized via Coomassie blue staining. **(A)** SGF was used at the standard concentration of pepsin (1X) and also 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 approximately 6 μ g of PMI. Molecular weight standards ($\times 10^{-3}$) are indicated.

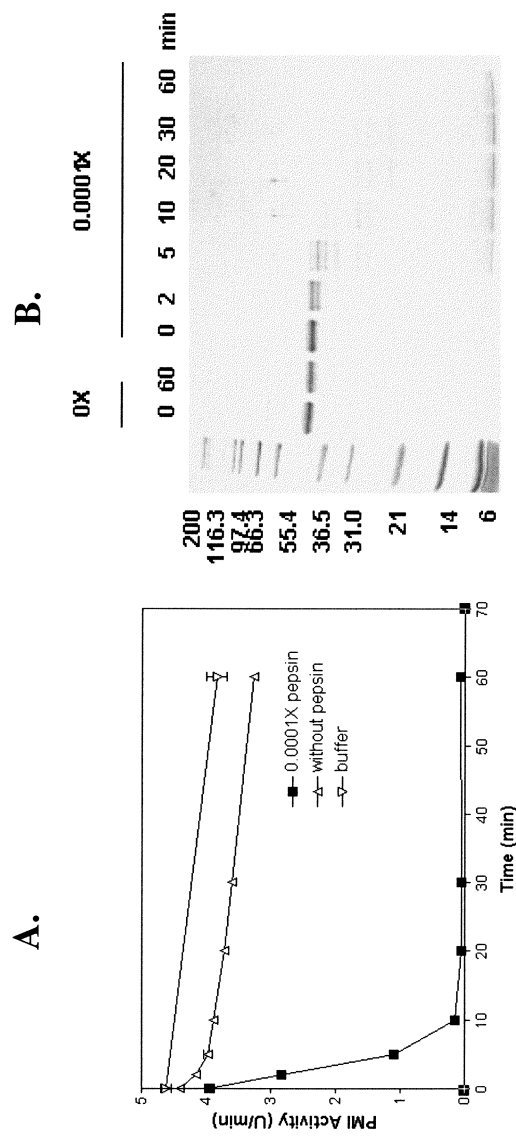


FIGURE 3. Time course of inactivation and digestion of PMI (approx. 45,000 MW) in 0.0001X pepsin in SGF. **(A)** Enzymatic inactivation. Reactions were without pepsin (—Δ—), with 0.0001X the standard pepsin concentration in SGF (—■—), or in buffer (—▽—). **(B)** Digestion. Coomassie blue staining was used to visualize proteolytic degradation of PMI. Samples were incubated for the indicated times in 0.0001X the standard concentration of pepsin in SGF. The control was incubated in SGF minus pepsin (0X) for the indicated times. Each lane represents a sample that initially contained 10 μg of sample PMI-0198, corresponding to approximately 6 μg of PMI. Molecular weight standards ($\times 10^{-3}$) are indicated.

Under these conditions, both PMI protein (FIG. 3B) and enzymatic activity (FIG. 3A) were undetectable after 10 min at 37°C. 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.

(5) The final approach was to determine whether the glycoprotein profiles differed in PMI transgenic maize and sugar beets from their negative segregants. (Negative segregants are progeny that, through Mendelian segregation, have not inherited the transgenes, despite having a transgenic parent[s].) This has relevance in the safety assessment of such transgenic plants because glycosylation is one common characteristic of known food allergens.³² The glycoproteins having affinity for concanavalin A–Sepharose from transgenic plants expressing PMI were compared 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 4. No differences in the silver-staining protein patterns of the glycoproteins isolated from the PMI-expressing and nonexpressing plants were detected. Similar results were found for sugar beets (data not shown). This

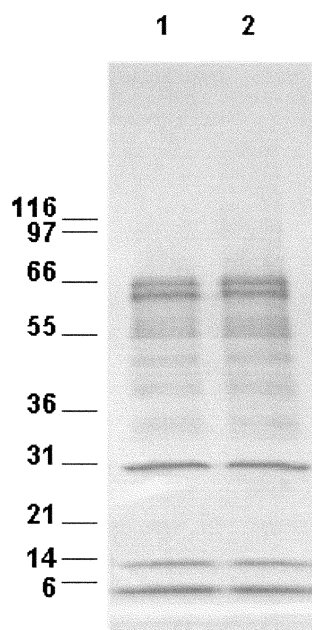


FIGURE 4. Silver-stained 8–16% polyacrylamide gel of proteins from PMI-positive and -negative segregants of maize that bound to a concanavalin A-bound Sepharose column. *Lane 1:* PMI-positive segregant, 1 µg protein. *Lane 2:* PMI-negative segregant, 1 µg protein. Molecular weights ($\times 10^{-3}$) are indicated.

suggests that no unintended effects on the process of protein glycosylation would be expected to occur in PMI transgenic plants.

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. *E. coli* PMI has been shown to be an effective selectable marker in multiple dicot and monocot crop species. As part of the preliminary safety assessment of PMI, its potential allergenicity was examined. No evidence of potential allergenic properties was found associated with PMI. It was readily digested as conventional dietary protein, possessed no sequence homology to known allergens, contained no *N*-glycosylation consensus sequence, and does not have multiple disulfide bonds. Additional safety-related studies have also been conducted,³³ and the results indicated no adverse effects; therefore, PMI is an ideal selectable marker for plant transformation.

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