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Effective selection of transgenic papaya plants with the PMI/Man selection system

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Abstract The selectable marker gene phospho-mannose isomerase (*pmi*), which encodes the enzyme phospho-mannose isomerase (PMI) to enable selection of transformed cell lines on media containing mannose (Man), was evaluated for genetic transformation of papaya (*Carica papaya* L.). We found that papaya embryogenic calli have little or no PMI activity and cannot utilize Man as a carbon source; however, when calli were transformed with a *pmi* gene, the PMI activity was greatly increased and they could utilize Man as efficiently as sucrose. Plants regenerated from selected callus lines also exhibited PMI activity but at a lower specific activity level. Our transformation efficiency with Man selection was higher than that reported using antibiotic selection or with a visual marker. For papaya, the PMI/Man selection system for producing transgenic plants is a highly efficient addition to previously published methods for selection and may facilitate the stacking of multiple transgenes of interest. Additionally, since the PMI/Man selection system does not involve antibiotic or herbicide resistance genes, its use might reduce environmental concerns about the potential flow of those genes into related plant populations.

Keywords Cell selection systems · Genetic transformation · Plant tissue culture · Mannose · Phospho-mannose isomerase (PMI)

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Abbreviations

BA	Benzyladenine
2,4-D	2,4-Dichlorophenoxyacetic acid
IBA	Indole-3-butyric acid
Man	Mannose
NAA	α-Naphthaleneacetic acid
PMI	Phospho-mannose isomerase
Suc	Sucrose

Introduction

Plant transformation technology usually relies on the introduction of a selectable marker gene to facilitate the identification and selection of the population of cells that are transformed from those that are not. For higher plants, there are only a few well-characterized selection systems, and these are generally limited to technologies for increasing resistance to antibiotics or herbicides (Brasileiro and Aragão 2001) and may not work efficiently with all species of interest. In addition, the paucity of selection options can be a constraint for the stacking of multiple transgenes in a given plant line and consequently may be a barrier to a wider adoption of transformation technology. Sequential "stacking" of multiple transgenes in elite cultivars will require either a new selectable marker gene for each new character gene introduced or a technology for removing the initial selectable marker gene so that the selectable marker gene can be used again for the subsequent transgenic event (Hare and Chua 2002). An additional consideration is that antibiotic and herbicide resistance genes in widely grown crops may pose real or perceived threats of transfer to weedy relatives or microorganisms. Lastly, although all evidence indicates that selection genes pose no health threat to human or animal consumers, there is a definite possibility of poor consumer acceptance of food products containing antibiotic-inactivating proteins. For all of these reasons, improved selection gene technology is critical for the advance of plant transgenic research and development.

Phospho-mannose isomerase (PMI, EC 5.3.1.8) is an enzyme that converts mannose (Man) to mannose-6-phosphate (Man-6-P). Because PMI is not present in many plants and as plant cells lacking PMI are not capable of surviving on synthetic medium containing Man, a PMI/Man selection system has been investigated for its potential in identifying transformed plant cells. The use of PMI as a selectable protein for plant transformation was first described by Bojsen et al. (1999), who showed that expression of the bacterial *pmi* gene would allow Man-sensitive plant cell cultures of potato (*Solanum tuberosum* L.), sugarbeet (*Beta vulgaris* L.), and corn (*Zea mays* L.) to grow on Man as their carbon source. This group patented the PMI/Man selection system (Bojsen et al. 1998, 1999) that has been used successfully with Arabidopsis (*Arabidopsis thaliana* L.) (Todd and Tague 2001), cassava (*Manihot esculenta* Crantz) (Zhang et al. 2000), corn (Negrotto et al. 2000; Wang et al. 2000; Wright et al. 2001), rice (*Oryza sativa* L.) (Datta et al. 2003; He et al. 2004; Hoa et al. 2003; Lucca et al. 2001), sugarbeet (Joersbo et al. 1998, 1999), sweet orange (*Citrus sinensis* L. Osbeck) (Boscariol et al. 2003), wheat (*Triticum aestivum* L.) (Wright et al. 2001), and pearl millet (O'Kennedy et al. 2004).

The selection gene, *pmi* (*manA* from *Escherichia coli*), is widespread in nature. It does not confer resistance to either antibiotics or herbicides and therefore has no potential for conferring a selective advantage to weeds or microorganisms. The transformation efficiency using the PMI/Man system has been reported to be as high and sometimes considerably higher (Boscariol et al. 2003; Joersbo et al. 1998; Lucca et al. 2001; Wright et al. 2001) than with more traditional antibiotic or herbicide selection. PMI is readily digested in a simulated gastric environment, has no adverse effects in an acute mouse oral toxicity study, generates no detectable biochemical changes in the Man-associated pathway (Privalle et al. 1999), lacks many of the attributes associated with known oral allergens (Privalle 2002), and may thus be considered as an ideal selectable protein for plant transformation.

In this paper, we evaluate the use of the PMI/Man system for papaya (*Carica papaya* L.) transformation using biolistic bombardment.

Materials and methods

Plant materials and embryogenic callus culture

Hypocotyls of aseptically grown 10-day-old seedlings of papaya (*Carica papaya* L.) cv. Kapoho were excised and sliced into 2- to 3-mm-thick sections (Fitch 1993). From each seedling, approximately 50 2.0-mm-thick sections were placed in 100×15-mm petri plates on 25 ml half-strength MS salts medium (Murashige and Skoog 1962), pH 5.8, containing 30 g l^{-1} sucrose (Suc), 2.5 g l^{-1} Phytagel, and 10 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), as previously described (Fitch et al. 1990). Tissue cultures developed in the dark at 27°C with monthly subcultures for 2 months or 3 months to produce somatic embryogenic calli that were multiplied for up to 6 months with monthly subcultures under the same embryogenic conditions.

Effect of Man on growth and development of papaya callus cultures

Non-transformed somatic embryogenic callus cultures were plated on the same half-strength MS salts medium used for callus initiation but for which Suc was replaced with Man at 0, 0.5, 1, 2, 5, 10, 20, or 30 g l^{-1} with the total always being 30 g l^{-1}. Five 100-mg fresh weight callus clusters were transferred to tissue culture plates, replicated five times for each Man level, then incubated in the dark at 27°C for 4 weeks. At the end of the first cycle of growth, each callus was weighed before a second subculture on fresh medium. At the end of the second cycle, calli were weighed again and then transferred to plates containing regeneration medium consisting of half-strength MS medium without 2, 4-D, but in its place were added 0.2 mg l^{-1} benzyladenine (BA) and 0.2 mg l^{-1} α-naphthaleneacetic acid (NAA), and 30 g l^{-1} Suc was substituted in place of Man. Regeneration was visually evaluated and recorded from five replicate plates containing three calli clusters that had been cultured on medium containing different levels of Man for 2 months with monthly subcultures.

Particle bombardment and selection of transformants

Plasmid pNOV3610, provided by Novartis (now Syngenta; http://www.syngenta.com), contains a plant-expressible *pmi* gene driven by the *Arabidopsis Ubq*3(*At*) ubiquitin promoter (with its first intron) for selection on Man. The plasmid DNA was coated onto 1.6-μm gold particles (Bio-Rad, Hercules, Calif.; http://www.bio-rad.com) and delivered into embryogenic calli as previously described (Fitch et al. 1990) using a PDS 1000 Helium (Bio-Rad) device. Each target plate containing approximately 1 g fresh weight embryogenic callus was shot three times. Bombarded embryogenic calli recovered on half-strength MS embryogenesis medium containing 10 mg l^{-1} 2, 4-D, pH 5.8, 3% Suc, and 2.5% Phytagel for 10 days. Each bombarded callus clump, considered as an independent event, was divided into five or six small pieces that were labeled as 'replicates' during selection and regeneration. The selection of bombarded embryogenic calli was performed on half-strength MS embryogenesis medium with Man (3%) replacing all Suc for 2 months with monthly subcultures. Regeneration from Man-resistant callus was for 1–2 months without selection by replacing the 3% Man with 3% Suc. Plants regenerated from any of the replicate lines were considered as arising from the same transformation event.

Growth of selected callus on Suc or Man

After 2 months of selective growth on Man, five equal-sized (approx. 100 mg) replicates of two of the PMI-selected

callus lines, PMI 51 and PMI 60, and one non-transformed callus line were plated on half-strength MS medium containing either Suc or Man at 30 g l^{-1} to compare fresh weight growth on the two sugars. At the end of 1 month, fresh weights of the individual calli were recorded, and calli were transferred to fresh media of the same composition for determining weights at the end of a second 1-month cycle.

Regeneration, micropropagation, rooting, and acclimation of plants

After 2 months of selection on 3% Man, the putatively transformed callus produced plantlets on half-strength MS medium with 3% Suc and containing 0.2 mg l^{-1} BA and 0.2 mg l^{-1} NAA. Proliferating shoots were cut into nodal sections and subcultured on the same medium once a month according to Fitch (1993). Roots were initiated by placing freshly cut shoots on MS medium containing 2 mg l^{-1} IBA for 7 days, followed by their removal to MS medium containing no growth regulators. Rooted plants were potted in sterilized vermiculite wetted with half-strength MS medium until dense masses of roots developed. Plants were transplanted into larger pots containing steam-sterilized potting soil for greenhouse acclimation to full sunlight before being transplanted to the field.

Molecular analysis of transgenic plants

Genomic DNA was extracted from approximately 20 mg of callus and leaf tissues using the method described by Wang et al. (2000). PCR analysis was carried out using the published procedure of Negrotto et al. (2000). Each 25-µl PCR reaction contained 1.5 U *Taq* DNA polymerase, 10 m*M* Tris-HCl (pH 9.0), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 200 µ*M* of each dNTP, 1.0 µ*M* of each primer, and bovine serum albumin (BSA) as a stabilizer. The PCR reactions were subjected to 30 cycles of 30 s at 94°C, 30 s at 55°C, and 45 s at 72°C using a Perkin-Elmer 9600 thermal cycler (PE-Applied Biosystems, Foster City, Calif.) The primers used to amplify a 550-bp fragment of the *pmi* transgene were: PMI-U (18 mer) 5′-ACAGCCACTCTCCATTCA-3′ and PMI-L (18 mer) 5′- GTTTGCCATCACTTCCAG-3′. The primers used as a positive control to amplify a 300-bp fragment of the *actin* endogenous gene were: ACT-U (20 mer) 5′-ACTACGAGTTGCCTGATGGA-3′ and ACT-L (20mer) 5′-AACCACCACTGAGCACAATG-3′. Reverse transcriptase (RT)-PCR was carried out using PMI-specific primers according to the standard protocols. The primers used to amplify a 250-bp fragment of the *pmi* cDNA were: Man-1 (20 mer) 5′-ACATCCGGCGATTGCTCACT-3′ and Man-2 (20 mer) 5′-GCAGGTAAGCGTGCGGTGTT-3′. The PCRs were subjected to 30 cycles of 30 s at 94°C, 30 s at 55°C, and 45 s at 72°C using a Bio-Rad iCycler thermal cycler.

PMI enzyme activity assay

The PMI assays of calli and leaf tissues were based on the modified protocol of Wang et al. (2000). Plant tissues (250 mg) were ground in 250 µl of 50 m*M* Tris-HCl in liquid nitrogen and centrifuged at 14,000 rpm for 30 min at 4°C. The supernatant (50 µl) was added to 100 µl 50 m*M* Tris-HCl (pH 7.5), mixed with 100 µl of reaction mixture, and incubated for 30 min at 37°C to remove any endogenous D-Man-6-P. The reaction mixture consisted of 25 µl β-nicotinamide dinucleotide phosphate (β-NADP, 10 m*M*; Sigma, St. Louis, Mo.), 25 µl phosphoglucose isomerase (PGI, 10 U/ml, EC 5.3.1.9; Sigma), 12.5 µl glucose-6-P dehydrogenase (G6PDH, 10 U/ml, EC 1.1.1.49; Sigma), and 32.5 µl Tris-HCl (50 m*M*, pH 7.5). At the end of a 30-min incubation at 37°C, the substrate of 5 µl of D-Man-6-P (50 m*M*) was added to the assay solution, and the optical density was measured over 30 min in an MRX plate reader (Dynex Technologies, Frankfurt, Germany; http://www.dynextechnologies.com) at 340 nm. As a positive control, PMI purified from *E. coli* (100 U/ml, EC 5.3.1.9; Sigma; http://www.sigmaaldrich.com) was substituted for the plant extract. PMI activity was calculated from the slope of absorbance over time using Biolinx (http://www.dynextechnologies.com) software. Protein concentration of the extract was determined with the Bradford method (Bradford 1976).

Data analyses

Data for callus growth and PMI activities were subjected to a one-way analysis of variance (ANOVA) using the SAS (http://www.sas.com) software followed by mean separation using the Waller-Duncan K-ratio *T*-test.

Results and discussion

Toxic effects of Man on growth and regeneration of non-transformed papaya cell cultures

When plant cells lacking the enzyme PMI are cultured on medium containing Man in place of Suc, the cells convert Man to Man-6-P but are unable to isomerize Man-6-P to fructose-6-phosphate (Fru-6-P) and are thus deprived of a carbon source and become depleted of phosphorous (Barb et al. 2003). The present research showed that non-transformed papaya callus has little or no PMI activity and that its growth was inhibited in culture by Man at any concentration greater than 5 g l^{-1} (Fig. 1). The inhibitory effects of Man were greater during the second month of subculture than during the first. Callus weight at the highest concentration of Man (30 g l^{-1}) was only about one-third that of the control, and this callus did not appear normal. It was brownish, watery, and had less turgor than did callus on control medium without Man.

Overall, there was an exponential decrease in the percentage of plant regeneration from non-transformed calli

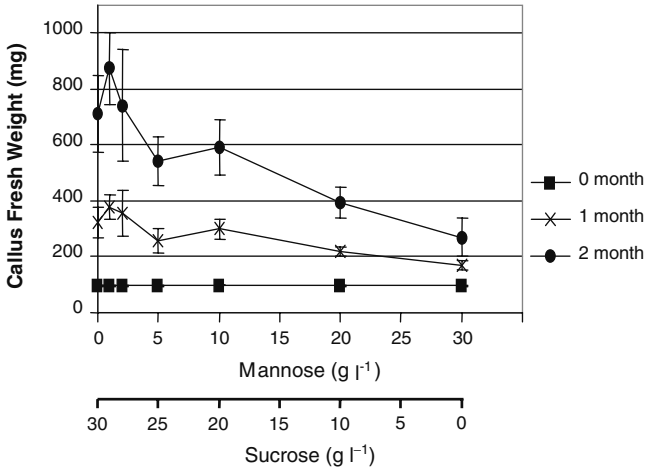

Fig. 1 Fresh weight ± standard error of non-transformed (wild type) papaya embryogenic callus grown in the dark for up to 2 months on half-strength MS salts medium containing 0–30 g l^{-1}mannose and 30–0 g l^{-1} sucrose. The initial fresh weight of callus was approximately 100 mg

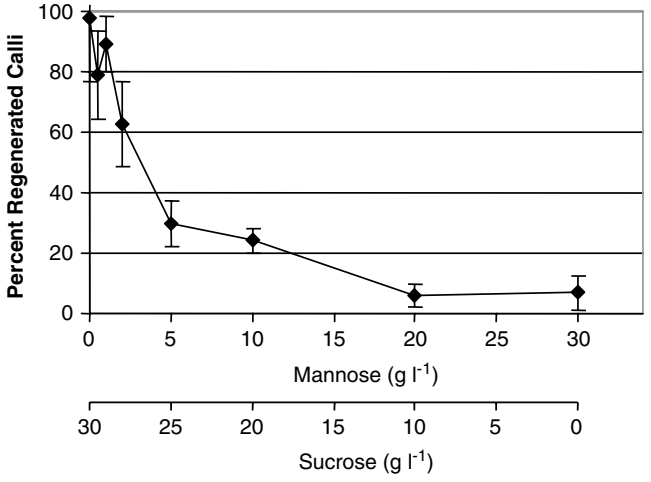

Fig. 2 Percentage ± standard error of callus clusters from which plants regenerated at the end of 2 months after transfer from 2 months of culture in the dark on half-strength MS salts medium containing mannose concentrations of 0–30 g l^{-1} and sucrose concentration of 30–0 g l^{-1} to fresh half-strength MS salts medium containing 30 g l^{-1} sucrose in place of mannose

that had been grown for 2 months on various levels of Man (0.5–30 g l^{-1}, Fig. 2). When Man was substituted for Suc at concentrations of 5 g l^{-1} or more, callus regeneration decreased to less than 50% of that of the controls, with the lowest regeneration, about 6%, occurring at 20 g l^{-1} and 30 g l^{-1} Man.

Effects of Man on growth and regeneration of PMI-transformed papaya cell cultures

The PMI-transformed and Man-selected callus appeared yellow and healthy and grew vigorously on 30 g l^{-1} Man without Suc, while the non-transformed callus turned watery and brownish and increased in fresh weight slowly

Table 1 Comparative fresh weights of non-transformed and PMI-selected callus lines of papaya grown on half-strength MS medium supplemented with either Suc or Man at 30 g l^{-1}. Initial fresh weight of each callus was 100 mg. *SE* standard error

Callus	Suc medium		Man medium	
	One-month (mg ± SE)	Two-month (mg ± SE)	One-month (mg ± SE)	Two-month (mg ± SE)
Non-transformed	324±55	711±137	170±18a	270±70a
Transgenic PMI 51	298±42	681±150	302±39b	674±122b
Transgenic PMI 60	274±35	639±130	269±40b	625±107b

[a]Weight values in the same column followed by different letters are significantly different ($P \leq 0.05$) as determined by ANOVA and the Waller-Duncan K-ratio *T*-test

or not at all under the same conditions. Plants regenerated from Man-resistant calli were micropropagated, rooted, and acclimated to pot culture under greenhouse conditions. The time required for the selection and regeneration steps varied slightly from experiment to experiment, but it was generally possible to recover Man-resistant plants within 13–17 weeks from bombardment, with 1 week in recovery, 8 weeks in Man selection, and 4–8 weeks in plant regeneration. Man-resistant plants did not exhibit any obviously aberrant morphology. We are currently growing these plants to maturity to test for sexual transmission of the *pmi* gene.

Comparative growth of non-transformed calli and PMI-selected calli on 30 g l^{-1} of either Man- or Suc-containing media indicated that *pmi*-transformed calli can utilize Man as effectively as Suc for its carbon source (Table 1). However, when replicates of these same lines of calli were grown on 30 g l^{-1} Man, fresh weights of the non-transformed line were only about one-half as large as when grown on Suc. This is in contrast to growth on Man by the PMI-selected calli, which increased in fresh weight by about threefold per month, which was the same as when they were grown on Suc. The weights of non-transformed and PMI-selected calli grown on 30 g l^{-1} Man differed ($P \leq 0.05$) as determined by ANOVA and the Waller-Duncan K-ratio *T*-test.

In the experiments to evaluate wild-type callus growth on Man, the media containing 5–10 g l^{-1} Man also contained 25–20 g l^{-1} Suc, which is sufficient carbon to support papaya callus growth (Fitch et al. 1990). Nevertheless, growth was inhibited. Therefore, the growth inhibition seen with Man is presumably due to a specific inhibitory effect of Man, perhaps phosphate and/or ATP depletion (Joersbo et al. 1998) rather than carbon depletion. With Arabidopsis, as little as 5 m*M* Man significantly inhibits seed germination, and this inhibition is not caused by ATP and/or phosphate depletion (Pego et al. 1999). In contrast to papaya callus growth, seed germination inhibition in Arabidopsis was significantly reversed by adding metabolizable sugars, including Suc, so it seems that Man inhibition in the two systems and/or tissues occurs by different mechanisms.

Table 2 Number of transformed papaya callus obtained from PMI selection

Trial	Number of culture plates bombarded	Transformed callus recovered (total)	Independent transformed lines per gram fresh weight of embryogenic calli (mean ± SE)
1	4	86	(20.6±1.2)
2	4	79	

Efficiency of the PMI/Man selection system for transforming papaya

Our PMI/Man selection protocol produced approximately 20 plant lines per bombarded plate of 1 g fresh weight callus, yielding a total of 165 transformed plants from 8 g callus (Table 2). These 165 plants, obtained from callus lines that had been cultured separately for 2 months on medium containing Man as the only carbon source, are tentatively considered as the minimum number of independent events, since the callus lines were isolated from one another 10 days after bombardment. Our frequency of recovery of resistant papaya lines using the PMI gene and Man selection is significantly higher than that reported with the neomycin phosphotransferase II (*npt*II) gene and G418 selection where 0.26 transgenic lines were recovered per gram of bombarded callus (Fitch et al. 1990). The present rate is also higher than that reported for visual identification of transgenic lines with the green fluorescence protein (GFP) gene, which produced eight lines per gram bombarded callus (Zhu et al. 2004). Reasonable rates of transformation (1.6 transgenic lines per gram embryogenic callus) have been reported using *Agrobacterium* (Cheng et al. 1996), but we have found it difficult to disinfect *Agrobacterium*-treated papaya cultures.

Calli surviving Man selection but not containing/expressing the PMI gene (escapes) were rare; of the 75 Man-resistant lines tested for PMI enzyme activity, all but two had activity significantly higher than that of the wild-type control, and 95% had at least fivefold more activity than the wild type (Fig. 3). Ten Man-resistant callus lines that were positive for PMI activity were tested by PCR; all contained the expected 550-bp PCR-amplified DNA fragment indicative of the *pmi* gene fragment (Fig. 4). The results of RT-PCR of the same ten lines using *pmi*-specific primers (data not shown) indicated that the *pmi* transgenes were transcriptionally active in plants. This apparently low rate or lack of escapes is an especially attractive feature of the PMI/Man system that translates into reduced time and expense for producing transgenic papaya lines.

PMI activity in transformed papaya

PMI activity was measured in a random sample of 75 of the 165 selected callus lines to evaluate the expression of the *pmi* gene in papaya callus. PMI activity varied by about 20-fold between the highest and lowest activity expressed in the PMI/Man-selected lines (Fig. 3). Of the 75 independent representative lines tested, 71 lines (95%) had PMI enzyme activity at least fivefold higher than that of non-transformed lines.

PMI activity was also measured in leaves of plants regenerated from selected callus. For all 71 of the fivefold higher selected callus lines, the PMI activity was lower

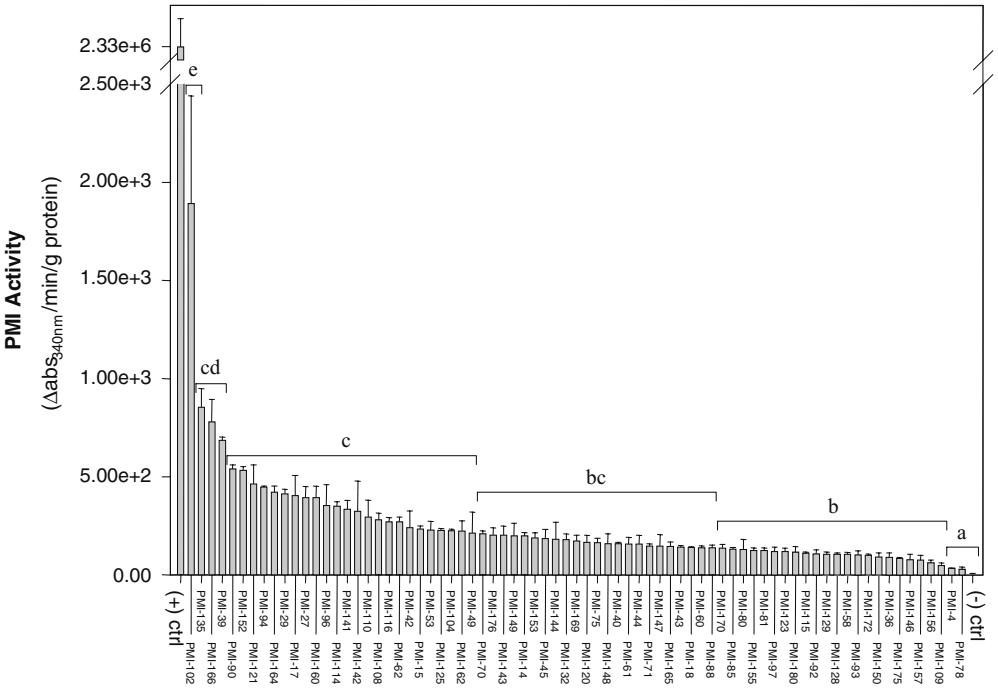

Fig. 3 Phospho-mannose isomerase activity ± standard error in 75 PMI transformed callus lines after 2 months of selective growth on culture medium consisting of half-strength MS salts and 30 g l^{-1} mannose. The cluster of callus lines included in *square brackets* (*a*–*e*) are significantly different ($P \leq 0.05$ as determined by ANOVA and the Waller-Duncan K-ratio *T*-test) from adjacent clusters. + Control, purified *Escherichia coli*-expressed PMI as a positive control, − control, non-transformed control

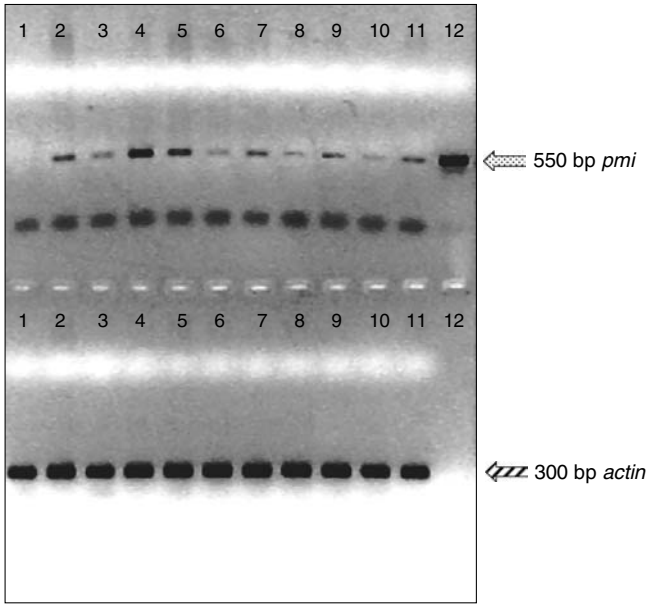

Fig. 4 Agarose gel electrophoresis image of PCR-amplified DNA from ten selected lines with PMI activity from transformed callus lines after 2 months of selective growth on culture medium consisting of half-strength MS salts and 30 g l^{-1} mannose. *Arrow* at the *upper half* of the gel shows an amplified 550-bp fragment of the *pmi* transgene. *Arrow* at the *lower half* of the gel shows an amplified 300-bp fragment of the endogenous *actin* gene. *Lanes*: *1* products of PCR amplification of non-transformed Kapoho callus, *2–11* products of PCR amplification of ten mannose-resistant callus lines that were positive for PMI activity. *Lanes 2–11* PMI callus lines 153, 115, 142, 123, 042, 148, 014, 060, 044, 125, respectively, *lane 12* products amplified from the transformation plasmid

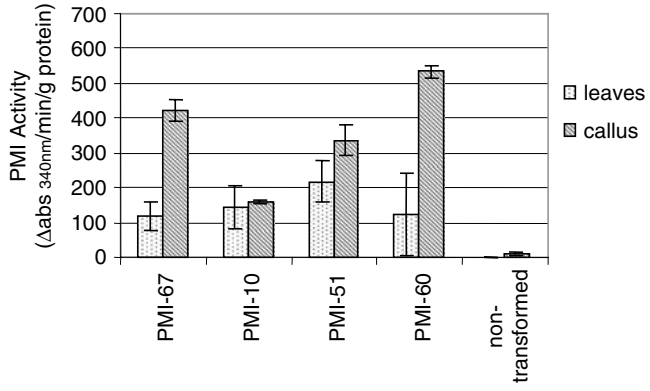

Fig. 5 PMI activity ± standard error in PMI transformed calli and the leaves of young plants regenerated from each callus

in the leaves than in the callus from which the plants had regenerated. In most cases where the PMI expression was relatively high in the callus, the difference in PMI activity was significantly lower in the leaves (Fig. 5). Only 45 of the 75 selected plant lines (60%) had leaf PMI activity that was at least fivefold higher than wild type. The positive control was PMI that had been purified from *E. coli* (http://www.sigmaaldrich.com).

For many of the lines tested, PMI activity decreased after plant regeneration. This change could reflect the developmental expression pattern for this particular promoter, or it might reflect partial silencing of the transgene during development. For selection markers, maintaining gene activity through the selection process is a primary requirement, and this was certainly achieved. Even the reduced expression levels seen in the regenerated plant lines are sufficiently high to confer resistance, so the observed decrease in expression should not in any way limit the effectiveness of this selection system.

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