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A Non-Antibiotic Selection System Uses the Phosphomannose-Isomerase (PMI) Gene for *Agrobacterium*-Mediated Transformation of Chinese Cabbage

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To establish a non-antibiotic selection system that utilizes the phosphomannose-isomerase (PMI) gene for Chinese cabbage transformation, we first determined the optimum mannose concentration for selecting transformed cells. Hypocotyl and cotyledon explants that were grown on media containing more than 5 g L^{-1} mannose did not induce green calli but, rather became chlorotic and withered before dying. In contrast, media containing 20 g L^{-1} sucrose plus 5 g L^{-1} mannose proved suitable for selection. We then used this particular level of mannose to transform hypocotyl tissues. Within 6 weeks, shoots were regenerated from some of the calli; subsequently, these plants were transplanted to pots and grown in the greenhouse. A 514-bp PCR fragment was obtained from most transformants but not from the non-transformed plants. Southern blot analysis also revealed the expected *PMI* gene in those PCR-confirmed transgenic plants. RT-PCR of total RNA was performed to confirm *PMI* expression. We have now demonstrated that this gene does not inhibit the growth of transgenic plants, and that this selection system can be applied to Chinese cabbage transformation.

Keywords: Agrobacterium tumefaciens, Chinese cabbage, non-antibiotic selection, phosphomannose-isomerase

Plant cells can be transformed using chimeric genes that confer resistance to toxins such as antibiotics and herbicides. In the presence of a selective agent, only those cells that are transformed and express the selectable marker gene will divide and regenerate. Plants regenerated from those surviving cells then contain the selectable marker gene joined to a gene of interest.

Various antibiotic resistance genes serve as selectable markers in the production of transgenic plants (Yoder and Goldsbrough, 1994). For *Brassica*, the most commonly used is *neomycin phosphotransferase* II (*npt*II) from transposon Tn5 (Bevan et al., 1983; Carrer et al., 1993; Murry et al., 1993; Omirulleh et al., 1993). This gene confers resistance toward aminoglycosides such as kanamycin, neomycin, geneticin (G418), and paromycin. Another effective selection marker for *Brassica* transformation is the hygromycin phosphotransferase gene (*hpt*) from *Escherichia coli*, which provides resistance to hygromycin (van den Elzen et al., 1985; Waldron et al., 1985; Armstrong et al., 1990; Ishida et al., 1996). Other types of selectable markers are obtained by using herbicide resistance genes. In that group, the most predominant one for *Brassica* transformation is the *bar* gene (Thompson et al., 1987), which confers resistance to the herbicides bialophos and phosphinotricin (De Block et al., 1989).

Recently, the use of antibiotics and herbicide selection agents has caused widespread public concern because of inadequate knowledge of their impact on the environment and on human health. This concern could be settled if researchers develop a non-antibiotic mannose selection system that uses the phosphomannose-isomerase (PMI) gene.

In plants, the aldohexose sugar, mannose, is a precursor in ascorbate synthesis, as well as a critical component of cell wall polymers and glycoprotein oligosaccharide moieties, which include cell membrane-bound receptors. Mannose is an especially important carbohydrate in plants that metabolize it, with mannose-6-phosphate being the immediate product of mannitol catabolism. Sucrose, which is often used as a carbon source in plant tissue culture media, can be replaced by other sugars, e.g., fructose or glucose, but generally not by mannose.

PMI is a key enzyme for mannose metabolism. This sugar is phosphorylated by hexokinase to mannose-6-phosphate and, in the presence of PMI, enters the

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glycolytic pathway after isomerization to fructose-6-phosphate. Although the PMI structural gene of *Escherichia coli* has been cloned (Miles and Guest, 1984), most vegetable crops do not contain such a gene. Nevertheless, *PMI* has been investigated in a variety of agronomic species, including rice (Lucca et al., 2001; He et al., 2004), wheat (Reed et al., 1999; Wright et al., 2001), maize (Negrotto et al., 2000; Wang et al., 2000), sugar beet (Joersbo et al., 1998), tobacco (Barb et al., 2003), and tomato (unpublished).

Chinese cabbage (*Brassica rapa* L.) is one of the most important vegetables for agricultural production, and is widely cultivated in Asia, especially Korea, Japan, and China. However, despite its importance, it had not yet been tested systemically. Therefore, the objectives of our study were to: 1) examine its capacity for regeneration on a mannose-containing medium for stable production of an adequate number of transgenic plants, and 2) use *PMI* in the transformation of Chinese cabbage plants.

MATERIALS AND METHODS

Plant Material and Preparation

We used a commercial F_1 hybrid of Chinese cabbage (*Brassica rapa* var. Seoul; Dong-bu Seed, Korea). Seeds were submerged first in 70% ethanol for 1 min, then in 30% commercial Clorox (1.6% hypochlorite) plus 0.1% Tween-20 for 20 min, with vigorous shaking. They were rinsed three times and germinated under lights in mayonnaise bottles (about 50 seeds each) that contained an MS basal medium with 3% sucrose and 8 g L^{-1} agar (pH 5.8). For our cotyledon cultures, seeds were grown for 5 d under a 16-h photoperiod until the cotyledons were fully expanded. To produce hypocotyl cultures, seeds were grown for 7 d under a 16-h photoperiod until the hypocotyls were 4- to 5-cm-long. They were then dissected, avoiding the shoot apex, and immediately cut into 10-mm-long segments.

Optimization of Mannose Concentration for Selecting Transformed Cells

The effect of mannose was examined by observing shoot induction under 20 combinations of regeneration media in 90-mm Petri dishes. Each combination included an MS medium containing 4 mg L^{-1} BA, 1 mg L^{-1} NAA, 2 mg L^{-1} $AgNO_3$, and 0.8% agar (pH 5.6), which was then supplemented with one of five levels of mannose (0, 1, 5, 10, or 15 g L^{-1}) and four levels of sucrose (0, 10, 20, or 30 g L^{-1}). Cotyledon explants were placed perpendicular to the medium surface, while hypocotyl explants were positioned so that a cut side was sufficiently buried in the medium. Explants were grown for 2 weeks at 25 ± 2°C under a 16-h photoperiod, then transferred to fresh media. Five plates (replicates) of 10 cotyledons or hypocotyls per treatment were arranged in a completely randomized design. After 6 weeks, regenerated shoots greater than 5 mm long were counted. All experiments described above were repeated four times, and data were combined and analyzed with the statistical program SAS (SAS Institute, 1985). F-tests were used to determine the significance of treatments, and mean separations were based on least significant difference (LSD).

Agrobacterium-Mediated Transformation with *PMI* Gene

The vector pNOV2819 (Syngenta; Fig. 1) was used to transform Chinese cabbage. This vector contains the *PMI* gene, which plays a role in converting mannose-6-phosphate to fructose-6-phosphate. Before transformation commenced, *PMI* was confirmed in pNOV2819 via restriction enzyme digestion and PCR analysis.

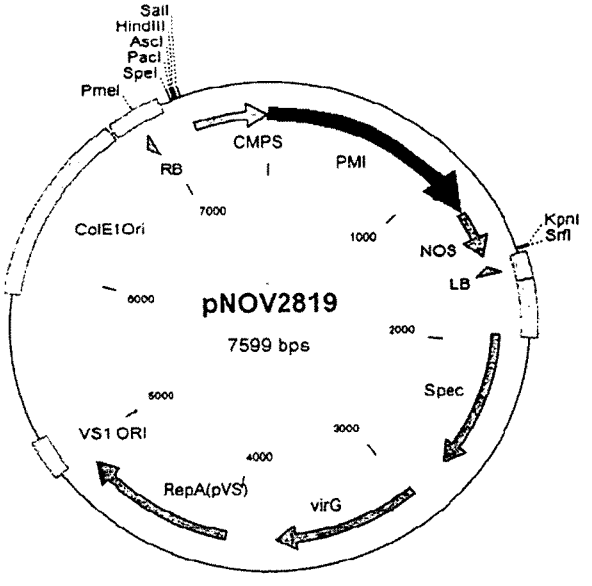

Figure 1. Plasmid map of pNOV2819 vector with phosphomannose-isomerase (PMI) gene used for transformation of Chinese cabbage. *PMI* was placed under CMPS promoter. CMPS, CmYLCV partial promoter; NOS, Nos terminator; RB, right border; LB, left border.

Agrobacterium tumefaciens strain LBA4404, containing pNOV2819, was grown on a solid YEP medium with kanamycin (50 mg L^{-1}) at 28°C for 48 h. After selection, several colonies of LBA4404 were placed in 5 mL of the same YEP liquid media, and shaken at 28°C for 48 h. A 200 µL aliquot of this bacterial culture was then grown in 50 mL of YEP medium (pH 7.0) for 48 h (OD_{600} = 0.8). After centrifugation at 5500 rpm for 8 min, the pellet was re-suspended in 50 mL of YEP medium. Hypocotyl explants were infected by immersion in the bacterial inoculum for 10 min. Following infection, they were placed on sterile filter paper and embedded in a co-cultivation medium (MS medium, no sucrose, 4 mg L^{-1} BA, 1 mg L^{-1} NAA, 2 mg L^{-1} $AgNO_3$, and 0.8% agar; pH 5.7). After co-cultivation at 25 ± 2°C under darkness for 3 d, the explants were washed with an MS liquid medium supplemented with 200 mg L^{-1} cefotaxime and 250 mg L^{-1} carbenicillin. They were then transferred (16 per plate) to a selection medium (MS medium, 20 g L^{-1} sucrose, 5 g L^{-1} mannose, 4 mg L^{-1} BA, 1 mg L^{-1} NAA, 2 mg L^{-1} $AgNO_3$, 200 mg L^{-1} cefotaxime, 250 mg L^{-1} carbenicillin, and 0.8% bactoagar; pH 5.8). Explants were grown for 2 weeks at 25 ± 2°C under a 16-h photoperiod before being sub-cultured to fresh medium. The regenerated green shoots were transferred to a rooting medium (1/2 MS medium, 20 g L^{-1} sucrose, 5 g L^{-1} mannose, 200 mg L^{-1} cefotaxime, and 0.8% agar; pH 5.8), then transplanted to soil after roots appeared.

Confirmation of Transformed Chinese Cabbage Plants

PCR was performed with 50 ng of genomic DNA and the following oligonucleotide primers: PMI-1 (18 mer), 5'-ACAGCCACTCTCCATTCA-3'; and PMI-2 (18 mer), 5'-GTTTGCCATCACTTCCAG-3'. These primers amplified a 514-bp fragment. PCR was performed in a 50 µL reaction mixture with *Taq* polymerase and a thermocycler (Biometra, USA). The samples were initially denatured at 95°C for 5 min; followed by 30 cycles of 30 s denaturation at 95°C, 30 s of primer-annealing at 55°C, and 1 min of synthesis at 72°C; and a final extension at 72°C for 5 min. The PCR products were analyzed on a 1.0% agarose gel. For Southern hybridization, 10 µg of genomic DNA was digested with *Bam*HI, separated by electrophoresis on a 0.9% agarose gel, and transferred to a Hybond N^+ membrane (Amersham, USA). The separated DNA was probed with a ^{32}P-labeled 514-bp PCR product of *PMI* from pNOV2819 plasmid DNA to confirm integration of the T-DNA into the plant genome. Random primer-labeling of the probe was conducted according to the manufacturer's protocol for the Takara Ladderman TM Labeling Kit (Takara, Japan).

RT- PCR Analysis for Transgene Expression

RT-PCR was performed with the Revers-iT™ One-step RT-PCR Kit (ReddyMix™ Version, ABgene, UK) according to the manufacturer's instructions (ABgene®). After combining 2 µL of total RNA, 2× ReddyMix™ RT-PCR Master Mix, 1 µL each of 10 pmol forward and reverse primers, and Reverse-iT RTase Blend (50 units μL^{-1}) in the 50 µL reaction mixture, it was placed in a thermal cycler. First, the strand was synthesized at 47°C for 30 min, followed by RTase Blend inactivation and denaturation at 95°C for 2 min, and PCR (35 cycles of 30 s denaturation at 95°C, 30 s of primer-annealing at 58°C, and 1 min of synthesis at 72°C; with a final extension step of 72°C for 5 min). The PCR products were analyzed on a 1.5% agarose gel.

Growth of Transformed Chinese Cabbage with *PMI* Gene

Transgenic plants were transplanted to pots and grown for 6 weeks in the greenhouse. Their heights were measured weekly.

RESULTS AND DISCUSSION

Determining Appropriate Mannose Concentration for Selection of Transformed Chinese Cabbage Cells

Mannose treatment significantly influenced callus formation and shoot regeneration of Chinese cabbage. Cotyledon and hypocotyl explants grown on sucrose-free media ordinarily showed chlorosis after 2 weeks of incubation. Calli formation on either type of explant was unaffected by 5 g L^{-1} mannose, but was dramatically inhibited when the concentration was increased to 10 g L^{-1}. When either explant source was placed on media containing more than 5 g L^{-1} mannose, green calli did not develop, but the tissues showed chlorosis, then withered and died (data not shown). Although shoot induction from cotyledons was not affected by 1 g L^{-1} mannose, significantly fewer shoots were formed when that concentration

Table 1. Number of shoots induced from different explant types of 'Seoul' Chinese cabbage (*B. rapa* L.) based on sucrose and mannose concentrations.

Explant type	Sucrose (g L^{-1})	Mannose (g L^{-1})				
		0	5	10	15	20
Cotyledon	0	0.0 f [z]	0.0 f	0.0 f	0.0 f	0.0 f
	10	2.6 c	1.2 e	0.0 f	0.0 f	0.0 f
	20	3.8 b	1.6 d	0.0 f	0.0 f	0.0 f
	30	4.4 a	4.0 b	0.0 f	0.0 f	0.0 f
Hypocotyl	0	0.0 d7	0.0 d	0.0 d	0.0 d	0.0 d
	10	0.0 d	0.0 d	0.0 d	0.0 d	0.0 d
	20	0.5 c	0.4 c	0.0 d	0.0 d	0.0 d
	30	1.6 a	1.2 b	0.0 d	0.0 d	0.0 d

[z], Each value represents a mean of 5 replicates for 10 cotyledons or hypocotyls. All experiments were repeated 4 times, and data were combined and statistically analyzed. Means followed by the same letter within a column are not significantly different at the 0.05 probability level, according to the LSD for each explant type.

was increased to 5 g L^{-1} (Table 1). Similar performances were observed with our hypocotyl explants. A level of 5 g L^{-1} caused cotyledon and hypocotyl explants to become bleached. Therefore, these results indicate that transformed cells of Chinese cabbage can be selected in media containing low levels of mannose.

Mannose can diminish respiration in wheat and tomato (Stenlid, 1954; Morgan and Street, 1959), perhaps because of the accumulation of mannose-6-phosphate, which inhibits phosphoglucose isomerase, thereby blocking glycolysis (Goldsworthy and Street, 1965). Other impacts include depletion of the pyrophosphate required for ATP production (Goldsworthy and Street, 1965; Herold and Lewis, 1977), transcriptional repression of genes associated with photosynthesis and the glyoxylate cycle (Jang and Sheen, 1994, 1997; Graham et al., 1997), and apoptosis in maize cells (Stein and Hansen, 1999).

Our study showed that a low concentration of mannose can be a useful selection marker for transformation and regeneration of Chinese cabbage from cotyledon and hypocotyl tissues. Compared with other *Brassica* members, this species is generally considered recalcitrant to *in vitro* shoot regeneration (Murata and Orton, 1987; Jain et al., 1988; Narashimhulu and Chopra, 1988). Although lower levels of mannose result in more transformants, but simultaneously allow for more untransformed escapes (Fry et al., 1987; Radke et al., 1988), those smaller concentrations can be successful as markers for reducing selection pressure.

Here, for either cotyledons or hypocotyls, the addition of 30 g L^{-1} of sucrose to the regeneration medium induced the formation of more shoots than did a supplement of only 20 g L^{-1}. However, because the former concentration is the standard amount used for most regeneration media, we could not determine the particular effect of incorporating mannose. Nevertheless, we suggest that a medium containing 20 g L^{-1} sucrose and 5 g L^{-1} mannose is the optimum for selection in Chinese cabbage transformation.

Agrobacterium-Mediated Transformation with *PMI* Gene

Within 3 to 4 weeks after co-cultivation, calli on the selection medium began to form at the cut ends of hypocotyls inoculated with *A. tumefaciens* (Fig. 2a). After another 2 weeks, the calli were transferred to fresh selection media of the same composition. Most shoots were regenerated within 4 to 5 weeks (Fig. 2b). These new shoots were then transferred to a root induction medium supplemented with 200 mg L^{-1} cefotaxime (Fig. 2c), where roots formed from the cut ends of the shoots within 4 weeks (Fig. 2d). The rooted plants were then transferred to the greenhouse for further growth (Fig. 3).

PCR analysis with the primer set of PMI-1 and PMI-2 produced a predicted 514-bp fragment in 8 out of 10 PCR-confirmed transgenics, but no such fragment was found in any of the non-transformed plants (Fig. 4). This represented an 83.3% selection efficiency for *PMI* transformation in Chinese cabbage.

Southern blot analysis also revealed the expected *PMI* gene in those PCR-confirmed transgenic plants. Of the seven independent T_0 lines identified here, four showed one copy of the transgene, one had two or three, and two had three to five copies (Fig. 5). This also demonstrates that our PMI selection system

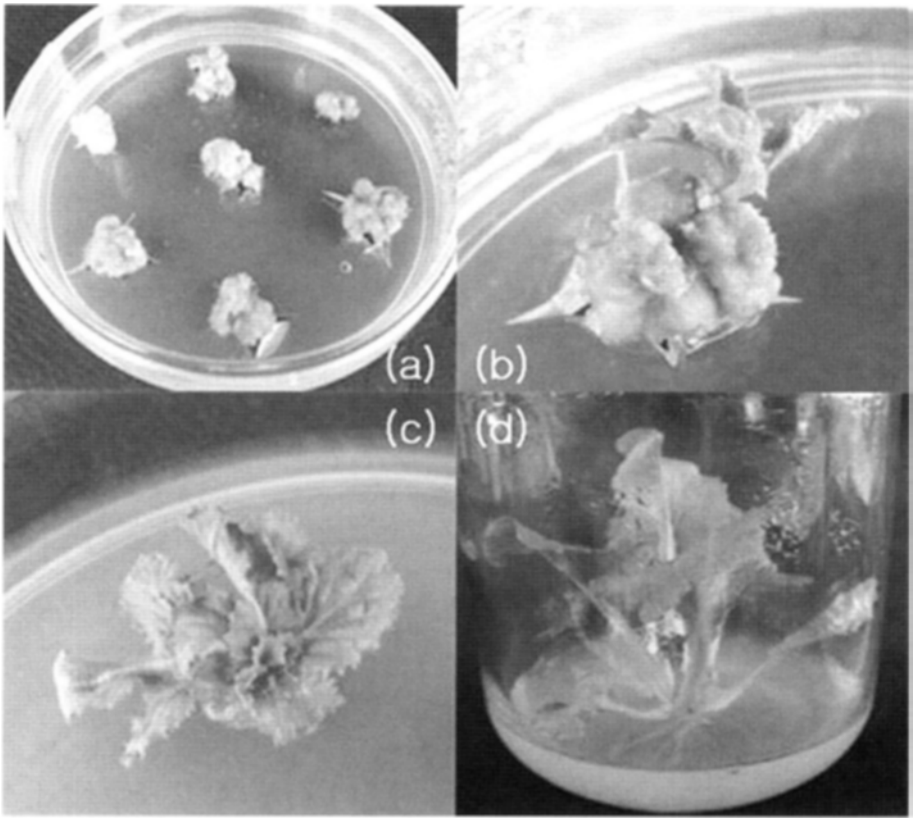

Figure 2. Transformation of Chinese cabbage with pNOV2819 containing *PMI* gene. (**a**) Transformed calli were induced from hypocotyl explant onto selection medium; (**b**) Transformed shoots were induced from calli onto selection medium; (**c**) Transformed shoot was transferred to rooting medium; (**d**) Roots were induced from transformed shoot in rooting medium.

Figure 3. Acclimation of transformed Chinese cabbage plants with *PMI* gene. Rooted plants from mannose-containing medium were transplanted to pots and acclimated in a greenhouse for one month.

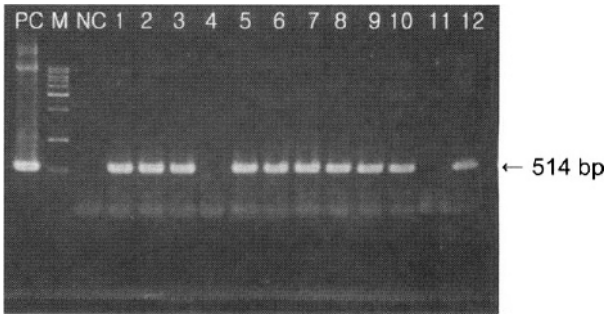

Figure 4. PCR analysis of transformed Chinese cabbage plants with *PMI* gene selected in mannose-containing medium. PMI-1 (5′-ACAGCCACTCTC- CATTCA-3′) and PMI-2 (5′-GTTTGCCATCACTTCCAG-3′) primers were used to identify PMI. Analysis revealed expected 514-bp fragment for *PMI* gene. PC, Plasmid with pNOV2819 (positive control); M, size marker (1-kb ladder); NC, non-transformed plant; Lanes 1, 2, 3, 5, 6, 7, 8, 9, 10 and 12, transformed plants confirmed by PCR; Lanes 4 and 11, selected shoots on selection medium without PCR product.

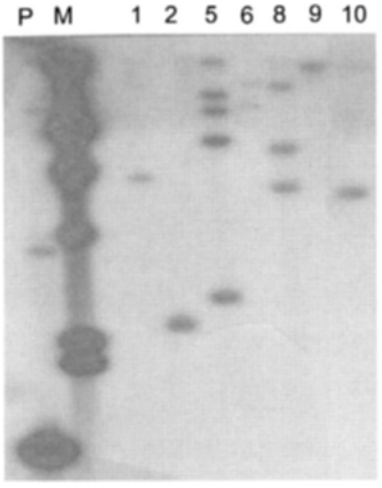

Figure 5. Southern hybridization analysis of transgenic Chinese cabbage plants with *PMI* gene selected in mannose-containing medium. Ten μg of genomic DNA was digested with *Bam*HI, separated by electrophoresis on 0.9% agarose gel, and probed with ^{32}P-labeled 514-bp PCR product of *PMI* gene from pNOV2819. P, *PMI* gene in pNOV2819 (positive marker); M, λ*Hin*dIII size marker; Lanes 1-7, transformed plants confirmed by PCR.

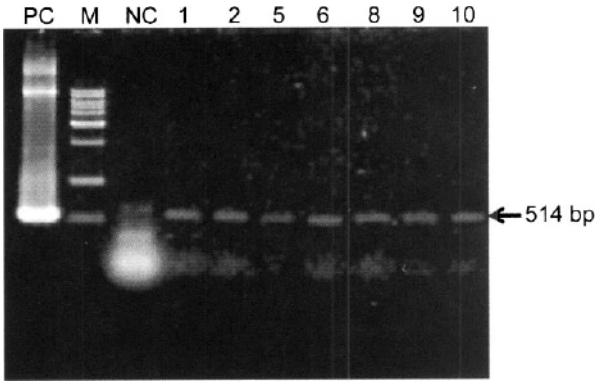

Figure 6. Confirmation of transgene expression from transgenic Chinese cabbage plants by RT-PCR. PMI-1 (5′-ACAGCCACTCTCCATTCA-3′) and PMI-2 (5′-GTTTGCCATCACTTCCAG-3′) primers were used. Analysis showed expected 514-bp fragment for phosphomannose isomerase gene. PC, Plasmid with pNOV2819 (Positive marker); M, Size marker (1-kb ladder); NC, non-transformed plant; Lanes 1, 2, 5, 6, 8, 9, 10, 514-bp product obtained from transformed plants by RT-PCR.

is effective for Chinese cabbage transformation.

RT-PCR of total RNA was performed to confirm *PMI* expression in the transgenic plants. The reverse transcripts were also amplified by the primer set of PMI-1 and *PMI*-2; non-transformed plants served as our negative control. Again, the predicted 514-bp RT-PCR fragments was obtained from each of seven transformed plants (Fig. 6), whereas the control plants lacked this fragment. Although we did not determine its degree, *PMI* expression could be detected in each transgenic line.

After 6 weeks, the average transgenic plant, containing the *PMI* gene, was taller (17.1 cm versus 15.7 cm) than the non-transformed plant (Fig. 7). In field trials, Privalle et al. (1998) have compared parameters for plants from seven independent, transformed *PMI* maize events with their isogenic, non-transformed counterparts, and have reported no statistical differences in heights. This indicates, therefore, that *PMI* expression has no adverse effect on growth or other characteristics. In our study, it is possible that differ-

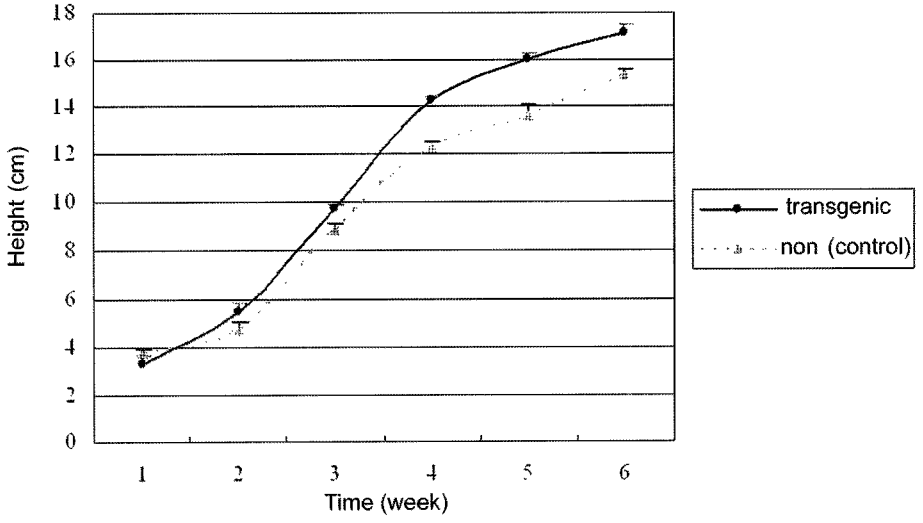

Figure 7. Growth curves for transgenic Chinese cabbage with *PMI* gene and non-transgenic plants. Heights are average of 7 transgenic lines or the average of 3 control (non-transgenic) plants. Bar indicates standard deviation.

ences in plant heights were due to a tissue-culture effect.

PMI catalyzes the reversible interconversion of mannose-6-phosphate and fructose-6-phosphate. Plant cells lacking this enzyme are incapable of surviving on synthetic media that contain mannose as a carbon source. Most vegetable crops that are genetically modified to express the *E. coli manA* gene (*pmi*) under the control of a plant promoter are able to survive selection on a mannose-supplemented medium (Bojsen et al., 1994; Joersbo et al., 1998). Traditional transformation protocols call for plant cells to be placed on culture media with sucrose as the carbon source. In contrast, the PMI/mannose selection system described here suggests that transformed plant tissues can be cultured on media enhanced either with mannose alone or with both sucrose and mannose as carbon sources. Although mannose has no direct, adverse effect on plant cells, the success of subsequent selections would probably depend on its phosphorylation to mannose-6-phosphate by hexokinase. That is, in the absence of *PMI*, mannose-6-phosphate would accumulate and cell growth would cease.

In conclusion, we have shown that the *PMI* gene is a suitable selective marker for Chinese cabbage transformation. *PMI* can be expressed in cells of that species, conferring the ability to utilize mannose as a carbohydrate source in our proposed transformation system.

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