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A novel plant code optimization phosphomannose isomerase (*pPMI*) and its application in rice (*Oryza sativa* L.) transformation as selective marker

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Abstract

The phosphomannose isomerase (PMI) gene has been developed as selective marker gene for plant transformation. This positive selection system does not use toxic compounds such as antibiotics or herbicides. In this study, we used *PMI* effectively and safely. The original nucleotide sequence of *PMI* was codon optimized as rice (*Oryza sativa* L.) synonymous codon usage bias. The nucleotides in the *PMI* were optimized according to the preferred codons in rice. This plant code optimized phosphomannose isomerase (*pPMI*) has higher GC content (61.31%), especially GC content at the third position in a codon (83.42%). The vectors harboring *pPMI* and *PMI* were transferred into rice by *Agrobacterium*-mediated transformation. There were higher transformation frequency (54.5%) and single copy rate (44.5%) using *pPMI* as selective marker than *PMI*. This work showed that *pPMI* could be a suitable substitution for *PMI* in production of transgenic crops. Moreover, *pPMI* could be used in gene editing technology for higher transformation frequency and safety.

Keywords: Gene modification; *pPMI*; GC content; Synonymous codons; Rice.
Abbreviations: AT_ adenosine and thymine; BAR_ bialaphos resistance; Cas_CRISPR-associated protein; CDSs_coding DNA sequences; CPR_chlorophenol red; CRISPR_clustered regularly interspaced short palindromic repeat; GC_guanosine and cytidine; GC_1_ guanosine or cytidine at the first position in all codons; GC_2_guanosine or cytidine at the second position in all codons; GC_3_guanosine or cytidine at the third position in all codons; gDNA_genomic DNA; GFP_green fluorescent protein; F-6-P_fructose-6-phosphate; GM_genetically modified; HPT_hygromycin phosphotransferase; HR_ homologous recombination; ISAAA_ International Service for the Acquisition of Agri-biotech Applications; M-6-P- mannose-6-phosphate; MS_Murashige and Skoog; OE_over-expression; pPMI_plant code optimization phosphomannose isomerase; PMI_phosphomannose isomerase; RNAi_ RNA interference; SCUB_synonymous codon usage bias.

Introduction

Transgenic technology can break reproductive isolation. The gene transfer among different species serves as an effective improvement method for crop yield, quality and resistance improvement. The GM (genetically modified) crops are considered as the fastest crop technology in the history of modern agriculture. According to the report of ISAAA (International Service for the Acquisition of Agri-biotech Applications), Global Status of Commercialized Biotech/GM Crops in 2013, the global area of biotech/GM crops continued to increase for the 18th year at a sustained growth rate of 3% or 5 million hectares, reaching 175.2 million hectares in 2013 (James C, 2013).

Antibiotic resistance genes, such as hygromycin phosphotransferase (HPT) gene, were extensively used as selective marker genes in transgenic technology (Hiei et al., 1994). These transformation systems always employed antibiotics as selectable agents to kill the non-transformed cells (Duan et al., 2012). However, these antibiotic resistance genes were mostly isolated from bacterium or fungi. Thus, there is a potential risk that the other microbial strains may obtain the resistance genes by gene exchanging. So, it may lower the effect of these antibiotics. Moreover, these genes may cause the public concern for the possibility of gene flow between GM crops and similar species (Lu and Yang, 2009; Ni et al., 2014). In order to replace these antibiotic resistance genes, other selective marker genes such as herbicide resistance gene *BAR* (bialaphos resistance), visual marker gene *GFP* (green fluorescent protein) have been researched. However, these genes are also deficient for the similar problems as antibiotic resistance genes or the low efficiency and high-cost in large scale application (Wang and Peter, 1997; Davis and Vierstra, 1998). Some methods such as marker-free strategies could eliminate marker genes. Up to date, these strategies have not been widely utilized in commercial production due to some disadvantages, such as laborious. So, it is urgent to produce GM cops by using safe selective marker genes and natural selectable agents.

PMI (phosphomannose isomerase) is a kind of enzyme in sugar metabolism, where widely exist in algae and some legumes. It can catalyze mannose-6-phosphate (M-6-P) into fructose-6-diphosphate (F-6-P). Mannose can be transformed into M-6-P in rice and other higher plant cells. But due to lack of PMI, M-6-P cannot be converted to F-6-P. The accumulation of M-6-P would inhibit phosphoglucose isomerase and block glycolysis. So, *PMI* can be used as a selective marker gene to distinguish transformed cells from non-transformed cells. Furthermore, unlike antibiotics and herbicides, *PMI*-mannose selection system is a positive selection system with higher selection efficiency to reduce the negative effects of died cells in the negative selection systems (Duan et al., 2012). Moreover, mannose is widespread in algae and fern. The F-6-P, the catalytic products of PMI, is the ingredient of honey and fruit juice. On the other hand, mannose and F-6-P are natural substances and environmental friendly. So, *PMI* has been used as a safe selective marker gene for the production of GM crops, including rice (Lucca et al., 2001), maize (Negrotto et al., 2000), wheat (Gadaleta et al., 2006).

But PMI used in current transgenic technology is isolated from *E. coli* K-12 (Miles et al., 1984). As we know, prokaryotes and eukaryotes have different base composition, synonymous codon usage bias (SCUB). There is a clear distinction between dicot and monocot codon (Campbell and Gowri, 1990). For example, monocot has higher content of guanosine and cytidine (GC), showing more obvious SCUB and more frequent guanosine or cytidine at the third position in all codons (GC_3) than prokaryotes and dicot (Tatarinova et al., 2010). Therefore, they can be used directly without artificial optimization design of *PMI*, which will affect its expression efficiency in eukaryotic cell, and the screening effect as selective marker gene (Cheng et al., 1998). Moreover, there may be a concern about safety of food and ecology, where the PMI is isolated from *E. coli* (Ni et al., 2014).

In this study, in order to use *PMI* more effectively and safely in production GM crops, the original nucleotide sequence of *PMI* was codon-optimized as rice (*Oryza sativa* L.) SCUB. This plant codon-optimized phosphomannose isomerase (*pPMI*) was transferred into rice as selective marker gene by *Agrobacterium*-mediated transformation.

Results

Difference in SCUB and GC content between rice and E. coli K-12

The SCUB between rice and *E. coli* K-12 was shown in Table 1. Obviously, there was different SCUB in 2 species as some codons were used more frequently than others. The preferred codons (the codons with the highest usage frequency) were different between rice and *E. coli* K-12. The preferred codons in rice were UUC, CUC, UCC, UAC, UGC, CCG, CAC, CAG, CGC, AUC, ACC, AAC, AAG, GUG, GCC, GAC, GAG, GGC, and UGA. The preferred codons in *E. coli* K12 were UUU, CUG, AGC, UAU, UGC, CCG, CAU, CAG, CGC, AUU, ACC, AAC, AAA, GUG, GCG, GAU, GAA, GGC, and UAA.

There was different GC content of coding DNA sequences (CDSs) and individual codon positions between 2 species (Fig 1.). The total GC content of rice CDSs was 55.26%, which was higher than the GC content of *E. coli* (52.35%). The GC content in 3 different positions of codons was also different between the 2 species. The GC_1 (guanosine or cytidine at the first position in all codons) in rice was 58.19% and the GC_1 in *E. coli* was 60.82%. The GC_2 (guanosine or cytidine at the second position in all codons) in rice and *E. coli* were 45.97% and 40.61%, respectively. The GC_3 had the largest difference between rice (61.61%) and *E. coli* (55.62%).

Design of pPMI and sequence analysis

The nucleotides in the *PMI* were optimized according to the preferred codons in rice (Table 1) to form a new *PMI* gene called *pPMI*. The comparison of the DNA sequences and amino acids sequences between *PMI* and *pPMI* was shown (Supplementary File 1, Fig S1). Total of 286 bases in *pPMI* were modified compared with *PMI*, accounting for 24.31% of the total length (1176 bp). *PMI* used 54 of the 61 codons for 20 amino acids, whereas *pPMI* only employed 37 codons. The amino acids sequences were same completely (Supplementary File 1, Fig S2).

There was a large difference in GC content between *PMI* and *pPMI* (Table 2.). The GC content of *PMI* was 50.77 %, whereas that of *pPMI* was up to 61.31%. There were no obvious different in the GC1 and GC2 between *pPMI* and *PMI* (Fig. 2). The GC_1 in *pPMI* was 61.73%. The GC_1 in *PMI* was 59.69% and the GC_2 in *pPMI* and *PMI* were 38.78% and 38.52%, respectively. But the GC_3 had the largest difference between *pPMI* (83.42%) and *PMI* (54.08%).

Rice transformation and PCR and real-time PCR assay of transgenic plants

To determine whether *pPMI* was functional in rice, we fused *pPMI* and *PMI* to the constitutive promoter *CaMV35S* and transferred them into rice via *Agrobacterium*- mediated transformation to obtain transgenic plants (Fig. 3). The gDNA samples of all transgenic plants were extracted for PCR and copy number assay. All gDNA samples were detected by PCR analysis, and the target band of 490 bp could be amplified from all of the transgenic plants (Fig. 4). To determine the copy number of the transgenic events, real-time PCR using TaqMan probe assay was performed (Supplementary File 1, Fig S3). The differences in transformation frequency and single copy rate between *pPMI* and *PMI* were shown in Table 3. There were higher transformation frequency (54.5%) and single copy rate (44.5%) using *pPMI* as selective marker than *PMI*.

Detection of PMI activity by chlorophenol red assay

Transgenic rice leaves were detected for PMI activity using chlorophenol red assay. Leaves from all transgenic plants (harboring *pPMI* or *PMI*) changed the color of the medium to yellow. In contrast, no sample control and the medium containing negative control leaves showed color change (Fig. 5).

Discussion

Selective marker genes accompanied with the objective genes, are co-transferred into the host genome and used to select out the transformed cells. However they are redundant after the generation of transgenic plants and may lead to the environmental and health risk (Rommens, 2004). Several techniques, such as site-specific recombination and co-transformation, could eliminate those selectable genes (Rommens et al., 2004). But these strategies were laborious and time-consuming, which not utilized widely. Therefore, a safe selectable marker gene, like *PMI*, would be highly desirable in the production of transgenic plants, avoiding the use of antibiotics or herbicides and the corresponding genes (Mehrotra and Goyal, 2012).

There are different codon usage biases and GC content among different species. Codon optimization has been widely

Table 1. The synonymous codon usage bias (SCUB) between rice (*Oryza sativa* L.) and *E. coli* K-12.

Codon	Amino acid	Rice		*E. coli* K-12	
		Codon number	Percentage (%)	Codon number	Percentage (%)
UUU	F	446,063	36.88	101	56.74
UUC		763,386	63.12	77	43.26
UUA	L	209,911	6.79	78	14.97
UUG		500,913	16.21	61	11.71
CUU		517,919	16.76	61	11.71
CUC		880,959	28.51	54	10.36
CUA		263,871	8.54	27	5.18
CUG		716,525	23.19	240	46.07
UCU	S	433,684	16.17	29	11.15
UCC		557,258	20.77	28	10.77
UCA		424,426	15.82	40	15.38
UCG		420,825	15.69	41	15.77
AGU		300,879	11.22	37	14.23
AGC		545,529	20.34	85	32.69
UAU	Y	339,638	39.65	86	53.42
UAC		517,042	60.35	75	46.58
UGU	C	211,609	33.35	30	42.25
UGC		422,851	66.65	41	57.75
UGG	W	472,543	100.00	55	100.00
CCU	P	463,459	23.47	43	17.41
CCC		411,848	20.86	33	13.36
CCA		486,283	24.63	34	13.77
CCG		613,159	31.05	137	55.47
CAU	H	385,174	44.99	81	54.73
CAC		470,960	55.01	67	45.27
CAA	Q	460,234	39.35	62	30.39
CAG		709,469	60.65	142	69.61
CGU	R	244,821	10.30	108	36.12
CGC		550,575	23.17	133	44.48
CGA		219,662	9.24	22	7.36
CGG		458,602	19.30	21	7.02
AGA		358,226	15.07	7	2.34
AGG		544,515	22.91	8	2.68
AUU	I	483,941	33.46	156	58.21
AUC		662,207	45.79	93	34.70
AUA		300,085	20.75	19	7.09
AUG	M	812,432	100.00	127	100.00
ACU	T	363,451	21.97	41	16.40
ACC		508,156	30.71	117	46.80
ACA		394,803	23.86	33	13.20
ACG		388,036	23.45	59	23.60
AAU	N	515,761	44.90	112	47.26
AAC		633,017	55.10	125	52.74
AAA	K	544,476	33.08	170	73.28
AAG		1,101,342	66.92	62	26.72
GUU	V	529,509	23.27	86	25.29
GUC		685,971	30.14	60	17.65
GUA		231,761	10.18	59	17.35
GUG		828,681	36.41	135	39.71
GCU	A	667,854	20.75	55	10.54
GCC		1,050,723	32.65	162	31.03
GCA		591,267	18.37	108	20.69
GCG		908,634	28.23	197	37.74
GAU	D	863,983	47.38	194	64.88
GAC		959,498	52.62	105	35.12
GAA	E	738,891	35.96	224	70.44
GAG		1,315,826	64.04	94	29.56
GGU	G	505,609	19.17	109	29.38
GGC		1,005,701	38.14	171	46.09
GGA		542,264	20.56	47	12.67
GGG		583,357	22.12	44	11.86
UAA	STOP	22,360	24.24	9	64.29
UAG		28,508	30.91	0	0.00
UGA		41,361	44.85	5	35.71
Total		34,132,283		5,122	

Note: The preferred codons with the highest usage frequency in rice or *E. coli* K-12 were indicated in green color.

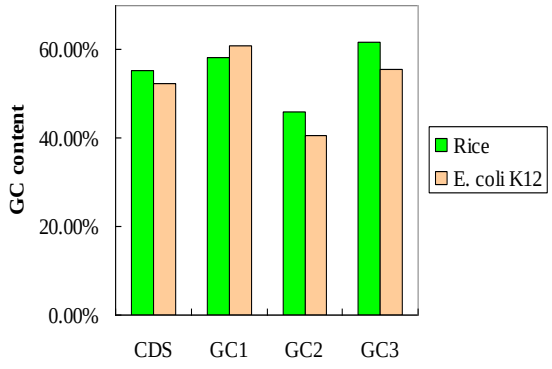

Fig 1. Differences in GC content of coding DNA sequences (CDSs) and individual codon positions between rice and *E. coli* K-12. The GC content of rice was showed in green. The GC content of *E. coli* K-12 was shown in pink. CDS, the GC content of coding DNA sequences. GC_1, the guanosine or cytidine at the first position in all codons. GC_2, the guanosine or cytidine at the second position in all codons. GC_3, the guanosine or cytidine at the third position in all codons.

used to enhance protein expression in diverse systems. In this study, we optimized the original nucleotide sequence of *PMI* as rice SCUB. The GC content of *pPMI* was 61.31%, higher than *PMI* (50.77%). Especially, The GC_3 content of *pPMI* was 83.42%, much higher than *PMI* (54.08%). High GC content would increase the stability of genome structure, though high GC content may cause the emergence of potential methylation signals (Tatarinova et al., 2010). Song et al. (2014) reported that high GC content may not be the limiting factor that affects efficient gene expression in rice. Moreover, the constitutively expressed exons in plants have a higher GC content than those that are alternatively expressed (Guo et al., 2007). There is a positive relationship between expression levels and GC_3 (Muyle et al., 2011). The genes with high GC_3 content have important functional features, such as, methylation, variable expression (Tatarinova et al., 2010). So, *pPMI* has higher GC content especially GC_3, which may increase the expression efficiency and produce more resistant calli under the mannose selection pressure, which in turn, improves the transformation frequency (Duan et al., 2012).

The *PMI*-mannose selection system has been an important tool in plant genetic transformation. By *Agrobacterium* or bombardment mediated genetic transformation, over-expression (OE) and RNA interference (RNAi) are routine technique to studying plant gene function. But sometimes it is hard to identify the specific phenotype just caused by the OE of exogenous gene, due to unprojected effects of OE or insertion sites of the transferred DNA (Wen et al., 2014). The RNAi also has some shortcomings, such as temporary, easy loss, heavy workload of molecular identification (Chew and Seymour, 2013). Very recently, the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) system has been widely used for gene editing (Cong et al., 2013; Xu et al., 2014). CRISPR/Cas system has attracted great attention due to its ability to induce sequence specific genome editing. Furthermore, compared with OE and RNAi, this system does not need the consistent work of transferred DNA. If the gene editing is completed, the transferred DNA can be eliminated easily by genetic segregation. Thus, it is very easy to obtain the transgenic events which have the target genome editing and no redundant transferred DNA. Moreover, gene knock-in can be accomplished mediated by homologous recombination (HR) using CRISPR/Cas system. Because several transformation vectors would be needed in HR, different selective marker genes are required.

In the future, *pPMI* selective marker gene could be used widely in the CRISPR/Cas system. It is helpful to improve gene editing efficiency, reduce public concerns about transgenic technique.

Materials and Methods

Plant materials

The *japonica* rice variety Nipponbare was used as the recipient of *Agrobacterium*-mediated transformation.

Analysis of SCUB between rice (Oryza sativa L.) and E. coli K-12

The codon information of rice and *E. coli* K-12 was acquired from the codon usage database (http://www.kazusa.or.jp/codon) (Nakamura et al., 2000). Rice had 92,188 coding DNA sequences (CDSs) with a total of 34,132,283 codons, and *E. coli* K-12 had 14 CDSs with a total of 5, 122 codons. The frequency of synonymous codons for each amino acid was calculated and the codons with the highest usage frequency among synonymous codons were chosen as preferred codons.

Design and synthesis of Ppmi

The nucleotides in the *PMI* were optimized according to the preferred codons in rice to form a new *PMI* gene named *pPMI*, which was synthesized by GENEWIZ Inc. (Suzhou, China). The restriction enzyme cutting sites of *Xho*I were added before the start codon and after the stop codon. Then *pPMI* was transferred into the PUC57-AMP vector to construct the PUC57- *pPMI* vector.

Construction of the expression vectors and rice transformation

The fragments of *PMI* and *pPMI* were then sub-cloned into the pCAMBIA 1381 vectors. EHA105 strains of *Agrobacterium tumefaciens* harboring p*35s*::*PMI* and p*35s*::*pPMI* vectors were used for the rice transformation. The *japonica* rice variety Nipponbare was used as the recipient. The rice *Agrobacterium*-mediated transformation were performed as described by Duan et al. (2012). The selection pressure is 12.5 g/L mannose + 5 g/L sucrose, and no mannose was supplied in regeneration media. The procedures from calli infection to regeneration were replicated three times for p*35s*::*PMI* and p*35s*::*pPMI* vectors. Four hundred calli were infected with *Agrobacterium* as one replication experiment. Lines of mannose- resistant transgenic plants were selected, and before being transplanted in the greenhouse, pieces from the transgenic plant leaves were sampled for DNA extraction and chlorophenol red (CPR) assay.

DNA extraction and PCR assays

For high-throughput genomic DNA (gDNA) extraction, 10-20 mg powder of rice leaf sample was placed in one well of a 96-well deep well block. The extraction procedure used was described in a previous report (Li et al., 2013).

Table 2. Differences in nucleotide composition between *pPMI* and *PMI*.

Gene	GC Content (%)	AT Content (%)
pPMI	61.31	38.69
PMI	50.77	49.23

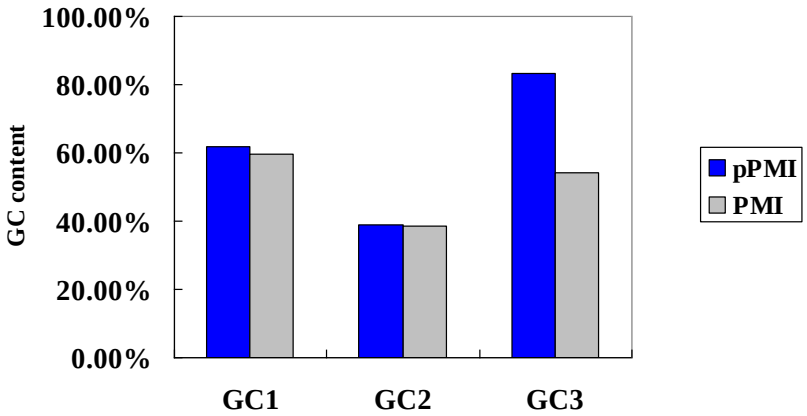

Fig 2. Differences in GC content of individual codon positions between *pPMI* and *PMI*. The GC content of *pPMI* was shown in blue. The GC content of *PMI* was shown in gray. GC_1, the guanosine or cytidine at the first position in all codons. GC_2, the guanosine or cytidine at the second position in all codons. GC_3, the guanosine or cytidine at the third position in all codons.

Table 3. Differences in transformation frequency between *pPMI* and *PMI*.

Selective marker	Resistant calli rate	Regeneration rate	PCR positive rate	Transformation frequency	Single copy rate
pPMI	75.2 ± 5.5%**	72.5 ± 6.0%	100%	54.5 ± 3.3%**	44.5 ± 7.6%
PMI	64.8 ± 4.7%	70.2 ± 4.4%	100%	45.5 ± 2.1%	41.4 ± 5.2%

Data are reported as means ± SD. **Represents significant difference at 0.01 confidence level.

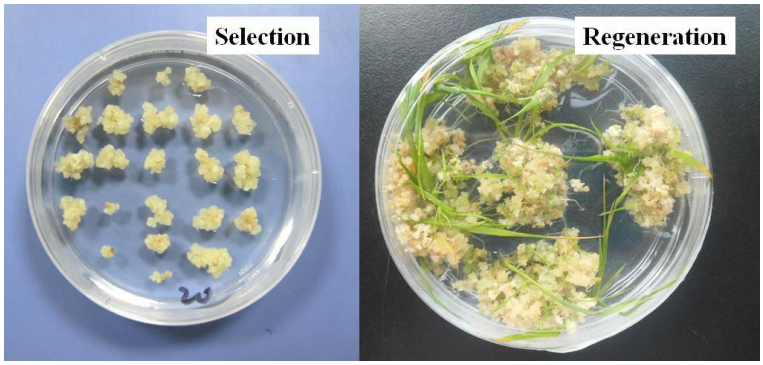

Fig 3. Production of transgenic rice using *pPMI* as selective marker gene.Selection, mannose resistant calli on selection media after 3 weeks of selection. Regeneration, mannose resistant calli on regeneration media for 3 weeks.

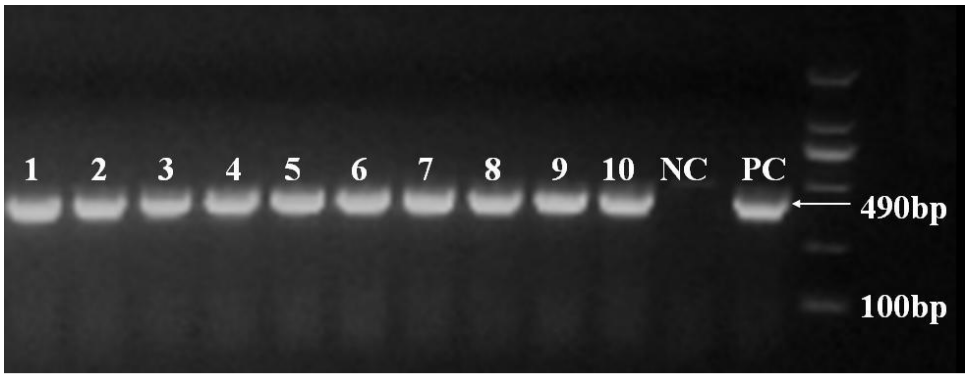

Fig 4. Electrophoresis analysis of PCR amplification of gDNA samples from transgenic plants. Lanes 1-5, trangenic rice samples harboring *PMI*; Lanes 6-10, trangenic rice samples harboring *pPMI*; NC, negative control; PC, positive control.

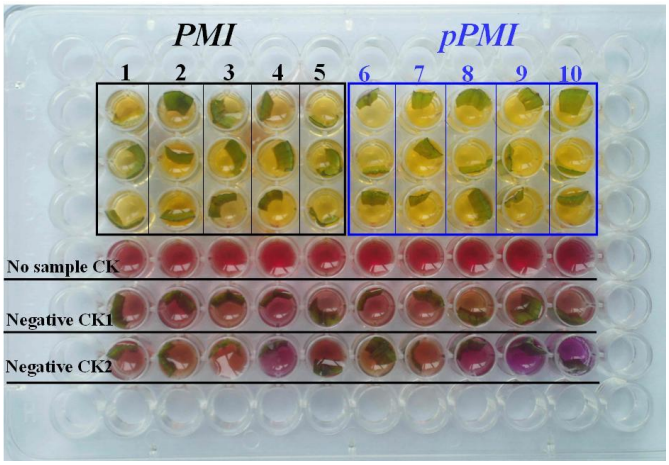

Fig 5. Chlorophenoln red assay of transgenic rice plants. Lanes 1-5, trangenic rice samples harboring *PMI*; Lanes 6-10, trangenic rice samples harboring *pPMI*; No sample CK, no sample control; Negative CK1-2, wide type negative control. Leaves from all transgenic plants (harboring *pPMI* or *PMI*) changed the color of the medium to yellow. No samples control and the medium containing negative control leaves did not change.

The DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) according to the manufacturer's protocol. The PCR primers for *PMI* amplification were 5′-CCGCCGGAGATATCGTTTCACTG-3′ (forward primer) and 5′-CACGGTTCACCCTGCTGGC-TATC-3′ (reverse primer). The PCR primers for *pPMI* amplification were 5′-CGGCGGGGGACATTGTGTCCCTC -3′ (forward primer) and 5′- CACGGCTCGCCTTGCTGG-CTATC -3′ (reverse primer). PCR amplification was carried as described by Duan et al. (2012).

Copy number assay by real-time PCR analysis

To determine the copy number of transgenic events, the TaqMan assay was performed for real-time PCR analysis (Duan et al., 2012; Gui et al., 2014). FAM (6-carboxy-fluorescein) was used as the reporter of *PMI* or *pPMI* target genes. TET (5- tetrachloro-fluorescein) was used as the reporter of the internal control, sucrose phosphate synthase (*SPS*). TAMRA (tetramethyl-6-carboxyrhodamine) was used as the quencher for either the target genes or the internal control. All the primers and probes were synthesized as described previously (Duan et al. 2012; Li et al., 2013; Gui et al. 2014). Real-time PCR were carried out in an Applied Biosystems 7500 Real-time PCR System (Life Technologies, USA) equipped with a 96-well plate using the following program: 5 min at 95°C for pre-denaturation, and then 30 cycles each of 10 s at 95°C and 30 s at 60°C.

PMI protein detection by chlorophenol red (CPR) assay

A CPR assay was used to identify transformed plants expressing the *PMI* or *pPMI* gene (Lucca et al. 2001). Chlorophenol red is the pH indicator. The medium with chlorophenol red has a deep red color at pH 6.0. The tissue was able to metabolize mannose acidified the medium and turned it from red to yellow. Rice leaf pieces from mannose resistant and wide type (as negative control) rice plants were immersed for 1-2 h in a solution containing Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog, 1962) and 20 g/L mannose at pH 5.8. Then the pieces were incubated in 200 µL of filter-sterilized assay medium, containing MS salts and vitamins supplemented with 5 g/L mannose and 50 mg/mL chlorophenol red at pH 6.0 in the 96-well plates. The plates were kept in the dark at 25°C for 3-4 days before evaluation.

Experimental design and statistical analysis

The procedures from calli infection to regeneration were replicated three times. Four hundred calli were infected with *Agrobacterium* as one replication experiment. Student's t-test was used for the statistical analysis of the results. The *P*-values (≤0.01) were calculated to determine the significance.

Conclusion

In this study, the original nucleotide sequence of *PMI* was codon-optimized as rice synonymous codon usage bias. The nucleotides in the *PMI* were optimized according to the preferred codons in rice. This plant code optimized phosphomannose isomerase (*pPMI*) had higher GC content (61.31%), especially GC content at the third position in a codon (83.42%). There were higher transformation frequency (54.5%) and single copy rate (44.5%) using *pPMI* as selective marker than *PMI*. This work showed *pPMI* could substitute the *PMI* in production of transgenic crops.

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