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Biolistic co-transformation of rice using gold nanoparticles

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ABSTRACT- In order to produce transgenic rice lines lacking selectable marker gene, biolistic co-transformation technique using gold nanoparticles was adopted. In the first step, the efficiency of different sizes of gold particles was evaluated. The results showed that the efficiency of the nanoparticles in the transformation was comparable to that of the micro particles. Subsequently, two separate plasmids including p UBC harboring *cry* IA (c) gene and pTRA132 carrying *hph* gene as selectable marker gene were mixed and coated with gold nanoparticles. Embryo genic calli originated from mature rice seeds were targeted with plasmid-coated nan projectiles. Putative transgenic cells were selected from selection media after three subcultures in MS medium were supplemented with 50 mg hygromycine B l^{-1} in three weeks' intervals. The selected calli were then grown into putative transgenic plantlets. Molecular analysis performed on the regenerated lines showed that at least one copy of both genes was inserted into the genome of some transgenic lines. RT-PCR analysis also confirmed successful transcription of the *cry* IA (c) gene. Accordingly, it could be concluded that the biolistic co-transformation method using gold nanoparicles could be a successful method for gene delivery into rice varieties. Nevertheless, expression of *cry* IA (c) gene and loci of both genes should still be investigated in future studies.

INTRODUCTION

Rice is one of the most important crops worldwide whose production is threatened by many biotic and abiotic stresses. For instance, lepidoptera pests and especially stem borer pests such as *Chilo suppressalis*, *Scirpophaga incertulas*, and *Sesamia inferens* are among the serious yield loss factors of rice which may lead to 100% loss of yield in some years (Ghareyazie et al., 1997). Unfortunately, a resistance gene against these pests in rice germplasm is yet to be found. Genetic engineering is one of the most powerful technologies of the 21st century driving the ‘new green revolution’ in the agriculture sector (Li et al., 2014). In fact, gene transformation is considered promising in offering solutions in order to overcome various stresses in plants. For instance, the potentials of *cry* genes to control Lepidoptera pests has led to producing resistant transgenic lines worldwide.

It has been reported that Cry protein is capable of successfully controlling pests including stem borer pests in rice. It is worth quoting that rice as a model plant and a staple food crop has been subjected to different transformation techniques. In the year 1991, successful biolistic transformation (also known as particle bombardment) in rice was reported by Christou et al. and the technique remained as the most common transformation method for many years. Later, *Agrobacterium*-mediated transformation using immature embryos as explant was successfully developed for both Japonica and Indica rice varieties (Chen et al., 2009).

However, due to some disadvantages associated with this method such as inaccessibility of immature embryos throughout a year, the biolistic transformation is still in use (Chen et al., 2009).

It should also be mentioned that particle bombardment is still the most efficient way in achieving plastid transformation in plants and it is the only method so far to achieve mitochondrial transformation (Johnston et al., 1988; Lightowlers, 2011; Larosa and Remacle, 2013). Moreover, the method has a unique advantage allowing the manipulation of the delivered DNA to influence the quality and structure of the resultant transgene loci (Sah et al., 2014). Despite these advantages, the biolistic transformation is also associated with some disadvantages such as the probability of DNA breakage. If these drawbacks are efficiently addressed, the attractive above-mentioned aspects as well as the possibility of co-transformation,

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i.e., simultaneous transferring of more than one gene into a host plant could be instrumental.

By removing marker genes from transgenic plants, development and marketing of genetically-modified products could be achieved at lower costs which might consequently speed up the commercialization of new products (Kuiper et al., 2001; Daniell, 2002; Smyth et al., 2002). In line with that, various strategies such as site-specific recombination, homologous recombination, transposition, and co-transformation have been developed in order to eliminate the marker gene from the nuclear or chloroplast genome after selection (Puchta, 2003).

Among the mentioned methods, co-transformation is defined as the combined delivery of two or more independently inserted DNAs into a recipient genome, capable of excluding marker genes in the next segregating generations. There are different co-transformation protocols developed for both *Agrobacterium*-mediated and biolistic transformations. Although an *Agrobacterium*-mediated co-transformation system was reported for rice with efficiencies ranging from 5-10% (Wakasa et al., 2012) to 86% (Sripriya et al., 2011), the challenges mentioned earlier for *Agrobacterium*-mediated techniques for rice still remained unresolved.

The first attempt to produce a marker-free transgenic line in rice was reported by Peng et al. (1990) who claimed stable transformation of protoplasts of the Indica rice variety, IR54, with both the *neo* and *gusA* genes. Later, *Agrobacterium* co-transformation method was also developed and Golden rice is a typical successful case produced using this technique (Beyer et al., 2002). Biolistic transformation method offers a simple way for co-transformation. More specifically, agronomically important and selectable marker genes are located in different constructs and then a microcarrier would be coated with these separate constructs. The coated microcarriers are then accelerated toward mature seed-driven embryogenic calli (Santosini et al., 2012). Hence, it is expected that two kinds of targeted explants harboring selectable marker gene or both genes can survive on the selection medium. A simple PCR analysis can distinguish between regenerated lines and finally, plantlets containing both genes can be selected. Moreover, the separate constructs may be inserted into different loci and therefore, the selectable marker gene can be eliminated from the transgenic line in the next segregating generation (Yau and Stewart, 2013).

As mentioned earlier, there are some problems associated with the application of biolistic transformation including risk of DNA breakage when micro projectile hits the target tissues. In the primary biolistic system, the micro projectile would be coated with DNA vector and hence, after acceleration of the projectiles toward explants, the first part of the micro projectile hitting target tissues is the DNA vector. This fact may lead to increased risks of DNA breakage during the process (O'Brien and Lummis, 2011). By theory, if the DNA vector can be coated with projectiles, the risk of DNA breakage would be decreased. Accordingly, Kneuer et al. (2000) successfully developed a system using silica nanoparticles for animal cell transformation. O'Brien and Lummis (2011) used gold nanoparticles for human embryonic kidney HEK293 cells arguing that although the transformation rate did not increase, the use of nanoparticles led to ~30% less damaged HEK293 cells following transfection (O'Brien and Lummis, 2011).

To the best of our knowledge, most experiences were on the application of nanoparticles for gene delivery in animal cells including human cells (Wang et al. 2014), and there are few reports on the application of gold nonoparticles for gene delivery in rice cells (Rai et al., 2012). Therefore, the aim of this study was to investigate the possibility of biolistic co-transformation in rice using gold nanoparticles.

MATERIALS AND METHODS

Plant Materials and Tissue Culture

De-husked mature seeds of ‘Hashemi’ rice cultivar were used as plant material. This cultivar is an aromatic commercial cultivar which showed excellent callus induction and regeneration abilities (Mortazavi et al., 2006). The seeds were surface sterilized with 95% ethanol for 2 min and 2.5% chlorax solution for 30 min. After each step, the seeds were rinsed three times with sterile distilled water. N6 medium (Chu et al., 1975) supplemented with 2 mg 2,4-D l^{-1} as growth regulator was used for callus induction. The seeds were sown in 10 cm Petri dishes containing the N6 medium so that their endosperms were soaked in the medium while the bottom surface of the embryos were contacted onto the surface of the medium. The Petri dishes were stored in a 25 °C dark incubator for 5-6 weeks (Mortazavi et al., 2006). Subsequently, the embryogenic calli were harvested and subjected to gene transformation.

Plasmid Vectors

The plasmids pUBC carrying *cry*IA(c) gene (kindly donated by I. Altosaar, Ottawa University, Canada) and pTra132 harboring a hygromycin B resistance gene, *hph* (as selectable marker) (Zheng et al., 1991), were used in co-transformation experiments. The pAct1D vector harboring *gus* gene (McElroy et al., 1990) was used for optimization of the transformation. Schematic maps of these plasmids are shown in Fig. 1.

Optimization of Biolistic Gene Transformation

Three µg of four different types of gold nanoparticles, i.e., 50, 200, 600, and 1000 nm in diameter, were separately mixed with 10 µg of pAct1D plasmid, 50 µl of 0.05 M spermidine, and 50 µl of 1 M $CaCl_2$. The mixtures were then agitated by vortexing for 5 min and were allowed to settle. The supernatants were then removed and the gold pellets were dried on macro carrier disks. The disks were subsequently used in the transformation as described below:

Two kinds of rupture disks comprising 1100 and 1550 psi were used to determine the optimum helium

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gas pressure. The gold-coated DNA was accelerated toward embryogenic calli with BioRad PDS-He1000 apparatus. Distance to the target plate was considered as much as 5 cm. Finally, the targeted calli were subjected to GUS staining and the number of blue spots on the target tissues were scored on the following day (Nishihara et al., 1993). A factorial experiment based on Completely Randomized Design with three replications was adopted as statistical model for data analysis.

Co-Transformation of Rice

Three µg of gold nanoparticles (50-100 nm in diameter) were mixed by 10 µg of each pUBC and pTra132 plasmids. Then, 50 µl of 0.05 M spermidine and 50 µl $CaCl_2$ 1 M were added to the mixture and the vial was agitated by vortexing for 5 min. The mixture was then allowed to settle and after removing the supernatants, the gold pellets were dried on macrocarrier disks. The gold-coated vectors were accelerated toward embryogenic calli using the same BioRad PDS-He1000 apparatus. Rapture disks with 1100 psi gas pressure threshold were used, and the shot gun was performed with a 5 cm distance.

The targeted tissues were transferred into the MS medium supplemented with 50 mgl^{-1} hygromycine B as selection agent. After 3 subcultures on the same medium with 3 weeks' intervals, white and fresh calli were transferred into the regeneration medium consisting of enhanced MS medium (Wang et al., 1987) supplemented with 50 mgl^{-1} hygromycin B, 20 grl^{-1} maltose, 2 mgl^{-1} kinetine and 3 mgl^{-1} NAA as growth regulators, as well as 50 mgl^{-1} triptophan and 4 gr l^{-1} agarose. The Perti dishes were incubated in 25/18 ˚C temperatures and 16/8 h day/night photoperiod until regeneration. The regenerated plantlets were transferred into hydroponic conditions (Yoshida et al., 1976) and were grown in the medium until seed production.

Molecular Analysis

DNA was extracted from leaf samples using the protocol proposed by Dellaporta et al. (1983). The extracted DNA was used in PCR to screen the regenerated plantlets. In summary, two specific primer pairs for amplification of the first and second half region of *cry*IA(c) gene, one specific primer pair for amplification of *hph* gene and one specific primer pair for amplification of the 18S rRNA gene, as internal control, were used in separate PCR reactions. The following temperature profile was used for the PCR: 300 seconds at 95 ˚C as pre-heating, 40 cycles with 45 seconds at 95 ˚C as melting phase, 30 seconds for primer annealing, 60 seconds for extension at 72 ˚C, and finally, 420 seconds at 72 ˚C for final elongation (Table 1). Each 25 µl PCR reaction consisted of 2.5 µl 10X PCR buffer (SinaClone, Cat. No. MM2101), 0.2 µl *Taq* polymerase (10 units per 30 µl, SinaClone, Cat. No. 1601), 1 µl $MgCl_2$ (50 mM, SinaClone, Cat. No. MM2091), 4 µl primer solutions (10 pM), 1 µl dNTPs (10 mM, SinaClone, Cat. No. 7604C), 1 µl DNA sample (30 µM), and finally 15.3 µl double distilled water.

Southern Analysis

Southern blot analysis was carried out to confirm gene insertion into the rice genome. Thirty µg of the purified DNA sample from each putative transgenic and control line, and about 30 ng of pUBC plasmid vector were digested with *Hin*dIII restriction enzyme and separated on an electrophoresis agarose gel. Traditional suction method was used for DNA blotting. Probe was prepared by PCR DIG Probe Synthesis Kit (Roche, Product No.

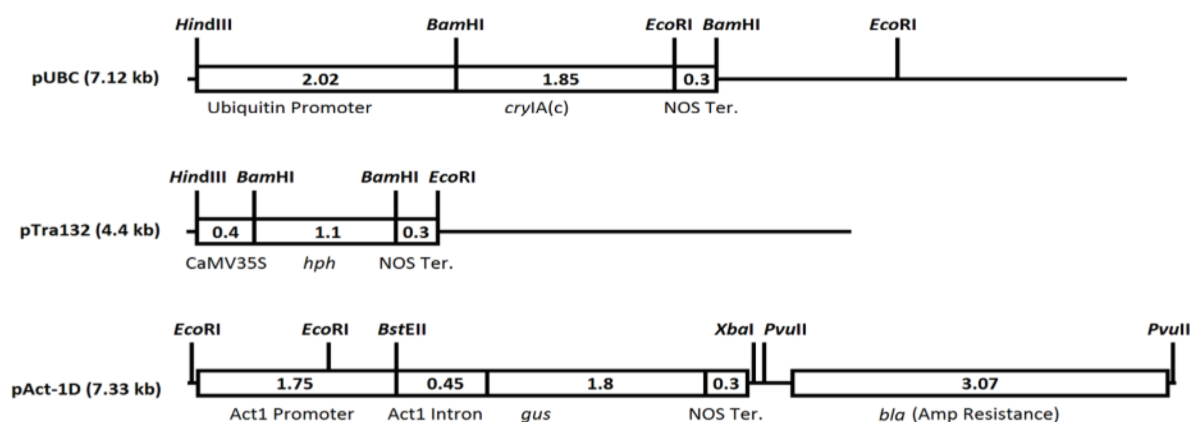

Fig. 1. Schematic map of pUBC, pTRA132 and pAct1-D plasmids. The pUBC plasmid was constructed based on p GEM-4 and was harboring a *cry*IA (c) gene under the control of the ubiquitin promoter. The pTRA132 was constructed based on pUC12 and was carrying a hygromycin phophotransferase (*hph*) gene under the control of the CaMV35S promoter. The pAct1-D plasmid harboring *gus* gene under the control of the Act1 promoter from rice plant was used for the optimization of the transformation system. The numbers in the maps show the size of gene parts in kb.

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Table 1. The Primers used in PCR and RT-PCR analyses in this study.

Primer	Orientation	Sequence (5' - 3')	Annealing Temprature
cry1A(c)1	Forward	CGG TGTAGT TTC CAA TCA GCC TAG	56 ˚C
	Reverse	ACA CTC CCA TCG ACA TCT CCT TGT	56 ˚C
cry1A(c)2	Forward	ATG ACT CCA GAA CCT ACC CTA TCC	56 ˚C
	Reverse	GTC ACA GAA GCA TAC CTC ACA CGA	56 ˚C
Hyg	Forward	AGA ATC TCG TGC TTT CAG CTT CGA	58 ˚C
	Reverse	TCA AGA CCA ATG CGG AGC ATA TAC	58 ˚C
18s	Forward	GTA ACC CGT TGA ACC CCA TT	56 ˚C
	Reverse	CCA TCC AAT CGG TAG TAG CG	56 ˚C

11636090910) based on *cry*IA(c)1 primers (Table 1). Other steps of the Southern blot analysis including blocking, probe hybridization, antibody treatment, and DIG staining were performed according to DIG DNA Labeling and Detection Kit procedure (Roche, Product No. 11093657910).

RT-PCR Analysis

Total RNA was extracted from fresh leaf tissues according to Chomczynski and Sacchi (1987). About 1 mg of each RNA sample was used in a two-step reverse transcription PCR (RT-PCR) reaction. In the first step, cDNA was synthesized using the Transcript or High Fidelity c DNA Synthesis Kit (Roche, Cat No. 05 091 284 001). In the second step, the cDNA was used as template in a PCR reaction using *cry*IA(c)2 gene primers. Control reactions included no transgenic control plant, pUBC plasmid, and artificial transgenic plant, a mixture of both plasmids and non-transgenic plant were considered in the second step of the analysis.

RESULTS AND DISCUSSION

Table 2 summarizes the results of analysis of variance on histochemical GUS staining data scored from targeted embryo genic calli by different gold particles (Fig. 2). The findings presented in the table show that the existing differences among blue spot numbers resulted from the different gold nanoparticles and rupture disc, and their interactions were not significant.

Herein, five out of 12 putative transformed lines resulted from biolistic co-transformation with gold nanoparticle-coated DNA were subjected to molecular analysis. All transgenic lines transformed simultaneously with the pUBC and pTra132 plasmids were confirmed to contain the entire transgene by PCR analysis using four specific primer pairs including Cry1, Cry2, Hyg and 18S (Table 1) which produced the expected 530 bp, 750 bp, 630 bp and 18 bp fragments, respectively (Fig. 3). The findings obtained confirmed that all the tested putative transgenic plants possessed bands related to both pUBC and pTra132 plasmids while the size of the amplified fragments was in agreement with that of the positive control. This result may imply that both plasmids successfully participated in the transformation process. Moreover, they could also be indicative of successful integration of the foreign genes into the rice genome.

Southern blot analysis of DNAs extracted from some putative transgenic plants harboring the vector pUBC indicated that all transgenic lines received at least one copy number of the plasmid (Fig. 4). Since *Hin*dIII cuts the pUBC vector only once, it was expected that the number of hybridization bands would be in agreement with the copy number of the integrated vectors into the transgenic lines. The findings presented in Fig. 4 revealed that one plant (Fig 4, line 2) received only one copy of the gene of interest. However, two other plants received four and three copies of the gene of interest, respectively (Fig 4, lines 1 and 3). Moreover, based on data presented in Fig. 4, it could be concluded that these transgenic plants were indeed originated from independent events. Insertion of plasmids in the respective genomes also demonstrated that gene transformation using gold nanoparticles as projectiles was a feasible and practical way for gene delivery into rice genome.

Reverse transcription-PCR is used to confirm that a transgene can be transcribed in transgenic lines (Dos Santos et al., 2003). The reverse transcription-PCR results obtained for the five transgenic lines investigated are summarized in Fig. 5, confirming successful transcription of the transgene in all transgenic lines. The data presented in this figure also show only one band associated with the *cry*IAc gene implying that no broken copies of the transgene existed in the lines or probably the broken copies of the transgene were too short to be transcribed to produce incomplete mRNAs. This finding confirmed those of O'Brien and Lummis (2011) and Kneuer et al. (2000) who demonstrated that the application of nanoparticles in transformation as projectiles could lower the incidence of inserting broken copies into the genome and could also decrease damaged cell rates during transformation.

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Table 2. Analysis of variance on histochemical GUS staining data scored from targeted embryogenic calli by different gold particles.

Source of Variation	DF	MS	F
Gold Particle Size	3	83.11	3.07 ^{ns}
Rupture Disk	1	03.95	0.15 ^{ns}
Gold Particle Size * Rupture Disk interaction	3	17.25	0.64 ^{ns}
Error	15	27.11	-

ns stands for non-significant at α=0.05.

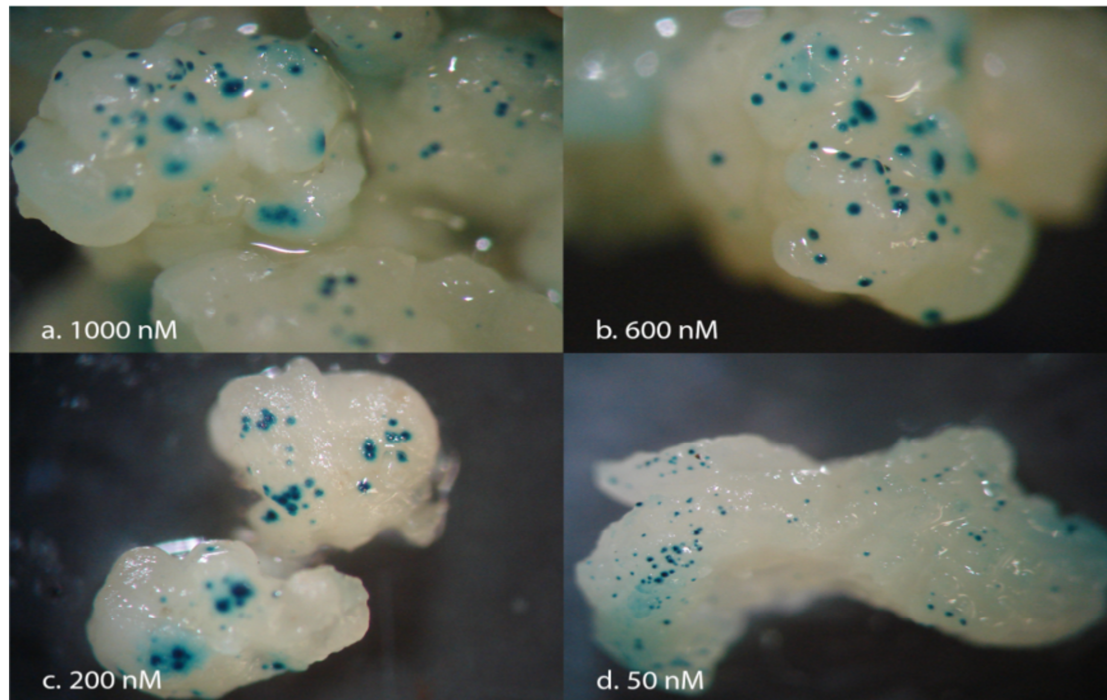

Fig. 2. Transient expression of *gus* gene in embryogenic calli targeted with, a) 1000 nm gold particles; b) 600 nM gold particles; c) 200 nM gold particles; and d) 50 nM gold particles.

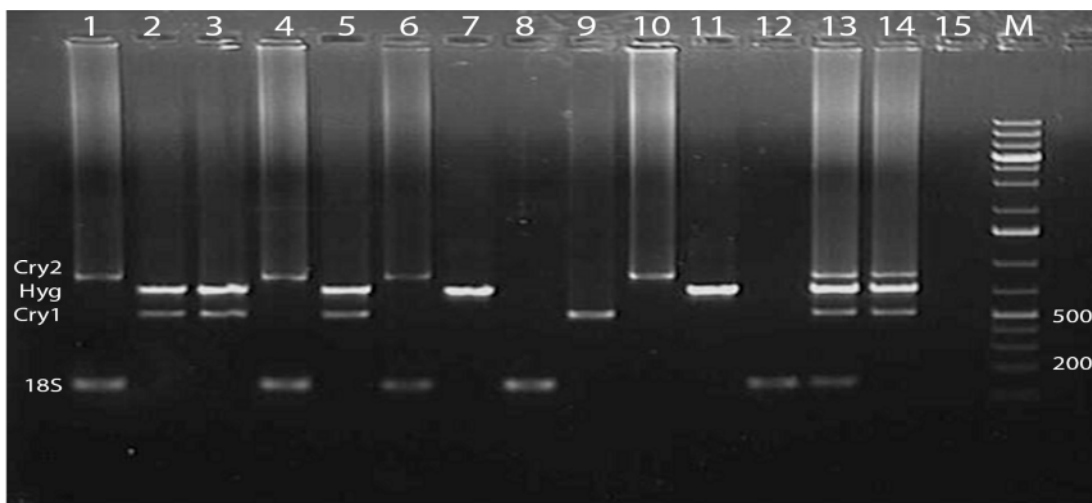

Fig. 3. Electrophoresis pattern of PCR products amplified from five putative rice lines transformed with pUBC and pTra132 plasmids using Cry2 and 18S primers (lines1, 4, 6, and 8) and Cry1 and Hyg primers (lines 2, 3, 5, and 7). Lines 9 and 10 show PCR products amplified from pUBC plasmid samples using Cry1 and Cry2 primers, respectively. Line 11 shows PCR product amplified from pTra132 plasmid using Hyg primer. Line 12, a non-transgenic plant sample was used as DNA template in PCR with a mixture of four primer pairs. 13 is the artificial transgenic plant, a mixture of both plasmids and non-transgenic plant, and line 14 shows PCR products amplified from a mixture of both plasmid vectors using four primer pairs. Line 15, negative control (water) and M, Fermentas 1kb weight marker (SM1333).

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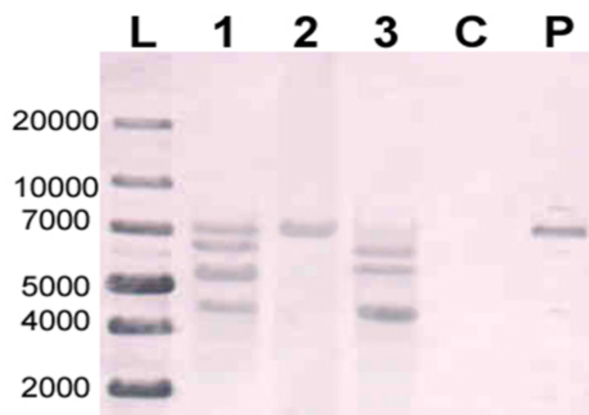

Fig. 4. Southern blot analysis of DNAs extracted from three putative rice plants transformed with pUBC and pTra132 plasmids (lines 1-3), from a non-transgenic plant (negative control, line C) and from a pUBC plasmid (line P) using a *cry*IA(c) gene probe. L= M DIG-labled weight marker

It is worth quoting that the application of gold particles of 1 or 0.6 µM in size in the transformation of plant tissues is common. Our results showed that gold nanoparticles could also be applied as nano projectile in biolistic transformation offering similar efficacy as that of micro particles. Similar observations were reported by O'Brien and Lummis (2011) who also investigated the application of nano-sized gold particles for the transformation of human cells, achieving transformation rates comparable with those of micro particles. They argued that the advantage of nanoparticles would be attributed to the lower number of broken copies during transformation.

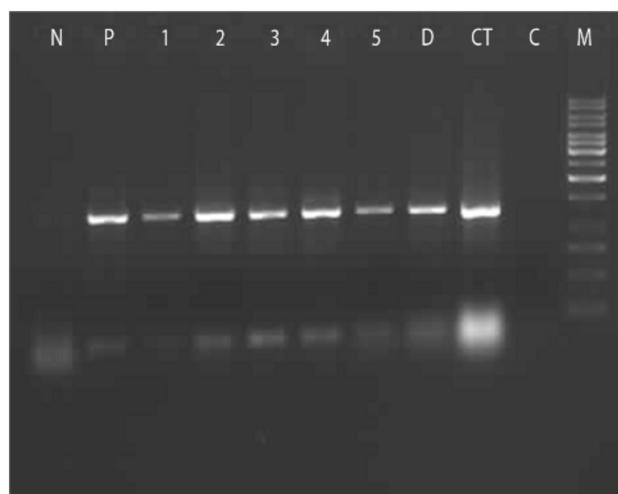

Fig. 5. RT-PCR analysis of five rice transgenic lines using CryIA(c)2 primers. Abbreviations are as below: N non-transgenic plant; P, pUBC plasmid; 1-5, transgenic plants; D, DNA from a known transgenic plant as positive control; CT, artificial transgenic sample; C, negative control or from RNA of non-transgenic plant; M, 1kb+ DNA ladder.

Although accumulation of Cry protein and its ability to control stem borers should be evaluated by further analyses such as western blot analysis and bioassay, mRNA production by the transgene in combination with the expression of hph gene during selection and regeneration processes could be indicative of the fact that the *cry*IA(c) transgene was active in the obtained transgenic lines. This finding also confirmed the intactness and stability of the transgenes in the genome of the rice transgenic lines. Overall, the results of this study demonstrated the suitability of gold nanoparticles asprojectiles for stable rice plant transformation.

Southern blot analysis revealed that two out of three plants tested were harboring multiple copy insertion (Fig. 4). Ghareyazie et al. (1997) showed that the integration of a few plasmids to each other through crossing over to produce a large plasmid would be a probable event during biolistic transformation. In those cases, Southern blot analysis reveals numerous bands which may take place as multiple bands along the lane, indicating that the amount of plasmids for transformation should be decreased. Therefore, the outcomes of Southern blot analysis would help to prevent coating of multiple copies of plasmid with gold nanoparticles. Indeed, if the Southern blot analysis showed multiple bands for a transgenic line, it means that the amount of the plasmid used for biolistics transformation should be decreased.

CONCLUSIONS

In general, the results obtained herein showed that biolistic co-transformation of rice using gold nanoparticle as projectile was a practical method. In this research, two separated plasmids were used in the transformation and were successfully integrated in the genome of an aromatic Iranian rice cultivar. Since the transgenic lines did not exhibit any abnormal physiologic traits and showed a growth pattern similar to that of the non-transgenic parental line, it is predictable that the transgenic lines were fertile. In practice, the plants produced a few seeds. The seeds could be well grown to the next generation to investigate the inheritance patterns of both *cry*IA(c) and *hph* genes.

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تراریزش توام گیاه برنج به روش زیست‌پرتابی با استفاده از نانوذرات طلا

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چکیده- به منظور تولید لاین‌های برنج تراریخته مقاوم به آفات که فاقد ژن نشانگر انتخابی باشد، تکنیک ریزپرتابی با استفاده از نانوذرات طلا مورد استفاده قرار گرفت. در نخستین گام، کارایی نانوذرات طلا با اندازه‌های مختلف مورد ارزیابی قرار گرفت. نتایج نشان داد که نانوذرات طلا می‌توانند به اندازه میکروذرات در انتقال ژن کارایی داشته باشند. در گام بعد، دو پلاسمید مجرا مورد استفاده قرار گرفتند. پلاسمید puBC حامل ژن cry1A(c) و فاقد ژن مارکر انتخابی به همراه پلاسمید pTRA132 حاوی ژن مقاومت به هیگرومایسین به عنوان نشانگر انتخابی مخلوط شده و سپس با نانوذرات طلا پوشش داده شدند. این مخلوط پلاسمیدها به کالوس‌های جنین‌زایی که از بذور رسیده منشا گرفته و دارای توان تقسیم سلولی و باززایی بالایی بودند، به روش ریزپرتابه وارد شدند. سلول‌های تراریخته احتمالی از بافت‌های بمباران شده پس از ۳ دوره گزینش در محیط کالوس‌زایی N6 حاوی ۵۰ میلی‌گرم در لیتر هیگرومایسین B مورد گزینش قرار گرفتند. نهایتاً کالوس‌های مقاوم به هیگرومایسین در محیط باززایی MS حاوی ۵۰ میلی‌گرم در لیتر هیگرومایسین B باززا شدند. در نهایت گیاهان تراریخته احتمالی حاصل با آنالیزهای مولکولی مورد بررسی قرار گرفتند. این آنالیزها نشان داد که حداقل یک نسخه از ژن مورد نظر به گیاهان تراریخته وارد شده است. آنالیز RT-PCR نیز تأیید کرد که ژن cryIA(c) در گیاهان تراریخته قابلیت رونویسی و بیان ژن را دارد. لذا می‌توان استنباط کرد که استفاده از روش ریزپرتابه و نانوذرات طلا می‌تواند یه روش کارا و موثر در انتقال ژن و تولید گیاهان تراریخته به ویژه در گیاه برنج مورد استفاده قرار گیرد.

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