Development of Transformation System for
Some Egyptian Rice Genotypes Using Particle Bombardment

M.M. Saker, S.M. Abdallah, S.S. Youssef, H.A. Moursy and A.M. El Sharkawy

Department of Plant Biotechnology, National Research Centre, Egypt
Genetic Engineering Research Centre, Faculty of Agriculture, Cairo University, Egypt

Abstract: Although several reports on rice transformation have been published, producing transgenic Egyptian rice is still in its infancy stage. We report an improved regeneration and transformation system for some superior Egyptian rice genotypes. The developed system involves the proliferation of highly regenerative embryogenic callus from either mature or immature embryo explants onto MS medium containing 3 mg/L 2,4-D (callus induction medium, CIM). Differentiation of embryogenic callus into plantlets occurred onto MS medium supplemented with 2 mg/L Kinetin + 1 mg/L NAA (shoot recovery medium, SRM). The regenerants were rooted onto basal MS medium and easily acclimatized to ex vitro conditions. Although all investigated genotypes responded to this regeneration system, the regenerative capacity of some genotypes (Giza 177 and Giza 178) showed the highest regenerative capacity, while others (Giza 171, 172 and 175) showed the lowest response. The obtained results of genetic transformation indicate that the origin of the shooted explants (recipient tissue), flight distance of microprojectiles and the applied pressure were the most effective parameters. Also it was found that hygromycin was more efficient than kanamycin as a selective antibiotic in the selection medium. The optimal conditions for the production of transgenic rice involve the shooting of explants with a microprojectile at applied pressure of 1 300 psi and flight distance of 9 cm. Biochemical and molecular analyses of putative transformants using both fluorescence and histochemical GUS assays and PCR amplification using specific primers flanking genes of interest confirmed the stable transformation events.

Key words: Oryza sativa • transformation • regeneration

INTRODUCTION

Rice is likely the most important food crop in the world and almost half of the world’s population depends on rice as their staple food [1]. Therefore, to meet the needs of the growing world population, conventional breeding methods need to be combined with recent achievements in rice biotechnology. Nowadays the available complete rice genome sequence has opened up a sea of opportunities and the current rice post-genomics era will likely change our approach towards problem solving in biology [2, 3]. In parallel, rice technology has moved beyond proof of concept and reached a stage where it can supplement existing breeding methods to improve production [4, 5].

In Egypt, rice production has reached a record due to the integrated management programs, but still there is a room to increase the potentiality of the varieties. One procedure to increase rice productivity and farmer revenues is to minimize the use of pesticides through the production of transgenic rice that autoresists notorious pests, namely rice blast and rice stem borer, which cause up to 50% yield loss in some areas. In this context, there are so many successful examples worldwide that employed genetic transformation approaches to enhance natural pest resistance of plants [6-11]. Practicing of this strategy in Egyptian rice improvement involves the development of reproducible regeneration and transformation systems for the economically important genotypes.

Rice genetic transformation has taken the rapid strides since the first transgenic rice plant was produced 15 years ago. Although the introduction of DNA into rice cells is easy, regenerating of fertile transgenic plants is difficult and limited to some Japonica and one indica cultivars. To overcome the regeneration problem, most of the previously published studies used immature embryos [13, 14]. Unfortunately, immature embryos are disadvantageous because collecting them is tedious and laborious and they must be collected in the reproductive

Corresponding Author: Dr. M.M. Saker, Department of Plant Biotechnology, National Research Center, Egypt, P.O. Box 11622
phase. To address this problem, transformation systems for some of japonica and indica varieties using mature embryo-derived callus were established [14]. During the last 10 years, great progress has been made to develop a high frequency, routine, genotype independent and reproducible genetic transformation protocol for rice, either through particle bombardment or Agrobacterium-mediated transformation technology [15]. There has been much debate on these two systems in terms of their efficiency and the pattern of integration, mode of inheritance and stable expression of the transgenes. However, experiments dealing with both systems keeping all other factors constant, like target explants, genes of interest, tissue culture cycle, are lacking [16].

In spite of the great progress in rice improvement using biotechnology, there is no published data regarding the improvement of Egyptian rice varieties using genetic transformation. This background necessitates the development of an efficient regeneration and transformation system for the Egyptian rice genotypes, as a key step towards the improvement of agronomical and nutritional quality of rice. To address this problem, the present study was suggested with the objective to establish a robust transformation system using particle bombardment as a prerequisite for the introduction of agronomically-important genes into commercially important superior Egyptian rice genotypes.

**MATERIAL AND METHODS**

**Plant material:** Eight rice varieties, namely Giza 171, 172, 175, 176, 177, 178, Sakha 101 and Sakha 102 were kindly provided by the Rice Research Program, Field Crop Institute, Agricultural Research Centre, Ministry of Agriculture, Egypt.

**Plasmids:** Two molecular constructs were used in the present study. The first one is the E. coli vector (pGL2) containing 1.1 kb chitinase coding fragment and hygromycin resistance under the control of the plant promoter 35S and NOS terminator [9] and the second is cholesterol oxidase gene cloned in the E. coli vector (pBC4) alongside GUS reporter gene and NPTII gene under the control of 35S promoter and NOS terminator [16].

**Tissue culture:** Immature seeds were collected after 15 days of anthesis. Both mature and immature seeds were dehusted and sterilized in 70% ethanol for 1 min and then in 75% commercial Clorox (%25% NaOCl) with 1-2 drops of Tween 20 for 30 min. Seeds were rinsed four times with sterilized distilled water. Aseptic seeds were cultured onto callus induction medium (MS + 3 mg/L 2, 4-D) and incubated in the dark at 27°C. After four weeks of cultivation onto callus induction medium, proliferated calli were separated from the endosperm and transferred to the same fresh medium. For shoot recovery from embryogenic callus, somatic embryos were transferred to MS supplemented with either 1 mg/L NAA or 2 mg/L BA and incubated in light photoperiod (16/8 light/dark, 3000 lux). Differentiated shoots were rooted onto basal MS medium.

**Bombardment conditions:** Bio-Rad Helium PDS-1000/He device was used in the bombardment experiments. Bombardment conditions were carried out as described by Valdez et al., with minor modifications [14]. Different ranges of applied pressure (900, 1100, 1300 and 1500 psi) and flight distances (3, 6 and 9 cm) were empirically optimized.

**Selection and recovery of putative transformants:** Lethal concentrations of hygromycin and kanamycin were identified based on the results of preliminary experiment, which indicated that the growth of the control callus was completely inhibited onto 50 mg/L hygromycin and also indicated that 100 mg/L kanamycin is the minimum concentration of kanamycin which could be sufficient for selection. Bombarded calli were transferred to CIM supplemented with 50 mg/L of hygromycin or 100 mg/L Kanamycin, followed by subculture every 14 days (for at least two subcultures) on the same fresh medium for propagating of the transformed calli and to obtain clear differential growth between antibiotic resistance and antibiotic sensitive calli colonies (escapes). Kanamycin and hygromycin resistant calli colonies were transferred to SRM supplemented with the same type and level of antibiotics as in CIM and incubated in 16/8 light/dark photoperiods. Proliferated shoots were multiplied onto MS medium supplemented with 2 mg/L BA and transferred to MS basal medium for rooting.

**Gus assays:** Histochemical GUS assays were performed typically as described by Jefferson et al. [17]. For fluorescence GUS assays, a small part of tissue (2-4 mm) was homogenized in 100 µL GEP solution in a well of an ELISA plate, then 100 µL MUG solution was added and the plate was incubated in dark at 37°C overnight. Positive samples have a strong blue fluorescence under UV light. GEP solution consists of 50 m M NaHPO₄,
10 mM DTT or 2-Mercaptoethanol, 1 mM Na 2 EDTA, 0.1% Na - Lauroylsarcosine and 0.1% Triton. MUG consists of 20 mg MUG in 50 mL GEP solution.

**PCR confirmation:** Genomic DNA was isolated from transformed callus and plantlets according to Doyle and Doyle [18]. The presence of CHI and CHO genes in transformants was confirmed by PCR amplification using specific primers. The sequences of CHI primers are 5'-CCCTCAACTGCTGATCA-3' forward primer and 5'-TGGCTGACTTCAGGTTGTG-3', reverse primer and the sequences for CHO gene are 5'-GACGGATCCATGAGAAGGTACATG-3' and 5'-TCGACCTGACAGCTCAAGATCAA-3' (forward and reverse, respectively). Amplification was conducted for 35 cycles using Biometra (Germany) thermal controller preheated to 92°C as follows: denaturation at 92°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 2 min. PCR products were separated by 1% agarose gel electrophoresis using TAE buffer and visualized with ethidium bromide.

**RESULTS**

**Callus proliferation and shoot regeneration:** Data of the preliminary studies indicated that 2,4-D at concentrations ranging from 1 to 5 mg/L induced callus proliferation from mature embryo explants of the eight tested varieties. Also immature embryo-derived callus was highly regenerative and responsive than mature embryo-derived callus in all tested varieties. The initiation of callus was observed after two weeks of cultivation onto CIM (MS + 3 mg/L 2, 4-D) under dark conditions. Most proliferated calli were compact and nodular. Some batches of soft calli were also proliferated and were discarded during subculturing alongside germinating seedling. Upon transfer of nodular embryogenic callus onto MS medium containing NAA at 0.5 or 1 mg/L in combination with 2 mg/L of BA or Kinetin (SRM), calli began to differentiate into shoots. Subculturing of the embryogenic callus onto the same fresh CIM medium led to repetitive cycles of somatic embryo formation (nodular structures) and increment in fresh weight of calli pieces. The frequency of calli responding for differentiation was genotype dependent. Two cultivars namely, Giza 177 and Giza 178 showed the highest regenerative capacity. The cultivars Sakha 101 and Sakha 102 also responded to the developed protocol. However, cultivars Giza 171, 172 and 175 showed poor response. Roots were developed upon transferring of the regenerants onto basal MS medium (growth regulator-free medium). The developed plantlets were easily acclimatized to ex-vitro conditions (green house). Practicing of this protocol led to the production of fertile and true-to-type rice plants from mature embryo explants of the eight investigated Egyptian rice genotypes (Fig. 1).

**Bombardment conditions and selection of putative transgenic calli:** For this purpose, the plasmid pBC4 harboring the economically important gene cholesterol oxidase, which confers the transgenic plants tolerant to insects and the selectable marker gene GUS was used in the bombardment experiments. Bioloistic parameters influencing gene delivery in rice were investigated. Data summarized in Table 1 indicate that a flight distance of 9 cm (24) gave the highest percentage of callus survival (20%) onto selective medium following shooting. It was also noticed that shooting of callus at shorter flight distance (2/3, ca 3 cm) led to destructive effects on the shoots callus. This observation can be noticed from the percentage of callus shooted with uncoated particle onto antibiotic free medium (Table 1). Regarding the effect of applied pressure, data of the Table 2 indicate that using rupture disks of 1300 and 1500 psi gave the highest survival.
Table 3: Effect of the origin of the recipient tissue ( explants) on the efficiency of gene delivery

<table>
<thead>
<tr>
<th>Exp. Type</th>
<th>Cholesterol oxidase gene</th>
<th>Cuscuta gene</th>
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<tbody>
<tr>
<td></td>
<td>No. of bombarded explants</td>
<td>% of survival</td>
</tr>
<tr>
<td>Callus</td>
<td>100</td>
<td>15</td>
</tr>
<tr>
<td>Immature embryo</td>
<td>200</td>
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<tr>
<td>Mature embryo</td>
<td>700</td>
<td>40</td>
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Fig. 1: Different stages of in vitro regeneration of Egyptian rice genotype Giza 177, A): Proliferation of embryogenic callus from mature embryo explants. B and C): Shoot recovery onto SRM. D): Rooting onto MS basal medium and E, F and G): Acclimatization to ex vitro conditions.

survival of callus onto selective media (40 and 42%, respectively). Regarding the type of target tissue (recipient tissue), the obtained data, tabulated in Table 3, indicate that the origin of recipient explants is of great importance on the efficiency of transformation. It was found that immature embryo explants gave the highest percentage of callus survival (15 and 3%) onto the selective media, in spite of the selection pressure applied (kanamycin and hygromycin, respectively). It may be concluded that flight distance of 9 cm and applied pressure of 1300 psi are the optimal physical conditions which gave the highest frequency of putative transgenic callus based on survival onto selective media.

The percentage of callus survival on kanamycin selective media was almost 3-20 folds greater than its survival on hygromycin selective media. Data of Table 3 also indicate that immature embryo-derived callus showed the highest percentage of survival, i.e. transformation frequency, onto hygromycin selective media (3). In both cases, transgenic shoots can be obtained onto kanamycin and hygromycin selective media. In some cases, albino shoots were regenerated onto kanamycin containing media (Fig. 2). The frequency of transformation events ranged from 1 to 40% based on the type of target tissue and the applied selective pressure. Although, the highest percentage of calli survival was recorded onto kanamycin selective medium for mature embryo-derived explants, the recovery of transformed shoots was higher in the case of immature-embryo derived callus.
Fig. 2: Proliferation of transgenic rice shoots (albino and normal) onto kanamycin containing selective medium A and B, respectively), C: Proliferation of transgenic shoots onto hygromycin containing selective medium.

Fig. 3: GUS blue fluorescence, visualized under UV light, of tissue sap of transformed rice shoots harboring the selectable marker gene GUS. A: Wells (1-5) transformed and well (6) untransformed (negative control), B: Histochemical assay of Gus positive rice embryogenic callus.

Biochemical and molecular analysis: Detection of GUS fluorescence under UV light was used to preliminary select transformed shoots following shooting with microprojectiles carrying the plasmid pBC4, which harboring the selectable marker gene GUS, in addition to the economically important gene cholesterol oxidase. The detection of GUS fluorescence is very simple and efficient, even at very low level of GUS expression. Further confirmation of GUS expression was performed histochemically. The most interesting observation was the confirmation of the results of fluorescence GUS assays by histochemical GUS assays (Fig. 3). The efficiency of this simple GUS assay was previously confirmed [19].

Fig. 4: PCR analysis of transgenic rice shoots harboring cholesterol oxidase (A) and chitinase (B) and M is 1 kbp marker, lane 1: non transformed control callus, lane 2: transgenic clone no. 1, lane 3: transgenic clone no. 2 and lane 4: positive control. Arrows point to the expected DNA fragment of interest.
Molecular confirmation was performed on the DNA level using PCR amplification. Specific primers flanking chitinase and cholesterol oxidase genes were used for PCR amplification. Data presented in Fig. 4 show GUS positive transgenic clones having the expected amplification products, as in the case of positive control, for chitinase gene (ca 450 bp) and cholesterol oxidase gene (ca 300 bp).

DISCUSSION

This study describes an efficient biolistic-mediated transformation system for economically important Egyptian rice genotypes using embryogenic callus derived from mature embryo explants. The developed protocol involved prior optimization of regeneration system for the investigated genotypes using mature embryos. The overall trend of type and level of growth regulators which were used in this study to induce shoot proliferation from immature and mature-embryo derived calli cultures is in accordance with the results of Oinam and Kothari [20]. Despite of the vast amount of literature on rice tissue culture [21], there are numerous difficulties in obtaining the desired response from rice genotypes of different origins and of different genetic make up, accordingly it is an essential task to develop sufficient plant regeneration from each genotype empirically. These results on totipotency of mature seed explants are very useful in any protocol aiming at genetic transformation of rice as the mature embryos can be obtained at any time of the year and it will be more convenient than the use of immature embryos which is routinely used in most of previously published studies [12, 13]. The results of optimization of the bombardment conditions indicate that flight distance of 9 cm and applied pressure of 1300 and 1500 psi (Tables 1 and 2). Such observation points to some sort of damage and/or inhibition of callus growth as a result of uncoated tungsten microparticles. Pervious studies pointed out to this disadvantage of biolistic and attributed it to the cytotoxicity of unknown nature [12, 24]. Recently, this side effect has been studied in details by Krysiak et al. [25], who reported the generation of DNA double-strand breaks and inhibition of somatic embryogenesis by tungsten microparticles in wheat.

It was also observed that hygromycin is more efficient as antibiotic for selection of transgenic rice callus in comparison with kanamycin (Table 3). This low efficiency on kanamycin may be due to high percentage of escapes, i.e. putative transformants. It is also possible that the cells competent for regeneration were not competent for transformation or cells competent for transformation lose their ability for regeneration due to stress caused by the impact of the particles [24, 26].

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