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Evaluation of Factors Impacting *Agrobacterium*-mediated *Indica* Rice Transformation of *IR58025B* - a Public Maintainer Line

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Abstract

In this study, we report our efforts to develop an efficient *Agrobacterium*-mediated transformation protocol for the transformation of *Indica* rice *IR58025B* with mannose as a selection agent. Various factors, such as basal medium, culture temperature, strains for infection, air-drying duration of calli after infection, and salt concentration of co-culture medium, were systematically optimized so that the highest transformation frequency (TF) reached to 74%. About 94% of mature embryos were induced to generate calli of high quality on modified NB medium. Both callus quantity and quality were improved when callus was induced from endosperm-free embryos in comparison to intact mature seeds. Higher temperature (32°C) showed improvements when compared to lower temperatures at (25°C and 28°C) for both callus induction and transformation. *Agrobacterium* strains of EHA105 produced the best TF in comparison with EHA101, LBA4404 and AGL1. However, LBA4404 produced the highest single copy frequency. Prior to co-cultivation, air-drying of inoculated calli was performed in a laminar flow hood for 4-7 hours and produced a higher TF. Lower salt concentration for co-cultivation medium enhanced both DNA delivery and TF.

Keywords: Agrobacterium-mediated transformation; Air-drying; Culture temperature; Co-transformation

Abbreviations: PMI: Phospho Mannose Isomerase; TF: Transformation Frequency; COC: Co-Cultivation Medium; T-DNA: Transfer DNA

Introduction

Rice is one of the most important cereal food crops in the world and *Indica*-type rice provides the staple food for more than half of the world's population (http://faostat.fao.org/site/339/default.aspx). In spite of its large scale production, a number of abiotic and biotic factors have limited its productivity. To meet the growing demands of an ever-increasing global population, genetic transformation of *Indica* rice, particularly for public breeding materials, has become a matter of increased urgency. Many factors are known to affect the efficiency of T-DNA delivery to plant cells, such as different plant varieties [1,2], different explant types [3-5], the quality of explants and *Agrobacterium* strains [6], the cell density of *Agrobacterium* for infection, inoculation period, co-culture medium and desiccation stress of the tissue before or after infection [6-9].

Indica rice is still known as recalcitrant for transformation although *Japonica* rice was transformed using the *Agrobacterium*-mediated method since 1994 [10] and *Indica* rice in 1996 [11]. Few reports on the *Agrobacterium*-mediated transformation of *IR58025B* are available [12]. This public cultivar is commonly applied to generate hybrids by breeders in the south Asia since it has higher combining ability and generates good hybrids. Therefore, there is a need to evaluate various factors that affect tissue culture and to develop a highly efficient *Agrobacterium*-mediated transformation protocol for this public cultivar. Hiei et al. [13] reported a protocol for Group I *Indica* rice transformation including *IR58025B* using immature embryos as explants. Our study mainly focused on callus explants derived from mature seeds and systematically optimized several factors that could influence transformation frequency. These factors include the basal medium for generating high quality calli, the optimized tissue culture temperature, different strains, desiccation after infection, different salt concentration in co-cultivation (COC) media, and the size of transformation constructs. By optimizing these factors, a highly efficient and reproducible *Agrobacterium*-mediated transformation protocol was developed for *Indica* rice-*IR58025B*, a public maintainer line used in hybrid rice production.

Materials and Methods

Callus induction

Tissue culture was performed as previously reported by our lab [14,15]. De-husked mature seeds of *IR58025B* were surface sterilized, soaked in autoclaved water for 4-6 hours at room temperature (28°C) before endosperms were removed with a scalpel blade. Embryos were cultured on callus induction medium with a scutellum side contacting the callus induction medium. Rice embryos were cultured at 32°C unless specified otherwise.

Transformation method

Transformation procedures were performed as previously reported [14]. PMI-09 (maize gene codon optimized *pmi*) driven by the maize Ubiquitin promoter was used in all of the transformation experiments with the exception of the strain comparison experiments in which PMI-01 (native *pmi* gene from *E. coli*) driven by the rice Actin promoter was used [14]. Embyogenic calli were inoculated with the *Agrobacterium tumefaciens* strain EHA105, LBA4404 and AGL1 respectively in those experiments to compare the three strains. After infection with *Agrobacterium*, the calli were placed onto an empty plate with 3 pieces of dry filter paper on the bottom and a piece of dry filter paper on the

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top of infected calli for 0, 4, 7 hours respectively. The petri dishes were covered with the plate lid during air-drying in a hood. After air-drying for 0-7 hours, the calli were transferred to the co-culture medium. Three types of co-culture media were tested; normal basal medium, 1/4 salts level and 1/8 salts level medium. All the calli on the co-culture media were cultured at 23°C for *Agrobacterium* infection. The infected calli were divided into three groups and maintained at 25°C, 28°C, and 32°C throughout all of the following transformation steps: recovery, selection, regeneration, and rooting (Figure 1).

Callus induction and COC media with treatments are introduced in corresponding experiments. The recovery medium is similar to the COC medium with following differences: 300 mg/l instead of 800 mg/lcasamino acids; 30 g/l instead of 20 g/l maltose; 3 g/l Phytagel to replace 8 g/l agarose; removal of 10 g/l glucose and 20 mg/l acetosyringone, with an additional 500 mg/l proline. The mannose selection pressure is the same as described in our previous report [14]. The shoot regeneration medium is similar to NBKA reported by Duan et al. [15] with the following modifications: 1 mg/l kinetin to replace 0.5 mg/l Kinetin, 200 mg/l Timentin to replace 250 mg/l Carbenicillin, and 3 g/l Phytagel to replace 2 g/l Phytagel, 20 g/l maltose and 5 g/l mannose to replaced 30 g/l sucrose and 30 g/l sorbitol. The rooting medium consisted of ½ MS basal salts and vitamins, MS iron, 30 g/l sucrose, and 4 g/l Phytagel. Figure 1 outlines our optimized process, with current standard operation conditions in bold and treatments in parenthesis.

Co- transformation

The map of vectors used in co-transformation is shown in Figure 2. The vector of 22189 is transformed with 4 mg/l bialaphos in the selection medium. OD660 of *Agrobacterium* suspension for 22189 and 21971 was adjusted to approximately 0.1, respectively, and then mixed together for co-transformation (Figure 2).

Analysis method

The TaqMan analysis was performed as previously reported by Gui et al. [14], using a relative quantification analysis to get the copy number of transgenic plants against a standard curve. Fam was used as the reporter of target genes and TET as the reporter of internal control and TAMRA was used as the quencher for either the target genes or the internal control.

PMI: Primer-forward: CCGGGTGAATCAGCGTTT

Primer-reverse: GCCGTGGCCTTTGACAGT

Probe: TGCCGCCAACGAATCACCGG

PAT: Primer-forward: TGGAAGCTCGAAATCTCTTTGC;

Primer-reverse: AACTCTTAGAGCTGCTGGATATAAGCA;

Probe: AGAATCCAACATCATGCCATCCTCCATG

GFP: Primer-forward: TTGCGCTGCCAGTTCGA

Primer-reverse: AAGTGCCAGTCGGGCATCT

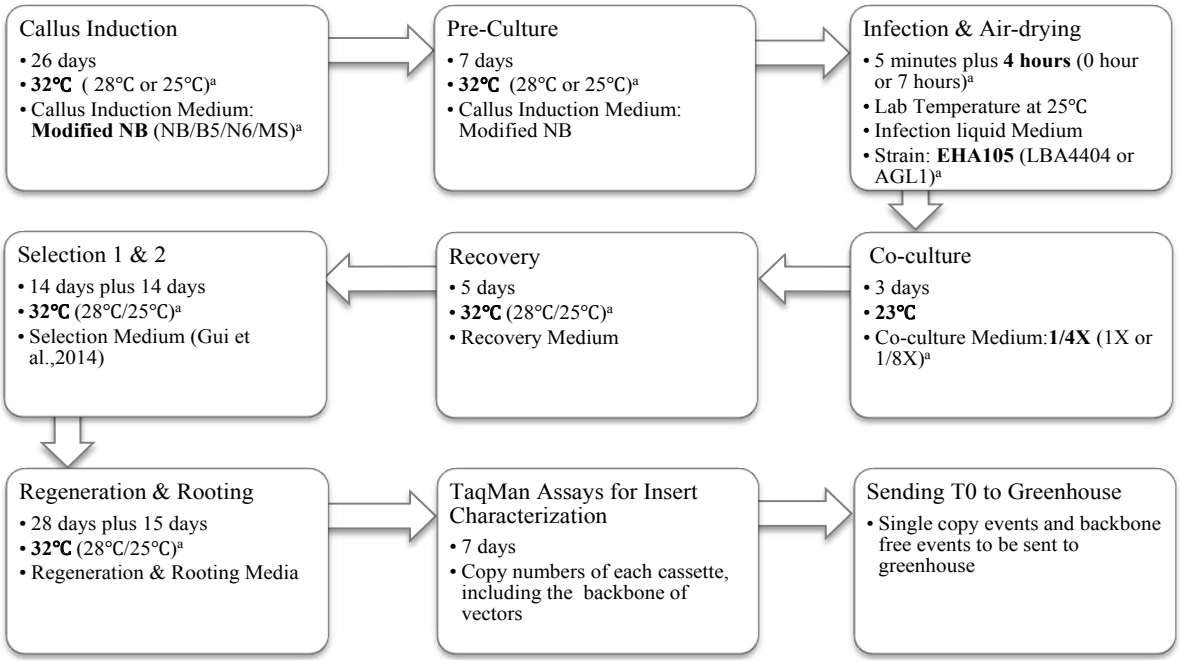
Probe: CCGTGTACAAGGCCAAGTCCGTGC

JMP statistical discovery software was used for statistical analysis.

Results

Callus quality affected by different media and explant types

Five types of basal media were tested: N6 [16], B5 [17], MS [18], NB (combination of N6 macronutrient components and B5 micronutrient and B5 organic components), and modified NB (with 10 times B5 micronutrient) (Figure 3). Based on the visible assessment of callus appearance, such as colour, texture and size, modified NB medium was observed as the best medium for callus induction of this cultivar. Calli grown on modified NB medium appeared to be of better quality



[a]Currently optimized protocol as the standard protocol in bold was in comparison with those treatments in parenthesis.

Figure 1: *IR58025B* transformation process and parameters tested.

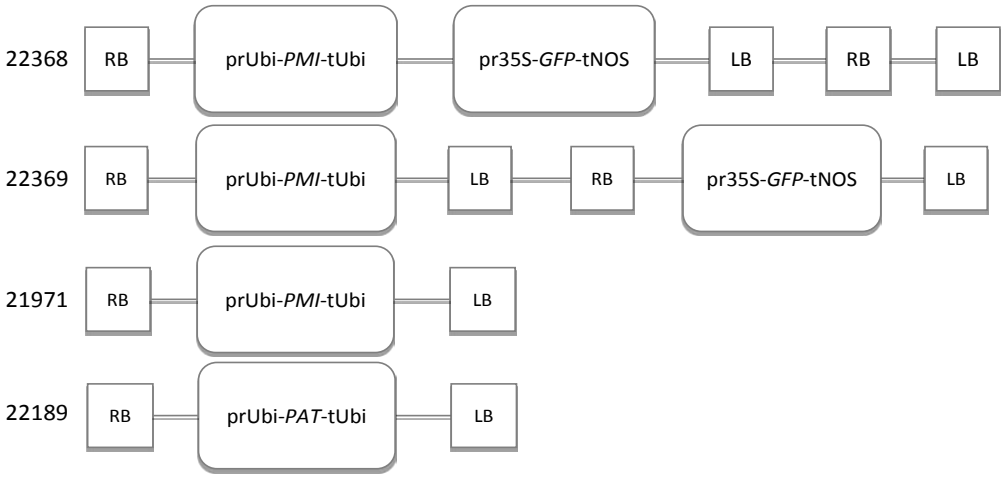


Figure 2: The vector maps used for co-transformation. LB and RB represent the orientation of left border and right border of T-DNA. Promoters and terminators are abbreviated as pr and t with genes in italic.

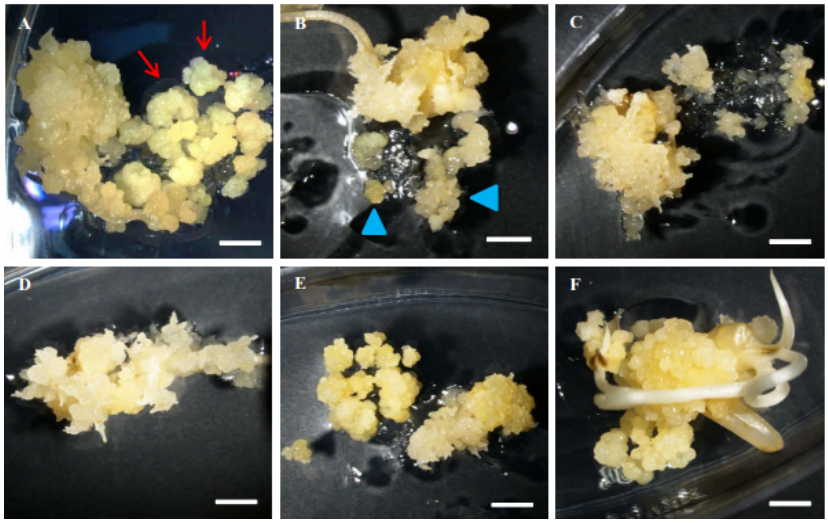


Figure 3: Callus induction on different media with or without endosperm. The pictures were taken after callus induction for 26 days. A-E, callus induction from embryos without endosperm on modified NB (A), NB (B), B5 (C), N6 (D), MS (E) medium or with endosperm on modified NB medium (F). Scale bar = 2 mm. Red arrows indicate good quality of calli as dry, yellowish white in colour, compact in structure and globular in & the blue triangles indicate poor quality of calli as being watery, frequently turning brown and necrotic.

as dry, yellowish white in colour, compact in structure and globular in shape (as shown with the red arrows in Figure 3A). Also, more calli were shown to be generated in modified NB medium than that induced on other basal salts. The other four kinds of media produced fewer embyogenic calli (Figure 3B-E) with poor quality as being watery, frequently turning brown and necrotic (as shown with the blue triangles in Figure 3B). Explant type is also a very important factor in the transformation of this line. The intact mature seed without removal of the endosperm generated a reduced number of embryogenic calli (Figure 3F) and introduced more contamination. Average callus induction frequency for mature embryos without endosperm indicated the highest callus induction frequency from modified NB medium and the least from B5 medium (Table 1).

Higher temperature for callus growth and transformation

Mature embryos without endosperm were cultured on modified NB medium at the three temperatures, 25°C, 28°C, and 32°C. For Treatment 32°C, embryogenic calli grew to about 2 mm in diameter after 26 days and were ready for sub-culture. To yield a similar size of callus to that at 32°C, it required 29 days at Treatment 28°C and 33 days at Treatment 25°C. More calli with higher quality were produced at higher temperature, averaging five embyogenic calli generated from each mature embryo at 32°C. Less embyogenic calli were generated at lower temperatures, with an average of three embyogenic calli grown from each embryo at 28°C and on average, less than one embyogenic callus produced at 25°C.

In addition, we compared the impact of culture temperatures on the following-up transformation steps after co-cultivation with *Agrobacterium*. The same callus lines induced at 32°C were infected with *Agrobacterium* and co-cultivated at 23°C for 3 days. Then, these infected calli were divided and grown at three different culture

Basic induction medium[a]	Macronutrient component	Micronutrient component	Organic component	Number of embryos tested	Callus Induction Frequency[b] (%)
Modified NB	N6	10× B5	B5	307	93.8
NB	N6	B5	B5	190	42.6
B5	B5	B5	B5	198	11.6
N6	N6	N6	N6	195	23.1
MS	MS	MS	MS	196	64.3

Table 1: Composition of callus induction media and its impact on callus induction frequency.
[a]In addition to macronutrients, micronutrients, and organic components, other components as 500 mg/l proline, 500 mg/l glutamine, 300 mg/l casamino Acids, 30 mg/l maltose, 2 mg/l 2, 4-D, MS iron salts, 3 mg/l Phytagel were included in all callus induction media.
[b]Callus induction frequency equals the percentage of any explants that produced good quality calli (as yellowish white, compact, and globular embryogenic calli) among total explants.

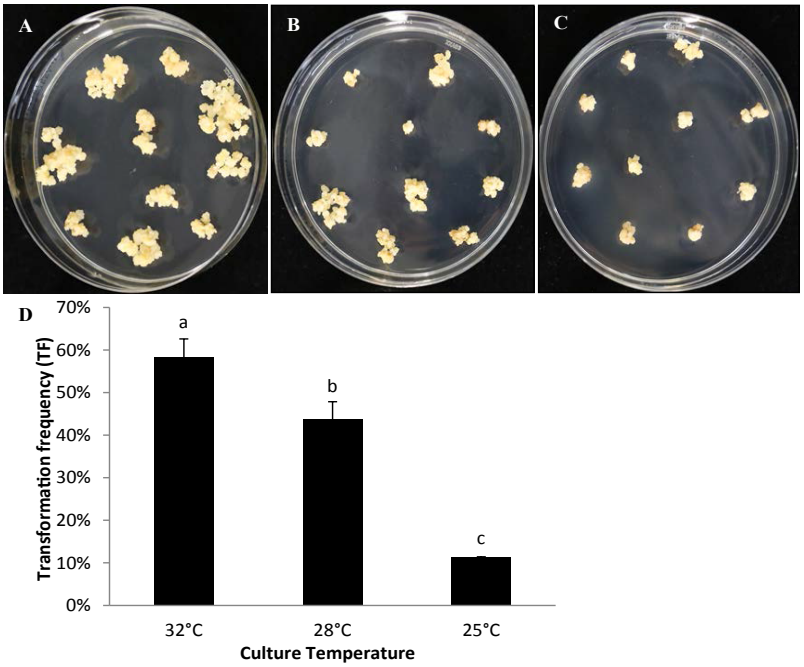


Figure 4: Effect of different culture temperatures on transformation. A-C, the same batch of infected and co-cultivated calli were divided into three treatments and kept at 32°C (A), 28°C (B) and 25°C (C) throughout four transformation steps as recovery, selection, shoot regeneration, and rooting. The pictures were taken right after the two rounds of selection steps with top views of Petri dishes as its diameter = 9 cm. D is for TF comparisons among three culture temperatures. Data represent the mean ± SD (n=4). Different lowercase letters indicate significant difference ($p \leq 0.05$). TF is defined as the percentage of positive events for PMI among total callus explants.

temperatures, 32°C, 28°C, and 25°C, throughout all remaining four transformation steps (recovery, selection, regeneration and rooting). The results show Treatment at 32°C generates the most resistant transgenic calli after the selection step, where the calli grow quicker and the whole transformation process requires about 86 days from infection to T0 plants ready for greenhouse. Treatment at 28°C requires 2 weeks longer than that at 32°C. The calli at Treatment at 25°C grew very slow and required 5 weeks longer than that at 32°C treatment. Treatments at 32°C, 28°C, and 25°C produces the TFs at 58%, 44%, and 11% respectively (Figure 4).

Strains tested for transformation of *IR58025B*

The transformation frequency of four *Agrobacterium* strains, EHA105, EHA101, LBA4404 and AGL1 were compared. EHA105 and EHA101 perform better than LBA4404 on transformation frequency. Considering the quality of transgenic events (single copy frequency) (Figure 5), LBA4404 is the best among four strains, as high as 59.5% single copy frequency, significantly higher than other three strains. Backbone free frequency is similar, around 60% for all four strains. The single copy frequency is defined as the percentage of single copy events among total positive events produced (Table 2).

Impact of air-drying treatment on TF

The rice calli were collected in a tube and mixed with *Agrobacterium* suspension culture for infection for 5 minutes. After infection, the liquid *Agrobacterium* solution was drained and then the infected calli were placed on COC medium for co-culturing. Different air-drying durations were tested immediately after infection and before co-cultivation. Air-drying for 4 or 7 hours in a laminar air flow hood improves TF to 62%, in contrast to poor TF of 46% without air-drying. Figure 6 displays the treatments of 4 and 7 hours air-drying, leading to more transiently expressing green fluorescence protein (GFP) cells (Figure 6B and 6C) in contrast to the zero hour (Figure 6A) and more resistant calli on selection medium (Figure 6E and 6F) in comparison with the zero hour (Figure 6D). We concluded that a longer air-drying time of 4-7 hours could benefit *Agrobacterium* infection compared to no air-drying treatment (Figure 6).

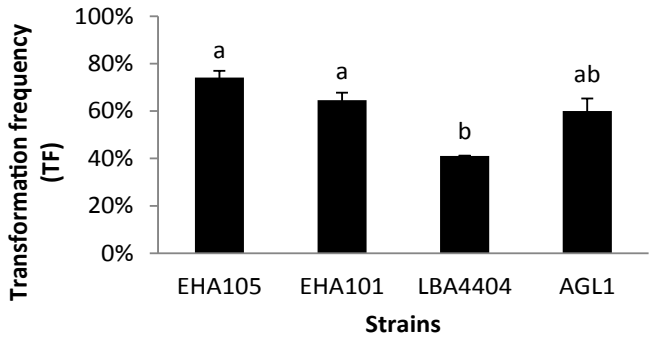


Figure 5: TF comparisons among EHA105, EHA101, LBA404, and AGL1. Data represent the mean ± SD (n=3). Different lowercase letters indicate significant difference (p≤0.05). TF is defined as the percentage of positive events for PMI among total callus explants.

	Copy number distribution			
	EHA105	**EHA101**	**LBA4404**	**AGL1**
0 copy (negative)	6.50%	4.60%	6.30%	7.10%
1 copy *pmi* gene	48.7% [b]	42.2% [c]	59.5% [a]	36.2% [d]
2 copies *pmi* gene	17.00%	21.50%	22.10%	23.50%
>2 copies *pmi* gene	34.30%	36.30%	18.50%	40.30%

Table 2: The quality of events delivered by the four strains. Putative events regenerated for EHA105, EHA101, LBA4404 and AGL1 are 244, 301, 208 and 238, respectively; TaqMan negative ones for *pmi* are escapes (0 copy); Different lowercase letters indicate significant difference (p≤0.05) among different strains for single copy percentage.

Effect of different salt concentrations of COC medium

We evaluated different salt concentrations in the COC medium. The normal salt concentration of basal medium (N6 macronutrients, B5 micronutrients, MS iron salts, and B5 organic components) in the COC medium was kept as 1X COC or reduced to 1/4 (1/4X COC) or 1/8 (1/8X COC). Other components as 500 mg/l glutamine; 800 mg/l casamino Acids; 20 g/l maltose; 10 g/l glucose; 2 mg/l 2, 4-D; 8 g/l agarose; 20 mg/l acetosyringone were kept the same. GFP expression indicated that lower salt concentrations of COC medium enhanced T-DNA delivery. Calli exposed to either 1/4 COC or 1/8 COC produce a higher TF of 63% than that in the normal 1X salt concentration (Figure 7).

Co-transformation efficiency

Marker free transformation is preferred since the selectable marker is not a trait but a tool in the transformation process. By co-transformation, marker-free transgenic plants are obtained by segregation in next generation due to two T-DNAs insertions which may integrate into different positions at different chromosomes. Co-transformation of 2 T-DNAs in one *Agrobacterium* strain or 2 T-DNAs in two different *Agrobacterium* strains was designed for generating marker free events. The co-TF was compared for 2 T-DNAs co-transformation (22369 in Table 3) vs. 2 *Agrobacterium* strains co-transformation (using PMI selection), and the 2 T-DNAs co-transformation was more efficient than the 2 strains co-transformation (Table 3).

Discussion

To establish an efficient *Agrobacterium*-mediated transformation system, high quality callus is a prerequisite and so choosing the best callus induction medium is one of the most important factors. Hiei et al. [19] demonstrated that the induction of embryogenic calli with the potential for higher rates of cell division is an important factor in determining transformation frequency. The commonly used basal medium for tissue culture is MS, N6, B5 and NB medium. Different plant species and genotypes require different kinds and amounts of nutrients, even requiring specific nutrient elements during specific stages [20]. The number of common medium is limited for our process; therefore, we decided to make some modifications based on the commonly used media for the specific variety.

In this study, the minor salts based on the NB medium were modified according to the report by Lin and Zhang [20] and resulted in enhanced callus proliferation and generated more embryogenic calli for this cultivar. As for the isolated mature embryos performing better than

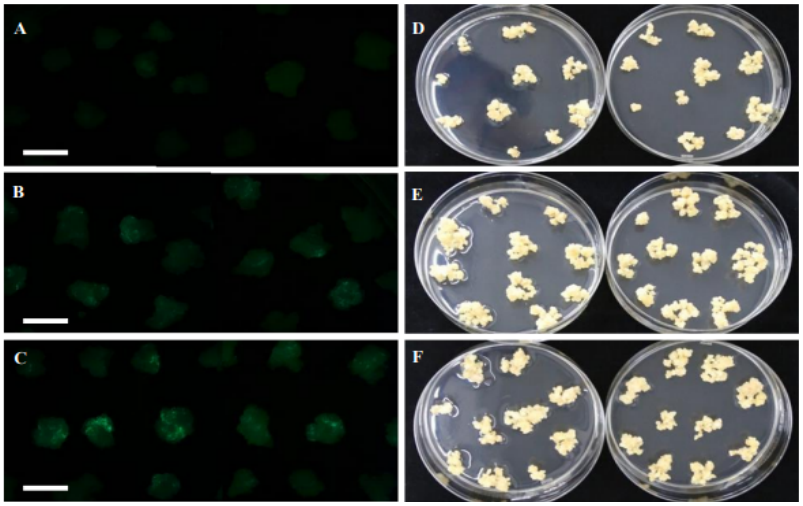


Figure 6: Effects of air-drying duration on GFP expression and selection. GFP expressions after co-cultivation for the treatments of air-drying durations (A=0, B=4, C=7 hours). The photos were taken 6 days after co-cultivation with scale bar=4 mm. Callus appearance after selection for air-drying durations (D=0, E=4, F=7 hours). Photos were taken after 4 weeks of selections with Petri dish diameter=9 cm.

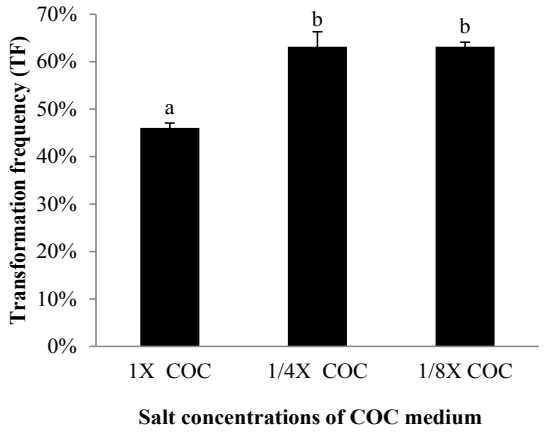


Figure 7: Effect of salt concentrations in the COC media on TF. Normal salt concentration of basal medium (macronutrients, micronutrients, Fe Iron and organic components) in COC medium as 1X COC or reduced to 1/4X COC or 1/8X COC. Other components were kept the same. Data represent the mean ± SD (n=3). Different lowercase letters show significant difference (p≤0.05). TF is defined as the percentage of positive events for PMI among total callus explants.

Vectors ID	Vectors information	TF only counting PMI or PAT TaqMan positive ones	Co-TF counting two TaqMan assays
22368	Control, 2 cassettes in one T-DNA	62.8% (PMI)	61.70%
22369	2 cassettes in two T-DNAs	60.0% (PMI)	42.00%
21971	1 cassette (PMI cassette)	53.0% (PMI)	N/A
22189	1 cassette (PAT cassette)	55.5% (PAT)	N/A
21971+22189	2 Agro strains	43.0% (PMI) + 5.0% (PAT)	5.00%

Table 3: Co-transformation efficiency Vector of 22189 contains PAT selection marker and all others contain PMI selection marker; Co-TF is defined as percentage of events positive for the two cassettes among total callus explants used; N/A: not applicable.

the intact mature seeds for callus induction, Lin et al. [21] considered that a regulation system or buffer action may exist in the mature rice seeds, which may protect the embryo from de-differentiating into calli if the endosperm was not removed. Removal of endosperms can break the buffer system so that embryos are more exposed to the culture medium, especially the growth hormone. Furthermore, improved quality and quantity of calli can be produced.

Temperature is one of the key factors which affect the growth rate of calli. A temperature of 32°C was deployed to be optimal for callus growth of this rice line. Higher temperature not only increases the callus induction and proliferation but also improves the quality of calli in comparison with lower temperature. However, temperatures higher than 32°C are not suitable for callus growth of this maintainer line (data not shown). Calli of higher quality can be infected by *Agrobacterium* more efficiently. It can also withstand the infection, recover better and produce a higher TF [22]. Air-drying treatment of calli after the *Agrobacterium* infection can improve the transformation frequency. We found that the air-drying of the calli after infection can significantly suppress the over growth of *Agrobacterium*, and reduce contamination of *Agrobacterium*. This process may protect the calli from the injury during *Agrobacterium* infection. Another possible explanation for improved TF is that air-drying may cause cell plasmolysis so that the T-DNA delivery is enhanced as well [23].

It has been reported that the use of low salt media during the infection and co-cultivation can enhance T-DNA delivery in many species such as maize [24,25], canola [7], and wheat [26]. In 2012, Tie et al. [27] reported that *Agrobacterium* infection appeared to have both up-regulatory and down-regulatory effects on thousands of genes. Fewer nutrients with starvation may lead to calli stress and trigger biochemical changes which can lead the plant cells to be more susceptible to *Agrobacterium* infection.

In a summary, an efficient Agrobacterium-mediated transformation protocol for the transformation of Indica rice IR58025B was developed. The modified NB medium was the best basal medium for induction with about 94% induction rate from mature embryos. Both callus quantity and quality were improved when callus was induced from endosperm-free embryos in comparison to intact mature seeds. Higher temperature (32°C) showed improvements when compared to lower temperatures at (25°C and 28°C) for both callus induction and transformation. Agrobacterium strains of EHA105 produced the best TF in comparison with other strains. Prior a higher TF Lower salt concentration for co-cultivation medium enhanced both DNA delivery and TF.

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References

1. Hinchee MVW, Connor-Ward DV, Newell CA, McDonnell RE, Sato SJ, et al. (1988) Production of transgenic soybean plants using Agrobacterium-mediated DNA transfer. Nat Biotechnol 6: 915-922.
2. Godwin ID, Ford-Lloyd BV, Newbury HJ (1992) In vitro approaches to extending the host-range of Agrobacterium for plant transformation. Aust J Bot 40: 751-763.
3. Sahoo RK, Tuteja N (2012) Development of Agrobacterium-mediated transformation technology for mature seed-derived calli tissues of indica rice cultivar IR-64. GM Crops Food 3: 1-6.
4. Hiei Y, Komari T (2006) Improved protocols for transformation of indica rice mediated by Agrobacterium tumefaciens. Plant Cell Tiss Org Cult 85: 271-283.
5. Karthikeyan A, Pandian SK, Ramesh M (2011) Agrobacterium-mediated transformation of leaf base derived calli tissues of popular indica rice (Oryza sativa L. subsp. indica cv. ADT 43). Plant Sci 181: 258-268.
6. Kumar KK, Maruthasalam S, Loganathan M, Sudhakar D, Balasubryamaniam P (2005) An Improved Agrobacterium-Mediated transformation protocol for recalcitrant elite indica rice cultivars. Plant Mol Biol Rep 23: 67-73.
7. Fry J, Barnason A, Horsch RB (1987) Transformation of Brassica napus with Agrobacterium tumefaciens based vectors. Plant Cell Rep 6: 321-325.
8. Jain RK, Jain S, Wu R (1996) Stimulatory effect of water stress on plant regeneration in aromatic indica rice varieties. Plant Cell Rep 15: 449-454.
9. Rance IM, Tian W, Mathews H, Kochko AD, Beachy RN, et al. (1994) Partial desiccation of mature embryo-derived calli, a simple treatment that dramatically enhances the regeneration ability of indica rice. Plant Cell Rep 13: 647-651.
10. Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (Oryza sativa L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. Plant J 6: 271-282.
11. Rashid H, Yokoi S, Toriyama K, Hinata K (1996) Transgenic plant production mediated by Agrobacterium in Indica rice. Plant Cell Rep 15: 727-730.

12. Ramesha S, Nagadharaa D, Pasalub IC, Padma AK, Sarmab NP, et al. (2004) Development of stem borer resistant transgenic parental lines involved in the production of hybrid rice. J Biotechnol 111: 131-141.

13. Hiei Y, Ishida Y, Komari T (2015) Rice, indica (Oryza sativa L). Methods Mol Biol 1223: 155-167.

14. Gui H, Li X, Liu Y, Han K, Li X (2014) The relationship between PMI (manA) gene expression and optimal selection pressure in Indica rice transformation. Plant Cell Rep 33: 1081-1090.

15. Duan Y, Zhai C, Li H, Li J, Mei W, et al. (2012) An efficient and high-throughput protocol for Agrobacterium-mediated transformation based on phosphomannose isomerase positive selection in Japonica rice (Oryza sativa L). Plant Cell Rep 31: 1611-1624.

16. Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, et al. (1975) Establishment of an efficient medium for another culture of rice through comparative experiments on the nitrogen sources. Sci Sin 18: 659-668.

17. Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 151-158.

18. Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15: 473-497.

19. Hiei Y, Ohta S, Komari T, Kubo T (1997) Transformation of rice by Agrobacterium tumefaciens. Plant Mol Biol 35: 205-218.

20. Lin YJ, Zhang QF (2005) Optimising the tissue culture conditions for high efficiency transformation of indica rice. Plant Cell Rep 23: 540-547.

21. Yongjun L, Yulan Z, Hua W, Qing L, Jiezhen L (1996) The effect of endosperm on the mature rice seeds cultured in vitro. Acta Agric Univ Jiangxiensls) 18: 259-263.

22. Hiei Y, Komari T (2008) Agrobacterium-mediated transformation of rice using immature embryos or calli induced from mature seed. Nature Protocols 3: 824-834.

23. Cheng M, Lowe BA, Spencer M, Ye X, Armstrong CL (2004) Invited review: factors influencing Agrobacterium-mediated transformation of monocotyledonous species. In Vitro Cell Dev Biol-Plant 40: 31-45.

24. Armstrong CL, Rout JR (2001) A novel Agrobacterium-mediated plant transformation method. Int Patent Publ.

25. Zhang W, Subbarao S, Addae P, Shen A, Armstrong C, et al. (2003) Cre/lox mediated gene excision in transgenic maize (Zea mays L.) plants. Theor App Genet 107: 1157-1168.

26. Cheng M, Fry JE, Pang S, Zhou I, Hironaka C, et al. (1997) Genetic transformation of wheat mediated by Agrobacterium tumefaciens. Plant Physiol 115: 971-980.

27. Tie W, Zhou F, Wang L, Xie W, Chen H, et al. (2012) Reasons for lower transformation efficiency in indica rice using Agrobacterium tumefaciens-mediated transformation: lessons from transformation assays and genome-wide expression profiling. Plant Mol Biol 78: 1-18.