Received : 29 Jan 2020 | Revised : 26 Feb 2020 | Accepted : 26 Feb 2020

Electronic Journal of Plant Breeding

Research Article

Standardisation of A*grobacterium*-mediated transformation in WP-22-2 – a semi-dwarf and early maturing rice mutant of improved white ponni

M. T. Andrew Peter Leon[1], K. K. Kumar[2], R. Pushpam[3], R. Kannan[4] and M. Arumugam Pillai[1*]

[1*]Department of Plant Breeding and Genetics, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Killikulam, Vallanadu, Tuticorin.Tamil Nadu, India.
[2]Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore. Tamil Nadu, India.
[3]Department of Rice, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore. Tamil Nadu, India.
[4]Department of Plant Pathology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Killikulam, Vallanadu, Tuticorin. Tamil Nadu, India.
E-Mail: mapillai1@hotmail.com

Abstract
Genome engineering using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR associated (Cas) system offers vast potential in the improvement of crop plants. For CRISPR/Cas9 mediated editing in rice, transformation protocols should be standardized for specific genotypes. We standardized the *Agrobacterium*-mediated transformation protocols for two genotypes – Improved White Ponni (IWP) and WP-22-2, a gamma-ray induced mutant of Improved White Ponni. Three different modifications of MS basal medium, different days old calli and bacterial culture of different optical density were evaluated. Callus induction with IWP-CI-medium-1 followed by callus proliferation for one month in IWP-CI-medium-2 produced transformable calli. Infection with 0.3 OD *Agrobacterium* culture for 10 min was less damaging and successful than higher density cultures. The transformation efficiency varied between the Improved White Ponni and WP-22-2 emphasizing the need for standardizing tissue culture protocols for every genotype. Transformation efficiency of 9.46 per cent and 3.53 per cent respectively were observed for Improved White Ponni and WP-22-2 which were higher than previous reports.

Keywords
Agrobacterium, transformation, rice

Introduction

Rice is the staple food for almost 50 per cent of the world's population. Being an important crop, improvements in rice varieties are made through conventional breeding, mutation breeding, molecular breeding and through genetic engineering. Though traits are improved through conventional breeding it involves more labour and time. Recent advancements in the field of genetic engineering, especially the CRISPR/Cas mediated targeted mutagenesis, is highly favoured due to its relative ease, scalability, less or no off-target mutations and ability to isolate 'transgene-clean' plants in two generations.Development of high-quality transformable material is the prerequisite for transgenic experiments. Though transgenics were developed through the particle gun method (Kloeti and Potrykus, 1999), protoplast transformation (Shimamoto *et al.,* 1989), *Agrobacterium*-mediated transformation in matured-callus is highly favoured. *Agrobacterium*-mediated transformation of immature embryos (Hiei and Komari, 2008) was found to be highly successful but is more tedious and requires additional treatments when compared to transforming calli developed from matured seed. Due to the wide genetic variability, the rice varieties behave differently in tissue culture media. Especially, the *indica* rice varieties are considered 'recalcitrant' and are difficult to regenerate after *Agrobacterium* infection (Hiei and Komari, 2008). Improved White Ponni (IWP) is a popular fine-grained rice variety grown in South India. However, it is lodging susceptible due to the tall stature and takes medium duration to maturity. *WP-22-2* is a semi-dwarf and early

maturing mutant developed from the Improved White Ponni through gamma-ray induced mutation. This genotype offers high potential to be improved through genetic engineering due to the high yield and grain quality traits. *Agrobacterium*-mediated transformation in Improved White Ponni has been reported already (Sridevi *et al.,* 2005) with a success rate of around 3% and the authors suggested the use of Maltose as the sugar source in the media. This study was conducted to standardize the tissue culture and transformation protocol in Improved White Ponni and WP-22-2 to get high-quality transformable calli in a short time.

MATERIALS AND METHODS

Plant material: Disease-free and high-quality rice seeds of Improved White Ponni and WP-22-2 were obtained from the Department of Plant Breeding and Genetics, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Killikulam. The protocols were standardised from 2018 to 2019.

Media and reagents:

Water: All reagents and media were prepared using deionised water.

Stocks of hormones used in tissue culture media: 1 mg mL^{-1} stocks of 2,4-dichlorophenoxyacetic acid (2,4-D), Kinetin, alpha naphthaleneacetic acid (NAA) and 6-benzylaminopurine (6-BA) were prepared by dissolving 50 mg of the chemical in a few drops of 1N NaOH followed by making up the volume to 50 mL with water.

100 mM Acetosyringone: 392 mg of 3',5'-Dimethoxy-4'-hydroxyacetophenone (Sigma, USA) dissolved in 20 mL of dimethylsulphoxide (DMSO). Stored at -20 °C in 1 mL aliquots.

50 mg mL^{-1} Hygromycin: Hygromycin solution (Merck, US) of concentration 50 mg mL^{-1} was used.

Antibiotics used in the media:

1. Cefotaxime (250 mg of Cefotaxime (Sigma, US) dissolved in 1 mL of molecular grade water (Himedia, India) and stored at -20 °C).

2. Rifampicin (20 mg of Rifampicin (Sigma, US) dissolved in 1 mL of 100% methanol and stored at -20 °C).

3. Kanamycin (100 mg of Kanamycin (Duchefa Biochemie, The Netherlands) dissolved in 1 mL of molecular grade water (Himedia, India) and stored at -20 °C).

AA media (for Agrobacterium infection): The modified AA medium was prepared according to Hiei *et al.*, (1994) and stored at 4 °C.

AB medium: The *Agrobacterium* minimal medium was prepared according to Hiei and Komari (2008) and stored at 4 °C.

IWP-CI-medium-1 (0.7X MS medium): 3.08 g of MS basal medium (Himedia, India) (Murashige and Skoog, 1962), 30 g of Maltose and 2.5 mL of 2,4-D dissolved in 800 mL of water and pH adjusted to 5.8. Volume made up to 1 L with water and Gel well (Tulip Diagnostics, India) 4.0 g and 2.0 g of Agar (Tulip Diagnostics, India) were added. Media were sterilised by autoclaving and dispensed in sterile Petri plates.

IWP-CI-medium-2 (0.7X MS medium with kinetin): IWP-CI-medium-1 added with 100 ìL L^{-1} of kinetin was utilized as IWP-CI-medium-2.

Selection medium: IWP-CI-medium-2 added with 50 mg L^{-1} of Hygromycin and 250 mg L^{-1} Cefotaxime was used.

Regeneration medium: 4.4 g of MS basal medium, 30 g of Maltose, 300 mg of casamino acids (SRL, India), 3 mL of 6-BA, 1.5 mL of NAA was dissolved in 800 mL of water and pH of the solution was adjusted to 5.8 and made up to 1 L with water. 4.0 g of Gelwell was added and autoclaved. At around ~60 °C, 1 mL of Hygromycin (50 mg mL^{-1}) and 1 mL of Cefotaxime (250 mg mL^{-1}) were added. The medium was poured in Petri dishes and Phytajars (Himedia, India).

IWP-medium-3: For the early *Agrobacterium* transformation of calli, the medium reported by Nguyen *et al.,* (2017) was evaluated.

Rooting medium: Half strength MS medium (2.2 g of MS medium) was prepared without any hormones. Hygromycin concentration was reduced to 40 mg L^{-1}. Autoclaved and poured in Phytajars.

Callus induction: 100 dehusked seeds of Improved White Ponni and WP-22-2 were surface sterilized with 70% ethanol for 45 seconds followed by 0.1% $HgCl_2$ for 3 min. 12 to 15 seeds per plate were placed on IWP-CI medium-1, IWP-CI-medium-2 or IWP-CI-medium-3. The plates were sealed with Phytawrap (Himedia, India) and incubated at 27 °C in dark (IWP-CI-medium-1 and IWP-CI-medium-2) or light (IWP-CI-medium-3).

Callus proliferation: After 7 days, the scutellum derived calli were excised and placed on fresh plates containing the same media and incubated for further 28 days (with subculture every 14 days) in case of IWP-CI-medium-1 and IWP-CI-medium-2. One set of calli from IWP-CI-medium-1 were subcultured on IWP-CI-medium-2 to check the effect of kinetin. The calli growing from seeds on IWP-CI-medium-3 was utilized as such for transformation after 5 days.

***Agrobacterium* culture:** The CRISPR plasmid pRGEB32 constructed with oligos targeting *tms5* genes of rice were mobilised into AGL-1 strain of *Agrobacterium*. Single colonies from freshly streaked AB plates were picked and grown in 50 mL of AB liquid medium with antibiotics (Rifampicin 20 and Kan 100). The culture was pelleted

and suspended with the AA medium containing 100 mM Acetosyringone to 0.3, 0.5 and 1.0 OD (optical density at 600 nm). Different incubation periods of 2 min, 5 min and 10 min were tried for the cultures.

Confirmation of transformation: Plant genomic DNA was isolated from putative transformants and the polymerase chain reaction was performed to confirm the presence of *hptII* gene.

Observations: The number of calli initiated after seven days, colour and hardness of the calli (friable nature), abnormal growth behaviour, the effect of *Agrobacterium* on calli and the number of *hptII* positive regenerated calli were observed. The transformation efficiency was calculated as follows: Transformation efficiency (%) = (no. of *hptII* positive regenerants / no. of calli cocultivated) × 100.

RESULTS AND DISCUSSION

Embryogenic calli developed from seeds remain a favourable choice in rice transformation. The efficiency of transformation relies not just on the vigour of calli produced but on the regenerating potential of the calli. The regeneration potential depends on the nutrients it received during the calli growth stages. The experiment reveals that the first step in the successful production of transformable material is in the use of good quality seeds. High-quality seeds would ultimately reduce the contaminations and respond well in the tissue culture media.

The N6 medium is commonly used in tissue culture of *japonica* cultivars (Hiei and Komari, 2008). Such cultivars respond well to the low N_2 content of the N6 media. However, for *indica* rice varieties, high nitrogen-containing MS media is preferred. However, the high nitrogen content may affect the hardening ability of tissue culture plants. To overcome this difficulty, the concentration of the callus induction medium was reduced. And the benefits of using maltose as the carbon source were reported elsewhere (Lentini *et al.,* 1995; Kumria *et al.,* 2001).The callus induction percentage was high in all the three media combinations for both IWP and WP-22-2 (**Table 1**). Addition of kinetin in minimal quantities improved callus proliferation ability (83 and 82 per cent respectively for IWP and WP-22-2) as earlier reported by Zaidi *et al.,* (2006). However, blackening of calli and abnormal growth were observed on calli grown solely on IWP-CI-medium-2 (**Fig 1C**). A simple modification of callus initiation with IWP-CI-medium-1 for 10 days followed by subcultures in IWP-CI-medium-2 resulted in vigorously growing calli (**Fig 1B**).

Table 1. Transformation efficiency of IWP and WP-22-2 genotypes of rice in different media composition

Genotype	Medium composition	No. of seeds inoculated	No. of calli co-cultivated	No. of calli survived the selection	No. of calli regenerated	Transformation efficiency (%)
IWP	IWP-CI-medium-1	100	73	3	1	1.37
	IWP-CI-medium-2	100	83	5	1	1.20
	IWP-CI-medium-1 and 2	100	74	10	7	9.46
	IWP-CI-medium-3	100	92	0	0	0.00
WP-22-2	IWP-CI-medium-1	100	71	4	1	1.41
	IWP-CI-medium-2	100	82	4	0	0.00
	IWP-CI-medium-1 and 2	100	85	6	3	3.53
	IWP-CI-medium 3	100	93	1	0	0.00

Agrobacterium infection was performed with calli of different age. Five days old calli grown on IWP-CI-medium-3 were infected with 0.3 OD culture for 10 min and cocultivated for 3 days. The calli responded poorly on the selection medium and none of the calli survived the selection which was contrasting to the results reported by Nguyen *et al.,* (2017). High phenol exudation, drying and stunting were observed (**Fig 1G**). This suggests that the genotypes IWP and WP-22-2 are unsuitable for a rapid method of transformation. Kumar *et al.,* (2005) found that two-month-old calli improved regeneration potential of recalcitrant *indica* type cultivars. In our experiment too, the results suggest prolonged incubation in callus induction medium increases the success rate of transformation in IWP and WP-22-2.

Calli grown on IWP-CI-medium-1 and IWP-CI-medium-2 for 35 days were infected with *Agrobacterium* cultures of OD 0.3, 0.5 and 1.0 for 10, 5 and 2 min respectively. The calli were co-cultivated for 3 days in the dark. The calli cocultivated with 0.3 OD bacterial culture alone were survived up to regeneration and this clearly shows that 0.3 OD culture was highly favourable in avoiding bacterial overgrowth and transformation in IWP and WP-22-2.After three rounds of selection, only the proliferating calli were transferred to regeneration medium containing 30 mg L^{-1} of Hygromycin. The transformation efficiency at the end of the experiment was below 10 per cent for both IWP and WP-22-2. Notably, IWP showed increased transformation efficiency than WP-22-2, suggesting genetic changes due to the effect of gamma rays by which

the mutant was originally developed. This further emphasizes the need to standardize tissue culture protocols for individual rice varieties before transgenic experiments. The regeneration of IWP was higher in this experiment than earlier reports (Sridevi *et al.,* 2005; Kumar *et al.,* 2005, Zaidi *et al.,* 2006). The transformation protocol suitable for CRISPR/Cas mediated genome engineering in IWP and WP-22-2 has been standardized in this experiment. The putative transformants obtained in this experiment would be further characterizedby the effect of CRISPR/Cas9 on the morphology.

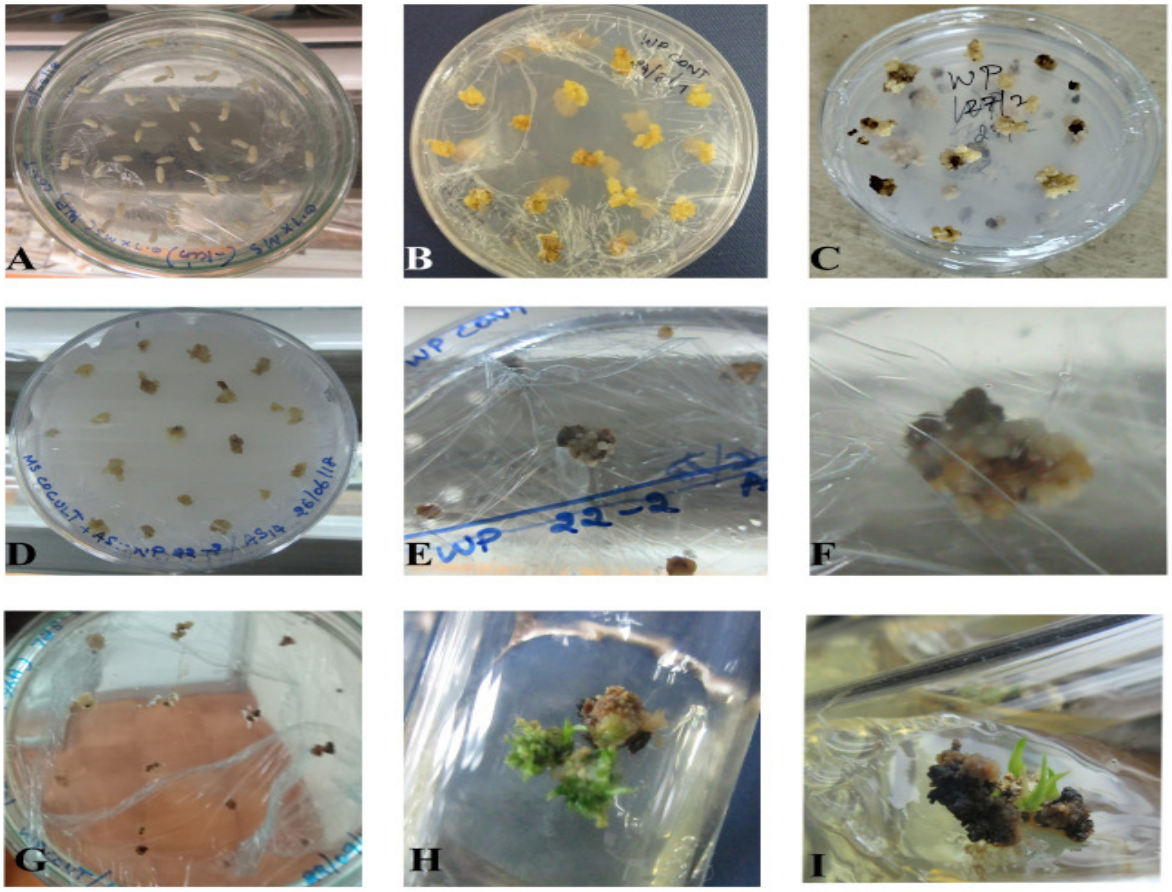

Fig. 1. Transformation protocol for IWP and WP-22-2. A) Matured seeds inoculated on IWP CI medium-1; B)Well proliferated calli ready to be transformed in IWP CI medium-2; C)Phenol exudation and blackening of calli grown solely with IWP CI medium-2; D) Cocultivation of calli with *Agrobacterium*; E & F) Proliferating calli on Selection medium containing hygromycin; G) Stunted growth of 5 days old calli used for infection (medium IWP CI media-3 – rapid method); H & I) Regenerating calli on regeneration medium

As a conclusion, Murashige and Skoog basal medium in reduced concentration (0.7X) improves the calli growth and regeneration ability in the rice genotypes IWP and WP-22-2. Use of basal MS medium with a high concentration of 2,4-D as the callus induction medium followed by subcultures in medium with 0.1 mg L^{-1} of kinetin produces vigorously growing calli. Infection with *Agrobacterium* in 0.3 optical density for 10 minutes reduces the bacterial overgrowth and increases transformation efficiency. However, a rapid method for *Agrobacterium*-mediated transformation may be standardized to reduce the time required for transgenic experiments in the genotypes Improved White Ponni and WP-22-2.

ACKNOWLEDGEMENT

We thank Mr Mohammed Yaseen, Dr Merina Prem Kumari and Dr D. Shoba for the help rendered during the study.

REFERENCES

Hiei, Y. and Komari, T. 2008. *Agrobacterium*-mediated transformation of rice using immature embryos or calli induced from mature seed. *Nat. Protoc.,* **3**:824-834. [Cross Ref]

Hiei, Y., Ohta, S., Komari, T. and 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. [Cross Ref]

Kloeti, A and Potrykus, I. 1999. Rice improvement by genetic transformation. In: Molecular Biology of Rice, Shimamoto, K (ed). Springer-Verlag, Tokyo, Japan, p. 283-301.

Kumar, K.K., Maruthasalam, S., Loganathan, M., Sudhakar, D. and Balasubramanian, P. 2005. An improved *Agrobacterium*-mediated transformation protocol for recalcitrant elite indica rice cultivars. *Pl. Mol. Biol. Rep.* **23:** 67-73. [Cross Ref]

Kumria, R., Waie, B and Rajam, M.V. 2001. Plant regeneration from transformed embryogenic callus of an elite indica rice via Agrobacterium. *Plant Cell Tissue Organ Cult.* **67:** 63-71. [Cross Ref]

Lentini, Z., Reyes, P., Martinez, C.P. and Roca, W.M. 1995. Androgenesis of highly recalcitrant rice genotypes with maltose and silver-nitrate. *Plant Sci.,* **110:** 127-138. [Cross Ref]

Murashige, T and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.,* **15**:473-497. [Cross Ref]

Nguyen, V.C., Nguyen, V.K., Singh, C.H., Devi, G.S., Reddy, V.S. and Leelavathi, S. 2017. Fast recovery of transgenic submergence tolerant rice cultivars of North-East India by early co-cultivation of *Agrobacterium* with pre-cultured callus. *Physiol. Mol. Biol. Plants*, **23**:115-123. [Cross Ref]

Shimamoto, K., Terada, R., Izawa, T. and Fujimoto, H. 1989. Fertile transgenic rice plants regenerated from transformed protoplasts. *Nature*, **338:** 274-276. [Cross Ref]

Sridevi, G., Dhandapani, M. and Veluthambi, K. 2005. *Agrobacterium*-mediated transformation of White Ponni, a non-basmati variety of *indica* rice (*Oryza sativa* L.). *Curr. Sci.,* **88:**128-132.

Zaidi, M.A., Narayanan, M., Sardana, R., Taga, I., Postel, S., Johns, R., McNulty, M., Mottiar, Y., Mao, J., Loit, E., and Altosar, I. 2006. Optimizing tissue culture media for efficient transformation of different indica rice genotypes. *Agron. Res.,* **4:** 563-575.