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Optimizing tissue culture media for efficient transformation of different indica rice genotypes

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This article is dedicated to the rye breeder Count Friedrich Georg Magnus von Berg, celebrating the 160th year of his birth.

Abstract. An efficient system was established for high frequency embryogenesis and regeneration of indica rice, *Oryza sativa* L. cv. MDU 5. Basic media, carbohydrate sources and concentrations, agar concentrations, amino acids, cytokinins and auxins were evaluated in callus induction and regeneration media to establish the most efficient regeneration protocol for MDU 5 indica rice. The optimized callus induction and regeneration media consisted of the basic MS salt mixtures and vitamin solutions supplemented with 4% maltose, 1g/L casein hydrolysate and 50 mg/L tryptophan, solidified with 1% agar. The callus induction medium was supplemented with 2,4–D 2 mg/L, kinetin 0.5 mg/L, indole acetic acid 1 mg/L, and 6–benzyl aminopurine 0.5 mg/L whereas the media for regeneration phase comprised kinetin 2 mg/L, indole acetic acid 1 mg/L and 6–benzyl aminopurine 2 mg/L. The media optimized for MDU 5 was analyzed for response of 73 other indica genotypes. Of these indica varieties 64 genotypes yielded 98.5% callus induction within eight days, 59% embryogenic calli formation, initiation of multiple green buds within eight days and a regeneration rate of 90%. The embryogenic calli derived were used for transformation with *Agrobacterium tumifaciens* (LBA 4404 or EHA 101 strains). Two binary vectors (pKHG4 and pIG121Hm) containing *hph* and *GUS* genes were used in these transformation studies. Fifty-one genotypes responded to the optimized media by producing hygromycin–resistant calli. The -histochemical test for ß–glucuronidase activity was positive from 32 genotypes with the transformation efficiencies ranging between 7.0% and 8.3%.

Key words: indica rice, regeneration, transformation, GUS
Abbreviations: BAP: 6–Benzylaminopurine; 2, 4–D: 2, 4–Dichlorophenoxyacetic acid; NAA: Naphthaleneacetic acid

INTRODUCTION

While *Arabidopsis* is a model for dicot genomes, *Oryza sativa* is a model for more agronomically important monocots including rice, wheat, oat, maize etc.

(Arabidopsis Genome Initiative, 2000; Considine et al., 2002). As excellent as it might be that different rice genomes have already been sequenced, the full assessment of biodiversity in the *Oryza* pool needs further exploration (Garris et al., 2005). Inherent in this system's biological approach to improvement of the food production capacity of the cereals is an appreciation of the ability to do forward and reverse genetics in as many different rice lines as possible. Transformation of both japonica and indica rice (*Oryza sativa* L) has been reported by several laboratories. Rice has been transformed by PEG, protoplast, electroporation, microprojectile bombardment and *Agrobacterium* transformation methods (Cheng et al., 1998; Datta et al., 2001; Repellin et al., 2001; Panahi et al., 2004; Vila et al., 2005). Most of the successful reports on transgenic production are limited to japonica, javanica and a few responsive indica rice genotypes like White ponni, IR 72, IR 64 (Datta et al., 2001; Sridevi et al., 2003), the aromatic Pusa Basmati–1 and Basmati 370 (Maqbool & Christou, 1999; Sridevi et al., 2005; for a recent review see Bajaj & Mohanty, 2005). Although the first reports of any rice transformation have been available since the late 1980s (Baba et al., 1986; Raineri et al., 1990), the paucity of reports describing transgenic indica lines may confirm that indeed indica varieties are difficult to transform. In these few reports, indica rice varieties are often considered to be sensitive to tissue culture and poorly responsive to transformation, most likely due to a poor response to tissue culture for callus production, somatic embryogenesis and regeneration (Lin & Zhang, 2005). Furthermore, indica rice varieties exhibit culture–specific genotype differences that render them recalcitrant to transformation.

Previous studies have described the evaluation of a few indica lines for their totipotency and have demonstrated their transgenic ability (Datta et al., 2001; Lin & Zhang, 2005). We report here the establishment and optimization of callus induction and regeneration media for the indica rice variety MDU 5. The effect of the optimized tissue culture media was analyzed on a large number of indica genotypes to obtain a high efficiency transformation system in these genotypes. A series of experiments was conducted with 74 genotypes, involving five media, two *A. tumifaciens* strains and two binary vectors.

MATERIALS AND METHODS

Mature rice seeds were sterilized in a 50% Javex solution (with a drop of Tween 20) for 30 min. After thorough rinsing with distilled water, the seeds were placed on solid callus induction medium. The cultures were incubated at 26°C in an unlighted growth chamber until calli became visible. The media for callus induction and regeneration were optimized for indica rice MDU 5. The effect of these optimized media was later analyzed on 74 rice genotypes listed below, all accessed via TNAU Madurai, India:

ACK 198, ACK 219, ACK 493, ACM 13, ACM 14, ACM 65, ACM 66, ACM 67, ACM 68, ACM 69, AD 92216, AD 93013, AD 93191, AD 95013, AD 95020, ADT 28, ADT 36, ADT 40, ADT 41, DT 42, AS 96006, AS 96070, AS 96142, ASD 1, ASD 2, ASD 3, ASD 4, ASD 5, ASD 6, ASD 7, ASD 8, ASD 9, ASD 10, ASD 11, ASD 12, ASD 13, ASD 14, ASD 15, ASD 16, ASD 17, ASD 18, ASD 19, ASD 20, CO 40, CO 43, CR 1009, IET 11443, IET 11675, IET 13483, IET 14086, IET 14177, IET 14329,

IET 3812, IR 64, IR 66, IR 72, MDU 4, MDU 5, Rasi, Salivahana, SSRC 91216, TKM 6, TKM 9, TM 91013, TN I, TNAU 91049, TNAU 93003, TNAU 93154, TNAU 95001, TP 88014, Vikas, Vikram, W/1263, White Ponni.

Tissue culture media

Media formulations were adjusted to determine an optimal milieu for callus induction and regeneration of indica rice MDU 5, developed by optimizing the carbohydrate source, agar concentration, amino–acid supplements and hormones of media for rice callus induction and regeneration. Sucrose, maltose or glucose at concentrations of 3%, 4% or 5% were used as carbohydrate sources in the callus induction and regeneration media. The media were solidified with 0.8%, 1% or 1.2% of agar. The callus induction and regeneration media used were with or without casein hydrolysate 1g/L and tryptophan 50 mg/L added to these media. To see the effect of the addition of cytokinins and auxin on the callus induction, the callus induction media were used with 2 mg/L 2,4–D singly or combined with 0.5 mg/L kinetin, 1 mg/L IAA and 0.5 mg/L 6–BAP. The regeneration medium was comprised of 2 mg/L kinetin, 1 mg/L IAA with or without 2 mg/L 6–BAP. After evaluating each of the above-mentioned agents singly, the combined effect of these ingredients was evaluated in a series of experiments measuring callusing days, callusing induction frequency (%), embryogenic calli formation frequency (%), green budding days and regeneration frequency (%).

The media optimized for MDU 5 was used to test the response of 73 other indica varieties for callusing days, callusing induction (%), embryogenic calli (%), green budding days and regeneration (%).

Callus induction frequency was calculated as follows:

$$\frac{\text{Number of calli}}{\text{Number of incubated seeds}} \text{ X } 100\%$$

Frequency of developing embryogenic calli was calculated as follows:

$$\frac{\text{Number of embryogenic calli}}{\text{Number of incubated seeds}} \times 100\%$$

After 3 or 4 weeks of subculture, pieces of callus (2–4mm in diameter) were placed on a Petri dish containing regeneration medium. Regeneration frequency was calculated as follows:

$$\frac{\text{Number of regenerated calli}}{\text{Number of calli incubated}} \times 100\% .$$

Transformation of embryogenic calli

The *Agrobacterium* based transformation vectors pKHG4 (Le Gall et al., 1994) and pIG121Hm (Ohta et al., 1990) (Fig. 1) were used to transform 64 indica rice genotypes. pKHG4 has proved indispensable in the creation of thousands of transgenic rice varieties, both japonica and javanica (tropical japonica) (Cheng et al., 1998). The binary vector pIG121Hm contains an intron in the N–terminal region of β–glucuronidase gene coding sequence. The GUS activity is expressed in plant cells but not in the cells of *A. tumefaciens*.

The embryogenic calli obtained from the optimized callus induction medium were used for transformation with *Agrobacterium tumefaciens*, LBA 4404 or EHA 101 as previously described by Cheng et al. (1998). The leaves from potentially transformed plants (hygromycin–resistant) were assayed for GUS activity by an improved histochemical staining protocol (Jefferson, 1987).

Transformation frequency was calculated as follows:

$$\frac{\text{Number of GUS}^{+}\text{ plants}}{\text{Number of calli inoculated with } \textit{Agrobacterium}} \times 100\%$$

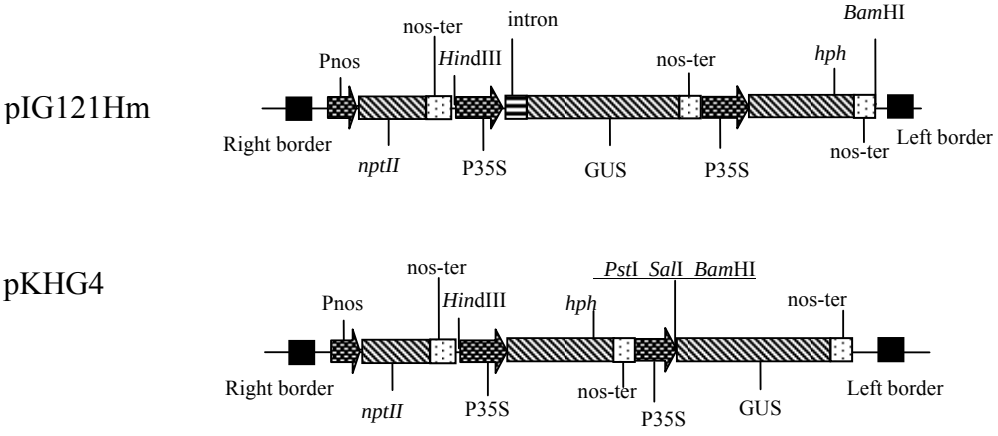

Fig. 1. T–DNA regions of the binary vectors pIG121Hm and pKHG4.

RESULTS

Response of indica rice cv. MDU 5

Three sources of carbohydrate were evaluated - sucrose, maltose and glucose. Maltose proved to be the most effective, resulting in the highest frequencies of callus induction, embryogenic calli and regeneration (Table 1).

Table 1. Effect of different carbohydrates on callus induction and regeneration of cv. MDU 5.

		Days to Callus formation	Callus induction (%)	Embryogenic calli (%)	Days to green bud formation	Regeneration (%)
Sucrose	3%	11	53	22	18	22
	4%	10	69	31	17	29
	5%	12	71	34	16	30
Maltose	3%	12	60	51	18	28
	4%	10	92	52	18	46
	5%	11	89	50	14	41
Glucose	3%	15	49	27	20	18
	4%	12	58	28	22	21
	5%	12	57	34	20	22

Table 2. Effect of agar concentration on callus induction and regeneration of cv. MDU 5.

	Days to Callus formation	Callus induction (%)	Embryogenic calli (%)	Days to green bud formation	Regeneration (%)
Agar 0.8%	11	53	34	20	28
Agar 1%	9	69	48	18	40
Agar 1.2%	8	71	46	18	37

Moreover, levels of maltose also influenced the response of MDU 5. The highest amount of callus induction (92%) and regeneration (46%) was achieved with 5% maltose. Media containing 3% maltose yielded 60% callus induction and 28% of regeneration. The presence of maltose in the media also lowered the number of days to callus formation and to green bud formation. Agar concentration in the callus induction and regeneration media was also important in enhancing the frequencies of callus induction, embryogenic calli and regeneration. Of different levels of agar evaluated, 0.8, 1.0, and 1.2%, the media containing 1% agar were the most favorable; those with 0.8% agar were the least (Table 2). However, the tissue culture response from media containing 1% and 1.2% agar was not significantly different. Media containing 1% agar led to 69% callus induction and 40% of subsequent regeneration. The 1.2% agar led to 71% callus induction and 37% of regeneration.

The addition of supplementary cytokinins (Kinetin and BAP) and auxin (IAA) to callus induction enhanced the frequencies of callus induction, embryogenic calli and regeneration. Furthermore, the presence of BAP in the regeneration medium also proved favorable. The highest frequencies of callus induction, formation of embryogenic calli and regeneration (98%, 41% and 53% respectively) were obtained when Kinetin, IAA and BAP were present in both callus induction and regeneration media (Table 3).

Table 3. Effect of cytokinins and auxin the on callus induction and regeneration of cv. MDU 5.

	Days to Callus formation	Callus induction (%)	Embryogenic calli (%)	Days to green bud formation	Regeneration (%)
MS without cytokinins♦ and auxin♥	10	57	31	18	23
MS + cytokinins and auxin in callus induction medium	9	93	35	13	38
MS + cytokinins and auxin in regeneration medium	12	51	33	11	39
MS + cytokinins and auxin in callus induction and regeneration medium	8	98	41	9	53

♦BAP and Kinetin
♥IAA

The application of supplementary amino acids to callus induction and regeneration media led to higher success rates where frequencies of 72% of callus induction, 41% formation of embryogenic calli and 40% of regeneration were observed (Table 4). The combined action of all supplements led to higher efficiency of callus

induction (96%), formations of embryogenesis calli (42%) and regeneration (71.4%) (Table 5). Seventy indica rice varieties were exposed to the media formulation (Table 6) to observe any genotype–specific response, and transformation efficiency was measured.

Table 4. Effect of amino acids on callus induction and regeneration of cv. MDU 5.

	Days to Callus formation	Callus induction (%)	Embryogenic calli (%)	Day to green bud formation	Regeneration (%)
MS without amino acids♣	11	50	31	21	23
MS + amino acids in callus induction medium	11	69	32	19	20
MS + amino acids in regeneration medium	11	51	31	18	31
MS + amino acids in callus induction and regeneration medium	11	72	41	19	40

♣Casein hydrolysate+L–tryptophan

Table 5. Combined effects of carbohydrate sources, agar, cytokinins, auxin and amino acids on callus induction and regeneration of cv. MDU 5.

Medium	Days for callu sing	Callus induction (%)	Calli weight (g)	Embryogenic calli (%)	Days for green budding	Regeneration (%)
MS, maltose 4%, agar 1%, amino acids♣, cytokinins♦ and auxin♥	7.3	96.0	2.740	42.0	8.0	71.4

♣Casein hydrolysate+L–tryptophan
♦BAP and Kinetin
♥IAA

Table 6. Tissue culture media.

Medium	Composition
MSICI	MS basal salts, vitamins, maltose 4%, agar 1%, kinetin 0.5 mg/L, IAA 1 mg, 6–BAP 0.5 mg/L, casein hydrolysate 1 g/L, tryptophan 50 mg, 2, 4–D 2 mg/L, pH 5.8
MSIR	MS basal salts, vitamins, maltose 4%, agar 1%, kinetin 2 mg/L, IAA 1 mg/L, 6–BAP 2 mg/L, casein hydrolysate 1 g/L, tryptophan 50 mg/L, pH 5.8

MSICI and MSIR are callus induction and regeneration media, respectively.

Table 7. Effect of MSICI and MSIR media on the callus induction and regeneration of indica rice.

No. genotypes with callus induction	Days to Callus formation	Callus induction (%)	Embryogenic calli (%)	No. of genotypes with green buds	Days to green bud formation	Regeneration (%)	Plantlets/callus
64	8	98.5	59	62	8	90	12

MSICI and MSIR are callus induction and regeneration media, respectively.

Table 8. Effect of MSICI and MSIR media on stable transformation of indica rice.

Experiment	Number of genotypes representing hygromycin resistant calli	Number of genotypes with GUS^+ plants	Transformation efficiency (%)
pKHG4–LBA	45	32	8.3
pKHG4–EHA	41	28	7.0
pIG121Hm–EHA	51	25	7.7

MSICI and MSIR are callus induction and regeneration media, respectively.

Response of 74 various indica genotypes

The analysis revealed that 64 of the 74 genotypes responded to the media optimized for MDU 5. The frequency of callus induction and regeneration was 98 and 90%, respectively. The results of this study showed that an average of 12 plantlets per callus could be achieved in these indica rice varieties (Table 7). Transformation of the 64 indica genotypes produced hygromycin–resistant calli from 45, 41 and 51 genotypes with pKHG4–LBA, pKHG4–EHA and pIG121Hm–EHA, respectively. An overview of the results achieved by using the different constructs is shown in Table 8. The leaves from hygromycin–resistant plants were assayed for GUS activity. Only the

leaves from representative positive transgenic plants are shown in comparison with those of non–transgenic control plants in Fig. 2. GUS assays revealed that it was possible to successfully transform 32 different genotypes with pKHG4–LBA, 28 genotypes with pKHG4–EHA, and 25 genotypes with pIG121Hm–EHA. Overall, 51 genotypes produced hygromycin–resistant calli and 32 genotypes had GUS–positive plants (Table 9 and 10, respectively).

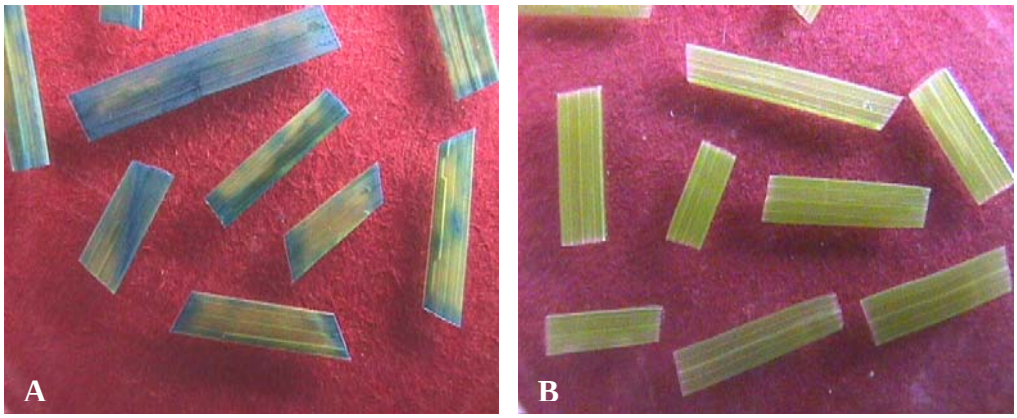

Fig. 2. Histochemical GUS expression in transgenic rice. **A**: leaves of transgenic rice plant, **B**: leaves of non–transgenic rice plant.

Table 9. Hygromycin B–resistant genotypes.

ACK 198	ACK 219	ACK 493	ACM 13	ACM 66
ACM 67	ACM 68	AD 92216	AD 93191	AD 95020
ADT 28	ADT 40	ADT 42	AS 96006	AS 96070
AS 96142	ASD 1	ASD 4	ASD 5	ASD 9
ASD 12	ASD 14	ASD 16	ASD 17	ASD 20
CO 40	CO 43	IET 3812	IET 11443	IET 11675
IET 14086	IET 14177	IET 14329	IR 66	MDU 4
MDU 5	Rasi	Salivahana	TKM 6	TKM 9
TM 91013	TN 1	TNAU 91049	TNAU 93003	TNAU 93154
TNAU 95001	TP 88014	Vikas	Vikram	W/1263
White Ponni.				

Table 10. GUS^{+} genotypes.

ACK 198	ACK 493	ACM 13	ACM 66	ACM 67
ACM 68	AS 96006	AS 96070	AS 96142	ASD9
ASD14	ASD16	ASD17	ASD20	CO 40
CO 43	IET 3812	IET 11675	IET 14086	IET 14177
IET 14329	MDU 4	MDU 5	Rasi	Salivahana
TKM 9	TN 1	TNAU 95001	Vikas	Vikram
W/1263	White Ponni.			

DISCUSSION

After nearly two decades of research on rice transformation, few indica rice varieties have been successfully transformed despite significant progress in this field (Lin & Zhang, 2005). Transformation of indica rice remains difficult for a number of reasons. One prerequisite for high efficiency transformation is an exceedingly effective and robust tissue culture system. Response of indica rice to callus induction and regeneration media is genotype specific. Therefore it is necessary to optimize these media individually for each genotype that is to be transformed. In this study, we have optimized media for callus induction and regeneration for high efficiency transformation of the indica variety MDU 5 which is cultivated on diverse climatic conditions in southern India (Peeran et al., 1999). A large number of indica rice genotypes responded to the same media, which facilitated efficient transformation of these rice genotypes as well.

The results presented here suggest that callus induction and regeneration of the MDU 5 genotype were influenced by the type and concentration of the carbon source. The response of this rice genotype to the media containing one of the carbohydrates–glucose, maltose or sucrose–indicated that maltose was the preferential carbon source for callus induction and regeneration. Maltose–containing medium induced 92% callus formation and 46% regeneration, suggesting that maltose promotes rice callus induction and regeneration more effectively than sucrose or glucose. Previous reports have also found maltose to be a better carbon source for regeneration (Kumria et al., 2001; Kumria & Rajam, 2002). In addition to supplying a carbon source to the calli, maltose has been described as an agent that may regulate osmotic potential of cellular environment of callus (Lentini et al., 1995). Moreover, because sucrose promotes the *in vitro* production of ethylene in excised tissues causing the browning of the callus,the substitution of maltose for sucrose may help protect the calli by reducing the production of ethylene.

Agar concentration also influenced callus induction and regeneration in MDU 5. The highest frequencies of callus induction and regeneration were obtained when the MS media were solidified with 1.2% agar. According to Lee et al. (2002) agar influences the shoot regeneration by regulating the humidity of *in vitro* culture conditions.

Another factor promoting callus induction and regeneration was the addition of amino acids. Casein hydrolysate provides a source of amino acids and has been shown to increase the production of embryogenic calli in rice (Moura et al., 1997). L–tryptophan is an essential amino acid which acts as a precursor of IAA (an auxin) in plants. In cereals, the process of somatic embryogenesis begins in the presence of high concentrations of auxin. Exogenous application of L–tryptophan may increase IAA synthesis in callus tissue (Sahrawat & Chand, 2004; Vikrant & Rashid, 2002). In the present study, the addition of casein hydrolysate and L–tryptophan to the callus induction and regeneration media enhanced callus induction and regeneration in MDU 5.

The presence of cytokinin in callus induction medium created a positive impact on the callus proliferation and subsequent regeneration of MDU 5. The auxin 2, 4–D is used routinely as a callus-inducing agent in rice, while very few reports indicate the

use of cytokinin. Previously, Rashid et al. (2001) have used cytokinin (BAP) in callus induction but have found it to have a negative effect on callus growth of indica rice. On the other hand, the effect of 2–isopentenyl adenine (2iP; another cytokinin) on rice callus growth and subsequent regeneration, reported by Zhu et al. (1996), was positive.

The seventy-four cultivars analyzed in the present study represent a wide range of indica rice (Peeran et al., 1999) but very little knowledge exists about their tissue culturability. Only a limited number of these cultivars have been previously investigated for their response to tissue culture (Giri & Reddy, 1994; Sridevi et al., 2003; 2005; Visarada & Sarma, 2004). The results indicated that the frequencies of callus induction, callus growth and green bud initiation could be increased by supplementation of cytokinins and auxins in callus and regeneration media. Improvement of callus induction and regeneration frequency in these indica varieties may open up opportunities to improve agronomic traits in these genotypes.

Embryogenic calli derived from the improved media were highly susceptible to *Agrobacterium* strains. LBA4404 and EHA101 *Agrobacterium* strains and pKHG4 and pIG121Hm binary vectors were effective in transferring T–DNA into the calli. The callus proliferation and subsequent regeneration using improved modified media was effective in obtaining successful subsequent transformation from a wide range of indica rice genotypes. This is evident from the results showing that 41–51 genotypes responded to the newly improved media by producing hygromycin–resistant calli. A transformation efficiency of 7.0%–8.3% could be obtained by using the optimized media. The results suggested that the use of improved media is effective in achieving efficient transformation of novel varieties of indica rice. Since the indica rice genotypes described here are widely grown in southern India, it seems likely that the regeneration protocols developed here might help in transformation of similar genotypes for crop improvement strategies.

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