Fast recovery of transgenic submergence tolerant rice cultivars of North-East India by early co-cultivation of *Agrobacterium* with pre-cultured callus

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Abstract Agro-climatic conditions of North-East India are very complex and rice cultivars present in the region have been adapted to grow under harsh environmental conditions. Germplasm present in the region is considered to possess several important and unique traits that are of importance in rice improvement programs. Genetic engineering is a powerful tool to introduce new traits into crop plants. However, not much information is available on the methods to introduce foreign genes into North-East rice cultivars. Therefore, the main objective of this study is to develop transformation procedures for fast recovery of transgenic plants from North-East rice cultivars. To achieve this objective, a systematic study was carried out to identify media components and culture conditions for efficient embryogenic callus induction from the mature seeds and differentiation of callus into plantlets from two North-East deep water rice cultivars, Taothabi and Khonggan. Also, role of preculture of callus on *Agrobacterium*-mediated transformation was studied. Co-cultivation of *Agrobacterium* with 1–5 days precultured callus was found to result in high frequency of transformation. Detailed

characterization of transgenic lines confirmed stable integration of transgenes and expression of reporter *gfp* gene. The whole process starting from callus induction to regenerating of transgenic rice plants that can be established in the soil was achieved in about 35–45 days. The procedures developed were found to be applicable to a popular variety IR 64. Therefore, methods developed in this study should be useful not only to introduce new traits quickly but also to validate the function(s) of several candidate gene(s) identified under the functional genomics of rice.

Keywords Rice transformation · *Agrobacterium* · North-East rice germplasm · Submergence tolerance

Abbreviations

MS medium	Murashige and Skoog medium
GFP	Green fluorescent protein
2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
NAA	Naphthaleneacetic acid
YEM medium	Yeast extract-mannitol medium
HYG	Hygromycin
OD	Optical density
EDTA	Ethylenediaminetetraacetic acid
CTAB	Cetyltrimethylammonium bromide
NaCl	Sodium chloride
HPT	Hygromycin phosphotransferase
UV	Ultraviolet

Introduction

Rice is a major cereal crop feeding more than two billion people worldwide. The conventional breeding methods have resulted in the development of high yielding rice

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varieties that contributed to green revolution. However, conventional breeding methods, limited by the gene pool available within the germplasm, need to be supplemented with modern biotechnological approaches in order to increase crop productivity and accomplish food security. Genetic engineering is widely used to widen the genetic pool of crop species that allow the introduction of genes from a wide range of organisms with new or improved functions. In order to accomplish such a goal, reproducible and simple methods to introduce foreign genes into required genotypes in a short period of time is highly desirable.

Production of a large number of independent transgenic events is a pre requisite for any successful introduction of new trait(s) through genetic engineering approaches. Like in several species, efficiency of genetic transformation in rice is highly dependent on the in vitro culture methods employed and the selection procedures followed (Hiei et al. 1994). Callus induction and plant regeneration of rice is highly dependent on the genotype and the composition of the medium and culture conditions used (Reddy et al. 1985; Abe and Futsuhara 1986; Mikami and Kinoshita 1988; Peng and Hodges 1989; Hartke and Lorz 1989; Oinam and Kothari 1993; Ge et al. 2006). Hiei et al. (1994, 1997) showed that 2–3 week-old scutellum derived calli are suitable for *Agrobacterium*-mediated transformation of japonica rice. Toki (1997) further improved the procedures that allowed the production of transgenic japonica rice in less than two months and later, by using a 1-day pre-cultured scutellum tissue, they (Toki et al. 2006) were able to transform japonica rice using *Agrobacterium* and recover transgenic plants in about 35–45 days.

North-East region of India along with Myanmar is rich in biodiversity and is considered as one of the centers of origin of Indica rice (Roy et al. 2015). Manipur, one of the North East states in India, is endowed with rice germplasm having several distinct agronomically important characteristics, such as biotic and abiotic stress tolerance including deep water submergence tolerance. Manipur being an area prone to floods, farmers rely on different varieties of rice like Murshi, Laiphou, Wainuchara, etc., although not high yielding, fragile and tedious to harvest as seeds fall off soon after maturity and are not economically viable but due to long history of adaptation to local agro-climatic conditions and other quality parameters. Among the local rice cultivars, Taothabi and Khongan are two varieties that show more tolerance towards deep water conditions caused by frequent and heavy rains. These cultivars are grown by the local farmers for many decades and some farmers still prefer cultivating these varieties despite low yields due to other desirable characters such as pest disease tolerance, stress tolerance, etc.

Only recently the attention is turned towards the importance of these special varieties of North-East India. Most of the wetland rice in Manipur survive floods through fast elongation of internodes. However, upon receding of floods these rice lodge and as a consequence over all yields are effected. Recent studies have shown that such fast elongation of internodes in deep water rice is regulated by Snorkel pathway (Hattori et al. 2009). On the other hand, some genotypes in rice were found to tolerate the submergence condition for up to few weeks without elongation, regulated by Sub1 pathway (Xu et al. 2006). We were interested to know, by over expression of sub1 pathway genes and/or down regulation of Snorkel pathway genes, if North-East rice cultivars can be converted into submergence tolerant but without elongation of the internodes. For this purpose one requires efficient procedures to transform and regenerate transgenic plants from the North-East indica rice cultivars. Therefore, the present study was undertaken to develop efficient genetic transformation methods for two North-East deep water rice cultivars, Taothabi and Khongan to recover plants in a short period. Here, we report on the development of *Agrobacterium* mediated transformation system where transgenic rice plants are generated in about 45 days. Such method should allow over expression or down regulation of genes involved in submergence tolerance. In addition, the methods developed for North East rice cultivars were also tested for their suitability to a popular indica rice variety IR 64. On the whole, the methods developed in this study should allow over expression of foreign genes and also facilitate characterization of several candidate genes that were identified based on “omics” studies. In addition, the procedures developed should be very useful in functional characterization of endogenous genes by over expression and/or down regulation to understand the adaptation of North-East rice cultivars to harsh environmental conditions and also to reveal genes involved in non-basmati aroma related traits. Therefore, we selected Taothabi and Khongan cultivars to develop transformation system to recover transgenic plants in a shorter period of time.

Materials and methods

Medium for callus induction and shoot regeneration

Mature seeds of indica rice cultivar Khongan, Taothabi and IR64 were de-husked, surface-sterilized with 70% ethanol for 1 min and then with mercuric chloride 0.1% for 15 min, followed by 5 washes in sterile distilled water. Surface-sterilized seeds were inoculated on MS medium (Murashige and Skoog 1962), supplemented with 30 g/l

Table 1 Effect of different combinations of 2,4-D and kinetin on callus induction of cultivar IR64 after 2 weeks of inoculation (150 seeds/medium formulation)

2,4-D (mg/l)	Kinetin (mg/l)	Seeds forming callus Number (%)			Seeds forming embryogenic calli Number (%)		
		IR 64	Khongan	Taothabi	IR 64	Khongan	Taothabi
0.0	0.0	0	0	0	0	0	0
1.5	0.0	62	25	20	40	15	18
1.5	0.25	66	48	36	46	32	30
2.0	0.0	78	56	51	54	47	50
2.0	0.25	80	64	70	65	58	63
2.5	0.0	84	78	85	68	69	74
2.5	0.25	90	84	98	71	73	90
3.0	0.0	86	93	95	72	68	87
3.0	0.25	82	85	91	70	74	82

sucrose and different combinations of 2,4-dichlorophenoxyacetic acid (2,4-D, 0–3.0 mg/l) and kinetin (0–0.25 mg/l) (Table 1). Cultures were kept at 32 °C in the continuous light (40–60 $\mu\text{mol s}^{-1} \text{m}^{-2}$) in the growth chamber. After 2 weeks of culture, calli (2–4 mm in diameter) were cultured on a shoot regeneration medium (Murashige and Skoog 1962) supplemented with different concentrations of 6-benzylaminopurine (BAP), naphthaleneacetic acid (NAA) and 30 g/l sucrose or maltose. Cultures were maintained under the regime of 16 h (light)/8 h (dark) at 26 °C cycle.

Agrobacterium-mediated transformation and regeneration of transgenic rice plantlets.

Callus induced from mature seeds after 1–5 days of cultivation in the callus induction medium under 2000 lx continuous light at 32 °C were used for transformation by *Agrobacterium*. *Agrobacterium* strain EHA105 harboring pCambia1390-sGFP (Toki et al. 2006) construct was grown in Yeast extract–mannitol (YEM) medium (Supplementary Table S1) containing 50 mg/l kanamycin, 50 mg/l streptomycin, 10 mg/l rifampicin at 28 °C in the dark. After reaching the growth to an optical density (OD_{600}) of ca. 0.5, the culture was centrifuged at 5000 rpm for 10 min at 4 °C. The bacterial pellet was re-suspended in equal volume of AAM medium (Toriyama and Hinata 1985) supplemented with 15–20 mg/l acetosyringone and the *Agrobacterium* were cultured at 28 °C with shaking at 200 rpm, in the dark for 1 more hour. Pre-cultured seeds on callus induction medium (MSD) for 1–5 days were immersed into *Agrobacterium* suspension for 15 min and blotted dry with sterilized filter papers to remove excess of bacteria. These seeds were transferred onto a filter paper that had been moistened with 0.5 ml of AAM medium and placed on MSD-AS co-cultivation medium.

After 3 days of co-cultivation at 25 °C in the dark, Calli along with seeds were washed 3 times in sterilized water and then washed once in sterilized water containing 500 mg/l carbenicillin to remove excess *Agrobacterium*.

The seeds along with small calli were blotted on sterilized filter papers and cultured on MSD-HYG selection (MSD medium containing 50 mg/l hygromycin and 500 mg/l carbenicillin) under continuous light at 32 °C for 2 weeks. The proliferating calli arising on selection medium were transferred to optimized shoot regeneration medium (MS-REM medium, MS medium containing 50 mg/l hygromycin, 500 mg/l carbenicillin). Cultures were kept at 26 °C under the regime of 16 h light and 8 h dark cycle. After 1–2 weeks, green buds, measuring more than 2 mm in length, arising from the freshly growing calli were transferred to hormone free MS medium containing hygromycin 40 mg/l. These green buds developed into plantlets with well developed roots on the same medium in about 5–10 days. These plantlets were transferred to culture bottles containing MS-HFM medium (Hormone free MS medium containing hygromycin 40 mg/l, Supplementary Table 2) for further growth. After 1–2 weeks putative transgenic plants were transferred to plastic pots and grown to maturity in the greenhouse.

Molecular characterization of transgenic plants

Genomic DNA was isolated as described by Murray and Thompson (1980), with minor modifications. Briefly, leaf tissue (0.5 g) was ground in liquid nitrogen with a pestle and mortar into fine powder. 750 μl DNA extraction buffer (including 0.1 M Tris–HCl, pH 8.0, 20 mM ethylenediaminetetraacetic acid (EDTA), 1.4 M sodium chloride (NaCl), 2% cetyltrimethylammonium bromide (CTAB), 0.2% β -mercaptoethanol) was added and the mixture was incubated at 65 °C for 30 min with gentle mixing by inverting the tubes. About 750 ml of phenol:chloroform:isoamylalcohol (25:24:1) was added and mixed by shaking for 10 min. The mixture was centrifuged at 13,000 rpm for 10 min at room temperature. The upper aqueous phase was transferred to a fresh tube. This step has been repeated once more and a third extraction was

performed with chloroform: isoamylalcohol (24:1). Genomic DNA was precipitated by the addition of 0.7 volume of isopropanol and DNA was collected by centrifugation at 13,000 rpm for 10 min at room temperature. The pellet was washed with 70% ethanol twice and air-dried at room temperature and dissolved in 50 µl TE buffer. About 30 µg of DNA was digested with *Hind*III and *Eco*RI enzymes for overnight, fragments were separated on the 0.8% agarose gel and transferred on to nylon membrane. The membrane was ultraviolet (UV) cross linked and then probed with 32P labeled hygromycin phosphotransferase (*hpt*) gene coding regions. Standard procedures were followed for hybridization (Sambrook et al. 1989) and membranes were subjected to autoradiography.

GFP expression was observed under a fluorescence Nikon stereo zoom microscope fitted with Digital Still Camera DMX 1200.

Results and discussion

Establishment of media and culture conditions for callus induction

Efficiency of genetic transformation in rice is also influenced by the composition of culture media and in vitro conditions employed during callus induction and differentiation stages. Indica rice has been shown to be even more sensitive to the presence of ammonium ions in the medium (Chu et al. 1975; Reddy et al. 1985). Therefore, a systematic study was conducted to identify composition of media and culture conditions for callus induction and its differentiation into plantlets from two North-East rice cultivars (Khongan and Taothabi) and a popular indica rice cultivar IR64. For callus induction, MS medium containing 30 g/l sucrose, 2 g/l casein hydrolysate, 5 mg/l proline and a combination of 2,4-D and kinetin was studied (Table 1). During callus initiation stage, cultures were placed under continuous light and at 32 °C. After 2 days of culture, formation of callus was observed from seeds placed on medium containing 1.5 and 2.0 mg/l 2,4-D from all three cultivars tested (Fig. 1), although the amount of callus produced after 5 days differed among the three genotypes used in the study. The highest frequency of callus induction was observed in the presence of 2.5 mg/l of 2,4-D. However, there is variation in terms of the amount of callus produced in 5 days on the same callus induction medium among the three cultivars. The amount of callus produced was relatively more in Khongan and Taothabi as compared to callus produced from IR 64 (Fig. 1). The frequency of explants developing calli decreased in the presence of 3 mg/l 2,4-D. Supplementation of 0.25 mg/l of kinetin to the medium increased frequency of embryogenic calli

formation compared to the medium containing 2,4-D alone. Highest frequencies (71%) of embryogenic calli was observed on the MS medium (MSD) containing 30 g/l sucrose, casein hydrolysate, proline, 2.5 mg/l 2,4-D, 0.25 mg/l kinetin for all three cultivars studied (Table 1). Therefore, MSD medium was used for the callus induction and proliferation throughout the transformation study.

Establishment of media and conditions for callus differentiation into plantlets

Influence of different concentrations and combinations of BAP and NAA along with two sources of carbohydrates, sucrose and maltose were studied on the plant regeneration (Table 2). The highest frequency of regeneration (14%) was recorded on MS medium (MSR) containing 30 g/l maltose supplemented with 2 mg/l BAP, 0.1 mg/l NAA. Comparative study on the efficiency of two carbohydrate sources i.e. sucrose and maltose on shoot regeneration showed that maltose enhanced shoot regeneration as compared to sucrose (Table 2). Maltose was used as a resource of carbohydrate in the shoot regeneration medium for several indica rice cultivars such as IR64, IR72 and Minghui 63 (Zhang et al. 1998), Pusa Basmati (Kumria et al. 2001), IR64 and IR72 (Kumar et al. 2005), IR8, IR24, IR26, IR36, IR54, IR64, IR72, Xin Qing Ai 1, Nan Jin 11 and Suewon 258 (Hiei and Komari 2006). Zaidi et al. (2006) also showed that maltose (30 g/l) is more effective as compared to sucrose (30 g/l) in shoot regeneration from 73 indica genotypes. Therefore, MS medium containing maltose 30 g/l maltose, supplemented with 2 mg/l BAP, 0.1 mg/l NAA, 2 g/l casein hydrolysate was found to be more suitable for all the three cultivars studied and hence this combination was used for regeneration throughout the transformation studies.

Agrobacterium mediated transformation of rice

Following the report of Raineri et al. (1990), *Agrobacterium* mediated transformation in rice was reported by several groups (Chan et al. 1992). Hiei et al. (1994) produced transgenic rice plants after co-cultivation with *Agrobacterium* and reported that the scutellum tissue is suitable for *Agrobacterium* infection. Till now, there were several reports of *Agrobacterium*-mediated transformation of indica rice (Aldemita and Hodges 1996; Zhang et al. 1998; Mohanty et al. 1999; Datta et al. 2000; Khanna and Raina 1999; Lin and Zhang 2005; Kumar et al. 2005; Hiei and Komari 2006). However, in most of these reports, the procedures followed are time consuming and it took about 2–4 months to generate transgenic rice.

Early co-cultivation of young calli developing from the scutellum tissue with *Agrobacterium* followed by early selection is the key step in obtaining rice transgenic plants

Fig. 1 Initial stages of callus induction during first 5 days of culture on MSD medium. Mature dehusked IR 64 seeds cultured on MS medium without 2,4-D (panel **a**) and with 2,4-D (2.5 mg/l), kinetin (0.25 mg/l) (panel **b**) and the callus formation was recorded every day 1D to 5D represent 1 day to 5 days of culture period. Note the induction of callus after 1 day of culture on MSD medium. No callus formation observed in the seeds grown on control medium (panel **a**). The callus produced after 1–5 days of culture was used to find out the suitable stage of callus to transform with *Agrobacterium* harboring desired gene construct (pCAMBIA1390-sGFP). Panel **c** and **d** show the callus formed after 1 to 5 days of culture on MSD medium from Khongan and Taotahi, respectively. Note that the amount of callus formed after 5 days of culture from Khongan and Taotahi is more as compared to IR 64

Table 2 Effect of different combinations of BAP, NAA and source of carbohydrate on callus differentiation

BAP (mg/l)	NAA (mg/l)	Sucrose (g/l)	Maltose (g/l)	Percentage of calli produced green shoots ^a		
				IR64	Taotahi	Khongan
0.0	0.0	30	0	0	1	2
2.0	0.0	30	0	7	8	10
2.0	0.1	30	0	8	25	26
2.0	0.5	30	0	5	20	23
0.0	0.0	0	30	0	2	2
2.0	0.0	0	30	12	11	14
2.0	0.1	0	30	14	27	30
2.0	0.5	0	30	9	24	25

^a 100 calli were cultured for each of media combination. Percentage of calli producing green shoots were estimated after 3 weeks of culture

in a short period of time. Toki et al. (2006) developed procedures that drastically reduced the time required to produce transgenic lines from japonica rice cultivar Nipponbare in about 35 days, starting from callus initiation to transferring the plantlet to pots following early infection of scutellum tissue with *Agrobacterium*. In this study, we have first identified the media composition and culture conditions for efficient callus induction and plant

regeneration. In the second step, we have identified suitable stage of scutellum derived callus from mature seeds for *Agrobacterium*-mediated transformation for two submergence tolerant North-East cultivars along with a popular indica rice cultivar IR64 and produced transgenic plantlets in about 35–45 days.

Young calli emerging from the mature seeds on MSD medium (Supplementary Table S2) for 1–5 days of pre-

Fig. 2 **a** Restriction map of vector used to transform North-East rice cultivars and IR 64. **b–d** The regeneration of plantlets from putative transgenic calli on MS-REM medium from IR 64, Khongan and Taotabhi, respectively. Southern blot hybridization to confirm stable integration of transgenes into genomic DNA of Khongan (**e**), Taotabhi (**f**) and IR 64 (**g**). Blots were hybridized with the coding region of *hpt* gene. Note the presence of different copies of *hpt* gene in different transgenic lines which is common when transformed with *Agrobacterium* mediated method. **h** Northern blot analysis to confirm the transcription of *hpt* gene in randomly chosen ten transgenic IR 64 plants. *M* Marker DNA in kbs, *C* control untransformed plant, 1–10 transgenic plants

culture (Fig. 1) were used as explants to co-culture with *Agrobacterium* harboring *hpt* and *gfp* gene constructs (Fig. 2a). After 3 days of co-cultivation at 25 °C in the dark, seeds along with calli were washed to remove *Agrobacterium*, and transferred to MS-HYG selection medium (Supplementary Table S2). Cultures were incubated under continuous light at 32 °C. After 14 days of culture on selection medium (MS-HYG), freshly growing calli were sub-cultured on MS-REM (Supplementary Table S2) medium containing 50 mg/l hygromycin to regenerate plantlets. Calli started differentiating into shoots in about 10–14 days (Fig. 2a–c). Appearance of a large number of green shoots could be seen in the calli at this

stage (Fig. 2a). These green shoots grow further into plantlets in about 5–7 days. Also could be seen at this stage is the simultaneous proliferation of differentiating callus during the regeneration process (Fig. 2a). Pre-culture of calli before *Agrobacterium* infection was found to improve transformation efficiency (Table 3). Calli transferred to MS-REM medium started differentiating within 3–4 days. All the differentiating calli with green shoots (above 2 mm in length) were sub-cultured onto same MS-HFM medium supplemented with 50 mg/l hygromycin. On this medium, majority of the green shoots grow further into plantlets in the next 5–7 days (Fig. 2b, c). During this period most of the plantlets also developed root system well. Such

Table 3 Effects of pre-cultured seed duration on *Agrobacterium*-mediated transformation in rice cv. IR64

No. of days seeds were pre-cultured before infection with <i>Agrobacterium</i>	No. of co-cultured seeds	Percentage of co-cultured seeds that produced hygromycin resistant calli ^a	Percentage of hygromycin resistant calli that are also positive for GFP expression ^a
1	140	20.0	12.1
2	130	26.2	16.9
3	120	31.7	30.8
4	110	38.2	37.3
5	110	50.9	45.5

^a After 14 days of culture on selection medium

plantlets with a shoot and root system were transferred first into small plastic cups for 10 days for acclimatization. Plantlets established in cups were transferred to pots placed in the greenhouse and grown to maturity to collect seeds.

Hygromycin was reported to be an efficient selectable marker to obtain transgenic rice plants with lesser escapes (Miki and McHugh 2004). In the present study, to select transformed calli, we have used 50 mg/l hygromycin in MS-HYG medium, supplemented with 300 mg/l L-proline and incubated the cultures in continuous light (2000 lx) at 32 °C. Such culture conditions were shown to allow rapid growth of transformed cells (Toki 1997). It is considered that L-proline provide protection against stress through various mechanisms (Hare and Cress 1997). Hence, L-proline might be promoting cell growth after co-culture with *Agrobacterium*. Furthermore, incubation of cultures at high temperature (32 °C) did not favour *Agrobacterium* growth but favoured the growth of transgenic calli as reported previously by Dillen et al. (1997).

It is important to keep hygromycin selection after initial period of callus growth. Hygromycin at 50 mg/l concentration was found to be sufficient to select transgenic calli in rice and at the same time stop the growth of untransformed cells. Therefore, putative transgenic rice plants were selected and regenerated on medium containing 50 mg/l hygromycin for two weeks and then grown on hormone-free MS medium supplemented with 40 mg/l hygromycin for 2–3 more weeks. Most of regenerated plants survived under the selection and growth conditions and were found to be positive for the presence of transgenes when tested using PCR and Southern hybridization.

Molecular characterization to confirm stable integration of transgenes

PCR and Southern blot analysis were performed to confirm the presence of transgenes and their stable integration

into rice genome. PCR analysis was carried out using genomic DNA as template and gene specific (*hpt* or *gfp*) primers. Presence of expected size bands were observed only in the putative transgenic plants growing in the presence of hygromycin (data not shown). Total genomic DNA digested with HindIII and EcoRI restriction enzymes with unique sites in the T-DNA region was separated on agarose gel and blotted onto Hybond-N membrane. Presence of specific bands could be seen only in the transformed plants that were absent in the untransformed control plant when the membrane was hybridized with the coding region of hygromycin as a probe (Fig. 2e–g), suggesting the stable integration of T-DNA into rice genome. Single (Fig. 2e, lanes 1, 2, 3, 4 and 6, Fig. 2f, lanes 3 and 4, Fig. 2g, lanes 1, 3 and 5–10) to multiple copies of transgene integration pattern (Fig. 2e, lane 5, Fig. 2f, lanes 1 and 2, Fig. 2g, lanes 2 and 4) could be seen in different independently transformed lines which is very common when transformed with *Agrobacterium* mediated method (Gelvin 2003). These results put together showed that the plantlets regenerated from the hygromycin resistant calli are stably transformed.

Expression of GFP at various stages of transformation

Expression of GFP was monitored continuously throughout the differentiation and dedifferentiation stages. The putative transgenic calli growing on the selection medium containing hygromycin (50 mg/l) were found to express GFP. Freshly growing calli were visualized under white light (WL) (Fig. 3, panel a) and blue light (BL) (Fig. 3, panel b) to check the expression of GFP. Expression of GFP could be seen in 2–5 day old calli after co-culture with *Agrobacterium* in Taothabi (Fig. 3, panel b). Similar observations were made with Khongan and IR 64 after transformation with *Agrobacterium*. Expression of GFP could be visualized at various stages of callus proliferation (after 5 days, Fig. 3, panel c, BL5 and 12 days, panel d, BL12) and during the differentiation of callus into plantlets in IR 64, after 10–12 days of culture on MS-REM regeneration medium (Fig. 3, panel d, BL35). Expression of GFP was observed in both shoot and roots in the well developed plantlets (Fig. 3, panel d, BL35) that are ready to be transferred to pots.

Following the procedures described in this study and outlined in Fig. 4 it is possible to transform and regenerate stably transformed North-East rice cultivars as well as IR 64 in about 35–45 days starting from callus induction to regeneration of transgenic rice plants that can be shifted to greenhouse.

Fig. 3 Expression of GFP in transgenic calli and plantlets. Seeds were cultured for 5 days on MSD medium, co-cultured with *Agrobacterium* for 3 days and then transferred to MS-HYG selection medium. Same procedure was followed for the controls but without co-cultivation step with *Agrobacterium*. Two to five day old callus produced on MSD-HYG medium from Taothabi was visualized under white light (panel a) and blue light (panel b). Five day and twelve day old callus of IR 64 visualized under white light (panel c WL5 and panel d WL12) and blue light (panel c BL5 and panel d BL12). Plantlets regenerated after 35 days of transformation visualized under white light (panel c WL35) and blue light (panel d BL35). Note the presence of GFP signal under blue light in the transformed calli and plantlets while no signal is observed in callus derived from untransformed controls. D days, WL white light, BL blue light

Conclusion

In the present study, we have identified media and culture conditions for efficient induction of embryogenic callus from the mature seeds of Taothabi and Khongan, and its differentiation into plantlets. Also, preculture conditions and their duration was established to use young mature seed derived calli as explants for *Agrobacterium* mediated transformation. Co-cultivation of *Agrobacterium* with 3–5 day pre-cultured calli was found to yield the highest frequency of transformation. The newly growing calli on selection medium was found to be PCR positive for the presence of *hpt* and *gfp* genes and also for GFP fluorescence. In addition, Southern blot analysis confirmed the stable integration of transgenes into plant genome. Following the methods described, stably transformed rice plants that can be transferred to green house can be achieved in about 35–45 days. The methods developed for North-East rice also worked equally well for other popular

Fig. 4 Flow chart showing final media, culture conditions and time lines used at various stages to recover transgenic lines from North-East rice cultivars

indica rice cultivar IR 64. Therefore, these methods should not only open up the possibility of introducing new traits through over of foreign genes in a very short span of time into the North-East rice cultivars but also be useful for down regulation of native genes to understand their role(s) using RNAi and other approaches.

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