Biotechnology Reports 7 (2015) 143–149

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Effect of antibiotics on callus regeneration during transformation of IR 64 rice

Thanh Ngoc Tran[a,b], Neeti Sanan-Mishra[a,*]

[a] Plant Molecular Biology Group, International Center for Genetic Engineering and Biotechnology, New Delhi, India
[b] National Key Laboratory for Plant Cell Technology, Agricultural Genetic Institute, Hanoi, Viet Nam

ARTICLE INFO

Article history:
Received 9 April 2015
Received in revised form 22 June 2015
Accepted 23 June 2015
Available online 27 June 2015

Keywords:
Antibiotics
Regeneration
Rice
IR64
Agro-bacterium

ABSTRACT

We report here the effect of antibiotics on the regeneration potential of recalcitrant indica rice cultivar, IR64. Different protocols reporting high-efficiency agro-bacterium-mediated transformation of mature seed-derived regenerative calli were used and compared. The putative transgenic (T_0) plants were analyzed for integration of the transgene through polymerase chain reaction and Southern blotting analyses. It was observed that the high-efficiency transformation of scutellar-derived regenerative calli could be obtained by using maltose as a carbon source and increased quantity of 2,4-D on a medium containing a higher concentration of gelling agent. The percentage of regeneration is greatly affected by the presence of antibiotics.

© 2015 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Rice is the most important crop and a model experimental plant in the world. Numerous transformation protocols are available to introduce a transgene into rice. These are based on direct gene transfer by chemical methods [12,18,37,49,56], electroporation [6,16,55] or particle gun delivery [24,26,48,58] and through *Agrobacterium* mediated transfer protocols involving *in planta* transformation [8] or callus mediated routes [19,20,40,43,45]. Direct gene transfer protocols have certain limitations as they may require specific equipment, time and skill labor, besides they are affected by the low rates of regeneration and high copy number integration [7]. The *Agrobacterium* mediated transformation of rice calli is the popular method for stable integration of the transgene in the rice genome. This normally results in low copy number integrations and plants having a single copy of transgene exhibit stable expression as compared to those containing multiple gene copies or scrambled inserts [22]. Moreover multiple gene copies frequently lead to co-suppression and gene silencing [51].

Several protocols are available that report the improvement in *Agrobacterium*-mediated transformation of rice. These are based on the use of different types of explants like mature seed [50], immature embryo [1], leaf [25], shoot apex [14] and root [5]. The protocols also describe the effects of changes in the nature and concentrations of phytohormones like BAP [27], Kinetin [21], TDZ [41], Zeatin [35] and Putrescine [44] in the culture medium. The protocols differ in the matrix [19,42] carbohydrate source [29] and the *Agrobacterium* strains [20]. It has been observed that, the transformation efficiency is generally low in the indica rice cultivars as compared to the japonica cultivars [9–11,26,58].

Most of the protocols employ antibiotic selection using one (Hygromycin) or two antibiotics on the regeneration medium. β-Lactam antibiotics are frequently used for *Agrobacterium* elimination from the transformed tissues [13]. β-Lactam antibiotics lyse and kill the bacteria by specifically interfering with the biosynthesis of the prokaryotic peptidoglycan component of the bacterial cell wall by binding to penicillin-binding proteins [3]. Carbenicillin and cefotaxime are the penicillin class and cephalosporin class, respectively, of the β-Lactam antibiotics group. The other categories of β-Lactam antibiotics include cephamycin, oxacephem, monobactam and carbapenem. Although eukaryotic plant cells do not have any known targets for β-Lactams, the chemicals can affect, positively or negatively, plant organogenesis, embryogenesis or callogenesis [33]. In the present study the effects of antibiotics on regeneration of the more popular and elite but recalcitrant cultivar, *Oryza sativa* cv IR64 was identified by comparing across different culture media varying in the nature and concentrations of phytohormones ensuing the pCAMBIA1300 constructs for *Agrobacterium*-mediated transformation of the regenerative calli.

* Corresponding author: Fax: +91 11 26742316.
E-mail address: Neeti@icgeb.res.in (N. Sanan-Mishra).

http://dx.doi.org/10.1016/j.btre.2015.06.004
2215-017X/© 2015 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

2. Methodology

2.1. Callus induction

Mature seeds of rice cultivar IR64 were de-husked and treated with 50% bleach supplemented with 2–3 drop Tween 20 for 45 min followed by 5–6 times washing with autoclaved distilled water. Surface-sterilized seeds were dried on autoclaved Whatman paper and incubated on callus induction media (CIM) in dark at 25–28 °C [42]. The CIM favoured the development of the scutellar region into a highly regenerative calli within a period of 3 weeks. The calli were excised and sub-cultured onto fresh CIM in dark for 5 days.

2.2. Agroinfection

The *Agrobacterium tumefaciens* LBA4404 cells carrying binary plasmid, pCAMBIA1300, were grown overnight in YEM medium [2] containing 50 mg/l kanamycin, 50 mg/l streptomycin and 25 mg/l rifampicin. About 20% of primary culture was inoculated into 100 ml YEM with 50 mg/l kanamycin, 50 mg/l streptomycin and 25 mg/l rifampicin in a 500 ml flask and allowed to grow for ~5 h till $OD_{600} \approx 0.8–1$. *Agrobacterium c*ells were harvested by centrifugation at 5000 rpm, 4 °C and the cells were resuspended in MS liquid media (MS salts [32] +10 g glucose +15 g/l sucrose and pH 5.8) to obtain $OD_{600} \approx 0.6–0.8$. *Agrobacterium* cells were activated by treating with 200 μM acetosyringone (AS) for 45–60 min in dark before infection.

The calli, after 5 days of sub-culturing, were immersed in the *Agrobacterium* suspension for 15–20 min with continuous shaking. The infected calli were transferred on sterile filter papers for drying and then were incubated on co-cultivation medium (CCM) at 26–28 °C for two days, in dark. After 2 days of co-cultivation, the infected calli were washed 3 times in sterile water and then washed once in sterile water containing 500 mg/l cefotaxime and again in sterile water containing 250 mg/l cefotaxime to remove *Agrobacterium*. The washed calli were dried on sterile filter papers and cultured on callus selection medium (CSM) kept in dark for selecting transgenic calli on 50 mg/l Hyg. After first round of selection for 20 days, brownish or black colored calli were discarded and white calli were transferred to fresh CSM medium for second selection cycle for 15 days. This step allowed the proliferation of micro calli and when small micro calli started growing on the mother calli, each micro callus was gently separated from the mother calli and transferred to fresh CSM medium for the third selection 15 days. Healthy calli were selected for regeneration.

2.3. Callus regeneration

After the third selection, healthy calli were transferred to the specific regeneration medium (RM1-4) and incubated in dark in culture room for 7 days. After which they were transferred to fresh regeneration medium and incubated at 26–28 °C under light. After 1–2 weeks, green buds were seen arising from the calli. The green buds developed into shoots and were transferred to rooting medium (RoM) in presence of Hygromycin (50 mg/l) under light for 20 days. The whole plants were transferred to vermiculite pots before being transferred to the soil pots and grown in the green house.

2.4. Media compositions

The CIM, CCM, CSM and RoM media compositions used are detailed below. These were adapted from Sahoo et al. [42] with some modification.

CIM: MS salts [32] with Vitamin B5 + 30 g sucrose + 0.3 g casein hydrolysate + 2.5 mg/l 2,4-D + 0.1 mg/l BAP + 0.65 g proline + 4 g phytagel, pH 5.8.

CCM: CIM + 200 μM acetosyringone.

CSM: CIM + 250 cefotaxime + 50 mg/l Hygromycin.

RM1: BAP medium: MS salts [32] with Vitamin B5 + 30 g maltose + 2.7 mg/l BAP + 1.2 mg/l kinetin + 0.5 mg/l NAA [42].

RM2: Kinetin medium: MS salts [32] with Vitamin B5 + 30 g maltose + 3 mg/l kinetin + 2 mg/l NAA [47].

RM3: TDZ medium: MS salts with [32] Vitamin B5 + 30 g maltose + 1 mg/l TDZ + 1 mg/l NAA [53].

RM4: Zeatin medium: MS salts [32] with Vitamin B5 + 30 g maltose + 1 mg/l zeatin + 0.5 mg/l NAA [35].

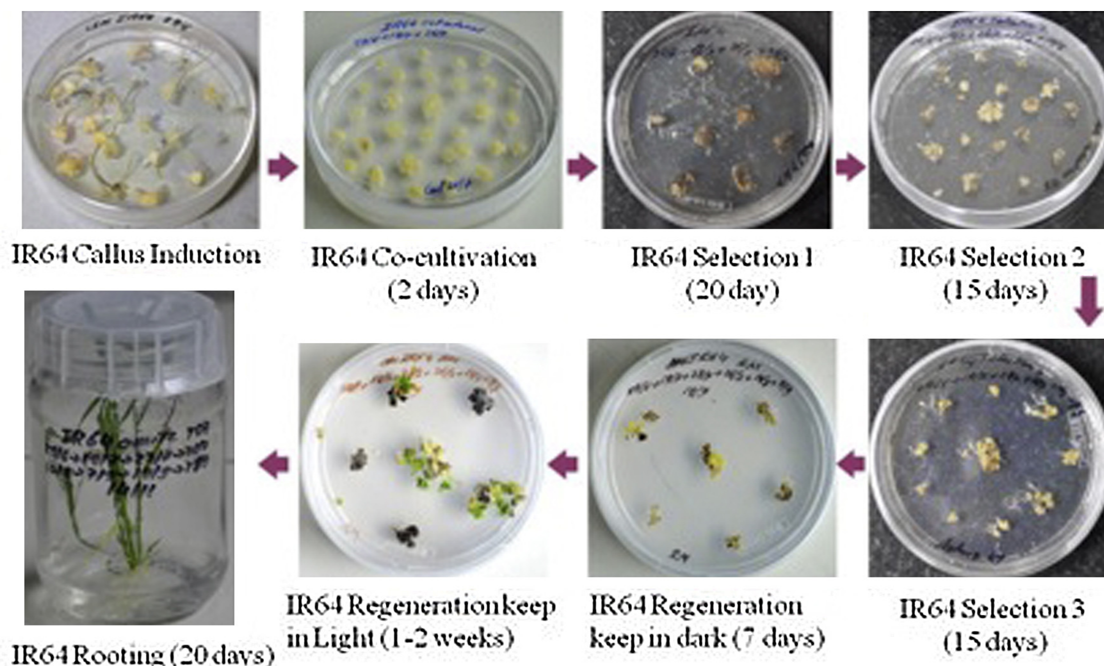

Fig. 1. Transformation of a pCAMBIA1300 based construct into IR64 Rice.

RoM: MS salts [32] with Vitamin B5 + 30 g sucrose + 3 g/l phytagel, pH 5.8.

2.5. Analysis of rice transgenic

T_0 plants were screened via PCR using 35S-Fwd primer 5′TGACGCACAATCCCACTATC3′ and SacI-Rev primer 5′AGCGAGCTCGGTACCGCTGCTGATGCTG3′. PCR-positive plants were checked to determine the copy number of transgenes using Southern blotting. To detect the copy number of the integrated transgene, Southern blot analysis of the PCR positive plants was carried out. For this 15 ng purified genomic DNA of rice was digested with restriction enzyme *BamHI* and run on 0.8% agarose gel. The DNA on the agarose gel was transferred to N^+ membrane. DIG-labeled DNA probes were used for Southern blot analysis and all procedures for the DIG application system (Roche) were performed according to the manufacturer's recommendations.

3. Result and discussion

In the present study the effect of antibiotics on regeneration of *Oryza sativa* cv IR64 transformed with pCAMBIA1300 was studied across different culture media using previously standardized protocols for *Agrobacterium*-mediated transformation. The details of the transformation protocol are described in the materials and methods. Briefly, the regenerative calli were produced from mature rice seeds on callus induction medium (CIM). The resultant calli were used as explants for co-cultivation with *Agrobacterium* on callus co-culture medium (CCM). The calli were washed to remove the *Agrobacterium* and transferred to the callus selection (CSM) medium. After three rounds of selection the micro calli were used for shoot regeneration by transferring them to the regeneration medium. The regenerated shoots were transferred to rooting media (RoM) (Fig. 1).

The shoot regeneration of cultivar IR64 was triggered on four distinctive media composed of different phytohormones viz. BAP [27], Kinetin [21], TDZ [41], Zeatin [35] and Putrescine [44]. In each case, the transgenic calli were selected in presence of only Hygromycin (Hyg) (30 mg/l) or a combination of Cefotaxime (250 mg/l) and Hyg (30 mg/l). The regeneration medium without any antibiotic was used as controls. The percentage of calli induced in each case is tabulated in Table 1 and pictorially represented in Fig. 2.

It was observed that in absence of antibiotic selection the maximum regeneration was obtained on the BAP medium followed by the Kinetin medium (Table 1). Whereas the regeneration percentage on the TDZ medium and Zeatin medium was almost half of that obtained on the other two media. In all the cases maltose was used as a carbon source as it has been reported that maltose showed better somatic embryogenesis in indica and japonica rice cultivars [23].

However, after applying the antibiotic selection the regeneration percentage came down as it supported the growth of putative transgenics only. In plant tissue culture, the nature and concentration of antibiotic used can extensively affect the regeneration potentials just as any other component of the culture media. The presence of antibiotic at exceeding high levels not only kill the non-transformed cells, it also inhibits growth of the transformed cells and plants, thereby leading to delay in regeneration [54]. The *nptII* gene, which confers kanamycin resistance, is the most popularly used selectable marker gene for generating transgenic dicotyledonous plants in research laboratories [54]. Rice callus shows natural resistance to Kanamycin and can survive on medium containing supplied kanamycin at doses up to ten times higher than that sufficient to kill many other species [4]. Hyg B is more effective in transgenic cell selection as compared to other agents, like Kanamycin [15,46]. Hyg B is an aminocyclitol antibiotic, which inhibits protein synthesis by causing mistranslation and interferes with protein translocation [17]. In plants, this antibiotic is very

Table 1
Effect of supplementing different phytohormones and antibiotics on shoot regeneration of cultivar IR64.

Phytohormone		BAP				Kinetin				TDZ				Zeatin			
Experiment number		1	2	3	Total	1	2	3	Total	1	2	3	Total	1	2	3	Total
Without antibiotic	No. of callus	23	17	16	56	23	17	16	56	23	17	16	56	23	17	16	56
	No. of shoots	5	3	3	11	3	2	3	8	4	2	1	7	2	2	1	5
	No. of plants	3	2	2	7	2	2	2	6	3	1	1	5	2	1	1	4
	Plants/callus (%)	13.04	11.75	12.50	12.44 ± 0.64	8.70	11.77	12.50	10.99 ± 2.02	13.04	5.88	6.25	8.39 ± 4.03	8.70	5.88	6.25	6.94 ± 1.53
Hygromycin (30 mg/L)	No. of callus	23	17	16	56	23	17	16	56	23	17	16	56	23	17	16	56
	No. of shoots	3	1	1	5	2	2	1	5	2	2	1	5	1	1	1	3
	No. of plants	2	1	1	4	1	0	0	1	1	0	0	1	1	0	0	1
	Plants/callus (%)	8.70	5.88	6.25	6.94 ± 1.53	4.35	0	0	1.45 ± 2.51	4.35	0	0	1.45 ± 2.51	4.35	0	0	1.45 ± 2.51
Cefotaxime (250 mg/l) and Hygromycin (30 mg/l)	No. of callus	23	17	16	56	23	17	16	56	23	17	16	56	23	17	16	56
	No. of shoots	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	No. of plants	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Plants/callus (%)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

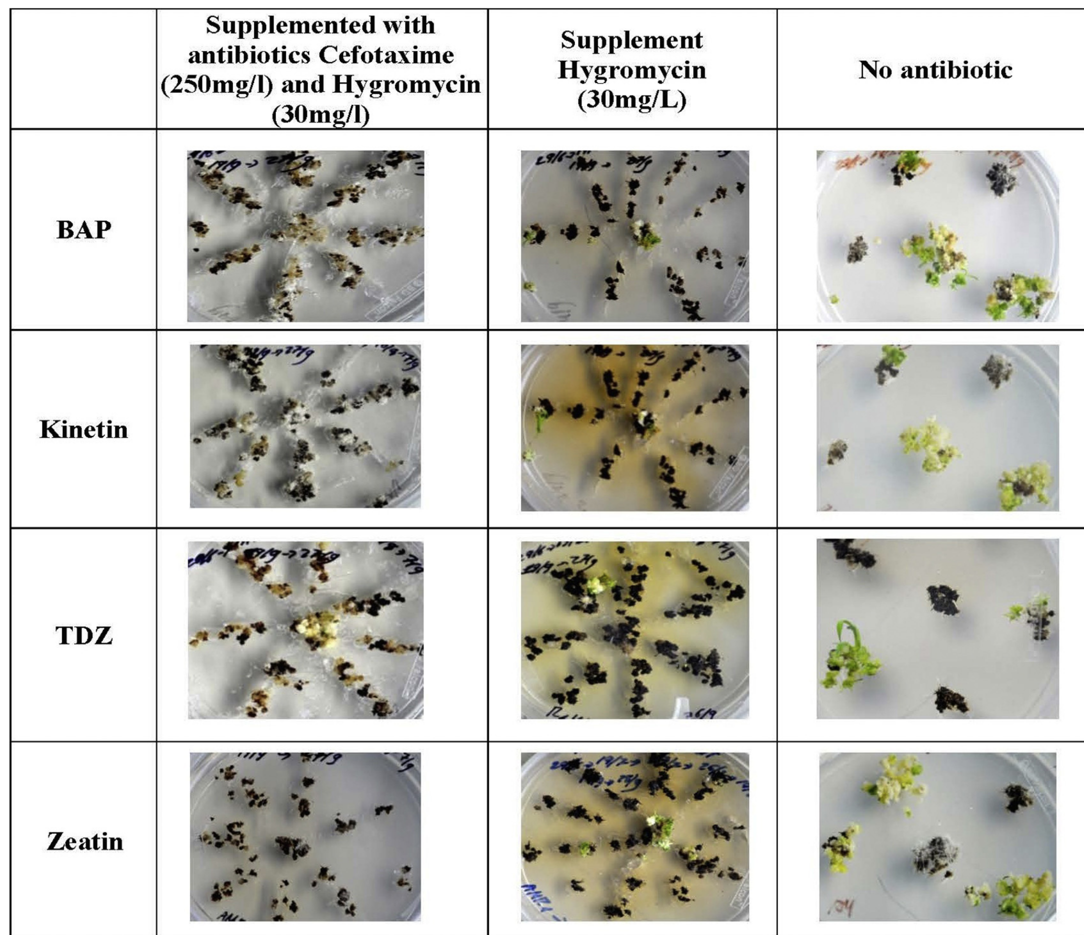

Fig. 2. Pictorial representation of the effect of phytohormones and antibiotic selection on the shoot regeneration of IR64 callus.

toxic [52] and transformation protocols in a number of crop plants, including dicots and monocots have successfully used Hyg as a selective maker for generating transgenic plants [30].

The effectiveness of the antibiotic selection is dependent on plant species, type of cultivars, tissue types, organs and even within different stages of development of the same species [15,30,31,36,57]. According to Li. et al. [28] non-transformed rice callus growth was greatly inhibited in medium supplied 30 mg/l of Hyg and the growth was completely inhibited in presence of when the concentration was raised to 50 mg/l. However, in some species much higher doses have been used for selection as in Minghui 63, an indica rice, the selection pressure was applied at 60 mg/l Hyg [29] while immature embryo-derived regenerative cultures of onion were selected on and 50–100 mg/l Hyg [15]. Lin and Zhang [29] also observed that during transformation of indica rice the overall transformation efficiency did not correspond with the regeneration frequencies observed in control sets with untransformed calli. The cultivars, which showed a high frequency of regeneration without transformation, exhibited much lower frequency of regeneration in the transformation experiment. Whereas cultivars that exhibited resistance to Hyg during regeneration of untransformed calli had greater frequency of regeneration after transformation. It was thus hypothesized that the regeneration frequency in the transformation experiment may be dependent the ability of the rice callus to resist the antibiotic, in this case Hyg [29].

In the case of IR64, it is recommended to use Hyg at a reduced concentration of 30 mg/L [27]. It was observed that in presence of Hyg alone the best results were obtained on the BAP medium where 7% regeneration was obtained on medium supplemented with Hyg while 1.5% regeneration was obtained on the other media with similar antibiotic selection (Table 1). These regenerated plants included the putative transgenic lines as confirmed by transgene integration using genomic PCR (Fig. 3) using specific primers. On increasing the selection pressure by using a combination of Cefotaxime (250 mg/l) and Hyg (30 mg/l) the regeneration potential of the calli was greatly affected. This is graphically represented in Fig. 2.

The inhibitory effect of cefotaxime on shooting and rooting was explained by Nauerby et al. [33]. The cefotaxime group of antibiotics contain 6-aminopenicillanic acid, phenylacetic acid and phenylmalonic acid in the β-Lactam ring and the side chain,

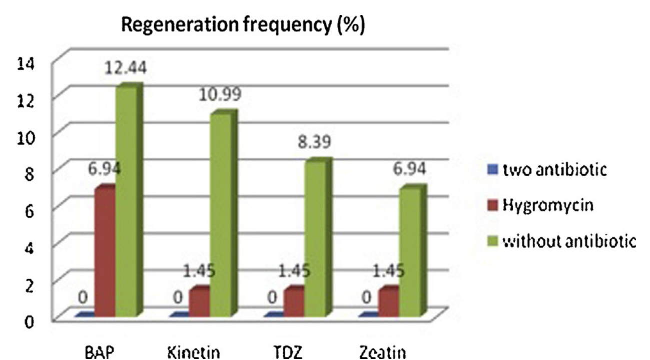

Fig. 3. Effect of different phytohormones and antibiotic selection on regeneration of shoots in rice (cultivar IR64) calli.

and may have important biological activities in plants and cause loss of phytohormone balance at high concentration, resulting in the low transformation and regeneration efficiencies [34]. It has been shown that the reduction of the time period for which the calluses are subjected to antibiotic selection could increase the regeneration potential of the transformed calli [50]. In RD6 indica rice a slight inhibitory effect was seen with carbenicillin and cefotaxime at 50 and 100 mg/l, respectively [38], however, the highest dose of both carbenicillin and cefotaxime (250 mg/l) completely inhibited regeneration [38,39].

The putative transgenic lines were screened for transgene integration by PCR using specific primers. The PCR amplified products of 390 bp indicated positive transgene insertion in all the putative transgenic lines selected except for line 3 of Zeatin medium supplemented with Hyg (Fig. 4). The copy number of transgenic IR64 rice lines was also confirmed using Southern blot analysis (data not shown). It was observed that most of the IR64 transgenic rice lines have single copy integrations while in some cases multi copy integrations were found.

Thus it has been shown that the high-efficiency transformation of scutellar-derived regenerative calli of recalcitrant indica rice cultivar IR64 can be obtained by using maltose as a carbon source and increased quantity of 2,4-D on a medium containing a higher concentration of gelling agent. However we observed that the percentage of regeneration is greatly affected by the presence of antibiotics. In the case of IR64, Hyg is required to be used at a reduced concentration and addition of a second antibiotic completely blocks regeneration. This suggests the need to develop protocols for antibiotic marker free selection to increase the yields of regenerates in such cultivars.

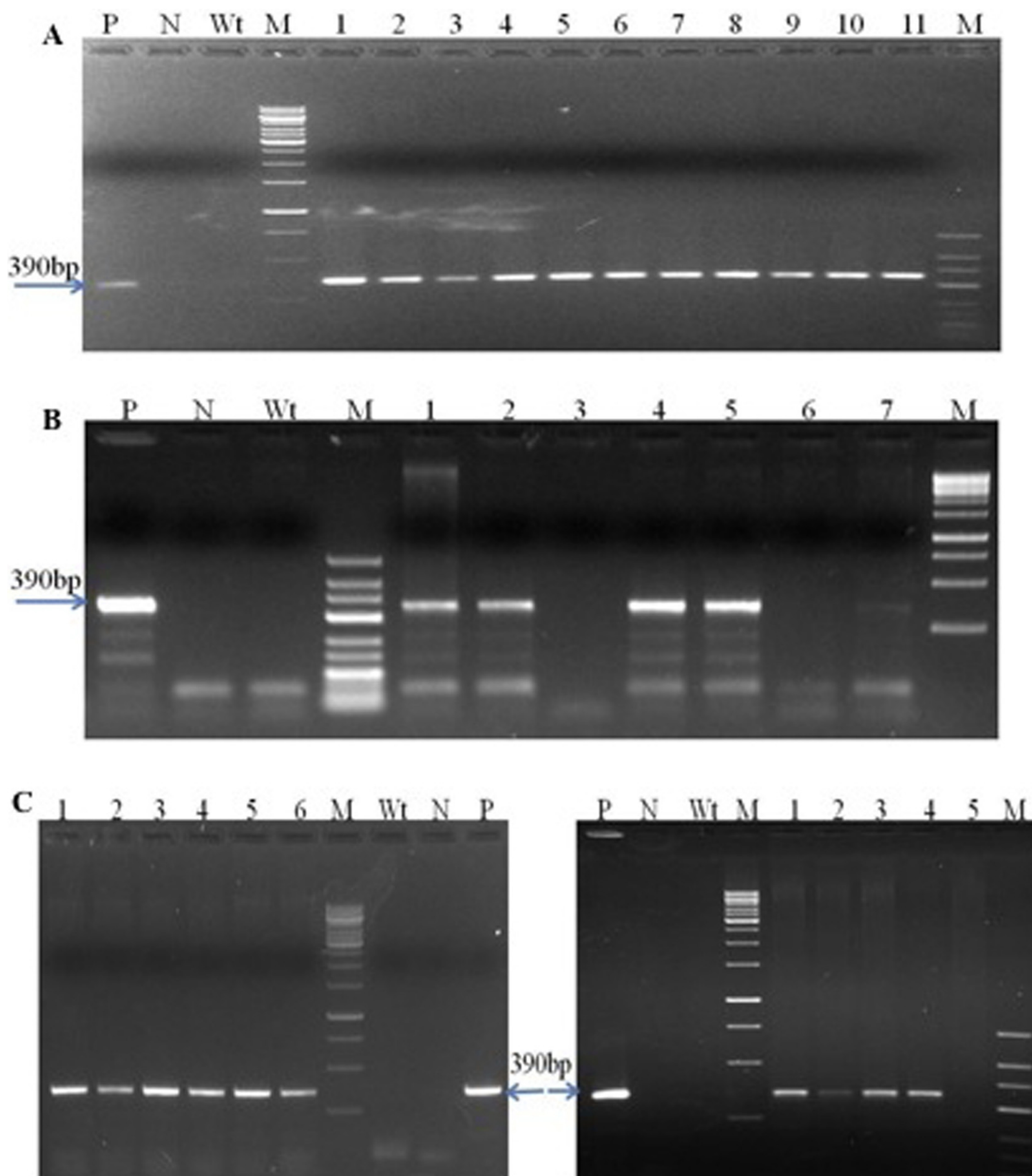

Fig. 4. Genomic PCR to screen the IR64 rice transgenics (A) Rice transgenics regenerated on BAP medium. 1 to 7: Lines 1, 2, 11, 24, 27, 41 and 43 regenerated on RM medium without antibiotic; 8-11 Lines 1, 2, 24 and 41 regenerated on RM medium with Hygromycin. (B) Rice transgenics regenerated on Kinetin medium. 1 to 6: Lines 1, 2, 25, 27, 41 and 44 regenerated on RM medium without antibiotic; 7: Line 1 regenerated on RM medium with Hygromycin. (C) Rice transgenics regenerated on TDZ medium. 1 to 5: Lines 1, 2, 5, 24 and 43 regenerated on RM medium without antibiotic; 6: Line 1 regenerated on RM medium with Hygromycin. (D) Rice transgenics regenerated on Zeatin medium. 1 to 4: Lines 1, 2, 24 and 41 regenerated on RM medium without antibiotic; 5: Line 3 regenerated on RM medium with Hygromycin. Wt: Wild type; N: Negative, P: Positive, M: Ladder.

Acknowledgements

This work was funded by grants from ICGEB, New Delhi and TTN received fellowship from ICGEB for carrying out this work.

References

[1] R. Aldemita, T. Hodges, *Agrobacterium tumefaciens*-mediated transformation of japonica and indica rice varieties, Planta 199 (1996) 612–617.
[2] E.K. Allen, O.N. Allen, Biochemical and symbiotic properties of the rhizobia, Bacteriol. Rev. 14 (1950) 273–330.
[3] L.E. Asbel, M.E. Levison, Cephalosporins, carbapenems, and monobactams, Infect. Dis. Clin. North Am. 14 (2000) 435–447 ix.
[4] R. Caplan, R. Dekeyser, M. Van Montagu, in: W. Ray (Ed.), Selectable Markers for Rice Transformation *Methods in Enzymology*, Academic Press, 1992, pp. 426–441.
[5] M.-T. Chan, T.-M. Lee, H.-H. Chang, Transformation of indica rice (*Oryza sativa* L.) mediated by *Agrobacterium tumefaciens*, Plant Cell Physiol. 33 (1992) 577–583.
[6] P. Christou, T.L. Ford, M. Kofron, Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos, Nat. Biotechnol. 9 (1991) 957–962.
[7] S. Dai, P. Zheng, P. Marmey, S. Zhang, W. Tian, S. Chen, R. Beachy, C. Fauquet, Comparative analysis of transgenic rice plants obtained by *Agrobacterium*-mediated transformation and particle bombardment, Mole. Breed. 7 (2001) 25–33.
[8] S. Das, N. Sanan-Mishra, A direct method for genetically transforming rice seeds modelled with FHVB2, a suppressor of RNAi, Plant Cell Tissue Organ Cult. 120 (2015) 277–289.
[9] K. Datta, Z. Koukolíková-Nicola, N. Baisakh, N. Oliva, S.K. Datta, *Agrobacterium*-mediated engineering for sheath blight resistance of indica rice cultivars from different ecosystems, Theor. Appl. Genet. 100 (2000) 832–839.
[10] K. Datta, J. Tu, N. Oliva, I. Ona, R. Velazhahan, T.W. Mew, S. Muthukrishnan, S.K. Datta, Enhanced resistance to sheath blight by constitutive expression of infection-related rice chitinase in transgenic elite indica rice cultivars, Plant Sci. 160 (2001) 405–414.
[11] K. Datta, R. Velazhahan, N. Oliva, I. Ona, T.W. Mew, G.S. Khush, S. Muthukrishnan, S.K. Datta, Over-expression of the cloned rice thaumatin-like protein (PR-5) gene in transgenic rice plants enhances environmental friendly resistance to *Rhizoctonia solani* causing sheath blight disease, Theor. Appl. Genet. 98 (1999) 1138–1145.
[12] S. Datta, K. Datta, N. Soltanifar, G. Donn, I. Potrykus, Herbicide-resistant Indica rice plants from IRRI breeding line IR72 after PEG-mediated transformation of protoplasts, Plant Mol. Biol. 20 (1992) 619–629.
[13] A. Demain, R. Elander, The β-lactam antibiotics: past, present, and future, Antonie Van Leeuwenhoek 75 (1999) 5–19.
[14] M. Dey, S. Bakshi, G. Galiba, L. Sahoo, S.K. Panda, Development of a genotype independent and transformation amenable regeneration system from shoot apex in rice (*Oryza sativa* spp. *indica*) using TDZ, 3 Biotech 2 (2012) 233–240.
[15] C.C. Eady, C.E. Lister, A comparison of four selective agents for use with *Allium cepa* L. immature embryos and immature embryo-derived cultures, Plant Cell Rep. 18 (1998) 117–121.
[16] H. Fujimoto, K. Itoh, M. Yamamoto, J. Kyozuka, K. Shimamoto, Insect resistant rice generated by introduction of a modified δ-endotoxin gene of *Bacillus thuringiensis*, Nat. Biotechnol. 11 (1993) 1151–1155.
[17] A. Gonzalez, A. Jimenez, D. Vazquez, J. Davies, D. Schindles, Studies on the mode of action of hygromycin B, an inhibitor of translocation in eukaryotes, Biochim. Biophys. Acta –Nuclic Acids Protein Synth. 521 (1978) 459–469.
[18] A. Hayashimoto, Z. Li, N. Murai, A polyethylene glycol-mediated protoplast transformation system for production of fertile transgenic rice plants, Plant Physiol. 93 (1990) 857–863.
[19] Y. Hiei, T. Komari, Improved protocols for transformation of indica rice mediated by *Agrobacterium tumefaciens*, Plant Cell Tissue Organ Cult. 85 (2006) 271–283.
[20] Y. Hiei, S. Ohta, T. Komari, T. Kumashiro, Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA, Plant J. 6 (1994) 271–282.
[21] K.M.A. Hoque, Z. Azdi, S.H. Prodhan, Development of callus initiation and regeneration system of different indigenous indica rice varieties, J. Biol. 1 (2013) 46–51.
[22] V.A. Iglesias, E.A. Moscone, I. Papp, F. Neuhuber, S. Michalowski, T. Phelan, S. Spiker, M. Matzke, A. Matzke, Molecular and cytogenetic analyses of stably and unstably expressed transgene loci in tobacco, Plant Cell 9 (1997) 1251–1264.
[23] R. Jain, M. Davey, E. Cocking, R. Wu, Carbohydrate and osmotic requirements for high-frequency plant regeneration from protoplast-derived colonies of indica and japonica rice varieties, J. Expt. Bot. 48 (1997) 751–758.
[24] J. Jiang, S.D. Linscombe, J. Wang, J.H. Oard, High efficiency transformation of US rice lines from mature seed-derived calli and segregation of glufosinate resistance under field conditions, Crop Sci. 40 (2000) 1729–1741.
[25] A. Karthikeyan, S.K. Pandian, M. Ramesh, *Agrobacterium*-mediated transformation of leaf base derived callus tissues of popular indica rice (*Oryza sativa* L. sub sp. *indica* cv. ADT 43), Plant Sci. 181 (2011) 258–268.
[26] H.K. Khanna, S.K. Raina, Elite indica transgenic rice plants expressing modified Cry1Ac endotoxin of *Bacillus Thuringiensis* show enhanced resistance to Yellow Stem Borer (*Scirpophaga Incertulas*), Transgenic Res. 11 (2002) 411–423.
[27] K. Kumar, S. Maruthasalam, M. Loganathan, D. Sudhakar, P. Balasubramanian, An improved *Agrobacterium*-mediated transformation protocol for recalcitrant elite indica rice cultivars, Plant Mol. Biol. Rep. 23 (2005) 67–73.
[28] L. Li, R. Qu, A. de Kochko, C. Fauquet, R.N. Beachy, An improved rice transformation system using the biolistic method, Plant Cell Rep. 12 (1993) 250–255.
[29] Y.J. Lin, Q. Zhang, Optimising the tissue culture conditions for high efficiency transformation of indica rice, Plant Cell Rep. 23 (2005) 540–547.
[30] Z.-H. Meng, A.-H. Liang, W.-C. Yang, Effects of hygromycin on cotton cultures and its application in *Agrobacterium*-mediated cotton transformation, In Vitro Cell. Dev. Biol. Plant 43 (2007) 111–118.
[31] J. Milojević, L. Tubić, V. Nolić, N. Mitić, D. Ćalić-Dragosavac, B. Vinterhalter, S. Zdravkovic-Korac, Hygromycin promotes somatic embryogenesis in spinach, Plant Cell Tissue Organ Cult. 109 (2012) 573–579.
[32] T. Murashige, F.A. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures, Physiol. Plant 15 (1962) 473–497.
[33] B. Nauerby, K. Billing, R. Wyndaele, Influence of the antibiotic timentin on plant regeneration compared to carbenicillin and cefotaxime in concentrations suitable for elimination of *Agrobacterium tumefaciens*, Plant Sci. 123 (1997) 169–177.
[34] Y. Ogawa, M. Mii, Meropenem and moxalactam: novel β-lactam antibiotics for efficient *Agrobacterium*-mediated transformation, Plant Sci. 172 (2007) 564–572.
[35] J.A. Paine, C.A. Shipton, S. Chaggar, R.M. Howells, M.J. Kennedy, G. Vernon, S.Y. Wright, E. Hinchliffe, J.L. Adams, A.L. Silverstone, Improving the nutritional value of Golden Rice through increased pro-vitamin A content, Nat. Biotechnol. 23 (2005) 482–487.
[36] Y.D. Park, D. Ronis, A.A. Boe, Z.M. Cheng, Plant regeneration from leaf tissues of four North Dakota genotypes of potato (*Solanum tuberosum* L.), Am. Potato J. 72 (1995) 329–338.
[37] J. Peng, F. Wen, R. Lister, T. Hodges, Inheritance of gusA and neo genes in transgenic rice, Plant Mol. Biol. 27 (1995) 91–104.
[38] T. Pipatpanukul, S. Bunnag, P. Theerakulpisut, M. Kosittrakul, Transformation of indica rice (*Oryza sativa* L.) cv. RD6 mediated by *Agrobacterium tumefaciens*, Songklanakarin J. Sci. Technol. 26 (2004) 1–13.
[39] A.M. Priya, S.K. Pandian, R. Manikandan, The effect of different antibiotics on the elimination of *Agrobacterium* and high frequency *Agrobacterium*-mediated transformation of indica rice (*Oryza Sativa* L.), Czech J. Genet. Plant Breed. 48 (2012) 120–130.
[40] D. Raineri, P. Bottino, M. Gordon, E. Nester, *Agrobacterium*-mediated transformation of rice (*Oryza sativa* L.), Nat. Biotechnol. 8 (1990) 33–38.
[41] V. Saharan, R.C. Yadav, N.R. Yadav, B.P. Chapagain, High frequency plant regeneration from desiccated calli of indica rice (*Oryza sativa* L.), Afr. J. Biotechnol. 3 (2004) 256–259.
[42] K.K. Sahoo, A.K. Tripathi, A. Pareek, S.K. Sopory, S.L. Singla-Pareek, An improved protocol for efficient transformation and regeneration of diverse indica rice cultivars, Plant Methods 7 (2011) 49.
[43] Y. Sato, S. Yokoya, Enhance tolerance to drought stress in transgenic rice plants overexpressing a small heat-shock protein, sHSP17.7, Plant Cell Rep. 27 (2008) 329–334.
[44] F. Shoeb, J.S. Yadav, S. Bajaj, M.V. Rajam, Polyamines as biomarkers for plant regeneration capacity: improvement of regeneration by modulation of polyamine metabolism in different genotypes of indica rice, Plant Sci. 160 (2001) 1229–1235.
[45] M. Shri, A. Rai, P. Verma, P. Misra, S. Dubey, S. Kumar, S. Verma, N. Gautam, R. Tripathi, P. Trivedi, D. Chakrabarty, An improved *Agrobacterium*-mediated transformation of recalcitrant indica rice (*Oryza sativa* L.) cultivars, Protoplasma 250 (2013) 631–636.
[46] G.-Q. Song, A. Walworth, J. Hancock, Factors influencing *Agrobacterium*-mediated transformation of switchgrass cultivars, Plant Cell Tissue Organ Cult. 108 (2012) 445–453.
[47] G. Sridevi, M. Dhandapani, K. Veluthambi, *Agrobacterium*-mediated transformation of White Ponni, a non-basmati variety of indica rice (*Oryza sativa* L.), Curr. Sci. 88 (2005) 128–132.
[48] D. Tobias, M. Manoharan, C. Pritsch, L. Dahleen, Co-bombardment integration and expression of rice chitinase and thaumatin-like protein genes in barley (*Hordeum vulgare* cv. Conlon), Plant Cell Rep. 26 (2007) 631–639.
[49] K. Toriyama, Y. Arimoto, H. Uchimiya, K. Hinata, Transgenic rice plants after direct gene transfer into protoplasts, Nat. Biotechnol. 6 (1988) 1072–1074.
[50] H. Tyagi, S. Rajasubramaniam, I. Dasgupta, Regeneration and *Agrobacterium*-mediated transformation of a popular indica rice variety ADT39, Curr. Sci. 93 (2007) 678–683.
[51] H. Vaucheret, C. Béclin, T. Elmayan, F. Feuerbach, C. Godon, J.-B. Morel, P. Mourrain, J.-C. Palauqui, S. Vernhettes, Transgene-induced gene silencing in plants, Plant J. 16 (1998) 651–659.
[52] C. Waldron, E. Murphy, J. Roberts, G. Gustafson, S. Armour, S. Malcolm, Resistance to hygromycin B, Plant Mol. Biol. 5 (1985) 103–108.
[53] T. Wenzhong, I. Rance, E. Sivamani, C. Fauquet, R.N. Beachy, Improvement of plant regeneration frequency in vitro in indica rice, Chin. J. Genet. 21 (1994) 15–99.
[54] A. Wilmink, J.J.M. Dons, Selective agents and marker genes for use in transformation of monocotyledonous plants, Plant Mol. Biol. Rep. 11 (1993) 165–185.

[55] J. Wünn, A. Klöti, P.K. Burkhardt, G.C.G. Biswas, K. Launis, V.A. Iglesias, I. Potrykus, Transgenic indica rice breeding line IR58 expressing a synthetic crylA (b) gene from *Bacillus thuringiensis* provides effective insect pest control, Nat. Biotechnol. 14 (1996) 171–176.

[56] S. Yoshimura, U. Yamanouchi, Y. Katayose, S. Toki, Z.-X. Wang, I. Kono, N. Kurata, M. Yano, N. Iwata, T. Sasaki, Expression of Xa1, a bacterial blight-resistance gene in rice, is induced by bacterial inoculation, Proc. Natl. Acad. Sci. 95 (1998) 1663–1668.

[57] P. Zhang, J. Puonti-Kaerlas, PIG-mediated cassava transformation using positive and negative selection, Plant Cell Rep. 19 (2000) 1041–1048.

[58] S. Zhang, W.-Y. Song, L. Chen, D. Ruan, N. Taylor, P. Ronald, R.N. Beachy, C. Fauquet, Transgenic elite indica rice varieties, resistant to *Xanthomonas oryzae* pv. *oryzae*, Mol. Breed. 4 (1998) 551–558.