DISCUSSION
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Transformation or rice has become the most important route for introgressing target genes from diverse sources into rice, these include both input traits and output traits. While the former include the genes conferring resistance to diseases, pests, high/low temperature, drought and salt, the latter include enriched vitamin production, iron, proteins of therapeutic importance and vaccines. It has become clear from the experiments carried out in the past decade or so that the rice transformation protocols are rather cumbersome and usually take more than 12 weeks from seed explant to plantlet. Among the rice genotypes, indica varieties are particularly known to exhibit high recalcitrance towards tissue culture methods and therefore, relatively few successful transformation cases were reported. Simple and rapid transformation protocols will save time and cost and greatly facilitate gene transfer into indica rices. Our objective is to improve the efficiency of transformation through the development of protocols that require less time and complex operations and can be applied to diverse genotypes.

In the present study an efficient and reproducible protocol that requires about 6 weeks to produce transgenic rice plants by *Agrobacterium*-mediated transformation was developed. All the experiments in establishing the indica rice transformation protocol were carried out using mature seed derived scutellar callus from an elite *indica* rice cultivar Manasarovar, unless otherwise stated. The efficiency of the developed protocol was tested on three randomly selected *indica* rice genotypes.
Nagina22W, Nootripattu and Pusa basmati.

5.1 Improved protocols for callus induction and regeneration

Among the three basal media tested for callus induction in cv "Manasarovar", NB medium with 2 mg/L 2,4-D (NBCI) was found to be marginally better with 96% callus induction (Fig.1) compared to MS with 91% and B5 with 81% respectively. Based on the phenotypic observations, the number of embryogenic calli produced and the frequency of embryogenic callus induction, NBCI medium was found to be an effective medium for the tested rice cultivars. The embryogenic and non-embryogenic calli were visually discriminated based on the criteria reported by Rueb et al., (1994).

The production of embryogenic callus with high regeneration potential appears to be largely dependent on 2,4-D concentration. It is well known that all calli will not be embryogenic. The frequency of embryogenic callus formation on NBCI medium was enhanced with 2.25 mg/L 2,4-D (Fig 2). It is clear from figure 2 that with the increase in 2,4-D concentration from 1.75 mg/L to 2.25 mg/L, the percentage of embryogenic callus also increased. However, a marginal decrease was observed using 2.5 mg/L 2,4-D, indicating that a concentration of 2.25 mg/L was ideal for the cultivar "Manasarovar". Also the three other indica rice genotypes (Nagina22W, Nootripattu and Pusa basmati) produced similar increased callus induction frequencies when tested on NBCI medium containing 2.25 mg/L 2,4-D (Fig.3). These results suggest that a combination of NBCI medium and 2.25 mg/L 2,4-D is ideal for embryogenic callus induction from the indica rice genotypes tested. This is somewhat different from the
earlier reports of Kumria et al., (2001) and Mohanty et al., (1999) which used less concentration of 2,4-D.

As the age of explant is a critical factor that significantly affects the embryogenicity in callus cultures, callus of 5 different age groups, that is, 14, 18, 21, 25 and 28 d old were tested for their regeneration efficiency upon transfer to NBRE medium (Table 1) having 3 mg/L BA. Among the calli of the tested age groups, 18 d old callus cultures showed a high regeneration efficiency of 60.86% (Fig. 4). However, the frequency of regeneration declined with increase in the age of the callus explant, indicating that 18 d old embryogenic cultures carry a high regeneration potential. This stage of the explant was found critical for all the indica rice cultivars tested. This result also signifies that 18 d old callus explants needs to be cultured on NBC1 medium containing 2.25 mg/L 2,4-D. These results are different from the earlier reports of (Kumria et al., 2001) and (Hiei et al., 1994; Khanna and Raina 1998) which suggested the use of either 21 d or 28 d old callus cultures for in vitro regeneration in rice.

The regeneration protocol described here is simple, efficient and reproducible, and the time needed to regenerate transgenic plants is relatively short. Plant regeneration is observable by the end of fifth week. Earlier reports on production of transgenic plants reveal that at least 12 weeks time is needed to initiate plant regeneration from transformed callus (Hiei et al., 1994; Rashid et al., 1996; Mohanty et al., 1999; Kumria et al., 2001; Lee et al., 2002). Most of the regeneration protocols mentioned in the above reports either have a pre-regeneration step or a subculture step.
before transferring callus to regeneration under light. However, in the present work, such in-between steps were eliminated since the 18 d old primary calli was directly used for regeneration or cocultivation. Use of primary callus directly for regeneration was recently reported by Kumria et al., (2001), however the time needed to regenerate transgenic plants using their protocol was longer than the optimized protocol described in the present work.

In vitro regeneration from callus cultures was carried out on three different media NBRE, MSRE and B5RE. Plant regeneration was relatively better on NBRE medium containing 3 mg/L BA (Fig. 5). This increased regeneration frequency was accompanied by a high RSYI (relative shoot yield index) value. A high RSYI value signifies higher regeneration with the formation of multiple shoots (Rubluo et al, 1984). Observations from the preliminary experiments on the effect of cytokinin on plant regeneration using BA and kinetin both independently and in combination (Fig 6) suggest that BA is more suitable for in vitro regeneration in the tested rice genotypes.

5.2 Improved protocols for Agrobacterium-mediated rice transformation

Agrobacterium-mediated transformation usually requires 3 d of cocultivation. Cocultivation was performed for 2.5 d instead of 3.0 d. This decrease in cocultivation time did not significantly affect the transformation efficiency (Fig 10). Instead, it turned out to be beneficial to transformed calli as this facilitated easy removal of Agrobacterium using less stringent washes.

Transformation frequency was significantly higher when calli were
cocultivated with *Agrobacterium* on NBCC liquid cocultivation medium (Fig 11). The results indicate that the semi-solid medium used in most of the transformation protocols reported till date was not obligatory for callus transformation. Also the need for a separate cocultivation medium, such as AA medium, was eliminated as callus transformation was achieved using liquid NBCC medium containing 100 μM acetosyringone. Eliminating such optional steps accelerated transgenic production, reduced cost and complexity of operation.

One of the major changes that reduced the time needed for production of transgenic plants is the use of selective regeneration medium. The transformed calli when transferred to selective regeneration medium for two weeks resulted in proliferation hygromycin resistant calli that showed stable GUS expression (Fig 17). Regeneration of transgenic shoots was observed after two weeks when the above hygromycin resistant calli were transferred to NBRE medium containing cefotaxim. Based on the frequency of transgenic plants produced, it is concluded that the developed rice transformation protocol was efficient in transformation of the three *indica* rice genotypes (Fig. 14) tested. Also, this protocol being efficient and relatively short, this protocol for the production of transgenic plants is suited for *indica* rice genotypes.

5.3 Ascorbate peroxidase (Apx1) rice transgenics

In order to assess the developed transformation protocol, we transformed rice
genotypes with maize *apxl* gene that encodes cytosolic ascorbate peroxidase. Accordingly, the maize *apxl* cDNA was cloned in sense and anti-sense orientation downstream to a super promoter that was reported to enhance gene expression by 156 fold (Ni *et al.*, 1995) in tobacco and *Arabidopsis*. The present study using such a promoter is the first report in rice.

Transgenic plants were initially tested for *in vitro* hpt assay using leaf sections (Fig. 27). Transgenic leaves remained healthy and green after one week on hygromycin selection medium that was supplemented with 100 mg/L hygromycin-B, while untransformed leaves showed severe necrosis and turned brown, indicating the constitutive expression of *hpt* gene in transgenic plants. These observations are in agreement with the reports of Wang and Waterhouse (1997).

The presence or *hpt* gene was confirmed in 7 independent To transgenic plants (S1, S2, S3, S5, S6, S7 and S8) out of 8 by PCR analysis using primers specific to *hpt* gene (Fig. 28). Similarly, in 6 independent *apxl* antisense expressing transgenic lines (AS1, AS3, AS4, AS5, AS6 and AS7) out of 7 tested revealed hpt specific amplicon. The transgenic lines S4 and AS2 failed to show the corresponding amplification of *hpt* gene and hence were assumed as escapes.

As the *apxl* gene of maize and rice share 80.26% sequence homology, care was taken in designing primers where a majority of the sequence was unique to maize *apxl and* not to rice. A primer sequence of 25 nucleotides was chosen which shared only 60% of its sequence with rice *apxl* gene. These primers specifically amplified a 512 bp amplicon from the maize *apxl* gene in transgenic rice plants (Fig. 29). Since
the primers designed were more specific for maize \textit{apxl} gene, no amplification was observed in untransformed control plants. Further, the rice \textit{apxl} homologue is a large gene with introns and therefore did not lead to amplification under the given conditions. Eight putative transgenic plants (S1, S5, S6, S8, AS1, AS3, AS5 and AS7) showed the expected amplicons. Five of these transgenic plants (S1, S5, S8 AS5 and AS7) showed maize \textit{apxl} specific transcript as revealed by RT-PCR experiments (Fig 30). Notably, both sense and anti-sense plants revealed the transgene. However, we have not detected any dramatic increase in transcript in any transgenic plant though all carry super promoter that was expected to enhance expression by more than 156 fold (Ni \textit{et al.}, 1995).

Transgene integration and copy number was confirmed by southern blot hybridization using the maize \textit{apxl} cDNA sequence as a probe. The \textit{T}$_0$ transgenic plants S1, S5, S8 and AS7 showed single copy insertions, while the plant AS5 showed multiple insertions (3 copies) of transgene (Fig. 31). Production of transgenic plants carrying multiple copy insertions of the transgene is not uncommon (Hiei \textit{et al.}, 1994). Since no hybridization signals were observed in untransformed DNA, it is inferred that the synthesized radio labeled probe corresponding to the cDNA of maize \textit{apxl} did not cross hybridize to rice \textit{apxl} gene sequence under the stringent hybridization conditions used in the experiments.

The above study demonstrates a well defined rice transformation protocol that results in production of \textit{indica} rice transgenics in the shortest possible time of 6 weeks. All the steps involved in rice transformation were critically evaluated and the protocol
was simplified, and made less cumbersome to a considerable extent to minimize the costs involved. The rapid rate with which the \textit{apxi} transgenics were produced is a direct proof for the efficiency of the developed protocol. Further, the general applicability of this protocol was proven by transformation of 4 different rice cultivars. This protocol, however, needs further evaluation by testing it on more \textit{indica} genotypes and analyzing transgenics beyond T\textsubscript{2} generation. We expect this procedure will accelerate the process of \textit{indica} rice transformation in a cost effective manner.