International Journal of Botany Studies
ISSN: 2455-541X; Impact Factor: RJIF 5.12
Received: 03-02-2021; Accepted: 29-02-2021: Published: 05-03-2021
www.botanyjournals.com
Volume 6; Issue 2; 2021; Page No. 44-47

Efficient and rapid method for the screening of transgenic rice plants

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Abstract
Rice is one of the most important cereals crops with great potential for improvement through biotechnological intervention. *Agrobacterium*-mediated transformation method for rice has been well established. In the transformation method, antibiotic resistant gene of *hpt* is routinely used as a powerful marker for transgenic rice plants selection. In this study, the hygromycin sensitivity test was executed on wild type Pusa Sugandh 2 (PS2) seeds, based on mesocotyl length, root development and leaf color, the most efficient optimal concentration of hygromycin 50mg/L was used for selection of the transgenic seeds and leaf assay. Wild type seedlings revealed reduction in mesocotyls lengths, root elongation and bleached leaf, as compared to transgenic seedlings on hygromycin selection media. Non-transgenic rice leaves (PS2) had phenotypic symptoms of necrosis, dark-brownish strips or bleached tips and entirely bleach out of lamina, while transgenic leaf remained green and healthy after one week of hygromycin selection. Based on the above results, a simple, rapid, inexpensive, time-saving method for effective evaluation of transgenic progeny containing *hpt* gene using leaf and germination assay of transformed seeds are described. This study confirms an unambiguous differentiation between transgenic and non-transgenic plants.

Keywords: *Agrobacterium*-mediated, hygromycin, leaf assay, rice, transgenic selection

Introduction
Rice is one of the major cereal crops in the world and staple diet for more than half of the world's population. Among the cereals, rice has smaller genome size, which has been considered as a model plant in monocots for genetic studies. *Agrobacterium* mediated plant transformation has been well established method that allows the development of transgenic rice plants (Komari and Hiei, 1996) [11]. Selection of the transformed explants using antibiotics or herbicides is the routine process of transgenic development and the resistance genes such as hygromycin phosphotransferase (*hpt*) (Brodersen et al., 2000) [3]; neomycin phosphotransferase (*nptII*) (Schroeder et al., 1993) [18], phosphinothricin acetyl transferase (Thompson et al., 1987) [20] and *CP4- EPSPS* (Steinrucken and Amrhein, 1980) [19] are widely used.
Among the two types of selectable markers, antibiotic resistant genes provided a more effective system for plant transformation in the various plant species, such as rice (Hiei et al., 1994) [8], maize (Van den Elzen et al., 1985) [21], wheat (Ortiz et al., 1996) [16] and barley (Bartlett et al., 2008; Hagio et al., 1995) [2, 6]. Antibiotic resistance marker genes provide significant advantages over herbicide resistance genes because; they do not affect regeneration and fertility of the transgenic plants (Aldemita et al., 1996) [1]. Moreover, hygromycin is widely used for transgenic selection in monocot plants, which usually show notable natural resistance to another antibiotic, kanamycin (Christou et al., 1995; Miki et al., 2004) [4, 14]. Presence of antibiotic resistant gene in transgenic plants provides a suitable and easy method of screening the transgenic material using leaf assay and germination of seeds on selective media.
Although, PCR technique is a fast and sensitive method for transgenic screening, cross-contamination and reaction conditions often give false positive results, and hence need to be extremely careful. GUS assay (β-glucuronidase assay) is another easy method to determine the delivery of the foreign gene into the plant after 16-48 hrs of the entry of DNA, by transient expression of the Reporter gene (Jefferson et al., 1986) [10]. This assay is based on chromogenic conversion of the substrate x-gluc by GUS. But large-scale screening of transgenic population using molecular techniques like Southern or Northern hybridization, dot blot analysis, enzymatic assay, polymerase chain reaction (PCR) and GUS assay are laborious, most expensive and time-consuming process.
The *hpt* gene has been used as selectable marker in as a model plant in monocot of rice by Ito et al (2006) [9], Latha et al (2019) [12]. During transgenic development process, large scale of transgenic progeny needs to be evaluated for advancement and identification of homozygous lines. In many plant species, the *hpt* selectable marker trait is used for screening of transgenic plants during progeny advancement. In the present investigation, we reported the transgenic seed germination and leaf assay for screening transgenic rice plants expressing the hygromycin resistance gene. This method is simple, rapid, and less expensive, requires lesser amounts of plant material as well as chemicals and allows clear discrimination between transgenic and non-transgenic plants.

Materials and Methods
Plant Material
Non-transgenic seeds of rice *cv* Pusa Sugandh 2 (PS2) were used for hygromycin sensitivity test. For the screening of transgenic lines, seeds were collected from *hpt* gene containing transformed PS2 rice plants produced by *Agrobacterium*-mediated transformation method (Unpublished data). For leaf assay, the primary transformants or their progeny and non-transgenic rice

plants (PS2) were grown on *in vitro* growth condition for one month or attaining eight leaf stages.

Hygromycin sensitivity test

Hygromycin sensitivity test was carried out at different concentration *viz*, 0, 10, 20, 30, 40, and 50 mg/L with the wild type (PS2) seeds. The effective concentration of hygromycin was found, when more than 90% of the non-transgenic seeds got suppressed in germination. Healthy wild type (PS2) seeds were surface sterilized by using 70% ethanol for 1min and washed thrice with sterile distilled water, followed by commercial bleach containing 1.5% sodium hypochlorite for 5min with vigorous agitation, at last washed thrice with sterile distilled water before germinating the seeds on each selection plate. Approximately 20 seeds were screened on each plate of MS media supplemented with hygromycin. Evenly spread seeds were left on selection medium at 28°C for 3days under dark condition, and continued with placing the plates under light and dark for 16/8h at normal growth conditions. The wild type seedlings grown were evaluated for hygromycin resistant and susceptible phenotypes after a week.

Screening on transgenic seeds

Transgenic seedlings were selected on MS media supplemented with the effective concentration (50mg/L) of hygromycin determined through the hygromycin sensitivity test. These transformed seeds were obtained from the *Agrobacterium*-mediated transformation. Similar surface sterilization, plating and observation of hygromycin resistant and sensitive phenotypes method were applied to screen these transgenic seedlings.

Rice leaf assay

The leaf assay for hygromycin screening of the transgenic progenies was carried out by the following protocol of Roy et al (2012) [17]. Approximately 2-3cm long 8 leaf segments were excised from one month old transgenic and non-transgenic plants with the help of sterilized blade and immediately dipped in MS medium (Murashige and Skoog, 1962) [15] supplemented with hygromycin B (50mg/L). Rice leaves segment were kept in petri dishes at 28°C under 16/8h of light/dark cycles. The hygromycin resistant and sensitivity of rice leaves phenotype were evaluated after seven days of hygromycin treatment.

Results and Discussion

In the present investigation, we found hygromycin had no effect on germination initiation in the wild type seeds. After one week of hygromycin selection, wild type (PS2) seedlings showed differences in mesocotyls lengths, due to the effect of hygromycin to the mesocotyls and root growth (Harrison et al., 2006) [7]. Wild type seedlings (PS2) on hygromycin had significant reduction in mesocotyls lengths as compared to those that were grown on MS basal media without hygromycin. Wild type seedlings grew on 30-50mg/L hygromycin selection medium exhibited progressively decreased mesocotyls length and root elongation (Figure 1D-F). Green leaves and root development were also observed in 20mg/L concentration of hygromycin (Figure 1C). However, as the concentration of hygromycin increased to 30mg/L, the leaves begin to change lighter green color and arrested root elongation (Figure 1D). At the same time, the leaves were entirely bleached out at 50mg/L (Figure 1F). The study of Ee et al (2014) demonstrated that based on hypocotyls length and leaf color of Arabidopsis seedlings, the most effective hygromycin concentration at 25mg/L was determined for the screening of transgenic Arabidopsis seeds. Based on hygromycin effects on mesocotyl length, root development and leaf color, the most efficient concentration of hygromycin was detected in the range of 30-50mg/L. Therefore, we thought 50mg/L hygromycin concentration was optimal for selection process. This phenotypic observation helps to distinguish the transgenic rice seedlings from non-transgenic seedlings.

The concentration of hygromycin selection at 50mg/L was used for assessment of transgenic rice seeds. We found that all the seedlings of non-transgenic plants and putative transgenic seedlings without transgene became completely bleached. Mesocotyl growth and root development were completely arrested. Moreover, the seedlings were died after seven days of hygromycin treatment (Figure 2B, 2G). Hygromycin-resistant transgenic seedlings had green leaves with long mesocotyls growth and established long roots on selection plates after a week period (Figure 2C-F). The seedlings were phenotypically appeared similar to the wild type seedlings grown on MS medium without hygromycin (Figure 2A). Similar results were also reported with Pusa basmati 1, which exhibited those hygromycin sensitive transgenic plants died after seven days of hygromycin treatment unlike the hygromycin resistant transgenic seedlings that remained green and produced leaves (Roy et al., 2012) [17].

The leaves of hygromycin susceptible putative transgenic rice showed necrosis, dark brownish strips or bleached, after 7days of hygromycin treatment, similar to that of non-transgenic plant leaves (Figure 3B, 3G); although, higher concentration of hygromycin (50mg/L or greater) resulted in more prominent and widespread symptoms. The leaf assay for sensitivity to hygromycin showed contrasting responses in *hpt*-positive and *hpt*-negative plants. Hygromycin-resistant transgenic rice leaves are remained green, healthy with very less pronounced bleached when subjected to hygromycin selection, which illustrated the resistance ability of the transgenic plants to hygromycin (Figure 3C-F). Some of the transgenic rice plants showed other kinds of phenotypic symptoms, such as localized bleaching, others turned blackish brown from tip and still others exhibited bleaching in strips etc (Table 1). From all of the above described symptoms, hygromycin resistant transgenic rice leaves could be distinguished from the non-transgenic rice leaves. Similarly, the study of Roy et al (2012) [17] showed the leaf tips of *hpt*-positive transgenic rice plants remained green or very less affected when exposed to hygromycin selection, whereas, the leaves of *hpt*- negative transgenics showed necrosis and brown patches similar to non-transgenic rice leaves. Different kind of symptoms in the transgenic lines (Table 1) may be due to the copy number of *hpt* gene. This suggests that the high copy number may have led to silencing of *hpt* gene expression (Linn et al., 1990) [13].

Table 1: Leaf Assay of Putative Transgenic Rice for Hygromycin Resistant Gene

S. No	Rice plant	Leaf assay	Phenotypic observations
1	Control-I	Neutral	Green, healthy
2	Control- II	Negative	Dead, dark brown strips and necrosis
3	TL-1	Positive	Green, healthy with less pronounced bleaching
4	TL-2	Positive	Greenish yellow
5	TL-3	Positive	Green with localized patches
6	TL-4	Positive	Green with very small necrotic patches
7	TL-5	Negative	Dead, brownish strips, necrotic

Control I: Non transgenic rice (PS2) leaves were placed on MS medium without hygromycin.
Control II: Non transgenic rice (PS2) leaves were placed on MS medium supplemented with 50mg/L hygromycin**.**
TL1-TL5: Different transgenic lines rice leaves were placed on MS medium supplemented with 50mg/L hygromycin.

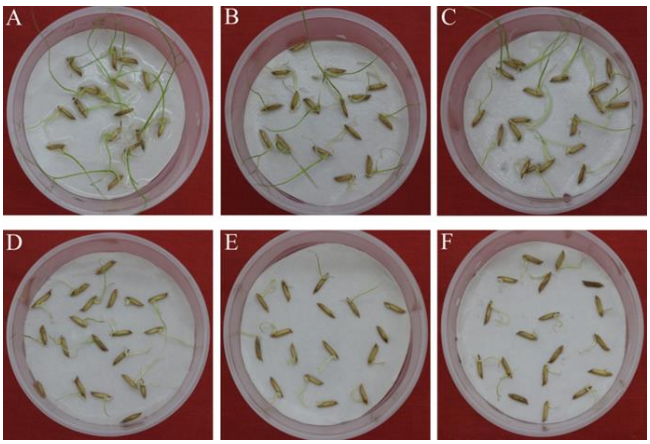

Fig 1: Optimization of hygromycin concentration using the non-transgenic PS2 rice seed germination.

(A) Wild type rice (PS2) seeds growing on MS medium without hygromycin. (B-F) Hygromycin sensitivity test was executed at different concentration *viz*, 10, 20, 30, 40 and 50 mg/L with the wild type (PS2) seeds, respectively.

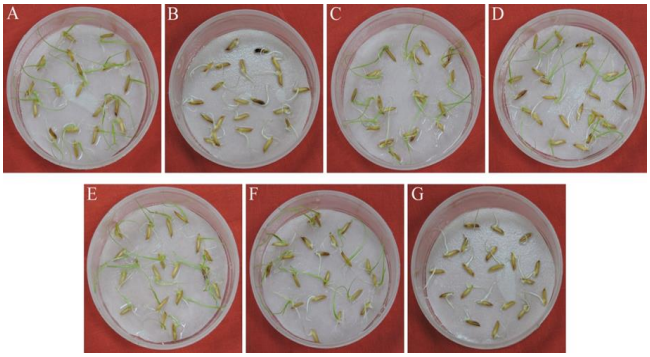

Fig 2: Hygromycin sensitivity test of the transgenic rice seedlings.

(A) Wild type rice seeds (PS2) growing on MS medium without hygromycin. (B) Wild type rice seeds (PS2) growing on MS medium supplemented with 50mg/L hygromycin. (C-G) Different lines of transgenic rice seeds growing on MS medium supplemented with 50mg/L hygromycin.

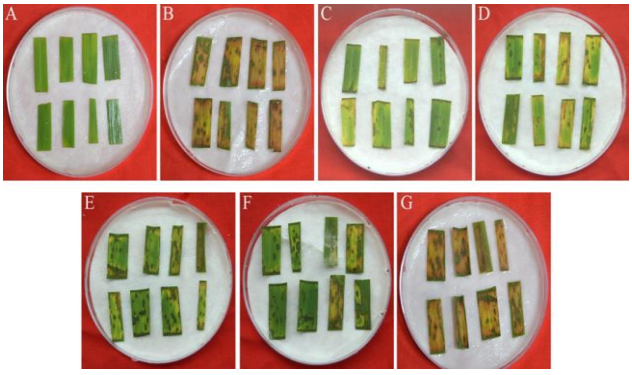

Fig 3: Leaf assay for hygromycin sensitivity using transgenic rice plants.

(A) Wild type rice (PS2) leaves placed on MS medium without hygromycin. (B) Wild type rice (PS2) leaves placed on MS medium supplemented with 50mg/L hygromycin. (C-G) Different lines of transgenic rice leaves placed on MS medium supplemented with 50mg/L hygromycin.

Conclusion

Our findings showed that the optimum concentration of hygromycin for selection of transgenic rice seedlings was in the range between 30-50mg/L. The concentration of hygromycin at 50mg/L was used to distinguish transgenic rice seedlings from the non-transgenic, which showed significant differences in mesocotyls length, root development and leaf color after a week of hygromycin selection. Leaf assay revealed that hygromycin-resistant transgenic leaves had healthy, green with very less pronounced bleached as compared to hygromycin susceptible transgenic leaf on hygromycin selection. The main advantage of this method is simple, rapid, use of minimal quantity of transgenic plant tissues, as well as chemicals which causes no permanent damage to the plants. Therefore, it can be used for screening large numbers of progeny populations of transgenic plants and preliminary selection of transgenics. A simple method could also be applicable to other transgenic plant species using *hpt* as the selectable marker genes.

Acknowledgements

The entire work was performed at ICAR-NIPB, New Delhi and funded by "ICAR-NFBSFARA project on "Phenomics of moisture deficit and temperature stress tolerance in rice". The authors are thankful to Project Director, NIPB, New Delhi for all support and the laboratory facility for this work.

Author contributions

JK performed the experiments and drafted the manuscript. AMS helped in manuscript editing. SRS helped in manuscript preparation. PKM designed the experiments, revised the manuscript, and supervised the entire work.

Abbreviations

GUS- β-glucuronidase,
EPSPS- 5-enolpyruvulshikimate-3-phosphate synthase,
hpt- hygromycin phosphotransferase,
npt- neomycin phosphotransferase,
PCR- Polymerase chain reaction,
PS2- Pusa Sugandh 2,
MS- Murashige and Skoog.

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