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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 7 (3): 318-323, 2004
ISSN 1028-8880

Hygromycin Based Selection of Transformants in a Local Inbred Line of *Zea mays* (L.)

Khurram Bashir, [1]Muhammad Rafiq, Tahira Fatima, Tayyab Husnain and Sheikh Riazuddin
National Center of Excellence In Molecular Biology (CEMB), 87-W,
Canal Bank Road, University of The Punjab, Lahore, Pakistan
[1]Tissue Culture Executive, Qarshi Research International (Pvt) Ltd.,
Hattar Industrial Estate, Haripur, N.W.F.P., Pakistan

Abstract: In present studies, level of toxicity of hygromycin was optimized for local inbred line of maize (*Zea mays* L.). The immature embryos were isolated and cultured on N6 media modified with 1.0 mg L^{-1} 2,4-D, 25 mM L-Proline and 100 mg L^{-1} Casein Hydrolysate and containing different concentrations of hygromycin. These immature embryos were observed for callus formation on working concentrations of 0, 20, 40, 60, 80, 100 and 120 mg L^{-1} hygromycin. The results showed that 97.3 38.5 and 32.5% calli survived on media containing 0, 20 and 40 mg L^{-1} hygromycin. Hygromycin concentration of 60 mg L^{-1} and above was effective for BR-6 as no callus formation was observed at this concentration or the immature embryos were completely dead. It was also observed that although 32.8% calli survived on media containing 40 mg L^{-1} hygromycin, these calli were slower in growth, smaller in size and pale in color as compared to calli containing (hph) gene on same media. On the basis of these results 40 mg L^{-1} hygromycin seems suitable for identification of putative transformants.

Key words: Hygromycin, *Zea mays* L., callus formation, transformation

INTRODUCTION

Plant biotechnology is based on the delivery, integration and expression of defined genes into plant cells, which can be grown to generate transformed plants. Efficiency of stable gene transfer is not high even in the most successful transfer systems[1,2] and only a fraction of the cells exposed, integrate the DNA construct into their genomes. Moreover, a successful gene transfer does not guarantee expression, even by using signals for the regulation of transgene expression. Therefore, systems to select the transformed cells, tissues or organisms from the non-transformed ones are indispensable to regenerate the truly genetically transformed organisms.

Antibiotic resistance genes allow transformed cells expressing them to be selected for out of populations of non-transformed cells. The antibiotic resistance genes can be the genes of interest in their own right or they can be operatively linked to other genes to be transformed into the organisms. The effectiveness of a particular antibiotic resistance system depends mainly on the following elements: The selective agent should fully inhibit growth of untransformed cells. The lowest concentration of the toxic compound that suppresses growth of the non-transformed cells and does not cause detrimental effects to the transformed ones as in monocotyledonous or dicotyledonous plants[3]. Among the most widely used antibiotic resistance genes, as selectable markers are hygromycin phosphotransferase (hph)[4,5] and neomycin phosphotransferase II (nptII)[6-8]. There are also other marker genes like gentamycin acetyltransferase resistance[9], bleomycin[10] and phleomycin resistance[11], but these are not as commonly used.

Hygromycin phosphotransferase is an amino glycoside antibiotic produced by *Streptomyces hygroscopicus* and suitable marker system for both plant and animal systems. It inhibits protein synthesis by interfering with translocation and causing mistranslation at the 80S ribosome[12,4]. The hph gene from *E. coli* confers resistance to hygromycin. The resistance gene codes for a kinase that inactivates hygromycin through phosphorylation. Cloning of the resistance gene and fusion with eukaryotic promoters has resulted in the development of vectors that permit selection for resistance to Hygromycin in both prokaryotic and eukaryotic cells[13]. Hygromycin is usually more toxic than kanamycin and kills sensitive cells more quickly. It is nowadays one of the preferred antibiotic resistance marker systems for transformation of monocotyledonous plants, particularly gramineae (cereals and forages).

Corresponding Author: Muhammad Rafiq, Tissue n Culture Executive, Qarshi Research International (Pvt.) Ltd., Hattar Industrial Estate, Haripur, N.W.F.P., Pakistan, Tel: +92-0995-617273, 617373, Fax: +92-0995-617275
E-Mail: rafijavid@hotmail.com; rafiq_75@yahoo.com

Hygromycin is normally used at a concentration of 50-200 μg mL^{-1} for selection in mammalian cells and 100 μg mL^{-1} in bacterial. In the case of plants, sensitivity to the selective agent depends on many factors, including the ex-plant type, the developmental stage, tissue culture conditions (e.g. pH) and the genotype[12]. Thus, the concentration of hygromycin required for complete growth inhibition of given cells can be reduced by increasing the pH of the medium. In addition, using low salt media whenever possible decreases the amount of Hygromycin needed[13,14].

Keeping in view all these factors a practical concentration for selection of putative transformants of local inbred line of maize was established at national center of excellence in molecular biology, University of the Punjab Lahore.

MATERIALS AND METHODS

Inbred line BR-6 was obtained from Department of Plant Breeding and Genetics, university of Agriculture Faisalabad. The line was sown in field in four replications at ten days interval to ensure the continuous supply of immature embryos. All plants were hand pollinated, to ensure the homozygosity of the material and ears were harvested 10 days post pollination. The ears were stored at 4^0C for 24 h, each ear was de-husked, surface sterilized for 20 min in 500mL of 50% (v v^{-1}) sodium hypo chloride (with 0.1% Tween 20), rinsed 3 times (5 minutes each) with sterile distilled water and then the tops of the kernels were excised with sterile scalpel blade and zygotic embryos were isolated with a fine tip spatula. Immature embryos with undamaged scutellar tissues were placed on media with embryo axis in contact with the N6 modified medium and incubated in the dark at 25±2°C.

Immature embryos were cultured on N6 medium[15] modified with 1.0 mg L^{-1} 2,4-D, 25 mM L-Proline and 100 mg L^{-1} Casein Hydrolysate. Media was divided in to seven treatments according to completely randomized design. These treatments were N6 modified media containing working concentration of 0, 20, 40, 60, 80, 100 and 120 mg L^{-1} Hygromycin. All treatments were divided in to three replicates and data was processed statistically. Media was changed each forth night and patterns of callus formation were recorded and updated weekly up to 45 days.

For transformation studies, immature embryos of maize inbred line BR-6 were isolated and cultured on N6 modified medium as described. After four days the explants were cultured on osmoticum medium (1.0 mg L^{-1} 2,4-D, 25 mM L-Proline, 2% sucrose, 0.2 M sorbitol, 0.2 M manitol and 0.4% phytagel) for four h. A plasmid containing hph gene under CaMV35S promoter and controlled by nos polyadenlylation signal was used for the transformation studies. DNA was coated on tungsten particles and bombarded from a distance of 15cm and He pressure of 60 kg $inch^{-2}$. Twenty hours after bombardment, calli were transferred to N6 modified medium containing 40 mg L^{-1} hygromycin. Calli were sub cultured on same media each forth night and transgenic and non-transgenic calli were identified on the basis of survival, growth rate, size and color.

RESULTS AND DISCUSSION

In Pakistan several reports are available on transformation of tobacco[16], chickpea[17,18], brassica[19], cotton[20] and rice[21,22] but the maize is still difficult to transform. The basic deficiency was a reproducible and efficient selection system and generation of fertile plants through callus culture as it is essential requirement for biolistic transformation system. Present study is an attempt to fill the gaps and to establish a reproducible system for transformation of local varieties of *Zea mays* L.

Immature embryos were isolated and cultured on N6 modified media containing different concentrations of hygromycin. During first week callus formation efficiency

Table 1: Callus formation response of inbred line BR-6 on media containing different concentrations of Hygromycin

Hyg. Conc. (mg L^{-1})	Callus formation (%age) 1st week	2nd week	3rd week	4th week	5th week
0	97.3±0.67	97.3±0.66	97.3±0.32	97.3±0.40	97.3±1.16
20	90.9±1.10	83.6±0.88	63.4±1.55	50.3±1.86	38.5±3.50
40	86.8±1.91	76.4±1.45	55.5±1.80	43.3±0.88	32.8±2.93
60	79.2±0.42	47.0±4.51	25.9±1.57	3.0±1.00	1.3±0.33
80	78.9±1.74	35.8±2.13	7.5±1.78	0.0±0.00	0.0±0.00
100	55.9±1.59	19.8±1.61	0.0±0.00	0.0±0.00	0.0±0.00
120	48.6±2.27	15.0±2.08	0.0±0.00	0.0±0.00	0.0±0.00

The values followed by±show standard error of the mean

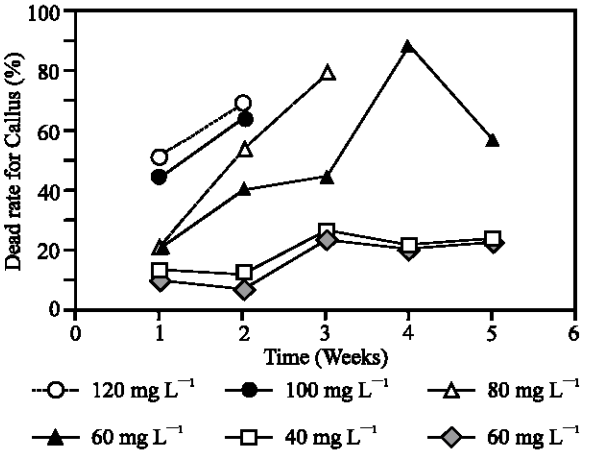

Fig 1: Effect of time period on survival of callus on N6 media containing different concentrations of Hygromycin

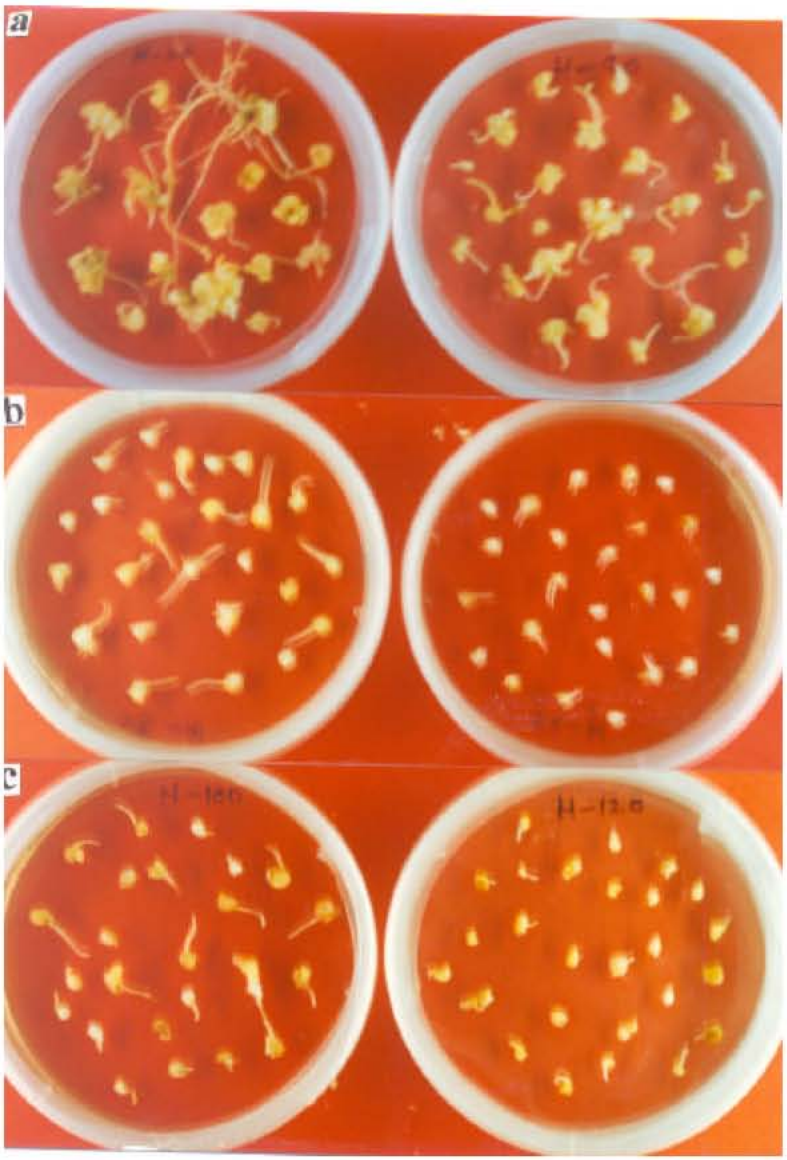

Fig. 2: Callus formation on N6 media containing different concentration of Hygromycin

a: N6 media containing 20 mg L^{-1} hygromycin
b: N6 media containing 40 mg L^{-1} hygromycin
c: N6 media containing 60 mg L^{-1} hygromycin
d: N6 media containing 80 mg L^{-1} hygromycin
e: N6 media containing 100 mg L^{-1} hygromycin
f: N6 media containing 120 mg L^{-1} hygromycin

on media without hygromycin was 97.3% while callus formation efficiency varied from 90.9, 86.8, 79.2, 78.9, 55.9 and 48.6% on media containing 20, 40, 60, 80, 100 and 120 mg L^{-1} hygromycin, respectively. Callus formation rate was constant on drug free media during 2nd, 3rd, 4th and 5th week while the values dropped up to 83.6, 76.4, 47.0, 35.8, 19.8 and 15.0% for 20, 40, 60, 80, 100 and 120 mg L^{-1} hygromycin, respectively during 2nd week (Table 1). This trend continued for 3rd, 4th and 5th week and callus survival rate after five weeks was 38.5, 32.8, 1.3, 0 and 0% for 20, 40, 60, 80, 100 and 120 mg L^{-1} hygromycin, respectively. These results showed that hygromycin might be effective selective agent for identification of transformants in *Zea mays*. It was also observed that the effectiveness of the drug increased with time as frequency of dead calli increased with passage of time (Fig. 1). The

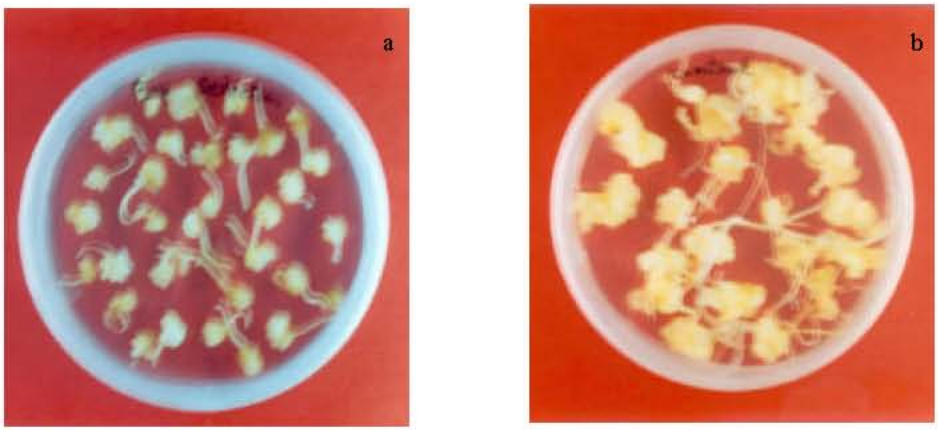

Fig. 3: Comparison of transgenic and control calli on media containing hygromycin
a: Calli containing hph gene
b: Control calli

death rate on media containing 20 mg L^{-1} hygromycin was 10.4 and 7.1% during first and second week while it reached to a value ranging from 20.7 to 24.2% during 3rd to 5th week. Similar trends were observed for media containing 40 mg L^{-1} hygromycin, as the death rate was 13.2 and 12.0% during first and 2nd week, respectively while it was 22.0 to 27.4% during last three weeks. It is clear from the data that hygromycin was more effective during the last three weeks for lower concentrations like hygro-20 and hygro-40 while for higher concentrations (hygro60 to hygro-120) it was very effective from first day as no callus survived more than three weeks on these media. Another important criterion for identification of transformants was the size, growth rate and color of the callus (Fig. 2). The calli on drug free media were larger in size, efficient in growth and bright in color while the calli surviving on media containing different concentrations of hygromycin were smaller in size, slower in growth and pale in color. On the basis of these results 40 mg L^{-1} hygromycin seemed suitable for identification of transformants. In a separate experiment calli bombarded with hph gene were placed on media containing 40 mg L^{-1} hygromycin. The calli containing hph gene were bright in color, larger in size and grow fast as compared to control calli growing on media containing hygromycin (Fig. 3). Plants regenerated from these calli proved positive in molecular analysis thus confirming the effectiveness of the selection system (Data not shown).

Most antibiotic resistance genes used in biotechnology were originally isolated from bacteria. To be used in plants these genes undergo a series of modifications as regulatory elements in the DNA sequence are exchanged for those used in plant cells and usually the gene sequence is also altered to reflect the preferred codon usage of plants. This would make horizontal gene transfer back to bacteria unlikely[23]. Hygromycin resistant gene of *Escherichia coli* origin[24,25] and optimized for expression in Eukaryotes was used as the selective marker gene during tissue culture, as it was necessary to use hygromycin in culture media to select resistant calli[26].

Hygromycin is an antibiotic with broad-spectrum activity against prokaryotes and eukaryotes[27]. This antibiotic was originally developed for veterinary use and is still added in animal food as an anthelmintic (Hygromix). Since the discovery in the early eighties of hygromycin-resistance genes[28-29], hygromycin has become a standard selection agent in gene transfer experiments in a wide variety of prokaryotic and eukaryotic cells. Hygromycin strongly inhibits protein synthesis through a dual effect on mRNA translation[30,37]. Like other aminoglycoside antibiotics, hygromycin induces misreading of aminoacyl-tRNA by distorting the ribosomal A site (decoding center)[30-32]. Hygromycin also affects the ribosomal translocation process[30,4]. In the presence of the antibiotic, mRNA is often mistranslocated, being moved more or less than the three necessary bases.

Hygromycin caused significant effects on the survival, growth, size and color of calli, which was directly proportional to concentration of hygromycin. Although a number of calli survived on low concentrations of hygromycin, yet the values were significantly different from control at all concentrations of hygromycin. Results strongly suggest that hygromycin could successfully be used in experiments involving transformation of local varieties of *Zea mays* L. and identification of putative transformants. Hygromycin has already been used in identification of transformants in this specie[8,33-35] It is also clear that an efficient and reproducible selection system involves continuous selection for approximately five

weeks. These results suggest use of 40 mg L^{-1} of hygromycin as the excess hygromycin may also have negative effects on the growth and proliferation of calli harboring hph gene[3]. These effects can even multiply when these genotypes are continuously screened for long periods so hygromycin may cause damage to transgenic calli and can produce abnormal genotypes[36]. Further decrease of hygromycin may be possible by changing pH condition, ex-plant type, the developmental stage, tissue culture conditions (e.g pH) and the genotype[13,14] but changing pH, salt or genotype conditions may raise further complexities as in our hands BR-6 gives best regeneration on N6 modified media among different regeneration media and different local varieties of *Zea mays* L. (Unpublished Data).

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