IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS)
e-ISSN: 2319-2380, p-ISSN: 2319-2372. Volume 8, Issue 9 Ver. II (Sep. 2015), PP 22-25
www.iosrjournals.org

Isolation & Screening of Phytase Gene by using Basta Selection Medium in transgenic Wheat Plants

S. Farooqi[1,2], A. Maqbool[2], H. Mubeen[1] , S. Raza[1]
[1]*Department of Biotechnology, Faculty of Biological Sciences, University of South Asia, Lahore*
[2]*Department of Biological Sciences, Forman Christian College, Lahore*

Abstract: *Wheat is one of the most widely grown crops in Pakistan. To improve the use of organic phosphate by wheat, phytase gene was expressed in wheat under the control of root specific promoter. The bar gene conferring resistance to the herbicide Basta (containing phosphinothricin) was transferred into the wheat plants by agrobacterium mediated transformation and transformants were selected on Basta medium. The transformed cells are capable to grow normally in presence of Basta while non-transformed cells could not grow. About 300 wheat plants were analyzed for continued tolerance to Basta. The number of plants survived after basta selection was 266. After DNA extraction of 150 plants, integration of phytase gene was checked with the help of PCR.*

I. Introduction

Transformation of crop plants to make them transgenic is completed in three steps: the transfer of genes to plant cells, selection and regeneration of fertile plants. Molecular and biological techniques carrying out detection of primary regenerants and their offspring are time consuming techniques of the same level. Identification of the transgenes should be done at the initial level. Scientists have put great efforts to shorten the screening process (Lee *et al.,* 2009).

To get appropriate transgenic event, large number of plants are infected in all transformed methods. The initial screening of putative transgenic plants from non-transformed is done on the basis of selection marker. For the screening of transgenic plants, selection systems are used. These selection systems work by allowing the selective growth of transformed cells. Genes offering resistance to specific antibiotics or herbicides are found to be highly effective for selection. Such genes allow rapid identification of transformed cells, tissues, and regenerated shoots. Non transformed cells are killed by antibiotics and herbicides by a variety of mechanisms. These resistance genes are in widely usage for transgenic plant production. There are almost fifty marker genes that are used for transgenic, transplastomic plant research and crop development (Darbani*et al*., 2007).

The main selectable markers that offers resistance to herbicides or antibiotics include bar and pat genes that are resistant to phosphinothricin, the active ingredient in BASTA and bialaphos herbicides, EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) that are resistant to the herbicide glyphosate, and the bla, nptII, hph, aadA, and cat genes, that are resistant to the antibiotics ampicillin, kanamycin, hygromycin, spectinomycin and cloramphenicol, respectively (Gadaleta*et al.,* 2006).

There are many ways to screen transformed plants but the strategy selected for screening depends on the type of selectable marker or reporter gene present in vector. If an antibiotic resistance gene is our selectable marker then for screening, culturing of the transformed cells on a medium containing that particular antibiotic is carried out (Soneji*et al*., 2007b, 2007a). For the distinction of phenotypes, reporter genes are used for screening. The presence of selectable marker and reporter genes in transgenic plants are checked and further integration and expression of the transgene is evaluated to minimize false selection (Chalfie*et al.,* 1994).

Mahmood*et al.,* (2012) developed an efficient protocol for rapid screening of tissue culture responsive genotype and establishment of high frequency regeneration system of wheat. They used immature embryos of seven elite wheat cultivars and they were in *in vitro* culture processes. Most appropriate cultivars for tissue culture responses by using auxins and cytokinins one at a time in solidified MS medium were selected. Callus formation and regeneration varied significantly among varieties and among phytohormone concentration. In this paper, selection methods used for the screening of phytase containing transgenic wheat plants have been discussed.

II. Materials & Methods

Transformation of plants: To improve phosphorus use efficiency, two plasmids C219-A and C219-B, having phytase gene under Arabidopsis and barley *Pht*promoter were transformed into wheat immature embryos by using Agrobacterium mediated transformation.

Screening and shifting of plants: For screening of transgenic plants, plants were shifted on MS-K regeneration medium containing kinetin hormone (1mg/L), basta (2mg/L) and timentin (160mg/L). Plants were kept on selection medium of basta for 2 weeks. The plants which survived were checked and they were shifted to MS-0 medium in jars for another 15 days. The second round of selection was done by using high concentration of basta (3mg/L). After selection the plants were transferred to MS-0 in jars for 2-3 weeks, until long and healthy roots were emerged.After good growth of shoots and roots, plants were shifted in plastic pots containing peat moss, vermiculite and perlite (2:1:1 respectively).

DNA extraction: DNA was extracted from plant from green leaves by following CTAB method (Kang & Yang, 2004). To check the integrity of the DNA, electrophoresis was done in 1%agarose gel. For this purpose, 2µl DNA sample and 3µl loading dye was used.

Confirmation of gene insertion by PCR: Integration of phytase gene in the plant chromosomal DNA was checked by amplifying phytase gene fragment from isolated genomic DNA. Primers were designed from border regions of the targeted sequences

Phytase activity assay: To check phytase activity from the rhizosphere of transgenic and non-transgenic plants, phytase was extracted from soil sample.

III. Results

Screening of putative transgenic plants on basta: Total 523 putative transgenic plants were regenerated. Among 523 plants, 95 plants were carrying C219-A contruct and 442 were the carrying C219-B construct. All of these plants were shifted on MS-K medium containing basta 2mg/L. In first round of selection (2mg/L), 450 plants were survived while during second round of selection (3mg/L), 300 plants were survived (figure1).

Figure: 1. Transformed callus on medium containing MS-K and basta

Shifting of plants in pots: Total 266 plants were shifted in plastic pots containing peat moss, vermiculite and perlite (2:1:1 respectively). Out of 266, 150 plants remain healthy. Shifting of transgenic plants in pots is shown in figure 2.

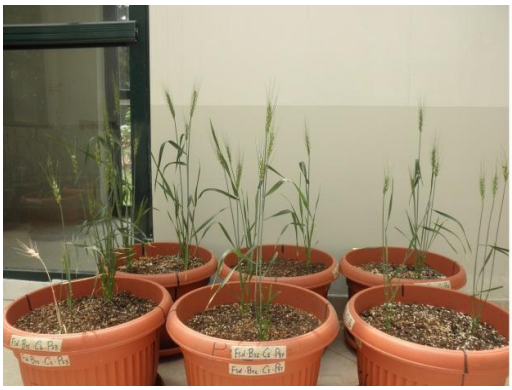

Figure 2: Transgenic wheat plants in pots.

Confirmation of gene insertion in transgenic plants: To confirm the presence of gene in transgenic plants, genomic DNA was extracted from 150 plants and then PCR amplification was carried out. A very good sharp band greater than 10kb was observed on the gel (Figure3) in each case. DNA concentration was measured by nanodrop.

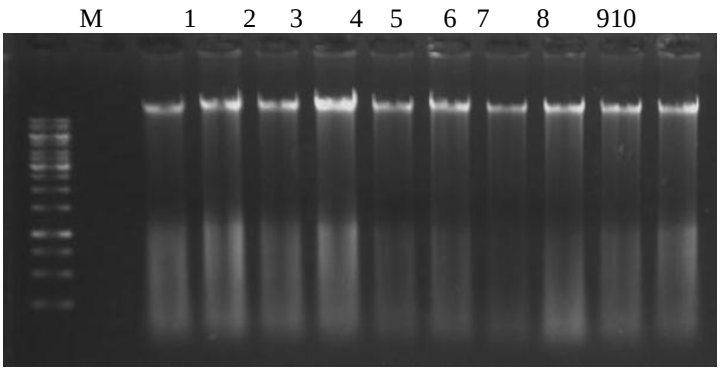

Figure 3: DNA extraction of putative transgenic plants.
M= 1kb DNA ladder; lane 1-10= DNA extraction of putative transgenic plants.

PCR analysis: Gene integration was confirmed by using gene junction primers at both ends of each cassette. An amplicon of 386bp and 493bp was amplified from promoter-gene and gene-terminator region of construct-B by using specific primers: Phy nosR1, Phynos F1 and CB phyF1, CB PR1 primers as shown in figure4. PCR was performed using 150-250ng/µl of genomic DNA.

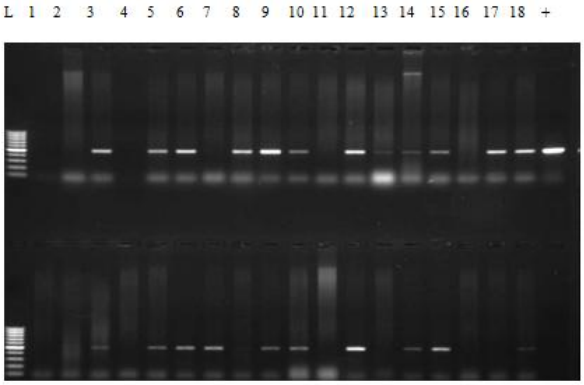

Figure 4: PCR amplification of promoter and gene portion with primer
L= 100bp DNA ladder; lane1-35 = putative transgenic plant samples; C= control sample; + = phytase gene positive control

Phytase activity: The phytase activity in soil of transgenic plants was calculated using standard phosphorous calibration curve.Phytase activity was checked for the soil samples of transgenic plants. From The absorbance measured by spectrophotometer, phosphorous liberated by the action of enzyme was measured. On the x-axis, plants are shown and on the y-axis, phytase activity is aligned. Phytase activity is expressed in U/min/100UI.About 15 plants exhibited higher phytase activity than non-transgenic control plants. Highest phytase activity was shown by plant 256 and that was 1.830U/min/100UI. This value is 1.08 folds higher than the control plant.

IV. Discussion

At the initial stage, putative transgenic plants were selected on basta selection medium (Figure 1). A reliable selectable marker is necessary for the screening of transgenic plants. A good gene marker can make the identification of transformed plants effective and fast (Zhao *et al.,* 1993). In the present study, in the T-DNA of phytase gene constructs (C219-A and C219-B), selectable marker used is *BAR* gene (Figure 1). This gene confers resistance to basta in medium when expressed in transformed cells. The transformed cells are capable to grow normally in presence of basta while non-transformed cells could not grow. The plants were shifted on selection plates containing basta / phosphinothricin (PPT) at the concentration of 2mg/L when they were about 2 and half inch in length to discriminate among transformed and untransformed plants. The second treatment of basta 3mg/L was given after root emergence to avoid any false selection at first round of basta (Figure 4.1). Ganasan and Huyop (2010) optimized the 0.2mg/L of basta in *Citrulluslanatus*but concentration varies from plant to plant for screening. Transgenic wheat containing the reporter gene *β*-glucuronidase and the selectable BAR gene confer resistance to the herbicide Basta. The techniques used for the confirmation of transformants were *β*-glucuronidase assay activity, PCR and Southern blot analysis. Among these techniques, PCR technique was found to be the best and efficient (Melchiorre*et al.,* 2002).

After basta selection, the well rooted and long shoot plants were shifted to mixture of peat moss, vermiculite and perlite (Figure 2). This mixture has the ability to provide plants with enough moisture and aeration to the plants and results in good root growth. Vermiculite possesses cation exchange properties, thus, it can hold and made available ammonium, potassium, calcium and magnesium to the growing plants. Vermiculite, when combined with peat moss promotes faster root growth and provides quick anchorage to young roots (Hartmann *et al.,* 2007). The addition of perlite to peat moss increases the amount of air held in the peat moss, as well as the amount of water retained by the peat moss. This obviously improves the growing conditions for plants (Donahue & Miller, 1990). This mixture provided them semi natural conditions for their growth (Hartmann *et al.,* 2007). They were also treated with rooting powder containing 0.01% IBA that is responsible for long healthy roots and make a net of roots to keep plant in erected position. Abdel-hussain and Salam (1988) studied that IBA treatment at 4000ppm gave the highest rooting percentage, greatest number of roots', root length and individual root weight of cuttings. Daud*et al.,* (1989) reported that dipping olive leaf cutting in 2000, 3000 and 4000 ppm IBA increased the number of roots. An essential step is development of the plantlets in soil like peat moss, vermiculite and perlite. To sustain the stability of foreign gene in plant genome, successive generations should be obtained. In this study this task was also kept in mind. Nearly one month later, the basta screened plants have been transferred from jars to this mixture. During this period, the number of plants was decreased depending on their growth and health (Ravanfar*et al.,* 2010).

Before the shifting of plants to green house, plants were first acclimatized. The plants in jars were very sensitive and delicate. Due to their sensitivity, they were kept in environmental growth chamber for one month. They were provided70% humidity with temperature 25°C. For few days, the pots were covered with plastic bags to shun too much evaporation and drying (Tuncer, 2006). The survived plants were shifted in green house where there was a natural light with appropriate day and night period (Figure 2). They were observed and monitored throughout their growth period. They were supplied with Hoagland's solution for the availability of some macronutrients and micronutrients so that they should not be grown under nutrient deficient conditions. Plants responded and grew well as they had all required growth conditions. A good number (266) of putative transgenic (To) plants of wheat variety (Faisalabad 2008) expressing phytase gene under root specific promoters were obtained.

Acknowledgements

This research was conducted at Biotechnology Departmant of Forman Christian College, Lahore. The authors would like to thank Dr. Kauser Abdullah Malik, Dr. Muhammad Irfan and all of the colleagues.

References

[1]. Lee, H.K., S.K. Cho, O. Son, Z. Xu, I. Hwang and W.T.Kim. 2009. Drought Stress-Induced Rma1H1, a RING Membrane-Anchor E3 Ubiquitin Ligase Homolog, Regulates Aquaporin Levels via Ubiquitination in Transgenic Arabidopsis Plants. Plant Cell. 21(2): 622–641.
[2]. Darbani, B., A. Eimanifar, C.N. Stewart and W. N. Camargo. 2007. Methods to produce marker-free transgenic plants. J. Biotechnol., 2: 83–90.
[3]. Gadaletaa, A., A. Giancasproa, A. Blechlb and A. Blancoa. 2006. Phosphomannoseisomerase, pmi, as a selectable marker gene for durum wheat transformation. J. Cereal Sci., 43:31–37.
[4]. Soneji, J.R., R.M. Nageswara, C. Chen and F.G. Gmitter. 2007a. Regeneration from transverse thin cell layers of mature stem segments of citrus. In: Plant and Animal Genome XV Conference, San Diego, California, USA.
[5]. Soneji, J.R., R.M. Nageswara, C. Chen and F.G. Gmitter. 2007b. Agrobacterium-mediated transformation of citrus using two binary vectors. ActaHortic., 738:261–264.
[6]. Chalfie, M., Y. Tu, G. Euskirchen, W.W. Ward and D.C. Prasher. 1994. Green fluorescent protein as a marker for gene expression. Sci., 263: 802–805.
[7]. Mahmood, I., A. Razzaq, M. Ashraf, I. A. Hafiz, S. Kaleem, A. Qayyum and M. Ahmad. 2012. In vitro selection of tissue culture induced somoclonal variants of wheat for drought tolerance. J. Agr. Res.,50(2).
[8]. Kang, T.J. and M.S. Yang. 2004. Rapid and reliable extraction of genomic DNA from various wild-type and transgenic plants. BMC Biotechnol.,4: 20.
[9]. Zhao, Z., K. Lowe and W. Marsh. 1993. Bar gene as a selection marker for maize transformation. MNL.67:54.
[10]. Ganasan, K. and F. Huyop. 2010. The sensitivity of plant tissue culture and Plant cell of citrulluslanatus cv. Round dragon against BASTA. Int. J. Agr. Res., 5, 11-18.
[11]. Melchiorre, M.N., R.H. Lascano and V.S. Tripp. 2002. Transgenic wheat plants resistant to herbicide BASTA obtained by microprojectile bombardment. BioCell, 26(2): 217-223.
[12]. Hartmann, H.T., F.T. Davis and F.L. GENEVE. 2007. Plant Propagation. In:Principles and Practices. London.
[13]. Donahue, R.L. and R.W. Miller. 1990. Soils: An Introduction to Soil and Plant Growth. Prentice Hall, Inc. Englewood Cliffs. New Jersey.
[14]. Abdel-Hussain, M. A. A. and M.A. Salman. 1988. Effect of some treatments on the rooting of cv. Nebali olive cuttings under mist. Mesopotamia J. Agr., 20(2): 59-72.
[15]. Daud, D. A., J. T. Agha, K. H. Abu-Lebda and M.S. AlKhaiat. 1989. Influence of IBA on rooting leafy olive cuttings. Olive.,6(27): 28-30.
[16]. Ravanfar, S.A., M.A. Aziz, M.A. Kadir, A.A. Rashid and F. Haddadi. 2011. In vitro adventitious shoot regeneration and acclimatization of Brassica oleraceasubsp. italicacv. Green Marvel. Afr. J. Biotechnol., 10(29): 5614-5619.
[17]. Tuncer, T. 2006. Transformation of tobacco (Nicotianatabaccum) with antimicrobial PFLP gene and analysis of transgenic plants. Middle East Technical University, (Graduate) s128.