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Analysis and amplification of Phytase gene in transgenic wheat by polymerase Chain Reaction

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Abstract: *In the present study, we report the conformational analysis of phytase gene containing transgenic wheat plants using polymerase chain reaction. 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. Phytase gene enhances the degradation of phytic acid by the release of phytase. In the present study, transgenic wheat plants containing phytase gene were screened. About 300 plants were selected for basta screening. All of these plants were shifted to pots and kept in green house carefully. DNA extraction was carried out from survived 150 plants. Integration of phytase gene was checked with the help of PCR using gene junction primers of all of these genomic DNA samples. PCR results showed that gene has been successfully integrated in 51 transgenic wheat plants.*

I. Introduction

During the last decades, the practice of molecular techniques to check gene integrity in transgenic plants has been progressively encouraged. Typically, polymerase chain reaction (PCR) is applied to amplify short fragments of DNA from samples, and the PCR products are then identified by gel electrophoresis. As compared to Southern blot analysis, the PCR results give valuable complementary information about gene integration pattern and also about the integrity of the T-DNA borders (Spertini et al., 1999). PCR is the preferred method for detection and screening of transgenic offspring. When time and cost may be two important parameters, then PCR is best choice. Processing of large number of samples in short span of time is one of the strains in case of PCR. To overcome this strain, crude DNA extracts must be selected for amplification by PCR.

Isolation of DNA from cotton and recalcitrant plant can be problematic as the yield of DNA isolated is often low and the quality can be poor. Despite their high potential, PCR based methods have some limitations in specific areas of application (Morisset et al., 2008).Among all of the techniques, conventional PCR with an analysis of specific PCR products is best and has proved useful in many scientific fields (Kota, 1999; Markus et al., 1999; Yongback et al., 2002).

The presence of the reporter gene inputative transgenic plants can be checked by PCR using primers specific to these genes (Xu et al., 2005, Soneji et al., 2007b, 2007a). Before PCR, Southern blots were performed to confirm the presence of foreign DNA and its correct integration into the plant genome. Large-scale preparations of pure genomic DNA, large amounts of expensive restriction endonucleases, and labelled probes were required. PCR is an efficient alternative to Southern blot analyses and its most convincing advantage is the observation that the integration of gene can be examined from as little as 0.2 ng of genomic DNA (Wassenegger, 2001).

Plants may not produce extracellular phytase (Richardson et al., 2004). Scientists have made efforts to give plants access to phosphorus stored in soils as phytate. For this, transgenic plants (Arabidopsis thaliana, Nicotiana tabacum L., Trifolium subterraneum L. and Solanum tuberosum L.) which express phytase genes from a soil fungus (Aspergillus sp.) have been developed. There is an important class of phosphatases called phytases (InsP6 phosphohydrolase). They carry out the sequential hydrolysis of phytic acid and release less phosphorylated myo-inositol derivatives and inorganic phosphate (Wyss et al., 1999; Brinch-Pedersen et al., 2002). Over expression of phytase genes in plant roots is one main strategy to increase phytase activities in the rhizosphere, and thus stimulate phosphorus assimilation from soil.

In this paper, we investigate the confirmation of putative transgenic wheat plants from genomic DNA using PCR. Wheat plants were made transgenic with phytase gene. The objective of the work was to determine by PCR whether the insertion of phytase gene has been successful or not.

II. Methods and Materials:

Transformation and shifting of plant to basta medium for selection: 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. 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. After selection the plants were transferred to MS-0 in jars.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 (Table2). DNA isolated from untransformed plants was used as negative control and that of plasmid as positive. Master mix was prepared according to Table1.

Table1: PCR reaction set up for amplification of phytase gene from the genomic DNA of wheat putative transgenic plant

Sr.No.	Reagents	Concentration
1	PCR Buffer	2.5µl
2	$MgCl_2$	2µl
3	DNTP	1µl
4	Forward primer	1µl
5	Reverse primer	1µl
6	Taq polymerase	1.5µl
8	Water	14µl
9	DNA template	2 µl
	Total volume	25µl

Gene junction primers were designed from both ends of the target sequence to screen transgene plants. The primer set 1 i.e., CBphyR1 and CBF1 primers were designed from the end of barley pht1 promoter and start of phytase gene. The primer set 2 i.e., PhynosF1 and PhynosR1 primers were designed from the lower part of the cassette (end of phytase gene and start of terminator) and used for both constructs. The primer set 3 i.e., CAphyR2 and CAF2 primers were designed from end of Arabidopsis promoter and start of phytase gene.

Initially, gradient PCR was run to optimize the annealing temperature for each set of primer. For primer set 1 (CBphyR1&CBF1), the annealing temperatures used were 40°C,42°C,44°C,46°C,48°C and 50°C. The optimized temperature was 46°C. For primer set 2 (PhynosF1&PhynosR1), gradient PCR was set with following annealing temperatures: 44°C,46°C,48°C,50°C and 52°C. The optimized annealing temperature for this set was 50°C. For primer set 3 (CAphyR1&CAF1), the annealing temperatures for gradient were 45°C,47°C,49°C and 51°C. The optimized temperature was 49°C. The PCR amplified products of each primer pair was run on 1% agarose gel containing 0.05% ethidium bromide along with 100bp standard DNA ladder to verify the size. The PCR set up and profile is given in (Table 3).

Table2: Gene junction primers with sequence and annealing temperature.

Primer Name	Primer Sequence	Annealing Temp
CAphyR2	TTTTCCCTTTGCTATCCGTT	49°C
CAPF2	CTTGGATTCTTTGCGTTGT	49°C
CBphyR1	GTTTCGGAAAAGCATTGG	46°C
CBPF1	CAAGTAGCGAGGAAATGT	46°C
Phy nosF1	ACTCTACAACGGGACTAA	50°C
Phy nosR1	GCGGGACTCTAATCATAA	50°C

PCR program:

Initial Denaturation ---------- 95°C for 5 min
Denaturation ----------95°C for 1 min
Annealing----------46°C for 1 min
Extension----------72°C for 30 sec
Final Extension----------72°C for 10min

} 40 Cycles

III. Results

Screening of putative transgenic plants on basta and shifiting to pots: Almost 71% putative transgenic plants were regenerated. In first round of selection (2mg/L), 450 plants were survived while during second round of selection (3mg/L), 300 plants were survived. 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 1.

Figure1:Transgenic wheat plants in pots&transformed callus on medium containing MS-K and basta

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 (Figure2) in each case. DNA concentration was measured by nanodrop.

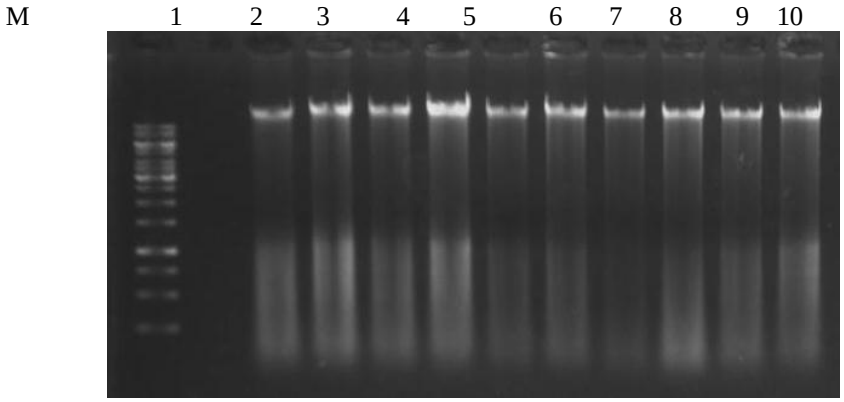

Figure 2: DNA extraction of putative transgenic plants.

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

1. **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, Phy nos F1 and CB phyF1, CB PR1 primers as shown in figure 4.4 and 4.5. PCR was performed using 150-250ng/µl of genomic DNA.

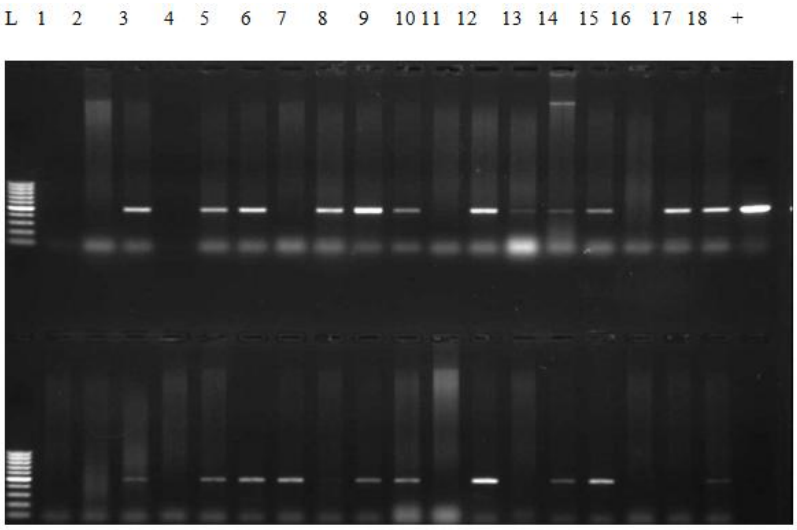

Figure 3: PCR amplification of promoter and gene portion with primer set1

L= 100bp DNA ladder; lane1-35 = putative transgenic plant samples; C= control sample; + = phytase gene positive control

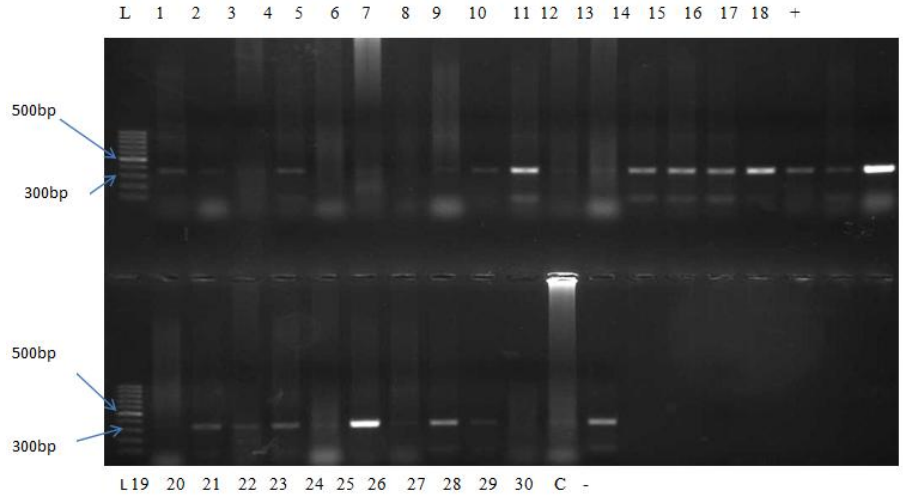

Figure 4: PCR amplification of promoter and gene portion with primer set 1

L= 100bp DNA ladder; lane 1-30= putative transgenic plant samples; C= control sample; +=phytase gene positive control and –ve=negative control.

PCR analysis was done for 150 plant genomic DNA samples. Out of 150, 51 plants were confirmed for having phytase gene in them.

IV. Discussion

When PCR technique is compared with Southern blotting, PCR shows advantages over Southern blotting. In case of PCR, small amount of DNA is required for multiple independent tests. The PCR technique is faster as it takes a total of less than two days, as compared to Southern blotting which takes at least the best part of two weeks. PCR is easy to perform and less costly. Still a highly degraded DNA can be amplified in PCR. Southern blotting requires large amount and fine DNA samples for good results (Li et al., 1988). The use of real-time PCR for checking trans-gene expression and copy number in transgenic plants has few drawbacks. As this technique is costly when done with gene specific primers and probes for each different transgene (Mason et al., 2002).

Present results showed that the DNA produced by this simple, low cost, fast and safe protocol can be used in PCR-based techniques on a wide range. Primers were designed from border regions of the targeted sequences. The biggest challenge was without doubt the fact to optimize the PCR condition for designed primers. Almost three months were gone in trying to get the desired results. DNA isolation from plant leaves was rather a simple task and a well-set protocol in our laboratory. PCR results reveal that phytase gene have

been successfully integrated into the genome of 52 plants. There were total seven plants containing C219-A construct. PCR method has been used for identifying phytase gene in transgenic corn (Peng, 2010). In transgenic soybean, PCR analysis was used to obtain an idea about the verification of gene transfer. PCR detection of the gene sequence among individuals confirmed that 72% carried the foreign gene (Wilcox et al., 2000). In transgenic rice containing phytase gene, integration of phytase gene was checked through PCR by using synthetic primers. Integration was further confirmed by Southern blotting (Qiao-quan et al., 2006).

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