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MOLECULAR ASSESMENT OF CHITINASE ACTIVITY IN TRANSGENIC WHEAT

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Fungal diseases especially rust of common wheat (*Triticum aestivum*) and durum (*T. turgidum* subsp. *durum*), historically was one of the most destructive wheat diseases. Significant losses occurred in the past when the disease developed into epidemic proportions in wheat crops (Roelfs, 1978). Plants naturally respond to fungal attack by a complex network of defense mechanisms, which are activated upon perception of a pathogen and designed to limit its penetration and development. Defense responses include structural and biochemical responses like reinforcement of the plant cell wall, accumulation of phytoxlexins with microbial toxicity, ribosome-inactivating proteins (RIPs) that inhibit protein synthesis, antimicrobial peptides and the synthesis of other pathogenesis-related (PR) proteins (Yang *et al*., 1997). Some PR proteins, such as chitinase and glucanases, have hydrolytic activities against structural components of fungal cell walls and may exhibit strong antifungal activities *in vitro* (Schlumbaum *et al*., 1986; Leah *et al*., 1991). *In vivo*, chitin oligomers released from fungal cell walls

function as elicitors that stimulate a general resistance response (Cote and Hahn, 1994). The induction of resistance responses by chitin-derived oligosacchrides has also been described for wheat (Barber *et al*., 1989).

Chitinase (poly [1,4-N-acetyl-/3-D-glucosaminid] glycan hydrolase, EC 3.2.1.14) catalyzes the hydrolysis of chitin polymer in fungal cell walls into N-acetylglucosamine oligomers (Toyoda *et al*., 1991). Plant chitinases are induced as a result of pathogenic infections as well as by abiotic agents (Lee and Hwang, 1996 and Punja and Zhang, 1993). Chitinases possess anti-fungal activity that causes *in vitro* lysis of hyphal tips as well as inhibition of spore germination in *Alternaria*, *Fusarium* and *Trichoderma* (Schlumbaum *et al*., 1986; Mauch *et al*., 1988). Because of variation in cell wall composition, pathogenic fungi differ in their sensitivity towards chitinases. Chitinases are induced locally in the infection sites or accumulate systemically in other tissues following a pathogenic attack (Pan *et al*., 1992). Chitinases also contribute indirectly to the induction of host defense responses. Mycelial wall fragments released as a result of chitinase activity may act as elicitors of plant defense mechanisms, i.e., accumulation of phenolic compounds, lignification and phytoalexin synthesis (Kurosaki *et al*., 1988). Nitzsche (1983) reported that chitinase is a possible resistance factor in wheat against yellow rust disease.

Several laboratories have been able to transfer plant- or microbial-derived chitinase genes into plants and develop transgenic crops with enhanced resistance to fungal diseases. These include transgenic tobacco and canola (Broglie *et al*., 1991; Terakawa *et al*., 1997), rice (Nishizawa *et al*., 1999), grapevine (Yamamoto *et al*., 2000), peanut (Rohini and Sankara, 2000), grapevine (Bornhoff *et al*., 2005), Italian ryegrass (Takahashi *et al.* 2005) and carrot (Jayaraj and Punja, 2007).

In wheat, Chen *et al.* (1998) introduced rice chitinase gene (*chi11*) into the spring wheat cultivar 'Bobwhite'. After inoculation with conidia of *F. graminearum*, the symptoms of scab developed significantly slower in transgenic plants of the T_1, T_2 and T_3 generations than in non-transformed control plants. Oldach *et al*. (2001) introduced barley class II chitinase and a barley type I RIP, all regulated by the constitutive *Ubiquitin*1 promoter from maize, into wheat. They found that the formation of powdery mildew (*Erysiphe graminis* f. sp. *tritici*) or leaf rust (*Puccinia recondita* f. sp. *tritici*) colonies was significantly reduced on leaves from *chitinase* II- expressing wheat lines compared with nontransgenic controls. The increased resistance of *afp* and *chitinase* II lines was dependent on the dose of fungal spores used for inoculation. Heterologous expression of the fungal *afp* gene and the barley *chitinase* II gene in wheat demonstrated that colony formation and, thereby, spreading of two important biotrophic fungal diseases is inhibited approximately 40 to 50% at an inoculum density of 80 to 100 spores per cm

This paper reports the production of two wheat transgenic lines stably expressing one of the pathogen related (PR) proteins, the barley *chitinase* gene (*chi*). The plant expression vector pbarley/chi/bar is harbouring the *chi* gene under the control of *ubi* promoter and *NOS* terminator and the *bar* gene under the control of *35S* promoter and *NOS* terminator. The integration and expression of the transgene(s) were proved using molecular analysis, i.e., PCR, Southern, RT-PCR. The activity of chitinase enzyme on colloidal chitin was measured in the protein extract of the transgenic plants.

MATERIALS AND METHODS

Plant expression vector

The plant expression vector used in transformation experiments was pBarley/chi/bar (Fig. 1). This vector is harbouring the barley chitinase (*chi*) and *bar* genes. *chi* gene is under the control of *ubi* promoter and NOS (nopaline synthase) terminator. While, the *bar* gene is under the control of 35S CaMV promoter and NOS terminator. To obtain the pBarley/chi/Bar expression vector, three construction steps were performed. First, using the appropriate primers, the *chi* gene was isolated from barley and subcloned in expression vector pET-30 obtaining pET-30/barley/chi. The correct orientation of the insert was verified by sequencing by the dideoxy chain termination method (Sanger *et al*., 1977) using an automated DNA sequencer (ABI Prism 310; Applied Biosystems, Foster City, CA, USA) and M13 universal primers and sequence specific primers. Second, *BamH*I fragment obtained from pET-30/chi was ligated into the *BamH*I site of pAHC17 vector to obtain pbarley/chi vector. To select the transformed *E. coli* colonies with the right orientation, pbarley/chi was cut using *Nco*I restriction enzyme. *Nco*I site is located 60 bp from the start codon of the *chitinase* gene. Right colonies are expected to give 4.80 and 0.93 kb in length, while the wrong ones give 4.17 and 1.56 kb. *Hind*III fragment containing *bar* gene cassette obtained from pAB8 vector was ligated to *Hind*III site of pbarley/chi.

Wheat transformation and regeneration procedure

Spikes from field grown wheat plants (*Triticum aestivum* L., cv. Hiline) were collected two weeks postanthesis. Immature caryopses were sterilized using 20% commercial bleach supplemented with few drops of Tween 20. Immature embryos were isolated and placed on TW callus induction medium (Weeks *et al*., 1993) with the epiblast exposed to the medium as modified by Bahieldin *et al*. (2000). After 4-7 days in culture, embryo-derived calli were exposed to osmotic treatment (TW medium supplemented with 0.4 M mannitol) for 4 h, and then bombarded with pbarley/chi/bar plasmid using the helium-driven DuPont Biolistic Delivery System (Bio-Rad) with a pressure of 1100 psi. Bombarded calli were left on the same medium overnight (16 h), then transferred to TW callus induction media for four weeks. Embryogenic calli were transferred to regeneration medium (MS containing the growth regulator

thidiazuron (TDZ). After 2-4 weeks, calli-derived shoots were transferred to hormone-free MS rooting medium. Well-rooted plants were transferred to the greenhouse potting mix soil: beatmos: sand (1:1:1). The greenhouse day/night temperatures were 25 ± 2°C / 19°C under a 16-h photoperiod.

Leaf painting

Herbicide resistance of putative transgenics and progeny was tested by a leaf painting assay, i.e., painting intermediate parts of the plant leaves from both sides with 1 g/L Basta (stock contains 20% ammonium glufosinate); one half the recommended dose.

Molecular analysis of transgenic plants

- ***Polymerase chain reaction (PCR)***

DNA was extracted from leaf material (300 mg) using a CTAB procedure (Sambrook and Russel, 2001). The reaction was done using oligonucleotide primers specific for the *chi* gene (expected size 1079 bp after its sequencing at MSU and Blast alignment with Gene Bank data bases) as well as for the *bar* gene (expected size 400 bp). The specific primers were synthesized at AGERI, ARC, Giza, Egypt, for the two genes and *NOS* terminator sequence with the following sequences: *bar* (Forward: 5` TACATCGAGACAAGCACGGTCAACT 3`; Reverse: 5`ACGTCATGCCAGTTCCCGTG3`), *chi* (Forward: 5`TATTATCATATGAGATCGCTCGCGGTGGTGGTG 3`; Reverse: 5`TATATACATATGGGATCCATAGGCGAAGGGTCT3′, *NOS* (reverse) 5′ TTATCCTAGTTTGCGCGCTA 3′. Amplification was carried out in a Hybaid PCR Express programmed for 40 cycles as follows: 94°C/4 min (1 cycle); 94°C/1 min, 58°C/1 min, 72°C/2 min (38 cycles); 72°C/8 min (1 cycle); 4°C (infinitive). Agarose (1.2%) was used for resolving the PCR products. Restriction fragment length polymorphism (RFLP-PCR) based analysis was carried out to proof the correct size of the PCR product using restriction enzymes (Louw *et al*., 1995; Wu *et al*., 2002). PCR products of both genes were digested with restriction enzymes. The restriction enzymes were chosen according to the genes restriction map.

- ***Southern analysis***

The Southern blotting steps followed the original method developed by Southern (1975). Ten micrograms of genomic DNA digested with *Eco*RI were size separated on a 1% (w/v) agraose and then transferred to nylon membrane (Hybond N+, Amersham). The whole construct pBarley/chi/bar was labelled by (α^{32}P) dCTP using the Prime it II random Primer labelling Kit (Stratagene, USA).

- ***Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis***

Total RNA was isolated from the putative two transgenic plants as well as the non-transgenic control plants using Promega SV Total RNA Isolation System (cat. no. Z3100). RT-PCR was carried out

using the Titan One Tube RT-PCR system (Roche, cat. no. 1888382). The primer used was the same specific primer for *chitinase* gene.

- ***Estimation of chitinase activity***

Total proteins were extracted from leaf tissue of positive leaf painted putative transgenic plants as well as non transgenic control plants (cv. Hi-line). The protein concentration was measured according to Bradford method (Bradford,1976) using bovine serum albumin (Sigma) as the standard. The activity of chitinase enzyme was assayed following the method of Reissig *et al.* (1955) by a colorimetric method using colloidal chitin as substrate. Colloidal chitin was prepared as described by Hsu and Lockwood, (1975) from crab shell chitin (Sigma). Chitinase specific activity was expressed as micromoles of N-acetylglucosamine mg^{-1} protein h^{-1} at 37°C.

RESULTS AND DISCUSSION

Development of transgenic plants

Wheat transformation was performed using biolistic bombardment protocol as established by Weeks *et al.* (1993) and modified by Bahieldin *et al.* (2000). Immature embryo-derived calli was used as target for bombardment. Calli were bombarded once using 1100 PSI at 6 cm distance. To increase the transformation efficiency, calli were subjected to osmotic treatment before and after the bombardment (Vain *et al.,* 1993). To increase the size, calli were transferred to TW medium for two subcultures, two weeks each. Then, the calli were transferred to regeneration media. All regenerated plantlets were transferred to half strength MS rooting medium. No bialaphos selection was performed during the course of tissue culture. However, this protocol is different from that published by (Sivamani *et al*., 2000) who used callus induction medium supplemented with 5 mg/L bialaphos and got 10 transgenic plants. Bahieldin *et al*. (2000) used two bialaphos concentrations of 0 and 5 mg/L and got three independent transgenics from no selection experiment. PPT (phosphinothricin, active ingredient of the herbicide bialaphos) inhibited glutamine synthetase, an important enzyme during photosynthetic electron transport. This detrimental effect of the herbicide cause cell death due to the accumulation of ammonia in non transformed cells. The problem with this irreversible non-selective effect of the herbicide is that the tissue is chimeric with transformed and non transformed cells. Ammonia tends to be diffused across the cell membrane from the non transformed cells into the neighbouring transformed cell, which leads to the low recovery rate of transformants (Jones, 2005).

Leaf painting

Herbicide resistance of putative transgenenics was tested by painting the middle green parts of the plant leaves from both sides with the herbicide Basta at one-half recommended dose (1 g/l). This procedure revealed the expression of *bar* gene in the genomic background of

wheat. The leaves of transgenic plants were resistant to the herbicide (stay green), while non transgenic as well as control plant leaves turned yellow and the cells died within two days. To detect transgenic plants in the segregated T_1 generation, plants were sprayed with the same Basta concentration. Herbicide Batsa is one of the most important criterions to assess the production of *bar* transformants (Anand *et al.* 2003). In this study, two independent transgenics were obtained. The presence of the transgene(s) in the T_1 plants was following the Mendlian segregation.

Polymerase chain reaction (PCR) analysis

PCR analysis using gene-specific primer combinations designed for detection of the *chitinase* and *bar* genes was used for characterization of the two transgenic lines. To detect the presence of *chitinase* gene, two different primer combinations were used. The *chi* forward and reverse primers were used in the first combination (expected band size 850 bp), while in the second one, the *chi* forward and *NOS* reverse were used (expected band size 1100 bp). As showed in (Fig. 2a), the putative transgenic plants (1 and 2) as well as positive control (+ve) showed the expected band sizes in both combinations. It was found that when using the combination of *chitinase* gene specific primers (*chi* forward and reverse), the wild type negative control (Hiline) gave a PCR product with the same expected band size like positive control and the two putative transgenic plants. This due to the fact that the barley *chitinase* gene has analogous in wheat genome (Gene Bank accession numbers: AY973229 and AY973230, (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db= nucleotide&val=62465513). So, using this combination cannot confirm the presence of this gene. To solve this problem, it was decided to use *NOS* reverse primer (Fig. 2b) instead of *chi* reverse primer. Moreover, the PCR product was confirmed using the restriction enzymes *(BamH*I, *Sac*II and *Sal*I). According to the *chi* gene restriction map, cutting the *chi*-PCR product with *BamH*I will give (Fig. 3).

Another PCR analysis was carried out to proof the presence of the *bar* gene in the background of the putative transgenic plants. Two different combinations of primers were used; *bar* forward and *bar* reverse primers to detect a product band of about 543 bp, while the other combination was *bar* forward and *NOS* terminator reverse primers to detect a product band of about 793 bp. (Fig. 4a & b, respectively).

Genomic southern analysis

Total genomic DNA isolated from leaf tissue of positive leaf painting with herbicide Basta and positive PCR transgenes were digested with *EcoRI* enzyme and hybridized with an *EcoRI* fragment (1.772 kb) of pBarley-chi plasmid containing *Chi* gene cassette used as a probe to confirm the integration of *Chi* gene in the wheat genome and to estimate the gene copy number in the genome of

transgenic plants. Figure (5) shows the hybridization of the probe pBarley/chi/bar plasmid containing *chitinase* gene with genomic DNA of the two putative transgenics after being cut with *EcoRI* to liberate 1.772 kb band. As expected, a fragment with detected size released from genomic DNAs of the two transgenics. The results also indicated that transgenes were integrated at one copy only.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Expression of the integrated gene was tested on the RNA extracts of the two Basta leaf-painting-positive T_1 plants utilizing the Titan-one tube RT-PCR gene expression system. The results of *chitinase* gene expression for the two T_1 plants indicated the presence of the expected cDNA band size (850 bp), (Fig. 6).

Protein expression analysis

Chitinase activity was measured in chi-transgenic lines as well as in non transgenic cv Hiline plants (Fig. 7). One unit of chitinase activity was defined as the amount of enzyme that released 1 µmol GlcNAc from colloidal chitin per minute. The non-transgenic plants showed low activity level (121.47 µM GluNAc min^{-1} g^{-1}). Comparing to non-transgenic plants, the relative increase in chitinase activity of transgenic line chi1 was (275.6%) and in chi 2 (212.1%). The difference in activity between the transgenic lines may be attributed to the transgene position in the genomic background resulting in suppression of gene expression in chi2 plant. On the other hand, observed activity exhibited by non-transgenic plants might refere to the basal levels of activity and/or due to another member of a possible multi gene family for chi gene.

Transgenic seeds will be maintained and increased in order to detect transgenic families with the introduced transgene in homozygous condition. Field experiments to test the performances of *chi* transgenics will be taking place in locations targeting the most infected areas with fungal diseases in Egypt. Transgenic events with the most resistance to fungal and the best agronomic performance will be identified and selected. Also, biosafety testing will be conducted according to the regulatory policies and procedure of the national biosafety system in the guidelines of the National Biosafety Committee in Egypt. Finally, transgenic plants will be incorporated into ongoing Egyptian breeding program. Economically significant benefits for farmers can be expected if the genetically engineered wheat plants are developed and be commercialized

SUMMARY

Barley chitinase gene (poly [1,4-N-acetyl-/3-D-glucosaminid] glycan hydrolase, 26 kd, EC 3.2.1.14), was introduced to common bread wheat (*Triticum aestivum*,cv. Hiline), by biolistic transformation using pBarley/chi/bar transformation vector which harbouring the barley chitinase (*chi*) under the control of *ubi* promoter and NOS terminator and *bar*

gene under the control of 35S CaMV promoter and NOS terminator. Two different transgenic lines were stably expressing the barley *chitinase* gene (*chi*), the integration and expression of the transgene(s) were proved using molecular analysis, i.e., PCR, Southern, RT-PCR. The activity of chitinase enzyme was assayed by a colorimetric method using colloidal chitin as substrate; the activity of chitinase enzyme on colloidal chitin was measured in the protein extract of the transgenic plants.

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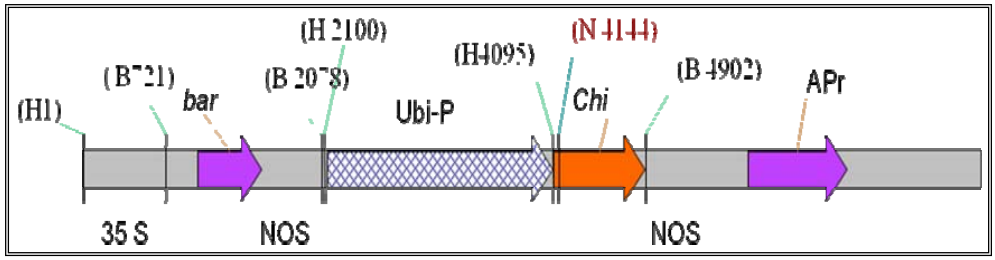

Fig. (1): Map of the plant expression vector pBarley/chi/bar. H, *Hin*dIII, B, *Bam*HI, N, *N*COI, Apr, Ampicillin resistance gene.

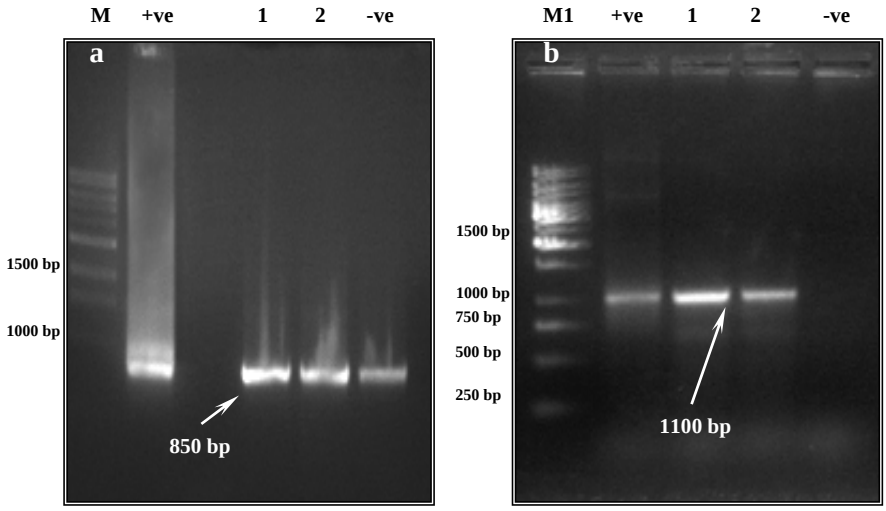

Fig. (2): PCR involvirward and reverse specific primers. b. Combination using *chitinase* forward and *NOS* reverse specific primer. +ve, the pBarley/Chi/bar positive control; -ve, non-transgenic cv Hiline; M, 1 kb DNA ladder (BioLabs); M1, 250 bp DNA ladder (Pharmacia).

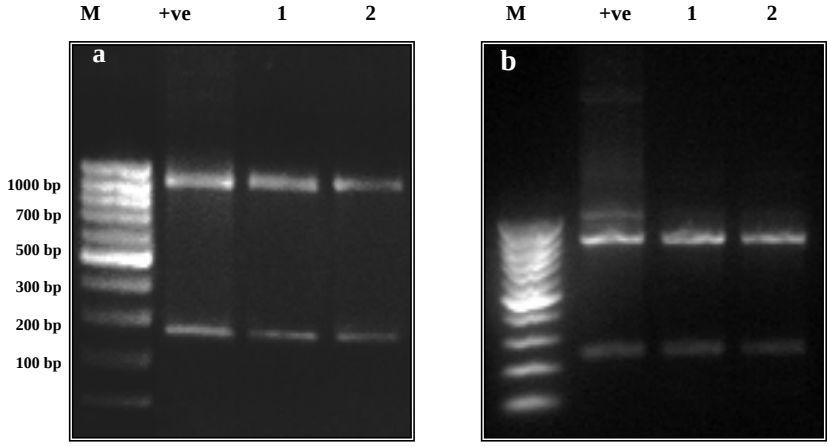

Fig. (3): PCR-based RFLP analysis for *chitinase* PCR product (1100 bp) digested with a) *BamH*I and b) *Sal*I. +ve, PCR product of pBarley/Chi/bar vector; 1 & 2, PCR product of putative transgenic plants; M, 100 bp DNA ladder (Fermentas).

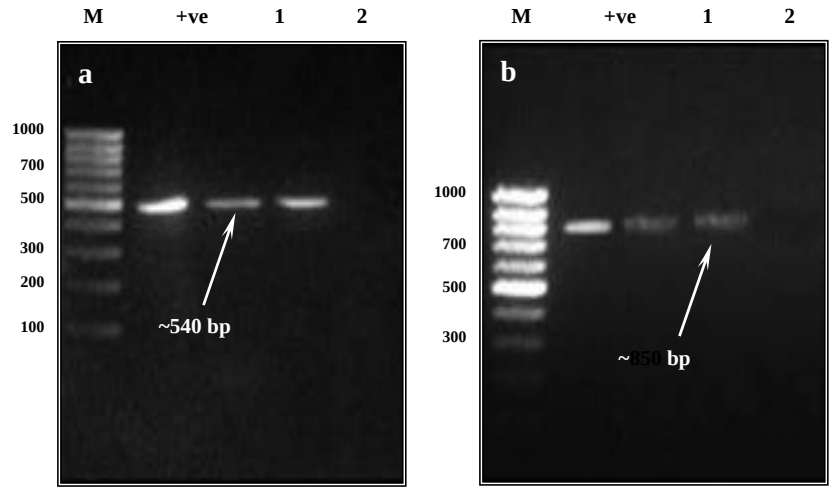

Fig. (4): PCR product for *bar* gene of the two putative transgenic plants (1&2). a. Combination using *bar* forward and *bar* reverse primers. b. Combination using *bar* forward and *NOS* reverse primers. +ve, the pBarley/Chi/bar positive control; -ve, non-transgenic cv Hiline; M, 100 bp DNA ladder (Fermentas).

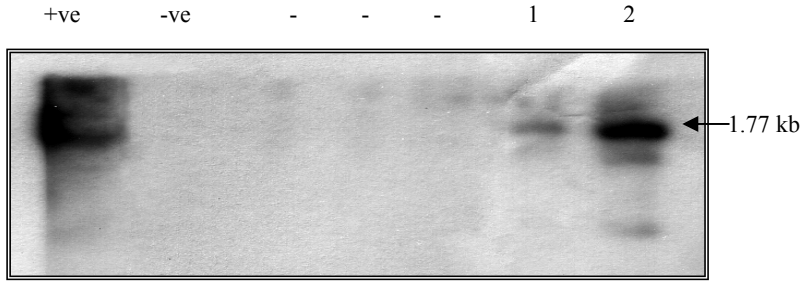

Fig. (5): Genomic Southern analysis for the DNA of the putative transgenic plants (*EcoRI* digest) utilizing chitinase gene cassette of pbarley/chi/bar as a probe (1.772 kb). +ve, positive control, 1, 2 putative transgenic plants, -ve, non-transgenic cv Hiline.

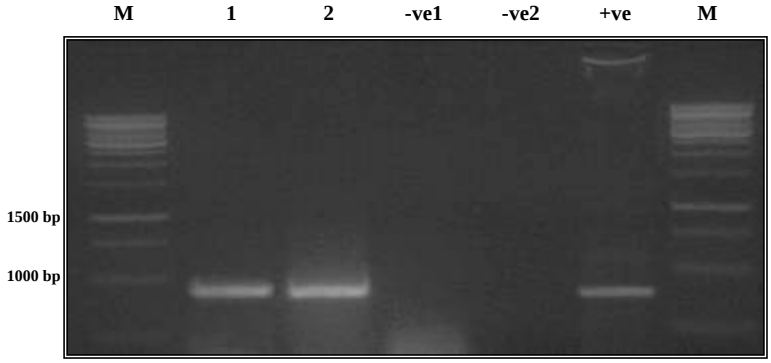

Fig. (6): RT-PCR involving *chitinase* gene for the two transgenic plants (1 & 2). +ve positive control (pBarley/Chi/bar plasmid); –ve1, non-transgenic cv Hiline; -ve2, negative control (no cDNA); M, 500 bp DNA ladder (Invitrogen).

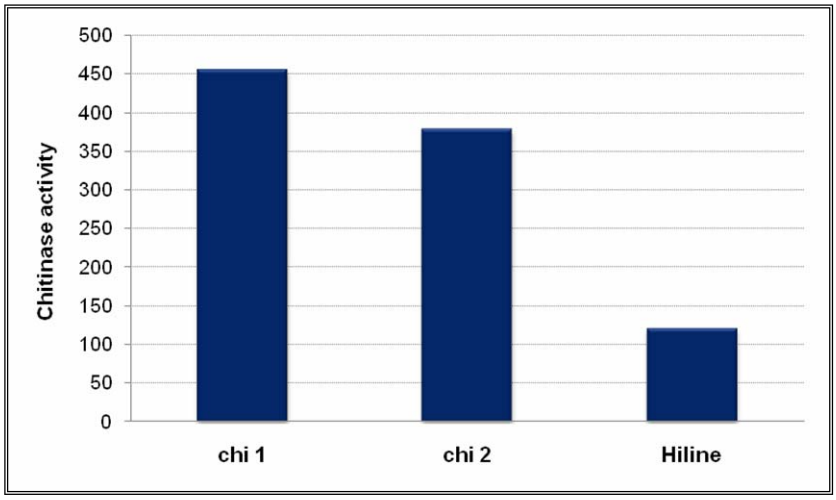

Fig. (7): Chitinase activity (μM GluNAc min^{-1} g^{-1}) in leaves of *chi*-transgenic wheat lines as well as non-transgenic cv. Hiline.