# TRANSGENIC EGYPTIAN WHEAT (*Triticum aestivum* L.) WITH *Arabidopsis NPR1* GENE *via* BIOLISTIC BOMBARDMENT

**A. H. FAHMY[1], K. EL MANGOURY[1], WALAA ABOU EL-WAFA[2], HODA M. S. BARAKAT[2], SOHEIR EL-SAEED EL-KHODARY[2], J. LI[3] AND S. MUTHUKRISHNAN[4]**

1. *Agriculture Genetic Engineering Research Institute, Agricultural Research Center, Egypt*
2. *Plant Science Department, Faculty of Science, Ain Shams University, Egypt*
3. *Department of Plant Pathology, Kansas State University, Manhattan, KS, USA*
4. *Department of Biochemistry, Kansas State University, Manhattan, KS, USA*

Wheat production needs to significantly increase to keep up with the growing demand (Bhalla, 2006). Unfortunately, this increase could not be achieved by plant breeding *via* conventional methods because of the limitation of available genetic pool. However, through genetic transformation technology this limitation can be overcome. Until date, the biolistic approach has been most successful in delivering foreign genes into wheat. Chawla *et al.* (1999) obtained transgenic wheat shoots following selection of cultures co-bombarded with a selectable herbicide resistance gene and gene of interest. Considerable progress has been made in the use of genetic engineering techniques for enhancing plant fungal disease resistance using this method. Chugh and Khurana (2003) bombarded two bread wheat (*Triticum aestivum* L.) varieties with pAHC25 (carries *bar* and *gus* genes). They recorded that transformation frequency of CPAN1676 variety was 4.4% while that for PBW343 was 3.6%. Fahmy *et al.* (2007) co-bombarded immature embryo-derived calli of wheat with two plasmids i.e. pK-Dy10 harboring *Dy10* gene and pAHC25 plasmid containing *gus* and *bar* genes. They detected *gus* expression using a histochemical gus assay. Ding *et al.* (2009) pointed to that the *gus* gene could be used as a screen able marker gene, to assess the performance of DNA delivery in transformation of wheat and it was efficient and practical.

Plant pathogens are actual threats to worldwide agriculture. Significantly, fungal attacks cause unlimited yield losses for most of agricultural species. Fungi cause more than 70% of diseases in major crops (Agrios, 2005). Numerous of fungal pathogens that infect wheat plants are responsible for considerable yield loss. Chemical fungicide applications which are not economical and detrimental to the environment are generally used in management of fungal plant diseases. A method for protecting plants against diseases is constructing and employing pathogen-resistant cultivars. Therefore, biotechnology integrated with traditional agricultural practices will be the backbone for

sustainable agriculture. Several classes of genes have been used in genetic engineering approaches to develop resistance in wheat to fungal pathogens utilized genes such as chitinase and *Arabidopsis thaliana NPR1* genmimi. Exogenously applied jasmonic acid enhanced *Arabidopsis thaliana* resistance to many necrotrophic fungi (Thomma *et al*., 2000). Although JA and At*NPR1* cooperate, providing a clue to the activation of induced systemic resistance (ISR) (Koornneef and Pieterse, 2008). *Arabidopsis* engages SA signaling *via* At*NPR1* to govern diseases. Genetic engineering technology's future potential remains promising given the progress towards enhancing resistance for a wide range of pathogens in several plant species (Punja, 2006). Chern *et al.* (2001) transformed the *Arabidopsis NPR1* gene in rice and this represents the first demonstration that *Arabidopsis NPR1* gene can enhance disease resistance in a monocot plant. They stated that these results also suggest that monocot plants include a pathway controlling At*NPR1*-mediated resistance. Chern *et al.* (2005) stated that *Arabidopsis thaliana NPR1* gene is a key regulator of systemic acquired resistance (SAR), which confers lasting broad-spectrum resistance. Also, they confirmed that transformation of *Arabidopsis NPR1* gene in rice resulted an enhanced resistance to *Xanthomonas oryzae* pv. *Oryzae* pathogen. Jiang *et al.* (2006) used optimized parameters for transforming into immature embryos of wheat cv. Yangmai 158 with genes for pathogenesis-related proteins and obtained forty-nine plants resistant to bialaphos. Two plants were identified to be positive by PCR. Makandar *et al.* (2006) showed that the *Arabidopsis thaliana NPR1* gene (At*NPR1*) regulates the activation of systemic acquired resistance (SAR), when expressed in the wheat cv. Bobwhite. Xing *et al.* (2008) transformed immature embryo-derived calli of common wheat *via* particle bombardment and after two rounds of bialaphos selection and regeneration; herbicide-resistant plants were obtained. Makandar *et al.* (2010) enhanced disease resistance in transgenic wheat and *Arabidopsis* plants that express the At*NPR1* gene. They declared that SA signaling through At*NPR1* is important for limiting the severity of diseases. On other hand, Fahmy *et al.* (2013) produced transgenic wheat developed using *chi* gene *via* particle bombardment of immature embryos and confirmed using polymerase chain reaction analysis that *chi* gene was transferred into 11 transformants with a transformation frequency of 1.8%. Recently, Silva *et al.* (2015) achieved genetic transformation in strawberry with the At*NPR1* gene and stated that the At*NPR1* gene confers broad-spectrum disease resistance when transformed. Introduction of disease resistance genes into wheat genome is considered necessary, especially when wheat production in Egypt is decreased severely due to infection with a wide range of fungal pathogens. This objective could be achieved *via* genetic transformation techniques. The goal of the current research is to improve Egyptian wheat (*Triticum aestivum* L.) cultivar cv. Giza 164 with improved disease resistance by introduction of At*NPR1* gene, which has a major

role in plant defense using recent biotechnology tools. Here, we reported a reliable transformation of the Egyptian wheat cv. Giza 164 with At*NPR1* gene using genetic transformation *via* microprojectile bombardment.

## MATERIALS AND METHODS

### *Sterilization and isolation of explants*

Grains of Egyptian wheat cultivar cv. Giza 164 were obtained from the Wheat Department, Field Crops Institute, Agricultural Research Center, Ministry of Agriculture and Land Reclamation, Egypt. Immature caryopses were removed from spikelets under aseptic conditions in a laminar flow hood. Grains were surface sterilized with 20% (v/v) commercial Clorox® (5.25% sodium hypochlorite) supplemented with few drops of Tween 20, and then rinsed five times with sterile double distilled $H_2O$. After that, sterile immature embryos (1-1.25 mm) were aseptically dissected.

### *Culturing of explants*

Immature embryos with scutellum side up were cultured on callus induction medium (CIM) (Weeks *et al*., 1993) containing Murashige and Skoog salts (Murashige and Skoog, 1962) supplemented with 0.15 g of L-Asparagine, 0.1 g of myo-inositol, 20 g sucrose, 2.5 g Phytagel as a solidifying agent and 2.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) as an auxin source. Then embryos were incubated in dark for five days before bombardment with gold particles coated with DNA.

### *Biolistic gene transformation*

Twenty five immature embryo-derived calli after one week from culturing were transferred to CIMS osmotic medium (CIM medium supplemented with 0.4 M sorbitol) for four hours before bombardment. Embryo-derived calli were placed in the center of a Petri dish (15x100 mm) and co-bombarded using the helium driven Biolistic Delivery system (Bio-Rad) with l.0 µ Golden microcarriers coated with the two plasmids; the first plasmid is pAHC25 containing the *gus* gene *(uidA)* and the *bar* gene that confers tolerance to phosphinothricin (ppt) both under control of the maize ubiquitin promoter (Fig. 1A), while the second plasmid pJS406 containing At*NPR1* gene (Fig. 1B). Target immature embryo-derived calli were bombarded with rupture disc strength of 1100 psi. Calli were kept for additional 16 hr on the same osmotic medium (CIMS) after bombardment. Calli were then transferred to CIM medium for an additional five days of recovery.

### *Histochemical analysis*

Histochemical staining analysis was conducted after seven days from bombardment to study *gus* gene expression according to Jefferson *et al.* (1987). Gus activity was assayed using callus tissues samples in *gus* buffer (Daniell *et al*., 1991). Then Petri dishes with calli were incubated at 37°C overnight. Transient *gus* gene expression was photographed under the microscope.

***Selection and regeneration of transformed calli***

After the five days of recovery period calli were transferred to CIMB selection medium (CIM medium with 3 mg/l bialaphos) for two subcultures every three weeks. Survived embryogenic calli after the six weeks, were then placed on MSRB regeneration selection medium (Murashige and Skoog's medium supplemented with 3 mg/l bialaphos and 0.125 mg/1 TDZ) for two weeks at 25°C and a 16 hr photoperiod. Green regenerated shoots were transferred for two more weeks onto FMSB rooting selection medium (Murashige and Skoog medium supplemented with 1 mg/l bialaphos). Finally, healthy rooted plantlets were transferred to soil pots in controlled growth chamber for acclimation and then transferred to greenhouse.

***Leaf painting assay***

A freshly prepared aqueous solution of 0.2% glufosinate-ammonium herbicide was applied on the mid-lamina portion (about 2.5 cm long) of the second/third youngest leaf according to Schroeder *et al.* (1993) for monitoring *bar* gene expression.

***DNA isolation and Polymerase Chain Reaction (PCR)***

Total genomic DNA of putative transgenic plants and controls (non-transgenic) were isolated using DNeasy Plant Mini Kit (Qiagen, Germany). Then, presence of transgenes (*gus*, *bar* and At*NPR1* genes) were detected, initially, by polymerase chain reaction (PCR). Specific oligonucleotide primers for *gus*, *bar* and At*NPR1* genes were used (Table 1). DNA amplifications were performed in a thermal cycler using initial denaturation at 94°C for 5 min, followed by 30 cycles of 1 min at 94°C, 1 min at 58°C (for *gus* gene)/62°C (for *bar* gene)/57°C (for At*NPR1* gene) and 2 min at 72°C. One additional complete extension cycle was performed for 10 min at 72°C.

***Dot blot hybridization analysis***

To confirm At*NPR1* gene integration in the genomic DNA of transgenic plants, Dot-Blot hybridization analysis was conducted. DNA from transgenic and non-transgenic plants (negative control) as well as the transformation vector pJS406 (positive control) were used in this experiment. DNAs were denatured by heating at 95°C for 10 min., and then directly spotted onto nitrocellulose membrane followed by a fixation step by exposure to UV waves for 1 min. The plasmid pJS406 was used as probe. Labeling and detection procedure was accomplished using DNA Labeling and Biotin Chromogenic Detection Kit (Thermo Scientific) by random primed DNA labeling with digoxigenin-dUTP and detection of hybrids by enzyme immunoassay according to the kit instructions manual.

## RESULTS AND DISCUSSION

Fungal and bacterial diseases are serious and destructive diseases of wheat.

In severe epidemic years in Egypt, diseases may reach 50-100% incidence with yield reduction from 10-40%. Also, contamination of harvested grain by toxins, reduce the grain value for food or feed consumption. Genetic variation for fungal and bacterial resistance is low in wheat germplasm pools and inadequate to provide resistance through traditional breeding approaches. Therefore, genetic engineering will provide an additional approach to enhance disease resistance. The At*NPR1* gene plays a crucial role in systemic acquired resistance in plants.

Here, we report a successfully genetic transformation of the At*NPR1* gene into the Egyptian wheat cultivar cv. Giza 164. In this experiment, plasmids pAHC25 and pJS406 were co-transformed into immature embryo derived calli which was used as a target tissue. A total of 625 calli were subjected to microprojectile bombardment at the same conditions as described for the transformation process. In a separate experiment, histochemical staining analysis of bombarded calli was achieved subsequent to the bombardment in order to study the *gus* gene expression which was monitored using a microscope and photographed (Fig. 2).

Following a recovery period of five days following bombardment, calli were sub-cultured twice at three weeks intervals onto selection medium (CIMB medium, Fig. 3). The total number of calli that survived after selection phase onto CIMB medium was 161 (Table 2 and Fig. 4).

The surviving calli (161) on CIMB selection medium were sub-cultured for more two weeks on regeneration selection medium (MSRB). Out of the 161 surviving calli, only 63 calli regenerated shoots on CIMB selection medium. Regenerated shoots were then transferred to FMSB rooting selection medium and maintained for two more weeks. Healthy rooted plantlets numbering 105 plantlets were established in the FMSB rooting selection medium, and were then transferred into soil pots and transferred to the controlled growth chamber (Conviron®) for acclimatization (Table 2 and Fig. 4 ).

During the acclimatization process, a total number of 48 plants survived and set seeds in a bio-containment greenhouse (Fig. 5B). All produced plants were fertile and set seeds which indicated that the insertion of the transgenes did not affect the fertility of plants or seed setting. Leaf painting assay was conducted to examine the expression of the *bar* gene in these plants as mentioned before (Table 2 and Fig. 6).

Putative transgenic plants were subjected to molecular analysis to verify the integration of foreign genes (*gus*, *bar* and At*NPR1*) into plant genome by the PCR analysis and Dot-Blot hybridization analysis. Total genomic DNA of putative transgenic plants and control (non-transgenic) were isolated using DNeasy Plant Mini Kit (Qiagen, Germany). The DNA was then subjected to the PCR analysis for *gus*, *bar* and At*NPR1* genes using appropriate primer pairs for each gene.

PCR products were then separated on 1% agarose gel. PCR results revealed products of the expected sizes for all transgenes; 1050 bp for *gus* gene, 443 bp for *bar* gene and 439 bp for At*NPR1* gene, as shown in Fig. (7). PCR analysis confirmed that 12, 14 and 12 plant's transgene insertion were positive for *gus*, *bar* and At*NPR1* genes, respectively (Table 3).

To confirm the integration of the At*NPR1* gene, Dot-Blot hybridization analysis was used and confirmed the integration of the At*NPR1* gene in all the twelve positive PCR plants (Fig. 8). PCR and Dot-blot analysis results indicated that the frequency gene transformation process of At*NPR1* gene was 1.9%, while transformation frequency of both *gus* and *bar* genes were 1.9% and 2.2%, respectively (Table 2 and Fig. 8). Similar results were obtained by Chern *et al*. (2001) whom transformed rice plants with At*NPR1* gene and confirmed At*NPR1* gene integration using polymerase chain reaction (PCR) and Dot-Blot hybridization. Also, Makandar *et al.* (2006) produced At*NPR1* transgenic wheat plants by co-transforming embryogenic calli with plasmid pJS406 expressing At*NPR1 gene* and plasmid pAHC20 that expresses the *bar* gene *via* particle bombardment and utilized PCR to monitor the presence of At*NPR1* and *bar* genes.

At*NPR1* gene is a key regulator of systemic acquired resistance (SAR) and can enhance disease resistance in plants such as *Arabidopsis* and monocot plants (Friedrich *et al.*, 2001; Chern *et al*., 2005). At*NPR1* gene confers lasting broad-spectrum disease resistance to viral, bacterial and fungal pathogens (Chern *et al*., 2001). Also, At*NPR1* gene is as a positive regulator of the salicylic acid (SA). Salicylic acid signaling through At*NPR1* gene is important for limiting severity of fungal disease (Loake and Grant, 2007; Bari and Jones, 2009). In wheat, recently, it was proved that At*NPR1* is involved in SA signaling and that it plays a key role in both SAR signaling and disease resistance (Makandar *et al*., 2012).

At*NPR1* gene promotes the cross-talk between the SA signaling pathway and the jasmonic acid (JA) signaling pathway. Moreover, the antagonistic effect of SA on JA signaling requires At*NPR1* (Spoel *et al*., 2003 and 2007; Liu *et al*., 2005). This is supported by the work of Makander *et al*. (2010) who reported that JA signaling promotes disease by attenuating the activation of SA signaling. Jasmonic acid (JA) also, contributes to defense presumably during the later stages of infection. Together SA and JA-dependent defenses could provide maximal protection against fungal disease.

Here we report for the first time, transformation of At*NPR1* gene in our Egyptian cultivar cv. Giza 164. The objective of this study is the developing of wheat cultivar cv. Giza 164 in plant defenses using tools of modern biotechnology *via* transformation with the At*NPR1* gene, which will be a valuable alternative to the exogenous application of SAR-inducing chemicals, and furthermore will

play a significant and a superior role in improving Egyptian wheat broad-spectrum disease resistance, including fungal, bacterial and viral resistance. Since, transgenic improved disease resistance Egyptian wheat plants production is a valuable component in a disease management program; it is desirable to transform wheat with genes that are competent for conferring broad and durable fungal resistance even if this resistance is partial.

In conclusion, genetic transformation of Egyptian wheat cv. Giza 164 with At*NPR1* gene paves the way for further studies on gene expression and for achieving both biotic and abiotic stress tolerance. Moreover, leading to increased wheat production, and also filling the huge gap between consumption and production of wheat in Egypt.

## SUMMARY

At*NPR1* gene plays a crucial role in biotic resistance in plants. Immature embryo derived calli of wheat cv. Giza 164 were co-transformed with the plasmid pAHC25 containing *bar* and *gus* genes and the plasmid pJS406 containing At*NPR1* gene *via* Biolistic bombardment. The produced calli were subcultured on bialaphos containing medium (CIMB). Surviving calli were then regenerated on selection medium (MSRB) to produce green regenerated shoots, which were then transferred onto rooting/selection medium (FMSB). Regenerated rooted plantlets were transferred to soil pots in the control growth chamber for acclimatization. Only 48 plants succeeded in acclimatization and reached seed setting in biocontainment greenhouse. Transgene integration was confirmed by PCR and dot-blot analyses. The overall transformation frequency was 1.9%.

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Table (1): Specific PCR primers for *gus*, *bar* and At*NPR1* genes.

| Gene | Sequence | Fragment size (bp) |
|---|---|---|
| *Gus R* | 5`-AGTGTACGTATCACCGTTTGTGTGAAC-3` | 1050 |
| *Gus F* | 5`-ATCGCCGCTTTGGACATACCATCCGTA-3` | |
| *Bar R* | 5`-CAGATCTCGGTGACGGGCAGGC-3` | 443 |
| *Bar F* | 5`-CCGTACCGAGCCGCAGGAAC-3` | |
| At*NPR1 R* | 5`-CATGATCGCAAAACAAGCCACTAT-3` | 439 |
| At*NPR1 F* | 5`-CGAACAGCGCGGGAAGAAT-3` | |

Table (2): Transformation characteristics of Egyptian wheat cv. Giza 164 with At*NPR*1 gene representing numbers of bombarded calli, surviving calli, shooted calli, regenerated plantlets on selection, acclimatized transgenic plantlets.

| Number of bombarded calli on selection | Number of surviving calli on selection | Number of shooted calli on selection | Number of regenerated plantlets on selection | Number of acclimatized plantlets |
|---|---|---|---|---|
| 625 | 161 | 63 | 105 | 48 |

Table (3): Transformation characteristics of Egyptian wheat cultivar Giza 164 representing *Gus*, *bar* and At*NPR1* genes positive PCR plants and At*NPR1* gene Dot Blot positive plants.

| | *Gus* gene +ve PCR plants | *bar* gene +ve PCR plants | At*NPR1* gene +ve PCR plants | At*NPR1* gene Dot Blot +ve plants |
|---|---|---|---|---|
| Transformed Plants Number | 12 | 14 | 12 | 12 |
| Transformation Efficiency% | 1.9 | 2.2 | 1.9 | 1.9 |

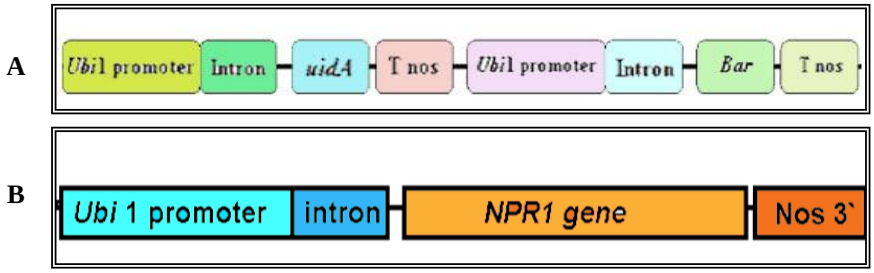



Fig. (1): Schematic representation of the two plasmids (A) pAHC25 plasmid and (B) pJS406 plasmid.

Fig. (2): Transient expression of the marker gene (*gus*) in wheat calli.

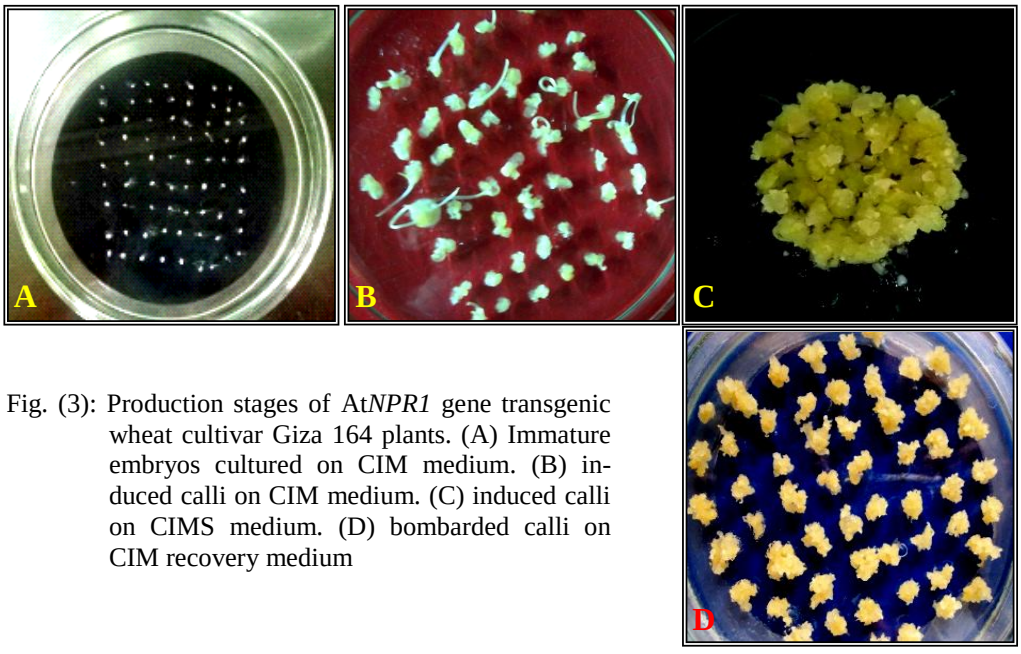
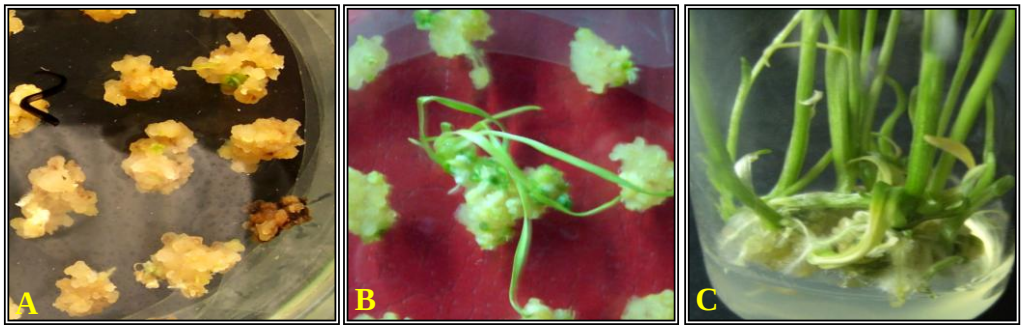



Fig. (3): Production stages of At*NPR1* gene transgenic wheat cultivar Giza 164 plants. (A) Immature embryos cultured on CIM medium. (B) induced calli on CIM medium. (C) induced calli on CIMS medium. (D) bombarded calli on CIM recovery medium



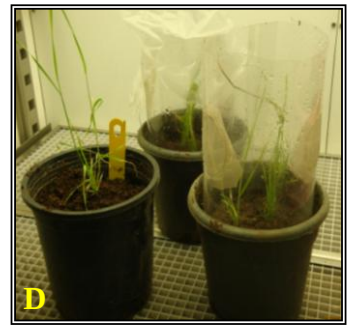




Fig. (4): Production stages of At*NPR1* gene transgenic wheat cultivar Giza 164 plants. (A) non-transformed calli on CIMB selection medium. (B) bialaphos tolerant calli (putative transgenic) on MSRB regeneration selection medium showing putative transgenic shoots. (C) putative transgenic plantlets on FMSB selection rooting medium. (D) putative acclimatized transgenic plants incubated in control growth chamber (Conviron®).

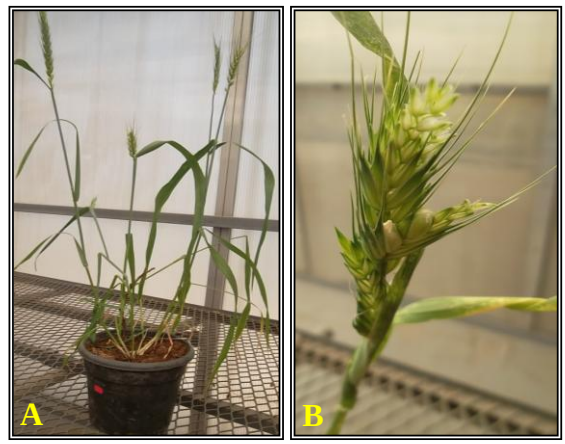



Fig. (5): Production stages of At*NPR1* gene transgenic wheat cultivar Giza 164 plants: (A) Fertile transgenic plants grown on soil pot in bio-containment greenhouse. (B) Transgenic spike showing seeds filling.

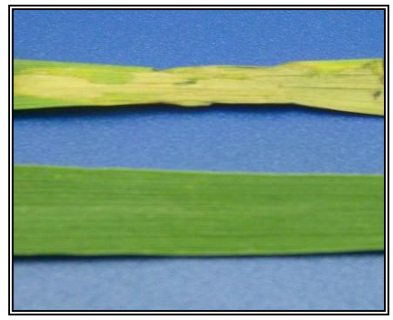


Fig. (6): Leaf painting assay examining the *bar* gene expression in transgenic At*NPR1* plants: the upper leaf from untransformed plant (control) showing leaf necrosis. The lower leaf from transgenic plant shows leaf resistance to 0.2% glufosinate-ammonium herbicide application.

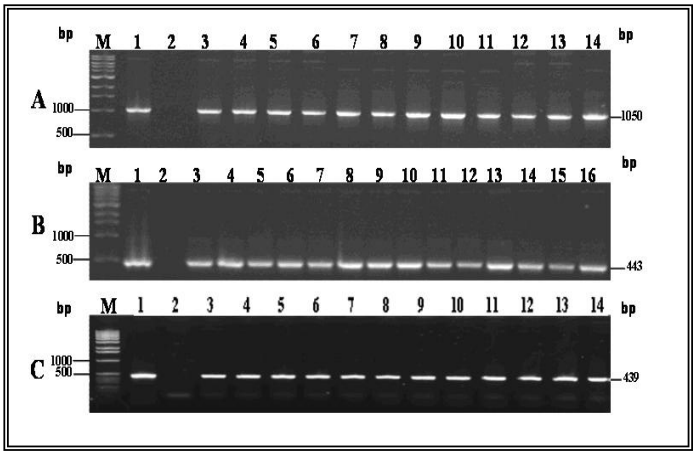



Fig. (7): PCR analysis. (A) Amplification product of *gus* gene (1050 bp). (B) Amplification product of *bar* gene (433 bp). (C) Amplification product of At*NPR1* gene (439 bp). Lane M is DNA marker (1 kb ladder). Lane 1: positive control (plasmid), Lane 2: non-transformed wheat cv. G164 (negative control). Other Lanes are the transgenic wheat plants.

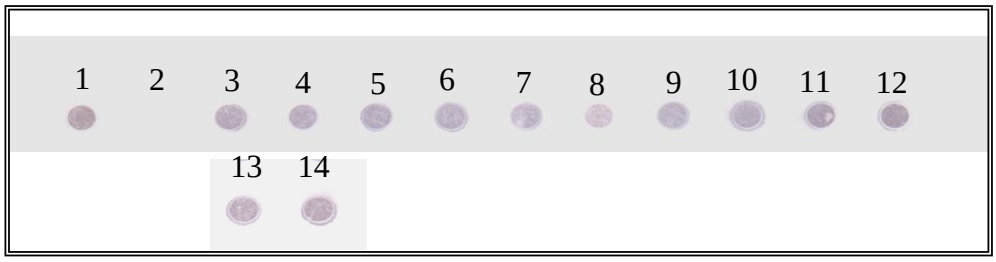



Fig. (8): Dot blot analysis of transgenic wheat cv. G164 plants; Dot 1 is pJS406 plasmid (positive control), Dot 2 is non-transformed wheat cv. G164 (negative control) and Dots 3-14 are the 12 wheat At*NPR1* transgenic plants.