IV. RESULTS AND DISCUSSION

Abiotic stresses initiate the production of reactive oxygen species (ROS) such as superoxide anions, hydroxyl radicals and hydrogen peroxide **(Millar *et al.*, 2001)**. These molecules can react with most cellular macromolecules and ultimately leading to oxidative damage affecting the yield and quality of plant products **(Allen *et al.*, 1999)**. The principle aim of the present study was the improvement of drought and salt stress tolerance of Egyptian hexaploid wheat (*Triticum aestivum* L.) cultivar; Giza 164, *via* genetic transformation with *Le-GST* gene which encodes the enzyme glutathione s-transferase. To achieve this goal, the plant expression vector pLe-GST/bar was introduced into wheat immature embryo derived-calli using micro-projectile bombardment protocol.

1. Plant expression vector construction

The plasmid pLe-GST/bar harbors *Le-GST* gene encoding glutathione s-transferase enzyme and *bar* gene encoding the enzyme phosphinothricin acetyl transferase (PAT). Both genes are driven by cauliflower mosaic virus *(CaMV) 35S* promoter and terminated by *NOS* terminator.

To obtain pLe-GST/bar, an intermediate construction step was done. HindIII fragment of *bar* cassette, obtained from pAB6, was integrated into HindIII site of plasmid pAM35S/Le-GST-T3 (Fig. 1). To proof ligation of *bar* gene cassette, plasmid DNA minipreps obtained from bacteria transformed with pLe-GST/bar plasmid, were checked by HindIII digestion. Colonies with the correct DNA size were expected to give 4.70 kb and 2.09 kb (lanes 2 and 3, Fig. 2).

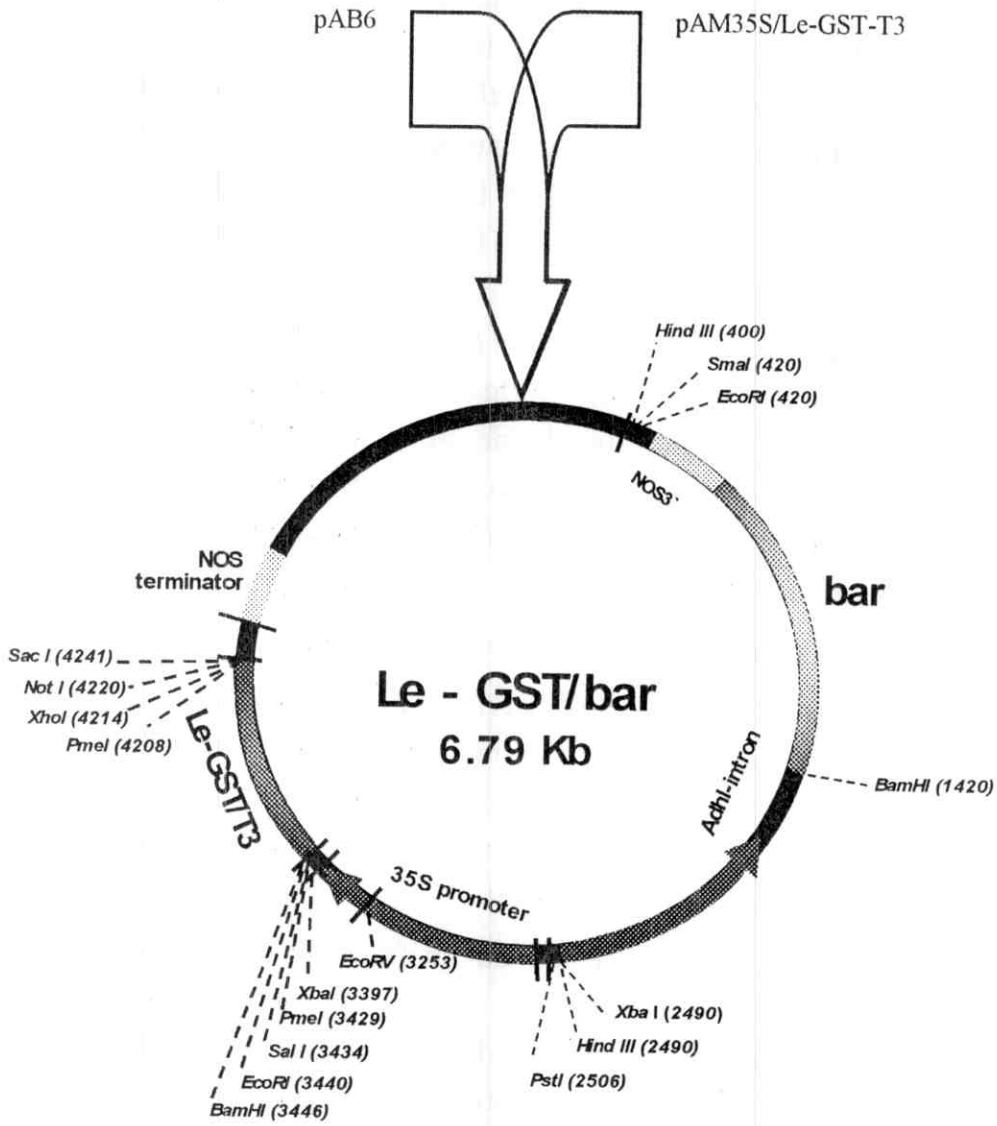

Fig. (1): Restriction maps of plasmids pAB6, pAM35S/Le-GST-T3 and pLe-GST/bar.

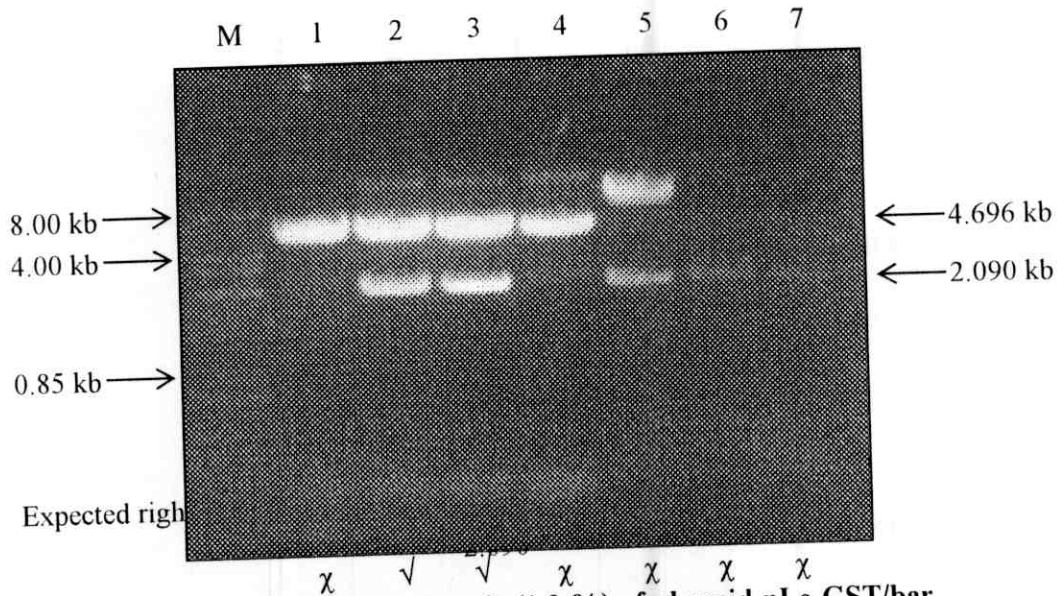

Fig. (2): Agarose gel electrophoresis (1.2 %) of plasmid pLe-GST/bar minipreps of different clones (lanes 1-7) digested with HindIII restriction enzyme along with the expected restriction fragment sizes (kb). M refers to AGE-marker I.

Selected colonies were checked for *bar* gene cassette orientation by EcoRI digestion. Right colonies were chosen based on promoter-promoter orientation. The correct DNA size was expected to give 4.70 kb and 2.09 kb (lane 3, Fig. 3).

Glutathione s-transferases (GSTs) are dimeric enzymes catalyzing the conjugation of the tripeptide glutathione to electrophelic compounds. They are involved in the detoxification of the reactive oxygen species (ROS) **(Dixon *et al.*, 1999)**. **Dixon *et al.* (2003)** reported that the tripeptide glutathione (GSH) (γL-glutamyl-L-cysteinyl-glycine) used as a substrate or coenzyme for GSTs, as they are predominantly expressed in the cytosol and protect cells from oxidative stress.

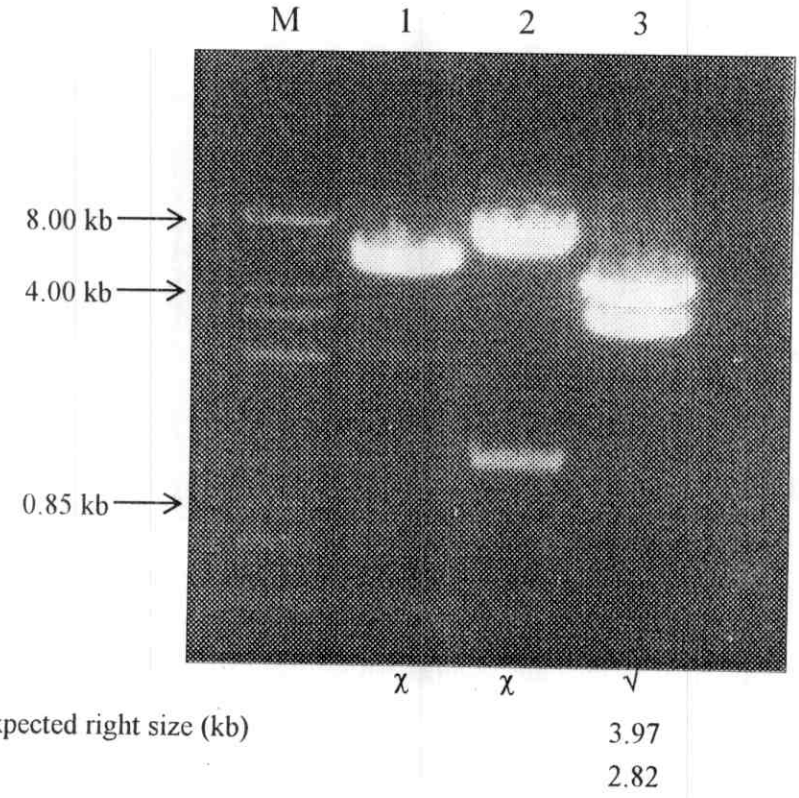

Fig. (3): Agarose gel electrophoresis (1.2 %) of plasmid pLe-GST/bar minipreps of different clones (lanes 1-3) digested with EcoRI restriction enzyme along with the expected restriction fragment sizes (kb). M refers to AGE-marker I.

The second gene present in the pLe-GST/bar vector is the *bar* gene. This gene is involved in the biosynthetic pathway of herbicide bialaphos, (commercial name, basta). Bialaphos consists of phosphinothricin (PPT), which is an irreversible inhibitor of glutamine synthetase (glutamate-ammonia ligase), and two L-alanine residues. Glufosinate ammonium, the chemically synthesized PPT, and bialaphos were used as non selective herbicides. The *bar* gene codes phosphinothericin acetyl transferase (PPT), which converts PPT with high affinity into an inactive

nonherbicidal acetylated form by transferring the acetyl group from acetyl-coA on the free amino group of PPT **(D'Halluim *et al.*, 1992)**. The *bar* gene has been successfully used as a selectable marker in wheat transformation using bialaphos as a selective agent **(Bahieldin *et al.*, 2000; Shawky, 2000; Eissa, 2001; Saleh, 2002)** resulting in a considerable rate of transformation efficiency when using the herbicide at an appropriate concentration.

The *CaMV 35S* promoter had the advantage of driving a constitutive activity of the gene downstream it in several plant species and was used to express a number of foreign genes in transgenic plants **(Takumi and Shimada, 1995)**. In pLe-GST/bar plasmid both genes were driven by *CaMV 35S*. In the *bar* gene cassette *CaMV35S* promoter was fused to an alcohol dehydrogenase *(Adh)* intron downstream the promoter. It was found that introns have a strong enhancing effect on gene expression when inserted down stream a promoter sequence within the transcriptional frame **(Sinibaldi and Mettler, 1992).**

2. Wheat transformation using immature embryos-derived calli

Wheat transformation using biolistic bombardment protocol was established by **Weeks *et al.* (1993)** and modified for Egyptian wheat by **Bahieldin *et al.* (2000)** and **Shawky (2000).** Many genes of economic values have recently been transferred to wheat. Examples of these genes are the rice chitenase gene for fungal resistance **(Chen *et al.*, 1998b)**, *HVA1* for drought tolerance **(Sivamani *et al.*; 2000, Bahieldin *et al.*, 2005)** and cDNA encoding the maize GST subunit GST-27 **(Milligan *et al.*, 2001)**.

During the course of regenerating transformed wheat cells (Fig. 4) the following steps were considered:

2.1. Callus induction and transformation

In this study, *Le-GST* gene was introduced to the genomic background of wheat calli derived from immature embryos and these calli have a potential ability to give fertile plants with normal spikes. This methodology opens up the possibility of introducing novel genes that may induce resistance to disease and abiotic stress.

In the present study, immature embryos were used for callus induction after 15 days from anthesis. Immature embryos are found to be the best starting material for obtaining embryogenic callus of wheat **(Maddock 1985; Vasil and Anderson, 1997).** The induction frequency of callus from immature embryos is generally quite high **(Maddock *et al.*, 1993; He *et al.*, 1986; 1988).** There were a strong correlation between transformation frequency and the age of wheat donor plants according to **Pastori *et al.* (2001)**.

In this study, embryos were cultured on callus induction medium (Fig 4a) with scutellum side up for five days according to **Bahieldin *et al.* (2000),** who found that embryos of American wheat cv Hi-Line placed with scutellum side exposed to the medium will not undergo cell regeneration in which cell division rate will be low. **Shawky (2000), Eissa (2001) and Saleh (2002)** used the same orientation in Egyptian cultivar Giza 164, the same cultivar used in the present study. This disagrees with the results of **Weeks *et al.* (1993)**, who cultured embryos with

scutellum side exposed to the medium and obtained high rate of transformation.

Also, the concentration of 2,4-D was an important factor which has an effective role in quality of callus. In this study, a concentration of 1.5 mg/L 2,4-D was used **(Bahieldin *et al.*, 2000; Shawky, 2000; Eissa, 2001; Saleh, 2002).** These results differed from those of **Ozias and Vasil (1983) and Chen *et al.*, (1998a)** who found that the optimum 2,4-D concentration was 2 mg/L for callus induction. While, **Alock *et al.* (1999)** used 2.5 mg/L 2,4-D for wheat callus induction. In contrast, **Barro *et al.* (1998)** found that the presence of picloram results in higher transformation efficiencies than 2,4-D.

The preculture of wheat embryos on TW callus induction medium for 5 days before bombardment allow selecting cell divided embryos with devided cells. However, transgenic wheat plants of both spring and winter cultivars were obtained from immature embryos bombarded after culture for more than five days, but not from those less than five days **(Takumi and Shimada, 1996)**. The preculture of wheat embryos on callus induction medium, TW **(Weeks *et al.*, 1993)** for 6 days before bombardment is important to select embryos active in cell division and allows for the exclusion of watery calli with no cell clusters (Fig. 4b).

The calli were placed on osmotic medium containing mannitol (0.4 M) for at least four hours before bombardment and left overnight after bombardment (Fig. 4c).The osmotic treatment before and after bombardment increases transformation efficiency **(Vain *et al.*, 1993; Zhou *et al.*, 1999)**.

Also, it allows the foreign DNA to be integrated in the bombarded cell genome more efficiently **(Altpeter *et al.*, 1996).** For the same purpose, **Dong *et al.* (2002)** used sucrose (0.4 M) instead of mannitol for osmotic pretreatment of wheat embryo calli.

In the present study, all calli were bombarded, either once or twice, with a gas pressure of 1100 psi at the shortest distance (6 cm). **Shawky (2000),** found that t 1100 psi is the best pressure for Egyptian cultivar Giza 164, since higher pressure (i.e., 1550 psi) showed higher mechanical damage of the transformed cells leading to a decrease in regeneration frequency.

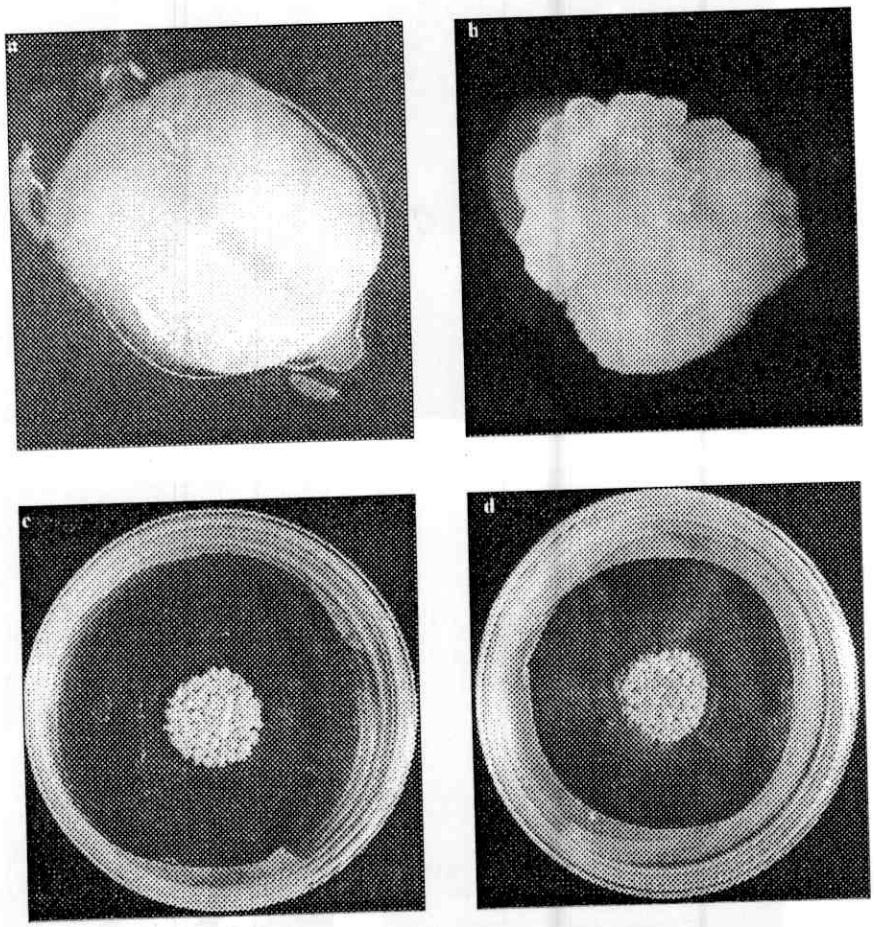

Fig. (4): Performance of different tissues before and after biolistic bombardment of the wheat cultivar Giza 164.

a. Immature embryo
b. Induced calli
c. Calli before bombardment
d. Bombarded calli

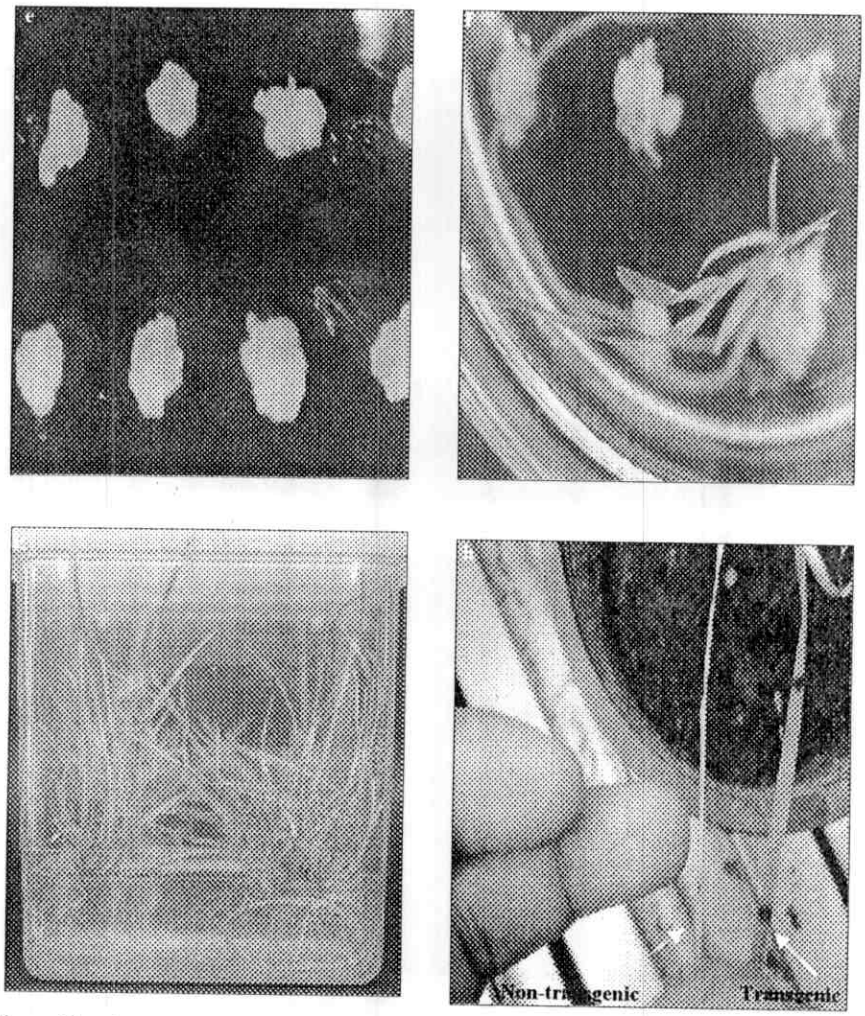

Fig. (4): Cont.

e. Callus growth performance
f. Regenerated palntlets
g. Rooted plantlets
h. Acclimatized and leaf painted plantlets

This agreed with **Chen *et al.* (1998a)**, who used gas pressure of 1100 psi to transform Bobwhite cultivar. However, this protocol is different from that published by **Bahieldin *et al.* (2000),** who used a gas pressure of 1550 psi to bombard the American wheat Hi-Line cultivar and had no problem with transformation and regeneration efficiencies.

The bombarded calli were cultured on TW medium without selection for five days. This recovery period, after bombardment, allows the bombarded cells to recover from mechanical damage caused by accelerated gold particles. Also, this period gave the transformed cells enough time to begin the expression of introduced selectable marker gene which allows for reliable selection **(Eissa, 2001)**. After the recovery period time it was found that induction of callus for four weeks was enough to select and maintain the regenerability of the transformed cells **(Bahieldin *et al.*, 2000; Eissa, 2001).**

In the present study, two bialaphos concentrations (0 and 2 mg/L) were used and two transgenic plants were obtained from no selection experiment (0 mg/L bialaphos). This agreed with **Bahieldin *et al.* (2000),** who used two bialaphos concentrations of (0 and 5 mg/L) and got three independent transgenics from no selection experiment. On the other hand, **Zhou *et al.* (1995)** used 2 mg/L bialaphos to select bombarded Bobwhite cultivar embryo calli. **Sivamani *et al.* (2000)** and **Zhang *et al.* (2000)** used 5 mg/L bialaphos for producing transgenic wheat plants. Also, **Eissa (2001)** and **Saleh (2002)** used 3 mg/L and obtained transgenic wheat plants with *sacB* and *Hal-2* genes, respectively. Furthermore, **Shawky (2000)** used 0, 3, 5 mg/L and got three

transgenics from 3 and 5 mg/L bialaphos during callus induction, while no transgenics were obtained from no selection experiment Also, **Chen *et al.* (1998a)** cultured the bombarded calli on medium containing 1 mg/L bialaphos for the first week, and then transferred calli on medium containing 3 mg/L for 2-3 weeks. However, bialaphos selection during callus induction greatly reduces the regenerability of wheat calli **(Ye *et al.*, 2001)**. This reduction increased with the higher bialaphos concentration (5 mg/L). On the other hand, the number of escapes increases with 0 mg/L bialaphos **(Eissa, 2001)**.

2.2. Regeneration of bombarded cells

In the present study, calli were plated on MS regeneration medium after two subcultures, 15 days each, on callus induction medium. The regeneration medium was supplemented with 0.2 mg/L TDZ phytohormone and 0 or 2 mg/L bialaphos to select the transgenic cells for two weeks (Fig. 4f). The two putative transgenic plants were obtained from 0 mg/L selection medium. This is in harmony with **Eissa (2001)**, who reported that the highest number of shoots was recorded when no selection pressure was extended during callus induction with one shot, while the lowest number was obtained from calli bombarded twice (2 shots) with 5 mg/L bialaphos.

Bahieldin *et al.* (2000) and **Saleh (2002)** used 1 mg/L bialaphos concentration during callus regeneration, while **Nehra *et al.* (1994)** and **Eissa (2001)** found that 3 mg/L bialaphos greatly affected the regenerability as it decreases embryoid formation of calli as they turned to brownish color when transferred to light and eventually died. The number of shoots at

the regeneration stage depended on the transformation parameters especially bialaphos concentration and the number of bombardments. **Eissa (2001)** found that the lowest number of regenerated plantlets was obtained from calli bombarded twice and treated with 5 mg/L bialaphos. While, other workers **(Nehra *et al.*, 1994; Chen *et al.*, 1998a; Zhang *et al.*, 2000)** used 5 mg/L bialaphos in regeneration medium to obtain transgenic plants and decrease the chance for escapes.

2.3. Rooting and acclimatization

In this study, the regenerated plants were transferred to hormone-free rooting medium containing 0 mg/L bialaphos for two weeks. This disagreed with **Eissa (2001) and Saleh (2002)** who used 3 mg/L bialaphos in rooting medium.

All rooted plantlets (Fig. 4g) were transferred to the greenhouse and acclimatized regardless of their rooting performance according to **Eissa (2001)** and **Saleh (2002)**. In contrast, **Chen *et al.* (1998a), Bahieldin *et al.* (2000)** and **Shawky (2000)** transferred only the well-rooted plantlets and they considered the root performance as an indication for transgenic plant. Our two transgenic plants which were obtained from this study have moderate root and good shoot systems.

3. Evaluation of putative transgenic plants

Herbicide resistance of putative transgenics was tested using the herbicide basta (1 g/L), by painting the middle green parts of the plant leaves from both sides. This procedure demonstrated the expression of *bar* gene, the selectable marker gene, in the genomic background of wheat. The transgenic plant leaves were resistant to the herbicide (stay green), while non-

transgenic as well as control plant leaves turned yellow and the cells died within three days (Fig. 4h). Two plants were shown to resist leaf painting and subsequently subjected to molecular analysis. Many authors **(Lörz *et al.*, 1997; Barcelo *et al.*, 1998; Witrzen *et al.*, 1998; Uze *et al.*, 1999; Zhou *et al.*, 1999; Zhang *et al.*, 2000; Eissa, 2001; Saleh, 2002)** used *bar* gene as a selectable marker gene in wheat transformation.

3.2. Molecular analysis

3.2.1. Molecular analysis on the structural level

3.2.1.1. Polymerase chain reaction (PCR) analysis and PCR/Southern

The two putative leaf painting-resistant transgenic plants and their progeny were screened by PCR and PCR/Southern to confirm the presence of the two genes, i.e., *bar* and *Le-GST*, in their genomic background. Figures (5) and (6) show PCR product corresponding to the expected sizes (400 bp) for partial-length *bar* gene. This result confirmed the presence of the *bar* gene as a selectable marker in the two putative transgenic plants (16 and 39) as well as in the six T_1 transgenic progeny (plants no. 16/2, 39/2, 39/3, 39/5, 39/6 and 39/7).

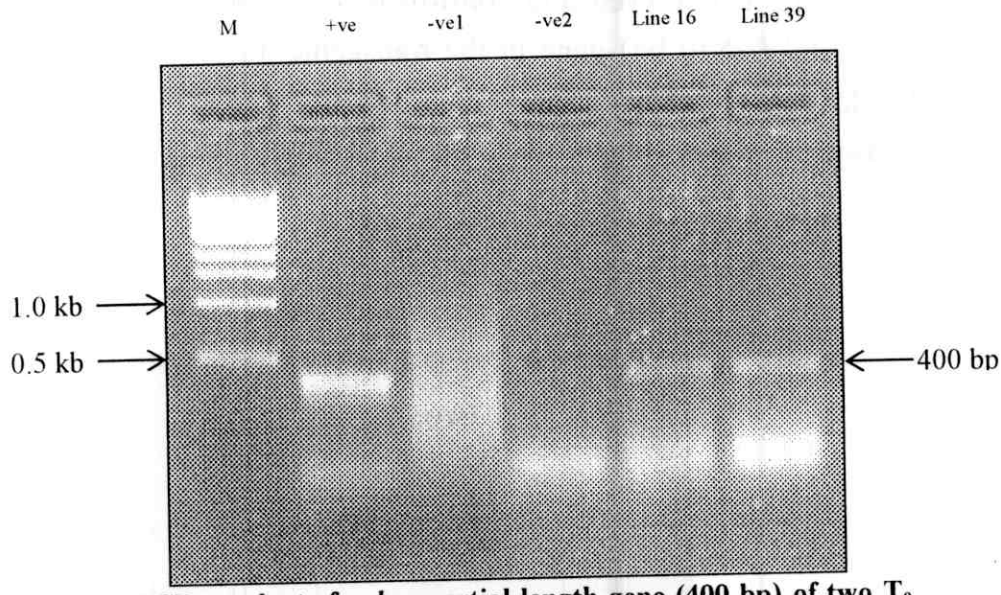

Fig. (5): PCR products for *bar* partial-length gene (400 bp) of two T_0 transgenic plants (16 and 39). +ve, pLe-GST/bar plasmid (positive control), –ve1, non-transgenic Giza 164. –ve2, no-DNA sample. M refers to 1kb DNA ladder.

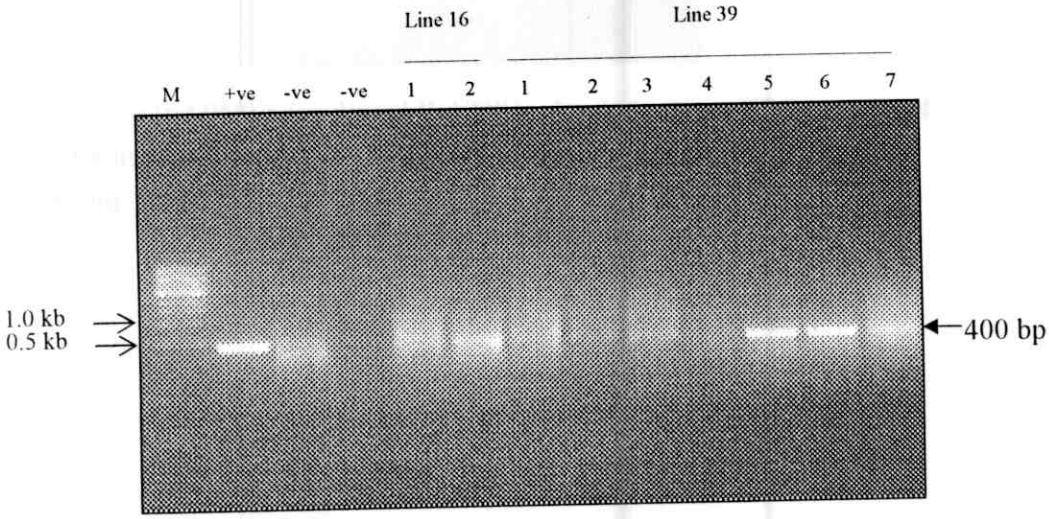

Fig. (6): PCR products for *bar* partial-length gene (400 bp) of nine T_1 transgenic plants (16/1-39/7). +ve, pLe-GST/bar plasmid positive control, –ve1, non-transgenic Giza 164. –ve2, no DNA sample. M refers to 1kb DNA ladder.

Fig. (7) showed and confirmed the expected band sizes for *Le-GST* (840 bp) gene in the transgenic T_0 plants (16, 39). While, Fig. (8) showed the PCR product of *Le-GST* gene in T_1 transgenic plants (plants no. 39/1, 39/2, 39/3, 39/4, 39/5, 39/6 and 39/7).

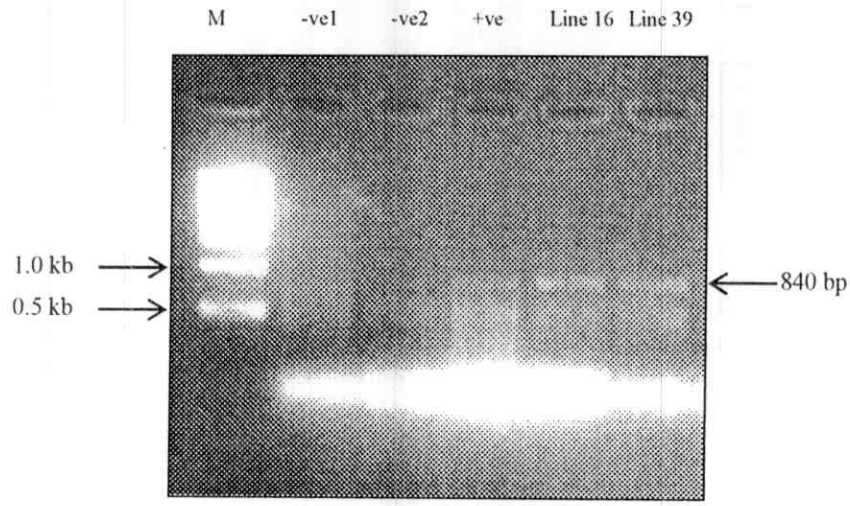

Fig. (7): PCR products for *Le-GST* full-length gene (840 bp) of the two T_0 transgenic plants, (16 and 39). –ve1, non-transgenic Giza 164. –ve2, no-DNA sample, +ve, pLe-GST/bar plasmid positive control. M refers to 1kb DNA ladder.

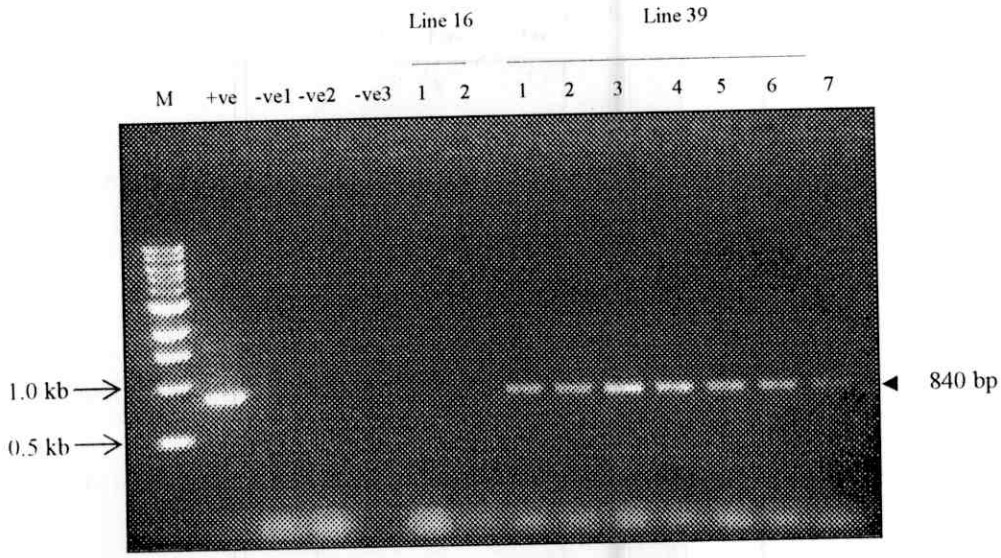

Fig. (8): PCR products for *Le-GST* full-length gene (840 bp) of nine T_1 transgenic plants (16/1-39/7). +ve, pLe-GST/bar plasmid positive control, –ve1, non-transgenic Giza 164. –ve2, no DNA sample. –ve 3, no primer sample. M refers to 1kb DNA ladder.

Also, PCR/Southern blotting was used to investigate the presence of pLe-GST/bar construct in the genome of the putative transgenic plants. PCR product using specific *bar* gene primers were hybridized with HindIII *bar* gene fragment obtained from pLe-GST. The results in Fig. (9) showed that the *bar* gene probe was hybridized with PCR product (400 bp) of transgenic plants as well as the two positive controls (pLe-GST/bar plasmid and previously obtained *HVA1*/*bar*-transgenic plant), while no hybridization occurred with the negative controls (non-transgenic plant and DNA template-free sample).

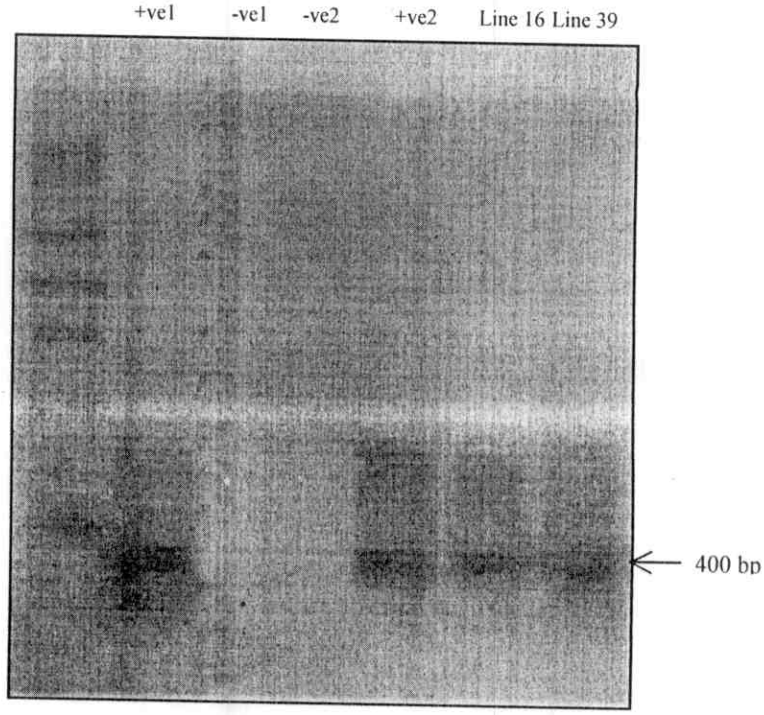

Fig. (9): R/Southern for the two T_0 transgenic plants (16 and 39) involving 400 bp PCR product of *bar* partial-length gene with *bar* gene cassette (2.09 Kb) of pLeGST used as a probe. +ve1, pLeGST, +ve2, *HVA1/bar* transgenic plant, 1 and 2, putative *Le-GST/bar* transgenic plants, -ve1, non-transgenic Giza 164, -ve2, no DNA sample.

Therefore, it is evidence that both *bar* and *Le-GST* genes were physically present in the genomic background of the transgenic plants. Using PCR as an indicator for the presence of foreign genes has been reported by many investigators **(Bahieldin *et al.*, 2000; Shawky, 2000; Eissa, 2001; Saleh, 2002; Archana *et al.*, 2003a; Linag *et al.*, 2003).** While, PCR/Southern analysis have been used by many workers to study the presence of the transgenes in the plant genomes **Takumi and Shimada (1996)** confirmed the integration and

inheritance of the *bar* gene as a selectable marker by the combination of PCR amplification and Southern analysis of T_0 and T_1 plants. **Saleh (2002)** used PCR/Southern to detect the integration of transgene in wheat genome.

3.2.1.2. Genomic Southern

Genomic Southern blotting to prove integration of our transgene(s) was done (Fig. 10). Genomic DNAs from different individuals at the T_1 generation were digested with *HindIII* to librate 2.09 kb *bar* gene cassette (*35S/bar/NOS*). *Le-GST* gene plus *NOS* terminator was used as a probe to detect the *bar* gene cassette and prove integration of *Le-GST* gene to the high-molecular weight genomic DNA. The results indicated the presence of the *bar* gene cassette in two T_1 individuals, while integration to the high-molecular whight DNA was negative. This conflict might be explained by the low genomic DNA concentration as well as the low sensitivity of the non-radioactive method of detection used in the present study. However, more experimentation ought to be done in the following generation(s) to get a final figure on the integration and expression patterns of different transgenic individuals. Many investigators studied the integration of the transgenes in the plant genomes using Southern analysis. **Lörz *et al.* (1998)** used Southern to analyze the integration pattern of wheat transgenic plants. **Uze *et al.* (1999)** used Southern blotting to improve that linearized DNA in both double strand and single strand forms integrated into the wheat genome with high frequency than circular DNA. **Eissa (2001)** used Southern analysis to improve the integration of *cpy/sacB* gene in transgenic wheat genome.

Also, **Archana *et al.* (2003a)** confirmed the presence of the transgene *nptII* in wheat T_0 plants using Southern hybridization.

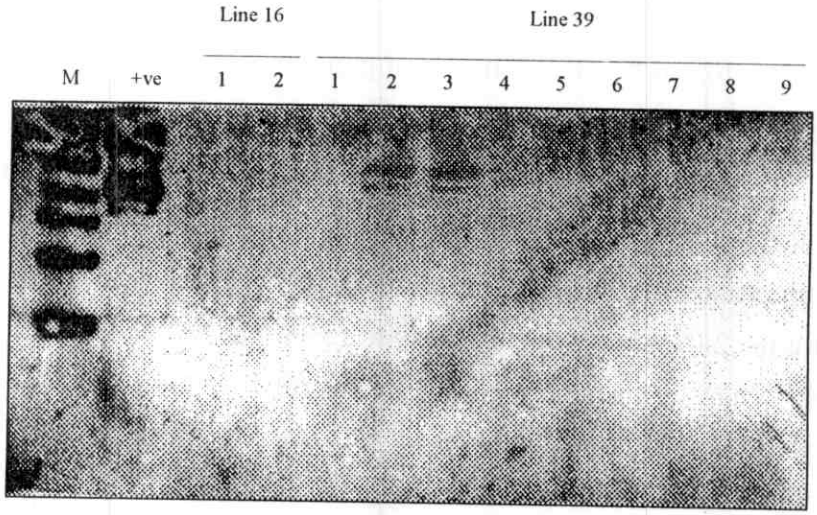

Fig. (10): Genomic Southern for the DNAs of different T_1 transgenic plants (1-9) digested with HinDIII to librate the *bar* gene cassette (2.09 Kb). PCR product (1.19 Kb) involving *Le-GST* gene plus NOS terminator of pLe-GST was used as a probe. +ve1, pLe-GST/bar.

3.2.2. Molecular analysis on the functional level

3.2.2.1. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Expression of the *Le-GST* gene was tested on total RNA extracts of the nine T_1 transgenic plants belonging to the two original transgenic events utilizing the Titan-One Tube RT-PCR Gene Expression System. The results of *Le-GST* gene expression for the nine T_1 plants indicated the presence of one cDNA band (over 1 Kb), with varying sizes, in only three T_1 plants (Figure 11). As expected, the band size is higher than the original gene size (840 bp) due to the presence of the PolyA tail. However, the

band differed in size due to the differences expected in the length of the PolyA tail in different RNA samples.

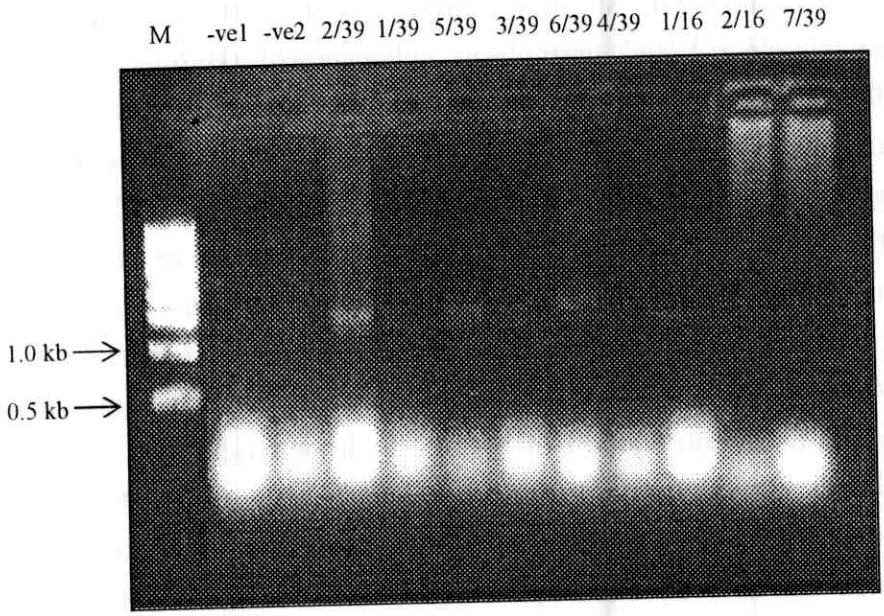

Fig. (11): RT-PCR involving *Le-GST* gene for the nine T_1 transgenic plants (2/39-7/39). –ve1, negative control (G164),-ve2, no DNA sample. M refers to 1Kb DNA ladder .

RT-PCR was used by many reaserchers to detect gene expression in transgenic plants. **Takumi *et al.* (1999)** used RT-PCR for the screening of excision and integration of a maize transposable element in wheat cells. **Pauk *et al.* (2000)** used it to detect the expression of *bar* and *nptII* genes in wheat. **Wang *et al.* (2000)** detected the expression of potato U1 snRNA in tobacco protoblast. **Bahieldin *et al.* (2003)** used RT-PCR to detect the expression of *bar* and *cpy/sacB* chimeric genes in transgenic wheat.

In general environmental stresses are a serious problem limiting crop productivity in rainfed agriculture. As the world's

population continues to expand, it is becoming increasingly important to increase food production by improving crop resistance to environmental stresses, through traditional breeding and biotechnology-based germplasm enhancement (**Bahieldin *et al.*, 2005**). Recent progress in transformation protocols has made it possible to transfer genes from other species into wheat to achieve agronomically desirable traits, such as drought (**Bahieldin *et al.*, 2003; Bahieldin *et al.*, 2005**) and salinity (**Abebe *et al.*, 2003**). Environmental stresses generate high reactive oxygenated compounds or species (ROS) that damage plants **(Lesser, 1997).** Whereas, the reactive oxygen species attack on cell components results in the production of organic hydroperoxides and activated alkenes, epoxides, and quinans, *GSTs* participate in oxidative stress mechanisms by catalyzing GSH-dependent reactions that inactivates such products by conjugation or reduction **(Kilili, 2004)**. S-conjugates and subsequently, ATP-dependent membrane pumps recognize the glutathione s-conjugates and shuttle them into the vacuolar or to the apoplast **(Flocco *et al.*, 2004)**.

In the present study, *Lycopersicon esculentum Le-GST* gene encoding glutathione s-transferase enzyme was introduced into wheat plants (*Triticum aestivum* L.) cv. Giza 164. The presence and expression of this gene was confirmed using molecular analysis.

The overall results of the transgene detection on the structural as well as the functional level were inconsistant. T_1 southern negative 2639/2, 2639/5 and 2639/6, whose RT-PCR results of *Le-GST* gene were positive, should indicate the integration of the transgene to the high-molecular whieght band. This contredication can be explained by the poor quality and low

concentration of genomic DNAs isolated from different individuals as well as the low sensitivity of the nonradioactive method of detection used in the present study. It is highly recommended that different molecular analyses of the transgenes are done in subsequent generation to prove both integration and expression of the transgenes and to test for possible silencing of either gene.

GSTs are soluble proteins composed of two subunits with molecular masses of 22-27 kDa each. Expression of GSTs in plants is highly responsive to biotic and abiotic stress and to a wide variety of stress-associated chemicals including salicylic acid, methyl jasmonate, abscisic acid and H_2O_2. Tobacco seedling over expressing tobacco Tau class GST are more tolerant to chilling and osmotic stress than wild type plants (**Roxas *et al.*, 1997**), whereas a GST participating in oxidative stress tolerance was discovered in black grass (**Cummins *et al.*, 1999**). Furthermore, a tomato Tau class enzyme, termed Bi-GST, was found to confer resistance to oxidative stress when expressed in yeast cells (**Kampranis *et al.*, 2000**).

In conclusion, the present study sugests that the introduction of *Le-GST* in transgenic wheat plant is a promising approach to improve crop productivity under drought and/or salt stress. As a recommendation, transgenic seeds ought to be maintained and increased in order to detect transgenic families with transgene introduced in homozygous condition. In addition, it is recommended to test these transgenic plants under stress conditions (either drought or salt stress). Moreover, it is planned in the future to accumulate more stress-related transgenes in Egyptian bread wheat by regular crossing to maximize its ability to tolerate salt as well as drought stresses.