International Journal of Botany

ISSN: 1811-9700

science alert

ANSInet
an open access publisher
http://ansinet.com

International Journal of Botany 1 (2): 175-182, 2005
ISSN 1811-9700

Construction of a Dehydrin Gene Cassette for Drought Tolerance from Wild Origin for Wheat Transformation

[1]Salwa Badr, [2]Ahmed Bahieldin, [2]Bassem Abdelgawad and [1]Abdelfattah Badr
[1]Department of Botany, Faculty of Science, Tanta University, Tanta, Egypt
[2]Agricultural Genetic Engineering Research Institute, Agricultural Research Center, Giza, Egypt

Abstract: Construction of the dehydrin gene was made in a plant expression vector carrying the bar gene as a selectable marker in order to use in transforming wheat cultivar Giza 164. Plant transformation was made to the immature embryo-derived calli using biolistic bombardment. A tissue culture and transformation protocol were performed and bar gene was used as a selectable marker against the herbicide bialaphos. Five putative transgenic plants were successfully detected by leaf painting with the herbicide. Molecular analyses were done for these plantlets at the structural level; three gave positive results with PCR. The integration of the dehydrin gene into the genomic background of two transgenic plants was confirmed by Southern blotting.

Key words: Dehydrin gene, tolerance, wheat transformation, construction

INTRODUCTION

The response of plants to abiotic stresses can be observed at different levels[1] and involves molecular, physiological and morphological processes[2]. At the molecular level, drought induces changes in the expression of several genes associated with drought; one of the most notable changes is the accumulation of dehydrins, a protein family that accumulate in response to water stress[3]. Dehydrins were first evident and also most concentrated, in the cytosol throughout the accumulation period suggesting that the primary function of dehydrins involves the cytosol and structures contained therein[4]. Dehydrins are evolutionarily conserved among photosynthetic organisms including angiosperms, gymnosperms, ferns, mosses, liverworts, algae and cyanobacteria, as well as in some non-photosynthetic organisms such as yeast[2].

In earlier reports, wheat suspension cell cultures were used as target tissues for microprojectile bombardment, but cells were unable to regenerate plants[5]. Wheat explants were then turned to regenerable callus but callus tissue occurred at very low frequency and was difficult to either identify or maintain[6]. More recently, callus tissues produced from immature zygotic embryos were frequently used as a target tissue of wheat for DNA delivery using biolistic bombardment[7]. This protocol was modified by Bahieldin *et al.*[8] and Shawky[9] to fit the Egyptian wheat cultivars and has been adopted in this study.

The main goal of this study is the introduction of a stress related dehydrin gene, in the genome of a cultivar of Egyptian bread wheat (*Triticum aestivum* L.) namely Giza 164 through genetic transformation, i.e. in order to improve its tolerance to drought stress. The gene of interest was isolated from *Vicia monantha* Retz., a wild bean plant growing in the western Mediterranean coast of Egypt. The full-length gene was directly isolated by PCR and constructed in $pHBN_1$ plasmid at AGERI, ARC and the gene was proven to function in *E. coli*.[10].

MATERIALS AND METHODS

Gene construction: A dehydrin gene in pHBN1 plasmid was isolated from *Vicia monantha* Retz,. (a wild plant growing in the western Mediterranean coastal region of Egypt) at AGERI and constructed in pPCR-Script Amp cloning vector[10] which was used in this investigation.

Release of the dehydrin gene from $pHBN_1$ plasmid: The purified plasmid was digested with BamHI to release the dehydrin gene DNA fragment with 590 bp harboring cohesive-ended termini suitable for further cloning. The reaction was made in 50 μL total volume containing 2 μg purified plasmid, 30 units of the enzyme at a final concentration of 50 mM NaCl, 10 mM Tris-HCl-pH 7.9, 10 mM $MgCl_2$ and 1 mM DDT. The reaction was incubated at 37°C for 1 h. The DNA fragment was then purified using Wizard® PCR Preps DNA Purification system from Promega according to the manufacture's instructions.

Cloning of the dehydrin gene: The $pAHC_{17}$ plasmid vector harboring the ubi promoter/Nos3' (4.9 kb) was digested

Corresponding Author: Salwa Badr, Department of Botany, Faculty of Science, Tanta University, Tanta, Egypt

with BamHI to create linear molecules of the vector with cohesive-ended termini. The purified fragment containing the dehydrin gene and the linearized $pAHC_{17}$ vector were ligated by T_4 DNA ligase for 16 h at 16°C. The ligation reaction contained 0.1 μg of the vector, 0.3 μg of insert DNA and 10 units of T_4 DNA ligase at a final concentration of 50 mM Tris-HCl (pH 7.8 at 25°C), 10 mM $MgCl_2$, 10 mM DTT, 25 mg mL^{-1} BSA, 1 mM ATP. Competent cells of DH5α *E. coli* were prepared for transformation of the recombinant plasmid by the titration procedure. For each ligation reaction, 200 μL aliquot of the competent cells was thawed on ice and 10 μg of the ligation was added and transformation was made by heat-shock. Recombinant colonies containing a new recombinant plasmid pDB_1 were then selected.

The confirmation of the orientation of the plasmid was carried out by restriction analysis using *Hind*III endonuclease. The restriction reaction was made in 50 μL containing 2 μg plasmid DNA, 3 units *Hind*III at 37°C for 1 h and restriction products were separated by electrophoresis in 1.5% agarose gel.

Construction of pDB_2 plasmid with the dehydrin DNA insert: A pAB_8 plasmid containing the bar gene cassette was digested with the HindIII to yield the bar gene cassette with cohesive-ended termini (2.09 kb) suitable for the cloning of the HindIII cohesive-ended cloning vector. A *Hind*III partial digestion of the pDB_1 recombinant plasmid was carried out to yield the linear form of the vector with cohesive-ended termini suitable for the dehydrin DNA insert. The bar gene insert and the partially digested pDB_1 vector were ligated by T_4 DNA ligase for 16 h at 16°C and the recombinant DNA plasmid (pDB_2) was used to transform the *E. coli* competent cells DH5α.

Confirmation of the recombinant plasmids using PCR: Two PCR reactions were performed to the two recombinant plasmids pDB_1 and pDB_2 using specific primers of the dehydrin gene and the bar gene. The specific primers were designed and synthesized using the DNA synthesizer 392, Applied Biosystems, Lincoln CA, USA. The sequences for the bar and the dehydrin genes are as follows:

A- *bar* gene:
bar1 (forward) TACATCGAGACAAGCACGGT
bar2 (reverse) ACGTCATGCCAGTTCCCGTG

B- dehydrin gene:
dehydrin1 (forward) CTACTTATACCTTTGGGT
dehydrin2 (reverse) TGGTTCTTCGAAGAGGAATTA

The reactions for both genes were carried out in total volumes of 50 μL each containing 30 ng plasmid DNA, 2.5 units of Taq DNA polymerase, at a final concentration of 20 pmol of each amplification primer, 200 mM each of dNTPs in a buffer containing 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl-pH 9, 0.1% Triton®X-100 and 0.1 mg mL^{-1} BSA.

The reaction mixture was mixed gently and centrifuged briefly. The PCR profile was as follows: denaturation of the plasmid DNA at 94°C for 4 min, followed by 40 cycles of amplification with denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 2 min and ending with a single cycle of extension at 72°C for 4 min. PCR products were separated in 1.5% agarose gel and visualizated using UV transilluminator.

Wheat transformation and regeneration: Immature embryos were isolated from the sterilized caryopses, cultured and bombarded according to the protocol published by Bahieldin *et al.*[8]. Bombarded calli were, grown on two selection media containing 3 and 5 mg L^{-1} bialaphos for callus induction, transferred to selective regeneration medium (1 mg L^{-1} bialaphos), then to a selective rooting medium, (2 mg L^{-1} bialaphos). Healthy rooted plantlets were transferred from the rooting medium to the greenhouse.

The leaf painting assay was carried out to 1 g L^{-1} basta for testing the herbicide resistance of the putative transgenics.

Molecular analysis of putative transgenic plants
PCR analysis: PCR was performed to the genomic DNA extracted from the putative transgenics to insure the presence of the bar and dehydrin genes in the genomic DNA. The primers used for both genes have the following sequences:

Dehydrin1 (forward)
GGTGGGTTTACTGGTGAAGCCGGCAGACAA

Dehydrin2 (reverse)
CTAGTGTCCAGTACATCCTCCAGTACCAGG

The PCR reaction for both genes was carried out in 50 μL volume containing 30 ng DNA, 2.5 unites of Taq DNA polymerase, 20 pmol of primers and 200 mM each dNTPs in buffer. The PCR profile was as follows: Denaturation of the DNA at 94°C for 4 min, followed by 35 cycles of amplification with denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 2 min and ending with a single cycle of extension at

72°C for 7 min. Subsequent electrophoresis of the PCR products was made in 1.5% agarose gel.

Genomic southern analysis: Genomic DNA was isolated and digested overnight using *Bam*HI restriction enzyme. The original method[11] was used with the exception of using the non-radioactive digoxigenin (DIG), for DNA labeling and detection. The digested DNA was electrophoresed in 1% agarose gel and the DNA was transferred to Hybond N^+ nylon membrane. Probe was prepared and labeled using the Dig labeling and detection kit from (Roche, Catalog No. 1093657).

Prehybridization was performed by soaking the gel in 20 mL prehybridization solution (5X SSC, 1%N-lauroylsarcosine, 0.02% SDS, 1% blocking reagent) for 30 min. The probe (20 ng mL^{-1}) was denatured by heating in a boiling water bath for 5 min then placed on ice to cool. The probe was added to the hybridization solution (2.5 mL per100 cm^2) and incubated at 68°C overnight with gentle shaking. The membrane was washed twice in 2X SSC for 5 min then washed in 0.1% SDS for 5 min, washed in 0.1X SSC for 15 min at 68°C then washed in 0.1% SDS for 15 min at 68°C two more times. Washings were performed according to manufacturer's recommendations. The blotted DNAs were probed with the labeled probe to confirm the transgenic nature of the plants.

RESULTS

Construction of dehydrin gene: The recombinant plasmid comprised of the $pAHC_{17}$ plasmid and the dehydrin DNA sequence, which has been named pDB_1, has a size of 5500 bp. The schematic representation of this plasmid is shown in Fig. 1.

Confirming of the orientation of dehydrin gene in pDB_1 plasmid: To prove the orientation of the dehydrin gene in the pDB1 plasmid, the plasmid was digested with HindIII because this enzyme has a unique site in the plasmid and a unique site in the gene (34 bp from the 3` end of the gene). The digestion products were electrophoresed in 1.5% agarose gel and visualized under UV transilluminator. The size of the digestion products was estimated with reference to 1 kb ladder as marker.

Electrophoretic profiles showing the result of pDB_1 plasmid digestion using *Hind*III is illustrated in Fig. 2. Lane 1, 4 and 6 show the presence of a 2566 bp and a 2944 bp fragments; the first fragment comprises the 556 bp of the dehydrin gene plus 2010 bp of the plasmid, the second fragment (2944 bp) is the rest of the plasmid (2910 bp) plus, 34 bp of the dehydrin gene. Lanes 2, 3, 5 and 7 show the presence of a 2044 bp fragment which

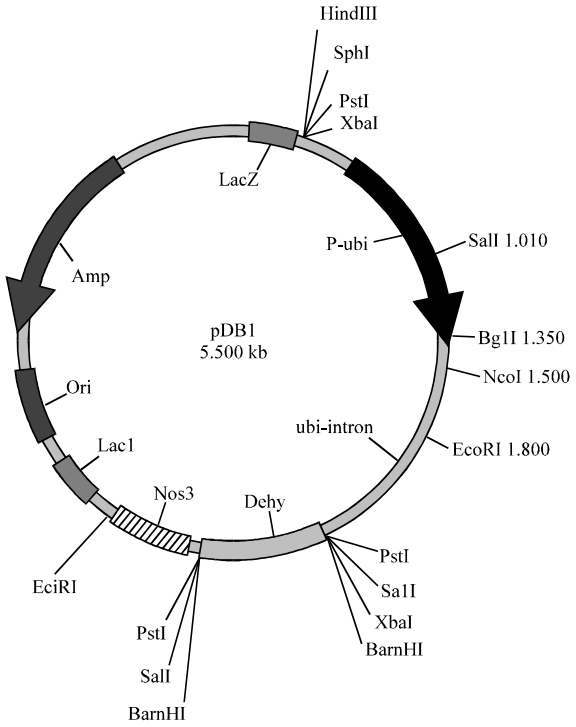

Fig. 1: Schematic representation of pDB_1 plasmid

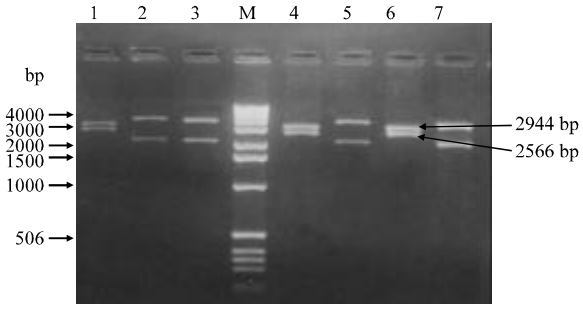

Fig. 2: Agarose gel electrophoretic profile of pDB_1 construct digested with *Hind*III enzyme, where (M) = DNA marker, Lanes 1, 4 and 6 are the new construct (pDB_1) in right orientation and Lanes 2, 3, 5 and 7 are self ligated plasmid ($pAHC_{17}$) in wrong orientation

represents the 34 bp of the wrong oriented dehydrin gene plus a 2010 bp fragment of the plasmid and 3466 bp fragment, which comprises the 556 bp that is the rest of the dehydrin gene in the wrong orientation plus 2910 bp, which represents the rest of plasmid.

Construction of pDB_2 plasmid: The plasmid pDB_1 was digested using HindIII endonuclease. The bar gene cassette fragment was then cohesive-end ligated into

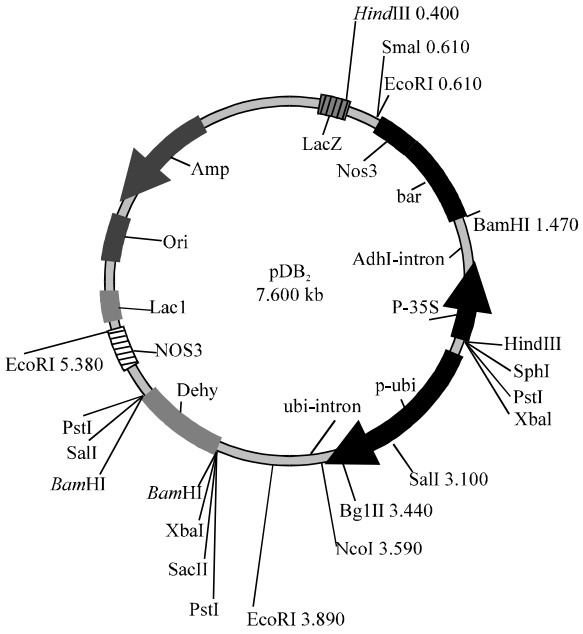

Fig. 3: Schematic representation of pDB_2 plasmid

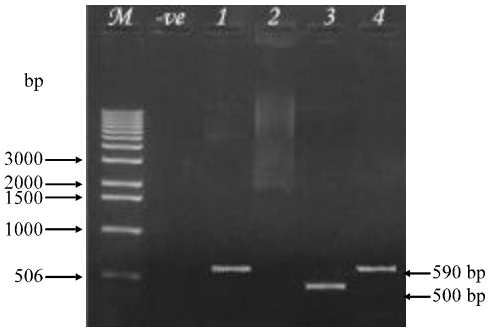

Fig. 4: Agarose gel electrophoretic profile of PCR products of the constructed pDB_1 and pDB_2 plasmids, where (M) = DNA marker, (-ve) = negative control, Lanes 1 and 2 are the PCR product of pDB_1 plasmid, Lanes 3 and 4 are the PCR product of pDB_2 plasmid

pDB_1 to yield a recombinant plasmid comprised of the pDB_1 plasmid and the bar gene cassette, which has been named pDB_2. Ligation was performed using a molar ratio of 3:1 (insert : vector). Transformation efficiency was expressed as colony forming units (cfu) per μg of plasmid DNA. The efficiency ranged between 10^6 and 10^7 cfu per μg of insert DNA. The pDB_2 plasmid is schematically represented in Fig. 3. The ligation product was introduced into *E. coli* DH5α cells and the transformed colonies were detected as ampicilline resistance colonies that were used for more analysis.

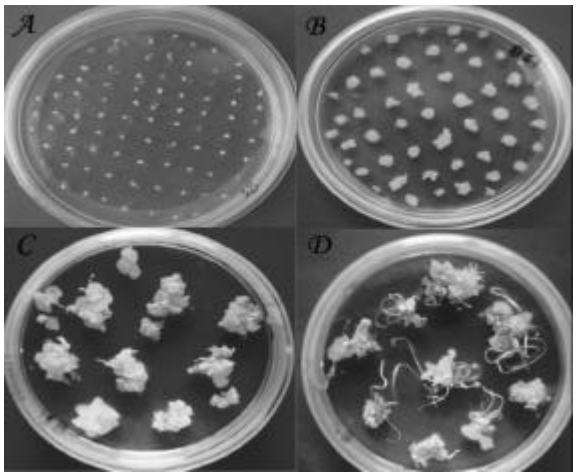

Fig. 5: Callus induction from immature embryos of wheat, where:
A = immature embryos on TW medium,
B = young calli in first subculture medium,
C = young calli in second subculture medium,
D = young calli forming shoots

Subculture on LB ampicilline containing medium was made for the transformed colonies.

Two specific primers were used for the amplification of pDB_1 and pDB_2 plasmids. The electrophoresis profile (Fig. 4) shows the PCR products of pDB_1 plasmid in lane 1, which clearly revealed the presence of a 590 bp DNA fragment that represent the dehydrin gene. Lane 2 is void because of absence of bar gene. Lanes 3 and 4 on the other hand show the PCR product of pDB_2 plasmid in which a 500 bp DNA fragment refers to the bar gene in lane 3 while the 590 bp DNA fragment in lane 4 represent the presence of the dehydrin gene. The lane -ve represents the profile of a negative control.

Transformation of wheat embryos: Immature embryos, aseptically excised from wheat spikes, 12-15 days postanthesis (Fig. 5A), were subcultured (Fig. 5B) for 15 days at 25°C in the dark. The calli, transferred from the first subculture, were cultivated for extra 15 days at 25°C in the dark (second subculture) under the same culture conditions (Fig. 5C). Plant regeneration was performed at 25°C under fluorescence light for two weeks on shoot proliferation medium (Fig. 5D).

Calli that succeeded to form shoots were transferred to root formation (Fig. 6) Putative transformed plantlets were then transferred to acclimatization, under high moisture content and transferred to the soil for subsequent analysis.

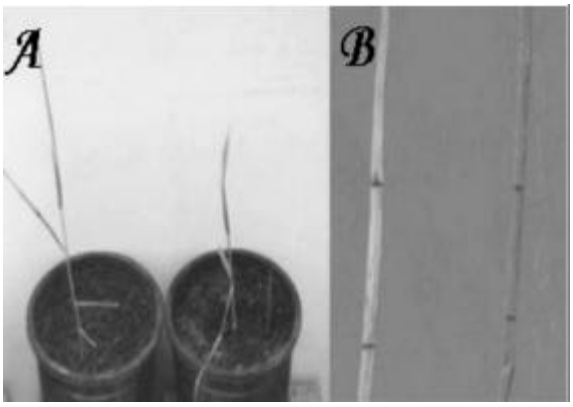

Fig. 6: A: Leaf painting to young plants in soil and B: two separate leaves painted with basta

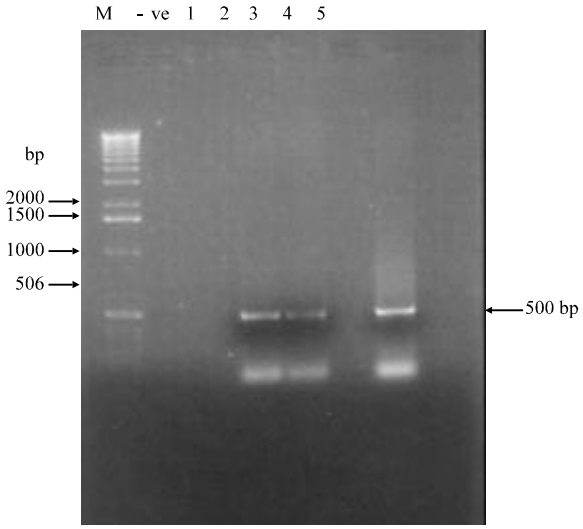

Fig. 7: Agarose gel electrophoretic profile of PCR products of transformed plants with bar gene primers, where (M) = DNA marker, (-ve) = negative control, Lanes 2, 3 and 5 are transformed plants and lanes 1 and 4 are non-transformed plants

Leaf painting : In putative transgenic plants, herbicide resistance was tested by painting the middle green part of the plant leaves from both sides with the herbicide basta (Fig. 6A). The result of this test clearly demonstrated the expression of the bar gene. The transgenic plant leaves that remained green are resistant to the herbicide, while leaves of non-transgenic plants turned yellow and died within two days (Fig. 6B).

Molecular analysis of transformed plants

Polymerase Chain Reaction (PCR): Genomic DNAs from transgenic plants were isolated and used as templates for

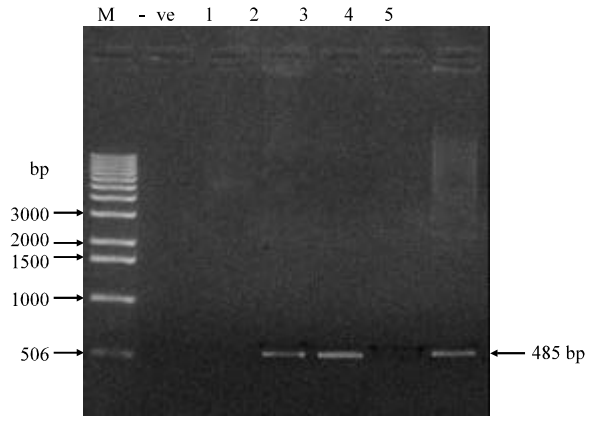

Fig. 8: Agarose gel electrophoretic profile of PCR products of transformed plants with dehydrin gene primers, where (M) = DNA marker, (-ve) = negative control, lanes 2, 3 and 5 are transformed plants and lanes 1 and 4 are non-transformed plants

PCR reactions using the bar gene primer and dehydrin gene primers to ensure the integration of the gene in the genome of transformed plants. Figure 7 shows the products of the PCR reaction for the *bar* gene. Lanes 1 and 4 are the PCR products for non-transgenic plants while lanes 2, 3 and 5 represent the PCR products of *bar* gene (500 bp) from the genome of transgenic plants.

Figure 8 shows the PCR products of derhydrin gene primers where lanes 2, 3 and 5 show the presence of 485 bp DNA fragment in the genome of transgenic plants, which represent dehydrin gene while lanes 1 and 4 represent PCR products of non-transgenic plants, the first (-ve) lane is the negative control. The size of the PCR products was estimated with reference to 1 kb ladder marker.

Genomic Southern analysis: A labeled probe was generated from the PCR reaction of the pDB_2 construct and used to detect the integration of the dehydrin gene in the transgenic genomes. The pDB_2 construct was digested using BamHI endonuclease to release the dehydrin gene fragment with 590 bp to be used as the positive control and the non transgenic wheat genome (cultivar Giza 164) was used as a negative control after it was digested with BamHI.

Figure 9 shows the Southern blotting for the dehydrin gene against a positive control presented in the +ve lane and a negative control in the –ve lane. Lanes 1 and 3 represent the blotting of DNA from two transgenic plants. Lane 2 represents the DNA blotting of a non-transgenic plant.

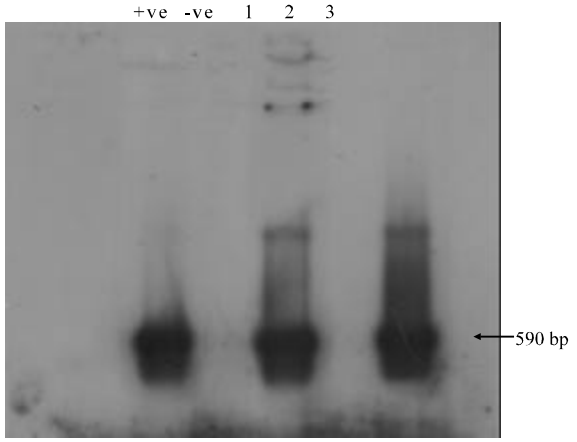

Fig. 9: Genomic Southern for the DNA of putative transgenic plants with dehydrin gene PCR of pDB_2 as a probe, where (+ve) = positive control, (-ve) = negative control, Lanes 1 and 3 are transgenic plants and lane 2 is non-transgenic plant

DISCUSSION

For transformation purposes, gene incorporation was affected in the plant expression vector pDB_2 by which it contained the dehydrin gene under the action of ubi promoter and NOS terminator and bar gene under the control of CaMV35S promoter and NOS terminator. The dehydrin gene was first inserted in the $pAHC_{17}$ vector between the ubi promoter and NOS terminator. The orientation of the dehydrin gene has to be checked to ensure that it was property inserted so that the proper ORF was downstream the promoter to produce the desired specific dehydrin protein. The second gene in the plant expression vector, i.e., the bar gene was used as a selectable marker to track the target gene during tissue culture and transformation processes.

Proof of successful construction of this plasmid was done by running a BamHI digested pDB_1 in agarose gel and observing the presence of a 4920 bp large fragment representing the $pAHC_{17}$ plasmid and a 590 bp fragment that represents the dehydrin gene of interest. The proper orientation of the dehydrin gene was also confirmed by running a *Hind*III digested pDB_1 in 1.5% agarose gel and observing the presence of 2566 and 2944 bp fragments under UV illumination. The first fragment comprised 556 bp of right-orientated dehydrin gene and 2010 bp of the plasmid and second fragment comprised the rest of the plasmid (2910 bp) plus 34 bp fragment, which represent the rest of the dehydrin gene.

The cloning procedure of the bar gene 2090 bp involved its release from the pAB_8 plasmid by digestion with *Hind*III endonuclease and its ligation to a *Hind*III digested pDB_1 plasmid to yield the recombinant plant transformation plasmid pDB_2. The pDB_2 was then isolated, purified and tested for the presence of the bar gene cassette. The analysis showed the presence of a 7600 bp fragment, which corresponds to a recombinant pDB_2 plasmid, which comprises the pDB_1 DNA (5500 bp) and the bar gene cassette (2090 bp). The construction of the pDB_2 plasmid was performed by its digestion using the HindIII endonuclease and electophoresis of the digestion products in 1.5% agarose gel. The viewing of the gel under UV illumination showed the presence of a 2090 bp, which represents the bar cassette and the two fragments of the pDB_1 plasmid DNA (2566 and 2944 bp). In the non-recombinant plasmids the digestion reaction in the two fragments of the pDB_1 plasmid only. The proper orientation of the pDB_2 plasmid was then confirmed by the electrophoretic analysis of its digestion with EcoRI endonuclease. The proper construction of the pDB_1 and pDB_2 plasmids was also confirmed using PCR specific primers for the dehydrin and bar genes. This revealed the presence of a 590 bp DNA fragment in the PCR product of pDB_1 plasmid that represent the dehydrin and a 500 bp fragment in the pDB_2 in the PCR products of the pDB_2 plasmids, which indicates the presence of the bar gene.

Immature embryos (0.5-1.0 mm) were aseptically excised from spikes 12-15 days post-anthesis and callus tissue was proliferated at the edges of embryos on TW medium after five days. At this stage calli were transferred to TW medium supplemented with 0.4 mannitol and bombarded four hour later. After five days recovery in the dark with no selection, calli were transferred on 3 or 5 mg L^{-1} biolaphos for additional 15 days in the dark. A second round of selection under the same culture conditions was performed for extra 15 days. Regeneration of plantlets was induced under fluorescence light for two weeks. Calli that successfully proliferated shoot were transferred to root formation medium for additional two weeks. Putative transformed plantlets were then acclimatized, transferred to potted soil and used for analysis in order to test the integration of the dehydrin and bar gene in their genome. The analysis for the presence of bar gene expression was made using the leaf painting protocol. The presence of the dehydrin and bar gene in the putative transgenic plants was confirmed by PCR using bar and dehydrin gene-specific primers.

The PCR products using bar gene primer yielded a 500 bp fragment on agarose gel which indicates the presence of the bar gene cassette. Meanwhile the PCR using the dehydrin gene-specific primer produced a 485 bp in the genome of the putative transgenic. The presence of this fragment has been regarded as indicative

of the integration of the dehydrin gene in the genome of transgenic plants.

In addition the integration of the pDB_2 construct in the genome of the putative transgenic wheat plants that gave positive results with leaf painting and PCR was further confirmed using Southern blotting. Genomic DNA isolated from transgenic plants was used as templates for overnight digestion using BamHI endonuclease, which released a 590 bp fragment of the dehydrin gene. PCR reaction was used to label the probe using dehydrin gene-specific primers of the pDB_2 construct. Blotting the genome of transgenic plants and the labeled probe clearly revealed the existence of 590 bp fragment that represents the dehydrin gene.

A low number of putative transgenics was recovered as a result of the low efficiency of DNA delivery system and reduced regeneration rate of microprojectile-bombarded tissues[12]. Besides there variation in regeneration of transformation frequencies has been reported due to differences in the *in vitro* culture responses with the genotype used as the target tissue for transformation[13,14]. However, the chance to get higher transformation efficiency is 3-4 folds when using embryos recovered from tissue culture-regenerated plants rather than seed-grown plants[15]. In addition, it was noted that the age of donor plants was a key factor in transformation efficiency, where the embryos derived from younger plants gave higher efficiency than those derived from older plants[16]. He and Lazzeri[17] found that the size of embryos (scutella) was also an important factor in the competence for DNA transfer, i.e., medium-sized embryos gave more GUS signals than the small or large embryos. Use of mannitol before and after bombardment is known to improve integration of bombarded DNA in the callus tissues[12].

In the present study, a selection of 3 mg L^{-1} bialaphos resulted in a rate of 94% plant escape frequency. It was noted that 35% escapes can be reached if selection was 4 mg L^{-1} or less at callus induction stage[18]. The results however show the efficiency of the applied protocol to transformation embryos and the successful regeneration of putative transgenic Egyptian wheat. The regenerated and tested transgenic plants are being tested for inheritance of drought tolerance.

REFERENCES

1. Close, T.J., 1996. Dehydrins: Emergence of a biochemical role of a family of plant dehydration proteins. Physiol. Plant., 97: 795-803.
2. Borovskii, B.G., I.V. Stupnikova, A.I. Antipina, S.V. Vladimirova and V.K. Voinikov, 2002. Accunulation of dehydrin-like proteins in the mitochondria of cereals in response to cold, freezing, drought and ABA treatment. BMC Plant Biol., pp: 2-5.
3. María, del M.P., O. del Pozo, R. Luna, J.A. Godoy and J.A. Pintor-Toro. 1996. Structure of the dehydrin tas14 gene of tomato and its developmental and environmental regulation in transgenic tobacco. Plant Molecular Biol., 32: 453-460.
4. Egerton, R., L.M. Warburton, R.A. Balsamo and T.J. Close, 1997. Temporal accumulation and ultrastructural localization of dehydrin in *Zea mays*. Physiol. Plant., 101: 545-555.
5. Vasil, V., S.M. Brown, D. Re, M.E. Fromm and I.K. Vasil, 1991. Stably transformed callus lines from microprojectile bombardment of cell suspension cultures of wheat. Bio. Technol., 9: 743-747.
6. Vasil, V., V. Srivastava, A.M. Castillo, E.F. Michael and I.K. Vasil, 1993. Rapid production of transgenic wheat plants by direct bombardment of culture immature embryos. Bio. Technol., 11: 1553-1559.
7. Weeks, J.T., O.D. Anderson and A.E. Blechl, 1993. Rapid production of multiple independent lines of fertile transgenic wheat (*Triticum aestivum* L.). Plant Physiol., 102: 1077-1084.
8. Bahieldin, A., R., Qu, W. Dyer, A.S. Haider and M. Madkour, 2000. A modified procedure for rapid recovery of transgenic wheat plants. Egypt. J. Genet. Cytol., 29: 11-23.
9. Shawky, A., 2000. Genetic studies on bacterial genes governing fructan accumulation for the production of transgenic wheat plants. M. Sc Thesis, Faculty of Science, Ain Shams University.
10. Nada, A.K., 1998. Thesis titled: Molecular studies on drought genes in legumes.
11. Southern, E.M., 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol., 98: 503-509.
12. Perl, A., M. Kless, A. Blumenthal, G. Galili and E. Galum, 1992. Improvement of plant regeneration and GUS expression in scutellar wheat calli optimization of culture conditions and DNA-microprojectile delivery procedures. Mol. Gen. Genet., 235: 279-284.
13. Takumi, S. and T. Shimada, 1997. Variation in transformation frequencies among six common wheat cultivars through particle bombardment of scutellar tissues. Genes Genet Syst., 72: 63-69.

14. Xia, G.M., C.X. He, H.M. Chen and R. Brettell, 1999. Transgenic plant regeneration from wheat (*Triticum aestivum* L.) mediated by *Agrobacterium tumefaciens*. Acta Ohytophysiologica Sinica, 25: 22-28.
15. Harvey, A., L. Moisan, S. Lindup and D. Lonsdale, 1999. Wheat regenerated from scutellum callus as a source of material for transformation. Plant Cell Tissue Organ Culture, 57: 153-156.
16. Pastori, G.M., M.D. Wilkinson, S.H. Steele, C.A. Sparks, H.D. Jones and M.A.J. Parry, 2001. Age-dependent transformation frequency in elite wheat varieties. J. Exp. Bot., 52: 857-863.
17. He, G.Y. and P.A. lazzeri, 1998. Analysis and optimization of DNA delivery into wheat scutellum and tritordeum inflorescence explants by tissue electroporation. Plant Cell Rep., 47: 390-97.
18. Rasco, G.S., A. Riley, M. Cannell, P. Barcelo and P.A. Lazzeri, 2001. Procedures allowing the transformation of a range of European elite wheat (*Triticum aestivum* L.) varieties via particle bombardment. J. Exp. Bot., 52: 865-874.