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Improvement of Flax Drought Tolerance Using Gene Transfer

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Abstract

Attempt was made to produce transgenic cell lines of flax cv. Blanka tolerant to drought stress. Genetic transformation systems were used to incorporate the DREB2A gene, as the specific gene for drought stress tolerance. In biolistic transformation, hypocotyl segments were bombarded with DREB2A and GFP genes at particle flight distance of 9 cm and rupture disc pressure of 1300 psi. The expression of the gene was observed under a light microscope after 24 and 48 hrs. In *Agrobacterium*-mediated transformation, the hypocotyl segments were incubated overnight with *Agrobacterium* culture at five optical density OD_{600} i.e. 0.2, 0.4, 0.6, 0.8 and 1 for 30 min with occasional stirring. Later, the explants were transferred to a selection regeneration medium supplemented with 50 mg/dm^3 hygromycin and 300 mg/dm^3 cefotaxime and subcultured every two weeks on a new selection medium. Molecular analysis confirmed the expression of the target DREB2A gene in flax genome.

Introduction

Flax (*Linum usitatissimum* L.) of the family Linaceae is an agriculturally and economically important species. It has industrial applications as a source of painting oil and, textile fiber. Flax also has medicinal uses as anticancer agent and is also used in livestock and human feeding. Flax is widely adapted to warm and cool temperate climates (Green 1986). It grows best on heavy loam soil that retains moisture well. Because of its limited root system, flax does not grow well on sandy, moisture-limited soils (Green 1986).

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Recently, Shysha et al. (2013) showed that there are a number of factors affecting the *Agrobacterium*-mediated transformation of flax, including as optical density (OD_{600}), the inoculation time of explants with *Agrobacterium* and co-culture conditions. The PCR analysis was used to confirm the transgenic nature of the resulting lines.

The dehydration responsive element binding (DREB) factor 2 protein is part of a plant-specific family of transcription factors that play important roles in regulating the expression of the genes rd29A, kin1 and erd10 in response to a variety of abiotic and biotic stresses (Agarwal et al. 2006). Ashraf et al. (2008) generalized that production of transgenic plants with DREB genes is useful for improvement of tolerance of environmental stresses in a number of species.

In the biolistic transformation of flax, GFP was used as fluorescent reporter marker according to Prasher et al. (1992) and Heim et al. (1995). Hraska et al. (2006) reported that green fluorescent protein (GFP) was very useful to obtain an efficient protocol for transformation.

The main goal of this study is to produce a transgenic flax cell lines tolerant to drought stress, which can adapt the newly reclaimed desert lands in Egypt. To achieve such goal, this work was designed to use transformation in combination with molecular techniques. Gene gun and *Agrobacterium*-mediated transformation systems have been applied and the cultivar Blanka of flax was used.

Materials and Methods

Seeds of the Blanka cultivar of flax (*Linum usitatissimum* L.) were provided by the Fibers Crop Research Section, Agricultural Research Center, Giza- Egypt. The seeds were used as the initial plant material for obtaining hypocotyl explants for gene transfer experiments.

Seeds were washed for one hour with liquid soap and then washed exhaustively with tap water. The washed seeds were immersed in 70% ethanol for 1 min, then in 30% commercial Clorox (1.5% NaCl) for 15 min, followed by rinsing 3 times in sterilized distilled water for 15 min.

The sterilized seeds were grown on basal MS which consisted of 4.4 g/dm^3 MS readymade medium purchased from Duchefa containing 3% sucrose and solidified with 2.8 g/dm^3 gelrite after pH adjustment to 5.8. The medium was dispensed into 40 ml aliquot in 370 ml covered glass jars, autoclaved at 121°C (1.2 kg/cm^2) for 22 min. and incubated in a growth chamber under photoperiod of 16/8 hrs light/dark (ca. 50 μmol/m^2/s) at a constant temperature 25 ± 1°C for 7 days.

In the laminar flow, 0.5 cm hypocotyl segments were excised from the central part of the hypocotyl of one-week-old seedlings and used as primary explants.

The cDNA *DREB2A* gene from pokkali rice was provided by Prof. S.K. Sopory of the International Centre for Genetic Engineering and Biotechnology (ICGEB), India under material transfer agreement. For biolistic transformation, the hypocotyl explants were subjected to osmotic medium (36.4 g/dm^3 mannitol + 4.4 g/dm^3 MS + 0.05 mM $FeSO_4$ + 170 mg dm^{-3} KH_2PO_4 + 0.5 mM $CuSO_4$ + 0.75 mM H_3BO_3 + 3 mg/dm^3 2, 4-D + 2.8 g/dm^3 gelrite) for 4 hrs in the dark at 25 ± 1°C, then ten explants were collected in the center of the plate to be "shot" with DREB2A and GFP genes using a gene gun.

All bombardment experiments were carried out with the Bio-Rad Biolistic, PDS 1000/He particle delivery system (Bio-Rad, Richmond, CA, USA). Bombardments were made under a partial vacuum of 25-inch Hg pressure. Particle bombardment was performed according to Michael et al. (2010) with some modifications.

Target tissues (hypocotyl segments) were placed on osmotic medium in Petri dishes and bombarded (2 shots per Petri dish) with the following parameters of particles delivery system: rupture disc that bursts at pressure of 1300 psi (pounds per square inch), gasp distance was set to 6 mm, macrocarrier flight distance (distance between macrocarrier and stopping screen) was 11 mm and particle flight distance (distance between stopping screen and target tissue) was 9 cm. The sealed Petri dishes were placed at 25ºC for 2 days in the dark to allow transformation and gene expression to occur as well as to detect GFP expression as fluorescent signals visualized using a stereomicroscope. The transient gene expression was observed 24 and 48 hrs after bombardment.

For *Agrobacterium*-mediated transformation of flax hypocotyl segments, the gene was cloned in pCAMBIA1301 vector under 35S promoter. This vector also carries the hygromycin resistance gene as a selectable marker controlled by the CaMV35S promoter and harbors the GUS (β-glucuronidase) intron as a visible marker gene in its T-DNA (described in the next section). This vector contains a marker gene for the kanamycin resistance serving for bacterial selection (Fig. 1).

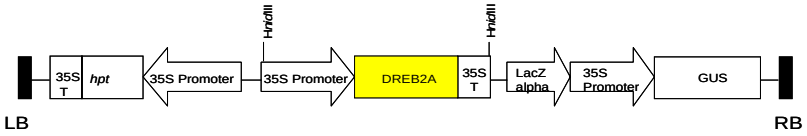

Fig. 1. Schematic diagram of the binary vectors (pCAMBIA1301 vector under 35S promoter).

The construction of this plasmid was done in ICGEB, India. Recombinant plasmids were introduced into *Agrobacterium tumefaciens* strain LBA4404 using the tri-parental mating procedure.

The hypocotyl segments were pre-cultivated on MS medium containing 4% maltose and supplemented with 2 mg/dm^3 BA, which is known to be regeneration medium for flax (Saker et al. 2009). Cultures were maintained in a growth cabinet under a 16 hrs photoperiod (ca. 50 μmol/m^2/s) at a constant temperature of 25 ± 1°C for 2 days before transformation process.

The overnight *Agrobacterium* culture was diluted 1 : 10 with YEB liquid (50 mg/dm^3 kanamycin and 25 mg/dm^3 streptomycin and 25 mg/dm^3 rifampicin) and supplemented with 100 mM acetosyringone for 4 hrs at 28ºC with shaking (200 rpm) to achieve the different OD_{600} at 0.2, 0.4, 0.6, 0.8 and 1.0.

Hypocotyl segments of flax from pre-cultivation media were incubated in this diluted culture at room temperature for 30 min with occasional stirring at OD_{600} of 0.2, 0.4, 0.6, 0.8 and 1. After incubation time, the hypocotyl segments were blotted on sterilized Whatman paper, and cultured on solid co-cultivation medium (regeneration medium supplemented with 100 mM acetosyringone) and incubated for 3 days at 25 ± 1°C in the dark. After three days of co-cultivation, the explants were gently cultured on selection medium, which consisted of the MS basal medium supplemented with 2 mg/dm^3 BA, 50 mg/dm^3 hygromycin (for plasmid selection) and 300 mg/dm^3 cefotax. The explants were incubated at 25 ± 1°C and 16 hrs photoperiod under light intensity (ca. 50 μmol/m^2/s) generated with a white fluorescent lamp. The infected explants were subcultured every two weeks on a new selection medium.

After four weeks, shoots developed on hygromycin resistance calli were rooted on basal medium. The transformation was evaluated by histochemical analysis of GUS gene expression, specific PCR and RT-PCR analysis.

For histochemical analysis, gus-assay was used according to McCabe et al. (1988). The expression events were identified visually and photographed. This gus-assay reflects the integration of vector DNA, and was a visual marker for vector DNA function in the plant as the GUS gene has the ability to break glucuronic acid which is used by *Agrobacterium* as carbon source. The product of this breakdown was detected by X-gluc in the presence of oxidative factors (such as Fe^{+2} and Fe^{+3}). When the plant cells contain the GUS gene, they will be stained blue indicating the formation of Cl-Br-indigo.

DNA was extracted from control, transgenic callus and transgenic plantlets using Quiagene kit (purchased from Clinilab). Specific PCR reactions with GUS and DREB2A primers) were performed as described by Koehler-Santos et al. (2003) with minor modifications. PCR amplification was performed in 25 μl reaction mixture each containing 0.5U (0.25 μl) Taq DNA polymerase, 0.2 mM (2.5 μl) dNTPs (dATPs, dCTPs, dGTPs and dTTPs), 5 μl (5X) colourless reaction buffer, 20.4 ng (3 μl) genomic DNA and 10 pmole primers (0.5 μl forward + 0.5 μl

reverse) and 13.25 μl sterile distilled water. The mixture was assembled on ice and overlaid with a drop of mineral oil. The GUS primer sequence was (5′-AGTGTACGTATCACCGTTTGTGTGAAC-′3) forward and (5′-ATCGCCGCTTT GGACATACCATCCGTA-3′) reverse. The DREB2A gene primer sequence was (5′-ATTGCTCCGTGCAAGTGAGGAAG-3′) forward and (5′-ATCTCAGCCAC CCACTTACCC-3′) reverse and was used for plasmid transformation. The DNA amplification was performed using Biometra Uno thermal cycler programmed as shown in Table 1.

Table 1. Temperature profile for the PCR amplification of GUS and DREB2A sequence.

Order	Action	Temp. (ºC)	Duration	No. of cycles
1	1st denaturation	94	5 min	
2	Denaturation	92	1 "	
3	Annealing	37	1 min 10 sec	42 cycles
4	Extension	72	2 min	
5	Last extension	72	5 "	
6	Incubation	4		

RNA was extracted from control, transgenic callus and transgenic plantlets with trizol (Chomczynski 1993). In a sterile microcentrifuge tube, cDNA was made by adding RNA (2 - 5 μg) and primer (200 - 500 ng) in a total volume of 15 μl water and the mixture was heated for 5 - 10 min in water bath and spinned for a few seconds and water and dNTPs were added. 5x RT buffer 1 or 5x RT reaction buffer 2 and DTT and $MnCl_2$ or 5x RT reaction buffer 2 and DTT and $MgCl_2$ were added. Then RNase inhibitors, M-MLV RT RNase H minus DNA polymerase were added and the mix was gently mixed and then incubated at 37ºC for 30 - 90 min (M-MLV RT Euromedex). The produced cDNA was used in the specific PCR, using GUS primer [forward (5′-GTGGGTCAATAATCAGGA AG-′3) and reverse (5′- CCAATCCAGTCCATTAATGC-3′)] and was also used to amplify the produced cDNA, as described by Koehler-Santos et al. (2003).

Results and Discussion

Fig. 6 shows fluorescent signals of transformed flax hypocotyl segments. Expression of genes was clearly observed as fluorescent spots in transformed hypocotyl segments (Fig. 2B), while non-transformed flax hypocotyl did not show any fluorescent spots (Fig. 2A). Expression of GFP and DREB2A genes on the transformed flax hypocotyl segments after seven days is evident (Fig. 3A, B).

Table 2 shows expression in the form of the total number of fluorescent signals after 24 and 48 hrs in hypocotyl segments containing the DREB2A gene.

The mean number of fluorescent spots after 24 hrs was 4.32 and increased to 5.45 after 48 hrs. This increase in fluorescent signals is higher by 26% after 48 hrs compared to fluorescent signals after 24 hrs indicating the success of transformation.

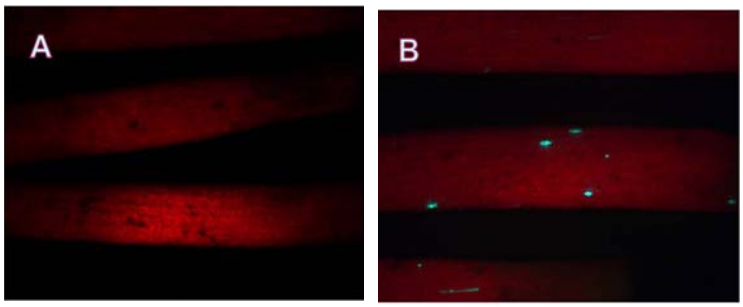

Fig. 2. Photographs illustrating the expression of GFP + DREB2A genes in flax hypocotyl explants after 24 hrs. A: Control, B: Transformed.

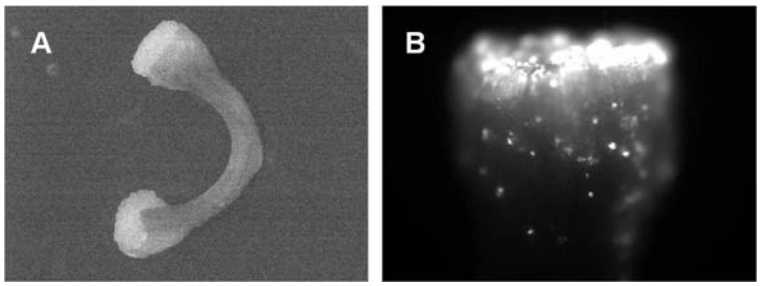

Fig. 3. Expression of GFP + DREB2A genes in flax hypocotyl explants after seven days under blue light microscope. A: Control. B: Transformed.

Callus induction, embryogenic calli and shoot embryos development on hygromycin selection medium were scored after four weeks of cultivation. Hypocotyl segments expanded during the first two weeks of culture followed by the appearance of swellings on the surface near to one end and compact callus at both cut ends of all hypocotyl segments**.** After that, buds and shoot embryos were developed on selection regeneration medium supplemented with BA. After 2 - 4 months of subculture, the hypocotyl segments, which were full of leaf primordia, produced many shoots (Fig. 4A, B). The highest callus induction per cent, somatic embryogenic induction per cent and number of somatic embryos occurred at OD 0.8 (Table 3).

The transformed tissues were uniformly expressed as indicated that, by the blue color stain in comparison with the non-transformed tissues, which did not exhibit any blue color (Fig. 5).

Transgenic flax calli and plants grown under antibiotic selection pressure were applied to GUS assay buffer. Blue color was observed in different tissues which indicate stable integration and expression of GUS gene (Fig. 6).

Table 2. Total means number of fluorescent signals after 24 and 48 hrs of hypocotyl segments contained DREB2A gene.

Plate number	Mean number of fluorescent signals after 24 hrs	Mean number of fluorescent signals after 48 hrs
1	1.5 ± 0.31	1.5 ± 0.36
2	2.1 ± 0.49	2.2 ± 0.43
3	11 ± 1.29	13.8 ± 0.67
4	5.1 ± 0.18	6.1 ± 0.63
5	3.5 ± 0.64	4.8 ± 0.62
6	2.7 ± 0.42	4.3 ± 0.20
Mean	4.32	5.4

Table 3. Callus induction, somatic embryogenic induction and number of somatic embryos per embryogenic calli of hypocotyl segments of flax (cv. Blanka) infected with different *Agrobacterium* concentration cultured for four weeks on selection regeneration medium supplemented with BA.

Optical density (OD_{600})	Callus induction (%)	Somatic embryogenic induction (%)	No. of somatic embryos per embryogenic calli
0.2	67[a]	60 [a]	7 [a] ± 0.74
0.4	47[b]	43 [b]	6 [a] ± 1.32
0.6	53[b]	38 [b]	3 ± 0.77
0.8	87	77	13 [b] ± 0.24
1.0	73[a]	46 [a]	12 [b] ± 0.62
F- value	8.4484	31.125	53.183
Propabilty level (p <)	0.0001	0.0001	0.0001

All values are means ± Sd. Similar letters mean non-significance ($p > 0.05$).

Putative lines: PCR analysis was used to confirm the integration of the DREB2A in GUS positive calli and plants. Genomic DNA of independently transformed and untransformed tissues was checked using specific primers for the GUS reporter gene. The expected amplicons (1200 bp) was amplified in transformed calli and plants, whereas; no amplification was detected in non-transformed tissues (Fig. 7). Also, specific primers for DREB2A gene were used and the expected amplicon (250 bp) was detected in transformed calli and plants, whereas; no amplification was detected in non-transformed tissues (Fig. 7).

RT-PCR analysis was used to confirm the integration of the DREB2A in GUS positive calli and plants. Genomic DNA of independently transformed and untransformed tissues was checked using specific primers for GUS reporter gene. As shown in Fig. 7, the expected amplicon (675 bp) was amplified in transformed calli and plants, whereas, no amplification was detected in non-transformed tissues.

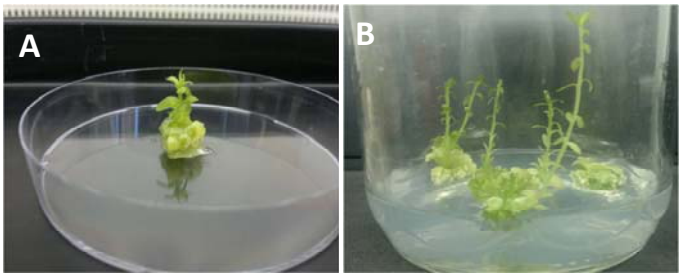

Fig. 4. Shootlet embryos of flax drerived from transgenic callus cultured onto selection regeneration medium. A: After two months. B: After four months.

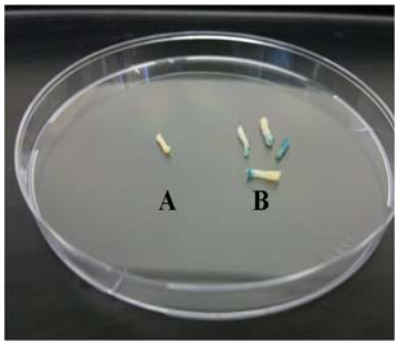

Fig. 5. GUS expression of *Agrobacterium* transformed flax hypocotyl segments after three days on co-cultivation medium. A: Control explants. B: GUS positive.

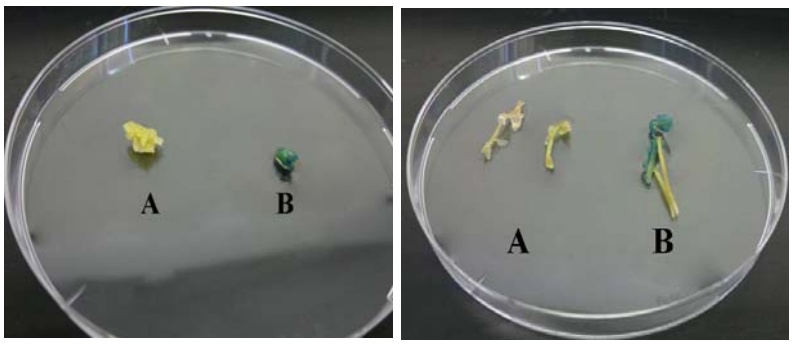

Fig. 6. Photograph illustrating GUS expression in transgenic flax calli and plants. A: Control. B: Positive.

The transformation of flax by bombarding hypocotyl tissues with gold particles coated with DREB and GFP genes was achieved and the expression of genes was confirmed by the presence of fluorescent signals under blue light microscope (Wijayanto and McHughen 1999).

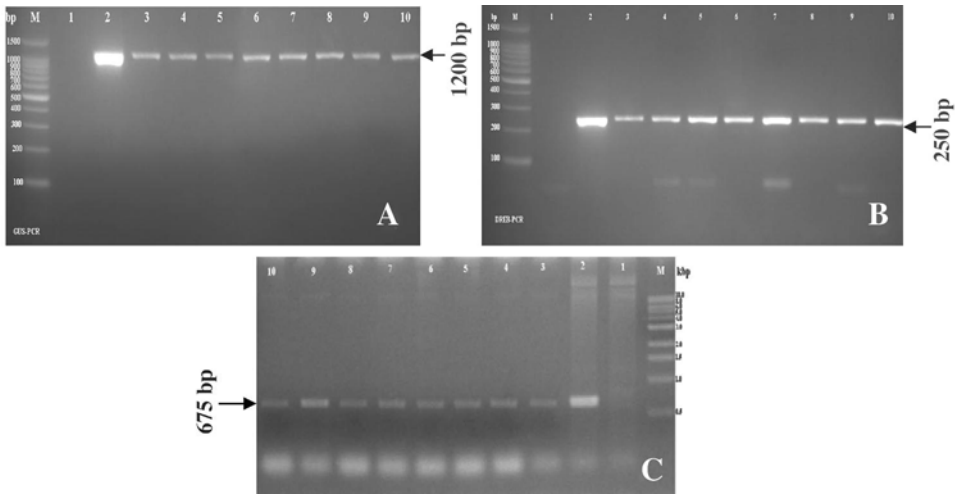

Fig. 7. A: Specific-PCR fingerprinting pattern of GUS primer. B: Specific-PCR fingerprinting for DREB2A gene amplified by DREB2A primer and C: RT-PCR fingerprinting pattern of GUS primer in flax putative lines. M is 100 and 500 bp DNA molecular weight in bp. Lane 1: Negative control (non-transformed), Lane 2: Positive control (plasmid DNA), Lanes (3 - 5): Transgenic calli, Lanes (6 - 10) : Transgenic plants.

Present results showed that the mean fluorescent signal increased over time. Signals after 48 hrs were higher by 26% in comparison with after 24 hrs. These results are congruent with the results of Bleho and Šamaj (2010). Transgenic calli and plants were subjected to histochemical GUS expression assay. Blue color was formed in different tissues indicating the stable integration and expression of GUS gene. The best substrate available for histochemical localization of β-glucuronidase activity in tissues and cells is 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc). The substrate works very well, producing a blue precipitate at the site of enzyme activity.

Genomic DNA of independently random transformed and non-transformed tissues was checked using specific primers for GUS reporter gene. The expected amplicons (1200 bp) were detected in transgenic calli and plants. Also, specific primers for the DREB2A gene were used and the expected amplicon (250 bp) for this gene was amplified in transgenic calli and plants. The expected amplicons (675 bp) for GUS gene was also amplified in transgenic calli and plants in RT-

PCR analysis. The results agree with those of Bhat and Srinivasan (2002), Lamblin et al. (2007) and Yemets et al. (2009).

More recently, Shysha et al. (2013) showed that there are a number of factors affecting on the *Agrobacterium*-mediated transformation of flax, including optical density (OD_{600}) the inoculation time of explants with *Agrobacterium* and co-culture conditions. The PCR analysis was used to confirm the transgenic nature of the obtained lines. The PCR analysis confirmed the transgenic nature of the lines selected on kanamycin, as well as their progenies in the first generation.

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