An efficient regeneration system via somatic embryogenesis in some Egyptian durum wheat cultivars mediated high-throughput transformation of durum wheat using *Agrobacterium tumefaciens*

Ahmed S. Ibrahim

Plant Biotechnology Research Laboratories (PBRL), Plant Physiology Division, Faculty of Agriculture, Cairo University, Egypt.

**ABSTRACT**

In this study, an efficient *Agrobacterium*-mediated transformation protocol for elite Egyptian durum wheat cultivars was established. The obtained results revealed that, the influence of dicamba on induction of embryogenic calli with somatic embryos was significantly high in comparison with 2,4-D and picloram in all tested cultivars. Thus, the callus induction medium (CIM1) supplemented with 2mg/l dicamba considered to be the optimum induction medium for somatic embryogenesis and high regeneration capacity in different genotypes of Egyptian durum wheat (Banisweef 1, Banisweef 4, Banisweef 5, Banisweef 6, Sohag 2 and Sohag 3). Thus, these results proved that, this protocol is less genotype-dependent plant regeneration system and this high level of regeneration in particular with cv. Banisweef 6 (89.66 %) and cv. Sohag 3 (83.66 %) was enabled to attain high frequencies of transformation efficiency ranged from 4.93 % to 14.20 % with an average value 10.06 % according to (Total No. of positive plants/No. of explants) deduced from 10 independent experiments using 1503 explants (scutellum of immature embryos) of cv. Banisweef 6. In Comparison to the deduced results from 10 independent experiments using 1583 explants of cv. Sohag 3 with transformation frequencies ranged from 2.58 % to 10.18% with an average value 6.73%. The obtained results in this study will sustain further development of transgenic plants of other commercial durum and bread wheat cultivars and other cereal crops such as maize, rice and sorghum.

**Key words:** Durum wheat (*Triticum durum* Desf.), immature embryo, somatic embryogenesis, regeneration, Dicamba, 2,4-D, picloram, Transformation, *Agrobacterium tumefaciens*.

**Introduction**

Plant engineering via gene transfer technology "Gene Revolution" is a powerful technique for direct improvement of commercial crops by developing of novel plants expressing a set of valuable traits. Although, the production of stable fertile transgenic cell lines expressing target genes from commercial varieties remains a major barrier, in particular with cereal crops (Ibrahim et al. 2010). Durum wheat (*Triticum durum* Desf.) is a major food crop of the Mediterranean Basin, with a cultivated area of 9% of the world total wheat crop; it possesses an ultra hard endosperm classification and is mainly used for making pasta and semolina (Shewry et al. 1995). It is also a vital part of the development of wheat functional genomic resources due to its ancestral AABB genome composition. During the last two decades many reports were published on wheat transformation via direct gene transfer using microparticle bombardment (Vasil et al. 1992, 1993; Weeks et al. 1993; Becker et al. 1994; Nehra et al. 1994; Zhou et al. 1995; Altpeter et al. 1996; Barro et al., 1998; Lazzeri and Jones, 2009) and *Agrobacterium*-mediated transformation (Komari et al. 1996; Hiei et al. 1997; Hansen and Wright, 1999; Gheyser et al. 1998; Shibata and Liu, 2000; Dai et al. 2001; Matthews et al. 2001; Miller et al. 2002; Jones, 2005; Jones et al. 2005; Travella et al. 2005; Sparks and Jones, 2009). Although, there has been significant progress in *Agrobacterium*-mediated transformation of wheat (*Triticum aestivum* L.) in recent years, it is still remains at low frequency and genotype dependent and confined mainly to a few responsive varieties with quite different transformation frequencies (He et al. 2010) such as the model spring genotype ‘Bobwhite’ (Cheng et al. 1997, 2003), other spring wheat varieties, Fielder, Cadenza, and Veery 5 (Weir et al. 2001; Khanna and Daggard, 2003; Wu et al., 2003; Jones et al., 2005), and the winter wheat variety Florida (Wu et al. 2003; Jones et al. 2005). Therefore developing of an efficient high throughputs *Agrobacterium*-mediated durum wheat transformation system may contribute to furthering our understanding of important physiological processes and elucidation of wheat gene functions (He et al. 2010).

Wheat has different regeneration systems such as somatic embryogenesis and *de novo* adventitious bud formation in *in vitro* tissue culture (Papenfus and Carman, 1987). Somatic embryogenesis has been observed in
several gramineous species such as maize, barley, rice, bread and durum wheat (Benkirane et al. 2000). In wheat species, different explant sources have been used for embryogenic callus formation and plant regeneration; mature and immature embryos (Özgen et al. 1998; Özgen et al. 1996), infloroscences (Redway et al. 1990; Benkirane et al. 2000), coleoptile (Benkirane et al. 2000), shoot apical meristems (Ahmad et al., 2002) and anthers (Asrmstrong et al. 1987). These tissues vary in their ability to regenerate whole plants (Delporte et al. 2001). Immature embryos and immature infloroscences gave the highest frequencies of regenerated plants in vitro (Benkirane et al. 2000).

High regeneration capacity from cultivated durum wheat varieties is a fundamental requirement for establishing efficient genetic transformation protocol of this important cereal crop.

Therefore, the aim of this study was establishment of an efficient Agrobacterium-mediated transformation protocol for elite Egyptian durum wheat cultivars based on a highly efficient regeneration protocol. Here, an efficient and reproducible protocol for regeneration via somatic embryogenesis of six cultivars of Egyptian durum wheat (Banisweef 1, Banisweef 4, Banisweef 5, Banisweef 6, Sohag 2 and Sohag 3) and Agrobacterium-mediated transformation of two cultivars Banisweef 6 and Sohag 3 was established.

Materials and Methods

Plant material, isolation and culture of immature embryos:

Egyptian durum wheat (Triticum durum Desf.) cultivars; i.e. Banisweef 1, Banisweef 4, Banisweef 5, Banisweef 6, Sohag 2 and Sohag 3 were obtained from the Department of Wheat, Field Crops Institute, Agricultural Research Center (ARC), Ministry of Agriculture and Land Reclamation, Egypt. Immature grains of six Egyptian durum wheat cultivars were collected approximately two weeks post anthesis. Grains were surface sterilized with 20% commercial Clorox® (5.25% Sodium hypochlorite) supplemented with few drops of Tween 20, followed by soaking of immature grains for 2 min in sterile de-ionized H₂O (pH 3.0) to equilibrate the high alkalinity of Clorox®, then washing five times in sterile de-ionized H₂O (pH 7.0). Semi-translucent immature embryos in size 1-1.25 mm were aseptically dissected from immature grains of each cultivar under a stereo binocular microscope and cultured on callus induction medium (CIM).

Callus initiation:

For callus induction, fifty immature embryos were cultured with the up-side of the scutellum (embryo axis side) placed in contact to the callus induction medium (CIM) to inhibit the germination of embryos, then after three days the embryo axis was removed using fine-tip forceps, three different callus induction media were used to evaluate their effects on number of regenerated plants per cultured callus derived from immature scutellum. The three tested media basically contains MS salts (Murashige and Skoog, 1962) supplemented by 1mg/l thiamine-HCl, 0.5 mg/l pyridoxine-HCl, 0.5 mg/l nicotinic acid, 250 mg/l myo-inositol, 1g/l casein hydrolysate, 0.69g/l L-Proline, 25 mg/l L-Methionine, 25 mg/l L-Cysteine, 5µM CuSO₄ .5H₂O, 20 g/l maltose, 500 mg/l MES monohydrate: 2-(N-morpholino) ethanesulfonic acid as a buffering agent, 2.5 g/l Gelrite as a solidifying agent and pH at 5.7. The first callus induction medium (CIM1) contains 2 mg/l dicamba (3,6-Dichloro-o-Anisic acid), the second medium (CIM2) contains 2 mg/l 2,4-D (2,4-Dichlorophenoxyacetic acid) and the third medium (CIM3) contains 2 mg/l picloram (4-Amino-3,5,6-tri-chloropicolinic acid). All media used in this study were filter-sterilized using Durapore PVDF 0.22 µm, WHPL 47 mm (Millipore Cat. No. GVWP04700), i.e. double concentrated CIM medium (2x) 250 ml was filter-sterilized then mixed with (2x) 500 ml of Gelrite® 70°C (2x Gelrite® = 5g/l was autoclaved for 20 min at 121°C and 15 psi) and poured 50 ml aliquots into Petri dishes (120 x 20 mm). Cultures were then incubated in the dark for two weeks in controlled growth chamber at 24°C. Subsequently, cultures were then subcultured onto same fresh medium for two more weeks.

Plant regeneration:

Four week-old calli with embryo-like structures were then transferred onto plant development medium (PDM) in Petri dishes (120 x 20 mm) containing 50 ml of MS medium (MS salts and vitamins, 40 g/l maltose and 3 g/l Gelrite®) supplemented with 1 mg/l BA and 0.2 mg/l IAA at 24°C and kept under cool white fluorescent light with 10000 lux under 16 hr / 8 hr light/ dark cycle. Calli of different cultivars were subcultured three times with two weeks interval, then the regenerated plantlets of each cell line were transferred onto magenta box containing 50 ml of root development medium (RDM) which was MS medium (MS salts and vitamins, 30 g/l sucrose and 2 g/l Gelrite®) supplemented with 0.1 mg/l IBA (Indole-3-butyric acid), and kept in the growth chamber under the same light and environmental conditions as previously stated for three weeks.
Acclimatization:

After development of a root system, plantlets were transferred to soil mixture; peat moss : sand (3:1) respectively, in small pots and covered with plastic pages, and then placed in a controlled growth chamber at 22 °C with high humidity 80% for 3 weeks, then successfully transferred to big pots and placed in the greenhouse until maturity.

Agrobacterium strain, vector and inoculum:

Agrobacterium tumefaciens strain AGL1 harbouring the supervirulent pTiBo542 disarmed Ti plasmid (Lazo et al. 1991) was used. The binary vector pWBVec10, Fig. 1 (Wang et al. 1997, 1998) was used for the transformation system using the reporter gene uidA and the selectable marker hpt gene for hygromycin selection. A full strength inoculum with (OD_{600} = 1.0) was obtained by growing standard inoculum in 10ml of MG/L medium (Garfinkel, 1980) for 16-20h with shaking (150 rpm) at 28°C.

Agrobacterium-mediated transformation of durum wheat:

Isolated immature embryos of durum wheat cv. Banisweef 6 and Sohag 3 were precultured for three days on CIM1 containing (2 mg/l dicamba) before the inoculation with Agrobacterium. The scutella of immature embryos were immersed in a full strength Agrobacterium suspension (OD_{600} = 1.0) with 100 mM acetosyringone for one hour, then transferred to a sterile Petri dish to remove excess bacterial solution, and then immediately transferred without rinsing, the scutellar surface placed in contact to the callus induction medium (CIM = 100 mM acetosyringone) 50 explants/Petri dish; 120 x 20 mm. Co-cultivation of immature scutella with A. tumefaciens was performed in darkness for 2 days at 24°C. After co-cultivation, explants were transferred to the rest medium (CIM1) supplemented with 2 mg/l dicamba, 150mg/l Timentin™ (Duchefa) for 7 days. Then the selection process was performed by transferring the explants to the selection medium (CIM) supplemented with 2 mg/l dicamba, 150mg/l Timentin™ (Duchefa) and 50mg/l hygromycin B for 4 weeks (subculture each 2 weeks). Resistant embryogenic callus lines were transferred to plant development medium (PDM) supplemented with 1 mg/l BA, 0.2 mg/l IAA, 25mg/l hygromycin B, and 75mg/l Timentin, and incubated at 24°C under florescent light 16h/day for 6 weeks (subculture each 2 weeks). Regenerated plantlets were transferred to magenta boxes containing 50 ml of root development medium (RDM) supplemented with 0.1 mg/l IBA, 75mg/l Timentin, 25mg/l hygromycin B and solidified by 2g/l Gerlite, then kept in the growth chamber under the same light and environmental conditions as previously stated for 4 weeks.

Acclimatization:

After development of a root system, plantlets were transferred to soil mixture; peat moss : sand (3:1) respectively, in small pots and covered with plastic pages, and then placed in a controlled growth chamber at 22 °C with high humidity 80% for 3 weeks, then successfully transferred to big pots and placed in a growth chamber at 22°C day/18°C night with 70% humidity until maturity.

Fig. 1: Structure and restriction map of the cereal transformation vector pWBVec10.

Abbreviations: Ubi1, promoter, first exon and first intron of the maize ubiquitin 1 gene; uidA, coding region of the E. coli β-glucuronidase gene; nos, 3’ transcript termination region of the Agrobacterium tumefaciens nopaline synthase gene; 35S, CaMV 35S promoter; hpt coding region of Streptomyces hygroscopicus hygromycin B phosphotransferase gene; CAT-1, intron of castor bean catalase gene; LB, T-DNA left border sequence; RB, T-DNA right border sequence; Tn7/SpecR, spectinomycin resistance gene.
Restriction sites are abbreviated as follows: B, BamHI; Ec, EcoRI; Ev, EcoRV; H, HindIII; K, KpnI; N, NcoI; No, NotI; P, PstI; S, SalI; Sa, SacII, Sc, SacI, Sn, SnaBI; Ss, SacI; Sp, SphI, X, XhoI, Xb, XbaI.

**Histochemical assay of GUS activity:**

Histochemical staining for GUS was performed using 5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid (X-gluc) (Jefferson *et al.* 1987).

**PCR analysis of transformants:**

To investigate the presence of transgenes in T₀ and T₁ generations of transgenic plants, standard PCR reactions were performed using 100 ng genomic DNA (Harwood *et al.* 2000). The presence of *uidA* gene was determined by amplification of a 326-bp fragment of the gene using the primer pair (GUS Ting 97) 5'-TAG AAA CCC CAA CCC GTG AAA-3' and 5'-TGG CGT ATA GGC GCC CTC AGT-3'. The primer pair 5'-CTT CGA TGT AGG AGG GCG TGG -3' and 5'- AGC TCC GGA TGC CTC CGC TCG -3' was used to amplify a 845-bp fragment of the *hpt* gene.

**Southern blot analysis:**

Genomic DNA was isolated from leaf tissue of individual transgenic plants; 15 µg of genomic DNA was digested with XbaI to determine the transgenic lines which contain the *uidA* gene as well as to detect the gene copy number in the genome of these lines. The restricted DNA was size-fractionated by agarose gel electrophoresis and transferred to a positively charged membrane (Nylon Membranes, positively charged; cat. no. 11209272001, Roche) according to Southern (1975). Hybridization of membrane was performed according to the method outlined in the (NEBlot® Kit cat. no. N1500S, Phototope®-Star Detection Kit cat. no. N7020S, and Biotinylated 2-Log DNA Ladder cat. no. N7554S, NEB) instruction manual with one modification; the probe of *uidA* gene was generated by PCR amplification using a specific primer pair 5'-CCT GTA GAA ACC CCA ACC GTG G-3' and 5'-GAG CAT CTC TTC AGC GTA AGG G-3' to amplify the 1kb biotinylated probe. The Phototope®-Star Detection Kit was used to detect the transgene sequences on hybridized filters. Then to detect emitted signals the filters were exposed to Fuji x-ray films at 37 °C for 1-4 hours before developing of x-ray films.

**Statistical Analysis:**

Data obtained was recorded and were exposed to the proper statistical analysis of complete randomized design (Snedecor and Cochran, 1969) in three replicates. Means obtained were differentiated using Duncan’s new multiple range test as described by (Duncan, 1955).

**Results and Discussion**

**Regeneration capacity of durum wheat cultivars:**

**Effect of auxin type:**

To date bread wheat due to its hexaploidy and also durum wheat (tetraploid) remain recalcitrant to regeneration and transformation in particular in elite commercial wheat cultivars and still need more efforts to develop efficient transformation protocols. In this study optimizing a protocol for enhancing the regeneration of durum wheat is the basic step towards establishing an efficient transformation system of this important plant. Immature embryos of six Egyptian durum wheat (*Triticum durum* Desf.) cultivars i.e. Banisweef 1, Banisweef 4, Banisweef 5, Banisweef 6, Sohag 2 and Sohag 3 were cultured on three different callus induction media to evaluate their response to the type of auxin; dicamba, 2,4-D and picloram at 2 mg/l for induction of somatic embryogenesis and regeneration capacity. After 3 days of culture of immature embryos on different callus induction medium (CIM1, CIM2 and CIM3) supplemented with 2 mg/l of auxin; dicamba, 2,4-D and picloram, respectively, the swelled embryo axis was removed and the scutellum of each embryo was subcultured on new Petri dish containing the same medium (25 explants/ Petri). Callus proliferation was observed on scutellum tissue after 5-10 days of subculture and the response of cultivars was vary with different media as shown in Fig. (2) and Table (1).

Embryogenic calli with embryoids like structures were observed on 18-28 day-old calli of different cultivars on different medium with high rate in particular on CIM1 supplemented with 2 mg/l dicamba. Four week-old calli were transferred to plant development medium (PDM) supplemented with 1 mg/l BA and 0.2 mg/l IAA and incubated at 24°C under florescent light 16h/day for 6 weeks (subculture each 2 weeks) somatic embryos were germinated and produced green plantlets Fig. (2). Regenerated plantlets were transferred to root development medium to complete the formation of root system of each plant, then after three week plants were transferred to...
small pots for acclimatization in growth chamber at 22 °C with high humidity 80 % for 3 weeks, then successfully transferred to big pots and placed in the greenhouse until maturity.

In addition to the above mentioned observations, the obtained results in (Table 1) revealed that the influence of dicamba on induction of embryogenic calli with somatic embryos was significantly high in comparison with 2,4-D and picloram in all tested cultivars. Thus, the callus induction medium (CIM1) supplemented with 2mg/l dicamba considered to be the optimum induction medium for durum wheat. In addition to the regeneration capacity which recorded the highest levels with calli cultured on (CIM1) followed by (CIM2) supplemented with 2mg/l 2,4-D and (CIM3) supplemented with 2mg/l picloram. The regeneration capacity recorded the highest level in Banisweef 6 (89.66) and Sohag 3 (83.66) followed by Sohag 2 (76.66), Banisweef 4 (73.66), Banisweef 5 (72.33) and Banisweef 1 (70.33).

Fig. 2: Regeneration of durum wheat plants from immature embryos via somatic embryogenesis. (A) Immature grains of Banisweef 6. (B) Callus induction from the scutellum of Banisweef 6 immature embryos after 5 days on callus induction medium (CIM). (C) The embryogenic callus derived from the scutellum of Sohag 3 immature embryo after 3 weeks on CIM. (D) The embryogenic callus derived from the scutellum of Banisweef 4 immature embryo after 4 weeks on CIM. (E) Germination of somatic embryos and development of green plantlets of Sohag 2 after 3 weeks on plant development medium (PDM). (F) Germination of somatic embryos and development of green plantlets of Banisweef 1 after 3 weeks on PDM. (G) In focus germinated somatic embryos of Banisweef 5 after 3 weeks on PDM. (H) In focus germinated somatic embryos of Banisweef 6 after 3 weeks on PDM. (I) Regenerated plants of cv. Banisweef 1, 4,5,6 and cv. Sohag 3 and 2 after 3 weeks on root development medium (RDM). (J) Fertile regenerated plants in greenhouse.
These results confirmed the impact of type of auxin on callus induction and regeneration in durum wheat and it could be proposed that the sensitivity of cells of each cultivar to growth regulators act a major role in induction of embryogenic callus and formation of somatic embryos which determine the regeneration capacity of the genotype. The impact of type of auxin on callus induction and regeneration in wheat has been studied by many researcher; He and Lazzeri (2001), Chen et al. (2006), Nasrclar et al. (2006), Sarker et al. (2007), Sikandar et al. (2007), Danci et al. (2008), Raja et al. (2008 ), Yu et al. (2008), Rashid et al. (2009), Aňzal et al. (2010), Raziuddin et al. (2010), Ren et al. (2010), Tao et al. (2011), Yin et al. (2011) Fahmy et al. (2012) and Murín et al. (2012).

Effect of Cultivar:

The variation in response of wheat genotypes in tissue culture was recorded in several reports, thus screening the amenability to tissue culture of different genotypes is fundamental to find out genotypes with high regeneration capacity. Wheat genotype effect plays an imperative role in plant regeneration, Karadimova et al. (1985).

The obtained results in Table (1) showed that, the variation between the cultivars in regeneration capacity recorded significant differences; i.e. the difference was enough to reach the 5 % level of significance between Sohag 3 and Sohag 2 in addition to the other cultivars except Banisweef 6. Moreover, the average number of regenerated plantlets per explants which derived from germinated somatic embryos recorder significant differences between cultivars, thus, Banisweef 6 recorded the highest value (4.78) which was significantly different than other cultivars even than Sohag 3 which recorded (3.44). These results indicate that Banisweef 6 and Sohag 3 cultivars with their high ability to regenerate through somatic embryogenesis which resulted in a regeneration capacity exceeded 80 % and with the high value of average number of regenerated plantlets per explants which ranged from (3.44 to 4.78) are the most amenable cultivars to be used for transformation experiments. Therefore, Banisweef 6 and Sohag 3 cultivars were used in the transformation experiments using Agrobacterium in this study. The obtained results here are in accordance with many reports on the existed genotype-dependency in wheat, that the variances among the studied durum wheat cultivars in plant regeneration may be regarded to gene action effect of the plant genotype, which in turn could adjust the endogenous hormonal reaction affecting this process.

Genotype dependency was focused to be particularly high in wheat plant regeneration as suggested by many investigator as Agarwal and Tiwari (1995), Gonzalez et al. (2001), Varshney and Altpeter (2001), Mzouri and Amssa (2002), Yadava and Chawla (2002), Li et al. (2003), Sharma et al. (2003b), Yu et al. (2003), Khatri et al. (2006), Nasrclar et al. (2006), Tiwari et al. (2006), Bi et al. (2007), Danci et al. (2008 ), Dodig et al. (2008), Vendruscolo et al. (2008), Shah et al. (2009), Aňzal et al. (2010), Raziuddin et al. (2010), Tao et al. (2011) Fahmy et al. (2012) and Murín et al. (2012).

Table 1: Effect of different type of auxin on embryogenesis and regeneration from immature embryos of durum wheat cultivars; Sohag 2, Sohag 3, Banisweef 1, Banisweef 4, Banisweef 5 and Banisweef 6.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Auxin</th>
<th>No. of explants (immaure Embryos)</th>
<th>No.of embryogenic calli</th>
<th>No. of calli producing green shoot</th>
<th>Average No.of plantlets/ explant</th>
<th>Percentage of regeneration</th>
<th>No. of calli producing shoots/total No. of explants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sohag 2</td>
<td>Picloram</td>
<td>300</td>
<td>213B</td>
<td>182C</td>
<td>175D</td>
<td>230B</td>
<td>205D</td>
</tr>
<tr>
<td>Sohag 3</td>
<td>Picloram</td>
<td>300</td>
<td>221B</td>
<td>210B</td>
<td>196C</td>
<td>251A</td>
<td>236B</td>
</tr>
<tr>
<td>Bensweef 1</td>
<td>Picloram</td>
<td>300</td>
<td>179C</td>
<td>161D</td>
<td>154E</td>
<td>211C</td>
<td>197D</td>
</tr>
<tr>
<td>Bensweef 4</td>
<td>Picloram</td>
<td>300</td>
<td>193C</td>
<td>166D</td>
<td>151E</td>
<td>221C</td>
<td>190D</td>
</tr>
<tr>
<td>Bensweef 5</td>
<td>Picloram</td>
<td>300</td>
<td>183C</td>
<td>174D</td>
<td>178D</td>
<td>217C</td>
<td>205D</td>
</tr>
<tr>
<td>Bensweef 6</td>
<td>Picloram</td>
<td>300</td>
<td>251A</td>
<td>229A</td>
<td>221B</td>
<td>269A</td>
<td>244B</td>
</tr>
</tbody>
</table>

Means followed by different capital letters in columns are significantly different at $P = 0.05$ according to Duncan's multiple range test.
Finally, successful regeneration from embryogenic calli with embryos like structures of different genotypes of durum wheat was established. Regeneration via somatic embryogenesis resulted in formation of green plantlets derived from germinated somatic embryos at high frequency, thus, each callus line produced many plantlets, with developed roots on PDM medium, as shown in (Figure 1 G, H) which proved evidently that most of these plantlets regenerated through somatic embryogenesis. The highest level of regeneration was recorded with Banisweef 6 and Sohag 3 cultivars, for this reason both cultivars were used in the transformation experiments using *Agrobacterium* as described in the following part.

*Agrobacterium tumefaciens*-mediated transformation of durum wheat:

An efficient transformation system of durum wheat cv. Banisweef 6 and Sohag 3, using AGL1 pWBVec10 was established through a set of experiments; ten independent experiments were carried out for each cultivar, as shown in Tables 2 and 3, Fig. (3). Ten successful experiments of durum wheat cultivar Banisweef 6 were performed and resulted in the production of 67 transgenic cell lines with 151 positive plants expressing *uidA* and *hpt* genes using 1503 explants (scutellum of immature embryos) with transformation frequencies % ranged from 2.11% to 6.25% with an average value 4.47% according to (No. of positive lines/No. of explants) and from 4.93% to 14.20% with an average value 10.06% according to (Total No. of positive plants/No. of explants). In Comparison to 55 transgenic cell lines of cv. Sohag 3 with 107 positive plants resulted from ten independent experiments using 1583 explants with transformation frequencies % ranged from 1.74% to 5.98% with an average value 3.52% according to (No. of positive lines/No. of explants) and from 2.58% to 10.18% with an average value 6.73% according to (Total No. of positive plants/No. of explants). The transgenic T₀ plants were tested using GUS assay and PCR analysis for *uidA* and *hpt* genes. Southern blot analysis of representative plants confirmed the presence of the *uidA* gene and integration into the genome of T₀ lines.

The obtained results in this study confirmed the success of this regeneration and transformation protocol which resulted in the production of transgenic durum wheat with the highest transformation rate of durum wheat recorded so far in particular for Banisweef 6 with an average value 10.06% and with a maximum value recorded ranged between 12 -14.20% in 5 independent experiments as shown in Table (2). These values were higher than that reported for bread wheat and other durum wheat varieties. For example, He et al. (2010), recorded an efficiency for *Agrobacterium*-mediated transformation of durum wheat cv. Stewart (in average 6.3%, with a maximum in one batch of 12.3%) and they concluded that the obtained result of transformation efficiency was higher than that reported for bread wheat and other durum wheat varieties by several researchers as follows; Khanna and Daggard (2003) reported 1.2–3.9% for the variety Veery-5 using the LBA4404/pHK21 strain and vector combination (pHK21 is also a superbinary vector containing additional vir genes), while Cheng et al. (1997) achieved 1.4-4.3% efficiency for the variety Bobwhite using C58ABI/pMON18365. Wu et al. (2008) used the same superbinary pGreen/pSoup system (pAL154/pAL156) for the *Agrobacterium*-mediated transformation of durum wheat cv. Ofanto and obtained final transformation efficiencies of between 0.6% and 9.7%, with an average of 3.1%, but transformation of bread wheat varieties Florida and Cadenza with the same *Agrobacterium* strain and plasmid combinations gave only 0.3-3.0% efficiency (Wu et al. 2003).

Also, Chinese bread wheat varieties (Xinchun 9 and Kenong 199) were transformed with the same *Agrobacterium* protocol of (Wu et al. 2003) and the efficiency was almost at the same level as reported by Wu et al 2003, ranging from 0.5% to 3.3%. Birika et al. (2012) reported that, two cultivars of wheat, Kontesa and Torka, and one cultivar of triticale, Wanad, were tested in *Agrobacterium*-mediated transformation experiments. The transformation rates for the wheat cultivars ranged from 0.00 to 3.58% and from 0.00 to 6.79% for triticale. The best values for wheat were 3.58% for Kontesa and 3.14% for Torka, and these were obtained after transformation with the pGreen vector carrying the nptII selection gene under the control of 35S promoter.

Finally, the results of this protocol using *Agrobacterium* are in accordance with several reports which confirmed the superiority of using *Agrobacterium* protocol for wheat transformation in comparison with biolistic protocols which resulted in low transformation rates as reported by many researcher; Wiley (2005) obtained an efficiency of 0.21%, Bell (2003), produced 10 lines at 0.06% efficiency, and Pellengerichei et al. (2002) used three elite CIMMYT durum wheat cultivars to generate an overall efficiency of 1.7%.

**Histochemical Assay of GUS Activity:**

Stable GUS assay was performed using embryogenic callus tissues 3 weeks after inoculation with *Agrobacterium* as shown in Fig. 4. After regeneration, GUS assay of leaf samples of the T₀ transgenic plants was carried out to detect the positive GUS lines (Fig. 4).
Fig. 3: Production of transgenic durum wheat plants using *Agrobacterium tumefaciens* AGL1 pWBVec10. (A) Immature embryo of Banisweef 6 after 3 days of preculture on CIM just before isolation of embryo axis and inoculation of the scutellum with *Agrobacterium*. (B) Scutella of Sohag 3 immature embryos after 2 days of inoculation with *Agrobacterium*. (C) Callus induction from the scutellum of Banisweef 6 immature embryo after 14 days on CIM+ Hygromycin B 50 mg/l + Timentin 150 mg/l. (D) The embryogenic calli with somatic embryos derived from the scutella of Sohag 3 after 4-week on CIM. (E) Distinguished germinated somatic embryos of Banisweef 6, arrow points to mature somatic embryos after two week on regeneration medium (PDM) + Hygromycin B 25 mg/l + Timentin 75 mg/l. (F) Regeneration of putative transformed plants of Sohag 3 after 3 weeks on (PDM) + Hygromycin B 25 mg/l + Timentin 75 mg/l. (G) Regeneration of putative transformed plants of Banisweef 6 after 3 weeks on (PDM) + Hygromycin B 25 mg/l + Timentin 75 mg/l. (H) Transgenic plants of Banisweef 6 after 2 weeks on root development medium (RDM) + Hygromycin B 50 mg/l + Timentin 75 mg/l. (I) Transgenic plants of Sohag 3 after 2 weeks on (RDM) + Hygromycin B 50 mg/l + Timentin 75 mg/l. (J) Fertile transgenic plants in growth chamber; Sohag 3 (left) and Banisweef 6 (right).
Table 2: Agrobacterium tumefaciens-mediated transformation of durum wheat cv. Banisweef 6

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>No. of explants (half embryos)</th>
<th>No. of callus lines</th>
<th>No. of lines with green plantlets</th>
<th>Average No. of positive plantlets / line</th>
<th>No. of positive lines</th>
<th>Total No. of positive plants</th>
<th>Transformation frequencies %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>uidA+ hpt+</td>
<td>uidA+ hpt+</td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>124</td>
<td>11</td>
<td>7</td>
<td>1.80</td>
<td>5</td>
<td>9</td>
<td>4.03</td>
</tr>
<tr>
<td>B2</td>
<td>147</td>
<td>18</td>
<td>11</td>
<td>2.11</td>
<td>9</td>
<td>19</td>
<td>6.12</td>
</tr>
<tr>
<td>B3</td>
<td>168</td>
<td>13</td>
<td>6</td>
<td>2.50</td>
<td>4</td>
<td>4</td>
<td>2.38</td>
</tr>
<tr>
<td>B4</td>
<td>138</td>
<td>22</td>
<td>13</td>
<td>2.57</td>
<td>7</td>
<td>7</td>
<td>5.07</td>
</tr>
<tr>
<td>B5</td>
<td>176</td>
<td>24</td>
<td>15</td>
<td>2.27</td>
<td>10</td>
<td>11</td>
<td>6.25</td>
</tr>
<tr>
<td>B6</td>
<td>169</td>
<td>13</td>
<td>12</td>
<td>2.16</td>
<td>6</td>
<td>6</td>
<td>3.55</td>
</tr>
<tr>
<td>B7</td>
<td>142</td>
<td>8</td>
<td>5</td>
<td>2.33</td>
<td>3</td>
<td>3</td>
<td>2.11</td>
</tr>
<tr>
<td>B8</td>
<td>139</td>
<td>14</td>
<td>9</td>
<td>2.42</td>
<td>6</td>
<td>7</td>
<td>5.04</td>
</tr>
<tr>
<td>B9</td>
<td>135</td>
<td>18</td>
<td>12</td>
<td>2.25</td>
<td>8</td>
<td>8</td>
<td>5.93</td>
</tr>
<tr>
<td>B10</td>
<td>165</td>
<td>15</td>
<td>10</td>
<td>2.14</td>
<td>7</td>
<td>7</td>
<td>4.24</td>
</tr>
<tr>
<td>Total</td>
<td>1503</td>
<td>156</td>
<td>100</td>
<td>2.26</td>
<td>65</td>
<td>67</td>
<td>4.47%</td>
</tr>
</tbody>
</table>

Table 3: Agrobacterium tumefaciens-mediated transformation of durum wheat cv. Sohag 3

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>No. of explants (half embryos)</th>
<th>No. of callus lines</th>
<th>No. of lines with green plantlets</th>
<th>Average No. of positive plantlets / line</th>
<th>No. of positive lines</th>
<th>Total No. of positive plants</th>
<th>Transformation frequencies %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>uidA+ hpt+</td>
<td>uidA+ hpt+</td>
<td></td>
</tr>
<tr>
<td>S1</td>
<td>155</td>
<td>8</td>
<td>5</td>
<td>1.33</td>
<td>3</td>
<td>3</td>
<td>4.94</td>
</tr>
<tr>
<td>S2</td>
<td>148</td>
<td>15</td>
<td>11</td>
<td>1.83</td>
<td>6</td>
<td>6</td>
<td>4.05</td>
</tr>
<tr>
<td>S3</td>
<td>163</td>
<td>12</td>
<td>8</td>
<td>2.40</td>
<td>5</td>
<td>5</td>
<td>3.22</td>
</tr>
<tr>
<td>S4</td>
<td>144</td>
<td>19</td>
<td>11</td>
<td>2.20</td>
<td>5</td>
<td>5</td>
<td>3.47</td>
</tr>
<tr>
<td>S5</td>
<td>172</td>
<td>11</td>
<td>6</td>
<td>1.66</td>
<td>3</td>
<td>3</td>
<td>1.74</td>
</tr>
<tr>
<td>S6</td>
<td>178</td>
<td>25</td>
<td>14</td>
<td>2.29</td>
<td>7</td>
<td>7</td>
<td>4.32</td>
</tr>
<tr>
<td>S7</td>
<td>167</td>
<td>21</td>
<td>15</td>
<td>1.70</td>
<td>8</td>
<td>10</td>
<td>5.98</td>
</tr>
<tr>
<td>S8</td>
<td>153</td>
<td>13</td>
<td>11</td>
<td>1.75</td>
<td>4</td>
<td>4</td>
<td>2.61</td>
</tr>
<tr>
<td>S9</td>
<td>147</td>
<td>18</td>
<td>13</td>
<td>2.25</td>
<td>4</td>
<td>4</td>
<td>2.72</td>
</tr>
<tr>
<td>S10</td>
<td>156</td>
<td>19</td>
<td>11</td>
<td>1.88</td>
<td>8</td>
<td>8</td>
<td>5.19</td>
</tr>
<tr>
<td>Total</td>
<td>1583</td>
<td>161</td>
<td>105</td>
<td>1.93</td>
<td>53</td>
<td>55</td>
<td>3.52%</td>
</tr>
</tbody>
</table>

Southern blot analysis of the uidA gene:

To determine the copy number of the uidA gene, the binary plasmid vector pWBVec10 and genomic DNA of putative transgenic cell lines (tested by PCR) were digested with XbaI (the expected fragments ≥ 2260 bp). Hybridization with the uidA probe detected the fragments in the digested plasmid as well as in digested genomic DNA of four putative transgenic plants of cv. Banisweef 6 representing 4 independent T0 transgenic cell lines (Lanes 1,2,3 and 4) and 3 putative transgenic plants of cv. Sohag 3 (Lanes 5,6 and 7) representing 3 independent T0 transgenic cell lines (Fig. 5a&b). The result of hybridization of the membrane with the uidA probe confirmed the presence of the uidA gene in one copy in 2 tested lines of cv. Banisweef 6 in lanes No. 1 and 3 in fragments ranging from 22 -23 kb in size, and 2 tested lines of cv. Sohag 3 in lanes No. 5 and 7 in fragments ranging from 19 -22 kb in size. The presence of the uidA gene in two copies was detected in 2 tested lines in the genome of the transgenic lines of cv. Banisweef 6 in lanes No. 2 and 3 in fragments ranging from 6 -19 kb in size, and one tested line of cv. Sohag 3 in lane No. 6 in fragments ranging between 10 -12.5 kb in size. In conclusion, these results of Southern blot analysis of randomly selected putative transgenic cell lines confirmed the presence of uidA gene at low copy number (1-2 copies) in genome of all 7 tested transgenic durum wheat plants derived from 7 independent cell lines. Also, these results are in accordance with several reports on low copy number of integrated transgenes in transgenic plants derived from Agrobacterium-mediated transformation protocols; Hiei et al. 1997, Gheysen et al. 1998, Hansen and Wright 1999, Shibata and Liu, 2000, Dai et al. 2001, Jones, 2005, Jones et al. 2005, Travella et al. 2005, Ibrahim 2006, Sparks and Jones, 2009, He et al. 2010, Ibrahim et al. 2010, Bińka et al. (2012).
Fig. 4: GUS assay of callus and leaf samples. (1) Stable expression of uidA gene in embryogenic callus derived from scutellum tissues of cv. Banisweef 6 immature embryos after after 3 weeks of inoculation with Agrobacterium tumefaciens pWBVec10 in comparison with non-transformed calli. (2) Stable expression of uidA gene in embryogenic callus derived from scutellum tissues of cv. Sohag 3 immature embryos after after 3 weeks of inoculation with Agrobacterium in comparison with non-transformed calli. (3) Histochemical GUS assay of leaves from T0 transgenic plants; (A- Banisweef 6, B- Sohag 3 and C- leaf of the control non-transgenic plant).

Inheritance of the uidA and hpt genes in the T1 generation:

PCR reactions were performed to detect the presence of both the uidA gene and the selectable marker hpt gene in the progeny of T0 plants (Fig. 6). Results of GUS assay and PCR reactions were used to assess the inheritance of uidA and hpt genes in the T1 generation of durum wheat cv. Banisweef 6 and cv. Soha3 as shown in (Table 4 and 5).

The results of PCR analysis for uidA and hpt genes using genomic DNA of T1 plants confirmed the inheritance of both genes in the T1 generation. The segregation of the transgenes uidA and hpt in the T1 generation (Segregation of uidA and hpt genes both + : both -) of both cultivars showed ratios near to 3:1 segregation ratio and in average (2.4:1) in cv. Banisweef 6 and (2.5:1) in cv. Soha3 and these results confirm the presence of the transgenes at low copy number and with high frequency of single copy in the generated transgenic plants in this study and these results are in agreement with several reports (Khanna and Daggard, 2003, Cheng et al. 1997, Wu et al. 2006, 2008 and 2009, He et al. 2010, Bińka et al. (2012).

Table 4: Segregation of the uidA and hpt genes in the T1 generation of durum wheat cv. Banisweef 6 transgenic plants

<table>
<thead>
<tr>
<th>Name of T0 Transgenic plant</th>
<th>Total No. of T1 plants tested by GUS assay and PCR</th>
<th>Segregation of uidA and hpt genes both (+) : both (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1-L1 (1)</td>
<td>16</td>
<td>14 : 2</td>
</tr>
<tr>
<td>B2-L1 (2)</td>
<td>11</td>
<td>10 : 1</td>
</tr>
<tr>
<td>B3-L1 (1)</td>
<td>10</td>
<td>6 : 4</td>
</tr>
<tr>
<td>B4-L1 (1)</td>
<td>8</td>
<td>5 : 3</td>
</tr>
<tr>
<td>B5-L3 (1)</td>
<td>16</td>
<td>11 : 5</td>
</tr>
<tr>
<td>B6-L1 (1)</td>
<td>12</td>
<td>8 : 4</td>
</tr>
<tr>
<td>B7-L2 (1)</td>
<td>16</td>
<td>9 : 7</td>
</tr>
<tr>
<td>B8-L1 (1)</td>
<td>14</td>
<td>11 : 3</td>
</tr>
<tr>
<td>B9-L1 (1)</td>
<td>13</td>
<td>9 : 4</td>
</tr>
<tr>
<td>B10- L1 (3)</td>
<td>17</td>
<td>11 : 6</td>
</tr>
<tr>
<td>Total</td>
<td>133</td>
<td>94: 39</td>
</tr>
</tbody>
</table>
Table 5: Segregation of the \textit{uidA} and \textit{hpt} genes in the T\textsubscript{1} generation of durum wheat cv. Sohag 3 transgenic plants.

<table>
<thead>
<tr>
<th>Name of T\textsubscript{0} Transgenic plant</th>
<th>Total No. of T\textsubscript{1} plants tested by GUS assay and PCR</th>
<th>Segregation of \textit{uidA} and \textit{hpt} genes both (+) : both (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1-L2 (1)</td>
<td>12</td>
<td>8 : 4</td>
</tr>
<tr>
<td>S2-L1 (1)</td>
<td>13</td>
<td>11 : 2</td>
</tr>
<tr>
<td>S3-L1 (1)</td>
<td>15</td>
<td>10 : 5</td>
</tr>
<tr>
<td>S4-L1 (1)</td>
<td>12</td>
<td>8 : 4</td>
</tr>
<tr>
<td>S5-L1 (2)</td>
<td>11</td>
<td>8 : 3</td>
</tr>
<tr>
<td>S6-L4 (2)</td>
<td>15</td>
<td>11 : 4</td>
</tr>
<tr>
<td>S7-L5 (1)</td>
<td>11</td>
<td>9 : 2</td>
</tr>
<tr>
<td>S8-L1 (1)</td>
<td>13</td>
<td>8 : 5</td>
</tr>
<tr>
<td>S9-L1 (1)</td>
<td>10</td>
<td>6 : 4</td>
</tr>
<tr>
<td>S10-L1 (1)</td>
<td>12</td>
<td>10 : 2</td>
</tr>
<tr>
<td>Total</td>
<td>124</td>
<td>89 : 35</td>
</tr>
</tbody>
</table>

Fig. 5a: T-DNA of the pWBVec10 vector and the expected fragments of the \textit{uidA} gene when plasmid or genomic DNA digested with XbaI.

Fig. 5b: Southern blot analysis of the \textit{uidA} gene in durum wheat genomic DNA isolated from four putative transgenic plants of cv. Banisweef 6 representing 4 independent T\textsubscript{0} transgenic cell lines (Lanes 1, 2, 3 and 4) and 3 putative transgenic plants of cv. Soha3 (Lanes 5, 6 and 7) representing 3 independent T\textsubscript{0} transgenic cell lines. Genomic DNA (15 µg) of each sample was digested with XbaI to determine the copy number of the \textit{uidA} gene (the expected fragments ≥ 2260 bp). Arrows point to the detected fragments of \textit{uidA} gene in the genome of transgenic plants. Fragments in (lanes 2, 4 and 6) represent the two copies of the \textit{uidA} gene in two transgenic plants of Banisweef 6 and one plant of Sohag 3, respectively. (M) DNA marker size. (P) Plasmid vector pWBVec10 digested with XbaI. (NT) Genomic DNA of non-transgenic plant digested with XbaI.
Fig. 6: PCR analysis of genomic DNA from leaf tissues of T1 transgenic durum wheat plants cv. Banisweef 6 and Sohag 3. (M) DNA ladder 1 kb. (H) Sterile H2O. (P) Plasmid pWBVec10. (NT) Genomic DNA from non-transgenic plant as a negative control. (Lanes 1-6) Genomic DNA from leaf tissues of six T1 transgenic plants of cv. Banisweef 6 derived from six individual T0 transformed lines were used in PCR reactions with the primer set GUS Ting 97 to amplify the 326 bp of the uidA fragment (arrow) and the primer set of hpt gene to amplify the 845-bp fragment (arrow). (Lanes 7-12) Genomic DNA from leaf tissues of six T1 transgenic plants of cv. Sohag 3 derived from six individual T0 transformed lines were used in PCR reactions.

Conclusion:

In Conclusion, an efficient transformation system for elite Egyptian durum wheat cultivars was established by applying this developed protocol which proved to be efficient with barley plant for the model cultivar Golden Promise in the previous work (Ibrahim 2006) and for barley commercial cultivars (Ibrahim et al. 2010).

In the present work regeneration via somatic embryogenesis was established and resulted in high frequencies of regeneration which exceeded 70 % for all tested genotypes. The obtained results revealed that, the influence of dicamba on induction of embryogenic calli with somatic embryos was significantly high in comparison with 2,4-D and picloram in all tested cultivars. Thus, the callus induction medium (CIM1) supplemented with 2mg/l dicamba considered to be the optimum induction medium for somatic embryogenesis and high regeneration capacity in different genotypes of Egyptian durum wheat. Thus, these results proved that, this protocol is less genotype-dependent plant regeneration system and this high level of regeneration in particular with cv. Banisweef 6 and cv. Soha3 which recorded (89.66) and (83.66) respectively, was enabled to attain high frequencies of transformation efficiency ranged from 4.93 % to 14.20 % with an average value 10.06 % according to (Total No. of positive plants/No. of explants) deduced from 10 independent experiments using 1503 explants (scutellum of immature embryos) of durum wheat cv. Banisweef 6. In Comparison to the deduced results from 10 independent experiments using 1583 explants of cv. Sohag 3 with transformation frequencies ranged from 2.58 % to 10.18% with an average value 6.73%. The obtained results in this study will sustain further development of transgenic plants of other commercial durum and bread wheat cultivars and other cereal crops such as maize, rice and sorghum.

References


Wiley, P.R., 2005. The use of genetic transformation to determine the molecular basis for grain texture in wheat. PhD thesis, Department of Biological Sciences and CPI Division, Bristol University and Rothamsted Research.


