Genetic Transformation of Immature Zygotic Embryos of Maize Genotypes via *Agrobacterium tumefaciens*

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Abstract: Many cereals crops are recalcitrant species to genetically modification through their resistance to Agrobacterium infection and recalcitrance to *in vitro* regeneration. However, a routine and efficient transformation protocol of Syrian maize (*Zea mays*) using an *Agrobacterium tumefaciens* standard binary vector system for year-round production of fertile transgenic maize plants was achieved. Immature zygotic embryos of Syrian genotypes and the control hybrid line Hi II were infected with *A. tumefaciens* strain EHA101 harboring a standard binary vector pTF102. The average stable transformation frequency (number of bialaphos-resistant events recovered per 100 embryos infected) of the present protocol was 14.5% for Hi II and 5.9 for Syrian genotypes. The expression of the beta-glucuronidase (GUS) gene was delivered with high efficiency to maize calli, roots and shoots by *A. tumefaciens* carrying the GUS gene was observed. Progeny analysis through polymerase chain reaction (PCR) analysis of total isolated DNA confirmed the integration, expression and inheritance of T-DNA carrying the selectable marker gene bar and reporter gene gus in the genomes of transgenic maize plants. More than 90% of transformants were normal in morphology. The protocol took about 3 months from the start of infection and co-cultivation to the planting of transformants into pots. It is anticipated that this study will assist further enhancement of maize transformation technology leading to develop an updated protocol for the Agrobacterium-mediated generation of maize. To our knowledge, this is the first report providing evidence of the transformation ability of Syrian genotypes via *A. tumefaciens*.
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1. Introduction

Maize (*Zea mays* L.) is one of the most important agronomic crops in the world and plays a pivotal role as a cereal crop for the nutritional intake of humans, via the direct utilisation as a food crop or through livestock as a fodder crop (CIMMYT 2002). In developed agricultural systems highly improved hybrid varieties dominate the market but in less developed agricultural systems such as Syria, indigenously developed varieties are more frequently used and whilst they may have lower yield potential than hybrids, they have often been selected for traits adapted to low productivity soils with low available water capacity.

Genetic transformation using direct or indirect methods is an effective means to integrate beneficial genes from wild relatives or unrelated species into crop plants for the production of genetically altered plants with improved specific traits. *Agrobacterium tumefaciens* mediated transformation is considered as an efficient and indirect method to transfer recombinant DNA into plant genomes (Ke et al., 2001, Cardoza and Stewart, 2004, Tzfira et al., 2002). Despite an initial recalcitrance to *Agrobacterium* in recent years, genes and techniques have become available using *A. tumefaciens* mediated transformation of cereaks (Ke et al., 2001; Repellin et al., 2001; Sahrawat et al., 2003). Transformation of foreign genes into explants such as immature embryos (Shrawat and Lorz 2006), embryogenic pollen (Kumlehn et al. 2006), somatic embryos (Lenis-Manzano et al. 2010) and isolated ovules (Holme, 2006) have proven useful in cereals. The results of *Agrobacterium*-mediated transformation of cereals recently confirmed that this technique is a reliable and a repeatable method for cereals. Frame et,al (2011) has reported an approach for an *Agrobacterium*-mediated transformation protocol used efficiently to transform of two distinct maize genotypes Hi II hybrid and inbred B104 line through direct targeting the immature zygotic embryos (IZE_s). However, *Agrobacterium* mediated genetic transformation of cereals has been largely confined to particular genotypes that combine the amenability to gene transfer by *Agrobacterium* with adequate *in-vitro* regeneration potential. Such restricted genotype limitation severely limits the wide use of this technique. To date it is not known whether or not indigenously bred Syrian maize genotypes have suitable *in-vitro* regeneration capability or transformation potential. In Syria there is keen interest to expand the area of Maize grown but yields are

frequently limited by water availability. Relatively little progress has been made to improve drought tolerance in conventional Syrian maize breeding programmes and the attraction of a genetic transformation approach utilising anti-stress genes is self evident.

Agrobacterium-mediated plant transformation frequency is influenced by several bacterial, plant and environmental factors (Tzfira et al. 2002). At the plant level, it has been reported that the type of plant tissue used was the critical factor of successful *Agrobacterium*-mediated transformation of cereals (Nadolska-Orczyk et al. 2000). On the bacterial side, the density of the bacterial culture (Cheng et al. 2004; Opabode 2006) and the strains ability to attach and transfer its T-DNA to the host cells (Cheng et al. 2004) were described to influence the transformation frequency. Hiei *et al*. (1994) reported that efficient transformation of rice mediated by *A. tumefaciens* requires the appropriate choice of starting materials, tissue culture conditions, bacterial strains and vectors to efficiently ensure gene transfer. Ishida *et al*. (1996) succeeded in integration of one to three copies of the transgenes into host maize plant chromosomes with little rearrangement using immature embryos co-cultivated with *A. tumefaciens* that carried "super-binary" vectors. It has also been shown that integration of T-DNA carrying the marker gene nptII in the genomes of diploid and haploid maize plants could be achieved by the treatment of pistil filaments with agro suspension during artificial pollination (Mamontova et al. 2010).

Here, we present an updated, efficient protocol based *Agrobacterium*-mediated transformation for maize, which has been successfully employed to produce transgenic lines of Syrian maize genotypes.

2. Materials and Methods

2.1 DNA plasmid constructs and *Agrobacterium tumefaciens strains* for plant transformation

Transformation of Hi II hybrid and *Syrian maize genotypes* was mediated by the *A. tumefaciens* strain EHA101 (Hood et al., 1986) harbouring the standard binary vector pTF102 (Frame et al. 2002). 11 *Agrobacterium*-mediated transformation experiments were carried out using EHA101 strain at different times. pTF102 is an 12.1 kb standard binary vector containing the *bar* selectable marker gene cassette which confers resistance to phosphinothrycin, the active ingredient in bialaphos (White et al. 1990), and the *gus*-intron reporter gene under the control of a doubled enhanced cauliflower mosaic virus *CaMV35S*-promoter (P35S). A spectinomycin-resistant marker gene (*aadA*) is carried in the vector for bacterial selection (Hood et al. 1986). The vector backbone is a derivative of the pPZP binary vector (Hajdukiewicz et al. 1994) with a broad host range (pVS1) origin of replication that contains borders derived from a nopaline Ti plasmid. In this vector the tobacco etch virus (TEV) translational enhancer was inserted at the 5' end of the *bar* gene (Carrington and Freed 1990). The soybean vegetative storage protein terminator (Mason et al. 1993) was cloned to the 3' end of the *bar* gene.

Bacterial cultures for weekly experiments were maintained on yeast extract peptone (YEP) medium (An et al., 1988) containing 100 mg L^{-1} spectinomycin (for pTF102 plasmid) and 50 mg L^{-1} kanamycin (for the *Agrobacterium* strain EHA101). Bacterial plates were grown at 28 °C in the dark for 3 days before use.

pTF102 was kindly provided by Professor Kan Wang (Iowa University, USA).

2.2 Maize germplasm

Hi II, the research standard germplasm for maize transformation (Armstrong and Green 1985; Armstrong et al. 1991), originating from the Maize Genetics Coop and four genotypes originating from the General Commission for Scientific Agricultural Research, Syria (GCSAR), were used in this study to test their response to *A. tumefaciens* mediated transformation (Table 1).

Table 1. Description of germplasm used for *Agrobacterium* transformation*

Base Germplasm ID	Pedigree	Cross for transformation	Origin
Hi II (F1)	Hybrid (Hi pA x Hi pB)	HiF1 x HiF1	Maize Genetics Cooperation - Stock Center, USA
Basil-1 (B.1)	Single hybrid	B.1F1X B.1F1	GCSAR- Syria
Basil-2 (B.2)	Double hybrid	B.2F1X B.2F1	
Ghota-1 (GH.1)	Synthetic varieties	GH.1F1 X GH.1F1	GCSAR- Syria
Ghota-82 (GH.82)		GH.82F1 X GH.82F1	

*Immature embryos for transformation experiments were achieved by sib or self pollination for each genotype. Hi F1 plants were sib or self pollinated to produce Hi F2 embryos for transformation of control material in this study.

2.3 Growth of donor plants

Germination of seeds was carried out in 9 cm pots (0.25 L) containing Multi-Purpose compost in a growth chamber (22/20 ^{o}C day/night, 13 hours light, 170 µmol s^{-1} m^{-2} PAR) for three weeks, at the end of which the seedlings had 3-4 leaves with a good root mass and were transplanted into 35 cm pots (20 L) with a substrate mix and 60 g Osmocote Pro slow release fertilizer per pot for fertilization (see Notes 1 & 2). Plants were grown and pollen was collected

from each genotype to pollinate the tassels of the same plant or other plants in the same genotype to produce immature embryos for *in-vitro* studies.

2.4 Tissue culture media

Infection (IM), callus induction (CIM), and selection (SM) media after Zhao et al., (2002) were used for the *Agrobacterium* protocol for maize transformation. Co-cultivation media (CCM) was modified from Zhao et al. (2001) and Olhoft & Somers, (2001) to contain 300 mg L^{-1} cysteine; callus induction and selection media contained a combination of cefotaxime (100 mg L^{-1}) and vancomycin (100 mg L^{-1}) for elimination of *Agrobacterium* after co-cultivation.

All media contained N6 salts and vitamins (Chu et al., 1975), 1.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid, and 0.7 g L^{-1} L-Proline in addition to the following ingredients: infection medium contained 68.4 g L^{-1} Sucrose and 36 g L^{-1} Glucose (pH 5.2) and was supplemented with Acetosyringone (AS, 100 mM) (Sigma, Aldrich) before use; co-cultivation medium contained 30 g L^{-1} Sucrose, 0.85 mg L^{-1} silver nitrate, AS (100 mM), and N6 Vitamins, and 3 g L^{-1} gelrite (pH 5.8); callus induction medium contained 30 g L^{-1} Sucrose, 0.5 g L^{-1} 2- (4-morpholino)-ethane sulfonic acid (MES), and filter sterilized N6 vitamins, 0.85 mg L^{-1} silver nitrate and 8 g L^{-1} purified agar (pH 5.8). Selection medium was identical to callus induction medium but with the addition of 1.5 or 3 mg L^{-1} bialaphos. Infection medium was filter sterilized, whereas all other media were autoclaved.

2.5 Isolation and preparation of immature embryos

Greenhouse-grown cobs were harvested 19 - 22 day after pollination and were stored at 4°C before being dissected. . 1 to 2 donor ears per genotype were dissected for every transformation experiment. The top of the kernel crowns was removed with a sterile scalpel in a laminar flow bench using aseptic technique..Then, F2 immature zygotic embryos IEs (1.5-2.0 mm in length) were aseptically excised, and up to 100 IEs collected in a 2 mL screw cap microtube containing 2 mL of IM. (Table 2).

2.6 Inoculation and co-cultivation of immature embryos

Agrobacterium tumefaciens cultures were grown for 2 to 3 days on solid YEP medium containing suitable antibiotics at 28 °C in the dark (Table 2). On the day of transformation, the *Agrobacterium* colonies were collected from the plate with a spatula and re-suspended in 5 mL of infection medium (IM) supplemented with 100 mM AS (acetosyringone) in a 50 mL tube and incubated at 28 °C on a shaker at 75 rpm for 2 to 4 hours, or at room temperature for 5 to 7 hours. The spectrophotometric optical density OD_{550} of the bacterial culture was measured and adjusted to 0.3 to 0.4 by the addition dilution of a fresh IM immediately before embryo infection. For inoculation, the collected IEs were washed twice with 2 mL of IM and 1 to 1.5 ml of *Agrobacterium* suspension was added to the embryos after the final wash and mixed by gently inverting the tube 20 times. After an incubation period of 5 minutes at room temperature, the IEs were transferred to four dry 4.5 cm filter paper disks to remove excess bacterial solution. Subsequently 40 IEs each were placed with the scutellum side up (embryo-axis side in contact with the medium) onto petri dishes containing CCM (Figure 1). Plates were wrapped with vented tape (leucopore tape, Melford) and incubated in the dark for 3 d at 20°C.

2.7 Callus induction, development and selection of transformed calli

After three days of co-cultivation, IEs were transferred and incubated first on callus induction media (CIM), to initiate callus formation, (Fig. 2, b). Embryogenic callus induction frequency (ECIF) was calculated as a percentage of targeted infected embryos that had initiated embryogenic callus at their scutellum base after 7 to 10 d on callus induction medium (Table 3).

For the first two weeks of selection, immature zygotic embryos (35 IZEs per plate) were transferred to Selection I media (SM_I) containing 1.5 mgL^{-1} bialaphos (Sigma Aldrich, UK). Plates were wrapped with parafilm throughout selection and incubated at 28°C in the dark. In the second selection step, bialaphos was increased to 3 mg L^{-1} for 2 weeks later on SM_{II}, Table 2. Individual clones (from selection I) were transferred and sub-cultured two more times (2 x 14 d) on selection II media.

Transformation frequency % was measured as the number of independent bialaphos-resistant callus events recovered after sub-culturing on SM_{II} with 3 mgL^{-1} bialaphos per 100 immature zygotic embryos infected and selected.

2.8 Regeneration and Rooting

For regeneration of R_0 transgenic plants from embryogenic callus, firstly; immature somatic embryos were produced by sub-culturing of bialaphos-resistant calluses on RM_I supplemented with 1.5 mg L^{-1} bialaphos for 2 weeks in the dark at 25°C, Table 2. Secondly; the regeneration was accomblished by production of mature somatic embryos through further maturation on RM I followed by germination in the light on RM_{II} as described by Frame *et al*. (2000). Petri dishes were sealed with air permeable adhesive tape (leucopore tape, UK).

Table 2. Details of the transformation procedures and media compositions*

Treatment/step	Procedure
***Agrobacterium* Preparation**	Streak agro out from the glycerol stock on YEP medium (5 g L^{-1} yeast extract, 10 g L^{-1} peptone, 5 g L^{-1} NaCl2, 15 g L^{-1} Bacto-agar, pH = 6.8 + 100 mg L^{-1} spectinomycin, 50 mg L^{-1} kanamycin) for 2-3 d, 28 ˚C, dark. Harvest colonies with 5 mL IM (with 100 mM AS) in 50 mL tube, incubate 2-4 h, at 28 °C, 75 rpm.
***Agrobacterium* Infection**	Collect up to 100 IEs in 2mL IM (N6 salts and vitamins, 1.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.7 g L^{-1} L-proline, 68.4 g L-1 sucrose, and 36 g L^{-1} glucose, 100 μM Acetosyringone (pH = 5.2). wash 2x, remove IM, add 1-1.5 ml *Agrobacterium* suspension OD_{550} = 0.3- 0.4, 5 minutes resting at RT, dark. Dry IEs on 4 filter papers (4.5 cm).
Co-Cultivation	72 hours, 40 IEs on CCM (N6 salts and vitamins, 1.5 mg L^{-1} 2,4-D, 0.7 g L^{-1} L-proline, 30 g L^{-1} sucrose, and 3 g L^{-1} gelrite (pH 5.8)+ 0.85 mg L^{-1} silver nitrate, 100 μM AS, 300 mg L^{-1} Cysteine, and N6 Vitamins "added after autoclaving" . 20 ºC, dark.
Resting (callus induction)	40 IEs each for 7 d on CIM (N6 salts and vitamins, 1.5 mg L^{-1} 2,4-D, 0.7 g L^{-1} L-proline, 30 g L^{-1} sucrose, 0.5 g L^{-1} 2- (MES), and 8 g L^{-1} purified agar (pH 5.8)+ N6 vitamins, 100 mg L^{-1} cefotaxime, 100 mg L^{-1} vancomycin, and 0.85 mg L^{-1} silver nitrate (added after autoclaving), 28 ºC, dark
Selection for stable transformation events	35IEs each for 2 x 14 d on SM (N6 salts and vitamins, 1.5 mg L^{-1} 2,4-D, 0.7 g L^{-1} L-proline, 30 g L^{-1} sucrose, 0.5 g L^{-1} MES, and 8 g L^{-1} purified agar (pH 5.8). + N6 vitamins, 100 mg L^{-1} cefotaxime, 100 mg L^{-1} vancomycin, and 0.85 mg L^{-1} silver nitrate, + 1.5 mgL^{-1} Bialaphos, 28 ºC, dark. 20 IEs each for 2-5x 14 d on SM + 3mg L^{-1} Bialaphos, 28 ºC, dark.
Regeneration/ Plantlet formation	10 calli each for 7- 14 d on RM_0 (MS salts and modified MS vitamins, 60 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol, 1.0 mg L^{-1} 2,4-D, 3 g L^{-1} gelrite (pH 5.8), 250 mg L^{-1} cefotaxime, + 1.5 mg L^{-1} Bialaphos. 25 ºC, dark. 2x 14 d on RM_I (no hormones), + 3 mgL^{-1} Bialaphos, 25 ºC, dark. 2x 14 d on RM_{II} (MS Salts and modied MS vitamins, 100 mg L^{-1} myo-inositol, 30 g L^{-1} sucrose, 3 g L^{-1} gelrite, (pH 5.8, no hormones, or Bialaphos), 24 ºC,16 hours light (170 μmol $s^{-1}m^{-2}$).
Plant establishment in soil/ Acclimatization	1 transgenic plant in a glass vials (150 x 25 mm) containing 2/3 strength MS solid medium, for 10- 14 d, 24 ºC,16 hours light (170 μmol $s^{-1}m^{-2}$). Transplant plantlets of 6-10 cm leaf length into soil in a growth chamber for 21 d (22/20 ºC day/night, 13 hours light, 170 μmol $s^{-1}m^{-2}$ photon flux density)

*MS (Murashige and Skoog, for example, Duchefa no. M0221), N6 (Gamborg B5 Vitamin Mixture, e.g., Duchefa no. G0415), IEs- immature embryos. MES (4-morpholino)-ethane sulfonic acid).

2.9 Acclimatization of transgenic plants

Regenerated R_0 plantlets formed roots and shoots and when they had a leaf length of 2 to 3 cm were transferred individually to glass vials containing 15 mL of 2/3 strength MS solid media for further elongation for up to 14 d until they reached a total shoot length of approximately 10 cm. Regeneration (%) was measured as the number of independent transgenic clones successfully regenerated to plants per 100 events for which regeneration was attempted.

Plantlets of 6 to 10 cm leaf length were transferred to soil in a growth chamber and cover with a plastic Humi-dome with one ventilation hole opened for 7 to 10 d. When the plants could be easily lifted out of the small pot (7.5 cm) with roots holding the soil, they were transplanted to a bigger pot (10 cm) and grown on for a further 7 d before removing from the growth chamber (Table 2).

2.10 Histochemical analysis of transient and stable GUS expression

Histochemical GUS assays (Wilson et al. 1995,Jefferson 1987) were carried out to assess transient expression of the GUS gene in immature zygotic embryos 4 or 5 d after infection. Histochemical GUS staining assays were also conducted on all bialaphos-resistant putative transgenic calli recovered from selection. Leaf tissues and male and female flowers of transformed plants and control progeny plants were tested to confirm expression of the GUS transgene in R_0 and R_I progenies of the studied germplasms. Samples were submerged in the substrate (see note, 3), vacuum infiltrated for 2 - 5 min, and incubated at 37°C overnight. Blue staining cells were visualized after removing the staining solution and soaking leaf tissues in 50% ethanol followed by several changes of 50% ethanol to remove chlorophyll. Plant tissue pieces were scored as positive or negative for GUS expression.

2.11 Isolation of DNA and PCR analysis

Genomic DNA was isolated from 100 mg of transformed callus and leaf tissue of individual regenerated plants according to manufacturer's instructions using liquid nitrogen (GenElute™ Plant Genomic DNA Miniprep Kit, sigma, cat: G2N70).

The expression of selected genes was quantified by PCR. Standard PCR reactions were performed using specific primers of the selection gene bar and the *gus*-intron reporter gene:

GUS-f 5' CAACGTCTGCTATCAGCGCGAAGT 3' and

GUS-r 5'TATCCGGTTCGTTGGCAATACTCC 3'
bar-f 5' TCTACACCCACCTGCTGAAGTC3 'and
bar-r 5' AAACCCACGTCATGCCAGTTCC3 '.

100 ng of pTF102 plasmid was used as a positive control and non-transformed DNA used as a negative control. Samples of genomic DNA were prepared usind REDTaq® DNA Polymerase kit (Sigma, cat: D4309-250UN); cycling was controlled by Applied Bio system (Step One Plus) programmed with the following conditions: initial denaturation at 94 °C for 2 minutes; denaturation, 94 °C for 30s; annealing, 60 for 1 min; extension, 72 for 30s; final extension, 72 for 7 min; and then held at 4. Samples were subjected to 40 cycles for denaturation, annealing and extinsion.

Amplified DNA was separated by electrophoresis in 1% agarose gel using 100 bp ladder (Fisher Scientific UK Ltd, BPE2581-200).

2.12 Progeny segregation analysis for *bar* gene expression

R1 and R_0 progeny of transgenic and control germplasm was screened for *bar* gene expression using a bialaphos (glufosinate) leaf-spray test (Brettschneider et al., 1997). Plants were sprayed three times 14 d after planting in the soil with a 250 mg L^{-1} solution of glufosinate prepared from the herbicide of Glufosinate-ammonium (Sigma Aldrich, CN. 45520). The glufosinate resistant (alive) or sensitive (dead) scores were recorded 3 days after the spray.

Leaf segments (10 mm long) from 30-day-old regenerated plantlets were also screened on Agar medium containing different concentrations of glufosinate. The Petri dishes were sealed with parafilm and incubated at 25 °C. Changes in the colour of the segments were examined 2, 3 and 4 days after the start of incubation. A resistant segment, which expressed the transgene, stayed green whereas a sensitive segment (non-transgenic) turned yellow.

3. Results

3.1 Genotypes response for callus formation

To assess the response of genotypes for callus formation on callus induction medium containing 300 mg L^{-1} Cysteine (Cys), embryos were infected and cocultivated as described (table. 2), then transferred to callus induction medium for 7 to 10 d, after which they were assessed for embryogenic callus initiation. The percentage of embryo response for callus formation (No. of embryos initiated to embryogenic callus per 100 embryos cultured) was assessed over eleven independent experiments. Sixty five percent of 573 hybrids embryos that *A. tumefaciens*-infected embryos produced embryogenic callus compared with 56 % of 361 varieties embryos and 72 % of 228 infected embryos of Hi II hybrid (Fig. 1). On the other hand, of 437 non-infected embryos (across all genotypes) cultured on cocultivation medium containing Cys, 73 % produced embryogenic callus while 62 % of 1162 infected embryos of all studied genotypes produced embryogenic calli.

3.2 Stable transformation frequency

To determine whether Syrian maize germplasm had suitable transformation ability, bialaphos-resistant callus events were identified by their ability to recover on a selection medium containing 3 mg L^{-1} bialaphos (Fig. 2, c), and by their sustained growth to produce embryogenic callus (Fig. 2, d), and mature somatic embryos (Fig. 2, f), that transferred to a regeneration medium as described in Table 2. Results from 23 transformation events (11 for hybrids, 7 for varieties and 5 for Hi II) showed that the stable transformation frequency (No. of bialaphos-resistant events recovered per 100 embryos infected) in these experiments averaged 5.2% and ranged between 2.2 % and 10.9 % for hybrids while averaged 6.5 % and ranged between 2.5 % and 10.1 % for varieties compared with average of transformation frequency 14.5 % of infected Hi II embryos (Tables 3 and 4).

Stable transformation frequency based on GUS gene expression was calculated and whereas 81% of bialaphos-resistant events recovered expressed the GUS gene for varieties, 92% of the bialaphos-resistant events of hybrids showed a positive GUS gene expression (Table, 3). However the percentage of recovered events expressing the GUS gene of total Hi II events resistant to bialaphos was 76%. All experiments produced transgenic events, emphasizing the reproducibility of this protocol for maize transformation.

3.3 Plant regeneration

Bialaphos-resistant embrogenic callus events were regenerated on regeneration medium containing bialaphos. Over 37 transgenic events were regenerated to plants (Fig. 2, g) and grown on. In contrast, seed harvested in glasshouses from crossing of 10 control plants representing 4 of non transferred events (Fig. 2, j). There were differences between genotypes regarding their regeneration ability to produce a plant from somatic embryos. Frequency of regeneration for varieties was higher (57%) than that for hybrids (11%) but of the 26 survived callus events with mature somatic embryos for which regeneration was attempted, 21 event of Hi II regenerated to transgenic plants successfully (Table, 4). The average percent of regeneration frequency for transferred events attempted of Hi II was the best (81%) compared with that from non transferred events (96%). However, Syrian varieties also showed a good efficiency of regeneration 54% - 60% for transgenic events from Ghota-1 and Ghota-82 respectively (Fig. 2, i).

Table 3. Efficiency of *Agrobacterium tumefaciens*-mediated transformation of Syrian maize hybrids, Basil-1 and Basil-2. (NA, not applicable; NT, not tested).

Genotypes/ construct	Experiment date	No. of inoculated immature embryos (A)	No. of callus formation [a]	No. of Bialaphos-resistant events recovered (B)	No. of callus events expressing GUS gene [e] (C)	No. of events regenerated to plants/ No. of events attempted (D)	Frequency of regeneration [b] D, %	Transformation Frequency [c,d]	
								B/A,%	C/A,%
B.1/pTF102	27/08/12	46	34	5	5	1/5	20	10.9	10.9
	30/08/12	36	24	3	NT	contam		8.3	_
	13/09/12	35	27	0	NA	NA		0.00	0.00
		138	96	3	3	1/3	33.3	2.2	2.2
	20/0912	25	8	2	2	0/2	0	8.00	8.00
	04/10/12	97	60	5	4	0/5	0	5.2	4.1
Total		377	249	18	14	2/15	13.3	4.8	4.1
B.1/ Control	27/08/12	35	26			3/13	23.08		
	30/08/12	12	7			0/10	0		
	20/09/12	25	18			6/12	50		
Total		72	51			9/35	25.71		
B.2/ pTF102	25/08/12	35	20	1	1	0/1	0	2.86	2.86
	30/08/12	40	30	3	2	0/3	0	7.5	5.00
	13/09/12	40	25	1	1	0/1	0	2.5	2.50
	20/09/12	25	15	1	1	1//1	100	4	4.00
	25/09/12	29	17	3	3	0/3	0	10.34	10.34
	04/10/12	27	17	2	2	0/2	0	7.41	7.41
Total		196	124	11	10	1/11	9.09	5.61	5.10
B.2/Control	25/08/12	33	32			1/19	5.26		
	27/08/12	13	10			1/3	33.33		
	30/08/12	59	41			4/30	10		
		20	NT			1/13	7.69		
	20/09/12	23	12			0/4	0.00		
Total		128	95			7/69	10.2		

a: Number of callus initiated on callus induction medium which based to calculate the callus formation %.
b: Frequency of regeneration= (no. of events regenerated to plants/ no. of events attempted) x 100.
c: Transformation frequency = independent bialaphos (3 mgL^{-1}) resistant events recovered / total of embryos infected (X100).
d: Transformation was calculated as the number of GUS positive callus or explants among the total number of embryos infected,%. Results were scored 3 – 5 subcultures after treatment with *A.tumefaciens* or when the explants were produced (Independent transgenic plants/inoculated immature embryos x100).
e: Intron gus: a β-glucuronidase (GUS) gene that carries an intron in the coding sequence (intron-gus) (Ohta et al. 1990; Jefferson 1987).

Table 4. Efficiency of *Agrobacterium tumefaciens*-mediated maize transformation of *Hi II and Syrian varieties, Ghota-1 & Ghota- 82.*

Genotypes/ construct	Experiment date	No. of inoculated immature embryos (A)	No. of callus formation [a]	No. of Bialaphos-resistant events recovered (B)	No. of callus events expressing GUS gene [e] (C)	No. of events regenerated to plants/ No. of events attempted (D)	Frequency of regeneration [b] D, %	Transformation Frequency [c,d]	
								B/A, %	C/A,%
Gh.1/ pTF102	21/08/12	40	22	1	NT	1//1	100	2.5	-
		33	20	0	NA	NA			0.00
	25/08/12	35	23	1	0	0/1	0	2.9	0.00
	13/09/12	32	20	3	2	0/3	0	9.4	6.3
	02/10/12	79	41	8	8	6/8	75	10.1	10.1
Total		219	126	13	10	7/13	53.9	5.9	5.6
Gh.1/control	25/08/12	66	50			20/29	69		
	13/09/12	15	9			2/7	28.6		
Total		81	59			22/36	61.1		
Gh.82/	30/08/12	57	30	2	2	1//2	50	3.5	3.5
pTF102	13/09/12	15	8	1	NT	0/1	-	6.7	0
	02/10/12	70	40	7	5	5/7	71.4	10	5.7
Total		142	78	10	7	6/10	60	7.04	5.50
Gh.82/Control	30/08/12	17	14			4/7	57.14		
	13/09/12	55	41			20/29	69		
	15/10/12	29	13			11/13	84.62		
Total		101	68			35/49	71.4		
Hi $_{II}$/ pTF102	22/09/12	78	53	15	12	5/9	55.6	19.2	15.4
	25/09/12	50	37	7	5	5/6	83.3	14	10
	15/10/12	44	32	7	6	7/7	100	15.9	13.6
	02/10/12	29	22	2	2	2/2	100	6.9	6.9
	04/10/12	27	20	2	0	2/2		7.4	0
Total		228	164	33	25	21/26	80.8	14.5	11.0
Hi$_{II}$/control	22/09/12	30	25			6/6	100		
	25/09/12	25	19			17/18	94.4		
Total		55	44			23/24	95.8		

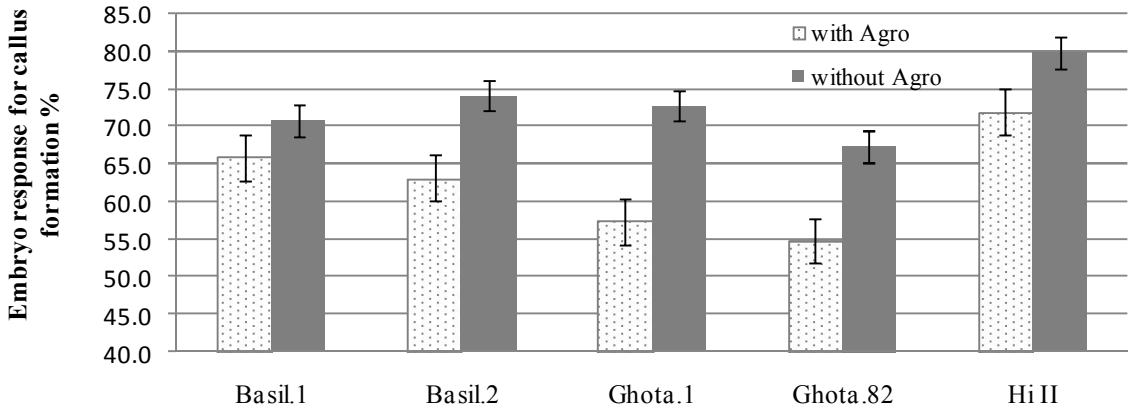

Figure 1. Response of *A. tumefaciens*-infected or noninfected embryos of maize genotypes for embryogenic callus formation. Data from 11 experiments (with *Agrobacterium* mediated transformation), and 7 experiments (without *Agrobacterium*).

3.4 Transient GUS expression

Transient expression of GUS gene was analyzed in the infected immature embryos.

Monitoring of the level of expression of a transgene in the immature embryos after 3 to 10 days of inoculation gave a very useful indicator for optimization of the protocol (Fig. 2, b). Also, a histochemical GUS staining assay was carried out on bialaphos-resistant callus events or on regenerated transgenic plants to determine whether those expressing the *bar* gene also expressed the *gus* reporter gene.

Of the 80 events analyzed for GUS staining, 66 events (83%) were GUS positive (Tables 3 and 4). All blue spots on the tissues derived from bialaphos-resistant callus (Fig. 2,e), or from leaves of transgenic plants (Fig. 2,h) are GUS-positive areas referring to an efficient transformation with pTF102- plasmid. In contrast, non transferred callus and control explants tissues showed no GUS-positive staining (Fig. 2, h). The positive results of the GUS assay in transgenic plants even appeared clearly in roots (Fig. 2, h) indicating constitutive expression.

3.5 PCR analysis of transgenic plants

Putative transformation events that are GUS-positive were further analyzed by standard PCR reactions with the appropriate primers using 100 ng of genomic DNA. DNA from bialaphos-resistant callus and from ten R_0 of transformed plants were subjected to PCR amplification of a 280 bp fragment within the Bar coding gene (Fig. 3). Four transformed plants of varieties and hybrids have been identified and DNA extracted from R_1 progeny of these plants was amplified by PCR (Fig. 3b). Results indicated the presence of bar gene in both R_0 and R_1 progeny of transformed plants. The PCR products were visualized following gel electrophoresis (Figure 3).

3.6 Stable GUS expression

To confirm that the *bar* gene is expressed in transgenic plants, 30–40 days after being transplanted into the soil, plants were sprayed with 350 mg L^{-1} glufosinate prepared from the glufosinate -ammonium /Phosphinothricin herbicide (Sigma- UK, CN. 45520) and 0.1% Tween 20 (v/v). A glufosinate leaf-spray test (Brettschneider et al. 1997) was curried out three to five times at 1 to 2 day intervals using a glufosinate solution. Then plants were scored for herbicide resistance 2, 3, 4 and 5 days after the spraying those which expressed the transgene, stayed green and alive (Fig. 2, j), whereas non-transgenic plants turned yellow and showed herbicide sensitivity and eventually died. The majority of resistant transgenic plants also expressed positive GUS expression.

3.7 Inheritance and fertility of R_1 generation

The expression of the GUS transgene in segregating offspring of the studied germplasms was confirmed by GUS assay. Some of the outcrossed progeny of variteis carring the transferred DNA with GUS gene were tested for stable GUS expression. Silks and tasels of R1 generation were expressed positive GUS expression (Fig. 4) reffering to a stable transformation within transgenic maize plants produced using Agrobacterium- mediated DNA transfer to plant cells. Only three of the twenty six transformed embryogenic calli obtained from hybrids produced transgenic maize plants. In contrast, thirteen transgenic plants of twenty three transformed embryogenic calli of the varieties were produced. Progenies of these plants contained and expressed the foreign genes (Fig. 2, 3 and 4) were fertile transgenic plants. Data of the two fertile plants of the initial events from each genotype are presented in Table 5.

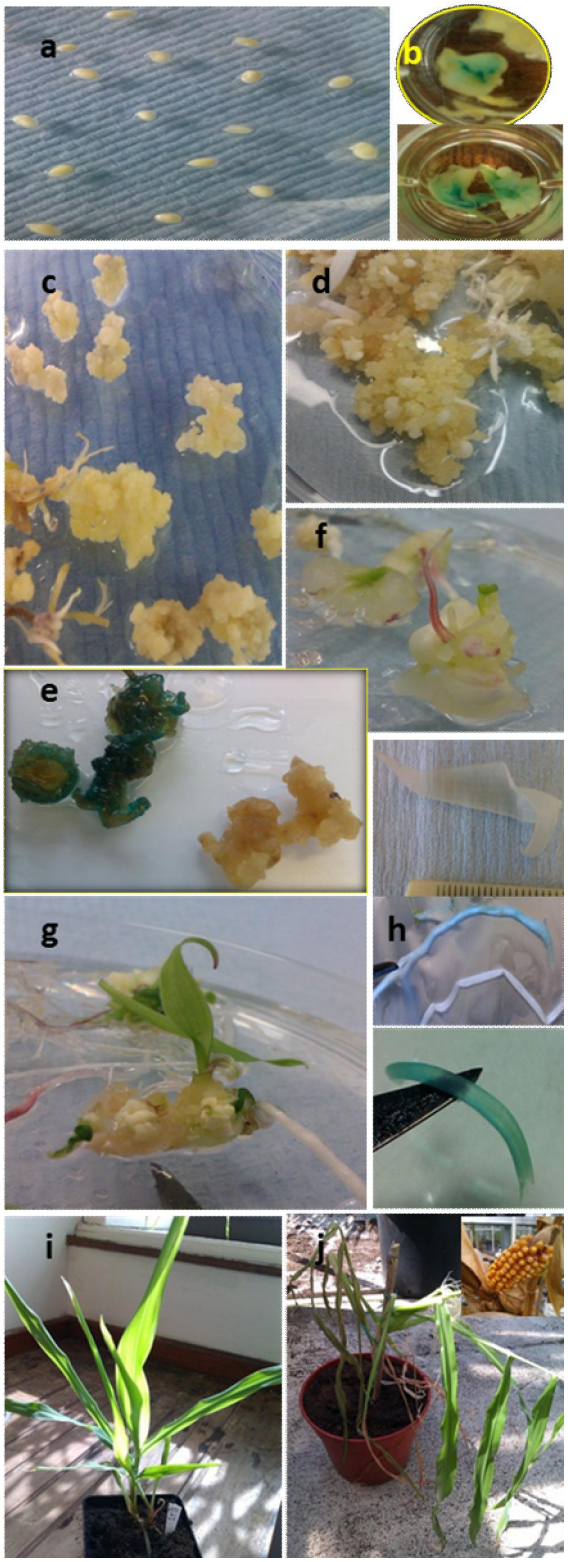

Figure 2. Immature embryos during co-cultivation with *A. tumefaciens* standard binary vector system, pTF102

(a) Transient Gus gene expression and distribution of blue foci in Syrian maize immature zygotic embryos infected with *A. tumefaciens* after 3- 4 days of cocultivation on medium containing 300 mg L^{-1} Cys, (b upper), and after 7 -10 days of culturing on callus induction medium (b, lower). Putative transformed callus growing on 3 mg L^{-1} bialaphos, (c) Bialaphos-resistant, embryogenic callus event emerging from a single clone, (d) GUS-positive staining of the *gus* gene expression in bialaphos-resistant callus event, (e left), whereas, non transformed callus (on the right) expressed GUS-negative staining. Mature somatic embryos derived from embryogenic callus on regeneration medium containing 3 mg L^{-1} bialaphos, (f) Regeneration of transformed mature somatic embryos (g) shooting and rooting of transformed clones. Transgenic plants production in the lab, (i) Stable *Gus* (h) and *bar* (j) transgene expression in transgenic plants. The leaf segment (down) was a GUS-expressing plant (positive), whereas that up was nonexpressing (negative) plant, (h) The surviving plant on the right was a *bar*-expressing plantlet (resistant) to glufosinate herbicide spray, whereas the nonexpressing plant on the left (sensitive) died (j).

Lane L, 100 bp ladder; lane 1, positive control pTF102; lanes 2, 3, 4, 5, and 6, DNA of transformed callus; lanes 7, 8, 9 and 10, transformed DNA from regenerated plants; lane c, negative control. Fig. 3B, containing the detection of transformed DNA of R_1 progeny. Lane L, 100 bp ladder, lane 1 and 2, transformed DNA from varieties; lane 3, non transformed DNA; lanes 4 and 5, transformed hybrids DNA; lane c, negative control.

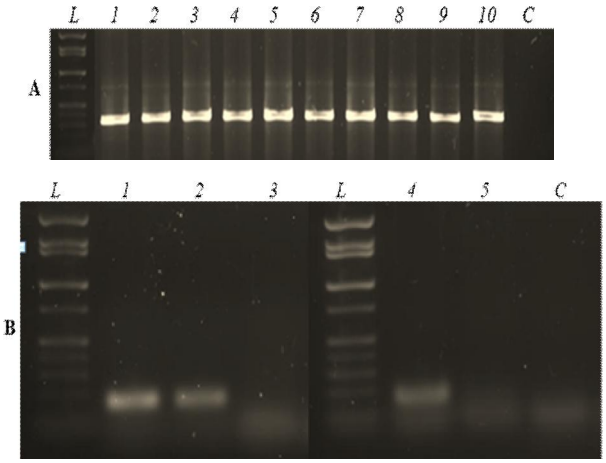

Fig. 3. PCR amplification of transformed progeny. Fig. 3A, detection of DNA fragments containing bar gene sequences of transformed and nontransformed DNA of R_0 progeny.

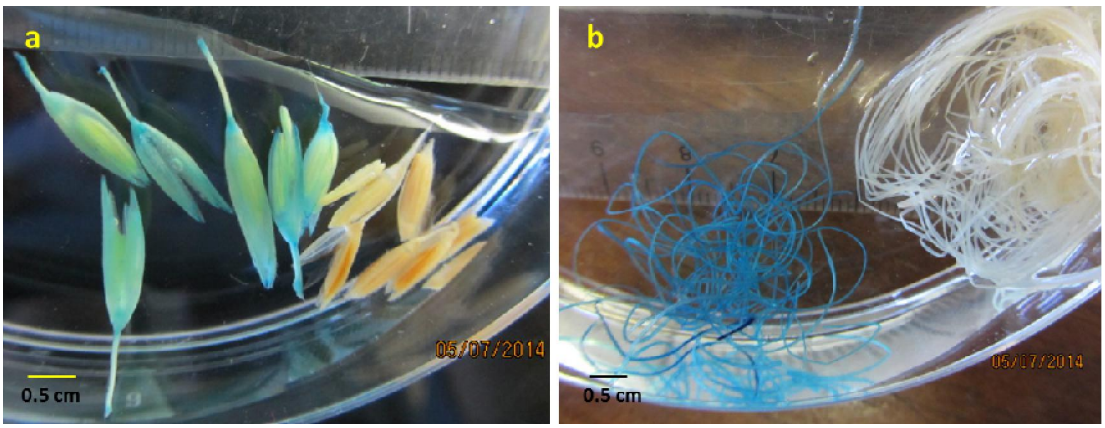

Figure 4. Stable GUS transgene expression in R_1 progeny of transgenic maize plants.

Transient GUS gene expression and distribution of blue foci in transformed tassels, GUS-positive staining (a, left), and GUS-negative staining of non transformed tassels (a, right). and female flowers derived frome R1 progeny of transformed maize plants. GUS staining assay of the gus gene expression in transformed silks(b left) and non-transformed silks expressed GUS-negative staining (b, right).

Table 5. Segregation data of fertile progienies of transgenic maize palnts and nontransgenic plants. NT, not tested.

Genotypes	Event number*	R_0 Progeny				R_1 Progeny			
		No. of rows/cob	Average seeds/cob	Weight of 1000 seed(g)[b]	% plants produced seed (n=13-17)	No. of rows/cob	Average seeds/cob	Weight of 1000 seed(g)	% plants produced seed[c] (n=3-4)
Varieties	56/Gh.1	12	216	284		10	180	311	
	9/Gh.1	12	156	296		14	224	288	
	60/Gh.82	10	180	319		12	192	301	
	61/Gh.82	14	252	311		14	280	325	
	Average	12	201	302	92	12	219	306	75
Control[a]		12	192	307	94	12	228	303	100
Hybrids	16/B.1	10	80	219		10	150	225	
	65/B.1	12	120	215		NT	NT	-	
	29/B.2	12	132	260		14	308	261	
	Average	12	111	231	88	12	229	243	100
Control		14	142	244	93	14	280	240	100
Hi II	53	10	200	297		NT	NT	-	
	54	8	160	302		10	200	299	
	Average	10	180	300	93	10	200	299	NT
Control	55	10	180	297	93	10	200	302	NT

*Plants were given unique numbers when transformed . Numbers denoting the plate that which the transgenic plant derived for each genotype. The female plant was pollinated with the same plant or crossed with another plant for each genotype. [a]Control data were collected from three non-transgenic plants. [b]In some cases, there was not a vailable pollen to pollinate all the silks properly (Fig. 4). [c]Percentage of R_1 plants produced seed obtained from data based on 3 or 4 plants.

4. Discussion

Results showed that the response of the hybrids for callus formation was greater than for the varieties. There were significant differences within the studied germplasm of maize according to their response to produce of somatic embryos derived from surviving embryogenic callus. Even though the hybrids were quicker than varieties in callus formation, they were the slowest in the production of embryogenesis callus and development of mature somatic embryos. The rate of recovery of bialaphos-resistant clones from regenerable callus was affected by maize genotype.

Different genotypes needed a different number of sub-cultured callus to produce embryogenic callus. However, depending on the genotype, embryogenic callus types were recommended for manual selection after 2-3 subcultures for hybrids and 4-5 subcultures for varieties. But, the varieties response of regeneration was different significantly and was better than the response of hybrids (Fig. 4). Of the 26 transformed clones of the control genotype, Hi II, 21 clones successfully regenerated to plants.

This report demonstrated that the Syrian varieties have gathered between the ability to suitable transformation and a regeneration. Thereby, local genotypes can be transformed via *A. tumefaciens*-mediated transformation. This ability opens the doors to improve future maize breeding by transformation with anti-stress genes and with the potential to acquire improved stress resistance. To our knowledge, this marks the first report in which a reproducible method for Syrian maize transformation using an *A.*

tumefaciens standard binary vector system has been demonstrated.

Notes

(1) The substrate mix consists of a 1:1:1 volumetric mixture of John Innes N^{o} 2 compost + peat-based multi-purpose compost and sand with 60 g Osmocote Pro per pot for fertilization.

(2) Osmocote Pro is a general long-term slow release fertilizer that contains 19% N, 7% P and 10% K.

(3) GUS buffer solutions (made fresh immediately prior to use): sodium phosphate buffer NaH_2PO_4 (pH 7.0) 0.1 M + NaEDTA, 10 mM + Triton X-100, 0.1 % + potassium ferricyanide K_3Fe (CN), 0.5 mM + X-Gluc, 2.0 mM + H_2O.

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