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Agrobacterium-Mediated Transformation and in Vitro Regeneration of Tomato (Lycopersicon Esculentum Mill.) Plants Cv. Castlerock

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ABSTRACT

A protocol for Agrobacterium-mediated transformation was developed for tomato plant (cv. CastleRock). Transformation was carried out using disarmed A. tumefaciens strain LBA4404 harbouring a binary vector pITB-AFP. Sequencing of plasmid DNA extracted from this strain indicated that it contains defesin gene (AFP), an antifungal protein-coding gene, under the control of a CaMV 35S promoter and nopaline synthase (NOS) terminator, hygromycin phosphotransferase (hpt) and β–glucuronidase (GUS) genes, as selectable and marker genes, respectively.

The factors that affect transformation/regeneration protocols were optimized in a series of experiments. Results indicated that exposure of cotyledonary explants to Agrobacterium inoculums of 0.8 $O.D_{600}$ for 30 mins, selection on Hygromycin-containing medium (Hygromycin concentration of 25mg/L) and subsequent regeneration on MS medium supplemented with 2.5mg/L BA as a cytokinin and 1.0 mg/L IAA as an auxin resulted in transformation efficiency of 11%. GUS expression was observed in transformed tomato shoots but never in the control plants. PCR amplification of DNA extracted from the transformed tissues demonstrated the generation of the expected amplicon, corresponding to AFP gene. This result strongly verifies the successful transformation of the tomato cultivar CastleRock, an endeavour which is reported for the first time in Sudan. Moreover, this protocol paves the way for problem solving-applications encompassing other Sudanese crops of economic importance.

Key Words: Agrobacterium, castleRock, transformation, defensin, PCR

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INTRODUCTION

Tomato (Lycopersicon esulentum Mill.) is the major vegetable crop grown worldwide, with a production estimates of 95 million Mt *(FAOSTAT, 2002)* and its production is concentrated in semi-arid regions *(Santa-Curz, et al. 2002).* Presently, tomato is becoming increasingly important in Sudan for local consumption and for export. It is cultivated throughout the year under irrigation in an area that exceeds 36540 hectares with an average yield of 17.57 tons per hectare *(AOAD, 2007)*. The most important grown cultivars are the canning types such as Strain B, Strain C, Peto86, Peto111 and CastleRock in addition to few local varieties.

In Sudan, cultivated tomatoes suffer from many diseases that are caused by viruses, bacteria and fungi. Among virus diseases, tomato mosaic root knot and tomato yellow leaf curl are considered as the most important. Bacterial diseases include bacterial wilt (Pseudomonas solanacearum), bacterial canker (Corynebacterium michiganense) and bacterial speck (Pseudomonas syringae). Major fungal diseases are Fusarium wilt (Fusarium oxysporum f. sp. lycopersici), Verticillium wilts (Verticillium dahliae), powdery mildews (Leveilula taurica) and early and late blights, which are caused by Alternaria solani/alternata and Phytophthora infestans, respectively.

While the use of disease resistant cultivars may present an effective way of controlling the above diseases, genetic engineering techniques continue to play a major role in the development of disease resistant cultivars *(Bhatia, et al. 2003)*. Various factors that affect the development of techniques for the isolation and identification of many genes involved in plant disease resistance, morphology and development have been studied *(Ling, et al. 1998)*. In addition, different factors such as Agrobacterium cell density *(Murray, et al. 1998)*, regeneration and co-cultivation conditions *(Hu and Phillips, 2001)*, addition of acetosyringone and cell competence after wounding *(Murray, et al. 1998)* and gene constructs *(Krasnyanski, et al. 2001)* were found to play a major role in tomato transformation. The first report of tomato transformation was forwarded by *McCormick, et al. (1986)* and since then, there have been numerous publications on transformation of various tomato cultivars *(Sree Vidya, et al. 2000; Hu and Phillips, 2001; Park, et al. 2003; Raj, et al. 2005; Sun, et al. 2006 and Saker, et al. 2007).* Standardization of tomato transformation procedures is, still, incomplete as different tomato cultivars vary in their response to specific treatment. The present study was undertaken to develop an efficient procedure for the production of fungal-resistant transgenic tomato (cv. CastleRock) plants expressing defensin gene.

MATERIALS AND METHODS

Agrobacterium-mediated tomato transformation was performed in accordance to *Park, et al. (2003)* protocol with some modifications.

Plant tissue:
Seeds of tomato (Lycopersicon esculentum Mill.) cultivar castleRock were surface sterilized in 10% (v/v) sodium hypochlorite solution, with 2 drops of Tween-20, for 15 min followed by three rinses in sterile water. Seeds were then germinated on MS (Duchefa Biochemie) inorganic salt (MSO) medium *(Murashige and Skoog, 1962)* with 30 g/L sucrose, pH 5.7 and solidified using 7g/L TC agar (GFS Chemicals and reagents, Texsas, USA). Hypocotyl explants and cotyledonary sections either with distal or proximal ends from 8-day-old seedlings were used. Explants sub-cuttings made in MSO liquid medium were blot-dried and placed on MS medium supplemented with 1 mg/L of both 6-benzyladenine (BA) and naphthalene acetic acid (NAA) for one day before co-cultivation with Agrobacterium.

Bacterial strain and plasmid:
A defensin (antifungal, AFP) gene cloned in Agrobacterium tumefaciens LBA4404 strain was used. This strain harbours the pITB-AFP plasmid vector which contains defensin (AFP) gene under the transcriptional control of cauliflower mosaic virus 35S promoter (CaMV-35S) and nopaline synthase, Nos, terminator, hygromycin phosphotransferase (hpt) gene and GUS-intron (uidA-β-glucuronidase) as a reporter gene. Bacteria were grown on LB medium supplemented with streptomycin (30 mg/L) and kanamycin (100 mg/L) with shaking at 200 rpm to an OD_{600} = 1.0. The bacterial suspension was then diluted with MSO medium in a ratio of 1: 5 and used for transformation experiments.

Transformation and regeneration protocol:
The precultured explants were carefully submerged in an Agrobacterium inoculum in a Petri dish (Ø9 cm) for 30 min with gentle swinging. The explants were then blot dried on sterile filter paper, transferred to Medium B and incubated in the dark for three days at 25°C. Explants were then transferred to plates containing a selection medium (Medium C) supplemented with 25mg/L hygromycin. The plates were sealed with parafilm and explants were left to regenerate at 25°C, with a 16h/8h (light/dark) photoperiod in the culture room. Hygromycin-resistant calli obtained after a second round of selection were transferred to a fresh selection medium. Once in every two weeks, the fresh and healthy looking hygromycin-resistant calli were sub-cultured in a fresh selection medium for shoot regeneration. After approximately 6–8 weeks, shoots were excised and transferred to Medium D for shoot development. The shoots obtained were transferred to rooting medium (Medium E) for root development. A set of explants which was not co-cultivated with Agrobacterium was also regenerated, as described above, as a negative control. Transformation frequency was expressed as a percentage of the number of shoots recovered from hygromycin-resistant calli relative to the total number of incubated hygromycin-resistent calli. Any rooting shoot on the selection medium containing 25 mg/L hygromycin was considered as a transformant.

Media composition:
The culture media used in the transformation and regeneration experiments were solidified with 7g/L TC agar and its pH was adjusted to 5.7.

Medium B (Co-cultivation medium):
MS salts and vitamins, 30 g/L sucrose, 1 mg/L BAP and 1 mg/L NAA.

Medium C (Selection and shoot regeneration medium):
MS salts and vitamins, 30 g/L sucrose, 2.5 mg/L BAP, 0.1 mg/L Indole-3-acetic acid (IAA), 250 mg/L cefotaxime and 25 mg/L hygromycin.

Medium D (Shoot eleongation medium):
MS salts and vitamins, 30 g/L sucrose, 1 mg/L BAP, 0.1 mg/L IAA and 250 mg/L cefotaxime.

Medium E (Rooting medium):
MS salts and vitamins, 30 g/L sucrose, 1 mg/L IAA and 250 mg/L cefotaxime.

Effect of Cefotaxime on callus growth and shoot regeneration:
To examine the influence of cefotaxime on callus growth and shoot regeneration (before and after transformation), 100 cotyledonary explants (25 pieces per Petri dish) were separately cultured on MS medium containing 0, 50, 100, 200, 250 and 300 mg/L cefotaxime. Regeneration frequency was calculated as a percentage of the number of regenerated explants/total number of explants *(Ling, et al. 1998)*.

Effect of the inoculation period:
To assess the effect of different periods of transfection (the period for which explants were immersed in the bacterial suspension) on the transformation frequency, explants were submerged in the bacterial broth for 10, 20, 30 and 35 mins.

Effect of Agrobacterium density:
Bacterial densities (OD_{600}) ranging from 0.3 to 2.0 were tested to determine the optimum density for transformation.

Sensitivity of tomato explants to hygromycin:
To determine the effect of hygromycin (water solution of hygromycin B) on the regeneration of tomato explants and to screen for an appropriate hygromycin concentration for transformants selection, cotyledonary and hypocotyl explants were transferred to the induction medium supplemented with different concentrations of hygromycin (0, 5, 10, 15, 20, 25, 30, 35 and 40 mg/L) in Petri dishes (10 cm in diameter) each containing 100 explants. The explants were incubated for 2 weeks in the dark at 25ºC and then transferred to a 16/8 h(light/dark) photoperiod of 80 lmol m^{-2} s^{-1} at 25ºC. The number of regenerated shoots was recorded after 5 weeks.

Effect of plant growth hormones combination on regeneration:
To evaluate the effect of growth regulators on transformation efficiency, different plant growth regulator combinations were examined. These combinations were in mg/ L: BA 1.0/ NAA 0.1 for 4 days (a one-day preculture period and a 3-day

co-cultivation period) followed by transfer to BA 2.5/IAA 0.1; Zeatin 1.0/IAA 0.1; Zeatin 2.0; or to Zeatin 2.0/IAA 0.1.

Histology and histochemical analysis:
GUS activity was analyzed in putative transformed primary explants, shoots and leaves from regenerated plants according to *Jefferson* (*1987*). Plant cells were incubated at 37°C for 24 hrs in GUS-staining solution (0.5 mM of X-gluc, 10 mM EDTA, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, in 0.1 M phosphate buffer, pH 7.0). GUS activity was visualized under stereoscope after washing the stained explants in 70% ethanol.

Molecular verification of the transformants:
To confirm the presence of the AFP gene in the regenerated transformed plants, total DNAs were isolated from both transformed and untransformed (negative control) plant samples and were used as templates for PCR. Plasmid DNA was also isolated and used as a positive control.

Rapid plant DNA extraction:
DNA extraction was carried out following CTAB (cetyl trimethyl ammonium bromide) protocol, which is a modification of the method of *Doyle and Doyle* (*1987*).

Polymerase Chain Reaction (PCR):
The presence of AFP gene was investigated by PCR amplification. Specific oligonucleotide primers for AFP gene were 'forward': 5′- CGC GGA TCC ATG GCG AGG TGT GAG AAT TTG GCT-3′ and 'reverse': 5′-TGC TCT AGA ATG GCG AGG TGT GAG AAT TTG GCT-3′.

Each PCR reaction was performed in 25 µl (total volume) of the reaction mixture that consisted of 1X reaction buffer, 10 ng plant DNA from a putative transgenic plant as a template, 200 mM dNTPs, 1.5 mM $MgCl_2$, 2 mM of each primer and 0.5 unit of Taq DNA polymerase. Amplification was carried out in a Thermal Cycler (Biometra) under the following conditions: 94ºC for 3 min. for initial denaturation, 94ºC for 1 min. for denaturation, 54ºC for 1.5 min. for annealing, 72ºC for 1 min for elongation, 7 min at 72ºC final extension and 35 cycles of amplification.

Amplified DNA fragments were electrophoresed on 1.0% agarose gel and detected by ethidium bromide staining and photographed under ultraviolet light. The presence of the target band in the transformants and its absence in the untransformed plants is considered as a proof of successful transformation.

RESULTS AND DISCUSSION

A protocol for transformation and regeneration of tomato plants (cv. CastleRock) has been developed on the basis of the results of the optimization experiments. A total of 270 cotyledonary leaves and 204 hypocotyls of tomato plants were used for transformation. The efficiency of gene transfer was evaluated by histochemical analysis of transient and stable gus-gene expression and determination of Hygromycin resistant calli. Initially, Hygromycin resistance was used for selection of transient gene expression. Transformation trials revealed great variations in the transformation frequency depending on the explant type (cotyledon or hypocotyl). The results of four independent experiments showed that cotyledonary explants were more efficient for regeneration and subsequently transformation than hypocotyl explants (Table 1). In both explant types, callus formation was observed in more than 90% of the cultured plant tissues within 15-25 days (Figure 1). Trials to detect the transformed cells were made, after 5 days from infecting the explants with Agrobacterium, by placing individual explant pieces on Hygromycin-containing medium. Green calli were obtained, on average, after 4 weeks of incubation. The percentages of explants that showed Hygromycin resistance after three selections was 50 and 40% for cotyledonary and hypocotyl explants, respectively. Transformed tissues were maintained on selection media until the appropriate size for transfer to shoot induction medium. Shoot induction was achieved in 6-8 weeks during which explants were transferred to fresh medium every two weeks. Well-grown shoots were cut off and were vertically inserted into root induction medium and incubated for 10 days. A representative result is given in (Figure 2). The highest transformation frequencies were 11% and 3% for cotyledonary explants in media containing BA and Zeatin, respectively (Table 2). The results of survival of transformed and untransformed plantlets at various stages are summarized in (Table 3). The frequency of shoot initiation for transformed plantlets was found to be 3.4% (7/204) in the medium containing Zeatin and 7.4% (20/270) in the BA medium. For untransformed plantlets, shoot initiation frequency was 78.0% and 86.0% in Zeatin and BA media, respectivelly. High frequency of shoot induction in the untransformed tissues may be attributed to the competence of the cells for regeneration *(Velcheva, et al. 2005).* Direct shoot initiation, rather than the callus, was observed at the edge of the cotyledonary proximal end. Similar observations were reported by *Raj, et al. (2005) and Peres, et al. (2001)* who attributed this to be due to hormonal metabolism and/or genetic background of organogenetic competence. The shoots were subsequently transferred on fresh selection medium containing BA for shoot elongation. Survival of the transformed shoots during shoot elongaton period indicated successful transformation. The possibility of non-transformed shoot survival on the elongation medium is generally reported in similar transformation trials giving a false positive indication *(Velcheva, et al. 2005)*. The regeneration of non-transformed plants was explained by non-efficient selection due to the protection of non-transformed cells presumbly caused by the occurrence of chimeric plants *(Velcheva, et al. 2005; Ghorbel, et al. 1999 and Almeida, et al. 2003)* or alternatively that the selection antibiotic was partially or completely phosphorylated by cells expressing hpt gene *(Velcheva, et al. 2005)*.

Previous studies aiming at improving shoot regeneration of transformed tissue from many tomato cultivars indicated the necessity of using a tobacco, petunia or tomato feeder layer for pre-culture and explant-Agrobacterium co-cultivation *(McCormick, et al. 1986; Fillatti, et al. 1987; Delannay, et al.1989; Van Roekel, et al. 1993; Agharbaoui, et al. 1995; Frary and Earle, 1996; Ling, et al. 1998 and Tabaeizadeh, et al.1999).* Hamza and Chupeau *(1993)* showed that pretreatment with feeder cells stimulated tomato cell transformation but regeneration of transformed cells was much reduced. Although, in this study, no feeder layer

was used for pre-culture or Agrobacterium co-cultivation, 11% transformation frequency was obtained. In short, this streamlined method achieved adequate transformation frequency on tomato plants. Park, et al. *(2003)* reported similar results on various tomato cultivars with a transformation frequency of 20%. Results on regeneration trials indicated that callus induction and regeneration from tomato explants are quite permissive over a range of plant growth regulators. Previuos studies *(McCormick, et al. 1986; Van Roekel, et al. 1993; Frary and Earle, 1996 and Costa, et al. 2000)* have suggested specific types and combinations of plant growth regulators for effective regeneration of transformed tomato plants.

Verification of the transgenic plants:

GUS histochemical assay:

The histochemical GUS activity of selected regenerants showed a uniform dark blue color in the transformed tissues. On the other hand, non-transformed tissue did not exhibit such a color under identical assay conditions. GUS expression was found in some parts of the cotyledonary leaves and the hypocotyl pieces either at high (dark blue) or low levels (light blue). The possibility of false GUS positive was ruled out because the GUS gene was interrupted by an intron.

Table 1: Effect of explants type on the rate of transformation.

Explant type	Cotyledons	Hypocotyls
Total No. of explants	270	204
Callus formation (%)	100	100
Regenerant (%)	98	92
Hygromycin-resistant calli (%)	50	40

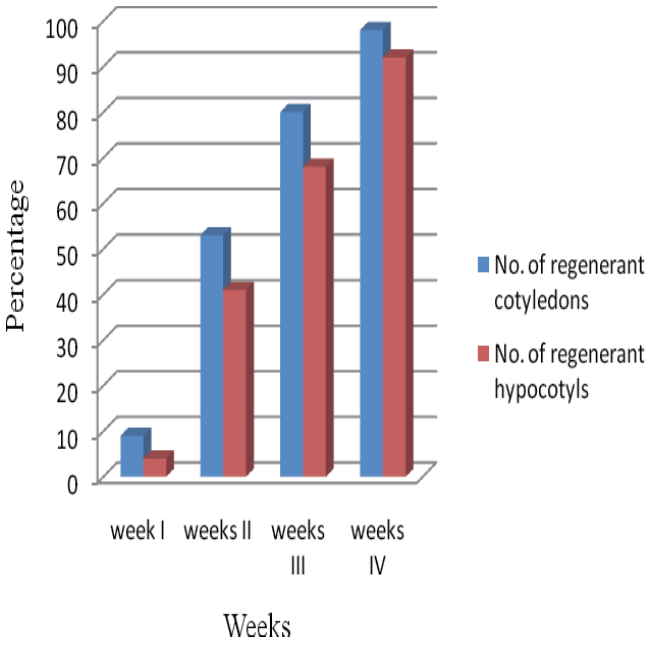

Figure 1: Percentages of regenerant tomato explants during 4 weeks.

(a)

(b)

(c)

Figure 2: Various stages during regeneration of transgenic tomato plantlets:

(a) callus induction and shoot initiation.
(b) shoot elongation.
(c) rooted plantlets

Table 2: Effect of explants type and growth regulators on the transformation frequency of tomato.

Plant growth regulators	**2.5 mg/L BA + 1.0 mg/L IAA**		**1.0 mg/L Zeatin + 0.1 mg/L IAA**	
Type of explants	**Cotyledonary explant**	**Hypocotyl explants**	**Cotyledonary explants**	**Hypocotyl explants**
Total No. of explants	138	98	132	106
No. of explants initiated shoot primordial	98	62	93	85
No. of shooted explants	15	5	5	2
No. of rooted explants	15	5	5	2
Transformation frequency with regeneration	11%	5%	3%	2%

Table 3: Survival percentage* of transformed (co-cultivated) and untransformed (control tomato plants at various stages of development.

Stage	**Co-cultivated**				**Control**			
	survival/total		**percentage**		**survival/total**		**percentage**	
	BA medium	**Zeatin medium**	**BA medium**	**Zeatin medium**	**BA medium**	**Zeatin medium**	**BA medium**	**Zeatin medium**
Co-cultivation	191/270	147/204	70.7	72.0	186/200	178/200	93.0	89.0
Shoot initiation	20/270	7/204	7.4	3.4	170/200	156/200	86.0	78.0
Shoot elongation	20/270	5/204	7.4	2.5	170/200	150/200	85.0	75.0
Rooting	15/270	4/204	5.6	2.0	164/200	148/200	82.0	74.0

*Results based on pooled data of four experiments.

DNA isolation and PCR analysis:

Genomic DNA from seven randomly selected transformed plants as well as from untransformed ones were isolated and subjected to PCR analysis. The putative transgenic plants, when screened by PCR using AFP-specific primers, gave the expected PCR amplicon (300 bp), whereas such an amplicon was not observed in untransformed (negative control) plants (Figure 3). This result clearly demonstrates the presence of the AFP gene in tomato planlets co-cultivated with Agrobacterium containing the AFP-disarmed plasmid construct.

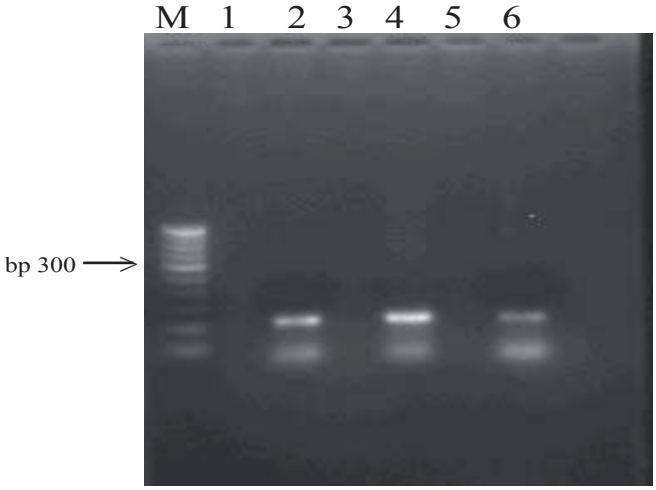

Figure 3: PCR confirmations of regenerated transformed plants using AFP primers. Lanes: M: 1 kb marker; 1: Negative control; 2 and 6: Transformed plants; 4: Positive control; 3 and 5: Untransformed plants.

Transformation of variuos tomato cultivars was previously reported by various authors. Transformation frequencies have ranged from 6% in cv. Pusa Ruby *(Sree-Vidya, et al. 2000)* to 40% in cv. Micro-Tom *(Sun, et al. 2006).* In spite of reported successes in tomato transformations, most of the transformation procedures were cumbersome and relied on either feeder layers (petunia, tomato, or tobacco), time consuming media formulations or successive subcultures *(Qiu, et al. 2007).*

We can generally conclude that no simple general procedure for tomato transformation exists due to many unknown factors that affect transformation efficiency. For example, genotypes vary in their response to specific treatments and standardization of the various procedures is incomplete *(Park, et al. 2003).* Van Roekel, et al. *(1993)* reported significant effects of culture media and Agrobacterium strains on the transformation frequency of tomato. High-frequency transformation using A. tumefaciens depends not only on the efficiency of the plant in vitro regeneration system but also on the subsequent elimination of bacterial cells from transformed tissues *(Tang, et al. 2004)*. However, it has been reported that Agrobacterium tumefaciens-mediated gene transfer is influenced by strain *(Stomp, et al. 1990)*, explant type (*Humara, et al. 1999*), temperature *(Dillen, et al. 1997),* size of T-DNA *(Park, et al. 2003)* and constitutive expression of the virulence genes *(Hansen, et al. 1994 and Rossi, et al.1996).*

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