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AGROBACTERIUM-MEDIATED TRANSFORMATION OF SUPER STRAIN B AND RIO GRANDE TOMATO CULTIVARS USING GV3101 BINARY VECTOR HARBORING PTDINS-PLC2 GENE

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ABSTRACT

The aim of this study is to deploy best conditions of previously published protocols on Agrobacterium-mediated transformation of tomato, to deliver PtdIns-PLC2 gene, which render plants tolerant to drought, to Money Maker, Super Strain B and Rio Grande tomato cultivars. The obtained results indicated that the efficiency of Agrobacterium-mediated gene transfer in tomato tissues is influenced by many factors; the most important are cultivars, explants type and optical density. The highest transformation efficiencywas 68% of Money Maker flamingo bill like explant andthe transformation efficiencySuper Strain B and Rio Grande cotyledons were 48% and 40%, respectively. On contrary, the transformation efficiency of Rio Grande hypocotyls and Super Strain B hypocotyls were 20% and 16 %, respectively. The best optical density for cotyledons and hypocotyls of the SSB and RG was 0.05 followed by 0.5 and 1.0. The presence of transgenes and their expression were confirmed by molecular analysis (PCR, RT PCR and Real Time PCR) and histochemical analysis (GUS assay). In conclusion, GUS assays and PCR affirmed the successful deliver and expression of PtdIns-PLC2 in Money Maker Super Strain B and Rio Grande tomato plants regenerated, elongated and rooted on selective media. The obtained transformants Money Maker plantexposed to droughtstress in vitro with different concentration of Mannitol to assess the activity of the gene and estimate some physiological variation

KEYWORDS: Drought, *Agrobacterium tumefaciensis,* PtdIns-PLC2 Gene, PCR, RT PCR and GUS Assay

INTRODUCTION

Abiotic stresses including drought were serious threats to the sustainability of crop yields accounting for more crop productivity losses than any other factor in rainfed agriculture (Bray et al, 2000). Success in breeding for better adapted varieties to abiotic stresses depend upon the concerted efforts by various research domains including plant and cell physiology molecular biology, genetics, and breeding. Use of modern molecular biology tools for elucidating the control mechanisms of abiotic stress tolerance, and for engineering stress tolerant crops is based on the expression of specific stress-related genes. Hence, genetic engineering for developing stress tolerant plants, based on the introgression of genes that are known to be involved in stress response and putative tolerance, might prove to be a faster track towards improving crop varieties (Bhatnagar-Mathur et al, 2008).

Today tomato is widespread throughout the world and represents the most economically important vegetable crop worldwide (Bergougnoux, 2014). However, tomato production, as compared to other vegetables, has always been associated with abundance of water. Tomato is very sensitive to water stress, the most sensitive being the stages of

flowering and fruit enlargement (Srinivasa Rao et al, 2000). And accordingly, extensive efforts have been done to produce transgenic tomato plants more tolerant to abiotic stresses with special focus on drought (Bergougnoux, 2014; Cortina & Macia, 2004; Park et al, 2003; Saker et al, 2008; Sivankalyani et al, 2014). Unfortunately, few successful transgenic tomato plants more tolerant to drought have been commercialized. This background necessitates the needs for more research on tomato transformation either to reach robust transformation systems for commercial cultivars or transferring new genes involved in eliciting biotic and abiotic stress tolerance in tomato (Saker et al, 2011 and Abd-El-Haliem et al, 2012).

The most widely used method for transferring genes into tomato is Agrobacterium-mediated transformation. The FLAVR-SAVR™ tomato variety was the first genetically engineered crop to be commercialized (Kramer & Boyer, 1995). For 30 years now, Agrobacterium has been used for research purposes, and it has also been used to produce genetically engineered crops for commercial purposes (Alimohammadi & Bagherieh-Najjar, 2009). The first report on transformation using Agrobacterium tumefaciens(Horsch et al, 1985)and the first specific report on tomato transformation by Agrobacterium was done in 1986 (McCormick et al, 1986). The explants used were as diverse as cotyledons, hypocotyls, stems and leaves of the tomato (Bird et al, 1988; Davis &Miller, 1991; McCormick et al, 1986). Tomato regeneration has been previously reported via organogenesis in several articles using different explants, such as, leaf (Gaffe et al, 1997; Oktem et al, 1999) and cotyledon (van Roekel et al, 1993).

PtdIns-PLC2 gene (Phosphatidylinositol-specific phospholipase C), plays a central role in the phosphatidylinositol specific signal transduction pathway. The importance of PtdIns-PLC2 is an elicitor of a battery of events that systematically control hormone regulation, and plant growth and development in what may be a preprogrammed mode (Georges et al, 2009). Several studies showed thatcellular defense signaling requires phosphatidylinositol-specific phospholipase C (PI-PLC) enzyme activity (Dowd & Gilroy, 2010; Hunt et al, 2003). PI-PLC enzymes are considered to be signal transducers, mainly due to the signaling roles attributed to their substrates and reaction products (Abd-El-Haliem et al, 2012). The present study was conducted to make use of previously published protocols for Agrobacterium-mediated transformation of tomato, and deploying the most efficient conditions to deliver PtdIns-PLC2 gene, which render plants tolerance to drought, to Super Strain B and Rio Grande tomato cultivars.

MATERIALS AND METHODS

Plant Materials

Seeds of tomato, L. esculentumcv Super Strain B and Rio Grande were kindly provided by Agriculture Research Center, Giza, Egypt and Center of Biotechnology, Sfax, Tunisia. Seeds were Surface sterilized by immersion in 70% ethanol for 1 min, thoroughly washed with sterilized distilled water and immersed in 10% commercial Clorox (active ingredient 5.25% Sodium hypochlorite) with 2 drops of tween 20 for 10 min, then rinsed three times with sterilized distilled water. Sterilized seeds were germinated on MS basal salt medium (Murashige & Skoog, 1962), supplemented with 30 g/l sucrose and 2.8 g/l gelrite. The pH of the medium was adjusted to 5.8 with 1M KOH, then autoclaved for 20 min at 121ºC. Seeds were cultivated for 72 h under darkness at 25±2 ºC, then under photoperiod of 16 h light and 8 h dark for 7-10 days.

Bacterial Strain and Plasmid

Agrobacterium tumification**GV3101** harboring binary vector **PBI121 was used in this study.** The plasmid PBI 121 harbor phospholipase C gene (PLC2) under the control of cauliflower mosaic virus (CaMV35S) promoter and nopaline synthase (NOS) terminator, kanamycin resistance gene (Kan) as selectable marker gene and β-glucuronidase (gus) as a

reporter gene. This strain was kindly provided by Prof. Fawzy George, Plant Biotechnology Institute, National Research Council, Canada through MTA (Material Transfer Agreement).

Agrobacterium Culture

Single colony from Agrobacterium **GV3101** harboring binary vector PLC2 was inoculated in 3 ml YEB liquid medium (Trypton 5 g/l + Beef extract 5 g/l + Yeast extract 1 g/l + Sucrose 5g/l + Mg4So4 X 7 H2O 0.5 g/l) supplemented with 50 mg/l kanamycin (kan) and 25 mg/l rifampicin (rif) and incubated on incubating shaker at 28 ºC and 200 rpm overnight, then 250µl of overnight culture were added to 250 ml LB liquid media with antibiotics and incubated overnight at 28 ºC in incubating shaker at 200 rpm. Then, AgrobacteriSum cells were collected by centrifugation for 10 min at 3000 rpm and re-suspended in MS liquid medium supplemented with 100 µM acetosyringone to a final OD_{600} = 0.05, 0.5 and1.0.

Transformation Protocol

The transformation procedure was carried out as described by Khoudi et al. (2009)**.** Cotyledon and hypocotyl explants of 7-10 days old seedlings were used. Two parameters affecting Agrobacterium-mediated transformation (bacterial densities and type of explants) were optimized. Germinated plantlets were collected from germination jars and transferred to sterile disposable Petri dish contains sterilized distilled water under aseptic conditions. Cotyledon and hypocotyl explants were separated using sterilized sharp blade, and transferred upside down to pre-culture media for 48 h. About 100 explants from each explants type for each variety were used in this experiment. Pre-cultured cotyledons and hypocotyls were immersed into Agrobacterium suspension for 30 min with occasional agitation, blotted on dry sterile Whatman filter paper to remove excess of bacteria and then blot explants upside down on co-cultivation media and incubated in dark for 48 h. At the end of co-cultivation period, the explants were washed three to five times with sterile distilled water supplemented with 250 mg/L cefotaxime, blotted on dry sterile Whatman filter paper. Equal numbers of each type of explants were transferred upside down (10 explants/Petri dish) to relevant regeneration and shoot elongation media under selection pressure of 50 mg/ml kanamycin. Shoot regeneration medium I (SRI, Table 1) for 6 weeks followed by shoot elongation I (SEI, Table 1) for 2months. Elongated shoots (approximately 3 cm) were excised from the rest of the explants and transferred to rooting medium (RM, Table 1). Shoot regeneration data were recorded 6 weeks after initiation of culture.

Table 1: Compositions of Tissue Culture Media Used in Transformation and Regeneration

Component	GM	PC	CO	SRI	SEI	RM
Basal	MS	MS	MS	MS	MS	MS
Sucrose (g)	30	30	30	30	30	30
Indole-3-acetic acid (IAA) (mg)	-	-	-	0.1	0.1	0.2
6-benzylaminopurine (BAP) (mg)	-	1	1	-	-	-
Zeatin (mg)	-	-	-	1	0.2	-
1-naphtalene acetic acid(NAA) (mg)	-	1	1	-	-	-
Cefotaxime (mg)	-	-	-	250	250	-
Kanamycin (mg)	-	-	-	50	50	50
Gelrite (g)	2.8	4	4	4	4	4
Acetosyringone (mg)	-	-	100	-	-	-

GM, germination medium; PC, pre-culture medium; CO, co-cultivation medium; SRI, shoot regeneration medium I; SEI shoot elongation medium I; SRII, shoot regeneration medium II; SEII, shoot elongation medium II; RM, rooting medium

DNA Extraction

Plants rooted on selective rooting medium and wild type plants were selected for DNA isolation and PCR analysis. DNA isolation from fresh leaves was carried out using GeneJET Plant Genomic DNA Purification Mini Kit.

Specific Nested-PCR

Screening of putative transformants was performed using specific primers for PLC gene. The plasmid was used as a positive control. First round RT-PCR was carried out using forward primer 5'-CATGTCGAAGCAAACGTACAAAGT-3' and reverse primer 5'-ACACAAACTCCA-CCTTCACGAGAA-3', and the size of the expected PCR product is (1700 bp). PCR reaction mix (25 µl) consisted of 5 µl of 10X buffer, 2.5 µl dNTPs (10 mM), 1.6 µl $Mgcl_2$(25 mM), 0.125 µl Taq polymerase (5 U/ µl), 0.5 µl (10 Pmole) forward primer, 0.5 µl (10 Pmole) reverse primer, 11.775µl ddH2O and 3 µl of DNA template (60 Pmole). PCR amplification using PCR Touchdown Program, i.e. 94°C for 5 min, 20 cycle of 94°C for 30 S, Tm+5 (65°C) for 45 S and 72°C for 2 min, then 20 cycle of 94°C for 30 S, Tm-5 (54°C) for 45 S and 72°C for 2 min, and72°C for 5 min (final extension). The PCR product was then undergoes a second round PCR using a new set of primers (forward primer 5'-ACTCCAGAACTTCAGTCCAAAGTTGCT-3' and reverse primer 5'-ATTGGCTCTAAACA-TTCCTTGCATTA-3') with the same PCR reaction mixture and touchdown program was 94°C for 5 min, 20 cycle of 94°C for 30 S, Tm+5 (69°C) for 45 S and 72°C for 2 min, then 20 cycle of 94°C for 30 S, Tm-5 (59°C) for 45 S and 72°C for 2 min, and 72°C for 5 min, and the expected size of PCR product is 700 bp.

RNA Extraction

Plants rooted on a selective rooting medium and wild type plants were selected for RNA extraction. RNA isolation from fresh leaf tomato tissues was carried out using Gene JET Plant Genomic RNA Purification Mini Kit

RT PCR

Preparation of cDNA from total RNA was carried out using M-MLV Reverse Transcriptase (Promega, USA). The PCR was carried out by forward primer 5'-CATGTCGAAGCAAACGTACAAAGT-3', reverse primer 5'-GATTGACTGCGCATCCTCTT-3' Using Phusion High Fidelity PCR Master Mix Kit, Thermo Scientific. PCR reaction mix (25 µl) consisted of 12.5 µl of 2 X phusion master mix, 1.5 µl (10 Pmole) forward primer, 1.5 µl (10 Pmole) reverse primer, 7.5 µl H2O and 2 µl of cDNA. The PCR cycler conditions was 98°C for 2 min then 40 cycle (98°C for 15 S, 55°C for 15 S, 72°C for 30 S), then 72°C for 2 min. The expected size of RT PCR product is 200 bp.

Agarose Gel Electrophoresis

The amplification products were analyzed by electrophoresis in 1% agarose gel in sodium borate buffer stained with ethidium bromide and photographed by gel documentation system.

Histochemical Assay (Transient GUS Assay)

Histochemical analysis of gus expression in transformed explants, young leaves and stem of transformed tomato plants using 5-bromo-4-chloro-3-indolyl-b-d-glucuronide(X-Glu) as a substrate was carried out as described by Jefferson

(1987). The GUS reaction buffer consisted of (200 mM phosphate buffer PH 7 + methanol+ Triton X-100 + 7µl/ml X-Glu). Explants were incubated in X-Glu buffer over night at 37 ºC, then chlorophyll was removed from tissues and leaves by soaking them in 70% ethanol for 4±6 h. The ethanol was replaced three or four times then washed with distilled water.

RESULTS AND DISCUSSIONS

Optimization of Transformation Conditions

Several investigators have demonstrated that cotyledon and hypocotyls were superior to leaves for promoting shoot organogenesis in tomato and The efficiency of Agrobacterium-mediated gene transfer to tomato cells is influenced by variety(Khoudi et al, 2009; Ling et al, 1998; Plastira & Perdikaris, 1997). Based on these reports, we used cotyledon and hypocotyl explants in our transformation experiment and used three optical densities of bacterial culture concentrations (0.05, 0.5, and 1.0), to identify the optimal bacterial concentrations and type of explants. The obtained data indicated that, cotyledon explants of Super Strain B cultivar were more responsive and gave the best results of transformation, expressed as number of proliferated shoots on selective media, compared with cotyledon explants of Rio Grande cultivar. On contrary, hypocotyl explants of Rio Grande were better than that of Super Strain B. Statistical analysis revealed a significant difference between proliferated shoots regeneration means of cotyledon and hypocotyl explants for both cultivars at different optical density, as show in table 2 and figure 1. Published data on optimization of bacterial concentrations, indicated that the best concentration of overnight grown Agrobacterium cultures, diluted prior to co-cultivation were ranged from 0.01 to 1.0 at OD_{600} nm (McCormick et.al. 1986 (Frary & Earle, 1996), accordingly, different optical densities ranged from (0.05 to 1.0) were tested in this study. The influence of different bacterial densities (OD_{600}) on the transformation efficiency was evaluated (Table 2) and (Figure 1). Bacterial concentration (OD_{600}=0.05) was more efficient than (OD_{600}=0.5) and (OD_{600}=1.0).

Table 2: Percentage of Explants Producing Transformed Proliferated Shoots on SRI (Table 1) Media 6 Week After Initiation of Culture

Cultivars	O.D / Explants	0.05	0.5	1.0
Rio Grande	Cotyledons	80 ± 7.1	70 ± 7.1	58 ± 8.4
	Hypocotyls	34 ± 11.4	30 ± 12.2	8 ± 8.4
Super strain	Cotyledons	88 ± 8.4	74 ±18.2	68 ± 17.9
	Hypocotyls	28 ± 8.4	18 ± 16.4	6 ± 5.5

Percentage mean ± SD (n=100)

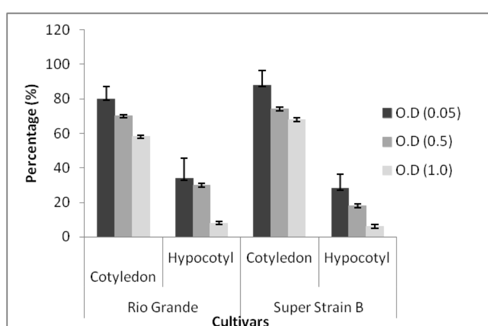

Figure 1: Histogram Illustrating the Percentage of Explants Producing Transformed Proliferated Shoots on SRI (Table 1) Media, After Six Weeks of Cultivation

Figure 2 shows shoot proliferation from cotyledon and hypocotyl explants Super Strain B and Rio Grande cultivars. Data of figure 2 shows clearly that the growth rate of proliferated shoots on cotyledon explants of Super Strain B at $O.D_{600}$0.05 was fast and had the highest number of shoots per explant (figure 2-J). Meanwhile, the growth rate of proliferated shoots on hypocotyl explants of Super Strain B at $O.D_{600}$1.0 was slow and had a few small numbers of shoots per explant.

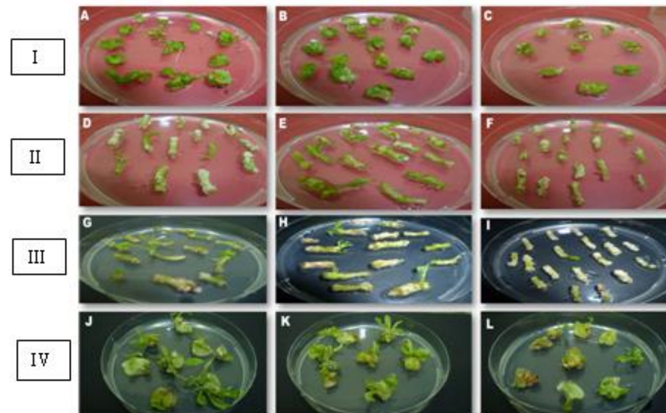

Figure 2: Shoot Proliferation from Cotyledon and Hypocotyl Explants of Super Strain B and Rio Grande Cultivars Inoculated with Different Concentrations of Agrobacterium with (Different Optical Bacterial Densities) Under Selection Pressure of 50 Mg/Ml Kanamycin on Shoot Regeneration Medium I (SRI) After Six Weeks of Cultivation

I: Cotyledon explants of Rio Grande at (A) $O.D_{600}$ 0.05. (B) $O.D_{600}$ 0.5 and (C) $O.D_{600}$ 1.0.

II: Hypocotyl explants of Rio Grande at (D) $O.D_{600}$ 0.05. (E) $O.D_{600}$ 0.5. (F) $O.D_{600}$ 1.0.

III: Cotyledon explants of Super Strain B at (G) $O.D_{600}$ 0.05. (H) $O.D_{600}$ 0.5. (I)$O.D_{600}$ 1.0.

IV: Hypocotyl explants of Super Strain at (J) $O.D_{600}$ 0.05. (K) $O.D_{600}$ 0.5. (L) $O.D_{600}$ 1.0.

Several studies have reported that cotyledon was the best explant for promoting shoot organogenesis in tomato (Pawar et al, 2012; Sharma et al, 2009; Velchevaa et al, 2005 and Pawar et al, 2012), and hypocotyl explants were not as efficient in generating transgenic shoots as cotyledons and that it was usually not worth the effort to use them in transformation experiments Similar (Schutze and Weiczorrek, 1987 and McCormick, 1991). In contrast, other researchers reported that hypocotyls and cotyledons were found to give equivalent transformation efficiencies of 5.2-7.4% (Frary & Earle, 1996). This contradiction necessitates empirical optimization of the best explants for the different cultivars. The results of the present study indicated that cotyledon is the best explant could be used for transformation of Super Strain B and Rio Grande cultivars.

The results of the present affirmed that the efficiency of cotyledon explants was higher than that of hypocotyl explants on all treatments and in the two cultivars under investigations. The obtained results also pointed to significant differences in the efficiency of transformation, expressed as percentage of proliferated, elongated and rooted shoots per explants on selective media, after six months of cultivation on rooting selection media. The highest efficiency of cotyledon explants (48%) was reported at $O.D_{600}$ 0.05 in Super Strain B, meanwhile the highest efficiency of hypocotyl explants (20%) was reported in Rio Grande at $O.D_{600}$ 0.05 (Table 3 and Figure 3). Reviewing of published data indicated that the transformation frequencies efficiencies had ranged from 6%-80% (Qiu et al, 2007, Rai et al, 2012 andYasmeen, 2009).

Table 3: Transformation Efficiency %

Cultivars	O. D / Explants	0.05	0.5	1.0
Rio Grande	Cotyledon	40.0±7.1	36.0±11.4	24.0±5.5
	Hypocotyl	20.0±7.1	20.0±7.1	4.0±5.5
Super strain	Cotyledon	48.0±8.4	38.0±8.4	32.0±8.4
	Hypocotyl	16.0±5.5	10.0±7.1	4.0±5.5

Percentage mean ± SD (n=100)

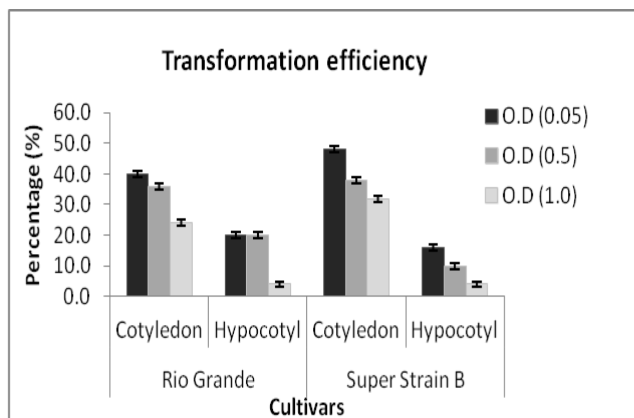

Figure 3: Transformation Efficiency %

Transient GUS Expression of Putative Transformants

Histochemical GUS assay (Jefferson et al, 1987) was used to monitor the expression of gus gene in developing shoots and leaves at different subculture on new fresh selection medium. The assay demonstrated the presence of green-blue color zones in the tissues of putative transformants only, meanwhile non-transformed tissues didn't exhibit any blue color (Figure 4). It is worth to mention that not all putative transformants gave positive results in GUS assays, and those shoots were excluded during sub culturing (multiplication process). Lacks of GUS expression in kanamycin resistant tissues may be due to genetic alternation in gus gene resulted from rearrangement of the coding sequence or methylation of the gene (Das et al, 2012).

Figure 4: Transient Gus Expression of Rio Grande and Super Strain B Cultivars

A. Control hypocotyl and cotyledon explants (non-transformed) show no color.

B. Transformed hypocotyls show blue spots. C. Transformed cotyledons show blue spots

Molecular Analysis

Polymerase chain reaction (PCR) was routinely used as a fast and reliable molecular test to check the insertion of

gene in the genome (de Vetten et al, 2003; Li et al, 2009). In this study, PCR tests were carried out on positive control (plasmid used in the transformation) and genomic DNA of both non- transformed and transformed tomato plantlets grown for six months on selective media and had positive gus expression. PCR tests were confirmed by nested-PCR and RT-PCR using specific primers for PtdIns-PLC2 gene as shown in figure (5, 6, 7 and 8). These results confirmed stable integration of PtdIns-PLC2 gene in tomato plant.

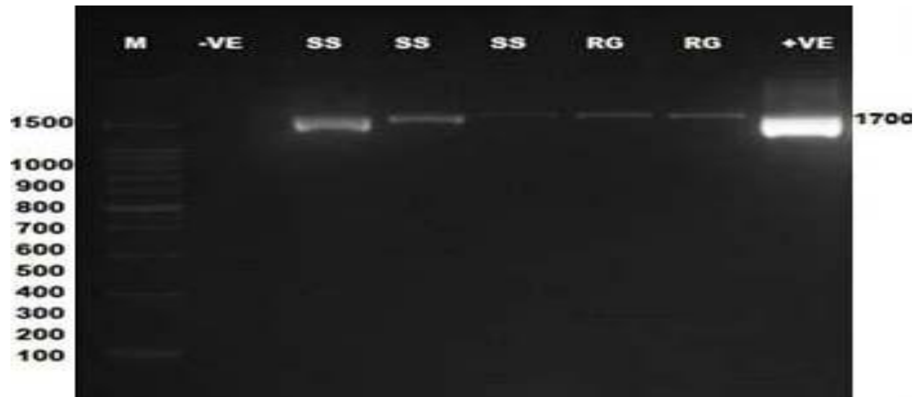

Figure 5: First Reaction of Nested PCR Analysis that Catch Whole PLC Gene Size at 1700 pb of Putative Transformants Tomato Plantlets Using Specific Primers for PLC Gene, M is 100 bp DNA Ladder, -VE is Negative Control (Non-Transformed),+VE is Positive Control (Plasmid DNA), SS is Transgenic Super Strain B Cultivar and RG is Transgenic Rio Grande Cultivar

Figure 6: Second Reaction of Nested PCR Analysis that Catch Specific Size Within PLC Gene at 700 bp of Putative Transformants Tomato Plantlets Using Specific Primers for PLC Gene, M is 100 bp DNA Ladder, -VE is Negative Control (Non-Transformed), +VE is Positive Control (Plasmid DNA), SS is Transgenic super Strain B Cultivar and RG is Transgenic Rio Grande Cultivar

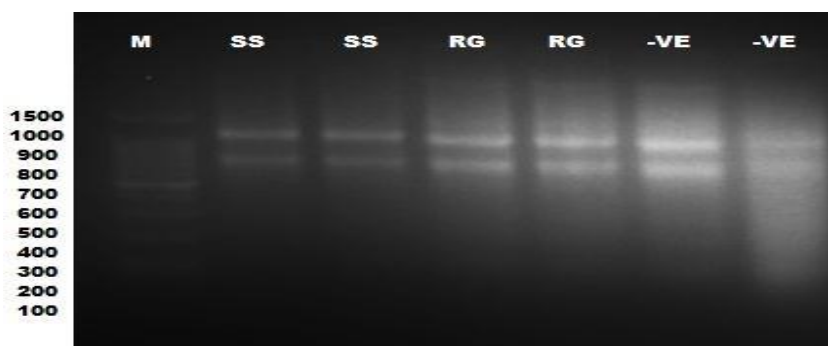

Figure 7: RNA of Three GUS Positive Transformants Tomato Plantlets, M Is 100 Bp DNA Ladder, -VE is Negative Control (Non-Transformed), SS is Transgenic Super Strain B Cultivar and RG is Transgenic Rio Grande Cultivar

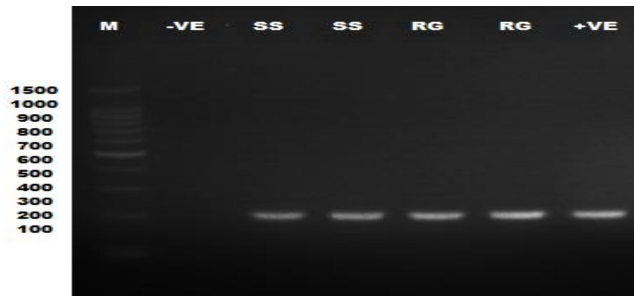

Figure 8: RT PCR Amplifications Using PLC Primers in Three Transformants, M is 100 Bp DNA Ladder, -VE is Negative Control (Non-Transformed), +VE is Positive Control (Plasmid DNA), SS is Transgenic Super Strain B Cultivar and RG is Transgenic Rio Grande Cultivar

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