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Effect of different factors on regeneration and transformation efficiency of tomato (*Lycopersicum esculentum*) hybrids

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Abstract: The current study aimed to produce rootstock material through micropropagation by developing efficient regeneration and *Agrobacterium*-mediated transformation protocols for three high quality commercial tomato hybrids (Felina, Siena and Don Jose) to overexpress the *GmGSTU4* gene from *Glycine max* L. previously shown to enhance antioxidant activity. We investigated the plant growth regulators zeatin (Z) and 3-idoleacetic acid (IAA) to determine their best combination for an efficient regeneration protocol for each hybrid. The highest regeneration efficiency was observed in Felina (94.4%) with 1.0 mg/l Z and 0.1 mg/l IAA. In contrast, Don Jose (92.5%) and Siena (83.3%) performed better with 0.5 mg/l Z and 0.1 mg/l IAA. The three hybrids did not differ in micropropagation index, however, Felina showed the highest number of *in vitro* rooted and *in vivo* acclimatized plants. Factors such as the age of explant, days in pre- and co-culture and the concentrations of acetosyringone and thiamine on *Agrobacterium*-mediated genetic transformation were assessed. The transformation indices were 37.04% for the Felina, 13.8% for Siena and 8.33% for Don Jose. We conclude that targeted genotype-specific regeneration protocols will provide an efficient and cost effective genetic transformation system for rootstock production and further incorporation into micropropagation and transgrafting systems.

Keywords: *Agrobacterium*-mediated transformation; glutathione-S-transferases; growth regulators; micropropagation; shoot regeneration, *Solanum lycopersicum*

Tomato (*Solanum lycopersicum*) is one of the most cultivated vegetables of the *Solanaceae* family, facing various problems related to environmental stresses (Lin *et al*. 2014). The most common genetic rootstock sources for grafted tomato are the tomato hybrids and interspecific tomato hybrids as virus-free micropropagated material. An efficient approach to reduce the negative effects of external stresses to commercially

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important horticultural crops is the development of tolerant rootstocks through genetic transformation (Lee *et al*. 2010; Schwarz *et al*. 2010) compared to the relatively slow methods of traditional breeding (Flores *et al*. 2010). The genetic transformation of an elite hybrid and its use as rootstock material to support the non-transformed scion (cis-grafting, Albacete *et al*. 2015), may eliminate any potential compatibility issues (Goldschmidt 2014) and provide an effective strategy to improve tolerance to abiotic and biotic stresses.

Genetic transformation is a key technology for functional genomics (Sun *et al.* 2006). Glutathione S-transferases (GSTs; EC.2.5.1.18) are multi-functional enzymes (Marrs 1996; Roxas *et al*. 2000; Cummins *et al*. 2011), that have been shown to enhance plant tolerance by participating in the antioxidant mechanism as glutathione peroxidases (GPx) and protect plants from abiotic stresses (Nianiou-Obeidat *et al*. 2017).

Our main research aim was to develop effective regeneration and transformation protocols for three commercial Greek tomato hybrids, Felina, Siena and Don Jose, that show good agronomic traits and fruit quality, yet have restricted adaptability to environmental stress factors, to be used as rootstock material in transgrafting systems, using a gene construct (Figure S1 in Electronic Supplementary Material (ESM)) previously shown to confer antioxidant tolerance to abiotic stresses such as salinity (Kissoudis *et al*. 2015b) and to herbicides in tobacco plants (Kissoudis *et al.* 2015a). Since both plant species are members of the Solanaceae family we were interested to test whether the overexpression of the same gene would also enhance stress tolerance in tomato plants.

MATERIAL AND METHODS

Plant material and growth conditions. Seeds of the tomato hybrids, Felina, Siena and Don Jose (provided by Agris S.A., Greece) were surface sterilized with 2.5% NaOCl for 12–15 min and germinated on MS medium (Murashige & Skoog 1962). *In vitro* plants were grown under 16 h light/8 h dark, at 1500 lux.

***In vitro* regeneration**. Cotyledon explants were excised from 12–15 days old seedlings and placed on 10 nutrient media (A1, A2, B1, B2, C1, C2, D1, D2, E1, E2) supplemented with a combination of different concentrations of IAA (3-idoleacetic acid; 0 and 0.1 mg/l) and Z (zeatin; 0, 0.5, 1, 1.5 and 2 mg/l) (Table , Figure 1). After 4 weeks, the *in vitro* survival and regeneration efficiency of the explants were measured as the number of explants that produced shoots, the rate of regenerated shoots per explant and the total number of regenerated shoots per treatment.

Genetic transformation. We used the *Agrobacterium tumefaciens* LBA4404 strain, carrying the pART27 plasmid with the CaMV 35S:*GmGSTU4* gene (Figure S1 in ESM) (Benekos *et al*. 2010). The genetic transformation protocol was developed initially on Felina. The effect of different parameters was investigated in two separate phases, as described in Table 2. The optimum conditions for the transformation, were applied to Siena (480 explants) and Don Jose (540 explants) (Figure 1).

Cotyledons were first incubated for 15 min into the bacterial culture of *A. tumefaciens* – *GmGSTU4* and later were immersed into the co-culture liquid MS medium. The incubation was carried out at 25°C under low light conditions for 24 or 48 h. The explants were placed on the selection media [C_2 or B_2 + 100 mg/l kanamycine + 250 mg/l cefotaxime (Cf)]. Transformation efficiency was calculated after 6 weeks, as the percentage (%) of the shoot regeneration (independent transformation events) (Patil *et al*. 2002) in comparison with the wild-type plants.

Verification of putative transgenic lines. Genomic DNA from the putative transgenic lines was isolated using the DNeasy Plant mini kit (Qiagen, Germany) and PCR reactions were carried out using the DyNAzyme II (DNA polymerase) kit (Finnzymes, Finland) according to manufacturers' instructions. *GmGSTU4* (*GST4*F5 and *GST4*R5) and *NPTII* (*nptII*F and *nptII*R) specific primers were used for the analysis (Table S1 in ESM).

Relative expression analysis of the 35S-*GmGSTU4*. Total RNA was extracted and DNase digestion was conducted as described in Benekos *et al*. (2010) using TRIzol Reagent 15596-026 (Invitrogen, USA) and RQ1 RNase-Free DNase kit (Promega, USA), respectively. For the RT q-PCR the SYBR Fast qPCR Kit (Kapa Biosystems, Switzerland) was used with the *GmGSTU4* (GST4F and GST4R) and actin (ACTF and ACTR) specific primers (Table S1 in ESM) at 95°C for 1 min, followed by 30 cycles of denaturation at 95°C for 5 s, annealing at 62°C for 20 s and elongation at 72°C for 3 s, with a final cycle of 10 min at 72°C. Relative expression was calculated with the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001).

***In vitro* micropropagation and *in vivo* hardening of transgenic lines**. The micropropagation rate and the mean number of plants that survived *in vitro* and *in vivo* were tested in the Don Jose and Siena and

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Table 1. *In vitro* regeneration of three tomato hybrids; the optimum PGR media per hybrid for all three parameters are shown in bold

Regeneration media (mg/l)	A1	A2	B1	B2	C1	C2	D1	D2	E1	E2
IAA	0	0.1	0	0.1	0	0.1	0	0.1	0	0.1
Z	0	0	0.5	0.5	1.0	1.0	1.5	1.5	2.0	2.0
Don Jose										
Regenerated explants	0.33 ± 0.33^{f} (1)	1.33 ± 0.33^{f} (4)	5.33 ± 0.66^{cde} (16)	**9.33 ± 0.88^{a} (28)**	6.33 ± 0.66^{bc} (19)	8 ± 1.00^{ab} (24)	4.33 ± 0.88^{cde} (13)	6 ± 0.57^{bcd} (18)	3.33 ± 0.33^{e} (10)	4 ± 0.57^{de} (12)
Total shoots	0.33 ± 0.33^{e} (1)	1.33 ± 0.33^{e} (4)	11.66 ± 0.88^{c} (35)	**27.6 ± 1.20^{a} (83)**	12.33 ± 1.45^{c} (37)	19.33 ± 2.33^{b} (58)	7.33 ± 0.88^{d} (22)	7.33 ± 0.88^{d} (22)	4.33 ± 0.88^{d} (13)	6 ± 0.57^{d} (18)
Shoots/explant	0.33 ± 0.33^{f} (1)	0.33 ± 0.33^{f} (1)	2.22 ± 0.14b^{c} (6.67)	**2.99 ± 0.15^{a} (8.98)**	1.95 ± 0.12^{bcd} (5.86)	2.42 ± 0.1^{ab} (7.28)	1.75 ± 0.14^{cde} (5.25)	1.23 ± 0.11^{bcd} (3.69)	1.27 ± 0.14^{e} (3.83)	1.54 ± 0.17^{de} (4.62)
Siena										
Regenerated explants	0.66 ± 0.33^{f} (2)	0.33 ± 0.33^{f} (1)	8.33 ± 0.33^{b} (25)	**10.66 ± 0.33^{a} (32)**	6.66 ± 0.33^{c} (20)	8 ± 0.57^{b} (24)	5.66 ± 0.33^{cd} (17)	4.66 ± 0.33^{de} (14)	3.66 ± 0.66^{e} (11)	4.33 ± 0.66^{de} (13)
Total shoots	0.66 ± 0.33^{f} (2)	0.33 ± 0.33^{f} (1)	20.66 ± 1.85^{b} (62)	**30.66 ± 1.33^{a} (92)**	12.33 ± 1.85^{c} (37)	20.33 ± 0.88^{b} (61)	10.33 ± 1.33^{c} (31)	8.66 ± 0.88^{cd} (26)	4.33 ± 0.88^{e} (13)	6.33 ± 1.45^{de} (19)
Shoots/explant	0.33 ± 0.33^{d} (1)	0.33 ± 0.33^{d} (1)	2.48 ± 0.18^{ab} (7.43)	**2.88 ± 0.18^{a} (8.65)**	1.84 ± 0.22^{bc} (5.52)	2.55 ± 0.08^{a} (7.66)	1.82 ± 0.19^{bc} (5.47)	1.85 ± 0.07^{bc} (5.55)	1.17 ± 0.09^{c} (3.53)	1.44 ± 0.18^{c} (4.33)
Felina										
Regenerated explants	0.33 ± 0.33^{f} (1)	0.33 ± 0.33^{f} (1)	0.33 ± 0.33^{de} (10)	5 ± 0.57^{d} (15)	9 ± 0.57^{b} (27)	**11.33 ± 0.33^{a} (34)**	4.66 ± 0.33^{d} (14)	7 ± 1.00^{c} (21)	2.66 ± 0.88^{e} (8)	4.66 ± 0.33^{d} (14)
Total shoots	0.33 ± 0.33^{g} (1)	0.33 ± 0.33^{g} (1)	6 ± 1.00^{ef} (18)	11.33 ± 1.20^{d} (34)	24.3 ± 1.33^{b} (73)	**34.3 ± 1.66^{a} (103)**	9.33 ± 0.88^{de} (28)	16.33 ± 2.33^{c} (49)	4 ± 1^{fg} (12)	9.66 ± 1.66^{de} (29)
Shoots/explant	0.33 ± 0.33^{d} (1)	0.33 ± 0.33^{d} (1)	1.8 ± 0.28^{c} (5.42)	2.27 ± 0.06^{bc} (6.82)	2.71 ± 0.09^{ab} (8.13)	**3.03 ± 0.13^{a} (9.09)**	2.01 ± 0.20^{bc} (6.05)	2.34 ± 0.11^{abc} (7.03)	1.64 ± 0.21^{c} (4.92)	2.06 ± 0.29^{bc} (6.20)

Data are mean ± SE (n = 3 with 12 cotyledons each); significant differences of each treatment at $P \leq 0.01$; values followed by the same letter are not statistically different; numbers in parenthesis demonstrate the total number of regenerated explants and total number of regenerated shoots; IAA – 3-idoleacetic acid; Z – zeatin

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Table 2. Two-phase development of an efficient genetic transformation protocol of tomato

Phase	Factor	Levels	No. of cotyledons
1	cotyledon age (days)	12, 15	432
	pre-culture duration (days)	0, 1, 2	
	co-culture duration (days)	1, 2	
2	acetosyringone (μM)	0, 100, 200	135
	thiamine (mg/l)	0.1, 0.4	

their best performing transgenic lines (C16, C26 and B3, B7 respectively). Nodal stems with a bud were placed in MS medium with 0.1 mg/l IAA, after 4 weeks, the number of rooted plants were measured. The rooted plants were transferred to plastic pots filled with compost : perlite (2 : 1) and the number of acclimatized plants that survived was measured 1 week after hardening.

Statistical analysis. All the experiments followed the Completely Randomized Design (CRD). Each value is presented in the form of mean ± standard error (SE). Analysis of variance (ANOVA) was performed using the SPSS Inc. statistical package (Ver. 11.5, 2012). Differences between means were evaluated for significance by using the least significant difference (LSD) and Duncan tests at level of 0.01 and 0.05, where noted.

RESULTS AND DISCUSSION

***In vitro* regeneration**. The regeneration of Felina was significantly higher for the C2 medium (0.1 mg/l IAA and 1.0 mg/l Z) with mean number of regenerated explants 11.33 and mean of total shoots 34.33 (Table 1). In comparison, significantly higher regeneration of the Siena and Don Jose was observed for the B2 medium (0.5 mg/l Z and 0.1 mg/l IAA), with 10.67 and 9.33 mean number of regenerated explants, respectively (Table 1). Felina required higher concentration of Z compared to Don Jose and Siena and showed greater regeneration frequency (94.4%), followed by Don Jose (92.5%) and Siena (83.33%) (Table 5, non-transformed plants). Similarly, high rates of regeneration were achieved using a combination of 0.5 mg/l Z and 0.1 mg/l IAA (Grigoriadis *et al*. 2005), whereas MS with 1 mg/l Z and 0.5 mg/l IAA showed the highest percentage of regeneration (30.4%) in tomato hybrid Rio Grande (Yasmeen 2009). Herein, the three hybrids were similar in terms of regeneration efficiency, when grown in the best

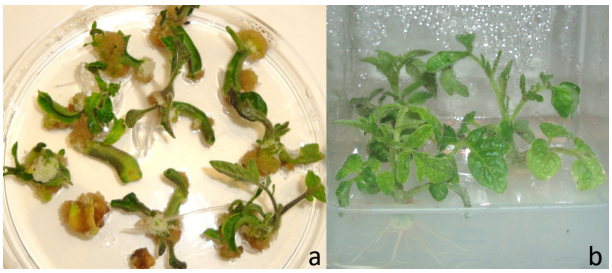

Figure 1. Regeneration of putative transformed tomato Don Jose: cotyledon explants (a) and putative transgenic plants (b)

regeneration media (Table 2) confirming that the selection of the appropriate medium for the *in vitro* regeneration of tomato is genotype specific, which affects not only the efficiency of the regeneration, but also the genetic transformation (Ajenifujah-Solebo *et al*. 2012). The micropropagation index was similar between the three hybrids, yet the *in vitro* rooting was significantly higher and the *in vivo* acclimatization successful in Felina compared to Siena and Don Jose (Table S3 in ESM).

Genetic transformation. During the first phase, using slightly older cotyledons (15-days compared to 12-days old), increased tolerance to the manipulation during the genetic transformation, which, combined with 1 day pre-culture and 2 days co-culture lead to a 16.7% success of regeneration of putative transformed cotyledons (Table 3). This is possibly due to the greater size and thickness of the explant and the accumulation of higher amounts of plant growth regulators (Velcheva *et al*. 2005). Tomato

Table 3. Effect of the cotyledon age and the length of pre-culture and co-culture on shoot regeneration during tomato transformation

Parameters	Days	Putative transformed shoots
Age	12	0.16 ± 0.077^{b}
	15	**0.58 ± 0.140^{a}**
Pre-culture	0	0.37 ± 0.150^{ab}
	1	**0.68 ± 0.170^{a}**
	2	0.06 ± 0.060^{b}
Co-culture	1	0.16 ± 0.077^{b}
	2	**0.58 ± 0.14^{a}**

Data are mean ± SE; significant differences at $P \leq 0.01$ for the cotyledon age and at $P \leq 0.05$ for the duration of pre-culture and co-culture; values followed by the same letter are not statistically different; $n = 4$ with 9 explants each; means ± SE in bold indicate the selected parameters for the best pre- and co-culture media

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Table 4. Effect of acetosyringone in the co-culture medium and thiamine in the selection medium

Parameter	Concentration	Putative transformed shoots
Acetosyringone (μM)	0	1.83 ± 0.16[b]
	100	2 ± 0.67[b]
	200	**3 ± 0.33[a]**
Thiamine (mg/l)	0.1	1.89 ± 0.28[b]
	0.4	**2.67 ± 0.27[a]**

Data are mean ± SE; significant differences of each treatment at $P \leq 0.01$; values followed by the same letter are not statistically different; $n = 3$ with 9 explants each; means ± SE in bold indicate the selected parameters for the best co-culture medium

cotyledons, between the ages of 8–18 days, are successfully used for genetic transformation indicating that the optimum age depends on the tissue, genotype and species (Gao *et al*. 2009).

Different concentrations of acetosyringone and thiamine in the selection medium affected the transformation efficiency (Table S2 in ESM). More specifically, the use of 15 days old cotyledons, pre-culture for 1 day, co-culture for 2 days with the addition of 200 μM As in the medium, and 0.4 mg/l T in the selection medium has led to a 37.04% transformation efficiency (Table 4), which is amongst the upper levels presented in the literature, varying between 6% and 43% (Wu *et al*. 2006). Different pre-culture duration between 0–3 days has been successfully applied in previous studies in various tomato hybrids (Qiu *et al*. 2007), increasing the success of the genetic transformation (Cardoza & Stewart 2003). Gao *et al*. (2009) previously reported that 1–3 days of co-culture have successfully increased the transformation efficiency. The addition of 200 μM acetosyringone in the co-culture media increased 4-fold the genetic transformation efficiency (Cortina & Culiáñez-Macià 2004). Similar results were observed with 100 μM (Ahsan *et al*. 2007), 200 μM (Raj *et al*. 2005) up to 400 μM acetosyringone (Fuentes *et al*. 2008). Wu *et al*. (2006) showed that 50, 75 and 200 μM acetosyringone combined with low pH values positively impacted the expression of the *vir* genes. The positive effects of the vitamin thiamine into the selection media is related to its antioxidant properties. In our study, higher transformation efficiency was achieved in treatments with 0.4 mg/l compared to 0.1 mg/l thiamine. Similar results were also found by Cortina and Culiáñez-Macià (2004).

The optimized genetic transformation protocol for Felina, that was also applied in Siena and Don Jose is summarized in Figure 2. The cotyledon explants of

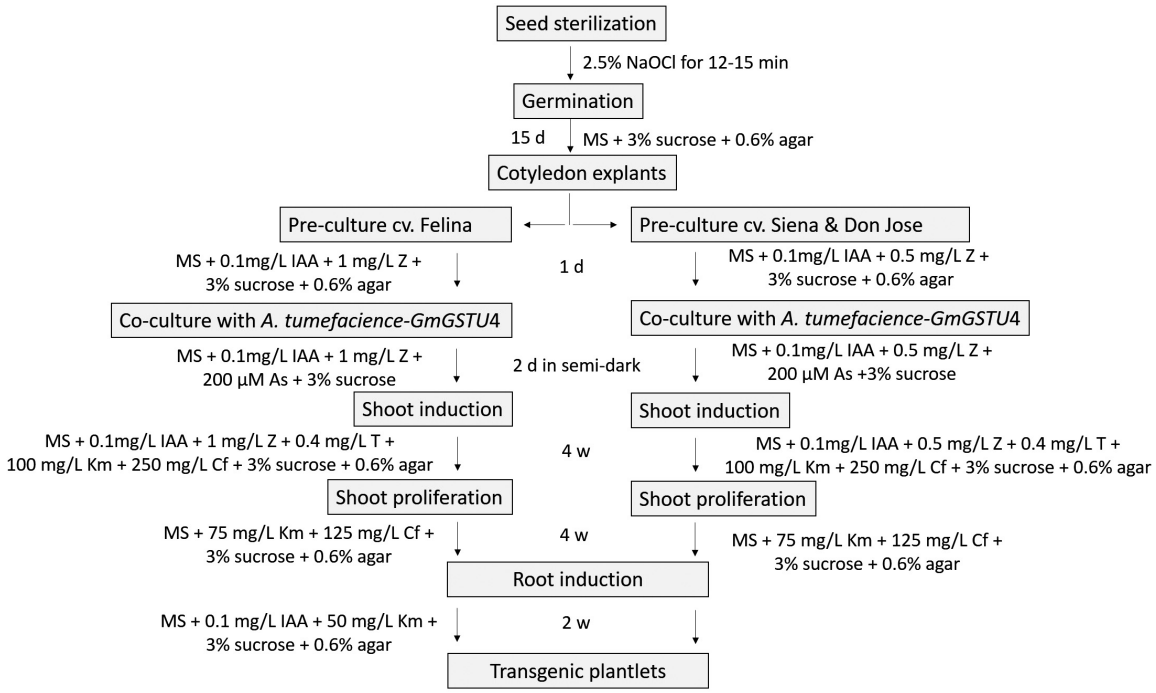

Figure 2. Procedure of overexpression of the *GmGSTU4* gene in the three commercial tomato hybrids

The lowercase letters d and w indicate days and weeks, respectively

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Table 5. Comparison between the transgenic and wild-type (WT) tomato plants in the mean number of regenerated explants and the regeneration frequency (%)

Hybrids		Mean No.*	Regeneration frequency (%)
DonJose	WT	8.33[a]	92.50
	*GmGSTU*4	3.66[b]	40.74
Siena	WT	10.00[a]	83.33
	*GmGSTU*4	4.00[b]	33.30
Felina	WT	11.33[a]	94.40
	*GmGSTU*4	3.33[b]	37.04

*Significant differences of each treatment at $P \leq 0.05$; values followed by the same letter are not statistically different ($n = 3$ with 12 and 9 cotyledons for Siena and Don Jose, respectively and $n = 4$, with 9 cotyledons Felina)

Table 6. Quantitative expression of the *35S-GmGSTU4* gene in transgenic lines C16, C26 (Don Jose), B3, B7 (Siena) and A1 (Felina)

Line	*35S-GmGSTU4* expression*
C16	3.11 (0.78–5.44)
C26	0.91 (0.29–1.53)
B3	1.95 (1.46–2.44)
B7	0.49 (0.28–0.69)
A1	3.13 (2.58–3.68)

*The values represent the fold difference in *GmGSTU4* gene relative to the endogenous *Solanum lycopersicum* actin gene in the transformed plants ($n = 3$)

Don Jose, after co-culture with the *A. tumefaciens-GmGSTU4*, presented higher percentage of shoot regeneration compared to Felina and Siena (Table 5).

Verification of putative transgenic lines. After the genetic transformation, cotyledon explants of Felina (54 out of 567), Siena (14 out of 480) and Don Jose (27 out of 540), were successfully regenerated and survived in selection medium supplemented with kanamycine. The transformation efficiency was 9.5% for the Felina hybrid and 5% for both the Siena and Don Jose hybrids, although in the best trial it reached 37.04%, 13.8% and 8.33% respectively. Each regenerated plantlet derived from independent transformation events on the selection medium. The 11 from Felina, 2 from Siena and 3 from Don Jose were positive transformed plants (Figure S2 in ESM). Two lines from each hybrid, except Felina with one line, were chosen based on their best performance and growth rate and were used for further experiments. All the selected transgenic lines were successfully acclimatised *in vivo*. The variation in the genetic transformation efficiency of the three hybrids may be explained by the development of the protocol was based on Felina and applied to the other two hybrids. Similar levels of transformation efficiency have been previously reported in different tomato model cultivars, such as Moneymaker, Micro-Tom and Rio Grande, at 5.1% (Guo *et al*. 2012), 6% (Vidya *et al*. 2000), 8% (Choi *et al*. 2011), 19.1% (Cruz-Mendivil *et al*. 2011), 20.83% (Girhepuje & Shinde 2011) up to 35.3-44.3% (Rai *et al*. 2012). This strengthens the evidence of the genotypic effect on the genetic transformation process (Ma *et al*. 2015) and the difficulty to develop a universal protocol.

Overexpression of the *GmGSTU4*. The selected transgenic lines (Figure S2 in ESM) were the A1 (Felina), B3 and B7 (Siena) and C16 and C26 (Don Jose). The lines A1, B3 and C16 showed variable *GmGSTU4* expression 3.13, 1.95 and 3.11-fold respectively in relation to the endogenous actin gene (Figure S3 in ESM, Table 6). This variation in the transgene expression may be attributed to position effect, silencing or presence of regulatory sequences at the site of integration, as reported previously (Butaye *et al*. 2005; Francis & Spiker 2005; Zhang *et al*. 2013).

CONCLUSION

In this research, we established genotype specific regeneration and *A. tumefaciens* mediated genetic transformation protocols for three tomato hybrids that produced several transgenic lines overexpressing the *GmGSTU4* gene. The streamlined procedure of combining the best regeneration protocol with a genetic transformation method that uses only two different media has achieved adequate genetic transformation frequencies on the three commercial hybrids tested for use as micropropagated rootstock material. This protocol pipeline is expected to be of commercial value for either mass production of virus-free micropropagation material of these specific varieties or in combination with the genetic transformation and cis-grafting system for use as rootstock material.

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