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Optimization of *Agrobacterium*-mediated transformation conditions for tomato (*Solanum lycopersicum* L.)

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Abstract

An orthogonal experimental design L16 (4^5) was used to optimize *Agrobacterium*-mediated transformation of cotyledon and stem explants of *Solanum lycopersicum* L. cv 'Zheza No.905'. Five factors influencing transformation efficiency including stage of explants, preculture, *Agrobacterium* density, infection time and co-cultivation duration were evaluated using the carotenoid biosynthetic gene *CsZCD* (Crocus zeaxanthin 7,8-cleavage dioxygenase), with an aim to establish a high-throughput transformation protocol for tomato. The result showed that the optimal transformation protocol for cotyledon explants consisted of 8-9 day age of seedling, 2 days of preculture, an *Agrobacterium* suspension of OD_{600} = 0.6, 3 days of con-cultivation and an infection time of 20 min. For stem explants, the protocol included 4-5 day age of seedling, 3 days of preculture, an *Agrobacterium* suspension of OD_{600} = 0.6, 4-day con-cultivation and an infection time of 15 min. Under these conditions, the transformation efficiency of the cotyledon and stem reached 26.33% and 28.00%, respectively. This protocol is also suitable to another local cultivar 'Shengya'with transformation efficiency of 19.33% and 23.33%, respectively.

Keywords: Tomato transformation; *Agrobacterium tumefaciens*; Orthogonal design; CsZCD gene.
Abbreviations: BA_6-benzyladenie; CsZCD_Crocus zeaxanthin 7,8-cleavage dioxygenase; LSD_least significant difference; MIC_ minimum inhibitory concentration; MS_Murrashinge and Skoog; NAA_naphthaleneacetic acid; *NPT*II_neomycin phosphotransferase II gene; PCR_polymerase chain reaction.

Introduction

Tomato (*Solanum lycopersicum* L.) is a major vegetable crop widely cultivated throughout the world and a genetic model for improving other dicotyledonous crop plants (McCormick et al., 1986, Ling et al., 1998), and is of great interest because variation in the carotenoid biosynthetic pathway can be readily observed by the accumulation of various carotenoid biosynthetic intermediates. *Agrobacterium*-mediated transformation has succeeded in the introduction of foreign genes into plants (Arshad et al.,2014;Wang et al., 2010), and antibiotics are widely used to suppress or eliminate *Agrobacterium tumefaciens* infecting in vitro cultures in tomato transformation in order to select transgenic tissues when its presence is no longer required, because the multiplication and rooting rates of the cultured plants can be reduced or induced to death with microbial contaminants (Grzebelus and Skop, 2014; Cassells, 1991). Moreover, elimination of *Agrobacterium* in transgenic plants is a pre-requisite to prevent gene release when these plants are transferred to the soil (Estopà et al., 2001; Barrett et al., 1997). Cefotaxime highly resistant to β-lactamases is the most commonly antibiotics employed to eliminate *Agrobacterium* from cultures (Farzaneh et al., 2013; Chevreau et al., 1997; Labia et al., 1986), and inhibits bacterial cell wall synthesis (Wang et al, 2010; Holford and Newbury, 1992), and did not have negative effects on bud regeneration when leaf explants were cultured (Chevreau et al., 1997). Kanamycin is the selection agents most widely used for plant transformation. The corresponding resistance gene (nptII) encoding an aminoglycoside 3'-phosphotransferase inactivates kanamycin by phosphorylation (Bowen, 1993). However, uses of these antibiotics to select transgenic shoots highly rely on the species, with escapes (false-positive shoots) being one of the major problems (Estop`a et al., 2001). In *Agrobacterium*-mediated tomato transformations, transformation frequencies are not only related to *Agrobacterium* density, co-cultivation time, and infection time, but also correlated with the explants, such as the seedling age and pre-cultivation time. Optimization of transformation protocols using an orthogonal experimental design reduces the number and cost of experiments, shortens selection time of the protocol, and increases transformation frequencies (Guo et al., 2012). Therefore, in this study, the factors influencing transformation efficiency such as type of explants, pre-culture, *Agrobacterium* density, infection time and co-cultivation duration were evaluated, using the carotenoid biosynthetic genes *CsZCD* encoding a chromoplast enzyme that initiates the biogenesis of these apocarotenoids by cleaving zeaxanthin (Qiu et al., 2007), with an aim to establish a high-proficiency transformation protocol for tomato through analyses resulting from orthogonal design.

Results and Discussion

Effect of cefotaxime and kanamycin on growth suppression of Agrobacterium tumefaciens

When *Agrobacterium .tumefaciens* cultured on MS with different cefotaxime concentrations, inhibitory effect on bacterial growth was observed at up 200mg/l after 2 days (Fig 1). 300mg/l of cefotaxime had shown less percentage of colony formation of *Agrobacterium tumefaciens* after 2 days (12%).

Table1 Composition of the various media.

Medium	GM	LM	PMc	PMs	SMc	SMs	RM
MSB_5 salts	0.5X	1X	1X	1X	1X	1X	0.5X
Sucrose	1%	3%	3%	3%	N	N	3%
Glucrose	N	N	N	N	1%	1%	N
Agar	0.6%	N	0.6%	0.6%	0.6%	0.6%	0.6%
pH	5.8	5.8	5.8	5.8	5.8	5.8	5.8
$AgNO_3$ (mg/L)			2.0	3.0	2.0	3.0	
Vc (mg/L)			100	100	100	**50**	
NAA (mg/L)	-	-	0.1	0.2	0.1	0.2	0.1
BA 2.0mg/L	-	-	+	+	+	+	+
Cefotaxime(mg/L)	-	-	-	-	400	400	-
Kanamycin(mg/L)	-	-	-	-	50	60	50

*N:Null

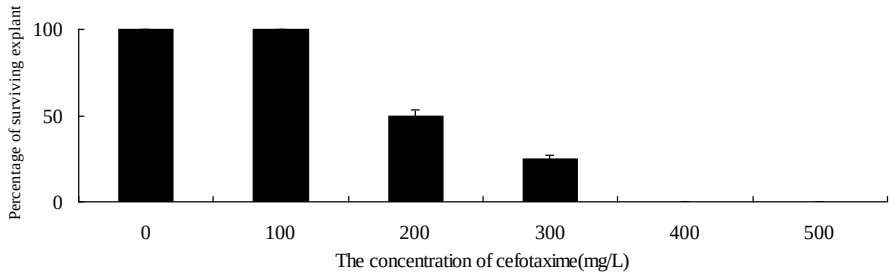

Figure 1 Effect of different concentration of cefotaxime on the growth of *Agrobacterium tumefaciens*

However, the bacterial growth was not observed at the concentration of cefotaxime 400 and 500 mg/l after 2days and till 2 weeks (Fig 1). From these observations it is concluded that the cefotaxime at up 400 mg/l completely inhibited the growth of bacteria. The results showed that 400 mg/l cefotaxime significantly decreased the callus induction and shoot generation (Table 3), and kanamycin at the concentration of 50 mg/l and 60 mg/l significantly inhibits callus induction, and shoot regeneration in the non-transformed tomato plantlets of cotyledons and stems, respectively (Table 4). Thus, 400 mg/l cefotaxime and 50 mg/l kanamycin were found to be the MIC for the selection of transformed callus and adventitious shoot buds from cotyledon explants, and 400 mg/l cefotaxime and 60 mg/l kanamycin found from stem explants.

Optimization of Agrobacterium-mediated tomato transformation

The optimum level of each factor can be got by comparing M1, M2, M3 and M4 (Table 5-6). The extreme deviation (R) was indicated in Table 5-6. R in columns showed the interaction effect on tomato efficiency, which can not be neglected. It was found that combination is better for them. By calculation, 8-9 day age of seedling + 2 days of preculture + an *Agrobacterium* suspension of OD_{600} = 0.6 + 3 days of con-cultivation + an infection time of 20 min is the best combination for cotyledon explants. Whereas, the best combination for stem explants is 4-5 day age of seedling + 3 days of preculture + an *Agrobacterium* suspension of OD_{600} = 0.6 + 4 days of con-cultivation + an infection time of 15 min. ANOVA was also shown in Table 5-6. The five factors, namely age of seedling, preculture duration, *Agrobacterium* density, infection time and co-culture condition, had significantly different effects on transformation efficiency ($P<0.01$). For cotyledon explants, age of seedlings had the largest effect on transformation efficiency, followed by preculture duration, *Agrobacterium* density, conculture condition and infection time. For stem explants, preculture duration had the largest effect on transformation efficiency ($P<0.05$), followed by *Agrobacterium* density, age of seedlings, conculture condition and infection time.

Selection and regeneration of transformants

The non-transformed explants of both leaf and stem lost their green colour and failed to develop callus and adventitious shoot buds when cultured in their correspondent selection medium. However, , the transformed explants of both leaf and stem produced calli (Figure 2C) and shoot buds in their correspondent selection medium after 3 days (Figure 2A) and 4 days (Figure 2B) of con-cultivation, respectively, and subsequently the adventitious shoots were developed from shoot buds in their selection medium (Figure 2D). Regeneration shoots were induced to root for about 4 weeks which were the main transformants (Figure 2E).The transformed shoots were successfully rooted in the root induction medium containing 50 mg L^{-1} kanamycin (Figure 2 E,F,G). The transformants were evaluated for the presence of the transformed T-DNA. The well-rooted transformed shoots were separated from the media and successfully hardened and acclimatized in the green house conditions (Figure 2 H,I).

Genotypic effect on transformation efficiency

The cotyledon explants of three tomato cultivars were used to evaluate the genotype effect on transformation efficiency with combination of the five factors including seedlings age of 8-9 days, pre-cultured for 2 days, the concentration of bacteria (OD_{600} = 0.6), con-cultivation duration of 3 days, infection time of 20 min. Whereas, the combination of the five factors for stem explants included seedlings age of 4-5 days, pre-cultured for 3 days, the concentration of bacteria (OD_{600} = 0.6), con-cultivation duration of 4 days, infection time of 15 mins.

Table 2. Orthogonal design for *Agrobacterium*-mediated transformation protocol of tomato L16(4^5).

Orthogonal combination	Age of seedlings	Preculture time (days)	Bacterium density (OD_{600})	Bacterium infection time (min)	Co-cultivation time (days)
1	2-3	0	0.2	5	1
2	2-3	1	0.4	10	2
3	2-3	2	0.6	15	3
4	2-3	3	0.8	20	4
5	4-5	1	0.2	15	4
6	4-5	0	0.4	20	3
7	4-5	3	0.6	5	2
8	4-5	2	0.8	10	1
9	6-7	2	0.2	20	2
10	6-7	3	0.4	15	1
11	6-7	0	0.6	10	4
12	6-7	1	0.8	5	3
13	8-9	3	0.2	10	3
14	8-9	2	0.4	5	4
15	8-9	1	0.6	20	1
16	8-9	0	0.8	15	2

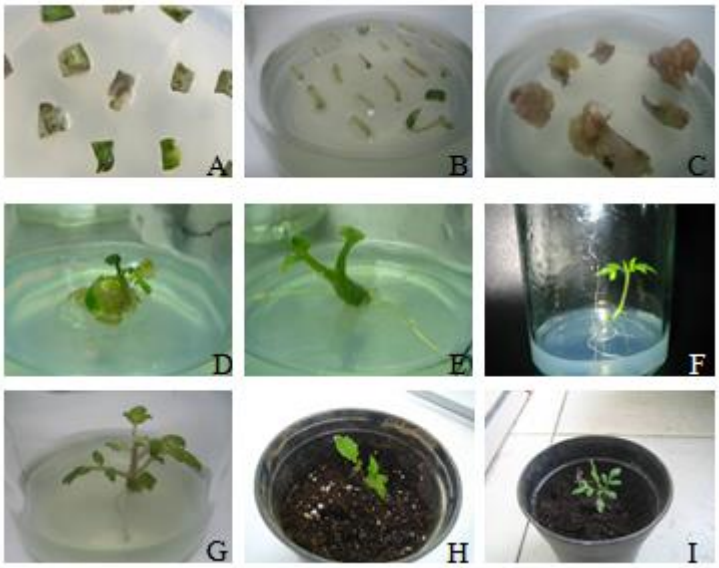

Fig 2. Adventitious shoot regeneration from putatively transformed cotyledon of tomato cv. 'Zheza No.905', in selection medium. (A) 3 days of con-cultivation of cotyledon explants after absorbing the bacterial suspension; (B) 4 days of con-cultivation of stem explants after absorbing the bacterial suspension; (C) Callus induction after 3 weeks of culture of . tomato cv.'Zheza No.905'; (D) Adventitious shoot bud induction after 8 weeks of culture of tomato cv.'Zheza No.905'.; (E) Root induction after 4 weeks in the putatively transformed shoots of tomato cv.'Zheza No.905'; (F) Root induction after 4 weeks in the putatively transformed shoots of tomato cv 'Shengya'; (G) Root induction after 4 weeks in the putatively transformed shoots of tomato cv'Fudan'; (H) Hardened transformed tomato plantlet.cv. 'Zheza No.905'; (I) Hardened transformed tomato plantlet.cv. 'Shengya'.

Among the three cultivars 'Zheza No.905', 'Fudan' and 'Yasheng' tested, the cotyledon and stem from 'Zheza No.905' showed the significantly maximum transformation efficiency with 26.33% and 28.00%, respectively. The cotyledon and stem from 'Fudan' produced the lowest transformation efficiency with 7.67% and 13.33%, respectively. (Figure 3).

Molecular analysis of transformed tomato plantlets

The transformants from 'Zheza No.905' (Lane 6-8) and 'Shengya' (Lane 12-14) showed the predicted band for *CsZCD* gene. However, the predicted band could not be detected in the transformants from 'Fudan' (Lane 9-11). Non-transformed plants of 'Zheza No.905' (Lane 3), 'Fudan' (Lane 4) and 'Shengya' (Lane 5) showed no amplification (Figure 4). In summary, the optimal transformation protocol for cotyledon explants consisted of 8-9 day age of seedling, 2 days of preculture, an *Agrobacterium* suspension of OD_{600} = 0.6, 3 days of con-cultivation and an infection time of 20 min. For stem explants, the protocol included 4-5 day age of seedling, 3 days of preculture, an *Agrobacterium* suspension of OD_{600} = 0.6, 4 days of con-cultivation and a 15 min. infection. Under these conditions, the transformation efficiency of the cotyledon and stem reached 26.33% and 28.00%, respectively. This protocol is also suitable to another local cultivar 'Shengya'with transformation efficiency of 19.33% and 23.33%, respectively.

Table 3. The effect of different concentration of cefotaxime on the generation of tomato explants.

Cefotaxime ($mg \cdot L^{-1}$)	Callus percentage（%）		Regeneration percentage（%）	
	Cotyledon	Stem	Cotyledon	Stem
0	93.67±5.13aA	89.33±2.52aA	74.67±5.13aA	74.33±2.52aA
100	94.00±3.46aA	85.33±4.73abA	65.67±4.93abAB	71.00±1.73abAB
200	87.33±9.61abAB	83.00±4.6135abA	65.67±3.21abAB	64.67±4.16bcAB
300	83.33±6.43abAB	76.67±7.10bA	62.67±8.96bAB	65.67±2.52bcAB
400	75.67±5.69bB	74.67±6.66bA	55.33±6.43bBC	61.67±3.79cB
500	57.67±8.39cC	76.67±9.07bA	43.00±7.21cC	51.33±5.51dC

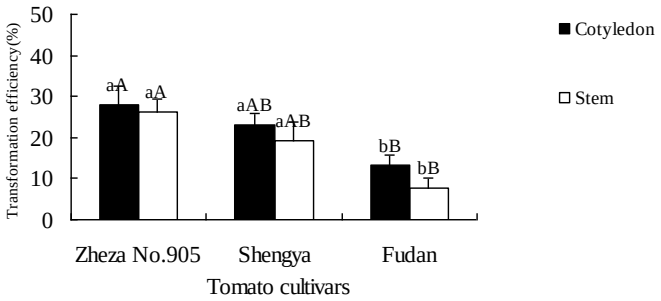

Figure 3 Effect of different cultivars on transformation efficiency

Table 4. The effect of different concentration of kanamycin on the survive of tomato cotyledon and stem.

Type of explants	Kanamycin ($mg \cdot L^{-1}$)	No. of explants	No. of explant with callus	Callus percentage（%）	Vigor and survival of the explants
Cotyledon	0	120	112	94.00±3.46aA	++++
	20	120	74	62.33±6.66bB	+++
	40	120	12	9.33±3.08cC	+
	50	120	0	0.00±0.00cC	-
	60	120	0	0.00±0.00cC	-
Stem	0	120	105	86.00±2.00aA	++++
	20	120	84	61.33±6.51bB	+++
	40	120	36	32.33±10.02cC	++
	50	120	2	1.67±0.58dD	+
	60	120	0	0.00±0.00dD	-

+ shows the vigor of explant. The more number of +, the better the vigor is.- shows non survival of explants.

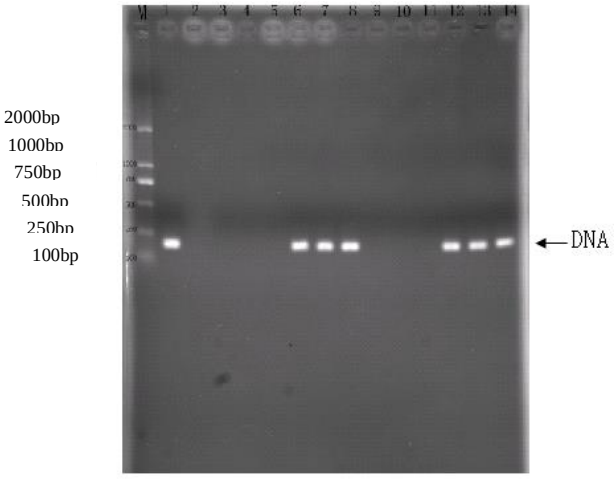

Fig 4. Analysis of the CsZCD gene in regenerated transgenic plants. Agarose gel of PCR-amplified 200bp CsZCD fragment M: 2000bp marker; 1:plasmic; 2:empty; 3:untransformed control ‘Zheza No.905’; 4: untransformed control ‘Fudan’; 5: untransformed control ‘Shengya’; 6-8: independent transgenic plants of ‘Zheza No.905’; 9-11: independent transgenic plants of ‘Fudan’; 12-14: independent transgenic plants of ‘Shengya’.

Table 5. Results of orthogonal test on the factors affecting the *Agrobacterium*-mediated transformation protocol of cotyledon of 'Zheza No.905'.

Orthogonal combination	Age of seedlings	Preculture time (days)	Bacterium density (OD600)	Bacterium infection time (min)	Co-cultivation time (days)	Transformation efficiency (%)
1	2-3	0	0.2	5	1	0.33±0.58i
2	2-3	1	0.4	10	2	1.33±1.53hi
3	2-3	2	0.6	15	3	16.33±4.51bcd
4	2-3	3	0.8	20	4	4.00±1.00ghi
5	4-5	1	0.2	15	4	11.00±2.00ef
6	4-5	0	0.4	20	3	13.33±1.53cde
7	4-5	3	0.6	5	2	4.33±1.53ghi
8	4-5	2	0.8	10	1	7.00±1.00fg
9	6-7	2	0.2	20	2	14.33±1.53cde
10	6-7	3	0.4	15	1	6.67±1.53fgh
11	6-7	0	0.6	10	4	18.67±1.53bc
12	6-7	1	0.8	5	3	12.67±3.21de
13	8-9	3	0.2	10	3	21.00±2.65b
14	8-9	2	0.4	5	4	27.00±7.00a
15	8-9	1	0.6	20	1	28.67±6.03a
16	8-9	0	0.8	15	2	18.00±2.65bcd
M1	5.50	12.67	11.67	11.08	10.67	
M2	9.00	13.50	12.08	11.92	9.58	
M3	13.08	16.17	17.00	13.17	15.75	
M4	23.67	8.92	10.50	15.08	15.25	
R	18.17	7.25	6.50	4.00	6.17	
F	110.82**	16.05**	14.74**	5.41**	17.65**	

M mean of frequencies at a certain level of each factor. e.g., M1 in the age of seedling column represents the mean transformation efficiency when age of seedling was at the level one (2-3 day age of seedling). R extreme deviation, i.e. maximum value of M minus minimum of M for each factor. Values in transformation efficiency column are means ± SD. Values followed by different letters indicating significant difference between treatments ($P < 0.05$). ** shows significant difference at $P = 0.01$ among age of seedlings, *Agrobacterium* density, infection time, con-cultivation time.

Table 6. Results of orthogonal test on the factors affecting the *Agrobacterium*-mediated transformation protocol of the stem of 'Zheza No.905'.

Orthogonal combination	Age of seedlings	Preculture time (days)	Bacterium density (OD600)	Bacterium infection time (min)	Co-cultivation time (days)	Transformation efficiency (%)
1	2-3	0	0.2	5	1	1.67±1.53i
2	2-3	1	0.4	10	2	14.00±1.00def
3	2-3	2	0.6	15	3	17.67±1.53cd
4	2-3	3	0.8	20	4	24.67±2.08b
5	4-5	1	0.2	15	4	15.33±3.05cdef
6	4-5	0	0.4	20	3	11.67±2.08fg
7	4-5	3	0.6	5	2	34.33±2.08a
8	4-5	2	0.8	10	1	16.33±2.08cde
9	6-7	2	0.2	20	2	13.67±1.53efg
10	6-7	3	0.4	15	1	27.67±3.51b
11	6-7	0	0.6	10	4	19.00±2.00c
12	6-7	1	0.8	5	3	11.67±1.53fg
13	8-9	3	0.2	10	3	17.00±2.00cde
14	8-9	2	0.4	5	4	10.00±1.00g
15	8-9	1	0.6	20	1	15.33±3.05cdef
16	8-9	0	0.8	15	2	6.00±1.00h
M1	14.50	9.50	12.00	14.17	15.17	
M2	19.25	14.00	15.75	16.50	16.92	
M3	17.83	14.50	21.33	16.58	14.42	
M4	12.08	25.67	14.58	16.42	17.17	
R	7.17	16.17	9.33	2.42	2.75	
F	34.26**	154.41**	50.58**	4.46*	5.85**	

M mean of frequencies at a certain level of each factor. e.g., M1 in the age of seedling column represents the mean transformation efficiency when age of seedling was at the level one (2-3 day age of seedling). R extreme deviation, i.e. maximum value of M minus minimum of M for each factor. Values in transformation efficiency column are means ± SD. Values followed by different letters indicating significant difference between treatments ($P < 0.05$). ** shows significant difference at $P = 0.01$, * at $P = 0.05$ among age of seedlings, *Agrobacterium* density, infection time, con-cultivation time.

Discussion

Antibiotics sensitivity

To develop a rapid and efficient *Agrobacterium*-mediated transformation method for tomato, the antibiotic cefotaxime is commonly explored for counter-selection of *Agrobacterium* (Wang et al., 2010; Hackelford and Chlan, 1996; Ellis et al., 1989). This antibiotic, at concentrations required for bacteria control, is not detrimental to the plant tissue in vitro (Wang et al., 2010). The effect of different cefotaxime concentrations on *Agrobacterium tumefaciens* growth was investigated to determine the optimal counter-selection conditions for *Agrobacterium*-mediated transformation of tomato. The results showed that cefotaxime is quite effective for inhibiting the growth of EHA105 at the relatively low concentration of 200 mg/l. The concentration of 400 mg/l cefotaxime in the medium not only effectively eliminated *Agrobacterium* growth, but also inhibited tomato differentiation, indicating that cefotaxime could be used as counter-selection of *Agrobacterium* in the *Agrobacterium*-mediated transformation of tomato. Kanamycin is an effective aminoglycosidic antibiotic used widely as a successful selective agent of transformed tomato plantlets ranges from 50 (Sivankalyani V. et al., 2014; Kaur and Bansal, 2010; Riggs et al., 2001) to 100 mg/l (Rai et al., 2013). In our study, kanamycin 50 mg/l and 60 mg/l completely blocked regeneration from untransformed explants of cotyledon and stem, respectively, and therefore, could be used to select for transformed cells. This result is in consistent with the findings of indian mulberry, *Morus indica* cv. K2 (Bhatnagar and Khurana, 2003).

Main factors influencing the transformation efficiency of Tomato

Transformation of various tomato cultivars was previously reported, and the various transformation frequencies were obtained from 6% in cv. Pusa Ruby (Vidya et al., 2000), 10-13% in Rio Grande (Safdar and Mirza, 2014) to 40% in cv. Micro-Tom (Qiu et al., 2007; Sun et al., 2006). Transformation efficiency is controlled by multiple factors such as *Agrobacterium* density, infecting time, co-cultivation time, which must be investigated to optimize the transformation procedure. The results of our study indicated that among the three factors, bacterial suspension concentration had the strongest effect on transformation in every combination. A bacterial suspension concentration with OD_{600}=0.6 optimized the transformation efficiency. Different bacterial optical densities were documented in various tomato cultivars by using different *Agrobacterium* strains (Sivankalyani et al., 2014; Singh et al., 2011; Gao et al., 2009; Qiu et al., 2007; Sun et al., 2006; Krasnyanski et al., 2001; Pozueta-Romero et al., 2001; Van Roekel et al., 1993). The second factor influencing transformation was co-cultivation time: too long a time resulted in multiplied bacteria and too short a time decreased transformation frequency. In our study, 3-day for cotyledon explants and 4-day for stem explants was an appropriate time span for co-cultivation, which differs from times documented elsewhere (Carolina and Francisco, 2004; Frary and Van Eck, 2005; Kou et al., 2007). These differences may be resulted from the genotypes of tomato and the use of different plant tissue. As for duration of *Agrobacterium* infection, 20- minute for cotyledon explants and 15-minute for stem explants was an optimum time of dipping the bacterial suspension. The present study showed that 4-5 day and 8-9 day age of seedling was optimum for the transformation of tomato ‘Zheza No.905’, and played more important role than pre-cultivation of explants where 2-day for cotyledon and 3-day for stem obtained the highest transformation efficiency. The orthogonal test showed that age of seedling played more important role in tomato transformation than bacterial suspension concentration did. A high-throughput transformation protocol was established for tomato ‘Zheza No.905’ using orthogonal design. The protocol presented here is useful for introducing functional genes into these plants.

Molecular confirmation

To confirm the presence of the CsZCD gene in putative transgenic plants, PCR was performed using its primers. Among the tested plants, six plants (three plants from ‘Zheza No.905’ and three plants from ‘Shengya’ exhibited the 200 bp band corresponding to CsZCD gene. However, the predicted band could not be detected in the transformants from ‘Fudan’ (Lane 9-11 in Fig 4), which might be resulted from that the concentration of cefotaxime was too low for explants of ‘Fudan’ to remove the *Agrobacterium* cells and the resulted adventitious shoots were somehow infected with these cells. Several authors reported similar results when regenerating transgenic shoots after *Agrobacterium* infection (Dolatabadi et al., 2014; Peña et al., 2008; Estop`a et al., 2001).

Materials and Methods

Plant materials, culture media and growth condition

The seeds of main tomato (*Solanum lycopersicum* L.) varieties ‘Zheza No.905’, ‘Shengya’, ‘Fudan’, purchased from the local market (Qiaobei Seed Company of Fuzhou), were surface sterilized according to Qiu et al. (2007). The sterilized seeds were germinated on Murrashinge and Skoog (MSB_5) germination medium (GM). Seeds were sown in a Magenta box and germinated at 25 ± 2℃ during a 16h light period. All media component are shown in Table 1.

Bacterial strains and plasmids

One binary vectors *CsZCD* containing a selectable marker neomycin phosphotransferase II gene (NPTII) that confers resistance to antibiotic kanamycin, and one *Agrobacterium* helper EHA105 were used. Plasmid were maintained in *E.coli* and bacteria were grown overnight in LB according to Qiu et al. (2007).

Sensitivity of explants to antibiotic

Various concentrations of kanamycin (0, 20, 40, 50, 60 mg L^{-1}) and cefotaxime (0, 100, 200, 300, 400, 500 mg L^{-1}) were used to test the sensitivity of cotyledon and stem explants, which were cultured in selection medium (SMc and SMs, respectively) containing respective diverse concentrations of kanamycin and cefotaxime. The minimum inhibitory concentration (MIC) of the cefotaxime and kanamycin was used for initial selection procedure. Medium without antibiotic was used as a positive control. The treatment was continued till the tissue became necrotic by sub-culturing at regular intervals of 30 days. Each experiment was carried out in triplicate.

Transformation procedure

All steps involving tomato transformation were the same as those presented by Qiu et al. (2007). *Agrobacterium* culture was centrifuged and the pellet was resuspended in liquid medium (LM) and density was set to different OD_{600}. The

cotyledons and stem explants from aseptically grown seedlings were excised and infected (Swing the plate gently) with *Agrobacterium tumefaciens* strain EHA105 harbouring CsZCD gene. They were blotted dry on sterile paper and the cotyledons explants were placed upside up onto selection medium for cotyledons (SMc) and stem (SMs). Approximately 40 explants were placed on a single Petri dish (Ø9cm). The explants were sub-cultured to the same medium every four weeks. Eight weeks later, shoots were excised and transferred to rooting medium (RM). The cultures were incubated at 25 ± 2℃ under 16h photoperiod. After 4 weeks, the well-rooted plantlets were transferred to pots containing soil:sand:vermiculite (1:1:1 ratio). Hardened transformants were maintained in the green house conditions.

PCR analysis

Plasmids were isolated using the Biocolors Prep Mini Kit (Biocolors, Shanghai, China) according to the protocol provided by the manufacturer. DNA was purified using the Biocolors Gel Midi Kit (Biocolors, Shanghai, China) according to the manufacturer's instructions.

The PCR reaction condition was selected according to Qiu et al. (2007). The primers 5'-GTCGAGTTTCGTGATG-3' and 5'-CCAGTGAATTCCCGATCTAGTAAC-3' were used for the *CsZCD* gene amplification. The predicted sizes of amplified DNA fragments were 200 bp. PCR was carried out in 25 µl volumes containing 2.5 µL 10× PCR buffer with $MgCl_2$, 800 µ*M* of dNTP mixture, 0.8 µ*M* of each primer, 0.625 U *Taq* polymerase (Biocolors), and 2µl of sample DNA. The reaction mixture was subjected to the following reaction conditions: a 5 min denaturation step at 94˚C, 35 cycles of 30 s at 94˚C, 30 s at 58˚C, 1 min at 72˚C. The amplified fragment were electrophoresed on a 1.0% (w/v) agarose gel, using Tris-borate buffer (containing 1.3M Tris, 0.7M Boric acid and 24.5 mM EDTA, pH8.4).

Experimental design and statistical analysis

Five factors were studied using a 16 (4^5) orthogonal design (Table 2). These factors are the age of seedlings, pre-culture, bacterium density, infection time and co-cultivation time. Optimal conditions for transformation efficiency were determined based on five parameters at four different levels. Every orthogonal combination contained 120 explants, and each experiment was repeated triple. Transformation frequency was recorded according to Qiu et al. (2007). Results are presented as means ± SD. Capital letters and small letters show the least significant difference (LSD) test at 0.01 and 0.05, respectively. ANOVA (Analysis of Variance) was carried out by SPSS and F value is shown.

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Reference

Arshad W, Haq IU, Waheed MT, Mysore KS, Mirza B (2014) *Agrobacterium*-mediated transformation of tomato with *rolB* Gene results in enhancement of fruit quality and foliar resistance against fungal pathogens. PLOS ONE. 9(5):1-11.

Barrett C, Cobb E, McNicol R, Lyon G (1997) A risk assessment study of plant genetic transformation using *Agrobacterium* and implications for analysis of transgenic plants. Plant Cell Tiss Org Cult.47: 135–144.

Bhatnagar S, Khurana P (2003) *Agrobacterium tumefaciens*-mediated transformation of Indian mulberry, *Morus indica* cv. K2: a time-phased screening strategy. Plant Cell Rep.21:669–675.

Bowen BA (1993) Markers for plant gene transfer. In: Kung S and Wu R(eds) Transgenic Plants, Vol 1, Academic Press Inc San Diego CA, , p 89–124.

Carolina C Francisco ACM (2004) Tomato transformation and transgenic plant production. Plant Cell Tiss Org Cult. 76: 269-275.

Cassells AC (1991) Problems in tissue culture: Culture contamination. In: Debergh PC & Zimmerman RH (eds).Micropropagation Technology and Application Kluwer Academic Publishers, Dordrecht, p 31–34.

Chevreau E, Mourgues F, Neveu M, Chevalier M (1997) Effect of gelling agents and antibiotics on adventitious bud regeneration from in vitro leaves of pear. *In Vitro* Cell Dev Biol -Plant,. 33: 173–179.

Dolatabadi B Ranjbar G, Tohidfar M, Dehestani A (2014) Genetic transformation of Tomato with three pathogenesis-related protein genes for increased resistance to *Fusarium oxysporum* f.sp. lycopersici. J Plant Mol Breeding. 2(1):1-11.

Ellis DD, Lazaroff W, Roberts DR, Flinn BS, Webb DT (1989) The effect of antibiotics on elongation and callus and bud formation from embryonic tissue of *Picea glauca*. Canadian J Forest Res. 19:1343-46.

Estopà M, Marfà V, Melé E, Messeguer J (2001) Study of different antibiotic combinations for use in the elimination of *Agrobacterium* with kanamycin selection in carnation. Plant Cell Tiss Org Cult. 65: 211–220.

Farzaneh A, Adel Y, Ali N, Younes G (2013) Determine effective concentrations of β-lactam antibiotics against three Strains of *Agrobacterium tumefaciens* and phytotoxicity on tomato and tobacco. Inter J Agro Plant Prod. 4 (11):2919-2925.

Frary A, Van Eck J (2005) Organogenesis from transformed tomato explants. Methods Mol Biol. 286: 141-150.

Gao N, Shen WS, Gao Y, Su Y, Shi WM (2009) Influence of bacterial density during preculture on *Agrobacterium*-mediated transformation of tomato. Plant Cell Tiss Org Cult. 98: 321-330.

Grzebelus E, Skop L (2014) Effect of β-lactam antibiotics on plant regeneration in carrot protoplast cultures. *In Vitro* Cell Dev Biol -Plant. 50(5): 568–575.

Guo M, Zhang YL, Meng ZJ, Jiang J (2012) Optimization of factors affecting *Agrobacterium*-mediated transformation of Micro-Tom tomatoes. Genet Mol Res. 11 (1): 661-671.

Hackelford S, Chlan CA (1996) Identification of antibiotics that are effective in eliminating *Agrobacterium tumefaciens*. Plant Mol Biol Rep. 14:50-57.

Holford P, Newbury HJ (1992) The effects of antibiotics and their breakdown products on the in vitro growth of *Antirrhinum majus*. Plant Cell Rep. 11:93–96.

Kaur P, Bansal KC (2010) Efficient production of transgenic tomatoes via *Agrobacterium*-mediated transformation. Biol Plantarum. 54:344–348.

Kou XH, Luo YB, Tian HQ, Shi Y (2007) Genetic transformation processing of tomatoes with anti-PG gene. Food Sci. 28: 187-191.

Krasnyanski SF, Sandhu J, Domier LL, Buetow E, Korban SS (2001) Effect of an enhanced CaMV 35S promoter and a fruit-specific promoter on *uida* gene expression in transgenic tomato plants. *In Vitro* Cell Dev Biol -Plant. 37:427- 433.

Labia R, Morand A, Peduzii J (1986) Timentin and β-lactamases. J Antimic Chemother. 17 (Suppl C): 17–26.

Ling HQ, Kriseleit D, Ganal MG (1998) .Effect of ticarcillin/potassium clavulanate on callus growth and shoot regeneration in *Agrobacterium*- mediated transformation of tomato (*Lycopersicon esculentum* Mill). Plant Cell Rep. 17:843–847.

McCormick S, Niedermeyer J, Fry J, Barnason A, Horsch R, Fraley R (1986) Leaf disc transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens*. Plant Cell Rep. 5: 81–84.

Peña L, Cervera M, Fagoaga C, Romero J, Ballester A (2008) Compendium of transgenic crop plants. In Kole C and Hall TC (eds).Tropical and Subtropical fruits and Nuts. Blackwell Publishing, Oxford, UK, p:1-62.

Pozueta-Romero J, Houlné G, Can˜as L, Schantz R, Chamarro J (2001) Enhanced regeneration of tomato and pepper seedling explants for *Agrobacterium*-mediated transformation. Plant Cell Tiss Org Cult. 67:173–180.

Qiu DL, Diretto G, Tavarza R, Giuliano G (2007) An improved protocol for *Agrobacterium* mediated transformation of tomato and production of transgenic plants containing carotenoid biosynthetic gene. Sci Hort. 112(2): 172-175.

Rai AC, Singh M, Shah K (2013) Engineering drought tolerant tomato plants over-expressing *BcZAT12* gene encoding a C_2H_2 zinc finger transcription factor. Phytochem. 85:44–50.

Riggs CD, Zeman K, DeGuzman R, Rzepczyk A, Taylor AA (2001) Antisense inhibition of a tomato meiotic proteinase suggests functional redundancy of proteinases during microsporogenesis. Genome. 44:644–650.

Safdar N, Mirza B (2014) An experimental comparison of different transformation procedures assessed in tomato cv Rio Grande with yeast *HAL1* gene. Turk J Biochem. 39(3):245–252.

Singh S, Rathore M, Goyary D, Singh RK, Anandhan S, Sharma DK, Ahmed Z (2011) Induced ectopic expression of At-CBF1 in marker-free transgenic tomatoes confers enhanced chilling tolerance. Plant Cell Rep. 30:1019–1028.

Sivankalyani V, Takumi S, Thangasamy S, Ashakiran K, Girija S (2014) Punctured- hypocotyl method for high-efficient transformation and adventitious shoot regeneration of tomato. Sci Hort. 165: 357–364.

Sun HJ, Uchii S, Watanabe S, Ezura H (2006) A highly efficient transformation protocol for Micro-Tom, a model cultivar for tomato functional genomics. Plant Cell Physiol. 47:426–431.

Van Roekel JSC, Damm B, Melchers LS, Hoekema A (1993) Factors influencing transformation frequency of tomato (*Lycopersicon esculentum*). Plant Cell Rep. 12:644–647.

Vidya CSS, Manoharan M, Kumar CTR, Savithri HS, Sita GL (2000) *Agrobacterium* -mediated transformation of tomato (*Lycopersicon esculentum* var. Pusa Ruby) with coat-protein gene of *Physalis* mottle tymovirus. J Plant Physiol. 156:106–110.

Wang P, Wang G, Teng YJ, Li XS, Ji J, Xu XH, Li YZ (2010) Effects of cefotaxime and kanamycin on thallus proliferation and differentiation in *Porphyra yezoensis* and their inhibition on *Agrobacterium tumefaciens*. Marine Biol Res. 6: 100-105.