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Optimization of factors affecting *Agrobacterium*-mediated transformation of Micro-Tom tomatoes

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ABSTRACT. Micro-Tom is the smallest known variety of tomatoes. An orthogonal experimental design $L_{16}(4^5)$ was used to optimize *Agrobacterium*-mediated transformation of cotyledon explants of *Lycopersicon esculentum* cv. Micro-Tom. Four parameters were investigated to determine their effect on transformation frequency: the concentration of bacterial suspension, time of dip in bacterial suspension, co-cultivation time, and concentration of carbenicillin. We also examined the effect of these parameters on contamination rate, necrosis rate, mortality, cut-surface browning rate, and undamaged explant rate. Both the bacterial and carbenicillin concentrations had a significant influence on the rate of infected explants. The time of co-cultivation also had a significant influence on the transformation parameters. The optimal transformation protocol consisted of an *Agrobacterium* suspension of 0.5×10^8 cells/mL (OD_{600} = 0.5) and an infection time of 5 min, one day of co-cultivation and 500 mg/L carbenicillin. Under these conditions, the transformation efficiency of the shoots reached 5.1%; the mean transfor-

mation frequency was 3.9% (N = 838).

Key words: Orthogonal design; Micro-Tom; Tissue culture; Hygromycin phosphotransferase gene

INTRODUCTION

A miniature dwarf tomato (*Lycopersicon esculentum*), Micro-Tom, is a model cultivar for tomato functional genomics. This cultivar has several unique features, including small size and short life cycle, which allow the mature fruit to be harvested within 70-90 days after sowing. Establishing a high-throughput transformation protocol for Micro-Tom was simple using *Agrobacterium* (Meissner et al., 1997).

Previous analyses of *Agrobacterium*-mediated tomato transformation protocol optimization examined only the merits of classical fractional factorial design, such as explants, feeder layers, time of preculture, inoculation with *Agrobacterium tumefaciens*, co-cultivation, and antibiotic concentration (Xia et al., 2004; Hui et al., 2007; Zen and Cheng, 2008; Ling et al., 2009). This method requires a large number of experiments that can become expensive, however, as mass transfer depends on several variables. In fact, both the main effects of the variables and the interactions among variables should be considered, because these interactions exist in many parametrical problems.

Orthogonal design, the theory and methodology as well as source and characteristics of which have been described elsewhere, is a sophisticated time- and cost-saving testing strategy that draws an orthogonal array to pinpoint areas in which variations may be successfully reduced (Ross, 1988). With probability theory, mathematical statistics, and experience as the basis, the use of a standardized orthogonal table to design experiment, calculate and analyze results, and quickly find an optimal scheme is an efficient treatment of multivariate optimization methods in scientific computing. In addition to keeping the merits of classical fractional factorial design, this strategy takes into account the interactive effects among variables. Optimization of transformation protocols using an orthogonal experimental design reduces the number and cost of experiments (Min, 2009), shortens the protocol selection time, and increases transformation frequencies.

Transformation frequencies were found to be very poor when the hygromycin phosphotransferase gene (*HPTII*) was used as the selection agent. Etiolation occurred when the regeneration shoots were growing for a time period, and obtaining surviving plants was difficult (Zen and Cheng, 2008). Therefore, the involvement of *HPTII* in the transformation protocol of Micro-Tom required further optimization. In this study, we established a high-throughput transformation protocol for Micro-Tom through design and analyses resulting from orthogonal design. This protocol could become a powerful tool for functional genomics in tomatoes.

MATERIAL AND METHODS

Plant materials

Seeds of *L. esculentum* Mill. cultivar Micro-Tom was surface sterilized in 70% (v/v) ethanol for 30 s and in 10% (v/v) sodium hypochlorite solution with one drop of Tween-80 for 10 min and then rinsed three times in sterilized distilled water. The seeds were germinated in

a conical flask with 40 mL Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with 15 g/L sucrose and 3 g/L plant agar. The pH was adjusted to 5.8. All cultures were maintained at 25°C under a 16-h light/8-h dark photoperiod with a light intensity of 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$. For the genetic transformation, the seedlings were used when the cotyledons expanded fully and the true leaves were 1 to 2 mm in length.

Orthogonal design

An orthogonal experimental design L_{16} (4^5) (Table 1) was used to evaluate the effects of bacterial suspension concentration, time of dip in bacterial suspension, co-cultivation time, and carbenicillin concentration on transformation frequencies after infection with *Agrobacterium*. Data analysis was performed with DPS v7.05. Optimal conditions for productivity were determined based on five parameters at four different levels. Every orthogonal combination contained 7-10 explants, and each experiment was repeated twice.

Table 1. Orthogonal design for *Agrobacterium*-mediated transformation protocol of Micro-Tom $L_{16}(4^5)$.

Orthogonal combination	Concentration of bacterial suspension (OD_{600})	Time of dip in bacterial suspension (min)	Co-cultivation time (days)	Concentration of carbenicillin (mg/L)
1	0.2	1	1	200
2	0.3	5	2	300
3	0.5	10	3	400
4	0.7	15	4	500
5	0.2	5	4	400
6	0.3	1	3	500
7	0.5	15	2	200
8	0.7	10	1	300
9	0.2	10	2	500
10	0.3	15	1	400
11	0.5	1	4	300
12	0.7	5	3	200
13	0.2	15	3	300
14	0.3	10	4	200
15	0.5	5	1	500
16	0.7	1	2	400

Agrobacterium preparation

Agrobacterium strain EHA105 with appropriate plasmids pCAMBIA1300-2A11-*INH* (constructed in our laboratory) was used for this study. The chimeric *HPTII* gene on pCAMBIA1300 conferred to transformed plant cells the capability to grow on medium containing hygromycin. This strain was grown overnight at 28°C and 200 rpm in 5 mL yeast extract beef medium containing 50 mg/L kanamycin and 100 mg/L rifampicin. One milliliter of the bacterial suspension (optical density at 600 nm [OD_{600}] ≈ 1.0) was placed in 50 mL yeast extract beef medium and centrifuged with various OD_{600} values (0.2, 0.3, 0.5, and 0.7). The bacteria were resuspended in the same volume of MS medium with 100 µM/L acetosyringone and used for *Agrobacterium* inoculation.

Transformation method

Cotyledons were sectioned into two halves across the midvein region. The end of each

cotyledon was cut to allow it to adsorb the bacterial suspension. The explants were dipped in the bacterial suspension for 1, 5, 10, or 15 min and blotted dry on a sterilized paper towel. The explants were placed in co-cultivation medium (pH 5.8) containing MS salts, 30 g/L sucrose, 3 g/L agar, and 1.5 mg/L zeatin. The plate was incubated in darkness for 1, 2, 3, or 4 days at 25°C. The explants were subcultured into callus induction medium (pH 5.8) containing MS salts, 30 g/L sucrose, 3 g/L agar, 1.5 mg/L zeatin, 24 mg/L hygromycin, and 200, 300, 400, or 500 mg/L carbenicillin sodium. When calli with shoot buds formed from the cotyledon fragment, the zeatin concentration was adjusted to 1 mg/L (shoot elongation medium). When calli with adventitious buds developed from the explants, the cotyledons were cut off and transferred to a new conical flask containing shoot elongation medium for shoot elongation. When a stem developed in addition to a leaf, the stem was cut off at the maximum possible length and transferred to new shoot elongation medium. When a stem developed in addition to a leaf, they were cut and transferred to rooting medium. The rooting medium (pH 5.8) contained MS medium with 0.05 mg/L NAA, 15 g/L sucrose, 3 g/L agar, 17 mg/L hygromycin, and 500 mg/L carbenicillin sodium.

Polymerase chain reaction (PCR) analysis

Genomic DNA was extracted from the *in vitro* grown leaves of putative transgenic plants using a plant total DNA extract kit (TIANGEN BIOTECH Co., Ltd., Beijing, China) and analyzed using PCR amplification using primers for *HPTII* (hpt-forward: 5'-TTGGCGACCTCGTATTGGGA-3', hpt-reverse: 5'-CAAGACCTGCCTGAAACCGAA-3'). Plasmid DNA and untransformed plant DNA were used as positive and negative controls, respectively. The amplification conditions consisted of pre-denaturation at 94°C for 10 min, denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s for a total of 30 cycles with a final extension at 72°C for 10 min. The reaction products (392 bp) were separated on 1.0% agarose with an electrophoresis system (Sub-cell model 192; Bio-Rad, USA).

Statistical analyses

Regeneration frequency was calculated from the ratio of explants with adventitious regeneration to bud total explants. Regeneration frequency of resistance bud was calculated from the ratio of the number of regeneration resistance adventitious buds to total explants. Transformation frequency was calculated from the ratio of successfully transformed transplanted Micro-Tom plants to total explants.

RESULTS

Several factors have been reported to affect transformation frequency. In this study, contamination rate, necrosis rate, mortality, cut-surface browning rate, and undamaged explant rate were tested.

Contamination rate comparison

We studied factors influencing contamination rate, and sorted in descending order, they

were as follows: co-cultivation time > concentration of carbenicillin > bacterial suspension concentration > time of dip in the bacterial suspension (Tables 2 and 3). In these experiments, we observed that the main factor affecting the contamination rate of transformation was co-cultivation time. Increasing co-cultivation time from 1 to 2 days markedly increased the contamination rate. Table 3 shows the highly significant difference between 1 and 2 days, whereas the differences between co-cultivation times of 1 day and 3 or 4 days were not significant. The second factor influencing contamination rate of transformation was concentration, both of carbenicillin and of bacterial suspension ($P < 0.05$). The time of dip in the bacterial suspension had no significant influence. The main factor influencing contamination rate of transformation was co-cultivation time. When carbenicillin reached a fairly high level, reducing the co-cultivation time decreased the contamination rate. A comparison of contamination rates showed that a bacterial suspension at a concentration of 0.5×10^8 cells/mL ($OD_{600} = 0.5$), co-cultivation of 1 day, and a bacterial concentration of 400 mg/L minimized contamination rate.

Table 2. Results of orthogonal test on the factors affecting the *Agrobacterium*-mediated transformation protocol of Micro-Tom.

Orthogonal combination	Contamination rate	Necrosis rate	Cut-surface browning rate	Mortality	Undamaged rate
1	34.8	71.5	33.1	21.5	0.0
2	100.0	71.5	100.0	0.0	0.0
3	73.8	83.8	93.8	0.5	0.0
4	100.0	74.1	100.0	6.3	0.0
5	94.5	88.9	100.0	0.0	5.6
6	81.3	68.8	87.5	12.5	0.0
7	100.0	91.7	100.0	0.0	0.0
8	6.3	78.6	26.8	0.0	0.0
9	100.0	92.9	100.0	0.0	0.0
10	0.0	56.3	50.0	0.0	18.8
11	100.0	69.1	100.0	0.0	0.0
12	100.0	81.3	100.0	0.0	0.0
13	100.0	73.0	100.0	0.0	0.0
14	100.0	58.6	100.0	0.0	0.0
15	6.3	50.0	18.8	18.8	31.3
16	100.0	92.9	100.0	0.0	0.0

Necrosis rate comparison

We studied factors influencing necrosis rate and observed that differences in the data range (R values) for factor co-cultivation time and bacterial suspension concentration were both significant ($P < 0.05$), but differences in carbenicillin concentration and the time of dip in the bacterial suspension were not significant for necrosis rate (see Tables 2 and 3). The necrosis rate increased with co-cultivation time and bacterial suspension concentration. The greater the necrosis, the fewer resistant calli we obtained. A comparison of necrosis rates showed that a bacterial suspension at a concentration of 0.5×10^8 cells/mL ($OD_{600} = 0.5$) and co-cultivation time of 1 day minimized necrosis rate.

Mortality comparison

We studied factors that influence mortality and observed that differences in the R values for co-cultivation time and carbenicillin concentration were both significant ($P < 0.05$),

Table 3. Orthogonal design-direct analysis for different transformation factors influence on K and R of contamination rate, necrosis rate, mortality, cut-surface browning rate, and undamaged explant rate.

	Level	Concentration of bacterial suspension (OD_{600})	Time of dip in bacterial suspension (min)	Co-cultivation time (days)	Concentration of carbenicillin (mg/L)
Contamination rate	K*1	82.313	79.013	11.825	83.700
	K2	70.313	75.175	100.000	76.563
	K3	70.000	70.000	88.750	67.050
	K4	76.563	75.000	98.613	71.875
	R*	12.313	9.013	88.175	16.650
	P	0.192	0.558	0.0001	0.0878
Necrosis rate	K1	81.550	75.525	64.063	75.725
	K2	63.750	72.900	87.200	73.013
	K3	73.613	78.425	76.688	80.438
	K4	81.688	73.750	72.650	71.425
	R	17.938	5.525	23.138	9.013
	P	0.302	0.953	0.209	0.833
Mortality	K1	5.363	8.488	10.050	5.363
	K2	3.125	4.688	0.000	0.000
	K3	4.813	0.125	3.250	0.125
	K4	1.563	1.563	1.563	9.375
	R	3.800	8.363	10.050	9.375
	P	0.836	0.305	0.176	0.162
Cut-surface browning rate	K1	83.263	80.138	32.150	83.263
	K2	84.375	79.688	100.000	81.700
	K3	78.125	80.138	95.313	85.938
	K4	81.700	87.500	100.000	76.563
	R	6.25	7.8125	67.85	9.375
	P	0.203	0.049	0.0001	0.038
Undamaged explant rate	K1	1.388	0.000	12.500	0.000
	K2	4.688	9.200	0.000	0.000
	K3	7.813	0.000	0.000	6.075
	K4	0.000	4.688	1.388	7.813
	R	7.813	9.200	12.500	7.813
	P	0.0039	0.0005	0.0001	0.001

K = mean value of the test corresponding to a factor in the same level; $R = K_{max} - K_{min}$. Asterisks (K*1) and (R*) mean the note of K and R.

but differences in the bacterial suspension concentration and the time of dip in the bacterial suspension were not significant (see Table 3). In these experiments, we observed that the main factor affecting mortality of transformation was co-cultivation time and carbenicillin concentration. Increasing co-cultivation time and carbenicillin concentration can lead to a significantly high rate of mortality. The results indicated that 2 and 4 days of co-cultivation and 300 and 400 mg/L carbenicillin concentration minimized mortality.

Cut-surface browning rate comparison

The R values in Tables 2 and 3 show that experimental factors influenced cut-surface browning rate in this order: co-cultivation time > carbenicillin concentration > time of dip in the bacterial suspension > bacterial suspension concentration. The R value of necrosis rate is also about much larger than other factors. Differences in co-cultivation time, carbenicillin concentration, and the time of dip in the bacterial suspension were highly significant ($P < 0.01$). Differences in bacterial suspension concentration also showed significance ($P < 0.05$). Consequently, all factors in this experiment showed significant effects on cut-surface browning rate, which was a key indicator of the ability of newborn resistant callus. The results indicated that a bacterial suspension at a concentration of 0.5×10^8 cells/mL ($OD_{600} = 0.5$), 1 day

of co-cultivation, an infection time of 5 min in the bacterial suspension, and a carbenicillin concentration of 500 mg/L minimized the cut-surface browning rate.

Undamaged explant rate comparison

The R values in Tables 2 and 3 show that experimental factors influenced the rate of undamaged explants in this order: co-cultivation time > time of dip in the bacterial suspension > bacterial suspension concentration > carbenicillin concentration, and the differences in all factors were highly significant. The results showed that a bacterial suspension at a concentration of 0.5×10^8 cells/mL (OD_{600} = 0.5), 1 day of co-cultivation, an infection time of 5 min in the bacterial suspension, and a carbenicillin concentration of 500 mg/L maximized the rate of undamaged explants.

Molecular identification and statistics of transgenic plants

Phenotypically normal and fertile plants were grown to maturity in the greenhouse. We obtained 10 lines of 33 transgenic tomato plants from 19 lines of regeneration (Figures 1 and 2). The optimized protocol was used to obtain a regeneration frequency averaging 9.1% and a 5.1% regeneration frequency of resistance bud (Figures 3 and 4). Currently, average transformation frequencies are approximately 3.9%.

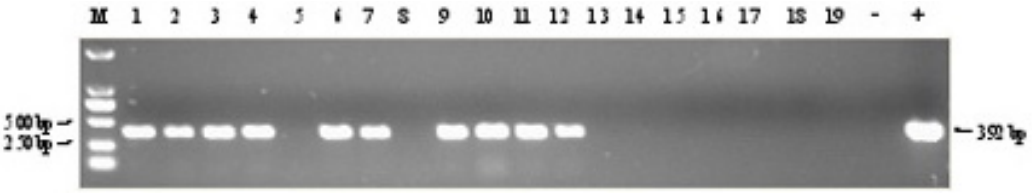

Figure 1. PCR identification of transgenic Micro-Tom plants with the *HPTII* gene. *Lane M* = DNA marker DL2000; *lanes 1-19* = independent hygromycin-resistant plants; *lane* + = plasmid of pCAMBLA1300-2A11-*INH* as the positive control; *lane* - = non-transgenic Micro-Tom plant.

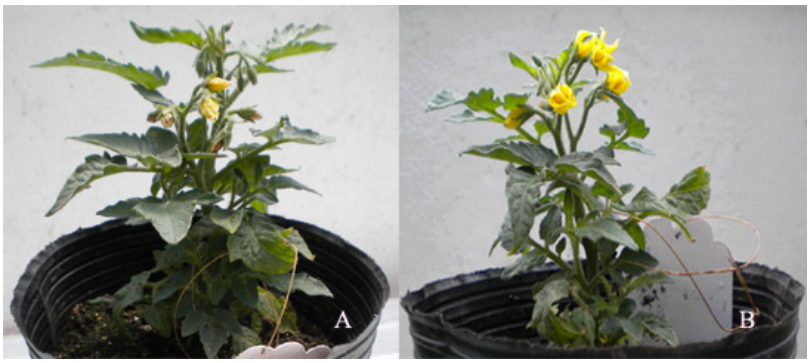

Figure 2. The growth of transgenic Micro-Tom plants. **A.** Control. **B.** Transgenic plant.

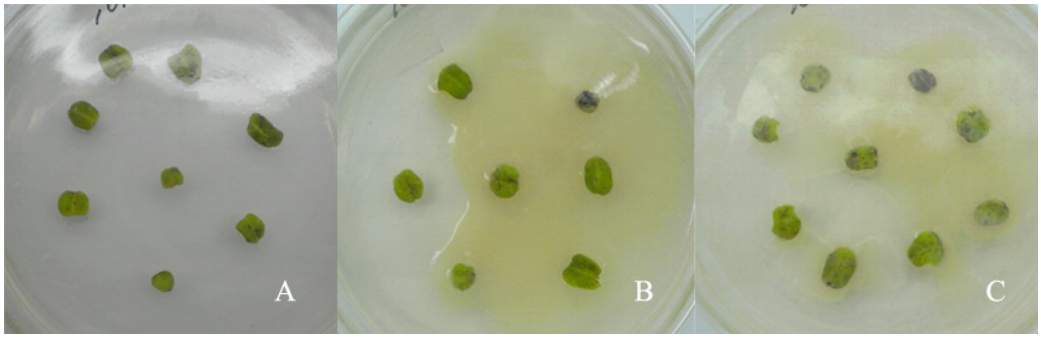

Figure 3. Influence of the bacterial suspension on Micro-Tom cotyledon for the orthogonal combination 15, 10 and 5. **A.** Orthogonal combination 15. **B.** Orthogonal combination 10. **C.** Orthogonal combination 5.

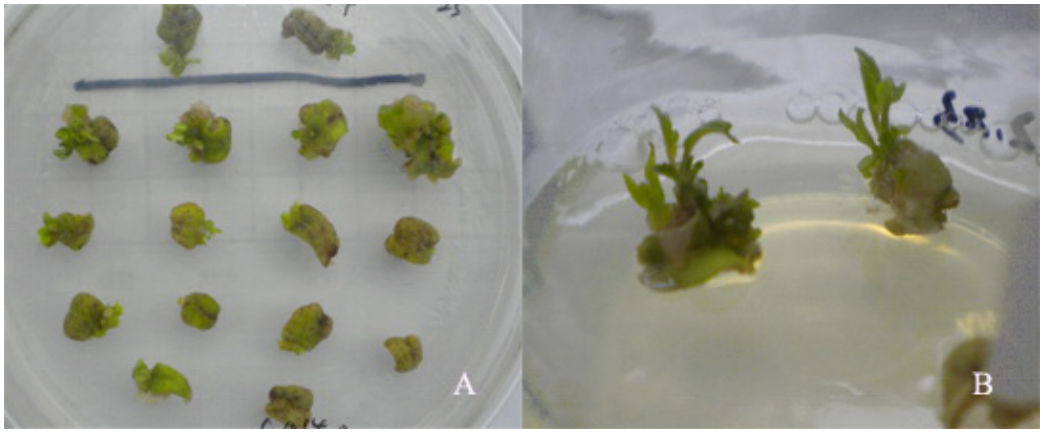

Figure 4. Regeneration of cotyledon growth after adsorbing the bacterial suspension. **A.** Infected regeneration callus. **B.** Infected regeneration shoots.

DISCUSSION

The results of our study indicated that co-cultivation time was the main factor influencing transformation: too long a time resulted in multiplied bacteria and too short a time decreased transformation frequency. In our study, 1 day was an appropriate time span for co-cultivation, which differs from times reported elsewhere (Biao et al., 1994; Liqiong et al., 2002; Carolina and Francisco, 2004; Yan et al., 2004; Frary and Van Eck, 2005; Kou et al., 2007), which may be related to the genotypes of tomato and the use of different plant tissue.

Carbenicillin concentration was the second factor that affected cut-surface browning rate, contamination rate, and mortality. In our trials, carbenicillin could not suppress the growth of *A. tumefaciens* when colonies were observed on the plate. Consequently, we needed to regulate the carbenicillin concentration to inhibit *A. tumefaciens* early in the selection culture. However, cut-surface browning rate showed strong effects on transformation frequencies. Infection caused explants to turn brown, induced necrosis, and reduced callus develop-

ment. Our results indicated that 500 mg/L carbenicillin was the optimal concentration for suppressing *A. tumefaciens* in early selection culture with minimal poisonous effects.

Bacterial suspension concentration had the strongest effect on necrosis rate in every combination. We conclude that the occurrence of necrosis might be associated with factors outside of our experimental variables. In other word, bacterial suspension concentration was not a key factor in successful transformation. A bacterial suspension concentration of 0.5×10^8 cells/mL ($OD_{600} = 0.5$) minimized necrosis rate.

The rate of undamaged explants was a comprehensive index of the association of infected explants and co-cultivation time, the time of dip in the bacterial suspension, bacterial suspension concentration, and carbenicillin concentration. This rate was the most important index of successful transformation, indicating whether explants remained green and exhibited vitality and whether they developed resistance calli. Orthogonal combination 15 was the best treatment with which to obtain a high rate of undamaged explants (see Table 4).

Table 4. Variance analysis of different levels for each factor.

Orthogonal combination	Average	5% significant difference	1% significant difference
15	31.25	a	A
10	18.75	b	B
5	5.55	c	C

In *Agrobacterium*-mediated transformations in tomato, transformation frequencies are not only related to co-cultivation time, the time of dip in the bacterial suspension, and bacterial suspension and carbenicillin concentrations but also correlated with the plasmid of transformation, which contains various selection marker genes such as kanamycin (*Kan*), that affect genetic transformation. In many traits of interest, because the enzymatic assay for *Kan* had minimal effects on plant regeneration compared with that for *HPTII*, most transformation experiments have used plasmids of *Kan* as selection markers (Li et al., 2007; Ying et al., 2008; Chen et al., 2010). The transformation efficiency is not a limiting factor; several authors have reported transformation rates with efficiencies ranging from 12 to 14% (Koonneef et al., 1986; McCormick et al., 1986; Hamza and Chupeau, 1993; Van Roekel et al., 1993; Ellul et al., 2003; Carolina and Francisco, 2004). Two publications report transformation frequencies of only approximately 1% (Chyi and Phillips, 1987; Pozueta-Romero et al., 2001), whereas others report efficiencies up to 40% (Hyeon et al., 2007; Zen and Cheng, 2008). Nevertheless, the only publication of *Agrobacterium*-mediated transformation with a plasmid harboring *HPTII* as the reporter gene reported resistance shoot transformation frequencies of only 3% (Zen and Cheng, 2008). In our trail, resistance shoot transformation frequency was 5.1%, with 33 transgenic plants obtained and transformation frequencies averaging 3.9%. This frequency is lower than those reported with *Kan* as the selection marker but higher than that reported with *HPTII* as the selection marker, indicating that the orthogonal design method established a high-throughput transformation protocol for an *HPTII* selection plasmid. The use of this technique opens new prospects for the selection of plasmids for plant transformation.

Orthogonal design is more widely used in industry than in agriculture. Using orthogonal design, we established a high-throughput transformation protocol for the Micro-Tom tomato. The results suggest that the protocol presented here is useful for introducing functional genes into these plants.

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