ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 7 (3): 537-543, 2008
ISSN 1682-296X

Transformation of Tomato with TYLCV Gene Silencing Construct Using Optimized *Agrobacterium*-Mediated Protocol

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Abstract: In this study, different tomato genotypes were transformed with silencing trigger construct that showed high efficiency in controlling *Tomato yellow leaf curl virus*. Cotyledons and hypocotyls of the tomato hybrid (981 X TY-6) were cultured on eight regeneration media containing different concentrations of auxins and cytokinins. Zeatin and Indole Acetic Acid (IAA) at 1 and 0.1 mg L^{-1}, respectively, were significantly enhanced shoot regeneration. Different concentrations of Phosphinothericin (PPT) were used to select transformed tomato plantlets; the lowest PPT concentration (5 mg L^{-1}) inhibited the growth of non-transformed plantlets but did not affect the growth of transformed plantlets. The inhibitory effects of four antibiotics (carbincillin, clavamox, cefotaxin and kanamycin) on the growth of *Agrobacterium tumefaciens* and the regeneration of shoots were investigated; carbincillin and clavamox at 500 mg L^{-1} showed high inhibitory effect. The transformation data indicated that the rate of regeneration from transformed tomato line (MP-1) was significantly higher than that of tomato hybrid (981 X TY-6).

Key words: Transformation of tomato (*Lycopersicon esculentum*), *Agrobacterium tumefaciens*

INTRODUCTION

Tomato (*Lycopersicon esculentum*) is one of the most important vegetable crops and is considered as a genetic model for improving dicotyledonous plants (Park *et al*., 2003). In basic and practical studies for tomato improvement, successful transformation is essential. However, tomato transformation is not routine nor reliable (Ling *et al*., 1998). Therefore, the development of an efficient and genotype independent tomato transformation method is crucial.

Tomato yellow leaf curl virus (TYLCV) belongs to the genus *Begomovirus* of the family Geminiviridae (Fauquet *et al*., 2000) and is transmitted by the whitefly *Bemisia tabaci* (Pant *et al*., 2001; Briddon and Markham, 1995; Pico *et al*., 2001). TYLCV was first reported in the Middle East in 1960s (Cohen and Harpaz, 1964). In Jordan, the disease was reported for the first time in 1978 (Makkouk, 1978) and recently, three viruses that belong to TYLCV complex were reported by Anfoka *et al*. (2005). These viruses are: *Tomato yellow leaf curl virus* (TYLCV, formerly TYLCV-Israel), *Tomato yellow leaf curl Sardinia virus* (TYLCSV, formerly TYLCV-Sardinia) and *Tomato yellow leaf curl virus*-Mild (TYLCV-Mild).

TYLCV is a very devastating disease throughout Africa, Middle East, South-East Asia and many other countries around the world (Pico *et al*., 1996). The severity of the disease is dependent on the epidemiology, distribution and the population density of the whitefly vector. In Jordan Valley, yield losses up to 100% are expected when tomatoes are grown in the open field between September and May (Makkouk, 1978).

Agrobacterium tumefaciens is a soil-born pathogen that plays a major role in the development of plant genetic engineering and the basic research in molecular biology. It accounts for about 80% of the transgenic plants that produced so far (Wei *et al*., 2000). *A. tumefaciens* can genetically transform plant cells by transferring the T-DNA from its (Ti) plasmid into the genome of infected plants (Zupan *et al*., 2000). The generation of transgenic plants having new traits is difficult to be obtained via traditional breeding programs. Advances in tissue culture and molecular biology techniques lead to the development of transformation protocols for both monocot and dicot plants with certain gene(s). Transformation requires the insertion of DNA into plant cells, incorporation of inserted DNA into chromosomes and expression in cells that could be induced to regenerate transgenic plants (Bradshaw *et al*., 1996; Jouanin *et al*., 1993; Kim *et al*., 1997).

The first attempt to transform tomato was achieved by McCormic *et al*. (1986). Since then, several studies

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improved the technique of tomato transformation (Fillatti *et al*., 1987; Fischhoff *et al*., 1987; Van Roeke *et al*., 1993; Frary and Earle, 1996; Ling *et al*., 1998; Vidya *et al*., 2000; Wei *et al*., 2000; Sun *et al*., 2005). Tomato genotypes vary in their response to specific regeneration and transformation treatments and standardization of the various transformation procedures needs to be improved (Wei and Gregory, 2001).

Recently, Abhary *et al*. (2006) showed, in a transient assay, the efficiency of gene silencing in controlling the disease caused by TYLCV complex. Tomato plants infiltrated with a silencing construct, deduced from the genome of three viruses belong to the TYLCV complex, were resistant to TYLCV infection. In this study, tomato was transformed with TYLCV gene silencing construct using optimized *Agrobacterium*-mediated protocol.

MATERIALS AND METHODS

Four experiments were designed to achieve the goal of this study. The first one was conducted to optimize the medium for shoot initiation from cotyledons and hypocotyls of the tomato hybrid (981 X TY-6) by using different concentrations of growth regulators. In the second one, the inhibitory effects of different antibiotics on the growth of *A. tumefaciens* were investigated. In the third one, the optimum concentration of PPT that can be used to select transformants was determined. In the fourth one, tomato plantlets were transformed with TYLCV gene silencing construct.

Plant materials: Seeds of the tomato hybrid (981 X TY-6) were surface sterilized with 10% sodium hypochlorite solution and 0.1% of Tween-20 for 15 min. After rinsing three times with sterile distilled water, 10-15 seeds were germinated in Petri dishes containing 20-25 mL of solid Nitsch medium (Nitsch and Nitsch, 1969). Petri dishes were incubated at 28°C under a 16/8 h (light/dark) photoperiod.

Cotyledons (up side down position) and hypocotyls from 8-9 day-old seedlings were transferred to Petri dishes containing 20-25 mL of feeder medium {MS salt (Murashige and Skooge, 1962) plus 100 mg L^{-1} m-inositol, 0.5 mg L^{-1} thiamine-HCl, 2 mg L^{-1} glycine, 5 mg L^{-1} nicotinic acid, 0.05 mg L^{-1} biotin, 1 mg L^{-1} 2, 4-D, 2.5 g L^{-1} phytagel and 30 g L^{-1} sucrose}; pH was adjusted to 5.8. The Petri dishes were incubated for 72 h at 28°C, under a 16/8 h (light/dark) photoperiod. Cotyledons (up side down position) and hypocotyls were then transferred to Petri dishes containing 20-25 mL of callus and shoot initiation medium.

Shoot initiation: Callus and shoot initiation media containing {MS salt plus 100 mg L^{-1} m-inositol, 0.5 mg L^{-1} thiamine-HCl, 2 mg L^{-1} glycine, 5 mg L^{-1} nicotinic acid, 0.05 mg L^{-1} biotin, 2.5 g L^{-1} phytagel, 30 g L^{-1} sucrose} and different growth regulators with the following concentrations: zeatin (2 mg L^{-1}); zeatin (1 mg L^{-1}) plus indole-3-acetic acid (IAA) (0.1 mg L^{-1}); zeatin (2 mg L^{-1}) plus IAA (0.1 mg L^{-1}); zeatin (1 mg L^{-1}) plus naphthalenacetic acid (NAA) (0.1 mg L^{-1}); 6-benzyl-aminopurine (BA) (2 mg L^{-1}) plus NAA (0.1 mg L^{-1}); BA (1 mg L^{-1}) plus NAA (0.1 mg L^{-1}); BA (2 mg L^{-1}) plus IAA (0.1 mg L^{-1}); BA (1 mg L^{-1}) plus IAA (0.1 mg L^{-1}) were used in this experiment. Each Petri dish contained 10 explants with ten replicates for each treatment. A total of 1600 explants (cotyledons and hypocotyls) were cultured. The experiment was evaluated using the following criteria: callus induction, shoots formation, indirect shoot formation and direct shoot formation.

Antibiotics treatments: Five concentrations (0.0, 250, 500, 750 and 1000 mg L^{-1}) of carbenicillin, cefotaxime and clavamox, were added to the callus and shoot initiation medium supplemented with 1 mg L^{-1} zeatin and 0.1 mg L^{-1} IAA. Each Petri dish contained 5 explants with 5 replicates for each treatment. The total number of explants (cotyledons and hypocotyls) used in this experiment was 750.

Phosphinothricin (PPT) treatments: To investigate the effect of PPT on shoot initiation, five concentrations of PPT (0.0, 2.5, 5, 7.5 and 10 mg L^{-1}) were added to the callus and shoot initiation medium supplemented with 1 mg L^{-1} zeatin and 0.1 mg L^{-1} IAA. Each Petri dish contained 5 explants with 5 replicates for each treatment. A total of 250 explants (cotyledons and hypocotyls) were used in this experiment.

Bacterial culture: The LBA4404 strain of *A. tumefaciens* with FGC5941 plasmid harboring the Silencing Trigger Construct (STC) (Abhary *et al*., 2006) was used. The bacterium was grown for 48 h in 250 mL Erlenmeyer flasks containing Luria-Bertani (LB) medium (10 g L^{-1} bacto-tryptone, 5 g L^{-1} bacto-yeast extract, 10 g L^{-1} NaCl, pH 7.5) plus 0.2 g L^{-1} $MgSO_4$ and 50 mg L^{-1} kanamycin sulfate. Flasks were kept at 26°C in a rotary shaker (220 rpm) until bacterial growth OD_{600} reached 0.25-0.35. The bacterial culture was used to transform tomato plants.

Transformation of tomato plantlets: Seeds of MP-1 tomato line and (981 X TY-6) tomato hybrid were grown on Nitsch medium. After germination, cotyledons and

hypocotyls were excised from 8-9 day-old seedlings and cultured in Petri dishes containing 20-25 mL of feeder medium and incubated for 24 h at 26°C. After that, explants were dipped in a solution of *A. tumefaciens* containing 375 μM acetosyringon. Explants were blotted and re-cultured on the feeder medium for 48 h at 26°C. The cotyledons and hypocotyls were transferred to callus and shoot initiation medium containing 1 mg L^{-1} zeatin + 0.1 mg L^{-1} IAA, 400 mg L^{-1} carbencillin or clavamox and 5 mg L^{-1} PPT. Plates were maintained at 26°C under a 16/8 h (light/dark) photoperiod.

Calli and shoots emerged from the explants were excised and transferred to the shoot elongation media (MS salt, 100 mg L^{-1} m-inositol, 0.5 mg L^{-1} thiamine-HCl, 2 mg L^{-1} glycine, 5 mg L^{-1} nicotinic acid, 0.05 mg L^{-1} biotin, 30 g L^{-1} sucrose, 1 mg L^{-1} zeatin, 0.5 mg L^{-1} zeatin riboside, 400 mg L^{-1} carbencillin or clavamox, 5 mg L^{-1} PPT and 2.5 g L^{-1} phytagel) pH 5.8. Plates were kept at 26°C under 16/8 h (light/dark) photoperiod. After that, healthy and normal looking shoots (2-3 cm long) were transferred to the rooting medium (MS salt, 100 mg L^{-1} m-inositol, 0.5 mg L^{-1} thiamine-HCl, 2 mg L^{-1} glycine, 5 mg L^{-1} nicotinic acid, 0.05 mg L^{-1} biotin, 30 g L^{-1} sucrose, 1 mg L^{-1} Indole Butyric Acid (IBA), 400 mg L^{-1} carbencillin or clavamox and 2.5 g L^{-1} phytagel) pH 5.8. Plates were kept at 26°C under 16 h light and 8 h dark conditions.

The total number of cotyledons used in this experiment was 400 for each genotype; each plate contained 20 cotyledons with total of 20 replicates. The total number of hypocotyls was 240 for each genotype; each plate contained 12 hypocotyls with total of 20 replicates. The variables studied were: callus induction, shoots regeneration, direct or indirect shoots formation, shoot elongation, transformation of plantlets and root formation from transformed plantlets.

Detection of the silencing construct in the transformed plantlets: Total DNA was extracted from leaf tissues of transformed tomato plantlets as previously described (Dellaporta *et al*., 1983). The primer pair STCv {TCATTCTAGAGGCGCGCCCGAAGGTT)/ STCc (ATATGGATCCATTTAAATATGCAACC), Abhary *et al*., 2006} was used to detect the presence of STC in transformed tomato plantlets by PCR. The PCR reaction consisted of 1X *Taq* DNA polymerase buffer (Promega, Co., Madison, WI, USA), 0.2 mM $MgCl_2$, 2.5 μM of each primer, 0.2 mM deoxynucleotied triphosphate (dNTPs) (Promega, Co., Madison, WI, USA), 3 μL diluted DNA (1:10) and 0.5 unit *Taq* DNA polymerase in a total volume of 25 μL. The PCR program was as follows: one cycle at 94°C for 5 min, 30 cycles at (94°C for 1 min, 55°C for 45 sec and 72°C for 1 min); then extension step at 72°C for 5 min. The PCR product was analyze by gel electrophoresis, visualized under UV transilluminator and photographed with the gel documentation system (Gel Doc 200, BIO-RAD, USA).

Statistical analysis: All data were statistically analyzed using one way analysis of variance (ANOVA). The significant differences among means were tested by using Least Significant Differences (LSD) at $p \leq 0.05$ (Steel and Torrie, 1960).

RESULTS

The effects of eight plant growth regulators on shoots formation and indirect shoots from tomato hybrid (981XTY-6) were significant, however callus induction and direct shoots formation were not significantly affected (Table 1). The highest rate of shoot regeneration was obtained using 1 mg L^{-1} zeatin and 0.1 mg L^{-1} IAA (Table 2).

As presented in Table 3, the inhibitory effects of three antibiotics with five different concentrations on shoot regeneration from cotyledons and hypocotyls showed no significant differences. The highest value of shoot regeneration (3.6) was obtained from cotyledons and hypocotyls when 250 mg L^{-1} carbincillin was used in the medium (Table 4). As indicated in Table 5, the effect of PPT concentrations on the growth of shoots regenerated from cotyledons and hypocotyls gave significant differences. Table 6 shows that the lowest PPT concentration that could inhibit the growth of non-transformed shoots was 5 mg mL^{-1}.

All investigated criteria for the effects of two tomato genotypes on *Agrobacterium*-mediated transformation from cotyledons showed significant differences (Table 7). The highest values for all the studied characteristics were produces from the tomato line MP-1 (Table 8). On

Table 1: Mean squares and degree of freedom (df) from analysis of variance for the effects of eight media on four characters of tomato callus and shoots regenerated from cotyledons and hypocotyls

Source of variation	df	Callus induction		Shoots formation		Indirect shoot formation		Direct shoot formation	
		Coty.	Hyp.	Coty.	Hyp.	Coty.	Hyp.	Coty.	Hyp.
Media	7	1.871	0.996	25.254*	22.825*	247.714*	125.129*	0.286	0.0
Error	32	4.625	2.688	2.113	1.225	3.325	2.125	0.263	0.0

*Significant at the 0.05 level

Table 2: Means for the effects of eight media on four characters of tomato callus and shoots regenerated from cotyledons and hypocotyls

	Callus induction		Shoots formation		Indirect shoot formation		Direct shoot formation	
Growth regulator (mg L^{-1})	Coty.	Hyp.	Coty.	Hyp.	Coty.	Hyp.	Coty.	Hyp.
Zeatin 2	8.2	8.6	6.8	4.6	14.2	8.8	0.6	0.0
Zeatin 1/IAA 0.1	9.6	9.8	7.2	7.0	21.6	16.0	0.4	0.0
Zeatin 2/IAA 0.1	9.0	8.8	4.4	4.0	11.8	8.8	0.2	0.0
Zeatin 1/NAA 0.1	9.0	9.2	3.6	2.2	6.8	4.4	0.0	0.0
BA 2/NAA 0.1	8.0	8.8	1.0	0.8	1.6	1.0	0.0	0.0
BA 1/NAA 0.1	7.8	8.6	1.8	0.6	2.6	1.0	0.0	0.0
BA 2/IAA 0.1	8.8	8.8	2.2	2.0	2.4	3.0	0.4	0.0
BA 1/IAA 0.1	9.0	9.4	4.0	2.6	5.4	3.4	0.0	0.0
LSD	NS	NS	1.307	1.0	1.64	1.311	NS	NS

Table 3: Mean squares and degree of freedom (df) from analysis of variance for the effects of different concentrations of three antibiotics on shoots regeneration from tomato cotyledons and hypocotyls

Source of variation	df	Coty.	Hyp.
Antibiotic (A)	2	0.416	0.014
Concentration (C)	4	1.246	0.933
A X C	8	0.750	0.520
Error	60	11.936	10.098

Table 4: Means for the effects of different concentrations of three antibiotics on shoots regeneration from tomato cotyledons and hypocotyls

	Concentration (mg L^{-1})									
	0.0		250		500		750		1000	
Antibiotics	Coty.	Hyp.	Coty.	Hyp.	Coty.	Hyp.	Coty.	Hyp.	Coty.	Hyp.
Carbencillin	4.0	3.6	3.6	3.6	3.4	3.0	2.8	2.6	2.8	2.4
Cefotaxim	4.0	3.6	3.2	3.4	3.0	3.0	2.4	2.2	2.0	2.0
Clavamox	3.6	3.6	3.0	3.4	3.0	3.2	2.2	2.8	2.0	2.6

Table 5: Mean squares and degree of freedom (df) from analysis of variance for the effects of different concentrations of PPT on shoots regeneration from tomato cotyledons and hypocotyls

Source of variation	df	No. of shoots regenerated from cotyledons	No. of shoots regenerated from hypocotyls
PPT concentrations	4	15.44*	12.96*
Error	20	0.24	0.58

*Significant at the 0.05 level

Table 6: Means of the effects of different concentrations of PPT on shoots regeneration from tomato cotyledons and hypocotyls

PPT (mg L^{-1})	No. of shoots developed from cotyledons	No. of shoots developed from hypocotyls
0.0	4.00	3.60
2.5	2.00	2.40
5.0	0.20	0.60
7.5	0.00	0.00
10	0.00	0.00
LSD	0.886	0.44

Table 7: Mean squares and degree of freedom (df) from analysis of variance for the effect of two tomato genotypes on six characters of transgenic plantlets produced from cotyledons

Source of variation	df	A	B	C	D	E	F
Genotype	1	50.625*	21.025*	18.225*	11.025*	5.625*	2.025*
Error	38	0.809	0.13	0.188	0.072	0.23	0.04

*Significant at the 0.05 level, A: Callus induction; B: Shoots regeneration; C: Cotyledons that produced direct or indirect shoots; D: Shoots elongation; E: Transformed plantlets; F: Transformed plants that produced roots

Table 8: Means for the effect of two tomato genotypes on six characters of transgenic plantlets produced from cotyledons

Genotypes	A	B	C	D	E	F
981 X TY-6	9.25	3.00	2.70	1.90	0.75	0.35
MP-1	11.50	4.45	4.05	2.95	1.50	0.80
LSD	0.574	0.241	0.277	0.242	0.306	0.127

A: Callus induction; B: Shoots regeneration; C: Cotyledons that produced direct or indirect shoots; D: Shoots elongation; E: Transformed plantlets; F: Transformed plants that produced roots

Table 9: Mean squares and degree of freedom (df) from analysis of variance for the effect of two tomato genotypes on six characters of transgenic plantlets produced from hypocotyls

Source of variation	df	A	B	C	D	E	F
Genotype	1	2.025*	12.10*	11.025*	6.40*	1.60*	0.40
Error	38	0.325	0.258	0.183	0.158	0.221	0.211

*Significant at the 0.05 level, A: Callus induction; B: Shoots regeneration; C: Hypocotyls that produced direct or indirect shoots; D: Shoots elongation; E: Transformed plantlets; F: Transformed plants that produced roots

Table 10: Means for the effect of two tomato genotypes on six characters of transgenic plantlets produced from hypocotyls

Genotypes	A	B	C	D	E	F
981 X TY-6	7.10	1.40	1.20	0.90	0.30	0.20
MP-1	6.65	2.50	2.50	1.70	0.70	0.40
LSD	0.364	0.324	0.273	0.253	0.30	0.293

A: Callus induction; B: Shoots regeneration; C: Hypocotyls that produced direct or indirect shoots; D: Shoots elongation; E: Transformed plantlets; F: Transformed plants that produced roots

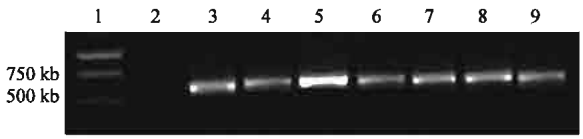

Fig. 1: Aliquots (10 µL) of the PCR products were analyzed on 1.5% agarose gel electrophoresis for the silencing trigger construct amplified from transformed tomato plantlets. Lane 1, 1 Kb DNA marker, lane 2 negative control, lane 3 positive control, lanes 4-9, amplified silencing trigger construct

average, 1.5 transformed plantlets were developed from MP-1 line while 0.75 plantlets developed from the hybrid 981XTY-6. The successful of transformation was confirmed by PCR; Fig. 1 showed that the silencing trigger construct exists in tomato plantlets produced from transformation of MP-1 and 981XTY-6 cotyledons.

The criteria for the effects of two tomato genotypes on *Agrobacterium*-mediated transformation from hypocotyls showed significant differences except the root formation (Table 9). The hybrid 981XTY-6 showed the highest response (7.1) of callus induction compared with (6.65) for MP-1; for the other characters, the differences were obvious and MP-1 gave the highest results comparing with 981 X TY-6 hybrid (Table 10).

DISCUSSION

Growth regulators usually used in the regeneration media affect the formation of adventitious shoots. Data of this study showed that cotyledons and hypocotyls from the tomato hybrid 981XTY-6 initiated callus and formed adventitious shoots on eight combinations of plant growth regulators. The medium containing zeatin as cytokinin and IAA as a weak auxin produced better results than the other media. The cellular differentiation and organogenesis in tissue culture are controlled by an interaction between auxin and cytokinin concentrations. The organogenesis in plants was promoted when cytokinin/weak auxin combinations were modified on cultured media (Venkatachalam *et al*., 2007). On the other hand, several attempts have been achieved to develop an efficient protocol for shoot regeneration from tomato plant (Van Roeke *et al*., 1993; Frary and Earle, 1996; Costa *et al*., 2000). Results of these studies indicated that callus induction and shoot regeneration from several tomato cultivars explants vary depending on the kind of the plant growth regulators used.

Several variables are involve in the improvement of transformation efficiency; the most important one is the influence of overgrowth-control antibiotics on both regeneration and transformation efficiency (Wei and Gregory, 2001). Three antibiotics (carbincillin, cefotaxin and clavamox) that belong to β-lactam class were used in this work. The results showed that these antibiotics had no significant effects on shoot regeneration from cotyledons and hypocotyls of the tomato hybrid 981XTY-6. β-lactam antibiotics have been shown to possess auxin-like structure feature and are broken down to physiologically active levels of the auxin phenyl acetic acid in cultures (Wei and Gregory, 2001; Holford and Newbury, 1992). The stimulation of cell division due to the release of auxin-like compounds enhances callus formation and shoots regeneration (Ling *et al*., 1998; Costa *et al*., 2000).

Cellular transformation dose not always lead to regeneration of transformed shoots, since not all transformed cells are competent for regeneration (Veltcheva *et al*., 2005). Data presented in this investigation provided clear evidence that MP-1 line was better than 981XTY-6 hybrid regarding to the period of regeneration and to the percentage of transformation efficiency. Similar results were provided by Rivka *et al*. (1997) who reported that transformation efficiency of MP-1 tomato cotyledons ranged from 4.3-7.4% depending on the plasmid types and *Agrobacterium* strain.

After transformation, some transformed tomato plantlets were died when transferred to the rooting medium containing 5 mg L^{-1} PPT. This is in agreement with the previous study (Benfey *et al*., 1990) which indicated a high expression of the 35S promoter in the vascular tissues but a significantly lower expression in meristematic tissues.

In conclusion, the best results of shoot regeneration were obtained from using 1 mg L^{-1} zeatin and 0.1 mg L^{-1} IAA and the highest shoot regeneration was produced from cotyledons and hypocotyls cultured on medium that contained 250 mg L^{-1} carbincillin. The performance of the tomato MP-1 line was better regarding all the studied characters except that for root formation from transformed hypocotyls.

ACKNOWLEDGMENTS

This research was supported by Grant No. M21-037 funded by the U.S. Agency for International Development, Middle East Research and Cooperation (MERC) Program.

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