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Genetic transformation of *Torenia fournieri* L. mediated by *Agrobacterium rhizogenes*

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Abstract

The transformation of *Torenia fournieri* L. mediated by *Agrobacterium rhizogenes* was studied. Almost all roots induced by four bacterial strains, R1000, R1601, A4 and R1205 were putative hairy roots. The effects of bacterial strains, bacterial concentration, acetosyringone, silver nitrate and co-cultivation pH on Torenia transformation were investigated. Strain R1000, co-cultivation for 3 days, 30 μmol L^{-1} acetosyringone, 4 mg L^{-1} silver nitrate and pH 6.5 in the cultivation medium provided the optimal conditions under which transformation frequency approached 90%.
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Keywords: *Torenia fournieri* L.; *Agrobacterium rhizogenes*; Hairy root; Genetic transformation; Acetosyringone; Silver nitrate

1. Introduction

Torenia fournieri L., known as Torenia or wishbone flower, is a common bedding ornamental plant during summer and belongs to the Scrophulariaceae family. It has become a model plant for biological research, its culture requirements in vitro are well established (Tanimoto and Harada, 1981). As Torenia is easy to grow in both field and laboratory, and its genetic transformation has potential for epocha cultivar breeding (Aida, 1998), many transgenic plants have been obtained by *Agrobacterium tumefaciens*-mediated transformation. The first transgenic plant of Torenia was reported in the mid-1990s (Aida and Shibata, 1995). Some of these transgenic plants were characterized by modified flowers with various longevities and colors (Aida, 1998; Aida et al., 2000, 2001).

Agrobacterium rhizogenes strains contain a single copy of a large Ri plasmid. In the Agropine Ri plasmid, T-DNA is referred to as left T-DNA (T_L-DNA) and right T-DNA (T_R-DNA). T_R-DNA contains genes homologous to the Ti plasmid tumor-inducing genes and the *tms* loci genes, two morphogenic loci of the T_R-DNA correspond to the tms loci of the Ti plasmids (White et al., 1985), can directly synthesize auxin (Capone et al., 1989). Genes of T_L-DNA, *rolA*, *rolB*, *rolC* and *rolD* direct the synthesis of a substance that reprograms the cells to differentiate into roots under the influence of endogenous auxin. T-DNA is transferred to wounded plant cells and becomes stably integrated into the host genome (Chilton et al., 1982). Transformants are selected by detecting the genes located in T-DNA such as *rolA*, *rolB*, *rolC* and *rolD.*

Hairy roots have been induced in many dicotyledonous plants by transformation with *A. rhizogenes* Ri T-DNA (Costamtino et al., 1994). Hairy roots are able to regenerate whole viable plants with high genetic stability. Most have an altered phenotype including hairy root syndrome, dwarfing, altered flowering, wrinkled leaves and increased branching, which have proven useful in ornamental plant breeding programs (Giovanni et al., 1997). However, the transformation of Torenia mediated by *A. rhizogenes* has not yet been reported.

In this study, we describe the Ri plasmid-mediated transformation of Torenia and investigate the effects of several major physical and chemical factors, on hairy root formation.

2. Materials and methods

2.1. Plant materials

Seeds of *T. fournieri* L. were soaked and sterilized according to a previous report (Li et al., 2001). Seeds were

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germinated on half-strength Murashige and Skoog medium (1/2 MS) (Murashige and Skoog, 1962) solidified with 0.8% (w/v) agar or on damp absorbent cotton, with or without light at 25 °C. Cultures were maintained under a 16-h photoperiod regime with fluorescent light at 25 °C.

For transformation experiments, leaves were excised from 4- to 7-cm-tall Torenia plants, with 4 expanded leaves.

2.1.1. Agrobacterium

A. rhizogenes strains R1000, R1601, R1205 and A4 belong to the agropine type and harbor the Ri plasmid with two parts of T-DNA (left T-DNA and right T-DNA) (Table 1). For the transformation experiments, each strain was inoculated into liquid YEB medium (sucrose 5 g L^{-1}, beef extract 1 g L^{-1}, yeast extract 1 g L^{-1}, peptone 5 g L^{-1}) (An et al., 1988) with 100 mg L^{-1} kanamycin and shaken for 30 h at 28 °C. The cultures were then centrifuged at 1500×*g* and resuspended in liquid MS medium containing 20 μmol L^{-1} acetosyringone before dilution to different concentrations for infection.

2.1.2. Kanamycin resistance test of normal roots

Roots excised from normal plants were cultured on selection media containing a range of concentrations from 0 to 300 mg L^{-1} kanamycin. The percentage of roots survived after 15 days culture was determined.

2.1.3. Transformation

Leaf explants were pre-cultivated on MS medium solidified with 0.8% (w/v) agar at a varying pH range (4.5, 5.5, 6.5, 7.5) and concentrations of acetosyringone (0, 10, 20, 30, 40 μmol L^{-1}) and silver nitrate (0, 1, 2, 3, 4 mg L^{-1}) for 2 days. The explants were then transferred to the *Agrobacterium* suspensions containing 20 μmol L^{-1} acetosyringone and incubated for certain time, then blotted dry on sterilized filter paper. The explants were returned to the same pre-cultivation medium for co-cultivation. After 0 to 4 days, these explants were transferred to resting media (1/2 MS) containing 500 mg L^{-1} carbenicillin and 200 mg L^{-1} kanamycin. Every 4 days, the explants were transferred to selection media (1/2 MS containing 250 mg L^{-1} kanamycin and 150 mg L^{-1} carbenicillin) until hairy roots formed. The numbers of explants with roots (20 days after infection) were recorded.

The standard condition referred to here is 15 min infection, 2 days pre- and co-cultivation at pH 5.5 using a bacterial concentration of 1.5 (OD_{600}). To determine the optimum conditions for transformation, one factor of the standard conditions was changed each time and the effects on hairy root formation measured.

Table 1
Bacterial strains and plasmids used in this work and their relevant characteristics

Strains	Ri plasmid	Type of plasmid	References
A4	pRiA4 and pB1121	Agropine	White and Nester, 1980
R1000	pRiA4b and pB1121	Agropine	White et al., 1985
R1601	pRi1500 and pTVK291	Agropine	Pythoud et al., 1987
R1205	Ri (*tms-I* and *tms-2* deletion)	Agropine	White et al., 1985

Table2
The effect of light and media on the germination frequency of *Torenia* seeds (40 seeds per test)

Medium	Treatment	Percentage of germination (%)
1/2 MS	Light	95.0
	Dark	52.5
Absorbent cotton	Light	100.0
	Dark	60.0

2.1.4. PCR amplification of the rolB gene

Total DNA was isolated from hairy roots according to the procedure of Edward et al. (1991). Two primers, 5′-CGCAAGCTACAACATCATAG-3′ and 5′-CAGTAGATCTCACTCCAGCA-3′, were used for PCR amplification of the 583 bp fragment of the *rolB* gene. DNA was amplified by 30 cycles of 30 s at 92 °C, 30 s at 52 °C and 1 min at 72 °C. The DNA size marker was DL 2000 (TaKaRa Biotechnology (Dalian) Co., Ltd.).

2.1.5. Southern blot analysis

Genomic DNA was digested with *nar I and ned I* and resolved by electrophoresis on 0.8% agarose gel before transferring to a nylon membrane by the method of Southern (1975). The membrane was pre-hybridized at 65 °C in 7% SDS and 0.25 M $NaHPO_4$ and then hybridized with the *rolB* gene fragment, which was isolated from restricted Ri plasmid with *nar I* and *nde I*, labelled with ^{32}P-dCTP by the method of Sambrook and Russell (2001). The hybridized blot was subjected to three washes in 20 m mol L^{-1} $NaHPO_4$ and 1% SDS. The blot was exposed to X-ray film (Kodak) at −70 °C for 3 days.

2.1.6. Statistical analysis

All data frequency on hairy root formation are the mean of three independent experiments (±S.E.) with a minimum of 20 explants per treatment. All roots that survived in the selection medium for 20 days were designated as hairy roots. All data among different treatment were tested by SSR testing (Duncan, 1955).

3. Results

Seeds were germinated on both 1/2 MS agar medium and damp absorbent cotton with or without fluorescent light after 4 days. Ten days later, the percentage of germinated seeds under light was much higher than that under dark on both media indicating that light strongly affected seed germination (Table 2).

Normal roots were placed on 1/2 MS medium containing a range of kanamycin concentrations (Fig. 1A) to examine their resistance to the antibiotic. All untransformed roots survived at kanamycin concentrations up to 100 mg L^{-1}. At higher concentrations, the survival frequency decreased and no roots survived at 250 or 300 mg/l. On this basis, 250 mg L^{-1}

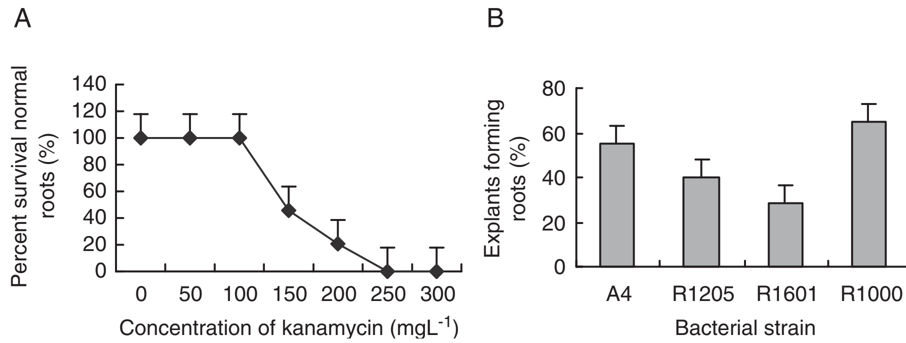

Fig. 1. (A) Effect of kanamycin on normal roots. (B) Efficiency of transformation to hairy roots by *A. rhizogenes* strains under standard conditions. Each value represents the mean of three different experiments.

kanamycin was used in the selection medium for further experiments.

Four strains of *A. rhizogenes*, A4, R1000, R1601 and R1205 were tested for their transformation efficiency (Fig. 1B). The transformation efficiency of R1000 and A4 were more efficient than that of R1205 and R1601 for Torenia transformation. R1000 (transformation frequency 65%) had the highest transforming ability and R1601 (transformation frequency 28.3%) had the lowest.

A. rhizogenes R1000 was used in subsequent experiments. Fig. 2A shows the effect of infection time on the frequency of hairy root formation. The transformation frequency strongly increased as the infection time increased from 5 to 20 min, rising to 80% at 20 min. As bacterial growth in the selection medium might be significant at times exceeding 20 min, the infection time was limited to 15 min in subsequent experiments.

The hairy root formation frequency increased four-fold as the bacterial concentration rose from 0.5 to 1.0 (OD_{600}), whereas slowly decreased at higher concentrations (Fig. 2B). This indicates that the optimum concentration is about 1.0.

The hairy root frequency increased progressively with increasing co-cultivation time from 2 to 4 days (Fig. 2C), becoming asymptotic towards the end. At longer co-cultivation periods, we also faced the problem of increased cell densities of bacteria in the selection medium. Therefore, co-cultivation was restricted to 2 or 3 days and this was found to be sufficient for transformation. The concentration of acetosyringone affected hairy root frequency, with an optimum at about 30 μmol L^{-1} (Fig. 2D).

The effects of varying some conditions in the pre- and co-cultivation medium were also investigated (Fig. 3). Silver nitrate between 0.5 mg L^{-1} and 4 mg L^{-1} caused little change

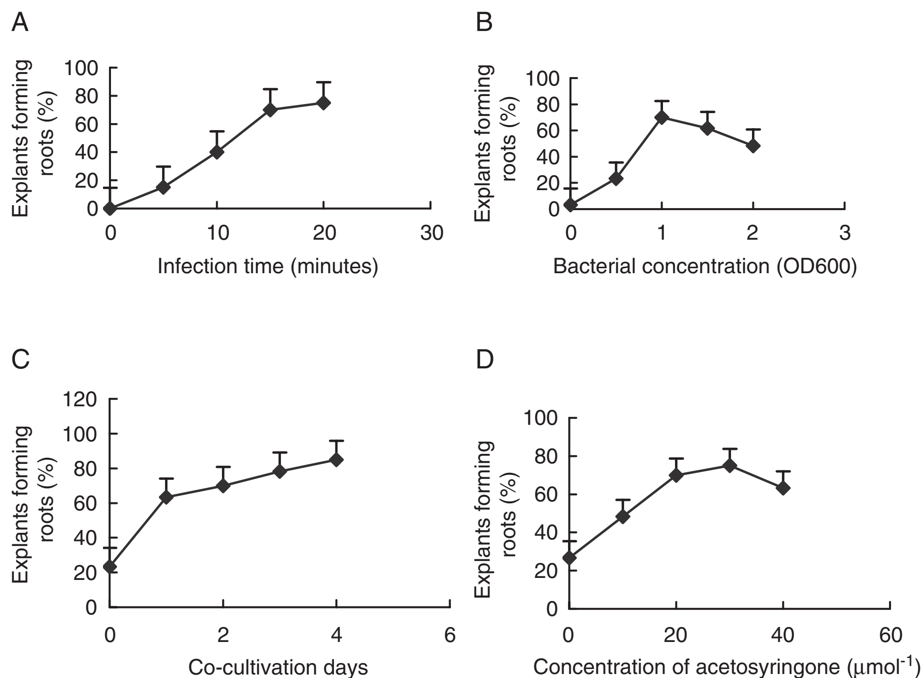

Fig. 2. Effects of infection period (A), bacterial concentration (B), co-cultivation period (C) and acetosyringone concentration (D) on hairy root formation of kanamycin-resistant roots. All root-inducing conditions were standardized in experiments except for the variables investigated. Each value represents the mean of three different experiments.

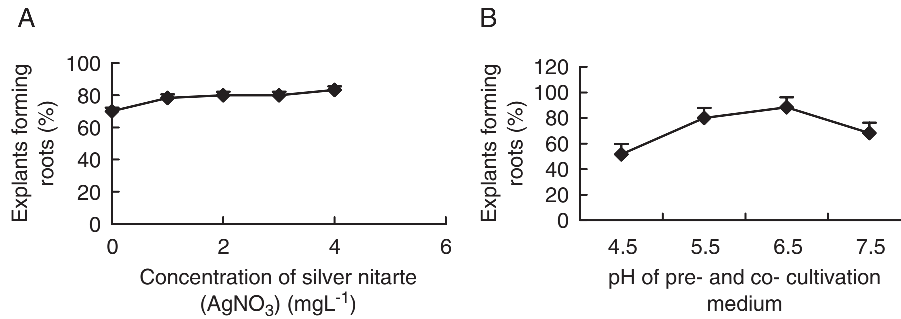

Fig. 3. Effects of silver nitrate (A) and pH (B) in the pre- and co-cultivation medium. (A) Different concentrations of silver nitrate were added to the standard composition. (B) All variables were standardized except pH of pre- and co-cultivation medium and 4 mg L^{-1} silver nitrate. Each value represents the mean of three different experiments.

in efficiency (Fig. 3A). At much higher concentration (above 10 mg L^{-1}), silver nitrate strongly inhibited hairy root induction (data not shown).

The pH of the pre- and co-cultivation medium strongly affected hairy root induction. Increasing pH from 4.5 to 6.5 strongly stimulated hairy root formation, with an optimum at about pH 6.5, but higher pH at 7.5 inhibited growth (Fig. 3B).

Hairy root production under the optimal conditions determined above was performed: bacterial strain R1000, co-cultivation for 3 days, 30 μmol L^{-1} acetosyringone, 4 mg L^{-1} silver nitrate and a pH 6.5 in the co-cultivation medium. Under this condition, the hairy root production frequency was 89.3%.

For amplification of the *rolB* gene, roots were excised from rooting explants that had been infected for 16 days (Fig. 4B) and cultivated on the selection medium. Four individual root clones were randomly selected and these survived on the selection medium after 10 days culture. All roots, except one

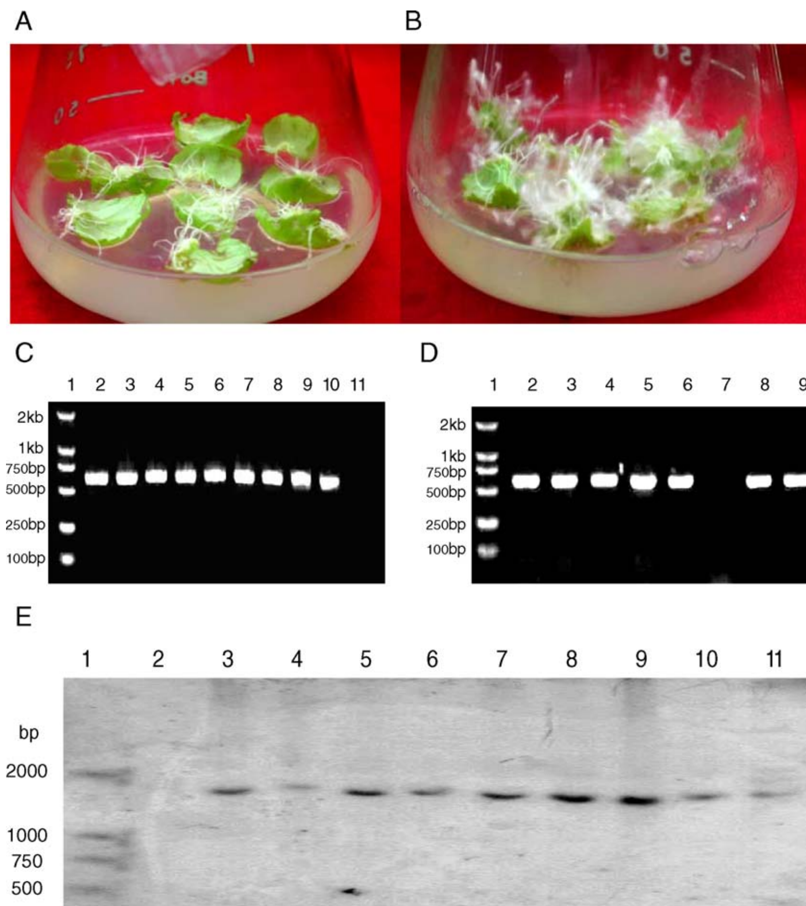

Fig. 4. Roots induced from infected *Torenia fournieri* leaf 12 days (A) and 16 days (B). PCR of DNA templates (C and D) and Southern blot analysis (E) from hairy roots induced by different bacterial strains. (C) Lane 1, DL2000 marker; lanes 2–5 roots induced by R1000; lane 6, positive control (Ri plasmid); lanes 7–10, roots induced by R1601. (D) Lane 1, DL2000 marker; lanes 2–5, root induced by A4; lanes 6–9, roots induced by R1205. (E) Lane 1, standard marker; lane 2, normal roots; lane 3, positive control (Ri plasmid); lanes 4–5, induced by R1000; lanes 6–7, induced by R1601; lanes 8–9, induced by A4; lanes 10–11, induced by R1205.

induced by R1205 (Fig. 4D), were found to harbor the *rolB* gene (Fig. 4C,D). This showed that almost all roots that could grow on selection medium containing 250 mg L^{-1} kanamycin were putative hairy roots. We randomly selected two root clones for Southern blot analysis out of four clones that were putative hairy roots. All roots analysed by Southern blot analysis were putative hairy roots, which indicated that the roots were transformed (Fig. 4E).

4. Discussion

The germination of Torenia seeds in MS medium was similar to MS medium solidified by agar and wettish absorbent cotton, and light strongly promoted this process. The later result confirmed previous findings (Li et al., 2001).

The genetic transformation mediated by *Agrobacterium* is affected by explant genotype and structure, chemical and physical factors, bacterial strains and signal molecules. Different strains of *A. rhizogenes* vary in their transforming ability (Kumar et al., 1991; Giri et al., 1997). This was confirmed in our study that the R1000 and A4 strains had greater hairy root generating capacity than R1205 and R1601 in Torenia transformation. This result could possibly be explained by the different plasmids contained by these strains (Nguyen et al., 1992), in which genes of Ri T_L-DNA direct the synthesis of a substance that induces the cell to differentiate into root formation under the influence of endogenous auxin (Ooms et al., 1986) and/or Ri T_R-DNA containing *tms* loci which directly synthesize auxin (Capone et al., 1989) and which then induce hairy root formation.

Bacterial concentration plays an important role in the production of transformed roots. Suboptimal concentrations resulted in low availability of bacteria for transforming the plant cells while high concentrations decreased their potential by competitive inhibition in soybean *A. rhizogenes*-mediated transformation (Kumar et al., 1991). Our results showed an optimum bacterial concentration 1.0 in Torenia of OD_{600}, which is similar to the effect of bacterial concentration on hairy root formation in soybean transformation, therefore possibly sharing the same mechanism.

Co-cultivation is very important in the transformation process. Bacteria attachment, T-DNA transfer and integration are occurred during this stage (Su et al., 2002). We can accelerate these processes by supplementing some ingredients in the co-cultivation medium or prolonging co-cultivation time so that these processes can terminate sufficiently. Our results indicated that if the co-cultivation time was too short these transformation processes cannot be completed, while long co-cultivation time could negatively affect transformation by reducing bacterial affinity to the plant cell or by competitive inhibition.

Some recalcitrant plant species can be transformed by inducing the *vir* genes of the bacteria by signal molecules or it can be achieved in vitro by co-cultivating *Agrobacterium* with wounded tissue or in media that contains signal molecules (Satchel et al., 1985). Acetosyringone or related compounds functioning as signal molecules have been reported to improve the *Agrobacterium*-mediated transformation in several plant species (Hu and Alfermann, 1993). In our work, exogenous acetosyringone at 10 to 30 μmol L^{-1} effectively enhanced hairy root formation, but the transformation frequency was not significantly increased by application of high (40 μmol L^{-1}) acetosyringone. We suggest that, in Torenia transformation, a relatively low acetosyringone concentration is sufficient for signaling, but at high concentration the molecule function is insufficient.

Most studies indicate that application of silver nitrate in co-cultivation medium causes strong promotion of *Agrobacterium*-mediated transformation (Cheng et al., 1994; Bu et al., 2000; Liu et al., 2003). However, an inhibitory effect has also been reported (Lin et al., 2003), which may be caused by different silver nitrate concentrations and different types of explants used in these experiments. This study shows that the genetic transformation frequency was increased at very low concentration of silver nitrate (Fig. 3A) and decreased at concentrations above 10 mg L^{-1} (data not shown).

Some studies indicate that silver can stimulate organogenesis in vitro by inhibiting ethylene signaling (Cheng et al., 1994) and can inhibit *Agrobacterium* growth (Lin et al., 2003). Our results are consistent with these findings, which suggest that the promotive effect of silver at low dose on hairy root formation may be due to inhibition of ethylene signaling, affecting *Agrobacterium* growth and stimulating some degree of *vir* gene expression.

However, some studies have indicated that ethylene also plays an essential role in crown-gall morphogenesis as well as in vascular differentiation and epidermal disruption. These ethylene-dependent processes cause a redirection of water flow and carbohydrate transport, which are necessary for tumor establishment induced by *A. tumefaciens* (Aloni et al., 1998; Wächter et al., 2003). Because *A. tumefaciens* and *A. rhizogenes* share a similar transformation mechanism, and some studies indicate silver nitrate in late co-cultivation inhibits *Agrobacterium*-mediated transformation, we predict that silver nitrate at a high concentration in co-cultivation inhibits hairy root formation by affecting some organogenesis-related enzymes.

Studies indicate that low medium (e.g. YEB medium) pH for activating bacteria promotes activation of some *vir* genes. *VirD*$_2$, for example, was effectively activated under pH 5.4–5.6, peaking at pH 5.1–5.2 (Satchel et al., 1985) and the transformation frequency was strongly affected when the pH changed by 0.3 (Godwin et al., 1991). When pH in the medium decreased from 7.2 to 5.8, the hairy root formation frequency strongly increased (Yu et al., 2001). However, the effects of pre- and co-cultivation medium pH on hairy root formation have not been previously reported. Fig. 3B shows that hairy root formation frequency increased with pre- and co-cultivation medium pH increasing from 4.5 to 6.5, but decreased when pH was over 6.5. The underlying mechanism of this is not clear.

The transformation of roots induced from Torenia leaves by *A. rhizogenes* was obtained with relative ease and it was demonstrated that the transformation frequency could approach 90% under optimal conditions. We therefore support the idea that Torenia could become an important model research plant with special reference to genetic transformation.

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