Development of Infectious cDNA Clones of Citrus Yellow Vein Clearing Virus Using a Novel and Rapid Strategy

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ABSTRACT

Yellow vein clearing disease (YVCD) causes significant economic losses in lemon and other species of citrus. Usually, citrus yellow vein clearing virus (CYVCV) is considered to be the causal agent of YVCD. However, mixed infection of CYVCV and Indian citrus ringspot virus (ICRSV) or other pathogens is often detected in citrus plants with YVCD. In this study, we re-examined the causal agent of YVCD to fulfill Koch's postulates. First, the full-length genome of CYVCV isolate AY (CYVCV-AY) was amplified by long-distance RT-PCR from a Eureka lemon (*Citrus limon*) tree with typical YVCD symptoms. The genomic cDNAs were then cloned into a ternary Yeast-*Escherichia coli*-*Agrobacterium tumefaciens* shuttle vector, pCY, using transformation-associated recombination (TAR) strategy, and 15 full-length cDNA clones of CYVCV-AY were obtained. Subsequently, four of these clones were selected

randomly and inoculated on Jincheng (*C. sinensis*) seedlings through *Agrobacterium*-mediated vacuum-infiltration, and it was found that 80 to 100% of inoculated plants were infected with CYVCV by RT-PCR at 20 to 40 days postinoculation (dpi) and by direct tissue blot immunoassay at 60 dpi. The progeny of CYVCV-AY from cDNA clones caused typical symptoms of YVCD such as yellow vein clearing, leaf distortion, and chlorosis, which were the same as that elicited by wild-type virus. Finally, the regeneration of CYVCV-AY genome was confirmed by long-distance RT-PCR in lemon trees inoculated with the infectious cDNA clone. These results proved that CYVCV was the primary causal agent of YVCD. This is the first report on the development of infectious cDNA clones of CYVCV, which lays the foundation for further studies on viral gene functions and virus–host interactions.

Yellow vein clearing disease (YVCD) was first discovered in 1988 on lemon (*Citrus limon* (L.) Brum. f.) and sour orange (*C. aurantium* (L.)) trees in Pakistan (Catara et al. 1993). Later in 1997, the disease was also found on Etrog citron (*C. Medica* (L.)), Rangpur lime (*C. limonia* (L.) Osbeck), sour orange, and some varieties of lemon in several areas of India (Alshami et al. 2003). Recent reports indicated that YVCD was spreading rapidly in Pakistan, Turkey (Önelge et al. 2011a), China (Chen et al. 2015), and Iran (Hashmian and Aghajanzadeh 2017). In China, YVCD was first observed in Eureka lemon trees in 2009, and it has been rapidly spreading in major citrus-growing provinces (Zhou et al. 2016).

Typical symptoms of YVCD on citrus trees are yellow vein clearing, leaf distortion, and occasional ringspots and veinal necrosis. The disease can be transmitted by graft and mechanical inoculations from affected field trees. YVCD can also be transmitted to other plant species by mechanical inoculation, resulting in local lesions on *Chenopodium quinoa* Willd. and *C. amaranticolor*, and systemic chlorosis, severe mosaic, and necrosis on *Phaseolus vulgaris* (L.) and *Vigna unguiculata* (L.) Walp., respectively (Alshami et al. 2003). In YVCD affected leaves from citrus or herbaceous plants, flexuous filamentous virus-like particles were observed under electron microscope to be 13 to 14 nm in diameter and 670 to 700 nm in length (Loconsole et al. 2012). Citrus yellow vein clearing virus (CYVCV) was considered as the causal agent of YVCD, although mixed infections with Indian citrus ringspot virus (ICRSV) or other pathogens were often detected in YVCD affected leaves (Alshami

et al. 2003; Loconsole et al. 2012). The putative viral genomic RNA copies in replicative form and several potential subgenomic RNAs, ranging from 0.8 to 10 kb, were detected in infected lemon and bean plants (Önelge et al. 2011b). Later, the genome sequence of CYVCV was obtained by next generation sequencing of small interfering RNAs (siRNAs) associated with an YVCD-affected source (Loconsole et al. 2012). The CYVCV genome had a positive-sense RNA of 7,529 nucleotides and contained six predicted open reading frames (ORF). So far, at least 25 CYVCV genomes from different geographic origins and hosts have been determined by siRNA or conventional sequencing method (Cao et al. 2015; Song et al. 2015; Zhou et al. 2016). Although the genome structure, viral particles, and herbaceous host were similar to that of ICRSV, CYVCV caused different symptoms in citrus and was serologically distinct from ICRSV (Alshami et al. 2003; Loconsole et al. 2012). Subsequently, CYVCV was adopted as a new species in the genus *Mandarivirus*, family *Alphaflexiviridae* (King et al. 2012). Although these properties of CYVCV have been reported, it is necessary to further validate the causal agent of YVCD.

Development of an infectious cDNA clone is the first step for fulfilling Koch's postulates for individual components of YVCD in citrus plants. But it is difficult to obtain such infectious cDNA clones, especially for viruses infecting woody host plants. In recent years, transformation-associated recombination (TAR) cloning was developed for rapid and efficient construction of full-length cDNA copies of viruses. With this technology, one or more fragments covering the entire genome of a virus can be simultaneously and directionally cloned into a linearized vector in a single transformation, resulting in infectious clones in a short time (Desbiez et al. 2012; Kouprina and Larionov 2016; Shang et al. 2017). Especially, when a ternary yeast-*Escherichia coli*-*Agrobacterium tumefaciens* shuttle vector was used in TAR cloning, the generated construct with viral cDNA can be used to infect host plants with *Agrobacterium*-mediated inoculation (Youssef et al. 2011), which could prevent the possible instability of viral cDNAs in *E. coli*, an obstacle for an array of viruses belonging to the family *Potyviridae* (Tuo et al. 2017), the genus *Tobravirus* (Constantin et al. 2004),

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In this study, full-length cDNA clones of CYVCV were constructed for the first time using TAR technology and inoculated onto citrus seedlings through *Agrobacterium*-mediated vacuum-infiltration. The progeny of cloned CYVCV elicited YVCD-like symptoms in citrus, suggesting that CYVCV is likely the causal agent of YVCD.

each cDNA clone was inoculated with 10 ml of LB liquid medium containing the antibiotic kanamycin (50 µg/ml), rifampicin (50 µg/ml), 10 mM MES (pH 5.85), and 200 µM acetosyringone. Overnight grown bacterial cells were pelleted and resuspended in induction buffer (10 mM MES, pH 5.85, 10 mM MgCl₂, 200 µM acetosyringone) with optical density at 600 nm (OD₆₀₀) of 1.0 for the vector-containing bacterium and 0.3 for RNA silencing suppressor Hc-Pro.

Citrus seedlings of 7-day post sowing were inoculated by the vacuum-infiltration method, in which the citrus seedling was submerged in bacterial suspensions for 20 s in a vacuum chamber, followed by a quick pressure release as described (Wang et al. 2017). Agroinfiltration was performed with 1-ml syringes without needle on plantlets at the four-leaf stage. Plants infiltrated with *Agrobacterium* containing empty vector pCY and the Hc-Pro expression plasmid were served as Mock. Inoculated plants were grown in a growth chamber with a 16 h light at 22°C and 8 h dark at 20°C.

CYVCV detection and resulting symptoms. Total RNA was extracted from the upper noninoculated leaves of inoculated plants at 20 and 40 dpi. RT-PCR was conducted to detect CYVCV using the protocol described previously (Chen et al. 2015) with primers CYVCY-F and CYVCV-R (Table 1). The direct tissue blot immunoassay (DTBIA) was performed at 60 dpi according to the protocol described previously (Bin et al. 2015) with CYVCV specific polyclonal antibody.

In addition, the inoculated plants were monitored every day for symptoms development. When symptoms were elicited on leaves of inoculated plants, long-distance RT-PCR was conducted with primer pair PCY-CYF/PCY-CYR to validate whether the genome of CYVCV was regenerated.

RESULTS

Construction of a ternary Yeast-*E. coli*-*A. tumefaciens* shuttle vector. To efficiently construct the full-length cDNA clone of CYVCV through TAR in yeast and to inoculate citrus seedlings mediated by *A. tumefaciens*, a ternary Yeast-*E. coli*-*A. tumefaciens* shuttle vector, pCY, was constructed based on pCambia1301, pXT1, and pYES1L. The pCY vector (GenBank accession number MG637043) is 9,656 bp, containing replication elements of yeast (ARS4/CEN5), *A. tumefaciens* (pVS1 oriV), and *E. coli* (pBR322 ori). The gene expression cassette 2x35S-MCS-HDV_{RZ}-NOS was located between the left and right arms of T-DNA insertion element (Fig. 1A).

As a validation, a full-length cDNA of potato virus X (PVX) genome was amplified using the primer pair PCY-PVXF/PCY-PVXR (Table 1) and was recombined with *Stu*I- and *Sma*I-digested pCY through TAR method in yeast. Five of six clones were positive and had the expected *Eco*RI restriction pattern in recombinant plasmids. Three of these clones (pCY-PVX) were then selected randomly and submitted to agroinfiltration on *Nicotiana benthamiana*.

Fig. 1. The full-length cDNA clone of citrus yellow vein clearing virus (CYVCV) generated by transformation-associated recombination (TAR) cloning based on long-distance RT-PCR and the ternary Yeast-*Escherichia coli*-*Agrobacterium tumefaciens* shuttle vector. **A**, Ternary shuttle vector pCY. The regulatory elements are indicated by different shaded arrows. **B**, The genome organization of CYVCV-AY. Arrow indicates the open reading frames (ORFs). **C**, Agarose gel electrophoresis of the long-distance RT-PCR products from CYVCV-AY-infected lemon using primer pair PCY-CYF/PCY-CYR. Lanes 1 to 4, samples from CYVCV-AY-infected lemon; and lane M, molecular weight markers. **D**, TAR Cloning strategy. The 30-bp overlapping regions for RT-PCR fragments and pCY vector are indicated, where homologous recombination occur. **E**, Identification of constructs carrying full-length cDNA clone of CYVCV genome by *Ssp*I digestion analysis. Lanes 1 to 4, construct of CYVCV-AY cDNA in pCY vector; and lane M, molecular weight markers.

Then typical symptoms were found at 7 dpi on all inoculated *N. benthamiana* plants and PVX was detected to be positive by RT-PCR, suggesting that all the selected plasmids were infectious (data not shown).

Therefore, pCY could be used for the seamless assembling of DNA fragments in yeast cells and for the subsequent transferring the assembled recombinant DNA molecule into *A. tumefaciens* or *E. coli*. Furthermore, the gene expression cassette 2x35S-MCS-HDV_{RZ}-NOS in the vector could ensure the viral genomic transcripts to be generated precisely during *Agrobacterium*-mediated inoculation on plant, once the virus full-length cDNA was recombined with *Stu*I- and *Sma*I-digested pCY.

Long-distance RT-PCR amplification of CYVCV. Full-length cDNA of CYVCV-AY genome was amplified successfully from infected Eureka lemon by long-distance RT-PCR with LA *Taq* DNA polymerase. And the expected ~7.5 kbp DNA was obtained and this fragment was gel purified for construction of CYVCV full-length clone (Fig. 1C). Only fragments less than full-length (0.5 to 3.0 kbp) could be amplified with *Taq* DNA or SpeedSTAR HS DNA polymerase under the same condition (data not shown).

TAR cloning and sequence analysis. TAR cloning was performed to assemble the ~7.5-kbp fragment into the *Stu*I- and *Sma*I-digested pCY. The experiment was repeated twice to testify its efficiency. The resulting colonies were screened, and a total of 21 positive colonies were obtained. Plasmids of these positive colonies were isolated from yeast cells and then examined for the presence of *Ssp*I restriction enzyme digestion pattern. A total of 15 plasmids had the expected *Ssp*I restriction enzyme digestion pattern, suggesting that the full-length cDNA of CYVCV-AY genome had been assembled into pCY vector. For the two TAR cloning repetitions, the constructs carrying the full-length cDNA of CYVCV-AY genome accounted for 40 and 58% of the total number of constructs examined, respectively (Table 2).

Next, we sequenced four randomly selected full-length cDNA clones of CYVCV-AY (101, 105, 202, and 204). All these four clones were 7,529 nt in length without a deletion or insertion. The consensus sequence of these clones had been deposited as GenBank accession number MG878869. The 7,529 nt sequence encodes six ORFs, which are the same as those of known CYVCV isolates (Cao et al. 2015; Loconsole et al. 2012; Song et al. 2015) (Fig. 1B). Alignment analysis showed that these sequences shared the highest nucleotide identity (99.15 to 99.40%) with CYVCV-RL (GenBank accession number KP120977), a CYVCV isolate from Yunnan Province of China.

A phylogenetic tree (Fig. 2) was constructed based on these sequences and genomic nucleotide sequences of available isolates from mandarinviruses and PVX, the representative members in the family *Alphaflexiviridae*. The phylogenetic tree showed that all the CYVCV isolates were clustered together into one clade, which were distinct from those of ICRSV. Moreover, isolates derived from China, CYVCV-AY, CYVCV-RL, CYVCV-YN, CYVCV-JX, CYVCV-CQ, and CYVCV-HU, were clustered into a separated subclade, while isolates from Pakistan and Turkey formed into another subclade, indicating that CYVCV has some divergence.

TABLE 2. Transformation-associated recombination (TAR) cloning for construction of citrus yellow vein clearing virus (CYVCV) full-length cDNA on pCY ternary shuttle vector

Trial	Number of colonies picked	Positive colonies ^a		Full-length cDNA clone of CYVCV ^b	
		Number	Rate (%)	Number	Rate (%)
Number 1	20	12	60	8	40
Number 2	12	9	75	7	58
Total	32	21	66	15	47

^a Detected by PCR with primer pair CYVCV-F/CYVCV-R.

^b Identified by restriction pattern of *Ssp*I.

***Agrobacterium*-mediated inoculation onto citrus and herbaceous plants.** The full-length cDNA of CYVCV-AY was cloned into the ternary vector pCY under the control of the enhanced 35S promoter, and these constructs were expected to be infectious by *Agrobacterium*-mediated inoculation on citrus or herbaceous hosts. In this study, both vacuum-infiltration and agroinfiltration inoculation strategies were therefore evaluated for these constructs following *A. tumefaciens* strain C58C1 transformation.

Vacuum-infiltration of Jincheng seedlings with *A. tumefaciens* cells carrying CYVCV-AY cDNA clones 101, 105, 202, and 204 caused 90, 100, 80, and 100% infection, respectively, based on RT-PCR detection at 20 and 40 dpi (Table 3; Fig. 3A). DTBIA assay (Fig. 3B) using specific antibody of CYVCV at 60 dpi indicated that 90, 100, 80, and 100% of seedlings were systemically infected by CYVCV-AY cDNA clones 101, 105, 202, and 204, respectively, which was consistent with that of RT-PCR. Typical symptoms of CYVCV infection such as yellow vein clearing and leaf distortion were developed gradually on leaves of most Jincheng seedlings after 15 dpi, which was indistinguishable from those incited by wild-type CYVCV-AY (CYVCV-wt) on lemon (Fig. 3C). In contrast, seedlings inoculated with empty vector pCY by vacuum-infiltration in parallel experiment were negative when tested by RT-PCR and DTBIA, and showed no visible symptoms. Therefore, these results revealed that CYVCV-AY cDNA clones 101, 105, 202, and 204 were all infectious on citrus, and virus recovered from these clones could replicate and move in citrus plants similar to that of CYVCV-wt. Also, CYVCV-AY cDNA clone 101 was inoculated on Eureka lemon seedlings by vacuum-infiltration, but 4 out of 10 seedlings died. The survived lemon seedlings were all infected and showed typical YVCD symptoms such as serious yellow vein clearing, leaf distortion, and chlorosis symptoms, which were the same as that induced by CYVCV-wt (Fig. 3D). Moreover, a ~7.5-kbp specific band was obtained by long-distance RT-PCR, indicating that the genome of CYVCV-AY was regenerated.

The same constructs used above were also inoculated into the leaves of Jincheng seedlings, *N. benthamiana* and three herbaceous hosts (*C. quinoa*, *V. unguiculata*, and *P. vulgaris*) of CYVCV by conventional agroinfiltration method. Surprisingly, none of these plants was CYVCV-positive in RT-PCR detection or showed

Fig. 2. Phylogenetic tree for isolates of citrus yellow vein clearing virus (CYVCV), Indian citrus ring spot virus (ICRSV), and potato virus X (PVX). The tree was constructed using MEGA 6 by neighbor-joining method with 1,000 bootstrap replicates. Bars represent 0.05 substitutions per site. Bootstrap support is indicated at branch points. Superscript a (*) indicates different full-length cDNA clone of CYVCV-AY. The accession numbers of reference sequences are as follows: CYVCV-RL (KP120977), CYVCV-Y1 (JX040635), CYVCV-HU (KT124646), CYVCV-YN (KP132424), CYVCV-JX (KX378154), CYVCV-CQ (KP313240), CYVCV-IS (KT345342), CYVCV-PK (KP13241), ICRSV-K1 (AY646511), ICRSV-Pu (HQ324250), and PVX-TW (AF272736).

TABLE 3. Infection rates of woody or herbaceous plants following *Agrobacterium*-mediated inoculation of citrus yellow vein clearing virus (CYVCV) full-length cDNA clones or empty vector pCY

Clone	Inoculation method	Infected/inoculated plants (infection rate (%))					
		<i>Citrus sinensis</i> (Jincheng)	<i>Citrus limon</i> (Eureka lemon)	<i>Nicotiana benthamiana</i>	<i>Chenopodium quinoa</i>	<i>Vigna unguiculata</i>	<i>Phaseolus vulgaris</i>
CYVCV-AY101	Vacuum-infiltration	9/10 (90)	6/6 ^a (100)	NI ^b	NI	NI	NI
CYVCV-AY105	Vacuum-infiltration	10/10 (100)	NI	NI	NI	NI	NI
CYVCV-AY202	Vacuum-infiltration	8/10 (80)	NI	NI	NI	NI	NI
CYVCV-AY204	Vacuum-infiltration	10/10 (100)	NI	NI	NI	NI	NI
pCY	Vacuum-infiltration	0/5 (0)	NI	NI	NI	NI	NI
CYVCV-AY101	Agroinfiltration	0/5 (0)	NI	0/5	0/5	0/5	0/5
CYVCV-AY105	Agroinfiltration	0/5 (0)	NI	0/5	0/5	0/5	0/5
CYVCV-AY202	Agroinfiltration	0/5 (0)	NI	0/5	0/5	0/5	0/5
CYVCV-AY204	Agroinfiltration	0/5 (0)	NI	0/5	0/5	0/5	0/5
pCY	Agroinfiltration	0/5 (0)	NI	0/5	0/5	0/5	0/5
pCY-PVX	Agroinfiltration	NI	NI	5/5 (100)	NI	NI	NI

^a Survived seedlings.

^b NI, not inoculated.

Fig. 3. Citrus yellow vein clearing virus (CYVCV) detection and resulting symptoms on citrus plants. Citrus seedlings were inoculated by vacuum-infiltration with *Agrobacterium* harboring full-length cDNA clone of CYVCV-AY or empty vector pCY, or infected by grafting with wild-type CYVCV-AY (CYVCV-wt). **A**, RT-PCR detection of CYVCV in Jincheng (*Citrus sinensis*) seedlings at 20 days postinoculation (dpi). Lane M, molecular weight markers; lanes 1 to 10, CYVCV-AY101; lanes 11 to 12, CYVCV-AY105; lanes P1 and P2, CYVCV-wt, positive control; and lane N, empty vector pCY, negative control. **B**, Direct tissue blot immunoassay (DTBIA) results for detecting CYVCV in Jincheng seedlings at 60 dpi. Lanes 1 to 3, CYVCV-AY101; P, CYVCV-wt, positive control; N1, empty vector pCY, negative control 1; and N2, health seedlings, negative control 2. **C**, Symptoms on Jincheng seedlings inoculated by vacuum-infiltration with CYVCV-AY101 (right) or empty vector pCY (left) at 20 and 40 dpi, respectively. **D**, Symptoms of CYVCV-AY101 (left) and CYVCV-wt (right) on Eureka lemon (*C. limon*).

CYVCV-like symptoms. These data suggest that conventional agroinfiltration into leaves of Jincheng and other CYVCV host plants failed to infect systemically. In contrast, vacuum-infiltration of *Agrobacterium* harboring CYVCV cDNA clones into Jincheng seedlings elicited CYVCV-like symptoms (Table 3).

DISCUSSION

YVCD causes economic losses in lemon and continuously spreading rapidly in Pakistan, India, Turkey, China, and Iran since it was discovered in 1988. The disease is always related to the presence of a filamentous virus and can be transmitted by graft inoculation. Moreover, the virus-derived siRNAs detected in a field sample were associated with CYVCV only, and the virus was detected in all natural and graft-inoculated lemons by RT-PCR analysis (Loconsole et al. 2012). Thus, CYVCV was considered as the causal agent of the disease (Alshami et al. 2003), despite the fact that Koch's postulates have not been completed. In fact, ICRSV, citrus tatter leaf virus (CTLV), and citrus tristeza virus (CTV) were often detected in CYVCV-infected citrus. In this study, an infectious full-length cDNA clone of CYVCV was obtained from a lemon tree with YVCD-like symptoms. When the infectious cDNA clone was inoculated onto seedlings of lemon or sweet oranges by *Agrobacterium*-mediated vacuum-infiltration, the cloned virus of CYVCV elicited YVCD-like symptoms including yellow vein clearing, leaf distortion, and chlorosis. Subsequently, genomic RNAs of CYVCV-AY were recovered from the plant infected with this infectious clone, which was confirmed by long-distance RT-PCR, serological tests, and the resulting symptoms. Therefore, we confirmed that CYVCV was the primary causal agent of YVCD in citrus.

Although agroinfiltration remains by far the most efficient and broadly applicable among plant inoculation techniques, it is necessary to be tailored for each virus–host combination, particularly for the woody hosts (Dolja and Koonin 2014). Delivery of viral cDNA clones through agroinfiltration into woody plants has been achieved only in a limited cases (Kurth et al. 2012). Efforts to agroinfiltrate alemow or Mexican lime plants with *A. tumefaciens* carrying a CTV cDNA clone in pCAMBIA were unsuccessful (Ambrós et al. 2011). Agroinfiltration with a citrus leaf blotch virus (CLBV) derived binary vector did not result in infection except for citron (Vives et al. 2008). We also failed to infect sweet orange or lemon plantlets by agroinfiltration with *A. tumefaciens* suspension harboring full-length cDNA clone of CYVCV-AY, but inoculation of citrus seedlings by vacuum-infiltration with the same agrosuspension resulted in infection rate of 80 to 100%. In fact, when citrus seedlings were inoculated with infectious clones of CTV or CLBV by vacuum-infiltration strategy, a high infection rate was also obtained (data not shown). These results suggested that vacuum-infiltration was an efficient strategy for inoculation of citrus trees with viral cDNA clones and may be used for other woody hosts. The high infection rate in citrus might be due to (i) undeveloped immune systems of the seedlings, (ii) RNA silencing suppressor coexpressed in infiltrated areas, (iii) high level of the recovered viral genome near the infection site, or (iv) a combination of these factors. Besides, CYVCV is a phloem-limited virus. Undeveloped phloem or protophloem cells in several-days old seedlings might be more susceptible for CYVCV infection than that in adult plants. In contrast, failure of viral cDNA clones to infect herbaceous hosts through agroinfiltration could be attributed to low level of the recovered viral genome near the infection site.

TAR is a rapid and efficient technology for assembling and cloning large DNA molecules that can be more than several million base pairs (Desbiez et al. 2012). In our experiments, this technology was employed to develop CYVCV cDNA clone based on long-distance RT-PCR amplification and the ternary shuttle vector pCY construction. Then, a total of 15 CYVCV full-length cDNA clones were obtained from two repetitive trials, and the obtained full-length cDNA clones accounted for 47% of the total clones.

Moreover, at least four of those clones are infectious when inoculated to citrus seedlings with vacuum-infiltration. These results suggested that TAR cloning is a powerful and efficient tool.

This is the first report on the development of infectious cDNA clone of CYVCV, followed by success infection of citrus seedlings with the vacuum-infiltration method. Furthermore, it was validated that CYVCV was the primary causal agent of YVCD. This study lays foundation for further studies on defining functions of CYVCV genes and for the development of a gene expression or VIGS vector. In addition, the combination of TAR cloning and vacuum-infiltration is testified to be a useful strategy for generating infectious cDNA clones of other woody plant viruses rapidly, particularly for viruses with large genomes.

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