INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
19–1446/2020/23–5–963–969
DOI: 10.17957/IJAB/15.1375
http://www.fspublishers.org

Full Length Article

Confirmation Incidence of *Tomato chlorosis virus* Naturally Infecting Tomato Crop in Egypt

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Received 19 September 2019; Accepted 27 December 2019; Published 03 March 2020

Abstract

During the 2017–2018 growing season, an unusual disease was observed in tomatoes grown in different locations in Fayoum, Giza, and Nobaria in Egypt. A total of 36 naturally infected tomato plants consists of twenty asymptomatic and sixteen symptomatic tomato leaf samples showing interveinal yellowing, reddish-bronze necrosis, downward rolling and bronzing symptoms, early senescence, leaf brittleness, interveinal were collected. All samples were tested with DAS-ELISA using polyclonal and/or monoclonal antisera against several important tomato viruses. The obtained results revealed that, 5 out of 36 samples, were singly infected with tomato chlorosis virus (ToCV) and one sample was singly positive for begomovirus. Potato virus Y (PVY), cucumber mosaic virus (CMV), tobacco mosaic virus (TMV), and pepino mosaic virus (PeMV) were singly detected in 4, 3, 4 and one out of 36 samples, respectively. Two samples had mixed infection of PVY with CMV and TMV with PVY. Reverse-transcriptase polymerase chain reaction results revealed that ToCV was detected in 5 ELISA positive samples, giving a 463 bp ToCV-specific DNA fragment while only one sample was amplified (587 bp) with begomoviruses primers. Sequence analyses of 27 ToCV isolates obtained from GenBank and compared with the five isolates found in this study. All the Egyptian isolates shared high nucleotide identity among themselves (96.8–100%), while their identity matched 93.4–100% with those published in NCBI-GenBank. There was low genetic diversity between all ToCV isolates examined. To our knowledge, this is the first analytical study regarding the presence of ToCV in tomato crops in Egypt.

Keywords: Criniviruses; DAS-ELISA; HSP70; RT-PCR; ToCV

Introduction

Tomato is host to more than 70 plant viruses worldwide (Jones *et al*. 1991; Duffus *et al*. 1996; Wisler *et al*. 1998; Barbosa *et al*. 2008, 2011; Massumi *et al*. 2009; Shakeel *et al*. 2017). Tomato yellows disease (TYD) is an emerging problem in open-field and greenhouse tomato crops worldwide and is attributed so far to two whitefly-transmitted Criniviruses, *Tomato chlorosis virus* (ToCV) and *Tomato infectious chlorosis virus* (TICV) (Tzanetakis *et al*. 2013). ToCV and TICV have a bipartite genome, consisting of two positive-sense RNAs, encapsidated in long filamentous virions of approximately 800–850 nm (Wintermantel and Wisler 2006). ToCV and TICV are genetically distinct viruses (Wisler *et al*. 1998) but cannot be distinguished on the basis of their symptoms in tomatoes (Wintermantel and Wisler 2006). Criniviruses are transmitted by whitefly species of the Genera Bemisia and Trialeurodes in a semi-persistent manner (Wintermantel 2004; Tzanetakis *et al*. 2013). ToCV and TICV have a moderate host range including hosts of seven families, *Amaranthaceae, Chenopodiaceae, Asteraceae, Plumbaginaceae, Aizoaceae, Solanaceae and Apocynaceae* (Trenado *et al*. 2007; García-Cano *et al*. 2010). Many cultivated crops such as sweet pepper (*Capsicum annuum*), potato (*Solanum tuberosum*), lettuce (*Lactuca sativa)*, tobacco (*Nicotiana tabacum)*, eggplant (*Solanum melongena)* and Zinnia (*Zinnia elegans*) were identified as natural hosts of ToCV (Barbosa *et al*. 2010; Fortes and Navas-Castillo 2012; Fiallo-Olivé *et al*. 2014; Orfanidou *et al*. 2014; Kil *et al*. 2015; Zhou *et al*. 2015).

ToCV has been reported to be a widespread virus worldwide (Ewsn 1999; Louro *et al*. 2000; Navas-Castillo *et al*. 2000; Acotto *et al*. 2001; Dovas *et al*. 2002; Hanafi 2002; Segev *et al*. 2004; Tsai *et al*. 2004; Dalmon *et al*. 2005; Abou-Jawdah *et al*. 2006; Anfoka and Abhary 2007; Barbosa *et al*. 2008; Wintermantel *et al*. 2008; Zhao *et al*. 2013; Al-Saleh *et al*. 2014; Salem *et al*. 2015; Shakeel *et al*.

To cite this paper: Amer MA, YE Ibrahim, AA Kheder, AH Hamed, AA Farrag, MA Al-Saleh (2020). Confirmation incidence of *Tomato chlorosis virus* naturally infecting tomato crop in Egypt. *Intl J Agric Biol* 23:963–969

2017; Abdel-Salam *et al*. 2019).

In Egypt several viral and viral-like agents were identified recently affecting tomato crops and lead to considerable yield losses and include *Tomato yellow leaf curl virus* (TYLCV), *Tomato ringspot virus* (ToRSV), *Cucumber mosaic virus* (CMV), *Tobacco mosaic virus* (TMV), *Tomato bushy stunt virus* (TBSV), *Tomato spotted wilt virus* (TSWV) and Tomato big bud phytoplasma (Ouf *et al*. 1991; Aref *et al.* 1994; Mazyad *et al.* 1994; Fegla *et al*. 1997; Aboul-Ata *et al*. 2000; Abdelkader *et al.* 2004; El-Afifi *et al*. 2004; El-Banna *et al.* 2007; Mahfouze *et al*. 2009; El-Dougdoug *et al.* 2010; Hafez *et al*. 2010; Mohamed 2010; Omar and Foissac 2012; Megahed *et al*. 2013; Ahmed *et al*. 2014; El-Banna *et al*. 2014; El-Dougdoug *et al.* 2014; AlKhazindar 2015; Rabie *et al*. 2017). The aim of this study was to investigate ToCV and identification of Egyptian isolates which caused tomato yellowing disease. We also conducted a phylogenetic relationship analysis between these isolates and other ToCV sequences obtained from NCBI-Gen Bank.

Materials and Methods

Sample collection

During 2017–2018 growing season, a total of 20 asymptomatic and 16 symptomatic tomato samples showing interveinal yellowing chlorosis, early senescence, leaf brittleness, interveinal reddish-bronze necrosis and downward rolling and bronzing (Fig. 1) were collected from the open fields from Fayoum, Giza, and Nobaria regions in Egypt.

DAS-ELISA

DAS-ELISA (Clark and Adams 1977) was used to detect the 14 viruses: *Tomato ringspot virus* (ToRSV), *Tomato mosaic virus* (ToMV), *Tomato chlorotic spot virus* (TCSV), *Tomato aspermy virus* (TAV), *Tomato chlorosis virus* (ToCV), *Pepino mosaic virus* (PeMV), *Potato virus* X (PVX), *Potato virus* Y (PVY), *Tomato black ring virus* (TBRV), TBSV, TSWV, TYLCV, CMV and TMV. ELISA kits were purchased from Agdia, Inc. (U.S.A.) and LOEWE® (Germany).

Detection of Begomoviruses and ToCV by PCR and RT-PCR

Total DNA was extracted from all tested samples using the Qiagen DNeasy Plant Mini Kit, and DNA was tested by PCR using universal begomovirus primers (Table 1) using the KAPA2Fast PCR Kit (KAPA BIOSYSTEMS) in a thermocycler (Eppendorf, Germany). The PCR conditions were conducted according to (Wyatt and Brown 1996).

Extraction of total RNA from all tested samples was carried out by a Plant RNA Mini Kit (Bioline, London, United Kingdom). My Taq RT-PCR Kit (Bioline) was used to amplify specific gene regions within the heat shock protein (HSP70) of tomato criniviruses using a pair of degenerate primers following by specific primer using a nested-PCR (Table 1) and KAPA2G Fast PCR kit to confirm the presence of either TOCV and/or TICV (Dovas *et al.* 2002).

Nucleotide sequence and phylogenetic analysis of ToCV

A total of five amplified PCR products (463 bp) of ToCV from the nested PCR obtained from infected tomato samples were purified using Nucleic Acid Purification Kit (Omega, Bio-Tek, Inc., G.A., U.S.A.) according to the manufacturer's protocol. The purified PCR product samples were sent to BGI Tech Solutions Co., Ltd. (Hong Kong), and were sequenced into both directions using specific primer for ToCV. Phylogenetic tree analysis was constructed using the Blastn, Muscle command, and Maximum Likelihood programs using Mega 7 software (Tamura *et al.* 2004). Egyptian isolates and representative sequences of ToCV isolates isolated from Japan, South Korea, China, Turkey, United Kingdom, Spain, France, Greece, South Africa, Brazil, Italy, Portugal, Tunisia, Lebanon, Saudi Arabia, and only one isolate from Egypt that was available in NCBI-GenBank were used to conduct the phylogenetic tree. TICV was used as an outgroup for rooting the tree. Percentage identity was checked among all isolates using DNASTAR.

Results

DAS-ELISA

Interveinal yellowing of lower leaves, early senescence, leaf brittleness, interveinal reddish-bronze necrosis and downward rolling, necrotic flecks, vein thickening (Fig. 1) were observed in the majority (16 samples) of tomato crop in many rural areas, mainly in Nobaria, Fayoum and Giza regions in Egypt. all collected samples were tested with DAS-ELISA against ToCV, TYLCV, TSWV, ToRSV, ToMV, TCSV, TBSV, TBRV, TAV, PVY, CMV, TMV, PeMV and PVX. The obtained results revealed that five out of 36 samples, were singly infected with ToCV, one sample was positive for begomovirus. PVY, CMV, TMV, and PeMV were singly detected in four, three, four and one samples out of total 36 samples, respectively. Two samples had mixed infection of PVY with CMV and TMV with PVY. Whereas, TSWV, ToRSV, ToMV, TCSV, TBSV, TBRV, TAV and, PVX, were not detected in any tomato tested samples.

Detection of begomoviruses and ToCV by PCR and RT-PCR

The obtained results using RT-PCR, revealed that ToCV was detected singly in five (13.9%) out of the 36 samples collected from different three locations in Egypt and was used to amplify specific gene of expected size (463 bp)

Table 1: Primers used for the detection of begomoviruses and criniviruses infecting tomatoes

Virus primers	Primer name	Sequence 5`-3`	Product size	References
Begomovirus	prV324	gcc(t/c) at(g/t) ta(t/c) ag(g/t) aag cc(a/c) ag	579 bp	Wyatt and Brown 1996
	prC889	gg(g/a) tt(g/a/t) ga(g/a) gca tg(a/t/c) gta cat g		
Crinivirus general	HS11	gg(g/t) tt(a/g) ga(g/t) tt(c/t) ggt act ac	587 bp	Dovas *et al*. 2002
	HS12	cc(g/t) cca cca aa(a/g) tcg ta		
Tomato chlorosis virus	TOC5	ggt ttg gat ttt ggt act aca ttc agt	463 bp	Dovas *et al*. 2002
	TOC6	aaa ctg cct gca tga aaa gtc tc		
Tomato infectious chlorosis virus	TIC3	ggg tta gag ttc ggt act act ttc agt	333 bp	Dovas *et al*. 2002
	TIC4	cgt cga aag att tct cat cga ct		

Table 2: Percentage identity, based on HSP70h sequences of five ToCV Egyptian isolates from the present study and 27 isolates available on GenBank, after aligning using the cluster W method

Accession No	Country	Host	Isolate	ToCV Egyptian Isolates				
				ToCV-EG-1	ToCV-EG-2	ToCV-EG-3	ToCV-EG-4	ToCV-EG-5
AB513442	Japan	Tomato	Tochigi	95.5	95.1	94.4	96.5	95.5
MG813910	South Korea	Tomato	JN2	96.5	95.1	94.4	96.5	95.5
KP114537	South Korea	Tomato	HP	96.5	95.1	94.4	96.5	95.5
MF278017	China	Tomato	LNLZ	96.5	95.1	94.4	96.5	95.5
KY679890	China	Eggplant	HSP70-2	96.5	95.1	94.4	95.5	95.5
KY679889	China	Eggplant	HSP70-1	96.2	94.8	94.1	95.2	95.1
KY419528	Turkey	Tomato	Kas	95.8	94.4	93.4	95.8	94.8
KY419527	Turkey	Tomato	Merkez	95.5	94.1	93.4	95.5	94.4
KY810787	United Kingdom	Tomato	FERA-160205	95.1	93.7	93.4	95.1	94.4
KJ200309	Spain	Tomato	Pl-1-2	95.1	93.7	93.4	95.1	94.4
KJ200307	Spain	Pepper	MM8	95.1	93.7	93.4	95.1	94.4
KJ740257	Spain	Tomato	AT80/99-IC	95.1	93.7	93.4	95.1	94.4
DQ355215	France	Tomato	305FR	95.1	93.7	93.4	95.1	94.4
EU284744	Greece	Tomato	Gr-535	95.5	94.1	93.4	95.5	94.4
KY471130	South Africa	Tomato	ToCR-186	95.5	94.1	93.4	95.5	94.4
JQ952601	Brazil	Tomato	ToC-Br2	96.2	94.8	94.1	96.2	95.1
KY400129	Brazil	Tomato	CR 152	96.5	95.1	94.4	96.5	95.5
KY400130	Brazil	Tomato	CR 161	96.5	95.1	94.4	95.5	95.5
AM231038	Italy	Tomato	Lulu-1	96.5	95.1	94.4	96.5	95.5
AF234029	Portugal	Tomato	-	95.5	94.1	93.7	95.5	94.8
KJ739308	Tunisia	Tomato	53	96.5	95.1	94.4	96.5	94.5
KJ739306	Tunisia	Tomato	29	95.8	94.4	93.7	95.8	94.8
DQ234079	Lebanon	Tomato	-	95.8	94.4	93.7	95.8	94.8
KT888042	Saudi Arabia	Pepper	TOC98-SA	95.5	94.1	94.4	95.5	94.4
KT888034	Saudi Arabia	Tomato	TOC380-SA	96.2	94.8	94.1	96.2	95.1
KT888033	Saudi Arabia	Tomato	TOC05-SA	95.5	94.1	94.4	95.5	94.4
MH667315	Egypt	Tomato	Giza-Egypt	97.2	99.0	99.3	97.2	98.6
MK161109	Egypt- Nobaria	Tomato	ToCV-EG-1		98.3	97.6	100	96.9
MK161108	Egypt- Fayoum	Tomato	ToCV-EG-2			99.3	98.3	98.3
MK161112	Egypt- Giza	Tomato	ToCV-EG-3				97.6	98.4
MK161110	Egypt- Nobaria	Tomato	ToCV-EG-4					96.8
MK161111	Egypt- Giza	Tomato	ToCV-EG-5					

regions within the HSP70 (Fig. 2) and only one sample collected from Giza region was detected using begomoviruses degenerate primers with the expected size of 579 bp (Fig. 2). No amplifications were observed with healthy tomato samples and no RT-PCR product was amplified with TICV when the specific TICV primers was used (Fig. 2).

Nucleotide sequence and phylogenetic analysis of ToCV

RT-PCR products obtained from five ToCV Egyptian isolates were purified and sequenced. The partial HSP70h gene of the isolates were determined and submitted to the NCBI-GenBank under the following accession numbers: MK161108 for Fayoum isolate, MK161109, MK161110 for Nobaria isolates, and MK161111, MK161112 for the Giza isolates.

The data obtained from phylogenetic tree revealed limited genetic variability among all Egyptian tomato ToCV isolates and the sequences of other isolates available in NCBI, isolated from different host species and different geographical origins (Fig. 3 and Table 2). The Egyptian isolates grouped together in one cluster that was supported with 81% bootstrap value. Also, the cluster that contained virus isolates from Portugal (AF234029), France (DQ355215), Spain (KJ200307, KJ200309, KJ740257), and one isolate obtained from the United Kingdom (KY810787) was supported with 62% bootstrap value. However, isolates from Japan (AB513442), Italy (AM231038), Tunisia (KJ739308), three isolates from China (MF278017, KY679889, KY679890), two isolates from each of South Korea (MG813910, KP114537) and Brazil (KY400129,

Fig. 1: Naturally different yellowing and interveinal chlorosis and leaf thickening symptoms observed in different tomato leaf samples collected from Nobaria (**A** and **B**), Fayoum (**C**), and Giza (**D**) regions, Egypt

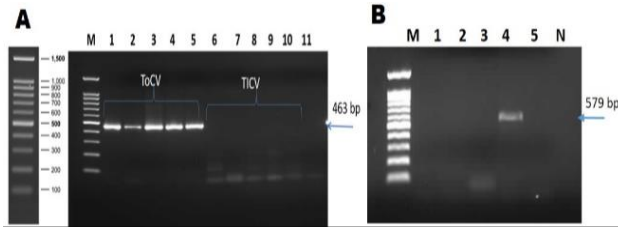

Fig. 2: (**A**) 1.5% agarose gel electrophoresis of nested-PCR amplified products (463 bp fragment) containing the heat shock protein (HSP70) gene using specific primers for ToCV and TICV in tomato samples collected from different locations in Egypt. Amplified products from symptomatic tomato leaves are shown in lanes 1, 2, 3, 4, and 5. No RT-PCR amplification was observed in infected tomato tissue when TICV primers were used (lanes 6, 7, 8, 9, 10) and no PCR amplification was observed in tissues from asymptomatic tomatoes (lane 11). **B:** 1.5% agarose gel electrophoresis of PCR amplified products (579 bp fragment) using universal primers for begomovirus on tomato samples collected from different locations in Egypt. The amplified product from symptomatic tomato leaves is shown in lane 4. No RT-PCR amplification was observed in other symptomatic tissues when the TICV primers were used (lanes 1, 2, 3, 5). No RT-PCR amplification was observed in uninfected sample tissue (lane N), Lane M: 100 bp DNA Ladder RTU (GenDirex)

KY400130) and nine different isolates including three from Saudi Arabia (KT888033, KT888034, KT888042), two isolates from Turkey (KY419527, KY419528), and one each from Greece (EU284744), Lebanon (DQ234079), Tunisia (KJ739306) and Brazil (JQ952601) grouped together in a cluster with less than 50% bootstrap support.

The nucleotide sequence identity for the Egyptian isolates shared a high similarity identity among themselves ranging from 96.8% to 100%, while databases comparisons

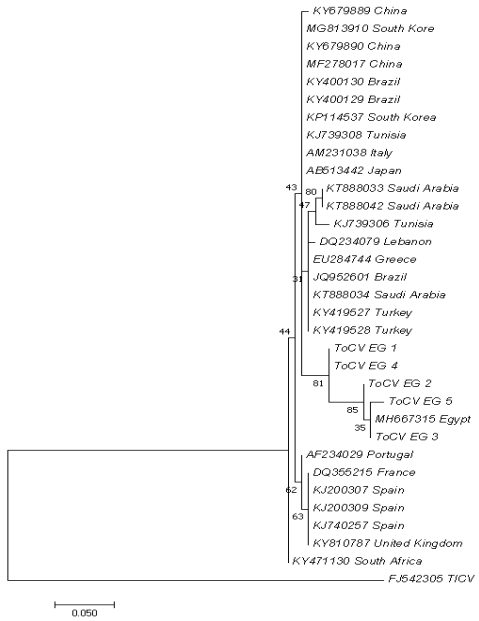

Fig. 3: A phylogenetic tree based on partial nucleotide sequences of HSP70h gene obtained from 33 ToCV isolates. EG 1-EG 5 virus isolates were obtained from the present study, while other isolates retrieved from NCBI-GenBank. The TICV sequence was used as an out-group. Bootstrap values generated from 1000 iterations are indicated on the tree

revealed a high degree of sequence identity (>94%) with other ToCV isolates. The lowest similarity (93.4–95.8%) was found between Egyptian isolates and isolates KY419528, KY419527 (Turkey); KY810787 (United Kingdom); DQ355215 (France); EU284744 (Greece); KY471130 (South Africa) and two isolates from Spain (KJ200309, KJ740257), all from tomato, and one from pepper (KJ200307), also from Spain.

Discussion

ToCV is widespread worldwide, and has caused severe epidemics in several countries in the Mediterranean basin (Louro *et al*. 2000; Acotto *et al*. 2001; Dovas *et al*. 2002; Hanafi 2002; Segev *et al*. 2004; Abou-Jawdah *et al*. 2006; Papayiannis *et al*. 2006; Anfoka and Abhary 2007; Al-Saleh *et al*. 2014; Salem *et al*. 2015). In Egypt, ToCV was found for the first time in a limited number of tomato samples in 2018 and it was the only virus detected in tomato plants suffering from TYD (Abdel-Salam *et al*. 2019). Tomato is a natural host of both ToCV and TICV, which cause TYD, resulting in heavy production losses (Wisler *et al*. 1998). Although ToCV does not cause any fruit symptoms, it causes a decline in plant vigor and reduces fruit harvest by reducing the photosynthetic area of the leaves (Wisler *et al*. 1998). As early as 2002, ToCV was reported to cause significant damage to tomatoes grown in greenhouses, with varying severity of symptoms in relation to cultivar being grown (Hanafi 2002).

Detection and differentiation of TICV and ToCV is based on molecular methods, as no antibodies are available due to low concentrations inside the phloem of diseased plants, (Livieratos *et al*. 1999; Rubio *et al*. 2001; Marco and Aranda 2005; Papayiannis *et al*. 2011). A polyclonal antiserum has been obtained for both ToCV and TICV using coat protein expressed in *Escherichia coli* and application for immunodiagnosis (Jacquemond *et al*. 2009), but for reliable routine diagnosis, RT-PCR is the main method currently used. Reliable ToCV diagnosis can be also done using dot-blot hybridization with ToCV specific probes and RT-PCR with ToCV-specific primers (Louro *et al*. 2000). Recently, commercial DAS-ELISA kits are available for these viruses.

In the present study, five isolates of a ToCV collected in tomato fields within a narrow agricultural area of North of Egypt and revealed that ToCV is so far the main Crinivirus associated with TYD. This study also revealed mixed infections of tomato with TMV and PVY, CMV, and TMV which normally results in more severe disease symptoms (Goodman and Ross 1974; Matthews 1991; Vance 1991; Hristova and Maneva 1999; Arocha *et al*. 2009; Hernandez-Gonzalez *et al*. 2011).

Detection of ToCV and TICV using RT-PCR is a more reliable method than the serological techniques in the case of criniviruses (Dovas *et al*. 2002; Barbosa *et al*. 2008). However, this is case in the current study, whereas, five samples were found to be infected singly by ToCV when RT-PCR analysis was used by comparing the ELISA method which gave positive results with only 3 out of the positive samples. Using general primers and further using nested-PCR techniques targeting HSP70 is preferred, as it helps simultaneous identification of both ToCV and TICV, while other primers are used for the specific detection of ToCV targeting its Heat shock protein, P22 gene, and minor coat protein (Vargas-Asencio *et al*. 2013).

Sequencing analysis of the Egyptian isolates showed limited genetic diversity, which is very common in most criniviruses, such as CYSDV, ToCV and TICV (Rubio *et al*. 1999; Orfanidou *et al*. 2014). Additionally, recent studies on the diversity of ToCV (Barbosa *et al*. 2013; Orfanidou *et al*. 2014) revealed that its low evolution rate is possibly correlated with the high negative selective pressure, a fact that facilitates the rapid spread of the virus throughout tomato-producing areas.

Conclusion

In this study, we confirmed that ToCV isolates in Egypt are grouped into one clade, based on phylogenetic analyses. This clustering makes it possible to hypothesize that the ToCV isolates found in Egypt have only one origin, which can be separated geographically. As such, it is necessary to obtain information on the inflow of viruliferous whiteflies or ToCV infected plants at the early stage of virus occurrence. Detection of TYD in eggplant and pepper and other cultivated crops should be further investigated in Egypt in a large scale. Although insecticide spray can reduce the whitefly populations, this is not very effective because whiteflies are very active, and transmit the virus before the insecticide killed the whiteflies and also the whiteflies develop resistance to insecticides. Therefore, control of TYD is so far only possible by using resistant cultivars.

Acknowledgements

We thank Prof. Nicolas I. Katis, Aristotle University of Thessaloniki, Faculty of Agriculture, Forestry and Natural Environment, School of Agriculture, Plant Pathology Lab, Thessaloniki, Greece, for his critically reviewing and valuable comments. The authors would like to extend their sincere appreciation to the Deanship of Scientific Research, King Saud University, Saudi Arabia, for its funding of this research group no. RG-1438-065.

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