Genet Resour Crop Evol (2013) 60:967–974
DOI 10.1007/s10722-012-9892-9

RESEARCH ARTICLE

Cytoplasmic diversity in *Brassica rapa* L. investigated by mitochondrial markers

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Received: 7 March 2012 / Accepted: 23 July 2012 / Published online: 8 August 2012

Abstract *Brassica rapa* L. is an important vegetable and oilseed crop. Cytoplasmic diversity of 36 *B. rapa* accessions was analyzed using the mitochondria-specific markers. Twelve representative materials including five additional Brassica species and one *Eruca sativa* Mill. were used as references. A modified multiplex PCR amplification using four pairs of primers was performed to test the mitochondrial types (mitotypes) of the tested materials. Ten accessions were detected with Cam-I mitotype which could amplify 500 and 800 bp bands, twenty-two accessions with Cam-II mitotype which could amplify 500, 800 and 906 bp bands, one accession with Pol mitotype. Interestingly, three *B. rapa* accessions were revealed with nap mitotype, two of them were local landraces in northern Shaanxi, the third one was a variety from Gansu province which was developed using one local landrace from Northern Shaanxi as female parent. The considerable cytoplasmic diversity in *B. rapa* provides useful information on studying the possible origin and evolution of *B. rapa* accessions, and conservation of the germplasm.

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Keywords *Brassica rapa* L. · Cytoplasmic diversity · Mitotype · A modified multiplex PCR

Introduction

The genus Brassica is one member of the Brassicaceae family, including a diverse range of the most important oilseed, vegetable and fodder crops worldwide (Labana and Gupta 1993). The major crop types comprises six crop species, three diploid species *Brassica rapa* L. (AA genome, n = 10), *B. oleracea* L. (CC genome, n = 9) and *B. nigra* (L.) Koch (BB genome, n = 8) and three amphidiploid species, *B. juncea* (L.) Coss. (AABB, 2n = 36), *B. napus* L. (AACC, 2n = 38) and *B. carinata* A. Braun (BBCC, 2n = 34). The relationships between the six major cultivated Brassica species were originally described by UN (1935). Each of the amphidiploids contains genomes from two diploid species. *B. rapa* is an important vegetable and oilseed crop. *B. rapa* vegetables are consumed worldwide and provide a large proportion of the daily food intake in many regions of the world, such as Chinese cabbage, turnip, and other leafy vegetable crops (Li 1981a, b). *B. rapa* has many types and sub-species, and is characterized by high seed yield with a high oil content, self-incompatibility, earlier maturity and disease resistance (Monteiro et al. 1988; Ren et al. 2000). China is

considered as one origin centre of *B. rapa* (Liu 1984), which has very rich genetic resources. Therefore, genetic diversity information on Chinese *B. rapa* will allow us to effectively maintain and utilize the germplasm in breeding program.

Mitochondrial and chloroplast genes are inherited in a strictly maternal fashion in most angiosperm plant species including *Brassica* (Palmer et al. 1983a;1983b; Soltis and Soltis 2000).The contents and structures of the chloroplast genome are highly conserved (Palmer 1991; Raubeson and Jansen 2005). In contrast to chloroplast, frequent homologous recombinations result in a complicated multipartite genome structure in the mitochondrial genome of higher plants (Avise 1994). The complete mitochondrial nucleotide sequence of *B. napus* (Nap and Pol), *B. rapa* (Cam), *B. oleracea*, *B. juncea* and *B. carinata* were determined by Handa (2003), Chen et al. (2011) and Chang et al. (2011), respectively. The entire chloroplast genome of rapeseed (*B. napus*) was also sequenced (Hu et al. 2010). With the ever-increasing number of *Brassica* cytoplasmic sequences, many cytoplasmic markers have been developed to analyze genetic diversity in *Brassica* genera and related species. Handa (2007) used PCR-based markers to investigate the origin and transmission of linear mitochondrial plasmid and mitochondrial genome. Zhao et al. (2010) distinguished the existing common cytoplasm resources, Pol, Nap, Cam, Ogu and Ogu-NWSUAF cytoplasm in one PCR-reaction using three pair mitochondria-specific primers in rapeseed. Flannery et al. (2006) designed ten pairs SSR primers according to intron and spacer regions of chloroplast DNA and indicated that eight of them showed polymorphism and detected a total number of 28 haplotypes in *Brassica* genera. Allender et al. (2007) designed six pairs SSR primers based on *Arabidopsis thaliana* chloroplast genome sequence or *B. napus* chloroplast sequence, and analyzed genetic diversity in *B. oleracea* and its wild relatives, and origins of the amphiploid species *B. napus* (Allender and King 2010). By using 24 chloroplast SSR markers, Lv et al. (2009) investigated chloroplast diversity in 90 *B. napus* accessions, three *B. oleracea*, and three *B. rapa*. More recently, Xu et al. (2011) used 10 chloroplast-specific SSR primers and 6 nuclear-specific SRAP primers to evaluate the genetic diversity and population structure of European wild *B. oleracea* accessions. Genetic diversity of *B. rapa* were extensively characterized at the nuclear DNA level (Zhao et al. 2005), however, to our knowledge, cytoplasmic diversity of *B. rapa* was not systematically investigated.

In this study, thirty-six accessions of *B. rapa* including *B. rapa* ssp. *pekinensis* (Lour.) Hanelt, *B. rapa* ssp. *chinensis* (L.) Hanelt, *B. rapa* ssp. *chinensis* var. *oleifera* and *B. rapa* ssp. chinensis var. tai-tsai were analyzed using a modified multiplex PCR assay. Four different mitochondrial types (mitotypes) were detected in *B. rapa* accessions. The results are very valuable for a wide range of applications in evolutionary study, Brassica breeding and improvement.

Materials and methods

Plant material and DNA extraction

In total, 48 Brassicaceae accessions including 36 *B. rapa* accessions were studied (Table 1, taxonomy according to Gladis and Hammer 1992). These 36 accessions consisted of 24 *B. rapa* ssp. *chinensis* var. *oleifera*, DC., 8 *B. rapa* ssp. *chinensis* (L.) Hanelt, 3 *B. rapa* ssp. *pekinensis* (Lour.) Hanelt and 1 *B. rapa* ssp. *chinensis* var. Tai-tasi. Five *B. napus* accessions representing five cytoplasm types Nap, Pol, Cam, Ogu and improved Ogu (Ogu-NWSUAF) (Chang et al. 2010), 2 *B. juncea*, 2 *B. oleracea*, 1 *B. nigra*, 1 *B. carinata* and 1 *Eruca sativa* Mill. accession were included as references. These accessions were sown in the experimental field of Northwest A&F University, Yangling, Shaanxi, People's Republic of China in 2010–2011.Ten three-leaf stage plantlets were randomly chosen from each accession for total genomic DNA isolation using the cetyltrimethylammonium bromide method (Murray and Thompson 1980).

A multiplex PCR analysis

Three pairs of primers specific to mitochondria genomes (Wei et al. 2005; Zhao et al. 2010) and one pair of primer specific to mitochondrial plasmid (Handa 2007) were used (Table 2). Multiplex PCR amplifications were carried out in a 20 μl volume containing 50 ng genomic DNA, 150 μM of each dNTP, 0.25 units of *Taq* DNA polymerase (TIANGEN, China), 1× PCR buffer and 0.15 μM of each primer. The following amplification protocol was

Table 1 List of 48 *Brassica* accessions used in this study and their mitochondrial types identified by the modified multiplex PCR assay

No.	Cultivar name	Taxa	Origin	Mitochondrial type
1	Parkland	*B. rapa* ssp. *chinensis* var. *oleifera*	Canada	CamII
2	Tobin_1	*B. rapa* ssp. *chinensis* var. *oleifera*	Canada	CamII
3	Tobin_2	*B. rapa* ssp. *chinensis* var. *oleifera*	Canada	CamII
4	200	*B. rapa* ssp. *chinensis* var. *oleifera*	Gansu	CamI
5	257	*B. rapa* ssp. *chinensis* var. *oleifera*	Gansu	CamII
6	703	*B. rapa* ssp. *chinensis* var. *oleifera*	Gansu	CamI
7	Hao You 11	*B. rapa* ssp. *chinensis* var. *oleifera*	Gansu	CamI
8	Long You No.6	*B. rapa* ssp. *chinensis* var. *oleifera*	Gansu	CamI
9	Long You No.8	*B.rapa* ssp. *chinensis* var. *oleifera*	Gansu	CamI
10	Long You No.9	*B. rapa* ssp. *chinensis* var. *oleifera*	Gansu	Nap
11	Tian You No.2	*B. rapa* ssp. *chinensis* var. *oleifera*	Gansu	CamII
12	Tian You No. 8	*B. rapa* ssp. *chinensis* var. *oleifera*	Gansu	CamI
13	Binxian Yimen 01	*B. rapa* ssp. *chinensis* var. *oleifera*	Shaanxi	Nap
14	Binxian Beiji 01	*B. rapa* ssp. *chinensis* var. *oleifera*	Shaanxi	Nap
15	Binxian Xinmin 01	*B. rapa* ssp. *chinensis* var. *oleifera*	Shaanxi	CamI
16	Baishui Youcai	*B. rapa* ssp. *chinensis* var. *oleifera*	Shaanxi	CamII
17	7D0488	*B. rapa* ssp. *chinensis* var. *oleifera*	Shaanxi	CamI
18	Fenyang Youcai	*B. rapa* ssp. *chinensis* var. *oleifera*	Shanxi	CamII
19	Linyi Youcai	*B. rapa* ssp. *chinensis* var. *oleifera*	Shanxi	CamII
20	Xinjiangxian Youcai	*B. rapa* ssp. *chinensis* var. *oleifera*	Shanxi	CamII
21	Yayou No.1	*B. rapa* ssp. *chinensis* var. *oleifera*	Sichuan	CamII
22	Jingning hongheizi	*B. rapa* ssp. *chinensis* var. *oleifera*	Zhejiang	CamII
23	Baiyu	*B. rapa* ssp. *chinensis* var. *oleifera*	Zhejiang	CamII
24	Huangze youcai	*B. rapa* ssp. *chinensis* var. *oleifera*	Zhejiang	CamII
25	Huai Nan Huang Xin Cai	*B. rapa* ssp. *chinensis*	Anhui	CamII
26	Yuan Zhong Hei You Bai Cai	*B. rapa* ssp. *chinensis*	Guizhou	CamII
27	Hei You Bai Cai	*B. rapa* ssp. *chinensis*	Henan	CamII
28	Si Ji Xiao Bai Cai	*B. rapa* ssp. *chinensis*	Henan	CamII
29	Shang hai Qing(Yu feng)	*B. rapa* ssp. *chinensis*	Shanghai	CamII
30	Shang hai Ji Mao Cai	*B. rapa* ssp. *chinensis*	Shanghai	CamII
31	Shang hai Qing(Yong an)	*B. rapa* ssp. *chinensis*	Shanghai	CamII
32	Longquan Heiyoucai	*B. rapa* ssp. *chinensis*	Zhejiang	CamII
33	Re Kang 50	*B. rapa* ssp. *pekinensis*	Shandong	CamI
34	Xia Lv Ming Xian	*B. rapa* ssp. *pekinensis*	Shandong	CamI
35	Chinese Cabbage Hybrid	*B. rapa* ssp. *pekinensis*	Shaanxi	Pol
36	Bai Ye Ta Cai	*B. rapa* ssp. *chinensis* var. tai-tsai Lin	Shanghai	CamII
37	Ganlan F_1	*B. oleracea*	Shaanxi	CamI-like
38	Ganlan CMS F_1	*B. oleracea*	Shaanxi	Ogu-like
39	Black mustard	*B. nigra*	Czech	Ogu-like
40	Westar	*B. napus*	Canada	Nap
41	Bronowski	*B. napus*	Poland	CamI
42	IP_Ogu CMS	*B. napus*	Shaanxi	Ogu-NWSUAF
43	Shaan 2A	*B. napus*	Shaanxi	Pol
44	Ogu CMS	*B. napus*	Shaanxi	Ogu

Table 1 continued

No.	Cultivar name	Taxa	Origin	Mitochondrial type
45	Wei yuan You Cai	*B. juncea*	Gansu	CamI
46	2598	*B. juncea*	Inner Mongolia	CamI
47	Dodolla	*B. carinata*	Canada	Ogu-like
48	Czech Yun Jie	*E. sativa* Mill.	Czech Republic	CamI-like

Cam typeI has 500 and 800 bp bands and Cam typeII has 500, 800 and 906 bp bands as shown in Fig. 1

Table 2 The sequences and information of the primers used in the multiplex PCR

Number	Name	Sequence 5′–3′	Target gene	Product length (bp)	References
1	P11	GAAACGGGAAGTGACAAT	Orf138	465	
	P12	GCATTATTTTCTCGGTCCAT			
2	P21	AGCTGTCTGGAGGGAATC	Orf222	1,102	Wei et al. (2005)
	P22	GCGGTCTCACGCACTAATC			
3	P21	AGCTGTCTGGAGGGAATC	Orf224	747	
	P32	ACGACATCAAGGAGGAAC			
4	ms31F	CCATGGATGATTCGACCCTCTTTCATAAG	Around orf3	906	Handa (2007)
	ms31R	CCCATGGAATAGAATGCCTTCTCCAATTC	(plasmid)		

carried out in C1000 thermal cycler (Bio-rad Co. Ltd. America). Initial denaturation was performed at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 50 s and extension at 72 °C for 70 s and a final extension at 72 °C for 7 min. The amplification products were analyzed on 1.5 % (w/v) agarose gels in 1× TAE buffer and visualized with ethidium bromide. The bands were photographed under UV light (Alphalmager EP, Alpha Innotech corporation, USA).

Result

The modified multiplex PCR

We first used one pair of primer specific to mitochondrial plasmid (Handa 2007, Table 2) to amplify total genomic DNA samples of 5 *B. rapa* (No.1, 21, 2, 3, 17 in Table 1) and 1 *E. sativa* Mill. (No. 48). PCR products were amplified from these five *B. rapa* accessions and the one *E. sativa* Mill. accession, however, there existed difference in their PCR product quantity (Fig. 1a). We then developed the original multiple PCR assay (Zhao et al. 2010) by increased one pair of primer specific to mitochondrial plasmid (Handa 2007). The modified multiplex PCR assay could reveal two Cam mitotypes, with Cam-I mitotype having the combination of 500 and 800 bp bands (Fig. 1c lane 5), and Cam-II mitotype having the combination of 500, 800 and 906 bp bands (Fig. 1c lanes 1–4). Amplification patterns for other mitotypes, Pol, Nap, Ogu, Ogu-NWSUAF were same as described by Zhao et al. (2010). The combination of a 747- and 500-bp band was specific to the accession with Pol cytoplasm (No.43 in Fig. 2), the combination of a 1102- and 800-bp band specific to the accession with Nap cytoplasm (No.40 in Fig. 2), A 465-bp band specific to the accession with Ogu cytoplasm (No.44 in Fig. 2), and the combination of a 465- and 1102-bp band specific to the accession with Ogu-NWSUAF cytoplasm (No.42 in Fig. 2).

Mitotypes of the accessions detected by the modified multiplex PCR

The PCR patterns of all accessions amplified with four pairs of mitochondria–specific primers are shown in Fig. 2, and the results summarized in the Table 1. Four mitotypes, Cam-I, Cam-II, Nap and Pol were detected

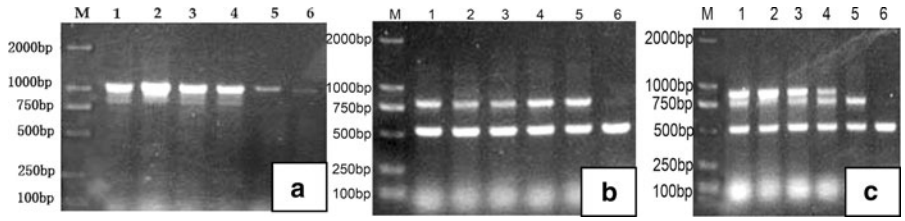

Fig. 1 The patterns of PCR were showed by one pair of primer specific to mitochondrial plasmid (Primer 4 in Table 2) (**a**), Three pairs of primers specific to mitochondrial genomes (Primer 1-3 in Table 2) (**b**) and these four pairs of primers (**c**) in 5 *B. rapa* (No. 1, 21, 2, 3, 17 in Table 1) and 1 *E. sativa* Mill. (No. 48) respectively. CamI has 500 and 800 bp bands showing in Fig. 1c *lane 5*; CamII has 500, 800 and 906 bp bands in Fig. 1c *lane 1* to *lane 4*

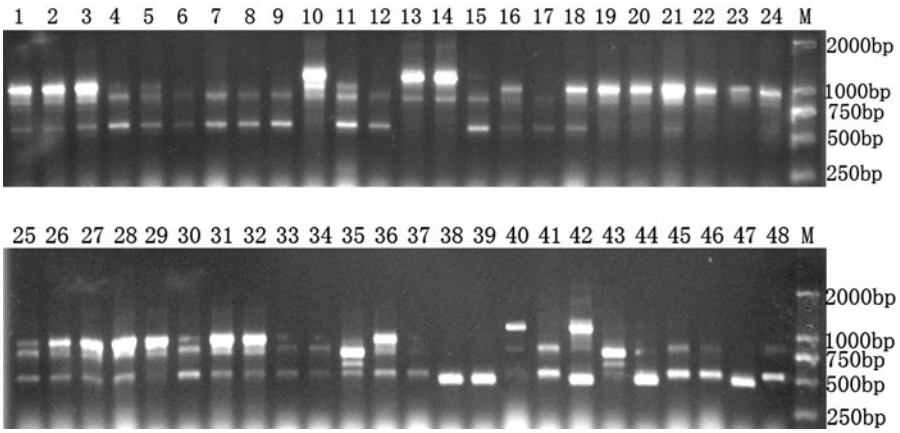

Fig. 2 The electrophoresis patterns of PCR products amplified with four pairs of mitochondria–specific primers. The number of each accession was shown in Table 1; M molecular weight marker

in all *B. rapa* accessions. 'Bronowski' (No. 41), a traditional check variety of Cam mitotype, was amplified a combination of 500 and 800 bp bands by the improved multiplex PCR, so, we classified it as Cam-I mitotype. Eight *B. rapa* ssp. *chinensis* var. *oleifera* and 2 *B. rapa* ssp. *pekinensis* (Lour.) Hanelt were found belonging to Cam-I mitotype. Thirteen *B. rapa* ssp. *chinensis* var. *oleifera*, all eight *B. rapa* ssp. *chinensis* (L.) Hanelt, and 1 *B. rapa* ssp. *chinensis* var. tai-tsai had Cam-II mitotype. Three *B. rapa* ssp. *chinensis* var. *oleifera* accessions (No.10, 13, 14) had Nap mitotype and one (No. 35) Pol mitotype. The amplification pattern of one *B. oleracea* accession (No. 38), one *B. nigra* accession (No. 39), and one *B. carinata* accession (No. 47) is similar to that of the accession No. 44 with Ogu mitotype, so their mitotypes are defined as Ogu-like. Similarly, the mitotypes of two *B. juncea* accessions (No. 37, 48) belong to Cam-I like.

Discussion

The characters of the modified multiplex PCR compared with the original multiplex PCR

Higher-plant mitochondria contain a variety of extra-chromosomal DNAs in addition to a large and complex main mitochondrial genome (Brown and Zhang 1995). These smaller DNAs have two forms, circular and linear. To date, 14 linear mitochondrial plasmids have been reported in only eight plant species, *Beta vulgaris* (Saumitou-Laprade et al. 1989), *B. napus* (Palmer et al. 1983a), *B. rapa* (Turpen et al. 1987; Handa et al. 2002), *Daucus carota* (Robison and Wolyn 2005), *Sorghum bicolor* (Pring et al. 1982; Dixon and Leaver 1982; Chase and Pring 1986), *Zea diploperennis* (Timothy et al. 1983), *Zea luxurians* (Grace et al. 1994), and *Zea mays* (Paillard et al. 1985; Levings III and Sederoff 1983; Weissinger

et al. 1982). The *Brassica* linear plasmid molecule of about 11.6 kb, the longest of all known mitochondrial plasmids in higher plants, showed a non-maternal inheritance, in contrast to mitochondrial genomes (Palmer et al. 1983a; Handa et al. 2002). The origin of this plasmid DNA remains unknown. Palmer et al. (1983a) and Handa (2007) reported that the presence of plasmid DNA was restricted to only two *Brassica* species, *B. rapa* and *B. napus*. *Brassica oleracea*, *B. juncea*, *B. nigra* and *B. carinata* do not have the 11.6 kb plasmid in their mitochondria. Handa (2007) postulated that the plasmid was originally present in *B. rapa*, one of the parent species of rapeseed (*B. napus*), and then transferred to *B. napus* through interspecific crosses in a modern breeding program. In the present investigation, PCR products were amplified from all *B. rapa* accessions with the single mitochondrial plasmid specific primer, however, there exists difference in their PCR product quantity (data was not shown). This phenomenon may be explained by the substoichiometrical difference of mitochondrial genome in different accessions, such as that observed by Chen et al. (2011) and Chang et al. (2011). We modified the original multiplex PCR assay (Zhao et al. 2010) by increasing one pair of primer specific to mitochondrial plasmid (Handa 2007) in this study. The modified multiplex PCR assay could reveal two Cam mitotypes, except that it has the capacity to distinguish the existing common cytoplasm resources, Pol, Nap, Ogu and Ogu-NWSUAF cytoplasm as the original one.

Different mitotypes existed in *B. rapa* accessions

Brassica rapa have highly morphological differences due to the long history of breeding and domestication for different traits along with natural selection for adaptation to different geographical regions. Oleiferous and turnip forms were developed in Europe while species in eastern Asia and western Asia have evolved into leaf form and oleiferous form. Leafy vegetables of *B. rapa* included Chinese cabbage (*B. rapa* ssp. *pekinensis*), non-heading pak choi (*B. rapa* ssp. *chinensis*), and mizuna which were widely found in China, Korea and Japan. Oleiferous form of *B. rapa* has advantage of having wide variability and great genetic potential for yield and other traits. *B. rapa* has been used to diversify *B. napus* germplasm. The development and use of molecular markers in *Brassica* started in late 1980s and since then different types of molecular markers have been developed and utilized for genetic diversity and evolutionary study in *B. rapa* and other *Brassica* species (Song et al. 1988a, b; Quiros et al. 1994; Kresovich et al. 1995; Demeke et al. 1992; Lowe et al. 2004; Choi et al. 2007; Kim et al. 2009; Zhao et al. 2005). In this study, a modified multiplex PCR assay including four pair of mitochondrial-specific primers was used to investigate the cytoplasm types in 48 *Brassica* accessions including vegetables and oleiferous *B. rapa* originated mainly from China. Among 36 *B. rapa* accessions tested, 10 accessions were detected with Cam-I mitotype, 22 with Cam-II mitotype, 3 with Nap mitotype and one with Pol mitotye. To our knowledge, three *B. rapa* ssp. *chinensis* var. *oleifera* accessions with Nap mitotype have not been reported in the previous investigations. Two of these three *B. rapa* accessions with Nap mitotye (No.13 and 14) were local landraces in northern Shaanxi, the third one (No.10) was a variety from Gansu province which was developed using one local landrace from Northern Shaanxi as female parent. Northern Shaanxi is characterized by extremely dry and cold climate in the winter, traditionally, *B. rapa* landraces are cultivated in this area, *B. napus* varieties can't survived during winter season in this area. In our experiment, both Nap and Cam mitotypes were found in *B. rapa* accessions, which may support the hypothesis of Chang et al. (2011), who inferred that the Nap mitotype has been inherited from an unidentified or lost mitotype of *B. rapa*. In addition, our result showed accession No. 35 (Chinese Cabbage Hybrid) and No. 43 (Shaan 2A) had the same mitotype (Pol), the result was consistent with the information provided by the breeder that cytoplasm of accession No. 35 came from accession No. 43. Further study on the differences between these four mitotypes existed in *B. rapa* can provide useful information on their possible origin and evolution of *B. rapa* accessions.

Acknowledgments We thank Profs Xiaoming Wu from Oil Crops Research Institute of Chinese Academy of Agricultural Sciences (Wuhan, Hubei) and Haohan Wang from Zhangye Academy of Agricultural Sciences (Zhangye, Gansu) for kindly providing some *B. rapa* accessions. This work was supported by the earmarked fund for China Agriculture Research System (CARS-13) and a grant from Northwest A&F University for S.W. Hu.

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