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国庆1号温州蜜柑珠心胚苗培育及四倍体发掘

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摘 要:【目的】温州蜜柑一般表现为雄性不育、果实无核,收集国庆1号温州蜜柑(*Citrus unshiu* Marc.,简称G1)种子,用于培育珠心胚苗并发掘多倍体新种质,为其提纯复壮和珠心胚变异育种提供基础材料。**【方法】**从G1成熟果实剥取种子,浸泡消毒后离体播种至播种培养基;待幼苗长至有3~5枚真叶时,采用流式细胞仪分析倍性和SSR分子标记鉴定遗传来源。**【结果】**从约12 500个果实中剥取获得106粒种子,离体播种后41粒种子萌发,经培养获得62株实生幼苗;对所有幼苗进行倍性检测,获得四倍体1株,其余61株均为二倍体;用4对多态性SSR引物对获得的四倍体和随机筛选的38株二倍体植株进行分子鉴定,表明发掘的四倍体和35株二倍体的带型与亲本G1完全一致,推测其均为珠心胚来源,且四倍体为G1珠心细胞自然加倍而形成;其余3株二倍体在部分SSR位点与G1带型不一致,推测由G1与周边其他品种杂交而来。**【结论】**这些珠心胚苗二倍体及四倍体种质为国庆1号温州蜜柑提纯复壮、珠心胚变异育种及相关基础研究提供了珍贵的种质资源。

关键词:柑橘;珠心胚;四倍体;流式细胞术;SSR分子标记

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Production of nucellar seedlings and exploration of tetraploid from Satsuma mandarin Guoqing No. 1

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Abstract:【Objective**】** Satsuma mandarin (*Citrus unshiu* Marc.) is a polyembryonic cultivar with typical cytoplasmic male sterile (CMS) trait, and it is widely grown in China because of its seedlessness and excellent fruit quality. Due to the CMS characteristic, the Satsuma mandarin has been used as female parent in sexual hybridization and contributed to the production of many excellent seedless cultivars such as Kiyomi tangor, Shiranui mandarin, Harumi mandarin and Setoka tangor. Satsuma mandarin Guoqing No. 1 (hereafter abbreviated as G1) is an early maturing variety selected from Satsuma mandarin Kamei *via* exploring spontaneous bud mutation by Huazhong Agricultural University. G1 fruit theoretically appears seedless, but it may produce a small number of seeds when it encounters high temperature during flowering period, indicating that its fertility may be partially restored under high temperature conditions. *Citrus* seedling production is usually based on asexual propagation, like grafting, which can keep the excellent characteristics of the original cultivar. However, long-term asexual propagation might result in the infection of viruses, viroids and bacterial diseases in citrus trees, leading to a certain degree of variety degeneration, and gradually show the decline of vigor, yield, quality and other phe-

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nomena. It is urgent to carry out purification, rejuvenation and variety improvement. Most virus diseases cannot be transmitted by seed, and citrus seedlings regenerated from the nucellar embryo can eliminate most virus diseases and usually show more vigorous growth than their parents, so as to achieve the purpose of purification and rejuvenation. In addition, selection breeding based on the variation of nucellar seedling is also an important approach in citrus breeding, and many new citrus varieties with different ripening stages, fruit types and peel colors were selected. The G1 trees were grafted on *Poncirus trifoliata* and planted in the citrus breeding orchard in Huazhong Agricultural University for more than ten years. In January 2021, due to the low temperature and freezing injury, G1 fruits were frozen and inedible, thus all the fruits were picked for seed collection. Because polyembryonic citrus has variable number of embryos and the nucellar embryos are usually more vigorous than zygotic embryo, mature seeds of G1 may regenerate more nucellar seedlings, which can be used as the valuable germplasm for tetraploid exploration. Here, we used G1 as the material, explored their nucellar seedlings for producing virus-free G1 and screened tetraploid germplasm for interploid breeding. It can be a model for the utilization of other Satsuma mandarin cultivars.【Methods】Following G1 fruits being picked, seeds were extracted and divided into developed and undeveloped types. By stripping the exopleura of all seeds, under aseptic conditions, the seeds were soaked and disinfected in 3% (ρ) NaClO solution for 15 min. In order to make the seeds germinate neatly, the endopleura was removed and then the seeds were sown *in vitro* on the germination medium. When the seedlings grew with 3−5 leaves, using flow cytometry and shoot tip chromosome counting to determine their ploidy level. Using a known diploid as a control, the fluorescence intensity of its leaves was set at 50, and then the peak at 100 indicated that the sample was tetraploid. In addition, because the basic chromosome number of citrus was 9, thus 18 and 36 chromosomes meant diploid and tetraploid, respectively. Whereafter, the seedlings were transplanted into plastic pots filled with nutrient soil and placed in a growth chamber. When the seedlings grew with 7−8 leaves, they were transferred to the greenhouse, and normal fertilizer and water management were guaranteed during this period. The genetic origin of the seedlings obtained in this study were further analyzed by automatic capillary electrophoresis system using four simple sequence repeat (SSR) markers, which were screened from the reported work.【Results】A total of 106 seeds were obtained from about 12 500 G1 fruits, with a seed setting rate of 0.85%. Among them, the numbers of developed and undeveloped seeds were 80 and 26, respectively, and the former was about three times as many as the latter. All seeds were sown *in vitro* on the germination medium. Due to the contamination of some developed seeds and the failure of germination of undeveloped seeds, only 41 developed seeds finally germinated, with a germination rate being 38.7%. From the germinated seeds, 62 seedlings were obtained, with an average of 1.5 plants regenerated per seed. By analyzing the ploidy level of 62 seedlings, one tetraploid plant was obtained, with the tetraploid occurrence rate of 1.61%. And the remaining 61 seedlings was proven to be diploid seedlings. These results determined by flow cytometry was also confirmed by root-tip chromosome counting. Four SSR markers were used to analyze the genetic origins of the tetraploid and 38 randomly selected diploid seedlings. The result showed that the bands of the tetraploid seedlings and 35 diploid seedlings were identical to that of G1, indicating that the tetraploid seedling might originate from the chromosome doubling of nucellar cells of G1 and all 35 diploid seedlings were the nucellar seedlings of G1. And the remaining three diploids showed some bands that G1 did not possess, indicating they might originate from the sexual hybridization of G1 with unknown pollen parent. In total, the seedlings derived from the nucellar embryo accounted for 92.11% of the 38 randomly selected diploid seedlings, indicating that nucellar embryos might be more vigorous than zygotic embryo.【Conclu-

sion】From the results above, it showed that in the seeds of polyembryonic citrus, certain proportion survival seeds was derived from zygotic embryos, and SSR molecular marker analysis can be an efficient tool to distinguish between nucellar and zygotic seedlings. The diploid and tetraploid nucellar seedlings obtained here may hold great potential for genetic improvement, polyploid breeding and related fundamental researches of Satsuma mandarin Guoqing No. 1.

Key words: *Citrus*; Nucellar embryo; Tetraploid; Flow cytometry; SSR molecular marker

温州蜜柑为细胞质雄性不育类型[1]，果实无核，易剥皮，品质佳，易栽培，是中国很多柑橘产区重要的主栽品种。国庆1号温州蜜柑是华中农业大学早期从龟井温州蜜柑芽变选出的早熟品种。在生产中，为保持品种特性，柑橘一般采用无性繁殖，但长期无性繁殖会在其体内积累一种或多种病毒、类病毒及细菌性病害[2]，导致出现一定程度的品种退化，逐渐表现出生活力降低、产量下降和品质衰退等现象，急需进行提纯复壮和品种改良。由于大多数病毒病不能通过种子传播，柑橘珠心胚苗能脱除大多数病毒，通常表现为生长势比亲本更旺盛，从而达到提纯复壮的目的[3-4]。

基于珠心系实生变异选择育种是柑橘的重要育种途径。日本育种家利用珠心系变异选育出大批熟期、果型、果皮色泽各异的温州蜜柑新品种，如兴津早生、三保早生等品种就是利用温州蜜柑的多胚性，从宫川温州蜜柑珠心系选育而来[3,5]。黄秀等[6]以由良温州蜜柑为母本与鸡尾葡萄柚等4个品种有性杂交，获得温州蜜柑种子用于珠心苗培育，以期选育符合市场需求的新品种。笔者在本研究中以国庆1号温州蜜柑为材料，采用离体播种方法培育珠心胚苗，以期为国庆1号温州蜜柑提纯复壮、珠心胚变异发掘和相关基础研究提供珍贵的种质材料。

1　材料和方法

1.1　研究材料

国庆1号温州蜜柑（*Citrus unshiu* Marc.，简称G1）嫁接于枳砧并定植于华中农业大学柑橘育种试验基地，其周围栽植有华农本地早橘等品种，树龄均10 a（年）以上。2021年1月因低温冻害影响，国庆1号温州蜜柑果实受冻而不可食用，全部采摘用于收集种子。

1.2　种子离体培养

将饱满种子置于1 mol·L^{-1} NaOH溶液中浸泡15 min去除果胶，用自来水冲洗干净后剥去外种皮；无菌条件下，将去除外种皮的种子置于3%（φ）NaClO溶液中浸泡消毒15 min；用无菌蒸馏水清洗至少3次后，将种子转移至灭菌的三角瓶；用镊子去除内种皮后，将其接种于播种培养基（MT + 25 g·L^{-1}蔗糖，pH = 5.8）并置于培养室光照培养。培养室条件：温度（25±1）℃；光照16 h。

1.3　倍性鉴定

待幼苗长至有3~5枚真叶大小时，用流式细胞仪和根尖染色体压片2种方法检测植株倍性。流式细胞仪（Cyflow space，Sysmex，Japan）倍性检测参考解凯东等[7]的方法。根尖染色体压片参考夏强明[8]的方法并适当修改。取生长旺盛的根尖，饱和对二氯苯溶液室温下预处理3 h后，用新鲜的卡诺固定液（$V_{乙醇}$∶$V_{乙酸}$ = 3∶1）固定24 h，最后转移至75%乙醇溶液4 ℃保存备用。制片前，根尖用酶液（1%果胶酶（Sigma- aldrich）、2%纤维素酶Onozuka（R-10，Yakult）和1%果胶酶（Y-23，Yakult））37 ℃水浴处理90 min后，用火焰干燥法进行染色体制片，并用荧光显微镜（Imager. M2，Zeiss，Germany）镜检，染色体图像用ZEN软件采集。

四倍体群体发生频率/%=四倍体植株数/群体植株总数×100。

1.4　植株移栽

将倍性分析后的所有植株移栽至装有营养土（$V_{进口泥炭土}$∶$V_{基质土}$∶$V_{蛭石}$∶$V_{珍珠岩}$ = 4∶4∶1∶1）的营养钵并置于生长室炼苗。待幼苗长至7~8枚真叶大小时，将其转移至温室，期间保证正常肥水管理。生长室条件：温度（25±1）℃；光照16 h。

1.5　SSR分子鉴定

基因组DNA提取和SSR分子标记分别参考Cheng等[9]和谢善鹏等[10]的方法。筛选的4对多态性SSR引物（表1）由生工生物工程（上海）股份有限公司合成。PCR反应体系10 μL：2× PCR mix 5 μL，正、反向引物各0.25 μL（10 μmol·L^{-1}），DNA模板1 μL，无菌水3.5 μL。PCR反应在ProFlex PCR仪（ABI，

表 1 SSR 引物序列

Table 1 Sequence of SSR primers

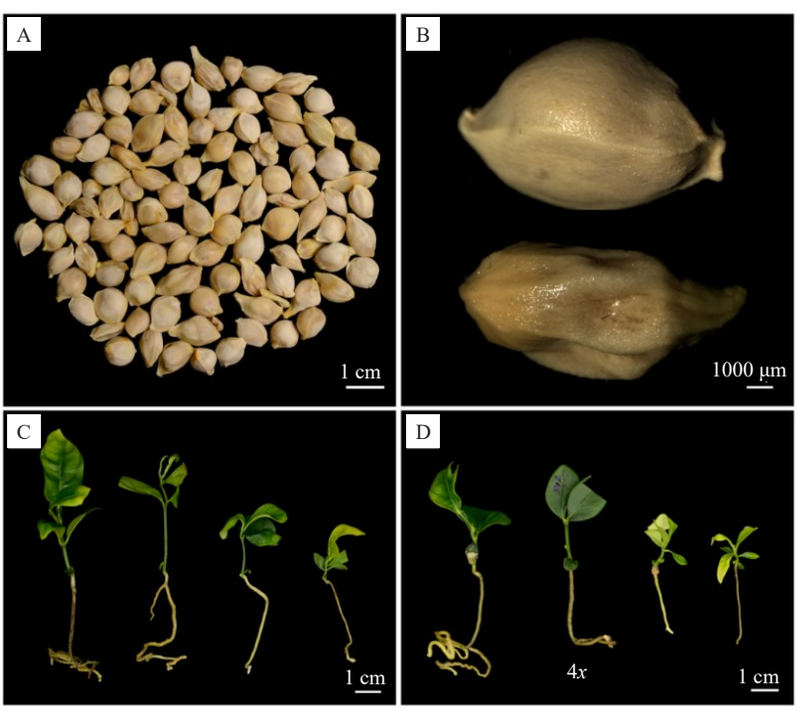
引物名称 Primer name	引物序列 Primer sequence	来源 References
TAA15	F:GAAAGGGTTACTTGACCAGGC;R:CTTCCCAGCTGCACAAGA	[11]
mCrCIR03G05	F:CCTTGGAGGAGCTTTAC;R:CCACACAGGCAGACA	[12]
Ma2_1480	F:CAATCACAGGAGCGACTTCA;R:CTCAATTCAGCAAACCGACA	[13]
Csin.0463	F:TTGTTCAAGTAAGGAAGAAACTCG;R:CACGCAAAGCAATGCTAAAG	[13]

USA)进行,扩增程序:95 ℃预变性5 min,95 ℃变性30 s,55 ℃退火30 s,72 ℃延伸10 s,35个循环,72 ℃延伸5 min,4 ℃保存。PCR产物由全自动毛细管电泳系统(QIAxcel Advanced,QIAGEN)电泳分离。

2 结果与分析

2.1 离体实生播种获得62株国庆1号温州蜜柑幼苗

温州蜜柑一般表现为雄性不育、果实无核,种子极少见;但若花期遇高温,育性能部分恢复,成熟果实可能偶尔有少量种子。基于该现象,笔者从国庆1号温州蜜柑约12 500个成熟果实中,剥取获得106粒种子(图1-A),其中80粒为饱籽,26粒为瘪籽(图1-B)。对所有种子离体播种培养,由于部分饱满种子污染和瘪籽未能萌发,最终有41粒饱满种子萌发,萌发率为38.7%;经培养获得再生实生幼苗62株,平均每粒种子再生植株1.5株(图1-C~D)。



A. 国庆 1 号温州蜜柑种子;B. 国庆 1 号温州蜜柑饱籽(上)及瘪籽(下);C. 国庆 1 号温州蜜柑 1 粒种子再生 4 株二倍体幼苗;D. 国庆 1 号温州蜜柑 1 粒种子长出 3 株二倍体和 1 株四倍体幼苗。

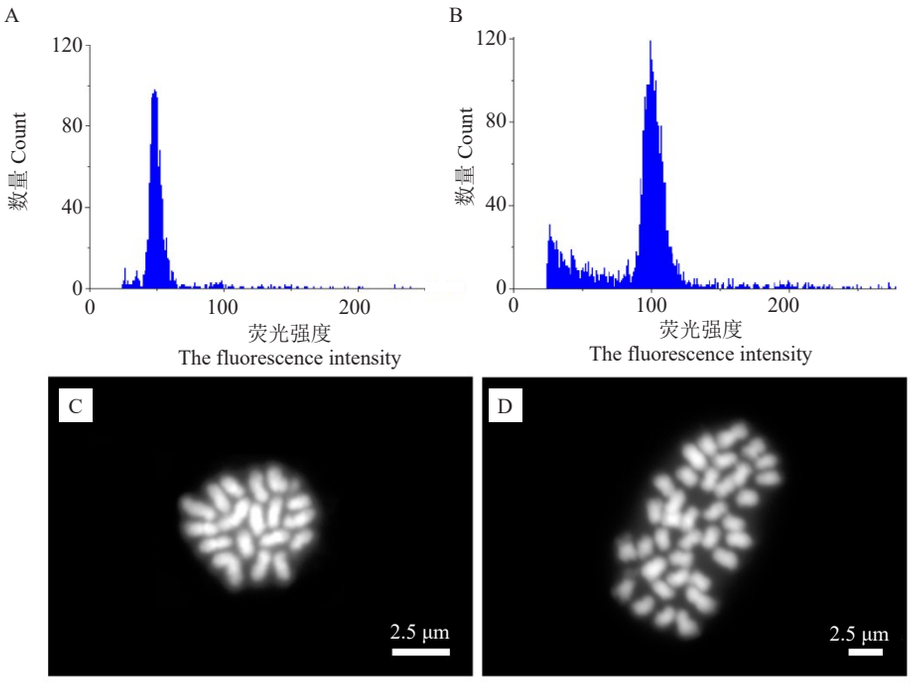
A. Seeds of Satsuma mandarin Guoqing No.1 (G1); B. A developed seed (upper) and an undeveloped seed (lower) of G1; C. Four diploid seedlings were regenerated from one G1 seed; D. Three diploid and one tetraploid seedling were regenerated from one G1 seed.

图 1 国庆 1 号温州蜜柑种子及实生幼苗形态

Fig. 1 Morphology of seeds and seedlings from Satsuma mandarin Guoqing No.1

2.2 倍性分析发掘1株国庆1号温州蜜柑四倍体植株

用流式细胞仪对所有62株实生幼苗进行倍性分析,其中61株为二倍体(图2-A),发掘获得1株四倍体植株(图2-B),四倍体发生频率1.61%。进一步用根尖压片法对获得的四倍体进行染色体数目分析,表明其染色体数为36条(图2-D),是二倍体(图



A~B. 流式细胞仪鉴定二倍体（A）和四倍体（B）子代植株倍性；C~D. 根尖染色体计数鉴定二倍体（C，2*n*=2*x*=18）和四倍体（D，2*n*=4*x*=36）。

A-B. Ploidy level histograms of the diploid (A) and tetraploid (B) seedlings determined by flow cytometry; C-D. Chromosome counting of diploid (C, 2*n* = 2*x* = 18) and tetraploid (D, 2*n* = 4*x* = 36) root tip.

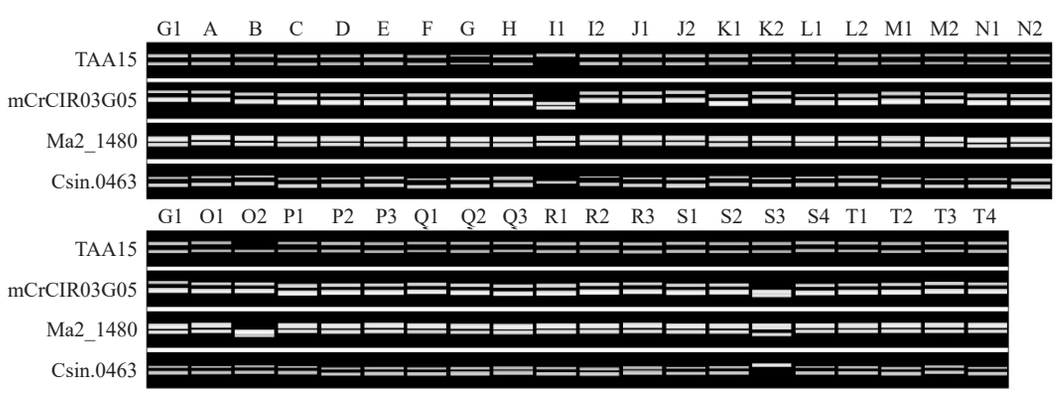
图 2　国庆 1 号温州蜜柑实生幼苗倍性分析

Fig. 2　Ploidy level analysis of the regenerated seedlings from Satsuma mandarin Guoqing No.1 using flow cytometry and shoot tip chromosome counting

2-C）染色体数目的2倍，验证了流式细胞仪倍性分析结果的可靠性。

2.3　SSR分子鉴定

将倍性分析后的所有实生幼苗移栽至温室。当幼苗长至一定大小后，选取4对扩增效果好的多态性SSR分子标记对随机选取的38株二倍体植株和1株四倍体植株进行遗传鉴定（图3）。结果表明，四倍体植株带型与其亲本国庆1号温州蜜柑完全一致（图3-T4），表明该四倍体可能由国庆1号温州蜜柑珠心细胞自然加倍形成，为双二倍体；而38株二倍



G1. 国庆 1 号温州蜜柑亲本；A~T. 来自不同种子的国庆 1 号温州蜜柑实生苗（不同字母表示不同种子，相同字母下的不同数字表示同一种子获得的不同实生苗）；T4. 四倍体植株。

G1. Satsuma mandarin Guoqing No.1; A-T. Seedlings of Satsuma mandarin Guoqing No.1 regenerated from different seeds (different letters indicate different seeds, different numbers under the same letter indicate different seedlings regenerating from same seed); T4. The tetraploid seedling.

图 3　国庆 1 号温州蜜柑实生幼苗 SSR 分子鉴定

Fig. 3　SSR profiles of the seedlings of Satsuma mandarin Guoqing No.1

体植株中，35株的条带与国庆1号温州蜜柑完全一致，推测由国庆1号温州蜜柑珠心胚发育而来；其余3株二倍体植株（I1、O2、S3）与国庆1号温州蜜柑条带不一致，为合子胚来源，可能为国庆1号温州蜜柑与其附近其他柑橘品种的天然有性后代。

3 讨 论

笔者在本研究中采用成熟种子离体播种，结合倍性分析和SSR分子鉴定，发掘出1株国庆1号温州蜜柑四倍体植株；随机挑选的38株二倍体实生幼苗，3株为国庆1号温州蜜柑有性胚苗，其余35株为珠心胚苗。这些种质材料将助力国庆1号温州蜜柑提纯复壮、珠心胚变异育种及相关基础研究。

温州蜜柑一般表现为雄性不育、果实无核；但若对其授粉或花期遇高温（育性会部分恢复），其果实偶尔会产生种子。如黄秀等[6]发现自然条件下，由良温州蜜柑果实基本无核，而用其他品种的花粉对其人工授粉，可获得少量种子（单果种子数介于0.36~1.66粒之间）；邓秀新等[14]对移入25 ℃温室的国庆1号温州蜜柑花粉育性进行统计，与对照相比表现出育性恢复现象，笔者在本研究中从约12 500个国庆1号温州蜜柑成熟果实获得106粒种子的结果与前人研究基本一致。推测国庆1号温州蜜柑种子可能由其与周围栽植的华农本地早橘等其他品种异花授粉形成或由于其花粉育性部分恢复后自交形成。笔者在本研究中离体播种了80粒饱籽和26粒瘪籽，由于操作不当，部分饱籽污染，仅41粒饱籽萌发；而瘪籽均未萌发，可能与瘪籽营养不足或低温受冻导致种子活力下降等有关。

笔者采用4对SSR引物对发掘的1株四倍体和38株二倍体实生幼苗进行遗传鉴定，发现四倍体带型与其二倍体亲本完全一致，与前人研究一致[15-17]，推测其由国庆1号温州蜜柑珠心细胞自然加倍形成。王江波等[18]用ISSR分子标记对由种子萌发来的7株芦柑实生苗进行遗传鉴定，其与母本条带完全一致均为珠心苗。笔者在本研究中随机挑选的38株二倍体植株中，35株二倍体植株条带与母本完全一致，为珠心胚苗；其余3株二倍体植株遗传背景与国庆1号温州蜜柑不一致，推测其为国庆1号温州蜜柑与周围其他柑橘品种异花授粉形成的有性胚苗。日本科学家最近报道宫川温州蜜柑四倍体与二倍体正反杂交时，均能产生饱满种子和三倍体后代，说明宫川温州蜜柑四倍体育性恢复，可以用作育种亲本[17]。笔者在本研究中发掘的国庆1号温州蜜柑四倍体，其开花结果习性有待观察评价，国庆1号温州蜜柑四倍体为细胞质雄性不育等相关基础研究和多倍体育种提供了珍贵的种质材料；培育的国庆1号温州蜜柑珠心胚苗将有助于该品种提纯复壮和二倍体水平珠心苗变异发掘。

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