

柑橘6个地方品种资源四倍体高效发掘及分子鉴定

谢善鹏¹,解凯东¹,夏强明¹,周锐¹,张成磊²,郑浩³,伍小萌¹,郭文武^{1*}

(¹华中农业大学园艺植物生物学教育部重点实验室,湖北武汉 430070; ²台州科技职业学院农业与生物工程学院,浙江台州 318020; ³常山县农业特色产业发展中心,浙江常山 324200)

摘要:【目的】基于柑橘多胚种子存在珠心细胞自然加倍的特点,发掘我国柑橘6个地方特色品种资源四倍体新种质。【方法】采摘柑橘成熟果实,剥取种子后催芽播种;待实生幼苗长至2~3片真叶大小时,采用“观根辨叶看油胞”形态初选法从实生幼苗群体筛选疑似多倍体;采用流式细胞仪鉴定疑似多倍体倍性并以SSR分子标记鉴定其遗传来源。【结果】基于幼苗形态初筛,分别从常山胡柚、温岭高橙、新会橙、橘血橙杂种、衢州香橙、酸橙890、72、373、709、303、1992株实生苗筛选获得疑似四倍体24、4、7、11、10和88株;流式细胞仪对上述疑似四倍体进行倍性检测,获得常山胡柚、温岭高橙、新会橙、橘血橙杂种、衢州香橙和酸橙四倍体14、2、4、3、6和24株;SSR分子鉴定表明,温岭高橙、新会橙、橘血橙杂种、衢州香橙和酸橙的39株四倍体植株全部由二倍体亲本珠心细胞自然加倍形成,而从胡柚发掘的14株四倍体,12株由珠心细胞自然加倍形成,其余2株为胡柚与其他柑橘的天然四倍体有性杂种。【结论】这些自然发掘的地方品种资源四倍体为我国柑橘品种改良及相关基础研究奠定了种质基础。

关键词:柑橘;多倍体;流式细胞仪;SSR分子标记

中图分类号:S666

文献标志码:A

文章编号:1009-9980(2022)01-0001-09

Efficient exploration and SSR identification of 53 doubled diploid seedlings from six local *Citrus* cultivars and germplasm resources

XIE Shanpeng¹, XIE Kaidong¹, XIA Qiangming¹, ZHOU Rui¹, ZHANG Chenglei², ZHENG Hao³, WU Xiaomeng¹, GUO Wenwu^{1*}

(¹Key Laboratory of Horticultural Plant Biology of Ministry of Education, Huazhong Agricultural University, Wuhan 430070, Hubei, China; ²College of Agriculture and Bioengineering, Taizhou Vocational College of Science & Technology, Taizhou 318020, Zhejiang, China; ³Agricultural Specialty Industry Development Center of Changshan County, Changshan 324200, Zhejiang, China)

Abstract:【Objective】To improve the quality of citrus seedling scion cultivars and the stress tolerance of citrus rootstocks, we explored and identified citrus doubled diploid seedlings from six local citrus cultivars and germplasm resources based on the presence of spontaneous doubling of nucellar cells in polyembryonic seeds. Citrus triploids are usually seedless and can be produced from the hybridization between diploid and tetraploid parents. However, the lack of citrus tetraploid germplasm resources has greatly limited the application of ploidy hybridization in citrus triploid improvement. And it has been extensively reported that tetraploids are more resistant and adaptable than diploids in higher plants. Therefore, it is urgent to explore tetraploid germplasm for the genetic improvement of local citrus cultivars.【Methods】Following the harvest of the citrus mature fruits, the seeds were extracted with seed coat stripped and placed in a thermostatic to accelerate germination. When the seeds germinated, they were sown in pots and cultured in a controlled growth chamber. After the seedlings grew with 2-3 leaves, putative tetraploids were visually screened based on the morphology of their root, leaf and oil

收稿日期:2021-07-15 接受日期:2021-09-12

基金项目:广东省科技计划(2018B020202009);国家重点研发计划(2019YFD1000100);国家自然科学基金(32172525);湖北省科技计划(2020BBA036,2020DFE017);中央高校基本科研业务费专项(2662019QD048)

作者简介:谢善鹏,男,在读硕士研究生,研究方向为果树生物技术与种质创制。Tel:027-87287393, E-mail:xieshanpeng@webmail.hzau.edu.cn

*通信作者 Author for correspondence. Tel:027-87281543, E-mail:guoww@mail.hzau.edu.cn

gland. The putative tetraploids usually have thicker and shorter taproots, fewer lateral root, smaller leaf index and decreased oil gland density. Their ploidy level was determined using flow cytometry and shoot tip chromosome counting. In addition, the morphological indicators of tetraploids and corresponding diploid seedlings were measured at the same developmental period (about 30-50 d after sowing), including root length, root diameter, number of lateral roots, stem diameter, leaf length, leaf width and leaf thickness. The genetic origin of tetraploids obtained in this study were further analyzed using 11 pairs of simple sequence repeat (SSR) primers. **【Results】** Changshan Huyou pummelo, Wenling Gaocheng, Xinhui sweet orange, a hybrid of blood orange with Clementine, Quzhou Xiangcheng and Sour orange respectively contains 1.4, 3.4, 8.0, 7.1, 5.6 and 5.8 embryos per seed, which indicated that these six genotypes were polyembryonic and different genotypes had variable number of embryos. Based on the morphological observations of root, leaf and oil gland of seedlings, we screened 24, 4, 7, 11, 10 and 88 putative tetraploids respectively from the 890, 72, 373, 709, 303 and 1992 seedlings of Changshan Huyou pummelo, Wenling Gaocheng, Xinhui sweet orange, the hybrid of blood orange with Clementine, Quzhou Xiangcheng and Sour orange. A peak at 50 of elative content of DNA was observed in the plants of the diploid, and a peak at 100 was observed in the plants of the tetraploid by flow cytometry. The number of diploid and tetraploid by shoot tip chromosome counting was 18 and 36 respectively. By analyzing the ploidy level of putative tetraploids, a total of 53 tetraploids were obtained, 14 from Changshan Huyou pummelo, 2 from Wenling Gaocheng, 4 from Xinhui sweet orange, 3 from a hybrid of blood orange with Clementine, 6 from Quzhou Xiangcheng and 24 from Sour orange, with the tetraploid pre-screening accuracy rate of 58.3%, 50.0%, 57.1%, 27.3%, 60.0% and 27.3% respectively. The natural occurrence rate of the tetraploid was 1.57%, 2.78%, 1.07%, 0.42%, 1.98% and 1.20% respectively in the above six genotypes. The morphological indicators of tetraploid and corresponding diploid seedlings were measured. The results showed that the taproot length and lateral root number of tetraploid seedlings from all genotypes were significantly lower than those of the corresponding diploid plants; root diameter and leaf thickness were significantly higher. For stem dimeter, the tetraploid seedlings of Wenling Gaocheng and Xinhui sweet orange were significantly smaller than that of diploid plants, the remaining four cultivars had no significant difference. For the leaf shape index, the tetraploid seedlings of Wenling Gaocheng were significantly larger than that of diploid plants, while the tetraploid seedlings of the hybrid of blood orange with Clementine were significantly smaller than that of diploid plants, the remaining four cultivars had no significant difference. These results provided theoretical support for improving the tetraploid pre-screening accuracy rate. Among the 11 polymorphic SSR markers, at least three SSR markers were successfully used for analyzing the genetic origins of tetraploid seedlings for each genotype. The results showed that the bands of 51 tetraploid seedlings were identical with that of their corresponding diploid genotypes, indicating that all of these 51 seedlings were originated from the chromosome doubling of nucellar cells of their corresponding diploids. And the remaining two tetraploids from Changshan Huyou pummelo showed some bands different from their corresponding diploid, indicating they might be originated from sexual hybridization with unknown pollen parent. **【Conclusion】** This study indicates that it is a simple and efficient approach to explore tetraploids from seedling populations of polyembryonic citrus genotypes based on preliminary morphological screening combined with flow cytometry and SSR analysis. These tetraploid germplasm resources explored from the study would be potentially valuable for scion and rootstock improvement and related fundamental researches in citrus crops.

Key words: *Citrus*; Polyploidy; Flow cytometry; Simple sequence repeat

果实无核是柑橘育种目标之一,而无核育种也是实现我国柑橘果品质提升的重要手段。我国柑橘资源丰富,地方品种繁多,但多数品种果实有核甚至多核,是导致近年来其市场竞争力降低和种植面积骤减的主要原因^[1]。三倍体一般雌雄配子不育,有性杂交难以形成种子,是天然的无核类型。实践证明,二倍体与四倍体倍性杂交创制三倍体是培育无核柑橘的最有效途径^[2]。但柑橘四倍体资源缺乏,极大限制了倍性杂交在柑橘三倍体育种中的应用。

除了柚、枸橼和少数宽皮橘外,柑橘多数品种具有珠心多胚现象^[3]。自然条件下,多胚种子珠心细胞存在一定概率的基因组加倍现象,种子萌发后可产生四倍体植株(也称为双二倍体),为发掘柑橘四倍体提供了一条便捷途径。Aleza等^[4]通过实生播种和流式细胞仪对实生苗倍性分析,从30余个珠心性柑橘品种的4442株实生幼苗筛选中选获得80株四倍体。但直接利用流式细胞仪对实生幼苗进行倍性检测筛选四倍体,工作量大,效率低。针对上述问题,梁武军等^[5]基于多倍体具备的独特形态学特征,提出了一种利用植株形态特征发掘柑橘四倍体的方法,即通过观察植株叶片形态特征先筛选疑似多倍体,再用流式细胞仪对其倍性快速鉴定,大大节约了直接对所有幼苗进行倍性分析的时间和劳动成本。在此基础上,周锐等^[6]通过优化播种过程和形态初选方法,将柑橘四倍体发掘平均准确率由35%提高至85%以上,过程缩短至40 d以内。利用该方法,目前已成功获得红橘^[5]、金柑^[6]、甜橙^[6-7]、枳橙^[8]等四倍体植株。

常山胡柚、温岭高橙、新会橙是我国地方特色品种,栽培历史悠久,风味独特,但种子较多;橘血橙杂种是克里曼丁橘与血橙的有性杂交后代优良单株,种子多胚,果实花青素含量极高;衢州香橙、酸橙是我国柑橘栽培的重要砧木类型。发掘上述品种资源的四倍体,可以为培育柑橘三倍体无核新品种提供优良的四倍体亲本,以及为矮化、适应性强的四倍体砧木培育提供珍贵的种质材料,具有重要的研究意义和实践价值。

1 材料和方法

1.1 实验材料

常山胡柚(*Citrus aurantium*)、温岭高橙(*C. grandis*×*C. sinensis*)、新会橙(*C. sinensis*)和衢州香

橙(*C. junos* Sieb. ex Tan.)成熟果实2020年秋季分别采自浙江省常山县、浙江省温岭市、广东省肇庆市德庆县柑橘研究所、陕西省陕南柑橘综合试验站;橘血橙杂种(*C. clementina*×*C. sinensis*)和酸橙(*C. aurantium*)成熟果实2020年秋季采自华中农业大学柑橘种质资源圃。

1.2 种子催芽播种和实生幼苗形态初选

种子催芽播种、实生幼苗形态学初选参考周锐等^[6]的方法。种子剥出并用清水洗净后,将其置于1 mol·L⁻¹ NaOH溶液浸泡15 min去除果胶。人工剥去外种皮后,将其均匀铺于垫有湿滤纸的培养皿,置于28 ℃恒温箱(暗培养)催芽。胚根萌发后,将其带至生长室播种于营养钵(约100粒/钵)。幼苗长至有2~3枚真叶时,利用“观根辨叶看油胞”法筛选疑似四倍体。生长室培养条件:温度(25±1)℃;光照16 h。

种子胚数统计:剥去种子内外种皮,在Olympus SZX7体视显微镜下统计胚数并拍照,每个品种至少统计10粒种子。

1.3 倍性鉴定

用流式细胞仪和根尖染色体计数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^[9]的方法。根尖染色体计数参考夏强明^[10]的方法并适当修改。取四倍体及其二倍体亲本生长旺盛的根尖,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%(φ)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 四倍体幼苗根、茎和叶等形态指标测定

以二倍体植株为对照,测量相同发育时期(约播种后30~50 d)四倍体实生幼苗形态指标,包括主根长、根粗、须根数目;茎粗;叶长、叶宽及叶片厚度。主根长用直尺测量;主根粗(根尖1 cm处)、茎粗(根颈1 cm处)、叶长、叶宽及叶片厚度用游标卡尺测量。每个品种的二倍体实生苗测量10株,对应四倍

体除温岭高橙、新会橙、橘血橙杂种测量所有株数外,其余品种均测量6株以上。

1.5 植株移栽

形态指标测定结束后,将四倍体植株及其二倍体分别独立移栽至装有营养土($V_{\text{进口土}}:V_{\text{基质土}}:V_{\text{蛭石}}:V_{\text{珍珠岩}}=4:4:1:1$)的营养钵,继续置于生长室培养。待幼苗长至7~8枚真叶时,将其转移至温室,期间保证正常肥水管理。

1.6 SSR分子鉴定

基因组DNA提取和SSR分子标记分别参考

表1 SSR引物序列

Table 1 Sequence of SSR primers

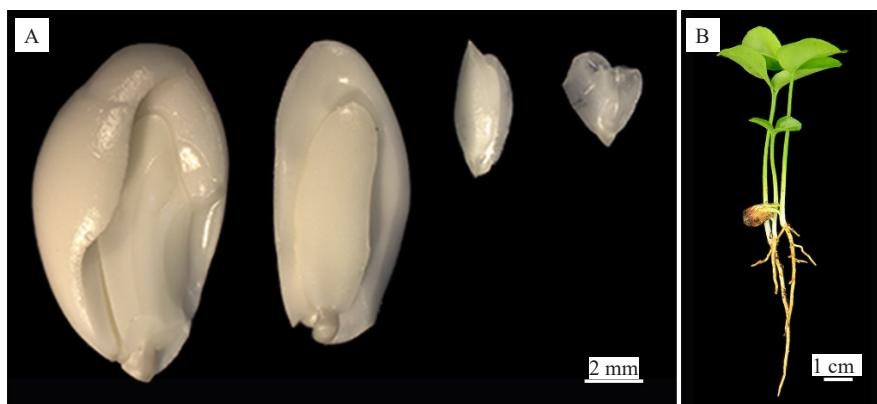
引物名称 Primer name	引物序列 Primer Sequence	文献 Reference
MEST256	F:CATTAATATCCGTGCCGC; R:GAGCAAGTGCCTGTTGTGT	[12]
MEST131	F:TACCTCACGTCAAACCA; R:GCTGTCACGTTGGGTGTATG	[12]
MEST86	F:CCTCTCTGGCTCTGGATTG; R:CCAAGTACACTAATCCTCTTCC	[12]
MEST88	F:GCCTGTTGCTTCTCTTCTC; R:ATGAGAGCCAAGAGCACGAT	[12]
MEST56	F:AGTCGCCCTTGCTTTCT; R:GGTGCAAAAGAGAGCGAGAG	[4]
mCrCI03D12a	F:GCCATAAGCCCTTCT; R:CCCACAACCATCACC	[4]
mCrCIR03G05	F:CCACACAGGCAGACA; R:CCTTGGAGGAGCTTAC	[13]
mCrCIR07D06	F:CCTTTACAGTTGCTAT; R:TCAATTCTCTAGTGTGT	[13]
mCrCIR01F08a	F:ATGAGCTAAAGAGAAGAGG; R:GGACTAACACAACACAA	[14]
mCrCI02D04b	F:AGCAAACCCCACAAC; R:CTCTTTCCCCATTAGA	[15]
Ma2_1480	F:CAATCACAGGAGCGACTTCA; R:CTCAATTAGCAAACCGACA	[16]

2 结果与分析

2.1 常山胡柚等6个品种的种子均为多胚性种子

从常山胡柚、温岭高橙、新会橙、橘血橙杂种、衢州香橙、酸橙的80、20、25、55、5、136个成熟果实中,

剥取获得736、62、328、452、164和1273粒种子,平均单果种子分别为9.2、3.1、13.1、8.2、32.8、9.4粒。为调查6个品种种子的胚性及其多胚程度,取每个品种10粒种子统计胚数。结果显示(图1),常山胡柚、温岭高橙、新会橙、橘血橙杂种、衢州香橙、酸橙平均



A. 酸橙多胚性种子;B. 酸橙一粒种子长出3株幼苗。

A. Polyembryonic seeds of Sour orange; B. Three seedlings generated from one seed of Sour orange.

图1 酸橙种子的多胚现象

Fig. 1 Polyembryony in Sour orange seeds

Cheng等^[11]和周锐等^[6]的方法。筛选的11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, USA)进行,扩增程序:95 °C预变性5 min,95 °C变性30 s,55 °C退火30 s,72 °C延伸10 s,35个循环,72 °C延伸5 min,4 °C保存。PCR产物由全自动毛细管电泳系统(QI Axcel Advanced, QIAGEN)电泳分离。

每粒种子包含1.4、3.4、8.0、7.1、5.6、5.8个胚,表明6个品种均为多胚类型且多胚性程度依次为:新会橙>橘血橙杂种>酸橙>衢州香橙>温岭高橙>常山胡柚。

2.2 形态学初选结合倍性分析发掘四倍体幼苗50余株

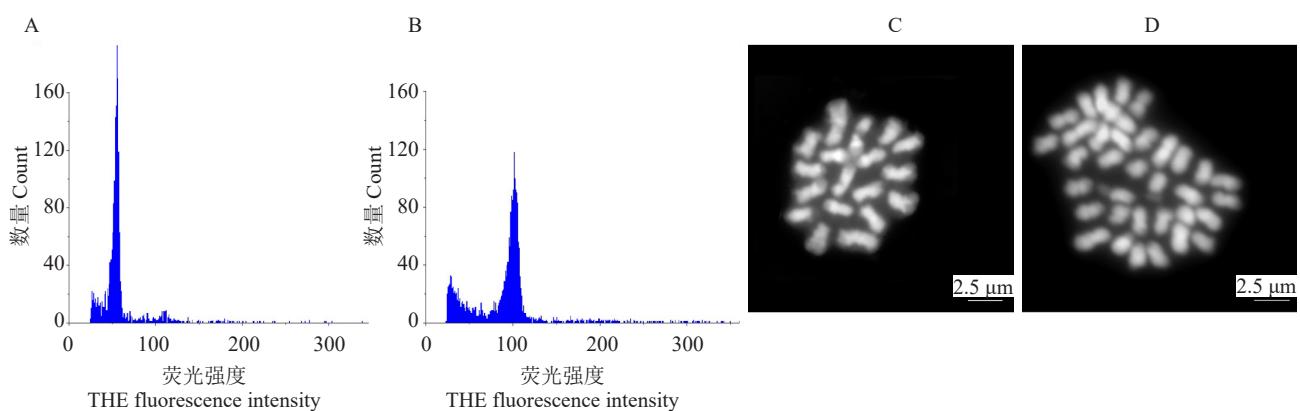
将常山胡柚、温岭高橙、新会橙、橘血橙杂种、衢州香橙和酸橙的种子通过催芽播种,分别获得890、72、373、709、303和1992株实生幼苗。依据多倍体形态特征(根粗、须根少;叶片厚、颜色深、叶形指数小;油胞密度低等),从常山胡柚、温岭高橙、新会橙、

橘血橙杂种、衢州香橙和酸橙分别筛选获得24、4、7、11、10和88株疑似多倍体(表2)。用流式细胞仪和根尖染色体计数对疑似多倍体进行倍性鉴定(图2),获得常山胡柚、温岭高橙、新会橙、橘血橙杂种、衢州香橙和酸橙四倍体14株、2株、4株、3株、6株和24株。常山胡柚、温岭高橙、新会橙、橘血橙杂种、衢州香橙和酸橙的形态初选准确率分别为58.3%、50.0%、57.1%、27.3%、60.0%和27.3%;四倍体群体发生频率分别为1.57%、2.78%、1.07%、0.42%、1.98%和1.20%(表2)。

表2 常山胡柚等6个地方品种的四倍体发掘情况

Table 2 The tetraploid seedlings identified from six citrus polyembryonic genotypes

品种 Cultivar	果实数 No. of fruits	种子数 No. of seeds	幼苗数 No. of seedlings	疑似多倍体数 No. of putative 4x plants	四倍体数 No. of confirmed 4x plants	准确率 Accuracy rate/%	群体发生率 Population incidence/%	耗时 Time duration/d
常山胡柚 Changshan Huyou pummelo	80	736	890	24	14	58.3	1.57	27
温岭高橙 Wenling Gaocheng	20	62	72	4	2	50.0	2.78	50
新会橙 Xinhu sweet orange	25	328	373	7	4	57.1	1.07	37
橘血橙杂种 Hybrid of blood sweet orange with Clementine	55	452	709	11	3	27.3	0.42	48
衢州香橙 Quzhou Xiangcheng	5	164	303	10	6	60.0	1.98	31
酸橙 Sour orange	136	1273	1992	88	24	27.3	1.20	36
总计/平均 Sum/Average	321	3015	4339	144	53	46.7	1.51	38



A-B. 流式细胞仪倍性鉴定(A. 二倍体;B. 四倍体);C-D. 根尖染色体计数(C. 二倍体, $2n = 2x = 18$;D. 四倍体, $2n = 4x = 36$)。

A-B. Ploidy histograms of the diploid and tetraploid plant determined by flow cytometry (A. Diploid; B. Tetraploid); C-D. Shoot tip chromosome counting (C. Diploid, $2n = 2x = 18$; D. Tetraploid, $2n = 4x = 36$).

图2 流式细胞仪和根尖染色体压片鉴定酸橙疑似四倍体植株倍性

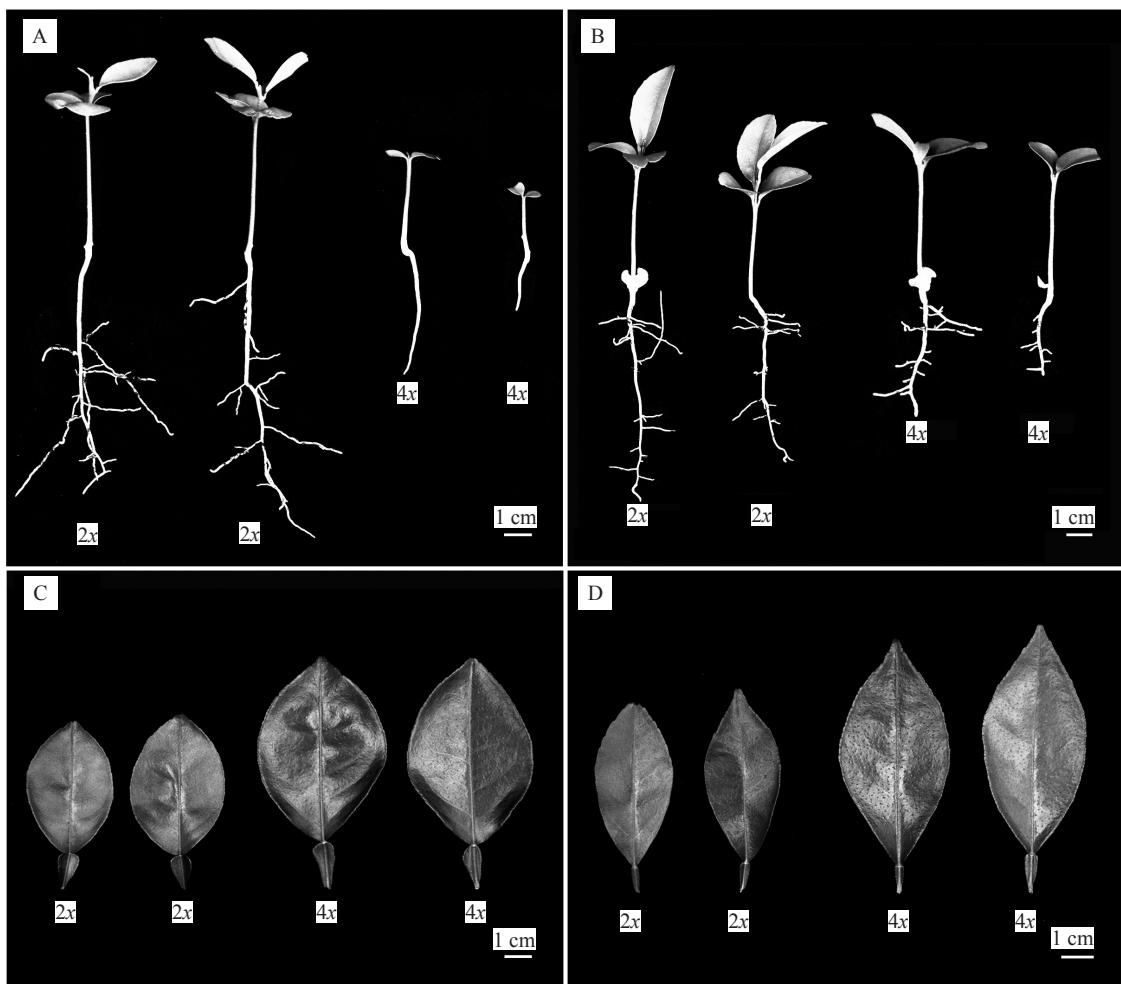
Fig. 2 Ploidy analysis of putative tetraploids screened from Sour orange using flow cytometry and shoot tip chromosome counting technique

2.3 四倍体与二倍体幼苗根、茎和叶片形态差异明显

对获得的四倍体及相应二倍体实生幼苗的根、茎和叶片形态指标进行测量,结果表明,四倍体植株的主根长、侧根数均显著低于相应的二倍体(图3);四倍体根粗、叶片厚度显著高于二倍体;对于茎粗,温岭高橙和新会橙的四倍体植株显著低于二倍体,其余4个品种的四倍体与二倍体之间差异不显著;对于叶形指数,温岭高橙的四倍体显著大于二倍体,橘血橙杂种的四倍体显著小于二倍体,其余4个品种的四倍体与二倍体之间差异不显著(表3)。

2.4 SSR分子鉴定

从筛选获得的11个多态性SSR标记中,每个品种至少筛选3个扩增效果好的SSR标记对发掘的四倍体植株进行分子鉴定(图4)。结果显示,温岭高橙、新会橙、橘血橙杂种、衢州香橙、酸橙所有四倍体的带型与其二倍体亲本完全一致,表现出高度一致的遗传背景,表明这些四倍体可能来自二倍体亲本珠心细胞自然加倍形成的双二倍体。而常山胡柚的14株四倍体中,12株四倍体的条带与其二倍体亲本完全一致,其余2株四倍体均扩增出了其二倍体亲本没有的条带,表明胡柚的14株四倍体有2个来源,推测其分别来自珠心细胞和合子细胞(胡柚与附近其他未知柑橘类型天然杂交)加倍形成的双二倍体。



A. 温岭高橙幼苗形态;B. 橘血橙杂种幼苗形态;C. 温岭高橙叶片形态;D. 橘血橙杂种叶片形态。

A. Morphological difference between the diploid and tetraploid seedlings of Wenling Gaocheng; B. Hybrid of blood sweet orange with Clementine; C. Leaf morphological difference between the diploid and tetraploid seedlings of Wenling Gaocheng; D. Hybrid of blood sweet orange with Clementine.

图3 温岭高橙和橘血橙杂种四倍体及其相应二倍体实生幼苗形态差异

Fig. 3 Morphological difference between tetraploids and the corresponding diploids seedling of Wenling Gaocheng and Hybrid of blood sweet orange with Clementine

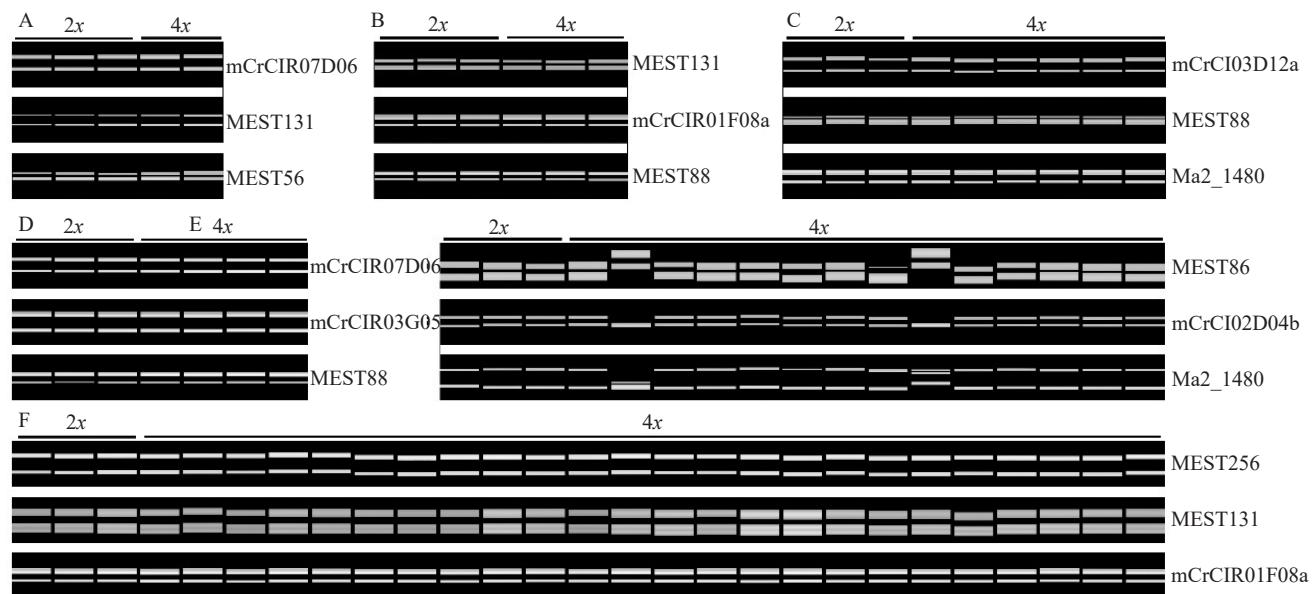
表3 四倍体与二倍体的根、茎和叶片形态特征比较

Table 3 Root, stem and leaf morphological comparison between tetraploid and their corresponding diploid seedlings

品种 Cultivar	倍性 Ploidy	主根长 Root length/ mm	侧根数 No. of lateral roots	根粗 Root diameter/ mm	茎粗 Stem diameter/ mm	叶片厚度 Leaf thickness/ mm	叶形指数 Leaf shape index
常山胡柚 Changshan Huyou pummelo	4x	49.12±22.83	2.30±1.64	0.80±0.33**	1.49±0.12	0.21±0.02**	0.97±0.18
	2x	98.71±8.85**	8.71±2.14**	0.23±0.04	1.50±0.11	0.15±0.01	1.04±0.14
温岭高橙 Wenling Gaocheng	4x	39.46±18.05	0.33±0.58	0.80±0.09*	1.30±0.30	0.18±0.01**	1.03±0.14*
	2x	88.87±12.05**	8.55±2.38**	0.64±0.15	1.52±0.20*	0.14±0.02	0.85±0.09
新会橙 Xinhui sweet orange	4x	24.39±7.50	1±1.41	0.88±0.15**	1.1±0.20	0.18±0.02*	1.56±0.36
	2x	78.21±5.58**	6.67±2.07**	0.50±0.12	1.40±0.10*	0.15±0.02	1.83±0.27
橘血橙杂种 Hybrid of blood sweet orange with Clementine	4x	28.71±15.14	7.00±6.56	0.99±0.03**	1.40±0.27	0.23±0.03*	1.19±0.13
	2x	73.60±22.76*	15.00±3.58*	0.51±0.07	1.35±0.16	0.19±0.01	1.93±0.11**
衢州香橙 Quzhou Xiangcheng	4x	44.42±8.64	2.00±1.87	1.04±0.09**	1.65±0.32	0.21±0.04*	1.37±0.08
	2x	102.64±6.22**	14.60±2.37**	0.65±0.16	1.90±0.18	0.18±0.02	1.41±0.10
酸橙 Sour orange	4x	44.91±17.12	3.31±1.38	1.17±0.27**	1.68±0.21	0.22±0.03**	0.98±0.12
	2x	89.75±12.76**	6.56±1.81**	0.55±0.08	1.53±0.09	0.17±0.01	1.12±0.19

注:表中数据(平均值±标准差)后面*表示二倍体和四倍体之间的显著差异(* $p < 0.05$; ** $p < 0.01$)。

Note: The values (mean±standard deviation) with * indicate significant difference between diploid and tetraploid (* $p < 0.05$; ** $p < 0.01$).



A. 温岭高橙;B. 橘血橙杂种;C. 衢州香橙;D. 新会橙;E. 常山胡柚;F. 酸橙。

A. Wenling Gaocheng; B. Hybrid of blood sweet orange with Clementine; C. Quzhou Xiangcheng; D. Xinhui sweet orange; E. Changshan Huyou pummelo; F. Sour orange.

图4 柑橘四倍体植株的SSR分子鉴定

Fig. 4 SSR profiles of the tetraploids with their corresponding diploid parents

3 讨论

笔者在本研究中以柑橘6个多胚品种常山胡柚、温岭高橙、新会橙、橘血橙杂种、衢州香橙、酸橙为材料,基于幼苗形态初选结合倍性快速鉴定,从实

生后代群体发掘出53株四倍体后代。虽然衢州香橙四倍体发掘前人已有报道^[17],即从400株实生群体发掘出3株四倍体;但本文报道的四倍体发掘效率更高,即从164粒种子发出的303株小苗中发掘出四倍体6株。衢州香橙、酸橙四倍体可用于培育柑

橘矮化、多抗的新型砧木；常山胡柚、温岭高橙、新会橙和橘血橙杂种的四倍体可作为亲本与二倍体倍性杂交创制三倍体无核新种质。

笔者用11个SSR标记对53株四倍体进行遗传鉴定，51株(96.2%)四倍体的带型与其二倍体亲本完全一致，表明多胚性柑橘种子中形成的四倍体更多偏向来自珠心细胞染色体自然加倍，这与前人报道的基本一致^[4,7]。但不同的是，常山胡柚中，还产生了2株遗传背景与二倍体亲本不一致的四倍体后代，且这2株四倍体均扩增出二倍体亲本没有的条带，表明其可能来自有性杂交。据报道，柑橘部分品种容易产生2n卵细胞^[4,18-19]，2n卵细胞与二倍体花粉受精可产生四倍体后代^[20]，但本文中所用胡柚母本树附近没有种植四倍体柑橘类型，可以排除其由2n卵细胞和四倍体植株产生的二倍体花粉受精形成的可能性。除2n卵细胞外，柑橘也可以形成2n花粉^[21]，2n卵细胞和2n花粉受精可以形成四倍体，但柑橘2n花粉发生频率一般较低^[4,22-23]；且与n花粉相比，2n花粉在柱头上萌发所需时间较长，同等条件下，与卵细胞受精的竞争力没有n花粉强^[24]。因此，基本上也可以排除上述2株四倍体由未减数2n卵细胞与外源2n花粉受精形成。种子珠心细胞染色体自然条件下能够加倍，而合子细胞与珠心细胞所处环境基本一致，推测合子细胞的染色体也能像珠心细胞一样，可以自发加倍形成双二倍体。前人未报道合子细胞染色体自然加倍的情况，可能原因是所选材料多胚程度较高，合子胚在种子发育后期已败育，即使发生了染色体加倍，也无法获得合子细胞加倍形成的四倍体。本文中的温岭高橙、新会橙、橘血橙杂种、衢州香橙和酸橙，种子多胚程度较高(分别为3.4、8.0、7.1、5.6、5.8个胚/种子)，可能是其实生后代没有获得由合子细胞加倍形成四倍体的重要原因。而常山胡柚由于多胚程度较低(1.4个胚/种子)，珠心胚干扰程度轻，使得部分合子细胞在加倍后得以存活。基于以上分析，推测发掘的与亲本遗传背景有差异的2株胡柚四倍体可能是胡柚与其附近的未知柑橘类型的有性杂交后合子细胞经染色体自然加倍而形成的。

致谢：广东省肇庆市德庆县柑橘研究所胡益波提供了新会橙果实，陕西省陕南柑橘综合试验站丁德宽提供了衢州香橙果实。

参考文献 References:

- [1] XIE K D, YUAN D Y, WANG W, XIA Q M, WU X M, CHEN C W, CHEN C L, GROSSER J W, GUO W W. Citrus triploid recovery based on $2x \times 4x$ crosses via an optimized embryo rescue approach[J]. *Scientia Horticulturae*, 2019, 252: 104-109.
- [2] OLLITRAULT P, DAMBIER D, LURO F, FROELICHER Y. Ploidy manipulation for breeding seedless triploid citrus[J]. *Plant Breeding Reviews*, 2008, 30: 323-352.
- [3] 张斯淇,徐强,邓秀新.无融合生殖与柑橘多胚现象的研究进展[J].植物科学学报,2014,32(1):88-96.
ZHANG Siqi, XU Qiang, DENG Xiuxin. Advances in apomixis and polyembryony research in *Citrus* plants[J]. *Plant Science Journal*, 2014, 32(1): 88-96.
- [4] ALEZA P, FROELICHER Y, SCHWARZ S, AGUSTÍ M, HERNÁNDEZ M, JUÁREZ J, LURO F, MORILLON R, NAVARRO L, OLLITRAULT P. Tetraploidization events by chromosome doubling of nucellar cells are frequent in apomictic citrus and are dependent on genotype and environment[J]. *Annals of Botany*, 2011, 108(1): 37-50.
- [5] 梁武军,解凯东,郭大勇,谢宗周,伊华林,郭文武.10个柑橘砧木类型同源四倍体的发掘与SSR鉴定[J].果树学报,2014,31(1):1-6.
LIANG Wujun, XIE Kaidong, GUO Dayong, XIE Zongzhou, YI Hualin, GUO Wenwu. Spontaneous generation and SSR molecular characterization of autotetraploids in ten citrus rootstocks[J]. *Journal of Fruit Science*, 2014, 31(1): 1-6.
- [6] 周锐,解凯东,王伟,彭珺,谢善鹏,胡益波,伍小萌,郭文武.依据多倍体形态特征快速高效发掘柑橘四倍体[J].园艺学报,2020,47(12):2451-2458.
ZHOU Rui, XIE Kaidong, WANG Wei, PENG Jun, XIE Shangpeng, HU Yibo, WU Xiaomeng, GUO Wenwu. Efficient identification of tetraploid plants from seedling populations of apomictic citrus genotypes based on morphological characteristics[J]. *Acta Horticulturae Sinica*, 2020, 47(12): 2451-2458.
- [7] 梁武军,解凯东,郭大勇,谢宗周,徐强,伊华林,郭文武.柑橘10个品种实生后代多倍体的发掘及SSR鉴定[J].园艺学报,2014,41(3):409-416.
LIANG Wujun, XIE Kaidong, GUO Dayong, XIE Zongzhou, XU Qiang, YI Hualin, GUO Wenwu. Spontaneous generation and SSR characterization of polyploids from ten citrus cultivars[J]. *Acta Horticulturae Sinica*, 2014, 41(3): 409-416.
- [8] 彭滢,李晓妍,肖璇.柑橘多胚性砧木枳橙同源四倍体的发掘与SSR鉴定[J].分子植物育种,2020,18(4):1211-1215.
PENG Ying, LI Xiaoyan, XIAO Xuan. Excavation and SSR identification of autotetraploids in citrus polyembryonic rootstock citrange[J]. *Molecular Plant Breeding*, 2020, 18(4): 1211-1215.
- [9] 解凯东,彭珺,袁东亚,强瑞瑞,谢善鹏,周锐,夏强明,伍小萌,

- 柯甫志,刘高平,GROSSER J W,郭文武.以本地早橘和慢橘为母本倍性杂交制柑橘三倍体[J].中国农业科学,2020,53(23):4961-4968.
- XIE Kaidong, PENG Jun, YUAN Dongya, QIANG Ruirui, XIE Shanpeng, ZHOU Rui, XIA Qiangming, WU Xiaomeng, KE Fuzhi, LIU Gaoping, GROSSER J W, GUO Wenwu. Production of citrus triploids based on interploidy crossing with Bendizao and Man tangerines as female parents[J]. *Scientia Agricultura Sinica*, 2020, 53(23):4961-4968.
- [10] 夏强明.基于 $2n$ 雌配子有性群体定位柑橘着丝粒及其序列特征分析[D].武汉:华中农业大学,2020.
- XIA Qiangming. Localization and sequence analysis of citrus centromeres based on a sexual population derived from $2n$ megagametophytes [D]. Wuhan: Huazhong Agricultural University, 2020.
- [11] CHENG Y J, GUO W W, YI H L, PANG X M, DENG X X. An efficient protocol for genomic DNA extraction from *Citrus* species[J]. *Plant Molecular Biology Reporter*, 2003, 21 (2) : 177-178.
- [12] GARCÍA-LOR A, LURO F, NAVARRO L, OLLITRAULT P. Comparative use of InDel and SSR markers in deciphering the interspecific structure of cultivated citrus genetic diversity:a perspective for genetic association studies[J]. *Molecular Genetics and Genomics*, 2012, 287(1):77-94.
- [13] CUENCA J, FROELICHER Y, ALEZA P, JUÁREZ J, NAVARRO L, OLLITRAULT P. Multilocus half-tetrad analysis and centromere mapping in citrus: evidence of SDR mechanism for $2n$ megagametophyte production and partial chiasma interference in mandarin cv. 'Fortune'[J]. *Heredity*, 2011, 107(5):462-470.
- [14] SNOUSSI H, DUVAL M F, GARCIA-LOR A, BELFALAH Z, FROELICHER Y, RISTERUCCI A M, PERRIER X, JACQUEMOUD- COLLET J P, NAVARRO L, HARRABI M, OLLITRAULT P. Assessment of the genetic diversity of the Tunisian citrus rootstock germplasm[J]. *BMC Genetics*, 2012, 13:16.
- [15] KAMIRI M, STIFT M, SRAIRI I, COSTANTINO G, EL MOUSSADIK A, HMYENE A, BAKRY F, OLLITRAULT P, FROELICHER Y. Evidence for non-disomic inheritance in a *Citrus* interspecific tetraploid somatic hybrid between *C. reticulata* and *C. limon* using SSR markers and cytogenetic analysis[J]. *Plant Cell Reports*, 2011, 30(8):1415-1425.
- [16] XU Q, CHEN L L, RUAN X A, CHEN D J, ZHU A D, CHEN C L, BERTRAND D, JIAO W B, HAO B H, LYON M P, CHEN J J, GAO S, XING F, LAN H, CHANG J W, GE X H, LEI Y, HU Q, MIAO Y, WANG L, XIAO S X, BISWAS M K, ZENG W F, GUO F, CAO H B, YANG X M, XU X W, CHENG Y J, XU J, LIU J H, LUO O J, TANG Z H, GUO W W, KUANG H H, ZHANG H Y, ROOSE M L, NAGARAJAN N, DENG X X,
- RUAN Y J. The draft genome of sweet orange (*Citrus sinensis*) [J]. *Nature Genetics*, 2013, 45(1):59-66.
- [17] 蒋景龙,阳妮,李丽,秦公伟,邓家锐,赵桦,任可心,丁德宽.衢州香橙四倍体种质发掘及形态特征性评价[J].果树学报,2021,38(5):655-663.
- JIANG Jinglong, YANG Ni, LI Li, QIN Gongwei, DENG Jiarui, ZHAO Hua, REN Kexin, DING Dekuan. Identification and characterization of tetraploids from seedlings of *Citrus junos* 'Quzhou xiangcheng'[J]. *Journal of Fruit Science*, 2021, 38(5):655-663.
- [18] ESEN A, SOOST R K. Unexpected triploids in *Citrus*: their origin, identification, and possible use[J]. *Journal of Heredity*, 1971, 62:329-333.
- [19] ALEZA P, CUENCA J, HERNÁNDEZ M, JUÁREZ J, NAVARRO L, OLLITRAULT P. Genetic mapping of centromeres of the nine *Citrus clementina* chromosomes using half-tetrad analysis and recombination patterns in unreduced and haploid gametes[J]. *BMC Plant Biology*, 2015, 15:80.
- [20] 解凯东,王惠芹,王晓培,梁武军,谢宗周,伊华林,邓秀新, GROSSER J W,郭文武.单胚性二倍体为母本与异源四倍体杂交大规模制柑橘三倍体[J].中国农业科学,2013,46(21):4550-4557.
- XIE Kaidong, WANG Huiqin, WANG Xiaopei, LIANG Wujun, XIE Zongzhou, YI Hualin, DENG Xiuxin, GROSSER J W, GUO Wenwu. Extensive citrus triploid breeding by crossing monoembryonic diploid females with allotetraploid male parents[J]. *Scientia Agricultura Sinica*, 2013, 46(21):4550-4557.
- [21] ROUISS H, CUENCA J, NAVARRO L, OLLITRAULT P, ALEZA P. Tetraploid citrus progenies arising from FDR and SDR unreduced pollen in $4x \times 2x$ hybridizations[J]. *Tree Genetics & Genomes*, 2017, 13(1):10.
- [22] LURO F, MADDY F, JACQUEMOND C, FROELICHER Y, MORILLON R, RIST D, OLLITRAULT P. Identification and evaluation of diplosgamy in clementine (*Citrus clementina*) for use in breeding[J]. *Acta Horticulturae*, 2004, 663(663):841-848.
- [23] 向素琼,龚桂枝,郭启高,汪卫星,李春艳,李晓林,梁国鲁.柑橘属 $2n$ 花粉自然发生与沙田柚 $2n$ 花粉诱导研究[J].西南农业大学学报(自然科学版),2005,27(5):51-55.
- XIANG Suqiong, GONG Guizhi, GUO Qigao, WANG Weixing, LI Chunyan, LI Xiaolin, LIANG Guolu. Spontaneous generation of $2n$ pollen in citrus and induction of $2n$ pollen in *Citrus grandis*[J]. *Journal of Southwest Agricultural University (Natural Science)*, 2005, 27(5):51-55.
- [24] ZHAO C G, TIAN M D, LI Y J, ZHANG P D. Slow-growing pollen-tube of colchicine-induced $2n$ pollen responsible for low triploid production rate in *Populus*[J]. *Euphytica*, 2017, 213(4): 94.