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以东试早柚为母本创制柑橘三倍体种质资源

刘承浪[1],冯 迪[1],曹宗洪[1],陶亚文[1],徐祥增[2],高世德[2],
岳建强[3],谢宗周[1],叶俊丽[1],柴利军[1],郭文武[1],邓秀新[1*]

([1]果蔬园艺作物种质创新与利用全国重点实验室•华中农业大学园艺林学学院,武汉 430070;
[2]云南省热带作物科学研究所,云南景洪 666100;[3]云南省农业科学院热带亚热带经济作物研究所,云南保山 678000)

摘 要:【目的】三倍体植物由于减数分离紊乱,难以形成可育的雌雄配子,属于天然的不育类型,配置以2*x*×4*x*多个杂交组合,旨在创制柑橘三倍体种质,丰富柑橘无核材料。**【方法】**以单胚性二倍体品种东试早柚为母本与四倍体柑橘材料ZP(纸皮,四倍体甜橙)、PT(四倍体葡萄柚)、NH(四倍体,诺瓦橘柚+HB柚体细胞杂种)为父本进行倍性杂交,授粉后85 d和100 d采摘幼果并对未成熟种子实施幼胚离体挽救培养,获得再生植株后用流式细胞仪和InDel标记对其倍性及遗传来源进行鉴定。**【结果】**从3个倍性杂交组合的168株再生植株中,通过倍性检测获得三倍体幼苗128株且均为双亲杂交后代,其中东试早柚×ZP共计60株、东试早柚×PT共计60株、东试早柚×NH共计8株。**【结论】**通过倍性杂交高效创制三倍体柑橘新种质,为柑橘早熟无核育种及相关基础研究提供了珍贵的育种材料。

关键词:柑橘;东试早柚;胚挽救;倍性育种;InDel

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Creation of triploid seedling plants of *Citrus* by crossing Dongshizao pummelo female with tetraploid male parents

LIU Chenglang[1], FENG Di[1], CAO Zonghong[1], TAO Yawen[1], XU Xiangzeng[2], GAO Shide[2], YUE Jianqiang[3], XIE Zongzhou[1], YE Junli[1], CHAI Lijun[1], GUO Wenwu[1], DENG Xiuxin[1*]

([1]National Key Laboratory for Germplasm Innovation & Utilization of Horticultural Crops/College of Horticulture and Forestry Sciences, Huazhong Agricultural University, Wuhan 430070, Hubei, China; [2]Yunnan Institute of Tropical Crops, Jinghong 666100, Yunnan, China; [3]Institute of Tropcal and Subtropical Cash Crops, Yunnan Academy of Agricultural Sciences, Baoshan 678000, Yunnan, China)

Abstract:【Objective】Citrus is mainly cultivated for fresh consumption in China, so seedless citrus has become the dominant position in market consumption and in breeding programs. In order to enhance the competitiveness of the citrus industry and meet the market demands, it has been the goal of breeders to cultivate seedless varieties of citrus. Triploids are naturally seedless material whose chromosomes are disrupted during meiosis, making it difficult to form normal fertile gametes, resulting in seedless fruits. Triploid citrus typically has larger fruits and possesses stronger resistance and adaptation to the environmental conditions due to chromosome doubling. Ploidy crosses is the most effective strategy to obtain seedless citrus varieties, using monoembryonic diploid and tetraploid ploidy as parents. Therefore, we performed several 2*x*×4*x* crosses to create triploid citrus.【Methods】In this study, we selected the Dongshizao pummelo [*C. grandis* (L.) Osbeck Dongshizao pummelo] as the female parent, which is a local specialty cultivar from Yunnan province, characterized by early-maturing, seedlessness, high sugar and low acidity. Then, we used a late-maturing and productive autotetraploid ZP [*C. sinensis* (L.) Osbeck Paperrind orange], PT [*C. paradisi* (L.) Osbeck grapefruit] and an allotetraploid somatic hybrids NH [(*C. reticulata* Blanco×*C. paradisi* Macf.) + *C. grandis* (L.) Osbeck Hirado Buntan pummelo], as

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作者简介:刘承浪,男,在读硕士研究生,研究方向为柑橘种质资源收集与利用。Tel:15687111894,E-mail:448827890@qq.com

*通信作者 Author for correspondence. Tel:027-87281712,E-mail:xxdeng@mail.hzau.edu.cn

the male parent for the artificial crosses. In the earlier step, the pollinated styles were stained with aniline blue to observe the cross-compatibility between the parents, and in the subsequent steps, the immature seeds obtained from young fruits at 85 and 100 days after pollination (DAP) were cultured *in vitro*. When seeds grew into seedlings, the ploidy levels were analyzed by flow cytometry. In addition, we also resequenced the maternal Dongshizao pummelo, then the data was mapped to the HWB [*Citrus grandis* (L.) Osbeck Wanbai pummelo] reference genome to obtain InDel (insertion/deletion) variant loci, which contained 50−200 bp differences. According to these loci, primers were designed upstream and downstream of them, and PCR amplification was performed using the DNA of Dongshizao pummelo, ZP, PT and NH as templates. The amplified products were detected using 2.5% agarose gel, and clear and stable InDel-specific band patterns were selected for the identification of the genetic origin of the polyploid regenerated plants.【Results】The aniline blue staining results of the pollinated styles showed that a large number of pollen tubes could grow down to the bottom of the styles, and showed cross-comptibility in crosses of Dongshizao pummelo × ZP, Dongshizao pummelo × PT and Dongshizao pummelo × NH. Due to the 1∶2 ($2x\times4x$) ratio of maternal and paternal genomes in the endosperm of the progeny, the seeds would be completely sterile in mature fruit. Therefore, the juvenile embryos were cultured *in vitro* before the seeds were sterilized. At 85 DAP, 771 immature seeds obtained from the young fruits of Dongshizao pummelo × NH cross were cultured *in vitro*. After shooting and rooting induction, 15 plants were regenerated. Similarily, when 570 and 482 immature seeds of Dongshizao pummelo × PT and Dongshizao pummelo × ZP crosses were rescued at 100 DAP, we obtained 96 and 117 regenerated plants respectively. The regeneration rates of 3 crosses were different, of which the two autotetraploids about 20% were 10 times higher than the allotetraploid (1.9%). We also checked the ploidy levels of the hybrid progenies, 168 out of 228 progeny obtained from the crosses between Dongshizao pummelo and three tetraploid citrus cultivars. In the Dongshizao pummelo × ZP cross, 94 plants were regenerated, in which 60 plants proved to be triploids by flow cytometry analysis, accounting for 63.8%. In the Dongshizao pummelo × PT cross, 1 tetraploid and 60 triploids were detected in 63 regenerated plants, accounting for 95.2% and 1.67% respectively. Similarily, eight plants were detected as triploids in 11 seedlings from the Dongshizao pummelo × NH cross, accounting for 72.7%. In this study, we also developed a group of specific InDel markers for the genetic identification of the polyploid seedlings. Using HWB genome as the reference genome, we developed 10 pairs of InDel primers for hybrid progeny screening using the resequence deta of Dongshizao pummelo, grapefruit and sweet orange, which were called after InDel01−InDel10 respectively. InDel05 and InDel10 could distinguish Dongshizao pummelo from ZP and PT but not NH; InDel07 and InDel08 could distinguish all the parental species, but some of their band patterns were not obvious. Accordingly, the InDel05 primers were selected for the identification of Dongshizao pummelo × PT and Dongshizao pummelo × ZP hybrid progeny, and the InDel07 primers were selected for the identification of Dongshizao pummelo × NH hybrid progeny. The results showed that all the 129 polyploids derived form the crosses were hybrids of both parents.【Conclusion】Using the local specialty cultivar Dongshizao pummelo of Yunnan as the female parent and two autotetraploids (ZP, PT) and one allotetraploid somatic hybrids (NH) as the male parents, three crosses of 128 citrus triploids and one tetraploid were created in a relatively short time after cross-pollination. Our work would lay a foundation for the selection and breeding of new seedless varieties and related molecular research in citrus.

Key words: Citrus; Dongshizao pummelo; Embryo rescue; Ploidy breeding; InDel

中国柑橘以鲜食为主，无籽柑橘成为市场消费的主流和育种方向[1]。为提升产业竞争力，满足市场需求，培育柑橘无核品种一直是育种者的目标。三倍体为天然的无核材料，其在减数分裂时期，染色体发生紊乱，难以形成正常可育的配子，导致果实一般为无核[2]。由于染色体加倍、倍性增加，三倍体果实较大、抗逆性增强，对环境也具有更强的适应性。通过倍性杂交以培育三倍体是获得柑橘无核品种最为有效的途径之一，其中又以单胚性二倍体品种为母本与四倍体杂交方式最为普遍[3]。Soost等[4]以单胚无酸柚与四倍体马叙葡萄柚杂交获得2个已广泛推广的无核品种Oroblanco和Melgold。Aleza等[5]配置以二倍体Fortune宽皮橘与四倍体Orlando柑橘品种等为亲本的77个杂交组合，获得4400多株三倍体再生植株，并从中筛选出一个综合性状优良的无核品种IVIA-600。近年来，华中农业大学柑橘团队也创制诸多以单胚性二倍体柑橘品种与四倍体体细胞杂种为亲本的杂交组合，获得一大批柑橘三倍体无核新种质[6]。

胚挽救在柑橘果树育种中是非常重要的技术，其核心是对由生理等因素导致种子不能成苗的合子胚人工接种于培养基上进行离体培育，并结合试管嫁接手段获得再生植株，极大地提升果树育种效率，加快育种进程[7]。彭珺[8]以2个二倍体柑橘品种与5个通过柑橘体细胞融合得到的四倍体品种进行倍性杂交，对幼胚进行离体培养，并结合流式细胞仪对再生植株进行倍性分析，获得三倍体141株。周锐[9]以不同柑橘材料为亲本，用相同的方法创制出147株三倍体。此外，随着全基因组重测序技术逐渐成熟，对于特定品系的柑橘品种，开发该品种特异性且扩增良好的InDel（Insertion/Deletion）分子标记对杂交后代进行遗传鉴定，具有低成本、高效、遗传稳定性好、准确性高且结果可靠的优势[10]。王沦[11]以甜橙的基因组为参考基因组挖掘到268个该品种存在的高质量InDel标记。宋谢天等[12]利用测序数据开发出7对InDel标记能区分柑橘有性后代和无性后代。

笔者在本研究中以云南地方特色早熟品种东试早柚为母本，与2个同源四倍体（ZP、PT）和1个异源四倍体（NH）为父本，杂交授粉后利用胚挽救、流式细胞仪及InDel标记等技术手段获得具有丰富遗传背景的柑橘三倍体材料，为柑橘早熟无核新品种选育和相关基础研究奠定材料基础。

1　材料和方法

1.1　试验材料

用于创制柑橘三倍体无核种质的材料：云南西双版纳州热带作物科学研究所柚试验基地的早熟品种东试早柚[*Citrus grandis*（L.）Osbeck‘Dongshizao pummelo’]，湖北武汉华中农业大学国家柑橘育种中心资源圃的纸皮[ZP，四倍体甜橙，*C. sinensis* (L.) Osbeck‘Paperrind orange’]、四倍体葡萄柚[PT，*C. paradise* Osbeck‘Grapefruit’]、NH[诺瓦橘柚+HB柚，*C. reticulata* Blanco×*C. paradisi* Macf. + *C. grandis*（L.）Osbeck‘Hirado Buntan pummelo’，四倍体，体细胞融合杂种]。

1.2　杂交授粉后亲和性鉴定方法

花粉的收集和保存参照朱晨桥[13]的方法，杂交授粉及授粉后7 d的花柱染色使用苯胺蓝染色法，通过倒置荧光显微镜观察花粉管的生长状态来鉴定杂交亲和性[14]。

1.3　杂交后代的胚培育及再生植株的倍性检测

胚挽救参照强瑞瑞[15]的方法并适当修改。授粉后85和100 d，采摘未成熟的果实暂置于4 ℃保存。无菌条件下，75%乙醇浸泡幼果15 min，立即置于乙醇灯上燃烧消毒灭菌，乙醇燃烧完全后用手术刀将果实剥开，取出种子。将种子尾部划一条缝并将其接种于萌发培养基（MT培养基+1 mg·L^{-1} GA_3）中，置于培养室培养。培养1个月后，未萌发的种子仍于萌发培养基中继续培养；将已萌发的种子形成的胚状体置于生芽培养基（MT+0.5 mg·L^{-1} BA+0.5 mg·L^{-1} KT+0.1 mg·L^{-1} NAA+40 g蔗糖+8 g琼脂）中增殖生芽，待其长出2~3枚叶片后将茎切下，接种于生根培养基（1/2 MT+0.1 mg·L^{-1} IBA+0.5 mg·L^{-1} NAA+0.5 g·L^{-1} 活性炭+8 g琼脂）中诱导生根。

流式细胞仪（Cyflow space，Sysmex，Japan）倍性鉴定参照谢善鹏[16]的方法并适当修改。以二倍体沙田柚为对照，从待测样品上取0.5 cm^{2}大小的新鲜叶片于干净的培养皿中，加约500 μL的细胞提取缓冲液（Nuclei extraction buffer，Cystain DNA 2 step），用刀片将其切碎，静置30~40 s后加入约1.5 mL的DNA染色液（Staining buffer，Cystain DNA 2 step）进行染色，最后用30 μm的微孔滤膜将样品过滤到2.5 mL试管中，用流式细胞仪（Cyflow space，Sysmex，Japan）进行上样检测，FloMax软件自动生成

DNA含量分布曲线。

1.4 植物基因组DNA的提取与检测

采集东试早柚、ZP、PT、NH及其各杂交子代成熟叶片并提取基因组DNA，提取方法采用改良CTAB法，具体步骤参照程运江[17]的博士学位论文，使用NanoDrop 1000超微量分光光度计对DNA进行质量检测，将质量合格的DNA初提液稀释至工作质量浓度（约200 ng·μL^{-1}），保存于−20 ℃冰箱备用。

1.5 InDel分子标记的筛选

利用母本东试早柚的重测序数据以晚白柚的基因组为参考基因组进行序列比对，获取InDel变异位点，筛选含有50~200 bp差异的位点。在InDel变异位点上下游设计引物（表1），以东试早柚、ZP、PT、NH的DNA为模板进行PCR扩增，PCR扩增反应体系20 μL，其中包括10 μL Mix，1 μL DNA，InDel上下游引物各0.5 μL，加入ddH_2O补足体积。PCR扩增产物使用2.5%琼脂糖凝胶检测，选择清晰稳定的特异性带型。

表1 东试早柚遗传鉴定所用的InDel引物信息

Table 1 InDel primers used in the genetic identification of Dongshizao pummelo

引物名称 Primer name	正向序列（5’−3’） Forward sequence（5’−3’）	反向序列（5’−3’） Reverse sequence（5’−3’）
InDel01	CATGACGATCACTGATTTGCTTTC	TGCAATTGAGAATCTATTGCTGC
InDel02	TCATAGCAAGCCGCTCTAGACTT	GTCAGTTTGGCGAAGTCAATGTTA
InDel03	CACCATAAGTGACGTCAATTTTGATAC	GCACTTGCTTGCTACCCAAGTAAA
InDel04	CTTCATTAAATTGGACATCTCTACTGATC	ACTATAATTAGCTTCAAAACTCCTGAAG
InDel05	CATTACATGAGAAGGTGCAGACA	AGCAAAATGGGAACTGAAAGGTAAC
InDel06	GAAGAATCGGAGGCACTTTATTTG	CTTATTCGAACTTGAGAAAATGTGTTC
InDel07	CTACTTTGTACAGGCCTTTTCAT	CTGTTGCACAGAAGAGTGGCT
InDel08	GGCTGTGTTTAAGGATTAATTAGAGC	GAACCTAATTGGGTTGAAAATGCG
InDel09	GCTCCAGAATATATGGCCACAGG	TGGAATTTGATAATCAAAGAATTTTCTAAGGAG
InDel10	CCCATGGGCTTAGAATTTGGGTC	AACACTACTCCTAAAAGCTACCG

2 结果与分析

2.1 东试早柚与四倍体柑橘杂交授粉后亲和性鉴定

以东试早柚作母本与2个同源四倍体（ZP、PT）、1个异源四倍体（NH）作父本进行倍性杂交。在东试早柚×ZP、东试早柚×PT、东试早柚×NH的杂交组合中，对授粉后的花柱进行苯胺蓝染色来观察花粉管的生长状态，发现大量花粉管能生长至花柱底部，均表现为亲和（图1）。

2.2 胚挽救获得再生植株

由于子代胚乳中母本和父本基因组比例为2∶2

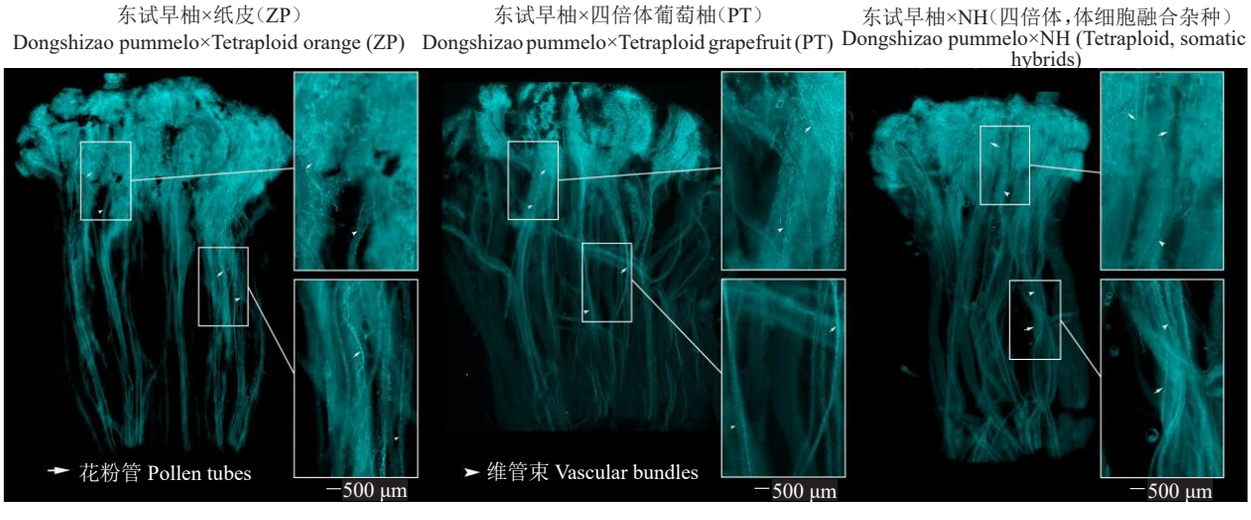
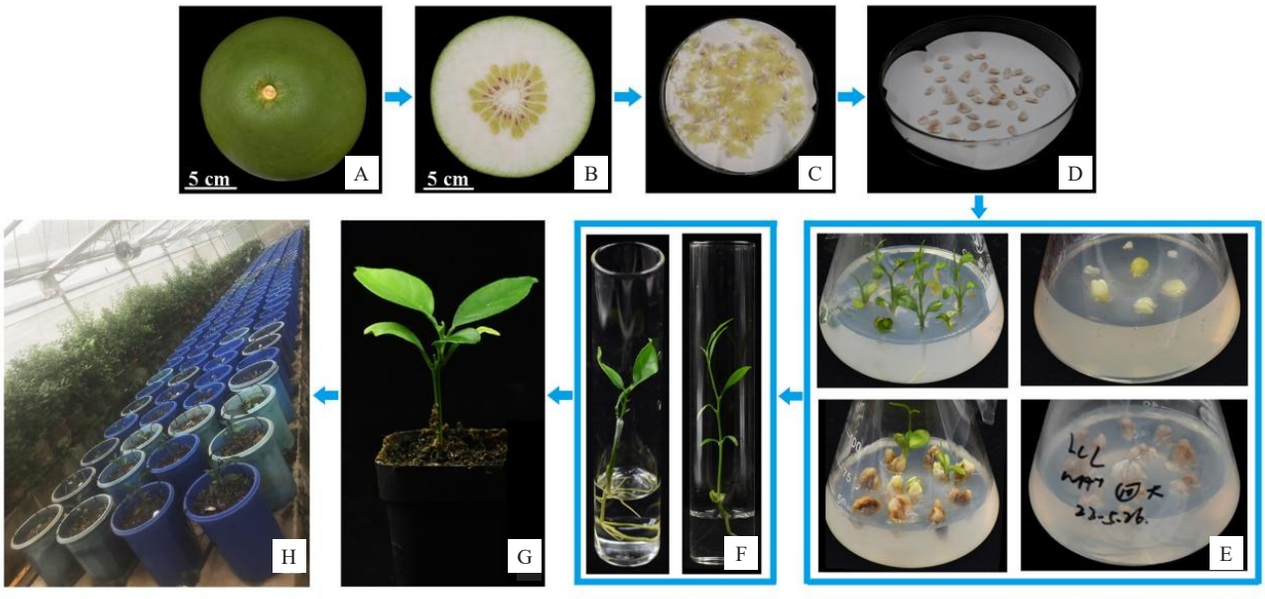


图1 杂交授粉后花柱的荧光成像

Fig. 1 Fluorescence images of pollen tubes in pistils after different pollinations

（2*x*×4*x*），后期种子完全败育[2]。故而在种子未败育之前，对幼胚进行离体挽救培养（图2）。授粉后85 d，取东试早柚×NH杂交组合的果实种子进行胚挽救，接种771粒种子，获得再生植株15株。授粉后100 d，取东试早柚×PT、东试早柚×ZP杂交组合的果实种子进行胚挽救，分别接种570、482粒种子，各获得再生植株96、117株（表2），组合间再生率存在差异，同源四倍体为父本的2个组合较高，在20%左右，异源四倍体为父本的组合较低，只有1.9%，相差近10倍。



A~B. 果实；C~D. 种子；E. 种子播种于 MT 培养基中；F. 炼苗；G~H. 移栽小苗。

A-B. Fruits; C-D. Seeds; E. The seeds sowing in MT medium; F. Seedlings hardening; G-H. Seedlings transferred to the pots.

图 2　胚挽救流程

Fig. 2　Process of embryo rescue

表 2　2*x*×4*x* 杂交组合授粉情况统计

Table 2　The results of embryo rescue and plant regeneration of 2*x*×4*x* crosses using cultivars

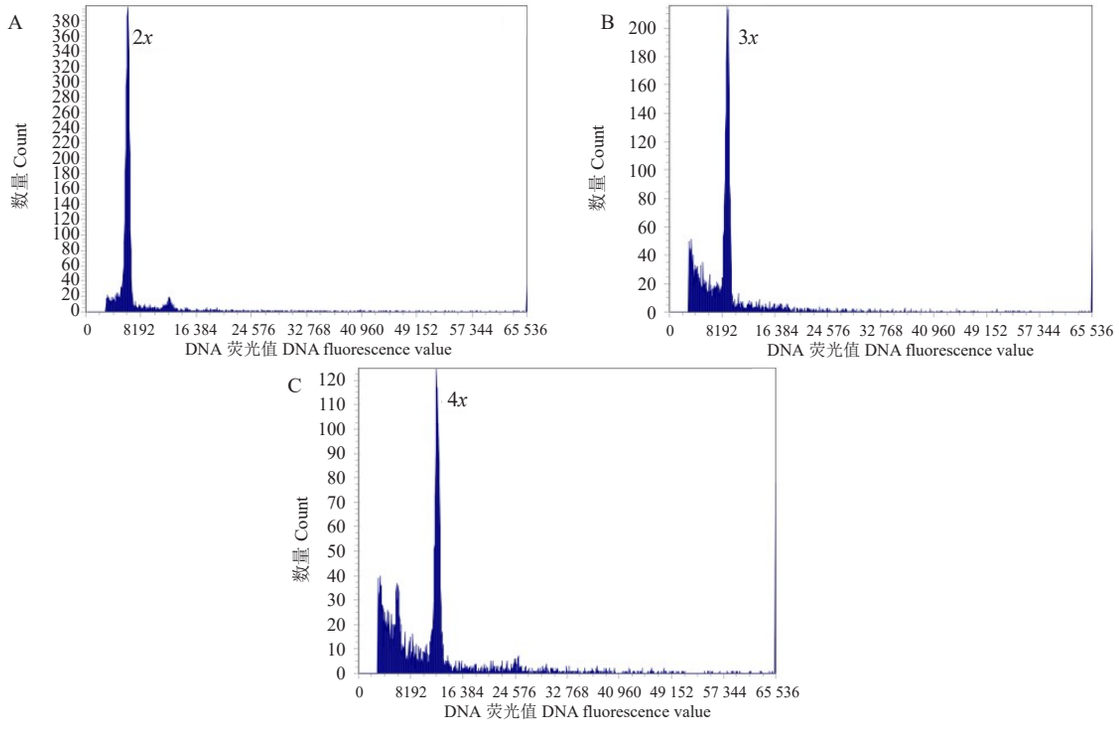
杂交组合 Cross combination	授粉数 No. of flowers	坐果数 No. of fruits	坐果率 Rate of fruit set/%	挽救果实数 No. of fruits for embryo rescue	总种子数 No. of seeds	再生植株数 No. of plantlets	成苗率 Rate of plantlets/%
东试早柚×PT Dongshizao pummelo×Tetraploid grapefruit	80	41	51.3	9	570	96	16.8
东试早柚×ZP Dongshizao pummelo×Tetraploid orange	80	54	67.5	10	482	117	24.3
东试早柚×NH Dongshizao pummelo×Tetraploid, somatic hybrids	80	62	77.5	10	771	15	1.9

2.3　再生植株倍性分析

利用流式细胞仪对东试早柚与3个四倍体柑橘杂交获得228株子代中的168株再生植株进行倍性检测（图3），在东试早柚×ZP杂交组合中，94株再生植株检测到三倍体幼苗60株，占再生子代的63.8%；东试早柚×PT杂交组合中，63株再生植株检测到三倍体幼苗60株和四倍体幼苗1株，分别占再生植株的95.2%和1.67%；东试早柚×NH杂交组合中11株再生植株检测到三倍体植株8株，占再生植株的72.7%（表3）。

2.4　再生植株的遗传来源分析

以晚白柚的基因组为参考基因组，利用东试早柚、葡萄柚及甜橙重测序数据开发10对InDel引物进行杂交子代筛选，其中InDel05和InDel10能区分东试早柚与ZP、PT，无法区分NH；InDel07和InDel08虽能区分所有的亲本，但是其部分特异性条带不明显（图4）。因此，选择InDel05引物对东试早柚×PT和东试早柚×ZP杂交子代进行鉴定，InDel07引物对东试早柚×NH杂交子代进行鉴定（图5）。结果表明129株多倍体再生植株均扩增出具有双亲差异性显著的带型。



A. 二倍体沙田柚，$2n = 2x = 18$；B. 东试早柚×四倍体柑橘的三倍体后代，$2n = 3x = 27$；C. 东试早柚×四倍体柑橘的四倍体后代，$2n = 4x = 36$。

A. Diploid，Shatian pummelo，$2n = 2x = 18$；B. Triploids obtained from Dongshizao pummelo × tetraploid citrus，$2n = 3x = 27$；C. Tetraploid obtained from Dongshizao pummelo × tetraploid citrus，$2n = 4x = 36$.

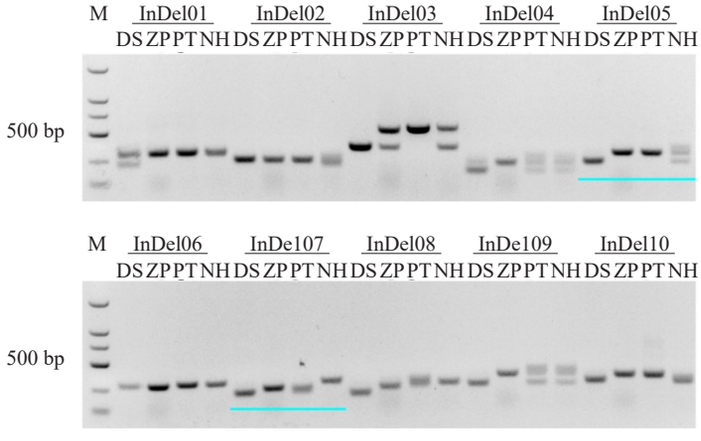
图 3 流式细胞仪倍性鉴定

Fig. 3 Ploidy level analysis by flow cytometry

表 3 东试早柚×四倍体柑橘授粉杂交组合再生植株倍性鉴定结果

Table 3 The results of seedlings ploidy identification of 2*x*×4*x* crosses using cultivars

授粉组合 Cross combination	检查的再生植株（系） No. of seedlings checked	3*x*（系） No. of triploids	4*x*（系） No. of tetraploids	多倍体（系） No. of polyploids
东试早柚×PT Dongshizao pummelo×Tetraploid grapefruit	63	60	1	61
东试早柚×ZP Dongshizao pummelo×Tetraploid orange	94	60	0	60
东试早柚×NH Dongshizao pummelo×Tetraploid, somatic hybrids	11	8	0	8

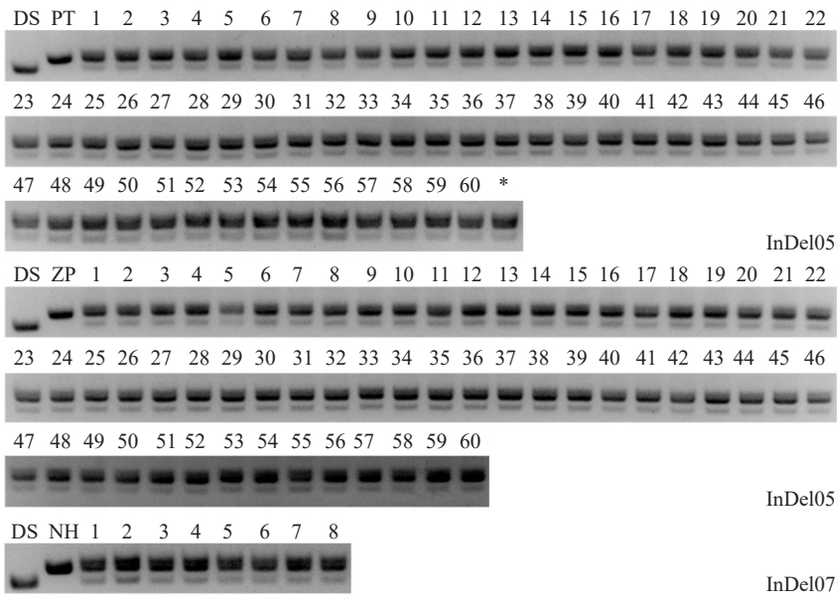


M. DL5000 DNA 分子标准；DS. 东试早柚；ZP. 纸皮，四倍体甜橙；PT. 四倍体葡萄柚；NH. 四倍体，体细胞融合杂种。

M. DL5000 DNA Marker; DS. Dongshizao pummelo; ZP. Tetraploid orange; PT. Tetraploid grapefruit; NH. Tetraploid, somatic hybrids.

图 4 不同柑橘种质 InDel 引物鉴定结果

Fig. 4 Results of InDel markers identification of different citrus germplasms



DS. 东试早柚，母本；ZP. 纸皮，四倍体甜橙，父本；PT. 四倍体葡萄柚，父本；NH. 四倍体，体细胞融合杂种，父本；*. 四倍体再生植株。

DS. Dongshizao pummelo, female parent; ZP. Tetraploid orange, male parent; PT. Tetraploid grapefruit, male parent; NH. Tetraploid, somatic hybrids, male parent; *. Tetraploid seedling.

图5　杂种 F_1 代植株 InDel 引物鉴定结果

Fig. 5　Results of InDel markers identification in F_1 generation plants of hybrid

3　讨　论

柑橘三倍体育种以获得果实无核为主要目标性状，同时能兼具不同成熟期、易剥皮、高糖低酸等优异性状。Fatta Del Bosco 等[18]以宽皮橘等为亲本通过倍性杂交，以期获得无核且易剥皮的新种质来取代 Avana 和 Tardivo di Ciaculli 多籽宽皮橘品种。西班牙则以无核且晚熟为目标性状，通过三倍体育种获得 Safor[19]和 Garbí[20]等具有无籽、中晚熟柑橘品种。染色体加倍也会导致三倍体植株表现出果皮增厚、果面粗糙、果实低糖高酸、枝刺增多且增长等诸多不利的性状。Grosser 等[21]以二倍体克里曼丁橘为亲本，倍性杂交获得的三倍体后代表现出枝刺长、枝刺增多等不利性状；但是以二倍体 Sugar Belle 为亲本，得到的三倍体后代表现出果实早熟、枝刺短、枝刺减少等优良性状。因此，杂交后代性状的表现与亲本的选择密切相关。笔者在本研究中选择果实早熟、无籽、高糖低酸的云南地方特色品种东试早柚为母本，融合双亲优良性状的四倍体体细胞杂种 NH、晚熟且丰产的同源四倍体 ZP 和 PT 为父本，通过三倍体育种以期获得果实早熟、丰产、品质更佳等诸多综合双亲优良性状的柑橘无核新种质。

在柑橘中，常以二倍体（♀）与四倍体（♂）、四倍体（♀）与二倍体（♂）进行倍性杂交，不正常的胚与胚乳的倍性比会致使杂交获得的合子胚提前败育[22]。胚挽救是对受各种不利因素影响而致使早期败育的合子胚离体培养，是大多数果树育种中普遍使用的一种方法[7]。解凯东等[23]创制以 8 个二倍体柑橘品种、6 个四倍体柑橘品种为亲本，通过倍性杂交获得再生植株 2832 株，并结合流式细胞仪对再生植株检测获得三倍体植株 401 株等一大批倍性材料。笔者在本研究中以二倍体单胚性东试早柚为母本，2 个同源四倍体和 1 个异源四倍体柑橘品种为父本，利用胚挽救手段获得 228 株再生植株，并使用流式细胞仪对 168 株再生植株进行倍性检测，获得三倍体植株 128 株，还意外获得 1 株四倍体材料。

目前，在柑橘育种中特异性 InDel 标记已成为鉴定杂交子代遗传来源的重要手段。汤雨晴等[24]利用金兰柚全基因组重测序数据开发出 24 对 InDel 标记，均能够有效区分金兰柚不同于其他的柚类品种。韩健等[25]基于基因组重测序数据开发出可有效检测沙田柚与枳杂交子代的 InDel 标记，从 1279 株子代中鉴定获得 698 株杂种苗。笔者在本研究中以晚白柚的基因组为参考基因组，开发 10 对候

选InDel标记用于区分东试早柚及ZP、PT、NH柑橘种质，筛选出2对高质量InDel标记对倍性检测获得的128株三倍体和1株四倍体再生植株进行遗传鉴定，均扩增出亲本特异性条带。因此，结合倍性检测的结果，获得的流式细胞仪129株多倍体植株均为双亲杂交子代，其中获得1株四倍体是由东试早柚×PT杂交而来，推测其可能是由母本产生未减数的雌配子与父本产生的二倍体雄配子杂交授粉受精而来的。

4 结 论

通过有性杂交，结合胚胎抢救、流式细胞仪及InDel分子标记等技术在较短时间内创制出3个组合的柑橘三倍体128株和四倍体杂种1株，为柑橘三倍体无核育种提供了材料。

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