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浙江地方特色品种温岭高橙无核潜力新种质创制及分子鉴定

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摘 要:【目的】温岭高橙是浙江台州地方特色品种,栽培历史悠久,且具有一定的保健和药用价值,但果实有核限制了其在台州以外地区的发展,因此利用细胞工程高效育种技术对温岭高橙进行无核化改良。**【方法】**通过2条育种途径,一是以二倍体温岭高橙为母本与异源四倍体为父本倍性杂交,授粉后90 d利用幼胚离体挽救培养和流式细胞仪倍性鉴定创制三倍体无核新种质;二是以雄性不育胞质杂种华柚2号为母本与温岭高橙有性杂交,通过成熟种子催芽播种,创制二倍体无核新种质。利用SSR分子标记对杂交后代进行分子鉴定。**【结果】**以温岭高橙为母本、异源四倍体NS和BDZNS为父本配置2个倍性杂交组合,授粉145朵花,坐果74个;胚抢救幼嫩种子1520粒,离体培养获得再生植株553株;用流式细胞仪对再生植株进行倍性鉴定,获得三倍体45株、四倍体8株;以华柚2号为母本与温岭高橙有性杂交,授粉69朵花,坐果20个,获得成熟种子312粒;通过催芽播种获得实生后代203株,倍性鉴定均为二倍体;用SSR分子标记对2个倍性杂交组合的21株三倍体和8株四倍体后代及华柚2号×温岭高橙随机选取的29株二倍体后代进行分子鉴定,显示所鉴定的三倍体及二倍体后代均含有父本特异条带,为其杂交双亲的有性后代,而倍性杂交获得的四倍体后代与母本条带完全一致,推测其为母本珠心细胞自然加倍形成的同源四倍体(双二倍体)。**【结论】**研究结果为温岭高橙无核新品种培育奠定了宝贵的材料基础。

关键词:柑橘;无核育种;倍性杂交;华柚2号

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Production and molecular identification of potentially seedless germplasms derived from Wenling-gaocheng, a citrus local cultivar in Zhejiang province

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Abstract:【Objective】Seedlessness is an important trait for citrus fresh fruits because consumers do not like fruits with seeds. Wenling-gaocheng, a natural hybrid of pommelo and orange, is an excellent local cultivar in Zhejiang Province because of its unique flavor and rich nutritional value. However, it failed to expand the market outside Taizhou city due to the abundance of seeds in the fruits. The fruits of the triploid plants are generally seedless. There are several ways to produce citrus triploid plants.

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Among them, using diploid varieties as female parents and allotetraploid as the male parents is the most efficient way. The triploid plants produced by interploidy hybridization with allotetraploid as the male parent generally possess abundant genetic variations, which is conducive to breed new cultivars with good fruit traits. Production of diploid progenies using cytoplasmic male sterile (CMS) cultivar Huayou No. 2 as the female parent crossed with the seedy diploids is another useful way to breed seedless citrus germplasm because the CMS trait in citrus is female inherited, and the CMS trait can be inherited to all progenies. Therefore, the above two strategies were employed in this study to produce seedless progenies with the lineage of Wenling-gaocheng, to produce promising materials for selecting seedless cultivars with the lineage of Wenling-gaocheng.【Methods】Two crossing strategies were employed in this study. The first one was to produce triploid progenies using Wenling-gaocheng as female parent and two alloteraploids as male parents. The pollination of the two interploidy crosses was conducted at the full-bloom stage and the immature fruits were collected on the 90th days after pollination (DAP). The immature seeds were removed from the fruits and cultured *in vitro* in the germination medium. When the seedlings regenerated, their ploidy level was determined by the flow cytometry. Another strategy was to produce diploid progenies with CMS traits using Huayou No. 2 as the female parent and the Wenling-gaocheng as the male parent. The pollination of this cross was conducted at the full-bloom stage and the fruits were collected at the mature stage. After the seeds were extracted from the fruits and the episperms were peeled, they were placed in a controlled chamber to accelerate germination. The seeds were sown in pots after germination in a plant growth chamber. After the seedlings grew with three or more leaves, their ploidy level was determined by the flow cytometry. At last, the genetic origin of all seedlings from the three crosses were determined by SSR markers.【Results】In the two interploidy crosses, a total of 145 flowers were pollinated and 74 fruits were set, with an average fruit setting rate of 51.03%. 1520 immature seeds were *in vitro* cultured *via* embryo rescue and 553 seedlings were obtained. Among them, 239 plantlets were regenerated from the cross of Wenling-gaocheng × NS and 314 plantlets were originated from the cross of Wenling-gaocheng× BDZNS. After determining their ploidy level, a total of 45 triploids and 8 tetraploid seedlings were screened out. Three SSR markers were used to determine their genetic origin. All the analyzed triploids were the hybrids of their parents and all the tetraploids had the same bands with Wenling-gaocheng, indicating that all the tetraploids might be derived from the nucellar doubling of Wenling-gaocheng. For the cross of Huayou No. 2 × Wenling-gaocheng, a total of 69 flowers were pollinated. From the 20 fruits, 312 mature seeds were obtained. After sowing and germination, 203 seedlings were obtained, all of them were identified as diploid by flow cytometry. Four polymorphic SSR markers were selected to determine their genetic origin of 29 randomly selected progenies and it showed that all the diploid progenies were parental hybrids.【Conclusion】The diploid hybrids derived from the cross of Huayou No. 2 × Wenling-gaocheng and the triploid hybrids derived from the two interploidy crosses could be used for seedless breeding of new varieties with Wenling-gaocheng genetic background. The autotetraploid progenies of Wenling-gaocheng also would be valuable for crossing with diploid Wenling-gaocheng to produce autotriploids.

Key words: *Citrus*; Seedless breeding; Ploidy hybridization; Huayou No. 2

中国是柑橘的重要起源中心，地方特色品种多，但多数因果实有核导致市场竞争力弱和种植面积缩减[1]。温岭高橙为柚和橙的天然杂种，果实风味独特、营养丰富，富含柠檬苦素和诺米林等黄酮类物质[2]，是一种集营养和保健功能于一体的优良地方特色品种；除鲜食外，温岭高橙还可作为加工品种进行榨汁等。在浙江台州温岭、玉环一带已有700余年栽培历史，但因其种子较多未能在台州市外打开

市场，制约了该品种的推广发展。

三倍体因减数分裂异常，不能形成正常配子，果实一般无核。因此，创制三倍体是培育柑橘无核新种质的重要途径。与芽变和实生选种相比，通过倍性杂交可大规模创制三倍体，并能根据基因型实现定向育种[3-4]。柑橘三倍体创制的策略中当属以二倍体为母本和四倍体为父本的倍性杂交最经典。以异源四倍体为父本倍性杂交创制的三倍体后代可能兼具三亲性状，变异丰富，有利于培育果实性状优良且无核的新种质。基于该策略，华中农业大学10余年来以异源四倍体为父本与二倍体为母本进行倍性杂交，结合幼胚离体挽救技术已创制获得3500余株具有丰富变异的三倍体有性后代，并筛选出了一批无核优系[5]，为中国柑橘无核新品种培育奠定了丰富的种质基础。

柑橘普遍存在珠心胚等生殖障碍，常规杂交育种效率低，雄性不育胞质杂种的创制为二倍体水平的柑橘无核改良提供了新思路。华中农业大学柑橘研究团队基于几十年的细胞工程育种实践，提出了柑橘二倍体胞质杂种创制的新思路，即以温州蜜柑为愈伤亲本、有核品种为叶肉亲本进行细胞融合培育二倍体胞质杂种，将温州蜜柑的雄性不育胞质定向转移至有核品种，在不改变有核品种倍性和核基因组的情况下实现果实无核化[6]。基于该策略，华中农业大学已经创制具有温州蜜柑雄性不育胞质的杂种10余例[5]。其中华柚2号已开花结果多年，与其叶肉亲本华柚1号相比，表现为雄性不育和果实无核（隔离种植），是国际首例利用细胞工程技术培育的无核新品种[7]。华柚2号保留了温州蜜柑的细胞质雄性不育胞质，除直接作为新品种推广外，作为超级母本与其他有核品种有性杂交，在二倍体水平改良其有核性状方面具有重要应用价值[8]，有望直接培育出果实无核且品质优良的柑橘新品种。因此，笔者在本研究中针对浙江台州地方特色品种温岭高橙存在的果实有核等问题，拟通过创制三倍体和带有雄性不育胞质的二倍体无核种质2条育种途径，创制一批具有无核潜力的温岭高橙新种质，为温岭高橙无核新品种培育奠定种质基础。

1 材料和方法

1.1 试验材料

温岭高橙三倍体无核新种质创制：以温岭高橙（*Citrus grandis* Osbeck × *C. sinensis* Osbeck）为母本、异源四倍体NS [由Nova橘柚（*C. reticulata* Blanco × *C. paradisi* Macf.）和无酸甜橙（*C. sinensis* Osbeck）细胞融合再生获得]和BDZNS[本地早橘（*C. succosa* Hort. ex Tanaka）与NS倍性杂交获得]为父本，配置2个倍性杂交组合，创制三倍体无核新种质。

温岭高橙二倍体无核新种质创制：以华柚2号（*C. grandis* Osbeck）为母本、温岭高橙为父本进行有性杂交，创制既有华柚2号雄性不育胞质又有温岭高橙核遗传物质的二倍体无核新种质。

温岭高橙定植于浙江省台州市温岭市四季海湖田园基地，树龄为10 a（年）；华柚2号定植于华中农业大学柑橘种质资源圃，树龄为15 a，树体健壮，无病虫害。

1.2 人工授粉和幼胚挽救离体培养

父本花粉制备及人工授粉参考解凯东等[9]的方法，花粉制备好后置于离心管中4 ℃低温避光保存备用。授粉时选取母本生长健壮、花量大的枝条进行人工去雄和授粉，授粉后进行套袋处理，避免外来花粉干扰。以温岭高橙为母本的倍性杂交组合授粉地点位于浙江台州市温岭四季海湖田园基地；以华柚2号为母本的二倍体间有性杂交授粉地点位于华中农业大学柑橘种质资源圃。

采摘倍性杂交组合授粉后约90 d的幼果，于超净工作台内采用乙醇浸泡后燃烧消毒，待乙醇完全燃烧后剥取未成熟胚珠进行幼胚离体挽救培养，胚珠处理及培养基制备参照Xie等[10]的方法，培养室温度（26 ±1）℃，相对湿度70%，光照时长12 $h\cdot d^{-1}$。

1.3 种子催芽播种

华柚2号×温岭高橙组合成熟种子的催芽播种参照周锐等[11]的方法。用1 $mol\cdot L^{-1}$的NaOH溶液浸泡处理成熟的授粉种子15 min，以除去其表面果胶；清水洗净后去除内外种皮，置于铺有湿纱布的培养皿，于恒温培养箱（28 ℃）催芽3~4 d；待胚根长度为1~2 cm时，将其集中播种于小营养钵；幼苗长到3~4叶龄时，再将其分别移栽至黑色塑料大营养钵，并置于温室集中管理。

1.4 植株倍性鉴定和移栽

植株的倍性用流式细胞仪（Cyflow space，Sysmex，Japan）进行测定，详细方法参照解凯东等[9]的

方法。倍性鉴定获得的多倍体植株经过3~5 d炼苗后，先移入装有营养土的小塑料杯并置于生长室培养1个月；待幼苗长出新叶，再移入黑色塑料营养钵，并置于温室培养。

1.5　SSR分子鉴定

杂交后代及其亲本基因组DNA提取参考Cheng等[12]的方法。从40对SSR引物中筛选5对父母本间有各自特异条带多态性SSR引物（表1），对获得的多倍体和二倍体后代进行杂种鉴定，根据后代在至少一个位点处的条带中是否出现父本特有的条带判断，如果出现则表明后代是杂种后代。引物由上海生物工程股份有限公司合成。PCR反应体系和扩增程序参考解凯东等[9]的方法。PCR扩增产物由全自动毛细管电泳（QIAxcel Advanced，Germany）分析，成像结果由仪器自带软件QIAxcel ScreenGel自动生成。

表1　SSR引物序列

Table 1　Sequence of SSR primers

引物名称 Primer name	正向序列 Forward sequence	反向序列 Reverse sequence	来源 Reference
Ci01C07	GTCACTCACTCTCGCTCTTG	TTGCTAGCTGCTTTAACTTT	[13]
Csin0191	GACTTGAACGGTTGCAATGA	TGATGTCCCAAAAGTGTGGA	[14]
Ma2-1480	CAATCACAGGAGCGACTTCA	CTCAATTCAGCAAACCGACA	[14]
Csin0032	TGCTAGGTAGCAATACTAAATGGAA	CGCCATTGTTAGAGCCTTTT	[14]
Csin0067	TTGATTTTTCAATTGGCCGT	AAAAATTGTTCTATCTTGAGCCTTT	[14]

2　结果与分析

2.1　人工授粉、幼胚离体挽救培养及催芽播种

以温岭高橙为母本、异源四倍体NS和BDZNS为父本进行倍性杂交，配置了2个组合创制温岭高橙三倍体无核新种质。温岭高橙 × NS组合授粉81朵花，坐果31个，坐果率38.27%，获得种子629粒，平均单果种子数20.3粒，经幼胚离体挽救培养（图1-A~H），获得再生植株239株；温岭高橙 × BDZNS组合授粉64朵花，坐果43个，坐果率67.19%，获得种子891粒，平均单果种子数20.7粒，经幼胚离体挽救培养，获得再生植株314株。

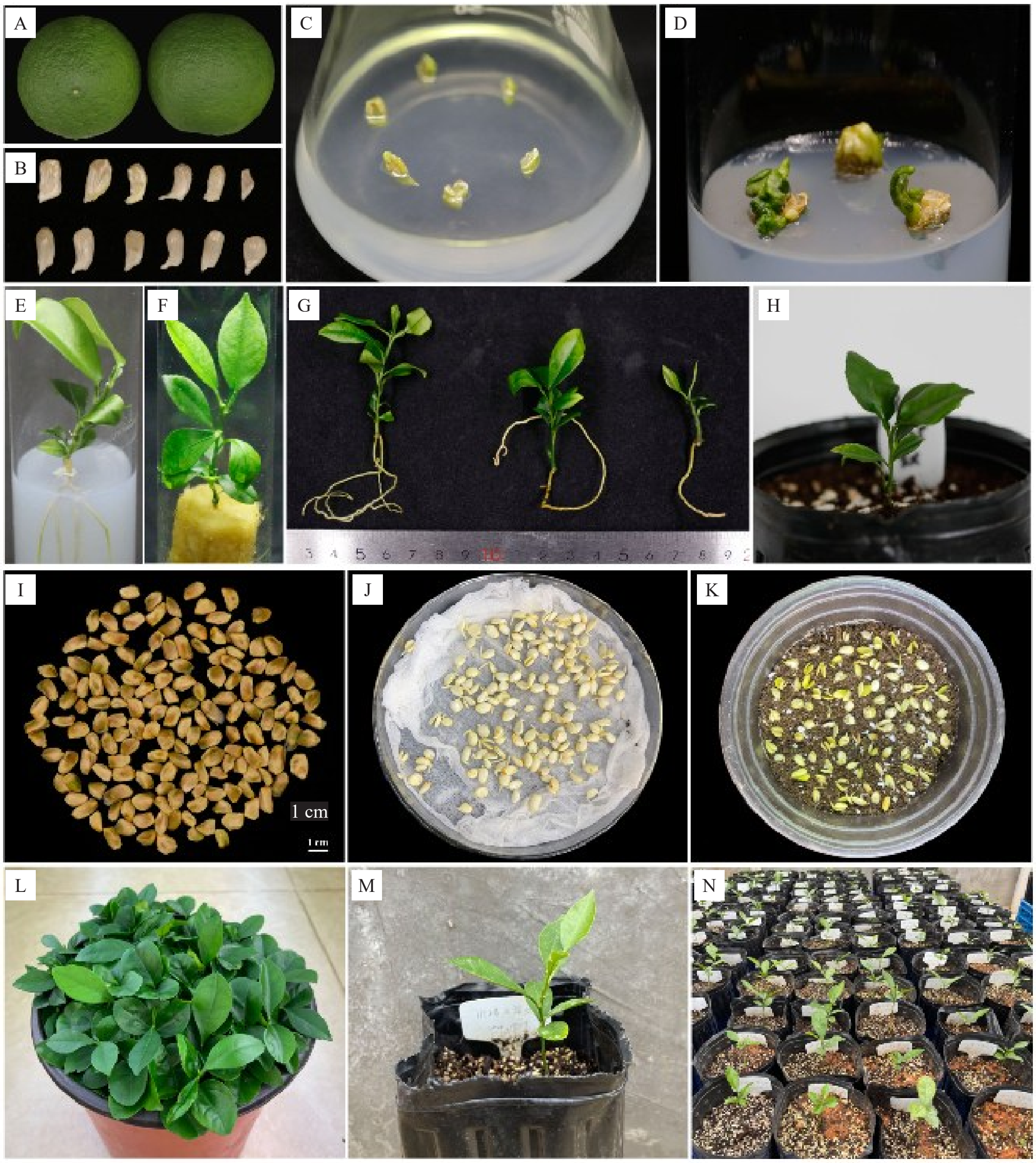
以华柚2号为母本、温岭高橙为父本进行有性杂交创制温岭高橙二倍体无核新种质，授粉69朵花，坐果20个，果实成熟期采果收集种子312粒，去种皮后催芽播种获得实生苗203株（图1-I~N）。

2.2　植株倍性鉴定

用流式细胞仪对以温岭高橙为母本的2个倍性杂交组合再生获得的553株幼苗（温岭高橙 × NS组合239株，温岭高橙 × BDZNS组合314株）和华柚2号 × 温岭高橙组合的203株实生苗进行倍性分析。结果（图2）显示，从2个倍性杂交组合的553株幼苗中鉴定获得三倍体45株，四倍体8株；其中，温岭高橙 × NS组合获得三倍体34株，四倍体3株，多倍体率为15.48%（图1-E-H）；温岭高橙 × BDZNS组合获得三倍体11株和四倍体5株，多倍体率为5.10%；华柚2号 × 温岭高橙组合实生播种获得的203株实生后代均为二倍体。

2.3　SSR分子标记鉴定

用5对SSR引物对3个组合获得的杂交后代进行分子鉴定，其中，3对SSR引物（Ci01C07、Csin0191、Ma2-1480）适用于温岭高橙 × NS和温岭高橙 × BDZNS组合，4对SSR引物（Ci01C07、Ma2-1480、Csin0032和Csin0672）适用于华柚2号 × 温岭高橙组合。由于2个倍性杂交组合后代中均有部分植株较小（未能取叶片提取DNA）的情况，仅对温岭高橙 × NS组合的16株三倍体和1株四倍体、温岭高橙 × BDZNS组合的5株三倍体和7株四倍体后代进行分子鉴定。结果显示，2个倍性杂交组合被鉴定的所有三倍体均含有父本特异条带，表明其均为双亲有性杂交后代；而被鉴定的四倍体后代扩增条带与母本温岭高橙均完全一致，表明这些四倍体可能为母本珠心细胞自然加倍形成（图3-A、B）。用4对SSR引物对从华柚2号 × 温岭高橙203株杂交后代中随机筛选的29个单株进行分子鉴定，结果显示，所有子代均含有父本特异条带（图3-C），表明随机挑选的29株后代均为父母本的有性杂交后代。

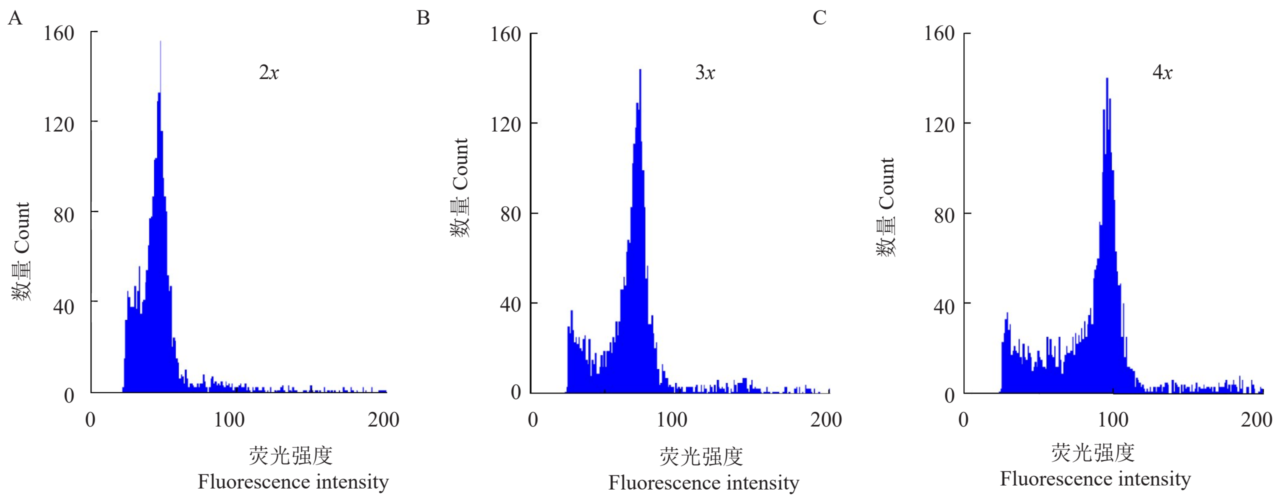


A~H. 温岭高橙 × NS 三倍体胚抢救及植株再生流程。A. 授粉 90 d 的果实；B. 授粉 90 d 的种子；C. 幼嫩种子离体接种；D. 种子萌发形成胚状体；E. 生根的三倍体植株；F. 三倍体植株炼苗；G. 不同倍性植株幼苗（左 . 二倍体，中 . 三倍体，右 . 四倍体）；H. 三倍体植株移栽；I~N. 华柚 2 号 × 温岭高橙催芽播种流程。I. 收获的成熟种子；J. 去掉种皮后的种子催芽；K. 催芽后的种子播种至基质；L. 实生幼苗；M. 实生幼苗移栽至大营养钵；N. 移栽后的群体。

A-H. Embryo rescue and triploid plant regeneration procedure of Wenling-gaocheng × NS. A. Pollinated fruits harvested at 90 d after pollination; B. Immature seeds obtained at 90 d after pollination; C. Embryo rescue culture of the immature seeds; D. Geminated seeds; E. Triploid seedling in rooting medium; F. Acclimatization of the triploid plantlet; G. Plant morphology of diploid (left), triploid (middle) and tetraploid plant (right); H. Triploid transplantation. I-N. Seed pregermination and sowing procedure of the cross of Huayou No. 2 × Wenling-gaocheng. I. Mature seeds; J. Seed pregermination after removing the seed coat; K. Seed sowing; L. Seedlings growing in the small pot; M. Seedings transplanted in the bigger pot separately; N. The hybrid population of the cross of Huayou No. 2 × Wenling-gaocheng.

图 1 温岭高橙三倍体胚抢救与植株再生及二倍体群体实生播种技术流程

Fig. 1 Procedure of triploid production by embryo rescue and diploid population production by seed sowing

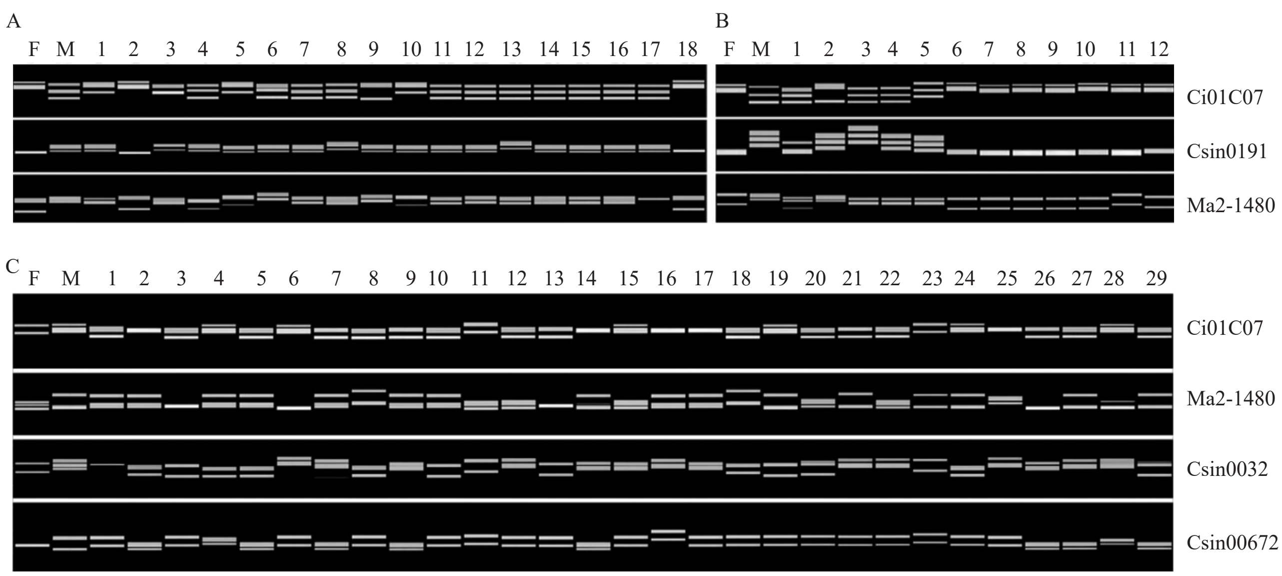


A. 二倍体后代（对照，荧光强度为 50）；B. 三倍体后代（荧光强度为 75）；C. 四倍体后代（荧光强度为 100）。

A. The diploid Wenling-gaocheng (Control, fluorescence intensity = 50); B. Triploid progeny (Fluorescence intensity = 75); C. Tetraploid progeny (Fluorescence intensity = 100).

图 2　用流式细胞仪对以温岭高橙为亲本所获再生植株的倍性鉴定

Fig. 2　Ploidy analysis of regenerated plants derived from Wenling-gaocheng as parents by flow cytometry



A. 温岭高橙 × NS 倍性杂交组合；F. 母本温岭高橙；M. 异源四倍体体细胞杂种父本 NS；1 和 3~17. 三倍体后代；2. 二倍体后代；18. 四倍体后代；B. 温岭高橙 × BDZNS 倍性杂交组合；F. 母本温岭高橙；M. 异源四倍体父本 BDZNS；1~5. 三倍体后代；6~12. 四倍体后代；C. 华柚 2 号 × 温岭高橙杂交组合；F. 母本华柚 2 号；M. 父本温岭高橙；1~29. 随机挑选的 29 株杂交后代。

A. SSR profile of the progenies from the cross of Wenling-gaocheng × NS; F. The female parent Wenling-gaocheng; M. The allotetraploid male parent NS; 1, 3-17. Ttriploid progeines; 2. Diploid progeny; 18. Tetraploid progeny. B. SSR profile of the progenies from the cross of Wenling-gaocheng × BDZNS; F. The female parent Wenling-gaocheng; M. The allotetraploid male parent BDZNS; 1-5. Triploid progenies; 6-12. Tetraploid progenies. C. SSR profile of the progenies from the cross of Huayou No. 2 × Wenling-gaocheng; F. The female parent Huayou No. 2; M. The male parent Wenling-gaocheng; 1-29. 29 randomly selected progenies derived from the cross of Huayou No. 2 × Wenling-gaocheng.

图 3　以温岭高橙为亲本的 3 个有性组合部分杂交后代 SSR 分子鉴定

Fig. 3　SSR molecular identification of progenies from three sexual crosses with Wenling-gaocheng as a parent

3 讨 论

通过应用倍性杂交和以雄性不育胞质杂种为母本有性杂交2条育种途径开展地方特色品种温岭高橙无核新种质的创制，获得了一批三倍体和二倍体新种质，为温岭高橙无核新品种培育提供了丰富的种质资源。

倍性杂交创制三倍体是培育温岭高橙无核新种质的重要途径。二倍体与四倍体倍性杂交是创制三倍体的有效策略，特别是异源四倍体的应用，相比以同源四倍体为父本，能够产生更加丰富的遗传变异[9]，有利于筛选品质优良且果实无核的三倍体新品种。前人尝试以温岭高橙为母本与2个异源四倍体柑橘杂交并获得了一批三倍体有性后代[15]，但因所用的异源四倍体体细胞杂种含有柠檬和酸橙等的遗传物质，果实品质均表现不佳，未能推广应用。笔者在本研究中所用异源四倍体体细胞杂种NS为Nova橘柚和无酸甜橙融合形成[4]，2个融合亲本均为优良的栽培品种；异源四倍体BDZNS是在NS基础上又融入了本地早橘的遗传物质，兼具三亲遗传物质，以其为亲本倍性杂交创制的三倍体，有望培育融合多亲性状且具有温岭高橙特性的无核新品种。笔者在本研究中除获得45株三倍体植株外，还获得了8株四倍体，分子鉴定分析其可能由珠心细胞染色体自然加倍形成，该结果与Xie等[10]报道结果一致。这些四倍体不仅可进一步作为亲本倍性杂交创制三倍体，作为砧木，可能还具有矮化树体和提升接穗抗性的潜力。

利用创制三倍体的策略虽可一步到位实现果实无核，但由于倍性增加，难免会带入一些不利性状，如刺变长、果皮和囊衣增厚等，往往需通过创制大量遗传变异丰富的群体才有望培育出优良且易栽培的无核新品种。柑橘二倍体间通过常规有性杂交不会改变后代的倍性，囊衣增厚和刺变长等不利性状表现不明显，较易培育出风味好且易栽培的品种，但难以解决果实有核的问题。柑橘CMS性状表现母性遗传，以具有CMS性状的品种为母本与其他有核品种常规有性杂交可创制无核种质。如日本利用温州蜜柑（具有CMS胞质基因）与其他品种有性杂交，培育出许多广泛栽培的无核或少核的新品种[16]。华中农业大学柑橘细胞工程与遗传改良团队利用温州蜜柑的CMS特性，提出并研发了创制柑橘二倍体胞质杂种无核新种质的育种途径[6-7,17]，并创制出雄性不育和果实无核的新品种华柚2号[18]、华柚3号[19]。华柚2号和华柚3号除可直接作为无核鲜食品种推广外，由于其均为单胚品种，果实成熟后播种即可得到大量杂种后代，育种效率高，因此作为超级母本与其他有核地方特色品种杂交改良其有核性状，具有重要应用价值。如华中农业大学以华柚2号为母本，分别与沙田柚和鸡尾葡萄柚杂交获得1018、687株有性后代[8]，其中华柚2号 × 沙田柚已有500余株后代开花结果，后代均表现雄性不育和果实无核（数据未发表），证实了该育种途径的有效性。笔者在本研究中以华柚2号为母本、温岭高橙为父本有性杂交获得203株有性后代群体，为培育温岭高橙无核新品种奠定了丰富的材料基础。

4 结 论

笔者在本研究中以浙江柑橘地方品种温岭高橙为亲本，通过与异源四倍体倍性杂交创制无核三倍体和以雄性不育胞质杂种有性杂交创制带有CMS性状的二倍体新种质两条育种途径，创制获得一批温岭高橙新种质，为温岭高橙无核新品种选育和品质改良奠定了丰富的材料基础。

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