

四倍体胡柚与鸡尾葡萄柚有性 杂交创制三倍体新种质

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摘要:【目的】柑橘三倍体具有无核或少核的特点, 通过 $4x \times 2x$ 杂交方式创制三倍体种质, 以弥补鸡尾葡萄柚种子多、抗寒性差的缺点。【方法】以四倍体(双二倍体)胡柚为母本与二倍体鸡尾葡萄柚杂交, 果实成熟后对种子大小进行分类并进行组织培养, 采用流式细胞仪、SSR分子标记鉴定再生子代的倍性及遗传组成。【结果】共授粉40朵花, 坐果19个, 坐果率为47.5%。获得的230粒种子中, 大种子占56粒, 再生49株, 2株为三倍体(占大种子再生后代的4.08%); 小种子117粒, 再生69株, 35株为三倍体(占小种子再生后代的50.72%); 败育种子57粒, 再生4株, 2株为三倍体。经SSR分子标记鉴定, 39株三倍体子代均为双亲杂交后代, 同时鉴定出1株杂种四倍体后代。【结论】创制了一批异源三倍体和四倍体新种质, 为选育抗寒、无核的杂种葡萄柚新品种奠定了种质基础。

关键词: 鸡尾葡萄柚; 胡柚; 倍性育种; 三倍体; 四倍体; SSR分子标记

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Regeneration of triploid citrus germplasm by the crossing between tetraploid Huyou and Cocktail grapefruit

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Abstract: 【Objective】Seed number has a crucial effect on the fruit quality for the fresh-fruit market and processing production in citrus. Triploid citrus can produce seedless fruits, and as polyploids, it usually shows the superiority of vigorous growth, enlarged fruits and enhanced stress resistance. In citrus breeding, the $4x \times 2x$ interploidy cross is an effective way to obtain triploids and to select new seedless species. Cocktail grapefruit (*Citrus paradisi* 'Cocktail') has excellent fruit taste with high total soluble sugar and low titratable acid contents but has the defect of abundant seeds and poor tolerance to cold. So reducing the seed number to enhance the economic value has raised the awareness among citrus breeders. Changshan Huyou (*Citrus aurantium* 'Changshanhuyou') is a native citrus species in Zhejiang province and exhibits rich flavor and excellent cold resistance, and its seed is a mixed type of single

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and multiple embryos. A tetraploid Changshan Huyou was identified and bear seedy fruits for several years. Therefore, we attempted to produce triploid hybrids by interploidy hybridization between the tetraploid Changshan Huyou and the diploid grapefruit. **【Methods】** The $4x \times 2x$ interploidy cross was conducted using a tetraploid Changshan Huyou ($4x$) as the female parent and the diploid Cocktail grapefruit ($2x$) as the pollen parent in this study. Pollen viability was determined by the result of Alexander staining and germination rate *in vitro* before artificial pollination. In the florescence stage of Cocktail grapefruit, the pollen was collected using a 2 mL centrifuge tube and stored in dry environment at $4\text{ }^{\circ}\text{C}$ for a short period. Seeds were extracted from the ripened fruit of hybrids and divided into developed seeds of normal size, developed seeds of small size ($1/3$ – $1/6$ of normal size) and undeveloped seeds. The normal size and small size seeds were sowed on Murashige and Tucker (MT) culture medium, and undeveloped seeds were sowed on $\text{MT}+1.0\text{ mg}\cdot\text{L}^{-1}\text{ GA}_3$ culture medium. The seedlings were transplanted after the plant growing 4–5 functional leaves. The ploidy of progenies was then measured by flow cytometry using young leaves. After DNA extraction, the genetic origin of the offspring was analyzed using 4 Simple Sequence Repeats (SSR) markers, which could display different profiles in Changshan Huyou and Cocktail grapefruit. The morphological differences were compared using one-year-old seedlings from offsprings and parents. **【Results】** The pollen grains of Cocktail grapefruit had a staining activity of 89.40% and *in vitro* germination rate of 30.02%, indicating that the pollen of Cocktail grapefruit had satisfactory vitality for further pollination. As a result, a total of 40 flowers of the tetraploid Changshan Huyou were used in pollination, and 19 hybrid fruits were harvested in November with a fruit-setting rate of 47.5%. A total of 56 developed seeds (average 3.0 seeds per fruit), 117 small developed seeds (6.2 seeds per fruit), and 57 undeveloped seeds (3.0 seeds per fruit) were obtained, and 49, 69 and 4 seedlings were germinated *in vitro* with an average germination rate as 87.50%, 58.97% and 7.02% for these seeds, respectively. Ploidy analysis showed that 39 (31.96%) and 83 progenies were proven triploids and tetraploids, respectively. Among the offsprings of each group, a total of 2, 35 and 2 triploid seedlings were identified from the developed seeds, small developed seeds and undeveloped seeds, respectively. The majority (89.74%) of triploids were originated from the small developed seeds. The hybrid nature of all the 39 triploids and 1 out of 22 tetraploids randomly determined were confirmed by the SSR marker of F14, P72, MEST86 and CAG01, and were indicated as the characteristic stripes of both Changshan Huyou and Cocktail grapefruit. The remaining 21 tetraploids were confirmed to be derived from selfing or nucellus embryos due to the presence of the characteristic stripe of the female parent. The leaf shape index of male parents, female parents, triploid F_1 seedlings ($3x$), and a tetraploid F_1 seedling ($4x$) were 1.73, 2.00, 1.83 and 1.65, respectively. The triploid and tetraploid offsprings obtained from hybridization were closer to the tetraploid female parent in terms of leaf shape index, without statistical difference from the female parent but with significant difference ($p < 0.05$) from the male parent. Leaf shape index of $3x$ hybrids was between $4x$ and $2x$ parents, however, the leaves of the $4x$ hybrid were wider and rounder than $4x$ female parent. The leaves of $4x$ hybrid offspring were significantly wider than the leaves of $3x$ hybrids. **【Conclusion】** The tetraploid Changshan Huyou was used as the maternal parent and hybridized with diploid pollen of Cocktail grapefruit in this study. After culture *in vitro*, ploidy identification and SSR molecular marker analysis, a total of thirty-nine heterozygous triploid offsprings were obtained, which mainly germinated from small developed seeds harvested from hybrid fruits in $4x \times 2x$ interploidy cross, providing an effective strategy to ensure the seedless germplasms in citrus breeding by ploidy hybridization.

Key words: Cocktail grapefruit; Huyou; Ploidy breeding; Triploid; Tetraploid; SSR markers

葡萄柚风味独特,可用作鲜食和加工,是一种具有市场潜力的柑橘品种。鸡尾葡萄柚(*Citrus paradisi* 'Cocktail')由暹罗甜柚和弗鲁亚橘杂交选育而来^[1],具有低酸,水分多,苦味轻的特点,受到消费者青睐,目前在浙江、广西、广东、江西、上海、湖北等长江以南地区有引种栽培^[2]。但鸡尾葡萄柚存在种子多、抗寒性差的缺点,单果种子数约30粒,不便鲜食。另外,鸡尾葡萄柚树体抗冻性弱,在低于-5℃环境下容易遭遇冻害^[3],在一定程度上阻碍了其扩大引种和栽培生产。

三倍体植株具有果实无核或少核的特征,作为多倍体,还具有生长旺盛、果实增大、抗逆性增强等优势。在柑橘育种中,通过四倍体和二倍体倍性杂交是获得三倍体无核后代的有效途径,选取优良且具有特定性状的亲本可以将亲本优势转移到子代^[3]。刘承浪等^[4]以早熟且高糖低酸的品种东试早柚作为二倍体母本,与3个四倍体柑橘品种杂交,培育并鉴定了128株三倍体杂交后代,以获得兼具双亲优良性状的无核新种质。为选育无核、抗寒、抗病的高酸柑橘品种,Viloria等^[5]使用柠檬及酸橙四倍体种质与二倍体进行杂交,从35个倍性杂交组合中获得了近650株三倍体植株。西班牙从1996年起,针对柑橘果实无核和丰富果实成熟期等育种目标,通过倍性杂交,培育了Garbi、Safor、Alborea、Albir等三倍体品种,满足了市场对无核柑橘周年供应的需要^[6]。近几年来,华中农业大学以异源四倍体体细胞杂种以及双二倍体为父本与大量性状优良的二倍体柑橘进行杂交,配置了80余个倍性杂交组合,创制了3000余份三倍体植株,为后续筛选优质无核柑橘新品种提供了丰富的三倍体资源^[7]。

常山胡柚(*C. aurantium* 'Changshanhuoyou')为中国特色地方柑橘品种,具有果实品质优良以及抗寒性强等特点,种子为单、多胚混合型^[8]。为弥补鸡尾葡萄柚种子多和树体不耐寒的缺点,以笔者课题组前期创制的胡柚双二倍体为母本、二倍体鸡尾葡萄柚为父本进行倍性杂交。通过杂交授粉、种子组织培养、幼苗流式细胞仪鉴定倍性和SSR分子标记鉴定遗传组成,培育并鉴定出了胡柚与鸡尾葡萄柚的三倍体和四倍体杂交后代,为选育抗寒、低酸、无核的杂种葡萄柚新品种奠定了种质基础。

1 材料和方法

1.1 材料

以定植于浙江农林大学校园内的四倍体胡柚(*C. aurantium* 'Changshanhuoyou')为母本、二倍体鸡尾葡萄柚(*C. paradisi* 'Cocktail')为父本,进行倍性杂交,创制三倍体无核新种质。

1.2 花粉采集与活力测定

鸡尾葡萄柚花粉收集参考谢善鹏^[9]的方法,在4℃避光环境下短时间保存备用。授粉前使用亚历山大染色法^[10]和花粉离体萌发法对鸡尾葡萄柚花粉活力进行检测,确保花粉活性足以进行杂交。花粉离体萌发法参考肖金平等^[11]的方法并作适当修改。将花粉均匀涂抹于含10%蔗糖+0.01%硼酸+0.03%CaCl₂+1%琼脂的固体萌发培养基上,用湿盒保湿,(25±1)℃温度下暗培养20h。各试验统计花粉总数在500个以上,花粉活力/%=有活力的花粉/花粉总数×100。

1.3 杂交授粉及胚培养

4月中旬在四倍体胡柚开花期挑选晴朗天气进行人工授粉,授粉参考解凯东等^[12]的方法。11月中旬果实成熟期,采集授粉得到的果实带回实验室剥取种子。根据种子大小以及发育程度,将其分为大种子(大小正常、内部充实)、小种子(大小约为正常种子1/6~1/3、内部充实)和败育种子(种子内部败育),并分别统计数量。所有种子用1 mol·L⁻¹ NaOH洗去表面残留果胶,流水冲洗3~4h后,在无菌环境下将大种子和小种子接种于MT培养基中,败育种子接种在MT+1.0 mg·L⁻¹ GA₃的培养基中。培养环境:温度(26±1)℃,湿度70%左右,日光照16h。待植株长出4~5枚功能叶和完整的根系时,将其移栽至温室继续培养。

1.4 再生植株倍性鉴定

再生植株的细胞倍性通过流式细胞仪(Cy-Flow® Ploidy Analyser, Sysmex, Germany)鉴定,样品处理使用CyStain™ UV Precise P倍性分析试剂盒(Sysmex, Germany),具体方法如下:以二倍体植株作为对照,用剪刀取0.5 cm²左右的新鲜叶片,置于塑料培养皿,在样品周围均匀加入200 μL细胞核裂解液(CyStain™ UV Precise P Nuclei Extraction Buffer),迅速用锋利的刀片切至匀浆,随后加入800 μL荧光染液(CyStain™ UV Precise P Staining Buffer)混

匀。混液用 50 μm 细胞滤网过滤至上样管,使用流式细胞仪上样分析。检测完毕后,检测结果使用 Origin 2021 进行数据分析以及作图。

1.5 基因组 DNA 提取与遗传来源分析

使用 CTAB 法提取叶片基因组 DNA,参考程运江等^[13]的方法并作适当修改。提取的 DNA 使用 1% 琼脂糖凝胶电泳检测后置于 $-80\text{ }^{\circ}\text{C}$ 冰箱保存。采用 SSR 分子标记鉴定后代遗传组成,4 对 SSR 引物源自

相关参考文献(表 1),由杭州有康生物科技有限公司合成。PCR 体系(10 μL)为:无菌水 2 μL ,正反向引物各 1 μL ,DNA 模板 1 μL , $2\times$ Accurate Taq 酶 5 μL 。PCR 扩增程序为:94 $^{\circ}\text{C}$ 预变性 5 min;94 $^{\circ}\text{C}$ 变性 1 min;58 $^{\circ}\text{C}$ 退火 30 s;72 $^{\circ}\text{C}$ 延伸 30 s;33 个循环后 72 $^{\circ}\text{C}$ 延伸 10 min,10 $^{\circ}\text{C}$ 保存。扩增产物加入 2 μL 6 \times Loading Buffer(TaKaRa)混合,使用 12% 聚丙烯酰胺凝胶电泳分离条带。

表 1 SSR 引物信息

Table 1 Information of SSR primers

SSR 引物名称 SSR primer name	正向序列(5'-3') Forward sequence (5'-3')	反向序列(5'-3') Reverse sequence (5'-3')	参考文献 Reference
F14	GCTCCTCGAATGAGAATGAAATGA	TGGTTGTGCGAAAATGAAGAGATA	[14]
P72	GTGAGGCAAAACGAAAAGAG	GGGCCATACAACGTAGAAG	[15]
MEST86	CCTCTCTGGCTTCTGGATTG	CCAAC TGACACTAATCCTCTTCC	[16]
CAG01	AACACTCGCACCAAATCCTC	TAAATGGCAACCCAGCTTTG	[17]

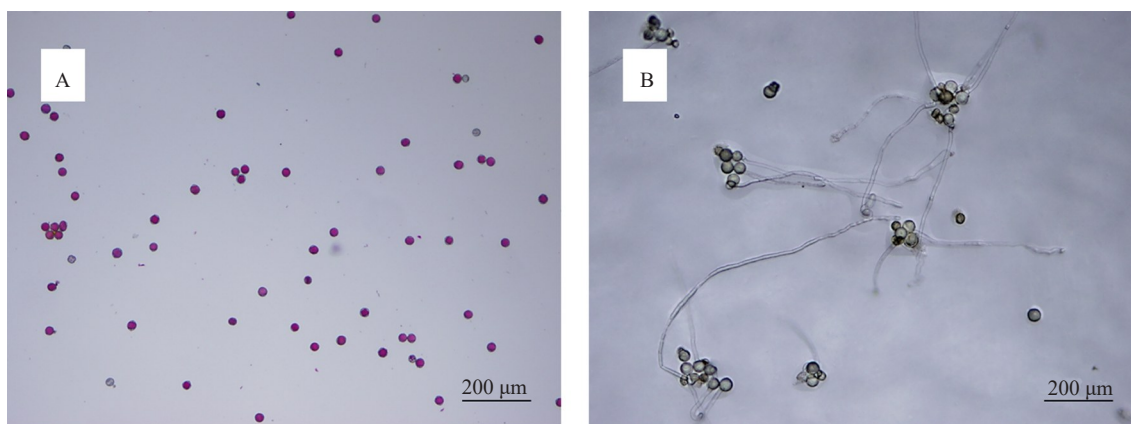
1.6 杂交后代形态学比较

杂交后代以父母本 1 年生实生苗为对照,对其植株上春梢成熟叶片的形状、长宽、叶形指数进行比较和测量。叶形指数计算公式为:叶形指数=主叶长度/主叶宽度,使用 SPSS 27.0 对计算结果进行多重比较和方差分析。

2 结果与分析

2.1 花粉活力测定

采用亚历山大染色法和花粉离体萌发法对父本鸡尾葡萄柚花粉活性进行检验(图 1),亚历山大染色法检测的花粉染色活力为 89.40%,花粉离体萌发



A. 亚历山大染色法;B. 花粉离体萌发法。

A. Alexander staining; B. Pollen germination *in vitro*.

图 1 两种方法下的花粉活力检测结果

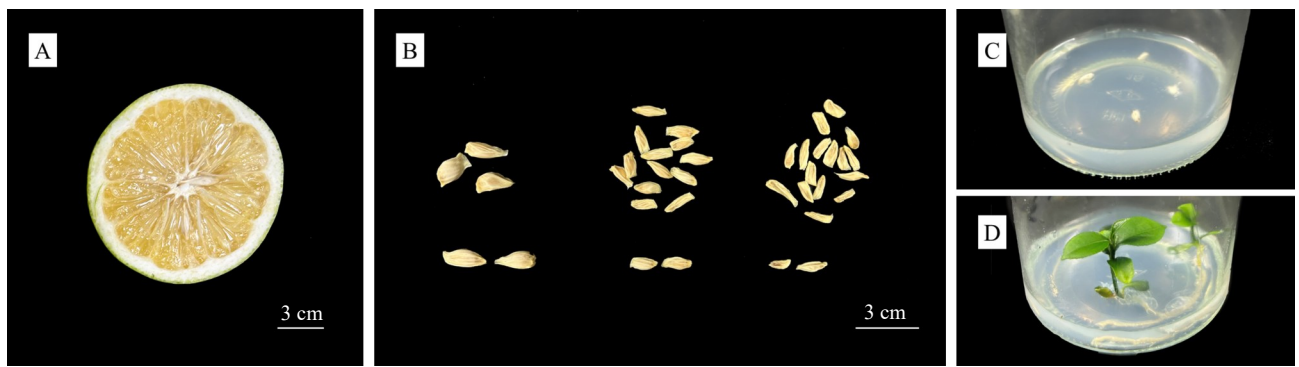
Fig. 1 Results of pollen vitality detection by two methods

法检测的花粉萌发率为 30.02%。两种检测方法均表明鸡尾葡萄柚花粉具有良好的花粉活力,能作为父本用于杂交。

2.2 杂交授粉结果

在四倍体胡柚花期使用鸡尾葡萄柚花粉授粉,共授粉 40 朵花,坐果 19 个,坐果率为 47.5%。11 月

采收授粉的果实后共获得种子 230 粒,种子分组后再接种于培养基内培养成苗(图 2)。其中大种子 56 粒(平均每果 3.0 粒),再生植株 49 株,成苗率 87.50%;小种子 117 粒(平均每果 6.2 粒),再生植株 69 株,成苗率 58.97%;败育种子 57 粒(平均每果 3.0 粒),再生植株 4 株,成苗率 7.02%。



A. 杂交果实; B. 种子分类(左-右: 大种子、小种子、败育种子); C. 播种; D. 出苗。

A. Hybrid fruit; B. Seeds (left-right: developed seeds, developed small seeds, undeveloped seeds); C. Sowing on the culture medium; D. Emergence of seedlings.

图2 胡柚(4x) × 鸡尾葡萄柚(2x) 杂交果实、种子分类及植株再生过程

Fig. 2 Fruits of Huyou (4x) × Cocktail grapefruit (2x), seeds sorting and regeneration of hybrid progenies

2.3 再生植株倍性分析

采用流式细胞仪对杂交获得的122株再生植株进行倍性分析(图3)。在122株检测植株中,有39株为三倍体,83株为四倍体,三倍体后代数量占31.96%。其中,大种子再生后代有4.08%为三倍体,

共2株;小种子再生后代有50.72%为三倍体,共35株。败育种子仅获得4株再生植株,其中三倍体后代2株,四倍体后代2株。在所有再生植株中,三倍体主要集中于小种子的再生后代,且数量和比例远高于其他两个种子类型。

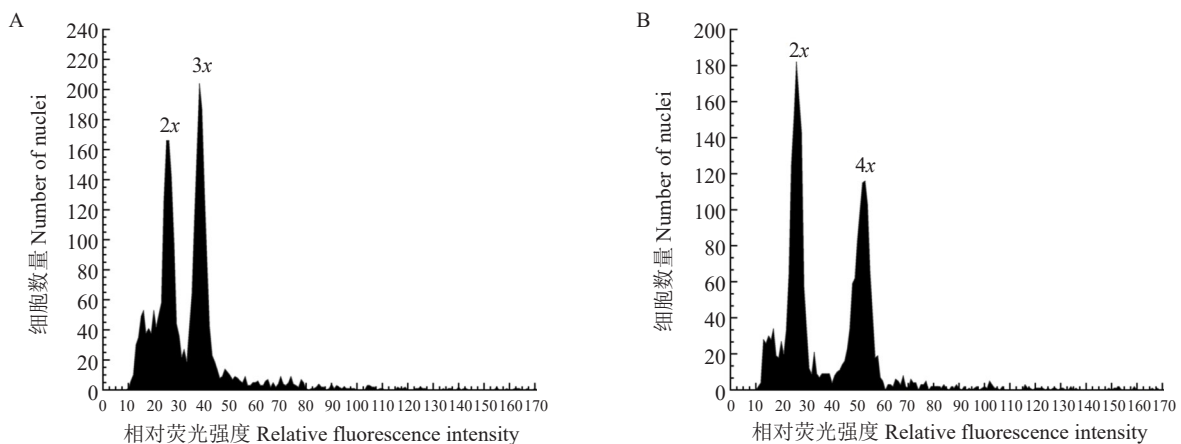


图3 三倍体后代和二倍体混样(A)以及四倍体后代和二倍体混样(B)的流式细胞仪峰图

Fig. 3 Ploidy analysis result of triploid(A) and tetraploid(B) progenies by flow cytometry with diploid as a control

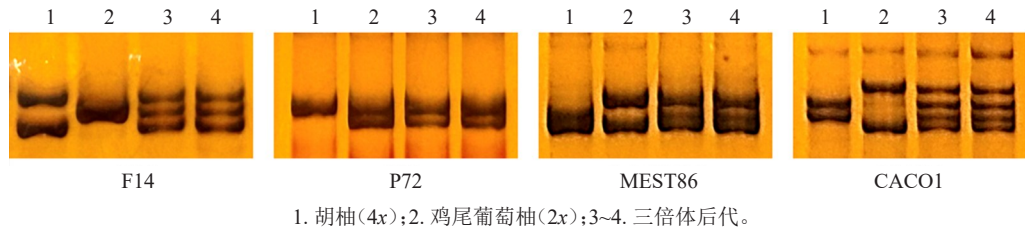
2.4 再生植株遗传来源分析

对再生获得的39株三倍体植株,以及随机挑选的22株四倍体植株,使用4对SSR引物进行杂合性分析(图4)。其中,SSR引物P72、MEST86、CAG01分别仅能鉴定部分后代,而SSR引物F14在鉴定的39株三倍体后代中均含有双亲差异条带。结果显示,所有三倍体后代均含有父母本的特征条带,为四倍体胡柚和鸡尾葡萄柚杂交所得。在检测的22株四倍体后代中,21株只含有母本特征条带,并非通过鸡尾葡萄柚杂交所得,仅有1株(7号)包含了父母

本的特征条带,为杂交产生的四倍体(图5)。

2.5 杂交后代植株形态及叶片特征比较

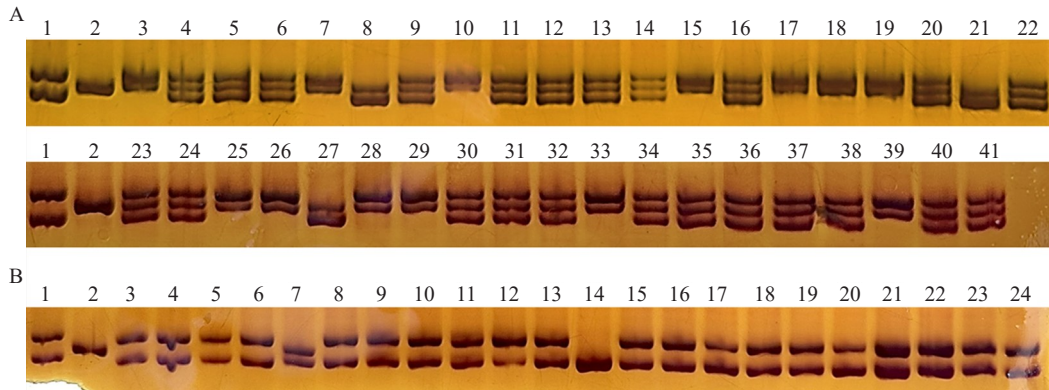
杂交后代以双亲1年生实生苗为对照进行植株形态与叶片形态对比(图6)。母本、父本、3x杂交后代以及本次获得的1株4x杂交后代的叶形指数分别为1.73、2.00、1.83、1.65。杂交获得的三倍体和四倍体后代叶形指数更接近四倍体母本,与母本差异不显著,但都与父本有显著差异。三倍体杂交后代叶形指数介于父母本之间,但本次鉴定得到的1株杂合四倍体植株叶片相对于四倍体母本更宽、更接近



1. 胡柚(4x);2. 鸡尾葡萄柚(2x);3~4. 三倍体后代。
1. Huyou (4x); 2. Cocktail grapefruit (2x); 3-4. Triploid progenies.

图 4 不同 SSR 引物对三倍体后代的遗传鉴定

Fig. 4 Identification of the genetic composition of triploid progenies by SSR marker

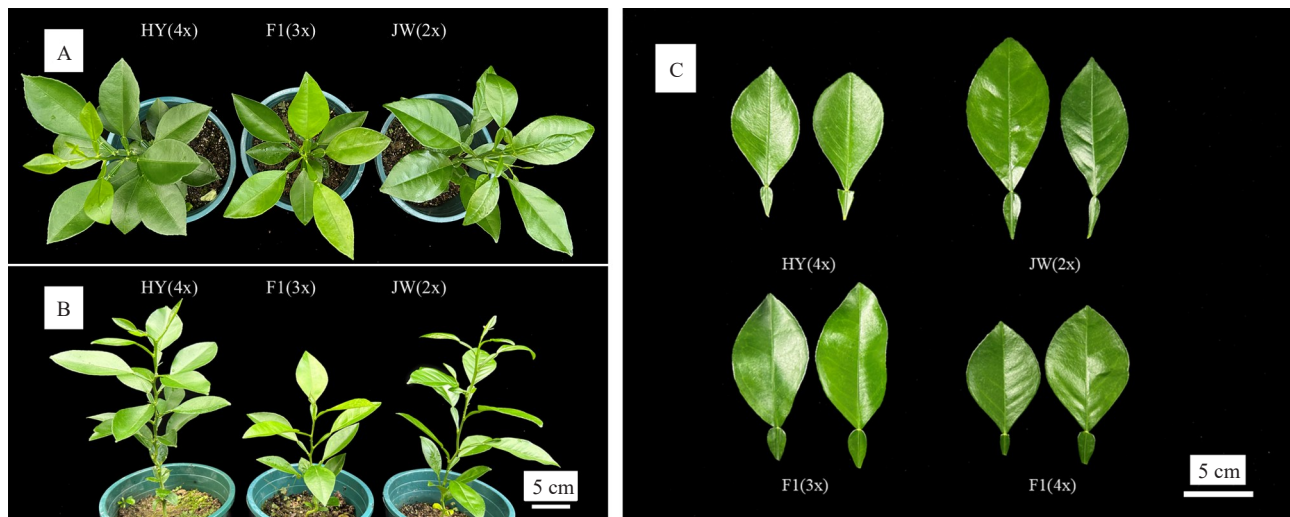


A. 三倍体后代鉴定结果[1. 胡柚(4x);2. 鸡尾葡萄柚(2x);3~41. 三倍体后代];B. 四倍体后代鉴定结果[1. 胡柚(4x);2. 鸡尾葡萄柚(2x);3~24. 四倍体后代]。

A. Identification of genetic composition of triploid progenies [1. Huyou (4x); 2. Cocktail grapefruit (2x); 3-41. Triploid progenies]; B. Identification of genetic composition of tetraploid progenies [1. Huyou (4x); 2. Cocktail grapefruit (2x); 3-24. Tetraploid progenies].

图 5 SSR 引物 F14 遗传鉴定结果

Fig. 5 Identification of the genetic composition using SSR marker F14



A, B. 3x 后代与亲本植株形态对比;C. 叶形对比[HY(4x). 胡柚(4x);JW(2x). 鸡尾葡萄柚(2x);F1(3x). 三倍体杂交子代;F1(4x). 四倍体杂交子代]。

A, B. Comparison of plant morphology of 3x progeny and parents;C. Comparison of leaf morphology [HY (4x). Huyou (4x); JW (2x). Cocktail grapefruit (2x); F1 (3x). Triploid hybrid progenies; F1 (4x). Tetraploid hybrid progenies].

图 6 杂交后代与亲本植株形态和叶片比较

Fig. 6 The plant and leaf morphology of hybrid progenies compared with parents

圆形(表2),且叶形指数显著小于三倍体。

表2 叶片形态指标比较
Table 2 Comparison of leaf indices

材料 Material	叶长 Leaf Length/cm	叶宽 Leaf width/cm	叶形指数 Leaf index
胡柚(4x)Huyou (4x)	8.99±0.89 b	5.20±0.52 b	1.73±0.12 bc
鸡尾葡萄柚(2x) Cocktail grapefruit (2x)	11.11±0.84 a	5.55±0.36 ab	2.00±0.09 a
F ₁ (3x)	9.78±0.84 b	5.37±0.47 b	1.83±0.13 b
F ₁ (4x)	9.98±1.30 b	6.02±0.74 a	1.65±0.11 c

注:不同小写字母表示差异显著($p < 0.05$)。

Note: Different small letters indicate significant difference at $p < 0.05$ level.

3 讨论

倍性杂交除了获得三倍体后代的无核特性外,亲本选配对获得符合育种目标的杂交后代有很大影响。植物抗寒性受基因的影响。章文才等^[18]研究发现,在来自不同亲本组合的柑橘自然杂交后代中,F₁代的抗寒性往往介于亲本之间,推测选择抗寒性较强的亲本可有机会提升后代的抗寒能力。通过抗寒性强的种质培育品质优良的抗寒后代在葡萄育种中已有大量实践以及成功案例^[19]。成年常山胡柚枝条致死温度在-13.7℃^[8],在冬季表现出较强的抗冻性。由于三倍体植物在解剖学及细胞学特性、光合特性和代谢产物等方面表现出良好的抗逆性能^[20-21],因此,鸡尾葡萄柚异源三倍体树体的抗冻性将可能得以提高。另外,鸡尾葡萄柚和胡柚的果皮和果肉内均含有丰富的类黄酮成分^[22],且均为低呋喃香豆素含量的品种^[23],杂交后代有望结出类黄酮含量较高、呋喃香豆素含量低的果实。

4x × 2x 杂交组合下,三倍体胚一般发育正常,无需提前进行胚挽救离体培养,与2x × 4x组合相比,在三倍体后代的获得上具有更高的效率,节约大量人力和物力。该杂交组合下获得的三倍体种子内胚和胚乳的倍性比为3:5,影响了胚发育的起止时间,导致其种子大小一般只有正常种子的1/6~1/3,故小种子可以作为筛选三倍体种子的特征之一^[24]。在本研究中,种子收集后根据大小以及发育程度将其分为大种子、小种子和败育种子,以观察种子形态与再生植株倍性的关联性。Esen等^[25]以Lisbon柠檬等四倍体柑橘为母本,授以四倍体花粉时,获得的种子全部或绝大部分为正常大小的种子;而在授以二倍体花粉时,所获得的种子全部为小种子。Aleza

等^[26]进行了多个4x × 2x柑橘组合的杂交,杂交果实中99.3%为小粒种子,且再生后代绝大部分为三倍体。在本研究中,89.74%的三倍体出现于小种子中,与前人研究结果基本一致。但小种子中仍有近一半的再生后代为四倍体,且在大种子中也获得了2株三倍体,表明不同基因型的柑橘作为亲本进行倍性杂交,三倍体后代不完全来自小种子,小种子发育而来的植株也不一定全部为三倍体。

简单重复序列(simple sequence repeat, SSR)标记是一种共显性遗传的分子标记,具有重复性好、多态性高、操作简单等优点,可用于鉴定杂交后代,提高育种效率。在本研究中,再生的39株三倍体后代均包含父母本的特征条带,可判断它们都是胡柚与鸡尾葡萄柚的杂交后代。除三倍体外,种子再生后还获得了部分四倍体。鉴定的22株四倍体中,21株仅含有母本胡柚的特征条带,非双亲杂交产生,来源可能为:(1)胡柚种子约60%为多胚^[8],四倍体后代为珠心胚再生而来。但试验中未对胡柚种子进行单多胚分级,无法较为准确地判断四倍体后代来自珠心胚还是为有性后代。(2)部分四倍体后代为母本自交而来,等位基因出现重组分离,如四倍体后代中的第14号单株。本研究中大部分果实内同时含有大种子和小种子,其中大种子再生后绝大部分为四倍体。Esen等^[25]在研究中同时授以四倍体母本4x及2x的混合花粉,也出现了大种子和小种子同时产生的情况,本次授粉过程中部分花的柱头可能受到了母本花粉的污染,导致同一果实内同时产生了四倍体和三倍体后代。二倍体柑橘中存在2n卵和2n花粉,四倍体在减数分裂时也可能产生n、2n和3n配子^[27],本试验中有1株四倍体后代包含了父本和母本的特征条带,且仅含有一条来自母本的等位基因,推测可能是由鸡尾葡萄柚未减数的2n雄配子与四倍体胡柚的2n雌配子杂交而来,四倍体后代详细的遗传来源还有待进一步研究。

4 结论

笔者在本研究中以四倍体胡柚为母本,与二倍体鸡尾葡萄柚有性杂交,经组织培养、流式细胞仪鉴定倍性、SSR分子标记鉴定遗传组成,共获得39株杂合三倍体后代和1株杂合四倍体后代,为今后筛选优质、耐寒、无核的杂种葡萄柚品种提供了种质基础。

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