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柑橘体细胞胞质遗传及叶绿体SSR引物开发*

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以38个组合的柑橘体细胞杂种(或胞质杂种)为试验材料,综合应用RFLP、CAPS和cpSSR分子标记技术,对这些杂种的线粒体和叶绿体遗传组成进行了分析;同时对试验技术体系进行了完善与拓展,开发了柑橘叶绿体SSR标记;并对柑橘愈伤组织长期继代保存过程中胞质基因组遗传变异进行了分析,主要结果如下:

1.完善了柑橘类作物的DNA提取方法。发现在DNA提取过程中高浓度NaCl结合水饱和乙醚处理能有效去除多糖,获得高纯度的DNA样品。用此方法能获得衰老、萎蔫或霜冻柑橘叶片的高质量DNA样品。在其他30余种热带、亚热带果树作物中试验也同样有效,证明该方法对这些富含蜡质层和多糖的作物普遍适用。

2.对4个属间组合的体细胞杂种和3个种间组合的胞质杂种(或体细胞杂种)的5个叶绿体区域和3个线粒体区域进行了CAPS分析。4个属间体细胞杂种组合检测到了线粒体多态性位点;而在3个属间组合和2个种间组合中检测到了叶绿体多态性位点。所有杂种的线粒体多态性位点与悬浮系亲本一致,叶绿体与叶肉亲本或悬浮系亲本一致。

3.用5种限制性内切酶对38个组合的杂种DNA进行酶切,并与12个线粒体探针随机组合进行RFLP分析。结果表明:柑橘体细胞杂种线粒体的来源偏向于悬浮系亲本,且普遍发生遗传重组;杂种线粒体的遗传组成受核背景的影响,异源四倍体体细胞杂种较二倍体胞质杂种的线粒体的遗传组成更为复杂;双亲共有的线粒体区域很容易传递给杂种后代。另外,线粒体的传递能力与亲本的基因型有关,在起源与进化中越古老的品种,将其线粒体传递给后代的能力越强。

4.对伏令夏橙 + 宁波金柑的体细胞杂种在1 a中按月连续采样,进行RFLP分析结合田间观察,发现线粒体DNA的丢失会导致植株的枯死,表明体细胞杂种的生长异常与线粒体遗传不稳定性有关。

5.综合RFLP和CAPS分析结果,38个组合中仅有14个组合检测到了叶绿体多态性,整体上呈现随机分离的趋势。现有的标记无法完成对其余组合叶绿体来源的分析,必须寻求新的叶绿体标记方法。

6.开展了体细胞杂种及胞质杂种的线粒体和叶绿体基因组的RT-PCR分析。并对线粒体基因组进行了Northern杂交分析,发现体细胞杂种线粒体表达量高于融合亲本,而胞质杂种与亲本之间无明显差异。

7.根据黑松(*Pinus thunbergii*)、水稻(*Oryza sativa*)、烟草(*Nicotiana tabacum*)和拟南芥(*Arabidopsis thaliana*)叶绿体基因组序列设计的33对引物,对柑橘类作物的总DNA进行扩增,经过筛选得到了14对能特异扩增的引物,分别命名为SPCC1-SPCC14。随即开展了以下研究:

1)选用34份柑橘及近缘属材料进行扩增,检测到等位位点的数目为1～8个,平均位点数为3.54个,平均多态性信息量(PIC)值为0.356。聚类分析结果显示,34份材料聚为7类:枸橼类、来檬类、柚类、宜昌橙类、金柑类、枳类和宽皮柑橘类。

2)对SPCC1的17个片段和SPCC11的21个片段进行克隆、测序,并与烟草和拟南芥中引物原始模体序列进行比对,结果表明所有特异性扩增片段均来源于叶绿体基因组,SSR是多态性产生的直接原因。

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3)根据引物归类结果选择合适引物，对柑橘体细胞杂种的叶绿体基因组进行了分析，所有组合都检测到多态性，进一步证实了柑橘原生质体融合过程中叶绿体的随机分离，并且在金诺橘 + 锦橙，朋娜脐橙 + 枸头橙等组合中检测到叶绿体呈 1∶1 的分离。

4)应用这些引物对 31 种热带、亚热带果树的 64 份样品进行了初步筛选，得到了 1 批通用性很强的叶绿体 SSR 引物，能在柑橘类、柿类，香蕉类、番石榴类、番木瓜类、葡萄类等作物的近缘属或种间检测到多态性，为这些作物开展叶绿体 SSR 标记提供了新方法。

8. 选取 23 份愈伤组织材料，以对应的田间叶片作对照，对他们在长期继代保存过程中线粒体和叶绿体(质体)基因组进行了遗传稳定性的 RFLP 分析、组织切片观察、cpSSR 分析及线粒体向核基因组转移等研究。结果如下：

1)愈伤组织在保存过程中线粒体变异的几率很小，但线粒体 DNA 含量普遍增加，而叶绿体(质体)基因组 DNA 含量无明显变化。

2)cpSSR 分析发现，引物对 SPCC4 在愈伤组织中检测到比叶片多了 1 条谱带，Southern 杂交和序列测定均表明，该谱带为特异扩增产物，说明愈伤组织中质体基因组普遍存在短核苷酸序列插入现象。

3)用线粒体探针与核基因组 DNA 进行斑点杂交，结果表明愈伤组织中线粒体基因可能存在向核基因组转移的现象。

关键词　柑橘；体细胞杂种；线粒体基因组(mtDNA)；叶绿体基因组(cpDNA)；叶绿体 SSR(cpSSR)；愈伤组织；分子标记

Cytoplasmic inheritance of somatic hybrids and development of primers for cpSSR in *Citrus*

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In the present study, cytoplasmic inheritance of *Citrus* somatic hybrids and cybrids from 38 intergeneric and interspecific fusion combinations was analyzed with restriction fragment length polymorphisms (RFLPs), cleaved amplified polymorphic sequence (CAPS) and chloroplast simple sequence repeat (cpSSR) markers. The experimental procedures were modified and optimized. A novel marker, cpSSR, was developed in *Citrus* and other tropical fruit crops. Meanwhile, genetic variations of organelle from 23 *Citrus* calli were analyzed. The main results were as follows:

1. A simple and efficient method for genomic DNA extraction from woody fruit crops containing high level of polysaccharides was developed. This method involves a modified CTAB or SDS procedure employing a purification step to remove polysaccharides by using water saturated ether at the presence of 1.25-1.30 mol/L NaCl. The quality of DNA samples extracted with this method was suitable for PCR and RFLPs analysis and for long-term storage. In addition, this procedure was successfully applied in DNA isolation from the freezed or withered or senile leaves of *Citrus* and more than 20 kinds of tropical and subtropical fruit crops.

2. Five universal pairs of chloroplast DNA (cpDNA) primer and 3 universal pairs of mitochondrial DNA (mtDNA) primers amplified monomorphic fragments among 4 intergeneric hybrids and 3 interspefic fusion combinations. After digested by restriction endonuclease, polymorphic mitochondrial CAPS markers were displayed in the 4 intergeneric combinations, while polymorphic chloroplast CAPS markers were found in 3 intergeneric and 2 interspecific fusion combinations. The results showed that the specific bands of mitochondria among all the hybrids were identical with embryogenic suspension parents, while

the chloroplast were identical either with the mesophyll parents or with the embryogenic suspension parents.

3. Detailed RFLPs analysis of mitochondrial genome among the hybrids of 38 fusion combinations were performed through digesting the genomic DNA with 5 restriction endonucleases and then hybridizing with 12 mitochondrial probes, results revealed that the mitochondrial genome of *Citrus* somatic hybrids and cybrids was biasly derived from the embryogenic suspension parent with commonly occurred rearrangements. The characterization of mitochondrial genome recombination was influenced by nuclear background of the hybrids, and mtDNA composition usually existed in allotetraploid somatic hybrids was more complicated than that in diploid cybrids. The common mtDNA fragments in the fusion parents could be usually detected in its offsprings. Mitochondrial inheritability was correlated with the genotype of the fusion parents. The ancient ancestor had stronger mitochondrial transferability than its offspring had. When both fusion parents are evolutionarily later than *C. reticulata*, there had the possibilities of *C. reticulata* like mitochondrial RFLP banding pattern displayed in their hybrids. When modern genotype acts as embryogenic suspension parent and ancient genotype as mesophyll parent, the mitochondria in the fusion hybrids may derived biasly from the mesophyll parent.

4. Leaves from the somatic hybrid of 'Valencia' orange and 'Meiwa' kumquat were harvested monthly for a whole year and analyzed with RFLPs marker combined with the field observation. Results revealed that the loss of the mtDNA fragments was correlated with the plant died back, suggesting that the genetic instability of mitochondrial genome is one possible reasons for the growth abnormality of the somatic hybrids.

5. Combined the data of CAPS with RFLPs in chloroplast genome analysis, 14 out of the 38 fusion combinations showed some degree of polymorphisms, chloroplasts were randomly segregated among these combinations. To further study the origin of chloroplast genome among other combinations, new markers are required to be developed.

6. To characterize the organelle at the expression level, RT-PCR and Northern hybridization were used in mitochondria. Results showed that expression of mitochondrial genes in somatic hybrids was higher than that in their corresponding fusion parents, while no clear difference existed among cybrids and their parents.

7. Thirty-three pairs of cpSSR primers designed according to the chloroplast genomes of pine (*Pinus thunbergii*), rice (*Oryza sativa*), tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana* were amplified with *Citrus* genomic DNA. After strictly screened, fourteen specific primers were obtained and named as SPCC1 to SPCC14, and subsequent studies were performed as follows:

1) Thirty-four representative accessions of *Citrus*, *Fortunella*, *Poncirus* and some of their hybrids were selected and amplified with 14 SPCC primers. Loci among the 34 genotypes ranged from 1 to 8 with an average of 3.54, and the mean PIC value was 0.356. Results of neighbor-joining cluster based upon the cpSSR data showed that all the samples were divided into 7 clusters, namely citron (*C. medica*), lime (*C. aurantifolia*), pummelo (*C. grandiss*), Yichang papeda: (*C. ichangensis*), kumquat (*Fortunella* spp.), trifoliate orange (*Poncirus* spp) and mandarin (*C. reticulata*) clusters.

2) Seventeen fragments of SPCC1 and 21 of SPCC11 were cloned, sequenced and aligned withsequences of the original primer motif from tobacco and *Arabidopsis*, the outcomes revealed that all the cloned sequences were from the chloroplast genomes and the polymorphism was caused by mononucleotide repeat of poly (A) or poly (T).

3) According to the cpSSR classification results, suitable SPCC primers were selected to analyze the

chloroplast genomes among the hybrids of the 38 fusion combinations, random segregation of chloroplast in *Citrus* fusion hybrids was further proved, and 1 ∶ 1 segregation ratio were revealed in the fusion combinations of Kinnow tangerine＋Jincheng orange and Bonanza navel orange＋Goutou sour orange, respectively.

4) Sixty-four DNA samples belonging to 31 kinds of tropical and subtropical fruit crops were amplifiedwith the synthesized cpSSR primers, and a set of wide adaptable universal primers were screened. For instance, SPCC1 was rich in polymorphism among intergenera or interspecies, such as persimmon (*Diospyros kaki*) and dateplum persimmon (*Diospyros lotus*), banana (*Musa* spp.), guava (*Psidium* spp.), papaya (*Carica* spp.) and grape (*Vitis* spp.).

8. Twenty-three genotypes of calli adequately representing the *Citrus* species and related genera were selected, and corresponding leaves were used as the control to analyze the stability of mitochondrial and chloroplast (plastome) genome. RFLPs, histological section, cpSSR and mtDNA transferring from the cytoplasm to the nuclei were analyzed. The results were as follows:

1) Low frequency of mtDNA mutation existed in *Citrus* calli, while the mtDNA contents increased in individual mitochondria, and no such phenomena were observed in chloroplast genome.

2) Analyzed with cpSSR, novel bands were detected among most of the calli. Both Southern hybridization and sequencing proved that the bands were amplified uniquely, suggesting that short sequences insertion occurred frequently in *Citrus* calli plastid genomes.

3) Dot blotting analyses were performed by using nuclear DNA hybridized with mitochondrial probes. Results showed that mitochondrial DNA transfering from cytoplasm into nuclei occurred during long-term subculture.

Key words　*Citrus*; somatic hybrids; mitochondrial genome (mtDNA); chloroplast genome (cpDNA); chloroplast SSR (cpSSR); callus; molecular marker

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