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利用 RAPD 分子标记对番茄杂交种纯度的鉴定研究

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摘 要: 应用 RAPD(Randomly amplified polymorphic DNA)分子标记对番茄京丹 1 号和毛粉 802 的 F_1 代杂交种纯度进行鉴定的实验研究。该项研究使用了 10 个碱基的单随机引物和 10 个碱基的双随机引物进行扩增。在 60 个单引物扩增反应中获得 7 个京丹 1 号父本特有的核酸标记片段。但在 14 个双随机引物对京丹 1 号和毛粉 802 杂交组合的扩增反应中获得了 7 个京丹 1 号 F_1 代杂交种特有的核酸标记片段和 5 个毛粉 802 父本特有的标记带。实验结果显示,双引物的扩增反应对鉴定双亲亲缘关系极近的杂交种纯度较单引物扩增反应更有效。其中,京丹 1 号的 14 个标记片段在北京蔬菜研究中心,种子纯度检测室又进行了重复扩增实验。实验结果为 87%的 RAPD 标记可以在使用不同的 PCR 仪和不同来源的 Taq 酶的实验条件下得到。RAPD 分子标记技术对鉴定双亲亲缘关系极近的杂交种纯度是真实可靠的。

关键词: RAPD; 单引物; 双引物; 京丹 1 号; 毛粉 802; 杂交种纯度

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Identification of hybrid purity of tomato (*Lycopersicon esculentum*) using RAPD markers

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Abstract: To determine the genetic purity of two commercial tomato (*Lycopersicon esculentum*) hybrids, Jingdan No. 1 and Maofen 802, randomly amplified polymorphic DNA (RAPD) markers were applied to discriminate hybrids from their parental inbred lines. Single primer and two-primer RAPD reactions were assayed. Sixty single primers were applied to discriminate the Jingdan No. 1 and Maofen 802 hybrids and their parental inbred lines. Six of them generated polymorphic bands that were different between Jingdan No. 1 and its female line. But no informative products produced between Maofen 802 and its parental lines. Jingdan No. 1 and Maofen 802 were screened with 14 two-primer reactions. Three two-primer reactions showed polymorphic bands discriminated Jingdan No. 1 hybrid from its parental inbred lines and Maofen 802 hybrid from its female inbred lines. The result demonstrate that two-primer RAPD reactions are more effective than the single primer reactions. Six single primer reactions and three two-primer X04-K13, K13-G12, X04-K11 reactions were repeated in National engineering research center for vegetable, Seed purity testing laboratory. 87% paternal-specific markers and hybrid-specific markers in the first experiment, which discriminated Jingdan No. 1 hybrid from his parental were generated on another PCR machine, MJ Research PTC-200 and with a different source

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Taq polymerase purchased from Katara company.

Key words: *Lycopersicon esculentum*; RAPD; two primer; hybrid purity

1 Introduction

In F_1 seed production, hybrid production is achieved by manual emasculation and pollination. In such a complicated procedure, there is a chance for self pollination due to incomplete emasculation. Thus, it is essential to screen the genetic purity of F_1 hybrid seeds before they are provided to the hybrid seed market.

We determine hybrid purity based on morphological markers, but it takes a whole season and it is costly. It is often difficult to find sufficient characteristic morphological traits distinguishing hybrids from their parental inbred lines since the cultivars are sensitive to changes in environmental conditions. The level of variation among domesticated tomato varieties is very low, especially because parental inbred lines commonly have close relationships.

Other methods such as isozyme analysis and electrophoresis of seed storage proteins have been successfully developed (Bassiri 1976, Ladizinsky and Hymowits 1979). However, these techniques can not differentiate the closely related genotypes due to limited polymorphism.

Molecular markers such as RFLP, AFLP, SSR, RAPD have been applied for the discrimination of the closely related genotypes of parental lines and genetic purity testing of tomato hybrid varieties. (Livneh 1989, Hoshizum 1993, Arens 1995, Paran 1998). Comparing these methods, RFLP and AFLP are lengthy, costly, and labour intensive. Simple sequence repeat (SSR) requires primer information for specific sequence on a crop special genome. The RAPD technique is advantageous due to its rapidity, simplicity, and need for small amounts of genomic DNA. Its disadventages is lack of lab to lab reproducibility. The key factor is different PCR machines and a different source of Taq polymerase. Schierwater and Ender's (1993) results demonstrated different thermostable DNA polymerases may amplify different RAPD products. Macpherson *et al.* (1993) assay showed that RAPD assay is likely influenced by multiple factors, especially that different PCR machines may produce very different data sets. So for Jingdan No. 1 the experiments were repeated in two labortories. The marker generated by K11, X04, X08, G09, G12, K13-G12, X04-K13, which discriminate hybrid from its parental line also repeated in the latter labortory. The reaction performanced in two different PCR machine and two source of Taq polymerase. The present study try some way to research the RAPD assay's reproducibility.

In the present study, we applied single primer and two-decamer primer sereening tomato. J. Hu *et al.* (1995) Southern analysis revealed that the two-primer reactions had no homology to the bands amplified in single-primer reactions involving the same primers. Furthermore, these new markers were not linked to markers amplified with the same primers in the standard RAPD reactions, suggesting that they were amplified from different genomic regions. And the two-primer RAPD tends to amplify more and smaller fragments than the standard RAPD technique. The present study investigated how to descriminate purity of hybrid seeds, and which one is more effective between the two-primer reactions and the single primer reactions. The results of this study indicated that the two-primer products is efficient for this aim.

2 Plant materials and Methods

2.1 Plant material

Two tomato (*Lycopersicon esculentum*) cultivars were commercial hybrid varieties, Jingdan No. 1(T26)and its two parental inbred lines designated T27 (male), T25 (female); Maofen 802(T19) and

its two parental inbred lines designated T21 (male), T20(female).

The young plants grew in the greenhouse for forty days, three fresh young leaves were collected from each single plant of every varieties. The total weight is about 30～40 mg. DNA from a single plant was isolated by a rapid method according to Dorokhov and Klocke (1996). The concentration of DNA was estimated by Pharmacia Biotech (Biochrom) Ltd. Spectrophotometer Ultrospec 2000. We bulked a pool from each single plant of every cultivars to ensure the uniformity of accessions. The concentration of a pooled DNA sample made from five of single plant DNA's was the same as that of each single plant DNA (10 ng/μL.)

2.2 PCR condition

The reactions were performed in a 12.5 μL solution containing Appligene 1x buffer (50 mM KCl, 10 mM Tris-HCl, pH8.8, 1% Triton X-100) 1.5 mM $MgCl_2$, 100 μM of each nucleotide, 0.2 μM primer, 0.25 Unit Taq DNA polymerase (Appligene Germany) or 1.0 Unit Taq DNA polymerase (Katara Japan, Sangon China) and 20 ng DNA. The reactions employing single decamer primer were conducted in sealed plates covered with plastic cover or 0.2 μL microtube and incubated for one cycle of 2 min. at 94 ℃, 45 cycles of 0.2 min. at 94 ℃, 1 min. at 36 ℃ (down-ramp rates was 50%), 1 min. at 72 ℃ (up-ramp rates was 50%), at the end, 9 min. at 72 ℃, then kept at 4 ℃. The reactions using two-decamer primer were incubated for one cycle of 0.5 min. at 94 ℃, 45 cycles of 0.5 min. at 94 ℃, 1 min. at 35 ℃ (down-ramp rates were 50%), 1 min. at 72 ℃ (up-ramp rates were 50%), 4 min. at 72 ℃, then kept at 4 ℃. The amplification reaction were performed in GeneAmp PCR System 9 700 (Perkin Elmer Corp. Norwalk. CT. USA) and MJ Research PTC-200 (MJ Research Inc., Watertown, MA, USA). After amplification, 5 μL of loading buffer (0.25% Bromphenol blue, 40 % sucrose in sterile bidest water) were added to each sample which was loaded into a 2.0% agarose gel in 0.5x TBE, pH8.0 (0.044 M Tris, 1.25 mM EDTA-Na2, 0.044 M boric acid) buffer submitted to electrophoresis at 3 V/cm. The amplified fragments were visualized by 0.2% ethidium bromide staining. The gel were saved in an image system with videocamera and computer software KS 400 (Zeiss Germany). First the primers were used to screen the parental line pools, then the amplifications which had been scored hybrid-specific and paternal-specific bands were repeated at least twice with DNA from the single plant both from hybrid variety and from single parental lines. Only bands that were intensely, reproducible, i.e., present or absent in both reactions were analysed.

2.3 Oligonucleotide primer

Sixty single primers (K01-K20, X01-X20, G01-G20; S371, S372, S373, S304, S374, S92, S89) were applied to discriminate Jingdan No. 1 hybrid and parental inbred lines. Twenty single primers (Y01-Y20) were applied to discriminate Maofen 802 hybrid and its parental inbred lines. Fourteen two-primer (K13-G12, K13-G09, G09-G12, Y06-Y07, Y01-Y06, Y01-Y07, Y01-Y10, K11-K13, X04-K13, X04-G09, X04-G12, K11-G09, K11-G12, K11-X04) were used for determining the two tomato hybrid varieties from their parental inbred lines. Only ten nucleotides primer were surveyed in this study. The primers (Roth Company, Germany. Sangon, Canada) discussed in this study are sited in Table 1. A GeneRulerTM DNA Ladder Mix (Fermentas, Germany) was used.

3 Results and Discussion

Maofen 802 was screened with twenty single primers, but there were no informative fragments that refered to paternal-specific and hybrid-specific markers generated

Hybrid 1 refers to informative bands of hybrid in the first experiments. Hybrid 2 refers to informative bands in repeated experiments.

Sixty single primers were used for Jingdan No. 1, primers K11, K12, K14, X04, X08, G09, G12

generated one paternal-specific markers, respectively. K11-1500, K12-1000, K14-300, X04-700, X08-1031, G09-700, G12-800 can distinguish hybrid T26 from female parent T25 (Table 1, Fig. 1). 12% of the primers reactions produced paternal-specific bands. A total of 182 fragments were produced, only 4% showed polymorphism.

Fourteen two-primer RAPD reactions were performed to distinguish the two tomato hybrid varieties from their parental inbred lines. Primer pairs K13-G12, X04-K13, X04-K11, generated two hybrid-specific bands, respectively, of sizes are 1250 and 1350 base pairs for K13-G12; 540, 820 and 900 base pairs for X04-K13; 1031 and 1200 base pairs for X4-K11. These fragments were able to distinguish hybrid T26 from its parents T27 and T25. (Table 1, Fig. 2).

Table 1 Primers used to discriminate two F_1 hybrids from their parental inbred lines in two experiments

Primer[1)]	Sequence	P^M	P^F	PM	PF	Hybrid 1	Hybrid 2
K11(S371)-1500	AATGCCCCAG	+	−	+	−	Jingdan No. 1 +	Jingdan No. 1 +
K12(S372)-900	TGGCCCTCAC	+	−	+	−	Jingdan No. 1 +	Jingdan No. 1 +
K14(S374)-300	CCCGCTACAC	+	−	+	−	Jingdan No. 1 +	Jingdan No. 1 +
K14(S374)-1000	CCCGCTACAC	−	−	+	−	Jingdan No. 1	Jingdan No. 1 +
X04(S304)-700	CCGCTACCGA	+	−	+	−	Jingdan No. 1 +	Jingdan No. 1 +
X08(S308)-1031	CAGGGGTGGA	+	−	+	−	Jingdan No. 1 +	Jingdan No. 1 +
G09(S89)-700	CTGACGTCAC	+	−	+	−	Jingdan No. 1 +	Jingdan No. 1 +
G12(S92)-800	CAGCTCACGA	+	−	+	−	Jingdan No. 1 +	Jingdan No. 1 +
S373-S92-1250	CAGCTCACGA			+	−		Jingdan No. 1 +
K13-G12-1350	GGTTGTACCC	−	+			Jingdan No. 1 +	
S373-S92-1350	CAGCTCACGA			−	+		Jingdan No. 1 +
X04-K13-900	CCGCTACCGA	+	−			Jingdan No. 1 +	
S304-S373-900	GGTTGTACCC			+	−		Jingdan No. 1 +
X04-K13-820	CCGCTACCGA	+	−			Jingdan No. 1 +	
S304-S373-820	GGTTGTACCC			+	−		Jingdan No. 1 +
X04-K13-800	CCGCTACCGA	−	+			Jingdan No. 1 +	
	GGTTGTACCC			−	+		Jingdan No. 1 −
X04-K11-600	CCGCTACCGA	+	−			Jingdan No. 1 +	
S304-S371-600	AATGCCCCAG			+	−		Jingdan No. 1 +
X04-K11-1031	CCGCTACCGA	−	+			Jingdan No. 1 +	
S304-S371-1031	AATGCCCCAG			−	+		Jingdan No. 1 +
K13-G12-1200	GGTTGTACCC	−	+			Maofen 802 −	
	CAGCTCACGA						
K13-G12-1100	GGTTGTACCC	−	+			Maofen 802 −	
	CAGCTCACGA						
X04-K13-620	CCGCTACCGA	+	−			Maofen 802 +	
	GGTTGTACCC						
X04-K11-600	CCGCTACCGA	−	+			Maofen 802 +	
	AATGCCCCAG						
X04-K11-1031	CCGCTACCGA	+	−			Maofen 802 +	
	AATGCCCCAG						

[1)] Primer number refers to Roth Ramdom Primer Kits and Sangon Canada. The number after primers refers to the band size in base pairs. P^M, PM = male parent; P^F, PF = female parent.

Primer pair X04-K13 generated the paternal-specific bands, X04-K13-620, that was different between Maofen 802 hybrid T19 and its female line T21. (Table 1, Fig. 3). Primer pair X04-K11 generated the bands, X04-K11-600 and X04-K11-1031 that were different between Maofen 802 hybrid T19 and both parents (T20, T21). (Table 1). K13-G12-1100 and K13-G12-1200 can distinguish Maofen 802 from its female parent (Fig. 3).

A total of 46 products performed by two-primer RAPD reactions were generated, 9 out of these fragments, i. e. 20% products showed polymorphism. 22% of the two-primer combination amplified the informative fragments.

RAPD technique has been developed for more

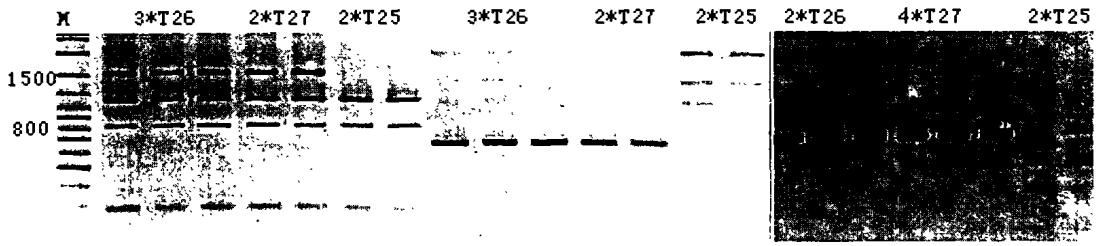

Fig. 1　The profiles generated by primer K11, G09, G12(from left to right)in Jingdan No. 1. T26: hybrid, T27: male parent, T25: female parent. M: GeneRulerTM DNA Ladder Mix. The number indacates molecular weight of polymorphic bands.

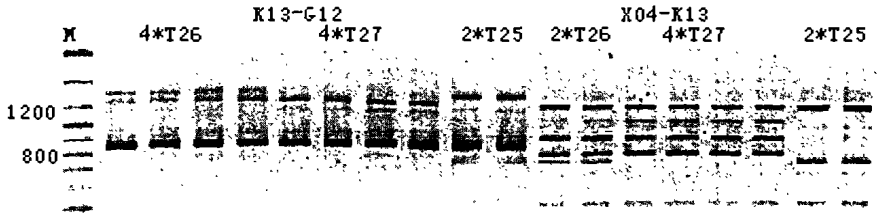

Fig. 2　The profiles generated by primer K13-G12, X04-K13 in Jingdan No. 1. T26: hybrid, T27: male parent, T25: female parent. M: GeneRulerTM DNA Ladder Mix. The number indicates molecular weight of hybrid-specific bands area.

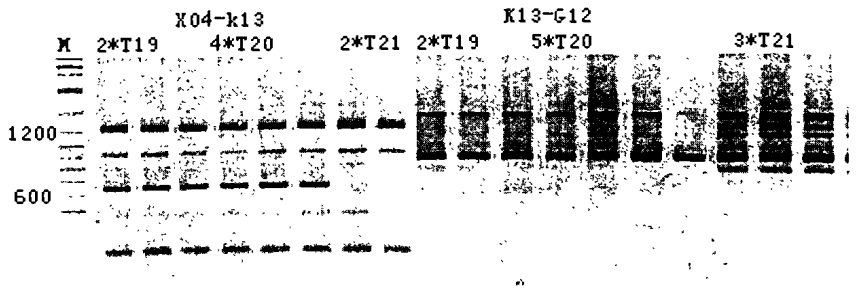

Fig. 3　The profiles generated by primer X04-K13, K13-G12 in Maofen 802. T19: hybrid, T20: male parent, T21: female parent. M: GeneRulerTM DNA Ladder Mix. The number indicates molecular weight of polymorphic bands area.

than 10 years. This study further investigated how to control the reaction condition which guarantee to get intensely and reliable RAPD markers. Macpherson *et al*. (1993) indicated that DNA concentrations between 10 ng and 30 ng and a primer concentration of 0.2 μM provided dependable amplification. Different thermal cyclers are generally capable of generating reliable, reproducible RAPD patterns. Since the RAPD assay is likely influenced by complicated factors because 10 oligonucleotide primers need a low annealing temperature. Different machines may produce very different data sets, so collaborating laboratories should consider this added variable when combining data generated using different machines. Research by Schierwater and Ender (1993) demonstrated that the outcome of a RAPD fingerprint pattern may depend on the type of polymerase used. In our experience, we al-

so need a standardization of the program as well as all of above mentioned factors. The present study investigated these problems of RAPD reactions in order to improve the RAPDs reproducibility. All of the paternal-specific markers and hybrid-specific markers were repeated in National engineering research center for vegetable, Seed purity testing laboratory. Primers were purchased from Sangon Company. Taq polymerase was coming from Katara Company (Japan). PCRs were performed using a Thermal Controller (MJ Research Inc., Watertown, MA, USA). We keep all of other factors including primers and DNA plates' concentration, Mg^{++} and dNTPs' concentration and performancing program were same as the first experiments. Primers X13-G12, X04-K13, X04-K11, X04, X08, K11, K14, K12, G09, G12 RAPD reactions generated 87% informative fragments of the first experiments. Only X04-K13 reaction failed to produce hybrid-specific band, X04-K13-800. K14 reaction produced another paternal-specific band, K14-1000. That mean collaborating laboratories could use the RAPD data sets.

The result used single primer suggested that there was a close relationship between the parental inbred lines of the two tomato hybrid varieties. The single primer amplification did not generate content informative fragments distinguished Maofen 802 hybrids from their parents. Sixty single primer reactions only produced 7 paternal-specific bands for Jingdan No. 1.

For this reason 14 two-primer reactions were used to amplify DNA fragments determined the hybrids from their parental lines and its number of reactions was achieved by possible combinations of the eight primers. After screening 14 two primers the reactions generated 7 hybrid-specific bands for Jingdan No. 1 and 5 paternal-specific bands for Maofen 802. Evidently the two primer reactions were more effective than the single primer reactions.

Hu *et al.* (1995) indicated a lack of hybridzation of single-primer to two-primer amplified products of different sizes suggested a lack of homology. So, two-primer reactions can amplify new, smaller, more informative fragments, and the amount of products were not increased so much that we can analysed clearly. Also, the two-primer RAPD increased the total number of reactions with a limited number of primers. 4% vs. 20% informative fragments indicated that two-primer reactions were more effective than single primer reaction. It is worth to develop its effectiveness.

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特别是扫描电镜方面提供微形态方面的资料，为系统学增加了新依据。

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