An Approach for Rapid Checking of Seed Purity by RFLP Analysis of Nuclear DNA in F₁ Hybrid of Cucumber (Cucumis sativus L.)

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Summary
In F₁ hybrid seed production, the elimination of contaminated self- and/or sib-crossed seeds from hybrid seeds is an important subject. We attempted to use RFLP analysis of nuclear DNA for checking the seed purity of the F₁ hybrid cucumber. Between the two parental lines which are promising for an F₁ cucumber cultivar, two RFLP clones were established. The frequency of RFLP clones in the genomic library was 1%. Two micrograms of DNA, which could be isolated from a 9-day-old seedling, were sufficient for RFLP detection with non-radioactive Southern hybridization. This system enabled a practical and a rapid check of the seed purity in an F₁ hybrid cucumber.

Introduction
In cucumber, the F₁ hybrid cultivars are advantageous because of the combined characteristics from two superior inbred lines. To produce the hybrid seeds, many female plants are crossed with a few male plants by hand pollination. The mistakes in artificial pollination sometimes cause the contamination of the self- and/or sib-crossed seeds of female lines in the hybrid seeds. At present, we have to check the purity of the harvested seeds by the field observation tests. However, these tests are costly and also risky, because the results may be affected by the conditions of cultivation and weather.

Instead of the field observation tests, the establishment of rapid laboratory techniques with high reliability and reproducibility have been desired and attempted. In some Cruciferous vegetables, certain isozymes were available are biochemical markers (Kim and Chang, 1986; Matsuura and Fujita, 1989; and Suurs, 1986). In the Cucurbitaceae species, Ishiki et al. (1992), Knerr et al. (1989), Perl-Treves et al. (1986) and Staub et al. (1985) reported polymorphisms of isozymes. However, there were only a few polymorphic loci within the parental inbred lines for F₁ hybrids (unpublished data).

We examined the availabilities of molecular techniques, i.e., restriction fragment length polymorphism (RFLP) in nuclear DNA and polymerase chain reaction (PCR) markers, for this purpose. In this report, we describe the establishment of RFLP clones which are available to detect the purity of the F₁ hybrid between two cucumber inbred lines. To establish a practical checking system using these RFLP clones, we examined the minimum amount of DNA for Southern analysis and the stage of the seedlings from which a sufficient amount of DNA was isolated.

Materials and Methods
1. Plant materials
We used two parental inbred lines of cucumber, NB-1 and GF-1, and the F₁ hybrid 'Aonaga-jibaifushinari', in this experiment. The F₁ hybrid is cultivated in the summer season in Japan. Sex expression in the cucumber is controlled by the multiple alleles of the locus acr (Kubicki, 1969). The female line, NB-1, is a gynoecious one with an acrF/acrF genotype. This line was derived from an F₅ plant between a gynoecious English forcing type, cv. Pandex, and a monoecious Japanese sum-
mer type, cv. Shindome, and shows a gynoeicous and Japanese summer type. The male line, GF-1, is a monoecious one with an acr⁺/acr⁺ genotype. This line is one of the Japanese local varieties in the Jibai group.

2. Construction of genomic and cDNA libraries

DNA and RNA were prepared from NB-1. Total DNA was isolated from fresh leaves by a protocol using cetyl trimethyl ammonium bromide (CTAB) solution (Murray and Thompson, 1980). The purified DNA was digested by Pst I. The digested fragments were separated by size using agarose gel electrophoresis, and the 0.5 ~ 1.5kb fragments were ligated with pUC18 vector digested by Pst I.

For the RNA purification, we sampled the growing leaves near the shoot tip. Plants were cultivated in a greenhouse and the sampling stage was about 30 days after sowing. Using the guanidine thiocyanate/CsCl method (Sambrook et al., 1989), the crude RNA was isolated from the leaves, and Poly(A)⁺ RNA was purified by oligo(dt)-latex (Nippon Roche Co.). The complimentary DNA was synthesized by a cDNA synthesis kit (Amersham Co.). The cDNA with Eco RI adapter was ligated with pUC18 vector digested by Eco RI.

Both libraries were transformed to JM109 bacterial competent cells. Probes for Southern analysis were isolated from both of the plasmid libraries by the alkaline lysis miniprep.

3. RFLP analysis

For whole genome Southern hybridization, DNA was isolated from the two parental lines using CTAB solution. They were digested by four restriction enzymes, Bam HI, Eco RI, Eco RV and Hind III. The digested DNA fragments were electrophoresed in 0.8% agarose gel with an 8 cm loading distance. After treatments of deprination (1N HCl, 5 min.) and alkaline denaturation (0.5N NaOH, 1.5M NaCl, 20 min.), the DNA fragments were transferred to a Hybond N+ nylon filter by capillary blotting. For the non-radioactive Southern hybridization, we used the ECL gene detection kit (Amersham Co.).

4. Determination of minimum amount of DNA for RFLP detection

DNA was isolated from the mature leaves of the F₁ hybrid, 'Aonagajibaifushinari' by the CTAB method. To prepare the filter of gradual amounts of DNA, 5.0, 4.0, 3.0, 2.0, 1.0, 0.5, 0.2 and 0.1μg DNA were digested by Eco RI and applied to agarose gel. The methods of blotting and Southern detection were the same as those mentioned above. An RFLP clone, P-061, was used for the probe of the Southern detection. This clone was established during the lead experiment in this study.

5. DNA isolation from different stages of seedlings

The seeds of F₁ hybrid 'Aonagajibaifushinari' were sown in a growth chamber controlled at 25°C, 24 hours day length. Four seedlings were sampled 3, 5, 7 and 9 days after sowing to isolate the total DNA by the CTAB method, and the yield of total DNA from each seedling was determined.

6. Preliminary checking of seed purity

The total DNA samples which were harvested from 9-day-old seedlings were digested by Bam HI. Using these samples, the filter was prepared for preliminary detection of purity checking. The probe for this detection was P-061.

Results and Discussion

1. Screening of RFLP clones

We had screened 196 genomic and 49 cDNA clones, respectively. Fifty-eight clones (29.6%) in the genomic library and 11 (22.4%) in the cDNA library did not show any signals on ECL Southern detection. The number of copies of these clones would be extremely few in the cucumber genome, so that we could not detect the signals by this non-radioactive Southern system. Patterns of hybridization with the remaining 138 genomic and 38 cDNA clones could be classified into four groups (Fig. 1). The first group, called "L", was characterized by ladder bands of weak hybridization from high to low molecular weight fragments. In this group, 35 genomic (17.9%) and 6 cDNA clones (12.2%) were found. The second group, "B", showed strong hybridization in certain fragment(s). In this group, 35 genomic (17.9%) and 6 cDNA clones (12.2%) were found. The third group was a type intermediate of "L" and "B", called "LB", which showed strong band(s) among the ladder of weak bands. In this group, there were 28 clones (14.3%) in the genomic and 8 ones (16.3%) in cDNA library. We classified the three groups,
“L”, “B” and “LB”, into monomorphic clones in this study, because we could not find clear singals of RFLPs. The last group, “PB”, showed RFLPs in combination with some restriction enzymes used. We obtained 2 RFLP clones (1.0%) in the “ PB” group from the genomic library, but the RFLP clone was not obtained from the cDNA library. One of the RFLP clone, P-051 (710bp), showed the polymorphism to the DNA digested by Hind III. The other one, P-061 (1050bp), showed the polymorphisms to the DNA digested by Bam HI, Eco RI and Eco RV. However the male line, GF-1, was the mixed line for the RFLP clone P-051. To use this clone for practical checking, the GF-1 must be purified. On the other clone, P-061, both parental lines were pure, so we used the P-061 only for the following experiments.

Using the RFLP clones, genetic maps were constructed in some plant species. In almost all of these studies, the distantly related varieties have been used for detection and mapping of RFLP. For example, in Brassica oleracea, Slocum et al. (1990) picked the cauliflower and cabbage, and in Brassica rapa, Song et al. (1991) used Chinese cabbage and spring broccoli. These strategies had an advantage for screening of the RFLP clones. For rice RFLP mapping, McCouch et al. (1988) assayed the varieties related distantly, Indica, Japonica and Javanica varieties; polymorphic clones were 58% at maximum. However, would these clones show the polymorphisms between some lines which were picked for the parental lines or recombination breeding programs? One of the purposes of this study was to clarify how often the RFLP clones could be isolated between the two lines which were picked by the plant breeders when some RFLP clones were wanted among the inbred lines maintained for their breeding materials. In this experiment, we used the inbred lines of cucumber, NB-1 and GF-1, as the model case for this purpose, and the rate of polymorphic clones between the two lines was clarified. Because the rate of the polymorphic clones has never been reported between closely related inbred lines, our data would be a useful standard for other pairs of inbred lines.

2. Establishment of the checking system

Based on the Southern detection using the P-061 clone, it was found that at least 2 μg of cucumber DNA was necessary (Fig. 2). It was also found that approximately 2 μg of DNA could be obtained from 7-day-old seedling (Table 1). In the experiment of Fig. 2, DNA was digested by Eco RI of which the digested fragments were the largest among three kinds of available restriction enzymes (Bam HI, Eco RI and Eco RV). If the largest fragment (11.2kb) of Fig. 2 is a single copy in the cucumber nuclear genome, the sufficient amount of DNA for electrophoresis could be theoretically calculated when a Southern hybridization system with the sensitivity less than 1pg is applied.

Amount of DNA for electrophoresis

\[
\text{Amount of DNA} = \frac{\text{Sensitivity of detection} \times \text{Genome size}}{\text{Size of target fragment}}
\]
This value was checked preliminarily.

Based on this calculation, the sufficient amount of DNA for electrophoresis was less than 0.1 μg, but 2 μg of DNA was needed for practical detection. This gap would result from the difficulty of the whole genome Southern hybridization.

As shown in Fig. 3, the smallest fragment (the 5.0 kb fragment digested by Bam HI) could be detected using 9-day-old seedlings and the purity was checked satisfactorily. In fact, we felt that the 9-day-old seedling was suitable to handle for this experiment.

From these results, it was clear that this checking system is available and convenient for practical use in purity testing of F₁ seeds. In this study, we examined only one F₁ hybrid of cucumber. But the data of this study will be the base to isolate the useful RFLP clones and to analyze the seed purity in other F₁ of Cucurbitaceae.

### Table 1. Yields of DNA from different stages of cucumber seedlings.

<table>
<thead>
<tr>
<th>Stage</th>
<th>No. of seedlings analyzed</th>
<th>Fresh weight (mg/Plant)</th>
<th>DNA yield (μg/Plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 days</td>
<td>4</td>
<td>170.0 ± 7.7</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>490.0 ± 9.7</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>761.6 ± 36.2</td>
<td>2.0 ± 0.8</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>1065.0 ± 33.3</td>
<td>2.9 ± 1.4</td>
</tr>
</tbody>
</table>

¹ Days after sowing.

² Mean ± S.E.

\[ \leq 1(\text{pg}) \times \frac{9.1 \times 10^6(\text{bp})}{11.2 \times 10^6(\text{bp})} = 0.081(\text{μg}) \]

1) This value was checked preliminarily.

2) Genome size of cucumber was obtained from Bennett and Smith (1976).


核 DNA の RFLP 分析によるキュウリ F1 品種の種子純度検定法の開発

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摘 要

RFLP マーカーをキュウリの F1 品種の種子純度検定に利用する上で必要となる、有効 RFLP クローン出現率、非放射性 DNA 検出システムにより検定する場合の必要 DNA 量ならびにその DNA 量が単離される生育ステージについて検討した。

F1 品種「青長地詰節成」の両親系統間で有効な RFLP クローンを見いだすために、母親系統のゲノミックおよび cDNA ライブラリーを作成して検索を行った結果、ゲノミックライブラリーのうち二つが RFLP クローンであることがわかった。RFLP クローンの出現率は 1% であった。cDNA ライブラリーからは RFLP クローンは見つからなかった。

キュウリ実生から CTAB 法で DNA を単離し、今回得られた RFLP クローンを用いて非放射性 DNA 検出システムにより検定する場合の必要 DNA 量は 2 μg であった。また 2 μg の DNA を単離できる生育ステージは、播種後 7 日目であったが、9 日目であれば取扱いも容易で充分量の DNA が単離できることが明らかとなった。以上の結果は、キュウリの一つの F1 品種と両親系統を用いてのモデル実験で得られたが、キュウリ実生の F1 品種について、種子の純度検定に RFLP マーカーを用いる際の基本データとなろう。