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Analysis of Fungal Genome
— Gene Analysis Technique using Orthogonal Field Alternation Gel Electrophoresis —

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眞菌의 게놈分析

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There is increasing trend among mycologists to investigate the precise genetic make-ups of diverse fungal taxon, especially when many of economically important fungi were used as primary model organisms of exploiting genetic manipulation for the production of such natural compound as antibiotics at the industrial scale.

Recently, a great deal of discussion and articles were devoted for the subject of modern biotechnology, molecular genetics and manipulation of genes in fungi. Many molecular markers for genetic analysis were exploited and introduced. For these described above, the readers could refer to recent review articles(1, 11, 16).

In this article, orthogonal field alternation gel electrophoresis(OFAGE) or Pulsed Field Gel Electrophoresis(PEGE) (3, 4, 5, 6, 13, 15) will be introduced and discussed; one of the new emerging techniques which is currently in use for the analysis of genome size and gene location(2, 8, 9).

This technique uses an agarose gel matrix to separate DNA molecule in a size range from 40 kb to 2,000 kb. The conventional gel electrophoresis separates DNA molecules up to 20 kb. The use of low percentage gel(0.3-0.5%) will be able to separate slightly larger DNA molecules of 50 kb in size. However, the electrophoresis uses a single pair of electrodes to generate the electric field. Such a field is to be uniform and oriented in a single direction. Also DNA molecules larger than 50 kb tend to co-migrate regardless of size.

Electrophoretic separation of DNA molecules larger than about 30 kb was extremely difficult until Schwartz and Cantor(13) demonstrated that separation of significantly larger DNA molecules could be achieved by forcing their periodic reorientation as they passed through pores in an agarose matrix. It has been found that periodic reversal of the polarity of the electrical field during electrophoretic run causes DNAs up to 1,000 kb to separate according to size in agarose gels. Reasons: Large DNA molecules can not migrate through a gel matrix under the influence of the electric field until they have oriented with a pore. Once oriented, the molecules move through the gel at the rate independent of size. Application of second electric field that acts at an angle to the first will push previously oriented molecules against a new set of pores in the gel matrix. The molecules can no longer migrate until they have reoriented with new pores. If these two electric fields are alternately activated over a period of time, molecules will be forced through a continuous cycle of orientation, migration and reorientation. Each molecule will spend a portion of the cycle time under going reorientation, with the remainder of time spent migrating. Because reorientation time is a function of molecular weight, smaller molecules will reorient faster and thus migrate faster than larger molecules. OFAGE devices are based on this principle(Fig.1).

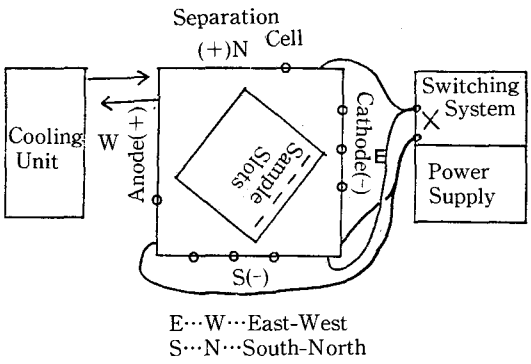

Fig.1. Schematic diagram of orthogonal field alternation gel electrophoresis apparatus(LKB 2015 model).

Separation of Genomic DNA of *Ustilago hordei*

In the author's laboratory, OFAGE(Pulsphor, LKB 2015) was used to separate chromosomal DNA in sporidia of *Ustilago hordei*. Most of the electrophoretic conditions used for this study was developed by us as there were not many published works on the separation of chromosomal DNA in fungi except yeast. Study on the separation of chromosomal DNAs of yeast and human genomes were carried out with OFAGE apparatus which was constructed by many investigators themselves according to the original design of Schwartz and Cantor(13).

The separation of fungal genomic DNA by OFAGE requires the following procedures.

1) Preparation of protoplasts-Chromosomal DNA to be analyzed should not be sheared during DNA extraction. To achieve this, the protoplasts were isolated and used. Conditions of protoplast isolation(0.6 M KCl, Novozyme 234 0.3-0.4%, 30℃, 60-90 min) vary with fungal materials used.

2) Embedding of protoplasts in low melting agarose block-The isolated protoplasts in osmoticum were embedded in an equal volumn of 2% low temperature melting agar in osmoticum and cast into 1.5-2.0 mm thick film of agar(final concentration of low melting agar-LMA 1%). There are other methods of preparing intact chromosomal DNA and embedding(7, 12).

3) Extraction of DNA-The thin-filmed agar block was treated with 260 mM EDTA in lysis buffer(150 mM NaCl, 50 mM EDTA, 10 mM Tris/HCl, pH 7.4, 20 mg/m*l* proteinase K) containing 1% sodium sarkasyl and additional proteinase K 1 mg/m*l*, and incubated at 50℃ for 24-48 hrs. The blocks were then washed 5 times with 5 m*l* TE buffer(10 mM Tris/HCl, 1 mM EDTA, pH 7.4), 5 min for each wash, finally TE buffer containing 1 mM phenylmethylsulfonylfluoride. The final preparation could be stored in 0.45 M EDTA, pH 8.0 at 4℃ for many months.

4) OFAGE run-Prepare 1-1.2% agarose gel in Tris/borate/EDTA buffer(22 mM Tris, 22 mM boric acid, 5 mM EDTA, pH adjustment is not necessary). Use teflon comb to mold the slot. Place a block(4 mm×1.5 mm×1 mm) of LMA containing protoplasts in the slot and semented with LMA, so that sample agar block should not be moved during the run. Gel running buffer is the same as the one used for the gel preparation.

5) The conditions of the electrophoresis very depending on the size of DNA molecules. By using the molecular size standard establish the running parameteres. For the separation of chromosomal DNA in sporidia of *Ustilago hordei*, it was set at 200 V(constant voltage), 4℃, pulsed 720 seconds, 72 hrs.

OFAGE separation of DNA of barley smut fungi is presented in Fig.2 and 3. Because of the large DNA fragments, the pulse was switched every 720 seconds for 72 hours at constant voltage 200 volts. Arrows in Fig.2 indicate chromosomal DNAs size range from 400 kb to 1,000 kb. The fast moving

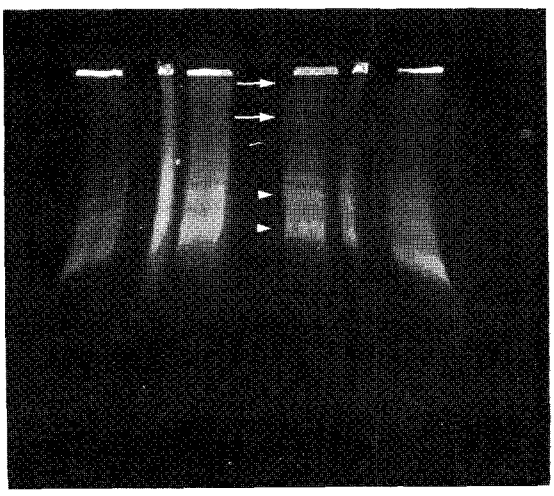

Fig.2. OFAGE of barley smut fungi DNA. See text for detail.

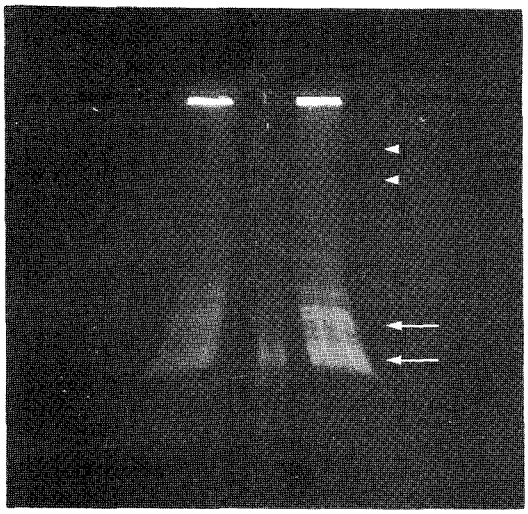

Fig.3. OFAGE of barley smut fungi DNA. See text for detail.

DNA fragments(arrowheads) ranges from 20 kb to 150 kb. By increasing running time from 72 hours to 96 hours, the resolution of DNA fragments of 20 kb to 150 kb increased(see arrows in Fig.3). These are not the perfect separation, but with improving the conditions, the good separation will be obtained.

The electrophoretic condition for separating chromosomal DNA of *Ustilago hordei* requires further improvement by changing extraction period to 48 hrs and the incubation temperature 50℃; increase the amount of proteinase K to 2 mg/ml; use the Novozyme to remove cell walls more thoroughly in preparing the protoplasts.

The interest of our laboratory in developing molecular karyotype would be to determine the genome size of economically important cereal rust fungus and eventually leading to characterize the product of gene for avirulence, the gene and its location on the chromosome.

Applications

Genome organization and molecular karyotype

The chromosomal and subchromosomal DNAs separated by OFAGE would indicate the size range of genomic DNAs. If there were 10 chromosomal DNA bands, it is an indication that 10 chromosomal DNA fragments constitute the genome of the organism examined. In parasitic protozoan, Trypanosome bruei(8) contains some 100 mini chromosomes of 50 kb to 150 kb in length and several DNA molecules in the 200 kb-1,000 kb size range. OFAGE was used to generate electrophoretic karyotype for yeast(5) and a complete physical map of *E. coli*(14) and large region from human chromosome(9).

Gene location

The restriction fragment length polymorphism of a DNA molecule would provide large numbers of genetic markers(see ref. 11 for RFLP markers in fungi). A unique DNA sequence is related to a certain enzyme production or protein, it is likely that this DNA could be containing the gene for this protein macromolecule. This DNA could be used as a probe to locate the gene on the chromosome. Once OFAGE was performed, the chromosomal DNA in the gel was Southern blotted and hybridized with this DNA probe. If one of the chromosomal DNA fragments hybridize to this probe, then that DNA fragment contains the gene.

If the presence of certain enzyme or protein is related to the expression of virulenge gene for pathogen, cDNA could be developed and find the location of this gene in the chromosome. There is evidence that certain virulence genes are linked on the basis of isozyme patterns in wheat stem rust, each of virulence gene can be located and determined whether the linkage could be demonstrated on the same chromosome. The gene for human leucocyte antigen is located on chromosome # 6(10), and this could be demonstrated by OFAGE, Southern blot and hybridization.

Molecular taxonomy

Many taxonomical criteria are not refine enough to delimit many fungal species. The genetic relationship between species which are considered related based on the conventional criteria such as morphology and life cycle can not be demonstrated if the genetic crosses are not possible. Under these conditions, OFAGE would be useful: chromosomal DNA of each species was separated by OFAGE. One species was Southern blotted and the other was eluted from the gel by Gene Clean method. The eluted DNA band would be used for a probe(through a nick translation procedure). The Southern blot of A species(for example) was cut into several strips, and probed with each DNA segment of B species. The hybridization would

reveal the degree of homology between these two species, which would be an indication of the degree of relatedness. Such an information could lead to provide evolutionary treands among related species, among which the genetic crosses were not possible.

Chromosomal rearrangement

The construction of physical map of DNA near the translocation break point should allow for identification and mapping of rearranged DNA fragments. Genes that are altered by such rearrangement can be isolated and identified. If there is a transposable element in a certain fungi, OFAGE/Southern blot/hybridization could be used to locate the chromosome which is homologous to this element or to which this element attached.

Further implication

OFAGE can not resolve entire genome because even in a small eukaryote like Trypanosome bruei, roughly 60% of the DNA in chromosome do not enter the OFAGE gels because their chromosomes are too large(over 2,000 kb) or their structures do not allow migration. The chromosomes of high eukaryotes are also far too large to be separated by this technique. Therefore, it requires to generate large DNA fragment by digestion with restriction endonucleases whose cleavage sites are not frequent. These large DNA fragments obtained by enzyme digestion could be further separated by OFAGE, and such large fragments could be cloned for developing artificial chromosome vector(2).

OFAGE is to provide versatile analysis for increasing understanding of genome structure and the organization of many organisms.

Note: This review was presented at the Seminar Series at Korea Advanced Institute of Science and Technology. Experimental details concerning the preparation of protoplasts, fungal culture, extraction of DNA, removal of RNA, protein and other non-essential macromolecules by enzyme digestion and the specific conditions used for OFAGE, please write to the author.

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