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Construction of a Pure Cryparin-null Mutant for the Promoter Analysis of Cryparin Gene

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Cryparin 유전자의 promoter 분석을 위한 cryparin 유전자 치환체의 순수 제조

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ABSTRACT: The cryparin of *Cryphonectria parasitica* belongs to a cell wall associated fungal hydrophobin. The cryparin, though it is encoded by a single copy gene, is known for the high expression during the liquid culture of *C. parasitica*, and it turns out that 22% of total mRNA was transcribed for cryparin at 48hr after the liquid culture. In addition, it is also known as one of down-regulated fungal proteins by the presence of double stranded RNA virus, *Cryphonectria hypovirus* 1. In previous studies (Kim *et al.*, 1999), we have constructed a cryparin-null mutant by replacing the cryparin gene with hygromycin B resistance gene due to site directed homologous recombination. In order for the promoter analysis of cryparin which seems to be very strong as well as mycoviral specific, it is preferable to have a strain with only a target promoter replaced and a discernable target site for incoming vectors. However, the cryparin-null mutant revealed the presence of an additional copy of transforming vector except the one which replaced the cryparin gene. In addition, the cryparin-null mutant did not contain any markers for targeted integration of incoming vectors. This prompts us to design an experiment to obtain a strain for promoter analysis of cryparin gene. A different mating type strain EP6(Mata, met$^-$) was mated with the cryparin-null mutant △Crp194-7(MatA, Crp△::*hph*) to make the progenies with only a single replacement vector and met$^-$ characteristic remained. Nutritional assay as well as Southern blot analysis revealed that the progeny, △Crp194-a6, was the methionine auxotroph with a single replacing vector in genome. Northern blot analysis and PAGE showed that there was no cryparin produced in this bred strain either.

KEYWORDS: *Cryphonectria parasitica*, Hypovirus, Gene replacement, Cryparin-null mutant, Promoter analysis

Hydrophobins are small, moderately hydrophobic proteins secreted by fungi and containing eight cystein residues in a conserved pattern (Wessels, 1996). Cryparin of *C. parasitica* is a member of fungal hydrophobin which is a protein conferring a hydrophobicity to mycelium (Carpenter *et al.*, 1992). Several studies have suggested the biological function of cryparin is involved in many different phenomena such as development, pathogenesis and symbiosis. However, none of them was clearly proved at the molecular level. Recent study using a cryparin-null mutant has shown that the biological function of cryparin is implicated in hydrophobicity as well as physical strength of hyphae in *C. parasitica* (Kim *et al.*, in press). Cryparin is unique in that it transcribs

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vigorously, probably the most vigorously transcribed gene in a fungus, and its expression is tightly regulated by the presence of double stranded RNA virus, *Cryphonectria* hypovirus 1 (Zhang *et al.*, 1994). Obtaining a cryparin-null mutant was demanded for several purposes; first, biological function of cryparin can be decided by comparing the null mutant with a wild type. Secondly it allows to perform the promoter analysis of cryparin gene to determine why this single copy gene is so active that it covers up to 22% of total mRNA and which cis-acting region(s), among those promoter regions, are important for the viral specific regulation. In the previous study (Kim *et al.*, 1999), it was possible to obtain a cryparin-null mutant. However it turned out that this mutant contained an additional randomly integrated transforming vector which needed to be removed for better promoter analysis (Ye and Wong, 1994). In addition, targeted integration of the fusion gene constructs containing various engineered promoters and reporter gene will be desirable to avoid the positional effects (Adams and Timberlake, 1990). Therefore, it prompts us to set up an experiment to breed a strain containing only a single replacing vector as well as discernable marker for targeted integration of incoming vector. This study explains how this strain is constructed.

Materials and Methods

Fungal strains and culture conditions

The cryparin containing strains EP6 ATCC 22508 (Mata, met$^-$), EP155/2(MatA) and cryparin-null mutant, △Crp194-7(MatA, Crp △::*hph*), were used in these studies. The cryparin-null mutant, △Crp194-7, was obtained by site directed homolougous recombination of wild type EP155/2 using the linearized transforming vector containing hygromycin phosphotransferase gene(*hph*) and benomyl resistance gene(*tub*-2) (Kim *et al.*, 1999) (Fig. 1). This strain appeared to have two copies of integrated transforming vector in the genome (Fig. 2). *C. parasitica* was grown on PDAmb plates for a week at 25°C in the continuous low light (Rigling and Van

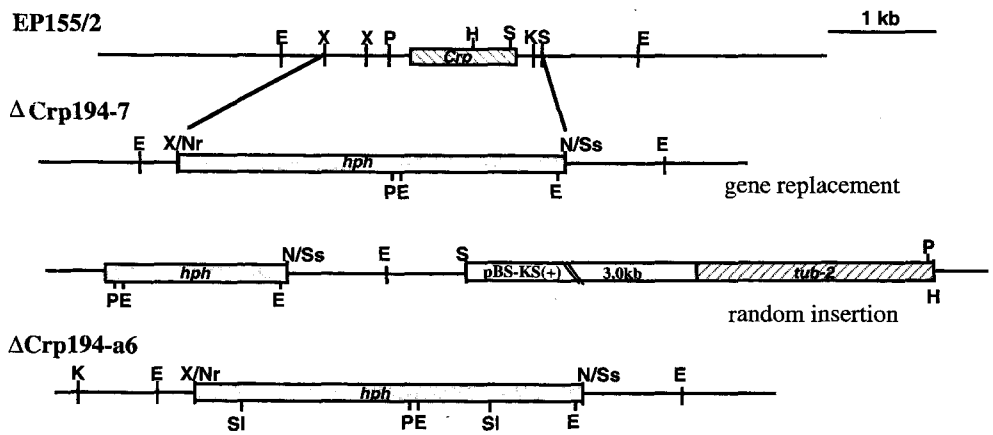

Fig. 1. Diagram for construction of a pure cryparin-null mutant. Genomic DNA of *C. parasitica* is represented by a single line. Genes are represented by open boxes. *Crp*=Cryparin gene, *hph*=hygromycin phosphotransferase gene cassette, *tub*-2=benomyl resistance gene. EP155/2 is the wild type parental strain for gene replacement of cryparin. △Crp194-7 has two copies of transforming vector in the genome while △Crp194-a6 has a single copy of transforming vector by gene replacement between *Crp* and *hph*. E(*Eco*RI), X(*Xba*I), P(*Pst*I), H(*Hin*dIII), K(*Kpn*I), N(*Nco*I), S (*Sac*I), Ss(*Ssp*I), Nr(*Nru*I), Sl(*Sal*I).

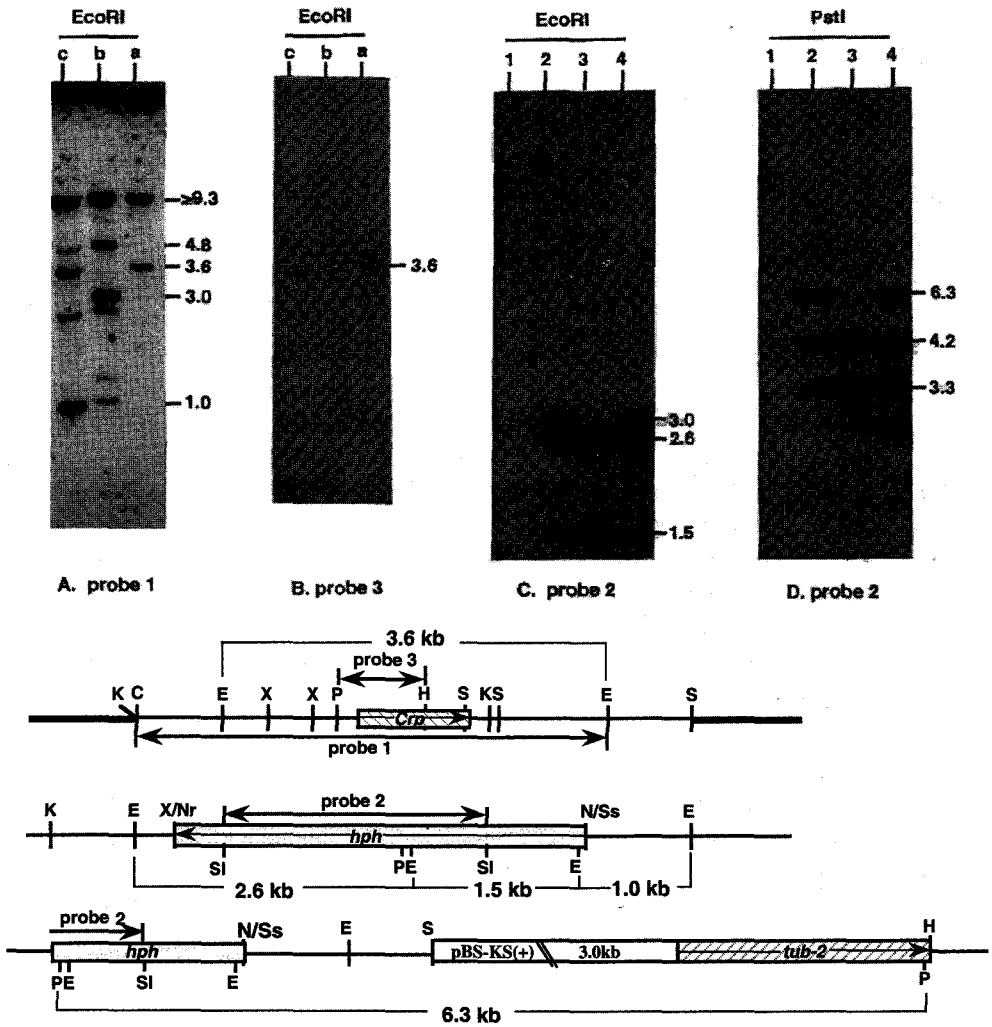

Fig. 2. Southern blot analysis for isolation of a pure cryparin-null mutant. Panel A and B; Lanes a, b and c represent EP155/2, △Crp194-7 and an ectopic transformant by random integration, respectively. Panel C and D; Lanes 1, 2, 3 and 4 represent EP6, △Crp194-7, △Crp194-a6 and △Crp194-a13, respectively. Numbers on right refer to the size of bands in kilobases. Probes and appropriate fragment sizes are shown in the restriction map represented at the bottom of panels.

Alfen 1993). For preparation of inoculum for liquid culture, the cultures of *C. parasitica* growing on plates were homogenized in 100 m*l* of sterile EP complete liquid media using a Warning blender, and resulting slurry was used to inoculate 1L of EP complete liquid media (Puhalla and Anagnostakis 1971).

Mating

Cryparin-null mutant of *C. parasitica*, △Crp194-7(MatA, Crp△::*hph*), was crossed with *C. parasitica* strain EP6(Mata). Sexual mating was performed on chestnut stem pieces and kept at room temperature until the characteristic perithecial neck became visible (Kim *et al.*, 1995). Each perithecium was harvested under stereomicroscope, broken down in distilled water by vortexing, then the exudating ascospores were spreaded on the PDAmb containing 50 μg/m*l* of hy-

gromycin B.

Genomic DNA isolation and Southern blot analysis

Genomic DNA from parental strains as well as resulting ascospore progenies were extracted. Lyophilized mycelium (1.0g) was ground to a fine powder in a mortar and pestle. This was transferred to a centrifuge tube containing 10 m*l* of lysis buffer (50 mM EDTA, 50 mM Tris pH 8.0, 0.7M NaCl, freshly added 1% CTAB and 1% mercaptoethanol). This lysate was mixed gently by shaking water bath at 68°C for 45 min. Addition of an equal volume of Chloroform : Isoamyl alcohol (24 : 1) to the lysate was followed by centrifugation for 10 min (Beckman JA14, 7000 rpm). The clean supernatant was transferred to a new tube and then an equal volume of isopropanol was added to precipitate the DNA by centrifugation for 10 min. The pellet was dissolved in 4 m*l* TE buffer. After purification with an equal volume of phenol and chloroform, the DNA was precipitated with the addition of 0.5 volume 7.5M ammonium acetate and two volumes of absolute ethanol. The DNA was pelleted by centrifugation at 7,000 rpm for 10 min, then dried under vacuum and resuspended in TE buffer (Borges *et al.*, 1990). Five to ten μg of genomic DNA were digested with either restriction enzyme *Eco*RI or *Pst*I, and blotted onto Nylon membrane. Southern blot analysis were conducted with radioactive probe containing cryparin gene (probe 1 and 3) and hygromycin B resistance gene(*hph*) (probe 2).

RNA isolation and Northern blot analysis

Total RNA was prepared from lyophilized and ground mycelia. 0.5g~1.0g of ground mycelia was added to 20 m*l* of lysis buffer [0.2M Tris-Cl pH 7.5, 0.25M EGTA, 4.8%(w/v) sodium *para*-aminosalicylate, pH adjusted to 8.8 with NaOH]. Two m*l* of phenol and a few drops of chloroform were added to lysis buffer. The mycelium was homogenized at 8,000 rpm for 1 min using a PowerGen-700D (Fisher Scientific). The homogenate was extracted several times with an equal volume of phenol and chloroform. Lithium chloride (LiCl) was added to a final concentration of 2M for precipitation of RNA, and the sample was incubated overnight at 4°C. The RNA was precipitated by centrifugation at 7,000 rpm for 30 min. The pellet was washed twice with 70% ethanol and suspended in RNase free water (Kim *et al.*, 1995).

Northern blot analysis were conducted with 30 μg of total RNA. The radioactive probe was a part of cryparin coding region.

Protein extraction and SDS-PAGE

Cryparin was extracted in 60% ethanol based on its hydrophobic properties (Carpenter *et al.*, 1992). The mycelia was lyophilized and ground. A fine powder was suspended in 4ml 60% ethanol. This was homogenized for 1 min at 8,000 rpm. The cellular debris were precipitated by centrifugation, and the supernatant was transferred to a new tube. Ethanol extracted samples were mixed with an equal amount of the protein loading buffer (0.125M Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol). Samples were boiled for 10 min and run on a 12% polyacrylamide gel. After the electrophoresis, the gel was stained with Coomassie Blue R-20, then destained in 30% methanol and 10% acetic acid solution.

Results and Discussion

For the promoter analysis of cryparin, the cryparin-null mutant, △Crp194-7, was obtained in the previous study (Kim *et al.*, 1999) by site directed homologous recombination of wild type EP155/2 using the linearized transforming vector containing hygromycin

phosphotransferase gene(*hph*) and benomyl resistance gene(*tub*-2) (Fig. 1). Southern blot analysis of △Crp194-7 with cryparin gene probe (probe 1) showed six bands instead of three (Fig. 2, Panel A, lane b) but no hybridizing band with probe 3 (Fig. 2, Panel B, lane b). However an ectopic transformant contained an additional three bands except those two bands at >9.3 kb and 3.6 kb originated from endogenous cryparin gene (Fig. 2, Panel A, lane c). This hybridizing pattern clearly showed that cryparin gene was deleted in a strain △Cpr194-7, but this strain appeared to have two copies of integrated transforming vector in genome due to one by homologous gene replacement and the other by random insertion (Fig. 2). Sexual mating of △Crp194-7 with EP6 was performed to remove the additional copy of transforming vector by random insertion but to harbor the methionine auxotrophic marker for the target site of incoming vector. *C. parasitica* strain EP6 was selected because it contained a site mutation at methionine gene which can be complemented by the part of wild type methionine gene due to the site-directed recombination (Churchill, 1994). Four to eight weeks after the mating, perithecial necks were distinctive and twelve well-separated perithecia were harvested for further analysis. Among those twelve ascospore suspension, eight did not produce any colonies on PDAmb containing 50 μg/ml of hygromycin B. However, four preparations yielded abundant colony forming units on hygromycin media. Two ascospore progenies for each perithecium were single-spored and transferred onto methionine deficient minimal media. Four of eight ascospores examined were methinine auxotrophic. Cryparin was prepared from representative two ascospore progenies, △Cpr194-a6 and △Cpr194-a13, and analyzed on the PAGE gel. Both ascospore progenies did not produce any cryparin (Fig. 3). Southern blot analysis was conducted to determine which of them was a pure cryparin-null mutant for the promoter analysis. The newly obtained cryparin-null mutants by sexual mating were digested with either restriction enzyme *Eco*RI or *Pst*I and hybridized to probe 2 (Fig. 2, Panel C and D, respectively). The *Eco*RI and *Pst*I digestion of *Crp* containing parental strain EP6 (Fig. 2, Panel C and D, lane 1) did not hybridize to probe 2 as expected. One of the ascospore progenies, △Crp194-a13, did show the same hybridizing patterns as the parental strain △Crp194-7 indicating that randomly integrated vector was not segregated in this strain (Fig. 2, panel C and D). Besides two *Pst*I-hybridizing bands by a replacing vector, it was somewhat unexpected to have only one additional band, instead of two, by randomly integrated vector because *Pst*I had a restriction site within the probe 2 (Fig. 2, panel C and D, lanes 2 and 4). This suggests that cross over of randomly integrated vector occurs within *hph* gene, and integration site is so close to *Pst*I that hybridization of probe 2 to resulting chimeric *Pst*I fragment does not occur. The correct sideness of integration around *Pst*I site appeared to be downstream of *Pst*I site within hph gene based on the facts that 6.3 kb fragment is possible only when the upstream region of hph gene remained intact. This was partly confirmed because we did not find any ascospore progenies which contained a randomly integrated vector alone. In other words, all the hygromycin resistant progenies did contain either two copies of vector or single copy of replacing vector because functional hph gene requiring for the growth on hygromycin media was encoded only by replacing vector. However △Crp194-a6 showed the characteristic two bands with expected sizes of 2.6 kb and 1.5 kb at *Eco*RI digestion and 4.2 kb and 3.3 kb at *Pst*I digestion, which suggests that △Crp194-a6 was a pure cryparin-

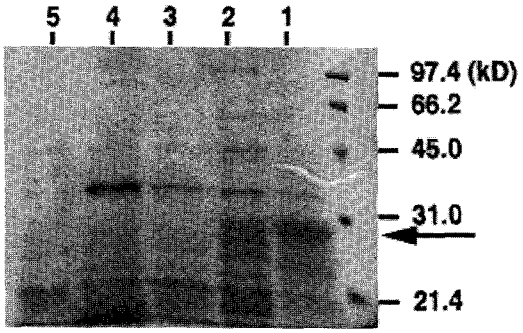

Fig. 3. SDS-PAGE of cryparin. Lanes 1, 2, 3, 4 and 5 indicate EP155/2, EP6, △Crp194-a6, △Crp194-7 and △Crp194-a13, respectively. Numbers on right refer to size (kD). Ep 155/2 and EP6 show the charateristic cryparin band at 30 kD indicated by an arrow.

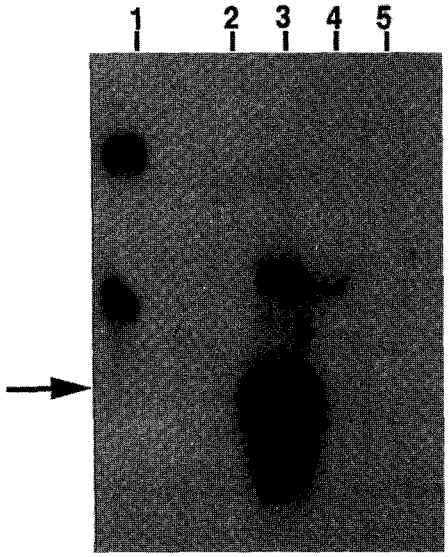

Fig. 4. Northern blot analysis to determine the absence of cryparin gene. Lane 1 contains digested-plasmid DNA encoding cryparin as positive control. Lanes 2 and 3 contain RNA from parental strains of △Crp194-7 and EP6, respectively. Lanes 4 and 5 contain RNA from ascospore progenies of △Crp194-a6 and △Crp194-a13, respectively. The arrow indicates the cryparin transcript.

null mutant with no additional copy of transforming vector in genome.

Northern blot analysis probed with a part of *Crp* indicated that no transcript of crp made from ascospore progenies, △Crp194-a6 (Fig. 4, lane 4) and △Crp194-a13 (Fig. 4, lane 5), and parental strain △Crp194-7 (Fig. 4, lane 2). While abundant Crp expression was detected in a parental strain EP6 (Fig. 4, lane 3).

To determine the deletion of *crp* in the protein level, cryparin was partially purified and SDS-PAGE was conducted (Fig. 3). △Crp194-a6, △Crp194-a13 and △Crp194-7 did not produce any cryparin as expected. However a

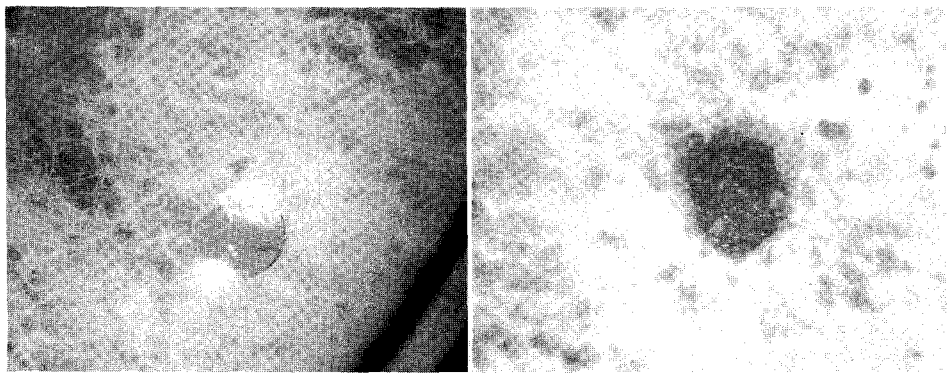

Fig. 5. A test for the "easily wettable" charateristics. A water droplet was placed on the top of aerial mycelia of the wild type strain EP155/2 (A) and the pure cryparin-null mutant strain △Crp194-a6 (B). The pure cryparin-null mutant shows the water-soaking phenomenon due to the "easily wettable" characterisitcs while the water on the top of wild type remains intact.

wild type EP155/2 and a parental strain EP6 produced cryparin as shown in Fig. 3.

The phenotypic characteristic of the cryparin-null mutant were compared with the wild type (Fig. 5). The newly bred strain △Crp194-a6 showed the characteristic of crypain-null phenotypes such as loss of hydrophobicity and weakened physical strength of aerial hyphae.

As the result of mating of cryparin-null mutant, △Crp194-7, with a different mating type strain EP6, it is possible to obtain a pure cryparin-null mutant, △Crp194-a6, with no additional copy of transforming vector and methionine-requiring auxotrophic marker in genome. This newly bred strain will be suitable to perform the promoter analysis of cryparin gene in that titration effect can be avoided and then various integrating vectors containing reporter gene will be escorted into site of mutated methionine gene because the corresponding transformants will be selected on minimal media which reinforce the intragenic complementation between residual mutated methionine gene and the incoming part of methionine gene.

적 요

Cryparin은 *Cryphonectria parasitica*의 세포벽에 풍부한 소수성 단백질에 속한다. cryparin은 비록 하나의 유전자에 의해 발현되지만 액체배양 후 48시간이 지나면 발현된 전체 유전자중에서 22%를 차지할 정도의 높은 발현 양상을 나타낸다. 또한 cryparin은 RNA mycovirus인 *Cryphonectria* hypovirus 1의 감염에 의해 발현이 현저히 억제되는 유전자로 알려졌다. 이미 지난 실험(Kim *et al.*, 1999)에서 상동염색체간의 재조합을 이용하여 cryparin 유전자를 항생제 hygromycin B 저항성 유전자로 치환한 치환체를 제조하였다. 발현율이 매우 높으면서도 virus에 의해 밀접하게 영향받는 cryparin 유전자의 promoter 분석을 위하여서는 대상이 되는 유전자 치환을 위한 vector만을 포함하며, 분석에 이용될 여러 유전자 운반체들이 어느 한곳에만 삽입되도록 하는 성질을 가진 균주의 개발이 필요하다. 그러나 지난번 실험의 결과 얻어진 cryparin 치환체는 치환용 vector외에도 무작위로 삽입된 vector가 존재하고 나아가 새로운 vector들이 어느 한곳에만 삽입되도록 하는 성질을 갖지 못하였다. 따라서 본 실험에서는 cryparin 유전자 치환체와 영양요구성 돌연변이체인 균주간의 교잡을 이용하여 분석 대상이 되는 유전자의 치환에 이용된 vector만을 포함하며, 분석에 이용될 여러 유전자 운반체들이 genome내의 어느 한곳에만 삽입되도록 하는 성질을 가진 균주를 제조하였다.

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References

Anagnostakis, S. L. 1979. Sexual reproduction of *Endothia parasitica* in the laboratory. *Mycologia* **71**: 213-215.

Adams, T. H. and Timberlake, W. E. 1990. Upstream elements repress premature expression of an *Aspergillus* development regulatory gene. *Mol. Cel. Biol.* **10**: 4912-4919.

Borges, M. I. Azevedo, M. O., Bonatelli, R. Jr., Felipe, M. S. S. and Astolfi-Filho, S. 1990. A practical method for the preparation of total DNA from filamentous fungi. *Fung. Genet. Newsl.* **37**: 10.

Calos, M. P. and Miller, J. H. 1980. Transposable elements. *Cell* **20**: 579-595.

Carpenter, C. E., Muellar, R. J., Kazmierczak, P., Zhang, L., Villalon, D. K. and Van Alfen, N. K. 1992. Effect of a virus on accumulation of a tissue specific cell surface protein of the fungus *Cryphonectria parasitica*. *Mol. Plant-Microbe Interact.* **4**: 55-61.

Choi, G. H., Larson, T. G. and Nuss, D. L. 1992. Molecular analysis of the laccase gene from the chestnut blight fungus and selective expression of its expression in an isogenic hypovirulent strain. *Mol. Plant-Microbe Interact.* **5**: 119-128.

Churchill, A. C. L. 1994. Molecular cloning and functional analysis of methionine gene from

Cryphonectria parasitica strain EP6. Ph.D. Dissertation. Uta State University.

Kim, D. H., Kazmierczak, P. and Van Alfen, N. K. 1999. The biological function of cryparin of *Cryphonectria parasitica*. *Mol. Plant-Microbe Interact.* **12**: in press.

Kim, D. H., Rigling, D., Zhang, L. and Van Alfen, N. K. 1995. A new extracellular laccase of *Cryphonectria parasitica* is revealed by deletion of LacA. *Mol. Plant-Microbe Interact.* **8**: 259-266.

Powell, W. A. and Van Alfen, N. K. 1987. Differential accumulation of Poly(a)$^+$ RNA between virulent and Double-stranded RNA-Induced hypovirulent strains of *Cryphonectria parasitica*. *Mol. Cell. Biol.* **7**: 3688-3693.

Puballa, J. E. and Anagnostakis, S. L. 1970. Genetics and nutrient requirement of *Endothia parasitica*. *Phytopathology* **61**: 169-173.

Rigling, D. and Van Alfen, N. K. *1993*. Extra- and intracellular laccases of the chestnut blight fungus, *Cryphonectria parasitica*. *Appl. Environ. Microbiol.* **59**: 3634-3639.

Wessels, J. G. H. 1996. Fungal hydrophobins: proteins that function at an interface. *trends in Plant Science* **1**: 9-15.

Ye, R. and Wong, S. -L. 1994. Transcriptional regulation of the *Bacillus subtilis* gluticol dehydrogenase gene. *J. Bacteriol.* **176**: 3314-3320.

Zhang, L., Villalon, D., Sun, Y., Kazmierczak, P. and Van Alfen, N. K. *1994*. Virus-associated down-regulation of the gene encoding cryparin, an abundant cell-surface protein from the chestnut blight fungus, *Cryphonectria parasitica*. *Gene* **139**: 59-64.