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Selection and Characterization of the Hypovirulent Symptom Micmicking Mutant in *Cryphonectria parasitica* Using Marker Rescuing

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*Cryphonectria parasitica*에서 mycovirus 감염 증상을 보이는 돌연변이 균주의 선발과 marker rescuing을 이용한 돌연변이의 특성화

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ABSTRACT: A mutant (HSM1) of *Cryphonectria parasitica* created during transformation reproduced the hypovirulent symptoms in virus-free wild type. Its phenomena have been proved with morphological marker such as reduced sporulation, pigmentation, and laccase production. In addition to the changes in phenotypic characteristics, down-regulations of *Lac*1, *Crp*1, *Vir*1 and *Vir*2 were also observed. The integration of transforming vector was confirmed and located within genome by marker rescuing. Vector integration occurred between two genes, *Cpg*2 and *Cpg*3, which resulted in the disruption of neither *Cpg*2 nor *Cpg*3. Both *Cpg*2 and *Cpg*3 genes, sized at 1.8 kb and 1.9 kb respectively, were rarely transcribed genes in *Cryphonectria parasitica*. *Cpg*2 expression was significantly overexpressed from 4 to 5 day old culture of both UEP1 and HSM1 while no differences were observed in *Cpg*3 expression. It appears that an aberration from the normal expression of *Cpg*2, not *Cpg*3, results in the hypovirulent symptoms in virus-free wild type.

KEYWORDS: ***Cryphonectria parasitica*, Hypovirus, Marker rescuing**

Cryphonectria parasitica (Murrill) Barr, the causal agent of chestnut blight, has been known for a devastating factor of chestnut forest in North America during the early of this century. Viral containing strains were isolated from nature and this double-stranded (ds) RNA virus was known for the biological control agent of this disease (Anagnostakis, 1979; Jaynes and Elliston, 1980). The Cryphonectria hypovirus 1 (CHV1) was best studied among dsRNA viruses in *C. parasitica* and CHV1-infected fungus showed phenotypic characteristics such as reduced virulence (hypovirulence), reduced sporulation and reduced pigment production with no detectable effects of virus on fungal growth in culture. By using infectious cDNA copy of CHV1, it was clearly demonstrated that the presence of viral genome is responsible for these symptoms in this fungus (Choi and Nuss, 1992a & b).

In addition to the changes of phenotypic characteristics in hypovirulent strains, it has been shown that expression of a small number of specific fungal genes are suppressed in hypovirulent strains (Powell and Van Alfen 1987a & b). These viral suppressed fungal genes are *Lac*1, a gene encoding an extracellular laccase (Choi and Nuss 1992; Rigling and Van Alfen 1991; Kim *et al.*, 1995), Crp1,

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a gene encoding an abundant tissue-specific physical strength related cell-surface hydrophobin (Zhang *et al.*, 1994; Kim and Van Alfen, in preparation), *Vir*1 and *Vir*2, genes involved in viral-induced sporulation-related symptoms (Zhang *et al.*, 1993). These viral specific fungal genes are down-regulated at transcription level, and are consequently thought to be coordinately regulated in normal strains of the fungus (Kazmierczak *et al.*, 1996).

Many fungal gene regulations have shown the presence of a coordinated regulatory cascade in life cycle (Fontana *et al.*, 1991, Gwynne *et al.*, 1987, Herskowitz 1989, Mirabito *et al.*, 1989). Larson *et al.* (1992) have shown that the modulation of an extracellular laccase expression in *C. parasitica* is involved in Ca^{++} dependent signal transduction pathway which is another example of regulatory cascade. Our hypothesis of fungal gene regulation by CHV1 is that a limited number of host gene(s) are normally coordinately modulated at some steps in the fungal growth cycle through the possible regulatory gene(s) in regulatory hierarchy and that a viral infection cause the perturbation of this regulation directly or indirectly through a regulatory proteins which occupy a key position in this hierarchy, which, in turn, results in viral symptom expression.

In order to obtain a putative fungal regulatory gene which modulate a set of fungal genes involved in development of hypovirulence symptom as well as affected specifically by the presence of mycovirus, CHV1, we considered phenotypic as well as molecular changes in hypovirulent strains as directional selection markers and have screened our fungal stock cultures produced during the previous transformation experiment for hypovirulent symptom mimicking. A transformant showing hypovirulent symptoms was selected. Marker rescuing was conducted to retrieve the mutated gene and to investigate the relationship between vector integration and phenotypic segregation.

Materials and Methods

Phenotypic characteristics of a hypovirulent simptom micmicking mutant (HSM1)

The transformants from virus-free strain EP155/2 of *C. parasitica* were collected from the previous studies (Kim *et al.*, 1995 and Zhang *et al.*, 1993). Hypovirulent symptoms associated with the presence of virus were examined among the transformants showing no pigmentation and reduced sporulation. Pigmentation was observed and recorded from cultures grown onto PDAmb plate (Kim *et al.*, 1995). Sporulation was measured from the 30 m*l* of PDAmb plate and compared with the wild type strain EP155/2 and its isogenic CHV1-containing hypovirulent strain UEP1 of *C. parasitica.* Extracellular laccase 1 (LAC 1) activity was estimated from the culture filtrate of HSM1, EP155/2 and UEP1.

Virulence test was conducted by using the dormant chestnut stems. The procedures and replications for conidiation, virulence and laccase activity were described previously (Kim *et al.*, 1995).

Molecular markers in HSM1

In addition to phenotypic characteristics of HSM1, molecular markers, those of which are specifically affected by the presence of virus, were also examined. The viral specific fungal genes which are specifically down-regulated by the presence of virus have been isolated previously and those are extracellular laccase gene (*Lac*1), Cryparin (*Crp*1), *Vir*1 and *Vir*2. Northern and slot-blot analysis of marker genes were conducted to examine the expression of those genes in mutants compared to wild type as well as hypovirulent strain.

RNA analysis

Total cellular RNA was prepared from the culture grown in EP complete media (Powell and Van Alfen, 1987). For Northern blot analysis, RNA was glyoxal denatured, fractionated on 1.2% agarose gels in 10 mM phosphate buffer, and transferred to GeneScreen Plus (DuPont). For slot blot analysis, RNA was glyoxal denatured, and 20 u*l* of 0.1 ug/u*l* RNA were loaded into slot. Conditions for hybridization and washing were described previously (Powell and Van Alfen 1987). The hybridization signals were scanned and quantified with a Ultrascan XL Laser Densitometer (Pharmacia LKB, Uppsala, Sweden). The rRNA probe was used to check for equal loading and transfer of RNA. RNA accumulation for each gene was compared by the ratio of the peak area of hybridizing band from each strain to that from the wild type EP155/2.

Marker rescuing

A transformant showing symptoms like very low pigment and low sporulation was screened onto PDAmb among our transformant stock cultures obtaining from the previous experiment using EP155/2 as DNA recipient. A transformant, HSM1, was selected and used to check other phenotypic characteristics as well as molecular markers which are specifically affected by the presence of virus.

The original transforming vector was examined and found an appropriate enzyme, BglII, which leave a intact pBluescriptIISK(-) part for the use of *E. coli* cloning. Genomic DNA was extracted from HSM1, digested with BglII, size-fractionated on the 0.6% agarose gel and electroeluted for fragment larger than 3.0 Kb from the gel. The electroeluted DNA fragment was ligated and used for the transformation of *E. coli* strain DH10B. Ampicillin resistant colony was screened for the plasmid containing an extra piece of DNA from the integration area and the plasmid was extracted to examine the presence of new flanking DNA. The resulting rescued DNA was then used to screen EP155/2 genomic library to obtain the intact clone. The rescued genomic clone was digested with EcoRI-SalI and the resulting fragments were subcloned into pBluescriptIIKS(+). Then, each ^{32}P-labeled subclone was used to determine the plasmid integration and screened for the RNA transcript.

Results

Phenotypic characteristics of HSM1

Among transformant stock cultures, a transformant, named as HSM1, from the set of experiment for Laccase 1 deletion (Kim *et al.*, 1995) was selected to have the phenotypes of reduced pigmentation and reduced sporulation on PDAmb. The symptoms associated with the presence of mycovirus, CHV1, were measured in this mutant to examine the effects of mutation on phenotypic markers. There was no typical bright yellow pigment in HSM1 (data not shown) and sporulation of HSM1 was significantly reduced from the wild type (Table 1). Laccase1 activity measured from culture filtrate was also significantly reduced from the wild type and it was similar or slightly higher than that of hypovirulent strain (Fig. 1). However there were no changes in virulence measured by

Table 1. Characteristics of HSM1 mutant compared with wild type (EP155/2) and hypovirulent (UEP1) strains

Strains	Canker area	Number of conidia
EP155/2	30.88 ± 9.95[a]	$4.38 \times 10^9 \pm 3.46 \times 10^7$
UEP1	1.04 ± 0.85*	$2.22 \times 10^7 \pm 0.71 \times 10^7$*
HSM1	31.86 ± 5.20	$4.77 \times 10^8 \pm 2.15 \times 10^7$*

[a]Values are means$\pm$standard deviation with four replications.

*are significant at p=0.01 using Duncan's multiple new range test.

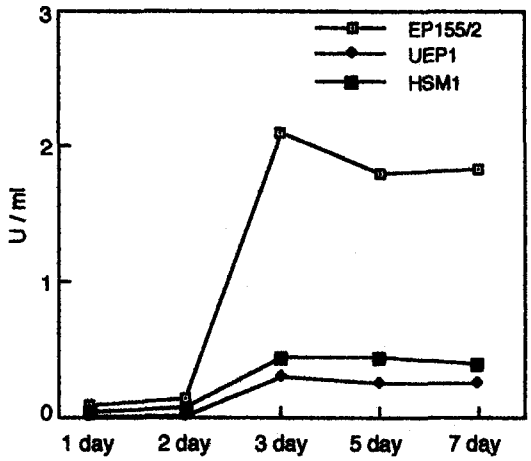

Fig. 1. Extracellular laccase activity of crude culture filtrate. Laccase activities were followed as a function of days after inoculation of liquid media.

pathogenicity against chestnut dormant stem (Table 1).

As a whole, this transformant seems to stay as juvenile stage as UEP1, which shows continuous vegetative growth without proper proceedings into normal developmental process such as sexual and asexual fruitbody formation.

Characteristics of molecular marker expression

Transformant HSM1 was further examined for the molecular marker genes by slot blot analysis. The expression level of marker genes was compared by the ratio of each gene's expression from HSM1 and UEP1 to that from EP155/2 after normalization by rRNA expression. The mRNA accumulation of early expressed marker genes, *Lac*1 and *Crp*1, which have a peak $\leq$72 hrs were represented in Fig. 2A. *Lac*1 expression from UEP1 peaked at 48 hrs and showed down-regulation from 77% to 23% from EP155/2. Lac1 expression from HSM1 did show a down-regulation from 78% to 45% from EP155/2 except the expression at 24 hrs which was not significantly different from EP155/2 (Fig. 2A). *Crp*1 expression peaked between 48 hrs and 72 hrs in this experiment, and both UEP1 and HSM1 showed a down-regulation from 30% to 60% and 70% to 80%, respectively at the peak. *Crp*1 accumulation was extended in EP155/2 upto 5 day but it was hardly detectable from both UEP1 and HSM1. The

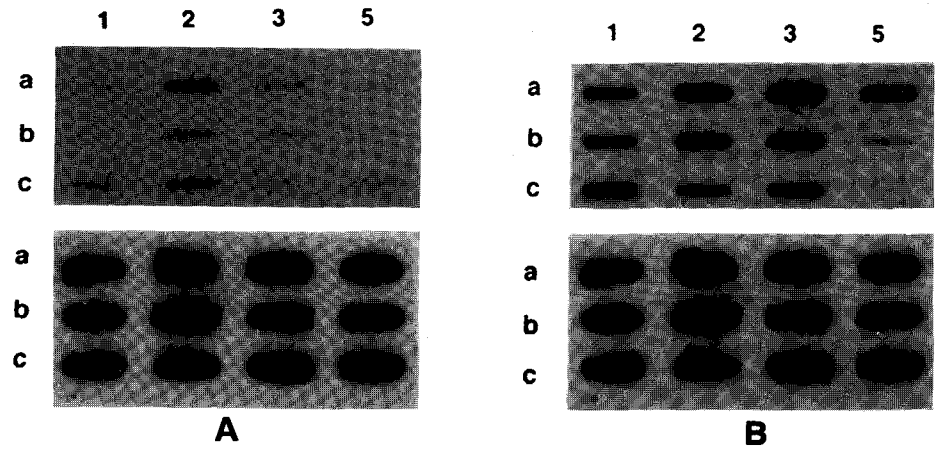

Fig. 2. Slot-blot analysis of *Lac*1 and *Crp*1 transcription. Numbers on the top represent harvesting days after inoculation. Rows a, b, and c contain a total RNA from isolates EP155/2, UEP1, and HSM1, respectively. (A) Down-regulation of *Lac*1 gene. The RNA was slot-blotted and probed with ^{32}P labeled *Lac*1 clone. (B) Down-regulation of *Crp*1 gene. The RNA was slot-blotted and probed with ^{32}P labeled *Crp*1 clone. Lower panel represents the hybridization patterns of each isolate to rRNA gene to normalize each gene's expression.

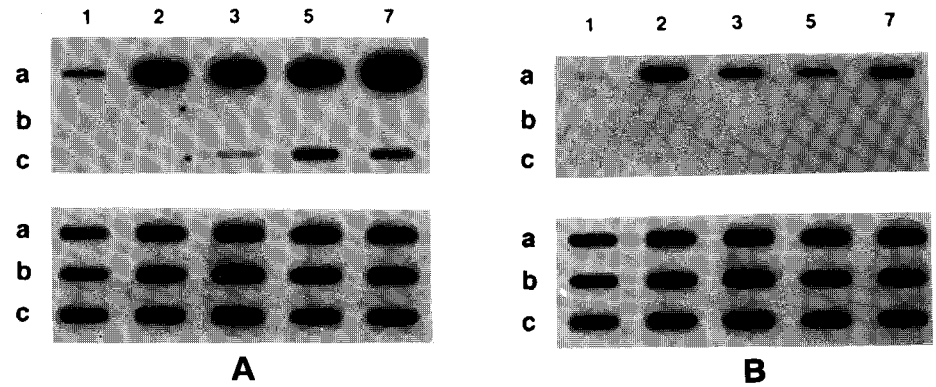

Fig. 3. Slot-blot analysis of *Vir*1 and *Vir*2 transcription. (A) Down-regulation of *Vir*1 gene. (B) Down-regulation of *Vir*2 gene. The RNA was slot-blotted and probed with ^{32}P labeled *Vir*1 and *Vir*2, respectively. Transcripts of *Vir*1 and *Vir*2 were analyzed as described above.

mRNA accumulation of late expressed marker genes, *Vir*1 and *Vir*2, were represented in Fig. 3. Both Vir1, a fungal pheromone, and *Vir*2, a *Vir*1's silent homologue, were either undetectable or significantly decreased from both HSM1 and UEP1.

Since most phenotypes associated with the presence of virus were reproduced in HSM1 and molecular markers in HSM1 were also down-regulated, when compared to that of wild type EP155/2, to similar extent to that of UEP1. HSM1 transformant prompt us to investigate which gene was affected by the integration of vector and how the virus make effects on disrupted gene by comparing the gene expression of UEP1.

Marker rescuing

Marker rescuing using 1 ug of recirculated DNA yielded three ampicillin resistant colonies. Recombinant plasmids were extracted from these colonies and analyzed with restriction enzyme to confirm the presence of new flanking DNA. Among the three plasmids, two gave a single fragment with *Bgl*II digestion and both showed the identical restriction patterns indicating that these are same clone (pRes1). The third plasmid showed the presence of an extra band besides all bands detected from the other two (pRes2). It also gave two fragments with *Bgl*II digestion, the enzyme used for the genomic DNA digestion, which suggest two independent *Bgl*II-digested fragments were dimerized first before they were put into plasmid by ligation. The size difference around *Bgl*II site representing the existence of new rescued DNA appears to be $\leq$250 bp (Fig. 4). The 1.2 Kb *Bgl*I-I-*Pst*I fragment containing a new rescued DNA along with DNA from the original vector (p$\triangle$lac84-1) were used for the Southern analysis to confirm that it contained the rescued DNA from the integrated area of HSM1 genome (Fig. 5). The intact original band was detected at 4.0 Kb in both EP155/2 and *Lac*1 null-mutant(84-1) due to the rescued DNA probe while there is no band at 4.0 Kb in HSM1, which suggest the rescued DNA came from the mutated region caused by original vector (p$\triangle$lac84-1) integration. The intact *Lac*1 gene was detected at 9.0 Kb from EP 155/2 and HSM1 while the mutation of *Lac*1, band at 12.0 Kb, was detected in *Lac*1 null-mutant(84-1) (Fig. 5). Southern analysis with different restriction enzymes suggested that the band at 9.0 Kb in HSM1 are double bands, one from the hybridization with *Lac*1 gene part and the other with the rescued

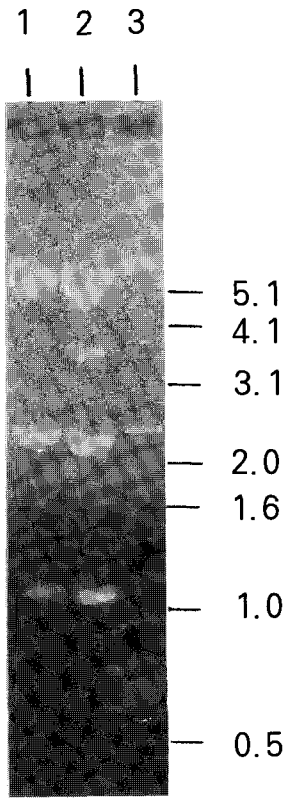

Fig. 4. Restriction analysis of rescued plasmid. Plasmids were digested by *Bgl*II and *Pst*I. Lanes 1, 2, and 3 contain the plasmid of pRes1, pRes2, and p△lac84-1, respectively. Arrows indicate the size difference due to the rescued DNA fragment. Numbers on the right are fragment sizes (Kb).

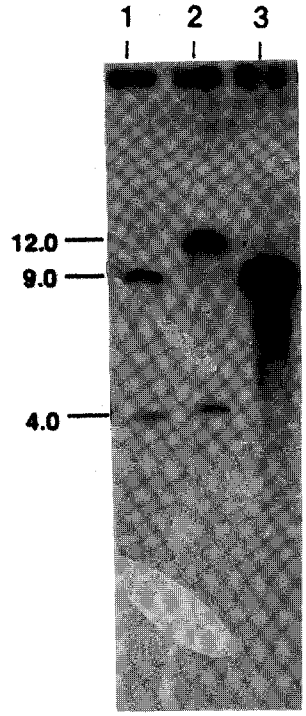

Fig. 5. Hybridization pattern of *Bgl*II-digested DNA to rescued DNA fragment. Lanes 1, 2, and 3 contain DNA from EP155/2, 84-1, and HSM1, respectively, and hybridize to the probe made by the rescued 1.2 Kb fragment. Numbers on the left are fragment sizes (Kb).

DNA part in the 1.2 Kb *Bgl*II-*Pst*I fragment probe (data not shown).

The 1.2 Kb *Bgl*II-*Pst*I fragment probe was used to screen the EP155/2 genomic λ library to identify the intact rescued area. Among the 14,000 plaques screened, eight λ clones gave the hybridizing signal and these were further analyzed with *Lac*1 gene probe. One (λRes1) out of 8 clones appears to be rescued DNA-specific clone based on the facts that the hybridization to the 1.2 Kb *Bgl*II-*Pst*I fragment probe but no hybridization to the *Lac*1 gene probe. The λRes1 were subcloned into pBluescriptIIKS(+) by using *Eco*RI and/or *Sal*I restriction site and the restriction sites were represented in Fig. 6. Each subclone then, was used as probe to locate where the transforming vector integrated. Fig. 7 suggests that subclone2 contained the integration area based on the different hybridizing pattern in HSM1. Furthermore, the sequence comparison between subclone2 and pRes1 confirmed that there is integration in this subclone and also indicated where it exactly integrated (Fig. 8). The restriction map of HSM1 with an exact integration point and the transcript from each subclone were represented in Fig. 6.

Northern analysis using each subclone as a probe was conducted to detect any transcript made from subclones. The transcript sized at 2.3 Kb and 1.7 Kb were detected from sub-

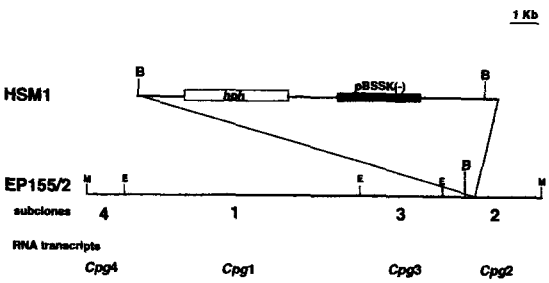

Fig. 6. Restriction map of λRes1 clone and its encountering area of HSM1 containing the integration site. Each subclone was generated by *Eco*RI digestion of λRes1 clone and referred as subclone 1 to 4 based on the fragment size. Transcripts from subclone1, 2, 3, and 4 were named as *Cpg*1, 2, 3 and 4, respectively. B, E, and M represent restriction enzymes of *Bgl*II, *Eco*RI, and *Mbo*I, respectively. *hph* and pBSSK(-) indicate hygromycin B phosphotransferase and pBluescriptIISK(-).

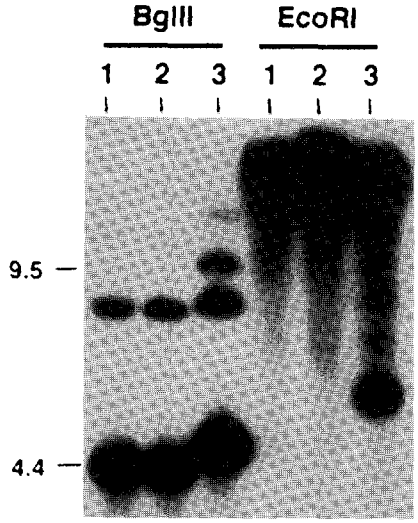

Fig. 7. Hybridization pattern of *Bgl*II and *Eco*RI-digested DNA to subclone 2. Lanes 1, 2, and 3 contain DNA from EP155/2, UEP1, and HSM1, respectively, and hybridize to the probe of subclone 2. Restriction enzymes are indicated on the bar and numbers on the left are fragment sizes (Kb).

clone1 and 4, respectively. However, there is no transcript detected in RNA extracted from 2 day old culture by using either subclone2

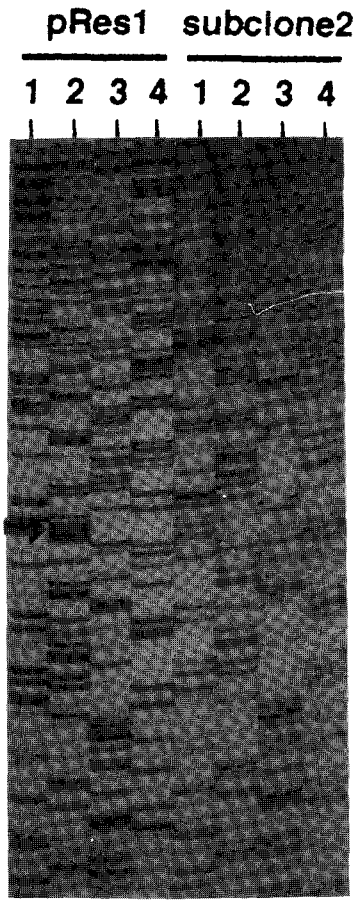

Fig. 8. Comparison of nucleotide sequences of pRes 1 and subclone 2 around integration site. Sequence comparison of pRes 1 and subclone 2 represent the nucleotide sequence of HSM1 and EP155/2, respectively. Arrow indicates the junction region containing of p△lac84-1 which is in agreement of the identical sequence before the junction and the deviating sequence after the junction. Lane 1, 2, 3, and 4 indicate the sequence of guanine, adenine, thymine, and cytosine, respectively.

and 3 as a probe. Therefore, northern analysis containing more time course was conducted with cDNA clones corresponding to subclone 2 and 3 as a probe. There was a transcript sized at 1.8 and 1.9 Kb from cDNA clone 2 and 3, respectively. Both gene's transcription were peaked at 4 or 5 days after liquid culture but made very rarely which re-

quire at least 3~4 weeks to see a faint band in the autoradiogram (data not shown). Interestingly both genes were transcribed in HSM1 with the same expression pattern as the EP155/2 which indicate that integration occurred in between gene *Cpg*2 and *Cpg*3.

Northern and Slot blot analysis of *Cpg*2 and *Cpg*3 with 1, 2, 3, 5 and 7 day old cultures revealed that both genes are transcribed from HSM1 and the difference between EP155/2 and HSM1 as well as UEP1 were maximized at the time around 5 day culture. Thus, the analysis for *Cpg*2 and *Cpg*3 were focused on 3, 4 and 5 day old cultures. The *Cpg*2 expression were slightly down-regulated at early culture time (2 day) but reached to normal at 3 day then significantly overexpressed from 4 to 5 day old culture of UEP1 and HSM1. The degree of *Cpg*2 expression from UEP1 and HSM1 reached maximum twice of EP155/2. However, *Cpg*3 did not show significant differences among the three strains. The *Cpg*1, another gene next to *Cpg*3 but further down from the integration point, was used as a internal control for comparison and its expression did not show any differences among the three strains (Fig. 9). These observations were consistent with the data from RNA extracted from three independent sets of culture preparation of all strains.

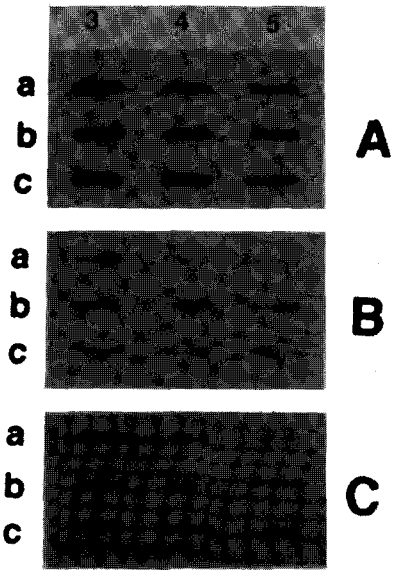

Fig. 9. Slot-blot analysis of *Cpg*1, *Cpg*2 and *Cpg*3 transcription. Expression of *Cpg*1, *Cpg*2 and *Cpg*3 are represented panel A, B, and C, respectively. Numbers on the top represent harvesting days after inoculation and rows a, b, and c contain a total RNA from isolates EP155/2, UEP1, and HSM1, respectively. Transcripts of *Cpg*1, *Cpg*2 and *Cpg*3 were analyzed as described at Fig. 2.

Discussion

Mutations have been used to study many genes involved in fungal metabolism as well as developmental process. Many of interesting mutant phenotypes were mapped to the particular linkage group through a either classical or parasexual genetics (Newell, 1978), but in a very few case it was possible to clone the mutated gene. Although functional complementation (Kim *et al.*, in preparation), antibody screening (Rigling and Van Alfen, 1993), differential hybridization (Powell and Van Alfen, 1987) and screening by heterologous probes or primer (Choi *et al.*, 1992; Varely *et al.*, 1992) were successful to isolate genes, it has not been successful to clone those genes without any clear directional selections such as regulatory genes resulting in pleotrophic responses or genes involved in developmental process. Transposon tagging was applicable to isolate a variety of genes in other systems (Cooley and Spradling, 1988; Greenward, 1985; Gridley *et al.*, 1987; Kleckner, 1981; Schmidt *et al.*, 1987; Sommer *et al.*, 1990) but it depends on the availability and mobility of the element (Calos and Miller, 1980). Marker rescuing as a means of isolation of tagged mutation by integrated bacterial plasmid carrying a selectable marker

was described in fungi (Kalpaxis *et al.*, 1991; Schiestl and Petes, 1991). Recently combination of marker rescuing and restriction enzyme named as restriction enzyme mediated integration (REMI) was efficiently used to clone developmental gene (Kuspa and Loomis, 1992).

Insertional mutagenesis and marker rescuing in *C. parasitica* was possible to identify the disrupted area with plasmid insertion and it was possible to identify an ectopic transformant with hypovirulent symptoms. Although transformation process carrying a spheroplast formation and DNA transfer under the presence of PEG and Ca^{++} can induce mutations in genome in addition to the insertional mutagenesis. No other detectable mutation other than the slight over-expression of *Cpg*2 due to the integration of vector was observed. It has been observed that continuous overexpression of *fos* results in eithe r aberrant differentiation (Curran and Franza, 1988; Muller and Wagner, 1984; Ruther *et al.*, 1985) or a block in differentiation (Ito *et al.*, 1989; Lassar *et al.*, 1989). Attempts to block c-*fos* expression using antisense RNA (Holt *et al.*, 1986; Nishikura and Murray, 1987) or microinjection of anti-FOS antibodies (Riabowol *et al.*, 1988) have resulted in an inhibition of cell growth. Thus, overexpression or underexpression of a regulatory gene can have a dramatic effect on growth and differentiation process.

As a conclusion, HSM1 phenotype seems to be the result of insertional mutation which suggests that a single mutation i.e., disruption of normal expression of *Cpg*2, appears to be responsible for all symptoms in virus-free wild type. These facts, altogether, prompts us to characterize *Cpg*2 gene itself further.

적 요

유전자의 삽입에 의해 발생하는 *C. parasitica*의 돌연변이체중 mycovirus에 감염된 것과 같이 색소와 포자를 적게 형성하는 균주(HSM1)를 선발하였다. 선발된 균주는 형태학적 병징외에도 laccase효소의 역가와 같은 생화학적 그리고 표지 유전자들을 통해 분자 생물학적인 특징도 virus에 감염된 균주와 동일한 특징을 나타냈다. HSM1에서 돌연변이가 일어난 부위를 cloning하여 조사한 결과, 유전자 삽입 부위는 *C. parasitica*의 두 유전자(*Cpg*2와 *Cpg*3)의 사이(intergenic space)이며 유전자의 삽입 결과, HSM1에서 *Cpg*2의 발현이 오히려 증가됨이 관찰되었고, 나아가 이와 같은 현상은 mycovirus 감염 균주(UEP1)에서도 일어나고 있음을 확인하였다.

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