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Ordered Differential Display from *Cryphonectria parasitica*

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Ordered differential display using RT-PCR (ODD-PCR) was conducted to have a profile of the differently expressed genes between a hypovirulent strain of *Cryphonectria parasitica* (UEP1) and its isogenic wild type strain (EP155/2). ODD-PCR has advantages of high sensitivity, reproducibility, proportional representation, and limited number of primer combinations comparing with other differential display methods. RNAs were prepared from 1 and 5 day liquid culture of both hypovirulent and wild type strains, and were further evaluated with the marker genes of *C. parasitica* such as cryparin and mating factor MF2-1, which were already proven to be specifically down-regulated by the presence of mycovirus CHV1-713. ODD-PCR was conducted using those RNAs and expressed genes were categorized to five groups according to their temporal and quantitative expression patterns. Those fives groups are CPC, CPE, CPL, CPD, and CPU which represent constitutively-expressed, early-expressed, late-expressed, down-regulated, and up-regulated, respectively. Ninety two primer combinations out of a total of 192 have been tested so far. Among the twenty to fifty distinct bands per each reaction, an average of four to ten genes was identified as viral-regulated fungal genes. Those viral-specifc genes were further analyzed by DNA sequencing followed by homology search. Characterization of 30 clones including all five groups were conducted as a preliminary data and more are under investigation.

Keywords **:** RNA differential display, hypovirulence, Cryphonectria parasitica.

The analysis of changes in gene expression in cells which underwent a particular step such as differentiation, dedifferentiation or pathogenesis is of prime interest of molecular biology. Until recently, the methods to differentiate gene expression for comparative studies rely largely on fingerprinting techniques for proteins by two-dimensional electrophoresis (O'Farrell, 1975) and subtractive hybridization (Lee et al., 1991). However, those methods were either not sensitive enough to detect the minute changes in gene expression or mainly qualitative methods which do not allow to determine quantitative changes (McLland et al., 1995). Thus, by comparing the patterns of expressed gene in a particular cell types, it is possible to detect both quantitative and qualitative changes. This allows not only to identify new genes but also to diagnose any changes in gene expression involved in a particular cellular process (Liang and Pardee, 1992; Bauer et al., 1993).

Recently, many variants of an approach generally termed as mRNA differential display have been developed. As an alternative approach to mRNA differential display using arbitrary primers, ordered differential display using RT-PCR (ODD-PCR) has been known to have advantages in that it is more quantitative, reproducible, accurate, sensitive and simple over the other methods (Matz et al., 1997).

Cryphonectria parasitica (Murrill) Barr, the causal agent of chestnut blight, was responsible for the destruction of the chestnut forests of North America during early of 1990's (Van Alfen, 1982). Furthermore, disease severity becomes more obvious in area such as Korea where the chestnut blight did cause only a mild problem. Recent studies have shown that i) more than 30% of necrotic lesion of chestnut tree in Korea were resulted from infection of *C. parasitica*, ii) the pathogenicity against Korean chestnut variety were varied, and iii) the putative mycoviral-containing strains were detected among the Korean *C. parasitica* population (Ju et al., 1999). In addition, variations on disease severity were observed among currently available chestnut varieties which were presumably considered as resistant ones (Lee et al., 1999).

However, strains containing double-stranded(ds) RNA viruses show the characteristic symptoms of hypovirulence, reduced sporulation and pigmentation (Van Alfen, 1982). In addition, symptoms caused by viral infection appear to be the results of aberrant expression of a small number of specific fungal genes in a hypovirulent strain (Kazmierczak et al., 1996). These viral regulated fungal genes are cutinase gene (Varley et al., 1992), *Lac*1, a gene encoding an extra-

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cellular laccase (Choi and Nuss, 1992; Rigling and Van Alfen, 1991; Kim et al., 1995), *Crp*1, a gene encoding an abundant tissue-specific physical strength related cell-surface hydrophobin (Zhang et al., 1994; Kim et al., 1998), and *Vir*1 and *Vir*2, genes involved in viral-induced sporulation-related symptoms (Zhang et al., 1993).

Several methods including two-dimensional gel electrophoresis (Powell and Van Alfen, 1987b), differential hybridization (Powell and Van Alfen, 1987a), and differential display using arbitrary primers (Chen et al., 1996) have been applied to compare the expression profiles between hypovirulent and its isogenic wild type strain. Here we attempt ODD-PCR of mRNA from hypovirulent and its isogenic wild type strain to analyze the fungal genes specifically involved in symptom development.

Materials and Methods

Fungal strains and culture conditions. The wild type *C. parasitica* strain EP155/2 and its isogenic CHV1-containing hypovirulent strain UEP1 were grown on PDAmb plates for a week at 25°C in the continuous low light (Kim, 1997). In order to prepare the primary inoculum for liquid culture, the cultures of *C. parasitica* grown on plates were homogenized in 100 ml 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).

RNA isolation and Northern blot analysis. Total RNA was prepared from lyophilized and ground mycelia. Twenty ml of lysis buffer [0.2 M Tris-Cl pH 7.5, 0.25 M EGTA, 4.8%(w/v) sodium *para*-aminosalicylate, pH adjusted to 8.8 with NaOH] was added to 0.5 g-1.0 g of ground mycelia. Two ml 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 2 M 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).

Among the molecular markers which are known to be specifically down-regulated by the presence of virus, *Crp*1 and *Vir*1 were examined first in RNA preparations before conducting ODD-PCR. 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) (Kim, 1997).

ODD-PCR. To obtain representative pools of 3' cDNA fragments from polyA-tract to the first occurrence of *Rsa*I recognition site, 2.0 μg of total RNA was reverse-transcribed with oligo-dT primer according to the protocols provided by the supplier (Stratagene), double-stranded, and digested with *Rsa*I enzyme followed by ligation with adaptor primer. The 3' cDNA fragments were PCR-amplified with adaptor-specific primer and such pool was then divided into simplified subsets by means of amplification with primer pairs consisted of ^{32}P-labeled AdE-xx (adapter specific extended primer with two-base extension) and primer TE-xx (T-extended with additional two bases at 3' end), thus selecting the corresponding 1/192 part of the total pool (Matz et al., 1997). Primers used for ODD-PCR are listed in Table 1.

Table 1. List of primers used for ODD

Primers	Sequences (5' to 3')
Tp	AACTGCAGTCGACCGTTTTTTTTTTTT
Adp	AACTGCAGGGCGTGGTGCGGAGGGCGGT
AdE-xx	AGGGCGTGGTGCGGAGGGCGGTCCxx[a]
TE-xx	CGCAGTCGACCGTTTTTTTTTTTTxx[b]

[a]xx, any combination of four nucleotides; [b]xx, any combination of four nucleotides except T on the first position

The PCR products were loaded on standard 6% (w/v) polyacrylamide-urea gels in Tris-borate buffer at 40 W. Gels were dried on the glass plate or transferred to 3MM filter paper and exposed to X-ray film overnight. The autoradiographs were visually examined and the bands were recorded as follows; CPC: constitutively-expressed, CPE: early-expressed, CPL: late-expressed, CPD: down-regulated, CPU: up-regulated.

For further characterization, individual bands of interest were excised, transferred into tube for DNA elution and DNA was reamplified by PCR using primers Tp and Inp followed by cloning of amplified products.

The cloned genes were sequenced and homology search was conducted. The expression pattern of cloned DNA was confirmed by Northern blot analysis again.

Results

Expression of marker genes. Before ODD-PCR, expression of viral-regulated fungal marker genes was tested with RNA preparations from 1 and 5 days cultures of both hypovirulent UEP1 strain and wild type EP155/2 strain. Cryparin gene, *Crp*1, which was known as the early-expressed gene was examined first and its expression pattern was as expected in that mRNA accumulation of *Crp*1 maximized at early stage ($\leq$ 3 days) and then it gradually decreased in EP155/2. In contrast, *Crp*1 expression in UEP1 was significantly down-regulated at both early and late stage (Fig. 1). Comparing with *Crp*1, mRNA accumulation of *Vir*1 reached the peak at late stage ($\geq$ 5 days) in EP155/2 and it also specifically down-regulated in UEP1 (Fig. 1). As an internal control for mRNA expression, glyceraldehyde-3-phosphate dehydrogenase gene was included.

ODD-PCR. Among the 192 possible primer pairs, 92 primer pairs were attempted for ODD-PCR. Before conducting ODD-PCR with all possible combinations of primer set, two combinations of primer pair were tested three times to check the reproducibility of PCR reaction. Major band patterns were so similar that it was not easy to

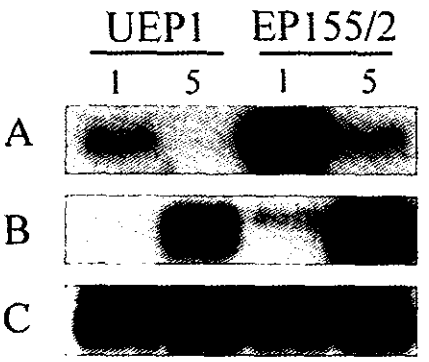

Fig. 1. Northern blot analyses with marker genes which were previously known as down-regulated fungal genes due to the virus infection. A, Northern blot analysis of cryparin gene. B, Northern blot analysis of *Vir*1 gene. C, Northern blot analysis using glyceraldehyde-3-phosphate dehydrogenase gene was used as a control for the loading amount of RNA. RNA was extracted from 1 and 5 day cultures of both hypovirulent strain (UEP1) and wild type strain (EP155/2). Numbers at the top represent the days of liquid culture.

tell the differences among them (data not shown).

Expression profile by a single primer pair revealed twenty to fifty discernable bands per each lane from a gel and these bands were categorized according to their temporal and quantitative expression patterns (CPC, CPE, CPL, CPD, and CPU) (Fig. 2). Those which revealed no difference between wild type EP155/2 strain and hypovirulent UEP1 strain were CPC, CPE, and CPL. Those which showed the difference between two strains were referred to CPD and CPU. Four to ten bands in each reaction were either CPD or CPU. They were excised from the gels and stored at -70°C for further analysis.

As an initial approach to obtain the expression profile, thirty bands including all five groups were randomly selected and characterized. (Table 2). Seven clones had no homology to the current database and the rest showed homologies to the sequence from various sources such as animal, plant, and microbes. In addition, one was from *C. parasitica* and five from other fungi.

The expression patterns of each of CPD and CPU were reevaluated by Northern blot analysis and the results were consistent with those of the ODD-PCR (Fig. 3).

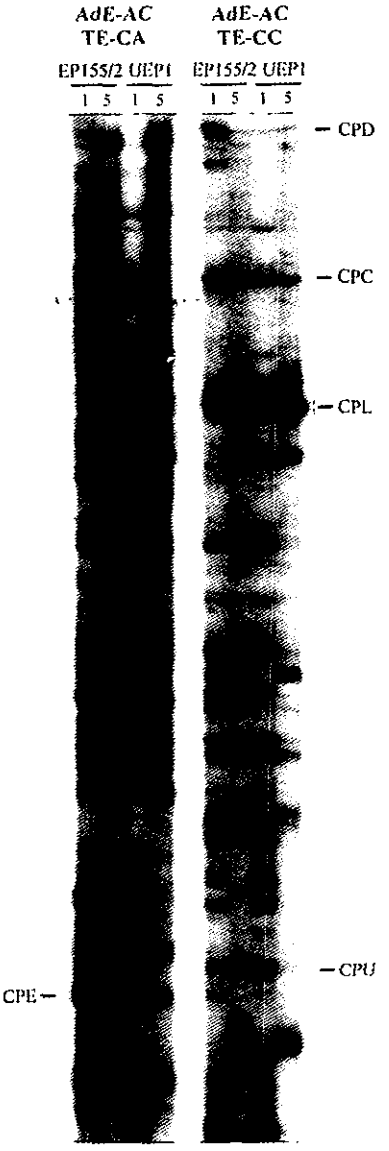

Fig. 2. Expression profiles of two different ODD-PCR subsets. Four RNA samples, extracted from 1 and 5 day cultures of both hypovirulent strain (UEP1) and wild type strain (EP155/2), were compared for each subsets. The respective combination of extended primers used for subset is indicated at the top of each panel. Bands of interest are represented as CPE, CPL, CPC, CPD and CPU for early-expressed, late-expressed, constitutively-expressed, down-regulated, and up-regulated gene, respectively. Numbers represent the days of liquid culture and primers are indicated at the top.

Discussion

It has been known that different culture conditions may result in alleviating the viral symptoms in *C.parasitica* (Hillman et al., 1990). Thus the expression patterns of known marker genes needed to be examined first and the results indicated that our culture conditions were similar to the other conditions allowing the characteristic morphological and molecular phenotypes.

Differential display using arbitrary primers has been used to compare fungal gene transcript accumulation in transformed *C. parasitica* with either its parental or isogenic hypovirulent strain (Chen et al., 1996). However no other study such as cloning and characterizing genes with altered expression has been conducted.

Unlike the differential display using arbitrary primers,

Table 2. Sequence similarity of cloned genes

Clone No.	ODD identifier	Accession number[1]	Putative identification	E value
0001	CPD	Y10012	*Klebsiella pneumoniae* cydA and cydB gene.	2.2
0002	CPC	AC000104	Sequence of BAC F19P19 from *A. thaliana* chrI.	1.1
0003	CPL	X13206	Cotton seed protein (late embryogenesis abundant)	1.0
0004	CPL	AC006299	*Homo sapiens*, clone hRPK.17_A_1, complete sequence.	4.0
0005	CPU	AF017138	*Rhodobacter capsulatus* cob(I)(cobO) and (cobW) genes	1.6
0006	CPL	S54007	Arylmalonate decarboxylase [*A. bronchisepticus*]	2.0
0007	CPC	L42307	*Neurospora discreta* mt-A1 gene	2e-25
0008	CPL		N.D[2]	1e-16
0009	CPD	AF085840	*H. sapiens* full length insert cDNA clone YI44E12.	0.66
0010	CPE		N.D	
0011	CPE		N.D	
0012	CPC	X53996	*C. parasitica* gpd-1 gene (EC 1.2.1.12)	4e-80
0013	CPD		N.D	
0014	CPC	AF222062	*Propionibacterium acnes* heat shock protein70 (dnaK)	3.8
0015	CPU	AF180731	*K. pneumoniae* dihydrofolate reductase (dhfrXII)	2e-35
0016	CPC	X62724	*H. vulgare* mRNA for ribosomal protein L17-1	3e-19
0017	CPC	X13254	*Neurospora crassa* crp-1 mRNA for ribosomal protein	7e-11
0018	CPC	AF003356	*P. aurantiogriseum* 28S large subunit rRNA gene	1e-64
0019	CPL	AL021492	*Caenorhabditis elegans* cosmid Y45F10D	2.9
0020	CPU		N.D	
0021	CPD	L25598	*C. elegans* cosmid C06G4.	2.7
0022	CPE	AJ002258	*M. musculus* mRNA for GC Binding Protein	0.20
0023	CPC	AL027362	*C. elegans* cosmid C15A7	0.22
0024	CPC	AJ01390.1	*E. nidulans* mtDNA between h2/h5 and bh2/b2 junction	1e-30
0025	CPC	AF034948	*Zea mays* ribosomal protein L17 (rpl17) mRNA	2e-08
0026	CPD		N.D	
0027	CPE	AB016876	*A. thaliana*, chromosome5, P1clone: MKM 21	0.10
0028	CPE	AF109907	*H. sapiens* FGF/int-2 gene upstream flanking region.	1.40
0029	CPL	M69077	*H. sapiens* DNA polymerase epsilon subunit B	0.51
0030	CPU	U05811.1	*T. reesei* QM9414 serine/threonine protein kinase (pkt1)	1e-30

[1] Accession number indicates Genbank entries already deposited.
[2] N.D. means "not determined".

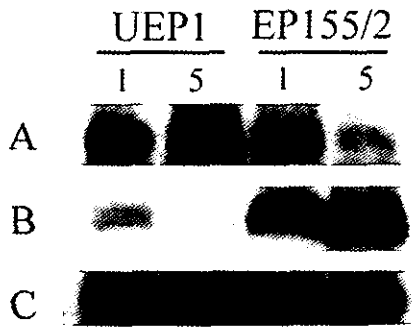

Fig. 3. Northern blot analyses of differentially expressed genes. A, Northern blot analysis using one of CPD clone (Clone No. 0009 in Table 2). B, Northern blot analysis using one of CPU clone (Clone No. 0020 in Table 2). C, Northern blot analysis using glyceraldehyde-3-phosphate dehydrogenase gene was used as a control for the loading amount of RNA. RNA was extracted from 1 and 5 day cultures of both hypovirulent strain (UEP1) and wild type strain (EP155/2). Numbers at the top represent the days of liquid culture.

each transcript is believed to be represented as a single band in ODD-PCR (Matz et al., 1997). Although the sample size is restricted, no repeat of sequence identity was observed, indicating that ODD-PCR was conducted as expected in this study. In addition, the result that glyceraldehyde -3-phosphate dehydrogenase known as the constitutively expressed gene (Kim et al., 1997) was identified as CPC verified the application of ODD-PCR as an appropriate method to obtain expression profiles of both EP155/2 and UEP1.

Twenty to fifty bands from each of 192 reactions may yield a sum of 3,840 to 9,600 possible mRNAs presented in the cell. Accordingly, four to ten viral infection-specific fungal genes per reaction can be resulted in a total of 768 to 1,920 genes, indicating that about 20% of total genes appears to be specifically modulated by the presence of virus. These results were not consistent with the previous studies showing fewer than 5% of total proteins (Powell

and Van Alfen, 1987b) or 400 PCR products (Chen et al., 1996) were affected by the presence of hypovirus. These discrepancies in numbers of viral infection-specific gene or gene products are assumed to be ascribed to the improved techniques allowing better sensitivity, reproducibility, and accuracy.

Currently, we are close to finish ODD-PCR with all the possible primer pairs, and genes of interest will be analyzed further by using forced expression of those cloned genes and/or making the loss-of-function mutants.

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