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Identification of Genes Expressed during Conidial Germination of the Pepper Anthracnose Pathogen, *Colletotrichum acutatum*

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Genes expressed during conidial germination of the pepper anthracnose fungus *Colletotrichum acutatum* were identified by sequencing the 5′ end of unidirectional cDNA clones prepared from the conidial germination stage. A total of 983 expressed sequence tags (ESTs) corresponding to 464 genes, 197 contigs and 267 singletons, were generated. The deduced protein sequences from half of the 464 genes showed significant matches (e value less than 10^{-5}) to proteins in public databases. The genes with known homologs were assigned to known functional categories. The most abundantly expressed genes belonged to those encoding the elongation factor, histone protein, ATP synthease, 14-3-3 protein, and clock controlled protein. A number of genes encoding proteins such as the GTP-binding protein, MAP kinase, transaldolase, and ABC transporter were detected. These genes are thought to be involved in the development of fungal cells. A putative pathogenicity function could be assigned for the genes of ATP citrate lyase, CAP20 and manganese-superoxide dismutase.

Key words : Expressed sequence tag, conidial germination, pepper anthracnose

Introduction

Pepper (*Capsicum annuum* L) is an important crop because of its high consumption as seasoning vegetable in Korea and many other countries. Anthracnose disease is one of the major limiting factors in pepper production [17]. Anthracnose causes lesions on immature green fruits, mature red fruits, leaves, and seedlings. Especially the yield loss is serious since pepper fruits with anthracnose have no value to market [18]. A number of different species of *Colletotrichum* such as *C. gloeosporioides*, *C. dematium*, *C. coccodes*, and *C. acutatum* were described to be involved in anthracnose symptom. *C. gloeosporioides* has been reported as the most dominant species as it represents 90% of the isolates associated with the disease [21]. Recently, however, a number of surveys on the composition of *Colletotrichum* species involved in pepper anthracnose indicate that *C. acutatum* is a major species involved [18].

Control of the pepper anthracnose has been dependent on the fungicide application due to the lack of resistance sources against the disease in Korea [18]. As the disease caused by *C. acutatum* increases in the fields, it needs to figure out the biological aspects of the species. There is little knowledge on the molecular events that occur during pathogenesis related development of *C. acutatum*. Identification of genes of plant pathogenic fungi provides the resources to understand the genetic basis of mechanisms involved during infection and development of the pathogen. The analysis of expressed sequence tags (ESTs) can provide a global scope of information on the genes expressed at certain conditions. EST technique was first introduced for human brain research [1]. Recently, ESTs are available with many phytopathogens [2, 3, 6, 7, 13-16, 19, 20, 22, 23, 25]. ESTs identified from pathogenic fungi have offered an efficient way to identify genes involved in pathogenicity in genome wide scale. *C. acutatum* has not been touched by genomic approaches yet for gene identification.

In this study, we describe a pilot-scale cDNA sequencing that provides the first opportunity for inspection of the gene expression during conidial germination of pepper anthracnose fungus *C. acutatum*. We have produced 983 ESTs from conidial germination stage cDNA library, resulting 464 genes. The result shows that sequencing of ESTs from a conidial germination cDNA library provides a view of gene activities during germination related differentiation and pro-

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vides a powerful way to identify new genes likely involved in fungal development and pathogenesis.

Materials and Methods

Culture conditions and RNA preparation

A virulent isolate of *C. acutatum* JC24 was isolated from pepper field. The isolate was maintained on Potato dextrose agar (Difco, USA) at 25±1°C under day/night lighting. Total RNA was prepared from 24 hr germinating conidia grinded in liquid nitrogen followed by Trizol based extraction method according to manufacturer manual (Sigma Chemical, St. Louis, MO, USA). To check integrity in a quick way, RNA was separated through a 1% agarose-TAE gel containing ethidium bromide. Poly(A) RNA was isolated using oligo(dT)-cellulose chromatography (Life Technologies, USA) following the manufacturer's recommendations.

cDNA library construction

cDNA synthesis and size selection were done using the cDNA synthesis kit (Stratagene) according to the manufacturer's instructions. Reverse transcription of the poly(A) mRNA was primed with a XhoI primer (5′-GAGAGAGA GAGAGAGAGAGAACTAGTCTCGAGT_{18}-3′) and, after second strand synthesis, a EcoRI adapter (5′-AATTCGG CACGAG-3′) was added (the underlined region was double stranded). The size-selected (1~3 kb) cDNA fragments were ligated unidirectionally into EcoRI-XhoI sites of pBluescript II SK+ (Stratagene), with the 3′ end of the cDNA adjacent to the XhoI site. Recombinant plasmid ligate then was transformed into *Escherichia coli* (DH10B, Gibco BRL) by electroporation. Individual white colonies were transferred to freezing media (36 mM $K_2HPO_4.3H_2O$, 13.2 mM KH_2PO_4, 1.7 mM $Na_3C_6H_5O_7.2H_2O$, 0.4 mM $MgSO_4$, 6.8 mM $(NH_4)_2SO_4$, and 4.4% glycerol) in 384-well microtiter plates and grown overnight at 37°C. The plates then were stored at -80°C after incubation.

Large scale DNA preparation

Plasmid DNA from cDNA clones were purified using a modified alkali lysis procedure. Clones were added to 1.2 ml of Terrific Broth (12 g of bacto-tryptone, 24 g of bacto-yeast extract, 4 ml of glycerol, 2.31 g of KH_2PO_4, and 12.54 g of K_2HPO_4 per liter) with ampicillin (100 mg/l) in 96-deep-well culture plates and cultured overnight at 37°C with shaking (300 rpm). Cells were separated by centrifugation (3,000 rpm for 10 min.) and resuspended in 100 µl of solution I (50 mM Tris-HCl, pH 8,0; 10 mM EDTA; 100 mg/ml RNase A). Cells were lysed with 200 µl of solution II (200 mM NaOH; 1% SDS) with gentle mixing. Solution III (3 M potassium acetate, pH 5.5) was added within 5 min. of solution II treatment. After gentle mixing, the lysed cells were removed by centrifugation (3,000 rpm for 10 min.). Supernatant was transferred into a Polyfiltronics Unifilter 800 (Cat. No. 7770-0062, Whatman) and collected by centrifugation in a receiver plate. DNA was precipitated by adding 0.6x volume of isopropanol and collected by centrifugation (3,000 rpm for 30 min.). After the pellet was washed with 70% ethanol and air dried, the DNA was dissolved in 50 µl of dH_2O. The quality and quantity of DNA for the cycle sequencing reaction was determined by agarose gel electrophoresis. The average insert size was determined by gel electrophoresis analysis after digestion with EcoRI and XhoI with randomly selected cDNA clones.

DNA sequencing

Partial nucleotide sequences of the cDNA inserts were determined using the dideoxy chain termination method. Purified plasmid DNA was sequenced using Applied Biosystems (ABI) Big Dye terminator kits (Perkin-Elmer) and the ABI model 3700 DNA sequencers in the NICEM (National Instrumentation Center for Environmental Management) of Seoul National University. T3 sequencing primers were used for 5′ end reading. Cycle sequencing reactions were performed as followed; DNA 200 ng, 3 µl of reaction buffer (200 mM Tris-HCl, 5 mM $MgCl_2$, pH 9.0), 1 µl of Big Dye, 1 µl of primer (3.2 pmole), and dH_2O up to 10 µl.

Nucleotide and protein sequence analyses

Raw sequence data were processed with the software programs, Phred-Phrap and Crossmatch [8, 9], to mask the vector sequences and to assemble the overlapping clones into contigs. Individual ESTs and consensus sequences of each contig in batch files were searched against the GenBank protein database using the BlastX algorithm through the web site of the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov).

Data access

Access to the sequence data is available at GenBank with accession numbers from GO613482 to GO614484.

Results

Generation of *C. acutatum* cDNA library

A conidial germination stage was selected since it is basal for further development in fungal growth. The cDNA library was constructed using mRNA isolated from germinated conidia of *C. acutatum*. At the time of harvest, ~90% of conidia were germinated. More than 95% of the cDNA clones contained inserts with an average insert size of 1.5 kb, ranging mainly from 1 kb to 2 kb (Fig. 1).

Sequencing and contig assembly

T3 primer was used to generate 5′ end sequence information from 1,032 randomly selected cDNA clones. Sequencing outputs were examined for quality with the Phred software program. A successful sequencing run was defined as a sequence containing at least 50 bp with a Phred score of 20 or higher. A total of 983 ESTs were considered successful based on these criteria. Contig assembly generated a set of 464 unique sequences (genes) including 716 ESTs in 197 contigs and 267 singletons. Number of ESTs in contigs ranged from 2 to 29 (Fig. 2). The redundancy of the library was determined to be 72.8%.

Homology search and functional annotation

The most redundantly detected genes with over 3 copies showed homology to genes encoding an elongation factor with a frequency of 3% (29/983), followed by histone protein H3, histone protein H4, ATP synthase, folate-dependent phosphoribosylglycinamide formyltransferase, 14-3-3 protein, clock-controlled protein 6, xylulose reductase, transaldolase, ribosomal protein, chitnase, cell division control protein, beta-1-3-glucanosyltransferase, ATP citrate lyase, NADH-ubiquinone oxidoreductase, ATP synthase 9, and 60s ribosomal protein (Table 1).

To understand the putative function of the genes expressed, 464 genes, unique sequence sets, were queried against GenBank using the BLASTX algorithm through the web site of the NCBI. Consequently, homology search revealed that 230 genes (49.6%) matched significantly ($E < 10^{-5}$) to non-redundant protein sequences in the GenBank (Fig. 3A). The number of 27 genes showed match against fungal originated homologs. A number of 10 genes matched to known *Colletotrichum* ones encoding proteins such as ATP synthase, actin, alpha-tubulin, 60s ribosomal protein, manganese-superoxide dismutase and MAP kinase kinase. The 464 genes including 230 genes matching to known protein sequences were categorized by molecular function (Fig. 3B). A molecular function assignment scheme was devised based on the Gene Ontology classification system (http://geneontology.org). The most major functional categories were represented by catalytic activity, binding and structural molecular activity. A total of 363 genes (78%) with matches that could not be fit easily to any category were classified to the unassigned.

Discussion

Conidial germination is the start point of pathogenic development of *C. acutatum* as fungal pathogen. The morphological and biochemical aspects of germination have not been well studied for this destructive fungal species. In the study here, numerous genes potentially involved in

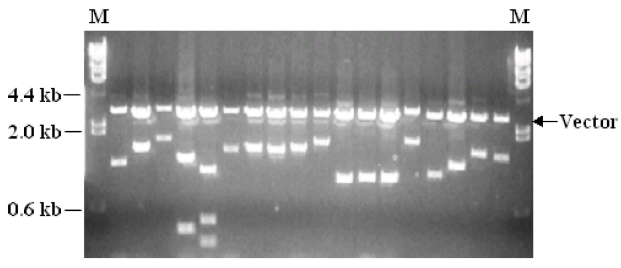

Fig. 1. Gel analysis of insert size of the *C. acutatum* conidial germination cDNA library. Randomly selected cDNA clones were analyzed by digesting plasmid DNA with EcoRI and XhoI to excise the insert DNA, and separated on 1% agarose gel with molecular marker DNA indicated by M. DNA fragments for vector, pBluescriptIISK+, are located at 2.9 kb position.

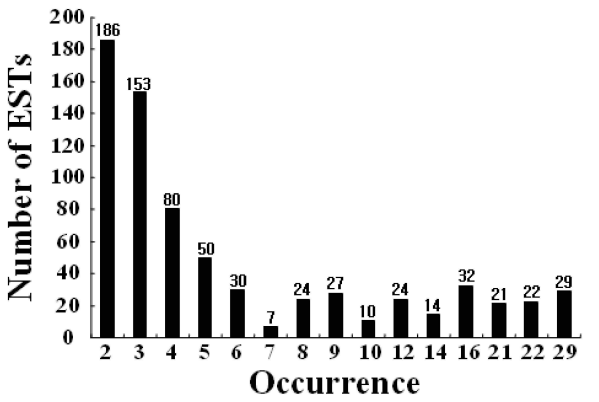

Fig. 2. Redundancy in ESTs from *C. acutatum* cDNA library from conidial germination stage. The occurrence of multiple sequences is given.

Table 1. Genes with putative identification of described function and redundantly expressed in the conidial germinating stage cDNA library of *C. acutatum*

Contig No.	Copy	Acc. No.	Species	Target name
Contig054	29	AAR16425.1	*Metarhizium anisopliae*	Translation elongation factor 1 alpha
Contig085	22	AAM76068.1	*Hypocrea jecorina*	Histone H3
Contig017	14	AAW69330.1	*Magnaporthe grisea*	Histone H4-like protein
Contig161	5	AAX07697.1	*Magnaporthe grisea*	ATP synthase gamma chain-like protein
Contig001	4	ZP_00222798.1	*Burkholderia cepacia* R1808	Folate-dependent phosphoribosylglycinamide Formyltransferase PurN
Contig033	4	EAA73638.1	*Gibberella zeae* PH-1	ATPB_NEUCR ATP synthase beta chain, Mitochondrial precursor
Contig096	4	CAC20378.1	*Hypocrea jecorina*	14-3-3-like protein
Contig139	4	CAD70877.1	*Neurospora crassa*	CLOCK-CONTROLLED PROTEIN 6
Contig147	4	AAM20896.1	*Hypocrea jecorina*	L-xylulose reductase
Contig050	3	AAW69342.1	*Magnaporthe grisea*	Transaldolase-like protein
Contig057	3	CAB88562.1	*Neurospora crassa*	Probable ribosomal protein l13a
Contig060	3	AAV98692.1	*Torrubiella confragosa*	Basic chitinase
Contig074	3	EAA62067.1	*Aspergillus nidulans* FGSC A4	CD42_CHICK Cell division control protein 42 homolog (G25K GTP-binding protein)
Contig083	3	CAD70754.1	*Neurospora crassa*	beta (1-3) glucanosyltransferase gel3p
Contig093	3	CAB76165.1	*Sordaria macrospora*	ATP citrate lyase, subunit 1
Contig106	3	AAX07707.1	*Magnaporthe grisea*	NADH-ubiquinone oxidoreductase-like protein
Contig179	3	CAC27323.1	*Colletotrichum gloeosporioides* f. sp. aeschynomene	ATP synthase 9
Contig190	3	EAA78050.1	*Gibberella zeae* PH-1	60S ribosomal protein L27a (L29)

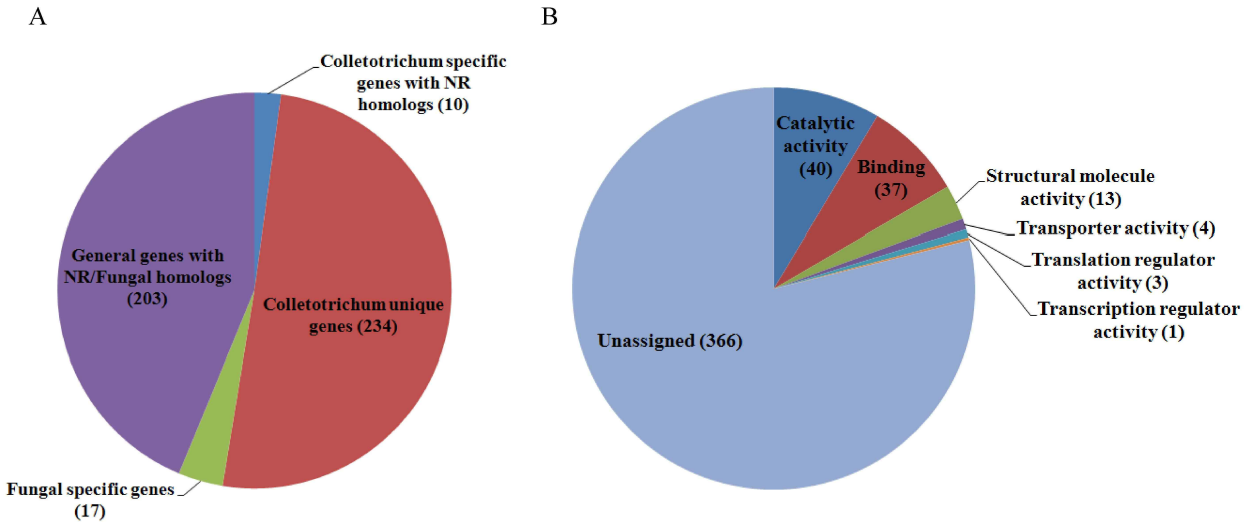

Fig. 3. A. Classification of 464 *C. acutatum* conidial germination genes according to groups of hits matching. B. Genes of 464 with no match or significant matches to known sequences based on BlastX (E value of $<10^{-5}$) were assigned into molecular function categories. The number of genes in each category is shown.

fungal development and pathogenicity were identified by sequencing a conidial germination stage cDNA library of *C. acutatum*. A total of 983 ESTs corresponding to 464 genes were generated in this pilot scale sequencing analysis. The number of genes assembled is likely to be an overestimate due to the low sequence quality for some sequences and the potential for lack of sequence overlap among mRNA originated form same gene.

The library used for EST sequencing was constructed by cloning the cDNA directly into a plasmid vector. Previously, the lambda phage vector was a popular choice for constructing cDNA libraries. Since it is now possible to obtain very high efficiency in *E. coli* transformation by electroporation, the library was directly ligated into the plasmid vector, pBlueScriptIISK+, which eliminates the need to use *in vivo* excision to create phagemids. A 96-well format plasmid DNA isolation method was established for these experiments. Several methods were evaluated including the Wizard system (Promega, USA), Polyfiltronics filter plate (Whatman, USA) and Qiagen miniprep kit (Qiagen, USA) for high-throughput DNA isolation. It was concluded that using the alkaline lysis method employing the Polyfiltronics filter plate for purification yielded DNA of acceptable quality/quantity in a cost-effective way. This method is now applied widely to isolate plasmid DNA or BAC DNA in a high-throughput manner. In these experiments, the amount of BigDye for EST sequencing also was evaluated. The standard protocol recommends use of 8 µl of BigDye in 20 µl of cycle sequencing reaction or 4 µl of BigDye in 10 µl of reaction volume. The BigDye is a major cost of any sequencing project. It was found that 1 µl of BigDye in a 10 µl reaction was minimally acceptable for detecting signals in this EST sequencing.

Based on Phred-Phrap analysis, 197 contigs containing 716 ESTs and 267 singletons were generated. The most redundant contig contained 29 ESTs and showed a high degree of similarity to the elongation factor. Comparison of the unique sequences to public databases in GenBank indicated that 50% of the genes did not share significant similarity with proteins in public databases. This result implies that EST sequencing is an efficient way to identify novel genes. Future functional analysis of these gene products may reveal previously unknown details in germination development of *C. acutatum* and lead to the creation of novel management strategies.

The EST approach also provides a measure of the level of expression of each gene. Therefore, it is valuable to investigate genes expressed abundantly to gain an understanding of their possible roles in conidial germination and further development. Based on the BlastX analysis, genes encoding an elongation factor, histone proteins, ATP synthases and ribosomal proteins were expressed frequently during conidial germination. These proteins are thought to have roles in general cell-maintenance and have been found to be highly redundant in other fungal species like *Neurospora* (www.genome.ou.edu), *Phytophthora* [14], and *Trichoderma* (www.fungalgenomics.ncsu.edu). A gene encoding folate-dependent phosphoribosylglycinamide formyltransferase PurN is also redundantly expressed and this protein is a multifunctional and has role in synthesis of purine, which is used for DNA synthesis. Other abundantly expressed gene encodes 14-3-3 protein, which has been identified in several fungi and is known as a family of putative kinase regulators originally characterized in mammalian brain tissue [26]. The 14-3-3 protein has a wide range of potential functions and pathological relevance. It regulates intercellular signal transduction to bind the target molecules including transcription cofactor. Also it prevents or mediates apoptosis by controlling potential signaling molecules. The function of this gene in *C. acutatum* during conidial germination and other development remains to be determined. Transaldolase detected in this EST data catalyzes the reversible transfer of a dihydroxyacetone moiety derived from fructose-6-phosphate to D-erythrose- 4-phosphate and is a rate limiting enzyme. The lack of transaldolase in some eukaryotic cells has been shown to make them more sensitive to oxidative stress [27]. Since conidial germination is a dynamic process in the life cycle of cell and needs a system for protect the cell from oxidative damage. Conidial germination requires the formation of new cell membrane and cell walls. Genes encoding chitinase and beta-1-3-glucanosyltransferase are abundantly detected and regarded to be involved in cell wall regeneration.

The advantage of analysis of ESTs derived from certain developmental stage is that the putative function of each gene expressed can be connected with the development process. A number of genes in the signal transduction pathway were identified. The involvement of signaling in development is well documented in several pathogenic fungi such as *Magnaporthe grisea* [5]. Present study identified gene of GTP-binding protein. GTP-binding proteins are known to be involved in signal mechanisms influencing many aspects of morphogenesis and pathogenicity of fungal pathogens. The involvement of MAP kinase pathway has been well examined in *M. grisea* [29] and a number of MAP kinase genes were also identified. The homolog of casein kinase II, alpha chain of *Giberella zeae* was also detected.

Germination is a dynamic stage in fungal development and may generate a high level of reactive oxygen. For successful growth and further development, cell must control

the effect of oxidative stress. Genes encoding manganese-superoxide dismutase and transaldolase protein were identified. This finding suggests that anti-oxidative stress is an important step for successful development of *C. acutatum*. The finding of ABC transporter ABC4 homolog may reflect the dynamic activity in transportation of molecules during germination.

The identification of ESTs showing homology to the genes related to pathogenicity in other plant pathogenic fungi is extremely interesting since these genes may have similar functions in the *C. acutatum*. ATP citrate lyase homolog was identified and isocitrate lyase is related to pathogenicty in *Leptosphaeria maculans*, causal agent of blackleg disease of canola [12]. The identification of CAP20 homolog is very interesting since CAP20 is known to be involved in appressorium formation and pathogenicity in *C. gloeosporioides* [10] and also detected in *M. grisea* during appressorium formation [4]. The function of CAP20 homolog is good candidate for studying a pathogenicity factor in *C. acutatum*. Manganese-superoxide dismutase homolog was also identified. Copper- and zinc- containing superoxide dismutase is required for the protection of human fungal pathogen *Candida albicans* against oxidative stresses and for the full virulence [11].

The main objectives of this project were to identify *C. acutatum* genes expressed during early development of conidia and to investigate their expression profile (abundance) by sequence analysis. The data presented here represent a preliminary survey. Additional sequencing efforts will focus on using different stage cDNA libraries or stage enriched subtractive cDNA libraries. Future efforts to characterize the genes involved in diverse pathogen-related developmental stages include several additional approaches such as microarray [24] and SAGE analyses [28].

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초록 : 고추 탄저병균의 포자 발아 단계 발현 유전자 동정

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고추 탄저병균의 포자 발아 단계에서 발현되는 유전자를 파악하기 위해 포자 발아단계cDNA library를 제작하고, 임의로 선택된 cDNA clone들에 대한 EST sequencing을 실시하였다. 총 983개 EST를 확보하여 contig assembly를 실시한 결과, 197개 contigs와 267개 singletons으로 조합되어, 최종적으로 464개의 유전자를 동정하였다. 464개 유전자 서열에서 유추한 아미노산 서열을 이용한 상동유전자 검색을 통해 절반의 유전자가 GenBank에 기존 등록된 유전자와 유의성 있는 유사성을 보였다. 가장 높은 빈도로 발현된 유전자는 elongation factor, histone protein, ATP synthease, 14-3-3 protein, clock controlled protein을 암호화하는 유전자들이었다. 그리고 고추 탄저병균의 세포 발달과정에 관여 하는것으로 추정되는 GTP-binding protein, MAP kinase, transaldolase, ABC transporter 유전자들도 검출되었다. 또한 고추 탄저병균의 병원성에 영향을 미치는 것으로 파악되는 ATP citrate lyase, CAP20, manganese-superoxide dismutase 유전자들도 검출되어, EST sequencing 을 통한 세포 발달 단계 발현 유전자 탐색이 효과적임을 알 수 있었다.