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Analysis of Genes Expressed during Pepper-*Phytophthora capsici* Interaction using EST Technology

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Pepper, consumed as a typical spice food around world, is mainly cultivated in warm countries, including Korea, China, and Mexico. *Phytophthora capsici* is a pathogen on several economically important crops, including pepper. The oomycete attacks the roots, stems, leaves, and fruit of the host plants. To understand the molecular mechanisms underlying development of the disease, the genes expressed during pepper-*P. capsici* interaction were explored by analyzing expressed sequence tags (ESTs). A cDNA library was constructed from total RNA extracted from pepper leaves challenged with *P. capsici* for three days, resulting in an early stage of symptom development for comparable interaction. A comprehensive analysis of single-pass sequencing of 5,760 randomly selected cDNA clones extracted 5,148 high-quality entries for contig assembly, which generated 2,990 unigenes. A homology search of the unigenes with BLASTX resulted in 2,409 matches, of which 606 showed classified functional catalogs.

Key words : Expressed sequence tag (EST), pepper, *Phytophthora capsici*

Introduction

Phytophthora blight of pepper (*Capsicum annuum* L.), caused by *Phytophthora capsici*, is one of the most destructive diseases in pepper production worldwide [10]. This *Phytophthora* disease has been responsible for major production losses of pepper [5]. The pathogen infects all parts of pepper including roots, stems, leaves, and fruits. The most common symptoms associated with the disease are wilting and a root and crown rot characterized by a dark brown stem lesion extending upward from the soil line [2]. The advancing lesion eventually girdles the main stem and kills the whole plant [3]. The control methods were limited due to the difficulty in dealing with this soil-borne disease. Controlling of this disease has mainly depended on chemical treatment, cultural methods, and crop rotation [10]. However, the frequent appearance of fungicide resistant isolates has reduced the effectiveness of chemical treatment [9]. Use of resistant pepper cultivars has been considered as the most profound way for protecting peppers from the *Phytophthora* blight. In Korea, however, many pepper cultivars susceptible to the disease have been cultivated intensively recently. The rational application of resistance in pepper protection could be achieved from the comprehensive understanding on the defense mechanisms during the interactions between pepper and *P. capsici*. And the knowledge of genes involved in pathogenicity may contribute to the development of novel strategies to control this devastating disease.

The pepper-*P. capsici* pathosystem is more complex comparing to other host-pathogen systems since *P. capsici* can infect virtually every part of the host plant. Host infection by virulent *P. capsici* can occur through stomata or by direct penetration into the epidermis of plant surface. The infected tissue can be heavily colonized by the pathogen in 24 hr after inoculation [21]. Until now, there is no pathogenicity gene characterized from *P. capsici*. On the other hand, a number of genes associated in defense have been cloned from host plant pepper and characterized. Pathogenicity related genes such as beta-1,3-glucanase [4, 12], chitinase [8, 11, 22], PR-1 protein, Thionins [20], pepper lipid transfer protein genes (LTP) [13], Sesquiterpene cyclase [17], and Hydroxymethylglutaryl-CoA reductase genes [7] have been studied

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in their expression profile during pepper-*P. capsici* interactions.

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 plants in response to a variety of conditions [6, 28, 30] and phytopathogens [14, 16, 19, 24, 26, 27]. ESTs identified from plant-pathogen interactions as Rice-*Magnaporthe grisea* [18, 25], Pine-*Heterobasidium* [15], Wheat-*Fusarium* [23], Wheat head-*Giberella zeae* [19], and Plant-nematode [29] have offered an efficient way to identify genes involved in plant defense and pathogenicity in genome scale.

In this study, we describe the results of EST analysis performed to explore the genes associated in pepper-*P. capsici* interactions. A comparable interaction was chosen to identify the defense-related genes from host plant and the pathogen genes necessary for survival in the host.

Materials and Methods

Plant, Pathogen, and Inoculation

Peppers (*Capsicum annuum* L.) of cultivar Bukang were grown in growth chambers under day-night illumination at 27°C. A virulent isolate PC1 of *P. capsici* was used in this study. The Oomycete was grown on oatmeal agar in the dark for 7 days at 25°C and exposed to fluorescent light for 7 days at 25°C for sporangial production. Sporangia were collected with sterile deionized water and incubated at 5°C for 1h to release zoospores. Zoospore suspension was adjusted to the concentration of 10^5/ml with sterile deionized water and sprayed to 6 week old pepper. The pepper plants after inoculation were placed in moist chambers at 22°C for 72 hr and the lesion areas were sampled for RNA preparation.

Construction of cDNA library

The pepper-*P. capsici* cDNA library, named KS13, was constructed from pepper leaves infected with virulent isolate of *P. capsici*. Total RNA was isolated using Trizole (Invitrogen, USA) following the manufacturer instructions. Poly(A)+ RNA was purified through oligo cellulose chromatography and used for cDNA synthesis and cloning with cDNA library system (Stratagene, USA). The cDNA products were size selected and inserted into the Uni-Zap XR vector. After *in vivo* excision, individual colonies were transferred to 96 well plates for further process.

EST sequencing

Plasmid DNA from cDNA clones was purified using a modified alkali lysis procedure in 96 well system (Qiagen, Germany). Prior to sequencing, all plasmids were checked for concentration. Sequencing of the cDNA clones was performed from the 5′ end with T3 universal primer using BigDye terminator kit (Perkin Elmer, USA) and the ABI 3700 DNA sequencer (Applied Biosystems, USA).

Data processing

Raw sequence data were treated in processing including vector trimming, quality screen, contig assembly, unique gene extraction and representative clone selection using the software stackPACK (STACK clustering system). In quality screen, sequences with over 100 bp after vector removal were used for further process. Non-redundant EST sequences (unigenes) from contig assembly were used for searching against GenBank protein database using BLASTX algorithm in local computing system. Sequences with homologs in known database with function were classified into functional categories following the MIPS system (www.mips.biochem.mpg.de). All sequences were deposited at GenBank (accession number 60489608~60494726).

Results

Constructon of cDNA library from pepper-*P. capsici* interactions

A cDNA library was constructed using mRNA isolated from pepper (cultivar Bukang) leaves harvested at 72 hr post-inoculation with virulent *P. capsici* isolate PC1. The average insert size was determined to be 1.2 kb approximately based on the agarose gel analysis of inserts that were PCR amplified using universal primers with randomly selected cDNA clones.

EST sequencing and contig assembly

Single pass, 5′-end sequences of 5,760 cDNA clones were generated using the T3 primer resulting 4,867 high quality ESTs. Redundant ESTs were grouped into 2,990 non- redundant genes (unigenes) including 2,049 singletons (single copy sequences) and 941 contigs (Table 1). There were 17 contigs with 10 or more ESTs, which were considered as highly expressed transcripts (Table 2). The most redundant

Table 1. Primary report on EST sequencing of pepper-*P. capsici* interaction

Items	Number
Total sequenced	5,760
Reliable sequenced	4,867
Contigs assembled	941
Singletons	2,049
Unigenes	2,990
Known unigene	2,409
Functionally classified unigenes	606
Functionally unclassified unigenes	1,803
Unknown unigene	581

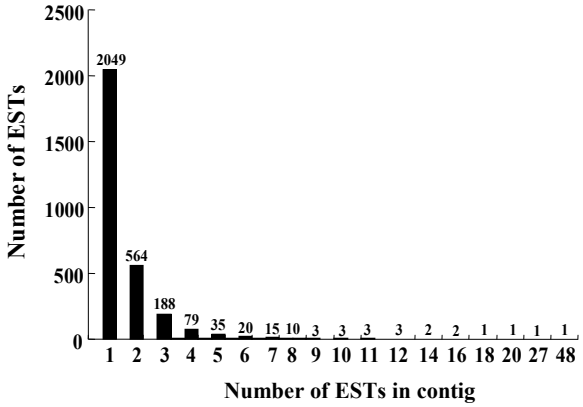

Fig. 1. Redundancy of 4,867 high quality ESTs expressed during peppr-*P. capsici* compatible interaction.

gene, antifungal protein gene, was assembled with 48 ESTs. Additionally, genes of oxidase (27 ESTs), sesquiterpene cyclase (20), catalse (18), formate dehydrogenase (14), oxidoreductase (12), divinyl ether synthase (11), esterase (11), lipoxygenase (11), allyl alcohol dehydrogenase (10), thionin like protein (10), and glyceraldehydes-3- phosphate dehydrogense (10) were also abundantly expressed. ESTs of 42.1% (2,049/4,867) were deteced only once in the data set (Fig. 1).

Functional categorizing

The data set of 2,990 unigenes was queried for homology search against public database by using BLASTX program with a threshold E-value of $<10^{-10}$. A number of 606 unigenes among the 2,409 encoding putative protein sequences were assigned in functional description by MIPS system (Fig. 2). Major genes belong to housekeeping roles such as metabolism (39%), protein synthesis (14%), and transport (12%). Also many genes were assigned to cell defense (7%), signal transduction (7%), transcription (6%) and energy (4%). Proteins belonging to function of biogenesis (3%), localization (3%), cell cycle (2%), and development (1%) were detected.

Discussion

Phytophthora blight of pepper caused by *P. capsici* is considered as a novel system for studying host-pathogen interaction due to the great economic importance and special aspects of pathogen as included in oomycetes. However, stud-

Table 2. Most redundant genes showing significant similarity to known protein database

Contig ID	Number of ESTs	Matching homolog	Source organism	Accession number
cn131	48	antifungal protein	*Capsicum annuum*	AAL73184
cn15	27	1-aminocyclopropane-1-carboxylate oxidase 3	*Petunia hybrida*	AAA33697
cn156	20	sesquiterpene cyclase	*Capsicum annuum*	AAC61260
cn56	18	catalase	*Capsicum annuum*	AAF34718
cn45	16	cristal-glass1 protein	*Capsicum annuum*	AAR83879
cn52	14	formate dehydrogenase	*Lycopersicon esculentum*	CAH60893
cn130	14	U-Lim protein	*Capsicum annuum*	AAR83883
cn381	14	cytosolic NADP-malic enzyme	*Lycopersicon esculentum*	AAB58728
cn70	12	oxidoreductase	*Arabidopsis thaliana*	NP_191681
cn150	12	3-hydroxy-3-methylglutaryl-coenzyme A reductase	*Capsicum annuum*	AAD28179
cn133	12	no match		
cn57	11	divinyl ether synthase	*Capsicum annuum*	ABH03632
cn84	11	esterase	*Capsicum annuum*	AAF77578
cn317	11	lipoxygenase	*Solanum tuberosum*	AAB67865
cn50	10	allyl alcohol dehydrogenase	*Nicotiana tabacum*	BAA89423
cn82	10	thionin-like protein	*Capsicum annuum*	AAF16413
cn202	10	glyceraldehyde-3-phosphate dehydrogenase	*Capsicum annuum*	CAC80375

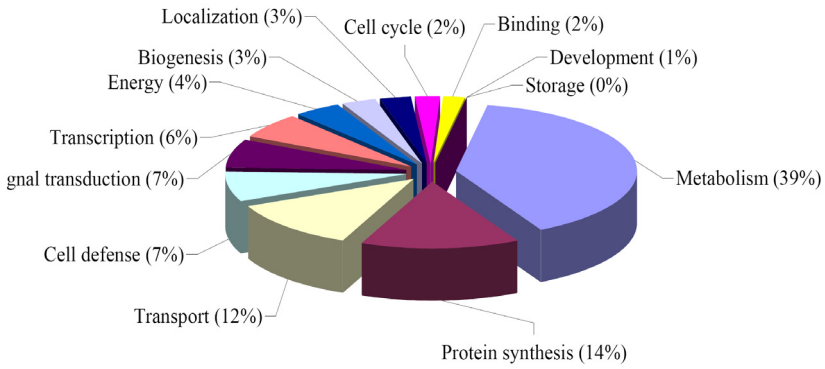

Fig. 2. Categorization on the 606 unigenes from KS13 cDNA library constructed from peppr-*P. capsici* compatible interaction with classified functions.

ies of the interaction have been barely conducted in genome wide scale. Here we report EST analysis revealing the genes involved in the interaction between pepper and *P. capsici*.

EST analysis has been a powerful tool for identifying large numbers of genes that are involved in certain conditions and also is a rapid and relatively efficient way to discover genes from interesting status of organism in study. EST based sequencing approaches are efficient comparing to whole genome sequencing in terms of gene discovery. EST analysis as a genomics based approach provides an efficient means of gene discovery in studying the plant microbe pathogen interactions like pepper-*P. capsici* interactions.

Randomly selected 5,760 cDNA clones were sequenced in 5′-ends, from which more information on genes could be achieved comparing to 3′-ends. About 15% of EST sequences were discarded for next process due to their low quality in base calling. Large numbers of genes were identified by examining 4,867 ESTs from cDNA library prepared from the comparable pepper-*P. capsici* interaction to provide insight into the molecular mechanisms of the plant defense and pathogenicity. Unnormalized library was chosen to explore the condition-dependent gene expression profiling during the interaction. Unigenes of 2,990 including 941 contigs and 2,049 singletons were assembled from 4,867 ESTs.

Gene of antifungal protein was most abundantly expressed in the condition of comparable pepper-*P. capsici* interaction. Also high expression was detected in the genes encoding proteins of oxidase, sesquiterpene cyclase, catalse, formate dehydrogenase, oxidoreductase, divinyl ether synthase, esterase, lipoxygenase, allyl alcohol dehydrogenase, thionin like protein, and glyceraldehydes-3-phosphate dehydrogense. Considering the functions of abundantly expressed genes during the contact of pepper and *P. capsici*, the interaction involves highly dynamic status in the life cycles of host and pathogen. Deeper dissection on the putative function of the genes involved could suggest clearer clues to understand the feature of the interaction.

Among the 2,990 unigenes queried in BLASTX program, 2,409(80.6%) showed matches in non-redundant database of GenBank (Fig. 1). The high portion of matched genes in database suggests the rich status of gene information of GenBank in plant genome area. However, most of matched genes, 1,803 from 2,409, showed homology with unclassified proteins, which indicated more work on functional studies of genes is necessary. The matched 606 unigenes showing classified functions were catalogued into 13 groups including metabolism, energy, storage, and cell cycle *et al*. Genes involved in ubiquitous metabolic pathways, protein synthesis, transport were prevalent. And also a high portion of genes were assigned to cell defense, signal transduction, transcription and energy. Proteins belonging to function of biogenesis, localization, cell cycle and development were relatively often detected. Genes involved in cell division, structure, differentiation were poorly represented in this library based on the functional classification. The normalized proportion of each gene group is not suggested since no comparative set was assigned in this analysis.

As more ESTs from diverse plant-pathogen interactions become available, comparative analysis on the genes associated in pathosystems will be possible in deep sense. Additionally, the value of this data will be fully appreciated with further analysis using functional genomics approaches such as microarray analysis and directed gene knockout mutant analysis. A better understanding of the molecular mechanisms involved in pepper-*P. capsici* should lead to novel approaches to manage the important plant disease.

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초록 : EST기법을 이용한 고추와 고추역병균간의 상호작용에서 발현되는 유전자들의 분석

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고추는 한국, 중국, 멕시코를 포함한 온대 및 아열대 지역을 중심으로 전세계적으로 전형적인 향신료로 식용되고 있으며 그 생산량 및 사용량은 해마다 증가하는 추세에 있다. 고추역병균인 *Phytophthora capsici*는 고추의 생산에 있어, 질적, 양적으로 많은 피해를 야기하는 식물병원균으로 알려져 있다. 난균강에 속하는 이 병원균은 기주식물의 뿌리, 줄기, 잎과 함께 과실에 이르기까지 식물체 전체를 가해한다. 고추역병의 발병을 분자수중에서 이해하기 위해서는, 발병과정에서 발현되는 유전자에 대한 연구분석이 필수적이며, 이를 위해 최근 개발되어 응용되고 있는 발현서열표지(expressed sequence tags, ESTs)의 분석을 시도하였다. 고추역병균을 접종한후 3일째 발병초기의 고추잎으로부터 추출한 total RNA를 이용하여 고추-고추역병균 발병초기 cDNA library를 구축하였다. 이 cDNA library에서 무작위로 선발된 5,760 clone에 대하여 말단 염기서열 분석을 수행하여 5,148개의 양질의 염기서열을 확보하고 contig assembly에 적용한 결과, 2,990개의 unigenes을 확보하였다. 이들 2,990개의 unigenes에 대한 BLASTX를 이용한 상동성 분석결과, 2,409개가 기존에 알려진 서열과 matching을 보였으며, 이중 606개가 기능적으로 구분되었다.