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Platform of Hot Pepper Defense Genomics: Isolation of Pathogen Responsive Genes in Hot Pepper (*Capsicum annuum* L.) Non-Host Resistance Against Soybean Pustule Pathogen (*Xanthomonas axonopodis* pv. *glycines*)

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Host resistance is usually parasite-specific and is restricted to a particular pathogen races, and commonly is expressed against specific pathogen genotypes. In contrast, resistance shown by an entire plant species to a species of pathogen is known as non-host resistance. Therefore, non-host resistance is the more common and broad form of disease resistance exhibited by plants. As a first step to understand the mechanism of non-host plant defense, expressed sequence tags (EST) were generated from a hot pepper leaf cDNA library constructed from combined leaves collected at different time points after inoculation with non-host soybean pustule pathogen (*Xanthomonas axonopodis* pv. *Glycines; Xag*). To increase gene diversity, ESTs were also generated from cDNA libraries constructed from anthers and flower buds. Among a total of 10,061 ESTs, 8,525 were of sufficient quality to analyze further. Clustering analysis revealed that 55% of all ESTs (4685) occurred only once. BLASTX analysis revealed that 74% of the ESTs had significant sequence similarity to known proteins present in the NCBI nr database. In addition, 1,265 ESTs were tentatively identified as being full-length cDNAs. Functional classification of the ESTs derived from pathogen-infected pepper leaves revealed that about 25% were disease- or defense-related genes. Furthermore, 323 (7%) ESTs were tentatively identified as being unique to hot pepper. This study represents the first analysis of sequence data from the hot pepper plant species. Although we focused on genes related to the plant defense response, our data will be useful for future comparative studies.

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As sessile organisms, plants have evolved due to local biotic and abiotic stressors to possess various defense mechanisms. The plant defense response is not simply the expression of defense-related genes, but involves orchestrated reactions of transcriptional activation of multiple genes, accumulation of secondary metabolites, activation of the hypersensitive response, and development of systemic acquired resistance (Lam et al., 1989; Dixon 1986; Dangl and Jones 2001; Ryal et al., 1996). This complexity of the plant defense responses rarely yields effective strategies leading to generation of plants with improved disease tolerance (Somssich and Hahlbrock 1998). Therefore, the identification and analysis of the genes involved in the defense processes is an essential step towards understanding the whole scheme of the plant defense mechanism and generation of disease resistant plants.

To gain insight into plant defense mechanisms, we performed random EST (Expressed Sequence Tag) sequencing to isolate genes expressed at the onset of the hypersensitive response (HR) during non-host pathogen, *Xanthomonas axonopodis* pv. *Glycines*, infiltration. Single pass, partial sequencing of 5' end of complementary DNA (cDNA) clones to generate ESTs represents a relatively inexpensive and rapid procedure for finding genes. Moreover, recent developments in DNA sequencing and sequence analysis tools have enabled single-pass cDNA sequencing to become a highly effective analytical method. Therefore, analysis of expressed sequence tags has become a popular method to examine the genes that are expressed in different species of plants at different stages of development (Cooke et al., 1996; Sasaki et al., 1994; Van de Loo et al., 1995; Allona et al., 1998; Mekhedov et al., 2000; Kwak et al., 1997; Zhang et al., 2001). As a result of these efforts, more

than 1,000,000 ESTs have been identified from more than 30 different plant species (http://www.ncbi.nlm.nih.gov/dbEST/).

The sequences of the ESTs were compared to sequences present in the Arabidopsis database (http://mips.gsf.de/desc/thal) to compare homology and classify their function. In addition, various sequence analysis tools and methods were used to study the sequences obtained. The results of this study will be useful not only for understanding the molecular mechanisms of plant defense, but also for understanding the mechanisms of other stress reactions, since the signal transduction pathways associated with the plant defense response partially overlap with other abiotic signaling pathways (Genoud and Metraux 1999; Thomma et al., 2001; Cheong et al., 2003). The results of EST analysis in this study are available through our web site (http://plant.pdrc.re.kr/Gene).

Generation, Quality Assessment and Clustering of ESTs

To isolate genes involved in the defense response of the hot pepper plant and get the expression profiles, we constructed three different cDNA libraries. One library (KS01) was generated using RNA prepared from hot pepper leaves inoculated with soybean pustule pathogen (*Xag*; *Xanthomonas axonopodis* pv *glycine*) and two others were generated from flower buds (KS07) and anthers (KS08).

Before performing detailed analysis of ESTs, sequences originating from non-nuclear organelles were identified by comparing EST sequences to mitocondrial, chloroplast, and ribosomal RNA sequences present in sequence databases using the BLASTN algorithm (cut-off value was e-10). For all three libraries, less than 4.3% of clones analyzed originated from organelles or ribosomal RNA. Ribosomal RNA sequences made up half of the non-nuclear sequences. Analysis of ESTs using an in-house developed program showed that 13% (1265 EST analyzed) contained complete open reading frames and were classified as tentative full-length cDNAs. These full-length cDNAs were divided into two groups. The first group, which made up 45% of the full-length clones, was characterized by the presence of a codon for Met (translation initiation codon) in the corresponding position to that of a homologue found by database search. In the second group there was no Met initiation codon present in the corresponding position to that of a homologue and the 5' untranslated region of each EST was generally more than three times the length of the corresponding homologue. Even though this approach is not very accurate (Ablett et al., 2000), it is a quick and convenient way to determine which cDNAs are possibly full-length, directly from the results of a BLASTX search.

The average G+C content of hot pepper ESTs was 42% which was similar to that of soybean and Arabidopsis ESTs (Qutob et al., 2000; TAGI 2000). G+C content could be a criterion for differentiating plant cDNA sequences from mixed sources such as cDNA from fungal pathogen-infected plant tissues when we sequencing fungal pathogen infected cDNA libraries. This method was used to distinguish soybean leaf cDNAs from those of the infecting fungus, *Phytophthora,* because the high G+C content of *Phytophthora sojae* cDNAs (60%), is 18% higher than that of soybean cDNAs. Single pass sequencing from the 5'-end of each cDNA clone produced an average of 516 bp of high quality sequence. After removing low-quality sequences (PHRED cut-off value 0.03), 8,525 cDNA sequences were selected and analyzed for redundancy. From this analysis, 4,685 clusters of different sequences were obtained which included 3,287 unique sequences. Nineteen percent of the clusters were composed of more than five redundant sequences. Since we have sequenced randomly selected ESTs, the redundancy gives an approximate indication the levels of mRNA expression. Our finding that 55% of the ESTs were redundant could be an under- or over-estimate. The former case could be explained by the use of an imprecise clustering algorithm because the ICATOOL program could group highly homologous gene family members as one cluster. The latter case is also possible because our EST data was obtained by 5'-end single pass sequencing and although most of our unique ESTs come from the 5'-end region of the transcripts, some of them probably come from internal regions of transcripts from the same gene due to the presence of partial length cDNAs. These would subsequently be clustered into different groups. In order to get more accurate information about clone redundancy, additional sequencing and detailed analysis will be needed.

Functional Categorization and Comparative Analysis of Hot Pepper ESTs

To assess the similarity between pepper ESTs and other eukaryotic genes or ESTs, we categorized the hot pepper ESTs into functional groups using MIPS Arabidopsis gene functional categories (http://mips.gsf.de/desc/thal). Since the putative translation products of 2035 ESTs did not match arabidopsis proteins using N2Tool (threshold 100), they were not included in the functional categorization, however, we were able to assign 2650 ESTs into functional groups. About half of the sequences were assigned into more than two groups. Forty-seven percent of the ESTs were assigned to their function group based on translated sequence similarity to categorized proteins, while the rest (53%) were homologous to unclassified proteins. Among

the 47% of ESTs with assigned function, about 30% were easily assigned by sequence similarity, but the remainder required careful assignment by manual inspection. The five main functional categories hot pepper ESTs were assigned to: signaling (13%), metabolism (8%), plant defense (5%), transcription (4%), and transport facilitation (4%). These major functional classes of hot pepper ESTs are similar to those of arabidopsis, although the percentages are different (TAGI 2000). Interestingly, we found for Arabidopsis genes, metabolism was the group with the highest proportion of genes but for hot pepper ESTs, the signaling group contained the highest proportion. In addition, growth-related genes were found to be abundant in the Arabidopsis genome, but were found rarely among hot pepper ESTs. This difference is likely to be caused by the direct comparison of Arabidopsis genomic information with expressed gene information from hot pepper. When we examined the functional distribution of ESTs from other plants, the percentage of genes present in the transport facilitation group was highest in hot pepper, *Phytophthora sojae* infected soybean, and NaCl treated *Suaeda salsa* ESTs (Zhang et al., 2001; Qutob et al., 2000; Ujino-Ihara et al., 2000; Ablett et al., 2000; Covitz et al., 1998). Early defense signals alter the activity of plasma membrane ion channels to stimulate ion fluxes across the plasma membrane (Zimmermann et al., 1997; Jabs et al., 1997; Lee et al., 2001, Blatt et al., 1999; EI-Maarouf et al., 2001). It is possible that signaling and transport facilitation are key process of the stress (pathogen) response, flowering bud formation, and/or anther formation and the genes encoding many of the unidentified ESTs could be involved in these processes.

Sequences of hot pepper ESTs were also compared to the sequences of 12 organisms in the NCBI nr DB that included eight different species of plants, as well as human, *C. eligans*, *D. melanogaster*, and *S. cerevisiae*. More than 58% of the ESTs were highly homologous to plant genes, however, about 3% of the ESTs were homologous to genes from non-plant sources. Since the size and diversity of information in the dbEST is much larger than the nr DB, searches for sequence similarities in the dbEST are likely to give more comprehensive results. All of the 8,525 hot pepper ESTs were compared to sequences present in the database and most clustered with ESTs of tomato, *medicago*, potato, arabidopsis, wheat, maize, and rice. As expected, tomato had the highest number of hot pepper homologues (74%) and rice had the least number of homologues (56%). We then tried to identify sequences that were tentatively specific to hot pepper plants. As a first step, we clustered hot pepper ESTs with other plant ESTs (Arabidopsis, Medicago, Maize, potato, rice, tomato and wheat) using N2tool (cut-off threshold 100). The unclustered sequences were subjected to BLASTX analysis against the NCBI nr DB, and the unmatched ESTs were classified as tentative hot pepper specific sequences. Results of this analysis indicate that based on the criterion used here, 7% (323 unique or clusters) of all clusters contained hot pepper-specific sequences. This number could be exaggerated because of the problems with random EST sequencing. In addition, the hot pepper genome may contain genes that are more divergent in sequence compared to other plant species, which could be demonstrated if a similar method of analysis was applied to the other plant ESTs.

We also carried out comparative analysis of ESTs obtained from plants infected with various pathogens. In the TIGR plant gene index (http://www.tigr.org/tdb/tgi.shtml), we found three sources of EST sequence information separately generated from *Phytophthora*-infected potato leaves and *Pseudomonas*-infected susceptible and resistant tomato leaves. Comparison of our ESTs with those in the database showed that Xcg-infected pepper leaves have about 10% more singleton and unique genes (Data not shown). We also compared pepper ESTs with 585,123 ESTs from 149 different libraries generated from nine different species of plants including tomato, rice, soybean, potato, medicago, maize, ice plant, and barley. We found that the proportion of singleton and unique sequences was higher in pepper than in other plant species (Data not shown). Although most plant genomes have a relatively lower proportion (below 50%) of single copy or unique DNA sequences compared to those in animal genomes, in pepper plants, it has been shown that 65% of the genome is composed of single-copy sequences (Walbot and Goldberg 1979; An et al., 1996). Based on An's previous work and our results here, we believe it is possible that the higher percentage of single and unique ESTs from pepper reflect its unique genome structure.

Comparison of Expression Analysis of ESTs in Different Libraries

The frequency a particular EST occurs in a specific library represents the expression level of its corresponding gene in a specific situation, and is called an electronic Northern blot (Ewing et al., 1999). The expression profile of the ESTs from three different libraries could be divided into seven categories. Eighty-eight percent of the ESTs were expressed only in a specific library, about 2% of the ESTs were expressed in all three libraries, and the remaining 10% of ESTs were expressed in two of the three libraries. Of the ESTs expressed in a specific library, 56%, 17%, and 15% of total ESTs were from KS01, KS07, and KS08 libraries, respectively. Since we are interested in genes related to cell death, defense and disease resistance, we sequenced more

clones from the KS01 library which was made from pathogen-infected leaf tissues, and focused on the analysis of the 2640 ESTs that were unique to this library. We selected the ESTs which appeared at least six times among the clones analyzed from the KS01 library. A total of 136 EST clusters were selected and categorized based on their putative functions. Seventy-two (53%) of the clusters could be categorized into functional groups. The three functional categories, defense (21%), metabolism (8%), and protein synthesis (7%) covered 36% of the KS01 derived abundant ESTs. This result is consistent with the fact that plants protect themselves by altering their metabolism or pattern of gene expression (Dixon 2001; Maleck and Dietrich, 1999; Bowles 1990). The most prominent changes in gene expression in plant tissues associated with disease resistance are those of the PR (pathogenesis-related) genes (Ward et al., 1991). Among our ESTs from the KS01 library, five different classes of genes encoding PR proteins including PR-1, PR-10, chitinase, SAR8.2, and glucanase, were abundantly expressed. Furthermore, several disease resistance-related genes encoding proteins, such as thionin, ubiquitin, catalase, glutathione-S-transferase, cytochrome P450, and 14-3-3 were also highly expressed (Oh et al., 1999; Becker et al., 2000; Wu et al., 1999; Levine et al., 1994; Whitbred and Schuler 2000; Roberts and Bowles 1999). During pathogen attack, plants rapidly produce ethylene (Dong 1998). The ethylene biosynthetic pathway is well characterized and ACC oxidase (ACO) has been identified as the ethylene-forming enzyme (Kende 1993). We found eight different ACO genes in the KS01 library. Among them, five were abundantly expressed (appeared more than six times). This result suggests differential regulation of ACO family members during plant-pathogen interaction leading to the disease resistance response in hot pepper plants.

Sixty-four (47%) clusters could not be categorized into a specific functional group. Among them, twenty EST clusters did not have any significant homology to sequences within established databases. These ESTs may play unique role(s) in the defense response of the hot pepper plant.

Limitation of EST Sequencing Analysis

While we have identified candidate 136 putative pathogen responsive ESTs, caution should be taken in interpreating this result based on the two reasons. First, most of ESTs are easily generated from moderate or highly expressed genes in specific cDNA libraries. Especially, when the total number of ESTs generated per individual cDNA libraries were significantly small, the biased identification toward to abundantly expressed genes was obvious. Therefore, the rarely expressed genes possibly excluded from the EST sequencing and expression analysis. Second, we didnt have reference (healthy leaves) cDNA expression information and had relatively small number of ESTs information form individual libraries. Therefore, we should apply other experimental methods to increase accuracy of our expression estimation. For this purpose, the cDNA microarray analysis could be used as a powerful tool.

Isolation and Functional Classification of *Xag* Responsive Genes Using cDNA microarray

In spite of efforts to identify the pathogen inducible genes using computational expression analysis, we could not validate pathogen inducible genes. Subsequently, we made the cDNA microarray containing 4,685 ESTs to screen the pathogen responsive ESTs following *Xag* infiltration. Previous study indicates that the rapid cell death, hypersensitive response (HR), followed by pathogen infection has been proposed to play an important role in disease defense (Heath, 1980). Consistent with this proposal, the hypersensitive cell death was observed approximately 18 hours after *Xag* infiltration on hot pepper plant (data not shown). Hence, we collected hot pepper leaves at 21 hours after *Xag* infiltration for probe preparation.

Upon *Xag* infiltration, approximately 453 ESTs (9.7%) of the genes were significantly altered in their expression with more than 3-fold changes. Among them 283 ESTs were induced and 170 ESTs were reduced by *Xag* infiltration. We then determined the possible functional roles for the genes corresponding to these 453 ESTs. Since only the limited information is available at the moment, about 44% (201 ESTs) of pathogen responsive genes were unable to classify their function. However, 253 ESTs (56%) could be categorized into 11 functional group; *e.g.*, metabolism, defense, cellular organization, energy, signaling, transcription, transport, protein synthesis, protein destination, transposable element and cell growth, division, and DNA synthesis. Interestingly, metabolism related genes that are composed of 21% of all *Xag* infection responsive genes were dramatically regulated following *Xag* infection via up- and down-regulation of their expression. This result probably represents that the dynamic changes of metabolic pathway in plant play an important role during plant defense reaction. The defense, signaling, transcription, and transporter were up-regulated during the defense reaction, while cellular organization, energy, protein synthesis and protein destination-related genes were relatively down-regulated during the defense reaction. Surprisingly, about 60% of pathogen responsive and functionally known genes were metabolism and defense-related genes, indicating that plants may achieve the resistance reaction through both direct expressions of defense-related genes and modulation of metabolic pathway.

Perspective

Although, some of pathogen responsive genes isolated in our study were known function in plant defense reactions, most of pathogen responsive genes isolated in our study were unknown function. Therefore, we need more attractive approaches for their function identification, such as generation of large number of transgenic plants. But, the extremely low efficiency of transformation in hot pepper plants forces us to use heterologous plants, such as tomato or tobacco plants. Alternatively, virus induced gene silencing (VIGS) using *Nicotiana bentamiana*, tomato could be an appealing substitution, because of the high degree of sequence similarity between genus *Nicotiana* and *Capsicum* (Kim et al., personal communication). Recently, several labs have showed that the VIGS could be applied to the hot pepper plants (Kang; Ryu. personal communication). High-throughput functional identification of uncharacterized pathogen responsive genes obtained in this study is being underway and it will eventually broaden our knowledge about plant pathogen interactions.

Database Information

The sequence data in this paper have been submitted to the dbEST database under the ID 10227604-10236595 and the Genebank under accession number BM059564-BM068555. The sequences and further detailed information are available at http://plant.pdrc.re.kr/Gene. The microarray data will be available at http://plant.pdrc.re.kr:8888/array/index.html.

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