작물유전체기능연구사업

고추 cDNA microarray 제작, 배포, 내병성관련 유용유전자 발굴
Construction and distribution of pepper cDNA microarray and isolation of defense-related genes

한국생명공학연구원

과학기술부
제출문

과학기술부 장관 귀하

본 보고서는 과학기술부 특성연구개발사업 21세기프로젝트이 연구개발사업 중 과학기술
부와 농촌진흥청이 지원하는 작물유전자능 연구사업단 "고추의 cDNA microarray
제작 배포 및 내병성관련 유용유전자 발굴 "과제 (과제번호 CG1221)의 단계보
고서로 제출합니다.

2004. 8. 30

주관연구기관명 : 한국생명공학연구원

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보고서 초록

본 과제는 고추를 대상으로 하여 유전자를 내양발굴하고 이를 대상으로 유전자 집합체를 제작하여 유전자 발현 프로파일링을 대량으로 발굴하여 실제 분자 육종 프로그램에 이용할 유용유전자를 발굴하는데 그 목적이 있다. 따라서 1차년도 과제 수행을 통해 약 500여 개의 생체 방어 관련 유전자를 집합 실험 데이터 분석을 통해 발굴했으며, 이들의 기능 분석을 위한 추가 실험이 진행중이다. 이와 더불어 본 과제 수행 결과는 모두 데이터베이스로 만들어져서 국내 연구진에게 공개가 되고 있으며, 팀실험 또한 국내 연구진에게 공공서비스 되고 있다. 본 과제 수행중 과생된 모든 결과물은 전단적으로 엽어서 본 보고서를 완성하였습니다.
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3종류의 cDNA library를 이용하여, 4,685개의 고효 유전자를 발굴하여 유전자집을 제작하였다. 제작된 유전자집을 이용하여 biotic stress, abiotic stress, 그리고 환경적 처리 등 다양한 실험을 실시하였다. 이중 비거주 저항성 유전자는 Xag를 접종한 결과를 보면 비거주 저항성이 유도되는 상황의 초기 (접종 6시간후)와 후기 (접종 21시간후)에서 발현이 3배 이상 빌리는 유전자가 약 600여개이며 이중 200개가 발현이 높아들며 나머지 400여개는 발현이 증가하는 현상을 보였다. 접종 초기에 발현이 증가하는 유전자는 약 370개 이상, 이중 307개는 발현이 높고 63개는 발현이 높아들었다. 접종 후기에는 295개의 유전자 발현이 늘었고 173개는 발현이 높아들었다. 접종 초기와 후기에 공통적으로 발현이 늘어난 유전자는 199개이고 26개가 공통적으로 발현이 뜨웠다. 약 50%의 유전자는 접종 전 시기에 공통적으로 발현이 증가했지만, 약 12%의 유전자만 공통적으로 발현이 감소하였다. 유전자의 functional class 용면에서 보면 defense 또는 stress responsive 한 유전자 그룹, cell-wall modification/biosynthesis 에 관련된 유전자 그룹, 2차 대사산물 합성에 관련된 유전자 그룹, 그리고 다양한 종류의 전자 조절 유전자 및 신호 전달에 관련된 유전자의 발현이 증가하였고 이에 반해 chloroplast organogenesis 에 관련된 유전자 그룹, 광합성과 탄수화물 대사에 관련된 유전자 그룹들의 발현이 감소하였다.

방지항성에 관련된 유전자 발굴 이외에도 다양한 처리를 이용한 수집 데이터를 서로 비교해 보았다. 그 결과로 다양한 그룹의 유전자들을 발굴할 수 있었다. 예를 들면 biotic이나 abiotic stress에 드러나적으로 조절되는 유전자들, 모든 stress 상황에 general하게 조절되는 유전자들, biotic이나 abiotic stress 중의 한 가지 stress에만 특이적으로 조절되는 유전자들을 발견할 수 있었으며 이들 유전자의 기능규명은 이러한 stress-resistance 기작을 이해하는데 중요한 시발점이며, 내재해성 작용을 개발하는데 큰 기여를 할 것이다.
요 약 문

I. 제 목
고추 cDNA microarray 제작, 배포 및 내병성 관련 유전자 발굴

II. 연구개발의 목적 및 필요성
- 병저항성 작물 육종을 위해서는 작물과 병원체의 상호작용, 작물의 생체 방어기작에 관한 정보, 병원체 진입시 작물의 유전자 발현 변화 등에 관한 정보가 필요하므로 이를 위해서는 작물의 생체방어에 관여된 유전자의 대량분리 및 기능 이해가 필수적이다.
- 이를 위하여 대량의 유전자 발굴, 유전자 발현 분석 및 유전자 기능 연구가 필 수적이다.
- 대량으로 확보된 고추의 EST DB를 기반으로 유전자 첨(5K) 제작을 통한 유전자 발현 분석이 유전자 기능 연구에 선행되어야 할 것이다.
- 집을 이용한 대규모 유전자 발현 profile 분석 서비스 및 public DB의 구축은 국내 식물 연구에 중요한 기반 정보로 이용될 것이다.

III. 연구개발의 내용 및 범위

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- 200 여가의 화학 스트레스 저항성 관련 유전자 발굴
- 다수의 유전자 기능 분석 완료

V. 연구개발결과의 활용계획
- 유용 유전자 활용 계획
지난 3년간의 1단계의 연구 결과는 생체 방어 관련 유전자를 유전자집을 이용해 대량으로 선발하는 것이므로 이후 선발된 유전자들 대상으로 유전자의 기능을 규명하여 상업적으로 이용될 유용 유전자를 발굴 하는 것이 필수적이다. 이를 위해서 일차적으로 유전자집의 발현 데이터를 분석한 결과로 생체 방어에 관련된 것으로 여겨지는 500 여가의 유전자들을 선발하였고 이들을 대상으로 다양한 데이터 분석을 실시하여 상업적으로 이용될 가능성이 높은 것으로 추정되는 유전자들 2차 선발하여 Virus-Induced Gene Silencing(VIGS)를 이용한 유전자 기능 검정이 실시 될 것이다. VIGS 결과로 기능이 확인된 유용 유전자들을 대상으로 과 발현(over-expression) 시킨 형질전환체를 만들어 유전자의 기능을 재검정하고 이의 실용성 검정을 실시 할 것이다.

- 데이터베이스 활용 계획
본격적으로 구축된 유전자 발현 연구 기법 및 유전자 발현 데이터베이스는 이후 국내 식물 기능유전체 연구에 모델 시스템으로 지속적으로 이용될 것이며, 이와 더불어 다양한 분야의 국내 연구진들이 직접적으로 데이터를 활용할 수 있을 것으로 여겨진다.
SUMMARY

I. Title: Construction and distribution of pepper cDNA microarray and isolation of defense-related genes

II. Objectives and need for research

The primary goal of this project is isolation and characterization of defense related pepper genes for utilization of modern breeding program. For this goal, two approaches have been applied.

First, 5K cDNA microarray was made from hot pepper EST clones. The gene expression profiles using several treatments including biotic and abiotic stresses have been obtained for isolation of useful genes related to plant defence mechanism.

Second, characterization of functional role(s) of isolated genes have been tried using multiple tools of molecular and biochemical approaches. Subsequently all the array data has been released to the public.

III. Research contents

   - Generation of 5K cDNA microarray
   - Development of microarray analysis
   - Distribution of cDNA microarray into domestic researchers
   - Analysis of gene expression profiles related to non-host resistance
   - Selection of more than 30 useful genes
   - Function characterization of more than two useful genes

2. Objective of first year (2002): Functional characterization of useful genes
   - Analysis of expression profiles related to Salicylic acid treatment
   - Analysis of expression profiles related to Jasmonic acid treatment
   - Construction of expression profile DB
   - Public services - DB and array experiments
   - Selection of more than 30 useful genes involved in defense reaction
   - Function characterization of more than two useful genes

   - Analysis of expression profiles related to biotic stresses
   - Construction and public service of expression profile DB
   - Public services - array experiments
   - Selection of more than 40 useful genes involved in defense reaction
   - Function characterization of more than four useful genes
   - Generation of 10K cDNA microarray
IV. Results
- Isolation of 10,000 pepper unigen
- Generation of pepper 10K cDNA microarray
- Generation of ca. 200 expression profile data
- Construction of iterative database between EST DB and Gene Expression DB
  - Public services
  - Invention of gene knock-out system in pepper
  - Isolation and functional study of more than 500 genes possibly involved in plant defense reaction
  - Isolation of ca. 200 genes putatively involved in environmental stresses
  - Completed functional characterization of several genes involved in plant defense reaction.

V. Application of results
  1. Application of selected useful genes
     Since our results are the selected genes from cDNA microarray analysis, the functional characterization and commercial utilization of the useful genes should be followed. For this purpose, we finally selected 500 defense related genes we selected from microarray data analysis. The functional characterization of these genes using VIGS (virus induced gene silencing) will be applied. The phenotype screening of VIGS will narrow down the number of useful genes. Finally selected genes will be over-expressed and re-evaluated their functional roles.
  2. Application of database
     The gene expression profile database will be serve as a model system of plant functional genomic researches. In addition, the array data will be directly used by researchers.
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제 1 장 연구개발과제의 개요

가. 연구 개발의 중요성

- 병에 의한 작물 생산성 저하로 경제적 손실이 심각하며 방제를 위한 화학 농약의 사용은 경제적 부담뿐만 아니라 환경오염을 야기하므로 다른 대안이 필요하다.

- 병차항성 품종 개발을 통한 작물 생산성 향상 및 환경, 생태 오염 경감을 이루기 위해서는 새로운 병원체의 출현 속도를 능가할 현대적 개념의 본자 육종 개념의 도입이 결실함.

- 병차항성 작물 육종을 위해서는 작물과 병원체의 상호작용, 작물의 생체 방어기작에 관한 정보, 병원체 집합시 작물의 유전자 발현 변화 등에 관한 정보가 필요하므로 이를 위해서는 작물의 생체방어에 관련된 유전자의 대량분리 및 기능 이해가 필수적이다.

- 대량으로 생산되는 유전자 중에서 유용 유전자를 선발하기 위해서는 전통적인 one-by-one 접근법을 탈피한 새로운 방법의 도입이 필요하다.

- 유전자의 집은 대량의 데이터를 parallel 하게 분석하기 때문에 여기서 생성되는 데이터는 병차항성 연구뿐만 아니라 다른 연구의 기초 데이터로 활용될 수 있다.

- 이러한 유전자 발현 및 조절 연구는 유전자 조작을 통해 작물을 개량하여 현대적 개념의 육종에 필수적인 기본 정보를 제공할 수 있다.

- 선천극을 중심으로 벌어지는 일련의 유전자(유전 자원 확보) 전쟁은 미래의 농업경쟁력을 유전자의 확보, 유전자 분석, 그리고 이들의 지식 재산권에 달려있을 것으로 예측되어있다.

- 영세성을 탈피하지 못한 우리나라 중자 산업은 오로지 신품종의 개발에 달려있으며 이를 위해서는 우수 유용 유전자원의 확보가 필요하다.

나. 지금까지의 연구개발 실적

1). DDRT-PCR을 이용한 고추 생체방어 유전자 발굴

고추의 비기주 저항성에 관련된 유전자를 분리하기 위해서 부작품종에 Xcg를 감체 접종한 후 과민성저항성 반응(Hypersensitive Response: HR)이 나타날 때까지 적정 시간대별로 석문조직을 체취하여 여기서 분리된 total RNA를 시료로 DDRT-PCR을 수행해 있어 약 240개의 서로 중복되지 않은 DNA 단편을 확보했다. 이중 50%는 Genebank database(DB)에서 기존을 유추할 수 있는 유전자이고 나
머지 뇌 기능이 알려지지 않은 유전자였다. 병원체의 침입에 의해 발현이 증가되는 유전자를 선택하기 위하여 reverse Northern hybridization을 수행하여 분리된 유전자와의 50%이상이 선택되었으며 분리된 유전자들과의 자연적 발병상황 또는 자연적 병착성 상황에서의 발현을 확인하기 위하여 고효 생물체인 Xanthomonas campestris pv vesicatoria를 주사 접종한 후 시간대별로 RNA를 분리하여 Northern blot을 수행하였다. 그 결과는 최소한 19개의 유전자의 발현이 병착성 및 감수성 반응에서 차이를 보이고 있었으며 그중 약 10개의 유전자는 병착성 반응에서만 특이적으로 발현되는 것을 확인할 수 있었다.

DDRT-PCR을 이용하여 분리된 유전자의 전체적인 발현양상을 알아보기 위하여 PCR로 증폭된 유전자 조각 (300개 유전자)을 GMC array를 이용하여 slide glass에 심은 뒤 비기주 병착성 반응과정의 식물 RNA를 추정으로 probe를 만들 어 chip hybridization을 수행하였다. 그림 1은 감염 후 4시간 뒤의 RNA probe를 이용한 하나의 hybridization결과를 보여주고 있다. 300개의 유전자 중 약 70% 이상의 유전자가 병원체 침입에 의한 비기주 병착상 과정에서 발현이 증가되는 것으로 나타났다. 그중 일부 유전자의 발현이 이미 수행했던 Northern blot 결과와 일치하는 것을 확인할 수 있었다.

2). Random EST analysis

EST 유전자 확보를 위해 3가지 서로 다른 cDNA library가 이용되었다. 하나는 부작 고추 종종에 Xcg를 강제 접종한 후 HR이 나타날 때까지 경정 시간대별로 식물조직을 채취하여 여기서 분리된 total RNA를 한데 모아서 cDNA library를 만들었다. 다른 library는 조작 특이적 cDNA library로 행진 (Capsicum annuum cv HappyDry) 종종을 이용한 flowerig bud 와 anther cDNA library 인데 다른 연구진으로부터 제공되었다. 각각 KS01 (Xcg 접종 library), KS07(flowering bud), KS08(anther)로 명명되었고 전체적으로 11,000개의 EST (6600, 2300, 2200개가 KS01, KS07, KS08 library로부터) 가 sequencing 되었는데 그 중에서 분석 가능한 수준의 quality를 지닌 EST로 약 9,400개 정도 확보 되었다. 9400개 EST를 clustering software를 이용해 clustering 한 결과 약 4,700 개 정도가 unique gene이며 이를 query 서열로 해서 NCBI의 non-redundant (nr) protein DB 에 BLASTX를 한 결과 36%가 DB에 존재하는 sequence와 상당한 상동성을 보이고, 20%는 중간 정도의 유사성을, 18%는 아주 약한 수준의 유사성을 보였다. 그리고 18%는 DB에 존재하는 sequence와 유사성을 보이지만 기 능이 알려지지 않은 것이었고, 14%는 기존에 밝혀진 sequence와는 유사성을 찾 을 수가 없었다. MIPS (http://mips.gsf.de)의 애기장대 유전자와 BLASTX를 하
여 애기장대 유전자가 지정된 기능별 분류로 따라 4700개의 unigene의 기능을 분석하였는데 약 35%의 유전자가 애기장대 유전자와 유사성이 없어서 분석대상 에서 제외되었고. 나머지 65% 유전자에 대한 유전자의 분류는 Figure 3에 자세히 나와있다.

분석 결과를 보면 signal transduction, metabolism, defense, transport facilitation, transcription 등에 관련된 유전자들이 많이 발굴되었다. EST에 관한 전반적인 사항은 다음의 web site (http://plant.pdr.c.re.kr/blast/pepper.html)를 통해 확인할 수 있을 것이다. 4700개의 unigene 중에서 1125개를 선발해서 microarray의 전 과정을 예비 실험해 보았으며 이런 trial and error 과정을 통해 spotting 및 hybridization 의 재현성을 확보하였으며 trouble shooting 에 대한 knowhow도 많이 축적되어 있으므로 별 무리 없이 유전자 접을 진행시킬 수 있을 것이다.

다. 앞으로의 전망
- EST를 통한 유전자 발굴 및 기능 유전체 연구를 이용한 유전자 기능 분석이 가속화 될 것이고, 그 대상 작물 및 연구 분야도 점차 확대될 전망이다.
- 유전자 첨 연구의 가장 취약점인 데이터 분석에 관해 보다 다양하고, 정교한 분석 기법이 속속 개발되는 추세이고 이와 더불어서 실험이나가 사용하기 편리한 사용 software가 개발되어 유전자 첨을 이용한 연구를 더욱 가속화시킬 전망이다.
- genomics, proteomics, gene expression profiling, metabolic profiling, bioinformatics, computation biology 등의 이질적인 정보들이 서로 통합되어서 정보를 간의 시너지효과를 유발해 유전자의 기능 분석을 가속화 시킬 전망이다.
- 이미 확보된 재배종 작물의 저항성 유전자가 실용화되어서 종자시장에 등장할 것으로 예측된다.
- 분자 생물학적 연구기법과 유전공학 기술의 발달로 짧은 시간내에 대량의 유용 유전자 확보가 가능해졌으며, 출원되는 특허 건수도 급증하는 실정이며, 이러한 유용 유전자를 이용한 새로운 내병, 내충성 식품종 개발될 것이며 화학농 약 사용 규제와 맞물려 시장점유를 확대해 나갈 것으로 예측되어진다.
- 다수의 생명공학 식물제품이 시장에 등장하고 있고 이들이 종자 및 농산물 시장의 주류를 이룰 것으로 전망되며 유용 유전자의 확보가 국가 농업 경쟁력을 결정할 것으로 예측된다.
제 2 장 국내외 기술개발 현황

가. 기술의 정의

식물유전자 기술은 각각의 유전자라 아니라 유전자전체 구성 및 내용을 밝혀내므로써 보다 효율적으로 유전자들의 기능을 밝히고 유용유전자를 발굴하는 기술이다.

나. 기술의 동향

- 유전자집은 DNA나 oligomer을 고형물질에 결합시킬 때 결합을 용이하게 하거나 비특이적 결합을 방지하기 위해서 다양한 처리가 유리 표면에 행해지는데 대표적인 것이 silanization 이며 최근 3차원적 고분자 메트릭스를 도입하는 방법도 시도되고 있다. 폴리머 메트릭스 방법은 목적물질 검출시 신호를 증폭시켜 분석이 용이하며 비특이적 결합이 좋은드는 장점이 있다. 이와 더불어 고체 비드에 DNA를 붙여 이 비드를 유리 표면에 부착하여 표면강력을 증가시키는 방법도 사용된다. 결국 DNA나 Oligomer 를 유전자집 기반에 고정화시키는 기술이 유전자집 기술의 핵심이므로 고정화율을 높이는 차열한 경쟁이 다수의 최사를 중심으로 벌어짐

- 이미지 분석의 기술 흐름은 주로 자동화 및 정밀도를 높이는 방향으로 진행되고 있는데 이를 위해 바코드식별기술, 다양한 통계 알고리즘 도입, 실험 에러 및 outlier 제거법등이 도입되고 있으며 최근에는 3개 이상의 유전자집 이미지 분석 (하나의 유전자집에 3가지 이상의 실험 처리를 동시에 시행)을 위한 기법도 활발히 진행중임

- 데이터베이스는 유전자집 실험의 전 과정과 밀접하게 연관되어 있으며, 다양한 연구기관 및 회사에서 독자적인 DB가 구축되고 있는 실정인데 affymetrix 사의 경우 초기에는 유전자 발현을 다루는 데이터베이스, 현 다자인을 목적으로 데이터를 관리하는 데이터베이스 구축에서 최근에는 유전자집 실험 전반에 관한 통합된 사항을 관리하는 생물정보학 데이터베이스가 구축되고있는 실정이다. 현재 다양한 종류의 생물학 정보가 산재되어 있는 유전자집을 이용한 데이터 분석에서 가치 있는 의미를 찾아내기 위해서는 사실 이처럼 다양한 데이터의 통합화가 필수적이며 앞으로 중요성이 점차 커지며 통합화를 위한 첫걸음으로 XML (Extensible Markup Language), JAVA, COBRA를 이용한 데이터 통합화가 시도되며 공극적인 체계화인 Ontology 연구도 가속화되고있음.

- 전체 유전자열 염기서열 분석이 끝난 예기준대의 경우, 현재 유전자 annotation (Finding ORF)의 정확도가 70% 정도로 추정되고 있는 상황이기 때문에 이의 보완으
로 애피짓대 전체 염기서열을 TILLING 한 유전자집을 이용해 유전자의 ORF (mRNA 발현부위)를 규명하는 작업이 진행중임.

- 유전자집 제조 시 중요한 점은 심야야 할 목적 유전자의 확보와 이들의 annotation 정도인데 이를 위해서 각 연구진은 유전체 서열이 밝혀진 애피짓대인 경우도 대량의 EST (유전자 발현 단편) 연구를 진행해서 유전자를 확보하며 이와 더불어 다양한 염기 서열 분석을 통해 이들의 기능을 추정 (annotation) 하는 작업을 병행중이므로 여러 목적 작물을 대상으로 유전자 발현자체가 발바로 진행중임.

- 현재 모델식물인 애피짓대를 중심으로 활발한 유전자집 연구가 수행되고 있고 최근에는 다양한 작물, 토마토, 감자, 밀기, 콩, 옥수수, 등을 대상으로 활발한 연구가 진행중임.

- 현재까지의 유전자집 연구는 expression profiling (유전자발현분석)을 이용한 여러 가지 stress 상황에서의 유전자 발현 분석 및 조성 특이적인 유전자 발현 패턴 분석에 차우쳤으며 표현 생물정보학의 통합된 기술을 바탕으로 식물체내의 생리적 변화 및 유전자 기능 발굴 등의 여러분 분석을 해결하려는 방향으로 진행됨.

- 고밀도로 집결된 유전자집 제조가 가능함으로 조밀한 전체 유전자를 한장의 집에 심음으로 전체 유전자 수준의 유전자 발현 패턴 분석이 가능함으로 각 유전자 기능 분석과 유전자 유전자간의 상호작용 분석에 상당히 도움을 주려다 하였음.

- 각기 독립적인 유전자집 실험을 하나의 통합된 데이터베이스에 등록시켜놓으면 독립된 실험에서 보지 못한 많은 정보를 얻을 수 있으므로 데이터베이스화 작업이 Stanford Microarray Database (SMD)와 NCGI의 Gene Expression Omnibus (GEO)를 중심으로 진행중임.

- 유전자 발현 분석을 통한 새로운 전자 조절자 결합 부위 (cis-regulatory elements) 발굴 및 조절 유전자 데이터베이스 (예: TRANSFAC)와의 통합 분석을 통한 전자 조절자 전자 조절자 결합 부위의 예측이 가능해지며, 최근에는 유전자집과 chromatin immunoprecipitation 방법을 혼합 이용해서 각 전자 조절자가 결합하는 목적 유전자 전체 유전자 수준에서 발현하는 것이 가능해짐.

- Oligo 퀘를 이용한 대규모의 genotyping가 가능해지면 "Tiling"된 Oligo 퀘를 이용해서 point mutation의 결출도 가능해짐.

- 여러 가지의 조작, 발달 단계, 및 처리에 따른 대규모의 유전자 발현 데이터베이스가 구축되며 한 새로운 수준에서 가능한 종류의 유전자 발현 세트의 fingerprint가 가능해지며 이를 reference로 이용해 각 형질전환체나 돌연변이체의 유전자 발현을 분석해 이들의 표현형 및 유전자 기능 예측이 가능해질 것임.

- 유전자집은 mRNA 전자 수준에서 분석을 하기 때문에 2-D gel이나 mass
spectrometry에서 나온 단백질 발현 자료와 통합되면서 최적 유용한 자료가 되므로 이
러한 방향으로 연구가 진행됨.
- 대사산물 수준 (metabolite level) 에서 보면 metabolic profiling 과 biochemical
  genomics 데이터가 유전자질 데이터와 통합되면 대사산물 조절기작 (metabolic
  pathway), 효소 (enzyme), 유전자 발현 무리 (gene expression cluster)들간의 상관 관
  계 규명을 총망하고 이를 이용한 식물의 metabolic engineering 분야에 큰 영향을 미
  졸 수 있습.
제 3 장 연구개발수행 내용 및 결과

가. EST and Microarray Analyses of Pathogen Responsive Genes in Hot Pepper (Capsicum annuum L.) Non-Host Resistance Against Soybean Pustule Pathogen (Xanthomonas axonopodis pv. glycines)

Abstract
Large-scale single-pass sequencing of cDNA libraries and microarray analysis have proven to be useful tools for discovering new genes and studying gene expression. As a first step in elucidating the defense mechanisms in hot pepper plants, a total of 8,525 expressed sequence tags (ESTs) were generated and analyzed *in silico*. The cDNA microarray analysis identified 613 hot pepper genes that were transcriptionally responsive to the non-host soybean pustule pathogen *Xanthomonas axonopodis pv. glycines* (*Xag*). Several functional types of genes, including those involved in cell wall modification/biosynthesis, transport, signaling pathways and divergent defense reactions, were induced at the early stage of *Xag* infiltration. In contrast, genes encoding proteins that are involved in photosynthesis, carbohydrate metabolism and the synthesis of chloroplast biogenetic proteins were down-regulated at the late stage of *Xag* infiltration. These expression profiles share common features with the expression profiles elicited by other stresses, such as fungal challenge, wounding, cold, drought and high salinity. However, we also identified several novel transcription factors that may be specifically involved in the defense reaction of the hot pepper. We also found that the defense reaction of the hot pepper may involve the deactivation of gibberellin. Furthermore, many genes encoding proteins with unknown function were identified. Functional analysis of these genes may broaden our understanding of non-host resistance. This study is the first report of large-scale sequencing and non-host defense transcriptome analysis of the hot pepper plant species.

[The sequence data in this paper have been submitted to the dbEST and Genebank database under the codes 10227604-10236595 and BM059564-BM068555, respectively. Additional information is available at http://plant.pdrc.re.kr/ks200201/pepper.html]

Introduction
Plants have developed various defense mechanisms to survive local biotic and
abiotic stresses. The plant defense response generally involves the orchestrated transcriptional activation of multiple genes and the accumulation of secondary metabolites, and often entails the activation of the hypersensitive response (HR) and the development of systemic acquired resistance (Ryals et al., 1996 Dangl and Jones, 2001). The identification and analysis of the genes that are involved in the defense responses is thus an essential step towards understanding the entire scheme behind the plant defense response, which in turn may aid the generation of disease-resistant plants.

During the past few years, much effort has been put into sequencing the genomes of many different organisms, including the Arabidopsis and rice (TAGI, 2000; Yu et al., 2002; Goff et al., 2002). However, despite the existence of such entire genomic sequences, the generation and analysis of cDNA sequences or expressed sequence tags (ESTs) remains invaluable in genomic research, especially for research into plant species with larger genome sizes. Moreover, recent developments in DNA sequencing and data analysis tools have enabled single-pass cDNA sequencing to become a highly effective method for use in genomic research. The subsequent analysis of the ESTs has become a popular method to examine the spatial or temporal expression patterns of genes in many plant species. As a result, over a million ESTs have been identified from more than 30 different plant species (http://www.ncbi.nlm.nih.gov/dbEST/).

To date, little attention has been paid to the hot pepper (Capsicum annuum), as less than 500 nucleotide sequences are present in NCBI database (http://www.ncbi.nlm.nih.gov/Taxonomy). Hot peppers are now known to be good sources of vitamins (11 mg/g is present in the dried fruit) and to have medicinal uses such as alleviating pain, arthritis and long-term inflammation (Bosland and Votava, 1999; http://www.chilepepperinstitute.org/hotpeppers.htm). This usefulness of the hot pepper fruit in human health has increased its consumption and cultivation (1.6 million ha) throughout the world (FAO, 2002). We have sought to characterize the hot pepper genome. Since the genome of the hot pepper is rather large (2.7 3.3 X 109 bp), we initially started with an EST sequencing project (Lee and Choi, 2002).

Plant resistance can be classified as host or non-host resistance. Host resistance is usually parasite-specific and is restricted to particular pathogen races and is commonly expressed against specific pathogen genotypes(Heath, 2000). In contrast, resistance shown by an entire plant species to a species of pathogen is known as non-host
resistance (Heath, 1985). Therefore, non-host resistance is the more common and broader form of disease resistance that is exhibited by plants (Heath, 2000). However, recent work in *Arabidopsis* has revealed that the gene expression profiles in non-host disease resistance are similar to those involved in host disease resistance (Tao et al., 2003). To better understand such plant defense mechanisms, we focused on isolating genes that are expressed during the non-host resistance of the hot pepper plant by generating ESTs from its pathogen-infected tissues and using these in microarray analysis. The pathogen we employed was *Xanthomonas axonopodis* pv. *glycines* (*Xag*), the casual agent of the bacterial pustule of soybean (Hwang et al., 1992).

In this study, we generated 8,525 ESTs from hot pepper, extensively analyzed their sequences using the *Arabidopsis* database (http://mips.gsf.de/desc/thal), and applied various sequence analysis tools and methods. These ESTs were also used in cDNA microarrays, which were tested with probes obtained from *Xag*-infiltrated hot pepper leaves. Some of the EST clones were also double-checked to verify the fidelity of the microarray analysis.

**Materials and methods**

**cDNA library construction and DNA sequencing**

The KS01 cDNA library was constructed from poly (A) RNA prepared from hot pepper (*Capsicum annuum* cv. Bukang) leaves that had been inoculated with *Xag* (Suh et al 2001). Flowering bud and anther libraries, designated as KS07 and KS08, respectively, were generated from *C. annuum* cv. HangKeun (http://www.eugentech.com). The cDNA synthesis, size selection, insertional ligation, and packaging into a vector (for the KS01 and KS08 libraries, Uni-ZAP XR from the ZAP-cDNA Gigapack III Gold Cloning Kit was used while the HybriZAP 2.1 vector from the HybridZAP 2.1 Two-Hybrid System was used for the KS07 library) were performed according to the manufacturer's instructions (Stratagene, USA). The *in vitro* excision was done into pBS SK II (+) (KS01 and KS08, Stratagene, USA) or pAD-GAL4-2.1 (KS07, Staratagene, USA). Plasmid DNA from 11,000 randomly selected colonies was prepared and subjected to fluorescence cycle sequencing using the ABI Big Dye Cycle Sequencing Kit (PE Applied Biosystems, USA) with T7 (5'-GTAATACGACTCACTATAGGG; for KS01 and KS08 libraries) or A1 primers (5'-CGGTTTGGGAATCAGCAGG; for KS07 library). The reactions were run and
analyzed on an ABI Prism 3700 DNA Analyzer.

**Analysis of sequence data**

Sequence data were analyzed using an SGI Origin 3200 Unix machine (SGI Korea, South Korea). The ABI-formatted chromatogram sequences were fed into PHREP (Ewing et al., 1998a; 1998b). The sequences that contained 97% or higher unambiguous bases and were longer than 100 bp were retained for further analysis. Before the clustering of ESTs, these sequences were analyzed using RepeatMasker software (http://ftp.genome.washington.edu/RM/RepeatMasker.html) with default parameters to screen out the interspersed repeats and vector and low-complexity DNA sequences. Possible non-pepper and non-nuclear sequences were eliminated by searching for BLASTN matches with the ESTs that showed strong homology (<E = e-10) to mitochondrial, chloroplast, and ribosomal RNA genes. To do this, the Mendel DB was used (www.mendel.ac.uk/genomedb.html). To remove redundant ESTs, we clustered them into groups that contained more than 100 bp of core sequence using CAP3 (http://genome.cs.mtu.edu/cap/cap3.html) and ICATool (Parsons, 1995).

**Functional classification of hot pepper EST and comparative analysis**

To classify the pepper ESTs into functional groups, the 4,685 unique ESTs of hot pepper were compared to Arabidopsis sequences using N2tool at a threshold of 100. Following the MIPS MatDB (http://mips.gsf.de/proj/thal/index.html) classification, 2,650 ESTs could be assigned into functional groups. To functionally assign the 613 Xag-responsive genes that were identified by cDNA microarray analysis, manual function assignment using MIPS MatDB (http://mips.gsf.de/proj/thal/index.html), TAIR (www.Arabidopsis.org) and KEGG (http://www.genome.jp/kegg/kegg2.html) databases was applied by using full contig sequence instead of using each EST sequence.

We also performed a BLASTN search against the ESTs present in dbEST of seven different species of plant, which included tomato, Medicago, potato, Arabidopsis, wheat, maize and rice sequences. The EST sequences from each plant species were combined with the pepper EST sequences generated in this study and clustered using N2tool at a threshold of 100.
Infiltration of Xag

Hot pepper plants (C. annuum cv Bukang) were grown in a controlled environment room at 26oC with a photoperiod of 16hr light. Xag was grown on a YEP plate and then resuspended in a sterile 1mM MgCl2 solution at a concentration of 0.04A600 (corresponding to 1108 colony-forming units/ml) followed by infiltration into the ten-week-old plants using a needleless syringe(1ml) (Hwang et al., 1992). Control plants were inoculated with only 1mM MgCl2 solution. Leaves were harvested 6hr and 21hr after infiltration, frozen in liquid nitrogen, and stored at -80 oC until RNA extraction.

Northern blot analysis

Total RNA was isolated from the Xag-infiltrated and control leaf tissues of hot pepper plants at various timepoints after the inoculation according to Choi et al. (1992) and 20g of total RNA from each timepoint was electrophoresed in formaldehyde-containing agarose gel (Sambrook and Russell., 2001) and transferred onto a Nytran membrane according to manufacturer’s protocol (Amersham Pharmacia, USA). Each cDNA clone was labeled with 32[P]-dCTP using a Prime-a-Gene System according to the manufacturer’s instructions (Promega, USA) and used as a probe for Northern analysis. Prehybridization (overnight) and hybridization (16-20hr) were performed at 42oC using 6X SSPE containing 50% formamide and the washing steps described by Kang et al., (1998). After washing, the filters were exposed to X-ray film.

cDNA microarray preparation

A hot pepper cDNA microarray was generated from the 4,685 unique hot pepper EST clones. Briefly, the PCR-amplified DNA fragments were prepared from the plasmid DNA using the following primer pairs: the T7 forward primer (5'-GTAATACGACTCACTATAGGG) for the KS01, KS07 and KS08 libraries, and the T3 reverse primer (5'-AATTAACCCCTCCTAAAGGG-3') for the KS01 and KS08 libraries or the A1 reverse primer (5'-CGCGTGGGAATCACCTACTAGGG) for the KS07 library. QIAquick 96 PCR purification kit was used for PCR product cleanup (Qiagen, USA) and the cleaned PCR products were run on an agarose gel for quality and quantity control before they were spotted. The products were resuspended into 50% DMSO and
50% H2O to give a maximal final concentration of 0.25g/l, and 6l was transferred onto printing source plates (384 well plate; Applied Biosystems, USA). The purified cDNAs were printed on the glass slide (FMB cDNA slide; Turner Designs, USA) using a 16-pin arrayer. The spots are 200m in distance and average 100m in diameter. After printing, the slides were immediately cross-linked under UV (400mJ) and stored at room temperature until further use.

**Probe preparation, hybridization and scanning of the cDNA microarrays**

Total RNA was extracted from the Xag- and mock- (reference) infiltrated leaves of six independent plants using the TRIzol method described by AFGC (Arabidopsis Functional Genomic Group). The mRNA was generated from the total RNA using the Oligotex mRNA Midi Kit (Qiagen, USA) and 2g mRNA was labeled by direct incorporation of Cy-3- or Cy-5-conjugated dUTP (Amersham Pharmacia Biotech, USA) following the protocol of the Pat Brown Lab (cmgm.stanford.edu/pbrown/protocols/4_yeast_RNA.html). The labeled probes were combined and purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). The purified and labeled probes were concentrated to a final volume of 5l. For hybridization, the labeled probes were mixed with 10l of formamide (Sigma, Germany) and 5l of 2X hybridization solution (Amersham Pharmacia Biotech, USA). These solutions were denatured for 3 min at 95oC, applied to the microarray slide, covered with a cover slip, and incubated in a 42oC water bath for 1hr. After incubation, the array was washed at 55 oC with 1X SSC/0.2% SDS, 0.1X SSC/0.2% SDS, and then 0.1X SSC for 10 min each. The slide was scanned with an Axon GenePix 4000A scanner (Axon, USA) to generate one TIFF image. The PMT voltage was adjusted to yield a Cy-3/Cy-5 signal intensity that was as close to 1.0 as possible.

**Microarray data analysis**

The spot intensities were measured using the Axon GenePix Pro 4.0 image analysis software and global normalization was applied using the calculated ratio of median factor. After the normalization, a number of quality control methods were applied. First, all the spots flagged as "bad" or "not found" by image analysis software were removed from the analysis. Second, all the spots smaller than 40m in diameter were
removed. Third, all the spots that have at least 3 validated data were collected. Fourth, all the spots whose standard deviation (SD) is lower than 50% of the average ratio were selected. This resulted in the selection of 613 unique ESTs were selected and analyzed based on the three times differential expression after Xag infiltration.

**cDNA microarray quality control**

To ensure the reliability of the microarray results, two slides (four replicates) were used to analyze the mRNA abundance of each sample pair. Since two identical arrays were spotted on a single glass slide, the first array was probed with cDNA labeled with Cy-3 dUTP (Xag-infiltrated leaves) and Cy-5 dUTP (control leaves). The reverse pair set was applied to a second array. The reproducibility of the array hybridization signals is shown in Supplementary Fig. 1A and two subarray images using the dye-swapped method are presented in Supplementary Figs. 1B and 1C. The average correlation coefficient of technical replication (between arrays) was 0.92 and 0.93, and that of biological replication (between slides) was 0.84 and 0.81 at 6hr and 21hr, respectively. In addition, the microarray data were validated and confirmed by comparison with Northern blot analyses. Although the absolute value of fold-induction observed with the cDNA microarrays did not match with the Northern data, the overall pattern of mRNA expression in the Northern blots was similar to that revealed by the microarray data (Supplementary Fig. 1D).

**Results and discussion**

**Generation, quality analysis and clustering of ESTs**

To reduce the redundancy during EST sequencing, we constructed three different cDNA libraries. One (designated as KS01) was constructed using RNA prepared from hot pepper leaves infiltrated with the soybean pustule pathogen (Xag) while the other two were generated from hot pepper flower buds (KS07) and anthers (KS08). These libraries yielded a total of 8,525 ESTs, with KS01, KS07 and KS08 bearing 5,368, 2,017, and 1,140 ESTs, respectively.

Before analyzing the ESTs further, the sequences that originated from non-nuclear genes were identified by using the BLASTN algorithm to compare the EST sequences to the mitochondrial, chloroplast, and ribosomal RNA sequences present in sequence databases (the cut-off value was e-10). As shown in Supplementary Table 1, approximately 4.3% of the clones originated from mitochondrial (1.3%), chloroplast
(0.9%) and ribosomal RNA (2.1%). These non-nuclear clones represented 2.0%, 6.6% and 6.8% of the clones in the KS01, KS07 and KS08 libraries, respectively.

It has been reported that the G+C content of an EST can be useful to distinguish plant-derived from fungal sequences in mixed cDNA population (Qutob et al., 2000). Our data showed that the average G+C content of the hot pepper ESTs was 42% (Table 1), which is similar to that of soybean and *Arabidopsis* ESTs (Qutob et al., 2000; TAGI, 2000). Of the 8,525 cDNA sequences that produced an average of 516 bp of high quality sequence beginning with 5' ends (Table 1), 4,685 unique sequences were obtained. Of these, 3,287 sequences were singletons (Table 1) while 1,398 clusters were composed of redundant sequences (Supplementary Fig. 2). To get more accurate information about clone redundancy, additional sequencing and detailed analysis is currently in progress.

**Table 1. Quality of hot pepper ESTs**

<table>
<thead>
<tr>
<th></th>
<th>KS01</th>
<th>KS07</th>
<th>KS08</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total ESTs</td>
<td>5368</td>
<td>2017</td>
<td>1140</td>
<td>8525</td>
</tr>
<tr>
<td>Clusters (%)</td>
<td>3056 (57%)</td>
<td>1189 (39%)</td>
<td>901 (79%)</td>
<td>4685 (55%)</td>
</tr>
<tr>
<td>Singleton (%)</td>
<td>2163 (40%)</td>
<td>853 (42%)</td>
<td>817 (72%)</td>
<td>3287 (39%)</td>
</tr>
<tr>
<td>G+C content</td>
<td>41%</td>
<td>43%</td>
<td>41%</td>
<td>42%</td>
</tr>
<tr>
<td>Average sequenced length</td>
<td>524 bp</td>
<td>548 bp</td>
<td>402 bp</td>
<td>506 bp</td>
</tr>
</tbody>
</table>

*Clusters* denote a group of ESTs that bear a high degree of sequence similarity. The cluster number includes the number of singletons.

*Singletons* denote the ESTs that do not bear a high degree of sequence similarity to other EST sequences.

**Functional categorization and comparative analysis of hot pepper ESTs**

To assess the similarity between pepper ESTs and other eukaryotic genes or ESTs, we first categorized the hot pepper ESTs into functional groups using the MIPS *Arabidopsis* gene functional categories (http://mips.gsf.de/desc/thal). Of the 4,685 unique hot pepper ESTs, 2,035 did not match *Arabidopsis* proteins using the N2Tool (threshold 100). Thus, only 2,650 ESTs were assigned into functional groups. Approximately half of the sequences were assigned into more than two functional groups. The hot pepper ESTs were mainly assigned to five functional categories, namely, signaltransduction (9%), metabolism (5%), defense/cell rescue/cell death (3%), transcription (2%) and transport facilitation (2%) (Supplementary Fig. 3).

In addition to functional classification, the hot pepper ESTs were also compared to
plant EST sequences that are present in the NCBI dbEST database, including tomato, Medicago, potato, Arabidopsis, wheat, maize, and rice sequences. As expected, the tomato had the most hot pepper homologues (74%) while rice had the least (56% Fig. 1). Next, we sought to identify the hot pepper sequences that do not have homologues in other organisms. We found that 323 of the 4,685 unique sequences (7%) appear only in hot pepper (Fig. 1). Interestingly, 446 hot pepper ESTs (9.5%) did not match any region of the Arabidopsis genome. A similar result was also obtained when rice was compared to hot pepper (447 hot pepper ESTs showed no match). Finally, 120 ESTs (2.6%) did not match any region of Arabidopsis and rice genome.

Isolation, expression analysis and functional classification of Xag infiltration-responsive genes

The ESTs from the three different libraries could be divided into seven categories on the basis of their presence in one or the other library. Thus, 88% of the ESTs were expressed only in one specific library, about 2% of the ESTs were expressed in all three libraries, and the remaining 10% were expressed in two of the three libraries. Of all the ESTs expressed in the three libraries, 56%, 17%, and 15% were unique to the KS01, KS07, and KS08 libraries, respectively. Since the KS01 library was generated from Xag infiltrated leaves, we focused on these genes. Of these ESTs, we found that 136 may be candidate pathogen-responsive genes based on the computational expression analysis
(data not shown). However, since we do not have reference sequences from a healthy leaf cDNA library, we could not confirm that these ESTs are pathogen-inducible genes. Furthermore, the number of ESTs used for this study was insufficient for computational expression analysis. Moreover, Northern blot data indicates that most of these candidate genes are not real pathogen-responsive genes (data not shown).

To screen for the pathogen-responsive ESTs that are induced after Xag infiltration, we constructed a cDNA microarray that contains the 4,685 unique ESTs from the three libraries. For this purpose, we collected hot pepper leaves at an early time point after Xag infiltration (6hr), when no symptoms were observed, and at a late time point (21hr), when the distinctive HR phenotype was observed. We then prepared and labeled the mRNAs from these samples, which served as microarray probes.

During the image analysis, 464 (10%; 6hr) and 603 (13%; 21hr) spots were automatically removed by the GenePix Pro 4.0 software (www.axon.com). Thereafter, 1,933 (41%; 6hr) and 2,527 (54%; 21hr) spots were removed either because there were insufficient replicates (less than 3) or the data were unreliable (the SD is more than 50% of the average ratio). As a result, 2,288 (49%; 6hr) and 1,555 (33%; 21hr) ESTs were available for analysis.

It was found that upon Xag infiltration, approximately 613 ESTs (13.1%) showed significantly altered transcription (more than 3-fold) at at least one time point. The pathogen-responsive genes that are identified by this study are unlikely to include genes that are transcribed at low levels in response to Xag infiltration because such genes are probably not sequenced in the EST sequencing procedure. Thus, such genes are probably excluded in the microarray analysis. Of the pathogen-responsive genes that were identified, 402 were induced by Xag infiltration while 210 showed reduced transcription at at least one time point. Interestingly, one EST that encodes pathogenesis related protein (PR-1) showed significantly reduced transcription at the early time point and then an increase in transcription at the late time point.

At the early time point, 307 and 63 ESTs showed more than 3-fold up- and down-regulation of their transcription, respectively. At the late time point, 295 and 173 ESTs showed more than 3-fold up- and down-regulation of their transcription, respectively. Altered transcription at both the early and late time points was shown by 225 ESTs. Of these, 199 were up-regulated and 26 were down-regulated ESTs. This result indicates that Xag infiltration induces a complex combination of up- and
down-regulation of a variety of genes. Interestingly, at least 50% of the up-regulated genes were up-regulated at both time points after Xag infiltration (Fig. 2C). In contrast, only 12% of the down-regulated genes showed the same pattern at both time points (Fig. 2C). This indicates that most of the up-regulated genes are consistently expressed at both time points whereas the down-regulated genes are down-regulated at a specific time point, especially at the late stage of infiltration.

We then determined the possible functional roles of the 613 pathogen-responsive ESTs. Although we were unable to classify the function of 44% of the ESTs (272), the remaining 341 ESTs could be assigned into 11 categories (Fig. 2). The proteins encoded by these Xag-responsive ESTs were found to be related to metabolism, defense, cellular organization, energy, transport, transcription, and signaling. Surprisingly, we found that the genes in the different functional groups were differently regulated at the early and late time points after Xag infiltration. Regarding the genes in the cellular organization category, most of the genes that were up-regulated were related to cell wall organization or modification and their up-regulation already occurred at the early time point (Supplementary Table 2; Fig. 2A). In contrast, most of the down-regulated genes in this category were involved in chloroplast organogenesis and their down-regulation occurred at the late time point (Supplementary Table 2; Fig. 2B). In addition, in the genes in the energy category, most of the up-regulated genes were involved in glycolysis and were up-regulated at both the early and late time points (Supplementary Table 2). In contrast, most of the down-regulated genes were involved in photosynthesis (Supplementary Table 2). In the case of the metabolism-related genes, most of the up-regulated genes encode secondary metabolite-related enzymes and their transcription was already induced at the early time point (Supplementary Table 2; Fig. 2A). In contrast, the genes that encode carbohydrate metabolism-related proteins were mildly down-regulated at the early time point and then dramatically down-regulated at the late time point after Xag infiltration (Supplementary Table 2; Fig. 2B). Moreover, the genes that encode signaling components, transcription regulators and transporters were already up-regulated by Xag infiltration at the early time point (Fig. 2A). In conclusion, at the early stage of Xag infiltration, genes that encode signaling components and transcription regulators are upregulated and these modulate both the primary and secondary metabolic pathways and the transporters that assure the proper compartmentalization of defensive metabolites and diverse
precursors. This eventually leads to the up-regulation of genes that encode
defense-related proteins and metabolic enzymes, which may fortify the cell walls and
induce defense-related protein accumulation and other defensive reactions. In contrast,
the genes that encode chloroplast organogenesis and photosynthetic proteins are
down-regulated at the late time point, which suggests that Xag infiltration may reduce
photosynthetic activity.
Figure 2.

Comparison of the hot pepper genes regulated by Xa8 infiltration with those regulated in other plants by other biotic or abiotic stressors

Non-host resistance confers robust protection against pathogenic invaders and bears many similarities to host resistance (Heath, 2000; Thordal-Christensen, 2003). For
example, a recent study indicates that the mRNA expression patterns associated with the non-host and host defense reactions of *Arabidopsis* are similar (Tao Y et al., 2003). In addition, several studies have revealed that a substantial number of genes are commonly regulated by different defense/stress signals, including infection with a fungal pathogen, wounding, cold, drought or high salinity (Reymond et al., 2000; Schenk et al., 2001; Seki et al., 2001; Kreps et al., 2002; Cheong et al., 2003).

To investigate whether the genes that are regulated during *Xag* infiltration are the same types of genes that are commonly regulated in other plants in response to a variety of biotic and abiotic stressors, we compared our array data to those of *Arabidopsis* and tomato (only one array data used) plants that are stressed by various stressors. *Arabidopsis* was used in this comparison because it is the only plant for which sufficient array data is available for this purpose. Although the DNA contents used with the different arrays are not the same, this approach is still very useful in helping elucidate non-host resistance in hot pepper plants. As we expected, we found that the genes that were regulated in *Xag*-challenged hot pepper extensively overlapped with the gene expression patterns in the variously stressed *Arabidopsis* plants with regard to the functional categories of defense, signaling, metabolism, photosynthesis and cell wall modification.

(1) Regulation of defense-related genes by *Xag* infiltration of hot pepper

About 10% of all the *Xag*-responsive genes (62 ESTs) were classified to have a defense-related function. We also found that 80% of these defense-related proteins were more than 3-fold up-regulated at both time points. In particular, a number of genes that encode enzymes that may be involved in hydrogen peroxide production and processing were up-regulated. These include genes that encode the peroxidase, glutathione-S-transferase (GST) and NADPH oxidase-like enzymes. The activation of the oxidative burst has been shown to participate in the defense reaction to fungal toxin (fusicoccin) by the tomato as well as in the defense reaction by *Arabidopsis* to fungal pathogen (*A. brassicicola*), SAR (Systemic Acquired Resistance), oxidative stress and wounding (Schenk et al., 2000; Desikan et al., 2001; Maleck et al., 2001; Frick and Schaller, 2002; Cheong et al., 2003; Narusaka et al., 2003). Our study thus proposes that the oxidative burst is an essential component of the anti-pathogen response and that it plays an important role in the HR of the defense reaction. This hypothesis should be
verified by the further functional experiments.

(2) Regulation of signal transduction-related genes by Xag infiltration of hot pepper

About 7% of all the Xag-responsive genes (42 ESTs) were classified to have a signal transduction-related function, such as transcriptional regulators and signaling components. Of these genes, 81% (34 ESTs) were more than 3-fold up-regulated by Xag infiltration, which indicates that these signaling components are transcriptionally regulated by Xag infiltration. Signaling components have also been shown to undergo transcriptional regulation in other stress conditions, including biotic and abiotic stress conditions (Schenk et al., 2000; Desikan et al., 2001; Maleck et al., 2001; Seki et al, 2001; Frick and Schaller, 2002; Ramonell et al., 2002; Cheong et al., 2003; Narusaka et al., 2003). Signaling component genes include genes that encode receptor protein kinases, protein kinases, protein phosphatases, calcium dependent protein kinases (CDPK), calmodulins, and a variety of transcription factors. Those genes that encode calmodulin and CDPK are upregulated by both biotic and abiotic stresses suggests that the calcium ion may be one of the key signals that initiate general stress resistance reactions.

Interestingly, our array data revealed new transcription factors that have never previously been found to be pathogen-responsive elements. For example, the genes that encode CCR4-associated factor (CAF), AT Hook DNA binding protein and SPF1 were abundantly expressed during Xag infiltration. The involvement of these genes in plant defense has not been reported previously. In addition, we found that some transcription factors showed a different expression pattern in Xag-stressed hot pepper compared to previous expression studies. For example, unlike a previous report (Lee et al., 2002), we found that two genes encoding bZIP transcription factors showed reduced transcription in the Xag-infiltreated hot pepper. This suggests that both bZIPs may serve as negative regulators in the non-host defense response of the hot pepper.

(3) Regulation of metabolism-related genes by Xag infiltration of hot pepper

About 21% of all the Xag-responsive genes (131 ESTs) were classified to have a metabolism-related function. This is the functional category that contained the most pathogen-responsive genes. This could be explained by the fact that the genes in this category occupied a higher proportion of the array contents (Supplementary Fig. 3).
However, it may also represent that the Xag-induced direct regulation of metabolism-related genes could serve a key defense mechanism.

Our array data revealed that the genes that encode enzymes that are involved in the shikimate and phenylpropanoid pathway and the biosynthetic pathway of alkaloids and terpenoids were highly up-regulated (more than 3-fold) during both the early and late time points after Xag infiltration. The shikimate pathway produces a precursor for the synthesis of not only aromatic amino acids but also of many secondary metabolites (Weaver and Herrmann, 1997). Xag infiltration also strongly increased transcription of genes that encode chorismate mutase-like and tryptophan synthase-like genes. It has been shown that genes encoding shikimate pathway enzymes are also up-regulated in tomatoes challenged with fungal toxin (fusicoecin) and in Arabidopsis subjected to a fungal pathogen (A. brassicicola) and wounding (Schenk et al., 2000; Frick and Schaller, 2002; Cheong et al., 2003).

Alkaloids are one of the key defensive phytoalexins and their biosynthesis from L-Tyr to berberine (BIA; benzylisoquinolone alkaloid) involves multiple enzymatic reactions (Facchini, 2001). One of these reactions is performed by reticuline oxidase, whose transcripts are 4-fold abundant in the Xag-infected hot pepper. This suggests that Xag infiltration may lead to the accumulation of benzylisoquinoline alkaloids (BIAs) in the hot pepper plant. Enzymes that are involved in the alkaloid biosynthetic pathway are also activated at the transcriptional level after wounding, during the fungal pathogen (A. brassicicola) defense reaction, and after ultraviolet (UV) radiation, cold, salt and ozonetreatment (Kreps et al., 2002; Seki et al., 2002; Cheong et al., 2003; Narusaka et al., 2003). Several studies have also shown that the synthesis of sesquiterpene phytoalexins, primarily capsidiol, occurs in tobacco and pepper in response to pathogens, fungal elicitors and UV irradiation (Chappel et al., 1987; Kuc, 1995; Back et al., 1998). Our array data consistently showed that Xag infiltration highly up-regulates several genes that encode enzymes that are involved in the biosynthesis of terpenoids. Interestingly, such up-regulation of a terpenoid biosynthetic enzyme has not been observed in Arabidopsis stressed with various stressors. Unlike Arabidopsis, the up-regulation of capsidiol biosynthetic genes suggests that capsidiol could be one of key defensive phytoalexin in hot pepper.

The array data also revealed that genes encoding enzymes that are involved in the phenylpropanoid pathway were highly expressed during the defense reaction to Xag.
The activation of the phenylpropanoid pathway has also been shown to occur in tomatoes treated with a fungal toxin (fusicoccin) and in *Arabidopsis* stressed by a fungal pathogen (*A. brassicicola*), oxidative stress, cold stress, wounding or SAR (Malenke et al., 2000; Reymond et al., 2000; Schenk et al., 2000; Desikan et al., 2001; Frick and Schaller, 2002; Kreps et al., 2000; Cheong et al., 2003). The most striking effects of the activation of the phenylpropanoid pathway is that it produces many secondary metabolites, such as lignins, flavonoids and isoflavonoids, (Whitbred and Schuler, 2000). In addition, the accumulation of salicylic acid (SA) appeared to depend on the activity of phenylalanine ammonium-lyase (PAL) in tomatoes (Schaller and Frasson, 2000). Therefore, increased expression of phenylpropanoid pathway-related genes may play important roles in the production of several precursors of secondary metabolites and SA.

(4) Regulation of cellular organization- and hormone signaling-related genes by *Xag* infiltration of hot pepper

The array data show that approximately 5.4% of the *Xag*-responsive genes (33 ESTs) are related to cellular organization. Interestingly, most of cell wall organization/modification-related genes were highly up-regulated (more than 3-fold) after *Xag* infiltration. Increased expression of cell wall biosynthesis- and modification-related genes has also been observed in *Arabidopsis* stressed by a fungal pathogen (*A. brassicicola*) or wounding (Schenk et al., 2000; Cheong et al., 2003). Our data further confirm the idea that cell wall biosynthesis, modification or fortification is essential for defense reaction. In contrast, most of the down-regulated (more than 3-fold) genes were related to chloroplast organogenesis and chlorophyll biosynthesis. In addition, we also observed that the genes encoding proteins that are involved in carbohydrate metabolism show more than 3-fold decreased transcription. These results suggest that plants that are infected with a pathogen redirect the carbon flux from the primary metabolic pathway to the secondary metabolic pathway and reduce their photosynthetic activity. Tomatoes treated with fungal toxin (fusicoccin) show similar down-regulation of genes in this functional category, as do *Arabidopsis* plants exposed to a fungal pathogen (*A. brassicicola*), chitin ologomer, cold, drought or high salinity (Schenk et al., 2000; Frick and Schaller, 2002; Ramonell et al., 2002; Seki et al., 2002).

Plant defense against pathogens and environmental stresses such as wounding is regulated through a complex network of signaling pathways that involve three
signaling molecules, namely, SA, jasmonic acid (JA) and ethylene (Schenk et al., 2000; Kunkel and Brooks, 2002 Cheng et al., 2003). *Xag* infiltration causes more than 3-fold increased transcription of genes that encode ACC oxidase, are involved in the phenylpropanoid and shikimate pathways, or encode lipase, lipoxygenase, and lipid biosynthetic enzymes. These enzymes and pathways are involved in the production of the SA, JA and ethylene plant hormones. A recent study has shown that brassinosteroid (BR) is involved in a broad range of disease resistance in tobacco and rice (Nakashita et al., 2003). The gene that encodes the biosynthetic enzyme of BR, namely, Sterol delta-7-reductase, was 4-fold up-regulated by *Xag* infiltration. This indicates that BR may be involved in the defense reaction shown by the hot pepper plant. Interestingly, the gene that encodes gibberellin 2-oxidase, which degrades the active form of gibberellin into non-active gibberellin, was also 6-fold upregulated by *Xag* infiltration. This suggests that the deactivation of gibberellin may also be involved in the defense response of the hot pepper plant.

**Conclusion**

Even though 4,685 genes represent only a small part of the hot pepper genome, we were still able to identify 613 *Xag* infiltration-responsive transcripts, which may aid the elucidation of the non-host defense functions of the hot pepper. Photosynthesis and carbohydrate metabolism-related genes were severely down-regulated at the late stage of the defense reaction. In contrast, most of the genes that encode enzymes involved in secondary metabolism, defense-related proteins, signaling components, transcription regulators, and transporters showed increased transcription during the entire defense reaction process. Interestingly, similar genes are also regulated during the defense response of other plants to fungal and environmental stresses. Despite these similarities, however, we also identified several novel transcription factors that may be specifically involved in the non-host resistance of the hot pepper. We also found that the non-host resistance of the hot pepper may involve the deactivation of gibberellin. Furthermore, we also identified many genes that encode proteins with an unknown function. Further functional characterization of these genes could broaden our understanding of the non-host resistance mechanisms of the hot pepper and other plants.
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Toward Functional Genomics of Plant-Pathogen interactions: Isolation and Analysis of Defense-related Genes of Hot Pepper During Resistance Against Pathogen

ABSTRACT

In order to understand of plant-pathogen interactions, we are pursuing to isolate complete set of the hot pepper genes that are differentially expressed during pathogen attack. As a first step, we used DDRT-PCR technique and isolated 300 differentially expressed cDNAs from hot pepper leaves showing non-host resistance against bacterial plant pathogens (Xanthomonas campestris pv. glycines, Pseudomonas syringae pv syringae). Reverse Northern and Northern blot analyses revealed that 50% of those genes were differentially expressed in pepper leaves during non-host resistance response. Among them, 245 cDNAs which present as an independent gene without redundancy were micro-arrayed for further analysis. We are also generating random EST sequence database from various cDNA libraries including pepper tissues library showing non-host hypersensitive response against bean pustule pathogen (Xanthomonas campestris pv. glycines). As a primary stage we sequenced 8,000 cDNA clones and analyzed EST data. These clones are being spotted on glass slide to study the expression profiling. These integrated information will broaden the knowledge of plant-pathogen interactions and will be presented.

INTRODUCTION

As an sessile organism, plants defend themselves against invading pathogens by exerting diverse cellular responses. The most distinctive phenotype of defense response is the rapid cell death of plant at the site of infection, so called hypersensitive response (HR), to limit spreading of the pathogen (Dangle et al., 1996). In the cellular level, a large set of defense responses, including generation of ROS (Levine et al., 1994; Mehdy, 1994), cell wall lignification (Wetten and Sederoff, 1995), and biosynthesis of antibiotics (Darvill and Albersheim, 1984; Dixon, 1986) is accompanied by HR. Furthermore resistance responses are developing in unaffected parts of plant,
SAR (systemic acquired resistance), which provide pre-formed resistance against further infection with a broad spectrum of pathogens (Ryals et al., 1996).

The plant defense responses are orchestrated consequence of transcriptional activation of defense-related genes (Lamb et al., 1989). In the process of local and systemic responses, a large group of pathogenesis-related (PR) proteins are synthesized to display a broad spectrum of anti-microbial activity (Bowles, 1990). In addition to the genes directly related to the defense responses such as PR-genes, transcription of the genes encoding enzymes involved in secondary metabolic pathways are stimulated. Most intensively studied secondary metabolisms in this regard are terpenoid and phenylpropanoid pathways for producing phytoalexins and phenolics (Dixon and Lamb, 1990, Choi et al., 1992 and 1994). Since the secondary metabolism cannot occur without related primary metabolism where large carbon fluxes are supplied, genes involved in primary metabolisms are also expressed. Similarly, genes for the activated methyl cycle have elevated transcriptional activity, possibly to provide the activated methyl groups to be used in ethylene production and numerous methylation steps for secondary product formation (Kawalleck et al., 1992).

The complexity of the plant defense mechanisms is becoming apparent, since pathogen defense entails a major shift in metabolic activity rather than altered expression of a few class of defense-related genes (Somssich and Hahlbrock, 1998). Therefore, identification of a complete set of genes involved in the defense process is an essential step toward understanding of whole scheme of plant defense mechanisms. In this regard, as recently revealed by genetic studies with Arabidopsis, many more functionally unidentified plant genes must exist whose products are also required for mounting an effective defense responses (Glazebrook, et al., 1996; Rogers and Ausubel, 1997).

Subtractive hybridization, differential screening, differential-display PCR analysis, random EST sequencing and micro-array have been developed to isolate differentially expressed genes. Differential display is a simple and highly sensitive to detect mRNAs of low abundance. EST sequencing is a good tool to randomly isolate lots of genes related to a specific
condition/tissue. In contrast, micro-array is relatively new technique and powerful tool in genomics era. Although micro-array needs relatively high cost compare to other methods, it can generate lots of parallel data.

To contribute to the goal of plant-pathogen interaction studies, we have performed experiments for isolation of a mass of genes expressed during plant defense responses. The isolated genes were classified into previously identified defense-related genes, genes encoding primary or secondary metabolic enzymes of known function, and novel genes. Expression patterns of isolated defense-related genes and computational analysis of 8,000 EST clones isolated from pathogen-induced pepper cDNA library will be presented in the symposium.

MATERIALS AND METHODS

Plant materials and pathogens. Four to six weeks old hot pepper plants (Capsicum annuum L. cv. Bukang) were grown in a culture room under a regime of 16 h light and 8 h dark at 25 ± 2 °C. Ten weeks old tobacco plants (Nicotiana tabacum cv Xanthi nc) were also used in some of our studies. Bacterial strains used in this studies, Xanthomonas campestris pv. glycines 8ra or X. c. pv. glycines 8-13 (Xcg8ra and Xcg8-13, Hwang et al., 1992), and X. c. pv. vesicatoria, were generous gifts of Dr. Ingyu Hwang (Dept. of Plant Pathology, Seoul National University).

Construction of cDNA library. For hot pepper cDNA library construction, poly(A)+ mRNA was isolated from the total RNA prepared from hot pepper leaf tissues following syringe infiltration with soybean pustule pathogen (Xcg 8ra). Complementary DNA was synthesized and cloned into the EcoRI-XhoI sites of the λZAPII vector (Stratagene, USA), according to the manufacturers instructions. For tobacco cDNA library construction, eight week-old tobacco plants (N. glutinosa) were inoculated by sap prepared from TMV infected tobacco plants and maintained for 24 h at 32 °C for systemic spreading of viruses. The plants were then shifted to 25 °C for 18 h to induce systemic HR. Total RNA was isolated from the inoculated leaf tissues showing HR by the method of Choi et
al., (1992). Isolation of poly (A+) RNA using oligo-dT cellulose (Boehringer Mannheim, Germany) and construction of a cDNA library using a λZAP cDNA synthesis kit were carried out according to the manufacturer's instructions (Stratagene, USA). In addition to pathogen infected library, flowering bud and anther specific libraries were purchased from Eugenotech (www.eugenotech.com).

**Differential screening.** After in vivo excision of a TMV-induced cDNA library, 900 recombinant plasmids were isolated from each single colony for slot blot hybridization. Two or three micrograms of each recombinant plasmid DNA was loaded in duplicated slot blots. One blot was hybridized with a [32P]-labeled cDNA probe prepared with mRNA from tobacco leaf tissues showing HR. The other blot was hybridized with a [32P]-labeled cDNA probe prepared with mRNA from mock treated healthy leaf tissue. Hybridization was carried out in 5 X SSC containing 50% formamide at 42 °C and washes were performed at 50 °C in 0.2 X SSC and 0.1% SDS.

**Differential display PCR.** Hot pepper plants were syringe-inoculated with Xcg 8ra and the leaves were harvested every 4 hr until 12 hr of inoculation. Total RNA samples prepared from the leaves were subjected to mRNA differential display analysis (Liang and Pardee, 1992) to select the cDNA bands that were reproducibly induced in response to the pathogen. Total RNA from healthy untreated leaves was used as a control. After DNase I treatment to remove DNA contaminant, total RNA (0.4 μg) was used for reverse transcription and subsequent PCR. Each RNA sample was reverse transcribed using Th1N1N2 followed by PCR amplification in the presence of Th1N1N2 and any of each of the twenty arbitrary 10-mer (Operon, USA). Forty cycles of PCR were performed at 94°C for 30 sec, 42°C for 2 min, and 72°C for 30 sec. PCR products were separated on a 6 % polyacrylamide sequencing gels and visualized by autoradiography. Bands of interest were purified from the gels and reamplified. The reamplified fragments were cloned into the pGEM-T easy vector (Promega, USA), sequenced, and used as probes for RNA gel blot analysis and cDNA library screening.
EST generation and computational analyses. After in vivo excision of a Xcg-induced hot pepper cDNA library, 8,000 recombinant plasmids were isolated from each single colony for DNA sequencing. Single pass 5'-end sequences of average 400 bp in length were determined using dye terminator sequencing method. Obtained sequences were processed using vector clipping and contig assembly software (Phred) for parent EST selection. The selected parent EST sequences were collected and constructed as FASTA DB for massive local BLAST analyses and identification of genes. From these multi-process (Fig. 6), the identified genes were clustered and classified into different group of functions.

Micro-array and fluorescent probe preparation, hybridization, and scanning. Total 245 ESTs were randomly selected for spotting on the glass slide. Each clones were subjected to PCR at 30 cycles of 94°C denature, 60°C annealing, and 72°C extension condition. The amplified inserts were ethanol precipitated and resuspended in X3 SSC (final concentration), then spotted on the poly-L-Lysine coated glass slides by Sigma (Missouri, USA). 100 µg of total RNA was isolated from control and pathogen treated plants. Each RNAs were labelled with fluorescent dye and washed following the manufacture's protocol (NEN
Micromax; NEN, MA, USA). The arrays were scanned using GSI Lunomics scanner following the manufacture's protocol (Watertown, Massachusetts). Overall processes for micro-array experiments are shown in figure 1.

RESULTS AND DISCUSSION
The patho-system: For the isolation of pathogen defense genome of hot pepper, we used the non-host resistance of pepper plant against soybean pustule pathogen, X. c. pv. glycines. Upon infiltration of this bacterial pathogen into hot pepper, the leaf tissues undergo cell death and ended up with typical hypersensitive lesion within 20 hr of inoculation (Fig. 1). The symptoms were almost identical with that caused by infection with its own incompatible pathogen such as X. c. pv. vesicatoria. Electron micrographic study revealed the dramatic microscopic level difference in pepper-Xcg interaction between HR-causing Xcg 8ra and HR- mutant of same pathogen Xcg 8-13 (Fig. 2).

![Image](image-url)

**Figure 2.** Symptom and electron micrograph of hot pepper plant following infiltration of soybean pustule pathogen, Xanthomonas campestris pv. glycines 8ra and 8-13. Leaf pictures and electron micrographs were taken 24 hr of infiltration.

Isolation of defense-related genes from hot pepper using DDRT-PCR: Our previous studies in tobacco, we presumed that host and non-host HR in plant probably share signalling pathway(s) leading to hypersensitive cell death (Oh et al., unpublished data). For that reason, we used non-host resistance of hot
pepper against soybean pustule pathogen for isolation of mass of genes expressed during HR cell death following infection. From DDRT-PCR procedures shown in Fig. 3, 300 differentially expressed pepper cDNA clones were isolated following inoculation with Xcg. DNA sequencing analysis revealed that some of them were known genes as induced by pathogen but many of them were not identified through gene bank search. Fig. 4 show that differential expression of some selected pepper genes during non-host resistance of pepper against soybean pustule pathogen. Most of the genes were upregulated in both HR+ and HR- conditions but not in buffer infiltration. Similar results were also observed in the responses of tobacco against HR-inducing or HR-non-inducing pathogens. To date, Northern blot analyses were performed with 40 isolated genes. The Northern blot analyses revealed that most of the genes induced in non-host HR also induced in host HR conditions. Fig. 5 show expression of genes isolated in this studies which show differential transcriptional activity in resistance and susceptible reaction of pepper against its natural pathogen Xanthomonas campestris pv. vesicatoria. From the DDRT-PCR isolated clones, 254 independent clones were arrayed on slide glass as a DNA chip. The gene expression analysis using the DNA chip is under way and the prototype analysis data will be presented in the symposium.

Figure 3. Schematic flow of defense-related gene isolation and confirmation
from hot pepper plant following inoculation with Xcg using differential display PCR, reverse Northern, Northern blot hybridization, and micro-array hybridization.

**Random EST sequencing and data analysis:** After *in vivo* excision of a pathogen-induced hot pepper cDNA library, 8,000 recombinant plasmids were isolated from each single colony for DNA sequencing. Single pass 5'-end sequences of average 400 bp in length were determined using dye terminator sequencing method (ABI, USA). Vector and unclear parts of the obtained DNA sequences were clipped using Phred software then all the sequences were constructed as DB of FASTA format. Clustering and removing of redundant sequences of the FASTA sequence DB were performed using Phred/Phrap contig-assembly algorithm (Fig. 6). After removing the redundant sequences, the unique DNA sequences were filtered through Local BLAST multi-process and classified according to the functional categories of the genes. Analysis and classification of 8,000 EST sequence data obtained in this study will be presented in the symposium.
Figure 4. Expression of some DDRT-PCR selected genes during non-host disease resistance of hot pepper against soybean pustule pathogen, *Xcg* 8ra or 8-13.

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Figure 5. Expression of some DDRT-PCR selected genes during host disease resistance and susceptibility of hot pepper against *X. C. pv. vesicatoria*.

As a conclusion, we are pursuing to isolate and characterize the function of all the genes induced in hot pepper plant during resistance against pathogen. As an initial stage, we isolated thousands of genes related to the expression of disease resistance in pepper. Computational tools for analysis of massively isolated cDNA sequences were developed and will be keep improving. Studies on the functional genomics of isolated hot pepper genes in relation to defense against pathogen using DNA microarray and virus-induced gene silencing (VIGS) methods will be performed along with massive EST data base generation.
Figure 6. Flow chart of clustering, BLAST analyses, cDNA microarray, and classification of randomly sequenced EST clones from pathogen-induced hot pepper cDNA library.

Acknowledgement

This work was supported by a grant (PF003301-00) from Plant Diversity Research Center (PDRC) of 21st Century Frontier Research Program funded by Ministry of Science and Technology of Korean government. We thank to the Plant Molecular Genetics and Breeding Research Center (PMGBRC) from Korea Science and Engineering Foundation (KOSEF).

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**Abstract**

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.

**Introduction**

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
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 notonly
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

**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 mitochondrial, 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/athal). 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 El-Maarouf et al. 2001). It is possible that signaling and transport facilitation are key processes 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. elegans*, *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 (Table 4). 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 interpreting 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 didn’t 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 benthamiana*, 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](http://plant.pdrc.re.kr/Gene). The microarray data will be available at [http://plant.pdrc.re.kr:8888/array/index.html](http://plant.pdrc.re.kr:8888/array/index.html).

**Acknowledgement**

This work was supported by grants from PDRC (PF003301-00) and CFGC (CG1221) of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology of the Korean government.

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EST and Microarray Analyses of Pathogen Responsive Genes in Hot Pepper
(Capsicum annuum L.) Non-Host Resistance Against Soybean Pustule Pathogen
(Xanthomonas axonopodis pv. glycines)

Non-host resistance confers robust protection against pathogenic invaders and bears
many similarities to host resistance (Heath, 2000; Thordal-christensen, 2003). For
example, a recent study indicates that the mRNA expression patterns associated
with the non-host and host defense reactions of Arabidopsis are similar (Tao Y et al.,
2003).

To investigate how the pathogen responsive genes that are regulated during Xag
infiltration involved in the defense reaction, we have analyzed the possible role(s) of
these genes using well known defense reactions including host incompatible
interactions.

Analysis of genes those are up-regulated during the defense reaction of hot
pepper to Xag

Interestingly, most of the up-regulated genes in the cellular organization category
were related to cell wall organization/modification. Unlike mammals, plants build
physical barriers to enclose pathogens so as to block their access to nutrients. This takes
place, for example, in the hypersensitive reaction (HR) of the affected leaf epidermal
cell of a resistant plant. The microarray data revealed that during the Xag-defense
response, the genes encoding proteins that resemble the following cell wall
organization proteins show marked up-regulation: UDP-glucose dehydrogenase
(UGDH) (KS01037H02), alcohol dehydrogenase (KS01011B03), aldehyde
dehydrogenase (KS07006A03), reversibly glycosylated polypeptides (RGP)
(KS01065B09, KS01007E09) and hydroxycinnamoyl-CoA:tyramine
N-(hydroxycinnamoyl) transferase (THT) (KS01041F09, KS01003H03). UGDH combines
functions of alcohol dehydrogenase and aldehyde dehydrogenase, and utilizes
UDP-glucose to form the precursor of hemicellulose and pectin, the key component
of cell walls (Hempel et al., 1994; Delmer and Stone, 1988; Gilbeaut and Carpita, 1994).
The RGP's are implicated in the biosynthesis of polysaccharide and thus also in cell
wall synthesis (Saxena and Brown, 1999; Langeveld et al., 2002). THT is responsible
for the synthesis of cell wall-bound phenolic amines and is involved in cell wall
strengthening (Back et al., 2001). Taken together, these results indicate that cell wall
biosynthesis, modification or fortification is essential for the Xag-induced defense reaction, probably because it allows the plant to build physical barriers that enclose pathogens and thereby block their access to nutrients.

Defense-related genes were also highly expressed during Xag infiltration. These genes are divided into three classes, namely, oxidative burst-related proteins, pathogenesis-related proteins (PRs), and pathogen- or stress-inducible proteins. The genes belonging to the first class encode peroxidase, glutathione-S-transferase (GST) and an NADPH oxidase-like enzyme (KS01009C04, KS01028G11, KS059C03, KS01034B05, KS01014G06, KS01056D06, KS01066A09, KS01048C08, KS01037B11, KS01023F03). Genes encoding homologs of the gp91phox subunit of the plasma membrane NADPH oxidase complex are hypothesized to be a source of reactive oxygen species during host defense responses (Sagi and Flutur, 2001). Interestingly, the gp91phox homolog has two Ca2+-binding (EF) hand motifs and ectopic expression of calmodulin and CDPK genes enhances NADPH oxidase activity and oxidative burst in tomatoes and Arabidopsis (Keller et al., 1998 Romeis et al., 2000; Xing et al., 2001). This indicates that Ca2+ may play an important role in the regulation of NADPH oxidase activity. These observations may explain the increased transcription during Xag infiltration of gp91 homologs (KS01023F03), calmodulin (KS01002B01) and calmodulin-dependent protein kinase (CDPK; KS01045C01, KS01071G11), as these proteins may be involved in the generation of the oxidative burst. The genes belonging to the PR class that are regulated by Xag infiltration encode PR-1, PR-10, chitinase class I, II, IV, peroxidase and beta-1,3- glucanase (KS01013G04, KS01034B05, KS01038D06, KS01059C03, KS01015A02, KS01006B04, KS01009C04, KS01014G06, KS01028G11, KS01053A09, KS01007F05, KS01007A09, KS01074F04, KS01007B02, KS01066C07). That genes encoding pathogenesis-related proteins (PRs) are induced during the defense reaction is now well known (Van Loon, 1999).

The most prominent functional category into which the Xag-responsive genes fell was metabolism. Many genes involved in various biochemical pathways of both primary and secondary metabolic processes were up-regulated during Xag challenge. The shikimate pathway is involved in primary and secondary metabolism and produces chorismate, which is a precursor of not only aromatic amino acids but also of many secondary metabolites (Weaver and Herrmann, 1997). The array data revealed that the expression of genes that encode chorismate mutase-like (KS01012B11,
KS01054D07, KS01017E06) and tryptophan synthase-like enzymes (KS010056A12) was strongly enhanced during the defense reaction. Since both enzymes produce three aromatic amino acids, namely, phenylalanine, tyrosine, and tryptophan, the shikimate pathway could be the source of carbon fluxes in the secondary metabolic process. For instance, the biosynthetic pathway of bezylisoquinoline alkaloids (BIAs) is initiated by converting tyrosine to (S)-reticuline, with the following steps being catalyzed by the reticuline oxidase that converts (S)-reticuline to (S)-scoulerine (Facchini et al., 1996; Bird and Facchini, 2001). Our array data also revealed that the reticuline oxidase-like gene (KS080008B04) is expressed at a high level, which may lead to the possible generation of BIAs in the Xag-infected hot pepper plants. Several studies have shown that the synthesis of sesquiterpene phytoalexins, primarily capsidiol, occurs in tobacco and pepper in response to pathogens, fungal elicitors and ultraviolet (UV) irradiation (Chappel et al., 1987; Kuc, 1995; Back et al., 1998). Our array data consistently showed that two genes encoding enzymes that are potentially involved in the biosynthesis of capsidiol are up-regulated by Xag infiltration, namely, the sesquiterpene cyclase-like (KS01054G02) and 5-epi-aristolochene synthase-like (KS01016H03, KS01006C05) proteins. In addition, it was shown that Xag infiltration increases the transcription levels of a gene that encodes a linalool synthase-like protein. This indicates that monoterpenes may also accumulate in Xag-infiltated hot pepper plants. These results suggest that terpenoid biosynthesis may have occurred and thus may play an important role(s) in the defense reaction of hot pepper. Our array data also revealed that the three key genes of the phenylpropanoid pathway are highly expressed during Xag infiltration, namely, those encoding phenylalnine ammonium-lyase (PAL; KS08004G05, KS01037E08, KS01016G11), cinnamic acid 4-hydroxylase (C4H; KS01002B07, KS01002D09, KS01041G03) and 4-coumarate-CoA ligase (4CL; KS01013C08, KS01006E01, KS08019G01). The product of this core phenylpropanoid reaction is funneled into one of several branch pathways that lead to the production of lignins, flavonoids, coumarins, isoflavonoids and furanocoumarins (Whitbread and Schuler, 2000). Thus, the increased expression of the phenylpropanoid pathway-related genes in Xag-infiltated hot pepper suggests they may play important roles in the synthesis of important secondary metabolites.

Plant defense responses are initially regulated through a network of phytohormone signaling pathways (Kunkle and Brooks, 2002). The role of ethylene in plant defense is
somewhat controversial as it contributes to resistance in some interactions (Bent et al., 1992; Lund et al., 1998; Hoffman et al., 1999 Thomma et al., 1999). Despite this controversy, it is known that ethylene can activate plant defense-related processes such as the production of phytoalexins and PR proteins, the stimulation of camptotheacin-induced H2O2 production, and cell death (Fan et al., 2000 de Jong et al., 2002; Tornero et al., 1994). Our array data show that \textit{Xag} increases the transcription of the genes that encode ACC oxidase (KS01017E08, KS01011F07, KS01012F05, KS01045A04). In addition, we found that the gene encoding a RAN1-like protein (KS01038E03) was highly expressed during the defense reaction. RAN1 are cation-transporting P-type ATPases that are involved in copper trafficking and may be required to form functional ethylene receptors (Woeste and Kieber, 2000). Thus, the defense response of hot pepper may involve both increased ethylene biosynthesis and the activation of the ethylene receptor. Another phytohormone is SA, which has long been known to play a central role in plant defense against pathogens since SA levels increase in plant tissues following pathogen infection, and exogenous application of SA results in enhanced resistance to a broad range of pathogens (Ryals et al., 1996). A recent study has revealed that the biosynthesis of SA is initiated from chorismate and phenylalanine, which are the products of the shikimate and phenylpropanoid pathways, respectively (Shah, 2003). Although there is no direct evidence for the accumulation of SA in \textit{Xag}-infiltrated hot pepper plants, the activation of both the phenylpropanoid pathway and the shikimate pathway do suggest that SA may accumulate in \textit{Xag}-challenged hot pepper plants. A third phytohormone is JA, a fatty-acid-derived signaling molecule that is involved in defense against insect pests and microbial pathogens (Farmer et al., 1998). Our array data revealed that a number of genes, such as those encoding lipase (KS01058G05, KS01050C08) and lipoxygenase (KS01038A12, KS01047D01, KS01039A01), which may be involved in JA biosynthesis, are up-regulated by \textit{Xag} infiltration. In addition, other genes that may be involved in lipid and fatty acid biosynthesis are also upregulated by \textit{Xag} infiltration, which suggests that this challenge may cause the accumulation of JA. Moreover, JA is known to inhibit the biosynthesis and activity of photosynthetic pigment as well as the expression of the ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) and chlorophyll a/b binding proteins (CAB) (Reinbothe et al., 1997; Frick and Schaller, 2002). That the genes encoding these proteins are down-regulated during \textit{Xag}
infiltration also supports the possibility that JA may accumulate during this stress. A recent study has shown that brassinosteroid (BR) is involved in conferring a broad range of disease resistance on tobacco and rice (Nakashita et al., 2003). The gene that encodes the enzyme that biosynthesizes BR, namely, Sterol delta-7-reductase (KS01002H05), was up-regulated by Xag infiltration, which indicates that BR may be involved in the defense reaction of the hot pepper plant.

The array data also suggest that the defense signaling pathway may overlap with othersignaling pathways. We found that the genes encoding the wound-induced protein (KS01017B02) and proteinase inhibitor II (KS01047E07) were highly up-regulated during the Xag-induced defense reaction. This suggests that a signaling pathway may be shared by the pathogen-defense response and the wounding response. This possibility was also shown to be true in Arabidopsis and tomatoes (Durrant et al., 2000; Cheong et al., 2003). Moreover, it has been observed that PR gene expression and the HR process during the defense reaction show light- and phytochrome-dependency, and some components of the light signaling pathways have been shown to also participate in the defense-mediated signaling pathway (Genoud et al., 2002; Liu et al., 2002). For instance, although the COP9 signalosome was originally described as an essential regulator of photomorphogenesis, the tobacco homologues of the COP9 signalosome subunits have also been shown to be involved in N-specific resistance to TMV (Wei et al., 1994; Liu et al., 2002). Similar to this functional overlapping of the COP9 signalosome components, it has been shown that the RAR1 and SGT1 proteins interact with components of the SCF (Skp1, Cullin, F-box) E3 ubiquitin liagase complex, which may explain the requirement for the protein degradation process in the R gene-mediated defense (Shirasu et al., 1999; Austin et al., 2002; Liu et al., 2002). Interestingly, the array data showed that the COP9-like gene (KS1074C01) is expressed at higher levels during Xag infiltration but that the expression levels of the SGT1- and SKP1-like genes do not change. This result may suggest that other components, such as E3 ubiquitin ligase-like zinc finger protein and F-Box motif-containing protein, may interact with the COP9 signalosome. Alternatively, different homologues of SKP1 and SGT1 could exist in the hot pepper genome that were not identified in our EST sequencing. Nevertheless, our data show that COP9-mediated protein degradation may exists in the non-host defense reaction of the hot pepper plant and that the light- and defense-mediated signaling pathways could
overlap, as they do in the tobacco plant.

Importantly, some transcription-factors (TFs) were induced during the defense reaction of the hot pepper. Two genes that encode a scarecrow (SCR)-like TF (KS01065G03, KS01063F12) were identified and their transcription was induced during the defense reaction. Previous reports show that SCR not only regulates asymmetric cell division during radial organization of the Arabidopsis root (Laurenzio et al., 1996) but also increases its expression in the tomato in response to Pseudomonas infection (Mysore et al., 2002). bZIP TFs are also known to be involved in the defense response (Lee et al., 2002). However, in contrast to previous reports, we found that Xag down-regulates two genes that encode bZIP TFs (KS01017A10, KS01048E10). This may indicate that both bZIPs work as a negative regulator in the hot pepper. Two zinc-finger type TFs (KS01059H07, KS01013E12) were induced during the defense reaction. To our knowledge, this is only the second report that indicates zinc finger type TFs may be involved in the plant defense reaction (Mysore et al., 2002). NAC proteins have been shown to mediate viral resistance and to be induced by wounding and Phytophthora infestans infection in the potato (Ren et al., 2000; Xie et al., 1999 Collinge and Boller, 2001). The array data showed that a single gene encoding NAC domain-containing TFs (KS01055E05) was induced during the defense response of the hot pepper to Xag infiltration. CCR4-associated factor (CAF) forms a complex with CCR4 and DBF2, resulting in a transcriptional activator that is involved in regulating diverse processes including cell wall integrity, methionine biosynthesis, and M/G1 transition (Liu et al., 1997). Our array data showed that two CAF-like genes (KS01018B10, KS01065B07) are induced during the defense response to Xag. The involvement of CAF-like TFs in plant defense has not been reported previously. AT Hook DNA-binding proteins bind the AT-rich region in the promoters of soybean glutamine synthetase, oat phytochrome A3, and pea plastocyanin gene (Reisdorf-Cren et al., 2002; Nieto-Sotelo et al., 1994; Webster et al., 1997). Our array data showed that the gene encoding an AT Hook DNA-binding-like protein (KS01070D12) was highly induced during the defense response of hot pepper, even though this gene has not been previously reported to be involved in plant defense. The mRNA levels of PIRIN (Le-PIRIN) are dramatically increased during camptothecin-induced PCD (programmed cell death) in tomatoes (Orzæez et al., 2001). The human protein PIRIN forms quaternary complexes with the transcription factors NF-κB and Bcl-3 and targets
sequences in the promoter regions of anti-apoptotic genes (Wulczyn et al., 1996; Dechend et al., 1999). We found one gene encoding a PIRIN-like protein (KS01068H101) was activated during the Xag-induced cell death in hot pepper. This suggests that PIRIN-mediated cell death could be existed in the hot pepper defense mechanism. The sweet potato SPF1 binds to the 5’ upstream regions of three different genes that encode sporamin and beta-amylase of tuberous roots (Ishiguro and Nakamura, 1994). The SPF1-like gene (KS01061F08) was abundantly expressed during the defense reaction of hot pepper, although its involvement in plant defense has not been reported previously.

**Analysis of genes those are down-regulated during the defense reaction of hot pepper to Xag**

In marked contrast to the types of genes whose expression is up-regulated by Xag infiltration, the genes that are down-regulated by Xag are involved in photosynthesis and carbohydrate metabolism. Several observations strongly support the concept that the control of gene expression plays a fundamental role in carbohydrate-mediated feedback or sink-regulated inhibition of photosynthesis (Cheng et al., 1992; Krapp et al., 1993; Jang and Sheen et al., 1994). Interestingly, infection of leaf tissues by viruses and pathogens may lead to elevated carbohydrate levels, possibly because the export of photoassimilates is blocked (Hall and Loomis, 1972). In addition, bacterial infection rapidly induces extracellular invertase expression, which can convert apoplastic sucrose to fructose and glucose and eventually triggers the repression of photosynthetic genes (Sturm and Chrispeels, 1990). In line with this, our array data also showed increased levels of invertase expression (KS08005C04). Alternatively, the down-regulation of carbohydrate metabolism-related and photosynthetic genes may be related to JA. JA is known to inhibit the biosynthesis and activity of photosynthetic pigment and the expression of the ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) and chlorophyll a/b binding proteins (CAB) (Reinbothe et al., 1997: Frick and Schaller, 2002). Interestingly, our array data show that the genes encoding Rubisco (KS01050B02, KS01065H02, KS01051B04) and CAB (KS01046G01, KS01004H02, KS01007F12, KS01067G02) exhibit decreased expression levels. Based on these observations, it appears that the Xag infection-induced suppression of the genes encoding carbohydrate metabolism and the photosynthetic apparatus may benefit the plant by
redirecting the carbon flux toward defense reactions, such as the activation of secondary metabolic processes. Furthermore, it appears that JA also directly or indirectly affects the redirecting of carbon flux. This notion should be verified by future studies.

Interestingly, ten genes encoding proteins involved in protein synthesis, such as large subunits of ribosomal protein, eukaryotic translation initiation factor and GTP-binding elongation factor, were significantly down-regulated by Xag (KS01035G07, KS01015F04, KS07003G10, KS07001D03, KS01008D03, KS07009A02, KS01042H04, KS01004B11, KS01049F10, KS01058F03). Of these ten genes, six appear to inhibit protein synthesis in the chloroplast (data not shown). This suggests that most of the genes involved in protein synthesis that are down-regulated by Xag play significant roles in the biosynthesis of chloroplast-related proteins.

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제 4 장 목표달성도 및 관련분야에의 기여도

가. 연구 목표 달성도

| 구분 | 연구개발 세부목표 | 추진 실적 | 달성도 (%)
|------|------------------|----------|----------
| 2001 | 유전자 집합(5K) 제작 및 유전자 발현 분석 기법 확립 | - 고추 EST 5000개를 집합한 유전자 집합 제작  
- 대량을 유전자 발현 분석기법 확립  
- 유전자 발현 매핑, 분석 및 공공 서비스  
- 비기도향성, 상황의 유전자 발현 패턴 발굴  
- 50개 이상의 유용유전자 선발 및 기능 유추 | 120 %
| 2002 | 2종의 유용유전자 기능 발굴 및 검정 | - 살리살산 처리 후 유전자 발현 패턴 발굴  
- 자스폰산 처리 후 유전자 발현 패턴 발굴  
- 유전자 발현 프로파일의 DB화  
- 유전자 칭 배포 및 분석서비스  
- 50개 이상의 생체방어 관련 유전자 선발 및 기능 유추  
- 2개 유전자 기능 검정  
- 30개의 pathogen-inducible promoter 분리 | 120 %
| 2003 | 4종의 유용유전자 기능 발굴 및 검정 | - 생체방어관련 유전자 발현 패턴발굴  
- Transcription profile DB 공개  
- 칭의 공공 서비스 및 연구지원  
- 500개 이상의 생체방어 관련 유전자 선발 및 기능 유추  
- 15종의 Novel inducible promoter 선발  
- 4종 이상의 유전자 기능 규명  
- 10K cDNA chip 개발 | 150 %

나. 연구 개발 성과를 목록

논문 (11건)


특허 (출원 5건)
1. 최도일 등. 담배에서 분리된 병저항성 반응시 특이적으로 발현되는 NgCDM1 단백질, 그를코딩하는 유전자 및 프로모터.
   대한민국특허출원번호 제 2002-67957 (2002. 11. 4.) (출원인: 한국생명공학연구원)
2. 최도일 등. 스트레스 저항성 전사인자 유전자, 단백질 및 이에의해
   형질전환된 스트레스 저항성 식물체. 대한민국특허출원번호: 10-2003-0028792, (2003. 05. 07.) (출원인: 한국생명공학연구원)
3. 최도일 등. 스트레스 저항성 전사인자 유전자, 단백질 및 이에의해
   형질전환된 스트레스 저항성 식물체. 대한민국특허출원번호: 10-2003-00202269, (2003. 03. 31.) (출원인: 한국생명공학연구원)
   gene, its protein and a transfected plant by the gene,  
PCT/KR03/01937, (2003. 05. 07.)
5. Choi, D. et al. A new stress-resistance transcription factors (CaPIF1
   and NbPIF1) gene, its protein and a transfected plant by the gene,  
PCT/KR03/01936, (2003. 03. 31.)

연구결과의 기업 기술이전 (계약조건 명기)
유전자 분양에 따른 MTA 체결: 고추에서 분리된 병저항성관련 전사조절
유전자의 고추 형질전환 및 내병성 분석. (주) 농우 바이오, 상업적 가치가
있을 경우 적절한 계약체결
제 5 장 연구개발결과의 활용계획

- 유용 유전자 활용 계획

지난 3년간의 1단계의 연구 결과는 생체 방어 관련 유전자를 유전자집합 이용해 대량으로 선발하는 것이므로 이후 선발된 유전자를 대상으로 유전자의 기능을 규명하여 상업적으로 이용될 유용 유전자를 발굴하는 것이 다툴어야 할 것이다. 이를 위해서 일차적으로 유전자집의 발현 데이터를 분석한 결과로 생체 방어에 관련될 것으로 여겨지는 500 여개의 유전자들을 선발하였고 이들을 대상으로 다양한 데이터 분석을 실시하여 상업적으로 이용될 가능성이 높은 것으로 추정 되는 유전자를 2차 선발하여 Virus-Induced Gene Silencing (VIGS)를 이용한 유전자 기능 검정이 실시 될 것이며, VIGS 결과로 기능이 확인된 유용 유전자들을 대상으로 표 발현 (over-expression) 시킨 형질전환체를 만들어 유전자의 기능을 재검토하고 이의 실용성 검정을 실시 할 것이다.

- 데이터베이스 활용 계획

분자체를 통해 구축된 유전자 발현 연구 기법 및 유전자 발현 데이터베이스는 향후 국내 식물 기능유전자 연구에 모델 시스템으로 지속적으로 이용될 것이며, 이와 더불어 다양한 분야의 국내 연구진들이 직접적으로 데이터를 활용할 수 있을 것으로 여겨진다.
제 6 장 연구개발과정에서 수집한 해외과학기술정보

특기사항 없음

제 7 장 참고문헌
**특정연구개발사업 연구결과 활용계획서**

<table>
<thead>
<tr>
<th>사업명</th>
<th>종사업명</th>
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| 과제명 | 고추 cDNA microarray 제작, 배포 및 내병성 관련 유용유전자 발굴 |

<table>
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<th>연구기관</th>
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<td>1. 기업화(✓)</td>
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<td>2. 기술이전( )</td>
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<td>3. 후속연구추진(O)</td>
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<td>4. 타사업에 활용( )</td>
</tr>
<tr>
<td>5. 신재 및 기초연구추진( )</td>
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<td>6. 기술개발사업 (교육/연구)</td>
</tr>
<tr>
<td>7. 활용중단(미활용)</td>
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<td>8. 기타( )</td>
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특정연구개발사업 처리규정 제 31조(연구개발결과의 보고) 제 2항에 의거 연구결과 활용계획서를 제출합니다.

점부 : 1. 연구결과 활용계획서 1부.
2. 기술요약서 1부

2004년 8월 31일

연구책임자 : 최도일 (인)

연구기관장 : 양규환 (직인)

과학기술부장관 귀하
[제목 1]

연구결과 활용계획서

1. 연구목표 및 내용
   - 대량 확보된 고주의 EST DB를 기반으로 유전자 접(5K) 제작 및 고주 연구자에게 배포
   - 칩을 이용한 유전자 발현 profile 분석 서비스 및 public DB의 구축
   - 5K 칩을 이용한 내병성관련 유전자 발현 분석 및 유전자 기능유추
   - 추정된 기능을 실험적으로 검정하여 유용 유전자 또는 프로모터를 발굴(6개 이상)
   - 주요 유전자들의 내병성을 증진시킬 육종 소재로 제공(3개 이상의 신규 유전자)

2. 연구수행결과 현황(연구종료시점까지)

가. 특허(실용신안) 등 자료목록
<table>
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<th>발명명칭</th>
<th>특허등록번호</th>
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<tr>
<td>담배에서 분리된 방해성 반응을 숙이적으로 발현하는 NgCDM1 단백질, 그를 담배, 유전자 및 프로포먼스</td>
<td>2002-67957</td>
<td>2002/11/04</td>
<td>최도일 김영철 정영희 이상철</td>
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<td>스트레스 저항성 전사인자 유전자, 단백질 및 이에의해 형질전환된 스트레스 저항성 식물체</td>
<td>2003-20269</td>
<td>2003/3/31</td>
<td>최도일 오상근 박정미 정영희 이상철</td>
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<td>2003-28792</td>
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<td>A new stress-resistance transcription factor (CAPF1) gene, its protein and a transfected plant by the gene</td>
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<td>Choi, D et al</td>
<td>PCT</td>
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나. 프로그램 등록목록
다. 노하우 내역

라. 발생품 및 시 작품 내역

바. 논문계재 및 발표 실적

논문계재 실적(필요시 별지사용)

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<td>Functional &amp; Integrative Genomics</td>
<td>EST and microarray analyses of pathogen-responsive genes in hot pepper (Capsicum annuum L.) non-host resistance against soybean pustule pathogen (Xanthomonas axonopodis pv. glycines)</td>
<td>2004</td>
<td>4</td>
<td>Springer-Verlag Heidelberg</td>
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<tr>
<td>Molecules and Cells</td>
<td>A method of high frequency virus-induced gene silencing in chilli pepper (Capsicum annuum L. cv. Bukang)</td>
<td>2004</td>
<td>17(2)</td>
<td>한국본자생물학회</td>
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<td>Plant Pathology Journal</td>
<td>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)</td>
<td>2004</td>
<td>20</td>
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<td>Plant Molecular Biology</td>
<td>The Ethylene-Responsive Factor Like Protein 1 (CaERFLP1) of Hot Pepper (Capsicum Annuum L.) Interacts in Vitro with Both GCC and DRE/CRT Sequences with Different Binding Affinities: Possible Biological Roles of CaERFLP1 in Response to Pathogen Infection and High Salinity Conditions in Transgenic Tobacco Plants</td>
<td>2004</td>
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<td>An International Journal on Molecular Biology Molecular Genetics and Biochemistry Press</td>
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<td>Plant Physiology</td>
<td>The pepper (<em>Capsicum annuum</em>) transcription factor, CaPFI, confer pathogen and freezing tolerance in <em>Arabidopsis</em></td>
<td>2004</td>
<td>In Press</td>
<td>American society of plant physiology</td>
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<td>Physiological and Molecular Plant Pathology</td>
<td>Expression of a novel tobacco gene, NgCDM1, is preferentially associated with pathogen-induced cell death</td>
<td>2003</td>
<td>62</td>
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<td>Molecules and Cells</td>
<td>Involvement of hydrogen peroxide in repression of catalase in resistant tobacco following tobacco mosaic virus infection.</td>
<td>2003</td>
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<td>Molecules and Cells</td>
<td><em>Cadhin</em> (<em>Capsicum annuum</em> dehydrin), an osmotic-stress gene in hot pepper plants</td>
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<td>Physiologia Plantarum</td>
<td>A gene encoding stellacyanin is induced in <em>Capsicum annuum</em> by pathogens, methyl jasmonate, abscisic acid, wounding, drought and salt stress</td>
<td>2002</td>
<td>115</td>
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<td>Plant Pathology Journal</td>
<td>Toward functional genomics of plant-pathogen interactions: Isolation and analysis of defense-related genes of hot pepper expressed during resistance against pathoge</td>
<td>2001</td>
<td>18</td>
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3. 연구성과
유전자 분양에 따른 MTA 채절: 고주에서 분리된 병기향성관련 전사조절 유전자와 고주 형질전환 및 내병성 분석. (주) 농우 바이오, 상업적 가치가 있을 경우 적절한 계약체결

4. 기술이전 및 연구결과 활용계획

가. 당해연도 활용계획(6차원칙에 따라 구체적으로 작성)
  - 유용 유전자 활용 계획
    지난 3년간의 1단계의 연구 결과는 생체 방어 관련 유전자를 유전자침을 이용해 대량으로 생산하는 것이었으므로 선발된 유전자들 대상으로 유전자의 기능을 규명하여 상업적으로 이용될 유전자를 발굴 하는 것이 뒤따라야 할 것이다.
    이를 위해서 일반적으로 유전자침의 발현 데이터를 분석한 결과로 생체 방어에 관련된 것으로 여겨지는 500 개의 유전자들을 선발하였으며 이들에 대상으로 Virus-Induced Gene Silencing(VIGS)를 이용한 유전자 기능 검정이 실시 될 것이다.

  - 데이터베이스 활용 계획
    본과학을 통해 구축된 유전자 발현 연구 기법 및 유전자 발현 데이터베이스는 향후 국내 식물 기능유전체 연구에 모델 시스템으로 지속적으로 이용될 것이며, 이와 더불어 다양한 분야의 국내 연구진들이 직접적으로 데이터를 활용할 수 있을 것으로 여겨진다.

나. 활용방법

다. 차년도 이후 활용계획(6차원칙에 따라 구체적으로 작성)
  VIGS 및 다양한 데이터 분석을 실시하여 상업적으로 이용될 가능성이 높은 것으로 추정 되는 유전자를 2차 선발할 것이다. 2차 선발된 유전자를 대상으로 과 발현 (over-expression) 시킨 형질전환체를 만들어 유전자 기능을 재검정하고 이의 설용성 검증을 실시 할 것이다. 최종적으로 선발된 유전자는 종묘회사등에 넘겨져서 상업화에
이용될 것이다.

4. 기대효과

가. 기술적 측면
   - 고추물 모델로 한 세계적 수준의 기능유전자 연구 기술 확보 및 이를 기반으로 해서 가지과 식물 및 기타 주요 작물에 확대 이용할 수 있는 기반 기술 확보
   - 대량의 유전자 분리, 유지, 기능 검정을 통한 유용유전자 pool의 확대 및 여타 작물의 병저항성 품종육성에의 계속적 이용
   - 농업의 중요성에 비해 세계적으로는 연구가 미진한 고추의 유용 유전자 발굴 및 기능 규명을 통해 다수의 지적재산권을 확보하여 신토자산업의 기반이 되는 원천기술을 개발

나. 경제·산업적 측면
   - 생체방어관련 유전자의 발굴 기능 검정을 통한 육종 소재 제공
   - 유용유전자를 이용한 고부가가치 품종개발을 통한 농가 소득증대
   - 내용성, 내충성 작물 개발로 화학농약 감소와 정정농작물 생산으로 인한 농가소득증대
   - 농약 사용량 감소로 인한 환경 및 생태 오염 감소
   - 우수 종자 개발 및 종자 수출로 국익증대
   - 유용유전자의 특허화 및 고급기술의 지적재산권화로 국가경쟁력확보
   - 농업 향단기술 개발로 선진국과의 농업경쟁력 확보
   - 발굴된 유용 유전자의 경제작물로의 형질전환을 통하여 품종개발을 위한 육종 소재로 개발

5. 문제점 및 건의사항(연구성과의 제고를 위한 제도·규정 및 연구관리 등의 개선점을 기재)
기술의 주요 내용
[기술의 개요]
유전자집합 유리, 실험, 혹은 채취된 해롭의 재질로 되는 기판 위에 정밀전자계어 기술 등을 이용하여 수백 개의 수십 만개의 DNA, Oligo 등을 결합시켜 유전자 발현 양상을 분석해낼 수 있는 생명과학 데이터 마이크로칩 (biological Microchip)을 말하며 유전자집합의 데시 할 수 있는 기존의 대표적인 유전공학적 방법은 Southern, Northern blot, Mutation detection, DNA sequencing 등이 있는데 기존 방법과의 가장 큰 차이점은 동시에 최소한 수백에서 수만개의 유전자를 빠른 시간에 검색할 수 있다는 것임

Expression Profiling 이란 유전자집합을 이용하여 대량으로 생산되는 염기서열정보로부터 유전자를 찾아내고 이들 유전자가 세포의 발현 기작에 의해 특정단로 전환되는 과정을 포괄적으로 검출하여 이들 유전자의 작용 과정에 관한 분석(기능 분석), 유전자들의 상호작용 및 중요 관련 유전자를 발견하는 핵심기술을 총칭하는 용어이며, 특히 짧은 시간에 대량의 정보를 나타낼 수 있고 자동화가 용이하기 때문에 현재 기능 유전체학의 흐름으로 크게 주목을 받고있음

<기술적 특성>
(1) 유전자 발현 분석 (Expression Profiling) 기술은 기능 유전체 연구의 핵심 기반 기술로 단순히 유전자 집합의 이용에 그치는 것이 아니라 유전자 확보라는 생명과학 연구의 전부, 경질 전자, 기계공학 및 화학 기술을 이용한 유전자집합의 제조과정, 통계학을 이용한 전자데이터 분석, 최종적으로 데이터의 생명과학적 해석 및 융합 및 데이터베이스화에 이르는 방대한 일련의 작업으로 여러 전문 분야가 통합되어 시스템화된 연구 기술임

(2) 유전자집합 기술의 최종 목표는 대량의 유전자 발현 양상을 동시에 관찰함으로써 얻어지는 데이터를 분석하여 유전자 발현의 연관성과 기능을 이해하는데 있고, 이에 따라 유전자집합 실험의 설계를 포함하는 시작에서부터 최종적인 결과 분석 단계까지의 전 과정을 통해 대량의 데이터를 효율적으로 저장, 관리, 분석할 수 있는 생물정보학이 필수적이며, 해당은 array informatics (유전자집합 정보학)로 따로 분류되기도 함

[용도 및 이용분야]
(1) 현재까지의 유전자집합 연구는 expression profiling (유전자발현분석)을 이용한 여러가지 stress 상황에서의 유전자 발현 분석 및 조건 특이적인 유전자 발현 패턴 분석에 초점났으나 정기 생물정보학의 통합된 기술을 바탕으로 생물체내의 생리학적 변화 및 유전자기능 발현 등의 여러문제를 해결하려는 방향으로 진행됨

(2) 여러가지의 조합, 발달 단계, 및 처리에 따른 대규모의 유전자 발현 데이터베이스가 구축되면 섭시 분석에서 가능한 종류의 유전자 발현 세트의 fingerprint가 가능해지며 이를 reference로 이용해 각각의 컨텐츠나 돌연변이체의 유전자 발현을 분석해 이들의 표현형 및 유전자 기능 예측이 가능해질 것임

(3) 유전자 발현 분석을 통한 새로운 전자 조절자 결합 부위 (cis-regulatory elements) 발굴 및 조절유전자 데이터 베이스 (예: TRANSFAC)와의 통합 분석을 통한 전자 조절자- 전자 조절자 결합 부위의 예측이 가능해지며, 최근에는 유전자집합과 chromatin immunoprecipitation 방법을 혼합 이용해서 각 전자 조절자가 결합하는 목적 유전자를 전체 유전체 수준에서 발현하는 것이 가능해짐

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■ 기술의 분류

[기술분야] (1개만 선택(√로 표시)하여 주십시오)

- 정보산업
- 기계설비
- 소재
- 정밀화학·공정
- 생명과학
- 원자력
- 자원
- 에너지
- 항공·우주
- 해양
- 교통
- 보건·의료
- 환경
- 기초·원천
- 기타

[기술의 활용범위] (1개만 선택(√로 표시)하여 주십시오)

- 신제품개발
- 신공정개발
- 기존제품개선
- 기존공정개선
- 기타 (유용 유전자 대량 발굴)

[기술의 용도] (복수 선택(√로 표시)가능합니다)

- 기계설비
- 부품소자
- 원료제료
- 소프트웨어
- 가공처리기술
- 자동화기술
- 불량품 감소 등 현장애로기술
- 제품설계기술
- 공정설계기술

■ 산업대신권 보유현황(기술과 관련한)

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* '권리유형'란에는 특허, 실용신안, 의장, 컴퓨터프로그램 등을 선택하여 기재
* '출원단계'란에는 출원, 공개, 등록 등을 선택하여 기재
■ 기술이전 조건

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<td>이전 소요기간</td>
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<td>기술이전시</td>
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* 기술이전시 선항요건 : 기술이전을 위한 사전준비사항(필수 설비 및 장비, 전문가 확보 등)을 기술
* 실험화예상시기 : 기술을 활용한 대표적인 제품이 최초로 생산이 시작되는 시기를 기재

■ 기술의 개발단계 및 수준

[기술의 완성도] (1개만 선택(✓로 표시)하여 주십시오)

| √ | ① 기초, 탐색연구단계 : 특정유도로 위해 필요한 신 지식을 얻거나 기술적 가능성을 탐색하는 단계 |
| ② 융용연구단계 : 기술적 가능성의 실증, 실험실실용화 가능성의 입증 등 실험실실용화 확신 단계 |
| ③ 개발연구단계 : Prototype의 제작, Pilot Plant Test 등을 행하는 단계 |
| ④ 기업화 준비단계 : 기업화에 필요한 양산화 기술 및 주변 기술까지도 확보하는 단계 |
| ⑤ 상품화 완료단계 |

[기술의 수명주기] (1개만 선택(✓로 표시)하여 주십시오)

| √ | ① 기술개발 정립단계 : 기술의 정 형적 가능성한 있는 단계 |
| ② 기술실험단계 : 기술개발에 성공했으나 아직 실용성, 경제성 등이 확실치 않은 단계 |
| ③ 기술적용 시장단계 : 최초의 기술개발국에서만 활용되고 있는 단계 |
| ④ 기술적용 성장단계 : 기술개발국 및 일부 선진국에서 활용되고 있는 단계 |
| ⑤ 기술적용 성숙단계 : 선진국사이에서 활발한 기술이전이 이루어지며, 기술의 표준화가 되어가는 단계 |
| ⑥ 기술적용 성숙단계 : 선진국에서 개도국으로 기술이전이 활발하게 이루어지고, 선진국에서는 기술의 가치가 저하되나 개도국에서는 아직 시장의 가치가 높은 기술 |
### 기술발견 과정상의 기술수준
(1개만 선택( □ 표시)하여 주십시오)

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<td>③ 외국기술의 개선·개량단계 : 성능이나 기능을 개선시킴</td>
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<td>④ 신기술의 혁신·발명단계 : 국내 최초로 개발</td>
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■ 본 기술과 관련하여 추가로 확보된 기술

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[ 기술을 도출한 과제현황 ]

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연구개발 주요내용