Identification of Defense-Related Cassava Genes by Subtractive Hybridization and cDNA Microarrays

Soto-Suárez[1], M., Restrepo[1-2], S., López[2], C., Mosquera[1], G., Tohme[1], J., and Verdier[2], V.
Biotechnology Research Unit, CIAT[1] A.A. 6713, Cali, Colombia, E-mail : s.restrepo@cgiar.org, Institut de Recherche pour le Developpement (IRD)[2], Universite de Perpignan, Perpignan, France, E-mail : vverdier@univ-perp.fr.

Introduction

Cassava (*Manihot esculenta*) is currently the fourth energetic food in the world after rice, maize, and wheat and feeds more than 1000 millions people. Cassava Bacterial Blight (CBB) caused by *Xanthomonas axonopodis* p.v *manihotis* (*Xam*) is a destructive disease in the South America and Africa and yield losses range between 12 and 100%. Cytochemistry and biochemistry of defense responses to CBB have been studied (Kpémoua, K. *et al.,* 1996). However, the response of the plant to pathogen attack at the molecular and cellular level remains uncharacterized. At this level, defense responses to the bacterial genera *Xanthomonas* in other plant species as rice, *Arabidopsis* and cotton have been investigated, common events such as oxidative burst, pathogenesis-related genes (PR) expression, Hypersensitive Response (HR) and Systemic Acquired Resistance (SAR) have been observed (Yuwei, S. Ronald, P. 2002). The objective of this research was to identify genes associated with cassava defense response to *Xam* combining subtractive library construction and cDNA microarrays.

Experimental Procedures

Plant material inoculation, cDNA synthesis and subtractive library

Young plants from two resistant varieties (MBRA 685 and SG 107-35) were inoculated by stem puncture with *Xam* strain CIO 151. Stem tissues were collected at 6, 12, 24, 48 and 72 hours post inoculation (pi), and 7 days pi. mRNA was isolated using Oligotex mRNA Midi kit (QIAGEN, CA). cDNA was synthesized using SMART™ PCR cDNA synthesis kit (CLONTECH, CA).

To identify differentially expressed genes during pathogen attack subtractive hybridization was performed. A pool of cDNA obtained from inoculated plants was used as "tester" and a pool of cDNA obtained from healthy non-inoculated plants and plants inoculated with sterile water was used as "driver".

cDNA Microarrays preparation, hybridization and data analysis

Cassava clones from subtractive libraries, cDNA-AFLP analysis and other clones were collected, amplified by PCR and printed on glass slides. The resulting microarray contained 3072 elements with each cDNA printed eight times as replicates.

Total RNA isolation and cDNA synthesis was performed as described above. Fluorescent-labeled probes were prepared using indirect labeling and microarray hybridization were performed with cDNA pool from 24-48 pi. v.s cDNA from healthy plants, using two duplicate slides with reverse labeling (dye-swap).

Spot intensities from scanned slides were quantified using the ArrayPro 4.0. software. Intensity-dependent normalization LOESS was performed and the differentially expressed genes were detected with bootstrap analyses using SAM (Significance Analysis of Microarrays) software.

Results and Discussion

Using the subtractive libraries, 820 cDNA clones were sequenced. Clustering and assembly resulted in a total of 141 unigenes with 77 contigs and 64 singletons. Homology search was conducted using the BLAST program. The sequence analysis showed that 29 unigenes were homologous to plant genes of unknown function, 52 showed no homology, and the remaining 60 unigenes showed homology with other plant genes (Table 1). Functional categories were manually assigned using the information gathered from the MIPS *Arabidopsis database* (Figure 1). Analysis showed that 45% cDNA clones shared homology with plant genes involved in defense responses (Figure 1 and Table 2). A hypothetical model of gene expression changes occuring in the cassava-*Xam* incompatible interaction is proposed (Figure 2).

Signalling pathways

Specific signal transduction pathways are activated. Previous studies revealed that the rapid accumulation of cytosolic Ca^{2+} is necessary for the production of oxidative burst and PR genes activation. Clone **SUS01** is homologous to a fungus-inducible Calmodulin *Mlo* family protein isolated recently from rice (Kim, M.C. *et al.,* 2002). Rho GTPase protein role in defense signaling mechanism has been studied (**SUS02**).

Oxidative Burst, Transcription factors and PR genes

During infection, cassava produce a biphasic or polyphasic oxidative burst that is probably regulated by proteins such as Catalase (**SUS03**), thioredoxin (**SUS04**), glutaredoxin (**SUS05**) and NDPKs (**SUS06**). These clones are previously known defense genes involved in oxidative burst. PR genes (**SUS07**, **SUS08**, **SUS09**) and other defense-related gene expression are probably regulated by transcription factors, such as MYB and Zinc Finger Protein (**SUS10**, **SUS11**).

Cell wall modification

A clone homologous to a brassinosteroid-regulated gene Xyloglucan Endotransglycosylase (XET)(**SUS12**) was reported. Brassinosteroids (BRs) were found to induce disease resistance in plants. BRs increase the abundance of mRNA transcripts for wall-modifying proteins such as XET that incorporate new xyloglucan into the growing cell wall. The pathogen spread is then hindered by physical strengthening of cell walls.

Protein degradation and protein-protein interaction

Protein degradation appear to play a role in cassava defense response, **SUS13**, **SUS14**, **SUS15** and **SUS16** genes activity may be coupled to HR development. Interactions between 14-3-3 proteins (**SUS17**) and proteins with defensive functions has been reported (Roberts, M.R. *et al.,* 2002).

Library	Number
Sequenced cDNAs	820
Unigenes	141
Singletons	64
Contigs	77
No. ESTs match	52
Known function	60
Unknown function	29

Table 1. Sequence analysis of subtractive libraries

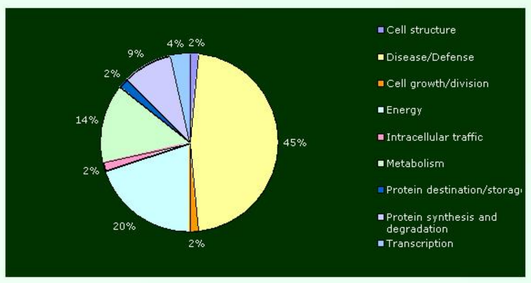

Figure 1. Functional categories

Clone	Highest Homology	Clone	Highest Homology
SUS01	Calmodulin	SUS14	Polyubiquitin (ubq10)
SUS02	Rho GTPase protein	SUS15	Zinc metalloproteinase
SUS03	Catalase	SUS16	Cysteine proteinase
SUS04	Thioredoxin m	SUS17	14-3-3 protein
SUS05	Glutaredoxin	SUS18	Annexin homolog RJ4
SUS06	Nucleoside-diphosphate kinase (NDPK)	SUS19	Naringenin-chalcone synthase
SUS07	Osmotin	SUS20	Calmodulin 7
SUS08	Pathogenesis-related protein 3	SUS21	Dormancy-associated protein
SUS09	Lipid transfer protein	SUS22	Pectinesterase
SUS10	MYB transcription factor	SUS23	Chaperone ANJ1 protein
SUS11	Zinc-finger protein	SUS24	Unspecific mono-oxygenase
SUS12	Xyloglucan endotransglycosylase	SUS25	LDJ2 protein
SUS13	Ubiquitin		

Table 1. Twenty-five cDNA clones from subtractive libraries shared homology with plant genes involved in defense responses

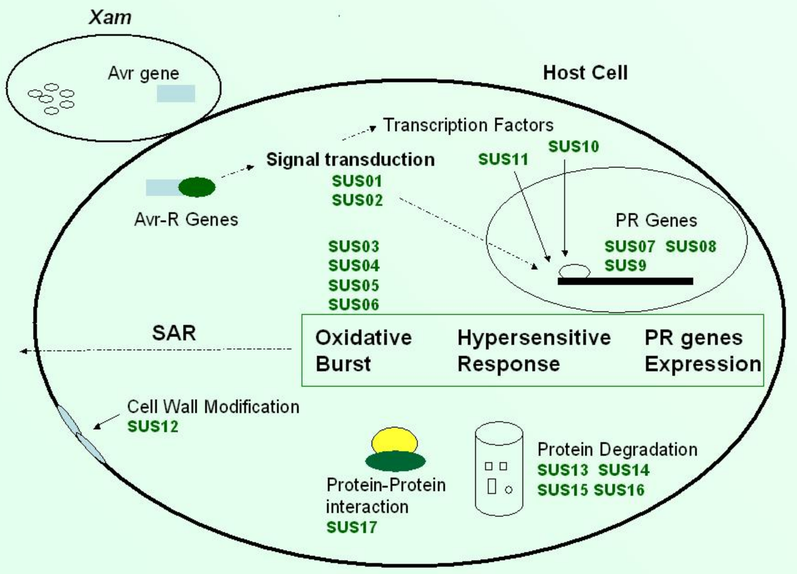

Figure 2. Hypothetical model of the cassava defense response to *Xam* infection. In green are the cDNA clones from subtractive libraries involved in defense responses.

cDNA Microarrays

A cDNA microarray enriched with genes involved in the cassava defense responses was constructed and hybridized to a cDNA pool from 24-48 pi. v.s cDNA from healthy plants. Data analysis revealed that some clones such as Glutaredoxin (**SUS05**), Lipid Transfer Protein PR gene (**SUS09**), Zinc-Finger Protein transcription factor (**SUS11**) Dormancy-associated protein (DOR)(**SUS21**) and Ubiquitin (**SUS13**) showed significant differential expression in response to pathogen attack (Figure 3). Other differentially expressed clones were homologous to *Arabidopsis* genes of unknown function (*A.th*) or showed no homology with sequences in the databases (NH), representing a new source of genes potentially involved in cassava defense responses.

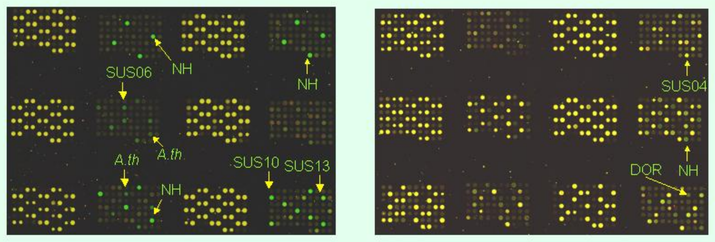

Figure 3. Three colors images showing differentially expressed clones **SUS05, SUS09, SUS11, SUS21**, **SUS13**, and *Arabidopsis* genes of unknown function (*A.th*) or without homology with sequences in the databases (NH).

Conclusions

Functional genomic tools such as subtractive libraries and microarrays permitted to give a first comprehensive overview of the molecular basis of the cassava defense response to CBB. Many defense signal transduction pathways lead to responses like oxidative burst, cell wall modification, protein degradation, and subsequent HR and SAR induction. A cinetic of the up and down regulated genes after inoculation will help in better understanding the mechanims of cassava defense response to different pathogens.

References

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