APPLYING BIOTECHNOLOGY TOOLS TO IMPROVE CONTROL DISEASES OF SOME TROPICAL CROPS

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SUMMARY

The application of biotechnology tools has made it possible to make significant advances in the detection of pathogens and in the study of the genetic variability of pathogen populations. As a result, adequate disease management strategies can be planned, and disease resistance markers for plants as well as DNA regions associated with resistance can be identified. Diseases, mainly those caused by microorganisms that cannot be cultivated in artificial media such as phytoplasmas, can be diagnosed using PCR. The bacterium *Xanthomonas axonopodis* pv. *manihotis* (*Xam)* can be identified using a specific probe and the identification of *Ralstonia solanacearum* in the soil is also facilitated by PCR. Species of *Phytophthora* can also be identified, phytoplasmas classified, and pathogen diversity established by using PCR-RFLP, thus improving the understanding of action mechanisms and how they co-evolve with the host. Other techniques such as RAPD, AFLP, and RAMS have made it possible to study the genetic variability of pathogens such as *Sphaceloma manihoticola, Colletotrichum gloeosporioides, C. acutatum, Sphaerotheca pannosa, Ceratocystis paradoxa,* and *X. axonopodis pv manihotis.* The identification of QTL markers associated with resistance to different species of *Phytophthora* in cassava has helped elucidate the genetics of resistance, whereas the identification of SSR markers associated with resistance to *Xam* is a tool that facilitates the selection of resistant genotypes. Functional genomics tools such as microarrays will give initial insight on the molecular basis of cassava's defense response to *X. axonopodis pv manihotis.* Also, the identification of resistance genes and resistance gene analogs in cassava contributes to the genetic improvement of this crop. Biotechnology is evolving continuously, offering modern tools that help solve plant health problems.

Keywords: diseases, diagnostic

RESUMEN

La aplicación de las herramientas que ofrece la biotecnología, han permitido lograr grandes avances en la detección de patógenos, adelantar estudios de variabilidad genética de poblaciones de patógenos, lo cual permite planear estrategias adecuadas de manejo de enfermedades, identificar marcadores asociados a la resistencia de las plantas a las enfermedades e identificar regiones de ADN asociadas con resistencia. Mediante la Reacción en Cadena de la Polimerasa (RCP) se pueden diagnosticar enfermedades, principalmente las causadas por microorganismos que no se pueden cultivar fácilmente en medios artificiales, como los fitoplasmas. Mediante una sonda específica es posible identificar la bacteria *Xanthomonas axonopodis* pv. *manihotis*. La identificación de *Ralstonia solanacearum* en suelo se facilita mediante PCR. Mediante PCR-RFLP es posible identificar especies de *Phytophthora*, clasificar fitoplasmas y establecer la diversidad de patógenos, los cuales permitirán un mejor entendimiento de los mecanismos de acción y su co-evolución con el hospedero. Otras técnicas como RAPD, AFLP y RAMS, han permitido estudiar la variabilidad genética de patógenos como *Sphaceloma manihoticola, Colletotrichum gloeosporioides, C. acutatum, Sphaerotheca pannosa, Ceratocystis paradoxa* y *X. axonopodis* pv *manihotis.* La identificación de marcadores QTLs asociados a la resistencia a diferentes especies de *Phytophthora* en yuca, han contribuido a dilucidar la genética de la resistencia, mientras que la identificación de marcadores SSR asociados a la resistencia a *X. axonopodis* pv *manihotis* es una herramienta que facilita la selección de genotipos resistentes. Herramientas de genómica funcional como los microarreglos permitirán una comprensión de la base molecular de la respuesta de defensa de la yuca a *X. axonopodis* pv *manihotis.*

Palabras claves: Enfermedades, diagnóstico

DIAGNOSING DISEASES

In the tropics, many diseases cause severe crop losses but their causal agents are not yet identified. Biotechnology tools are useful for efficiently detecting pathogens. Below we describe the detection and identification of the pathogens causing or associated with the following diseases in specific crops: lethal wilt of oil palm (*Elaeis guineensis*), frogskin disease and superelongation disease of cassava (*Manihot esculenta*), phyllody of lulo (*Solanum quitoense*), crispiness of coffee (*Coffea arabica*), and anthracnose of soursop (*Annona muricata*). Also discussed are pathogens that can affect several crops such as *Phytophthora* spp. (cassava, cacao, and *Heliconia* sp.) and *Ralstonia solanacearum*, which affects plantain (*Musa* AAB), *Heliconia* sp., tobacco (*Nicotiana tabacum*), tomato (*Lycopersicon esculentum*), hot pepper (*Capsicum* sp.), potato (*Solanum tuberosum*), eggplant (*S. melongena*), and canna (*Canna indica*).

Bacterial diseases

Plant diseases caused by bacteria can be severe, especially in warm, humid zones. Cassava bacterial blight (CBB), caused by *Xanthomonas axonopodis* pv. *manihotis*, is a major constraint for cassava production, causing losses of the entire crop. *X. axonopodis* pv. *manihotis* can be detected by polymerase chain reaction (PCR), on amplifying an 898-bp fragment. Using a specific probe, Verdier and Mosquera (1999) detected the bacterium in affected leaf and stem extracts, fruits, and sexual seed.

Bacterial wilt is caused by different races of *Ralstonia solanacearum*. It is a serious soil-borne disease of many economically important crops, including tomato, potato, tobacco, banana, plantain, and eggplant. The pathogen can also be transmitted through contaminated irrigation water, equipment, or personnel, and spreads very easily by transplanting infected plants and propagules. Bacteria in plant tissues and soil can be accurately detected by PCR, using specific primers that amplify a 288-bp fragment on a 16S rRNA gene (Martins, 2000). CIAT (2004a). reported, and registered at the GenBank database sequences obtained through PCR from different crops affected by different *R. solanacearum* races (Table 1).

Phytoplasma diseases

Phytoplasmas are bacteria that have no cell walls. Being plant pathogenic, they are associated with diseases in more than 300 plant species. Because culturing them *in vitro* is not yet possible, their detection and identification have been based largely on their molecular characteristics. Furthermore, preliminary classification of unknown phytoplasmas can be accomplished through RFLP analysis of PCR-amplified 16S rRNA groups. Some phytoplasmas have been found to cause diseases previously considered to be viral.

Based on the similarity of observed symptoms and reported diseases caused by phytoplasmas, researchers at CIAT amplified DNA fragments of phytoplasmas from oil palm, cassava, lulo, and coffee, using nested PCR. Moreover, these phytoplasmas could also be classified, using RFLP (CIAT 2002, 2004b; Álvarez *et al*. 2003a, 2003b, 2004).

In addition, CIAT (2004b) confirmed the association of a phytoplasma with cassava frogskin disease through DNA amplification with specific primers. This pathogen was detected in affected roots and leaves by nested PCR and transmission electron microscopy. A total of 320 DNA samples were obtained from infected plant tissues and another 80 from healthy tissues (Table 2). DNA sequencing of PCR products and banding patterns obtained after digestion of the amplified products with the enzymes *Alu*I, *Rsa*I, *Taq*I, and *Mse*I enabled us to identify the products as belonging to the 16SrIII group (or X-disease group). Of the methods used in this study, PCR was the most sensitive for detecting, identifying, and classifying phytoplasmas.

The first report of a phytoplasma being associated with lethal yellowing disease of oil palm was described by CIAT (2004b). A protocol was developed to identify the phytoplasma through nested PCR and DNA sequence analysis of the 16S rRNA region obtained from oil palm, glands and intestines of insect vectors, and weeds. Likewise, DNA sequence analysis revealed that X-disease group phytoplasmas were associated with coffee crispiness (Galvis *et al.* 2003) and "machorreo" of lulo (Álvarez *et al.* 2003a).

Table 1. DNA sequences registered at the Gen Bank database of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). These sequences from plant-disease organisms were reported by the " Centro Internacional de Agricultura Tropical" (CIAT)

GenBank accession	Size DNA sequence (bp)	Organism	Host	Collection site
AY525125	941	Phytoplasma X-disease group	*Coffea arabica*	Colombia
AY737647	1298	Phytoplasma X-disease group	*Manihot esculenta*	Colombia
AY731819	1567	Phytoplasma X-disease group	*Solanum quitoense*	Colombia
AY739024	1424	Phytoplasma aster yellows	*Elaeis guineensis*	Colombia
AY737648	438	*Glomerella acutata*	*Annona muricata*	Colombia
AY739025	524	*G. cingulata*	*A. muricata*	Colombia
AY739018	644	*Sphaceloma manihoticola*	*Manihot esculenta*	Brazil
AY739020	629	*Sphaceloma* sp.	*Euphorbia heterophylla*	Brazil
AY737489	255	*Ralstonia solanacearum*	*Solanum melongena*	Kenya
AY745758	225	*R. solanacearum*	*Canna indica*	Colombia
AY745759	223	*R. solanacearum*	*Capsicum* sp.	Philippines
AY745760	216	*R. solanacearum*	*Heliconia* sp.	Costa Rica
AY745757	235	*R. solanacearum*	*Solanum tuberosum*	Colombia
AY745755	203	*R. solanacearum*	*Musa*, banana	Colombia
AY745761	223	*R. solanacearum*	*Musa* AAB, plantain	Colombia
AY737488	220	*R. solanacearum*	*Nicotiana tabacum*	Colombia
AY745756	225	*R. solanacearum*	*Lycopersicon esculentum*	USA
AY745749	860	*Phytophthora cryptogea*	*Heliconia* sp.	Colombia
AY739022	747	*P. tropicalis*	*Manihot esculenta*	Colombia
AY739021	920	*P. melonis*	*M. esculenta*	Brazil
AY745754	839	*P. nicotianae*	*M. esculenta*	Colombia
AY745753	869	*P. melonis*	*M. esculenta*	Brazil
AY745751	791	*P. palmivora*	*Theobroma cacao*	Colombia

Table 2. Infected and healthy tissues evaluated, using nested PCR and DNA sequencing, to determine the incidence of phytoplasmas in cassava plants infected with cassava frogskin disease

Tissue health	Variety	Samples processed[a]		No. of positive samples	% nested PCR	PCR[b] sequencing	
		Roots	P and MR			Roots	P and MR
Infected	Catumare[c]	80	80	124	77	8	10
Infected	Manzana[d]	80	80	138	86	10	12
Healthy	Catumare	20	20	0	0	–	–
Healthy	Manzana	20	20	0	0	–	–

[a]Total number of samples processed from plants obtained from the field and grown in the greenhouse and screenhouse. P = leaf petioles; MR = leaf midribs. [b]The same number of samples was taken for both greenhouse and screenhouse. P = leaf petioles; MR = leaf midribs. [c]Moderately infected. [d]Severely infected.

Fungal diseases

Fungi and pseudo-fungi (Cromista, Oomycota) cause diseases that result in serious yield losses in many crops around the world. One such disease is *Phytophthora* root rot (PRR), which attacks the roots, stems, and sometimes leaves of cassava crops grown in many different agroecological areas of Africa, Latin America, and Asia.

Sequence-based identification of fungi is actually based on an rDNA gene complex, which is present in all microbial pathogens. Fungi carry three genes (18S, 5.8S, and 28S), which have spacers (ITS1 and ITS2) located between the genes. Within the rDNA gene complex are (1) highly variable sequences that provide unique signatures that identify species, and (2) conserved regions that contain genomic codes for the structural restraints present within organism groups. The ITS regions contain the major variability and, under most circumstances, these regions are useful for species recognition.

Knowledge of an unreported *Phytophthora* species, *P. tropicalis*, and *P. melonis* in cassava was generated by a DNA sequence analysis of the ITS region of the 18S rRNA gene. An alternative technique, the PCR-RFLP, was also applied, using amplification with ITS1 and ITS2, or ITS4 and ITS6, and digestion with the enzymes *Alu*I, *Taq*I, and *Msp*I. *Phytophthora palmivora* from cacao and *P. cryptogea* from heliconia were also identified by ITS-region sequencing (Table 1) (CIAT 2004b).

Superelongation disease of cassava (SED), caused by *Sphaceloma manihoticola*, is an important foliar and stem disease, causing crop losses of more than 80% in different areas in South America and the Caribbean. PCR fragments, obtained with ITS4 and ITS5, were isolated, cloned, and sequenced (CIAT 2004b), the sequences reported in the GenBank database (Table 1). PCR fragments, obtained with ITS1 and ITS4 from DNA of *Glomerella* spp. isolated from soursop were also cloned and sequenced (CIAT 2004b), and the sequences reported in the GenBank database (Table 1).

The successful identification of pathogens through molecular tools such as PCR and DNA sequence analysis will help improve host resistance and accelerate the identification of potential insect vectors and alternative hosts. Thus, it will encourage the development of new and effective practices for controlling these diseases.

DETERMINING THE GENETIC DIVERSITY OF PATHOGEN POPULATIONS

Once we have identified a pathogen, we can conduct genetic diversity studies to enable us to better understand it, its mechanism of action, and its co-evolution with its hosts. With such knowledge we can direct management strategies towards increasing crop productivity through disease resistance to that pathogen. That is, we can develop plants that resist targeted populations of the pathogen rather

than fulfill a specific objective. Thus, we can prevent the potential economic imbalances that so often negatively affect the poorest farmers, who are the main producers of many important crops such as cassava.

We now describe some of the genetic diversity studies carried out by CIAT researchers on several pathogens causing major crop diseases such as CBB, SED, powdery mildew, anthracnose, and bud rot.

Cassava bacterial blight

By characterizing *Xanthomonas axonopodis pv manihotis*'s genetic diversity, we can select a set of strains to search for resistance factors in cassava varieties targeted for an area carrying a given *X. axonopodis* pv *manihotis* population. Through RFLP and AFLP analyses, pathogen diversity was found to be limited in Africa but broad in South America, cassava's center of origin. High genetic diversity was also found in Brazil, Venezuela, and Colombia, with different pathotypes specific to each ecozone. The very high genetic diversity is possibly related to the also very high levels of genetic diversity observed for the host plant (Verdier et al. 1993; Restrepo et al. 1996; Restrepo 1999; CIAT 2002). The *X. axonopodis pv manihotis* isolates also showed that the structure of bacterial populations is also shaped by the spatial and temporal distributions of their diversity.

Cassava Superelongation disease

Using molecular techniques such as RAPDs, AFLP, and RAMs, Álvarez *et al* (2003c) detected variability in isolates of *Sphaceloma manihoticola* from south-central Brazil and Colombia, although genetic variation in the Brazilian population was narrower than that of the Colombian population. The RAPD analysis revealed high polymorphism, that is, considerable intraspecific genetic diversity, indicating that the pathogen may be able to adapt readily to changes in the environment, overcome resistance of new cassava varieties, or develop resistance to fungicides. The groups of isolates identified by genetic and pathogenic characterization of the pathogen showed no strong correlations, although high correlations existed between geographic origin (country and municipality) and genetic variation, hence indicating a possible center of diversity.

For Brazilian isolates, we could distinguish between isolates of *S. manihoticola* from cassava and those from milkweed, and also between *S. manihoticola* and *S. krugii*. In addition, RAPD analyses clearly differentiated among the levels of genetic variation among the host-plant populations of *S. manihoticola*. One study (Álvarez and Molina 2000) of Colombian isolates collected from cassava plants affected with SED and from *Euphorbia heterophylla* (a Euphorbiaceae species like cassava) showed how useful molecular markers are to better understand the movement of pathogen populations between geographically isolated regions and the effectiveness of host resistance (Álvarez *et al*. 2003c). These results would have been difficult or impossible to obtain on the basis of morphology alone.

Rose powdery mildew

Powdery mildew is caused by the fungus *Sphaerotheca pannosa* var. *rosae*, and is a major constraint to rose production. In Colombia, this disease is widely distributed, affecting the quality of *Rosa* spp. grown in greenhouses and causing serious economic losses. Molecular and pathogenic characterization of isolates generates information on the pathogen's genetic structure, thereby helping to make the most appropriate and durable crop management decisions.

Using RAPD and RAMS techniques, DNA polymorphisms were detected among isolates obtained from six rose cultivars grown on farms located in Cundinamarca (Colombia). Molecular analyses revealed considerable genetic diversity among the populations of *S. pannosa* var. *rosae*. The relationship between origin and genetic diversity suggests that different disease management practices will be needed according to farm and environment. The genetic variability observed with the analyzed primers proved that the pathogen is able to mutate and, depending on the environment in which it is found, can adopt several forms by which to survive in different tissues of the rose plant (Álvarez *et al*, 2000).

Anthracnose in soursop (*Annona muricata*), and citrus

Broad genetic variability was detected by AFLP and RAMS in isolates of *Colletotrichum acutatum* and *C. gloeosporioides*, collected from citrus and soursop (*Annona muricata*), respectively. In soursop, these results showed a relationship of the variables "pathogenic variability" and "colony diameter" with "genetic variability". The genetic variability observed with this technique was high. If we consider that sampling was restricted in terms of the study area, then we can deduce that (1) high variation exists among the pathogen's populations in different agricultural and climatic regions with geographic isolation, and (2) high variation exists within groups of isolates selected previously for their morphological traits such as colony diameter. This suggests that a broader, more systematic sampling must be planned to answer doubts arising from this preliminary study of *Colletotrichum* populations (Ospina y Álvarez, 2003).

To evaluate genetic variation in *C. acutatum*, the causal agent of citrus anthracnose, isolates were collected from a wide range of citrus species under varying conditions and from different geographic zones of Colombia. We used AFLP to analyze them and found considerable genetic variation, but no consistent relationship between the groups and host species or collection zones. These results confirmed the variability, both phenotypic (i.e., morphological traits) and genetic (molecular traits), that exists within the causal agent of citrus anthracnose. The results also confirmed that different species of the pathogen are associated with the disease (Ospina, 2002; CIAT 2003).

Dry rots in oil palm and other crops

Isolates of *Ceratocystis paradoxa* and related fungi were collected from oil-palm plantations and other crops in Colombia and characterized. Morphological comparisons, mating systems, and phylogenetic analyses (including the use of molecular markers) revealed a complex of five different taxa: *C. paradoxa* (banana), *C. radicicola* (date palm), *C. fimbriata* (cacao), *C. paradoxa sensu stricto* (oil palm) and a clearly distinct species in its ITS sequence and morphology (oil palm) intermediate between *C. paradoxa* and *C. radicicola* that should be described as new (CIAT 2000). These findings demonstrated that a disease may result from the activities of a complex of pathogens. Another eX. axonopodis pv manihotisple is the *Phytophthora—Pythium* soft rots that attack oil palm. Disease management strategies should therefore be oriented towards dealing with such complexes.

MARKER-ASSISTED SELECTION

Molecular markers also comprise an important tool for breeders. In cassava, for example pv manihotis, they were studied to identify their association with genetic resistance to cassava bacterial blight (CBB). More than 400 simple sequence repeats (SSRs) were evaluated, leading to the identification of one SSR marker. This was associated with CBB resistance in a segregating cassava backcross family under field conditions (Hurtado 2004).

Another example is QTL mapping for resistance to root rot in cassava, which was performed in family K, using simple marker analysis. We detected and mapped nine QTLs associated with *P. tropicalis* resistance, two QTLs with *P. palmivora*, and two with *P. melonis* (Álvarez et al, 2002; Llano 2003; Loke 2004). The QTLs were located on different linkage groups. Some QTLs would be expressed in one crop cycle but not in another, depending on the environmental conditions for crop development. The low phenotypic variance explained by the identified QTLs support the hypothesis of minor genes controlling *Phytophthora* resistance, as suggested by Llano et al. (2004).

OBTAINING RESISTANCE GENOME SEQUENCES

The presence of conserved domains permitted grouping of resistance genes (*R* genes) into at least four classes and to propose their possible function in the defense response as part of signal transduction pathways. *R* genes are clustered in the genome of several species and different genes within the same cluster can determine resistance to diverse pathogens. Based on sequence similarity between *R* genes, Llano et al. (2004), using degenerated primers designed from resistance genes of different crops, obtained four resistance gene analogs (RGAs), corresponding to NBS candidate genes, whereas Hurtado and Álvarez(2003) obtained 10 RGAs (NBS, Pto, kinase, amd LRR). One RGA showed association with CBB resistance in a segregating cassava population. The sequences were reported in the GenBank database.

Identifying genes associated with defense responses is a highly critical step, leading to the elucidation of disease-resistance mechanisms in cassava. Soto-Suárez *et al*. (2004b), using subtractive hybridization, identified genes differentially expressed during CBB pathogen attack. From two resistant varieties, 1536 clones were isolated. Sequence analysis of these showed that 16 cDNA clones shared homology with plant genes involved in defense responses. Another 70 clones were either homologous to plant genes of unknown function or showed no homology, thus representing potentially new genes involved in cassava's defense responses.

Soto-Suárez *et al*. (2004a) performed microarray hybridization, using as treat ent cDNA from resistant cassava plants collected at different times after infection and, as control, cDNA from healthy plants. Functional genomic tools such as the cassava microarray provided a preliminary comprehensive overview of the molecular basis of the cassava defense response to the CBB pathogen. It will help in the future understanding of the defense mechanisms used against other important pests and diseases.

A PCR approach was used, with specific primers for the *Xa21* gene (which confers resistance to *Xanthomonas* in rice), to isolate a 900-bp fragment (PCR250) in cassava cultivars showing high resistance to *X. axonopodis* pv *manihotis* (López et al, 2004). The PCR250 fragment showed 56% similarity with the *Xa21* gene, and was located in the linkage group X and associated with a QTL that explained 13% of resistance to *X. axonopodis* pv *manihotis* (Jorge et al. 2000). The PCR250 fragment was also used by López et al. (2004) to screen a BAC library. The complete sequence of cassava *Xa21* homologue gene was identified and named *RX. axonopodis pv manihotis-1*. It was 3600 bp long and contained an ORF of 1181 amino acids. This gene was induced 72 h after infection by *X. axonopodis* pv *manihotis.*

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