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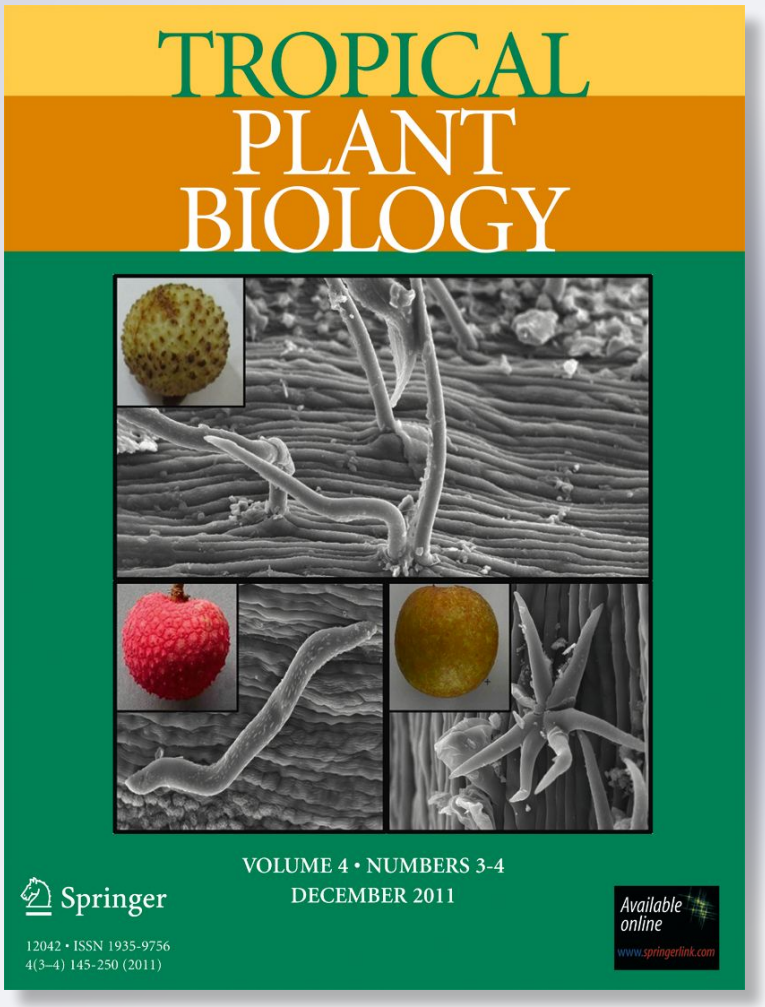

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Cassava Bacterial Blight: Using Genomics for the Elucidation and Management of an Old Problem

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Abstract Bacterial Blight is an important disease of cassava, causing losses that have resulted in historical famines in certain growing zones. The disease is caused by *Xanthomonas axonopodis* pv. *manihotis*, a gram-negative rod that belongs to the gammaproteobacteria. In this review, we describe the pathosystem and the recent studies that have been undertaken to elucidate both susceptibility and resistance mechanisms in cassava, with the hope of generating resistant plants using biotechnology. We first describe studies of the pathogen, including pathogen population changes through time as well as genomic tools that have recently been generated to determine pathogenicity factors. Secondly, we discuss mechanisms of disease resistance that have been elucidated in recent years and how these mechanisms could be used for the generation of improved plants resistant to CBB.

Keywords Cassava bacterial blight · Cassava genomics · Pathogenomics · Effector proteins · Disease resistance

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Abbreviations

CBB	Cassava bacterial blight
Xam	*Xanthomonas axonopodis* pv. *manihotis*
RFLP	Restriction fragment length polymorphism
AFLP	Amplified fragment length polymorphisms
RAPD	Random amplified polymorphic DNA
TTSS	Type III secretion system
TAL	Transcriptional activator-like
QTL	Quantitative trait loci
MAMP	Microbe-associated molecular pattern
MTI	MAMP-triggered immunity
ETI	Effector-triggered immunity
NBS	Nucleotide binding site
TIR	Toll/interleukin receptor
CC	Coiled coil
LRR	Leucine-rich repeat
HR	Hypersensitive response
GST	Glutathione S-transferase
IRP	Immunity-related protein
IRG	Immunity-related gene
PR	Pathogenesis-related proteins

Introduction

Cassava bacterial blight (CBB), causal agent *Xanthomonas axonopodis* pv. *manihotis* (*Xam*), is a destructive disease present in all areas where cassava (*Manihot esculenta*) is cultivated. CBB causes yield losses of over 50 to 75%, depending on the environmental conditions (Lozano 1986; Wydra and Verdier 2002), and in severe cases has contributed to situations of starvation in certain African countries (Lozano 1975; Ogunjobi and Dixon 2006). Prevalence and

severity of the disease have diminished in the last 10 years in areas of the world, such as South America and certain regions of Africa, perhaps due to implementation of adequate cultural practices and the use of resistant cultivars. However, in other areas, such as Southeast Asia, a considerable increase in the incidence of CBB has been observed in the past couple of years (http://ciatnews.cgiar.org/en/index.php/tag/outbreak/), probably due to an increase in rainfall and temperature related to global climate change. Despite its importance for food security in Africa and South America, research on CBB has been neglected, as reflected in the scarcity of recent publications on several topics ranging from the biology of the pathogen to the molecular interactions it has with the cassava plant. Our groups are attempting to close this gap and present this review to highlight those topics where advances have been made and those where not too much information is available, underscoring the importance of continuing research on this disease.

CBB is characterized by a wide range of symptoms. Young plants wilt and die shortly after planting when contaminated propagative material is used (Lozano 1975). When plants are infected at later stages, usually from secondary infections, the disease symptoms start with appearance of angular leaf spots on the abaxial side of the leaf, which are accompanied by orange exudates (Lozano 1986). As the disease advances other symptoms such as blight and wilting appear, with dieback and death of the plant in cases where the host and environmental conditions are highly conducive. Environmental conditions that are optimal for disease include temperatures around 28°C, wind and rain. Cassava cultivars range from very susceptible to moderately resistant. This resistance is quantitative but strain-specific (see below).

CBB is caused by *Xanthomonas axonopodis* pv. *manihotis* (*Xam*), a gram negative rod closely related to bacteria that cause disease in other crops such as citrus, tomato and pepper (Swings et al. 1993). Despite their relatedness, however, each bacterium has strict host specificity, with *Xam* affecting only cassava and closely related plant species. The bacterium lives as an epiphyte and, only when environmental conditions such as temperature and humidity are conducive to population increases (Boher and Verdier 1995), enters the leaves through wounds or natural openings (mainly hydathodes and stomata). Once inside, it multiplies to high levels of up to 10^8cfu/ml in the intercellular spaces of the mesophyll and subsequently in the vascular tissue of the plant, where it acquires the ability to move systemically (Verdier et al. 2004). *Xam* is dispersed from plant to plant in water droplets, a process that is more efficient under high wind conditions (Lozano and Sequeira 1974). Bacterial dissemination between fields occurs mainly with the exchange of infected propagative material (Lozano 1986).

The disease is usually managed by the use of resistant cultivars and cultural practices (Lozano 1986; Boher and Verdier 1995). Cultural practices such as the use of clean propagative cuttings, crop rotation, planting towards the end of the rainy season and removal of infected material are aimed at reducing bacterial populations in the field (Lozano 1986). The use of resistant cultivars has proven to be the most effective control measure for CBB. However, as further explained below, this resistance has not been bred into the cultivars best adapted to each Agro-Ecological Zone (ECZ; Restrepo et al. 2004). In addition, this resistance can be broken through the selection of sub-populations of the pathogen that escape recognition by the plant (Restrepo et al. 2004). Although not universally applicable, there is a tendency for a reduced durability of resistance as the diversity of pathogen populations increases. For this reason, in addition to implementing varietal resistance, it is important to assess the diversity of pathogen populations in the field and to deploy cultivars that are resistant to the local pathogen populations.

Diversity of *Xam* Populations in South America and Africa

CBB likely originated in South America, based on the high diversity of the pathogen in this region (Verdier et al. 2004). Today the disease is present in all areas of the world where cassava is cultivated. However, studies on genetic diversity of the pathogen populations have been performed only in certain areas of the world, including Colombia, Venezuela, Brazil, Nigeria and Togo (Berthier et al. 1993; Verdier et al. 1993; Restrepo and Verdier 1997; Verdier et al. 1998; Restrepo 1999; Restrepo et al. 1999a, b; Restrepo et al. 2000b). Historically diversity in *Xam* populations was assessed using metabolic tests, such as the ability to metabolize certain carbohydrates (Fessehaie 1997). However, the low reproducibility of these methods and their inability to determine genetic relationships among isolates has caused their replacement by more modern molecular technologies (Louws et al. 1999; Restrepo et al. 2004; Ogunjobi et al. 2010).

The first molecular population studies in *Xam* analyzed RFLPs (Restriction Fragment Length Polymorphism) and tested variability in chromosomal, plasmidic and ribosomal RNA regions (Verdier et al. 1993). The highest power of differentiation between strains with the use of these markers was observed when a fragment of the pathogenicity gene *pthB* (see below) was used as a probe (Verdier et al. 1993; Restrepo et al. 1999b, 2000b). However, this probe did not discriminate between closely related strains in certain populations. PCR-based methods, such as AFLP (Amplified Fragment Length Polymorphism), REP (Repetitive Element PCR) and ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus), were tested for their discriminatory ability because they are reproducible and provide a considerable amount of information in each assay. Interestingly, AFLP

markers had the highest power to discriminate between closely related strains (Restrepo et al. 1999a; Verdier et al. 2004) and were complementary to RFLP markers using *pthB* as a probe (Restrepo et al. 2000a).

Initial studies of the genetic diversity of *Xam* were concentrated in African populations. The first report, which included 49 strains collected in the 1970s and 1980s from Nigeria, Benin, Ivory Coast, Congo, Cameroon, Uganda, Kenya, Niger, Togo and Zaire, reported the absence of polymorphisms using RFLP probes against the rRNA region and very low levels of polymorphisms using a probe against a pathogenicity region (Verdier et al. 1993). The results suggested a clonal nature of the African populations. Higher levels of diversity were detected in South American populations (Verdier et al. 1994), the likely center of origin. More recent studies, which have concentrated on *Xam* populations in Nigeria and Togo, have described higher diversity (Verdier et al. 2004; Ogunjobi and Dixon 2006; Ogunjobi et al. 2007, 2010) with a higher number of haplotypes than previously reported. This reflects the fact that initial studies (Verdier et al. 1993) did not differentiate haplotypes among *Xam* strains from different countries, whereas the recent studies can differentiate several haplotypes in comparable numbers of strains from a single country (Verdier et al. 1993). While caution must be taken when comparing these studies since different molecular markers were used in the more recent studies (AFLP and RAPDs), it is clear that *Xam* diversity has increased over the past 30 years. It is possible that these new haplotypes have appeared with the introduction of new cassava cultivars in the field. This increase in bacterial diversity makes the deployment of resistant cultivars an increasingly complex task and underscores the importance of continuous studies of bacterial populations in the field.

In contrast with the case in Africa, populations of *Xam* in South America are diverse, based on results from strains collected in the 1970s and 1980s (Ogunjobi et al. 2007). A notable set of studies on the diversity of *Xam* populations was performed by Restrepo and collaborators in the 1990s in Colombia, Venezuela and Brazil (Verdier et al. 1993; Restrepo and Verdier 1997; Verdier et al. 1998; Restrepo 1999; Restrepo et al. 2000b, 2004). Results from these studies indicated a high genetic diversity of the pathogen in South American populations mainly using RFLP from a plasmidic region containing the *pthB* gene (Restrepo and Verdier 1997; Verdier et al. 1998; Restrepo 1999). In general, strains were not geographically differentiated, with some exceptions where a haploytype was characteristic of a specific Edaphoclimatic zone (Restrepo and Verdier 1997; Restrepo et al. 2000b). Migration of these latter haplotypes could also be detected between different cassava growing zones in Colombia (Restrepo et al. 2000b). In addition, bacterial populations had the capacity to change from year to year (Restrepo et al. 2000b) such that peaks of diversity were obtained in times and locations where environmental conditions were conducive for disease (Restrepo 1999; Restrepo et al. 2004). The diversity was also greater when fields were planted with a higher number of cassava varieties, suggesting host selection pressure (Restrepo et al. 2004). Our research group has assessed the diversity of populations collected in the northern coast of Colombia between 2008 and 2010. Results suggest a sustained, high level of diversity with some haplotypes being present since the 1990s and some new haplotypes detected in recent collections (Trujillo and Bernal, unpublished data). Higher levels of diversity were found in subregions where cassava has been cultivated longer and also in regions where environmental conditions are conducive to disease (Trujillo and Bernal, unpublished data).

In pathogen population studies, it is important to determine the virulence of isolates in diverse plant cultivars. Several attempts to define races in *Xam* using this approach have made it clear that no discrete races can be differentiated in this pathosystem (Restrepo et al. 2000a), although different levels of resistance are present in cassava accessions. A classification into groups of strains with the same spectrum of virulence on sets of cassava cultivars (i.e. strains that are able to cause disease in the same range of cultivars) has been implemented. In addition, virulence groups are not correlated with geographical origin of the isolates at continental or local scales (Elango and Lozano 1981; Verdier et al. 1998; Restrepo et al. 2000a) or with molecularly defined haplotypes (Verdier et al. 1994), although Latin American and African isolates had a tendency to be more virulent than the Asian ones (Verdier et al. 1993). Certain cassava cultivars showed resistant to most but not all of the strains, allowing recommendations for use of these cultivars, at least in Colombian fields (Restrepo et al. 2000a). A thorough study of pathotypes present in African populations is pending.

In summary, studies on the diversity of the pathogen have produced results that are relevant for cassava breeders in different zones of the world, underscoring the importance of closely following the populations of the pathogen both at the molecular and the pathogenic level.

Xam Enters the Genomic Era

Traditionally the identification of pathogenicity determinants in plant pathogenic bacteria, including some pathovars of *Xanthomonas*, was achieved using classical genetic approaches such as random mutagenesis and complementation (Staskawicz et al. 1995; Keen 1990). These and other studies defined certain clusters of genes important for pathogenicity in *Xanthomonas* species. As in other well-studied plant pathogenic bacteria, important pathogenicity factors included genes in the Type III secretion system (TTSS), which

secretes and translocates effector proteins (see below) into the plant cells (reviewed by Alfano and Collmer 2004). Other pathogenicity determinants in *Xanthomonas* include cell wall degrading enzymes, exopolysaccharides (EPS), and toxins (Boher et al. 1995; Boher et al. 1997; Kemp et al. 2004). However, the use of classical genetic screens is labor intensive, time consuming, and uninformative for redundant genes that lack a phenotype as a single mutant. The advent of more accessible genomic technologies has accelerated the discovery rate of pathogenicity genes.

There has been a special genomics focus on the genus *Xanthomonas* during the last decade, with several species now fully sequenced. The first genomes sequenced are pathogens from solanaceous plants and rice (Thieme et al. 2005; Lee et al. 2005; Ryan et al. 2011). Currently, there are sixteen genomes from this genus publicly available, representing bacteria with diverse lifestyles and host ranges (Da Silva et al. 2002; Qian et al. 2005; Salzberg et al. 2008; Vorhölter et al. 2008; Pieretti et al. 2009; Bogdanove et al. 2011; Potnis et al. 2011). This has allowed comparative studies that generate hypotheses on phenomena such as host specificity and lifecycle within the host plant (Studholme et al. 2010; Potnis et al. 2011; Bogdanove et al. 2011).

Our group has recently led the genome sequencing of *Xam* strain CIO151 using Illumina and 454 Technology (Ronaghi 2001). We have assembled the genome to approximately 40 fragments and performed automatic and manual annotation in an international effort with scientists from Universidad de los Andes, the Institute de Recherche pour le Développement (IRD) in Montpellier, Institut National de la Recherche Agronomique (INRA) in Angers and Toulouse, Universidad Nacional in Colombia, Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD) in La Reunion, Université de Angers, Marin Luther King University, The Institute of Microbial Technology in India, University of California at Berkeley, University College Cork and Universidade de Sao Paulo (Arrieta et al., unpublished data). The availability of this genome has allowed the identification of pathogenicity determinants as well as several genes encoding for effector proteins in *Xam* similar to other *Xanthomonas* species. Preliminary studies of colinearity between *Xam* and other *Xanthomonas* have suggested a high degree of conservation in gene content and order. Important pathogenicity factors have been detected by basic comparative genomics with other *Xanthomonas*. These include a cluster for secretion systems belonging to Types II, III, IV and VI, all of which share high levels of identity and synteny with those from other *Xanthomonas* in the *X. axonopodis* clade. In addition, the full cluster for Xanthan gum production and for the main Quorum Sensing system (Rpf, for Regulation of Pathogenicity Factors) were both detected. No prominent differences have been found in important pathogenicity factors in this genus. Therefore, the ability to infect cassava must lie in differences in specific gene sequences and/or regulatory regions. We are currently pursuing this hypothesis by experimentally testing the importance of some of these genes in the virulence of *Xam* using mutagenesis.

Effectors from *Xam* Involved in Pathogenicity

As stated above, the most important pathogenicity weapon in gram-negative plant pathogenic bacteria is the Type III secretion system (TTSS; reviewed by Alfano and Collmer 2004). This system injects effector proteins into the host cell during the infection process. These effectors modify normal processes in the plant and have been demonstrated to suppress host defense responses (see below). Within a resistant plant, one or several of these effector proteins are recognized by the plant's resistance gene product(s), and this recognition triggers strong defense responses in the host that leads to resistance (Jones and Dangl 2006).

It is estimated that each bacterium injects 35–50 different effector proteins into the host cell (Alfano and Collmer 2004). We have identified 25 effector genes in the sequenced genome of *Xam* CIO151, based on comparisons with other species of pathogenic bacteria from plants and animals (Arrieta et al., unpublished data). In addition, pthB, an effector that was previously used for assessment of bacterial diversity, has been demonstrated to be an important pathogenicity determinant in *Xam* (Castiblanco and Bernal, unpublished data). This gene belongs to a family of effectors classically called the AvrBs3/PthA, but more recently renamed Transcriptional Activator-Like (TAL) because of the activities demonstrated for this family within host cells (Kay et al. 2007; Boch et al. 2009; Moscou and Bogdanove 2009).

After secretion and translocation into the plant cell by the TTSS, TAL effectors travel to the host nucleus due to the presence of Nuclear Localization Signals (Bonas et al. 1989). Once in the nucleus, these effectors bind to promoters with specific DNA target sequences (Kay et al. 2007; Boch et al. 2009; Moscou and Bogdanove 2009) and lead to transcriptional activation using eukaryotic domains also present in the proteins.

Another effector, PthB, is important in the pathogenicity of *Xam* strain CFBP1851 (Castiblanco and Bernal, unpublished data). A non-pathogenic mutant lacking this gene acquired the ability to cause disease in cassava upon transformation with PthB. Therefore, the genes activated by this strain in susceptible cassava plants must be important for colonization and multiplication of the pathogen. Because of their importance, we are currently elucidating the gene targets of PthB in cassava plants using bioinformatics and transcriptomic strategies. In addition, the fact that this gene is important for pathogenicity makes it an important target for the generation of plants resistant to CBB by its use as a

pathogen-inducible promoter in transgenic plants. We are also studying other TAL effectors in *Xam* populations.

Genetics of CBB Resistance

Most commercial cassava cultivars are susceptible to the majority of and most prevalent *Xam* strains (Verdier et al. 2004). While there are a few cassava cultivars that show relatively high levels of natural resistance to CBB (Restrepo et al. 2000a, 2004), they are either not well adapted to particular agroecological regions where cassava is cultivated or do not exhibit the farmer- and/or consumer-preferred characteristics such as yield, starch content, processing quality and flavor. In the absence of chemical agents to control CBB, identifying the underlying genetic resistance present in such non-commercial cassava cultivars and transferring them to the commercial cultivars is the most promising way to manage this disease (Verdier et al. 2004).

Cellular responses to *Xam* has been characterized in stems of resistant and susceptible plants using histochemistry and gold cytochemistry (Kpémoua et al. 1996). Lignification, suberization and callose deposition in the parenchyma cells of phloem and xylem were the main responses. Xylem vessels showed an accumulation of pectic and/or lignin-like material after infection with *Xam*. However, these responses occurred in resistant as well as susceptible plants, although they were faster and more intense in resistant cassava. The vascular tissues and tyloses from resistant cultivars produced molecules of phenolic origin and showed a hyperplasia of the phloem cells. These responses probably are a result of attempts by the plant to isolate the bacterium and prevent infection of the surrounding cells (Kpémoua et al. 1996). Additionally, transmission electron microscopy studies on CBB-infected cassava plants showed that the main cell responses are localized to the vascular tissues (Boher et al. 1995; Kpemoua et al. 1996).

Resistance to CBB is considered to be polygenic and additive, with a variability of 25 to 66% (CIAT 1980; Jorge et al. 2000, 2001). As mentioned above, the different responses to infection by resistant and susceptible cultivars are expressed as a variation in the rate of colonization by the bacterium. Taken together, these results suggest that the resistance is quantitative. A Quantitative Trait Loci (QTL) mapping approach was employed to identify genomic regions involved in resistance, making use of the first cassava genetic map generated in 1997 (Fregene et al. 1997). QTL analyses were carried out employing five different strains of *Xam* (CIO84, CIO1, CIO136, CIO295 and ORSTX27), which represent its diversity. Several QTLs were identified, some of which were strain-specific while others were common for different strains. The variance explained by these QTLs fluctuated between 9 to 27% (Jorge et al. 2000). In addition, resistance was evaluated under field conditions in a region under high disease pressure during two consecutive crop cycles (Jorge et al. 2001). The *Xam* population was monitored in parallel. Several QTLs were identified; some of which changed during the two-year study period and could be correlated with changes in the bacterial population structure (Jorge et al. 2001). Interestingly, some QTLs on the D linkage group were identified in both studies and were constant over the two crop cycles (Jorge et al. 2000, 2001). More recently, QTLs for resistance to CBB were identified employing a different mapping population and using African strains of *Xam* (Wydra et al. 2004). Some QTLs were different from those detected in the previous studies, suggesting a strong specificity in the QTLs associated with resistance to particular *Xam* strains.

The next challenge is to improve fine mapping strategies in order to identify the genes present in the QTLs, as has been done in wheat and rice for the isolation of QTL-genes *Lr34*, *Yr36* and *pi-21* (Fu et al. 2009; Krattinger et al. 2009; Fukuoka et al. 2009). Increasing the number of molecular markers in the cassava genetic map through the use of new sequencing technologies could reduce the size of the QTL genomic region and allow the cloning of the corresponding genes. The isolation of genes corresponding to QTLs opens the possibility of employing them in biotechnological strategies to obtain broad and durable resistance. In addition, the isolation of the resistance genes in the QTLs will further the understanding of the molecular basis of these kinds of complex disease resistance traits.

Identifying Immunity Genes in Cassava

Studies in model systems such as *Arabidopsis thaliana* have sketched a general scheme of how plants defend themselves against disease. The first branch of plant immunity depends on membrane receptors with an extracellular LRR or LysM domain and an intracellular kinase domain named PRRs (Pattern Recognition Receptors; Zipfel 2009). These receptors recognize Microorganism Associated Molecular Patterns (MAMPs), which are highly conserved molecules present in pathogenic as well as non-pathogenic microorganisms (Nicaise et al. 2009). MAMP Trigger Immunity (MTI) is efficient to control non-adapted pathogens. However, some pathogens have evolved effector proteins, which block MTI, allowing the colonization of specific plant hosts by the pathogen. In order to trigger the second branch of immunity, plants employ intracytoplasmic Resistance (R) proteins, which directly or indirectly recognize particular effectors in a specific manner. Plant host proteins called pathogenicity targets mediate the indirect recognition. R proteins sense the action of effector proteins on the pathogenicity targets or their activity on other host proteins and trigger an immune response (Jones and Dangl 2006). These

effectors were classically named Avr proteins (Chisholm et al. 2006), because they rendered the pathogen avirulent, or unable to produce disease on those plants expressing the R gene. R proteins have a combination of some conserved domains. Most have a TIR (Toll Interleukin Related protein) or CC (Coiled-Coil) domain in their N-terminus, a NBS (Nucleotide Binding Site) domain in the central region and a LRR (Leucine Rich Repeat) in their C-terminus (Jones and Dangl 2006).

Based on the presence of these conserved domains, degenerate primers have been designed to amplify DNA sequences coding for proteins containing the NBS and TIR domains (Meyers et al. 1999). Employing this strategy in cassava, several RGC (Resistance Gene Candidates) were identified, and some were mapped (Lopez et al. 2003). In particular, one called *RXam2* co-localized with a QTL that accounts for 62% of the resistance to the *Xam* strain CIO151 (Lopez et al. 2007). In a similar manner, a cassava PCR fragment was obtained employing primers based on the *Xa21* rice resistance gene, which confers resistance to *Xanthomonas oryzae* pv. oryzae. When this fragment was mapped in cassava, it co-localized with a QTL that explains 13% of the cassava resistance to the *Xam* strain CIO136 (Jorge et al. 2000). This gene has been called *RXam1*. In our laboratory we have determined the complete sequence of these genes, and several functional studies are being carried out to test their role in CBB resistance. We have cloned small fragments of *RXam1* and *RXam2* into the binary pHellsgate vector, which allows cloning in sense and antisense directions with the objective to silence the corresponding genes. Cassava plants of the variety 60444 have been transformed, but only a few transgenic lines were obtained for the *RXam2* construction and none for the *RXam1* construction. It is likely that the siRNAs (small interfering RNAs) generated from the *RXam1* construction silenced other members of the LRR-kinase protein family that are essential for plant development. Preliminary results on the *RXam2*-silenced transgenic plants show that these plants are partially compromised in resistance to *Xam* evidenced by *i*) an increase in the symptoms after inoculation and *ii*) reduction in the expression level of the defense marker genes.

With the recent release of the genome of cassava, new opportunities to identify putative immunity related genes (IRG) are available to researchers. It is now possible to interrogate any proteome to identify proteins containing the conserved domains known to be present in plant immunity proteins. Using this approach, catalogs of the immunity related proteins (IRP) in the genomes of Arabidopsis (AGI 2000), poplar (Tuskan et al. 2006), papaya (Ming et al. 2008), potato (Xu et al. 2011) and castor bean (Chan et al. 2010) have been nearly completed. The number of IRPs varies significantly between different species. In papaya, for example, there are only 54 NBS-coding genes, 18 of them have just an NBS, 23 are NBS-LRR and 7 have an additional TIR domain (Porter et al. 2009). In contrast, there are 159 CC-NBS-LRR coding genes in rice and 183 (119 CC-NBS-LRR + 64 TIR-NBS-LRR) in *Populus*. A bioinformatics search in the cassava proteome has allowed identification of a preliminary set of cassava IRGs. These data will allow us to establish possible phylogenetic relationships among the cassava genes and between cassava and other cassava-related and non-related species. The next challenge will be to associate these IRPs with phenotypic resistance to particular strains of *Xam* and/or other cassava pathogens. For this reason it will be important to map these IRGs to the cassava genetic map. We have begun to explore this possibility through the sequencing and identification of SNPs of some of these genes in TMS30572 and CM2177-2, the two cassava cultivars employed as parents to construct the first cassava genetic map. Even though the target region to amplify and sequence was predominantly UTRs or introns, a low level of intervarietal polymorphisms were detected (Vasquez and Lopez, unpublished data). Alternatively, the search and mapping of SSRs close to IRGs will allow us to map these regions relatively easily.

Gene Expression

The recognition of MAMPs or Avrs by PRRs or R proteins, respectively, triggers a set of resistance-related responses (Tao et al. 2003). The most important events during a resistance reaction include ion fluxes, production of ROS (Reactive Oxygen Species), activation of MAP kinases, reinforcement of the cell wall with callose deposition and, frequently, a hypersensitive response (HR) or programmed cell death at the infection site (Dodds and Rathjen 2010). These events are consequence of the reprogramming of plant gene expression, which includes the induction of *PR* (Pathogenesis Related) genes (Van Loon et al. 2006). To monitor changes in gene expression, new approaches have been developed in the last few years. The generation of ESTs followed by the production of microarrays were reliable techniques used to build a global plant defense transcriptome (Malone and Oliver 2011). Employing cDNA-AFLP, Santaella et al. (2004) and Kemp et al. (2005) identified several genes expressed during *Xam* infection or during the cassava hypersensitive response, respectively. In cassava, several cDNA libraries were constructed from resistant and susceptible cassava cultivars challenged with *Xam*. The set of ESTs generated from these libraries was the base for construction of the first cassava microarray containing approximately 5700 unique cassava genes (Lopez et al. 2004). This microarray was employed to study changes in gene expression during a resistant reaction in cassava after inoculation with *Xam* strain CIO151. In total, 199 genes were de-regulated, 126 induced and 73 repressed at different times after inoculation (0.5, 1, 2, 7 and 15 days;

Lopez et al. 2005). The deregulated genes encode proteins involved in different host processes, such as cell wall reinforcement, production/degradation of ROS (peroxidases and cationic peroxidases), protein degradation and transcription regulation (Lopez et al. 2005).

The cassava genome annotation predicts approximately 35 000 cassava genes (www.phytozome.com). Therefore, the cassava microarray described above contains only one seventh of the cassava gene repertoire. As a result, new efforts have been directed toward elaboration of a cassava chip carrying 59,079 probes designed from approximately 73,000 target sequences (Utsumi et al., this issue). This resource provides new opportunities to explore the cassava transcriptome in response to different abiotic and biotic stresses, such as infection by *Xam*. However, with the advent of Next Generation Sequencing (NGS) technologies, it is now easier to directly sequence the complete RNA molecules present under particular conditions (Marguerat and Bahler 2009). This approach, named RNAseq, has been employed in several organisms to study changes in gene expression (Wang et al. 2009). It would likely be easier to map the reads obtained with the NGS, which are relatively short (75–100 bp by Illumina or 300 bp using 454). The combination of RNAseq and the cassava genome will allow us to assign a putative function for some of the ~35,000 cassava genes and to gain insights concerning the molecular processes that trigger response to *Xam* infection. The sequencing of RNA obtained from the parents employed to construct the cassava genetic map after inoculation with *Xam* will not only identify induced/repressed genes in response to the bacteria, but will also provide high redundancy in the number of reads obtained for each transcript and allow SNP identification. This information will be valuable for developing strategies to map these particular genes and to establish association between SNPs and QTLs of resistance.

MicroRNAs in Plant Immunity

As described above, during plant immune responses a reprogramming in gene expression is activated (Tao et al. 2003). Although the first point of control for gene expression occurs at the transcriptional level, new concepts in gene expression control have emerged in the last ten years. One control of gene expression at the transcriptional and post-transcriptional level occurs through microRNAs (miRNAs), which are small, non-coding RNAs (Brodersen and Voinnet 2009). miRNAs are encoded in the nuclear genome and when expressed cause the degradation of a transcript. The control of gene expression by miRNAs is relatively well studied during plant growth and development, but functions of these non-coding RNAs in abiotic and biotic stress responses have only been studied in the last few years (Ruiz-Ferrer and Voinnet 2009). In particular, during the plant immune response, miRNAs can negatively control pathogen invasion. In Arabidopsis, it was demonstrated that miR393 is induced in response to the MAMP flagellin (Navarro et al. 2006). The induction of this miRNA represses the expression of TIR1, an F-box-containing auxin receptor, and thus inactivates the expression of auxin-responsive genes (Navarro et al. 2006). Consequently, the pathogen is able to manipulate the auxin pathway to its benefit. In addition, some bacterial effectors from *Pseudomonas* have been shown to target and disrupt the miRNA silencing pathway with the aim of colonizing plant cells (Navarro et al. 2008).

Some cassava miRNAs have been described, however to date none have been deposited in the miRbase, the database for validated miRNAs (Zeng et al. 2010). We have sequenced two small RNA libraries, obtained from mock-inoculated cassava and cassava inoculated with *Xam,* employing Illumina. This allowed us to determine the cassava miRNome and to identify a subset of miRNAs induced and repressed in response to *Xam* (Perez and Lopez, BMC Plant Biol *in press*). Targets of these miRNAs include genes involved in transcription or in response to hormones, but also genes encoding for proteins probably involved in pathogen recognition (Perez and Lopez, BMC Plant Biol *in press*). The discovery and full understanding of cassava miRNAs involved in resistance to *Xam* will help to identify the target genes for these miRNAs, which might be key to plant defense. These genes could be modified through genetic engineering to either evade or be targeted by specific miRNAs, creating new possibilities to improve resistance to CBB.

Interactome

Plant-pathogen encounters involve the interaction between proteins from both organisms. In addition, during activation of the resistance signal transduction pathways, countless protein-protein interactions are disrupted while others are promoted (Nicaise et al. 2009). These interactions constitute a complex network integral to cell homeostasis. Previous studies have shown that pathogen effector proteins are directed to the "hubs" of these networks to deregulate the normal cell host physiology (Mukhtar et al. 2011). We have begun to elucidate some of the cassava protein-protein interactions during the cassava responses to *Xam*. Selected genes showing induction in expression after *Xam* inoculation were screened against a cassava cDNA library in order to detect their partners in function. We have detected, for example, interaction between Glutathione S-Transferase (GST) and chitinase and sulfite reductase. These three proteins form a loop of interaction. The availability of both the cassava and *Xam* genomes, together with other functional studies, will allow us to identify the most relevant cassava and *Xam* proteins to be selected for these interaction studies. In this

manner, we will obtain a complete picture of the cassava-*Xam* network. This knowledge will guide the strategies for biotechnological modification of some genes with the aim of generating CBB resistance in cassava crops.

Conclusions

CBB is an important disease in cassava that can severely compromise food security for more than 600 million people in regions of marginal food resources around the world. In the face of global climate change, new challenges arise for cassava researchers in the effort to control the potential outbreaks of this disease. Cassava's high heterozygosity, breeding depression and long reproduction cycle make conventional breeding complicated and time-consuming. The study of this pathosystem using molecular biology, functional and comparative genomics, and proteomics will be key in the generation of novel disease management strategies and molecular breeding for durable disease resistance.

The application of new molecular biology technologies to this pathosystem will allow us to sketch a genomic picture of both actors in this interaction. Sequencing of several *Xam* strains will allow the identification of the most conserved effector proteins that play important roles in the fitness of the pathogen. These effector proteins are key targets of plant resistance proteins, whether natural or engineered, that recognize the pathogen and trigger a plant immune response. The knowledge of the genome of *Xam*, together with the availability of inexpensive sequencing technologies, will allow for genome-scale genotyping of this pathogen. It will be possible to routinely and continually monitor the population dynamics of *Xam* in the different regions where cassava is cultivated. This will allow us to pinpoint migratory routes for the pathogen and to define other epidemiological characteristics that could be useful for disease management. On the other hand, the new sequencing technologies will allow us to easily obtain the genome sequences of several cassava cultivars. This information will be useful in both association mapping and conventional mapping projects, which will accelerate the discovery rate of resistance proteins for this and other diseases. As we have seen in the classical and more recent studies cited in this review, *Xam* is a diverse pathogen, which makes it necessary to identify a number of resistance proteins to pyramid resistance genes in order to obtain broad and durable resistance to CBB. The possibility of comparing the repertoire of immunity genes between individual cultivars will shed light on the molecular mechanisms that have molded the structure of genes involved in pathogen recognition. However, the challenge is in matching the rate of phenotypic data generation with that of the molecular data (gene repertoires, gene structure, nucleotide polymorphisms) for both cultivars and segregating populations. New data from a broad number of individuals in different environments, with different characteristics - including resistance to CBB - will be the bridge necessary to link genes to breeding programs. Since CBB results from the interaction between two actors, the information generated for both sides should be linked. The interactome between plant and pathogen proteins will highlight the function of particular proteins, but more importantly, will allow the identification of the most important targets of breeding. Alternatively, but not exclusively, the knowledge of plant immunity on model plants and other crops, such as Arabidopsis and rice, could be brought to cassava. The introduction of *PRR* genes, such as the *Xa21* from rice or *EFR* (*Elongation Factor Receptor*) in cassava, could provide a broad and durable resistance to CBB, as has been demonstrated for other plants (Mendes et al. 2010; Lacombe et al. 2010). Surely, cassava breeding programs will benefit from the great advances that will be achieved in the knowledge of plant-pathogen interactions in this and other diseases in the next decade. Ideally, this will have a positive effect on yield in this crop. In the coming years, cassava must do the jump necessary to close the gap with the knowledge base in model crops. Only in this way will cassava fulfill its potential to supply the food for a world population that continues to grow.

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