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Transference of *RXam2* and *Bs2* genes to confer resistance against cassava bacterial blight (CBB)

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A ti querido lector

When the Nazis came for the communists,
I remained silent;
I was not a communist.

When they locked up the social democrats,
I remained silent;
I was not a social democrat.

When they came for the trade unionists,
I did not speak out;
I was not a trade unionist.

When they came for the Jews,
I remained silent;
I wasn't a Jew.

When they came for me,
there was no one left to speak out.

Martin Niemöller

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Abstract

Cassava bacterial blight (CBB), caused by *Xanthomonas axonopodis* pv. manihotis (*Xam*), is a major disease in all regions where cassava is cultivated. To generate broad spectrum and durable CBB resistance it is necessary pyramiding several *R* genes. We have identified two candidate resistance genes, named *RXam1* and *RXam2* which colocalize with QTLs associated to resistance against *Xam*. *RXam1* codes for a RLK (Receptor-Like Kinase) protein. We generated several transgenic cassava lines overexpressing *RXam1*. *In vitro* plants of three lines showed reduced symptoms and reduced bacterial growth after *Xam* infection with strain CIO136 compared to empty vector transgenic plants. The second cassava gene, *RXam2,* codes for a NB-LRR protein. Using RNAi we generated *RXam2*-silenced transgenic plants, which were more susceptible to several *Xam* strains than non-transformed plants. On the other hand, plants overexpressing *RXam2* showed reduced symptoms to *Xam* strains. These data collectively suggest that *RXam2* is a resistance gene against *Xam*. In addition, an autoactive version mutated in the MHD motif (NB domain) of *RXam2* was generated through site directed mutagenesis and was able to generate a Hypersensitive Response (HR) by transient agroinfiltration in cassava and tobacco leaves. The autoactive version of *RXam2* was cloned under a TALE1$_{Xam}$-inducible promoter and transient expression in tobacco showed a strong HR when co-infiltrated with a plasmid containing the *TALE1*$_{Xam}$ gene. Several independent transgenic stable lines are being evaluated to assess TALE1Xam inducibility. Finally, several cassava transgenic plants overexpressing *Bs2* from pepper were obtained and showed constitutive, typical immune responses.

Keywords: cassava, *Xanthomonas*, bacterial blight, resistance genes

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List of abbreviations

Abbreviation	Meaning
AAD	Acidic activation domain
ARC	Apaf, resistance proteins, CED-1
BAK1	BRI1-associated receptor kinase-1
BAP	Benzylaminopurine
BIK1	Botrytis induced kinase-1
CBB	Cassava bacterial blight
CC	Coiled-coil
CDS	Coding sequence
CEBiP	Chitin elicitor binding protein
CERK1	Chitin elicitor receptor kinase-1
CFU	Colony forming units
CMD	Cassava mosaic disease
CNL	Coiled-coil NB-LRR
CBSD	Cassava brown-streak disease
EFR	Elongation factor receptor
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
FEC	Friable embryogenic calli
FLS2	Flagellin sensing-2
GA3	Gibberellic acid
GD	Gresshof and Doy basal medium
GUS	β-glucuronidase
HR	Hypersensitive response
IBA	Indole-3-butyric acid
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase

Abbreviation	Meaning
MTI	MAMP-triggered immunity
MS	Murashige and Skoog basal medium
NAA	Naphthalene acetic acid
NIL	Near isogenic line
NLR	NOD-like receptor/ nucleotide-binding leucine-rich repeat
NLS	Nuclear localization signal
NB	Nucleotide-binding
NOD	Nucleotide-binding oligomeric domain
OD	Optical density
OES	Organized embryogenic structures
PAMP	Pathogen-associated molecular pattern
PR	Pathogenesis-related
PRR	Pattern-recognition receptors
PTI	PAMP-triggered immunity
QTL	Quantitative trait loci
RBOHD	Respiratory burst oxidase homolog D
RIL	Recombinant inbred line
RLK	Receptor-like kinase
RLP	Receptor-like protein
RNAi	Interference RNA
ROS	Reactive oxygen species
RVD	Repeat variable diresidue
SERK	Somatic-embryogenesis receptor-like kinase
TALE	Transcription activator-like effector
TIR	Toll interleukin receptor
TNL	TIR NB-LRR
TTSS	Type three secretion system
UN	United Nations
Xam	*Xanthomonas axonopodis* pv. manihotis

Introduction

During decades, staple crops from the first world such as wheat, barley and maize have experienced elaborated breeding programs to solve the constraints imposed by pathogens. However, in countries from the third world other staple crops, such as cassava, sugarcane and banana account for caloric intake. Cassava was initially domesticated near the Amazon basin and now is cultivated in tropical countries across sub-Saharan Africa, South-East Asia and South America. Among tuber crops, cassava is the main source of calories in tropical countries. Colombia is the third largest cassava producer in South America with a forecast 2,5 million tonnes in 2015 (FAO, 2015). Cassava faces diverse limitations in terms of low current yields, nutritional quality, pests and diseases, among others. The most severe diseases are CMD (cassava mosaic disease), CBSD (cassava brown-streak disease) and CBB (cassava bacterial blight). While CMD and CBSD are endemic in sub-Saharan Africa, CBB is present in all three continents. CBB is caused by the Gram-negative bacterium *Xanthomonas axonopodis* pv. manihotis (*Xam*) and affects mainly the stems and leaves. The most reliable method to control this disease is through the use of varietal resistance. Several studies have focused on the dissection of cassava-*Xam* interactions in susceptible and resistant cultivars. However, at the present time no functional resistance genes have been fully characterized in cassava. Our group aims to understand molecular determinants involved in disease and resistance responses in cassava to *Xam*.

The work presented in this thesis covers the last results on the efforts conducted to identify and characterize resistance genes to CBB, mainly through functional studies of two previous CBB candidate resistance genes and to evaluate, as a proof of concept, the strategy based on interfamily transfer of a resistance gene from pepper to cassava. The present document contains four main chapters. The first one is a bibliographical revision on conceptual topics addressed in the present thesis. It starts with the biology and constraints of cassava, with a special highlight on pests and diseases and notably on CBB. In a second part, the historical background and conceptual aspects of plant-microbe interactions are revised, followed by a final description of some strategies for crop improvement based on the knowledge of plant immunity. The remaining three are focused on the employment of distinct research approaches to introduce a resistance

gene from pepper in cassava and validate the function of two candidates genes and their potential use to achieve resistance to CBB. These three chapters are written in a format of research articles.

More in detail, in the second chapter, a transgenic-based approach was employed for the interfamily transfer of the *Bs2* gene from pepper into susceptible cassava plants. Two main aspects are considered, first the conservation of NLR downstream signaling in distant species and second, the distribution of AvrBs2 as a core effector in species of the genus *Xanthomonas*.

In the third chapter, the overexpression of a gene associated with a QTL to CBB, called *RXam1* is reported. Data found indicate *RXam1* overexpression confers a decrease in susceptibility only to *XamCIO136*. In contrast, no contribution to resistance was found to other *Xam* strains tested.

In the fourth chapter, a functional analysis of *RXam2*, a gene associated with a major QTL is presented. Results obtained from *RXam2* silencing and overexpression suggests an important contribution for resistance to several *Xam* strains. In addition, an autoactive mutation of *RXam2* was used to develop an activator trap promoter induced by a TAL (transcription activator-like) effector from *Xam*.

Finally, a last chapter presents a general overview of the main results presented in each previous chapter, connecting them and considering the advantages, limitations and perspectives of this work.

Chapter 1

"Poco a poco, estudiando las infinitas posibilidades del olvido, se dio cuenta de que podía llegar un día en que se reconocieran las cosas por sus inscripciones, pero no se recordara su utilidad"

"Little by little, studying the infinite possibilities of a loss of memory, he realized that the day might come when things would be recognized by their inscriptions but that no one would remember their use."

Gabriel García Márquez

1 Review of related literature

Cassava and global food security in the context of climate change

Considering the exponential growth of human population compared with a linear increase on food production, it is has been estimated that human population will soon reach earth's carrying capacity leading to food shortage. United Nations population projections has predicted the world total population will reach 9,15 billion by 2050 (Alexandratos and Bruinsma, 2012). Although this is a slow rate compared with the 3,3 billion growth seen during the last four decades, a major correlation with decreased population growth is increased wealth and a concomitant higher food demand per capita (FAO, 2009; Godfray et al., 2010). This implies a need to produce from 70% to 100% more food to meet the population demands for 2050 (Tilman et al., 2011; Alexandratos and Bruinsma, 2012). An important challenge is to increase food production in the middle of a changing climate. This requires sustainable intensification to reduce environmental impact while producing more food. A combination of changing agronomic practices, reducing waste production, integrated pest management methods and a sustainable use of land destined for agriculture could help to achieve this goal (Godfray et al., 2010).

Increasing temperatures, CO_2 and ozone levels are generating awareness among scientific community to develop new strategies to manage this situation in terms of food security. Estimations indicate climate change is expected to have a major impact on food insecure regions (Rosenthal and Ort, 2012). Many of these undernourished countries depend on staple crops

different from rice, maize, wheat, and barley for their subsistence. Root and tuber crops consumption in the least developed countries account for most of the caloric intake (http://faostat3.fao.org/). When compared with other cereals, cassava's yield per hectare is significantly higher, and in many countries is the cheapest source of calories available (FAO, 2013b). Furthermore, since cassava can be efficiently produced in marginal areas at small scale, is grown almost exclusively by smallholder and low-income farmers with no need of mechanization. In addition, since it is clonally propagated by 15-30cm stem cuttings (stakes), the planting material is easily available and low-cost (FAO, 2013b). Unlike other staple crops, cassava inherent adaptability to diverse environments (limited water supply, acid soils, low soil fertility, difficult terrain) (El-Sharkawy, 2004; FAO, 2013b) suggests this crop is potentially highly resilient to future climate change (Jarvis et al., 2012). In addition, model-based predictions suggest cassava production is not severely affected by high CO_2 levels, moreover field trials have shown increased yield compared to other C3 plants (Rosenthal and Ort, 2012; Rosenthal et al., 2012). This has allowed cassava, the "food of the poor", to become a priority crop for food security in developing countries.

Cassava, from Amazonia to the world

The genus *Manihot* (Euphorbiaceae) is distributed through the Neotropic and is composed of 98 species (Rogers and Appan, 1973). This angiosperm family comprises several species with economic importance including *Jatropha curcas, Ricinus communis* and *Hevea brasiliensis*. One of the most representative species of this genus is cassava (*Manihot esculenta* Crantz). Cassava (known as yuca, manioc, tapioca, mandioca) is the principal source of calories among tuber crops (Cock 1982) (http://faostat3.fao.org/) and is native from South America (Allem, 1994, 1999; Bredeson et al., 2016). Cassava domestication began 5000-7000 years B.C. near the southern border of Amazon River. The Caribe and Arawak Indians were probably the first to cultivate cassava in the Caribbean and Northern South America, and some of their cultivation practices remain almost intact along the Amazon basin (Henry and Hershey, 2002). European colonialist and slave traders introduced it to Africa 500 years ago where it spread relatively slow (Gibbons, 1990; Allem, 1994, 1999; Olsen and Schaal, 1999). More recently, cassava was introduced to Asia

(India, Java and the Philippines) between the late 18th and early 19th century. Today cassava is intensive-cultivated in tropical and subtropical regions of Central and South America, Sub-Saharan Africa and South-East Asia (FAO, 2013b).

The accepted hypothesis for cassava botanical origin postulates *M. esculenta* ssp*. flabellifolia*, a woody perennial shrub from the Amazon basin, as the wild progenitor of cultivated cassava (Allem, 1994, 1999; Olsen and Schaal, 1999; Nassar and Ortiz, 2008). In terms of its domestication origin, recent analysis on genome genetic diversity of *Manihot* species have confirmed previous results from Olsen and Schaal (1999), who found cassava was domesticated once in the South-Western part of Amazon region from *M. esculenta* ssp*. flabellifolia* (Bredeson et al., 2016).

Cassava is a perennial vegetative-propagated shrub with a highly heterozygous genome. Despite it was frequently considered a polyploid species, studies based on general meiosis, indicate cassava is a diploid with 18 chromosomes (2*n*= 36) (Magoon et al., 1969; Hahn et al., 1990). However, since certain regions of the genome harbor duplications, it has been postulated a segmental allotetraploid origin of cultivated cassava (Magoon et al., 1969). Recent studies based on the conserved synteny between chromosomal pairs of the genus *Manihot* have shown the impact of paleotetraploidy on cassava genome (Bredeson et al., 2016). Furthermore, comparative genomic studies with *Jatropha curcas* (2*n*=22)*, Ricinus communis* (2*n*=20) and *Hevea brasiliensis* (2*n*=36) reveal paleotetraploidy is shared with *H. brasiliensis* which is estimated to diverged from cassava ~35 million years ago (Bredeson et al., 2016).

Reproductive biology

Cassava is diclinous and monoecious, meaning that both female and male flowers are produced on the same branch, female in the basal portion and male in the apex (Ceballos et al., 2015). The plant produces indeterminate inflorescences called racemes. Inflorescence develops in the apex of the steam with female flowers opening 10-14 days before the male flowers (Jennings and Iglesias, 2002; Ceballos et al., 2015). If female and male flowers from different branches or from different plants of the same genotype open simultaneously, it can lead to self-pollination

(Jennings and Iglesias, 2002). Flowering and the production of self and cross-pollinated seeds depends on the genotype and environmental conditions (pollinating species presence and planting design) (Jennings and Iglesias, 2002; Ceballos et al., 2010).

Since branching always takes place when an inflorescence is developed, the availability of flowers is influenced by plant growth. Cassava plants display apical dominance and indeterminate growth. Once apical dominance ceases and sympodial branches are formed (forking), the flowering occurs, although some non-terminal inflorescences have been observed (El-Sharkawy, 2004; Ceballos et al., 2015). Therefore, late branching types display less flowering compared with early-branching and low-growing ones. Although some genotypes display frequent flowering during a growing cycle, some others show little flowering or do not flower at all. Farmers usually prefer late-branching, tall and erect plant architectures due to the facilities in terms production, transport and storage. However this trait is not desirable for planned crosses when synchronization of flowering is needed since some genotypes could flower 4 months after planting while for some others it may take more than 10 months (Jennings and Iglesias, 2002; Ceballos et al., 2010, 2015).

Due to the difficulties related with flowering synchronization and the time required for a seed to mature, it takes usually more than a year to obtain seeds from a controlled cross (Ceballos et al., 2010). The fertility of the clones is variable and can be very low, between one and two seeds are usually obtained per pollination from the trilocular ovary (Jennings and Iglesias, 2002; Ceballos et al., 2010). Fresh harvested seeds are dormant and require a storage period of 3-6 months before germination. Due to the absence of known self-incompatibility effects, it is technically possible to obtain viable seeds from self-pollinations. However, the production of homozygous lines from repeated self-pollinations would require around 12-15 years (Ceballos et al., 2015). Since virtually all known improved varieties or landraces are derived from crosses between heterozygous parents, cassava plants grow today are clonally propagated hybrids (Ceballos et al., 2015).

Considering all the characteristic of this crop, it is difficult to establish controlled crosses, RILs (recombinant inbred lines), NILs (near isogenic lines) and introgression of genes for plant breeding as it is done in other crop species.

Adaptations to abiotic stress

Cassava has features that make it highly appealing in marginal areas with poor soils and unpredicted rainfall seasons where farmers usually lack resources for agrochemical input. As mentioned above, cassava is considered a crop with a great adaptability to adverse environmental conditions. A distinctive fact of this crop is that this plant do not have a critical period of adequate soil moisture essential for flowering and seed production (FAO, 2013b). On the contrary, cassava relies on several adaptations based on water-use efficiency to deal with drought stress. Actually, cassava leaves are well adapted to periods with limited water supply. One of its responses to increase water-use efficiency is a rapid reduction in the evaporative surface and partially closing stomata (El-Sharkawy, 2004). Another physiological important trait of cassava is the long leaf retention under water stress and the partial recovery when water becomes again available (El-Sharkawy, 2004). Also, its roots are known to prolong to great depths to gain access to subsoil water reserves. In consequence, cassava plants once established, can resist relative prolonged periods of drought, but it must be planted during periods of adequate water in the soil (Henry and Hershey, 2002; El-Sharkawy, 2004; FAO, 2013b). Cassava photosynthetic behavior is complex, it was previously reason to behave like both a C3 and C4-like plant and now is considered a C3-C4 intermediate (El Sharkawy, 1984; Edwards et al., 1990; El-Sharkawy, 2004, 2006). Cassava photosynthetic responses suggest an adaptation to tropical environmental conditions, requiring high solar radiation and high temperatures to express its full photosynthetic potential (Angelov et al., 1993; Alves, 2002). At morphological level, cassava leaves display novel adaptions such as papillae in the epidermal cells of the lower mesophyll surface that is proposed to maintain leaf water status under drought conditions (Angelov et al., 1993). In addition, cassava grows well in 25-29ºC, although it can tolerate temperatures up to 38ºC. When grown on temperatures above the optimum (between 30-40ºC) an increase in photosynthetic rates have been reported, moreover decreases in root yield are very little even when the optimal temperature range is exceed by 5-10ºC

Cassava does not only possess and exceptional tolerance to prolonged periods of droughts but also can grow and produce reasonable yields on acid, infertile soils and also on land with minimal preparation (FAO, 2013b). Some of the adaptations that allow this crop to grow on poor and degraded soils is the tolerance of low phosphorus (P) concentrations on the soil, low pH and high levels of exchangeable aluminium (Al) (Howeler, 2002). Since cassava roots form beneficial symbiotic associations with vesicular-arbuscular mycorrhizae (VAM) naturally occurring in the soil, it can generally grow with no application of P-fertilizers (Howeler, 2002; FAO, 2013a). Beside

performing better than other crops on infertile soil, cassava also responds efficiently to fertilizers application (Howeler, 2002).

Constraints for food security

Despite its advantages as a cheap caloric source and high yields, cassava relevance as a food security crop faces diverse challenges in terms of nutritional limitations, loss of genetic variability, diseases management, low number of vegetative seed per plant, among others. Although under the marginal conditions in which cassava is grown it produces more energy per area than most other crops, it has the lowest protein/carbohydrate ratio among world's 10 major crops (Sayre et al., 2011). Cassava roots contain on average 1%-2% protein by dry weight, even though some hybrids may contain near 5% protein (Nassar and Ortiz, 2008; Sayre et al., 2011). A typical cassava meal (500 g) provides enough calories for an adult but is an insufficient source of iron, zinc, vitamin A and protein, leading to micronutrient malnourishment (Sayre et al., 2011). Interestingly, tender cassava leaves contain 25% of protein (dry weight basis) and a valuable source of calcium, iron and vitamins A and C (FAO, 2013b). For this reason, in some countries of Central Africa, notably Democratic Republic of the Congo, Liberia, Cameroon and the United Republic of Tanzania, young tender leaves are harvested and consumed as a rich protein source (FAO, 2013b). Distinct approaches based on conventional breeding and genetic modification has been developed to address high carotenoids content in cassava. Since carotenoid content is a trait with high heritability in cassava, researches have been able to produce roots with the double carotenoid content trough breeding (Ceballos et al., 2012). On the other hand, the expression of enzymes involved in the synthesis of vitamin A precursors (carotenoids), through transgenic technologies have allowed to increase extensively total carotenoids content up to 30 times compared with WT white roots (Welsch et al., 2010; Failla et al., 2012). Even though the transformation system has produced several cv. 60444 plants as a proof-of-concept to address questions regarding nutritional improvement; this transformation model is not a genetic background preferred by farmers (Sayre et al., 2011). This denotes a need to develop transformation technologies in elite cultivars.

Due to the long crop cycle, asynchronous flowering and long breeding cycles, cassava improvement through conventional breeding is a demanding and time-consuming task. Since many landraces and cultivars display late or poor flowering, there is a recurrent use of the same genotypes as parents for breeding efforts, leading to a reduction of genetic diversity in cassava. Recent studies based on genomic data from wild and cultivated cassava revealed relationship among cultivated varieties that will aid for developing diverse germplasm for breeding efforts through genomic selection (Bredeson et al., 2016). Breeding decisions based on this knowledge will aim to restore lost variation on cultivated cassava.

Different studies have address the importance to clarify the position of cassava along the genus not only to resolve the phylogeny of Euphorbiaceae but also to study the genetic variability of cultivars aiming to identify possible genes loosed or gained during domestication of cassava (Allem, 1994, 1999; Olsen and Schaal, 1999; El-Sharkawy, 2004; Bredeson et al., 2016). Comparative analysis of genome variation between domesticated and cultivated cassava have revealed not only that starch accumulation has been positively selected during domestication, but also that genes associated with tolerance to extreme environments were lost (Wang et al., 2014). This implies biotic and abiotic stress can be weaker in cultivated conditions than natural environments.

Although during the period of 1980-2011 the global annual production has doubled from 124 million to 252 million tonnes of fresh root, current average yields are still lower than cassava's potential (FAO, 2013b). A study from the International Center of Tropical Agriculture (CIAT) estimates cassava could produce 22,3 tonnes per Ha, which accounts for more than 450 million tonnes a year considering the current harvested area (FAO, 2013b). In 2014, global production reached 288 million tonnes, (FAO, 2015), indicating we are still far from reaching cassava production full potential. All these data highlight the need to make an efficient use of soil and water, and to use varieties with high yields, tolerant to drought, resistant to pest and diseases in order to reach cassava production potential.

Pests and diseases

As many other crops, cassava is vulnerable to many pests and diseases that can produce severe yield losses, especially in Africa. Reliance on vegetative propagation makes this crop particularly prone to pest and diseases. Yield losses may change depending on the region where is cultivated and how intensively cassava is cultivated. Since cassava has a long crop cycle, is exposed to pathogens during prolonged periods, thus pesticides are not very effective and above all expensive (FAO, 2013b). A measure that can help farmers to avoid losses by diseases and also protects the agro-ecosystem is the use of tolerant or resistant cultivars. In addition, the use of planting material taken from healthy plants is also an efficiently control method. The main threats for cassava are arthropod pests, viral and bacterial diseases and root rot.

More than 200 arthropod species has been reported to affect cassava (Bellotti et al., 2012b). Some of these are specific for this crop, and some other attacks other crops as well. Since cassava botanical and domestication origin is the Neotropics, arthropods in this region have co-evolved for hundreds of years with this crop, therefore diversity of cassava pest is higher on Latin America compared to Africa and Asia (Bellotti et al., 2012b). In America the major arthropods that attack cassava are whiteflies, mite, mealybugs and hornworms (Bellotti et al., 2012a). In Africa, *Bemisia tabaci* is considered the major pest since it is the vector of Cassava Mosaic Disease (CMD) and Cassava Brown Streak Disease (CBSD), the most relevant diseases in Africa (Alvarez et al., 2012; Bellotti et al., 2012b).

The most devastating diseases in cassava are CMD, CBSD and Cassava Bacterial Blight (CBB). CMD is caused by a begomovirus complex, was first reported in Tanzania in 1894 and is endemic in Sub-Saharan Africa (Calvert and Thresh, 2002; FAO, 2013b). Symptoms usually include chlorosis mixed with normal green tissue, mosaic, mottling, twisted leaves and plant stunting (Calvert and Thresh, 2002). CMD is also the most relevant cassava disease in India and Sri Lanka. Host plant resistance derived from an interspecific cross between cassava and a wild relative, *M. glaziovii,* was identified in IITA (International Institute for Tropical Agriculture is the main method of control for CMD) and has been deployed in the field for many years (Fregene and Puonti-Kaerlas, 2002; Ceballos et al., 2012). Several studies have aimed to address genetic determinants of resistance to CMD and two genetic regions have been identified, called CMD1 and CMD2 (Fregene and Puounti-Kaerlas, 2002). CMD1 is thought to confer recessive and polygenic resistance while CMD2 is a dominant and monogenic resistance (Akano et al., 2002; Fregene and Puonti-Kaerlas, 2002). On the other hand, CBSD is caused by a Ipovirus and was first recognized

as a new disease different from CMD in 1930s (Calvert and Thresh, 2002; Alvarez et al., 2012). It has a diversity of symptoms affecting leaves, stem and tuberous roots. The most distinctive symptom is a corky necrosis on roots that render them unsuitable for consumption (FAO, 2013b). The integrated disease management for both CMD and CBSD include the use of non-infected (asymptomatic mother plants) material for propagation. However, since the disease is transmitted by *B. tabaci* whiteflies the use of clean planting material does not guarantee posterior infection. A more efficient control practice is the use of resistant or tolerant cultivars (Alvarez et al., 2012; FAO, 2013b). To date, no *R* genes have been cloned to confer resistance to CBSD.

Other minor diseases of cassava includes stem and root rots caused by several oomycetes, fungal and bacterial pathogens, anthracnose (Colletotrichum spp.), leaf spots (*Cercosporidium henningsii, Cercospora vicosae*), frogskin diasease (Phytoplasmas) and super-elongation disease (*Sphaceloma manihoticola*), among others (Alvarez et al., 2012). Reported cassava bacterial diseases includes bacterial blight (*Xanthomonas axonopodis* pv. manihotis), bacterial stem rot (*Erwinia carotovora* pv. carotovora) and bacterial stem gall (*Agrobacterium tumefaciens*) (Alvarez et al., 2012).

Cassava bacterial blight

CBB is the most devastating bacterial disease in America, Africa and recently in Asia. It was first reported in Brazil in 1912 and later in Colombia and Venezuela (Lozano, 1986). It has produced starvation in Democratic Republic of the Congo in 1970s where cassava is extensively cultivated. Losses produced by CBB can range between 50-75% when susceptible cultivars are used and depending on environmental conditions (Wydra and Verdier, 2002). CBB affects mainly leaves and stems. Due to the systemic nature of the disease, the primary symptoms, such as wilting of emerging shoots, appear when infected tools or plant material is used for cultivation. This leads to infection from plant to plant and also between crop cycles. When contaminated material is planted, primary symptoms include wilting of the newly emerged sprouts followed by die-back (Lozano, 1986). CBB manifestations appear after the dry season and when the first rains take place. In this case, previously infected plants can spread the bacteria to healthy plants. Splash caused by windy rain is the main method for bacterial mobilization, distribution and penetration of the inoculum to healthy plants (Lozano, 1975). High relative humidity coupled with windy rain

promotes the development of secondary symptoms including angular leaf spots, leaf blight, defoliation, wilting of the shoot and dieback, showing the typical "candle stick" appearance (Maraite and Meyer, 1975; Hillocks and Wydra, 2002). Leaf spots first appear as clearly distinguishable watersoaked angular areas on the abaxial surface. These lesions become brown and dark brown and could appear surrounded by a yellow halo, depending on the susceptibility of the plant (Lozano, 1986). Afterwards, angular leaf spots are enlarged and have a tendency to coalesce along the veins or on the edges of the leaf producing large blighted areas (Maraite and Meyer, 1975; Lozano, 1986). Leaf spots often exudates a sticky, yellow gum near the veins. Blighted areas spread over the entire leaf and the leaf withers and dries, afterwards the petiole collapses, but the dried leaf can remain attached to the stem for some time before it falls (Lozano, 1986). A yellowish gum is also observed in young stems and petioles. When infected shoots are observed under the microscope they show tyloses and mucilaginous substances, this is accompanied by the disintegration of the tissue and the formation of lysis pockets filled by latex, cell debris and bacteria (Maraite and Meyer, 1975). Rooting is faster in young green stems than mature ones and old stem tissue usually remain apparently healthy, this result in the typical dieback symptom of the immature portion of the steam (Lozano, 1975). With the exception of young susceptible infected cultivars that show necrotic vascular strands, roots from infected plants usually remain healthy (Lozano and Sequeira, 1974).

CBB etiology: *Xanthomonas axonopodis* pv. manihotis

The causal agent of CBB, *Xanthomonas axonopodis* pv. manihotis (*Xam*), is a Gram-negative, non-encapsulated rod, motile, with a single polar flagellum bacteria. *Xam* is aerobic and grows in medium containing sucrose producing slimy and unpigmented bacteria (Lozano and Sequeira, 1974). Aside from the lack of pigmentation, other physiological and biochemical characteristics are typical from other *Xanthomonas*. *Xam* is a systemic and epiphytic pathogen, it survives poorly in the soil however this can be a source of inoculum if contaminated with infected plant material (Lozano, 1975; Boher and Verdier, 1994). *Xam* produces disease symptom in cassava and also in other related *Manihot* species and can survive epiphytically on weeds for up to one month (Hillocks and Wydra, 2002).

The infection process in cassava begins with the epiphytic multiplication of *Xam* in the leaf forming microcolonies protected by mucus (Boher and Verdier, 1994). This increases bacterial population on the leaf surface where it contributes to build the inoculum. Its entry occurs through stomatal openings and epidermal wounds on the leaf produced by high winds. The preliminary phase occurs in the intercellular spaces on leaf spongy mesophyll where it multiplies rapidly by cell division, destroying the tissue and producing large amount of exopolysaccharides (Lozano, 1986; Boher and Verdier, 1994). This rapid multiplication of *Xam* coupled with the lysis of the host lamella in the leaf tissue produces a rapid colonization of the lamina and leads to the formation of angular leaf spots (Boher and Verdier, 1994). Subsequently *Xam* enters through the internal vessels on the leaf, enabling its movement to the petiole and afterwards to the stem. This allows bacteria to advance systemically through the vascular tissue including xylem and phloem vessels and pith cells (Lozano, 1986). Wilting is produced when bacteria and its matrix block the vessels impeding the movement of substances along the xylem vessels. Lysis pockets in phloem vessels and pith cells are also observed as a result of *Xam* growth (Boher and Verdier, 1994).

CBB integrated disease management

The losses caused by CBB can be drastically reduced with a combination of varietal resistance, cultural practices and sanitation measures (Lozano, 1986). Cultural farming practices including the use of clean cuttings, planting close to the end of the rainy season and crop rotation have proved to be efficient to control pathogen populations on the field (Lozano, 1986; Boher and Verdier, 1994; FAO, 2013b). The emergence and spread of the disease in a crop is mainly due to the use of tools infected with *Xam*, which are used as contaminated tools for the following production cycles. As a result, the pathogen can easily diffuse from cuttings taken from healthy asymptomatic stems (Lozano, 1986; Boher and Verdier, 1994; Wydra and Verdier, 2002). If the woody stems are infected, the bacteria are kept in vascular tissues where it can survive for up to 30 months, for this reason chemical methods are not very effective for controlling this disease. Other sanitation measures consist of soaking stakes in hot water at 50ºC during 50 minutes and sterilize tools used on contaminated fields on hot water or with disinfectant solutions (Alvarez et al., 2012; FAO, 2013a). To reduce plant-to-plant contamination by rain splash, intercropping is

recommended with fast growing species such as maize (FAO, 2013b).

The most effective and economic method to control CBB is to use resistant varieties (Boher and Verdier, 1994; Alvarez et al., 2012; FAO, 2013a). Some resistant varieties have been described (Restrepo et al., 2000a; Alvarez et al., 2012), however the disease is prevalent in areas where these varieties are not well-adapted or do not possess desirable agroculinary qualities (López and Bernal, 2012). This implies a need to transfer resistance to varieties adapted to specific agroecological zones where this crop is cultivated (Verdier et al., 2004). However, since the crop cycle of cassava is long, the process to obtain new resistant varieties through conventional breeding is time-consuming. The knowledge on molecular mechanisms of resistance combined with non-conventional strategies for gene transfer or modification may lead to the development of plants with improved resistance.

An historical background on plant-microbe interactions: the discovery of elicitors and receptors

In 1970s scientists discovered plants could recognize pathogen- derived molecules and induce the production of antimicrobial compounds named phytoalexins (Anderson-Prouty and Albersheim, 1975; Keen, 1975). Later it was discovered that similar defense responses were elicited by endogenous plants cell wall polysaccharides during the infection process (Hahn et al., 1981). Altogether, these pathogen-derived or endogenous molecules were called elicitors (Schwessinger and Ronald, 2012). A decade later many elicitors were identified and a new challenge started to look for their corresponding receptors.

By the time mentioned, it was known that plants relied on their organs as passive defense barriers to resist to a wide range of pathogens. Since plants lack specialized cells for immunity, each cell is capable of mounting a robust defense response (Spoel and Dong, 2012). This information lead to suggest receptors must be present in all plant cells to recognize elicitors from the pathogen. In the late 1980s biochemical approaches demonstrated high-affinity binding sites for elicitors in the plasma membrane (Cosio et al., 1988; Shibuya et al., 1993), however they were unable to further purify this plasma membrane receptors. Studies on vertebrate immunity helped

plant pathologist to understand the nature of these receptors. By the same time immunologists reasoned and discovered that microbes must possess pathogen-associated molecular patterns (PAMPs) that are recognized by pattern-recognition receptors (PRR) from the host (Janeway, 1989). Afterwards, several conserved molecules including LPS were identified as PAMPs in animals (Medzhitov and Janeway, 1997). The existence of these conserved molecules in animal pathogens and the parallels with previously described elicitors led plants pathologists to adopt the terms PAMPs and PRRs to explain plant-microbe interactions (Nürnberger and Brunner, 2002). However, this recognition of widely conserved pathogen molecules was likely to be broad-spectrum resistance and did not reconcile with Flor's hypothesis.

The gene-for-gene hypothesis and the zigzag model

In the early 1940s, H. Flor proposed the gene-for-gene hypothesis based on genetic analysis of flax (*Linum usitatissimum*) and the flax rust fungus (*Melampsora lini*) populations. This hypothesis determines that plants and pathogens gene products interact in specific combinations explaining disease resistance specificity. He was the first to employ the terms avirulence (*avr*) and resistance (*R*) genes, to designate those genetic determinants from the pathogen and the host, respectively. Flor found that single Mendelian dominant *R* genes from flax could confer resistance to specific rust strains carrying the corresponding *avr* gene (Flor, 1971). These findings let him to reason that interactions of matching Avr-R pairs, lead to the recognition of the pathogen and the activation of defense responses. Gene-for-gene hypothesis suggested *R* genes product functions as surveillance sensors of microbial molecules. However, many *R* genes did not confer broad-spectrum resistance, instead resistance was limited to some races of the pathogen. This started debates among the plant biologist community about the concept of Avr and elicitors, along with the fact that elicitor molecules could not explain gene-for-gene resistance (Albersheim and Valent, 1978). It appeared that elicitors were responsible for broad-spectrum resistance triggered and had little to do with gene-for-gene resistance. For this reason *R* genes are thought to govern a type of response referred to as "race-specific resistance", "major gene resistance" and "gene-for-gene resistance". Afterwards in the early 1990s the first plant *R* genes were isolated and the architecture of *R* gene product was elucidated (Martin et al., 1993; Whitham et al., 1994;

Lawrence et al., 1995). All the discoveries of distinct classes of R proteins combined with previous debates about apparently unrelated concepts were somehow unified in the zigzag model.

As mentioned above, similarities in the nature and structure of elicitors and receptors between plants and animals led to the adoption of the terms PAMPs and PRRs to explain plant-microbe interactions (Dangl and Jones, 2001; Nürnberger and Brunner, 2002; Ausubel, 2005). To reconcile these terms with the gene-for-gene hypothesis proposed by Flor, a two-layered plant immunity system was proposed. The first layer called PAMP-triggered immunity (PTI) is induced by the recognition of highly conserved molecules named PAMPs by transmembrane PRRs (Chisholm et al., 2006; Jones and Dangl, 2006). Since these conserved molecules are also present in non-pathogenic microorganisms these terms were later changed to MAMPs (microbe-associated molecular patterns) and MTI (MAMP-triggered immunity). A second layer called effector-triggered immunity (ETI) is activated once nucleotide-binding leucine-rich repeats (NLRs) proteins encoded by *R* genes recognize specific effector proteins from the pathogen, following the gene-for-gene hypothesis.

In the zigzag model the plant immune system is represented in four phases (Jones and Dangl, 2006). In the first phase the recognition of MAMPs by PRR triggers the activation of MTI leading to a signaling cascade that is effective against non-adapted pathogens. In a second phase, pathogenic bacteria inject effector proteins to the host cell using the type-3 secretion system (T3SS). Effector proteins interfere with MTI resulting in effector-triggered susceptibility (ETS). In a third phase, plant evolves NLRs proteins to recognize pathogen particular effectors activating a stronger resistance response called ETI. This response is rapid and specific, and is usually associated with the development of a hypersensitive response (HR). In a fourth phase, diversification of effectors is selected to avoid recognition by NLRs. Similarly, the generation of new *R* genes variants is favored to recognize newly evolved effectors and trigger ETI once again (Jones and Dangl, 2006; Chisholm et al., 2006). Under this model the plant immunity is observed as a classical “arms-race” scenario between evolutionary forces from the pathogen and the host.

Plant PRR complex associations

According to the zigzag model, the first layer of defense against pathogens is the recognition of MAMPs by PRRs. Plant PRRs known so far are surface localized receptors from two classes: receptor-like kinases (RLKs) and receptor-like proteins (RLPs). PRR extracellular domain (ECD) may contain a leucine-rich repeat (LRR), lectin motifs, lysine-motifs (LysM) or epidermal growth factor (EGF)-like domains (Macho and Zipfel, 2014). First characterized PRRs were FLS2, EFR and CEBiP-CERK1, known to recognize flagellin (Flg), elongation factor-tu (EF-tu) and chitin, respectively (Gómez-Gómez and Boller, 2000; Kaku et al., 2006; Zipfel et al., 2006; Miya et al., 2007; Liu et al., 2012b).

Flg, a component of the bacterial flagellum, is the archetypal elicitor known to activate MTI. A 22-aa epitope in the N- terminal of Flg (flg22) is recognized by FLS2 and triggers a defense response in *Arabidopsis* (Felix et al., 1999; Gómez-Gómez and Boller, 2000). FLS2 is a RLK with an ECD composed of 28 LRRs involved in ligand binding, a single-pass transmembrane (TM) domain and an intracellular kinase domain. It has been shown that once FLS2 perceives flg22, it quickly forms a complex with a regulatory RLK named BAK1 (BRI1-associated receptor kinase 1) (Chinchilla et al., 2006, 2007). The role of BAK1 or other RLKs from the somatic-embryogenesis receptor-like kinase (SERK) family as coreceptors for MAMP perception is not exclusive to the flagellin perception by FLS2. Moreover, a growing number of PRRs are shown to associate with BAK1 or related SERK proteins for signaling activation (Roux et al., 2011; Liebrand et al., 2013). Although differences in the Flg epitope exists between different species, FLS2 is conserved across plant linages (Boller and Felix, 2009; Mueller et al., 2012).

Chitin, a component of fungal cell wall, is known to act as an elicitor of MTI in both monocots and dicots (Newman et al., 2013). In *Arabidopsis* chitin perception requires homodimerization of the LysM RLK, AtCERK1 (Miya et al., 2007; Wan et al., 2008). AtCERK1 harbors three LysM in the ECD that interact with chitin (Petutschnig et al., 2010; Miya et al., 2007). The intracellular kinase domain probably initiates homodimerization of CERK1 induced by chitin, resulting in downstream signaling (Petutschnig et al., 2010). In contrast to FLS2, BAK1 association is not required for chitin perception by CERK1 (Albrecht et al., 2012). On the other hand, in rice, the chitin elicitor binding protein (CEBiP) is the major LysM RLK involved in chitin perception (Kaku et al., 2006). OsCEBiP is a GPI-anchor protein that harbors three LysM and a C-terminal domain (Kaku et al., 2006). Since OsCEBiP lacks an intracellular kinase or another signaling domain, it requires the formation of a hetero-oligomeric complex with OsCERK1 for chitin-induced signaling

(Kaku et al., 2006; Shimizu et al., 2010). Interestingly, OsCERK1 harbors a single LysM and an intracellular kinase domain but does not bind chitin (Shimizu et al., 2010). Even though FLS2 heterodimerization with BAK1 is important for signal transduction upon Flg perception, for chitin perception the associations with different kinases are required for signaling.

In recent years, several MAMPs and their corresponding PRRs have been identified. Interestingly, from the set of *R* genes isolated in the 1990s, one named *Xa21* encodes a receptor protein with an extracellular LRR domain, a transmembrane domain and an intracellular non-RD kinase domain (Song et al., 1995). Non-RD kinases lack the two conserved residues arginine and aspartate, a motif typical of most kinases that is associated with immune receptor including FLS2 and EFR (Dardick et al., 2012). Usually RD-kinases appear to regulate responses different from immunity or serve as coreceptors of PRRs (Chinchilla et al., 2006; Roux et al., 2011; Schwessinger et al., 2011), with the notable exception of the RD kinase CERK1 in *Arabidopsis* (Liu et al., 2012a). Unlike AtFLS2 and EFR, XA21 does not bind to the RD-kinase BAK1/SERK3 family but is known to associate with rice SERK proteins in the plasma membrane (OsSERK2). XA21 forms a complex with XB3 and XB25 for stability (Wang et al., 2006; Chen et al., 2010c). Recent studies suggest that after ligand binding, a series of conformational changes are induced in the XA21-OsSERK2 complex that induce XA21 transphosphorylation by OsSERK2 (Chen et al., 2014). Although XA21 ligand has not been identified yet, studies have identified several *Xoo* genes that are required for activation of XA21 (*rax* genes) (da Silva et al., 2004; Pruitt et al., 2015). These genes encode for components of a putative type I secretion system (T1SS): RaxA, RaxB and RaxC along with a tyrosine sulfotransferase, RaxST (da Silva et al., 2004). A recent study found *RaxX* as a gene located upstream *RaxST* which encodes for a small peptide that is subjected to tyrosine sulfation by RaxST to activate XA21-mediated immunity (Pruitt et al., 2015).

MTI signaling

Once PRR complexes and receptor-like cytoplasmic kinases (RLCKs) are activated they lead to a rapid production of reactive oxygen species (ROS) during the respiratory burst, activation of mitogen-activated protein kinase (MAPK), activation of Ca^{++}-dependent protein kinases (CDPK), callose deposition, SA/ET/phytoalexin production and the expression of defense-related genes

(Godfrey and Rathjen, 2012; Wu et al., 2014; Li et al., 2016). Earlier responses include an increase in cytosolic Ca^{++} concentrations that can be different in amplitude and duration depending on the MAMP perceived, leading ultimately to ROS production and the activation of CDPKs and MAPK cascades (Tena et al., 2011).

Although in higher plants there are no canonical Ca^{++} selective voltage-gated ion channels, some others ionic channels might allow Ca^{++} entry to the cytosol (Tsuda and Somssich, 2015). Ca^{++} sensor proteins (calmodulin, calcineurin B-like and Ca^{++}-dependent protein kinases) detect Ca^{++} signatures in the cytosol implicated in the generation of ROS, nitric oxide (NO) and transcriptional reprogramming (Tsuda and Somssich, 2015). The burst of ROS produced during immune signaling activation is controlled by plant oxidases called respiratory burst oxidase homolog (RBOHD) (Canton and Grinstein, 2014). Recent findings indicate that two signaling pathways, a Ca^{++} -dependent and Ca^{++} -independent pathway, control the activity of RBOHD (Canton and Grinstein, 2014; Kadota et al., 2014; Li et al., 2014). Interestingly, both pathways of RBOHD activation are mediated by BIK1, a RLCK associated to the PRR complex (Li et al., 2014). Upon MAMPs perception BIK1 directly binds and phosphorylates RBOHD (Ca^{++}-independent) and also positively regulates the increase in cytosolic Ca^{++} concentration (Ca^{++}-dependent) leading to the MAMP-induced ROS production (Kadota et al., 2014; Li et al., 2014). The direct RBOHD phosphorylation by BIK1 occurs in two serine residues (Ser39 and Ser343) while the Ca^{++}-dependent pathway is caused through the activation of CPK5 which possibly phosphorylates RBOHD in different residues (Kadota et al., 2014; Li et al., 2014).

In regard to other MTI signaling components, MAPK cascades play essential roles in signal amplification and for transducing the signal from PRR complexes to downstream components (Tena et al., 2011; Meng and Zhang, 2013). Both genetic and biochemical studies have identified two different MAPK cascades activated upon flg22 perception. The first consist of MEKK1 (a MAPKKK), MKK4/MKK5 (two MAPKKs), and MPK3/MPK6; and the second cascade is constituted by MEKK1, MKK1/MKK2 (two MAPKKs), and MPK4 (Meng and Zhang, 2013). In the first cascade, MPK3/MPK6 directly phosphorylate cyclin-dependent kinase Cs (CDKCs), which in turn phosphorylate the C-terminal domain (CTD) of RNAPII leading to a simultaneous transcriptional activation of a different subset of genes (Li et al., 2016). However, MAPK can also phosphorylate individual TFs such as WRKY33, ERF6/ERF104, BES1 and ASR3, which are involved in the activation of primary MTI responses (Li et al., 2016). One of the best studied substrates of MAPKs is the family of WRKY plant-specific transcription factors which play both positive and negative

roles during the plant defense response (Buscaill and Rivas, 2014). The *WRKY* gene family harbors >100 members in rice and >70 members in *Arabidopsis* (Tsuda and Somssich, 2015). Members of this family are induced under pathogen attack and abiotic stress, suggesting a wide role in stress responses (Tsuda and Somssich, 2015; Li et al., 2016). For example WRKY members are known to control the expression of genes encoding for *RBOHs* responsible for the respiratory burst. Some particular examples includes *WRKY22* and *WRKY29* that are induced upon flg22 treatment in *Arabidopsis*, while *WRKY33* is induced by necrotrophic fungus to modulate genes involved in the production of phytoalexins (Li et al., 2016). Another MTI response is the formation of callose deposits for the reinforcement of the cell wall. The regulation of genes involved in the biosynthesis and modification of precursors for callose production depend on the MYB51 transcription factor (Li et al., 2016).

Bacteria ingenious strategies: type 3 effectors and the suppression of plant immunity

MTI responses are sufficient to restrict most microbes, while plant pathogens are those able to suppress or evade this first layer of immunity in order to cause disease. One successful adaptation employed by pathogens is the deployment of diverse effectors to suppress the first plant surveillance system at distinct levels (Jones and Dangl, 2006; Lee et al., 2013). As mentioned above, bacterial type 3 effectors (T3Es) are injected through the T3SS into the cytoplasm of the plant cell. Bacterial pathogens can code for around 20-30 effectors proteins while oomycetes or fungi genomes can code hundreds of them (Kamoun, 2006; Lindeberg et al., 2012). The pangenome, full collection of genes, of *P. syringae* code for 57 families of effectors designated as Hop (Hrp outer protein) (Lindeberg et al., 2012). Most of the knowledge of T3E targets inside the host came from the study of Hop proteins. Once inside the cytoplasm they are able to move to distinct organelles to interfere with plant cell immune responses. Among the signaling pathways known to be suppressed by T3Es are PRR complex activation, MAPK signaling, callose deposition, vesicle trafficking, microtubule networks, SA and ROS responses in the chloroplast, NLR signaling activation and RNA metabolism (Block and Alfano, 2011; Deslandes and Rivas, 2012; Lindeberg et al., 2012; Lee et al., 2013; Bigeard et al., 2015). However, despite targeting several host immune processes, effectors seem to target host highly connected proteins (hubs) to subvert plant

immune responses. Recent studies of protein-protein interaction networks from effectors and *Arabidopsis* proteins, revealed effectors from three evolutionary diverse pathogen converged on a limited set of highly interacting host proteins (Mukhtar et al., 2011; Webling et al., 2014). Interestingly, this data implies effector target convergence evolved independently in the three kingdoms, suggesting convergence is an important feature in host-pathogen interactions (Webling et al., 2014).

Nevertheless, not all T3Es virulence target are proteins that reside in the cytoplasm, some of them travel to the plant nucleus where a direct interaction with promoters of particular genes explains its virulence function. Members of this particular family of T3Es act as bona fide transcription factors in the plant nucleus, conferring them the name of transcription-activator like (TAL) effectors (Boch et al., 2014). Once in the plant nucleus, TAL effectors bind to promoter regions of *susceptibility* (*S*) genes and thereby promote disease development (Schornack et al., 2013; Boch et al., 2014). In contrast, the plant strategy for the recognition of TAL effectors is considerably different from the recognition strategies for other T3Es and involves the expression of executor *R (E)* genes in a TAL effector-dependent fashion (Schornack et al., 2013; Boch et al., 2014). Notably, *E* genes identified to date code for proteins with diverse structure, these proteins have been conceptually classified in two groups (Zhang et al., 2015). The first one includes proteins with a possible function in plat development and physiology, such as Bs3 which is a flavin mono-oxygenase (Römer et al., 2007). The second group consist of small proteins with multiple hydrophobic domains and includes the rest of the identified E proteins (Zhang et al., 2015).

TAL effectors are modular proteins that consist of N-terminal type three secretion signal followed by a unique central variable repeat region and a C-terminal region harboring an acidic activation domain (AAD) and nuclear localization signal (NLS) (Schornack et al., 2013). Both the N-terminal and the C-terminal regions are highly conserved among TALs, while the central repeat region is highly variable and determines the DNA binding and specificity (Mak et al., 2013). Collectively the repeats mediate the direct base-specific interaction with the plant promoters in the so-called effector binding elements (EBEs). Central DNA-binding repeats are usually 33-35 amino acids long with a conserved sequence except in the position 12 and 13 where a high variation in the identity of residues have been found, for this reason these two positions are termed repeat variable diresidues or RVD (Boch et al., 2014). Interestingly, crystal structure studies showed that the base-specific contact with the plant DNA is mediated only by the residue 13 (Deng et al., 2012;

Mak et al., 2012). As a result, this residue has also been called the base-specifying residue (BSR) (de Lange et al., 2014).

Although the functional activity of TAL effectors in plant transcription modulation has been well documented during the last years, its role in bacterial virulence has only been recently elucidated. Despite the fact that TAL effectors are non-core T3Es in *Xanthomonas*, in some cases TAL effector mutants are severely compromised in virulence, and are therefore named "major TALs" (Yang and White, 2004). Interestingly, to date only gene activation, not repression has been proved to be generated through TAL effector binding. To date, plant *S* genes induced by TAL effectors are transcription factors, sugar transporters, sulfate transporters and genes involved in miRNA stability (Boch et al., 2014; Hutin et al., 2015a). The first reported and most studied host *S* genes are members of the *MtN3/saliva/SWEET* family, which encode for membrane proteins with diverse cellular functions (Yang et al., 2006; Liu et al., 2011). It has been showed that at least four different TAL effectors from *X. oryzae* pv. oryzae *(Xoo)* induce *OsSWEET14* at overlapping or entirely different binding sites (Boch et al., 2014; Hutin et al., 2015a). Since *OsSWEET14* codes for a hexose transporter in the plasma membrane that transports sugars to the intercellular space, it has been proposed that it might re-mobilize carbon source to the nutrient poor intercellular spaces for *Xanthomonas* growth (Chen et al., 2010a; Chen, 2014). Interestingly, TAL effectors from *X. citri* ssp. *citri* and *Xam* also target other members of the SWEET family in citrus and cassava, respectively (Cohn et al., 2014; Hu et al., 2014). Thus, functional convergence of TAL effectors targeting *SWEET* genes reinforces its role as crucial *S* genes in the *Xanthomonas* genus.

Curiously TAL effectors have only been widely identified in species of the genus *Xanthomonas*. Also, TAL effector-like proteins have been identified in *Ralstonia solanacearum* called Ralstonia injected proteins similar to TAL effectors (RipTALs) (de Lange et al., 2013, 2014). RipTAL are known to contain functional NLS and AAD and their base specificity is also defined by the amino acid residue 13 (de Lange et al., 2013). However, compared with *Xanthomonas* TAL effectors, non-RVD residues are also polymorphic with an effect on DNA binding (de Lange et al., 2013). TAL effector-like proteins with variable non-RVDs have also been identified in the fungal endosymbiont *Burkholderia rhizoxinica* (Juillerat et al., 2014). Nevertheless, despite repeat divergence the use of RVDs in both *Xanthomonas* and *B. rhizoxinica* is very similar (Schornack et al., 2013; Juillerat et al., 2014). Functional studies of these TAL effector-like proteins will aid to further understand the relevance of non-RVDs in TAL effector DNA binding and specificity.

Genomic organization and diversification of NLRs

As mentioned above, one of the main strategies pathogens employ to proliferate within host tissues is by sending virulence factors trough the T3SS, called T3Es. In return, plants developed R proteins to halt pathogen proliferation resulting in disease resistance. This "endless arms race" between the pathogen and the host implies a high diversification of both effectors and R proteins. Plant genomes harbor from dozens of *R* genes coding for NLRs (e.g. 54 in *Carica papaya*) to almost a thousand of them (e.g. 962 in *Malus* x *domestica*) (Porter et al., 2009; Velasco et al., 2010). Genomic analyses indicate both pathogen effectors and plant *R* genes are located in regions with exceptionally high mutation rates resulting in a rapid generation of diversity (Karasov et al., 2014). Comparative genomic studies have revealed *NLR* genes are among the most rapidly evolving genes and that new specificities arise as a response to pathogen pressure (Meyers et al., 2005; Guo et al., 2011; Karasov et al., 2014). One possible explanation for the high polymorphic variation in NLR sequences is their genomic location. *R* loci in the genome are present as either single genes or clusters (Meyers et al., 2005). Single *R* genes may consist of a unique resistant allele (e.g. *RPM1* in *Arabidopsis*) or multiple alleles (e.g. *L* locus in flax). In *Arabidopsis*, it has been shown that approximately two-thirds of NLR coding genes are located in 43 clusters (Meyers et al., 2003). Interestingly these clusters of NLRs are associated with high levels of polymorphisms (Clark et al., 2007). Similarly, *Medicago truncatula* and japonica rice harbor an 80% and 76% of their *NLR* within clusters, respectively (Zhou et al., 2004; Ameline-Torregrosa et al., 2007). Some of the molecular processes that contributes to *NLR* diversification include segmental duplications, non-homologous recombination, unequal crossing over, gene conversion and slippage of DNA polymerase (Zhou et al., 2004; Ameline-Torregrosa et al., 2007; Yang et al., 2008; Chen et al., 2010b; Guo et al., 2011). Nevertheless, the complete NLR sequence is not subjected to high variation, the C-terminal domain is the most variable region of NLRs and the central region the most conserved (Zhou et al., 2004).

Molecular architecture of NLRs

NLRs are modular proteins with three different evolving building blocks: a variable N-terminal region followed by a conserved central NB domain and a C-terminal LRR domain with variable

repeat number (Takken and Goverse, 2012). According to the domains present in their N-terminal region, NLRs fall in two major groups. NLRs with a Toll–interleukin 1 receptor (TIR) domain are called TNLs, and those with a Coiled-coil (CC) are called CNLs (Griebel et al., 2014; Takken and Goverse, 2012). LRR is the most variable domain and is mainly involved in effector recognition (Griebel et al., 2014; Michelmore et al., 2013). Although the LRR motif is present in proteins with diverse biological functions, structural studies of this motif suggest it provides a suitable framework for protein-protein interactions (Kobe, 2001; Bella et al., 2008). The domain is composed of repetitive LRR motifs from of 20-29 residues in length with interspaced hydrophobic residues (Bella et al., 2008). The number of repeats can vary dramatically. In *Arabidopsis* NLRs range from 8 to 25 repeats (Meyers et al., 2003; Tamura and Tachida, 2011). To date, there is a lack of crystal structure for plant NLRs, nevertheless structural studies from receptors harboring LRR in other species have been used for homology-based modeling (Sela et al., 2012). Canonical LRRs display a banana-shape with β sheets on the concave surfaces and α helices on their convex surfaces harboring solvent exposed residues for ligand interactions and hydrophobic residues conferring structure stability (Bella et al., 2008). The high sequence similarity and repetitions predisposes the LRR domain to diversification via duplications, deletions and fusion events. In particular, the uncommon phenomenon of illegitimate recombination have been proposed to drive initial duplication events in the LRR domain (Wicker et al., 2007).

In contrast, the central and N-terminal domains are more conserved regions involved in NLR activation and signal transduction (Takken and Goverse, 2012; Jacob et al., 2013). Plant NLRs belong to the AP-ATPase family of STAND (signal transduction ATPases with numerous domains) proteins (Lukasik and Takken, 2009; Bonardi et al., 2012). The ATPase activity of NLR is encoded in the NB domain which possess several conserved motifs important for its ADP/ATP binding function (van Ooijen et al., 2008a). Since the NB domain of R proteins is shared with the human apoptotic protease-activating factor 1 (Apaf-1) and its *Caenorhabditis elegans* homolog CED-4, this domain is also referred as the NB-ARC domain (nucleotide binding domain shared between Apaf-1, resistance proteins and CED4) (van der Biezen and Jones, 1998). No crystal structure is available for a plant NB-ARC domain, but the structure of animal NLRs including APAF-1 and recently NLRC4 have provided insights into the NB-ARC structure and dynamics (Stefan et al., 2005; Hu et al., 2013). These structural studies indicate NB-ARC domain is subdivided in four subdomains NB, ARC1, ARC2 and ARC3. However, plant NLRs lack the ARC3 subdomain which is important for animal NLRs oligomerization (Griebel et al., 2014). The NB subdomain has five

conserved motifs: hhGRExE, Walker-A/P-loop, RNBS-A, Walker-B/kinase 2 and RNBS-B/hhhhToR (Meyers et al., 2003; Leipe et al., 2004). Among this five, Walker-A and Walker-B are highly conserved motifs for P-loop NTPases (Takken et al., 2006). Near the N- terminus of the Walker-A motif, one conserved motif called hhGRExE is present. This motif has a predicted helical structure and may represent a linker region between the N-terminal domain and the NB subdomain (Leipe et al., 2004). Walker-A motif has the GVGKTT consensus sequence for both TNLs and CNLs (Lukasik and Takken, 2009). The lysine residue in this sequence might be responsible for binding to α-phosphate moiety of the bound nucleotide, while the next two threonine residues possibly interact with Mg^{++} ion (Walker et al., 1982; Takken et al., 2006). On the other hand, the first aspartate in the Walker-B motif is required for nucleotide binding and ATPase activity and also coordinates the Mg^{++} ion (van Ooijen et al., 2008b; Bonardi et al., 2012).

In the ARC subdomains ARC1 forms a four-helix bundle and ARC2 adopts a winged-helix fold (van Ooijen et al., 2008b; Griebel et al., 2014). In contrast to the NB and ARC2 subdomains, no mutations to produce a gain or loss of function have been reported in the ARC1 subdomain (van Ooijen et al., 2008b). A single motif with the consensus sequence GLPLA is found in the ARC1 and two in ARC2 named RNBS-D and MHD (Lukasik and Takken, 2009). The majority of mutations that produce HR in the absence of the pathogen or a cognate Avr proteins, called autoactivating mutations, map to the MHD motif in the ARC2 subdomain (van Ooijen et al., 2008b). An aspartate (D) to valine (V) substitution in the third residue of this motif in the potato NLR Rx induced an HR when transiently expressed in *N. benthamiana* leaves (Bendahmane et al., 2002). The D to V substitution has shown to induce autoactivity in other NLRs such as L6, I-2, M, MLA10, RPM1, ADR-L2, Rp1-D21 and PM3F (De La Fuente Van Bentem et al., 2005; Howles et al., 2005; Rairdan and Moffett, 2006; Gao et al., 2011; Williams et al., 2011; Bai et al., 2012; Roberts et al., 2013; Stirnweis et al., 2014; Wang et al., 2015c).

The N-terminal region is highly variable for both TNLs and CNLs (Takken and Goverse, 2012; Griebel et al., 2014). Interestingly CNLs are present in both monocots and dicots, whereas TNLs are restricted to dicots (Yue et al., 2012; Jacob et al., 2013). Crystal structure from the N-terminal region of CNL of barley MLA10 and L6 a TNL from flax are now available. In MLA10, two CC protomers forms a rod-shape homodimer with an extensive interactive surface (Maekawa et al., 2011a). Homology-based remote modeling suggest RPM1 and Lr10 CC domain produce similar structures (Sela et al., 2012). Similarly, structural analysis of the TIR domain from L6 indicates it forms an asymmetrical globular shape unit with two monomers (Bernoux et al., 2011b). This

structure is similar to other proteins consisting only on a TIR domain (Chan et al., 2010). These molecular and structural studies suggest that regardless of the N-terminal domain, both TNLs and CNLs adopt dimer conformation important for downstream immune signaling.

Pathogen recognition by NLRs

Once Flor's hypothesis settles the genetic basis of plant-pathogen interactions, the next step was to decipher how plant R proteins detect pathogen effectors. One molecular explanation for this hypothesis implies a direct interaction between the NLR and the effector proteins (Cui et al., 2015). Several *in vitro* interaction assays and yeast two-hybrid have demonstrated direct NLR-effector interactions in genes from flax (AvrM-M; AvrL567-L5/L6), *Arabidopsis* (RRS1-PopC2) and rice (AvrPita-Pi-ta; AvrPik-Pik) (Jia et al., 2000; Deslandes et al., 2003; Dodds et al., 2006; Catanzariti et al., 2010; Kanzaki et al., 2012; Ravensdale et al., 2012). Nevertheless, not all studied NLRs have shown to directly interact with its cognate effector and sometimes there is a lack of one to one correlation between NLR/Avr interactions.

Recent studies suggest an emerging panorama whereas one NLR or NLRs pair can recognize more than one effector protein. A second explanation for Avr recognition is an indirect detection of pathogen effectors by sensing its action on a set of host proteins (Khan et al., 2016). Three models for indirect recognition have been proposed, the guard model, the decoy model and the integrated decoy model. In the guard model, a NLR is bound and monitoring (guarding) a host factor involved in immunity that is modified by the effector (Jones and Dangl, 2006). Molecular evidence for this model comes from studies of two plasma membrane CNL receptors: RPM1 (resistance to *Pseudomonas syringae* pv. maculicola 1) and RPS2 (resistance to *Pseudomonas syringae* 2). Both RPM1 and RPS2 are constitutively guarding RIN4 (RPM1-interacting protein 4), which is targeted by three different *P. syringae* effectors: AvrB, AvrRpt2 and AvrRpm1 (Axtell and Staskawicz, 2003; Mackey et al., 2003; Kim et al., 2005). In the absence of AvrB, AvrRpt2 or AvrRpm1, RIN4 is a regulator of MAMP defense signaling (Kim et al., 2005; Liu et al., 2009). Once *P. syringae* deliver AvrB, AvrRpt2 or AvrRpm1 they target RIN4 and associated proteins to disrupt MTI signaling. This perturbation in RIN4 is sensed by RPM1 and RPS2 eliciting a resistance response. However, the guard model does not explain well the fact that pathogen effectors target

some monitored host factors that are not involved in immunity. In this scenario, the decoy model states these host factors, that are similar in structure to defense factors, serve as baits to trap effectors and trigger ETI (van der Hoorn and Kamoun, 2008). The biological function of AvrAC/XopAC, a T3E from *X. campestris* pv. campestris (*Xcc*), represents an interesting example with molecular support of this model. AvrAC virulence function relies on the uridylylation of receptor-like cytoplasmic kinases, including BIK1, in order to inhibit its kinase activity and consequently to block the downstream immune signaling in *Arabidopsis* (Feng et al., 2012). Similarly, a paralog of BIK1, PBL2 is also a substrate of AvrAC. Once AvrAC uridylylates PBL2, it is recognized by a pseudokinase-NLR complex (RKS1-ZAR1) to trigger ETI (Wang et al., 2015b). Thus, *Arabidopsis* has evolved decoy substrates with any other apparent function, such as PBL2, solely to recognize effector activity and trigger immunity.

Lately an integrated decoy model has been proposed as a third mechanism to explain effector recognition by NLRs pairs (Cesari et al., 2014). At least eight physically linked NLRs pairs have been identified in several genomes from both monocots and dicots, but perhaps the most studied are the CNL pair RGA4/RGA5 (resistance gene analog 4 and 5) from rice and the TNL pair RPS4 (resistance to *Pseudomomas syringae4*)/RRS1 (resistance to *Ralstonia solanacearum*1) from *Arabidopsis* (Cesari et al., 2014; Griebel et al., 2014). In this model two NLRs forming a heterodimeric complex are necessary for the recognition of effector proteins. One NLR acts as a receptor for Avr recognition (RRS1 and RGA5) and the other for downstream signaling (RPS4 and RGA4) (Cesari et al., 2014). In the case of RGA5 the bait is integrated in the C-terminal region (RATX1 domain) working as a decoy for effector recognition while in the case of RRS1 there is WRKY domain.

NLR activation and signaling

Several structural and biochemical studies indicate in the absence of pathogens NLRs are in a resting auto-inhibited state. As a consequence of pathogen perception by LRR domain, a signal is transduced to the rest of the protein to lift the steady-state intramolecular inhibition conferred by

the N-terminal region of the LRR domain (Takken and Goverse, 2012). A role of the LRR domain in auto-inhibition of NLRs was demonstrated with domain swap studies in RPS5 where the LRR domain was shown to suppress the activity of RPS5 in the absence of PBS1 cleavage (Qi et al., 2012). As mentioned earlier, mutations in several motifs on NB-ARC domain usually result in autoactivity, suggesting that in the absence of a pathogen, this domain exert a negative regulatory activity (Howles et al., 2005; Rairdan and Moffett, 2006; Gao et al., 2011; Williams et al., 2011; Roberts et al., 2013; Stirnweis et al., 2014). Studies on I-2 and Mi-1 from tomato, L and M from flax, and MLA27 from barley have shown these proteins function as molecular "switch" where the resting "off" state is bound to ADP, while the active "on" conformation is bound to ATP (Tameling et al., 2002; Maekawa et al., 2011a; Williams et al., 2011). Upon pathogen recognition, the LRR domain releases the intramolecular inhibition and a distinct configuration is adopted facilitating ADP exchange to ATP leading to NLR activation. Evidence of this model has been observed in I-2 from tomato and the M from flax were ATP bound states results in activation (Tameling et al., 2006; Williams et al., 2011). In addition, the open state conformation seem to expose certain areas that might be involved in the interaction with other proteins (Takken and Goverse, 2012). Supporting this idea, yeast-two hybrid assays indicate ADP, ATP and empty states display distinct interaction patterns confirming the intermolecular interactions in activation model (Lukasik-Shreepaathy et al., 2012).

Despite the variability of the C-terminal region of NLRs, its role in NLR function seems to be restricted to effector recognition and intramolecular molecular inhibition of the NB-ARC and N-terminal domains in the absence of the pathogen (Takken and Goverse, 2012). Structural studies suggest the N- and C-terminal regions of the LRR domain forms two subdomains that might be involved in two different functions (Sela et al., 2012). The LRR domain from Lr10 from wheat, Rx from potato, MLA10 from barley and L from flax possess solvent exposed highly variable regions under positive selection which is in concordance with a role in pathogen recognition and ligand binding (Dodds et al., 2006; Farnham and Baulcombe, 2006; Seeholzer et al., 2010; Sela et al., 2012). Perhaps one of the most studied interactions is the direct recognition of AvrL567 by L5 and L6. The presence of polymorphisms in the N- and C-terminal in the LRR domain of *L* alleles and its requirement for pathogen specificity of AvrL567 variants, suggest AvrL567 interact with both ends of the banana-shaped structure of the LRR domain of L5/L6 (Ravensdale et al., 2012). Also, single, double and triple mutants in AvrL567 showed additive and cumulative contribution to recognition specificity. Furthermore, polymorphisms in the NB- ARC and TIR domains affects effector binding, suggesting AvrL567 competes for binding with intramolecular interactions that

maintain the NLR in an inactive signaling state (Ravensdale et al., 2012). This is in accordance with the current model of NLR activation (Takken and Goverse, 2012; Cui et al., 2015).

Crystal structure studies of the CC and TIR domains from MLA10 and L6, respectively, indicate both proteins forms homodimers that result in induction of a cell-death response (Maekawa et al., 2011a; Bernoux et al., 2011b). However, it seems that MLA CC forms a constitutive dimer, while TIR dimers from L6 and N are formed after pathogen perception, demonstrating important differences in the activation of TNL receptors compared with CNL receptors (Mestre, 2006; Maekawa et al., 2011a; Bernoux et al., 2011b). An HR was also evidenced when the CC and TIR domains alone were transiently expressed in the absence of the pathogen, indicating oligomeric complexes are required for both CNL and TNL signaling (Bernoux et al., 2011b; Maekawa et al., 2011a). These results also suggest the N-terminal domain harbor self-sufficient signaling activities. Similarly, the NB-ARC domain of the CNL receptor from Rx in potato, which confer resistance to Potato virus X (PVX) has also showed to be sufficient to induce a cell-death response (Rairdan et al., 2008). Nevertheless, the elicitation of a hypersensitive cell-death response as a truly indicative of resistance via NLR signaling activation is still not clear. Several studies indicate a restriction in pathogen growth is uncoupled from the elicitation of host cell death in the infection site, suggesting plant immunity does not require cell death *per se* (Bendahmane et al., 1999; Gassmann, 2005; Coll et al., 2010; Heidrich et al., 2011; Bai et al., 2012). Interestingly, it appears that nucleocytoplasmic traffic is required for MLA10 from barley, N from tobacco and RPS4 and SNC1 from *Arabidopsis* to induce a resistance response (Shen and Schulze-Lefert, 2007; Bernoux et al., 2011a; Heidrich et al., 2011; Bai et al., 2012). Even though nuclear localization signals (NLS) have not been identified in many of these receptors, their nuclear localization is required for resistance (Burch-Smith et al., 2007; Meier and Somers, 2011; Bai et al., 2012). For example, after pathogen detection MLA10 is known to localize in the nucleus where is recruited by WRKY transcription factors leading to immune transcriptional reprogramming (Shen et al., 2007). In the absence of the pathogen, these WRKY transcription factors act as negative regulators of PTI. Posterior work showed that cell death signaling activity of MLA10 is suppressed in the nucleus but enhanced in the cytoplasm (Bai et al., 2012). Interestingly, nuclear MLA10 is capable of conferring disease resistance despite cell death signaling was found to be suppressed in this compartment (Bai et al., 2012). These findings provide a model where cell death signaling uncouples from disease resistance in a compartment- dependent manner. The opposite seems to occur with potato Rx where the cytoplasmic Rx is required for signaling activation, however both nuclear and cytoplasmic Rx pools are necessary for the correct

regulation of defense signaling (Slootweg et al., 2010). In this scenario, a unique model of NLR activation and resistance signaling cannot be established, instead different models of subcellular localization and NLR signaling activation have been proposed (Bernoux et al., 2011a), suggesting multiple downstream signaling pathways once NLRs are activated.

NLRs regulation

Since the activation of NLRs is related with the induction of an HR they are under strict molecular regulation with critical and low NLRs expression levels. One of the early regulatory nodes in NLR activation is their transcriptional control. Recent studies of natural epigenomic variation in *Arabidopsis* indicate the NLRs gene family display the higher frequency of differentially methylated regions, suggesting a high transcriptional regulation (Schmitz et al., 2013). An example that supports this idea is the transcriptional regulation of RPP4 and SNC1, two TNLs from *Arabidopsis*. Both SNC1 and RPP4 require the nuclear protein MOS9 to regulate its expression levels through the histone methyltransferase ATXR7 (Xia et al., 2013). Global analysis of *Arabidopsis* NLRs show general low expression levels with tissue-specific expression patterns (Tan et al., 2007). Similar results were recently obtained from genome-wide analysis of NLRs in sorghum were they showed low basal expression levels (Wang et al., 2016). This is consistent with the notion that NLRs are low expressed to constitutively respond to pathogen attack and subsequently induce a rapid signaling response (Michelmore et al., 2013). Recent transcriptomic analysis suggests some NLRs expression is induced after pathogen perception in *Malus x domestica* while in Eucalyptus some NLRs are differentially expressed in resistant compared with susceptible cultivars (Arya et al., 2014; Christie et al., 2016). An induced expression after pathogen treatment has been demonstrated for the RPP family through WRKY transcription factors (Mohr et al., 2010). A pathogen-induced expression has also been demonstrated for CNLs in rice such as *Xa1, Xa3, Xa27* and *Pib*; the barley *Mla6* and *Mla13*; and in Solanaceus *N* and *RB* TNL genes (Yoshimura et al., 1998; Halterman, 2003; Levy et al., 2004; Cao et al., 2007; Kramer et al., 2009). This data indicate NLRs display a low basal expression in unchallenged plants and that several stimuli during compatible or incompatible interactions might lead to their upregulation. However this was not the case in a global expression analysis in *Arabidopsis* where most of NLRs

studied did not significantly change their expression levels when treated with SA or JA (Tan et al., 2007). This conflictive data also suggest NLRs expression could be regulated by several molecular mechanisms.

Furthermore some NLR RNAs are known to exhibit co-transcriptional and post-transcriptional regulation. RNA regulation in NLRs includes alternative splicing, RNA silencing via microRNAs and siRNAs (small interfering RNAs) and nonsense-mRNA decay (Staiger et al., 2013). Genome wide analysis indicate at least 30 genes *Arabidopsis* are known to suffer alternative splicing (Wang and Brendel, 2006; Tan et al., 2007). In wheat some alleles of Lr19 show alternative splicing sites in the LRR domain that results in a difference of five LRR motifs. However, the shorter alternative variant still produces an arc-shaped LRR suggesting it might be involved in the recognition of other pathogen races (Sela et al., 2012). Several families of microRNAs have been identified to target mRNA of TNLs and CNLs from Solanaceus plants and legumes (Zhai et al., 2011; Li et al., 2012a; Shivaprasad et al., 2012). In particular, the superfamily that includes miR482 and miR2118 target the coding sequence of the P-loop motif in NLRs mRNA from tomato (Shivaprasad et al., 2012). In *M. truncatula* microRNAs regulate more than 60% of NLRs through phased trans-acting siRNAs, a regulation network important for their symbiosis with Rhizobia that is apparently absent in *Arabidopsis* (Zhai et al., 2011). Recently, nonsense-mediated mRNA decay (NMD) was shown to control the turnover of several TNLs from *Arabidopsis* (Gloggnitzer et al., 2014). Thus, NMD function at the post-transcriptional level controlling the threshold for activation of TNLs. In addition to co- and post-transcriptional regulation of mRNA, the protein degradation machinery further regulates NLR protein levels. Some of the proteins required for proper folding of NLRs are HSP90, an ATP-operated molecular chaperone, co-chaperones RAR1, PP5 and SGT1, and ATP-independent small HSP (Cheng and Li, 2012; Takken and Goverse, 2012). As an example, silencing of RSI2, a small HSP, compromised the development of an HR and the accumulation of I-2R, suggesting its importance for the stability and function of I-2R (Van Ooijen et al., 2010). However, not all chaperones are positive regulators for NLR stability, SGT1 appears to be a negative regulator of NLR accumulation (Takken and Goverse, 2012). SGT1 is known to interact with the ubiquitin E3 ligase SCF (Skp1–Cullin–F-box) complex that target proteins for proteasome degradation (Cheng and Li, 2012; Takken and Goverse, 2012).

Enhancing plant resistance to diseases through the knowledge of molecular plant-microbe interactions

One of the main goals for crop improvement is to obtain durable and broad-spectrum resistance (DBSR). Durable resistance (DR) is a concept used to refer the efficiency in large land extensions during a prolonged period of time, suitable for the development of the disease (Kou and Wang, 2010). On the other hand, broad-spectrum resistance (BSR) is defined to as the resistance for two or more variants of a pathogen species or to most of the races of the same species (Kou and Wang, 2010). To accomplish DBSR in crop plants distinct approaches have been successfully employed. Notably, the study of the genetics and molecular determinants of qualitative and quantitative resistance combined with pathogen virulence have proven to be valuable and robust strategies to achieve DBSR (Boyd et al., 2013; Dangl et al., 2013; Michelmore et al., 2013).

Understanding the role of plant immune receptors in disease resistance have allowed to develop several strategies which have proven to be successful to improve resistance to diseases (Boyd et al., 2013). One strategy is based on the use of PRRs to recognize conserved MAMPs to species that lack them. The first example was the use of EFR. EFR is restricted to the Brassicaceae family and was transferred to tobacco and tomato (Solanaceae) where it showed enhanced resistance to different bacterial pathogens (Lacombe et al., 2010). Recently, transgenic introduction of XA21 from rice to tomato, banana and sweet orange was shown to confer interfamily resistance to diseases caused by *Xanthomonas* and *Pseudomonas* (Mendes et al., 2010; Afroz et al., 2011; Tripathi et al., 2014). Also, the PRR called *Verticillium 1 (Ve1)* from tomato was transferred to *Arabidopsis* conferring resistance to *Verticillium* isolates (Fradin et al., 2011). These results suggest a relatively high degree of conservation of the PTI transduction pathways and the ability of PRRs from different plant species to activate an effective immune response.

A second strategy for crop improvement has been the cross-species introduction of NLRs (Mukhtar, 2013). The knowledge of effector distribution across distinct pathogens led to an effector-based approach for NLR cross-species transference (Dangl et al., 2013). The first notable example of this rationality was the transference of *Bs2* from pepper to tomato (Tai et al., 1999). This approach has been extensively used among related Solanaceae species and also within Poaceae species (Wulff et al., 2011). However, when NLRs are stably transferred to distant related species they have failed to show the expected improved resistance (Tai et al., 1999; Wulff et al., 2011). As mentioned earlier, our understanding of NLR implies a co-evolution between the host and adapted pathogens, leading to the so-called gene-for-gene or race-specific resistance. This

denotes a taxonomical restriction on NLR function, by which NLRs functional range is limited to the same species or close-related species within the same family, a phenomenon called restricted taxonomic functionality (Tai et al., 1999; Wulff et al., 2011; Mukhtar, 2013). Nevertheless, three studies have shown enhanced resistance conferred by stable transgenic expression of NLRs from distant families (Xiao et al., 2003; Maekawa et al., 2012; Narusaka et al., 2013). The first was achieved using two atypical *R* genes from the RPW8 (resistance to powdery mildew8) locus called *RPW8.1* and *RPW8.2* that confer BSR to powdery mildew fungi in *Arabidopsis* (Xiao et al., 2001). *RPW8* belong to a distinct class of *R* genes coding for receptors harboring an N-terminal transmembrane domain and a rare CC domain, however are known to recruit proteins required for NLR activation (Xiao et al., 2001, 2005). Remarkably, transgenic plants of *N. benthamiana* and *N. tabacum* expressing *RPW8.1* and *RPW8.2* from *Arabidopsis* showed enhanced resistance to powdery mildew pathogens *Erysiphe orontii* and *Oidium lycopersici* (Xiao et al., 2003). Besides this report, two recent studies showed further support of NLRs downstream signaling conservation across distant plant lineages (Maekawa et al., 2012; Narusaka et al., 2013). As mentioned above, RPS4 and RRS1 are two genetically linked TNLs from *Arabidopsis* that function together to confer resistance against *Colletotrichum higginsianum* and *Pst DC3000* carrying the effector AvrRps4 and *R. solanacearum* strains, which express the PopP2 effector (Deslandes et al., 2003; Narusaka et al., 2009). Importantly, the transference of RPS4 and RRS1 to other species from the Brassicaceae family (*Brassica rapa* and *Brassica napus*), but also to other species from the Solanaceae family (*S. lycopersicum* and *N. benthamiana*) and to cucumber (Cucubirtaceae) conferred resistance to bacterial and fungal pathogens (Narusaka et al., 2013). A remarkable example was the functional transference of a CNL receptor from monocots to dicots, lineages that diverged nearly 200 million years ago (Maekawa et al., 2012). The receptor MLA1 from barley was expressed in partially immunocompromised *Arabidopsis* plants where it showed to be fully functional and conferred isolate specific resistance to the barley pathogen *Blumeria graminis* f. sp. hordei (Maekawa et al., 2012). All together these studies have shown the possibility of *R* gene transfer between related and distant species.

A third strategy to engineer disease resistance has been achieved through the knowledge of TAL effector biology. As mentioned earlier, TAL effectors work as transcription factors to induce the expression of genes via binding to EBEs in the promoter region of host genes. TAL effectors dependent *R* genes have been classified as recessive, dominant non-transcriptional and dominant transcriptionally induced by TAL effectors (Zhang et al., 2015). The first group, recessive *R* genes, emerge from loss-of-function of susceptibility *(S)* alleles (Schornack et al., 2013; Hutin et al.,

2015a). In rice, two recessive resistance alleles, *xa13* and *xa25*, arise from EBE variants in the promoter region of *OsSWEET11* and *OsSWEET13* that avoid TAL effector binding leading to loss of susceptibility (Chu et al., 2006; Liu et al., 2011; Zhang et al., 2015). Recently, a study of natural diversity on the promoter region of *OsSWEET14* reveals an allele named *xa41* with deletion in a region with overlapping effector-binding sites (Hutin et al., 2015b). The knowledge of loss-of-function of *S* alleles in plant defense have been successfully employed to engineer resistant rice plants by genome editing of EBE regions on *S* alleles promoter (Li et al., 2012b). The second group, dominant non-transcriptional *R* genes, is represented by traditional *R* genes encoding for NLR proteins that are also able to recognize TAL effectors to trigger defense responses (Zhang et al., 2015). Intriguingly only one NLR, the TNL from tomato *Bs4*, have been reported to recognize TAL effectors to activate defense responses (Schornack et al., 2004; Kay et al., 2005). The third and perhaps the most studied group of TAL effectors dependent *R* genes are dominant and transcriptionally induced by TAL effectors, also called executor *R (E)* genes (Schornack et al., 2013; Boch et al., 2014; Zhang et al., 2015). To date, five *E* genes along with their cognate TAL effector gene have been cloned: *Xa27-avrXa27, Xa10-avrXa10*, and *Xa23-avrXa23* from rice and *Bs3-avrBs3* from pepper and *Bs4C-avrBs4* from tomato (Gu et al., 2005; Römer et al., 2007; Strauss et al., 2012; Tian et al., 2014; Wang et al., 2015a). Interestingly, *E* genes code for proteins with diverse structure and no homology to traditional NLR resistance proteins (Zhang et al., 2015). On the contrary, its specificity relies in their direct transcriptional activation by cognate TAL effectors which is known to induce an HR response and the restriction of pathogen growth in the infection site (Schornack et al., 2013; Zhang et al., 2015). For this reason, *E* genes are considered as modular systems, with a promoter that works as a TAL effector sensing domain and a downstream coding sequence involved in defense execution (Boch et al., 2014). This modularity of *E* genes have been exploited to engineer TAL inducible promoter for downstream *E* genes known as activator promoter traps (Schornack et al., 2013; Boch et al., 2014). Two studies have successfully employed promoter traps with customized EBEs from different pathogens to induce the expression of *Xa27* with broaden functional specificity to distinct pathogens (Römer et al., 2009; Hummel et al., 2012a). Recently a similar strategy was employed in rice where BSR to *Xoo* was obtained by engineering a modification of the *Xa10* promoter using five tandemly arranged EBEs leading the expression of *Xa10* (Zeng et al., 2015).

Taken together, these different strategies show the importance on how the comprehension of molecular plant-microbe interactions might lead to achieve broad-spectrum disease resistance. Although, BSR has been achieved primarily through individual PRR, NLR and *E* genes, it will be

interesting to conceive as a long term goal to stack PRR, NLR and *E* genes in a single loci to confer DBSR.

Quantitative resistance on durable and broad-spectrum resistance

Plant resistance can be determined by monogenic *R* genes conferring full or complete resistance. This type of resistance, given its genetic determinism, has been largely studied. However, very often plants show different levels of resistance. In this case, phenotypes range from partial to full resistant. Partial or incomplete resistant phenotypes are governed by several loci, each with a minor contribution to resistance. Quantitative disease resistance (QDR), also called partial or polygenic resistance, is referred to as the resistance determined by several genes with small additive effects (St.Clair, 2010; Niks et al., 2015). In contrast to qualitative disease resistance, QDR confers a reduction rather than a lack of disease (St.Clair, 2010). Since QDR is governed by several loci, is difficult to exploit it in breading programs compared with qualitative resistance. Nevertheless, given its polygenic nature, QDR is more robust and durable through years and less likely to be overcome by evolving pathogens populations (St.Clair, 2010; Boyd et al., 2013). Loci governing QDR have obtained attention recently since it can represent valuable sources for the deployment of DBSR through gene pyramiding (Singh et al., 2011).

To understand the molecular mechanisms involved in QDR several quantitative trait loci (QTL) have been recently identified. Although durable resistance has no particular molecular basis, to date durable genes isolated from QTLs are structurally diverse (Poland et al., 2009; St.Clair, 2010; Niks et al., 2015; French et al., 2016). Based on the structure and putative function of these genes, as well as on the nature of the quantitative resistance, several hypotheses have been proposed to explain its underlying molecular mechanism (Poland et al., 2009). Some hypothesis suggest that QDR might be related with a quantitative variation of MTI or ETI components, and others hypothesis imply completely different mechanisms for QDR loci such as defense transduction or developmental and morphological characters involved in non-host resistance (Poland et al., 2009). To date, at least eleven genes have been isolated to contribute to QDR (French et al., 2016). The lists of QDR genes cloned include at least five distinct types of kinases, two transporters and four NLRs. In wheat, *Yr36* was the first QDR gene coding for kinase that conferred a BS and

temperature-dependent resistance to wheat stripe rust (Fu et al., 2009). In maize, two non-RD wall-associated receptor-like kinases (WAK) have been identified as QDR genes. The first, ZmWAK was identified from a major QTL locus, qHSR1, and it is located in the plasma membrane and acts as a receptor to perceive and transduce extracellular signals (Zuo et al., 2015). The second, the maize *R* gene *htn1* was identified as a wall-associated receptor-like kinase (WAK-RLK) responsible for broad-spectrum QDR in maize (Hurni et al., 2015). Interestingly, an atypical kinase, RKS1, associated with BSR in *Arabidopsis* is involved in the recognition of AvrAC and triggers an ETI-dependent response (Huard-Chauveau et al., 2013; Wang et al., 2015b).

In contrast, four reports have shown NLRs underlying QDR. First, two studies reported *RGC1* and *RLM1* as genes conferring QDR to anthracnose stalk rot in maize and blackleg disease in *Arabidopsis*, respectively (Staal et al., 2006; Broglie et al., 2009). Recently, two genes coding for NLRs, *Pi35* and the NLR pair *RPS4/RRS1* have been identified as QDR genes from rice and *Arabidopsis*, respectively (Fukuoka et al., 2014; Debieu et al., 2016). *Pi35* is a durable race-specific QTL used in rice breading programs since 1961 (Nguyen et al., 2006). Interestingly, functional polymorphisms in *Pi35* were found to contributive in a cumulative fashion to enhance resistance (Fukuoka et al., 2014). In addition to *Pi35* in rice, the well-known *Arabidopsis* RPS4/RRS1 pair of NLRs was recently found to be involved in QDR race-specific resistance to *X. campestris* (Debieu et al., 2016).

In summary, during the last decade different studies have provided new information regarding QDR underlying molecular mechanism. Notably, recent studies showed how the knowledge of genes underlying QDR to rice blast has been nicely employed in the development of durable resistance trough gene pyramiding (Fukuoka et al., 2015; Yasuda et al., 2015). This reinforces the substantial value of the study of quantitative resistance for crop breading.

Recent progress aiming to understand cassava-*Xam* interactions

The knowledge of plant immunity have mainly derived from studies on the model plant species *Arabidopsis thaliana* and few crop species of economic interest such as rice and tomato. However, food security of most of the tropical countries rely on non-well studied plant species (orphan

crops), including tuber crops as cassava, as a main caloric source. Several studies have shown the limitation to extrapolate the knowledge and information of model plants to economical important crops. This highlights the necessity to perform research to gain information on the molecular basis of resistance for each particular crop to incorporate this data in the breeding programs.

As early mentioned, cassava clonal multiplication render this crop highly prone to different pest and diseases. A source of cassava resistance to both CMD and CBB are known to be present in the wild relative, *M. glaziovii*, for this reason cassava breeding efforts have exploited this resistance source into the development of elite cultivars (Hahn et al., 1979, 1980; Ceballos et al., 2012). Regarding bacterial disease, cassava resistance to CBB has been proposed to be quantitative, polygenic and additively inherited. In fact, several QTLs have been identified to different *Xam* strains (Hahn et al., 1979, 1980; Jorge et al., 2000). This notion of CBB quantitative resistance is supported by the fact that CBB phenotypes in cassava cultivars range from very susceptible to moderately resistant to different *Xam* strains (Jorge et al., 2000; Wydra et al., 2004; López and Bernal, 2012). Interestingly, cytochemical studies on inoculated cassava tissues suggest there is a variation in colonization and penetration rates into the vascular tissue that explains the difference between resistant and susceptible cultivars (Kpemoua et al., 1996).

Studies on *Xam* population structure have provided information about its geographic distribution and diversity, important factors for the selection of host resistance in breading programs. During the 1980s and the 1990s *Xam* populations were characterized in South America, Africa and Asia, and it was found that South American populations were the most diverse (Verdier et al., 1993, 1994; Restrepo and Verdier, 1997; Verdier et al., 1998; Restrepo et al., 2000b). Among *Xam* populations from South America, Brazilian populations were found to be the most diverse, compared with Colombia and Venezuela, which is in accordance with the origin center of cassava. In Colombia, the geographical regions that harbor higher diversity were the Caribbean coast and the Eastern plains (Restrepo and Verdier, 1997). More recently, during 2008-2010, the current status of *Xam* populations was assessed in the Caribbean coast and the Eastern plains (Trujillo et al., 2014b, 2014a). A comparison of *Xam* populations in both ecozones indicates that pathogen populations are more diverse in the Caribbean coast (Trujillo et al., 2014a). A pathotype analysis was also conducted in different cassava cultivars, but a lack of correlation was observed when compared with AFLPs haplotypes (Trujillo et al., 2014b).

Regarding *Xam* pathogenicity factors, analysis derived from *XamCIO151* genome sequence reveals the presence of genes coding for proteins involved in the six types of secretion systems described so far in Gram-negative bacteria (Arrieta-Ortiz et al., 2013). In terms of the T3Es, twenty-eight genes were identified in *Xam*, nine of these seemed to be conserved in all strains evaluated from three continents (Bart et al., 2012; Arrieta-Ortiz et al., 2013). Recent results derived from mutagenesis of T3Es have revealed that XopZ, XopX, XopAO1, XopN, XopQ and AvrBs2 constitute important virulence factors (Medina et al., in press). In the last years, information about *Xam* TAL effectors biology has become available. The first TAL effector described in *Xam* is TALE1 (previously named pthB) and is a major virulence factor in the *Xam* strain CFBP1851 (Castiblanco et al., 2013). Transcriptomic analyses of cassava plants have identified a heat shock transcription factor as a gene induced in response to TALE1 (Muñoz-Bodnar et al., 2014). In addition, *S* genes targeted by TAL effectors from *Xam668* and *XamCIO151* have also been identified (Cohn et al., 2014, 2016). Interestingly, a member of the *SWEET* family in cassava, *MeSWEET10a*, was reported to be induced by TAL20 from *Xam668* (Cohn et al., 2014).

In relation to molecular determinants of resistance to CBB, studies based on conserved domains among NLRs proteins has led to the identification and mapping of resistance genes candidates (RGCs) in cassava (López et al., 2003). Additional mapping strategies led to the identification of one RGC associated with a QTL in the linkage group U, that explained 62% of resistance to *XamCIO151* and was called *RXam2* (Resistance to *Xam* 2) (López et al., 2007). The availability of the cassava genome sequence allowed the identification of this RGC as a CNL protein. On the other hand, but also based on QTL analysis to a different *Xam* strain, a gene similar to *Xa21* was identified to co-localize with a QTL explaining 13% of resistance to *XamCIO136* (Jorge et al., 2000). This gene was called *RXam1* (Resistance to *Xam* 1). More recently, two studies have identified and mapped immunity related-genes in the cassava genome (Lozano et al., 2015; Soto et al., 2015). Still, to date no *R* genes have been cloned and functionally validated in cassava.

For the reasons mentioned above, this work aims to contribute to the functional characterization of two resistance gene candidates, *RXam1* and *RXam2*, and also to evaluate the feasibility of interfamily transfer of *Bs2* to cassava in order to confer resistance to CBB. To achieve these goals, in a first time, *Bs2* was overexpressed in stable transgenic cassava plants and the ability to produce defense response to *Xam* strains was evaluated. Second, the *RXam1* gene coding for a RLK, was overexpressed in susceptible plants and showed to restrict *XamCIO136* growth on *in vitro* plants. Third, both silencing and overexpression of *RXam2*, an NLR resistance candidate, was

conducted in a susceptible cultivar to evaluate the contribution of *RXam2* to CBB resistance. In addition, *RXam2* was employed in an activator trap promoter containing an EBE induced by TALE1 as an alternative strategy to induce an active NLR in the presence of a TAL effector from *Xam.*

Chapter 2

"Si no conoces a tu enemigo ni a ti mismo, correrás peligro en cada batalla"

"If you know neither the enemy nor yourself, you will succumb in every battle"

Sun Tzu

2 Interfamily transfer of *Bs2* from pepper to cassava (*Manihot esculenta* Crantz)

Paula Díaz Tatis, Juan Camilo Ochoa, Lina García, Adriana Bernal, Paul Chavarriaga and Camilo López

2.1 Abstract

Cassava bacterial blight (CBB) caused by *Xanthomonas axonopodis* pv. manihotis (*Xam*) is the most prevalent bacterial disease in cassava. Genome analysis of *Xam* strains showed the presence of an effector similar to *avrBs2* from *Xanthomonas euvesicatoria* among the core set effectors. Mutagenesis analysis suggests this effector is important for *Xam* virulence. According to *avrBs2* wide distribution and contribution to *Xam* virulence, we proposed a useful strategy to control *Xam* based on the overexpression of Bs2, the AvrBs2 cognate resistance protein. To accomplish it, Friable Embryogenic Calli (FEC) from the susceptible cultivar cv.60444 was transformed with *Bs2* gene from pepper under a 35S promoter. Unchallenged *Bs2* over-expressing cassava *in vitro* plants showed constitutive, typical immune responses. However, the overexpression of *Bs2* did not confer wide-spectrum resistance to *Xam* strains harboring *avrBs2*.

2.2 Introduction

Cassava (*Manihot esculenta* Crantz) belongs to the Euphorbiaceae family (Malpighiales) and is native from South America (Allem, 1994, 1999; Bredeson et al., 2016). Among root and tuber crops, cassava ranks first as a source of calories in the tropics (Cock 1982) (http://faostat3.fao.org/). Cassava was initially domesticated near the southeast rim of Amazon River but at the present time is cultivated in tropical and subtropical regions of South America,

Sub-Saharan Africa and South-East Asia (Allem, 1994, 1999; Olsen and Schaal, 1999). Cassava production is severely affected by different viral and bacterial diseases. The most important bacterial disease is cassava bacterial blight (CBB) caused by *Xanthomonas axonopodis* pv. manihotis (*Xam*) (López and Bernal, 2012). CBB resistance is considered to be quantitative and cultivars fluctuate from very susceptible to moderately resistant to different *Xam* strains (Jorge et al., 2000; Wydra et al., 2004; López and Bernal, 2012). Histological studies on inoculated cassava plants have shown the difference between resistant and susceptible cultivars is expressed as a variation in colonization rates and penetration into the vascular tissue. In addition, both resistant and susceptible plants have shown similar defense responses in vascular tissues, nevertheless these responses were more intense and faster developed in the resistant cultivar (Kpemoua et al., 1996; Sandino et al., 2015). This data support the idea that cassava resistance to CBB is considered to be quantitative and polygenic (Kpemoua et al., 1996; Jorge et al., 2000). One of the best control strategies employed to manage CBB has been the use of resistant cultivars and cultural practices. The most reliable approach for controlling CBB is the deployment of *Resistance (R)* genes into elite cassava cultivars. Although different strategies to identify candidate *R* genes are now been undertaken based on the availability of cassava genome and the presence of conserved domains (Lozano et al., 2015; Soto et al., 2015), to date, no *R* gene has been characterized and cloned in cassava.

Among optimal strategies to achieve resistance is to exploit the natural mechanisms plants have employed to defend against pathogens attack. In contrast to the innate and adaptive immune system present in animals, plants rely solely on their innate immune system in each individual cell for the recognition of non-self molecules (Spoel and Dong, 2012). Two types of plant immune receptors, surface-localized or intracellular receptors, typically detect invading pathogens.

Well-defined plant pattern-recognition receptors (PRRs) are surface-localized receptors belonging either to receptor-like kinases (RLK) or receptor-like proteins (RLP) (Wu and Zhou, 2013; Zipfel, 2014). PRRs usually detect well-conserved signatures of the pathogen, such as flagellin, chitin or elongation factor-Tu, collectively known as pathogen or microbe-associated molecular patterns (PAMPs/MAMPs) (Schwessinger and Ronald, 2012; Newman et al., 2013). On the other hand, typical *R* genes encode for intracellular receptor structurally similar to nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) that detect pathogen proteins delivered by the type-III secretion system (TTSS). This pathogen-derived effector proteins (commonly referred to as an avirulence protein or Avr) can be sensed by plants NLRs in

a direct fashion or indirect by their biochemical activity in plant targets (Maekawa et al., 2011b; Qi and Innes, 2013). This recognition activates an immune response known as effector-triggered immunity (ETI) (Jones and Dangl, 2006; Cui et al., 2015).

Although barely defined, the molecular conformation of NLRs is known to switch between a resting, transition and active state (Takken and Tameling, 2009; Bernoux et al., 2011a; Bonardi et al., 2012). Biochemical analysis of flax M and L6, barley MLA27, tomato Mi-1 and I-1 reveal that a resting or auto-inhibited "off" state has ADP bound on the NB-ARC domain (Tameling et al., 2006; Maekawa et al., 2011a; Williams et al., 2011). After pathogen recognition, ADP is exchanged for ATP rendering the protein in an active "ON" state and the initiation of ETI signaling (Takken and Tameling, 2009; Bernoux et al., 2011a; Cui et al., 2015). Further ATP hydrolysis will result in a subsequent inactive "OFF" state of NLR (Takken and Tameling, 2009). The role of oligomerization or heterodimerization between NLRs molecules to activate ETI is poorly understood. For some NLRs oligomerization of the N-terminal domain has been reported to initiate disease resistance signaling (Bernoux et al., 2011b; Maekawa et al., 2011a; Chang et al., 2013).

Activation of NLRs usually leads to a particular programmed-cell death called hypersensitive response (HR) that will ultimate serve for prevention against pathogen dispersal, although in some cases resistance is not associated with the presence of HR (Heath, 2000; Coll et al., 2011). Some of the molecular responses associated with the activation of plant defense are depolarization of the plasma membrane, activation of mitogen-activated protein kinases (MAPK) and Calcium-dependent protein kinases (CDPK) cascades, induction of WRKY transcription factors and induction of gene expression coding for pathogenesis-related proteins, oxidative burst and modulation of hormone signaling pathways (Elmore et al., 2011; Meng and Zhang, 2013; Buscaill and Rivas, 2014). The majority of these defense responses are induced by the activation of both RLKs and NLRs, although these mechanisms are utilized distinctly in PTI and ETI (Tsuda and Katagiri, 2010).

The knowledge of the role of plant immune receptors has lead to the development of novel strategies to account for broad-spectrum resistance (Mukhtar, 2013; Dangl et al., 2013). One of such promising strategies has been the interfamily transfer of PRRs recognizing well-conserved ligands. One successful example of this strategy was performed with EF-TU RECEPTOR (EFR). This PRR was first identified in *Arabidopsis* to confer immunity against bacteria harboring the Elongation Factor Tu (Zipfel et al., 2006). EFR is taxonomically restricted to the family

Brassicaceae, however it was transferred to tobacco and tomato (Solanaceae) where it conferred bacterial broad-spectrum resistance (Lacombe et al., 2010). EFR interfamily transfer has also been shown in wheat against bacterial pathogens (Schoonbeek et al., 2015). Similarly, Xa21, a RLK from rice has not only been overexpressed in monocots, e.g. banana to confer resistance to banana *Xanthomonas* wilt (BXW) (Tripathi et al., 2014), but also in dicots such as sweet orange and tomato against *Xanthomonas axonopodis* pv. citri (*Xac*) and *Ralstonia solanacearum*, respectively (Mendes et al., 2010; Afroz et al., 2011). These results suggest signaling activation downstream of PRRs recognition is functionally conserved in both monocot and dicots linages. New evidence supporting this idea is the recent demonstration that EFR-XA21 quimera receptors are able to associate with EFR complex in a ligand-dependent manner (Holton et al., 2015).

A second strategy has been the cross-species introduction of NLRs for broad-spectrum and durable resistance. *Rxo1*, an *R* gene from maize that confers a non-host resistance to *X. oryzae* pv. oryzae (*Xoo*) was transferred to rice. Notably this gene is involved in resistance to distinct pathogens, *Xoo* and *Burkholderia andropogonis* (Zhao et al., 2005). Alternatively, in dicots the NLR transfer has been carried out widely among Solanaceae species. In the case of potato, one of the most used strategies has been the introduction of *R* genes from wild species (Bradeen et al., 2009; Foster et al., 2009). The *Pto* gene from tomato has also been expressed in tobacco where enhances resistance to *Pseudomonas syringae* pv. tabaci (Thilmony et al., 1995). Another example of *R* gene transferred to a close related Solanaceae species has been the case of *Mi1-2* from tomato into eggplant, rendering this plant species resistant to the nematode *Meloidogyne javanica* but not to aphids (Goggin et al., 2006).

A notable example of cross-species introduction of an NLR is the case of *Bs2* transgenic tomato plants. *Bs2* is a bacterial spot resistance gene from pepper that activate immune response through the recognition of the AvrBs2 protein from *Xanthomonas euvesicatoria* (*Xeu*) strains (Tai et al., 1999). Although *Bs2* occurs commonly on pepper plants, this *R* gene does not naturally occur on tomatoes, despite been a close related species. Accordingly, *Bs2* was transferred to tomato and shown to enhance resistance to *Xeu* in both laboratory and field tests (Tai et al., 1999; Horvath et al., 2012). The cognate Avr recognized by Bs2, AvrBs2, is considered a core type-III effector involved in virulence in *Xanthomonas* spp. (Roux et al., 2015; Hajri et al., 2009; Kearney and Staskawicz, 1990). Recently, mutational analysis of *avrBs2* on *X. oryzae* pv. oryzicola (*Xoc*) and its heterologous expression in rice has shown this effector suppress rice immunity and contributes to the development of disease (Li et al., 2015). This knowledge about *avrBs2* wide distribution

across the genera has lead to new approaches based on transient expression of *Bs2* in *Citrus lemon* (Rutaceae) to improve resistance against citrus canker disease caused by *Xanthomonas axonopodis* subsp. citri (Sendín et al., 2012).

Genome sequence data from *Xam* strains revealed a proteome that comprises around 28 type-III effector proteins (Arrieta-Ortiz et al., 2013; Bart et al., 2012). Comparative analysis among *Xam* strains showed only one strain out of 65 strains surveyed showed a premature stop codon on *avrBs2* coding sequence, for this reason it is not considered a core type-III effector in *Xam* (Bart et al., 2012). However, all *Xam* strains evaluated shared >99% nucleotide identity in the *avrBs2* sequence between them and 58% identity with the gene from *Xeu*. The N-terminal of AvrBs2 is a highly conserved region between *Xam* and *Xeu* and the more relevant differences are found in the C-terminal (Medina et al. *in press*). Since *avrBs2* is highly conserved across *Xam* strains and apparently *Bs2* transient expression results in immunity activation in citrus, we decided to evaluate the feasibility of interfamily transfer and stable expression of *Bs2* in cassava susceptible plants.

2.3 Results

2.3.1 Generation of transgenic cassava plants overexpressing *Bs2* from pepper.

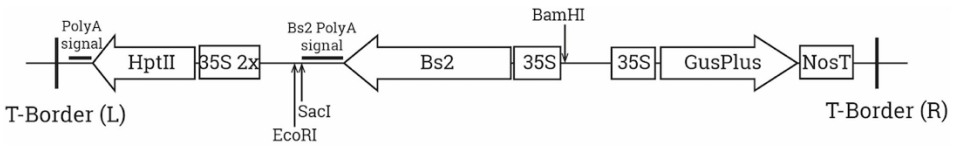
Previous studies have shown *avrBs2* is present in almost all *Xam* strains sequenced to date (Bart et al., 2012; Arrieta-Ortiz et al., 2013). In contrast, Bs2 appears to be restricted to pepper. In order to identify a putative orthologue of *Bs2* on cassava based on sequence similarity, we carried out a search on the cassava proteome v6.1 (41381 sequences) using the Bs2 protein sequence reported on the GenBank (accession number AAF09256.1). BLAST results from the first 20 hits are shown in Supplementary table 2-2. The hit with the higher score is annotated as an NLR that showed only 32% identity with Bs2 (250/765). This result indicates cassava proteome does not contain NLRs that share high homology with Bs2 from pepper, confirming a restricted presence of this gene in pepper. Accordingly, we hypothesized *Bs2* transferred to cassava could confer resistance to *Xam* strains harboring *avrBs2*.

To accomplish this, a complete cassette containing *Bs2* under 35S promoter and its native terminator was cloned in pCAMBIA1305.2 (Figure 2-1). Friable embryogenic calli (FEC) from the susceptible cassava cultivar cv.60444 was isolated, multiplied and transformed with the

aggressive *Agrobacterium* strain AGL1 containing the cassette with *Bs2*. Growing FECs clusters were subcultured on regeneration medium containing hygromycin for the selection of transgenic events. Approximately 50 embryos were obtained; although some of them showed abnormal phenotypes and some others did not developed apices. Healthy apices were selected for elongation and finally selected for antibiotic resistance. Fresh leaves from putative transgenic plants were then selected for GUS histochemical assay. In total, 12 plants gave positive results for this test. All shoots of *GUS* positive plants were clonally multiplied on cassava propagation medium for subsequent assays (Supplementary Fig 2-1).

Figure 2-1: Schematic diagram of the T-DNA region of pCAMBIA1305.2 used for cassava transformation.

Boxes shows promoter or terminator regions and arrows indicate ORFs. Thin vertical arrows indicate restriction sites used for *Bs2* cloning (SacI and BamHI). EcoRI digestion was used for Southern-blot analysis.



2.3.2 Molecular characterization of *Bs2* cassava transgenic plants.

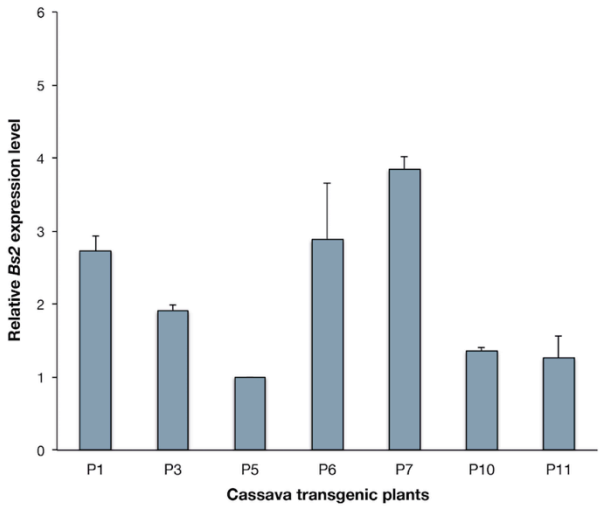
In order to confirm the presence of the transgene on cassava plants, genomic DNA was isolated from *in vitro* plants and a PCR was performed using specific primers for *Bs2*. The *PP2A* gene was used as an internal control. All the tested plants showed a fragment of 120pb corresponding to the *Bs2* gene (Suppl Fig 2-2). These plants were subsequently analyzed to identify independent transgenic events by Southern-blot using an *hptII* amplification PCR product as a probe (Suppl Fig 3). Genomic DNA was digested with EcoRI, in consequence each insertion event is represented by a single band in the Southern-blot. Most of the plants appeared to have inserted two copies of the T-DNA and it seems to be at least three distinct transgenic events. Plants showing similar insertion pattern were considered as derived of the same transgenic event as follows: Plant 1 (P1) (Event 1, 2 copies); P2, P3 and P4 (Event 2, 2 copies); P5 and P8 (Event 3, 2-3 copies), P6 and P7 (Event 4, 2 copies); and P9, P10 and P11 (Event 5, 2 copies).

In order to evaluate *Bs2* expression levels on transgenic plants, fresh leaves from 1 month-old *in vitro* plants were used for Reverse Transcription-PCR (RT-PCR) analysis. *Bs2* expression levels

were detected using specific primers for *Bs2*. All transgenic plants tested displayed high *Bs2* expression levels when compared to the constitutive endogenous control *tubulin* (Suppl Figure 2-4). Additionally, to quantify *Bs2* expression levels in the different plants obtained, a quantitative RT-PCR (qRT-PCR) was performed. qRT-PCR results showed *Bs2* expression levels varying among the different cassava transgenic plants evaluated (Figure 2-2). Since transcription levels of *Bs2* are not detected on E.V. or WT control plants, transgene expression of each plant is shown relative to the plant with less *Bs2* expression (P5). Fold change of *Bs2* expression levels between plants range from 2 (P3) to 4 times (P7). These results indicate *Bs2* is been efficiently expressed in all transgenic plants recovered.

Figure 2-2: Relative *Bs2* expression levels in cassava transgenic plants.

Total RNA was isolated from fresh leaves of unchallenged *in vitro* transgenic plants. *Bs2* expression was calculated by qRT-PCR and normalized to *tubulin* reference gene. Data shown is measured relative to P5 expression level. Mean of three replicates + SE is indicated.



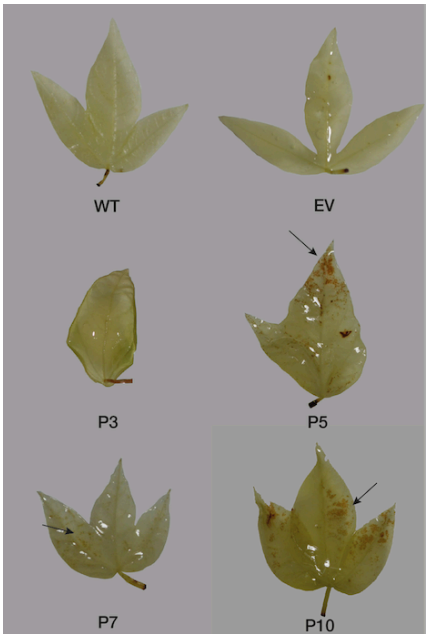
2.3.3 Evaluation of constitutive defense activation: ROS production and PR expression.

To assess whether cassava plants overexpressing *Bs2* could enhance the production of defense responses, leaves from unchallenged *in vitro* plants were detached and stained for reactive oxygen species (ROS) production using 3'-3 Diaminobenzidine (DAB). In contrast to WT and E.V.

control, *Bs2* transgenic leaves P5, P7 and P10 showed ROS production, evidenced as dark-brown colored dots (Figure 2-3).

Figure 2-3: Detection of reactive oxygen species (ROS) on *Bs2* cassava plants.

Leaves from challenged *in vitro* plant were detached and stained for reactive oxygen species (ROS) production using 3'-3 Diaminobenzidine (DAB). Stained leaves treated with ethanol to remove chlorophyll and photographed. Brown deposits pointed by black arrows indicate the presence of H_2O_2.

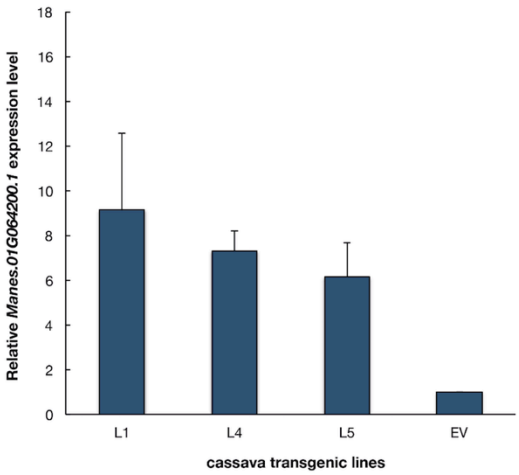


To further explore other defense responses that could be constitutively activated in these plants, the expression of three pathogenesis-related (*PR*) genes homologues in cassava was evaluated. For this, based on PR homologues in *Arabidopsis thaliana* and *Nicotiana benthamiana,* a set of PR candidates for cassava were selected and evaluated by qRT-PCR on unchallenged *in vitro* plants. A *PR5* cassava homologue (thaumatin-like protein, *Manes.01G064200.1*) exhibited high expression levels on *Bs2* transgenic plants compared with E.V. control (Figure 2-4). In contrast, a chitinase class one (*Manes.04G067900.1*, *PR3* homologue) is repressed on *Bs2* transgenic plants (Supplementary figure 2-6). These results suggest that some putative cassava *PR* homologues might be induced in transgenic plants over-expressing *Bs2*. Similarly, a *PR1* homologue, annotated as a cysteine-rich secretory protein (*Manes.07G050700.1*) showed very low expression levels in *Bs2* transgenic plants and no detectable levels on E.V. control plants (data not shown). Taken

together, these results indicate over-expression of *Bs2* in cassava induces an activation of defense responses as evidenced by basal ROS production and a putative *PR*s transcriptional activation.

Figure 2-4: Relative *PR5* expression level in cassava transgenic lines overexpressing *Bs2*.

Total RNA was isolated from fresh leaves. Expression levels were calculated by qRT-PCR and normalized to *tubulin* reference gene. Data shown was measured relative to WT expression level. Mean of three replicates + SE are shown.



2.3.4 *Xam* inoculations

To address whether over expression of *Bs2* on cassava could confer resistance to *Xam* strains, *in vitro* leaves were inoculated and bacterial growth was assessed after 3 and 7 days post inoculation (dpi). For this, leaves were dipped-inoculated with a 10^7 bacterial suspension of four *Xam* strains (CIO151, 668, 1344 and 232). The growth of the strain *XamCIO151* is reduced at 7 dpi on *Bs2* cassava plants, mainly in P2 and P3 (Figure 2-4). However, this decreased bacterial titer at 7 dpi was not observed for other *Xam* strains evaluated (Supp Fig 7, 8 and 9). To further evaluate defense responses during *Xam* inoculation, inoculated leaves were stained with DAB to detect ROS at 2dpi. There is an increase in ROS accumulation for *Bs2* cassava lines inoculated with *XamCIO151*, compared with non-inoculated control plants near the inoculation point (black arrows) (Figure 5). Again, ROS accumulation is observed as small brown deposits along the leave on *Bs2* cassava plants (white arrows), suggesting constitutive defense responses. This is not observed in E.V. control plants. Taken together, these results suggest despite these plants appear to produce defense responses, is not enough to restrict *Xam* growth on *Bs2 in vitro* cassava plants.

Figure 2-5: Bacterial growth of *XamCIO151* on *Bs2* transgenic cassava plants.

One month-old cassava *in vitro* plants were inoculated by piercing and dipped on both abaxial and adaxial side of the leave with a 10^7 CFU/mL suspension of *XamCIO151*. The number of bacteria was quantified at 0, 3 and 7 dpi, data represent a mean Log_{10} CFU/cm^2 from three biological samples ± SE. Statistical significance was assessed at 7 dpi at $P < 0.05$ using a Tukey–Kramer HSD test for post-ANOVA analysis. Control E.V. plants (red squares) and three *Bs2* lines: L11 (golden circles), L17 (dark-green triangles) and L19 (blue diamonds). No significance is designated as "n.s.". Three independent experiments gave similar results.

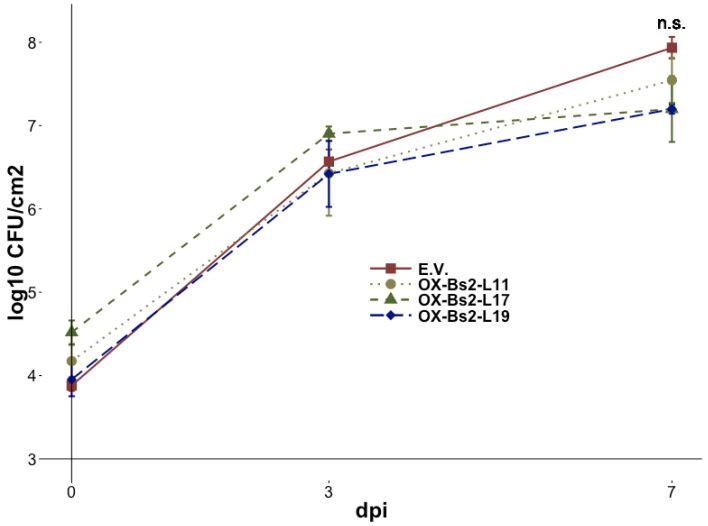
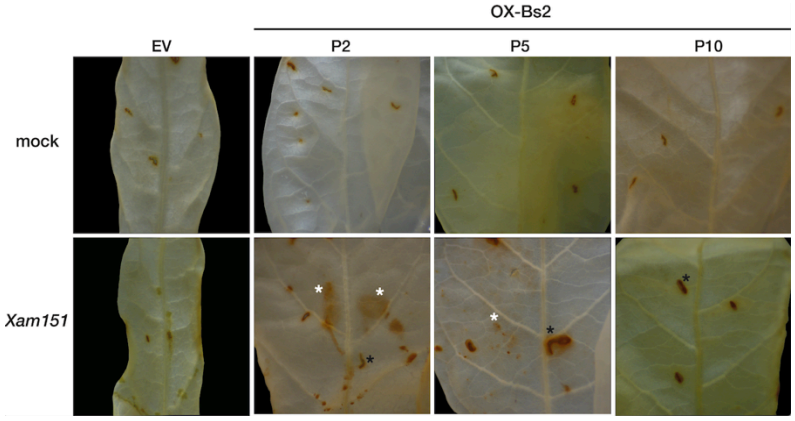


Figure 2-6: Reactive oxygen species accumulation on *Bs2* cassava plants inoculated with *Xam.*

Leaves inoculated with a 10^7 inoculum of *XamCIO151* were stained with DAB after 2 dpi. Asterisks indicate ROS accumulation near the inoculation point (black) and in other regions (white).



2.4 Discussion

The aim of this study was to evaluate the feasibility of interfamily transfer of *Bs2* from pepper, a species from the Solanaceae, to cassava, which belongs to the Euphorbiaceae, to generate resistance to *Xam* strains. Our results show that transgenic cassava plants obtained functionally express *Bs2* and that this overexpression is related to the accumulation of ROS in the leaves and activation of putative *PR* genes (Figure 2-2, Figure 2-3). Nevertheless, no significant differences in bacterial growth were observed in *Bs2* transgenic plants when inoculated with four different *Xam* strains.

The *Bs2* transgenic plants showed a strong constitutive *Bs2* expression. Aberrant activation of resistance signaling has been implicated in cases of hybrid necrosis as a way to avoid gene-flow between plant species or inbreed (Bomblies et al., 2007; Bomblies and Weigel, 2007). Although *Bs2* cassava plants showed a slight lack of vigor compared with control transformed plants, regenerated cassava transgenic lines showing *Bs2* constitutive overexpression did not produced autoimmunity deleterious phenotypes, associated with spontaneous cell-death. Nevertheless, some morphological changes were observed in *Bs2* cassava plants, such as decreased stem diameter and reduced leaf area. These effects could be associated with the continuous *in vitro* sub-culturing steps in auxin-rich media as is frequently observed (Chavarriaga, personal communication).

An increase in ROS production and expression of a *PR5* cassava homologue was observed in unchallenged cassava *Bs2*-transgenic plants. We found a slight and irregular ROS accumulation in

some leaf areas on non-inoculated *Bs2* cassava plants (Figure 2-6) and *Xam*-inoculated leaves showed an enhanced accumulation of ROS near the inoculation point. This result suggests the overexpression of *Bs2* is activating ROS signaling in the absence of biotic or abiotic stress. However, HR was not observed as a consequence of a possible activation in ROS signaling. No PRs have been described or functionally studied in cassava, although some candidates have been reported in previous studies (Lopez et al., 2005; Román et al., 2014). Based on this data and on a new bioinformatics search using PRs from *Arabidopsis* and *N. benthamiana* as queries in the cassava genome new PRs candidates were identified. The expression of some of them was studied on the *Bs2*-transgenic plants as molecular markers for the induction of plant immunity. We found that a *PR5* homologue that codes for a thaumatin-osmotin like protein in cassava is induced in *Bs2* cassava plants. The thaumatin-like PR5 proteins belong to a large family of proteins with diverse function, including permeabilization of fungal cell walls, inhibition of mammalian trypsin and α-amylase from insects (Loon et al., 2006). In *Arabidopsis* and grape wine, the induction of PR1, PR2 and PR5 (thaumatin) has been related with resistance against biotrophs (Develey-Rivière and Galiana, 2007; Spoel and Dong, 2012). Also, the leaf spurge non-host resistance to *Xam* appears to be associated with PRs induction, including chitinases and thaumatin-like proteins (Horvath et al., 2013). The induction of a *PR* homologue suggests an activation of an immune response as a consequence of the activation of *Bs2* signaling responses in cassava. This implies cassava defense responses could also depend on *PR* gene induction as it has been reported in other species (Seo et al., 2008). Thus, even though the constitutive overexpression of *Bs2* did not produce an implicit cell death leading to a lethal phenotype, some immune responses, including ROS and *PR*s can be activated in the absence of the pathogen.

As mentioned before, the constitutive *Bs2* expression did not result in a plant cell death. Previous studies have shown HR is triggered only when *avrBs2* and *Bs2* are simultaneously co-expressed, and not when *Bs2* is overexpressed alone (Tai et al., 1999). Similarly, when the CNL RPM1 is overexpressed in *N. benthamiana* it does not produce an ectopic spontaneous HR (Chung et al., 2011). However, other studies have reported an effector-independent HR when NLRs are overexpressed (Mukhtar, 2013). Interestingly, effector-independent HR have been frequently observed in CNL proteins, including Lr10 (Feuillet et al., 2003), Pi54 (Gupta et al., 2012), RPP13 (Rentel et al., 2008), RPS2 (Tao et al., 2000), RPS5 (Ade et al., 2007), although there is the case of an TNL, RPS4, where this phenomenon was also observed (Zhang et al., 2004).

In this study, the stable overexpression of *Bs2* in a highly susceptible cassava cultivar did not conferred resistance to *Xam* strains harboring *avrBs2*. Nevertheless, the stable overexpression of *Bs2* in the highly susceptible tomato cultivar VF36 did showed reduced disease to very low levels when inoculated with *X. euvesicatoria* strains harboring *avrBs2* (Horvath et al. 2012; Tai et al. 1999). These results might indicate the signaling responses after the activation of *Bs2* might be conserved only in species of the *Solanaceae* family. Indeed, the coexpression of *avrBs2* and *Bs2* triggers HR in solanaceus plants (tobacco, tomato, potato, eggplant), however HR is not observed when *avrBs2* and *Bs2* are coinfiltrated in non-solanaceus species, including *Arabidopsis,* broccoli, cucumber and turnip (Tai et al., 1999). The *Bs2* gene seems to be restricted to pepper and homologous genes have not been reported even in other Solanaceae plant species (Tai et al., 1999). Accordingly and considering the large evolutionary divergence between cassava (Malpighiales) and pepper (Solanales) no *Bs2*-homologue gene was found in the cassava genome. This phenomenon called restricted taxonomic functionality (RTF) has also been observed for the RPS2 gene from *Arabidopsis* where it was shown to be nonfunctional on transgenic tomato plants (Tai et al., 1999). Although it has been observed that NLR function is not confined to specific host-pathogen interaction, since a single NLR can confer resistance to non-related pathogens (Narusaka et al., 2014; Zhao et al., 2005, 2015), NLRs transfer between non-related species has not been extensively studied. A notable example is the barley (monocot) NLR MLA1 transfer to the dicot *Arabidopsis* (Maekawa et al., 2012). This supports the hypothesis that NLR do not always display RTF.

The lack of a resistance response to *Xam* despite the overexpression of *Bs2* and the apparent constitutive immunity (ROS production and induction of *PR5* gene expression) can be explained from the fact that Bs2 from pepper is not able to recognize AvrBs2 from *Xam.* Although AvrBs2 is considered as a well-conserved effector in *Xam*, its sequence appears to diverge from *avrBs2* of *X. euvesicatoria* and its nearly identical to *avrBs2* from *X. translucens* and *X. axonopodis* pv. glycines (Medina et al. *in press*). Preliminary results showed AvrBs2 homologue of *Xam* does not trigger an HR in pepper plants harboring *Bs2*, indicating Bs2 is not able to recognize avrBs2 from *Xam* to induce HR (Bart, personal communication). Recent results have shown although the catalytic site for the glycerophosphoryl diester phosphodiesterase domain (GDE) is conserved among *avrBs2* homologs, in *Xam* two residues involved in activation are not fully conserved in *Xam* homologs (Medina et al. *in press*). In order to confirm if our results are caused by the divergence of *Xam avrBs2* homologs and thus by the incapacity of Bs2 to recognize and trigger a defense response in cassava plants, *X. euvesicatoria* mutated in *avrBs2* and *WT* should be inoculated on *Bs2* cassava

plants. An additional strategy is to transform *Xam* WT strain and *ΔavrBs2* with *avrBs2* from *Xeu* and evaluate the appearance of an HR and the development of a resistance response in cassava *Bs2* plants. It remains to be determined whether AvrBs2 from *Xeu* is indeed recognized in cassava *Bs2* transgenic plants. Results obtained raised the question if it is possible that interfamily overexpression of a NLR may cause resistance without recognizing its cognate avirulence protein.

2.5 Materials and methods

2.5.1 Plasmid constructions

Bs2 cloned in pMDC32 (35S::*Bs2*-Bs2term) was kindly provided by Brian Staskawicz, University of California (UC), Berkeley. To overexpress Bs2 in cassava plants, the complete cassette containing 35S::*Bs2*-Bs2term was cloned in the Multiple Cloning Site (MCS) of pCAMBIA1305.2 (CAMBIA, Canberra, Australia). For this, pMDC32 was double digested with *BamHI* and *SacI* and ligated in the Multiple Cloning Site (MCS) of pCAMBIA1305.2. The ligation product was transformed in *E. coli DH10B* and positives colonies were confirmed using primers flanking the *Bs2* genes. The clones were further confirmed by sequencing of the complete T-DNA region. *Agrobacterium tumefaciens* aggressive strain AGL1 was transformed with pCAMBIA1305.2-*35S::Bs2*-Bs2term by electroporation. AGL1 cells were grown on Luria-bertani (LB) containing 50 mg L^{-1} kanamycin and 100 mg L^{-1} carbenicillin. PCAMBIA1305.2 vector contains *GUSPlus* gene as a reporter gene and *hygromycin phosphotransferase II (htpII)* as a selection marker. This vector has been previously used in cassava transformation.

2.5.2 Cassava FEC transformation

Cassava Friable Embryogenic Callus (FEC) production

FEC from the genotype cv. 60444 (NGA11 in the CIAT germplasm collection) was obtained as previously described (Taylor et al., 2012, 1996). *In vitro* cassava plants were grown and maintained in Murashige and Skoog (MS) basal medium supplemented with 1 ppm tiamine, 100 ppm myo-inositol, 2 μM $CuSO_4$, 0.002 ppm NAA, 0.05 ppm GA3, 0.04 ppm BAP and 20 g L^{-1} sucrose. For primary embryogenesis, axillar buds from 4-weeks old plant were excised and grown in MS supplemented with 2 μM $CuSO_4$, 50 mg L^{-1} casein, 20 g L^{-1} sucrose and 12 mg L^{-1}

picloram. Somatic embryos were grown during 30 days in dark conditions. For FEC induction, organized embryogenic structures (OES) were injured using a small blade and transferred to Gresshof and Doy medium with vitamins (GD) supplemented with 20 g L^{-1} sucrose and 12 mg L^{-1} picloram. FEC clusters were transferred to new medium every 20 days and maintained at 28°C and 12h light during 3 months until transformation.

FEC transformation and transgenic recovery

Cassava transformation of FEC was performed based on protocols previously described (Taylor et al., 2012; Li et al., 1996; Bull et al., 2009). Briefly, AGL1 strain carrying pCAMBIA1305.2-*35S::Bs2* was grown on LB broth supplemented with 50 mg L^{-1} kanamycin and 100 mg L^{-1} carbenicillin until the cell suspension reached an optical density at 600nm (OD_{600})= 0.5. Cells were resuspended in GD supplemented with 12 mg L^{-1} picloram and 100 µM acetosyringone and grown until an OD_{600}= 0.8. Clusters of FEC with a diameter around 10mm were soaked with 20ul of the cell suspension and cocultured for 4 days at 24ºC in dark conditions. FEC clusters were washed with liquid GD medium containing 500 mg L^{-1} cefotaxime to control *A. tumefaciens* overgrowth. After five days of subsequent washing steps, FECs were plated on GD medium supplemented with 20 g L^{-1} sucrose, 500 mg L^{-1} cefotaxime, 12 mg L^{-1} picloram and hygromycin 2 mg L^{-1}. Growing FEC clusters were transferred every four weeks to fresh medium with increasing hygromycin concentrations (4, 7, 10 and 15 mg L^{-1}). For the development of transgenic somatic embryos, clusters were transferred to MS medium supplemented with 20 g L^{-1} sucrose, 1 g L^{-1} BAP, 0.5 g L^{-1} IBA, 2µM $CuS0_4$, 500 mg L^{-1} cefotaxime and 15 mg L^{-1} hygromycin. Obtained embryos were grown on the same media without hygromycin selection and after that to MS medium containing 0.5 % activated charcoal to promote elongation of apices. Recovered putative transgenic plants were subsequently multiplied on MS medium with vitamins containing 20 g L^{-1} sucrose and 2µM $CuS0_4$.

2.5.3 Molecular characterization of putative transgenic plants

GUS staining

Leaf tissue from *in vitro* plants was immersed in a microcentrifuge tube with 1.5 mL of X-GLUC buffer (0.02 M NaH_2PO_4, 0.03 M Na_2HPO_4, 0.25 mM K_3FeCN_6, 0.25 mM K_4FeCN_6, 0.5 % triton X-100, 10 % DMSO and 1 mg mL^{-1} X-Gluc (5-bromo-4-cloro-3-indolyl β-D-glucuronide) (Sigma, St. Louis, MO, USA). Leafs were incubated at 37°C during 16 h, destained with 96% ethanol and photographed.

Genomic DNA extraction and PCR

For characterization of transgenic plants, leaf tissue was collected from regenerated *in vitro* plants and total plant genomic DNA was extracted with CTAB. PCR was performed using gene-specific primers for *b-tubulin* (tubex F and tubex R) and *Bs2* (Bs2qPCR F and Bs2 QPCR R). DNA from non-transgenic plants was used as negative control and plasmid DNA as a positive control. PCR was carried out in a Bio-Rad thermal cycler programmed for an initial denaturation of 3 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 56°C and 30 s at 72°C, and final extension of 10 min at 72°C. PCR reactions were performed in a 10ml final volume (0.2 mM of dNTPs, 0.1 mM of each primer, 1X DreamTaq buffer, 1 U of DreamTaq DNA Polymerase (ThermoScientific, Waltham, MA, USA) and 100 ng of genomic DNA).

Southern blot analysis

10ug of genomic DNA were digested with the restriction enzyme *BamHI*, enzyme that cuts once the T-DNA at the start of 35S promoter in the MCS (multiple cloning site). A 490pb *hptII* probe was synthetized by PCR (hptII probe F and hptII R) using plasmid DNA as a template and following recommendation by the DIG-labelling PCR DIG probe synthesis kit (Roche Applied Science, Indianapolis, IN, USA). Correct DIG-labelling was confirmed by running the labeled probe and unlabeled probe in a gel electrophoresis. The blots were hybridized at 42°C and detection was carried out according to manufacturer's instructions using anti-DIG antibody and CDP-star (Roche Applied Science, Indianapolis, IN, USA).

2.5.4 DAB staining

To detect ROS in *Bs2* transgenic lines, leaves from *in vitro* unchallenged with *Xam* plants were excised, submerged in 3, 3'-Diaminobenzidine (DAB) buffer as previously reported (Thordal-Christensen et al., 1997) and incubated for 6 hours in the dark at rt (room temperature). Leaves were destained from chlorophyll through at least three washing steps with 96% ethanol. Accumulation of H_2O_2 was detected in a stereoscope and leaves were photographed.

2.5.5 qPCR analysis

For quantitative real-time PCR (qPCR) total RNA was extracted from *in vitro* leaves of unchallenged plants using Invitrap ® Spin plant RNA minikit (STRATEC, Berlín, GER). To degrade

DNA molecules from RNA sample, 1 ug of total RNA was treated with DNaseI, RNAse-free (ThermoScientific, Waltham, MA, USA). cDNA synthesis was carried out using the First Strand cDNA Synthesis Kit (ThermoScientific, Waltham, MA, USA).

For qPCR the 7500 Fast Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA) and the Bio-Rad SsoFast EvaGreen supermix were used. PCR reactions were carried out as follows: initial denaturation at 95°C for 3 min, 40 cycles of 95°C for 10 s, 58°C for 10 s, 72°C for 10 s. PCR reactions were performed in a 10ml final volume using 0.2 mM of dNTPs and 0.1 mM of each primer. Primers used are listed in Supplementary Table 1. The expression levels of target genes were normalized to the *tubulin* gene expression level using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) as follows:

ΔC_t (*target gene*)= C_t (*target gene*) - C_t (*tubulin*)

$\Delta\Delta C_t$ (*target gene*)= ΔC_t (transgenic line) - ΔC_t (EV control)

2.5.6 *Xam* inoculation of *in vitro* plants

To measure bacterial growth in planta, two months-old *in vitro* plants were inoculated using a leaf-dipping method. Briefly, a Q-tip was submerged in a *Xam* cell suspension with an OD_{600nm} (optical density at 600mn)= 0,02,~10^7 colony forming units (CFU ml^{-1}). Young leaves were pierced with an insulin syringe and then wiped with a Q-tip previously immersed in the bacterial suspension. Both sides of the leaves were carefully dipped until a thin layer of the cell suspension was observed in the surface. Bacterial growth was monitored at 0, 3 and 8 days post-inoculation (dpi). For this, leaves from three technical replicates were detached and grounded independently in 10mM $MgCl_2$. The average bacterial titer was reported +/- standard error.

2.6 Supplementary information

Supplementary table 2-1: List of primers used in this study.

primer name	Sequence (5' to 3')

tubex F	GGAAAGATGAGCACCAAGGA
tubex R	ACCAGTATACCAGTGCAAGAAG
Bs2 qPCR F	CCTTGAGCGCCTCATGATTA
Bs2 qPCR R	TCCGAGGTCTTCTTGTTCTTTC
hptII probe F	GCGTCTGCTGCTCCATACAAG
hptII probe R	AGCGAGAGCCTGACCTATTGC

Supplementary table 2-2: First 20 hits of a BLASTP against the cassava proteome using the Bs2 sequence from pepper as a query.

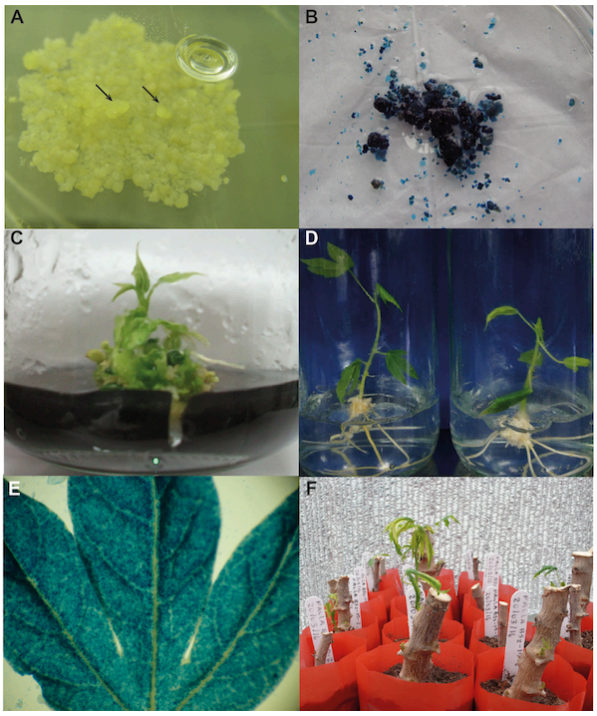
Subject ID	% identity	alignment length	mismatches	gap opens	q.start	q.end	s.start	s.end	E-value	bit score
Manes.18G105800.1 (M=2) PTHR23155:SF402 - DISEASE RESISTANCE PROTEIN RPP13-RELATED	32.68	765	434	21	148	862	165	898	2,00E-88	304
Manes.09G034700.1 (M=9) PTHR23155//PTHR23155:SF634 - LEUCINE-RICH REPEAT-CONTAINING PROTEIN	33.16	748	398	22	177	878	190	881	2,00E-87	301
Manes.10G023300.1 (M=9) PTHR23155//PTHR23155:SF634 - LEUCINE-RICH REPEAT-CONTAINING PROTEIN	29.05	809	454	22	131	863	154	918	6,00E-83	289
Manes.07G121100.1 (M=9) PTHR23155//PTHR23155:SF634 - LEUCINE-RICH REPEAT-CONTAINING PROTEIN	28.70	770	414	17	175	847	188	919	2,00E-78	276
Manes.10G025400.1 (M=9) PTHR23155//PTHR23155:SF634 - LEUCINE-RICH REPEAT-CONTAINING PROTEIN	31.24	765	402	25	175	862	185	902	3,00E-77	273
Manes.07G121300.1 (M=9) PTHR23155//PTHR23155:SF634 - LEUCINE-RICH REPEAT-CONTAINING	28.95	791	435	21	175	864	183	947	5,00E-75	267

PROTEIN										
Manes.10G022500.1 (M=9) PTHR23155//PTHR23155:SF634 - LEUCINE-RICH REPEAT-CONTAINING PROTEIN	29.44	754	387	22	175	814	184	906	4,00E-71	254
Manes.18G117800.1 (M=7) PTHR23155//PTHR23155:SF592 - LEUCINE-RICH REPEAT-CONTAINING PROTEIN	30.47	791	456	32	116	858	129	873	8,00E-66	238
Manes.07G121200.1 (M=9) PTHR23155//PTHR23155:SF634 - LEUCINE-RICH REPEAT-CONTAINING PROTEIN	29.79	678	382	20	232	857	329	964	9,00E-66	239
Manes.07G121200.1 (M=9) PTHR23155//PTHR23155:SF634 - LEUCINE-RICH REPEAT-CONTAINING PROTEIN	27.71	166	79	3	88	253	141	265	2,00E-10	65.5
Manes.10G102500.1 (M=4) PTHR23155//PTHR23155:SF633 - LEUCINE-RICH REPEAT-CONTAINING PROTEIN	27.86	829	490	25	96	868	104	880	1,00E-65	238
Manes.13G061300.1 (M=7) PTHR23155//PTHR23155:SF592 - LEUCINE-RICH REPEAT-CONTAINING PROTEIN	28.54	841	487	28	96	863	83	882	5,00E-65	236
Manes.10G023100.2	27.39	836	456	25	155	876	43	841	9,00E-65	235
Manes.10G023100.1 (M=9) PTHR23155//PTHR23155:SF634 - LEUCINE-RICH REPEAT-CONTAINING PROTEIN	27.39	836	456	25	155	876	43	841	9,00E-65	235
Manes.18G102800.1 (M=4) PTHR23155//PTHR23155:SF633 - LEUCINE-RICH REPEAT-CONTAINING	27.12	848	513	30	96	886	106	905	8,00E-64	233

PROTEIN										
Manes.18G102700.1 (M=4) PTHR23155//PTHR23155:SF633 - LEUCINE-RICH REPEAT-CONTAINING PROTEIN	27.23	797	484	25	122	868	135	885	2,00E-63	231
Manes.10G023200.1 (M=9) PTHR23155//PTHR23155:SF634 - LEUCINE-RICH REPEAT-CONTAINING PROTEIN	36.10	349	178	6	175	479	184	531	5,00E-63	224
Manes.18G117800.2	30.60	621	372	21	116	714	129	712	1,00E-59	218
Manes.18G117600.1 (M=7) PTHR23155//PTHR23155:SF592 - LEUCINE-RICH REPEAT-CONTAINING PROTEIN	26.76	811	505	29	98	861	105	873	2,00E-59	219

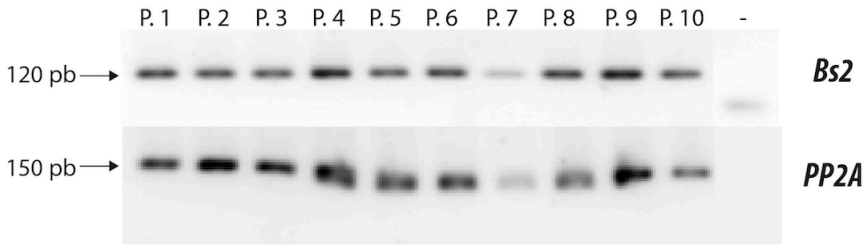
Supplementary figure 2-1: Production and characterization of *Bs2* cassava transgenic plants.

(A) Friable embryogenic calli from cv.60444 transformed with AGL1 strain, arrows point at growing embryonenic units. **(B)** FECs units from remaining clusters strongly express *GUS*. **(C)** Apices from regenerated embryos. **(D)** Regenerated *in vitro* plants. **(E)** Histochemical *GUS* assay on leaves from regenerated plants. **(F)** Shoots growing from stakes in the greenhouse.



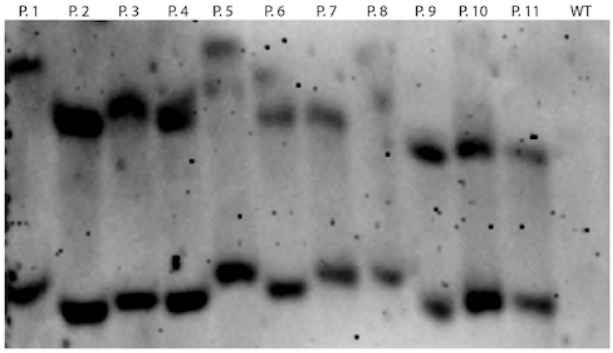
Supplementary figure 2-2: PCR analysis of *Bs2* cassava transgenic plants.

Genomic DNA was isolated from fresh leaves and a PCR was performed using specific primers for *Bs2*. *PP2A* was used as an endogenous control. P.1-P.10, genomic DNA from the different transgenic plants obtained; -, negative control.



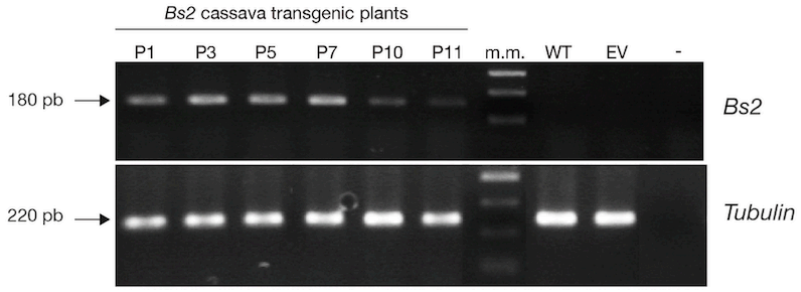
Supplementary figure 2-3: Southern-blot analysis of *Bs2* cassava transgenic plants.

Genomic DNA was isolated from fresh leaves and a southern Blot was performed using *hptII* as a probe. P.1-P.11, genomic DNA from the different transgenic plants obtained; WT, genomic DNA from wild- type plants used as a negative control.



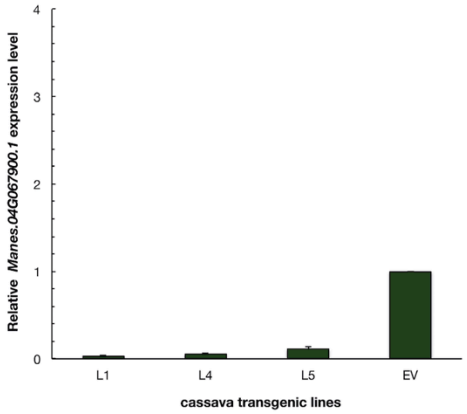
Supplementary figure 2-4: RT-PCR analysis of *Bs2* cassava transgenic plants.

Total RNA was isolated from fresh leaves and a RT-PCR was performed using specific primers for *Bs2*. *Tubulin* was used as an endogenous control. P.1-P.10, different *Bs2* transgenic plants obtained; WT, wild type non-transformed plants; EV, empty vector transgenic plants; -, negative control.



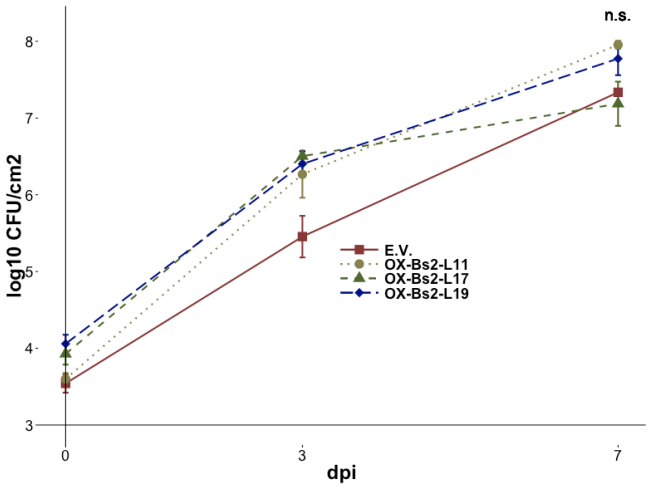
Supplementary figure 2-5: Relative *Manes.04G067900.1* expression levels in cassava transgenic lines overexpressing *Bs2*.

Total RNA was isolated from fresh leaves. Expression levels were calculated by qRT-PCR and normalized to *tubulin* reference gene. Data shown is measured relative to WT expression level. Mean of three replicates + SE are shown.



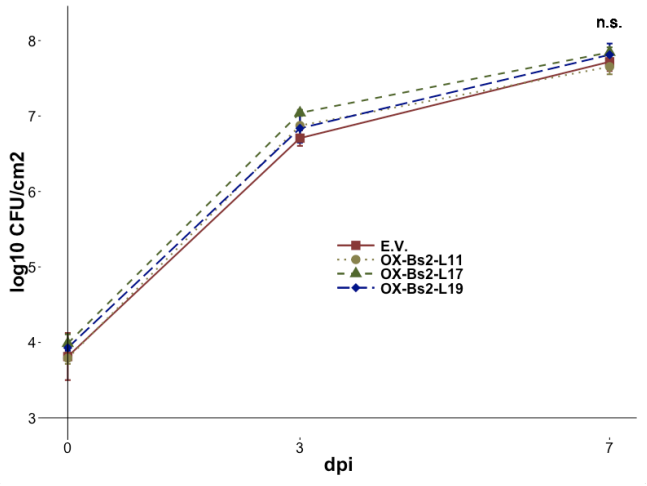
Supplementary figure 2-6: Bacterial growth of *Xam668* on *Bs2* transgenic *in vitro* cassava plants.

One month-old cassava plants were inoculated by piercing and dipping on the leaf with a 10^7 inoculum of *Xam668*. The number of bacteria was quantified at 0, 3 and 7 dpi, data represent a mean of three biological samples ± SE.



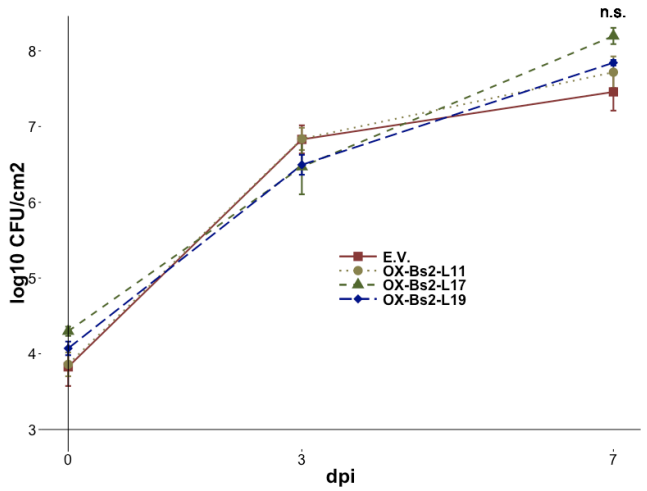
Supplementary figure 2-7: Bacterial growth of *Xam1344* on *Bs2* transgenic *in vitro* cassava plants.

1 month-old cassava plants were inoculated by piercing and dipping on the leaf with a 10^7 inoculum of *Xam1344*. The number of bacteria was quantified at 0, 3 and 7 dpi, data represent a mean of three biological samples ± SE.



Supplementary figure 2-8: Bacterial growth of *Xam232* on *Bs2* transgenic *in vitro* cassava plants.

One month-old cassava plants were inoculated by piercing and dipping on the leaf with a 10^7 inoculum of *Xam232*. The number of bacteria was quantified at 0, 3 and 7 dpi, data represent a mean of three biological samples ± SE.



Chapter 3

"Fortitudine vincimus"

"Venceremos por resistencia"

"By endurance we conquer"

Ernest Schackleton

3 The overexpression of RXam1, a RLK from cassava, confers less susceptibility to *Xanthomonas axonopodis* pv. manihotis

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3.1 Abstract

Cassava bacterial blight (CBB) is a prevalent disease in all regions where cassava is cultivated. CBB is caused by the Gram-negative bacterium *Xanthomonas axonopodis* pv. manihotis (*Xam*). Previous studies found different QTLs explaining resistance to several *Xam* strains. Interestingly one QTL called XM5 explaining 13% of resistance to *XamCIO136* was associated with a fragment similar to the rice *Xa21* resistance gene (PCR250). In this study we aimed to identify and characterize this fragment and it role in resistance to CBB. Screening and hybridization of a BAC library using the molecular marker PCR250 as a probe let to the identification of a receptor-like kinase similar to *Xa21* and was called *RXam1* (*Resistance to Xam 1*). Here we report the functional characterization of susceptible cassava plants overexpressing *RXam1*. Our results indicate the overexpression of *RXam1* leads to a reduction in bacterial growth of *XamCIO136*. This suggests *RXam1* might be implicated in strain-specific resistance to *XamCIO136*.

3.2 Introduction

The activation of plant immune responses depends on the recognition of pathogen molecules or pathogen derived-molecules by plant receptors. The first group of plant receptors carries an

extracellular LRR (Leucine-Rich Repeat) and an intracellular kinase domain referred to as Receptor-Like Kinases (RLKs) (Wu and Zhou, 2013). Examples of these proteins are FLS2 and EFR, which recognize flagellin and the Elongation Factor Tu (EF-Tu) respectively, two of the best studied MAMPs (Microbial Associated Molecular Patterns). Another example of this group of proteins is Xa21 from rice which confers resistance to strains of *Xanthomonas oryzae* pv. oryzae containing the corresponding *AvrXa21* gene (Song et al., 1995). In other cases the extracellular domain consist of a LysM domain, which is involved in the perception of sugar-derived molecules as chitin or LPS (Lipopolysaccharides), among the best known are the CERK1, LPY4 and LPY6 proteins from *Arabidopsis* (Liu et al., 2012a; Miya et al., 2007). Recently, an Arabidopsis B-type lectin SD-RLK was identified as a receptor for LPS from *Xanthomonas* and *Pseudomonas* (Ranf et al., 2015). Another group of receptors contains only the LRR domain and are named Receptor Like Protein (RLP), with members playing a function in other process different from plant immunity such as plant development (Zipfel, 2014). In the group of plant cytoplasmic receptors are the proteins containing a NB (Nucleotide-Binding) and a LRR domain and are referred as to Resistance (R) proteins (Meyers et al., 1999). This large group of proteins can be classed according to the presence of a Coiled-Coil (CC) or a Toll Interleukin Related (TIR) domain in their N-terminus. NB-LRR proteins have evolved to recognize pathogen-derived effector proteins in a specific manner and trigger the so called Effector Triggered Immunity (ETI) (Chisholm et al., 2006), which corresponds to the classical gene-for-gene model proposed by Flor (Flor, 1971). In the absence of R proteins, effectors are able to suppress plant immunity by a large diversity of mechanisms (Lee et al., 2013; Jones and Dangl, 2006). Once pathogen-derived molecules are recognized by extracellular or cytoplasmatic receptors, a plant immunity program is activated which include ion fluxes, Reactive Oxygen Species (ROS) production, activation of Mitogen Activated Protein (MAP) Kinases, activation of transcription factors which leads to a complex reprogramming of gene expression, including the activation of transcription of Pathogenesis Related (PR) genes (Kawano and Shimamoto, 2013; Macho and Zipfel, 2014; Elmore et al., 2011). In most of the cases a hypersensitive response (HR) occurs, which is a type of programmed cell death, having as purpose to avoid the expansion of the pathogen to uninfected cells.

Quantitative disease resistance (QDR) depends on the concerted action of several genes, each contributing in a different extent to the total phenotypic response (St.Clair, 2010; Roux et al., 2014). Although less intense, given its polygenic nature, QDR is considered durable and broad-spectrum (Kou and Wang, 2010). In recent years important efforts have culminated with the cloning of some genes belonging to Quantitative Trait Loci (QTL) associated to pathogen

resistance in wheat, rice, maize, soybean and *Arabidopsis* (French et al., 2016). Interestingly, most of these genes code for proteins with diverse biochemical function whose molecular mechanism in plant resistance has not been well established, given in part to its complex nature (Roux et al., 2014; Poland et al., 2009). However, based on the structure and putative function of these genes, as well as on the nature of the quantitative resistance, several hypotheses have been proposed to explain QDR molecular basis (Poland et al., 2009). One hypothesis, for example, considers *R* genes as weak alleles. Other postulation is that QDR is a consequence of the intensity in the expression level of genes involved in plant defense transduction signal (Poland et al., 2009).

Cassava (*Manihot esculenta* Crantz) is considered as a staple crop representing one of the main sources of carbohydrates for more than one billion people, notably in tropical region (http://faostat3.fao.org/). Cassava is produced by small farmers who depend on this crop not only for local consumption but also as a main income source. Cassava starch has multiple applications in different industries including textile, oil, paints, and recently in bioethanol production (FAO, 2013a). Cassava Bacterial Blight (CBB) is the most important bacterial disease affecting this crop (López and Bernal, 2012) and it was recently included in the top 10 plant bacterial diseases (Mansfield et al., 2012). CBB is caused by the Gram-negative bacteria *Xanthomonas axonopodis* pv. manihotis (*Xam*). CBB is present in all regions where cassava is cultivated and a higher incidence has been recently reported in some African and Asian countries (López and Bernal, 2012). There is no chemical control to CBB and the best approach for disease management is to employ resistant cultivars. Although there are some resistant cultivars most of them are not well adapted to the different agroecological regions where cassava is cultivated (Verdier et al., 2004). In addition, in most of the cases the resistant cultivars have shown low agronomical qualities, as low production. CBB resistance has been considered quantitative (Hahn et al., 1980, 1979) and some QTLs have been reported (Jorge et al., 2000; Wydra et al., 2004). No functional resistance genes to CBB or other cassava diseases have been cloned, although candidate genes and QTLs with major effects to CBB (Lopez et al., 2003; López et al., 2007) and CMD (Cassava Mosaic Disease) resistance have been reported (Gedil et al., 2012; Rabbi et al., 2012).

Previously, employing primers from the *Xa21* gene it was possible to amplify a fragment from cassava DNA. This fragment was mapped and shown to colocalize with a QTL explaining 13% of the cassava resistance to the *Xam* strain CIO136 (Jorge et al., 2000). Here we report the isolation

of *RXam1* and the characterization of cassava plants overexpressing *RXam1* demonstrating a role in defense against this particular *Xam* strain.

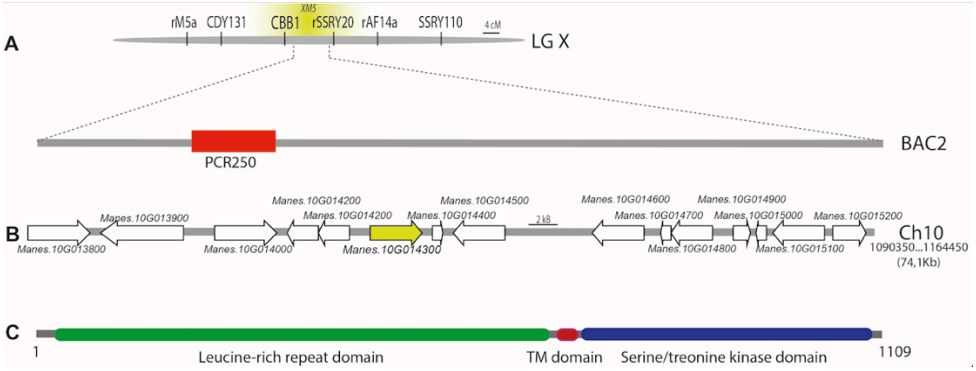
3.3 Results

3.3.1 Isolation of an RLK candidate in cassava for resistance against CBB

In a previous work, using primers from the rice *Xa21 R* gene an amplification product from cassava DNA was obtained and named PCR250. This fragment showed close similarity with the *Xa21* rice gene and it was mapped on linkage group X, where it showed a significant association with a QTL that explained 13% of cassava resistance to *Xam* strain CIO136 (Jorge et al. 2000). To identify the complete cassava gene, we screened a cassava BAC library from the cultivar TMS30001, using PCR250 as a probe. Four positive BAC clones were obtained and based on fingerprinting analysis we selected BAC 2 (containing one copy of the gene) to identify the putative full-length gene. Sequencing of this BAC, using partial shotgun sequencing and primer walking, resulted in the assembly of one contig of 4.6 kb, corresponding to the complete gene. BLASTx analysis of this contig showed close similarity to LRR-STK-like proteins and to the *Xa21* resistance gene. However, no ORF corresponding to a full-length, RLK was detected in this contig. Potential coding regions were interrupted by several stop codons. The cultivar used for the BAC construction was TMS30001, which is susceptible to *Xam* strain CIO136 (data not shown). To obtain the sequence of the possible functional gene, we used DNA from the cultivar MBRA685 that is resistant to *Xam* strain CIO136 with primers from the start and end of the predicted gene. In this case a PCR product of the expected size (3.1 kb) was generated and sequenced. The comparison between the sequences obtained from both MBRA685 and TMS30001 cultivars showed a high percentage of identity between both genes (94%). These genes showed about 34% identity with the rice *Xa21* gene (Supplementary figure 3-2). The 5' and 3' ends of the gene were obtained by rapid amplification of cDNA ends (RACE) from cDNA obtained from MBRA685 plants challenged with *Xam* strain CIO136. Sequences from the partial cDNA clones and the RACE products were assembled to produce a deduced transcribed sequence of 3,153 bp, containing an ORF of 1019 amino acids with 5' and 3' UTRs of 72 and 24 bp, respectively. This putative gene was called *RXam1* (*Resistance to Xam1*). A comparison of the *RXam1* genomic sequence with the cDNA sequence revealed the presence of one intron of 95 bp in the coding region (2,749 to 2,844 nt).

Figure 3-1: Schematic illustration of *RXam1* region on both the genetic and physical maps.

(A) Genetic map of linkage group X showing XM5 QTL based on Jorge et al., 2000 and BAC2 representation showing a region (pcr250) that colocalizes with XM5. **(B)** A region of chromosome 10 near rxam1. **(C)** Graphical representation of RXam1 domains.



The predicted 1019 amino acids (aa) protein contains an LRR domain located within the N-terminal sequence between 75 and 584 aa (Figure 3-1). The LRR consists of 20 imperfect repeats ranging in size from 23 to 26 aa. The putative STK domain is located between residues 703 and 975. A putative transmembrane domain was identified between the STK and LRR domains (Figure 3-1).

Although the *RXam1* gene product has more similarity to a putative LRR transmembrane protein kinase from *Arabidopsis thaliana* (46% identity at the aa level, GenBank accession NP_190295 with E-value of 0.0), it presents a high similarity to the *Xa21* gene product from rice (40% identity at the aa level). The most divergent regions are located at the 5' end of the proteins. In Xa21, the residues Ser686, Thr688 and Ser689 are involved in autophosphorylation (Xu et al., 2006b). From the alignment between Xa21 and RXam1 it was possible to identify the corresponding residues Ser-686 and Ser-689 in the positions 681 and 684, respectively. Residue Thr-688 in RXam1 is an Asn. Biochemical studies of RXam1 would further clarify whether these changes are involved or compromise the putative autophosphorylation activity.

RXam1 is annotated in cassava genome V6.1 as *Manes.10G014300* and is located in chromosome 10 between the positions 1122770-1126278. The markers SSRY20 and GY222 flank XM5, the QTL region containing RXam1 (Figure 3-1). Based on the available sequence for SSRY20 it was

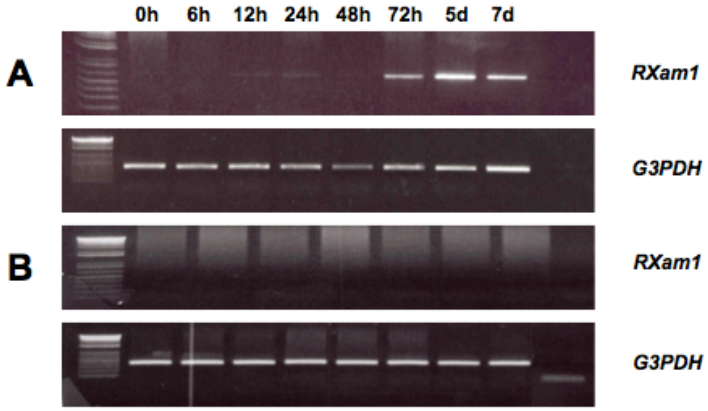
possible to localize *RXam1* on chromosome 10 on the cassava genome V6.1 (https://phytozome.jgi.doe.gov/) at positions 1071905-1072047, 50,7kb from the ATG start codon of *RXam1*. A region of 20 kb around SSRY20 and *RXam1* was analyzed to identify other candidate genes. No typical genes coding for proteins containing NB-ARC domain were found (Supplementary table 3-2). However, there are several genes coding for proteins with LRR and kinase domains, which are typically present in proteins involved in plant immunity (Supplementary table 3-2).

3.3.2 *RXam1* expression is induced after *Xam* inoculation

In order to evaluate if *RXam1* is induced after *Xam* inoculation, the expression was assessed by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Primers were designed in regions flanking the intron, which helped distinguish amplified products from mRNA and contaminating genomic DNA. Cultivars TMS30001 and MBRA685 were inoculated with *XamCIO136* and leaves collected at different times post inoculation. *RXam1* expression was induced by pathogen inoculation in the resistant but not in the susceptible cultivar. Indeed, no expression of *RXam1* was detected at any point of evaluation in the susceptible cultivar (Figure 3-2). *RXam1* mRNA was detected in the MBRA685 at 72 h post inoculation with a maximum at 5 days post inoculation (dpi) to decreased at 7 dpi (Figure 3-2).

Figure 3-2: *RXam1* expression analysis by RT-PCR in a susceptible and resistant cultivar upon *Xam* inoculation.

(A) Resistant cultivar MBRA685 was inoculated with *XamCIO136* and leaves were collected at 0, 6, 12, 24, 48 and 72 h, and 5 and 7 days post inoculation. **(B)** The same experiment carried out on the susceptible cultivar TMS30001.



3.3.3 STK domain of RXam1 interacts with a protein with finger domains in Y2H assays

As an initial approach to identify putative RXam1 interactors, the STK domain was used as bait against a cassava cDNA library made from the resistant cultivar SG107-35 inoculated with *XamCIO151*. A screen of $3x10^8$ clones was carried out founding one hundred fifty positive colonies. To verify these putative interactions, plasmid DNA was extracted and used to transform *E. coli*. To re-test the interaction, new plasmid DNA was extracted from this bacteria and used to retransform yeast, obtaining only five positive colonies. The sequence of these plasmids allowed to determinate that all five corresponded to the same gene (*Manes.03G064600*). This gene codes for a small protein of 358 aa containing a central CHY zinc finger domain followed by a C-terminal Ring finger domain and a Zinc-ribbon.

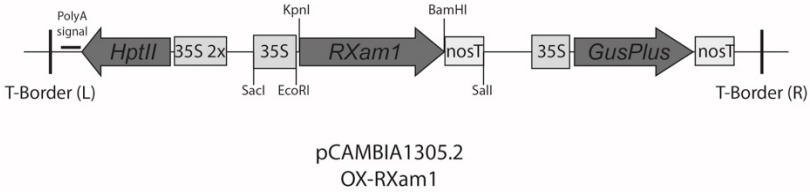
3.3.4 Production and validation of susceptible cassava plant overexpressing RXam1

In a first attempt we sought to validate the function of *RXam1* in cassava resistance to CBB following a silencing-based approach. For this, a fragment of 400pb corresponding to the STK region was amplified from the MBRA685 cultivar and cloned in a sense/antisense orientation into the pHellsgate12 binary vector. This construction was employed to transform CEF from cv. 60444. However after several assays and protocol modifications no transgenic plants were recovered.

As an additional strategy to validate the biological role of *RXam1* in resistance against CBB, we decided to overexpress *RXam1* in a susceptible cultivar. After several attempts to clone the full-length sequence of *RXam1* from MBRA685 cDNA, we decided to used *RXam1* from another resistant cultivar, MBRA902, which share high identity levels with the sequence of MBRA685 (data not shown). For this, we amplify the complete gene from MBRA902 using genomic DNA and cloned the complete CDS in the binary vector pCAMBIA1305.2 under a 35S promoter (Figure 3-3). FEC from the susceptible cassava cultivar cv. 60444 was transformed with *A. tumefaciens* strain LBA4404 containing pCAMBIA1305.2-35S::*RXam1-NosT* (designated OX-RXam1). As a transformation control, pCAMBIA1305.2 Empty Vector (E.V.) was also transformed. More than 100 embryos were regenerated and a total of 21 transgenic lines were recovered for *RXam1* and 39 for E.V. Since pCAMBIA1305.2 carries *GusPlus* as a reporter gene, these plants were initially characterized by *GUS* staining of young leaves (Supplementary figure 3-3). Nineteen out of twenty-one OX-RXam1 plants and thirty-two out of thirty-nine plants containing the E.V. were positive for the *GUS* histochemical test. Further, GUS positive plants were characterized by PCR using *G3PDH* as an endogenous control and *htpII* for transgenic plants detection (Supplementary figure 3-4). From these, in total eighteen plants were positive for *htpII* amplification (Supplementary figure 3-4). To identify independent transgenic lines and low copy number of insertions, Southern-blot analysis was conducted. At least three transgenic plants showed a single insertion event (one band) and ten transgenic plants showed double transgenic insertion events (two bands). From this, three single copy lines, L15, L16 and L18, were selected for subsequent functional assays (Supplementary figure 3-5).

Figure 3-3: Graphical representation of the T-DNA region from pCAMBIA1305.2 used for stable transformation of cv. 60444.

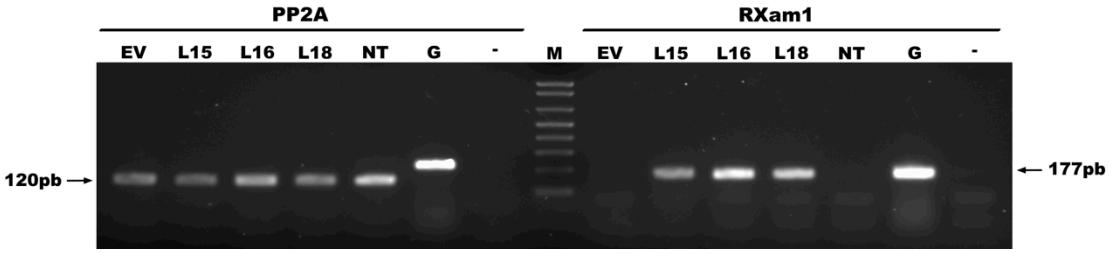
Boxes indicate promoter and terminator regions, arrows indicate open reading frames (ORFs). Enzymes used for molecular cloning on pCAMBIA1305.2 are showed as vertical lines.



To evaluate *RXam1* transcript levels in OX-RXam1 *in vitro* transgenic plants, a reverse transcriptase-PCR (RT-PCR) was performed using *RXam1*-specific primers. In this case PP2A primers were used to amplify a constitutive gene confirming the quality of cDNA (Moreno et al., 2011). The E.V. control plants and the no transgenic plants did not show expression of the *RXam1* gene, indicating very low expression levels of this gene in cassava unchallenged *in vitro* plants (Figure 1-3). On the other hand, in the three transgenic lines tested (L15, L16, L18), a band corresponding to *RXam1* expression with a size of 177pb was observed. This result indicates that OX-RXam1 transgenic lines (L15, L16, L18) displayed a constitutive and strong transgenic expression of *RXam1*.

Figure 3-4: Reverse transcriptase-PCR (RT-PCR) of *RXam1* on transgenic cassava plants.

Total RNA was isolated from fresh *in vitro* leaves on three OX-RXam1 cassava transgenic lines (L15, L16, L18). cDNA was synthetized and *RXam1* and *PP2A* (reference control) expression levels were detected by PCR. NT, Non-transgenic cv. 60444 plants. G, genomic DNA of cv. 60444 plants. -, negative PCR control.

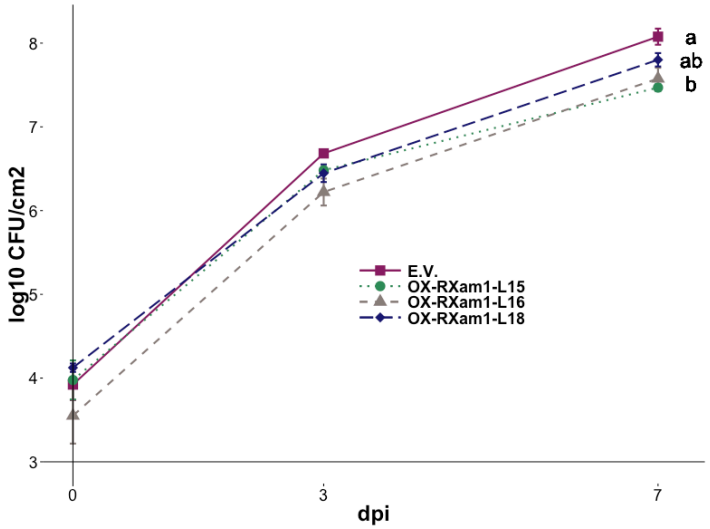


3.3.5 Phenotypic evaluation of transgenic plants overexpressing *RXam1* for resistance against CBB.

In order to assess the response of OX-RXam1 plants to *Xam*, leaves from *in vitro* plants were pierced in the mesophyll and dipped with a 10^7 CFU ml^{-1} bacterial suspension. *Xam* growth was assessed at 0, 3 and 7 days post inoculation (dpi). As control, plants transformed with E.V. were employed and the three transgenic OX-RXam1 lines were evaluated. Figure 3-5 shows *XamCIO136* growth on *in vitro* cassava leaves after 3 and 7 dpi. Interestingly, all OX-RXam1 exhibited reduced bacterial titers at 3 and 7 dpi (Figure 3-5). This indicates overexpression of *RXam1* causes less susceptibility in a highly susceptible genetic background. To test if *RXam1* could confer resistance to other *Xam* strains, other set of the same group of plants was inoculated with *Xam226*, *Xam668* and *XamCIO151*. However, *Xam* growth was similar in both control and OX-RXam1 lines (Supplementary figure 3-6). These results suggest the reduction in growth seems to be specific to *XamCIO136* (Figure 3-5).

Figure 3-5: Bacterial growth of *XamCIO136* on transgenic plants overexpressing *RXam1* is decreased compared with control plants.

Growth of *XamCIO136* on transgenic *in vitro* cassava plants carrying E.V. (purple squares) and three lines OX-RXam1: L15 (green circles), L16 (gray triangles) and L18 (blue diamonds). Leaves were inoculated with a 10^7 CFU/mL suspension of bacteria. The number of bacteria was quantified at 0, 3 and 7 dpi. Data points represent mean Log_{10} CFU/cm^2 ± SE from 3 technical replicates. Statistical significance was assessed at 7 dpi at $p < 0.05$ using a Tukey–Kramer HSD test for post-ANOVA analysis. Letters indicate significant different groups. This experiment was repeated twice with similar results.



On the other hand, quicker disease symptoms development was also evidenced in E.V. control plants when compared with OX-RXam1 lines. This was noted as an enhanced plant wilting in E.V. plants compared with OX-RXam1 lines at 15 dpi (Supplementary figure 3-7). In addition, to characterize possible resistance responses to *Xam*, leaves inoculated with *XamCIO136* were stained with DAB (3,3'-diaminobenzidine) in order to detect ROS (reactive oxygen species) production in L16 and E.V. control plants. Leaves were detached at 2 dpi, stained with DAB and destained with ethanol. However, both OX-RXam1-L16 and E.V. control plants produce similar brown deposits, indicating *RXam1* overexpression does not induce ROS production (Supplementary figure 3-8).

3.4 Discussion

The purpose of this work was to functionally characterize a gene, called *RXam1,* associated with a previously described QTL explaining 13% of resistance to *XamCIO136* in cassava.

RXam1 was first identified with primers designed from the *Xa21* sequence, a RLK from rice, thought to recognize a PAMP from *Xanthomonas oryzae* pv. oryzae. Indeed, *RXam1* codes for a receptor-like kinase harboring a N-terminal LRR, followed by a central TM and a C-terminal ST kinase domain. We found that *RXam1* expression appears to be transcriptionally induced upon *Xam* inoculation after 3 dpi in a resistant cultivar (Figure 3-2). In fact, in the absence of the

pathogen is troublesome to detect mRNA levels of RXam1 by RT-PCR. This gene expression profile is in concordance with previous results in our group where *RXam1* was also induced in stem and leaves in the resistant cultivars MBRA685 and SG107-35 after inoculation with *XamCIO151* (Contreras and López, 2008). Other studies have shown genes coding for RLKs, including *FLS2* (Boutrot et al., 2010), *EFR* (Zipfel et al., 2006), *CERK1* (Miya et al., 2007) and *XA21* as induced after pathogen perception. In contrast, genes coding for NLRs have a low basal expression and posses several distinct regulatory mechanisms, primarily at the protein level (Qi and Innes, 2013; Marone et al., 2013; Takken and Goverse, 2012). The induction of RLKs after pathogen perception might suggest the overexpression of RLK could produce deleterious effects when constitutively overexpressed in plants. However, we obtained transgenic cassava plants with strong constitutive expression of *RXam1* and relatively normal phenotypes. Similarly, other studies have shown the interfamily expression of *EFR* in tomato and tobacco produce normal plants (Lacombe et al., 2010). In tobacco a native *EFR* promoter was used while for tomato the strong constitutive 35S promoter was employed to produce *EFR* transgenic plants (Lacombe et al., 2010). Nevertheless, in both cases transgenic plants overexpressing a RLK with normal phenotypes were regenerated, indicating that RLKs overexpression does not necessarily produce a constitutive cell-death response. In the OX-RXam1 cassava plants a differential ROS production compared with the E.V. control plants was not observed, supporting the hypothesis that this RLK does not generate an implicit cell death response.

Through Y2H it was possible to identify a protein harboring a central CHY zinc finger domain followed by a C-terminal RING finger domain and a Zinc-ribbon as an interactor of the STK domain of RXam1. RING finger domains are common in Ubiquitin E3 ligases and are involved in distinct plant processes (Mazzucotelli et al., 2006; Troy et al., 2005). Interestingly, the Xa21 rice protein also interacts with an E3 ubiquitin ligase named XB3 to regulate their activity (Wang et al., 2006). This fact suggests a conserved mechanism between monocotyledonous to dicotyledonous plants to regulate some RLK proteins. Notably, a study found that a RING Zinc finger protein is involved in defense responses induced by chitin perception (Berrocal-lobo et al., 2010). Also, it is well known some PRRs appears to require ubiquitination for its regulation. For example once FLS2 activates the immunity-signaling pathway, it interacts with PUBs, a family of E3 ligases, to direct FLS2 to the proteasome machinery, avoiding thus the prolonged activation of plant immunity. Our results suggest RXam1 regulation could also be mediated by the protein degradation machinery.

In a first instance we anticipated *RXam1* might code for a PRR involved in broad-spectrum resistance given its similarity to XA21 and other RLKs involved in PTI. In addition, this gene corresponds to a QTL, which are usually related to broad spectrum disease resistance (French et al., 2016; St.Clair, 2010). Accordingly, we evaluated the response of the OX-RXam1 transgenic plants to four different *Xam* strains. However, a reduction in *Xam* growth was only observed in plants inoculated with *XamCIO136* (Figure 3-5 and Supplementary figure 3-6). Thus, *RXam1* seems to confer resistance only to the strain corresponding to the QTL previously reported (Jorge et al., 2000). Nevertheless, before concluding on the strain-specific nature of *RXam1* it should be convenient to evaluate the response of the OX-RXam1 plants to other *Xam* strains. Three of the strains used in this study were collected in Colombia (*XamCIO136* and *XamCIO151*) in 1995 (Restrepo and Verdier, 1997), and *Xam226* in 2008 (Trujillo et al., 2014c); while *Xam668*, was collected in Indonesia in 1978 (Bart et al., 2012). Interestingly, the marker associated to the QTL where *RXam1* is located, comes from the female parental (TMS30572) used for generating the genetic cassava map. This genotype is derived from an interspecific cross between *Manihot esculenta* and the wild relative, *M. glaziovii* (Fregene et al., 1997). Since the Brazilian Amazonian forest is considered the botanical origin of cassava and other wild relatives as *M. glaziovii*, it will be interesting to evaluate the response of the OX-RXam1 transgenic plants to Brazilian strains. In addition, a silencing based approach or gene edition through CRISPR/Cas of *RXam1* in *M. glaziovii* might aid to understand the role of this gene in a wild species background.

In this work, even that it was possible to observe a significant diminution in susceptibility in the OX-RXam1 transgenic plants to *XamCIO136*, it was not dramatically different to the E.V. transgenic control plants. In addition, no effect in disease resistance was observed for other *Xam* strains. However, it is important to consider the cassava variety employed to conduct the transformation experiments and the contribution of the QTL associated to *RXam1* to the phenotypic variation. Transgenic plants were obtained from cv. 60444, a highly susceptible cultivar not only to CIO136 but also to other *Xam* strains (Trujillo et al., 2014c). In addition, the QTL associated to *Xam* resistance (XM5) explained only 13% of the phenotypic variation (Jorge et al., 2000). These two elements made difficult to observe slight differences in response to other *Xam* strains and in consequence could mask the real effect of *RXam1* in CBB resistance and also lead to catalogue *RXam1* as a strain-specific gene.

Although *RXam1* seems to confer some degree of resistance to *Xam* strain CIO136, it is important to note that other genes present in the QTL region can be also participating in response to CBB. Since a simple regression method was employed for mapping XM5 (Jorge et al., 2000), the interval containing *RXam1* is not well delimited. There are several genes in a region spanning 350 kb around *RXam1* and although no genes coding for NLRs were found in this interval, other genes coding for proteins harboring LRR and kinase domains were identified (Supplementary Table 3-2). The gene edition of each of these candidate genes using the CRISPR/Cas system, for example, could help to clarify if they have a role in CBB resistance not only to *XamCIO136* but also to other *Xam* strains.

In this work we demonstrated that RXam1 seems to have role in resistance to *Xam* strain CIO136 and although no effect was observed to other *Xam* strains, several considerations should be taken into account before considering this gene a strain-specific resistance gene. No resistance gene has been isolated in cassava to any of the different diseases affecting this crop. Thus, *RXam1* represent the first resistance gene isolated in cassava.

While in the recent years several genes have been isolated from QTLs (Roux et al., 2014; French et al., 2016), this is the first report of a typical immunity-RLK involved in QDR. Several authors based on the co-localization of genes coding for NBS with QTLs have proposed that genes underlying QDR can be shared with genes involved in ETI. In this work we propose to expand this idea proposing genes involved in PTI (classical RLKs, as RXam1) can be also participating in QDR, helping to the understanding of the molecular bases of quantitative resistance.

3.5 Materials and methods

3.5.1 *RXam1* identification and isolation

BAC screening and hibridization

PCR250 corresponds to a DNA cassava fragment amplified with primers from the *Xa21* showing similarity to the rice *Xa21* gene (Jorge et al. 2000). A vector-free PCR250 was labeled with ^{32}a-ATP, using the Multiprime DNA Labeling System (Amersham Biosciences, Arlington Heights, IL, USA) and used as probe to screen a cassava BAC library constructed from TMS30001 (Tomkins et al. 2004). Hybridization was performed as described previously (Amersham Biosciences, Arlington Heights, IL, USA). Blots were exposed to X-ray films (Kodak) at -80°C, with intensifying

screens. Positive BAC clones were isolated and DNA was extracted, using the R.E.A.L. Prep 96 Plasmid Kit (QIAGEN, Inc., Valencia, CA, USA). DNA was digested with *Hin*dIII, separated on 0.9% agarose gels and transferred onto nylon membranes (Hybond-N+, Amersham Pharmacia Biotech, Ltd., Buckinghamshire, UK) and hybridized with the probe PCR250 as described above. BAC sequencing was performed with 10 ul of DNA using vector-derived primers. From the sequence generated new sets of primers were designed with the software Primer3 (Rozen and Skaletsky 2000). Sequencing reactions were carried out, using the ABI PRISM® BigDye™ Terminator Kit (Applied Biosystems, Foster City, CA, USA). The products were run on an ABI PRISM® 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The sequences were edited with the Sequencher program (Gene Codes Corporation, Ann Arbor, MI, USA). Similarity searches were carried out, using the BLASTx algorithm (Altschul et al. 1997) and alignment and bioinformatics analyses conducted with ClustalW (Chenna et al. 2003).

Isolation, amplification and sequencing of RXam1 from MBRA685

Genomic DNA was extracted from the resistant cultivar MBRA685 following a previously described protocol (Dellaporta et al., 1983). Based on the *RXam1* sequence obtained from the BAC clone, different primers were designed and *RXam1* was amplified by fragments to produce a complete contig. PCR reactions were performed in a 50 μL volume consisting of 0.2 mM of dNTPs, 0.1 mM of each primer, 1X PCR buffer, 2.5 mM $MgCl_2$, 1 U of Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA), and 100 ng of genomic DNA. Amplification was carried out in a Perkin-Elmer thermal cycler programmed for an initial denaturation at 93°C for 2 min, followed by 35 amplification cycles (93°C for 45 s, with T_m depending on each primer, for 45 s and 72°C for 1 min and 20 s), and a final extension step at 72°C for 10 min. The amplified fragments were eluted from a 1.2% low-melting agarose gel (Invitrogen Life Technologies Carlsbad, CA, USA). Bands were excised from the gel and purified, using a PCR-preps kit (Promega, Madison, WI, USA). Each purified DNA fragment was cloned, using pGEM®-T Easy cloning kit (Promega, Madison, WI, USA). In some cases, the PCR fragment was purified and sequenced directly.

To obtain the complete sequence of *RXam1*, including the UTR regions, 5′ and 3′ RACE was carried out with 3-months-old MBRA685 plants inoculated with *XamCIO136*. Leaves were inoculated as described previously (Restrepo and Verdier, 1997), and then collected at 6, 12, 24,

48, and 72 hpi, and 5 and 7 dpi. Total RNA was extracted from inoculated and non-inoculated tissues, using the RNeasy Mini Kit (QIAGEN, Inc., Valencia, CA, USA). 5′ and 3′ RACE were carried out using the First Choice RLM-RACE system (Ambion, Inc., Austin, TX, USA). Double-stranded cDNA was prepared, using the ProSTAR® First-Strand RT-PCR kit (Stratagene, La Jolla, CA, USA). For PCR amplifications, 1 μL of this cDNA was used. The synthesized cDNA was amplified, using glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as quality control for cDNA synthesis and to normalize. As negative control, the same RNA preparation was subject to cDNA synthesis but in the absence of the RT enzyme.

3.5.2 Expression analysis of *RXam1*

Semi-quantitative RT-PCR analysis was carried out in MBRA685 and TMS3001. 3-months-old plants were inoculated in the leaves, total RNA was extracted and cDNA was obtained as described in the previous section for 3' and 5' RACE assays. For RT-PCR *G3PDH* was used as a reference control.

3.5.3 Yeast two hybrid assays

A fragment of *RXam1* that contained a region coding for STK domain was cloned in PLAW11 and transformed in yeast cells AH109. Clones containing this fragment were used as bait against a cassava cDNA library made from the resistant cultivar SG107-35 inoculated with *XamCIO151* containing inserts ranging from 600 to 1500 bp cloned in pDONR222. The library was transfered to the pLAW10 vector and transformed in yeast AH109. Positive colonies from the first screen were plated again in selection medium to confirm its growth. Plasmid DNA was extracted from these secondary positive colonies and the interactions were again re-confirmed individually. Plasmids obtained from this test were extracted, transformed in *E. coli* and sequenced by standard Sanger sequencing.

3.5.4 *Agrobacterium*-mediated transformation of a susceptible cassava cultivar

Plasmid construction and bacterial strains for RXam1 overexpression

For cassava transformation a plasmid containing *35S::RXam1-NosT* in the MCS of the binary vector pCAMBIA1305.2 (Canberra, Australia) was developed. This vector contains *hygromycin*

phosphotransferase gene (htpII) as a selection marker and *B-glucoronidase* (*GusPlus*) as a reporter gene. To create pCAMBIA1305.2 (*35S::RXam1-NosT*) the 35S promoter of Cauliflower mosaic virus (35Sp), full-length *Manes.10G014300 (RXam1)* and Nopaline synthase terminator (NosT) were first amplified with primers containing restriction enzyme sites and cloned separately using TOPO-TA subcloning kit (Life Technologies GmbH, Darmstadt, Germany). The 35Sp was amplified with a set of primers containing restriction sites for *EcoRI* (35SEcoRI F) and *SacI* (35SSacI R), while the primers for the NosT amplification contained the restriction sites *BamHI* (NostBamHI F) and *SalI* (NostSalI R). *RXam1* was amplified from genomic DNA of a cassava resistant cultivar (MBRA902) using primers containing restriction sites for *KpnI* (RXam1KpnI F) and *BamHI* (RXam1BamHI R). Primers sequences used for cloning are showed in Supplementary Table 1-1. Once the clones were confirmed by PCR and sequenced. 35SP and NosT clones were independently digested with the respective enzymes (*EcoRI* and S*acI* for 35SP and *BamHI* and *SalI* for NosT) and ligated in the Multiple Cloning Site (MCS) of pCAMBIA1305.2. When the insertion of 35SP and NosT on the MCS of pCAMBIA1305.2 was confirmed by PCR and sequencing, the clone containing *RXam1* on TOPO-TA was digested with *KpnI* and *BamHI* and ligated in between 35SP and NosT. The final binary vector containing *35S::RXam1-NosT*, was confirmed by PCR and standard Sanger sequencing.

All PCR reactions used for molecular cloning and confirmation were performed in a 10 μl volume consisting of 0.2 mM of dNTPs, 0.1 mM of each primer, 1X DreamTaq buffer, 1 U of DreamTaq DNA Polymerase (ThermoScientific, Waltham, MA, USA) and 100 ng of DNA. Amplification was carried out in a Bio-Rad thermal cycler programmed for an initial denaturation at 95°C for 3 min, followed by 35 amplification cycles (95°C for 30 s, with T_m depending on each primer, for 45 s and 72°C for 1 min and 20 s), and a final extension step at 72°C for 10 min.

All digestion reactions of TOPO-TA clones and pCAMBIA1305.2 were carried out at 37ºC on a water bath during 3 hours using 10 U of restriction enzyme in a final volume of 20 μl (ThermoScientific, Waltham, MA, USA). The corresponding fragments were run and eluted from a 1% agarose gel. Bands were excised from the gel and purified, using The Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Purified fragments were used for ligation on pCAMBIA1305.2 using a T4 DNA ligase following the manufacturer's instructions (ThermoScientific, Waltham, MA, USA). Ligation reactions were transformed into *E. coli DH10B* and plasmids from PCR positive clones were obtained using GeneJET Plasmid Miniprep Kit

(ThermoScientific, Waltham, MA, USA). One the clones were confirmed by PCR and standard Sanger sequencing, were transformed into *Agrobacterium tumefaciens* strain LBA4404 by electroporation.

E. coli cells were cultivated at 37ºC in LB (Luria-Bertani) broth medium and *A. tumefaciens* LBA4404 at 28ºC in YEB medium (10 g yeast extract. 10 g Bacto peptone, 5 g NaCl) supplemented with streptomycin 50 ug ml^{-1} rifampicin 100 ug ml^{-1} and kanamycin 50 ug ml^{-1}.

FEC isolation and transformation

Friable embryogenic callus (FEC) from the susceptible cassava cultivar 60444 was isolated and used for *Agrobacterium*-mediated transformation as previously described (Bull et al 2009; Taylor et al, 2012;) with modifications. Briefly, organized embryogenic structures (OES) were generated from axillar meristems of four weeks-old *in vitro* plants and maintained through cycles of secondary embryogenesis. OES were purified by removing non-embryogenic tissue and hovered through a metallic mesh for induction of FEC. After three cycles of subcultures, FECs were co-cultured for three days in the dark at 21ºC with *A. tumefaciens* strain LBA4404 carrying the constructs of interest [optical density (OD) at 660nm= 0,05]. Empty vector pCAMBIA1305.2 was used as control.

3.5.5 Recovery of transgenic plants

Transgenic FEC were first selected on Greshoff and Doy (GD) medium (Greshoff & Doy 1974) supplemented with Picloram (12 mg l^{-1}), cefotaxime (250 mg l^{-1}) and hygromycin (20 mg l^{-1}) for two cycles of 21 days each. Next, selection proceed on Murashige and Skoog (MS) medium (Murashige & Skoog 1962), supplemented with naphthalene acetic acid (NAA; 0,19 mg l^{-1}), cefotaxime (100 mg l^{-1}) and hygromycin (20 mg l^{-1}) for two subcultures of 21 days each, the second of which was carried out in the dark, without cefotaxime, NAA at 0,04 mg l^{-1}, and hygromycin at 20 mg l^{-1}. Elongated embryos were then transferred to MS medium supplemented with sucrose (2% w v^{-1}) and 0,45 mg l^{-1} benzylaminopurine (BAP). Plantlets were rooted on MS supplemented with sucrose (2% w v^{-1}) and hygromyicin 20 mg l^{-1}.

3.5.6 Molecular evaluation of transgenic lines

β-Glucuronidase (GUS) assays

Since the T-DNA of pCAMBIA1305.2 contains *GusPlus* as a reported gene, a GUS assay was employed for a preliminary characterization of putative transgenic plants. For this, fresh leafs from *in vitro* plants were selected and immersed in X-Gluc buffer (NaH_2PO_4 0.02 M, Na_2HPO_4 0.03 M, K_4FeCN_6 0.25 mM, K_3FeCN_6 0.25 mM, DMSO 10 % (p/v), triton X-100 0.5 % (v/v) and X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide) 1 mg/mL (Sigma, St. Louis, MO, USA). Leafs were incubated at 37º C during 16 h in dark conditions. Leafs were destained and photographs were taken.

DNA extraction and PCR

Genomic DNA was extracted from fresh leafs of *in vitro* plantlets using the Plant DNA Extraction Protocol for DArT, (Available online: http://www.diversityarrays.com/sites/default/files/pub/DArT_DNA_isolation.pdf). For preliminary characterization of transgenic lines a PCR was performed from genomic DNA using gene-specific primers for *RXam1* (RXam1RT F and RXam1RT R) and the selection marker gene *hptII* (hptIIprobe F and hptIIprobe R). PCR reactions consisted of an initial denaturation step of 3min at 93ºC, followed by 35 cycles of 93ºC for30 s, 56ºC for 30s, 72ºC for 1 min, and a final extension at 72ºC for 5 min.

Southern Blot analysis

For the identification of independent transgenic events, southern-blot assays were performed as previously described (Taylor et al, 2012). DNA was digested using *KpnI,* which cuts once the T-DNA at the start of *RXam1*. A 587pb *hptII* probe was synthetized by PCR (hptIIprobe F and hptIIprobe R) using the PCR DIG probe synthesis kit (Roche Applied Science, Indianapolis, IN, USA). Hybridization and detection was carried out according to manufacturer's instructions using anti-DIG antibody and CDP-star (Roche Applied Science, Indianapolis, IN, USA).

RT-PCR analysis

Total RNA was extracted from *in vitro* leaves using Invitrap ® Spin plant RNA minikit (STRATEC, Berlín, GER). 1 μg of total RNA was treated with DNaseI RNase-free (ThermoScientific, Waltham, MA, USA), this was confirmed by PCR. First strand synthesis was performed with oligo(dT)$_{18}$ primers using the First Strand cDNA Synthesis Kit (ThermoScientific, Waltham, MA, USA). *RXam1* expression levels were assessed using *β-tubulin* as reference control. RXam1RT F and RXam1RT R were used for *RXam1* and tub3exRT F and tub3exRT R were used for*β-tubulin*.

Plant inoculations

Cassava *in vitro* plants were grown in a growth chamber at 28ºC under 16h of light on MS medium supplemented with sucrose (2% w v^{-1}). *Xam* strains were grown at 28ºC on liquid LPG medium (5 g yeast extract, 5 g dextrose and 5 g peptone per L of distilled water) and resuspended in 10mM $MgCl_2$. 2 month-old *in vitro* cassava plants were selected for Q-Tip leaf inoculation. Briefly, young leaves were punctured in the mesophyll (apoplast) using an insulin-syringe. Each leaf was then wiped on both adaxial and abaxial side using a Q-Tip previously immersed in a *Xam* cell suspension [optical density at 600nm (OD_{600nm})= 0,02,~10^7 colony forming units (CFU ml^{-1})]. Bacterial growth was assessed after 3 and 8 dpi. The complete leaf was ground in 100 μL of 10mM $MgCl_2$. Serial dilutions were plated on LPGA (5 g yeast extract, 5 g dextrose, 5 g peptone and 15 g agar per L of distilled water).

3.6 Supplementary information

Supplementary table 3-1: List of primers used in this study.

Primer	Sequence (5' to 3')	Gene_ID
35SEcoRI F	GCAGAATTCTCAACATGGTGGAGCAC	*35S promoter*
35SSacI R	TGCGAGCTCGTCCCCGGTGTTCTCTCC	
NostBamHI F	GCAGGATCCCGTTCAAACATTTGGC	*Nos terminator*
NostSalI R	TGCGTCGACCCCGATCTAGTAACATAG	
RXam1KpnI F	GCAGGTACCATGGGGTGTGGATGCTTCTG	*Manes.10G014300*
RXam1BamHI R	TGCGGATCCCTTAGGTGTAGATTTC	
RXam1RT R	ACTGGTAGATGCAACCACTCCTCA	*Manes.10G014300*
RXam1RT F	TGGAGCAGCTAAGTCTTTCATGGC	
PP2A F	TGCAAGGCTCACACTTTCATC	*Manes.09G039900*

PP2A R	CTGAGCGTAAAGCAGGGAAG	
tub3exRT F	GGAAAGATGAGCACCAAGGA	*Manes.08G061700*
tub3exRT R	ACCAGTATACCAGTGCAAGAAG	
G3PDHcassava F	CTGGTGGTTCAGGTATCAAAGA	*Manes.11G072400*
G3PDHcassava R	CCATATCATCTCCCATCACCATT	
hptIIprobe F	CGTCTGCTGCTCCATACAAG	*Hygromycine phosphotranferase*
hptIIprobe R	ATAGCTGCGCCGATGGTT	

Supplementary table 3-2: Genes located near *RXam1* QTL (XM5) in a region spanning 350 kb.

Locus name	**Position/ orientation**	**Annotation**
Manes.10G012100	946748..949615 forward	SERINE/THREONINE-PROTEIN KINASE-LIKE PROTEIN CCR3-RELATED
Manes.10G012200	953600..960188 reverse	Transcription factor NF-X1, contains NFX-type Zn2+-binding and R3H domains
Manes.10G012300	961201..962782 reverse	SERINE/THREONINE-PROTEIN KINASE-LIKE PROTEIN CCR3-RELATED
Manes.10G012400	963646..965704 forward	SERINE/THREONINE-PROTEIN KINASE-LIKE PROTEIN CCR3-RELATED
Manes.10G012500	966843..968646 forward	SERINE/THREONINE-PROTEIN KINASE-LIKE PROTEIN CCR3-RELATED
Manes.10G012600	993046..995138 forward	SERINE/THREONINE-PROTEIN KINASE-LIKE PROTEIN CCR3-RELATED
Manes.10G012700	996068..1001324 reverse	Phosphoinositide phospholipase C / Triphosphoinositide phosphodiesterase
Manes.10G012800	1008787..1012412 reverse	PHOSPHOINOSITIDE PHOSPHOLIPASE C 6
Manes.10G012900	1020020..1022711 forward	PEPTIDYL-PROLYL CIS-TRANS ISOMERASE
Manes.10G013000	1024740..1025361 reverse	PROTEIN EARLY FLOWERING 4
Manes.10G013100	1033551..1033712 forward	
Manes.10G013200	1033832..1037377 reverse	GDSL ESTERASE/LIPASE CPRD49
Manes.10G013300	1042308..1049745 reverse	
Manes.10G013400	1050244..1057032 reverse	PAX6 NEIGHBOR PROTEIN PAXNEB
Manes.10G013500	1067407..1070005 forward	ALPHA/BETA HYDROLASE RELATED PROTEIN
Manes.10G013600	1070710..107839	NUCLEOPORIN NUP84-RELATED

	1 forward	
Manes.10G013700	1079045..1083025 reverse	CYTOCHROME P450 90A1
Manes.10G013800	1091586..1096160 forward	PROTEIN YIPPEE-LIKE
Manes.10G013900	1100163..1106464 reverse	Arginyltransferase / Arginyl-tRNA--protein transferase
Manes.10G014000	109271..1114113 forward	
Manes.10G014100	1115463..1118178 reverse	CLATHRIN LIGHT CHAIN 2
Manes.10G014200	1118508..1120762 reverse	OVARIAN CANCER GENE-2 PROTEIN-RELATED
Manes.10G014300	1122770..1126278 forward	Protein kinase domain (Pkinase) // Leucine Rich Repeat (LRR_1) // Leucine rich repeat N-terminal domain (LRRNT_2), (RXam1)
Manes.10G014400	1127583..1128321 forward	PLEKHH PROTEIN
Manes.10G014500	1129990..1133312 reverse	Protein kinase domain (Pkinase) // Leucine rich repeat N-terminal domain (LRRNT_2) // Leucine rich repeat (LRR_8)
Manes.10G014600	1142192..1145798 reverse	NUCLEOLAR HISTONE METHYLTRANSFERASE-RELATED PROTEIN
Manes.10G014700	1147516..1147806 reverse	
Manes.10G014800	1148281..1150645 reverse	NUCLEOLAR HISTONE METHYLTRANSFERASE-RELATED PROTEIN
Manes.10G014900	1152921..1154046 forward	Protein of unknown function (DUF724) (DUF724)
Manes.10G015000	1155564..1155821 reverse	
Manes.10G015100	1157151..1160519 reverse	NUCLEOLAR HISTONE METHYLTRANSFERASE-RELATED PROTEIN
Manes.10G015200	1160579..1163637 forward	Protein of unknown function (DUF724) (DUF724)
Manes.10G015300	1167594..1172603 reverse	AUXIN TRANSPORTER PROTEIN 1-RELATED
Manes.10G015400	1169884..1170743 forward	
Manes.10G015500	1197588..1200790 forward	Leucine Rich Repeat (LRR_1) // Protein tyrosine kinase (Pkinase_Tyr) // Leucine rich repeat N-terminal domain (LRRNT_2) // Leucine rich repeat (LRR_8
Manes.10G015600	1203529..1206932 forward	Protein kinase domain (Pkinase) // Leucine rich repeat N-terminal domain (LRRNT_2) // Leucine rich repeat (LRR_8)
Manes.10G015700	1208420..1209859 reverse	MONOOXYGENASE

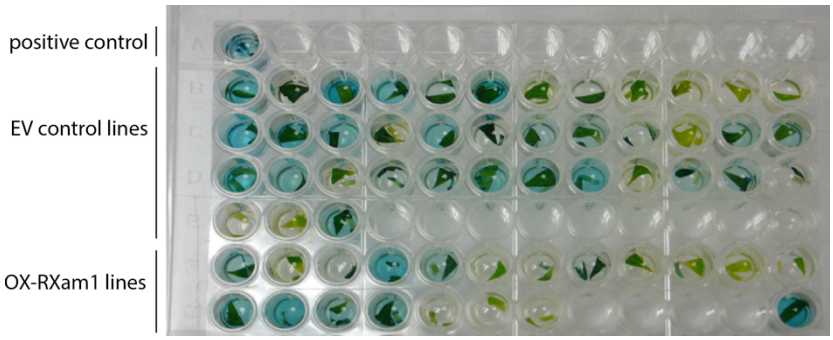
Manes.10G015800	1211558..1214856 forward	Protein tyrosine kinase (Pkinase_Tyr) // Leucine rich repeat N-terminal domain (LRRNT_2) // Leucine rich repeat (LRR_8)
Manes.10G015900	1226833..1229565 forward	Protein kinase domain (Pkinase) // Leucine Rich Repeat (LRR_1) // Leucine rich repeat N-terminal domain (LRRNT_2) // Leucine rich repeat (LRR_8)
Manes.10G016000	1259267..1262752 forward	Protein kinase domain (Pkinase) // Leucine rich repeat N-terminal domain (LRRNT_2) // Leucine rich repeat (LRR_8)
Manes.10G016100	1266018..1268949 forward	60S ACIDIC RIBOSOMAL PROTEIN P0
Manes.10G016200	1275042..1276844 forward	Hs1pro-1 protein C-terminus (Hs1pro-1_C)
Manes.10G016300	1295954..1298628 forward	
Manes.10G016400	1300434..1306020 reverse	Glucuronosyl-N-acetylglucosaminyl-proteoglycan 4-alpha-N-acetylglucosaminyltransferase / acetylglucosaminyltransferase
Manes.10G016500	1302609..1303994 forward	
Manes.10G016600	1312629..1314244 forward	AQUAPORIN PIP1-4-RELATED

Supplementary figure 3-1: Basic local alignment between *RXam1* and *Xa21* at amino acid level.

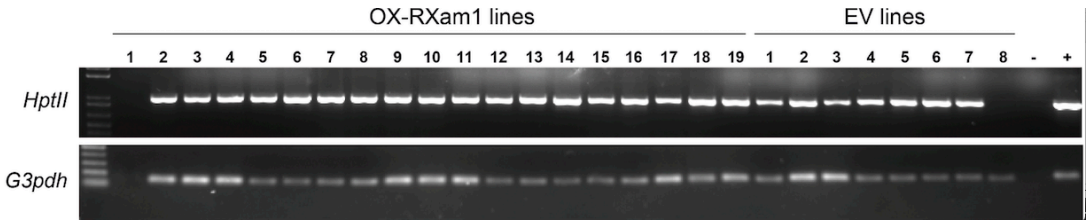
A period, indicates conservation between groups of weakly similar properties; a colon, indicates conservation between groups of strongly similar properties; an asterisk, indicates positions that have a single and fully conserved residue.

```
Xa21            --MISLPLLLFVLLFSALLLCPSSSDDDGDAAGDELALLSFKSSLLYQGGQSLASWNTSG 58
RXam            MGCGCFWSCFYIHAVVYLLFFPSTTYATGGNETDRLALLEFMGKIDADPFGVLSSWNSSV 60
                    .:   :::  .  **: **::   *.   *.****.* ..:  :    *:***:*
Xa21            HGQHCTWVGVVCGRRRRRHPHRVVKLLLRSSNLSGIISPSLGNLSFLRELDLGDNYLSGE 118
RXam            H--FCQWYGVTCSRR----HQRVTVLDLNSLKLAGTIPPHIGNLSFLKVLDLKNNSFRQN 114
                *  .* * **.*.**     :**. * *.* :*:* *.* :******: *** :* :  :
Xa21            IPPELSRLSRLQLLELSDNSIQGSIPAAIGACTKLTSLDLSHNQLRGMIPREIGASLKHL 178
RXam            IPPEVGRLRRLHTLYLYNNSLSGEIPSNLSRCSGLVRLSLVNNILAGEIPVELG-LLSKL 173
                ****:.** **: * * :**:.*.**: :. *: *. *.* :* * * ** *:*  *.:*
Xa21            SNLYLYKNGLSGEIPSALGNLTSLQEFDLSFNRLSGAIPSSLGQLSSLLTMNLGQNNLSG 238
RXam            QFLSVSRNNLSGIIPPSLGNLSSLTTLSAGLNNFVGEVPEALGQLKNLRFLGLFMNQLSG 233
                . * : :*.*** **.:****:**  :. .:*.: * :*.:****..*  :.*  *:***
Xa21            MIPNSIWNLSSLRAFSVRENKLGGMIPTNAFKTLHLLEVIDMGTNRFHGKIPASVANASH 298
RXam            TIPSSVFNLSSITLFDIASNQFEGKLPRDLFIFLPKLETFSIAVNQFTGPIPNSASNASN 293
                 **.*:::****:  *.: .*:: * :* : *  *  **.:.:..*:* * ** *.:***:
Xa21            LTVIQIYGNLFSGIITSGFGRLRNLTELYLWRNLFQTREQDDWGFISDLTNCSKLQTLNL 358
RXam            LLHLQLQGNKLTGKVPS--LEMLNIVAFFIDNNIIGNGEFDDLGFLCSLTNASSLQLLDI 351
                *  :*: ** ::* :.*   .: *:. ::: .*:: . * ** **:..***.*.** *::
Xa21            GENNLGGVLPNSFSNLSTSLSFLALELNKITGSIPKDIGNLIGLQHLYLCNNNFRGSLPS 418
RXam            SRNKLGGEIPECIGNLSSNLAMLSLHQNQISGKIPDGIGNLVNLESIEAYKNKLSGIIPP 411
                ..*:*** :*:.:.***:.*::*:*. *:*:*.**..****:.*: :   :*:: * :*.
Xa21            SLGRLKNLGILLAYENNLSGSIPLAIGNLTELNILLLGTNKFSGWIPYTLSNLTNLLSLG 478
am              SIGNLQNLVKLILDENKISGPIPSSLGNLTSLNRLHSADNDLQGTIPSSLADCKNLQVLD 471
                *:*.*:**  *:  **::**.** ::****.** *  . *.:.* ** :*:: .**  *.
Xa21            LSTNNLSGPIPSELFNIQTLSIMINVSKNNLEGSIPQEIGHLKNLVEFHAESNRLSGKIP 538
RXam            FSRNNLTGTLPPQVIGLSSLSIYVGFAQNNLRGSIPREVENLKNLGIFDVSDNKLSGEIP 531
                :* ***:*.:*.:::.:.:*** :..::***.****:*: :****  *....*:***:**
Xa21            NTLGDCQLLRYLYLQNNLLSGSIPSALGQLKGLETLDLSSNNLSGQIPTSLADITMLHSL 598
RXam            SSLGSCISLEYLYMQGNQFQGPIPSSLSSLRGLQVLNLSYNNLSGQIPEFLAGFSFIY-L 590
                .:**.*  *.***:*.* :.*.***:*..*:**:.*:** ********  **.::::: *
Xa21            NLSFNSFVGEVPTIGAFAAASGISIQGNAKLCGGIPDLHLPRCCPLLENRKHFPVLP--I 656
RXam            NLSFNNFEGRAPTDGIFKNASIVSVTGNSKLCGGIPEFQLPACNFKRSEKRRVKVIVGII 650
                *****.* *..** * *  ** :*: **:*******:::** *    .::::. *:   *
Xa21            SVSLAAALAILSSLYLLITWHKRTKKG-APSRTSMKGHPLVSYSQLVKATDGFAPTNLLG 715
RXam            AGGLGAILVVLSFIFLLRLRKKSRKPSSSYSENSLLELPKVSYRDLYKATDGFSSENLIG 710
                : .*.* *.:** ::**   :*  * . : *..*:   * *** :* ******:. **:*
Xa21            SGSFGSVYKGKLN-IQDHVAVKVLKLENPKALKSFTAECEALRNMRHRNLVKIVTICSSI 774
RXam            TGSFGSVYKGILDEGGPVVAVKVLNLQHHGAAKSFMAECEALRNIRHRNLVKILTACSGV 770
                :********* *:     ******:*::  * *** ********:********:* **.:
Xa21            DNRGNDFKAIVYDFMPNGSLEDWIHP---ETNDQADQRHLNLHRRVTILLDVACALDYLH 831
RXam            DYQGNDFKALVYEYMDNGNLEEWLHLPVSADRNHGEPKNLNLLQRVNIAIDVASAIEYLH 830
                * :******:**::* **.**:*:*      .::.: ::*** :**.* :***.*::***
Xa21            RHGPEPVVHCDIKSSNVLLDSDMVAHVGDFGLARILVDGTS-LIQQSTSSMGFIGTIGYA 890
RXam            HHCGNPIXHCDLKPSNVLLDGQMTAHIGDFGLAKFLLENMHNNSTNQFSSIGLRGTIGYA 890
                :*  :*: ***:*.******.:*.**:******::*::.      :. **:*: ******
Xa21            APEYGVGLIASTHGDIYSYGILVLEIVTGKRPTDSTFRPDLGLRQYVELGLHGRVTDVVD 950
RXam            PPEYGLGSEVSTYGDVYSYGVLLLEMFTGKRPTDDMFKEGLNLHKFAESALPNRVNEIVD 950
                .****:*  .**:**:****:*:**:.*******. *: .*.*:::.* .* .**.::**
Xa21            TKLILD--SENWLNSTNN--------------------SPCRRI---------------- 972
RXam            PILFQESHSEKPMNGRSNRIMMEFLISIVGIGIACSAELPANRMEINDAAKKLCLIRDKL 1010
                . *: :  **: :*. .*                      *..*:
Xa21            ---------
RXam            MAPDEEIYT 1019
```

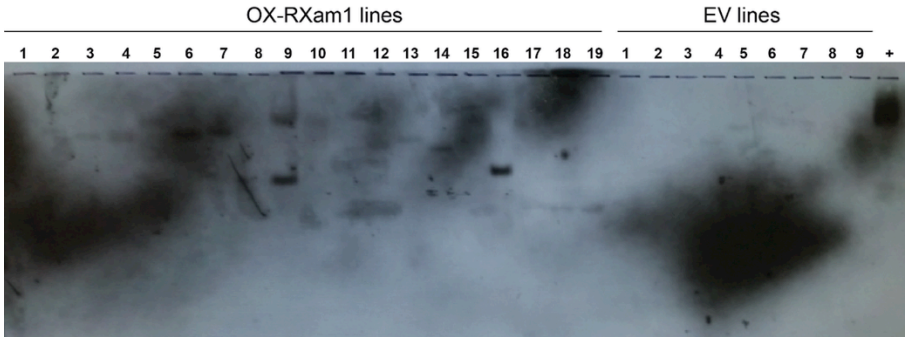
Supplementary figure 3-2: Histochemical *GUS* assay on *in vitro* leaves from transgenic cassava plants.



Supplementary figure 3-3: PCR from genomic DNA isolated on *in vitro* leaves from transgenic cassava plants.

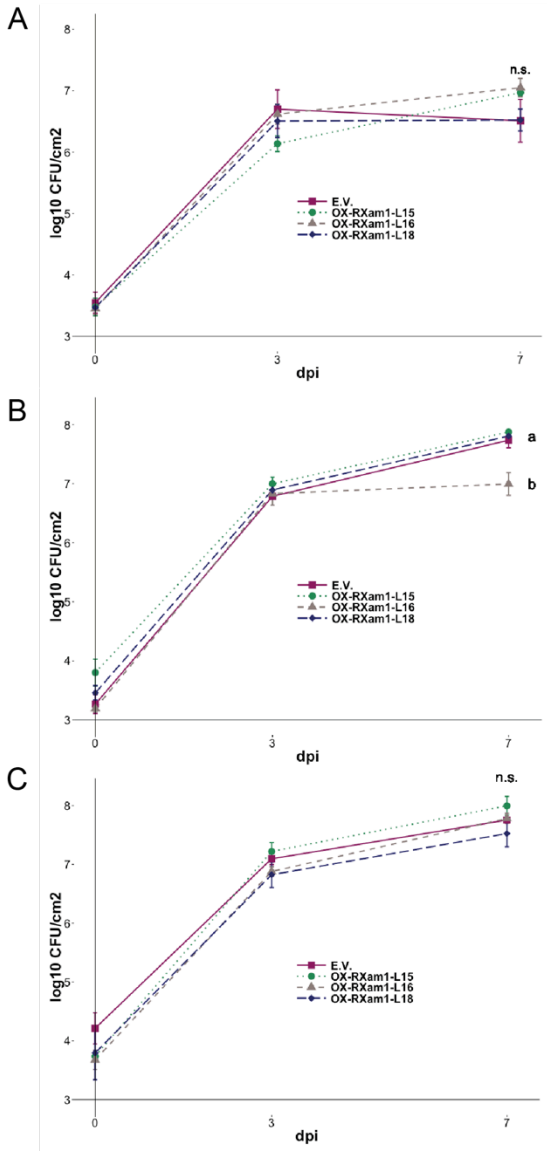


Supplementary figure 3-4: Southern-blot analysis of OX-RXam1 putative transgenic plants

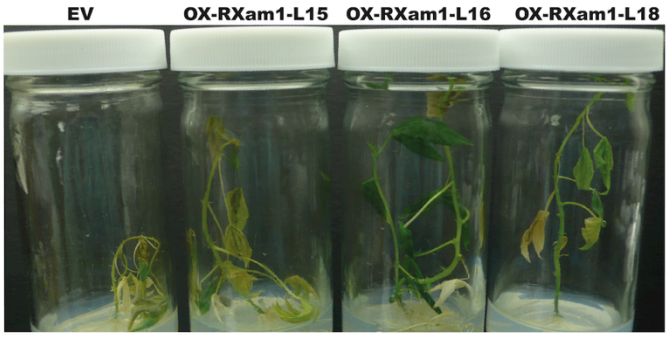


Supplementary figure 3-5: Bacterial growth of three *Xam* strains on OX-RXam1 *in vitro* transgenic plants.

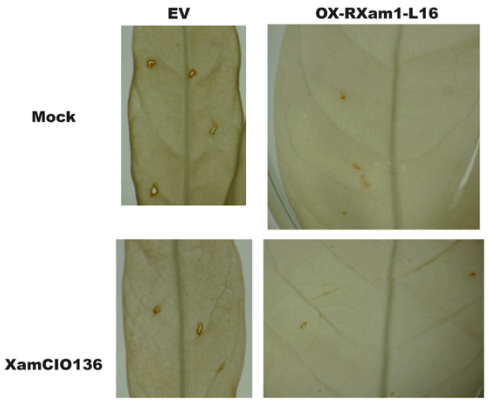
A *Xam226.* **B** *XamCIO151*. **C** *Xam668*.



Supplementary figure 3-6: Disease symptoms at 15 dpi on OX-RXam1 *in vitro* transgenic plants.



Supplementary figure 3-7: DAB staining on leaves inoculated with *XamCIO136* at 2 dpi in OX-RXam1 on L.16 and E.V. control plants.



Chapter 4

"No hables a menos que puedas mejorar el silencio"

"Don't talk unless you can improve the silence"

Jorge Luis Borges

4 Functional analysis of RXam2, a NLR from cassava (*Manihot esculenta* Crantz) involved in resistance to *Xanthomonas axonopodis* pv. manihotis.

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4.1 Abstract

Cassava (*Manihot esculenta* Crantz) is a tropical crop that ranks first as a source of calories among root and tuber crops. Cassava production is severely compromised by a diverse array of pathogens. A bacterial disease known as cassava bacterial blight (CBB) is present in all regions where cassava is cultivated. The causal agent of CBB is *Xanthomonas axonopodis* pv manihotis (*Xam*). Previous analyses have identified QTLs and RGC associated with resistance to *Xam* strains. Here we sought to validate the functional role a NLR associated with a QTL to *XamCIO151* called *RXam2* (*Resistance to Xam 2*). Transgenic cassava plants expressing small interfering RNA (siRNA) derived from *RXam2* were generated and analyzed. *RXam2* silenced plants showed enhanced susceptibility to all *Xam* strains tested. In addition, transgenic cassava plants overexpressing *RXam2* were produce. Our results suggest the overexpression of *RXam2* confers less susceptibility to *Xam*. Furthermore, as a proof of concept, an activator trap promoter was developed to induce the expression of a mutated autoactive version of RXam2. Altogether, our results indicate *RXam2* contributes to broad-spectrum resistance to *Xam* strains.

4.2 Introduction

On a traditional genetic basis, plant disease resistance has been divided in two broad categories: qualitative and quantitative resistance. The former refers to a complete or monogenic resistance that is determined by major *R* genes, while the latter explains partial, incomplete or polygenic resistance that is governed by several genes, each one contributing at different level to resistance

(St.Clair, 2010). Due to the complex nature of quantitative disease resistance (QDR), is more challenging to be employed in breeding programs compared with qualitative resistance (Michelmore et al., 2013). Nevertheless, considering its polygenic nature, QDR has always been associated with durable and broad-spectrum resistance (Poland et al., 2009; Roux et al., 2014). Remarkably, in the last ten years several strategies have lead to the identification of at least eleven genes coding for structurally diverse proteins underlying QDR in wheat, rice, maize, soybean, *Arabidopsis* and barley (Roux et al., 2014; French et al., 2016). However, to date the molecular mechanism underlying QDR is not clear. On the other hand, qualitative resistance has been relatively well studied. The most important contribution to this knowledge comes from the identification and function of classical *Resistance* (*R*) genes. These genes code for intracellular receptors that sense pathogen effector proteins using diverse molecular mechanisms (Cesari et al., 2014; Duxbury et al., 2016). These intracellular receptors are called nucleotide-binding domain and leucine-rich repeat (NLRs) for the presence of these two groups of conserved domains. Upon pathogen perception by NLRs a series of signaling events are rapidly triggered leading to the development of a programmed cell death called hypersensitive response (HR) (Coll et al., 2011). This implies a strict molecular control of NLR activation in the absence of invading pathogens. One of the most studied are the intra- and inter-molecular steady state inhibition of NLRs required for robust immune signaling (Takken and Tameling, 2009; Takken and Goverse, 2012; Duxbury et al., 2016).

NLRs contain an N-terminal domain putatively involved in signaling, a conserved central NB-ARC (nucleotide-binding shared with APAF-1, resistance proteins and CED-4) domain and a divergent C-terminal domain containing leucine-rich repeats (Takken and Goverse, 2012). The NB-ARC domain comprises three subdomains: NB, ARC1 and ARC2 (van Ooijen et al., 2008b; Lukasik and Takken, 2009; Qi et al., 2012). A substitution of aspartate (D) for valine (V) in MHD motif of the ARC2 subdomain has proven to result in the activation of NLRs in the absence on the pathogen in multiple species (Bendahmane et al., 2002; De La Fuente Van Bentem et al., 2005; Howles et al., 2005; Rairdan and Moffett, 2006; van Ooijen et al., 2008a; Gao et al., 2011; Maekawa et al., 2011a; Williams et al., 2011; Bai et al., 2012; Roberts et al., 2013; Stirnweis et al., 2014; Wang et al., 2015c). In accordance, this autoactivating mutation often leads to lethal or severe dwarf phenotypes (Howles et al., 2005; Gao et al., 2011; Roberts et al., 2013).

Cassava is a perennial shrub native from South America and domesticated in the southwestern part of the Amazon region (Allem, 1999; Olsen and Schaal, 1999; Bredeson et al., 2016). Its

starchy roots provide calories for around 1000 million people in tropical and sub-tropical regions from America, Africa and Asia (Cock, 1982) (http://faostat3.fao.org/). This crop is severely affected by several diseases ranging from insects, virus, fungi and bacterial pathogens (Lozano, 1986; Alvarez et al., 2012). Cassava bacterial blight (CBB) is the most frequent bacterial disease of cassava. CBB is a vascular and foliar disease caused by the Gram-negative bacterium *Xanthomonas axonopodis* pv. manihotis (*Xam*). Despite the constraints in production caused by pathogens that commonly infect cassava, there are few studies aiming to understand the genetic and molecular basis of resistance. Resistance to CBB is polygenic and additively inherited with an estimated heritability ranging from 25-65% (Hahn et al., 1979, 1980). Based on the availability of the first genetic linkage map (Fregene et al., 1997), several quantitative trait loci (QTL) with different contribution to CBB resistance were mapped in different linkage groups (Jorge et al., 2000, 2001). Later studies were focused on the identification of *R* genes in the cassava genome. Using conserved motifs on the NB (nucleotide-binding) domain among NLRs, a resistance gene candidate (RGC) approach was employed to identify putative *R* genes in the cassava genome (López et al., 2003). Afterwards, and trough SSRs and EST mapping strategies, some RGCs were shown to colocalize with new QTLs (López et al., 2007). Interestingly, a BAC containing an RGC (RGC7) was shown to colocalize with a QTL explaining 61.6% of resistance to *XamCIO151* (López et al., 2003, 2007). Recently, using bioinformatic and mapping SNPs strategies the repertoire of immunity genes were identified in the cassava genome and mapped (Leal et al., 2013; Lozano et al., 2015; Soto et al., 2015). However, to date no functional *R* genes have been identified and cloned in cassava.

Recent genome sequence data from *Xam* strains revealed a proteome that comprises around 28 type-III effector proteins (Bart et al., 2012; Arrieta-Ortiz et al., 2013). Among these, TAL (transcription activator-like) effectors from *Xam*, TALE1, TAL14 and TAL20 have shown to promote *Xam* virulence (Castiblanco et al., 2013; Cohn et al., 2014). TAL effectors are highly modular proteins that travel to the plant nucleus to modulate host gene expression to its own benefit (Boch et al., 2014). TAL effector activity is determined by a central domain with a variable number of repetitions, C-terminal nuclear localization signals (NLS) and an acidic activation domain (AAD) (Schornack et al., 2013). The central repetitive domain is responsible for DNA binding specificity. The number of repeats is variable between different effectors, although amino acids are conserved across repetitions with the exception of the hypervariable residues 12 and 13 (RVDs; repeat variable di-residue), that define the base-specific interaction with the host DNA (Boch et al., 2009; Moscou and Bogdanove, 2009; Deng et al., 2012; Mak et al., 2012). Hence, it is

possible to predict the binding site in the DNA of the host plant (EBEs; effector binding elements) from the given sequence of RVDs (Boch et al., 2014). Promoter regions of *susceptibility (S)* genes and executor *R (E)* genes naturally harbor one or more EBEs (Hutin et al., 2015a; Zhang et al., 2015). This knowledge has been recently used to engineer resistance by introducing different EBEs in the promoter region of well-known *E* genes (Strauss et al., 2012; Hummel et al., 2012b).

In this study we aimed to functionally characterize the previously identified candidate *R* gene (RGC7), renamed *RXam2,* associated with a major QTL explaining 61.6% of resistance to *XamCIO151* (López et al., 2007, 2003). For this, we silenced *RXam2* using an RNAi- based approach and found that *RXam2*-silenced plants showed enhanced susceptibility to several *Xam* strains. Further, *RXam2* was overexpressed and a small reduction in *Xam* growth was observed for three *Xam* strains. In addition, a promoter activator trap using the EBE of TALE1 from *Xam* was employed to activate an autoactive version of RXam2 (D492V). Taken together, these results suggest *RXam2*, a gene coding for a NLR from cassava might confer partial and broad-spectrum resistance to different *Xam* strains.

4.3 Results

4.3.1 *RXam2* (RGC7) codes for a typical NLR protein with CC motifs

In a previous study a BAC containing an RGC, called RGC7, was found to colocalize with a QTL explaining 61.6% of resistance against *XamCIO151* (López et al., 2007). The partial sequencing of this BAC by primer walking allowed the identification of a complete gene coding for a NLR, which was named *RXam2* (Resistance to *Xam* 2). The posterior sequencing of the BAC containing RGC7 showed to comprise a region of a gene coding for a NLR and was named *RXam2* (Resistance to *Xam* 2). *RXam2* is annotated in cassava genome V6.1 as *Manes.07G048100* (previously annotated as cassava4.1_031234m) and is located in chromosome 7 between the positions 4853478 and 4857038. Towards identifying other putative resistance gene candidates around the QTL, a region spanning at least ~ 5 Mb around *RXam2* was analyzed. In total sixty-three annotated genes were identified but with exception of *RXam2*, no other genes coding for proteins containing NB-ARC domains were found in this region (Supplementary table 4-2). We focused our research on *RXam2* as the best candidate explaining the resistance to *XamCIO151* to validate its function.

RXam2 codes for a predicted 1186 amino acids (aa) protein, which contains a putative coiled-coil motif domain, located within the N-terminal sequence between 35 and 55 aa. The NB domain is positioned between 170 and 323 aa and comprises five motifs: P-loop (201-207), RNBS-A (221-234), kinase 2 (273-281) and RNBS-B (303-312). ARC1 ranges from 323 to 402 aa and harbors two motifs: RNBS-C (324-344) and GLPL (367-373). ARC2 ranges from 402 to 519 aa and contains the motifs RNBS-D (425-437) and MHD (492-495). The LRR domain is located within the C-terminal sequence between 519 and 1186 aa (Figure 4-1).

Figure 4-1: Schematic representation of RXam2 protein sequence.

N-terminal, NB-ARC and C-terminal domains are depicted in bold letters on the right. The motifs present in each domain are highlighted in bold colored letters and the name of each motif is shown in bold letters above.

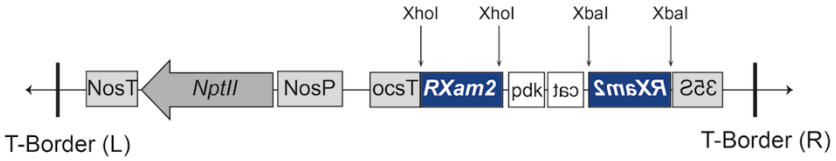
4.3.2 *RXam2* silenced plants display enhanced susceptibility to several *Xam* strains

In order to assess to role of *RXam2* on CBB resistance, a silencing construct (Figure 4-2) was developed with an intron-hairpin RNA interference (RNAi) construct derived from a fragment of

the NB region of RXam2. Friable embryogenic calli (FEC) from the model cultivar cv.60444 was produced and transformed with the RNAi construct. In total thirty-eight putative transgenic plants were regenerated. To detect positive transgenic plants, a PCR was performed using primers to amplify the selection marker *nptII*. In total, six plants showed an amplification of *nptII* (Supplementary figure 4-1). Posterior Southern-blot analysis indicates they all represent the same transformation event (Supplementary figure 4-2). With the aim of detecting whether *RXam2* mRNAs were in fact targeted by construct-derived small interfering RNAs (siRNAs), an RT-PCR analysis was performed. We expected to detect less *RXam2* transcription levels in silenced plants compared with WT control plants. However, *RXam2* expression in non-transgenic control plants was too low that was almost undetectable even with a high number of amplification cycles (data not shown). For this reason it was not possible to detect a measurable difference in *RXam2* mRNA levels on RXam2-RNAi plants compared with WT control plants using qRT-PCR analysis. To investigate mRNA levels of *RXam2* in the cassava transcriptome, RNAseq data from non- and inoculated material was analyzed and around 5-10 counts per million (CPM) mapped reads were shown to map against *RXam2* (data not shown). No differential expression of *RXam2* was observed as a result of *Xam* inoculation. Interestingly, two *RXam2* homologs (*Manes.10G091500* and *Manes.07G107800*) showed ten-times more CPM values compared with *RXam2* (data not shown). Taken together, these results indicate *RXam2* is a gene with constitutively low expression, even among other genes coding for NLRs.

Figure 4-2: Schematic diagram of the T-DNA region of the construct used for *RXam2* silencing.

A fragment corresponding to the NB domain of *RXam2* was cloned first on pENTR/D-TOPO and further subcloned into pHELLSGATE12 at XhoI and XbaI sites in a sense and antisense orientation. The silencing cassette is under 35S strong constitutive promoter and an OCS terminator sequence. Transgenic cassava plants were selected for resistance to G418.



To determine whether RXam2-RNAi transgenic plants display enhanced susceptibility, distinct approaches were addressed. Since *Xam* is a foliar and vascular pathogen, two different inoculation methods were assessed. First, a stem inoculation was performed and disease symptoms progression was evaluated. For this, adult plants were stem punctured with

XamCIO151 and symptoms progression was measured at 7, 14, 21 and 28 dpi (days post inoculation). A scale of disease symptoms from 0 to 5 was used, where 0 indicates no symptoms and 5 indicates plant wilting and the sum of mean AUDPC (area under disease progress curve) score at 28 dpi was calculated. Our results showed RXam2-RNAi plants display faster symptom development compared with control plants (Table 4-1). Given that some genes associated with QTLs are known to confer broad-spectrum resistance; other *Xam* strains were subsequently evaluated. Table 4-1 shows that RXam2-RNAi plants exhibit significant higher AUDPC scores for almost all strains (*P*-value <0,05) except for plants inoculated with *Xam686*. However, despite the fact that *RXam2* silenced plants inoculated with *Xam686* showed higher AUDPC scores, no statistical significant differences were found when compared with control plants. Nevertheless, at 28 dpi nearly all RXam2-RNAi plants showed wilting compared with control plants (Figure 4-3). Altogether, these results suggest *RXam2* has a possible role in resistance since silenced plants displayed enhanced susceptibility.

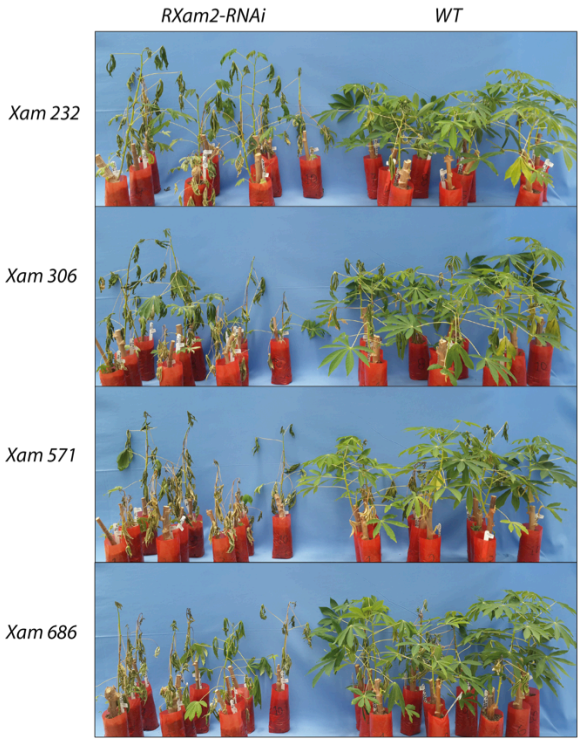
Table 4-1: Area under disease progression curve (AUDPC) analysis of *RXam2*-silenced plants inoculated with five different *Xam* strains.

Two month-old cassava plants grown from adult stakes were inoculated by stem puncturing with a *Xam* cell suspension [optical density at 600nm (OD_{600nm})= 0,02,~10^7 colony forming units (CFU ml^{-1})]. [a] Data shown is the mean of the sum of AUDPC in arbitrary units (AU) at 28 dpi from ten replicates. Standard error (SE) was calculated from ten biological samples per genotype. [b] An unpaired Student's t-test was used to calculate *P*-values. [c] One asterisk (*) indicates significant differences (*P*-value 0.05-0.01), two asterisks (**) indicate strong significant differences (*P*-value 0.01-0.001), three asterisks (**) indicate very strong significant differences (*P*-value < 0.001) and N.S. indicate not significant, compared with WT control plants for each strain.

Strain	Genotype	Mean AUDPC (AU)[a]	SE	*P*-value[b]	Significance[c]
XamCIO151	WT	30.7	3.8	0.00941	**
	RNAi_RXam2	45.0	3.7		
Xam306	WT	45.2	2.1	< 0.001	***
	RNAi_RXam2	64.3	2.4		
Xam232	WT	37.7	2.1	< 0.001	***
	RNAi_RXam2	56.2	3.0		
Xam571	WT	52.6	8.3	0.02473	*
	RNAi_RXam2	67.9	5.9		
Xam686	WT	49.4	6.4	0.147	N.S.
	RNAi_RXam2	56.7	5.3		

Figure 4-3: Disease symptoms of *RXam2*-silenced plants (left) compared with WT control plants (right) at 28 dpi.

Plants were inoculated in the stem as mentioned in Table 4-1.

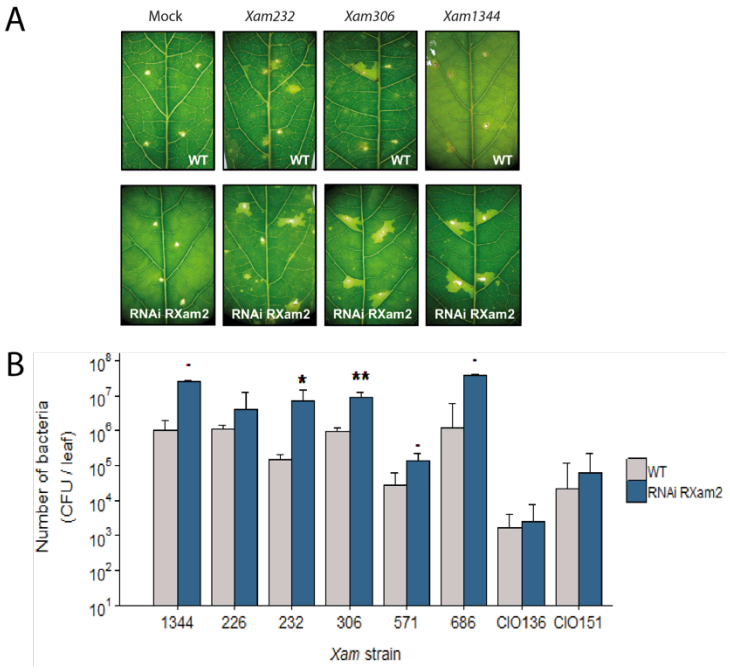


To test if the enhanced susceptibility observed for *RXam2* silenced plants was dependent on the inoculation method used, RXam2-RNAi plants were additionally inoculated on the leaves. For this, leaf inoculation was performed on greenhouse plants with a bacterial suspension at OD_{600nm}= 0,002,~10^7 colony forming units (CFU ml^{-1}). *Xam* strains were inoculated on young leaves in the mesophyll near the midvein. Three more strains were tested in addition to the five strains previously used for stem inoculation. Figure 4-3A shows disease symptoms observed at 8 dpi. Disease symptoms appear as yellow zones or water soaked areas near the inoculation point. The severity of disease symptoms was higher in RXam2-RNAi plants compared with control plants (Figure 4-3A). In addition, bacterial numbers were quantified. Our results reveal bacterial titers were increased in RXam2-RNAi plants for virtually all strains tested (Figure 4-3B). Nevertheless, greater differences in bacterial titers were observed for *Xam232* and *Xam306*. These results resemble data from stem inoculation where strong significant differences in AUDPC scores were

obtained on plants inoculated with *Xam232* and *Xam306*. Collectively, these results indicate *RXam2* silencing enhances susceptibility to practically all strains evaluated. This effect is particular prominent for *Xam232*, *Xam306* and *Xam1344* compared with the other strains evaluated.

Figure 4-4: *RXam2*-silenced plants display enhanced susceptibility to different *Xam* strains.

Two month-old cassava plants grown from adult stakes were inoculated by leaf dipping with a *Xam* cell suspension [optical density at 600nm (OD_{600nm})= 0,02,~10^7 colony forming units (CFU ml^{-1})]. (**A**) disease symptoms observed at 8 dpi is shown here for strains *Xam232, Xam306* and *Xam1344*. The first row of photographs shows leaves from the WT control genotype and the second row are photographs from RXam2-RNAi plants. The first column shows mock (10mM $MgCl_2$) inoculated plants. (**B**) Bacterial numbers were measured at 3 (data not showed) and 8 dpi. Mean bacterial titers from three biological samples per genotype at 8PDI ± standard error (SE) is shown here. One-tail unpaired Student's t-test was used to calculate statistical significance for RXam2-RNAi plants compared with WT control plants for each strain used. Lines (-) indicate marginally significant differences (*P*-value 0.1-0.05), one asterisk (*) indicates significant differences (*P*-value 0.05-0.01) and two asterisks (**) indicate strong significant differences (*P*-value 0.01-0.001).

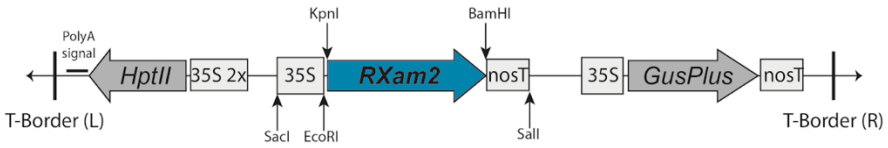


4.3.3 *RXam2* overexpression confers less susceptibility to *Xam*

To further assess *RXam2* role in cassava resistance to CBB, transgenic cassava plants overexpressing *RXam2* were produced. For this, *RXam2* full-length was amplified from the resistant cultivar CM6438-14 and cloned into the binary vector pCAMBIA1305.2. The T-DNA graphical representation of the construct employed for *RXam2* overexpression (OX-RXam2) is shown in Figure 4-5. Forty-three putative transgenic plants were regenerated on selection medium. To rapidly detect positive transgenic plants a histochemical *GUS* assay was performed. Twenty-eight out of forty-three showed a strong blue staining (Supplementary figure 4-3). Genomic DNA was isolated from *GUS* positive plants and a PCR of the selection marker *hptII* further confirmed positive transgenic plants (Supplementary figure 4-4). Afterwards, a Southern-blot analysis was performed from some lines using *hptII* as a probe (Supplementary figure 4-4) and single copy lines were selected for expression analysis. A reverse transcriptase-PCR (RT-PCR) analysis using *RXam2* specific primers showed *RXam2* was indeed overexpressed in the single copy lines evaluated (Supplementary figure 4-5).

Figure 4-5: Schematic representation of *RXam2* overexpression construct in pCAMBIA1305.2 employed to transform cassava plants.

Boxes indicate promoter and terminator regions, arrows indicate open reading frames (ORFs) and thin vertical arrows indicate restriction sites used for cloning.



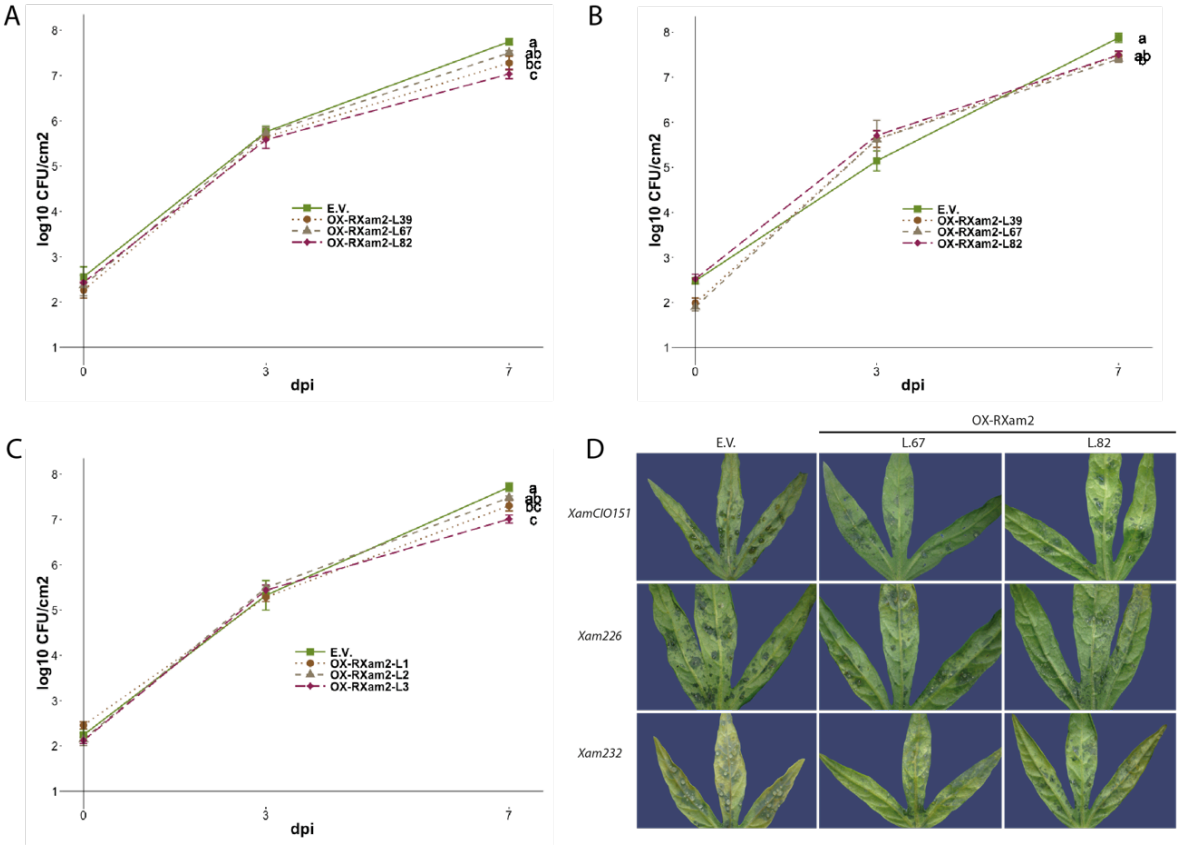
To determine the effect of *RXam2* overexpression on CBB resistance, *in vitro* plants from single-copy lines were inoculated on the leaf using a Q-tip dipped with a bacterial suspension at OD_{600nm}= 0,002,~10^6 colony forming units (CFU ml^{-1}). Empty vector (E.V.) control plants and three independent single copy OX-RXam2 lines (L.39, L.67 and L.82), were inoculated with four different *Xam* strains, *XamCIO151, Xam226, Xam232* and *XamCFBP1851*. Bacterial numbers were measured at 0, 3 and 7 dpi. Figure 4-6 shows bacterial growth for three strains *XamCIO151, Xam226* and *Xam232* and disease symptoms observed at 7 dpi. After 3 dpi *XamCIO151* grew at similar rates in all genotypes tested, while at 7 dpi all OX-RXam2 lines showed less bacterial

numbers (Figure 4-6A). Similar results were obtained for bacterial numbers of *Xam226* and *Xam232* that display a tendency of a higher bacterial count in E.V. control plants at 7 dpi compared with all OX-RXam2 lines (Figure 4-6B, C). In contrast, bacterial numbers of *XamCFBP1851* were similar in all the genotypes tested (Supplementary Figure 4-6).

In addition, disease phenotypes were evaluated on inoculated leaves and a correlation was observed with bacterial growth. Figure 4-6D shows inoculated leaves symptoms observed at 10 dpi. Disease is observed as yellow and wilted leaf regions, water soaked lesions around inoculation points and also around natural small wounds (Figure 4-6D). Although water soaked lesions are observed in all genotypes, sticky *Xam* exudates protruding from the inoculation point and natural wounds were prominent on E.V. control plants. Another symptom observed exclusively on E.V. control plants is the presence of a yellow and partial leaf wilting, due to *Xam* colonization of vascular tissues on the leave. This was observed on plants inoculated with *XamCIO151* and *Xam232* (Figure 4-6D, first column).

Figure 4-4: Bacterial growth of *Xam* and disease symptoms on transgenic cassava plants overexpressing *RXam2.*

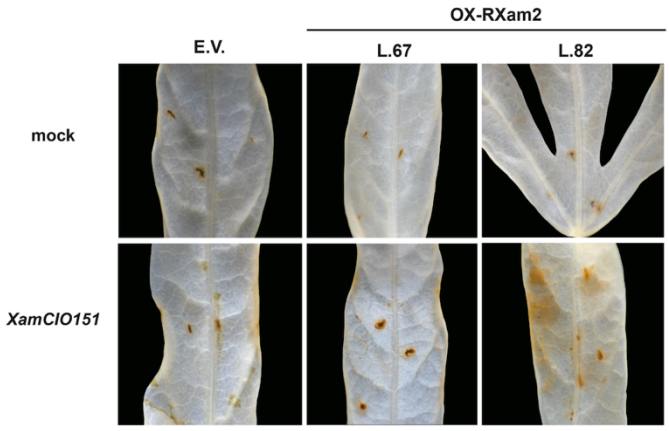
Two month-old *in vitro* cassava plants were inoculated by piercing and dipped on both abaxial and adaxial side of the leaf with a 10^6 inoculum of *XamCIO151* (**A**), *Xam226* (**B**) and *Xam232* (**C**). Leaf-discs around inoculation points were selected and the number of bacteria was quantified at 0, 3 and 7 dpi, data represent a mean of Log_{10} CFU/cm^2 from three technical replicates ± SE. Statistical significance was assessed at 7 dpi at $P < 0.05$ using a Tukey–Kramer HSD test for post-ANOVA analysis. Letters indicate significant different groups. (**D**) Disease symptoms produce at 10 dpi by different *Xam* strains is diminished in *RXam2* over-expression plants. 2 month-old *in vitro* cassava plants were inoculated by piercing and dipped on both abaxial and adaxial side of the leave with a 10^6 inoculum of *XamCIO151, Xam226* and *Xam232*.



To further evaluate possible defense responses induced by the overexpression of *RXam2*, inoculated leaves were stained with DAB in order to detect reactive oxygen species (ROS). Figure 4-7 shows in the first row control leaves inoculated with $MgCl_2$ and the second row leaves inoculated with *XamCIO151* collected at 2 dpi. ROS production (observed as brown deposits after DAB staining) is commonly induced with the mechanical stress of the needle, for this reason is observed in the inoculation point. Interestingly, both lines of OX-RXam2 plants showed enhanced ROS accumulation in the inoculation point and also in distal regions (Figure 4-7). All together, these results indicate *RXam2* overexpression render cassava plants less susceptible to distinct *Xam* strains.

Figure 4-5: ROS accumulation on *in vitro* cassava plants overexpressing *RXam2* inoculated with *XamCIO151*.

Leaves were inoculated with a 10^6 inoculum of *XamCIO151* were stained with DAB after 2 dpi. Control plants were inoculated with 10mM $MgCl_2$ (mock).

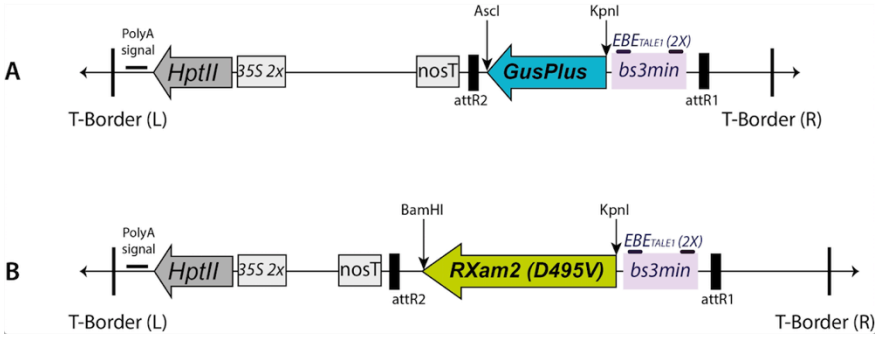


4.3.4 Developing trap promoters to induce autoactive RXam2

As a proof of concept for the development of a new strategy to engineer resistance to CBB, an activator trap promoter was produced to induce the expression of an autoactive version of RXam2. First, an autoactive missense mutation on the MHD motif located in the ARC2 subdomain of RXam2 (Figure 4-1) was generated. For this, a substitution of aspartate (D) to valine (V) in the position 495 was produced using overlapping PCR. In order to evaluate the autoactivity of RXam2, transient assays were performed in *N. benthamiana* and cassava leaves. The *RXam2* autoactive mutant (*RXam2*$_{(D495V)}$) was first cloned into the binary vector pBAV139 under the strong 35S promoter. An HR was produced only when *RXam2*$_{(D495V)}$ was overexpressed; this was not observed when WT RXam2 was overexpressed (Supplementary figure 4-7).

Figure 4-6: Schematic representation of the T-DNA from TALE1 activator trap constructs.

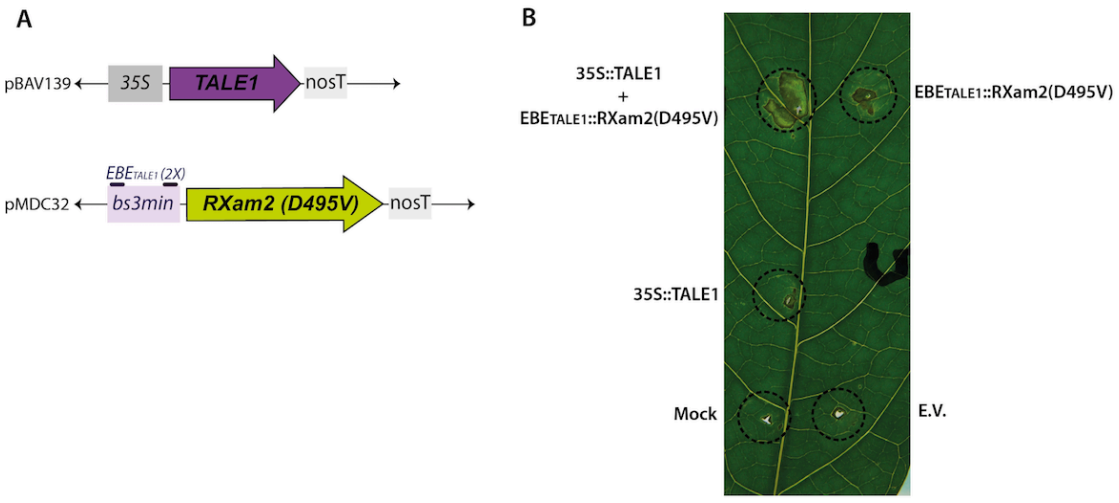
The complete CDS of *GusPlus* (**A**) and *RXam2*$_{(D495V)}$ (**B**) were cloned in pMDC32 under the expression of a minimal Bs3 promoter containing two EBEs for TALE1. Vertical thin arrows indicate restriction sites used for cloning. Transgenic cassava plants were selected for resistance to hygromycin.



Afterwards, we decided to clone *RXam2$_{(D495V)}$* under an activator trap promoter containing an EBE for TALE1 in the binary vector pMDC32. The gene *GusPlus* under the *Bs3* minimal promoter containing the same EBE was used as control (Figure 4-8). To evaluate whether TALE1 was able to induce the activation of *RXam2$_{(D495V)}$* transient coinfiltration assays were performed on *N. benthamiana*, *N. tabacum* and cassava leaves. Notably, TALE1 induces a strong HR on *N. benthamiana* and *N. tabacum* leaves, for this reason it was not possible to detect an HR produced exclusively by TALE1 and RXam2$_{(D495V)}$ coexpression (Supplementary figure 4-8). Nevertheless, when TALE1 and RXam2$_{(D495V)}$ were transiently coexpressed in cassava leaves, an HR-like response was observed (Figure 4-9). Altogether these results suggest TALE1 is able to induce *RXam2$_{(D495V)}$* expression.

Figure 4-7: Transient expression of TALE1 and RXam2 (D495V) on cassava leaves.

(**A**) Summarized diagram of the constructions used for transient expression assays. (**B**) Cassava leaves were infiltrated with *A. tumefaciens* strain AGL1 harboring pMDC32 (E.V.), EBE$_{TALE1}$::RXam2(MHV)-pMDC32, 35S::TALE1-pBAV139 and coinfiltration of EBE$_{TALE1}$::RXam2(MHV)-pMDC32 and 35S::TALE1-pBAV139. Infiltrated area is enclosed in a circle. Photographs were taken at 5 dpi.

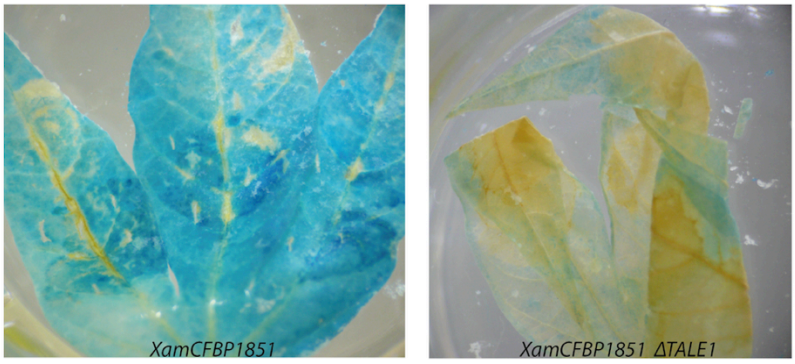


In addition and to further explore TALE1 induction of RXam2$_{(D492V)}$, transgenic cassava plants with *EBE$_{TALE1}$::RXam2$_{(D492V)}$* were produced. The schematic diagram of the construct used to transform cassava plants is shown in Figure 4-8, *GusPlus* was used as a reporter control for *EBE$_{TALE1}$* induction. In total, eighty-two plantlets for *EBE$_{TALE1}$::GusPlus* were regenerated and

forty-five for EBE_{TALE1}::$RXam2_{(D492V)}$. Since previous results from transient expression suggested the promoter containing EBE_{TALE1} has a basal activity (data not shown) the following strategy was carried out: first, EBE_{TALE1}::*GusPlus* plants were selected for GUS staining to test whether these plants showed a constitutive expression of the reporter gene. Indeed, most of the plants evaluated showed varying levels of GUS activity; nevertheless fifteen lines did not exhibit *GusPlus* basal activity (Supplementary figure 4-9). Second, these plants were further characterized by PCR and Southern-blot to confirm they represent transgenic events and were not false positives (Supplementary figure 4-10 and 4-11). In total twelve lines showed amplification of *hptII* (Supplementary figure 4-10). A summary of the regenerated plants and molecular characterization of the transformants are shown in Supplementary table 4-3. In order to detect if *Xam* strains carrying TALE1 could induce the EBE_{TALE1} promoter, EBE_{TALE1}::*GusPlus* lines with no basal *GUS* activity plants were infiltrated in the leaves to evaluate *GUS* activity. Figure 4-10 shows that in the line L.37 *GusPlus* is being induced by the strain *CFBP1851* that is known to contain TALE1. Nevertheless, a slight basal *GusPlus* expression is observed when inoculated with *CFBP1851ΔTALE1*. These results indicate TALE1 is being effectively injected to plant cells, travel to the nucleus and activates *GusPlus* at 48 hpi.

Figure 4-8: Histochemical *GUS* staining on transgenic EBE_{TALE1}::*GusPlus* plants inoculated with *XamCFBP1851*.

Leaves from a EBE_{TALE1}::*GusPlus* L.37 were inoculated with *XamCFBP1851* (left) and *XamCFBP1851ΔTALE1* (right). Leaves from *in vitro* plants were infiltrated with a *Xam* cell suspension [optical density at 600nm (OD_{600nm})= 0,3, ~10^8 colony forming units (CFU ml^{-1})]. After 48 h *GUS* histochemical staining was assessed.



On the other hand, it was assumed that plants showing a basal $RXam2_{(D492V)}$ expression were lethal and therefore not regenerated. In total forty-five plantlets carrying EBE_{TALE1}::$RXam2_{(D492V)}$ were obtained. Southern-blot analysis of these plants indicates twenty-two out of forty-five were

single-copy lines (Supplementary figure 4-11). Transgenic plants were further selected for subsequent experiments. To test whether EBE_{TALE1}::$RXam2_{(D492V)}$ could induce resistance when inoculated with *Xam*, stem inoculation of *CFBP1851* and *CFBP1851ΔTALE1* was performed on *in vitro* plants. *CFBP1851* harbors two TALs, one of 14 RVDs (TALE1) and a second one with 21 (Zárate et al., unpublished results). Table 4-2 shows disease resistance determination for five independent EBE_{TALE1}::$RXam2_{(D492V)}$ transgenic lines (L.12, L.44, L.51, L.53 and L.54). Since *CFBP1851* is a virulent strain compared with *CFBP1851ΔTALE1* (Castiblanco et al., 2013) we focused our attention on EBE_{TALE1}::$RXam2_{(D492V)}$ lines with less disease rating when inoculated with *CFBP1851* compared with *CFBP1851ΔTALE1* and also when compared with control plants (Table 4-2 and Supplementary figure 4-11). Interestingly, L.51 and L.54 showed enhanced resistance when inoculated with *CFBP1851*, suggesting TALE1 is inducing $RXam2_{(D492V)}$.

Table 4-2: Disease resistance determination on EBE_{TALE1}::$RXam2_{(D492V)}$ plants.

In vitro plants were inoculated by stem puncturing with a *Xam* cell suspension [optical density at 600nm (OD_{600nm})= 0,02,~10^7 colony forming units (CFU ml^{-1})]. Disease resistance determination is based on ∑AUDPC at 28 dpi (Supplementary table 4-4). R, resistant; MR, moderately resistant; MS, moderately susceptible; S, susceptible. NT, non-transgenic control.

***Xam* strain/ plant genotype**	**NT**	***EBE::GusPlus***		***EBE::RXam2 (D495V)***				
		L.61	**L.2**	**L.12**	**L.44**	**L.51**	**L.53**	**L.54**
CFBP1851	MS	MR	S	S	S	R	S	MS
CFBP1851 ΔTALE1	R	MR	R	MS	S	S	R	S

4.4 Discussion

The implementation of host *R* genes is considered to be the most effective method to control CBB. Six years ago the first draft of the cassava reference genome was made available, even though, to date no *R* genes have been cloned in cassava. The gene reported in this study, called *RXam2*, comes from a previously identified RGC (López et al., 2003, 2007) and belongs to the CNL class of NLR proteins. Our results suggest *RXam2* might confer partial and broad-spectrum resistance to CBB.

Recently, the cassava repertoire of NLRs was identified and most of them were mapped on thirty-nine clusters located on eighteen chromosomal groups (Lozano et al., 2015). *RXam2* (previously

annotated as cassava4.1_031234m) was found to be located on the pseudomolecule IV as a single not clustered gene (Lozano et al., 2015). In a region spanning ~5 Mb around *RXam2* there are no other genes coding for NLRs. The only exception is a possible pseudogene (cassava4.1_030826) not annotated in the new genome version located 411590bp away from *RXam2*. The next two genes coding for NLRs are located at positions 1221680 and 6174556 and are annotated as *cassava4.1_000323* (*Manes.07G012100*) and *cassava4.1_000192*, respectively (Lozano et al., 2015). In the recent cassava genome V6.1 *RXam2* is annotated as *Manes.07G048100* and is located in chromosome 7. Since typical *R* genes code for NLR proteins and also the molecular marker associated with the QTL comprises a region of *RXam2*, is highly probable that *RXam2* is involved in disease resistance. Nevertheless, we cannot exclude other genes in this region to contribute to resistance associated with this QTL. The production of truncated *RXam2* variants using the CRISPR/Cas system could help to verify if *RXam2* is indeed a resistance gene for CBB.

4.4.1 *RXam2* silencing using RNAi lead to enhanced susceptibility to CBB

In a first instance, susceptible cassava plants containing small interfering RNA (siRNA) derived from *RXam2* were developed as a first approach to validate the role of *RXam2* on resistance to CBB. Our results indicate *RXam2* RNAi-mediated gene silencing in a susceptible cultivar results in increased susceptibility to several *Xam* strains. Others studies have shown NLR silencing causes the loss of resistance measured as the loss of an HR produced after a gene-for-gene interaction (Christopoulou et al., 2015). In the *Xam*-cassava pathosystem no gene-for-gene interaction have been described to date. Preliminary yeast-two hybrid assays indicate the LRR domain and the full-length RXam2 interacts with XopAK, XopC2, XopE1 and XopV from *Xam* (unpublished results). The inoculation of single KO mutants for XopAK, XopC2, XopE1 and XopV might aid to detect possible effector-specific resistance responses in RXam2-RNAi plants XopAK, XopE1 and XopV are among *Xam* core-effectors present in all strains evaluated, this could explain how *RXam2* silencing caused an enhanced susceptibility in all *Xam* strains tested. This suggests RXam2 might be implicated in broad-spectrum resistance. As an approach to confirm if XopAK, XopC2, XopE1 and XopV are truly recognized by RXam2 and also to detect if there is an effector-specific resistance response, transient co-expression assays between candidate interactors effectors and RXam2 in *N. benthamiana* will be conducted to evaluate the development of an HR phenotype. In addition, recently these effectors have been cloned with tags in binary vectors and coIP or BiFC experiments will be soon conducted in order to reconfirm interactions and to evaluate cellular colocalization.

Nevertheless, we could not detect differential mRNA levels by qPCR analysis in RXam2-RNAi plants when compared with non-transgenic control plants due to basal low expression levels (data not shown). Genes coding for NLRs have been reported to display a constitutive low expression and some studies suggest several regulatory mechanisms mainly at the protein level trough intramolecular domain interactions (Takken and Goverse, 2012; Qi and Innes, 2013). Other studies have reported NLR silencing in order to validate its role in disease resistance (Christopoulou et al., 2015; Wu et al., 2016). Virus-induced gene silencing (VIGS) have been employed in solanaceus plants to silence NLRs (Gabriëls et al., 2007; Wu et al., 2016). However this approach has reported to be problematic due to off-target gene silencing (Xu et al., 2006a; Wu et al., 2016). Hairpin RNAi approach has also been previously used to silence NLR genes in lettuce, although qPCR was not a reliable approach for quantifying mRNA levels of NLRs on silenced plants (Wroblewski et al., 2007). Instead, these authors used a *GUS* fragment concatenated with the NLR fragment in one cassette in order to confirm the feasibility of the RNAi construction (Wroblewski et al., 2007). In order to quantify possible differences in mRNA levels of *RXam2* in RXam2-RNAi plants compared with control plants, we propose to use previously developed artificial TALs (arTALs) to target *RXam2* promoter regions (unpublished results) in order to deliberately induce *RXam2* transcriptional levels. While the induction in transcription by arTALs will be detected in control plants, we expect *RXam2*-derived siRNA will target mRNA levels induced by arTALs and therefore allow the detection of differential mRNA levels.

4.4.2 Does *RXam2* overexpression leads to enhanced resistance?

Regarding the implication of RXam2 overexpression, we found cassava transgenic lines recovered showed relatively normal appearance, indicating no harmful phenotypes were produced after *RXam2* overexpression. Genes coding for NLRs usually display low and constitutive mRNA expression levels and there are several reports showing contrasting evidence about phenotypic responses caused by NLR transgenic overexpression. Dwarf phenotypes have been observed as a result of transgenic overexpression in *Arabidopsis* (Stokes et al., 2002). Previous reports have found that lines with the highest NLRs mRNA levels are targeted by the posttranscriptional gene silencing machinery (Oldroyd and Staskawicz, 1998). In contrast, potato lines with high expression levels of the *RB* gene showed enhanced resistance phenotypes (Kramer et al., 2009). Correspondingly, our results show that on transgenic lines evaluated, RXam2 mRNAs were not likely to be silenced since elevated mRNA levels of *RXam2* were detected by RT-PCR.

Our results reveal the overexpression of *RXam2* leads to a slight reduction in susceptibility to *Xam* strains. Also, ROS were produced after inoculation on transgenic plants overexpressing RXam2 (Figure 4-7). These results suggest that defense responses are activated after pathogen inoculation in OX-RXam2 plants. Indeed, NLR overexpression has been extensively reported to result in enhanced resistance responses (Oldroyd and Staskawicz, 1998; Tai et al., 1999; Tao et al., 2000; Xiao et al., 2001; Feuillet et al., 2003; Ade et al., 2007; Rentel et al., 2008; Bradeen et al., 2009; Kramer et al., 2009; Brunner et al., 2012; Narusaka et al., 2013). In addition, we demonstrated that RXam2 transient overexpression does not leads to constitutive HR response by itself (Supplementary figure 4-7). Similarly, in other studies NLR overexpression alone does not results in HR induction, this is the case for Bs2, Prf, RPM1 and RPS5 (Oldroyd and Staskawicz, 1998; Tai et al., 1999; Ade et al., 2007; Chung et al., 2011). In contrast, other studies have reported a strong HR induction and constitutive defense responses after transgenic NLR overexpression in the absence of the pathogen, this has been observed for RPS2, Rx, RPS4 and RPP13 (Tao et al., 2000; Bendahmane et al., 2002; Zhang et al., 2004; Rentel et al., 2008).

Altogether, our results showed that RXam2 overexpression does not leads to a constitutive HR, but still conferred a slight resistance response after *Xam* inoculation. Despite the fact that NLRs are usually associated with resistance produced by a gene-for-gene interaction, is possible that RXam2 overexpression leads to an unspecific resistance response that results in broad-spectrum resistance to CBB. One possible explanation for this it that RXam2 overexpression leads to defense activation that results in enhanced resistance but is not sufficient to trigger an spontaneous HR cell death response. Similar reports have proven that the overexpression of Prf, RPS2 and RPW8 leads to broad-spectrum resistance in the absence of HR (Oldroyd and Staskawicz, 1998; Tao et al., 2000; Xiao et al., 2001). As already mentioned, preliminary data suggest RXam2 might interact with *Xam* core-effectors and this could indicate plants overexpressing RXam2 are recognizing this effectors present in all *Xam* strains tested so far. Nevertheless, broad-spectrum resistance observed in the case of Prf, RPS2 and RPW8 does not involves a gene-for-gene interaction (Oldroyd and Staskawicz, 1998; Tao et al., 2000; Xiao et al., 2001). Under this scenario, it is possible that RXam2 overexpression lead to an overdose effect that is independent from a gene-for-gene interaction. It has been reported that the overexpression of one signaling component might activate the complete signaling pathway (Tang et al., 1999; Oldroyd and Staskawicz, 1998; Tao et al., 2000). However, this might be contradictory with the absence of an HR response. Even so, it has been reported that resistance and NLR activation is not always associated with the presence of HR (Heath, 2000; Coll et al.,

2011). Despite the fact our results showed the overexpression of *RXam2* leads to a slight reduction in disease symptoms to *Xam,* no clear reduction in *Xam* growth is observed when this experiment is repeated (data not shown), indicating a very small contribution to resistance when *RXam2* is overexpressed in the highly susceptible cultivar used.

Taken together, although *RXam2* silencing indicates this gene has an important role for resistance to CBB, the lack of a strong resistance response by overexpression of *RXam2* suggest there might be another level of regulation of this gene, perhaps at protein level. Indeed, gain-of-function mutations in the NB-ARC domain of NLRs support the fact that NLRs activation is regulated mainly trough intra or intermolecular interactions (van Ooijen et al., 2008a). This suggests that even though mRNA levels of *RXam2* are considerably higher than under normal conditions, RXam2 is in auto-inhibited conformation. This might explain why we did not observe a strong resistance response when *RXam2* was overexpressed.

4.4.3 Using TALs to engineer resistance using autoactive NLRs

The substitution of aspartate (D) to valine (V) in the MHD motif has been reported to induce autoactivity in several NLRs. We found that the transient overexpression of RXam2$_{(D492V)}$ produced an HR phenotype in *N. benthamiana* (Supplementary figure 4-7A). Similarly, an HR-like necrotic phenotype was observed when RXam2$_{(D492V)}$ was transiently overexpressed in cassava leaves (Supplementary figure 4-7B). This suggests the RXam2 activation triggers a signaling pathway that leads to HR that is conserved in *N. benthamiana* and cassava. HR has not been previously reported in cassava as a resistance response to *Xam*. However, our results demonstrates that a single gain-of-function mutation in the MHD motif of RXam2, called RXam2$_{(D492V)}$, produces and HR, as it has been widely reported for other NLRs. Interestingly, an HR was not observed when the WT RXam2 was overexpressed. This result indicates the activation of RXam2 is mainly regulated at protein level instead of transcriptional regulation.

Since RXam2$_{(D492V)}$ produces an HR and *E* genes in cassava are yet unknown, we used this NLR variant to develop a novel strategy to engineer disease resistance to CBB using an NLR autoactive executor trap. For this, a trap promoter using the EBE from TALE1 called EBE_{TALE1} was cloned to induce the expression of $RXam2_{(D492V)}$. The transient expression of EBE_{TALE1}*::RXam2*$_{(D492V)}$ in *N. benthamiana* and cassava leaves produced a HR phenotype when coexpressed with $TALE1_{Xam}$ suggesting an activation of RXam2 mediated by TALE1$_{Xam}$. Still, a slight constitutive activation of the promoter is observed when EBE_{TALE1}*::GusPlus* is coinfiltrated with $TALE1_{Xam}$ (data not shown).

This might indicate cis-regulatory elements present in the EBE activate the gene in the absence of $TALE1_{Xam}$ (Adam Bogdanove, personal communication). Interestingly, our results show that two transgenic cassava plants carrying EBE_{TALE1}*::RXam2*$_{(D492V)}$ showed enhanced resistance when inoculated with a strain harboring $TALE1_{Xam}$ (Table 4-2 and Supplementary figure 4-11). This is the first report of an engineered autoactive NLR to be transcriptionally induced in the presence of a TAL effector.

Despite $TALE1_{Xam}$ was the first TAL effector identified in *Xam* (Castiblanco et al., 2013), to date the repertoire of TAL effectors have been described in a small collection of *Xam* strains (Zárate, unpublished results). Based on this knowledge, new strategies could be designed where different EBEs from a set of prevalent strains can be arranged in tandem to induce executor genes, facilitating the detection of multiple strains carrying different TAL effectors. The isolation and cloning of *R* genes not only contributes to our understanding of the molecular mechanisms that underlies disease resistance but also provides significant tools for breeding programs. Strategies based on TAL effectors, such as engineering multiple EBEs or by combining *E* genes under prevalent EBEs in a determined pathogen population, might help to achieve broad-spectrum resistance in a similar manner to *R* gene pyramiding.

4.5 Materials and methods

4.5.1 Plasmids construction and cassava transformation

Molecular cloning of RXam2 on pHELLSGATE12

For the *RXam2* silencing construct on pHELLSGATE12, primers were designed in the NB region of a putative gene coding for a NB-LRR protein identified in the RGC7 (López et al., 2003) and was named *RXam2* (Fig. 1). Two sets of primers to produce an amplification product of 618pb (Table 1). A PCR product from cv. 60444 was first subcloned in pENTR/D-TOPO® (Invitrogen, Carlsbad, CA, U.S.A.) and then an LR reaction was performed to clone *RXam2* in pHELLSGATE12 to produce a sense-antisense orientation of the gene for silencing (Fig 2).

A 618bp fragment corresponding to the NB domain of *RXam2* was cloned first on pENTR/D-TOPO and further subcloned into pHELLSGATE12 in a sense and antisense orientation. Fragments are

separated by two introns in opposed orientation (cat and pdk), necessary for the formation of the hairpin structure. Arrows indicate restriction sites used for cloning confirmation. The silencing cassette is under 35S strong constitutive promoter and an OCS terminator sequence. Transgenic cassava plants were selected for resistance to *NptII*.

The PCR for pENTR™/D-TOPO cloning was performed in a final volume of 50 ul containing 1x PCR buffer (50 mM KCl, 100 mM Tris-HCl, 0,1% TritónX-100), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 µM of each primer and 2.5 U Taq polymerase (CIAT, Cali, Colombia) using 50ng of genomic DNA. The PCR protocol as follows: initial denaturation at 94ºC for 2' followed by 35 amplification cycles of 30" at 94ºC, 30" at 50º and 45" of extension at 72ºC, with a final step of extension at 72ºC for 5'. Amplification product was checked in an agarose gel electrophoresis and the plasmid was send for sequencing. The construct was transformed into *E. coli* and *Agrobacterium tumefaciens* strain AGL1 by electroporation and confirmed by PCR. *E. coli* cells were cultivated at 37ºC in LB (Luria-Bertani) broth medium and *A. tumefaciens* AGL1 at 28ºC in YEB medium (10 g yeast extract. 10 g Bacto peptone, 5 g NaCl) supplemented with carbenicillin 100 ug ml^{-1} and spectinomycin 50 ug ml^{-1}.

Molecular cloning of RXam2 on pCAMBIA1305.2

To create pCAMBIA1305.2 (*35S::RXam2-NosT*) the 35S promoter of Cauliflower mosaic virus (35Sp), full-length *Manes.07G048100 (RXam2)* and Nopaline synthase terminator (NosT) were first amplified and cloned separately using TOPO-TA subcloning kit (Life Technologies GmbH, Darmstadt, Germany). The 35Sp was amplified with a set of primers containing restriction sites for *EcoRI* (35SEcoRI F) and *SacI* (35SSacI R), while the primers for the NosT amplification contained the restriction sites *BamHI* (NostBamHI F) and *SalI* (NostSalI R). *RXam2* was amplified from genomic DNA of the cassava resistant cultivar CM6438-14 using primers containing restriction sites for *KpnI* (RXam2KpnI_F) and *BamHI* (RXam2BamHI_R). For cassava transformation with the *RXam2* overexpression construct a plasmid containing *35S::RXam2-NosT* using the backbone of the binary vector pCAMBIA1305.2 was generated (Canberra, Australia). This vector contains *hygromycin phosphotransferase gene (htpII)* as a selection marker and *B-glucoronidase* (*GusPlus*) as a reporter gene. The same plasmid lacking *RXam2* was used as a transformation control. PCR reactions were performed in a 50-ml volume consisting of 0.2 mM of dNTPs, 0.1 mM of each primer, 1X DreamTaq buffer, 1 U of DreamTaq DNA Polymerase (ThermoScientific, Waltham, MA, USA) and 100 ng of genomic DNA. Amplification was carried out

in a Bio-Rad thermal cycler programmed for an initial denaturation at 95°C for 3 min, followed by 35 amplification cycles (95°C for 30 s, with T_m depending on each primer, for 45 s and 72°C for 1 min and 20 s), and a final extension step at 72°C for 10 min. The amplified fragments were eluted from a 1% agarose gel. Bands were excised from the gel and purified, using The Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Each purified DNA fragment was cloned in TOPO-TA subcloning kit (Life Technologies GmbH, Darmstadt, Germany) following the manufacturer's instructions. Plasmids from the positive clones were obtained using GeneJET Plasmid Miniprep Kit (ThermoScientific, Waltham, MA, USA). To confirm the clones a PCR was conducted prior to digestion and ligation.

To produce the final binary vector containing *35S::RXam2-NosT*, the clones were independently digested with the respective enzymes and ligated in the Multiple Cloning Site (MCS) of pCAMBIA1305.2. This was confirmed by PCR and standard Sanger sequencing. The constructs were transformed into *E. coli* and *Agrobacterium tumefaciens* strain LBA4404 by electroporation and confirmed by PCR. *E. coli* cells were cultivated at 37ºC in LB (Luria-Bertani) broth medium and *A. tumefaciens* LBA4404 at 28ºC in YEB medium (10 g yeast extract. 10 g Bacto peptone, 5 g NaCl) supplemented with streptomycin 50 ug ml^{-1} rifampicin 100 ug ml^{-1} and kanamycin 50 ug ml^{-1}.

Molecular cloning of RXam2$_{(D495V)}$ on pMDC32

For the development of the autoactive variant of RXam2, a substitution of aspartate (D) to valine (V) in the MHD motif coding sequence was done by overlapping PCR using primers with mismatch regions. *RXam2* was amplified from the clone used for overexpression in pCAMBIA1305.2. The autoactive variant of RXam2 was first cloned in pCR®2.1-TOPO using primers with restriction sites for *KpnI* and *BamHI*. This variant was then double digested with *KpnI* and *BamHI* and ligated in a modified pENTR™/D-TOPO harboring $EBE_{TALE1Xam}$ on the minimal Bs3promoter (kindly provided by Megan Cohn, UC Berkeley). Later, an LR reaction was conducted to clone the complete *$EBE_{TALE1Xam}$::RXam2(D495V)* into a modified pMDC32 that has a deletion in the 35S promoter located before the attR sequence.

FEC isolation and genetic transformation

Friable embryogenic callus (FEC) from the susceptible cassava cultivar 60444 was isolated and used for *Agrobacterium*-mediated transformation as previously described (Taylor et al, 2012).

Briefly, organized embryogenic structures (OES) were generated from axillar meristems of 8 weeks-old *in vitro* plants and were maintained through cycles of secondary embryogenesis. OES were removed from non-embryogenic tissue and injured for induction of FEC. After three cycles of subcultures, FECs were co-cultured for three days in dark conditions with *A. tumefaciens* strain LBA4404 carrying the constructs of interest [optical density (OD) at 660nm= 0,8]. Empty vector pCAMBIA1305.2 was used as control.

4.5.2 *Agrobacterium tumefaciens*-mediated transient expression assays

For leaf agro-infiltration assays in *N. benthamiana* and *N. tabacum, A. tumefaciens* strain GV3101 was transformed with binary vectors containing $EBE_{TALE1Xam}$*::RXam2(D495V)* and $EBE_{TALE1Xam}$*::GusPlus*. For infiltration assays in cassava leaves the strain AGL1 carrying the same binary plasmids were used. An isolated colony was grown in LB containing the respective antibiotic resistance until the culture reached OD_{600nm}= 0,8-1.0. Bacteria was collected by centrifugation at 3000rpm for 10 min and washed twice in agro-infiltartion solution (10 mM MES, 10 mM $MgCl_2$, 150 µM acetosyringone, pH 5.6). The bacterial solution was left at room temperature during 3 h and the OD_{600nm} was adjusted at 0,3 and 0,03 depending on the experiment. Each infiltartion experiment presented was repetated at least three times.

4.5.3 Molecular evaluation of transgenic lines

β-Glucuronidase (GUS) stain

Since the T-DNA of pCAMBIA1305.2 contains *GusPlus* as a reported gene, a GUS assay was employed for a preliminary characterization of putative transgenic plants. For this, fresh leafs from *in vitro* plants were selected and immersed in X-Gluc buffer (NaH_2PO_4 0.02 M, Na_2HPO_4 0.03 M, K_4FeCN_6 0.25 mM, K_3FeCN_6 0.25 mM, DMSO 10 % (p/v), triton X-100 0.5 % (v/v) and X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide) 1 mg/mL (Sigma, St. Louis, MO, USA). Leafs were incubated at 37º C during 16 h in dark conditions. Leafs were destained with 96% ethanol and photographed.

PCR and Southern Blot analysis

Genomic DNA was extracted from fresh leafs of *in vitro* plantlets using the Plant DNA Extraction Protocol for DArT, (Available online:

http://www.diversityarrays.com/sites/default/files/pub/DArT_DNA_isolation.pdf). For preliminary characterization of transgenic lines a PCR was performed from genomic DNA using gene-specific primers for *RXam2* (RXam2RT F and RXam2RT R) and the selection marker gene *hptII* (hptIIprobe F and hptIIprobe R). PCR reactions consisted of an initial denaturation step of 3min at 93ºC, followed by 35 cycles of 93ºC for30 s, 56ºC for 30s, 72ºC for 1 min, and a final extension at 72ºC for 5 min. For the identification of independent transgenic events, southern-blot assays were performed as previously described (Taylor et al, 2012). DNA was digested using *KpnI,* which cuts once the T-DNA at the start of *RXam2*. A 700pb *hptII* probe was synthetized by PCR (hptIIprobe F and hptIIprobe R) using the PCR DIG probe synthesis kit (Roche Applied Science, Indianapolis, IN, USA). Hybridization and detection was carried out according to manufacturer's instructions using anti-DIG antibody and CDP-star (Roche Applied Science, Indianapolis, IN, USA).

RT-PCR analysis

Total RNA was extracted from *in vitro* leaves using Invitrap ® Spin plant RNA minikit (STRATEC, Berlín, GER). 1ug of total RNA was treated with DNaseI RNase-free (ThermoScientific, Waltham, MA, USA), this was confirmed by PCR. First strand synthesis was performed with oligo$(dT)_{18}$ primers using the First Strand cDNA Synthesis Kit (ThermoScientific, Waltham, MA, USA). *RXam2* expression levels were assessed using *B-tubulin* as reference control. RXam2RT F and RXam2RT R were used for *RXam2* and tub3exRT F and tub3exRT R were used for *β-tubulin*.

4.5.4 Plant inoculations assays

RXam2-RNAi plants were first grown *in vitro*, transferred to a greenhouse and posterior to soil in the field. Once this plants produced woody stems, stakes were cut and placed on soil pots for inoculations assays in the greenhouse. Inoculation assays for OX-RXam2 plants were performed on *in vitro* plants. OX-RXam2 cassava *in vitro* plants were grown in a growth chamber at 28ºC under 16h of light on MS medium supplemented with sucrose (2% w v^{-1}). *Xam* strains were grown at 28ºC on liquid LPG medium (5 g yeast extract, 5 g dextrose and 5 g peptone per L of distilled water) and resuspended in 10mM $MgCl_2$. Two month-old *in vitro* cassava plants were selected for Q-Tip leaf inoculation. Briefly, young leaves were punctured in the mesophyll (apoplast) using an insulin-syringe. Each leaf was then wiped on both adaxial and abaxial side using a Q-Tip previously immersed in a *Xam* cell suspension [optical density at 600nm (OD_{600nm})= 0,002,~10^6 colony forming units (CFU ml^{-1})]. Bacterial growth was assessed at 0, 3 and 8 dpi.

The complete leaf was ground in 100uL of 10mM $MgCl_2$. Serial dilutions were plated on LPGA (5 g yeast extract, 5 g dextrose, 5 g peptone and 15 g agar per L of distilled water). For stem inoculation assays for plants carrying *$EBE_{TALE1Xam}$::RXam2(D495V)*, two month-old *in vitro* cassava plants were punctured in the stem using an insulin-syringe. The bacterial inoculum [optical density at 600nm (OD_{600nm})= 0,002,~10^6 colony forming units (CFU ml^{-1})] was spread in the wound using a Q-Tip. A scale of symptoms from 0 to 5 was used and symptoms development was monitored at 7, 14, 21 and 28 days after inoculation as reported by (Trujillo et al., 2014b).

4.5.5 DAB staining

To detect reactive oxygen species (ROS) in *RXam2* transgenic lines, leaves from *in vitro* naïve plants were excised, submerged in 3, 3'-Diaminobenzidine (DAB) buffer as previously reported (Thordal-Christensen et al., 1997) and incubated for 6 hours in the dark at RT. Leaves were destained from chlorophyll doing al least three washing steps with 96% ethanol and incubation at 37ºC. Accumulation of H_2O_2 was detected in a stereoscope and leaves were photographed.

4.6 Supplementary information

Supplementary table 4-1: List of primers used in this study.

Primer	Sequence (5' to 3')	Gene_ID
RXam2-RNAiF	CACCTGGGAAAATTATAGGGGATGTT	
RXam2IR	TGCACATCCAACCAGTCC	
35SEcoRI F	GCAGAATTCTCAACATGGTGGAGCAC	*35S promoter*
35SSacI R	TGCGAGCTCGTCCCCGGTGTTCTCTCC	
NostBamHI F	GCAGGATCCCGTTCAAACATTTGGC	*Nos terminator*
NostSalI R	TGCGTCGACCCCGATCTAGTAACATAG	
RXam1KpnI F	GCAGGTACCATGGGGTGTGGATGCTTCTG	*Manes.10G014300*
RXam1BamHI R	TGCGGATCCCTTAGGTGTAGATTTC	
RXam1RT R	ACTGGTAGATGCAACCACTCCTCA	*Manes.10G014300*
RXam1RT F	TGGAGCAGCTAAGTCTTTCATGGC	
PP2A F	TGCAAGGCTCACACTTTCATC	*Manes.09G039900*
PP2A R	CTGAGCGTAAAGCAGGGAAG	

tub3exRT F	GGAAAGATGAGCACCAAGGA	*Manes.08G061700*
tub3exRT R	ACCAGTATACCAGTGCAAGAAG	
G3PDHcassava F	CTGGTGGTTCAGGTATCAAAGA	*Manes.11G072400*
G3PDHcassava R	CCATATCATCTCCCATCACCATT	
hptIIprobe F	CGTCTGCTGCTCCATACAAG	*Hygromycine phosphotranferase*
hptIIprobe R	ATAGCTGCGCCGATGGTT	

Supplementary table 4-2: List of genes located near *RXam2 (Manes.07G048100,* 4853478-4857038) in a region that spans ~4,8 Mb.

Source: (*M. esculenta* v6.1, http://phytozome.jgi.doe.gov).

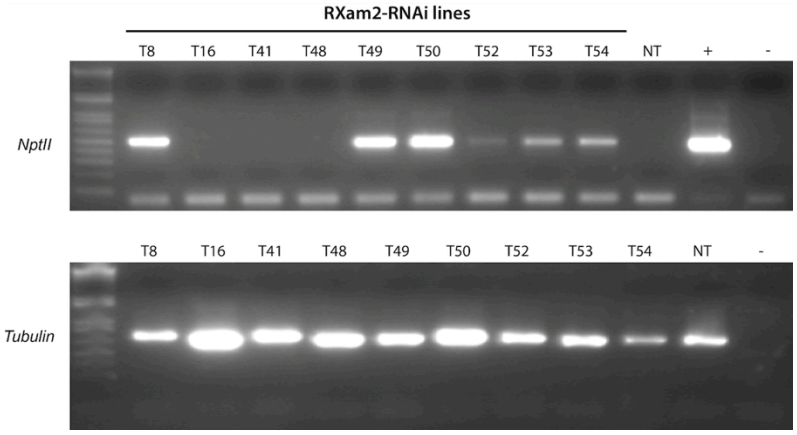
Locus name	position/orientation	Annotation
Manes.07G044300	419867..4423734 reverse	LEUCINE-RICH REPEAT-CONTAINING PROTEIN
Manes.07G044400	4426928..4427044 forward	
Manes.07G044500	4427368..4427613 reverse	
Manes.07G044600	4428108..4428402 reverse	
Manes.07G044700	4429053..4431421 reverse	LEUCINE-RICH REPEAT-CONTAINING PROTEIN
Manes.07G044800	4432440..4432556 forward	
Manes.07G044900	4449282..4453105 reverse	Ubiquitin family (ubiquitin) // HECT-domain (ubiquitin-transferase) (HECT) // Protein tyrosine kinase (Pkinase_Tyr)
Manes.07G045000	4453343..4455433 reverse	LEUCINE-RICH REPEAT-CONTAINING PROTEIN
Manes.07G045100	4456991..4458186 forward	ALCOHOL DEHYDROGENASE-RELATED
Manes.07G045200	4463561..4463713 forward	
Manes.07G045300	4464262..4464556 reverse	
Manes.07G045400	4465211..4468685 reverse	LEUCINE-RICH REPEAT-CONTAINING PROTEIN
Manes.07G045500	4476713..4476964 reverse	60S RIBOSOMAL PROTEIN L23A
Manes.07G045600	4484275..4486362 forward	L-TYPE LECTIN-DOMAIN CONTAINING RECEPTOR KINASE IX.1-RELATED
Manes.07G045700	4488189..4490297 reverse	L-TYPE LECTIN-DOMAIN CONTAINING RECEPTOR KINASE IX.1-RELATED
Manes.07G045800	4494810..4498164 reverse	Legume-like lectin family (Lectin_leg-like)
Manes.07G045900	4501329..4503194 reverse	L-TYPE LECTIN-DOMAIN CONTAINING RECEPTOR KINASE IX.1-RELATED
Manes.07G046000	4531471..4538065 forward	MADS-BOX TRANSCRIPTION FACTOR ANR1
Manes.07G046100	4538396..4539538 forward	
Manes.07G046200	4544658..4545120 reverse	Protein of unknown function (DUF2647) (DUF2647)

Manes.07G046300	4548113..4554400 forward	MADS BOX PROTEIN
Manes.07G046400	4575502..4577634 reverse	RECEPTOR-LIKE PROTEIN KINASE ANXUR1-RELATED
Manes.07G046500	4578478..4583512 reverse	OLIGOPEPTIDE TRANSPORTER 4
Manes.07G046600	4597998..4602139 forward	ZINC TRANSPORT PROTEIN ZNTB
Manes.07G046700	4603347..4607252 forward	HEAT SHOCK 70 KDA PROTEIN 10, MITOCHONDRIAL
Manes.07G046800	4610380..4611038 reverse	Cysteine-rich TM module stress tolerance (CYSTM)
Manes.07G046900	4622132..4625665 reverse	PB1 domain (PB1)
Manes.07G047000	4645902..4650136 reverse	PHOSPHOGLYCERATE MUTASE-LIKE PROTEIN
Manes.07G047100	4667244..4670045 forward	Leucine Rich Repeat (LRR_1) // Leucine rich repeat N-terminal domain (LRRNT_2) // Leucine rich repeat (LRR_8)
Manes.07G047200	4691061..4694364 reverse	
Manes.07G047300	4694999..4695264 reverse	
Manes.07G047400	4702923..4706068 forward	Leucine Rich Repeat (LRR_1) // Leucine rich repeat N-terminal domain (LRRNT_2) // Leucine rich repeat (LRR_8)
Manes.07G047500	4731876..4732052 forward	
Manes.07G047600	4814973..4815392 reverse	
Manes.07G047700	4817790..4818294 forward	
Manes.07G047800	4818422..4821365 reverse	Protein kinase domain (Pkinase) // Leucine rich repeat N-terminal domain (LRRNT_2) // Leucine rich repeat (LRR_8)
Manes.07G047900	4828363..4830396 reverse	L-TYPE LECTIN-DOMAIN CONTAINING RECEPTOR KINASE IX.1-RELATED
Manes.07G048000	4847831..4850599 forward	Peptide-methionine (R)-S-oxide reductase / Selenoprotein R
Manes.07G048100	4853478..4857038 reverse	LEUCINE-RICH REPEAT-CONTAINING PROTEIN (*RXam2)*
Manes.07G048200	4860488..4860683 forward	
Manes.07G048300	4865364..4869547 reverse	INORGANIC PYROPHOSPHATASE
Manes.07G048400	4871525..4888093 forward	PROTEIN S-ACYLTRANSFERASE 23-RELATED
Manes.07G048500	4888464..4891790 reverse	L-galactose 1-dehydrogenase / L-galDH
Manes.07G048600	4900404..4902789 forward	TRANSCRIPTION FACTOR BHLH18-RELATED
Manes.07G048700	4959766..4960887 forward	PHOSPHOLIPID-TRANSPORTING ATPASE 10-RELATED
Manes.07G048800	4974566..4975885 forward	F-box domain (F-box) // Protein of unknown function (DUF295) (DUF295)
Manes.07G048900	4984884..4986145 forward	F-box domain (F-box) // Protein of unknown function (DUF295) (DUF295)
Manes.07G049000	4988359..4990679 forward	
Manes.07G049100	4990746..4994590 reverse	MALATE DEHYDROGENASE, GLYOXYSOMAL
Manes.07G049200	5003877..5007483 forward	Protein kinase domain (Pkinase) // Leucine rich repeat N-terminal domain (LRRNT_2) // Leucine rich repeat (LRR_8)
Manes.07G049300	5016190..5024087 forward	PROLINE IMINOPEPTIDASE
Manes.07G049400	5029941..5032189	Adenovirus IVa2 protein (Adeno_IVa2)

	forward	
Manes.07G049500	5032428..5035678 reverse	
Manes.07G049600	5037402..5039694 forward	Squalene monooxygenase / Squalene epoxidase
Manes.07G049700	5046733..5049978 forward	MuDR family transposase (DBD_Tnp_Mut) // SWIM zinc finger (SWIM) // MULE transposase domain (MULE)
Manes.07G049800	5050882..5056333 reverse	LL-diaminopimelate aminotransferase / LL-diaminopimelate transaminase
Manes.07G049900	5075174..5078537 forward	
Manes.07G050000	5083674..5091152 reverse	ANCIENT CONSERVED DOMAIN PROTEIN-RELATED
Manes.07G050100	5171609..5175780 forward	
Manes.07G050200	5201765..5202373 forward	F2J10.6 PROTEIN-RELATED
Manes.07G050300	5210408..5211110 reverse	F2J10.6 PROTEIN-RELATED
Manes.07G050400	5233947..5234589 reverse	F2J10.6 PROTEIN-RELATED
Manes.07G050500	5241605..5242267 reverse	DNA-DIRECTED RNA POLYMERASE 3, CHLOROPLASTIC

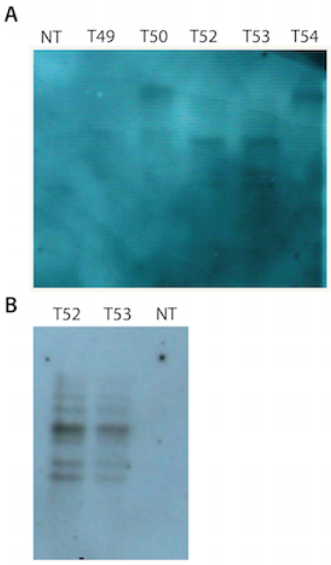
Supplementary figure 4-1: PCR of the selection marker *NptII* from genomic DNA of putative transgenic plants.

Genomic DNA was isolated from fresh leaves of unchallenged *in vitro* putative transgenic plants. *NptII* expression was evaluated and *tubulin* was used as a reference gene. NT, non-transgenic control; +, plasmid DNA; -, negative control.



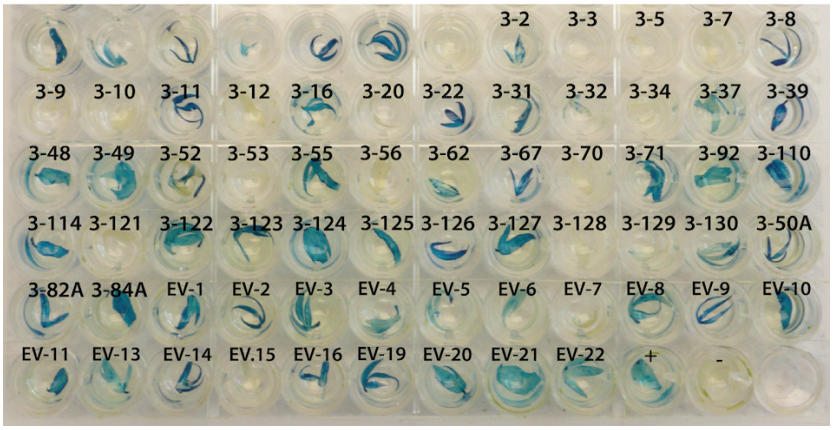
Supplementary figure 4-2: Southern-blot analysis of RXam2-RNAi transgenic cassava plants.

Genomic DNA was isolated from fresh leaves and a southern Blot was performed using *NptII* as a probe. T49-T54, genomic DNA from the different transgenic plants obtained; NT, genomic DNA from non-transgenic plants used as a negative control.



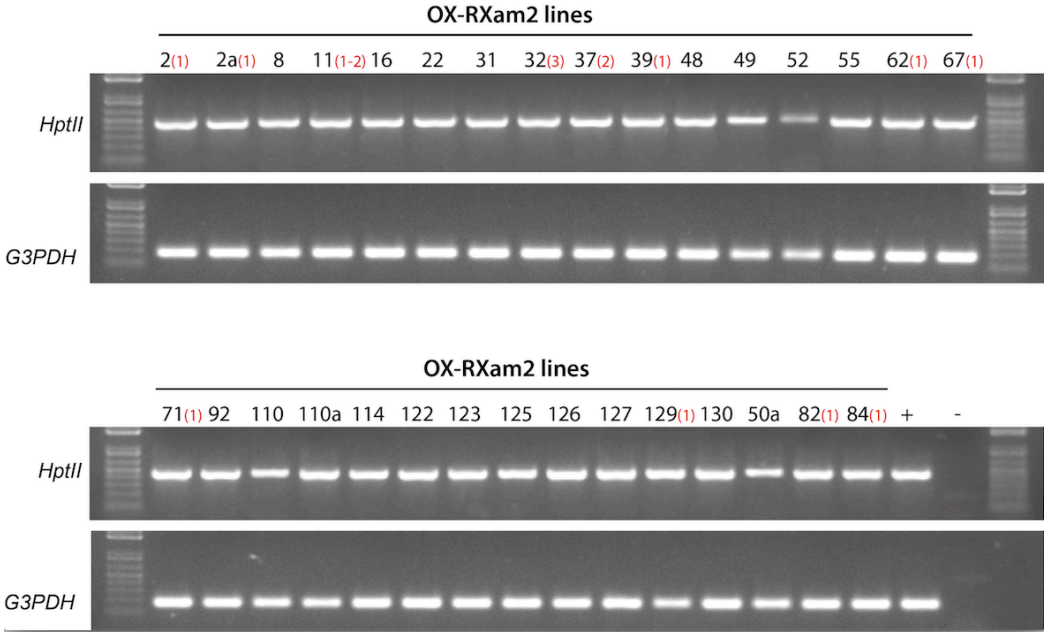
Supplementary figure 4-3: Histochemical *GUS* staining of putative OX-RXam2 transgenic plants.

Leaves from in vitro plants were detached and stained in X-Gluc buffer for 16h at 37ºC and destained in 96% ethanol. OX-Rxam2 plants are labeled from 3-2 to 3-84A; and empty vector control plants are labeled from EV-1 to EV-22; +, indicates a transgenic plants carriyng *GUS* as a reporter gene; -, indicates non-transgenic control plant.



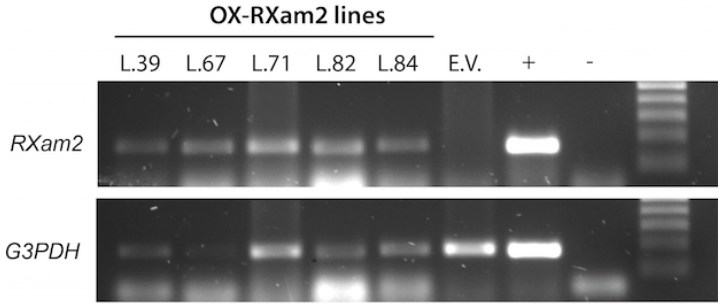
Supplementary figure 4-4: PCR from genomic DNA of putative OX-RXam2 transgenic plants.

Genomic DNA was isolated from fresh leaves from *in vitro* transgenic plants that were positive for the *GUS* staining. *HptII* expression was evaluated and *G3PDH* was used as a reference gene. Small red letters indicate the number of insertion events confirmed by Southern-blot analysis; +, confirmed transgenic plant; -, negative control.



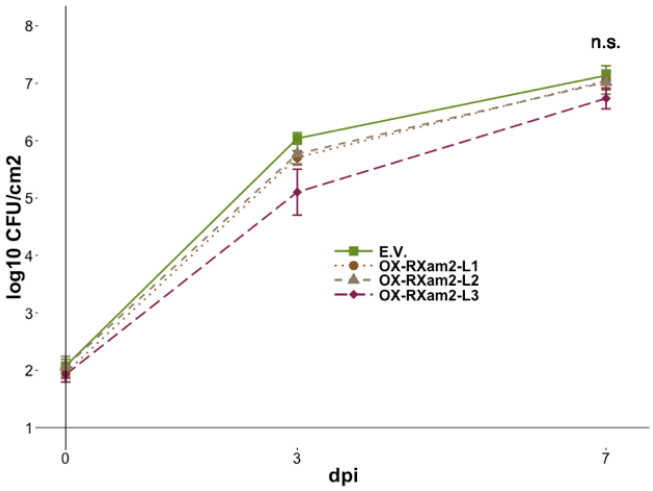
Supplementary figure 4-5: Reverse transcriptase (RT)-PCR analysis of OX-RXam2 transgenic cassava plants.

Total RNA was isolated from fresh *in vitro* leaves on five OX-Rxam2 cassava single copy transgenic lines (L39, L67, L71, L82 and L84). cDNA was synthetized and *Rxam2* and *G3PDH* (reference control) expression levels were detected by PCR. EV, Empty vector transgenic plants; +, genomic DNA of cv. 60444 plants; -, negative PCR control.



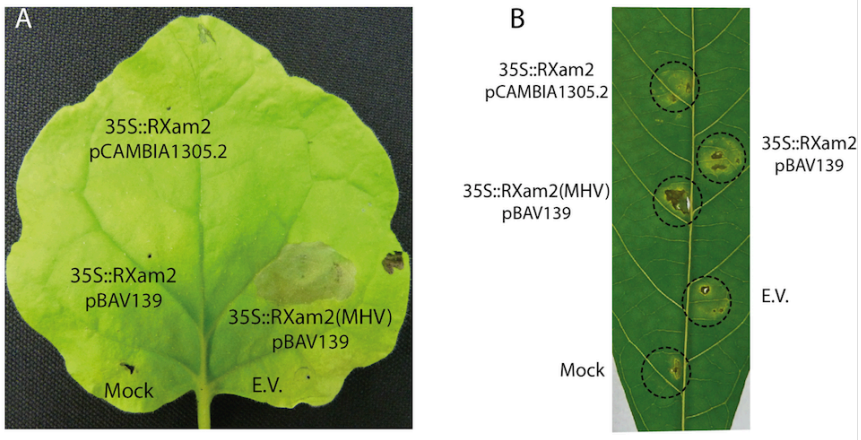
Supplementary figure 4-6: Bacterial growth of *XamCFBP1851* on OX-RXam2 plants.

Two month-old cassava plants were inoculated by piercing and dipped on both abaxial and adaxial side of the leaf with a 10^6 inoculum of *XamCFBP1851*. Number of bacteria was quantified at 0, 3 and 7 dpi, data represent a mean of three biological samples ± SE.



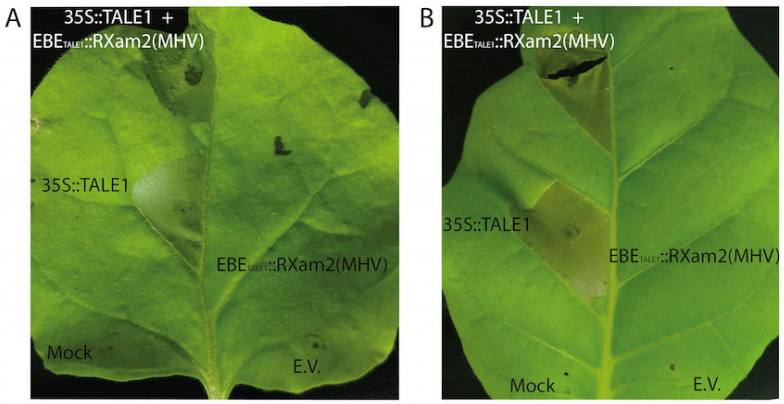
Supplementary figure 4-7: Transient expression assays of RXam2 (MHV) on *Nicotiana benthamiana* and cassava leaves.

(A) *N. benthamiana* leaves were infiltrated with *A. tumefaciens* strain GV3101 harboring pBAV139 (E.V.), 35S::RXam2-pBAV139, 35S::RXam2(MHV)-pBAV139 and 35S::RXam2-pCAMBIA1305.2 and photographed at 3 dpi. (B) Cassava leaves were infiltrated with the same constructs as (A) but the strain AGL1 was used instead of GV3101, photographs were taken at 5 dpi.



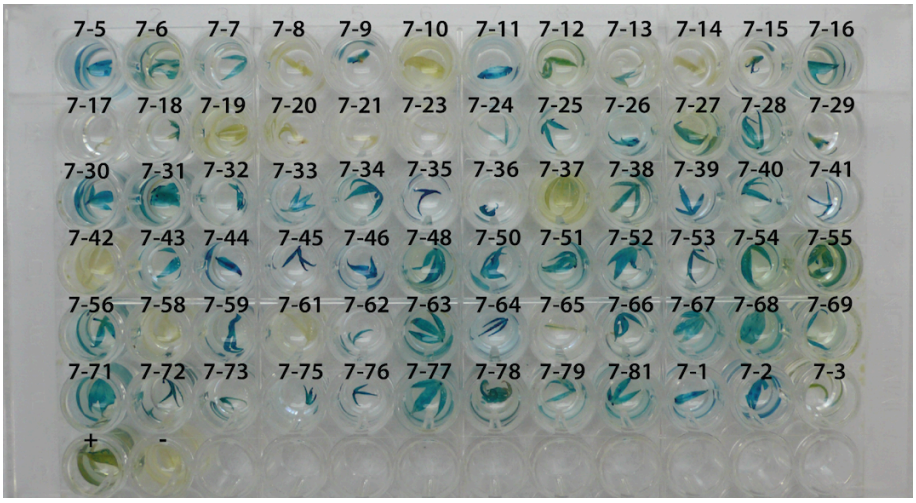
Supplementary figure 4-8: Transient expression assays on *N. benthamiana* and *N. tabacum* to evaluate the induction of the activator trap promoter EBE $_{TALE1}$.

(A) *N. benthamiana* leaves were infiltrated with *A. tumefaciens* strain GV3101 harboring pMDC32 (E.V.), EBE$_{TALE1}$::RXam2(MHV)-pMDC32, 35S::TALE1-pBAV139 and coinfiltration of EBE$_{TALE1}$::RXam2(MHV)-pMDC32 and 35S::TALE1-pBAV139 (B) *N. tabacum* leaves were infiltrated with the same constructs as (A).



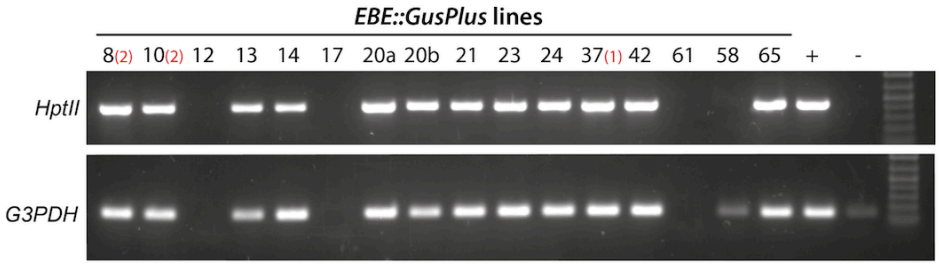
Supplementary figure 4-9: Histochemical *GUS* staining of putative *EBE$_{TALE1}$::GusPlus* transgenic plants.

Leaves from *in vitro* plants were detached and stained in X-Gluc buffer for 16h at 37ºC and destained in 96% ethanol. *EBE$_{TALE1}$::GusPlus* plants are labeled from 7-1 to 7-81; +, transgenic plant carriyng *GusPlus* as a reporter gene; -, non-transgenic control plant.



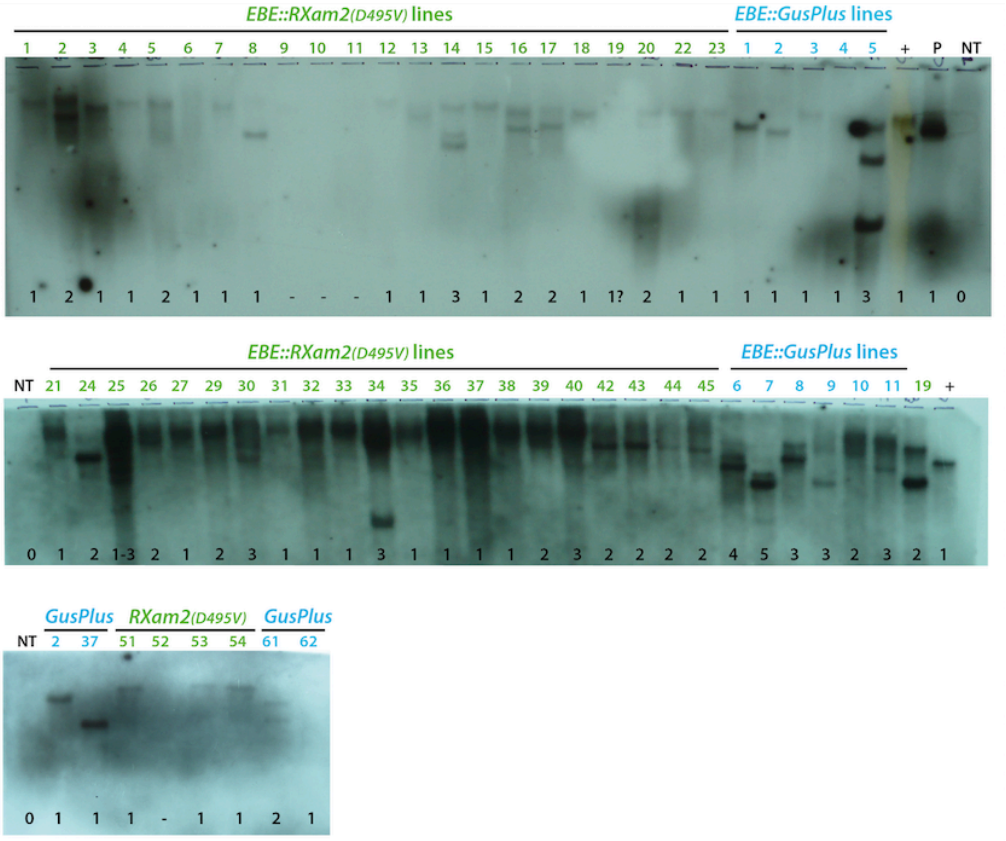
Supplementary figure 4-10: PCR from genomic DNA of *EBE$_{TALE1}$::GusPlus* transgenic plants.

Genomic DNA was isolated from fresh leaves from *in vitro* transgenic plants that were negative for the *GUS* staining. *HptII* expression was evaluated and *G3PDH* was used as a reference gene. Small red letters indicate the number of insertion events confirmed by Southern-blot analysis; +, confirmed transgenic plant; -, negative control.



Supplementary figure 4-11: Southern-blot analysis of *EBE$_{TALE1}$::RXam2(D495D)* and *EBE$_{TALE1}$::GusPlus* transgenic plants.

Genomic DNA was isolated from fresh leaves and a southern Blot was performed using *HptII* as a probe. Bold numbers below indicate the number of insertion events for each line analyzed. NT, genomic DNA from non-transgenic plants used as a negative control; +, genomic DNA from a transgenic plant used as a positive control; P, plasmid pMDC32.



Supplementary table 4-3: Summary of the transformation process and molecular characterization.

Construct	FEC transformed (mg)	Nº regenerated plantlets	GUS staining	PCR hptII	Single copy events
EBETALE1::GusPlus	68	82	Negative: 15/72	14/15	At least 1 from 3 plants evaluated
			Positive: 57/72	57/57	At least 5 from 9 plants evaluated
EBETALE1::RXam2(D495V)	68	45	-	45/45	22/45

Supplementary table 4-4: Evaluation of disease resistance in *EBE$_{TALE1}$::RXam2(D495V).*

Two-month-old *in vitro* plants were inoculated in the stem with a *Xam* cell suspension [optical density at 600nm (OD_{600nm})= 0,02,~10^7 colony forming units (CFU ml^{-1})]. Two lines of *EBE$_{TALE1}$::GusPlus* were evaluated (G-61 and G-2), five lines of *EBE$_{TALE1}$::RXam2(D495V)* were evaluated (R-44, R-51, R-53, R-54) and one non-transgenic control (NT) was evaluated. [a] Data shown is the mean of the sum of AUDPC in arbitrary units (AU) at 28 dpi from three replicates and the standard error (SE) was calculated from three replicates per genotype. [b] A genotype was considered to be resistant if the ∑AUDPC value <39 and susceptible when the ∑AUPDC value >49 according to

(Trujillo et al., 2014). [c] Mean disease rating is the mean of the symptoms observed at 28 dpi using a scale from 0 to 5 as reported by Jorge and Verdier, 2002. [d] A genotype was considered to be resistant if the mean disease rating ≤3. [e] Four categories were assigned based on previous studies (Trujillo et al., 2014). Resistant (∑AUPDC ≤39), moderately resistant (39<∑AUDPC<44), moderately susceptible (44<∑AUDPC<49) and susceptible (∑AUDPC≥49).

Strain	Genotype	∑AUDPC[a]	SE	Criteria 1[b]	Mean Disease rating[c]	Criteria 2[d]	New criteria[e]
CFBP1851 ΔTALE1	NT	30,83	2,33	R	3,00	R	R
	G-61	32,33	3,83	R	3,33	S	MR
	G-2	31,33	8,57	R	2,00	R	R
	R-12	48,33	8,35	S	4,00	S	MS
	R-44	54,00	5,00	S	4,00	S	S
	R-51	50,67	6,12	S	4,00	S	S
	R-53	32,33	2,05	R	3,33	S	R
	R-54	49,83	3,35	S	3,67	S	S
CFBP1851	NT	46,83	3,49	R/S	3,67	S	MS
	G-61	43,00	5,39	R/S	3,33	S	MR
	G-2	52,33	7,37	S	3,33	S	S
	R-12	54,50	3,00	S	3,67	S	S
	R-44	49,83	0,83	S	3,67	S	S
	R-51	34,67	10,98	R	2,67	R	R
	R-53	56,00	4,92	S	4,00	S	S
	R-54	40,33	2,62	R/S	3,33	S	MS

Chapter 5

"One never notices what has been done; one can only see what remains to be done"

"Uno nunca se da cuenta lo que se ha hecho; uno sólo puede ver lo que falta por hacer"

Marie Curie

5 General conclusions and perspectives

Among the most promising strategies for plant disease control is to employ the mechanisms selected by plants themselves to avoid or limit the attack of pathogens. Six years ago, the first version of the cassava genome was made available. This has unlocked new possibilities to the study of the molecular basis of host disease resistance. In this thesis we have explored three distinct approaches intending to solve the constraints caused by *Xanthomonas axonopodis* pv. manihotis, a bacterial pathogen from cassava. Here we report the interfamily transfer of the *Bs2* gene from pepper to cassava and the functional analysis of two candidate resistance genes, *RXam1* and *RXam2* coding for a RLK and a NLR protein respectively.

We produced transgenic cassava plants that functionally express the *Bs2* gene from pepper. Our results showed the overexpression of *Bs2* on a highly susceptible cultivar leads to a constitutive activation of immune responses in cassava. We also found that the overexpression of *Bs2* does not result in the development of an HR in cassava neither to a reduced *Xam* growth on *in vitro* plants tested. These results suggest that Bs2 is activating defense-signaling pathways in cassava still, is not sufficient to restrict *Xam* growth. Inoculation assays with *avrBs2* mutants on transgenic *Bs2* cassava plants will aid to demonstrate the recognition of the cognate effector AvrBs2.

The deployment of host resistance is mainly used to control CBB. Until now, there is a lack of reports for the isolation and cloning of *R* genes in cassava. Here we report that a gene coding for a RLK is associated with a minor QTL that contributes to strain specific resistance to CBB. Our results demonstrate that the stable overexpression of *RXam1* leads to a reduction in bacterial growth but this response is specific for strain CIO136 and it was not observed in other strains tested. This suggests that *RXam1* might be a gene involved in strain-specific resistance to CBB.

A complete functional analysis of a second cassava gene, *RXam2,* that codes for a NLR protein is presented here. Results obtained indicate *RXam2* silencing results in enhanced susceptibility to nearly all strains tested. In a similar way as observed for *Bs2*, the overexpression of *RXam2* does

not results in the development of HR. Nevertheless, a slight reduction in *Xam* growth was observed for several strains. These data collectively suggest that *RXam2* is involved in a broad-spectrum resistance

As a proof-of-concept for the development of a novel strategy for CBB resistance, we design an RXam2 variant under the control of an activator trap promoter. We found that an autoactive version mutated in the MHD motif of *RXam2* called RXam2$_{(D495V)}$ was able to trigger an HR phenotype. Based on this result, *RXam2$_{(D495V)}$* was cloned under a TALE1$_{Xam}$-inducible promoter and cassava transgenic plants were produced. Results obtained from transgenic lines evaluated indicate some lines display less disease symptoms to *Xam* strains carrying TALE1$_{Xam}$.

The information derived from the isolation and cloning of *R* genes has been usefully incorporated to breeding programs for several crops from the first world. However, most of the conventional breeding strategies developed for crops such as maize, rice, wheat or tomato are hardly suitable for vegetative propagated crops like cassava. Genetic transformation has emerged as a useful tool to overcome some constraints related to the reproductive biology of cassava.

Since the pioneering reports on cassava transformation technologies near 1990's, several other groups mainly in North America and Europe have demonstrated relevant improvements in the development of more efficient transformation protocols. Notably, several recent studies have reported important advances in the establishment of transformation protocols for industry and farmer-preferred cultivars in Asia and Africa. However, efforts are still needed to establish transformation technologies or other methods that allow genetic modification in commercial cultivars from South America.

The above results provide resources for the deployment of *R* genes, particularly *RXam1* and *RXam2,* to account for durable and wide-spectrum disease resistance in cassava. Although these genes were evaluated for resistance to CBB, it would be interesting to test if these plants are also tolerant to CMD and CBSD. The next step will be the further introduction of these genes by traditional or non-conventional breeding strategies on improved cassava varieties.

Finally, the availability of cassava plants transformed with an activator trap promoter controlling the expression of *GusPlus* might be employed as a bio-detector system for *Xam* strains containing TALE1 or other TAL effector containing similar RVDs sequences.

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7 Annex. Publications and presentations

Publications

Díaz-Tatis PA, Bernal A, López C. 2014. Transient GUS gene expression in cassava (*Manihot esculenta*, Crantz) using *Agrobacterium tumefaciens* leaf infiltration. Revista Mvz Córdoba 19, 4338-4349.

Chavarriaga-Aguirre P, Brand A, Medina A, Prías M, Escobar R, Martínez J, **Díaz P**, López C, Roca W, Tohme J. 2016. The potential of using biotechnology to improve cassava. In Vitro Cellular and Developmental Biology – Plant. ACCEPTED.

Díaz-Tatis PA, Zárate CA, Bernal A, López C. 2016. Infección de callo embriogénico friable de yuca con Xanthomonas axonopodis pv. manihotis. Revista Colombiana de Biotecnología. SUBMITTED.

Medina C**, Díaz-Tatis PA**, López C, Bernal A. 2016. Transient expression of type three effectors from *Xanthomonas axonopodis* pv. manihotis to screen for new sources of resistance in cassava.

Oral presentations in scientific events

Paula Díaz-Tatis, Mariana Herrera, Juan Ochoa, Adriana Bernal, Paul Chavarriaga, Camilo López. World Congress on Root and Tuber Crops. Nanning, Guangxi, China. January 2016. Title: Enabling resistance against cassava bacterial blight through transgenic and TAL technologies. Oral presentation.

Paula Díaz-Tatis, Mariana Herrera, Juan Ochoa, Adriana Bernal, Paul Chavarriaga, Camilo López. V Xanthomonas genomics conference. Bogotá, D.C. Colombia. July 2015. Title: Engineering Resistant Cassava (Manihot esculenta, Crantz) Plants Against Cassava Bacterial Blight. Oral presentation.

Paula Díaz-Tatis, Adriana Bernal, Paul Chavarriaga, Camilo López. XXXI Congreso Colombiano de Fitopatología. Pereira, Colombia. September 2013. Title: Expresión de la proteína de resistencia Bs2 en plantas de yuca para conferir resistencia a bacteriosis vascular. Oral presentation.

Paula Díaz-Tatis, Adriana Bernal, Camilo López. GCPII: The Second Scientific Conference of the Global Cassava Partnership for the 21th Century. Kampala, Uganda. June 2012. Title: Expressing diverse versions of disease resistance proteins for protection against cassava bacterial blight. Oral presentation.