



Identification of Disease Resistance Genes in Cassava using NBS Transcriptome Profiling

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Abstract

Cassava production is seriously affected by cassava mosaic disease (CMD), which is one of the most important problems in India and Africa. The disease can be kept under control by deploying resistance genes through marker assisted selection (MAS). Nucleotide Binding Site (NBS) profiling is a motif directing profiling technique which specifically targets chromosomal region containing resistance genes (*R*) and can produce markers tightly linked with *R*-genes. The present study aims to target disease resistance (*R*) genes in cassava using NBS transcriptome profiling technique. For this purpose, two cassava varieties viz., MNga-1 (resistant variety) and CI - 732 (susceptible variety) were selected on the basis of CMD resistance and reproducible polymorphic multi-locus banding pattern were generated with NBS primers (NBS2 and NBS5). The polymorphic bands present in the resistant variety were eluted and directly sequenced using ABI3500 automated sequencer. The functional annotations of the sequences were carried out in both Phytozome v10 and NCBI using blastn program. The sequences showed homology to *RCa6* gene, non-specific lipid-transfer protein (ns-LTPs) and heat shock proteins (Hsp 90, Hsp 89-1, Hsp 81-2).

Key words: Cassava mosaic disease, NBS transcriptome profiling, lipid-transfer protein, heat shock proteins

Introduction

Cassava, the third most important source of calories in the tropics is infected with various pests and diseases. One of the major constraints in cassava production is cassava mosaic disease (CMD) causing a yield loss of 20 to 95 per cent (Hahn *et al.*, 1980), which is exacerbated by clonal propagation, infected planting material and prolonged cropping cycle. CMD is caused by bipartite geminivirus and transmitted by whitefly, *Bemisia tabaci*. During 1990s, cassava production in Uganda was devastated by cassava mosaic geminivirus resulting in famine-related deaths (Legg *et al.*, 2004). Cassava saved the people of erstwhile Travancore province from the clutches of famine during II World war when the import of rice from Burma was stopped and during the subsequent times of food scarcity. The use of resistant cultivars has become a valuable strategy to control crop diseases. Host plant resistance is a long term effective

strategy to tackle CMD and resistance genes can be introgressed from *Manihot glaziovii* to cassava. This resulted in the development of many promising CMD resistant varieties (Tropical Manihot Selection-TMS and Tropical Manihot Esculenta-TME) in Africa. The nature of resistance in TMS lines (*CMD1*) was found to be recessive and polygenic whereas in TME lines (*CMD2*) were single dominant respectively. Later these varieties were used in cassava breeding program for the development of CMD resistant varieties.

One of the most important inbuilt characters found in crop plants is its ability to defend themselves from the invasion of pathogens. When plant is attacked by a pathogen, numerous defense responses occur in plants to avoid infection (Yang *et al.*, 1997). Most important is pathogen related proteins (PR proteins) found to be localized in almost all plant organs, though maximum abundance is found in leaves (Loon and Strien, 1999).

Presently, 17 families of PR proteins have been identified and are induced by various types of pathogens like virus, bacteria and fungi (Ebrahim *et al.*, 2011; Agrios G N, 1997). In the last few years, genetic studies on plant-pathogen interaction have allowed the identification of several resistance (*R*) genes which were proposed to function as receptors of race-specific elicitors (Ebel and Casio, 1994). When a plant recognizes a pathogen, there are several signaling cascades activated by *R* proteins which co-ordinate the initial plant defense response to prevent pathogen entry. The first *R*-genes isolated were *Hm1* from maize which confers resistance to the leaf spot fungus *Cochliobolus carbonum*. In plant viral infections, the replicase, the coat protein or the movement protein have been found to function as recognition determinants for *R* proteins (Padgett *et al.*, 2001). A previous study in cassava resulted in the isolation of 12 classes of resistance gene candidate (RGCs) and mapped on the framework cassava linkage map (Lopez *et al.*, 2000). Studies revealed the presence of about 150 NBS-LRR genes in *Arabidopsis* (Meyer *et al.*, 2003; Tan *et al.*, 2007) and 500 in rice (Zhou *et al.*, 2004) and at least 333 in *Medicago truncatula* (Ameline-Torregrosa *et al.*, 2008). However a better understanding of NBS in cassava in relation to CMD would provide a base for developing cassava resistance to CMD. This study was therefore carried out to isolate and characterize NBS-LRR type *R* gene sequences in cassava.

Materials and Methods

In this study, two plant materials were selected which are highly contrasting in CMD resistance *viz.*, Sree Padmanabha (MNga-1: resistant to CMD) and a local cultivar CI-732 (susceptible to CMD).

RNA isolation

Total RNA was isolated from young leaves collected from two cassava varieties MNga-1 and CI-732 using Spectrum™ Plant Total RNA isolation kit (Sigma). To avoid DNA contamination, the total RNA was treated with DNase (Thermo Scientific) and it was cleaned using RNeasy kit with RNA clean up protocol (Qiagen). RNA quality and purity were analyzed using 1.5% agarose gel electrophoresis (Fig.1).

Double stranded cDNA and restriction digestion

First strand cDNA was synthesized from total RNA using Revert Aid First strand cDNA synthesis kit (Thermo

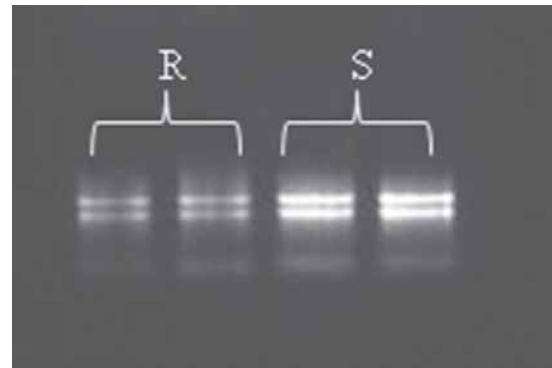


Fig. 1. RNA isolated from MNga-1 (resistant to CMD-R) and C1 - 732 (susceptible to CMD-S) checked in 1% agarose

Scientific) and this was then used for the second strand synthesis using RNase H and DNA Polymerase 1. Then the double stranded cDNA was purified by chloroform extraction. The quality of cDNA was checked in 1.0% agarose (Fig.2). A total of 200ng cDNA was digested with *Mse1* FastDigest restriction enzyme (Thermo Scientific) and incubated at 37 °C for 5 minutes in water bath. Finally the reaction was stopped by keeping the restricted samples at 65 °C for 5 minutes. The quality of digested products were checked in 1.0% agarose gel.

Adapter ligation

An adapter was ligated to the ends of restricted fragments which was based on the one described by Fischer *et al.*, (1995). For *Mse1* ligation short strand of adapter (5' TGGGATCTATACTT 3') was used and the adapter ligation was performed using high concentrate T4 DNA ligase (5 U/μl) at 22°C overnight. The reaction was terminated by heat inactivation.

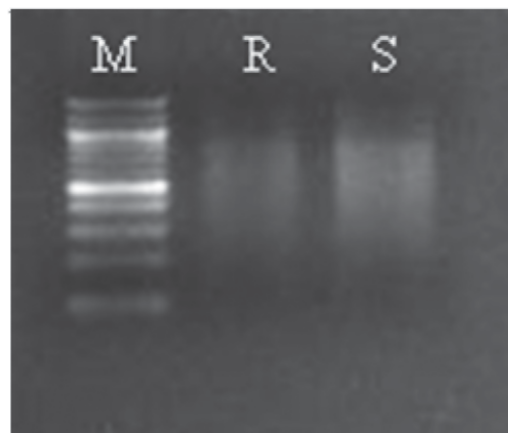


Fig. 2. Double stranded cDNA synthesized and checked in 1% agarose. M-100bp NEB DNA molecular weight ladder

A two-step PCR procedure was employed in the NBS amplification. The first step was a linear (asymmetric) PCR with a limited amount of the NBS-specific primer (1.5 mM), 10 μ M dNTPs, 0.4 U *Taq* DNA polymerase and 2.5 μ l PCR buffer in a reaction volume of 25 μ l. The program consisted of 30 cycles of 30 s at 95°C, 1 min 40 s at two different annealing temperatures one at 55°C for NBS5 and NBS7 primers and other at 60°C for NBS2, and 2 min at 72°C. The PCR reactions were done in Bio-Rad thermal cycler. The second step was an exponential PCR with NBS primer and an adapter primer by adding to the linear PCR product. A total of 50 μ l reaction volume consists of 15 pmol of each primer, 200 μ M dNTPs, 0.4 U *Taq* DNA polymerase, and 2.5 μ l PCR buffer and the cycling program were similar to that of the linear PCR. The amplified products were resolved in 6 per cent denatured PAGE (Fig.3. Table 1.).

Gel elution and purification

Fragments were excised from gel using a sharp razor blade and eluted in TE buffer. About 40 μ l of TE buffer were added to the eluted product, incubated at 65°C for 45 minutes. Then the eluted products were re-amplified using



Fig. 3. NBS profiling in PAGE

the adapter primer and NBS primer with the same conditions given for exponential PCR. The PCR products were checked in 2 per cent agarose to confirm the product size (Fig. 4). Then the PCR bands of corresponding size were eluted and purified using the QIA quick® gel extraction kit (Qiagen). These products were directly sequenced using ABI 3500 automated sequencer.

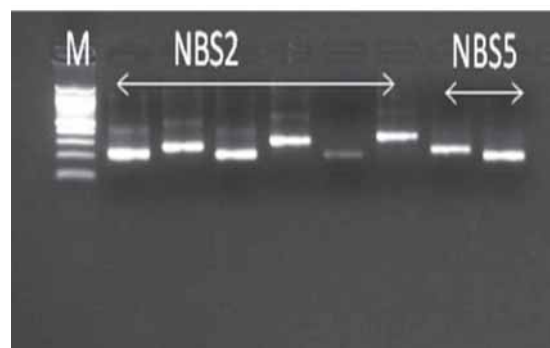


Fig. 4. Reamplified product separated in agarose

Sequence analysis

BLASTN was performed with the identified sequence and cassava genome in Phytozome with default parameters. Functional annotations of the resulted sequences were also carried out using Phytozome v10 and NCBI databases.

Results and Discussion

NBS profiling is an important technique used in the efficient targeting of disease resistant loci in many plants. The present study adopted the same procedure with little modification as reported by Gerard *et al.* (2004). In this study multiple bands (nearly 10 to 12) were obtained in both resistant and susceptible variety with NBS primers. The six bands in NBS2 and two bands in NBS5, which were found to differentiate between resistant and susceptible lines were isolated and characterized by sequence similarity searches in public nucleotide databases using BLASTN program (NCBI) and Phytozome v10. No differential band was obtained with NBS7 primer.

Table 1. List of NBS primers used for profiling

SL.No	Primer	Sequence (5'-3')	Targets
1	NBS2	5' GTWGYT'TICCYRAICCISSCAT 3'	Kinase-2 motif
2	NBS5	5' YYTKRTHGTMITKGATGATGTTTGG 3'	P-loop motif
3	NBS7	5' ATTGTTGGRATGGGMGGIMTIGG 3'	P-loop motif

Functional annotation using Phytozome v10

Functional annotations of sequences done using Phytozome v10, provides access to 48 sequenced and annotated green plant genomes including cassava which have been clustered into Malvidae gene family. Out of the eight bands sequenced, two protein coding regions were identified with NBS2 primers and one with NBS5 primers. First protein coding regions identified with NBS2 primer were found to be in two scaffolds *viz.*, scaffold 06872 (335059-335258) and scaffold 03623 (93154-93307) which comes under linkage group 1, encode mainly two functional proteins. One is bifunctional inhibitor/lipid-transfer protein/seed storage 2s albumin superfamily which showed similarity towards *A. thaliana* (AT5G38195.1_GX8P, AT1G07747.1_GX5P, AT1G48750.1_GX8P, AT1G73780.1, AT5G38170.1_GX6P, AT5G18280.1_GX9P, AT3G57310.1_GX4P, AT5G38160.1, AT1G48750.1) and another is a protease inhibitor/seed storage/LTP family protein precursor which showed resemblance towards *Oryza sativa* (LTPL154 (LOC-OsO6g49190.1_GX5P) LTPL159 (LOC-Os10g36160.1_GX3P), LTPL158 (LOC-Os10g36110.1_GX2P), LTPL152 (LOC-OsO5g47700.1_GX9P), LTPL153 (LOC-OsO5g47730.1_GX8P), LTPL162 (LOC-Os11g40530.1_GX5P).

Detailed analysis of structure and function revealed that bifunctional inhibitor/ LTP/ 2s seed storage albumin is an eight cystein motif domain which belongs to protein family that share signal peptide mainly involved in lipid metabolism. The studies suggest that DIR1 is a bifunctional inhibitor/ LTP/ 2s seed storage albumin found in *A. thaliana* and is mainly involved in the peptide and lipid signals in systemic disease resistance response. DIR1 encodes a putative apoplastic lipid-transfer like protein (LTP) (Maldonado *et al.*, 2002). Some protein members present in this group are involved in the regulation of plant type hypersensitivity response (AT3G18280.1). It was also reported that certain plant storage proteins like 2S albumins, Kunitz proteinase inhibitors, plant lectins, glycine-rich proteins, vicilins, patatins, tarins, and ocatins found in the storage vacuoles of the plant cell may act as antimicrobial agents in response to pathogen (Cândido *et al.*, 2011). LTPs are lipid-transfer proteins which have membrane permeability effect that enhance the *in vitro* transfer of phospholipids between cell membrane and can bind acyl chains (Kader J C, 1996). Non-specific lipid-

transfer proteins (nsLTPs) were identified as pathogen related-14 proteins (PR-14) implicated in plant defense against viral, bacterial and fungal plant pathogens (Blein *et al.*, 2002).

The second protein coding region (NBS2) was placed in the scaffold 11243 (95894-95966) and mainly encoded Heat shock protein (Hsp) which is similar to the different Hsp of *A. thaliana* and *Oryza sativa*. Important Hsp includes Hsp 81-2 (AT5G56030.2), Hsp 89-1 (AT3G07770.1), Hsp 90 (LOC_0s09g36420.1). The functional analyses of Hsp 81-2 revealed that these were mainly involved in the defense response towards insect vector whitefly (Bohorquez *et al.* 2012) and Hsp 89.1 was mainly involved in plant-pathogen interaction. Hsp 90 is 90KDa heat shock protein conserved in all living organism that are expressed ubiquitously and abundantly (Csermely *et al.*, 1998). The studies suggest that Hsp 90 is required for the resistance mediated by *R* proteins Rx1 and N as well as RPM1 and RPS2. Hsp 90 is also found to be associated with SGT1 and Rar1, two proteins required for the function of many *R* genes (Liu *et al.*, 2004). Analysis of the transcript of the NBS5 encodes universal stress protein.

Functional annotation using NCBI (BLASTN)

The functional annotations of the sequences were also carried out in NCBI using BLASTN. Protein coding region (NBS2) showing much similarity to RCa 6 NBS type resistant protein found in cassava (AY187296.1, AY271886.1, AY271885.1, AY271883.1), also cyanohydrins UDP-glucosyl transferase, respiratory burst oxidase H, water dikinase-3, ribulose 1,5- biphosphate carboxylase mRNA and TME 3 chloroplast complete genome were identified. The complete analysis of TME 3 chloroplast genome showed that chloroplast play a very important role in innate immunity by producing defense signaling molecules like salicylic acid, nitric oxide, ROS, NO and jasmonic acid. Respiratory burst oxidase is important for the generation of ROS (Mittler *et al.*, 2004; Keller T *et al.*, 1998), which in conjunction with NO is required for pathogen induced programmed cell deaths (Delledonne *et al.*, 1998).

Conclusion

The present study helps to identify and isolate RCa 6 NBS type resistant protein which confers disease resistance in cassava. The study also helps to understand the importance of LTPs, Hsp proteins in innate defense

mechanism in cassava. Therefore, identification of these genes in cassava is useful in the development of new markers for CMD resistance breeding.

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