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Transcriptomic analysis by cDNA RAPD profiling of differentially expressed genes upon infection of *Mungbean Yellow Mosaic Virus*.

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Abstract

Development of cultivars resistant to yellow mosaic virus disease has long been a major breeding objective, since this disease affects the production and productivity of many legumes including mungbean. Functional genomics tools can play a major role in genetic improvement for resistance to biotic stresses in crop plants. In this study, cDNA-RAPD approach was employed to analysis the transcript profile of the genes expressed in *Vigna radiata* (green gram) variety VRM (Gg) 2 and *Vigna umbellate* (rice bean) variety TNAU RED. The TDFs were compared to find the differential transcripts involved in the resistance of mungbean yellow mosaic virus upon infection. Agroinfection experiments were carried out using infectious clones and it was found that rice bean variety did not show the disease symptomotology, while the mungbean variety showed the typical yellow mosaic virus disease symptomotology. 61 random primers were used for this analysis, out of which 23 primers have identified 152 differentially expressed cDNA in TNAU RED. 23 TDFs(transcriptionally derived fragments) were sequenced. Results of BLASTP algorithm have shown that these cDNAs have significant homology with NBS-LRR protein, Pathogenesis related protein, Lipid transport protein, Photosystem II protein; the stress induced isozymes namely, Catalase, NADPH oxidase, Ascorbate peroxidase, Superoxide dismutase, Ribulose-1,5-bisphosphate carboxylase as well as Trypsin inhibitor gene T44 xyloglucan, Proteinase inhibitor and Sucrose synthase, These proteins are involved either directly, or indirectly, in stress tolerance in plants. Further characterization of these genes may show they have potential application for development of mungbean varieties with improved yellow mosaic resistance in mungbean

Key words: cDNA RAPD profiling mungban and yellow Mosaic Virus.

Introduction

Munbean is one of most preferred pulse crop by indian farmers. India is the biggest producer of mungbean with production of 710 million tons in 2013-14. Mungbean protein is easily digestable without flatulence and is important source of protein for people in the cereal-based society. It adapt well to various cropping systems as it has ability to fix atmospheric nitrogen rapid growth, and matures early.However, mungean production is being limited by various biotic and abiotic stress factors. Among various diseases, mungbean yellow mosaic viral disease is the most destructive and widely distributed one, causing10–100% yield loss depending on crop growth stage at the infection time [1]. Yield loss per annum due to YMD was estimated to be $ 300 million.Yellow Mosaic is caused by a virus belonging to the family Geminiviridae and and the genus Begomovirus and possess monopartite or bipartite circular, single-stranded DNA genomes (DNA-A and DNA-B) components encapsidated in geminate particles. The DNA A component of begomoviruses contains six partially overlapping open reading frames (ORFs) that are present on both the viral- and the complementary-sense strand. In DNA A,the pre-coat protein (AV2) and coat protein genes (AV1) are present on viral- sense strand, and genes encoding replication initiation protein (Rep,AC1),replication enhancer protein (REn,AC3) and transcription activation protein (TrAP,AC2) are present on the complementary ϕ sense strand.DNA B encodes two ORFs, nuclear shuttle protein(NSP,BV1) on the viral-sense and movement protein (MP,BC1) on the complementary-sense strand. Development of mungbean yellow mosaic virus resistant mungbean cultivars has long been a major mungbean-breeding objective. Knowledge of genes governing resistance mechanisms can enhance progress in this direction. Functional genomics tools such as cDNA RAPD profiling can be utilized for identification and characterization of resistance genes. The main

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objective of this study was, to identify gene(s) that are specifically expressed in mungbean yellow mosaic virus disease resistance and susceptible greengram and ricebean varieties.

Materials and methods:

Plant Material and Agroinoculation

To identify genes that are specifically expressed on mungbean yellow mosaic virus infection, seeds of ricebean variety TNAU RED (resistant to MYMV), and the susceptible mungbean variety VRM(Gg)2, were agroinoculated. The parital tandem dimeric constructs of the virus were mobilized from *E.coli* strain DH5α to *A. tumefaciens* strain EHA 105 [2] using pRK 2013 as helper plasmid in a triparental mating system [3]. After 48 h *Agrobacterium* cultures containing constructs were mixed in equal proportion according to the combination of viral genomic components to be inoculated, 30 µl of culture was used to inoculate each seedling. Both, VRM (Gg)2 and TNAU RED seeds were agroinoculated according to Mandal[4] by seed-sprout method. The plants were maintained at 16/8 h light/dark periods, 18,000 lx, 85% relative humidity for 3-4 weeks at 28-30^{o}C in pathology glass house until they were scored for symptoms and analyzed for viral DNA. The symptoms were recorded every day after the day of inoculation.

RNA Isolation

Total RNA was extracted from young leaf samples of mungbean and ricebean from infected and control plants by TRIsoln (GeNei™) method.The quantity of RNA quantified by NanoDropTM 1000 (NanoDrop Technologies, USA). The RNA quality was checked using 0.8% agarose gel electrophoresis. To avoid DNA contamination during cDNA conversion total RNA was treated with DNase. The total RNA (DNase treated) was reverse transcribed to cDNA using Maxima H Minus Double-Stranded cDNA Synthesis Kit (Thermo Scientific).

DD-RT PCR using cDNA-RAPD approach
Standradization of cDNA RAPD reaction using mungbean and ricebean DNA

Differential Display Reverse Transcriptase Polymerase Chain Reaction (DDRT-PCR), reaction protocol was standardized using three different DNA stock diltutions (1:10; 60ng/µL, 1:20; 40ng/µl and 1:30; 20 ng/ µL) and 61 different random primers (Table 1. The PCR amplification was performed using cocktail mixture containing 2µl DNA (for all three dilutions), Taq DNA Polymerase 0.3U, arbitrary 10-mer primer 0.5 µM, dNTPs 250 µM, 1X PCR buffer, and cDNA 1.0 µl in a thermocycler. Products were separated by electrophoresis on a 1.5% agarose gel

Polyacrylamide gel electrophoresis

After standardizing the reaction protocol, the cDNA products of the cDNA-RAPD PCR reactions were resolved on an 8 per cent denaturing (urea) polyacrylamide gel [5]. The PCR fragments of the differentially expressed genes were excised on a UV transilluminator (Fotodyne, USA) source and purified using a Gel Elution Kit (GeNei™ Bangalore, India). Twenty (20) such eluted, reamplified and purified TDFs (transcriptionally derived fragments) were sequenced (SciGenom, Kerala).

Sequence analysis

BLASTX sequence similarity search for twenty TDF sequences was performed against GenBank non-redundant (nr) public sequence database from NCBI at default parameters (http://www.ncbi.nlm.nih.gov). TDF sequences were translated into amino acid sequences in six frames using ExPASy - Translate tool available at SIB Bioinformatics Resource Portal**.** Motif pattern identification was done using ExPASy -Prosite Database. Locations of proteins in tissues were identified using ProtCompV.9.0 program (available at http://www.softberry.com/berry.phtml). Nucleotide sequences were BLAST searched using BLASTN and BLASTP algorithms (http://www.ncbi.nlm.nih.gov)

Results

Early infection in mungbean

Agroinfection of mungbean and ricebean seeds with plasmids containing tandem dimers of both DNA A and DNA B of MYMV resulted in the development of disease symptomotology in mungbean variety VRM(Gg)2 by showing the stunted growth, leaf curling, network of yellow veins and thickening of leaves. Rice bean variety showed no mosaic symptomotology.(Fig.1). To ensure successful infection, accumulation of coat protein and movement protein were checked by performing PCR. Coat protein primer i.e. MYMVCP-F (5'GCGGAATTACGATAC CGCC3')/MYMV CP-R (5'GATGCATGAGTACATGCC3') produced amplification of 750 bp. Movement protein primer i.e., MYMVMP-F (ATGGAGAATTATTCAGGCGCA3')/MYMVMP-R (5'TTACA ACGCTTTGTTCAC ATT3') amplified 900 bp band. DNA isolated from greengram variety infected

with MYMV. No amplification was observed in ricebean samples (results not shown).

RNA isolation and cDNA RAPD

Total RNA was isolated from control and agroinoculated resistant lines as well as control and agroinnoculated susceptible lines. The intactness of the isolated RNA was checked in an agarose gel (1.5per cent). The discrete bands of 25S and 18S RNA and their 2:1 ratio (a visual approximation) and the relatively clear inter band zone indicated the intactness of the RNA. About 20ng of cDNA from all the above four samples were taken for cDNA-RAPD analysis. Oligo dT primers were used for synthesis of the double stranded cDNA. Each double stranded cDNA product was used for the amplification with sixty one (61) different RAPD primers. The amplified products were resolved on 8 per cent denaturing urea-polyacrylamide gels and the gel was silver stained.

A total of 152 differentially expressed TDF's were identified with 23 different random primers, selected TDFs are shown in Fig.2. Out of 152 TDFs, 15 TDFs were amplified by the random primer OPBC 02, 14 TDFs were amplified by OPBE 01; 12 TDFs by OPBE 11; 11 TDFs ; by OPBB 01,OPBC 05 and OPBA 06. 10 TDFs were amplified by OPBA 01; 8 TDFs each by OPBE 03 and OPBE 03. 7 TDFs were by OPBB 15. 5 TDFs each by OPBB 06, OPBE 07 and OPBA 11. 4 TDFs were obtained from random primer OPBE 04. 3 TDFs each were obtained from random primer OPBE 02, OPBE 08,OPBE 10 and OPBA 17. 2 TDFs each were obtained from random primer OPBA 10,OPBB 19,OPBA 14,OPBA 13,OPBA 12,OPBA 07 and OPBE 06.Of 152 TDFs 38, 43,42 and 29 TDFs were identified exclusively in resistant infected, resistant control, susceptible infected and susceptible control respectively.

Elution and reamplification of selected TDFs

Twenty (20) TDFs (each three from OPBB06,OPBA13,and OPBE03; each two from OPBB15,OPBA 07,OPBE 04,OPBB01 and ;OPBC 05 and one from OPBA 06-1) were eluted from the gel and reamplified with respective same primers as in the initial cDNA-RAPD reaction (Fig 3a and 3b) and analyzed electrophoretically.

Sequence similarity search of TDFs against non redundant protein database

Sequence similarity analysis against the non-redundant protein database allowed assignment of putative functions to fourteen TDFs. For remaining six TDFs no significant similarity was found with the proteins available in the database. (Table 2). Five TDFs were exclusively present each in resistant (control) and resistant (infected) samples. Two TDFs each was expressed in susceptible (control) and susceptible(infected) samples .TDFs were functionally classified based on their putative function along with predicted cellular localization and protein motif. TDFs expressed in resistant lines were matched with proteins of nonredundant protein database such as with NADPH oxidase, Ascorbate peroxidase, Lipid transport protein, NBS-LRR protein, Ribulose-1,5-bisphosphate carboxylase, T44xyloglucan, Proteinase inhibitor, Sucrose synthase, and Cu/Zn Superoxide dismutase.And those expressed in susceptible lines had similarity with with Catalase, Trypsin inhibitor gene, Pathogenesis related protein and Photosystem II protein .Analysis of the predicted location of these proteins in tissues revealed that twelve proteins were predicted to be located in extra cellular matrix as secreted proteins, one protein found to be distributed in plasma membrane and one protein was predicted to be having nuclear location. Sequence similarity analysis of the seven TDFs against non-redundant protein database of NCBI showed similarity with proteins involved in disease resistance, biotic and abiotic stress tolerance, transcription, plant defence signalling and metabolism.

Identification of conserved motif in the translated proteins and their localisation in tissue

A total of ten conserved motifs were identified in translated proteins of fourteen transcripts; MYRISTYL, PKC_PHOSPHO_SITE, CK2_PHOSPHO_SITE ,CAMP_PHOSPHO_SITE , CATALASE_3 , ASN_GLYCOSYLATION, BOWMAN_BIRK, ATP_GTP_A, CYS_RICH and SOD_Cu_ZN_1.(Table 2).

Discussion

Identification and functional categorization of differentially regulated mungbean transcripts

In the present investigation total of 152 differential transcripts were identified through cDNA-RAPD analysis. This implies regulation of expression of different genes during MYMV infection and disease development. The possible role of these differentially expressed genes during the early infection periodis discussed here under according to their functional categories.

Catalase

It has been reported that catalases may play a critical role in plant defense mechanisms [6,7].These isoenzymes are involved in the specific detoxification of ROS. In the present investigation transcripts encoding Catalase were up-regulated in resistant plants infected with MYMV, this result is consistent with the previous result reported in *Capsicum*.[8]. Alteration in Catalase activity after pathogen infection or treatment with salicylic acid (SA) suggests that catalase has a specific role in the plant signal transduction cascade during plant-pathogen interactions [9]. On inhibition of catalase [9,10, 11]and ascorbate peroxidases by SA [12] the existence of possible link between SA and the oxidative burst were suggested. Reduced catalase activity was observed in infected with WCIMV [13].Similar observations were recorded in tobacco plants infected with TMV [14,15]

Proteinase inhibitor

Protease inhibitors in plants are usually considered to work as storage proteins (nitrogen source) and has a role defense mechanism[16].In the current study proteinase inhibitor gene was found to be up regulated in both resistant and susceptible infected plants suggesting that they may be hindering replication of viruses as mentioned by Gutierrez-Campos et al., 1999. [17]

NADPH oxidases (NOX)

Higher abundance of transcripts for respiratory burst oxidase i.e NADPH oxidase in this study suggested activation of hypersensitive response in the infected tissues of susceptible variety NADPH oxidases (NOX) catalyze the production of superoxides, a type of reactive oxygen species (ROS). ROS has been suggested to be a mediator of local resistance for incompatible pathogen –interaction [18] and are also associated with symptom development in compatible interaction.[19,20]

Superoxide dismutase and Ascorbate peroxidases

In the present investigation antioxidant enzymes Cu/Zn superoxidedismutases and Ascorbate peroxidase was found to be upregulated in resistant genotype suggesting that plant tries to maintain ROS homeostasis in order to protect host cells from oxidative damage/burst. Riedle-Bauer [21] studied oxidative stress in *Cucumis sativus* infected by *Cucumber mosaic virus*- and *Cucurbita pepo* plants infected by *Zucchini yellow mosaic virus* , and concluded that virus-enhanced peroxidation by the formation of ROS is involved in the development of both mosaic and yellowing symptoms in virus-infected tissues

NBS LRR

Disease resistance (R) genes are used by plants to recognize and confer resistance against a specific pathogen .In turn pathogens also suppress these disease resistance genes for their proliferation.NBS LRR is the conserved domain present in proteins involved in conferring resistance against viruses, bacteria and fungus. Recognition of elicitor produced by pathogen induce resistant gene thus initiating downstream defense signalling to stop pathogen proliferation [22]. Upregulation /downregulation of expression of genes encoding specific NBS-LRR proteins upon virus infection has already been reported [23,24,25,26]. Sahu *et al* [27] reported up-regulation of NBS-LRR gene in tomato cultivar tolerant against ToLCNDV. The present observation is also in accordance with these results. On MYMV infection, expression of NBS-LRR gene was upregulated in resistant genotype.

Rubisco and Photosystem II protein

Upregulation of photosynthetic proteins such as Rubisco and photosystem II proteins in the resistant plants suggests that plants try to enhance photosynthetic efficiency and protect photosynthetic machinery upon virus infection. These findings are in accordance with those of Subrata *et al* 2013 [28].On increasing photosynthetic efficiency extra energy is generated to avoid pathogen effectors

Sucrose Synthase

Sucrose and its cleavage products glucose and fructose are central molecules for metabolism and sensing in higher plants. Mobilization of these carbohydrates play important role in determining the outcome of plant-pathogen interactions. In the present investigation sucrose synthase enzyme was upregulated in resistant plants infected with MYMV suggesting that plant sugars are reallocated during infection by pathogen for their use, in turn, leading plants to modify their sugar content and triggering their defense responses.

Lipid Transfer proteins

The question that how viruses change and use plant membrane systems remains unclear. In present work LTP was down regulated in susceptible variety infected with MYMV. Sohal et al., 1990 [29] also reported that the expression of lipid transfer proteins, which are

implicated in defense responses to bacterial and fungal infections;are stimulated on CaMV infection of *Arabidopsis.*

Xyloglucanendo-transglycosylase

The xyloglucanendo-transglycosylase (XET) is an enzyme that is involved in the metabolism of xyloglucan, which is a component of plant cell walls.It forms a network that strengthens the cell wall. Reduced expression of xyloglucanendo-transglycosylase genes is common observation in case of virus infection. In current the investigation this gene was found to be downregulated in infected susceptible variety. Multiple genes of this family were found to show consistently reduced expression from early to late stage of infection of SACMV in *Arabidopsis* [24],Arabidopsis leaves infected with Turnip mosaic virus[30] and tomato leaves infected with ToLCNDV[27].

Pathogenesis related proteins

PR-10 family proteins are intracellular proteins with unknown enzymatic function. Some proteins of PR-10 family are induced under various stress conditions and act as common allergens [31, 32].PR 10 was upregulated in the present study in case of susceptible variety .

Conclusion:

This study identified differential transcripts in compatible and incompatible host-pathogen interaction during MYMV infection and indicates activation of innate immune response .The results presented in this study provide valuable information for understanding molecular mechanisms by which MYMV infected resistant and susceptible varieties respond to infection.

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Table 1: **List of arbitrary primers used in the study**

SNo.	RAPD Primer	SNo.	RAPD Primer	SNo.	RAPD Primer	SNo.	RAPD Primer
1.	OPBA07	17	OPBC04	33	OPBD04	49	OPBA10
2.	OPBA12	18	OPBD03	34	OPBD05	50	OPBA14
3.	OPBA13	19	OPBD04	35	OPBD07	51	OPBA17
4.	OPBB15	20	OPBD14	36	OPBD08	52	OPBA 18
5.	OPBB19	21	OPBC01	37	OPBD09	53	OPBA19
6.	OPBB06	22	OPBC02	38	OPBD10	54	OPBA20
7.	OPBB01	23	OPBC03	39	OPBD12	55	OPBE01
8.	OPBE03	24	OPBC05	40	OPBD20	56	OPBE02
9.	OPBE04	25	OPBC07	41	OPBA01	57	OPBE06
10.	OPBE05	26	OPBC08	42	OPBA02	58	OPBE07
11.	OPBE09	27	OPBC09	43	OPBA03	59	OPBE08
12.	OPBE11	28	OPBC10	44	OPBA04	60	OPBE10
13.	OPBE13	29	OPBC11	45	OPBA05	61	OPBE 14
14.	OPBE18	30	OPBD11	46	OPBA06		
15.	OPBC17	31	OPBD01	47	OPBA08		
16.	OPBC18	32	OPBD02	48	OPBA11		

Table. 2: List of TDFs and their features

TDF name	BLAST results against nr protein database	Location in tissue	Molecular function	Biological process	Motif
TDF 01	Vigna mungo catalase mRNA	Extracellular (Secreted)	oxidoreductase activity ion binding	response to stress catabolic process	CATALASE_3 PKC_PHOSPHO_SITE MYRISTYL CK2_PHOSPHO_SITE
TDF 02	Vigna radiata cultivar PusaBaisakhi NADPH oxidase (NOX) mRNA	Extracellular (Secreted)	oxidoreductase activity ion binding	immune system process response to stress signal transduction homeostatic process biosynthetic process	PKC_PHOSPHO_SITE MYRISTYL
TDF 03	Vigna mungoascorbate peroxidase mRNA	Extracellular (Secreted)	oxidoreductase activity ion binding	Antioxidant activity	PKC_PHOSPHO_SITE MYRISTYL ASN_GLYCOSYLATION CK2_PHOSPHO_SITE
TDF 04	Vigna radiata trypsin inhibitor gene, complete cds	Extracellular (Secreted)	enzyme regulator activity	------	BOWMAN_BIRK MYRISTYL PKC_PHOSPHO_SITE CK2_PHOSPHO_SITE CAMP_PHOSPHO_SITE
TDF 05	Vigna radiata pathogenesis-related protein 10 mRNA	Extracellular (Secreted)	------	Response to stress	PKC_PHOSPHO_SITE CK2_PHOSPHO_SITE MYRISTYL CAMP_PHOSPHO_SITE
TDF 06	Vigna radiata mRNA for lipid transport protein (ltp gene), variety HN2	Extracellular (Secreted)	Lipid binding	Transport	PKC_PHOSPHO_SITE CK2_PHOSPHO_SITE MYRISTYL
TDF 07	Vigna radiata NBS-LRR protein fjv-3 gene	Plasmamembrane	nucleic acid binding transcription factor activity ion binding DNA binding	biosynthetic process cellular nitrogen compound metabolic process immune system process response to stress cell death	PKC_PHOSPHO_SITE MYRISTYL ATP_GTP_A CK2_PHOSPHO_SITE
TDF 08	Vigna radiata ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene	Nuclear	Catalyzes the carboxylation of ribulose-1,5-bisphosphate	Carbon fixation	PKC_PHOSPHO_SITE MYRISTYL CK2_PHOSPHO_SITE
TDF 09	Vigna radiata cultivar T44 xyloglucanendotransglycosylase (XET) mRNA	Extracellular (Secreted)	hydrolase activity, acting on glycosyl bonds transferase activity, transferring glycosyl groups	carbohydrate metabolic process cell wall organization or biogenesis anatomical structure development	PKC_PHOSPHO_SITE MYRISTYL ASN_GLYCOSYLATION CK2_PHOSPHO_SITE
TDF 10	Vignaradiata var. radiata proteinase inhibitor mRNA, complete cds	Extracellular (Secreted)	enzyme regulator activity	-------	CYS_RICH PKC_PHOSPHO_SITE MYRISTYL CK2_PHOSPHO_SITE

TDF 11	Vignaradiata cultivar PusaBaisakhi sucrose synthase (SS) mRNA, partial cds	Extracellular (Secreted)	transferase activity, transferring glycosyl groups	biosynthetic process carbohydrate metabolic process symbiosis, encompassing mutualism through parasitism	PKC_PHOSPHO_SITE MYRISTYL ASN_GLYCOSYLATION CK2_PHOSPHO_SITE
TDF 12	Vigna radiata photosystem II protein (psbA) gene	Extracellular (Secreted)	photosynthetic reaction centres of bacteria and plants	Photosynthesis	PKC_PHOSPHO_SITE AMIDATION ASN_GLYCOSYLATION CK2_PHOSPHO_SITE
TDF 13	Vigna radiata ltp2 gene for lipid transfer protein, cultivar VC1973A	Extracellular (Secreted)	Lipid binding	Transport	PKC_PHOSPHO_SITE CK2_PHOSPHO_SITE MYRISTYL
TDF 14	Vigna radiata cultivar PusaBaisakhi Cu/Zn superoxide dismutase (CSD) mRNA	Extracellular (Secreted)	oxidoreductase activity ion binding	Antioxidant	SOD_Cu_ZN_1 ASN_GLYCOSYLATION MYRISTYL PRENYLATION CK2_PHOSPHO_SITE

A. TNAU RED 15 Days post infection **B. TNAU RED 15 Days post infection**

Figure. 1 Plants agroinoculated with DNA 'A' and DNA 'B' components of MYMV.
A : Rice bean variety TNAU RED. B: Greengram variety VRM(Gg)2

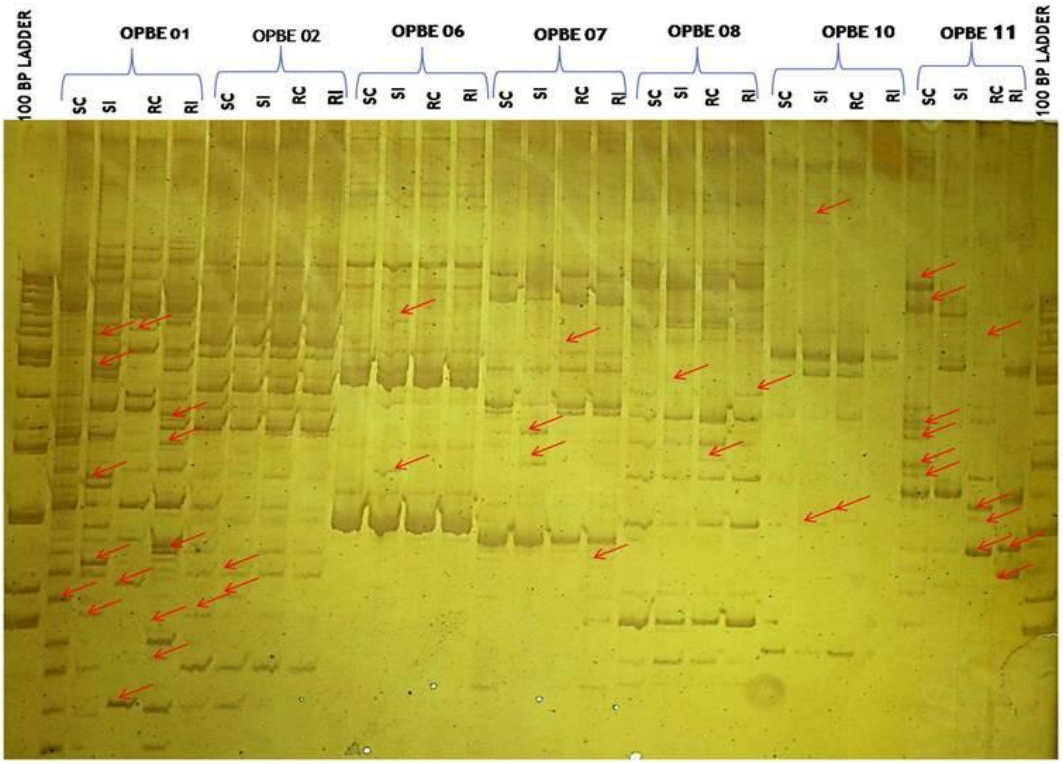

Figure 2: 8% Urea PAGE showing amplification of differentially expressed transcripts derived fragment obtained in susceptible and resistant variety.
***SC: Susceptible control , SI:Susceptible infected, RC:Resistant control, RI: Resistant infected**

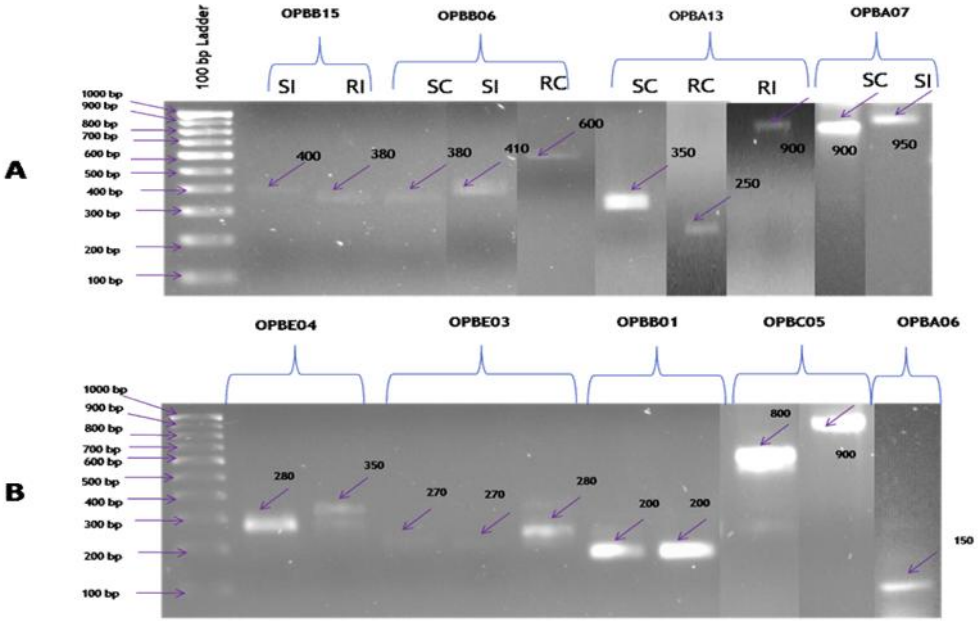

Figure.3 Agrose gel showing amplification of eluted differentially expressed transcripts derived fragment obtained in susceptible and resistant variety.
SC: Susceptible control SI: Susceptible infected, RC: Resistant control, RI: Resistant infected
A:Fragments eluted from primers: OPBB15,OPBB06,OPBA13 and OPBA 07
B: Fragments eluted from primers: OPBE 04,OPBE 03,OPBB01,OPBC05 and OPBA06

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