

Original Research Article

<https://doi.org/10.20546/ijcmas.2019.803.073>

## Expression Profiling of Resistance Gene Analogs from French Bean (*Phaseolus vulgaris* L.) for the Manifestation of Moong Bean Yellow Mosaic Virus

B. Divya\*, B. Fakrudin and C. Ashwat

College of Horticulture, Bengaluru, University of Horticultural Sciences, Bagalkot  
 Indian Institute of Horticultural Sciences, Hesarghatta, Bengaluru, India

\*Corresponding author

### ABSTRACT

#### Keywords

French bean, RGAs, Arka Anoop, MYMV and Expression analysis

#### Article Info

Accepted:  
 07 February 2019  
 Available Online:  
 10 March 2019

The popular and susceptible french bean genotype Arka Anoop was used for expression profiling of RGAs for the manifestation of MYMV. Leaf tissue was collected from both disease free and artificially disease challenged plants at 7<sup>th</sup>, 15<sup>th</sup> and 30<sup>th</sup> DAI and synthesized cDNA. The expression level of selected 10 RGA genes of french bean was measured in both disease free and artificially inoculated leaf tissues separately at 7<sup>th</sup>, 15<sup>th</sup> and 30<sup>th</sup> DAI. 10 RGA genes were tested under MYMV infested condition in french bean. All 10 selected RGA genes were expressed in leaf tissues at different days after inoculation with MYMV. COHFBRGA-2, 6, 8 and 10 genes of french bean were up-regulated in leaf of susceptible genotypes at disease inoculation condition at all the intervals. COHFBRGA-3, 4, 5 and 7 was down-regulated among all the intervals of disease development compared to control. Whereas, COHFBRGA-9 expressed only at 30 DAI.

### Introduction

French bean, *Phaseolus vulgaris* L. (2n = 22) is a member of the family Fabaceae. It is an important legume vegetable grown for its tender green pods either for fresh consumption or for processing as canned, frozen or freeze dried product. It is a nutritive vegetable which supplies protein (1.8 g), calcium (132 mg), thiamin (0.08 mg), riboflavin (0.06 mg) and vitamin C (24 mg) per 100 g of edible pods. Its pods can be used to strengthen diuretic, flushing of toxins from the body and also

infused in the treatment of diabetics (Prajapati, 2003).

It is native of new world, principally Central and South America (Kalpan, 1981) with small genome 633 Mbp (Arumuganatham and Earle, 1991). It is originated from wild species *Phaseolus aborigineus* L. and domesticated in Mexico, Peru and Colombia about 8000 years ago. In world, french bean is grown over an area of 1.48 million ha with annual production of 17.65 million MT and the productivity of 11.95 t/ha. In India, its cultivation is in 0.21

million ha with production of 0.58 million MT and productivity of 2.8 t/ha (Anon., 2015).

Like any other crops, legume vegetables are also susceptible to various biotic and abiotic stresses. Among the biotic stresses, Moong Bean yellow mosaic virus (MYMV) has become epidemic in bean growing areas and especially in locations where humid to moderately humid conditions, long dew periods and cool conditions prevail during the growing season of beans. It spreads through white flies (Rangaswamy, 1975). The Virus infects leaves, pods, petioles, rarely stems and branches. Initial symptoms appear usually on yellowing mosaic discoloration, followed by defoliation (Harter and Zaumeyer, 1941).

The yield loss due to MYMV ranges from 18 to 98 per cent (Mohan *et al.*, 1993). This disease is more severe in tropics than in temperate region (Coyne and Schuster, 1975). But, genetic resistance always has an edge over the other means of disease control as it is eco-friendly. Host plant resistance is very important because of high virulence and diversity of pathogen (Lopez *et al.*, 2003).

Many defense responses are initiated by resistance gene/genes, providing a mechanism by which the plant can recognize a pathogen and execute a defense response against it. Plant resistance (R) genes are thought to be one of the components of the genetic resistance mechanism in plants (Flor, 1956). Development of plant organs is determined by differential gene expression which can be regulated at different levels. Numerous R genes and RGAs have now been cloned, determination of activity and specificity against a given pathogen for development of durable resistance is important in french bean and other crop species (Madsen *et al.*, 2003). Advancement in technologies such as DNA sequencing methodologies, throughput platform DNA array, northern blotting,

subtractive hybridization, real-time PCR *etc.* have tremendously increased our knowledge of transcriptomes. But, the advent of real-time PCR technology has significantly changed the field of measuring gene expression in both the animal and plant molecular biology research.

Real-time PCR is the technique of collecting data throughout the PCR process as it occurs, thus combining amplification and the detection into a single step. It has become one of the most widely used methods of gene quantitation because of its high sensitivity, good reproducibility and wide dynamic quantitation range. It is the most sensitive method for the detection and quantitation of gene expression levels, in particular for low abundant transcripts in tissues with low RNA concentrations, from limited tissue sample and for the elucidation of small changes in mRNA expression levels (Mackay *et al.*, 2002). Keeping these in view, we conducted an expressional analysis of resistance gene analogs in response to rust disease manifestation in French bean.

## **Materials and Methods**

### **Disease induction and tissues collection from pot experiment**

French bean genotype Arka Anoop was raised in pots containing a mixture of soil, sand and well decomposed Farm Yard Manure (FYM) in the ratio of 2:1:1. The filled pots were kept in polyhouse. The pot mixture was sterilized before use. In replicated trials one seed was sown in each pot. To collect tissues from MYMV infected plants, the insect (vector) transmission protocol developed by Aidawaati *et al.*, (2002) was used. MYMV transmission experiment with *Bamisia tabaci* were conducted using rectangular nylon cages with mesh top. Around 50-100 *B. tabaci*/ plant were introduced into the cage through a hole made. After 24 and 48 h acquisition access

period *B. tabaci* adults were removed from MYMV agro infected french bean plants and transferred to separate cage containing healthy without virus inoculated french bean (Arka Anoop). After 24 h inoculated access period *B. tabaci* were removed and tissues were collected targeting different stages of disease manifestation both from challenged and control plants i.e., Arka Anoop plants (7th, 15th and 30th DAI). The tissues were frozen and stored at -800 C for isolation of total RNA (Plate 1).

### **RNA isolation and cDNA synthesis**

Total RNA was isolated from leaf tissues of Arka Anoop and Arka Sharath genotypes from both rust infected and non infected conditions using TRIzol reagent and driver cDNAs were prepared from the total RNA of each treatment by using SuperScript<sup>®</sup> VILO™ cDNA Synthesis Kit (Cat.no.11754-050, Invitrogen) as per the manufacturer's protocol (Plate 2-4).

### **Candidate-gene selection and primer design**

For 10 selected sequences of RGAs cloned in the our previous study the primer pairs were designed using Primer3Plus software and primers were synthesized by Eurofins Genomics India Pvt. Ltd. Bengaluru. A predicted melting temperature (T<sub>m</sub>) of 60+2°C, primer lengths of 20-24 nucleotides, guanine-cytosine (GC) contents of 45-55 per cent and PCR amplicon length of 90-200 base pairs (bp) were adopted for designing the primer pairs. The specificity of primer pairs were reconfirmed by searching homology in NCBI, BLAST search. The list of candidate genes and their respective primer pairs are shown in Table 1. PCR amplification of RGAs was optimized for different components using gradient PCR by Eppendorf master cycles gradient. PCR reactions were performed for genotype in a total volume of 20 µl containing 100 ng of cDNA, 1× PCR buffer, 2.5 mM

MgCl<sub>2</sub>, 0.2 mM dNTPs, 0.1 µM of each primer, and 2.5 units of *Taq* polymerase (Invitrogen Life Technologies, Carlsbad, CA). Cycling conditions were initial denaturation at 95°C for 10 min, followed by 40 amplification cycles (95°C for 15s, annealing temp °C for 20s, and 68°C for 20s) and a melting curve step at 95°C for 10 min before holding at 4°C).

The master mix of different components of real-time PCR was prepared fresh to avoid handling errors. The reaction mixture of 10 µl containing 1.0 ng cDNA, 200 nM of each gene specific primer and 5 µl of 2x SYBR green reagents (Cat.#4368706, Ambion, USA) were used in the experiment. Individual components of reaction mixture were standardized for 10 µl reaction volume. In our experiment we selected *Arabidopsis thaliana* housekeeping gene *actin* as an internal control (Caldana *et al.*, 2007 and Czechowski *et al.*, 2004).

The mathematical model delta-delta Ct method (Livak and Schmittgen, 2001) was used to determine relative expression ratio (fold change). In real-time PCR, fluorescence was recorded at each cycle to monitor the generation of amplified product. For proper calculation of initial target levels, differences in efficiency of amplification (E) must be taken into consideration. Even small differences in amplification efficiencies (E) will get added up making large apparent differences in mRNA levels. The absolute quantification requires a set up of standard curves from which PCR efficiency will be deduce; the disadvantages of standard curves are (i) the extra efforts and cost needed to set up additional samples (ii) Non matching E due to presence of inhibitors and serial dilutions. The relative quantification with PCR efficiency correction was adopted to calculate the fold change expression. PCR efficiency of all the RGAs was obtained from the exponential phase of each individual

amplification plot using the equation  $(1+E)^{-\Delta Ct} = 10^{\text{slope}}$  (Ramakers *et al.*, 2003). The LinReg PCR (<http://www.bioinfo@amc.uva.nl>; subject: LinRegPCR) software based on the above equation proposed a linear regression on the log fluorescence per cycle number data as an assumption-free method was used to calculate starting concentrations of mRNA and PCR efficiencies for each sample. The log-linear part of the PCR data was determined for each sample by selecting a lower and an upper limit of a “window of linearity”. Linear regression analyses was used to calculate the intercept and the slope, log (No) and log (eff.) respectively, from the straight line that fits best to the included data points. The individual PCR efficiency follows from the slope of the linear regression line ( $\text{Eff} = 10^{\text{slope}}$ ) and used as a quality check to exclude possible contained samples. To ensure unambiguous selection of data point within the “window of linearity”, the lines consisting of at least 4 and not more than 6 data points with the highest R2 value (0.99) and slope close to the maximum slope were selected.

### Processing the raw fluorescence data

Pre-requisite for LinRegPCR to achieve maximum PCR efficiency is background corrected fluorescence data points of each well. Raw fluorescence data was obtained from the Applied Biosystems stepone RT-PCR and this background was due to residual fluorescence of the dye, differences in tube transparency, dust, noise of the electronics etc. In majority of cases, a variable background makes a near-linear contribution to the curves generated by the amplifier and it should be subtracted from the raw fluorescence without distorting the data considerably. For background correction, the baseline fluorescence data was collected from 3-15 cycles. The fluorescence increments (raw fluorescence -  $Y_0$ ) were normalized to reaction fluorescence background ( $Y_0$ ) for each sample reaction as below (Yu *et al.*, 2006).

Normalized fluorescence = raw fluorescence -  $Y_0 / Y_0$

The proposed method minimized the influence of the initial vertical background shift of reaction. The background corrected or normalized fluorescence data was used to calculate PCR efficiency by LinRegPCR software. The calculated PCR efficiency was used to derive fold expression of TFs gene using the following method:

$$\text{Ratio} = \frac{(E \text{ target})^{-\Delta Ct}}{(E \text{ control})^{-\Delta Ct}}$$

E target = PCR efficiency of target gene in sample.

E control = PCR efficiency of target gene in control.

$\Delta Ct$  = (Ct of target gene - Ct of reference gene).

### Results and Discussion

Predicted features and functions of 10 cloned RGA genes were selected in this experiment for their expression analysis. The total RNA from each treatment was treated with *DNase I* enzyme to eliminate traces of genomic DNA (Plate 2). Actual confirmation of complete degradation of genomic DNA in RNA preparation was done through PCR amplification using total RNA as template. There was no amplification from the total RNA preparation indicating absence of traces of genomic DNA as contamination (Plate 3 and 4). However, elimination of contaminating genomic DNA enzymatically is very important in gene expression analysis using qRT-PCR (Chini *et al.*, 2007). Presence of genomic DNA/genetic copies of genes seriously alter the precision of expression quantitation of genes in target tissues. Generally, 18S rRNA, EF-1,  $\alpha$  actin,  $\beta$  tubulin and ubiquitin (UBQ) genes are considered as

good reference genes for any gene expression experiment (Caldana, 2007; Czechowski *et al.*, 2004). The gene expression stability measure (M) was estimated to identify the most stable reference gene among actin (AC1),  $\beta$ -tubulin, 18S rRNA and elongation factor-1 through qRT-PCR in a set of 3 different cDNA samples corresponding to different interval of day after flowering *i.e.* 7 DAI, 15 DAI and 30 DAF tissues from french bean leaves inoculated with rust (where inoculated samples were collected from both resistant and susceptible genotypes at different intervals). The NormFinder software which uses model-based variance estimation approach was used; the M value should be <1.5. The M value, 0.298, 0.311 and 0.326 for *actin* (AC1), *18S rRNA* and  *$\beta$ -tubulin* respectively, based on M value *actin* (AC1) gene was selected as endogenous reference gene for rest of qRT-PCR experiments.

In several instances these gene has been tested and used as reference genes in qRT-PCR experiments, and the M values of these reports are within the range of present experimental results (Claus *et al.*, 2004; Ruth *et al.*, 2008; Kakar *et al.*, 2008). It is the most stable combination indicating the absence of significant differences in the expression levels of reference genes in varied experimental conditions. In several instances of plant gene expression analysis by qRT-PCR these genes with similar combination have been adopted (Marino *et al.*, 2003).

PCR efficiency correction was used to calculate the fold change expression in the relative quantification of gene expression. The PCR efficiency of selected genes was calculated from the exponential phase of individual amplification plot using the equation  $(1+E) = 10^{\text{slope}}$  (Ramakers *et al.*, 2003). Subsequently, the average PCR efficiencies were computed for each individual primer pairs across all analyzed

samples. The range of PCR efficiency determined was in consistent with the results reported by Kakar *et al.*, (2008), Caldana *et al.*, (2007) and Czechowski *et al.*, (2004). Further, PCR efficiency was used to calculate final fold change of selected genes. The delta-delta Ct method (Livak and Schmittgen, 2001) was used to determine relative expression ratio of 27 genes (fold change). The delta-delta mathematical model of determining fold changes in the expression of genes is widely adopted in qRT-PCR (Czechowski *et al.*, 2004; Buchanan *et al.*, 2005; Caldana *et al.*, 2007; Yang *et al.*, 2010). In this method an amplification efficiency of each gene specific primer pairs from the log slope of fluorescence versus cycle number in the exponential phase and the same is used to calculate fold expression using the delta-delta Ct method. Similarly, Caldana *et al.*, (2007) and Yang *et al.*, (2010) used delta-delta Ct method to calculate relative fold change in rice and common bean respectively.

The technical precision of qRT-PCR was assessed by performing replicated measurements in separate PCR runs. The same pool of cDNA to account the precision in technique employed and two different pools of cDNA obtained independently from two different batches of total RNA under same condition to test precision of biological responses of plant to different day after inoculation were used. Precision, as reflected by the correlation coefficient, was high in both cases; technical and biological replicates recorded correlation coefficient values greater than 0.970 and 0.968 in different day after inoculation tissues indicating high precision of Melting curve analyses was performed for all PCR products to confirm the occurrence of specific amplification peaks and the absence of primer-dimer formation. Melting curve analysis showed that all 10 genes were giving specific amplification and there was absence of primer-dimer formation.

**Table.1** Specific primer pair sequences of french bean RGAs analyzed in response to MYMV disease manifestation using qRT-PCR

Sl. no.	Primer name	Primer sequence (5'-3')	Length (bp)	Tm (°C)	GC (%)	Product size (bp)
1	COHFBRGA1_F	ATGCAGGCCTCTGCAGTC	18	60.1	61.1	163
	COHFBRGA1_R	ACCTCGCGAATGCATCTA	18	57.9	50.0	
2	COHFBRGA2_F	GAGTCAGTGAGCGAGGAAGC	20	60.3	60.0	263
	COHFBRGA2_R	AGCTTGGCGTAATCATGGTC	20	60.1	50.0	
3	COHFBRGA3_F	ACCATGATTACGCCAAGCTC	20	60.1	50.0	245
	COHFBRGA3_R	CAGCAGCAGAAGCACAACTC	20	59.9	55.0	
4	COHFBRGA4_F	CAGGCGACGTTCGAGATCTAT	20	60.4	55.0	162
	COHFBRGA4_R	GTGCTGCAAGGCGATTAAGT	20	60.4	50.0	
5	COHFBRGA9_F	GAGTCAGTGAGCGAGGAAGC	20	60.3	60.0	263
	COHFBRGA9_R	AGCTTGGCGTAATCATGGTC	20	60.1	50.0	
6	COHFBRGA25_F	GTCGAGGAAATGGCCAAA	18	59.6	50.0	154
	COHFBRGA25_R	CACAGTCCCAGCAGCAGA	18	59.7	61.1	
7	COHFBRGA26_F	CGAGGAAATGGCCAAAAGTA	20	60.1	45.0	179
	COHFBRGA26_R	CGCTGGAAGAAGAGAAATGC	20	60.1	50.0	
8	COHFBRGA27_F	CGAGGAAATGGCCAAAAGTA	20	60.1	45.0	179
	COHFBRGA27_R	CGCTGGAAGAAGAGAAATGC	20	60.1	50.0	
9	COHFBRGA32_F	CTCCGCCTAGGAGTGAGTTG	20	60.0	60.0	217
	COHFBRGA32_R	GCCGTGCCTAAAGACTGAAC	20	59.9	55.0	
10	COHFBRGA38_F	AACGTCGTGACTGGGAAAAC	20	60.0	50.0	145
	COHFBRGA38_R	AATTTCCATTGCCATTTCAG	20	59.9	40.0	

**Table.2** Relative change in the expression pattern of selected R genes found in MYMV manifested leaf tissue at 7, 15 and 30 DAI in french bean

Sl. No.	Genes	Normalize $\Delta$ Ct values (control)			Normalize $\Delta$ Ct values			Fold expression			Log10 ratio			Cal. t value		
		7DAI	15DAI	30DAI	7DAI	15DAI	30DAI	7DAI	15DAI	30DAI	7DAI	15DAI	30DAI	7DAI	15DAI	30DAI
1	RGA1	1.939	12.588	12.629	12.927	12.629	2.040	0.010	0.005	4.139	-2.002	-2.308	0.617	0.397	0.343	0.169
2	RGA2	6.276	4.960	4.089	5.500	4.089	-1.404	110.268	82.983	76.283	2.042	1.919	1.882	0.401	0.347	0.171
3	RGA3	12.285	10.464	4.850	10.462	4.850	1.002	0.559	41.897	86.366	-0.253	1.622	1.936	0.405	0.351	0.172
4	RGA4	9.623	10.238	7.434	-24.446	7.434	9.837	40.534	0.795	0.031	7.131	-0.099	-1.514	0.411	0.356	0.174
5	RGA5	-0.756	7.104	4.809	7.889	4.809	1.349	0.025	6.880	0.734	-1.600	0.838	-0.134	0.363	0.363	0.363
6	RGA6	2.575	7.591	0.902	2.661	0.902	0.272	20.634	121.312	26.367	1.315	2.084	1.421	0.374	0.374	0.374
7	RGA7	7.028	7.825	4.993	8.231	4.993	3.817	0.048	0.553	0.423	-1.322	-0.257	-0.374	0.391	0.391	0.391
8	RGA8	3.841	4.138	2.576	4.028	2.576	-4.409	5.048	3.915	88.851	0.703	0.593	2.949	0.423	0.423	0.423
9	RGA9	6.363	4.545	5.386	4.886	5.386	0.959	0.972	0.976	5.851	-0.013	-0.010	0.767	0.568	0.599	0.568
10	RGA10	4.844	5.351	3.508	5.722	3.508	6.240	38.116	68.619	79.239	7.581	8.248	7.425	0.675	0.565	0.357

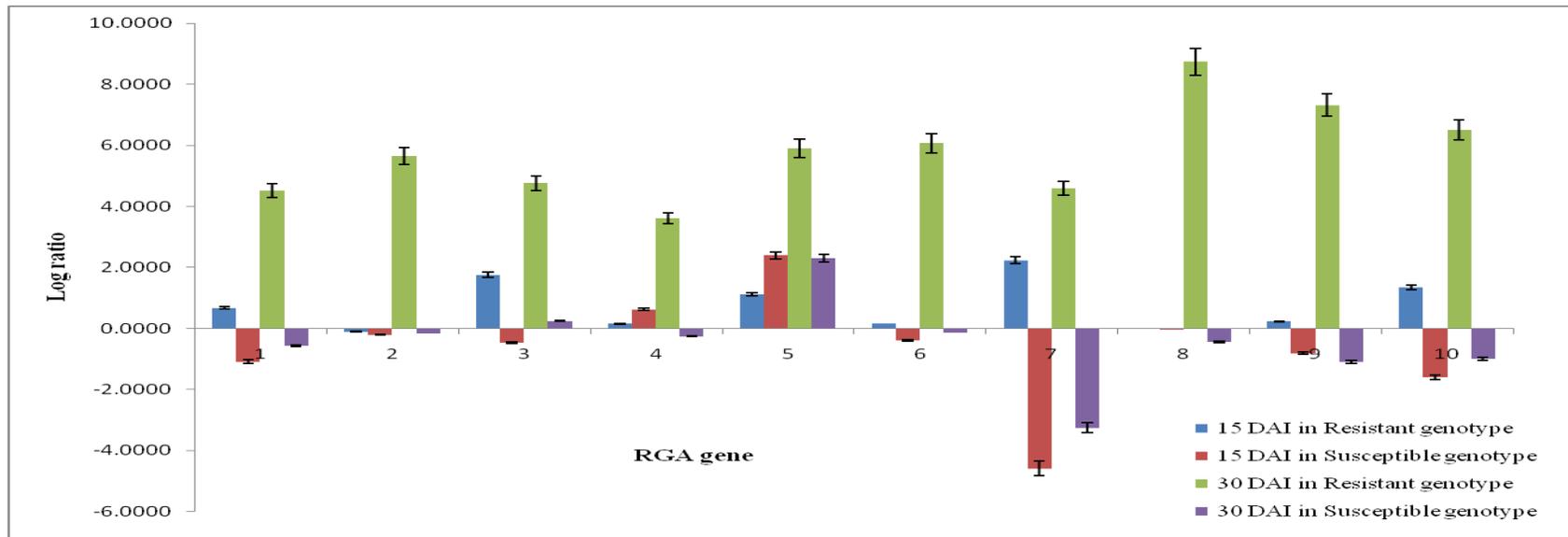
DAI: Days after inoculation

$\Delta$ Ct: Normalized Ct value

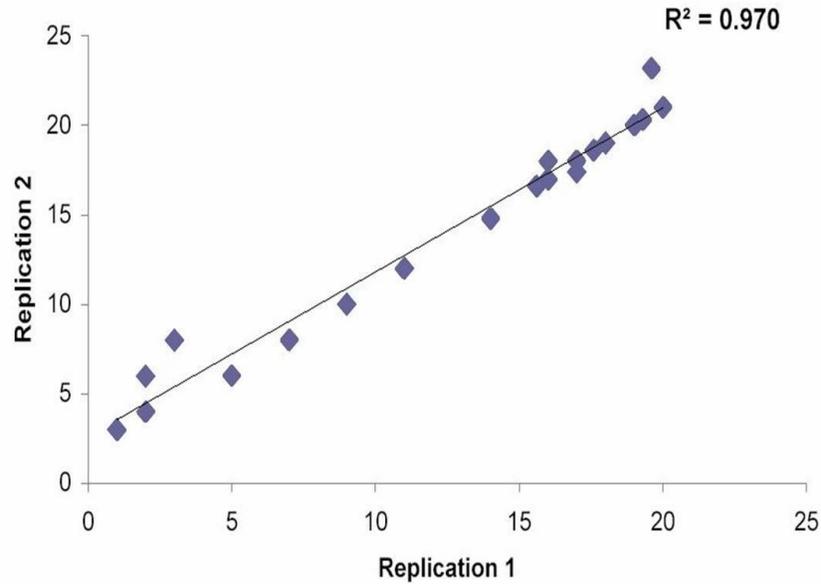
$\Delta$ Ct: (Ct of target gene-Ct of reference gene)

Table t value (1 %, df: 3) = 5.8409

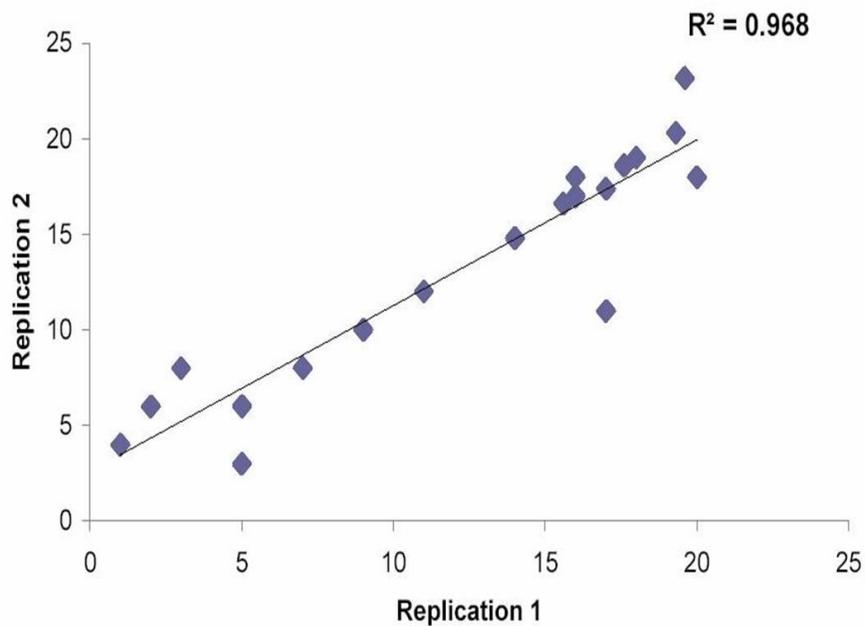
**Fig.1** Relative change in the expression pattern of selected RGA genes found in rust manifested leaf tissue at 15 and 30 days after inoculation of resistant and susceptible genotypes in french bean



**Fig.1a** Technical precision of real time PCR reflected as correlation coefficient between the duplicate measurements of cDNA levels of genes from the same reverse transcription reaction (biological replicates)



**Fig.1b** Technical precision of real time PCR reflected as correlation coefficient between the duplicate measurements of cDNA levels of genes from the same reverse transcription reaction (technical replicates)





**Plate 1a:** With out disease inoculation of *MYMV*

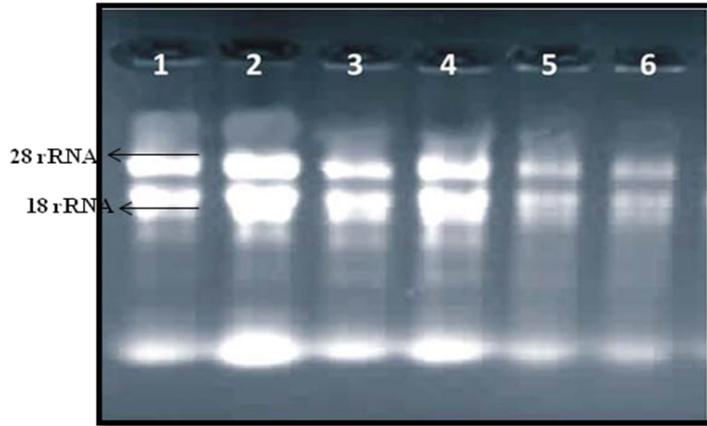


**Plate 1b:** Inoculation with *MYMV*



**Plate 1c:** Disease free and infected plant showing *MYMV* symptom 30DAI

**Plate 1:** General view of pot culture for *MYMV* infected tissues collection



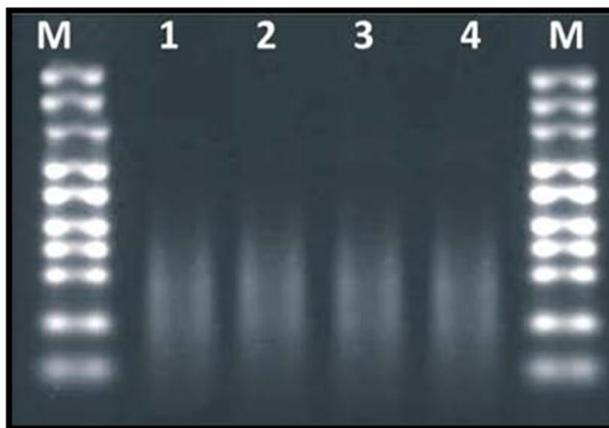
1-3-TOTAL RNA samples of rust infected leaf  
4-6-TOTAL RNA samples of *MYMV* infected leaf

**Plate 2: Confirmation of integrity and quality of total RNA on formaldehyde denaturing agarose gel**



M: 100 bp ladder  
1-22:MYMV infected leaf samples

**Plate 3: PCR confirmation of predicted genes with total RNA for complete degradation of genomic traces upon *Dnase I* treatment**



M: 1000 bp ladder

**Plate 4 cDNA from different stage of *MYMV* infected leaf sample on 1.2 per cent agarose**

Similar technical and biological precision in drought stress experiments leading to qRT-PCR testing of RGA gene was reported in *Arabidopsis* (Liu *et al.*, 2007; Huang *et al.*, 2008; Seo *et al.*, 2009), soyabean (Stolf-Moreira *et al.*, 2010) and maize (Li technical and biological treatments and response of french bean tissues (Figure 1a and 1b). A similar strategy to monitor the technical and biological precision of experiment was adopted by Czechowski *et al.*, (2004) in *Arabidopsis thaliana* and by Kakar *et al.*, (2008) in *Medicago* and Wang, 2007; Xiaofeng *et al.*, 2009).

10 RGA genes were tested under MYMV infested condition in french bean (Table 1). All 10 selected RGA genes were expressed in leaf tissues at different days after inoculation with MYMV. COHFBRGA-2, 25, 27 and 38 genes of french bean were up-regulated in leaf of susceptible genotypes at disease inoculation condition at all the intervals with a fold change ranged between 5.048 to 110.268 (7 DAI), 3.915 to 121.312 (15 DAI) and 26.367 to 88.851 (30 DAI). COHFBRGA-26 was down-regulated among all the intervals of disease development in susceptible genotype with highest fold change of 0.553 at 15 DAI and range was 0.048 to 0.423. At 7 and 15 DAI, COHFBRGA-1 was down-regulated with 0.01 to 4.139 range of fold expression and it was up-regulated at 30 DAI (0.005). Down-regulation of COHFBRGA-3 was observed at 7 DAI with 0.559 fold change and it was inversely expressed at both 15 and 30 DAI 41.897 and 86.366 fold changes was observed for both intervals. Whereas, in case of COHFBRGA-4 down-regulation exhibited for 15 and 30 DAI with fold change of 0.795 and 0.031 respectively, but was up-regulated at 7 DAI with 40.534 fold expressions. Down-regulation at 7 and 30 DAI and up-regulation at 15 DAI was notice for COHFBRGA-32 with fold change ranged between 0.025 to

6.88. But COHFBRGA-9 did not get expressed in 7 and 15 DAI and expressed only at 30 DAI with 5.851 fold change (Table 2 and Figure 1).

Emediato *et al.*, (2013) revealed that, gene expression for RGAs in both compatible and incompatible interactions across the infection time course with *Mycosphaerella musicola* in banana showed low levels of RGA expression in the early infection stages and up-regulation in late stages. In contrast, other RGAs displayed high expression levels in the resistant cultivar during the early infection stage, strong down-regulation in the intermediate infection stage, and recovery to higher levels in the final stages of the infection time course. Wang *et al.*, (2006) reported that differences in transcript levels could be correlated with the reaction to susceptibility/resistance of the host plant to pathogen. Gao *et al.*, (2006) found that some RGAs were expressed only after challenge with *Verticillium dahlia* in cotton.

In conclusion, this study strongly suggests that in common bean Assessing expression of RGAs in other tissues apart from leaf tissue at two time points to gain deeper understanding of these genes in manifestation of other disease.

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#### **How to cite this article:**

Divya, B., B. Fakrudin and Ashwat, C. 2019. Expression Profiling of Resistance Gene Analogs from French Bean (*Phaseolus vulgaris* L.) for the Manifestation of Moong Bean Yellow Mosaic Virus. *Int.J.Curr.Microbiol.App.Sci.* 8(03): 596-608.  
doi: <https://doi.org/10.20546/ijemas.2019.803.073>