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RGAP molecular marker for resistance against yellow mosaic disease in ridge gourd [*Luffa acutangula* (L.) Roxb.]

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ABSTRACT

Yellow mosaic disease caused by *Tomato leaf curl New Delhi virus* (ToLCNDV) causes 100 per cent losses in ridge gourd under epidemic conditions, particularly in the tropics and sub-tropics of India. Plant breeding approaches led by virus resistance marker-assisted selection have gained increased momentum in breeding for hasten the development of resistant varieties. In the present study, an effort had been made to identify molecular markers linked to yellow mosaic disease resistance loci in an F_2 population derived from a cross between susceptible 'Arka Prasan' and resistant 'IIHR-Sel-1' of ridge gourd. All the molecular markers were amplified in parents, and one polymorphic marker clearly distinguished the contrasting parents. The primers LaRGAP 63 produced a polymorphic DNA fragment that co-segregated with yellow mosaic disease reaction phenotypically in the F_2 population. The identified marker will be helpful to the breeders for introgression of resistance loci into the elite background.

Keywords: molecular breeding, resistance, Ridge gourd, ToLCNDV and yellow mosaic

INTRODUCTION

Yellow mosaic disease caused by the tomato leaf curl New Delhi virus (ToLCNDV) is a major production constraint in ridge gourd [*Luffa acutangula* (L.) Roxb.] cultivation. It results in heavy crop losses, especially in the tropical and sub-tropical regions as high disease dissemination through whitefly (*Bemisia tabaci*). It was first observed in India, causing viral disease symptoms on tomato (*Solanum lycopersicum* L.) (Padidam *et al.,* 1995), and recently it has invaded many new countries with extended host range (Zaidi *et al.,* 2016). Considering the potential of pathogen and health and environmental risk associated with pesticide use, identification, and introduction of genetic resistance in ridge gourd cultivars is a priority for breeders. Source of genetic resistance have been confirmed in gourds and other cucurbits (Islam *et al.,* 2010; Lopez *et al.,* 2015; Prasanna *et al.,* 2015a; Prasanna *et al.,* 2015b; and Saez *et al.,* 2016). Romay *et al.* (2019) reported a single recessive gene (*bgm-1*) with two independent minor genes (*Bgm-2* and *ToLCDV*) inheritance in melon accession IC-274014. Rai *et al.* (2014) reported monogenic recessive control for leaf curl virus in an inter specific cross (PBC 535 × Bhut Jolokia) of chili. In tomato, one major recessive gene (*ty-5*) with one additional gene was reported in resistant source 'Fla 8753' (Hutton *et al.,* 2012). Islam *et al.* (2010) observed single dominant gene action for yellow mosaic virus resistance in sponge gourd, DSG-6 and DSG-7 (*L. acutangula* L).

Incorporation of resistance alleles in the cultivated varieties require some biotechnological intervention as it recover resistant material fast with high precision. Molecular marker linked to various traits has been identified in gourd (Islam *et al.,* 2011; Karthik *et al.,* 2016). Genetic markers have been identified and leaf curl resistance genes were mapped on genetic map of tomato (Zamir *et al.,* 1994; Chague *et al.,* 1997; Ji *et al.,* 2007b; and Ji *et al.,* 2009). De Castro *et al.* (2007b) identified co-dominant cleaved amplified polymorphic sequence (CAPS) marker linked to tomato leaf curl virus resistance genes, *Ty-1* in tomato. The resistant genes were introgessed from *S. habrochaites*, *S. lycopersicon*, *S. pimpinellifoium*. Garcia *et al*. (2007) reported two sequence characterized amplified region (SCAR) markers (TG0302 and TY-2R1) linked to Tomato leaf curl virus resistance gene (*Ty-2*) in tomato. Ji *et al. (2007a)* detected co-dominant SCAR primer FLUW25 linked to leaf curl virus resistance genes (*Ty-3/Ty-3a*) in the LA2779 lines derived from tomato wild species *S.*

chilense. Identification and mapping of the mungbean yellow mosaic virus resistance gene have been reported by several researchers (Souframanien and Gopalakrishna, 2006; Gupta *et al.,* 2013; Alam*et al.*, 2014; Singh *et al.*, 2020). With this background, here we report a RGAP marker linked to yellow mosaic disease resistance in *L. acutangula* (L.) Roxb.

MATERIALS AND METHODS

Plant materials

This experiment was conducted in the F_2 population developed from a cross between Arka Prasan and IIHR-Sel-1. Arka Prasan is a commercial high yielding ridge gourd variety with long and tender fruits, however susceptible to yellow mosaic disease. *L. acutangula* genotype IIHR-Sel-1 is a yellow mosaic disease resistant advanced breeding line maintained as inbred after at least six generations of self-pollination at the Division of Vegetable Crops, ICAR-Indian Institute of Horticultural Research, Bengaluru (Kaur *et al.,* 2021b). Both the genotypes were true-to-type with highly uniform plant types due to continuous self-pollination.

Controlled crosses were made between the susceptible 'Arka Prasan' (used as a female parent; P_1) and the resistant 'IIHR-Sel-1' (used as a male parent; P_2) ridge gourd genotypes to develop F_1 generations. The F_1 population was self-pollinated to produce the subsequent F_2 population in the spring-summer season. In the field, 30 plants of each P_1, P_2, and F_1 (Arka Prasan × IIHR-Sel-1), whereas 252 plants of F_2 population were evaluated under high disease pressure conditions with high epidemics of whitefly, the insect vector for disease transmission during the spring-summer season under field conditions (Kaur *et al.,* 2020). Randomized complete block design with three replications was followed for current experiment. Plants were evaluated on a standardized 6-point interaction phenotype scale (Sohrab, 2005) where 0= no symptoms, 1= mild mosaic pattern in young leaves covering >10% area, 2= mosaic pattern in young leaves covering >25% area, 3 = mosaic pattern in young leaves covering>50% area, blistering and puckering of leaves, 4= widespread mosaic pattern in young leaves covering >75% area, distortion of leaves and 5= widespread mosaic pattern in young leaves covering >75% area, distortion of leaves and stunting of the plants. Eight scorings were taken at the weekly interval after the first disease symptoms (18 days after transplanting) on susceptible lines and last week data were used to link with the marker.

DNA extraction and primer selection

DNA of parents (Arka Prasan and IIHR-Sel-1) F_1's and segregating population (F_2) was extracted from young and healthy leaves using the procedure of cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990). The two-sequence related amplified polymorphism (SRAP) primers in four combinations previously reported (Islam *et al.* 2010) were used for molecular marker validation. Fourteen resistance gene analog polymorphism (RGAP) primers were designed using Primer 3.0 from nucleotide-binding sites leucin rich repeat (NBS-LRR) sequence analogs (Karthik, 2016) and screened in the current study. PCR reaction was performed in 15.0 µl aliquots containing 10X assay buffer, 15.0 mM MgCl2, 1.0 mM dNTPs, 3U Taq DNA polymerase, five µm primers, 30 ng template DNA, and sterile double distilled water in an Eppendorf thermocycler. The PCR profile starts with an initial denaturation at 95°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing as per the primers for 30 sec, extension at 72°C for 1 min followed by a final extension at 72°C for 5 min and storage at 4°C. The amplified products separated on 4 % agarose gel were visualized with ethidium bromide staining under the gel documentation system (UVITECH limited, Cleaver Scientific, United Kingdom).

Scoring of molecular profiles

The molecular markers with polymorphism between resistant and susceptible parents were selected. Primers showing polymorphism between the resistant and susceptible parents were used to screen the segregating F_2 population. The goodness of fit of the observation from the F_2 plants' genotyping with the expected Mendelian ratio was tested using chi-square analysis (Panse and Sukhatme, 1985).

Cloning and sequencing of PCR amplicons

Target amplified products of predicted size were gel excised and purified using MinElute Gel extraction kit (Cat# 28704, QIAGEN, GmbH and Hilden, Germany). The purified PCR products were cloned into the plasmid vector pTZ57R/T using InsT/A clone PCR product cloning kit (Cat# K 1214, MBI, Fermentas) following the manufacturer's instruction and transformed into competent cells of *E. coli* strain

DH5a using the standard heat-shock method. Recombinant clones were selected through blue/ white colony screening on LB plates supplemented with antibiotic ampicillin (1000 mg/ml), IPTG (0.1 M) and X-gal (20 mg/ml). The positive clones were confirmed for the presence of inserts by EcoRI digestion of the plasmids and electrophoresis on ethidium bromide-stained 2.0% (w/v) agarose gel. The clones showing expected inserts were sequenced from the Medaxin DNA Sequencing facility, Bangalore, Karnataka India.

The sequences obtained were subjected to NCBI (www.ncbi.nlm.nih.gov) BLAST N search, and the sequence showing the highest scores were retrieved. The sequence data were assembled and analyzed using the software BioEdit version 7.0.9.

RESULTS AND DISCUSSION

Three SRAP and RGAP markers previously found to be co-segregating with yellow mosaic disease resistant loci were monomorphic between the contrasting genotypes. Since linked markers failed to differentiate resistant and susceptible genotypes, attempts were made to identify molecular markers linked to yellow mosaic resistance loci. All the markers amplified in the parental lines, of which most of the primers were found monomorphic. One primer, LaRGAP 63, showed polymorphism and distinguished the contrasting parents (Fig 1). The marker LaRGAP 63 showed amplified product size (460 bp) in susceptible plants, while a smaller product size (455 bp) was observed in the resistant plants. Similar to susceptible parent, 460bp sizes were detected in F_1 plants (Fig 1). Marker was of the dominant nature, hence failed to detect any heterozygous band in the hybrid. The marker LaRGAP 63 was used for individual plant genotyping of 252 plants in the F_2 population. PCR amplification of 252 DNA samples with LaRGAP 63 showed the segregation pattern of 3:1 (susceptible: resistant) with ($\chi 2 = 0.09$; $p = 0.77$) indicating co-segregation of markers with phenotypic data where F_2 was fitted in 3:1 (susceptible: resistant) and single recessive gene action was observed for trait inheritance (Table 1). LaRGAP 63 marker amplified at 455bp in resistant genotype (IIHR-Sel-1) and 61 F_2 individuals whereas 460 bp band size was detected in susceptible genotypes, F_1 hybrid and 191 F_2individuals (Fig. 1). Marker LaRGAP 63 was found to be co-segregated with phenotypic observations (80.95 %) for yellow mosaic resistance in the genetic background of IIHR-Sel-1 (Table 1). Upon cloning and sequencing of amplified products, LaRGAP 63 in resistant and susceptible parents, the five nucleotides difference was confirmed. In the resistant parent, the deletion of 5 nucleotides was observed compared to the susceptible parent (Fig 2). Further sequence analysis with BLASTN tool (www.ncbi.com) and BioEdit 7.0, LaRGAP 63 showed 85.4% homology with mosaic virus resistance gene in cucurbits (Fig 3).

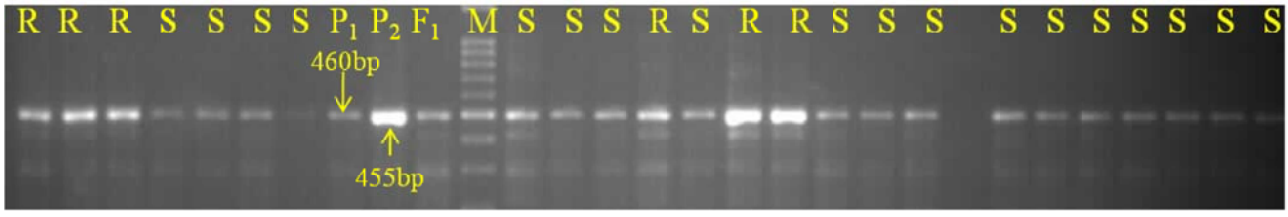

Figure 1. Genotyping of F_2 population with LaRGAP 63
(P_1- Arka Prasan, P_2- IIHR-Sel-1, F_1-Hybrid, S-Susceptible, R-Resistant and M-100 bp ladder)

Table 1. Segregation pattern of LaRGAP 63 marker locus using 252 F_2 plants derived from the cross of Arka Prasan × IIHR-Sel-1

Marker	Observed F_2 plants (Genotypic)		Expected ratio	$\chi 2$ value	P value at 5%	Observed F_2 plants (Phenotypic)		Co-segregation with trait (%)
	Resistant (R)	Susceptible (S)	R:S			R	S	
LaRGAP 63	61	191	1:3	0.09	0.77	68	184	80.95

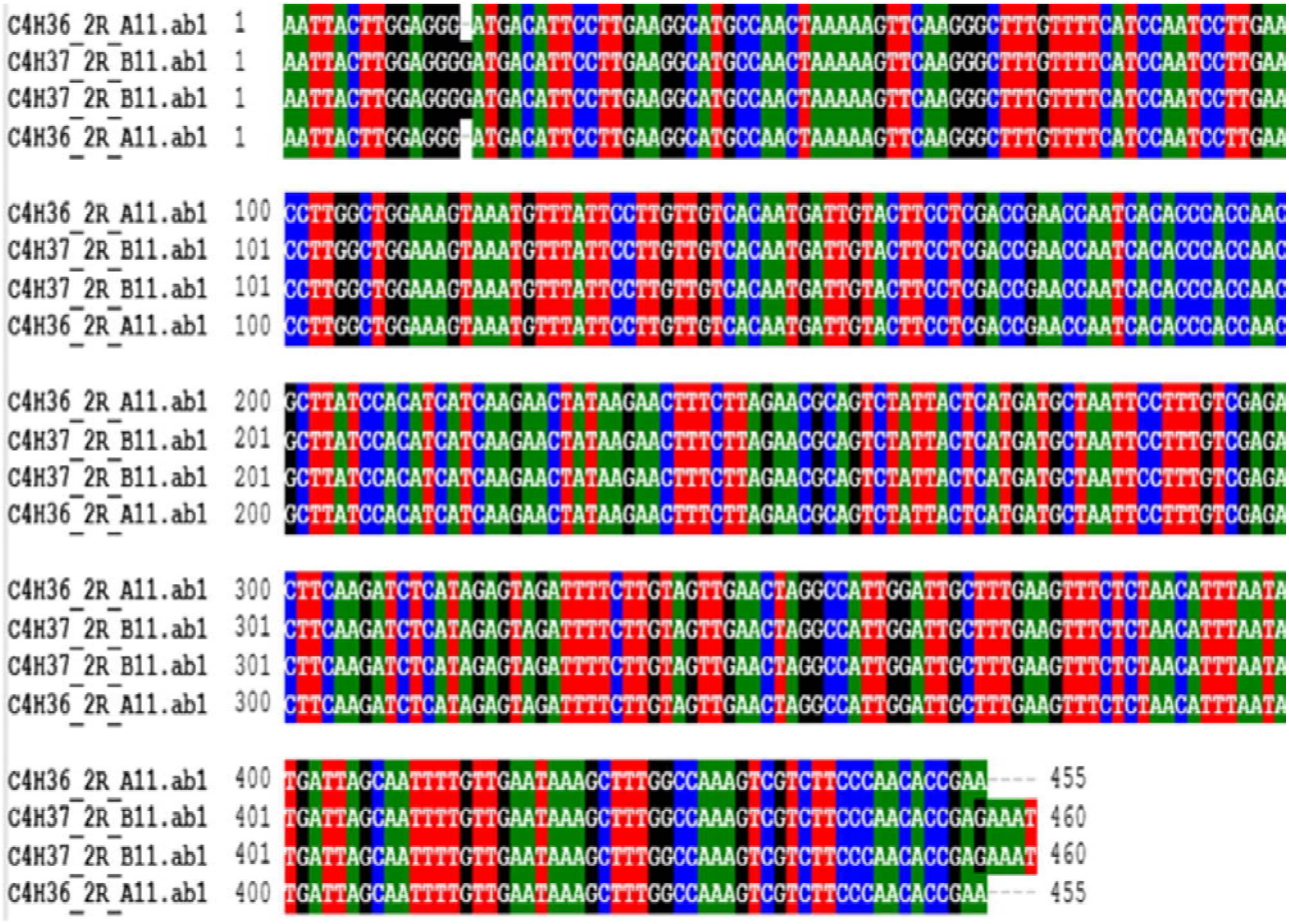

Figure 2. Sequence analysis of LaRGAP 63 displaying nucleotide difference between the resistant and susceptible parents

(C4H36_2R_All.ab1 and C4H36_2R_All.ab1 corresponds to genomic sequence of the amplified segment of the resistant genotype, IIHR-SEL-1 and susceptible genotype, Arka Prasan respectively)

Phenotypic evaluation combined with marker aided selection is important in molecular breeding. Molecular mapping of the ToLCNDV resistance gene was carried out using F_2 segregating population. Based on phenotypic evaluation for disease reaction in the F_2 population, a segregation pattern was found the best fit for 3:1 (susceptible: resistant). Thus, indicating that inheritance of resistance was under the control of a recessive gene (Kaur *et al*., 2021a). Islam *et al.* (2011) reported two SRAP markers linked to ToLCNDV resistance gene in sponge gourd. Due to genome synteny between ridge gourd and sponge gourd (Wu *et al*., 2016), both the previously identified markers were employed for validation in resistant *Luffa* genotype IIHR-Sel-1. Both the markers were amplified in susceptible and resistant genotypes but did not show any polymorphism. One RGAP marker (LaRGAP 63) has shown polymorphism between resistant and susceptible genotypes. PCR amplification of derived F_2 population with LaRGAP 63 showed the segregation pattern of 3:1 (susceptible: resistant) indicating the involvement of a recessive gene for marker inheritance (Table 1). Sequence analysis of LaRGAP63 showed 85.40 and 84.29 per cent homology with mosaic virus resistance protein and disease-resistant protein RPP-4, respectively. A number of RGAs were found to be linked with virus, bacteria and nematodes resistance in many crop species (Kanazin *et al*., 1996; Leister *et al*., 1996; Yu *et al*., 1996; Speulman *et al*., 1998; Spielmeyer *et al*., 2000). Saha *et al*. (2013) also identified one molecular marker (sgRGC 18) linked with ToLCNDV resistance in sponge gourd. Cultivated *Luffa* species are cross-compatible; however, fruit-related traits need to be studied. Pandey *et al*. (2018) found 56 polymorphic microsatellite markers for diversity analysis among *Luffa* species. Cucumber derived SSR markers were highly transferable to *L. hermaphrodita* (60.2%), *L.*

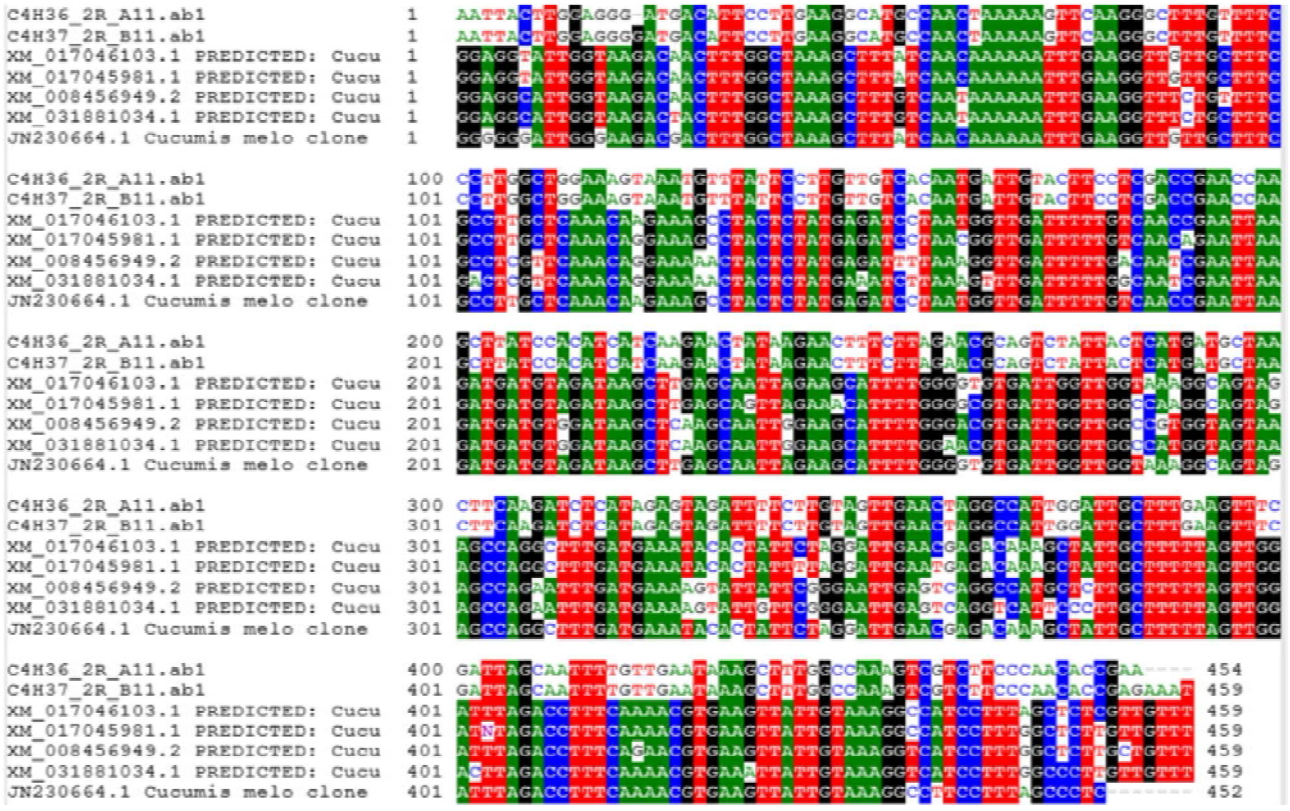

Figure 3. LaRGAP 63 nucleotides sequence similarity with known mosaic virus resistance protein available in public domain

(C4H36_2R_All.ab1 and C4H36_2R_All.ab1 corresponds to genomic sequence of the amplified segment of the resistant genotype, IIHR-SEL-1 and susceptible genotype, Arka Prasan respectively. Amplified genomic sequence was aligned with mosaic virus resistance genes available in public domain www.ncbi.com)

acutangula (68%) and *L. aegyptica* (61.16%) and therefore can be used for marker assisted selection in *Luffa* species.

Wu *et al.* (2016) constructed a genetic linkage map using an F_2 population derived from an interspecific cross between S1174 (ridge gourd) and 93075 (sponge gourd) spans over 1436.12 cM and the average distance between the markers is 8.11cM. Total 177 EST-SSR markers were employed which were distributed over 14 linkage group with an average of 102.58 cM per linkage group. These studies are more relevant for interspecies horticultural traits incorporation.

Saez *et al*. (2020) reported that major QTLs linked to ToLCNDV resistance was located at chromosome 8. Souframanien and Gopalakrishna (2006) identified ISSR marker (ISSR8111357) linked to mungbean yellow mosaic virus resistance gene in black gram. ISSR8111357 was validated using diverse germplasm differing in the disease reactions. ISSR8111357 marker was converted to co-dominant SCAR marker. Upon screening, the SCAR marker clearly differentiated the resistant and susceptible plants. Hoque and Rabbani (2009) found four polymorphic RAPD markers distinguishing 28 accessions of ridge gourd. Wu *et al*. (2014) designed 641 expressed sequence tags- simple sequence repeats (EST-SSR) primer pairs with sponge gourd transcriptome analysis out of which 494 were amplified and two hundred and one (40.69%) revealed polymorphisms between the sponge gourd [*L. cylindrica* (L.) Roem.] and ridge gourd [*L. acutangula* (L.) Roxb.]. These polymorphic markers were found highly transferable in six other cucurbits *viz*. pumpkin, cucumber, wax gourd, bitter gourd and bottle gourd. Therefore, these can be employed inmarker-assisted selection for the improvement of *Luffa* species.

The identified yellow mosaic disease linked marker LaRGAP 63 sharing homology with the mosaic virus resistance protein in cucurbits is vital to facilitate early plant selection and further pyramiding for resistance

loci for different virus isolates in common ridge gourd background. As the identified marker is dominant, it needs to be converted into a co-dominant to increase its efficiency for the ridge gourd's molecular resistance breeding program.

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