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Marker-assisted pyramiding potyvirus resistance genes into Rwandan common bean (*Phaseolus vulgaris* L.) genotypes

Markör destekli piramit potivirüs direnç genleri, Ruanda fasulye (*Phaseolus vulgaris* L.) genotipleri

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ABSTRACT

Bean common mosaic virus and Bean common mosaic necrosis virus belong to Potyvirus and the disease poses significant threat to bean yield especially in East and Central Africa. Combining the dominant and recessive resistance genes is the most effective disease control against all known strains of the virus, a strategy to enhance the durability and stabilize the yield. The aim of the study was to transfer both the dominant *I* and recessive *bc-3* genes into susceptible Rwandan common beans via marker-assisted backcross breeding. The markers specific for *I* gene (BCMV-48289723-CAPS) and for *bc-3* gene (ENM-CAPS) were used for selection in BC progenies. Successful gene combination was established in 33% of BC1F1 and BC2F1, 43% of BC3F1 and 16% of BC3F2 progenies. The seed color was fully recovered on some of the BC3 progenies with combined resistance genes. The advanced lines are expected to shield the crop against both BCMV and BCMNV under East and Central African conditions.

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***Anahtar Kelimeler*:**

Geri melezleme
Fasulye adi mozaik virüsü
Fasulye adi mozaik nekroz virüsü
Fasulye

ÖZ

Özellikle Doğu ve Orta Afrika'da bulunan ve fasulye veriminde önemli bir tehdit olan fasulye adi mozaik virüsü ve fasulye adi mozaik nekroz virüsü potirviruse aittir. Dayanıklılığı arttırmak ve verimi stabilize etmek için dominant ve resesif direnç genlerini birleştirmek virüsün bilinen tüm suşlarına karşı en etkili hastalık kontrol stratejisidir. Çalışmanın amacı, hem dominant *I* hem de resesif *bc-3* genlerini, marker destekli geri melezleme ıslahı yoluyla hassas Ruanda fasulyelerine transfer etmektir. *I* geni (BCMV-48289723-CAPS) ve *bc-3* geni (ENM-CAPS) için spesifik markerler BC progenlerinde seçilim için kullanıldı. Başarılı gen kombinasyonu progenilerde BC1F1 ve BC2F1'in %33'ünde, BC3F1'in %43'ünde ve BC3F2 %16'sında başarıyla gerçekleştirilmiştir. Tohum rengi, bazı BC3 progenilerinde kombine direnç genleriyle tamamen geri kazanılmıştır. Gelişmiş hatların, mahsulü Doğu ve Orta Afrika koşullarında hem BCMV hem de BCMNV'ye karşı koruması beklenmektedir.

1. Introduction

Common bean (*Phaseolus vulgaris* L.) is one of leguminous crops grown worldwide (Schmutz et al. 2014). Rwanda was ranked highest in bean consumption per capita (Blair et al. 2010). It plays an essential role for income generation and food security in Rwanda (Larochelle and Alwang 2014). Bean production continues to be under attack of destructive potyviruses causing significant yield loss across the globe (Drijfhout 1978; Provvidenti et al. 1984; Saiz et al.1995; Spence and Walkey 1995; Njau and Lyimo 2000).

Bean common mosaic virus (BCMV) and Bean common mosaic necrosis virus (BCMNV) belong to the genus of Potyvirus and are most destructive viruses that attack common beans. The BCMV and BCMNV are seed borne and transmitted by several aphid species (Kelly et al. 2003). Use of resistant cultivars in plant breeding with broader host plant resistance proves to be the best durable and economic effective way to control pathogens from attacking crops (Drijfhout 1978; Kelly et al. 1995; Miklas et al. 2000).

Resistance to BCMV is governed by both dominant inhibitor (*I*) gene and a number of recessive genes, namely *bc-u*, *bc-1*, *bc-1*^{2}, *bc-2*, *bc-2*^{2} and *bc-3* (Freyre et al. 1998; Strausbaugh et al. 1999).

Use of marker-assisted selection (MAS) for indirect selection of specific resistance genes in the absence of the pathogen has been used successfully to breed common bean (Miklas et al. 2006). Pyramiding of dominant and recessive genes which have distinctly different mechanisms of resistance, a strategy to enhance the durability of broader array possible to host plant resistance would enable farmers to achieve greater yield stability (Kelly et al. 1995; Mukeshimana et al. 2005).

A number of molecular markers tightly linked and specific to *I* and *bc-3* genes have been employed in MAS for identifying and selecting bean cultivars with enhanced resistance in segregating populations. In earlier work, Haley et al (1994) identified RAPD markers $OS13_{690}$ linked to the *I* gene in common bean across a wide range of germplasm in both Mesoamerica and Andean gene pools. Johnson et al (1997) developed SCAR markers from the $OC11_{350/420}$ (ROC11) and $OC20_{460}$ RAPD markers linked to the *bc-3* gene to improve their utilization.

Nobody has done research on pyramiding potyvirus resistance genes into Rwandan beans yet. The objective of this study was to transfer *I* and *bc-3* resistance genes against BCMV and BCMNV into Rwandan susceptible beans using marker assisted backcross gene pyramiding method.

2. Materials and Methods

2.1. Plant materials

Seeds from four recurrent parent materials of *P. vulgaris* cultivars; G54, RWR 1668, RWR 2355 and RWV 2361 obtained from RAB (Rwanda Agriculture Board, Rwanda) and donor parents; AC-HENSAL and USCR-7, both carrying a combination of *I* and *bc-3* resistance genes, obtained from National Plant Germplasm System (GRIN),USAID,USA were sown. Characteristics of parental materials were recorded at the field, under green house conditions in Antalya, Turkey (Table 1).

2.2. Segregating populations

Segregating populations derived from crosses between parental materials; G54xAC-HENSAL, RWR 1668xAC-HENSAL, RWR 2355xAC-HENSAL and RWV 2361xUSCR-7 were developed. The F1 individuals were both self-pollinated to produce F2 and backcrossed to their respective recurrent parents to generate BC1, BC2, and BC3 populations. The selected BC3F1 lines were selfed to BC3F2 to obtain homozygous resistant plants (Figure 1).

2.3. DNA extraction

The gDNA of parents, F2 and BC populations was extracted from young leaves of seedlings. A few milligrams of fresh tissues were ground and dispersed in 500 µl of extraction buffer [1.4 M of NaCl, 20 mM of EDTA, 100 mM of Tris-HCl (pH 8), 2% CTAB, and 0.2% of beta-mercaptoethanol] (Doyle and Doyle 1990). The suspension was mixed well, incubated at 65°C for 2 h, the homogenate was extracted with 500 µl chloroform–isoamyl alcohol (24:1), centrifuged for 30 min at 12000 rpm in a microcentrifuge.

The upper (aqueous) phase was transferred to a new set of 1.5 ml microtubes and precipitated with 350 µl ice-cold isopropanol at -20°C for overnight. The pellet formed after centrifugation at 12000 rpm for 10 min was washed twice with 200 µl of 70% ethanol, and then resuspended in 100 µl sterile distilled water. The DNA concentration was calibrated on 2% agarose gel using lambda DNA as standard. The DNA was stored at -20°C until use.

Table 1. Characteristics of parental plant materials used.

Cultivar	Growth habit	Gene pool	Response to BCMV/BCMNV
AC-HENSAL	Bush	MA	+
G 54	Semi-climber	MA	-
RWR 1668	Bush	A	-
RWR 2355	Semi-climber	A	-
RWR 2361	Climber	A	-
USCR-7	Bush	A	+

A: Andean, MA: Mesoamerican, +: Resistant to BCMV/BCMNV, -: Susceptible to BCMV/BCMNV.

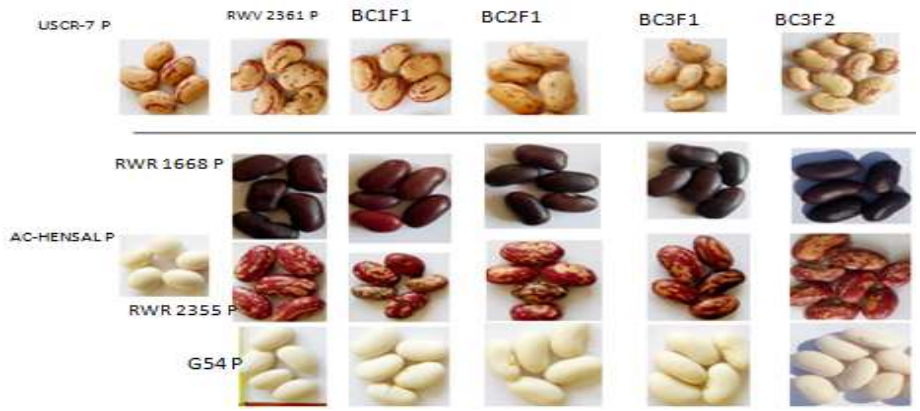

Figure 1. Breeding strategy: Plates show the seed color recovery in some of backcross generation lines (BC1F1, BC2F1, BC3F1 and BC3F2).

2.4. Marker analysis and PCR amplification

BCMV-48289723-CAPS and ENM CAPS markers were used to identify *I* and *bc-3* genes, respectively. A total of two CAPS markers were screened against parents and progenies. The PCR products generated were digested with *RsaI* and *TaqI* enzymes in separate sets (Table 2).

PCR reactions were performed in a total volume of 12 µl containing 1X *Taq* buffer $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.1 mM dNTPs, 5 Units of Taq polymerase, 4 µM of each primer and 2 µl gDNA in a PTC-200 thermocycler (Bio-Rad, Hercules, CA).

PCR Amplifications involved initial denaturation step at 95°C for 5 min, programmed for 35 cycles at 95°C for 1 min; 53°C for 1 min; 72°C for 2 min and a final phase at 72°C for 10 min. Eight µl of PCR amplicon was digested with *RsaI* and *TaqI* enzymes separately in a final volume of 15 µl, incubated for 2 h at 37°C and 65°C, respectively. The products were separated on a 2% agarose gel with ethidium bromide, run in 1XTBE buffer. The DNA fragments were viewed under ultraviolet light and the image was captured using the *DNR* Bio-imaging systems.

BCMV-48289723-CAPS marker specific to *I* gene (Bello et al. 2014) and ENM CAPS marker specific to *bc-3* gene (Naderpour et al. 2010) were used for selection of the resistant plants among segregating F2 and BC populations. In each generation of MABC, resistant plant materials carrying a combination of *I* and *bc-3* were identified and selected to develop the next backcross generation.

2.5. Segregation analysis

Goodness-of-fit test was performed at the significance level of 0.05 to check for deviation from the expected ratio 3R:1S (R: resistance allele, S: susceptible allele) for *I* gene and 1R:3S for *bc-3* gene in the F2 population and 1R:1S: 1R:1S for the two genes in BC populations with co-dominant markers. The *p*-values were calculated from chi-square scores (Tables 3).

3. Results and Discussion

Using Markers representing the *I* and *bc-3* genes, Successful gene combination was established in 33% of 17/52 BC1F1, 33% of 19/51 BC2F1, 43% of 19/44 of BC3F1, and 16% of 12/76 BC3F2 progenies. The seed color was fully recovered on some of the BC3 progenies with combined resistance genes (Figure 1).

BCMV-48289723-CAPS primer amplified a single DNA fragment of the expected size (301 bp) in all bean materials analyzed. The individual plants with *I* gene were determined by using *TaqI* restriction digestion enzyme which recognizes the restriction site TCGA and cleaves the resistance allele generating two bands of 201 bp and 110 bp by size. Heterozygous individuals for *I* gene found to have both uncut and cut fragments; 301 bp, 201 bp and 110 bp band patterns. However, the susceptible allele remained uncut with one band of 311 bp fragment by size and this was present in all susceptible individuals (Figure 2).

Furthermore, using ENM-FWe/RVe CAPS primers on the *P. vulgaris* gDNA template, PCR amplified a single DNA fragment of 541 bp in all bean materials analyzed. *RsaI* restriction endonuclease enzyme cleaves the resistance allele. Digestion of PCR products by *RsaI* enzyme resulted in cleavage into 381 bp and 160 bp fragments in all bean materials carrying the *bc-3* gene, and one fragment, 541 pb was observed in susceptible individuals. Heterozygous individuals for *bc-3* gene found to have both uncut and cut fragments, 541 bp, 381 bp and 160 bp band patterns (Figure 2).

Common bean genotypes carrying *bc-3* gene has been found to carry homozygous mutations at codons 53, 65, 76, and 111 in a PveIF4E[2] coding sequence. The mutated forms of translation initiation factor 4E (eIF4E) and/or its isoform eIF(iso)4E disrupt the interaction between cap-binding proteins and potyvirus genome-linked protein (Pvg), consequently plants loose susceptibility (Kang et al. 2005; Beauchemin et al. 2007).

In the *Arabidopsis thaliana*, two proteins, namely AteIF4E-1 and AteIF (iso)4E are associated with potyviral infection and their homologues are linked to potyviral resistance in other plant species (Robaglia and Caranta 2006; Hwang et al. 2009). The *bc-3* gene confers resistance to all known strains of BCMNV and BCMV in the presence of the dominant *I* gene (Kelly et al. 2003).

In addition to MAS, backcross progenies with a combination of *I* and *bc-3* genes were selected based on seed color.

Table 2. Molecular marker sequences used for selection of *I* and *bc-3* genes.

Gene	Marker type	Enzyme	Primer sequence (5'---3')	LG	Fragment (bp)	Reference
I	CAPS	*TaqI*	F: AGGAGGAAGAACGGTGGTC	2	311(201/110)	Bello et al. (2014)
			R: TTTGGTGGTAATTTGAAAATGG			
bc-3	CAPS	*RsaI*	F: ACCGATGAGCAAACCCTA	6	541(381/160)	Naderpour et al. (2010)
			R: CAACCAACTGGTATTCGGATT			

L G: linkage group; F: Forward primer; R: Reverse primer, bp: base pair. CAPS: Cleaved Amplified Polymorphic Sequence.

Table 3. Chi square analysis of F2 and BC segregation populations for *I* and *bc-3* genes.

Generations	Expected	Observed	$\chi 2$	*p-value*
F2 (*I*)	23:46:23	25:48:19	0.62	0.73
F2 (*bc-3*)	23:46:23	28:45:19	0.41	0.82
BC1F1	26:26:26:26	18:34:24:29	0.13	0.98
BC2F1	25.5:25.5:25.5:25.5	22:29:24:27	0.77	0.86
BC3F1	22:22:22:22	20:24:22:22	0.95	0.81

All *p*-values are greater than 0.05. Combined ratios for *Ibc-3* genes observed column *I-/-bc3*.

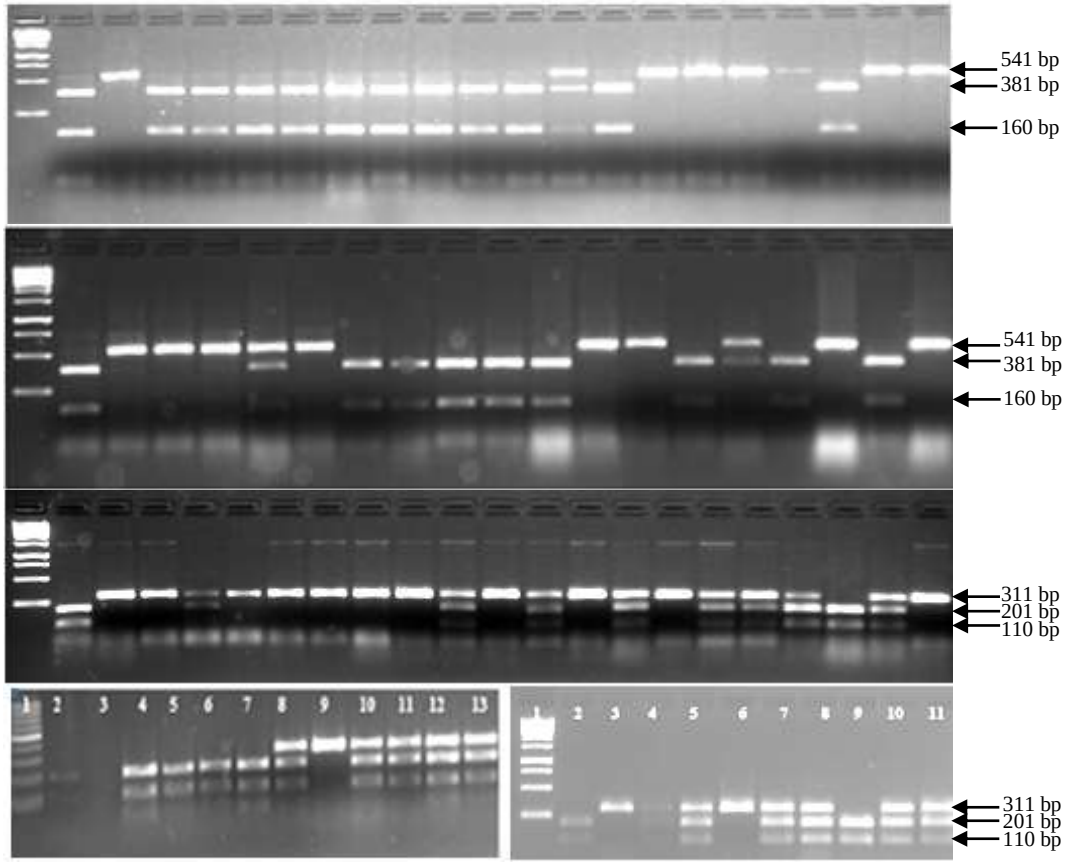

Figure 2. A representative figure showing segregation of resistance genes in F2 and backcross populations by CAPS markers. Lane 1, DNA ladder; Lanes 2 and 3: donor and recurrent parents in all plates. Lanes from 4 through the right side represent the F2 and BC lines. Both 381 bp and 160 bp: resistance bands (*bc-3*/*bc-3*); 541 bp: susceptibility band (*Bc-3*/-). Both 201 bp and 110 bp: resistance bands (*I* gene); 311 bp: susceptibility band (i/i).

The results of marker-assisted backcross (MABC), divided bean populations into the following categories; parents (*ii/Bc-3Bc-3* or *II /bc3bc3*), individuals with only *I* gene *(I-/Bc3-)*, individuals with only *bc-3* gene (ii/*bc-3bc-3*) and individuals with both *I* and *bc-3 (I-/bc3bc3*) in combination (Table 3). The $\chi 2$ values for F2 populations fit expected Mendelian ratio (3R:1S) for *I* gene and 1R:3S for *bc-3* gene. Also ratios of BC populations fit 1R:1S:1R:1S ratio (Tables 3).

Segregation ratios in the four F2 populations did not deviate from 3:1 ratio for either *I* or *bc-3* genes (Table 3). Along with F2 population findings, BC populations were evaluated and their observed ratios fit the expected segregation ratio of 1R:1S:1R:1S for both dominant and recessive genes. All *p*-values were greater than 0.05 (p>0.05) indicating monogenic inheritance of genes.

These results support previous findings reported by Kelly et al. (1995) and Mukeshimana et al. (2005) that *bc-3* inherited as a monogenic resistance gene. Our findings on segregation analysis of *I* gene are in agreement with previous studies on inheritance of resistance to potyviruses in *Phaseolus vulgaris L.* (Fisher 1994).

CAPS markers were present in parents, F2 and BC individuals possessing *I* and *bc-3* genes and absent in those lacking the resistance alleles. The findings of this study are in agreement to the results reported by Bello et al. (2014) who developed and validated the co-dominant marker, BCMV-48289723-CAPS from one of the SNP markers tightly linked to *I* gene in a RIL population, Melotto et al. (1996) who identified SCAR marker, $Sw13_{690}$ linked to the *I* gene in common beans and SW13 amplified a single 690 bp fragment linked to the *I* gene that more consistently permitted the identification of resistant common beans.

Further, ENM CAPS marker results of this study provided the opportunity to reconfirm previous results of Naderpour et al. (2010) who developed gene specific to ENM-FWe/RVe CAPS marker for *bc-3* gene. There are several studies reported markers linked to recessive gene (*bc-3)* in common beans, Mukeshimana et al. (2005) reported SEACAMCGG-134/137 STS and OG6575 RAPD markers linked to *bc-3*. Robaglia and Caranta (2006) reported resistance against potyviruses in three other species: *C. annuum*, *L. sativa* and *P. sativu*.

Importantly these markers now offer the unique opportunity to develop cultivars with the *I* and *bc-3* genes combination which is recognized to confer resistance to all known strains of BCMV and BCMNV in common beans. The advanced potyvirus resistant lines are expected to protect small farmers against yield losses.

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