Australian Journal of Crop Science AJCS
AJCS 7(3):354-360 (2013) ISSN:1835-2707

# Identification of molecular markers associated with the deleted region in common bean (*Phaseolus vulgaris*) *ur-3* mutants

**Kalpalatha Melmaiee[1], Antonette Todd[1], Phillip McClean[2], Rian Lee[2], Jessica Schlueter[3,†], Scott Jackson[4], and Venu Kalavacharla[1, 5]***

**[1]Department of Agriculture & Natural Resources, Delaware State University, Dover, DE 19901, USA**
**[2]Department of Plant Sciences, North Dakota State University, Fargo, ND 58105, USA**
**[3]Department of Agronomy, Purdue University, West Lafayette, IN 47907, USA**
**[4]Center for Applied Technologies, University of Georgia, Athens, GA 30620, USA**
**[5]Center for Integrated Biological and Environmental Research (CIBER), Delaware State University, Dover, DE 19901, USA**

**[†]Present address: Department of Bioinformatics and Genomics, University of North Carolina at Charlotte, Charlotte, NC 28223, USA**

***Corresponding autor: vkalavacharla@desu.edu**

**Abstract**

Common bean *(Phaseolus vulgaris* L.*)* is one of the most widely cultivated legumes grown for its dietary fiber and protein. Bean rust caused by the fungal pathogen *Uromyces appendiculatus* is a major constraint for bean production. *Ur-3*, one of several race specific loci in the bean genome contributing to rust resistance, provides resistance to 44 of the 89 rust races curated in the United States. Thus, development of markers linked to this locus is very important. The bean cultivar "Sierra" exhibits hypersensitive resistance response to race 53. The susceptible mutants ur3-Δ2 and ur3-Δ3 were derived by fast neutron bombardment of Sierra, and have deletions in the *Ur-3* region of the genome. This research is aimed at finding molecular markers associated with the deleted region in the mutants as a tool to better understand *Ur-3*-mediated rust resistance. Until now, only two RAPD-derived sequence characterized amplification region markers SK14 and SAE 19 are available for the *Ur-3* locus. We discovered that these markers are present (as PCR amplicons) in Sierra but are absent in the ur3-Δ2 and ur3-Δ3 mutants. We attempted to develop new molecular markers for characterization and tracking of this genomic region. Hybridization of a SK14-derived overgo probe showed erratic results by identifying BACs in and outside of the *Ur-3* region. Therefore, we developed a new SCAR marker named DESU-G1 utilizing the amplified fragment length polymorphism (AFLP) methodology for this deleted region in the ur-3 rust susceptible mutants. This is the first attempt to develop AFLP markers associated with the *Ur-3* locus in common bean.

**Keywords:** Rust; AFLP; *Uromyces appendiculatus;* SCAR marker; markers.
**Abbreviations:** AFLP- amplified fragment length polymorphism, BAC- bacterial artificial chromosomes, *Crg- C*omplements *r*esistant *g*ene, EST- expressed sequence tags, PCR- polymerase chain reaction, RGA- resistance gene analogs, SSR- simple sequence repeats, SCAR- Sequence-characterized amplified region.

## Introduction

Common bean *(Phaseolus vulgaris* L.*)* is one of the most widely cultivated crops contributing to 75% of traded food legumes in the world (Broughton et al., 2003). According to the United States Department of Agriculture statistics, the estimated value of common bean was $1.5 billion for the year 2010 in the United States alone. Common bean is mainly grown for human consumption, and serves as a primary source of protein to the vegetarian diet and to low-income families, and also as a staple food in South America and Africa. It is a good source of nutritionally rich complex carbohydrates, fiber, phyto-chemicals, flavonoids, and minerals such as iron, phosphate, magnesium, and manganese (Broughton et al., 2003). Common bean is a diploid species with a chromosome number of 2n =2x =22 and an estimated genome size of 650 million base pairs (Arumuganthan and Earle, 1991). The estimated linkage map in common bean ranges from 1259 centimorgan (cM) to 1545.5 cM with an average distance between any two markers ranging from 3.0 cM to 7.23 cM (Grisi et al., 2007; Hanai et al., 2010). Molecular marker development in common bean began in the early 1990s. Most of these early molecular markers are derived from Restriction Fragment Length Polymorphisms (RFLPs) (Adam-Blondon et al., 1994; Vallejos et al., 1992) and Randomly Amplified Polymorphism DNAs (RAPDs) (Freyre et al., 1998). Since this early development, other classes of molecular markers have also been developed in common bean which include amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSRs), resistance gene analogs (RGAs), and expressed sequence tags (ESTs) (Hanai et al., 2010; McConnell et al., 2010). Currently, only two RAPD-derived SCAR markers SK14 and SAE 19 are available for exploring the area around the *Ur-3* locus (http://www.css.msu.edu/bic/PDF/SCAR_Markers_2009.pdf) When the PCR amplified SK14 product was sequenced and compared to the NCBI-GSS database

(http://blast.ncbi.nlm.nih.gov), common bean BAC end sequences of PV_GBa 0085k23.r and PV_GBa 0100p20.r partially aligned with SK14. Further analyses of these BACs and overgo hybridization of BAC library PV_GBa with the SK14 probe provided unreliable results. Along with our research, previous researchers have also found SK14 to be variable in its use at the molecular level (Madubanya et al., 2009). Since our goal was to identify markers that could clearly track the *Ur-3* genomic region, we explored the use of other markers linked to this region. The marker SAE19 derived by the RAPD method has been reported to be linked in coupling phase with the *Ur-3* locus (Liebenberg et al., 2009; Johnson et al., 1995; Queiroz et al., 2004). However, SAE19 also produced similar erratic results as SK14 when analyzed with BAC library PV_GBa (data not presented), leaving a need for development of more reliable molecular markers to explore the *Ur-3* region. AFLP markers are often more reliable than RAPD markers because of RAPD's randomness and low reproducibility (Shasany et al., 2005). They also stated that AFLP markers exhibit more polymorphisms with fewer primer combinations than RAPD markers. Another advantage of the AFLP methodology is its high multiplexing capabilities and the possibility of generating higher marker densities. The AFLP methodology has been used in fingerprinting of genomic DNA in several crops, *e.g.* identification/analysis of resistant genes for root-knot nematodes and potato aphids in tomato (Vos et al., 1998), identification of markers for the Stripe rust resistance gene YrcC591 in wheat (Li et al., 2008), and development of markers for sterility mosaic virus resistance in pigeonpea (Ganapathy et al., 2009); additionally, McNeil et al (2011) converted AFLP markers to SCAR markers in sugarcane. AFLPs have been specifically used in common bean to develop molecular markers associated with resistance to bean pathogens such as bacterial blight (Naidoo et al., 2003), Golden Mosaic Virus, web blight, ashy stem blight (Kelly and Miklas 1998; Kelly et al., 2003), white mold (Kolkman and Kelly, 2003), and for the rust resistance locus *Ur-13* (Mienie et al., 2005). One of the major constraints of bean production is the fungal disease rust caused by the pathogen *Uromyces appendiculatus* affecting yield up to 75% under severe cases. Rust resistance in common bean is controlled by many race specific host-pathogen interactions following the gene for gene model. Race-specific resistance genes are supposed to be derived from Meso-American (*Ur-3, Ur-5, Ur-7, Ur-11)* and Andean *(Ur-4, Ur-6, Ur-9, Ur-12*) gene pools (http://www.css.msu.edu/bic/pdf/rust.pdf). The *Ur-3* locus controls hypersensitive resistance to 44 of the 89 races curated in the United States. The bean cultivar Sierra carrying the dominant *Ur-3* locus (genotype = *Ur-3 ur-4 ur-6 ur-11*) shows an incompatible reaction by exhibiting hypersensitive resistance response when infected with *U. appendiculatus* race 53 (Kalavacharla et al., 2000). In order to better understand the resistance mediated by the *Ur-3* locus, a forward genetics approach was utilized by developing disease susceptible mutants through fast neutron bombardment (Kalavacharla et al., 2000) of seed from the resistance genotype Sierra. Approximately 24,000 $M_2$ mutant plants were screened with race 53 and three plants crg, ur3-Δ2 and ur3-Δ3 that have a susceptible reaction to the pathogen were discovered (Kalavacharla et al., 2000). Further segregation analysis of $F_2$ plants derived from crosses between Sierra and the mutants revealed that the deletion in the genome of the susceptible mutant, crg, occurred in a separate gene called *Crg* (Kalavacharla et al., 2000) that is required for *Ur-3*-mediated rust resistance. The same study also showed that the ur3-Δ2 and ur3-Δ3 mutants *(ur-3 ur-4 ur-6 ur-11)* carry mutations at the *Ur-3* locus. Therefore, the main focus of this study was to develop molecular markers to identify the deleted regions in ur3-Δ2 and ur3-Δ3.

**Results**

***SK14 and SAE19 marker utilization***

The marker SK14 produced an amplification product of ~600 bp from Sierra and crg; however, no product was seen from the susceptible cultivar "Olathe" and the susceptible mutants ur3-Δ2 and ur3-Δ3 (figure 1a). The marker SAE19 produced an amplification product of ~800 bp with a deletion pattern similar to SK14 (figure 1b). Sequences of SK14 and SAE19 derived from Sierra are presented in figure 2. The SAE19 sequence derived from Sierra shows 99 percent sequence similarity with previously reported sequence (http://phaseolusgenes.bioinformatics.ucdavis.edu) (Johnson et al., 1995; Alzate-Marin et al., 2004; Queiroz et al., 2004).

***Overgo hybridization of SK14 to BAC library***

From the overgo hybridization, 63 BACs produced weak to normal signal intensities with the SK14 probe. Among these, 42 BACs were assigned to nine different FPC contigs and 21 BACs were not contained in any contig in WebFPC (supplementary material table 1). For further confirmation of hybridization, DNA from BACs with a positive hybridization pattern, and randomly chosen negative BACs were subjected to PCR amplification with the SK14 primers. PCR amplification was seen in both negative and randomly selected BACs (supplementary material figure 1) thus demonstrating that the SK14 marker was unreliable.

***AFLP gel analysis***

Selective amplification was performed with several combinations of *Eco*RI and *Mse*I specific primers to identify polymorphisms between Sierra and the mutants. The primer combination E+AAC/M+CAC produced clear polymorphic fragments (figure 3) in repeated experiments with a size range of 100 bp to 750 bp.

***Sequence analysis***

Each polymorphic fragment was cloned. Twelve colonies were isolated per fragment to capture sequence variation within the fragment. Clones of the fragments EAACMCAC-1, EAACMCAC-2, EAACMCAC-4 and EAACMCAC-5 were sequenced. Majority of the AFLP fragment sequences aligned into a sequence contig. The sequence of EAACMCAC-1 matched with the *P. vulgaris* genomic survey sequence Pv_GBa $125C_{21}$ 3' with a score of 279 bits and probability value of $3e^{-72}$ with 99% identity. However we were unable to find any other matches to bean transcriptomic sequences from NCBI or the 454 sequences derived from three Sierra transcriptomes (Kalavacharla et al., 2011).

***SCAR marker development***

The AFLP technique is very useful for developing molecular markers from genotypes with wide or narrow genetic differences. However, the application of these markers for marker-assisted selection is tedious and not economically feasible. For easy and practical use of these polymorphic fragments in common bean marker-assisted breeding programs, we wanted to develop SCAR markers. PCR

**Table 1.**Primer and linker sequences utilized in the study.

| Oligo sequences | References |
|---|---|
| SK14 Forward : 5'-CCC GCT ACA CAC CAA TAC CTG-3'<br>SK14 Reverse: 5'-CCC GCT ACA CTT GAT AAA ATG TTA G-3' | Haley et al. 1994; Miklas et al. 2002 |
| SAE19 Forward : 5'-CAG TCC CTG ACA ACA TAA CAC C -3'<br>SAE 19 Reverse : 5'-CAG TCC CTA AAG TAG TTT GTC CCT A-3' | Johnson et al., 1995; Miklas et al., 2002; Queiroz et al., 2004 |
| *Eco*RI linker1: 5'-CTC GTA GAC TGC GTA CC-3'<br>*Eco*RI linker2: 5'-AAT TGG TAC GCA GTC TAC-3' | Vos et al.,1995 |
| *Mse*I linker1: 5'-gac gat gag tcc tga g-3'<br>*Mse*I linker2: 5'-TAC TCA GGA CTC AT-3' | Vos et al.,1995 |
| EAACMCAC-1 Forward: 5'-CATGAAATTGTACGCCCAAA-3'<br>EAACMCAC-1 Reverse: 5'-CTCGACGGATGCAAACACTT-3' | Current study |

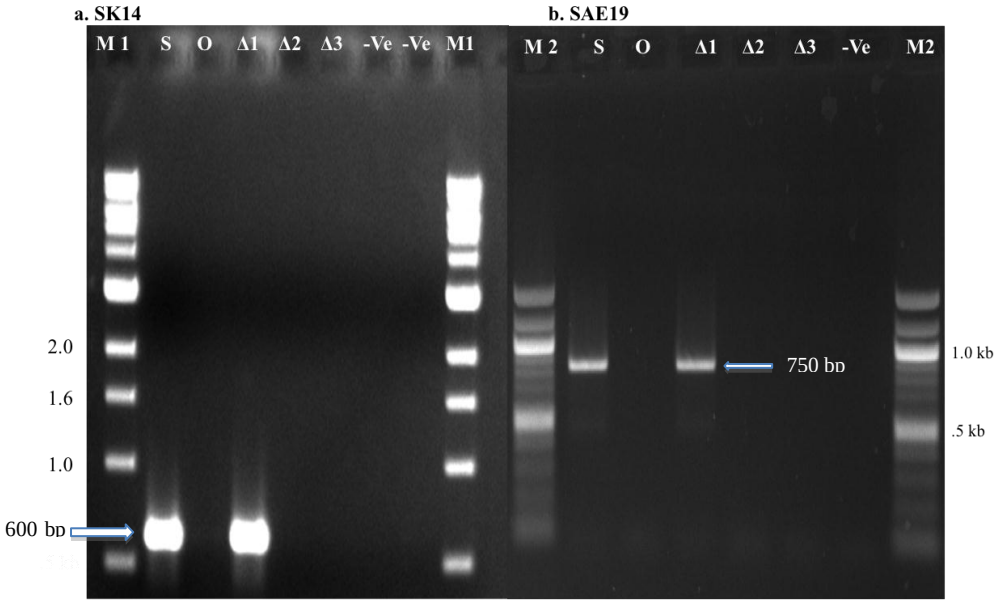


**Fig 1.** SK14 and SAE19 amplification patterns in rust resistant, susceptible and mutant genotypes for race 53. Lanes are as follows S: Sierra, O: Olathe, Δ1: crg, Δ2: ur3-Δ2, Δ3: ur3-Δ3, -ve. Negative PCR control, in which water was used instead of DNA. M1: 1kb DNA ladder, M2: 100bp DNA ladder a. Amplification pattern with SK14 marker. Approximately 600 bp amplicon can be seen in Sierra and crg lanes. No amplification was observed in Olathe and mutants ur3-Δ2 and ur3-Δ3 lanes when genomic DNA was used for PCR amplification. b. Amplification pattern with SAE19 marker. Amplification pattern is similar to SK14 marker and a 750 bp amplicon can be seen in Sierra and crg, no amplification in mutants ur3-Δ2 and ur3-Δ3 and in susceptible genotype Olathe

primers were designed for each fragment by selecting the representative sequence, and PCR products were amplified from genomic DNA of Sierra, Olathe and susceptible mutants. The primers designed from the EAACMCAC-1 fragment produced a 750 bp amplification for Sierra, Olathe, and crg similar to the AFLP results and the amplification product was not obtained for the susceptible mutants ur3-Δ2 and ur3-Δ3 (figure 4). This newly developed SCAR marker is named as DESU-G1 and the sequence of AFLP fragment is presented in figure 5.

## Discussion

### *Utilization of existing markers*

Previous studies reported that the SCAR marker SK14 was linked to the rust resistant locus *Ur-3* in common bean (Haley et al., 1994; Miklas et al., 2002) and our results show that this sequence is deleted in the two *ur-3* deletion mutants. Hybridization of the SK14 overgo probe to the BAC library shows that 64 BACs hybridized with weak to normal signal intensities. Based on WebFPC analysis, 14 of these BACs (with normal hybridization signals) were contained in contig 570. For further confirmation, we performed PCR screening with SK14 redesigned primers using DNA from these BACs along with DNA from randomly chosen BACs as negative controls. Surprisingly, both the positive and negative control BACs showed corresponding PCR products on the gel thus showing indiscriminate results. This may be due to the fact that SK14 is derived from a repetitive sequence region of the genome. The marker SAE19 also produced similar results as SK14 with our preliminary analyses of hybridization of BAC filters and further PCR screening. However, the sequences of SK14 and SAE19 do not have significant sequence similarity.

### *AFLP marker development and analysis*

AFLPs combine the advantages of RAPDs and RFLP finger printing (Belaj et al., 2003; McGregor et al., 2000). One of the major advantages of AFLPs is the ability to produce a greater number of fragments per amplification. Because polymorphisms can arise from deletions, insertions, and rearrangements at the restriction site, many of the AFLP-derived markers are dominant (Brugmans et al., 2003; Meudt

>SK14
CCCGCTACACACCAATACCTGCAGGGCTGCTACTCCAAGGCACCAAACTGAAAATAAAAAAAAGACAACCAACTGCTTG
GCTTGTTCTCCACCACTTAATCTGTGTCTCCAGCAGCACCAATTACCTACGCCAAGAGGACCGTGCCTGCAAGGGAGTA
TAGAATAGCCTAGATAACCAATATATTGCCGATTATCTTTTTCTTCACCATATATAACCAATATATTGCTTACTGTTAA
AGTTTTTCTGTCTTAATTCCAAATCAACTTGATAATTTGGTAAAGTTATTACATTTAAATTTGAGTCAAAATAAATTTA
AAATATTTTCGAATTCTTGATCTGAGCATTAACATTTTGAGTGGAAAAATACCATCATGAGAGGGTAACAAGGACCTGA
AATCAATTCCTTCAATTTGGTTCATGGAAAACATTATTACTAAAAAAAATATATGTAAATGTTAGAACAATAATCTTGG
ATGTATTAAGTACACTGTAATTTCTTATTGTTTTGACATATAGACACTACTACATATATAATAATTAGTAACATCAAAA
ATGTTACTAAATCAAAGAAAATGTTACTAATAAGATTTACTAACATTTTATCAAGTGTAGCGG

>SAE19
CAGTCCCTAAAGTAGTTTGTCCCCTAGGATTTGAATGACATTCGACCCATATTTCTAATTGGCTGTTTGTACAAAATTTC
CAATTTTTTTAGTAAATAGGCTAGAAAAGGTCATCCCAAAGGTTATTGACTAATCTCAATCAGCTTGCTTAGAAGGAAAC
CCTTCTTGATAGTGTTTTGATGACAAATGAGACACTCGATGAGGTCAAAAGTAAGGGTAAGAAGTGCATTATTGTAAGGC
TGATTTTGAAAAAGTTTATGACATGGTGAGATGGGATTATTTGGAACATATGATGTCTAGACTTGGTTTTTGTAGTACTT
GATTAGGTGGATGATGAGTTGTCTTAAATCTGCATCTATCTCGCTTTTAGTCAATGGTAGTCCAACTAAGGAGTTTAAGC
ACAAAAAGGTCTTAGACAAGGAGACCCTAACACCTTTTCAGTTTCTTGTTGGGGTAGTTAGGCAAGCATTGGAAACTGTT
TCATTAAAGGTATCAGAGTTGGACATAATGAGATTCAAATGTTTTCACAGTTTGCATATGACACTTTCTTCATACGTGAA
CGGGTATGGGTGTGTTGTAGCAATAAAAAACAATTTAAGATGTTTCCAATTTAAGATGTTGACCAATCTTTGGAAAATAT
AATGGCTTGTTTATGTTTTCAAACACGCAAGTGAAACAGATTTGAGTTTTTCAATTGTGAAGAAGTTGATTCTAAAGATT
GAACATTCACATCAAGCTTTGGGTTTTTCAAAGAGAGTGGAATAGGATTGCAATTGTATTGATTTCAGTTTGTACTGTGA
CAAGTGTAATCCTTTCTCAATCTACTGTATTTCTGGTGTTATGTTGTCAGGGACTGAA

**Fig 2.** Nucleotide Sequences of the RAPD derived SCAR marker SK14 and SAE19 linked to the *Ur-3* locus amplified and sequenced in the bean cultivar Sierra.

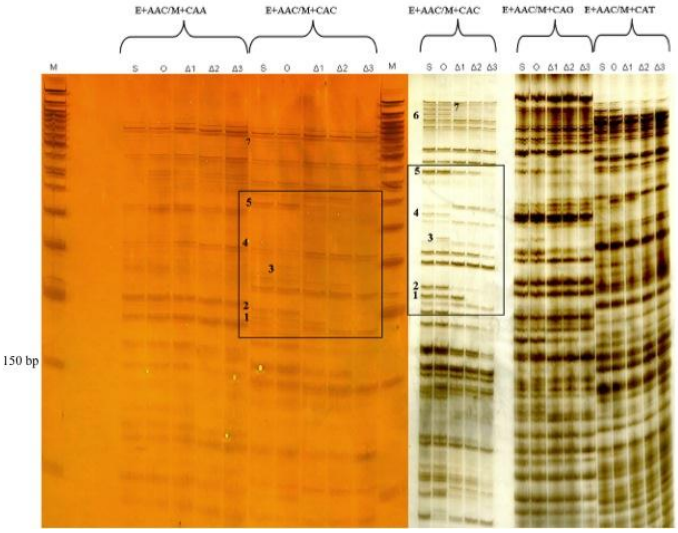


**Fig 3.** Scanned AFLP gels after silver nitrate staining. Experiment 1: EAAC/MCAA and EAAC/MCAC primer combination shown. Experiment 2: EAAC/MCAC, EAAC/MCAG and EAAC/MCAT primer combination shown. The fragments that are similar in both experiments are given the same number. Lanes S: Sierra, O: Olathe, Δ1: crg, Δ2: ur3-Δ2, Δ3: ur3-Δ3 and M: 50 bp DNA marker. Highlighted boxes shows similar fragment migration pattern from both the experiments. The numbers represent the polymorphic fragments as follows 1. EAACMCAC-1 2. EAACMCAC-2 3. EAACMCAC-3 4. EAACMCAC-4 5. EAACMCAC-5 6. EAACMCAC-6 7. EAACMCAC-7.

and Clarke, 2007). Another advantage of AFLP methodology for marker development is that no prior sequence knowledge is necessary. On the other hand, one major limitation from this method is that fingerprints may share common fragments (Archak et al., 2003; Shirasawa and Kishitani, 2004). Although converting AFLP markers to simple PCR- based markers makes it easier for marker-assisted breeding programs, it is a challenging task for several reasons. Among these, one reason is that some of the AFLP fragments are very small in size. From these short sequences, it is difficult to design optimal PCR primers that can produce polymorphic amplicons from large genomic DNA. Another reason is co-segregation of fragments of similar size in the gel, which makes separation and isolation of single fragments from this complex difficult. One way of minimizing this problem is by increasing the length of primers used in AFLP amplification so that they can produce more stringent and reliable fragments.

***The SCAR marker DESU-G1 and its application in identifying deleted region in the ur-3 mutants.***

Understanding the nature of the deleted region in the ur-3 mutants is critical as we may be able to derive markers that are closely linked to the *Ur-3* locus (Kalavacharla et al., 2000). In our study, the SCAR marker DESU-G1 shows dominant polymorphism between the resistant genotype Sierra and the susceptible mutants ur3-Δ2 and ur3-Δ3. A similar sized PCR product can be seen from the susceptible genotypes Olathe and crg. The DESU-G1 product in crg is seen as this susceptible mutant has a deletion at the *Crg* locus but not in the *Ur-3* region. The mutants ur3-Δ2 and ur3-Δ3 have a deletion at the *Ur-3* locus (Kalavacharla et al., 2000), which maps to linkage group 11 and are therefore missing this marker. There is a disparity in sequence size correlation between the AFLP fragment EAACMCAC-1 and the SCAR marker DESU-G1. A possible reason may be that the primers are anchoring at two adjacent places in the deleted region of the bean genome and producing two different PCR products. The larger (~750bp) product was not seen in the AFLP-PAGE gel possibly because of low concentration of this product or poor resolution. However, a 200 bp fragment corresponding to EAACMCAC-1 can be seen on the agarose gel along with this larger DESU-G1 fragment when the annealing temperature was reduced to 48°C from 51°C during PCR. Since the mutants are derived from the genotype Sierra by fast neutron bombardment, it is difficult to derive more polymorphic bands with a technique that relies on restriction sites to produce polymorphic bands. There is a need for further characterization of the deletion region and its correlation to *Ur-3* resistance as a cluster of resistance genes are present in this region of chromosome 11 in common bean (Hanai et al., 2010; Liebenberg et al., 2009), and we are in the process of generating further mutants by using gamma irradiation and EMS. These mutants can serve as good genetic tools to study the resistant genes present in and around the *Ur-3* region.

**Materials and methods**

***Plant material***

The resistant genotype Sierra, susceptible genotype Olathe and three susceptible mutants (crg, ur3-Δ2 and ur3-Δ3) derived from Sierra were used in this study. Sierra and Olathe belong to the Meso-American gene pool and exhibit contrasting phenotypes with respect to the *Ur-3* locus. Sierra, which carries the *Ur-3* locus, is resistant to race 53, a race that is avirulent on plants containing the *Ur-3* locus, while Olathe exhibits a susceptible reaction to race 53. Reaction of the five genotypes used in this study to race 53 can be seen in figure 6. Plants were grown according to the methods of Kalavacharla et al (2000) in the greenhouse. Leaves from the above five genotypes were collected at trifoliate stage and flash frozen in liquid nitrogen, and either used immediately for DNA extraction or stored at -80°C for later use.

***DNA extraction***

Genomic DNA was extracted using the CTAB method as described by Kalavacharla et al (2000) and Todd (2008). DNA was further purified by RNAse treatment (RNAse A

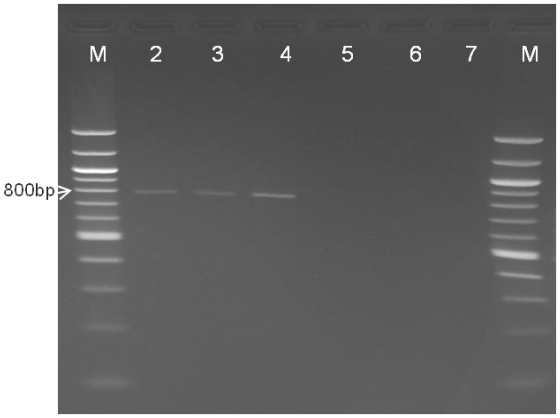


**Fig 4.** The SCAR marker DESU-G1 on 2% agarose gel. M. 100bp DNA size marker 2. Sierra 3. Olathe 4. crg 5. ur3-Δ2 6. ur3-Δ3 and 7. Negative PCR control in which water was used instead of DNA. Amplification pattern is in consistent with the AFLP fragment EAACMCAC-1.

```
> EAACMCAC-1
TGATGAGTCCTGAGTAACACATGAAATTGTACGCCCAAACTATGCGGGGCACCTCCTCAGCCCAACT
TCCCTTGGCTTTCTCAAGCCTTCCCTTCAAACCTCTCAACAACACCCGATTAGCTGATTCCACCTGG
CCATTTGTTTGAGGGTGCTCGACGGATGCAAACACTTGTTGAATTGGTACGCAGTCA
```

**Fig 5**. The AFLP sequence of EAACMCAC-1 fragment (191bp). Underlined sequences are the sequences of primers designed for the development of SCAR marker DESU-G1.

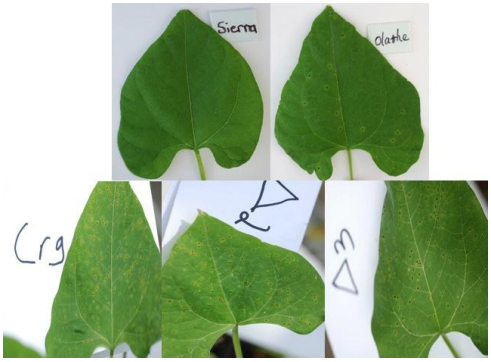


**Fig 6.** Plant material used in this experiment. Common bean (*Phaseolus vulgaris L.)* genotypes inoculated with spores of the rust fungus *Uromyces appendiculatus.* The picture is a close-up view of the leaves of common bean plants 10 days after inoculation. The genotype Sierra shows resistance, while the genotypes Olathe, and susceptible mutants crg, ur3-Δ2 and ur3-Δ3 produced grade 4, 5, 6 uridenia with race 53 as observed by Kalavacharla et al (2000).

10mg/mL; Sigma-Aldrich, St. Louis, MO) followed by phenol chloroform extraction (Sambrook et al., 1989). RNAse treatment was carried out at 37°C for 30 min. After isopropanol precipitation, the DNA pellet was washed with 75% ethanol, dried overnight, and re-suspended in autoclaved water. DNA integrity and quality was checked on 1% agarose gel, and concentration was measured with the NanoDrop 2000 (Thermo Scientific, Wilmington, DE).

***Utilization of RAPD derived SCAR markers***

The SCAR markers SK14 and SAE19 were utilized to amplify genomic DNA of the five genotypes mentioned earlier by PCR. PCR conditions were as follows; initial denaturation at 94°C for 2 minutes (min) followed by 35 cycles of denaturing at 94°C for 30 seconds (sec), annealing at 60°C for 1 min and extension at 72°C for 2 min followed by a final extension step of 5 min. PCR products were analyzed by electrophoresis on a 1% agarose gel (figure 1). The primer sequences are listed in table 1.

***Overgo hybridization of BACs with SK14 probe***

The SK14 probe "CTCCACCACTTAATCTGTGTCTCCAGCAGCACCAAT " was hybridized with filters from the PV_GBa BAC library at Purdue University. The PV_GBa library was developed from the genotype G19833 (Schlueter et al., 2008) that consists of 55, 296 clones and was obtained from the Clemson University Genomics Institute (CUGI; https://www.genome.clemson.edu), Clemson, SC. Southern hybridizations were carried out as per the methods described in Hass-Jacobus et al (2006).

***Screening Sierra and mutant plants with primers from BAC end sequences***

Overgo hybridization of the BAC library with the SK14 probe yielded 63 BACs with signals ranging from weak to normal intensities (data not shown). These BAC clones were assigned to corresponding contigs with the help of WebFPC (http://phaseolus.genomics.purdue.edu/WebAGCoL/Phaseolus/WebFPC/). Contig 570, which has the highest number of hybridized BACs was chosen for further study. DNA was extracted from the clones that produced normal signal intensities during hybridization using the large construct kit protocol (Qiagen, Valencia, CA cat# 12462) and amplified with redesigned SK14 primers. SK14 primers were redesigned as follows:
Original SK14 primers were derived from the resistant *Ur-3* genotype, and they do not produce PCR product from G19833. In order to screen the BAC library with the SK14 marker, we redesigned primers from the sequence of the PCR product amplified with the original primers, so that the new primers can anchor 50 to 60 bp inside the sequence and to amplify different regions of the 630 bp sequence. This subsequently amplified products ranging from 100 to 400 bp in G19833, Sierra and crg. No amplification was seen in mutants ur3-Δ2 and ur3-Δ3 and in the susceptible genotype Olathe with these redesigned primers.

***AFLP procedure***

AFLP fragments were generated following the protocol of Vos et al (1995) with some modifications as described in Hazen et al (2002). Genomic DNA (300 ng) from all the genotypes was double digested with *Eco*RI and *Mse*I restriction enzymes and ligated with corresponding linkers. Linker sequences are shown in table 1. Pre-amplification was performed with 2 µL of the above digested and ligated product under the following conditions: 1x PCR buffer (10 mM tris-HCl, pH 7.2, 50mM KCl and 0.1% TritonX-100), 0.5mM dNTPs, 0.5 U Taq polymerase, 1.5 mM $MgCl_2$ to a total volume of 20 µL. PCR conditions were as follows: an initial denaturing temperature of 94°C for 2 min followed by 26 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min with a final extension step of 72°C for 5 min. The pre-amplified product was diluted six times by adding 100 µL of sterile water. Final selective amplification was performed utilizing 2 µL of the above diluted, pre-amplified product

under the following PCR conditions; 94°C for 2 min, 12 cycles with the annealing temperature decreased by 0.7°C for every step (94°C for 2 min, 65°C for 30 sec, 72°C for 1 min) and 23 cycles (94°C for 30 sec, 56°C for 30 sec 72°C 1min) followed by an extension step of 1 min at 72°C in the final cycle.

***Polyacrylamide gel electrophoresis (PAGE)***

Six percent polyacrylamide gels were prepared on 33×42 cm plates (CBS Scientific, San Diego, CA. cat # DASG-600-33). PCR products containing 10 µL of selective amplified product with an equal volume of formamide loading dye (98% formamide, 10mM EDTA pH 8.0, 1.0 mg/mL bromophenol blue and 1.0mg/mL xylene cyanol) were loaded per well. A 50 bp DNA size marker was loaded alongside wells and electrophoresed for 3 hours (h) at 60W. Fragments were visualized by silver staining according to the manufacturer's protocol (Promega, Madison,WI. Cat # Q4134). Gels were then air dried, and stored at room temperature for further analyses.

***Cloning and sequencing of polymorphic fragments***

A small portion of selected polymorphic fragments were excised from the gel and directly transferred to PCR mix, and allowed to rehydrate on ice for 15 min prior to PCR amplification. The PCR product was verified on a 1.5% agarose gel, corresponding fragments were gel extracted using Qiaquick gel extraction system (Qiagen, Valencia, CA. Cat# 28106) and subsequently transferred to Invitrogen's TOPO TA vector (Invitrogen, Carlsbad, CA. Cat#K457540). For each selected fragment, 12 clones were isolated and verified on the gel for confirmation of presence of a single insert and sequenced.

***Sequence characterization***

AFLP fragment sequences were analyzed using the software DNASTAR (DNASTAR Inc., Madison, WI). PCR primers were designed using Primer3 software (www.frodo.wi.mit.edu*)* and amplified using 70 ng of genomic DNA from the genotypes Sierra, Olathe, and mutants crg, ur3-Δ2, and ur3-Δ3 following the PCR conditions shown in selective AFLP amplification. A 10 µL of PCR product was visualized on a 2% agarose gel (figure 5).

**Conclusions**

There is a need for development of molecular markers linked to rust resistant loci in common bean. The mutants ur3-Δ2 and ur3-Δ3 show a susceptible response to bean rust race 53. We utilized these mutants to develop molecular markers to identify deleted regions in the bean genome. Based on our present analyses and previous studies (Kalavacharla et al., 2000), both of these mutants have deletions in or around the *ur-3* region. The RAPD-derived SCAR markers did not provide consistent results for physical mapping with BAC library. Since there are no additional markers available for *Ur-3*, this is the first attempt to develop AFLP markers for both the *Ur-3* locus as well as for characterizing the deleted region in the susceptible ur-3 mutants. The polymorphic AFLP fragment EAACMCAC-1 was the only fragment that was polymorphic between Sierra and the ur-3 mutants. We were able to successfully convert this fragment to a SCAR marker (~750 bp). Further this marker can be utilized for chromosomal landing and physical mapping to understand the deleted region surrounding the *Ur-3* locus in these mutants.

**Acknowledgements**

The authors wish to express their sincere thanks to Michelle Graham for advice, Sathya Elavarthi for critical review of the manuscript, and USDA-CSREES for grant no 2007-03-409 to VK for support of KM and USDA-CSREES grant 2006-03558 to SAJ.

**References**

Adam-Blondon AF, Sévignac M, Bannerot H, Dron M (1994) SCAR, RAPD and RFLP markers linked to a dominant gene (*Are*) conferring resistance to anthracnose in common bean. Theor Appl Genet 88: 865-870.

Alzate-Marin AL, TL de Souza PO, Ragagnin VA, Moreira MA, de Barros EG (2004) Allelism tests between the rust resistance gene present in common bean cultivar ouro negro and genes *Ur-5* and *Ur-11*. J Phytopathol 152: 60-94.

Archak S, Gaikwad AB, Gautam D, Rao EVVB, Swamy KRM, Karihaloo JL (2003) Comparative assessment of DNA fingerprinting techniques (RAPD, ISSR and AFLP) for genetic analysis of cashew (*Anacardium occidentale* L.) accessions of India. Genome 46: 362-369.

Arumuganathan K, Earle E (1991) Nuclear DNA content of some important plant species. Plant Mol Biol Rep 9: 208-218.

Belaj A, Satovic Z, Cipriani G, Baldoni L, Testolin R, Rallo L, Trujillo I (2003) Comparative study of the discriminating capacity of RAPD, AFLP and SSR markers and of their effectiveness in establishing genetic relationships in olive. Theor Appl Genet 107: 736-744.

Broughton WJ, Hernández G, Blair M, Beebe S, Gepts, Vanderleyden J (2003) Beans (*Phaseolus* spp.) – model food legumes. Plant Soil 252: 55-128.

Brugmans B, van der Hulst RG M, Visser RGF, Lindhout P, van Eck H J (2003) A new and versatile method for the successful conversion of AFLP™ markers into simple single locus markers. Nucleic Acids Res 31: e55.

Freyre R, Skroch PW, Geffroy V, Adam-Blondon AF, Shirmohamadali A, Johnson WC, Llaca V, Nodari RO, Pereira PA, Tsai SM, Tohme J, Dron M, Nienhuis J, Vallejos CE, Gepts P (1998) Towards an integrated linkage map of common bean. 4. Development of a core linkage map and alignment of RFLP maps. Theor Appl Genet 97: 847-856.

Ganapathy KN, Byre Gowda M, Venkatesha SC, Ramachandra R, Gnanesh BN, Girish G (2009) Identification of AFLP markers linked to sterility mosaic disease in pigeonpea *Cajanus cajan* (L.) Millsp. Int J Integ Biol 7: 145-149.

Grisi MC, Blair MW, Gepts P, Brondani C, Pereira PA, Brondani RP (2007) Genetic mapping of a new set of microsatellite markers in a reference common bean (*Phaseolus vulgaris*) population BAT93 x Jalo EEP558. Genet Mol Res 6: 691-706.

Haley SD, Afanador L, Kelly JD (1994) Selection for monogenic pest resistance traits with coupling-phase and repulsion-phase RAPD markers. Crop Sci 34: 1061- 066.

Hanai L, Santini L, Camargo L, Fungaro M, Gepts P, Tsai S, Vieira M (2010) Extension of the core map of common bean with EST-SSR, RGA, AFLP, and putative functional markers. Mol Breeding 25: 25-45.

Hass-Jacobus BL, Futrell-Griggs M, Abernathy B, Westerman R, Goicoechea JL, Stein J, Klein P, Hurwitz B, Zhou B, Rakhshan F, Sanyal A, Gill N, Lin JY, Walling JG, Luo MZ, Ammiraju JS, Kudrna D, Kim HR, Ware D, Wing RA, San Miguel P, Jakson SA (2006) Integration of hybridization-based markers (overgos) into physical maps for comparative and evolutionary explorations in the genus *Oryza* and in Sorghum. BMC Genomics 7: 199.

Hazen S, Leroy P, Ward R (2002) AFLP in *Triticum aestivum* L.: patterns of genetic diversity and genome distribution. Euphytica 125: 89-102.

Johnson E, Miklas PN, Stavely JR, Martinez-Cruzado JC (1995) Coupling- and repulsion-phase RAPDs for marker-assisted selection of PI 181996 rust resistance in common bean. Theor Appl Genet 90: 659-664.

Kalavacharla V, Stavely J, Myers J, McClean P (2000) *Crg*, a Gene required for *Ur-3*-mediated rust resistance in common bean, maps to a resistance gene analog cluster. Mol Plant Microbe Int 13: 1237-1242.

Kalavacharla V, Liu Z, Meyers BC, Thimmapuram J and Melmaiee K (2011) Identification and analysis of common bean (Phaseolus vulgaris L.) transcriptomes by massively parallel pyrosequencing. BMC Plant Biol, 11:135.

Kelly JD, Gepts P, Miklas PN, Coyne DP (2003) Tagging and mapping of genes and QTL and molecular marker-assisted selection for traits of economic importance in bean and cowpea. Field Crops Res 82: 135-154.

Kelly JD, Miklas P (1998) The role of RAPD markers in breeding for disease resistance in common bean. Molecular Breeding 4: 1-11.

Kolkman JM, Kelly JD (2003) QTL conferring resistance and avoidance to white mold in common bean. Crop Sci 43: 539-548.

Li Y, Niu YC, Chen XM (2008) Mapping a stripe rust resistance gene YrC591 in wheat variety C591 with SSR and AFLP markers. Theor Appl Genet 118: 339-346.

Liebenberg M, Lebogang M, Mienie C, Kelly JD (2009) A Closer Look at the Resistance Gene Cluster on Common Bean Chromosome 11. Annu Rep Bean Improv Coop 52: 90-91.

Madubanya L, Liebenberg M, Mienie C, Kelly JD (2009) Mapping rust resistance genes on the distal portion of bean chromosome 11. Annu Rep Bean Improv Coop 52:78-79.

McConnell M, Mamidi S, Lee R, Chikara S, Rossi M, Papa, R, McClean P (2010) Syntenic relationship among legumes revealed using a gene-based genetic linkage map of common bean (*Phaseolus vulgaris*). Theor Appl Genet 121: 1103-1116.

McGregor C E, Lambert CA, Greyling M M, Louw JH, Warnich L (2000) A comparative assessment of DNA fingerprinting techniques (RAPD, ISSR, AFLP and SSR) in tetraploid potato (*Solanum tuberosum* L.) germplasm. Euphytica 113: 135- 144.

McNeil MD, Hermann S, Jackson P, Aitken K (2011) Conversion of AFLP markers to high-throughput markers in a complex polyploid, sugarcane. Mol Breeding 27: 395-407.

Meudt HM, Clarke A (2007) Almost forgotten or latest practice? AFLP applications, analyses and advances. Trends Plant Sci 12: 106-117.

Mienie C, Liebenberg M, Pretorius Z, Miklas P (2005) SCAR markers linked to the common bean rust resistance gene. Theor Appl Genet 111: 972-979.

Miklas PN, Pastor- Corrales MA, Jung G, Coyne DP, Kelly JD, McClean PE, Gepts P (2002) Comprehensive linkage map of bean rust resistance genes. Annu Rep. Bean Improv Coop 45: 125-126.

Naidoo R, Fourie D, Mienie CMS, du Plessis S, Van Rensburg L (2003) Identification and development of molecular markers linked to common bacterial blight resistance genes from Wilk 2. Annu Rep Bean Improv Coop 46: 220-221.

Queiroz VT, Sousa CS, Souza, TLPO, Costa MR, Sanglad DA, Ragagnin VA, Barros, EG, Moreira, MA (2004) SCAR marker linked to the common bean rust resistance gene Ur-11. Annu Rep Bean Improv Coop 47: 271-272.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Schlueter JA, Goicoechea JL, Collura K, Gill N, Lin J, Yu Y, Kudrna D, Zuccolo A, Vallejos CE, Muños-Torres M, Blair MW, Tohme J, Tomkins J, McClean P, Wing RA, Jackson SA (2008) BAC-end sequence analysis and a draft physical map of the common bean (*Phaseolus vulgaris* L.) genome. Tropical Plant Biol 1: 40-48.

Shasany AK, Darokar MP, Dhawan S, Gupta A K, Gupta S, Shukla A K, Patra, NK, Khanuja SPS (2005) Use of RAPD and AFLP Markers to Identify Inter- and Intraspecific Hybrids of Mentha. J Hered 96: 542-549.

Shirasawa K, Kishitani STN (2004) Conversion of AFLP markers to sequence-specific markers for closely related lines in rice by use of the rice genome sequence. Mol Breeding 14: 283-292.

Todd A (2008) Development of molecular tools for isolation of the common bean (*Phaseolus vulgaris*) rust resistance gene *Ur-3*. master's thesis. Department of Agriculture & Natural Resources Delaware State University, Dover, DE.

Vallejos CE, Sakiyama NS, Chase CD (1992) A Molecular Marker-Based Linkage Map of *Phaseolus vulgaris* L. Genetics 131: 733-740.

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Friters A, Pot J, Paleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA finger printing. Nucleic Acids Res 23: 4407-4414.