Theor Appl Genet (2006) 112: 913–923
DOI 10.1007/s00122-005-0195-9

ORIGINAL PAPER

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Molecular mapping of genes for resistance to the bean pod weevil (*Apion godmani* Wagner) in common bean

Received: 13 August 2005 / Accepted: 30 November 2005 / Published online: 6 January 2006

Abstract The bean pod weevil (*Apion godmani* Wagner) is a serious insect pest of common beans (*Phaseolus vulgaris* L.) grown in Mexico and Central America that is best controlled by host-plant resistance available in Durango or Jalisco genotypes such as J-117. Given unreliable infestation by the insect, the use of marker-assisted selection is desirable. In the present study, we developed a set of nine molecular markers for Apion resistance and mapped them to loci on chromosomes 2, 3, 4 and 6 (linkage groups b01, b08, b07and b11, respectively) based on genetic analysis of an $F_{5:10}$ susceptible × resistant recombinant inbred line population (Jamapa × J-117) and two reference mapping populations (DOR364 × G19833 and BAT93 × JaloEEP558) for which chromosome and linkage group designations are known. All the markers were derived from randomly amplified polymorphic DNA (RAPD) bands that were identified through bulked segregant analysis and cloned for conversion to sequence tagged site (STS) markers. One of the markers was dominant while four detected polymorphism upon digestion with restriction enzymes. The other markers were mapped as RAPD fragments. Phenotypic data for the population was based on the evaluation of percentage seed damage in replicated trials conducted over four seasons in Mexico. In single point regression analysis, individual markers explained from 3.5 to 22.5% of the variance for the resistance trait with the most significant markers overall being F10-500S, U1-1400R, R20-1200S, W9-1300S and Z4-800S, all markers that mapped to chromosome 2 (b01). Two additional significant markers, B1-1400R and W6-800R, were mapped to chromosome 6 (b11) and explained from 4.3 to 10.2% of variance depending on the season. The latter of these markers was a dominant STS marker that may find immediate utility in marker-assisted selection. The association of these two loci with the *Agr* and *Agm* genes is discussed as well as the possibility of additional resistance genes on chromosome 4 (b07) and chromosome 3 (b08). These are among the first specific markers developed for tagging insect resistance in common bean and are expected to be useful for evaluating the mechanism of resistance to *A. godmani*.

Keywords Insect resistance genes · Seed coat peroxidase · Hypersensitive response

Communicated by F. J. Muehlbauer

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Introduction

The bean pod weevil (*Apion godmani* Wagner) (Coleoptera: Curculionidae) is a destructive insect pest of common beans (*Phaseolus vulgaris* L.) grown in Mexico and Central America, especially the highland regions of Guatemala, Honduras, El Salvador and Northern Nicaragua between 800 and 2,000 m above sea level. In Mexico, it is particularly important in the States of Puebla, Mexico, Durango, and Chiapas (Sifuentes 1981; Garza and Muruaga 1993). The life cycle of the bean pod weevil alternates between forest soils and crop refuse where the adults overwinter and fields of common bean where they reproduce (Cardona 1989). Bean pod weevils generally appear in bean crops during flowering and consume small amounts of young leaves and flowers before they begin to reproduce. Damage occurs when the bean pod weevil females lay their eggs in the mesocarp of 1–4 cm long developing common bean pods and the resulting larvae burrow into the immature seeds

inside causing yield loss, reduced seed quality and lower seed viability (Kornegay and Cardona 1991). Bean pod weevil females usually lay one egg at a time and do so right above the developing seed. Eggs hatch within 8–9 days and the first instar penetrates the seed developing through second and third instar larvae in a period of approximately 3 weeks. The last instar forms a pupation chamber inside the pod where the pupal stage usually lasts 10 days. Newly emerged adults disperse to forested areas until returning when the next bean crop is about to flower. There is one generation per cropping season. When infestation is high, more than one bean pod weevil larvae can occupy a single seed. Yield loss caused by the insect is variable depending on the climatic conditions of the previous year but can be as high as 90% (Cardona and Kornegay 1999).

Chemical control of the bean pod weevil involves utilization of organophosphate insecticides, which while effective, increase production costs and can lead to environmental contamination and health problems (Cardona 1989; Kornegay and Cardona 1991). Furthermore, the bean pod weevil is difficult to detect and control in a timely fashion, given that the insect pest damages the seed hidden within normal looking pods and the damage is usually discovered on harvest when the adults have already emerged and control is no longer needed. For this reason, host resistance is a more effective strategy of controlling damage from the bean pod weevil. Sources of resistance are found within *P. vulgaris* in mostly Jalisco landraces such as Amarillo 153, Amarillo 154, Amarillo 155, Amarillo 169, de Celaya, Hidalgo 84, J-117, Mexico 332, Negro 150, Pinto Texcoco, Pinto 168 and Puebla 36 which are not adapted in lowland tropics (Cardona and Kornegay 1999; Garza et al. 1996, 2001). Additional sources include breeding lines such as APN 18, APN 83, APN 108 (Beebe et al. 1993).

Mechanisms of resistance to the bean pod weevil in common beans are thought to be either antibiosis involving a hypersensitive response that encapsulates the oviposition sites, insect eggs or larvae within necrotic tissue; or antixenosis that affects the preference for oviposition sites (Garza et al. 2001). Epistasis between two independent dominant genes, *Agr* and *Agm*, both of which are needed for high levels of resistance has been hypothesized to control the hypersensitive response as shown from a partial diallel with seven of the resistant sources carried out by Garza et al. (1996). In that study, the *Agr* gene provided intermediate levels of resistance while the *Agm* gene alone did not have any effect. These are some of the few known major genes for insect resistance and the only known case of hypersensitive response to oviposition known in common bean; hypersensitivity being more typical of disease resistance than insect resistance in both this crop and other species (Yencho et al. 2000). The fact that a few genes control resistance may explain the observation that it was relatively straightforward to transfer resistance from Mexican landraces to new breeding lines with Central American grain types and high yields (Beebe et al. 1993). In addition to qualitative resistance based on the hypersensitive response, quantitative resistance affected by genotype × environment interaction has been observed for some Guatemalan sources of resistance (Garza et al. 1996). While the hypersensitive response is stable across geographical areas and planting seasons, quantitative resistance may not be stable.

Marker-assisted breeding using a range of genetic markers has proven to be useful for the moving resistance genes from source genotypes to recipient germplasm in common bean (reviewed in Kelly et al. 2003). Many of the genetic markers in use today sequence tagged site (STS) markers, either sequence characterized amplified region (SCARs) markers or cleaved amplified products (CAPs) that have been developed from randomly amplified polymorphic DNA (RAPD) markers. However, almost all STS markers developed to date for common bean tag disease resistance genes rather than insect resistance genes or other traits (Kelly and Miklas 1998). The insect resistance genes in common bean that are currently tagged include the major gene resistance to the bruchid *Zabrotes subfasciatus* (Boheman) encoded by the Arcelin gene (Osborn et al. 1986) and the quantitative trait loci (QTLs) for resistance to *Thrips palmi* Karny (Frei et al. 2005), the former gene with a protein based marker and the latter QTLs with microsatellite and RAPD markers. In contrast, quite a few insect resistance genes have been tagged in other crops especially rice and wheat (Biradar et al. 2004; Dwiekat et al. 1997; Katiyar et al. 2001; Liu et al. 2001, 2002) and bulked segregant analysis has been useful in developing molecular markers for insect resistance (Yencho et al. 2000).

Given the major genes thought to be involved in resistance, our objective in this study was to develop STS markers for Apion resistance using bulked segregant analysis of recombinant inbred lines derived from the cross Jamapa × J-117, where 'J-117' is a resistance landrace source and 'Jamapa' is a susceptible black-seeded cultivar from Mexico. In this study, we developed a total of nine SCAR and CAPS markers, confirmed their linkage to Apion resistance genes and mapped them to their genomic position based on integrated genetic maps of common beans.

Materials and methods

Plant material

A recombinant inbred line population was developed from a cross of Jamapa (susceptible) and J-117 (resistant) through single seed descent to the F_5 generation followed by bulking for line development to create $F_{5:10}$ recombinant inbred lines. Jamapa also known as the variety 'Negro Jamapa 81' is a black-seeded (race Mesoamerica) cultivar that was released in Mexico in 1958. Jamapa was selected by F. Cárdenas from a

landrace collection made in Veracruz and held at the Experimental Station in Cotaxtla (Voysest 2000). This variety is known for its performance stability across a range of tropical environments but is susceptible to *A. godmani*. J-117 is also a stable genotype but was developed from a landrace originating in highland Mexico. J-117 is from the Jalisco race within the Mesoamerican gene pool. J-117 is pink striped and medium-seeded (30 g per 100 seeds), and was initially collected by the Mexican national Program in Atlacomulco, Mexico State. It was shown by Garza et al. (1996) to be highly resistant to *A. godmani*. A total of 50 recombinant inbred lines were developed for use in the experiments. Total genomic DNA was extracted for both parents and lines from three young trifoliates harvested in 1.5 ml Eppendorf tubes according to Afanador et al (1998). Briefly, the protocol involved grinding in liquid nitrogen and extraction using the following extraction buffer (2% CTAB; 1.4 M NaCl; 0.02 M EDTA pH = 8.0; 0.1 M Tris–HCl pH = 8.0; 1% β-mercaptanol). Organic phase extraction consisted in chloroform; isoamyl alcohol (24:1) followed by precipitation in 100% isopropanol and washing in 70% ethanol before re-suspension in 100 µl of Tris–EDTA buffer (pH = 8.0) and RNAse treatment. DNA was quantified on a Hoefer DyNA Quant 200 flourometer (Pharmacia Biotech, USA) and diluted to 5 ng/µl final concentration for all subsequent experiments.

Field screening

The population was evaluated for Apion resistance over four consecutive seasons (1994–1997) at the Santa Lucía de Prías Experiment Station of the Mexican Instituto Nacional de Investigaciones Agropecuarias (INIFAP) near Texcoco. This site usually has a heavy and uniform infestation of *A. godmani* with little or no interference from other pests. The elevation of the test site is 2,250 m and the mean annual precipitation is 670 mm, distributed between May and October. Mean annual temperature and relative humidity are 16°C and 70%, respectively. All experiments consisted in randomized complete block designs with four replicates each. Each test plot consisted of one row, 4 m in length, with spacing of 10 cm between plants within rows and 85 cm between rows. In each year, the experiments were planted under rain-fed conditions in late May and harvested in October. No fertilizers were added and weeds were controlled by hand. *A. godmani* was the only major insect pest observed causing damage to bean plants during the experiments and no pesticides were used during the experiments. To measure resistance levels, a random sample of 30 pods per plot was taken at crop maturity. Each pod was examined by carefully opening the pod along the ventral suture and removing each seed, which was then examined for *A. godmani* damage. The number of damaged seeds and total seeds were recorded, and the percentage of seeds damaged was calculated. As described by Garza et al. (1996) genotypes were classified as susceptible (> 50% damaged), intermediate (30–50% damaged) or resistant (< 30% damaged).

RAPD analysis and band cloning

Randomly amplified polymorphic DNA reactions were carried on a PTC-100 thermocycler (MJ Research Inc.), with 50 ng of DNA as template and 25 µl reaction volumes that contained 0.2 mM dNTP, 2.5 mM MgCl2, 1 × BSA (Sigma, Haverhill, UK), 1 × PCR buffer (Promega, Madison, WI, USA), 1 U *Taq* DNA polymerase and 0.4 µM of the corresponding primer (Operon, Alameda, CA, USA). PCR conditions consisted in hot start denaturation at 92°C for 1 min, followed by 37 cycles of denaturation at 92°C for 15 s, annealing at 42°C for 15 s and extention at 72°C for 1 min and 10 s. All RAPD reaction products were run at 110 V, 55–60 mA, on 1.5% agarose gels stained with ethidium bromide. RAPD bands that were polymorphic and which were significantly associated with the resistance phenotype were selected for cloning. RAPD bands were extracted from 1% low melting point agarose gels using a Wizard PCR prep purification system (Promega). In the case of Z4-800R, the band was purified on a 4% polyacrylamide gel, amplified with a second round of PCR and then cleaned in a 1% low melting point agarose gel. The purified insert DNAs were cloned into the PGEM-T easy vector system I using T4 DNA ligase (Promega) in a reaction at 20°C for 1 h followed by 16 h incubation at 4°C. Several recombinant clones were picked per ligation reaction and their DNA extracted with Qiaprep Miniprep kits (Qiagen, Valencia, CA, USA) which were checked for insert size with PCR amplification and or *Eco*RI digestions. The inserts of positive clones were fingerprinted with *Alu*I, *Cfo*I, *Hae*III, *Hsp*92II or *Mbo*I (Promega) digestion and sequenced using standard techniques, T7 and Sp6 primers, Big Dye sequencing kits and an ABI377 DNA sequencer (Applied Biosystems Inc., Foster City, CA, USA). Sequences were compared using the software Sequencher v.4.1 (Gene Codes corporation, Ann Arbor, MI, USA) and analyzed for sequence homology at BLAST-NCBI (http://www.ncbi.nlm.nih.gov/BLAST). Polymorphic RAPD markers and the bands cloned from them received a designation based on the primer that amplified the band, the size in nucleotides of the band and the parental source of the band coded as either from the resistant (R) or susceptible (S) parent; for example, where the resistant parent produced a 1,400 bp band with primer OPB-01, the marker was B11400R.

SCAR primer design and CAPS assays

Specific primers were designed for each unique cloned RAPD sequence using Primer 3.0 software (http://www.frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)

and these were tested for their ability to amplify single-copy SCAR products. Each primer was designed to be 24 nt or above in length and to have a melting temperature 58–65°C with approximately 50% GC content. PCR reaction conditions for the markers SK16, W6800R, C1800S, F10500S, M12800S and Z4800R were: denaturation at 92°C for 2 min followed by 35 cycles of 30 s denaturation, 30 s annealing at the indicated temperature and 1 min extension at 72°C. For the CAPS markers B11400R, R201200S, U11400R and W91300S, the denaturation and annealing stages were extended to 1 min each while the extension stage was extended to 2 min. Any monomorphic SCAR products were digested with the frequently cutting restriction enzymes *Alu*I, *Bfa*I, *Bst*u1, *Cfo*I, *Hae*III, *Hsp*92II, *Mbo*I, *Mse*I, *Rsa*I, *Sau*3AI, *Taq*I (Promega) to convert the markers into CAPS (Cleaved Amplified Polymorphic Sequences). Digestion was conducted in 30 µl volumes at recommended incubation temperatures for 4 h each.

Data analysis and genetic mapping

Phenotypic data for Apion resistance were transformed to arcsine square root of proportion and analyzed using the general linear model (GLM) procedure in Statistix (Analytical Software 2003). Scheffe's method of significance testing for arbitrary simultaneous linear contrasts was used to test for differences between selected susceptible and resistant groups of genotypes. Genotypic data for the SCAR and CAPS markers were used for linkage analysis in the Jamapa × J-117 population and in two additional populations for which genetic maps have been constructed, DOR364 × G19833 (Blair et al. 2003) and BAT93 × JaloEEP558 (Freyre et al. 1998). All genetic mapping was conducted using the software Mapmaker v. 3.0b (Lander et al. 1987) and a minimum LOD of three under the 'assign' command. Marker-trait associations in the Jamapa × J-117 population were determined with single point regression analysis of the phenotypic data onto the marker genotypes using the software program qGENE (Nelson 1997). Based on this last analysis, coefficients of determination (R^2) are reported for all significant marker QTL associations with a probability threshold of $P > 0.001$.

Results

Screening for resistance

Highly significant differences between genotypes were observed in all four seasons for seed damage percentage (Table 1) with coefficients of variation ranging from 9.6 to 15.7%. Although reliability of field testing was high as evidenced by the low coefficients of variation, variability in insect pressure could have caused the significant genotype × season interactions that were observed in the combined analysis and would have been more important in seasons when damage was low such as season 2 compared to seasons when damage was high such as season 4 (Table 2). Differences between seasons were significant for average seed damage with highest damage in 1997 (59.8%), followed by 1996 (52.5%), 1994 (44.6%) and 1995 (43.0%). Despite the difference between seasons, genotype effects were more significant than genotype × season effects (data not shown), Pearson correlations for seed damage were high between seasons

Table 1 Analysis of variance for percentage seeds damaged by *Apion godmani* in the recombinant inbred line population 'Jamapa' × J-117 screened over four consecutive seasons (1994–1997) at Santa Lucía de Prías Experiment Station near Texcoco, Mexico

Source	*df*	Sum of squares	Mean square	*F*	*P*	C.V. (%)
Season 1						
Rep	3	0.03524	0.01175			
Treatment[a]	51	5.97427	0.11714	23.8	0.0000	15.7
Error	153	0.75286	0.00492			
Total	207	6.76237				
Season 2						
Rep	3	0.03539	0.01180			
Treatment	51	3.90742	0.07662	22.0	0.0000	13.7
Error	153	0.53274	0.00348			
Total	207	4.47556				
Season 3						
Rep	3	0.01130	0.00377			
Treatment	51	4.27538	0.08383	20.1	0.0000	12.3
Error	153	0.63710	0.00416			
Total	207	4.92378				
Season 4						
Rep	3	0.00769	0.00256			
Treatment	50	6.63201	0.13264	40.2	0.0000	9.6
Error	150	0.49431	0.00330			
Total	203	7.13402				

Percentages transformed to arcsine square root of proportion

[a]Treatments included the two parents (Jamapa and J-117) and the 50 recombinant inbred line progeny from the Jamapa × J-117 cross

Table 2 Percentage seed damage by *Apion godmani* in recombinant inbred lines (RILs) and parents

Genotype	1994	1995	1996	1997
Mean 10 most resistant RILs	5.2b ± 0.57	6.7b ± 0.53	11.6c ± 0.84	12.5b ± 0.68
Mean 10 most susceptible RILSs	38.0a ± 0.96	34.2a ± 1.08	42.0b ± 1.56	55.6a ± 1.60
'Jamapa' (susceptible parent)	44.8a ± 2.84	35.6a ± 3.19	56.3a ± 2.80	61.2a ± 2.78
J 117 (resistant parent)	1.1b ± 0.45	4.0b ± 0.74	9.1c ± 0.82	6.6b ± 1.31

Means ± SEM of four replicates per genotype per season. Means within a column followed by different letters are significantly different; separation by Scheffe's *F* method of significance testing for arbitrary linear contrasts ($P < 0.01$)

($r = 0.617$ to 0.684, $P = 0.0000$) and the differences between resistant and susceptible parents were large. Population histograms showed evidence for a binomial distribution in the season with the highest level of infestation and a continuous distribution in the three other seasons with lower levels of infestation (Fig. 1). Significant differences between the parents were observed in every season with J-117 having an average seed damage rate of 5.2% and Jamapa having an average seed damage rate of 49.5%. In all seasons, the mean seed damage of the ten most resistant RILs was not significantly different from that of the resistant parent and, likewise, the mean seed damage of the ten most susceptible RILs was not significantly different from that of the susceptible parent except in the third season when the susceptible RILs did not present the same damage level as the susceptible parent (Table 2).

Bulked segregant analysis

DNAs from the four most resistant and four most susceptible recombinant inbred lines were combined into their respective bulks in equal concentrations. A total of 131 decamer primers were used to discover random amplified (RAPD) polymorphisms among the parents that were also observed in the resistant and susceptible bulks. Both repulsion and coupling markers were considered as long as the presence or absence of the bands was consistent between the bulks and the parents. Out of a total of over 1,200 RAPD bands produced by the RAPD primers, 35 bands detected differences between the parents and the same polymorphism with the bulks (Fig. 2). All of these markers were validated in the full set of 50 individuals from the population to determine association with Apion resistance and to define linkage groups. Among the polymorphic bands, a subset of nine was selected for cloning and conversions to SCARs or CAPS markers because they were significantly associated with Apion resistance in the regression analysis ($P < 0.05$) (Table 3). The amount of variance explained by each of these RAPD markers ranged from 3.5 to 22.5% depending on the marker and the season analyzed with the most significant markers overall being W9-1300S and Z4-800S; and the least significant markers being C1-800S and M12-800S.

SCAR development

All nine RAPD bands, B1-1400R, C1-800S, F10-500S, M12-800S, R20-1200S, U1-1400R, W6-800R, W9-1300S and Z4-800R, were successfully cloned (Table 3; Fig. 2). Approximately half of these were cloned from the

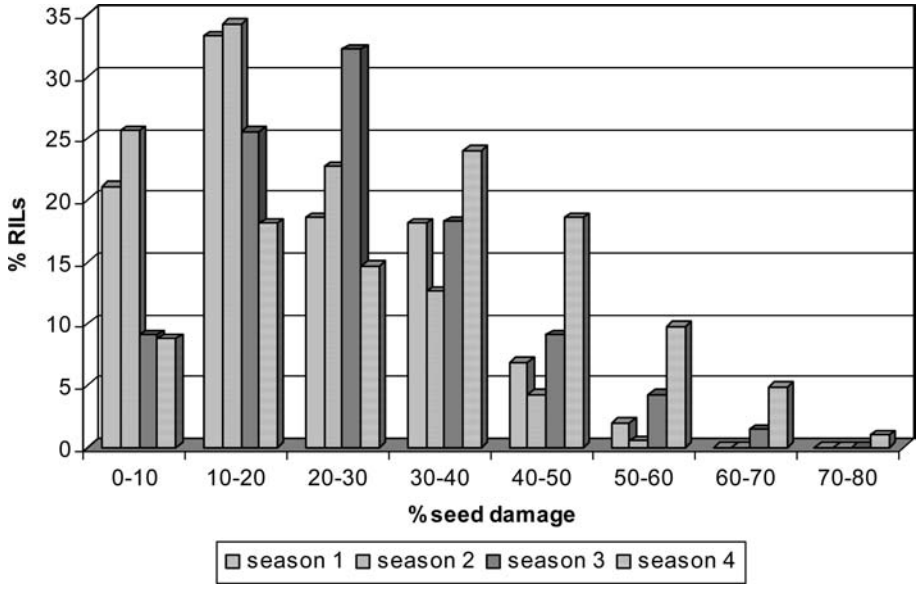

Fig. 1 Population distribution for 50 recombinant inbred lines from the Jamapa × J-117 population for *Apion godmani* resistance tested over four seasons in Santa Lucía de Prías Experiment Station near Texcoco, Mexico

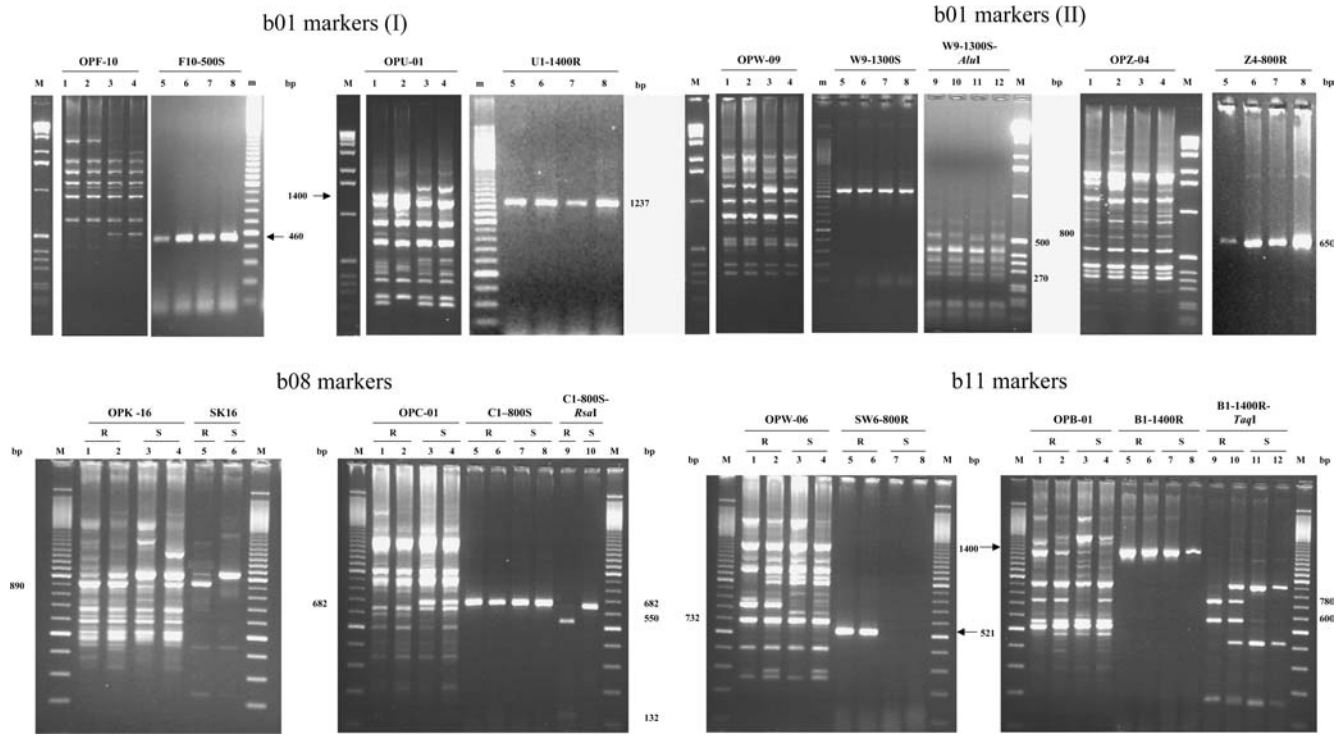

Fig. 2 SCAR and CAPS markers developed from polymorphic RAPD bands found in bulked segregant analysis of *Apion godmani* resistant and susceptible parents and individuals of the Jamapa × J-117 population. SCAR markers are indicated by fragment size according to name in Table 2. CAPS markers are indicated by restriction enzyme used for cleavage. Molecular weights (in bp) are indicated for cloned band in the case of the RAPDs and expected PCR or cleavage products in the case of the SCAR and CAPS markers. *Lanes 1*, *5* and *9* resistant parent (J-117); *2*, *6* and *10* resistant bulk; *3*, *7* and *11* susceptible parent (Jamapa); *4*, *8* and *12* susceptible bulk; *M* molecular marker size standard (1 kb and 100 bp ladders)

susceptible parent and half were cloned from the resistant parent. Where RAPD markers were putatively co-dominant given their segregation in the Jamapa × J-117 population, as in the case of Z4-800R and Z4-850S, the band from the resistant parent was cloned. All other RAPD bands targeted were dominant markers in the Jamapa × J-117 population.

BLAST searches using both blastn and blastx procedures identified nucleotide–nucleotide and/or nucleotide–protein homologies for several of the cloned RAPD bands (Table 3). Several clones had high homologies to retrotransposons or transposon elements from a range of dicotolydenous species, including (1) R201200S with similarity to the long terminal repeat (LTR) of a retrotransposon from *Arabidopsis thaliana* (Genbank entry A71444, probability of 3e-14); (2) W91300 with similarity to the Gag–Pol polyprotein of a retroelement from *P. vulgaris* (AY508120, 1e-65); and (3) B11400R with similarity to the polyprotein of a transposon from *Cicer arietinum* (CAC44140, 3e-06 for). Homologies with retrotransposons is a common feature of cloned RAPD bands in common bean. Two other clones were similar to expressed gene sequences from other legumes. The cloned band Z4-800R was similar to an EST from soybean, *Glycine max* (CA799444; 6e-73) representing an unknown gene from that crop (H85112; $P = 0.003$). Meanwhile the cloned band F10500S was similar to several ESTs from *Medicago truncatula* expressed during insect herbivory (BI266328, 2e-18), elicited cell culture (BF646172, 7e-21), mycorhizal infection (AW584349, 7e-21) and leaf development (BG454278, 5e-21). At the protein level, this clone was similar to the seed coat peroxidase 1 precursor gene from *Glycine max* (AAL77517, $P = 0.006$) and *P. vulgaris* (AAD37427, $P = 0.007$).

Sequence characterized amplified region and CAPS development is also summarized in Table 3. A total of 15 primer sets were designed for the nine RAPD band sequences and these were tested on the population parents and on the bulks. A single primer set was selected per RAPD band for marker analysis. The marker derived from W6-800R was dominant as a SCAR in the Jamapa × J-117 population, while all other amplification products were monomorphic as SCARs. The W6-800R marker showed a clear polymorphism consisting of positive and negative signals in PCR amplification with a band present for J-117, the resistant parent, and absent for Jamapa, the susceptible parent (Fig. 2). The remaining monomorphic SCARs were tested with frequently cutting restriction enzymes (all with 4 bp recognition sites) to identify CAPS polymorphisms. Restriction fragment differences were revealed for four of the PCR fragments (B1-1400R, C1-800S, R20-1200S

Table 3 Development of SCAR and CAPS markers from RAPD bands that were significantly associated with *Apion godmani* resistance in the common bean population Jamapa × J-117 as ordered by linkage group through comparative genetic mapping

RAPD band[a]	Association with Apion resistance (R2) per season[b]				Linkage group[c]	Genbank no.	Blastx results[d]	SCAR primer sequence (L/R)	Tm (°C)	SCAR polym	CAPS polym[e]
	A	B	C	D							
1. F10-500S	13.6	13.4	11.8	17.3	b01	BV676535	Peroxidase *Glycine max*	GGAAGCTTGGGGAGTAGGGATTC AACCAGGTAGCTTGACAGGCAGA	65.2	–	–
2. R20-1200S	NS	6.8	NS	NS	b01	BV676533	Retrotransposon *Arabidopsis*	CCTGCAGATTGTCCAAGATCATCC CCAATCCACAATGCCATGTATCAA	61.6	–	AluI,MboI
3. U1-1400R	11.2	11.4	12.3	16.0	b01	BV676530	NS	GACGATGCACAGTGCATCAACT TCCCCTAACTTCGGTATTTTTCCA	66.0	–	–
4. W9-1300S	22.5	18.6	14.0	19.2	b01	BV676528	Retrotransposon *Phaseolus*	GTGACCGAGTTACACGAAGGGATA AGGGATAAAACTACCGAGCGAGGA	74.0	–	*AluI*
5. Z4-800R	17.0	20.0	10.9	10.5	b01	BV676529	Unknown gene *Glycine max*	GGCTGTGCTGTTGAACCTC AGGCTGTGCTCAATGGGTT	55.0	–	–
6. M12-800S	8.1	6.8	3.5	8.5	b07	BV676532	NS	GGGACGTTGGATGGGTGAGT GGGACGTTGGTCTAGTCTTTTGG	55.9	–	–
7. C1-800S	NS	4.3	NS	NS	b08	BV676534	NS	CGAGCCAGCAAAGAACACCTAAATG TTCGAGCCAGGGGTTTTCCT	63.2	–	*RsaI*
8. B1-1400R	9.1	10.2	4.3	5.5	b11	BV676531	Retrotransposon Cicer	GTTTCGCTCCCACAAGTGAATCTG GTTTCGCTCCAAGCCTGACCTAGTG	59.8	–	TaqI
9. W6-800R	7.7	8.5	NS	4.7	b11	BV676536	NS	AGGCCCGATGCCCCCTTAT TGGAGTCGGTCAAACCCATGTT	56.8	+	NA

[a]Polymorphic RAPD bands received a designation based on the primer that amplified the band, the size in nucleotides of the fragment and the parental source of the band coded as either from the resistant (R) or susceptible (S) parent

[b]Association of RAPD marker with Apion resistance determined by single point regression analysis ($P \leq 0.05$)

[c]Linkage group determined in either of two mapping populations: (1) BAT93 × Jalo EEP558 (Freyre et al. 1998) or (2) DOR364 × G19833 (Blair et al. 2003). Other markers linked in the Jamapa × J-117 population to markers mapped in the other two populations also assigned based on comparative genetic mapping (Fig. 3)

[d]Highest-significance BLASTx hits (significance value and Genbank entry given in text, *NS* non significant)

[e]Restriction enzyme used to generate cleaved amplified polymorphism (–indicates monomorphic; *NA* indicates not applicable in case of dominant SCAR markers)

and W9-1300S) when digested with different restriction enzymes, two of the fragments being polymorphic with *Alu*I digestion and one each polymorphic with *Rsa*I or *Taq*I digestion. Four of the SCAR fragments remained monomorphic (F10-500S, M12-800S, U1-1400R, Z4-800R) despite digestion with the frequently cutting restriction enzymes (Fig. 2).

Linkage analysis and single point regression

Genetic mapping of the RAPD markers and the SCAR or CAPS markers derived from them was conducted in a total of three recombinant inbred line mapping populations: the first population being the Jamapa × J-117 population itself followed by two additional populations useful for marker placement on the integrated map for the species, DOR364 × G19833 (Blair et al. 2003) and BAT93 × JaloEEP558 (Freyre et al. 1998). Of a total of 35 polymorphic RAPDs, 29 markers were linked in groups of two or more in the Jamapa × J-117 population while nine markers remained unlinked at an LOD 3.0. The linked markers formed a total of six linkage groups of which four linkage groups contained markers with significant association with Apion resistance and are shown in Fig. 3. The significant linkage groups ranged from 3.2 to 62.3 cM in length and contained between 2 and 11 individual markers. When the RAPDs were converted to SCAR or CAPS markers, they mapped to the same location as the RAPD (for B1-1400R and W6-800R) or to a closely linked locus (for C1-800S, R20-1200S and W9-1300S).

Mapping in the DOR364 × G19833 or BAT93 × Jalo EEP558 populations was used to place a total of nine RAPD and one SCAR marker on the integrated map for the species as shown in Fig. 3. Chromosomes and corresponding linkage groups were named according to the conventions of Pedrosa et al. (2003) and Freyre et al. (1998), respectively. A total of four chromosomes (linkage groups) were identified by the Apion resistance gene markers: chromosome 2 (also known as linkage group b01) contained the RAPD markers F10-500S, R20-1200S and U1-1400R, the first two mapped in DOR364 × G19833 and the latter mapped in the BAT93 × JaloEEP558 population; chromosome 4 (linkage group b07) contained the three closely linked RAPD markers, K12-1100S, M12-800S, and W20-600S, that clustered around the Phaseolin locus; chromosome 3 (linkage group b08) contained the previously developed SCAR marker SK16 (N. Weeden, personal

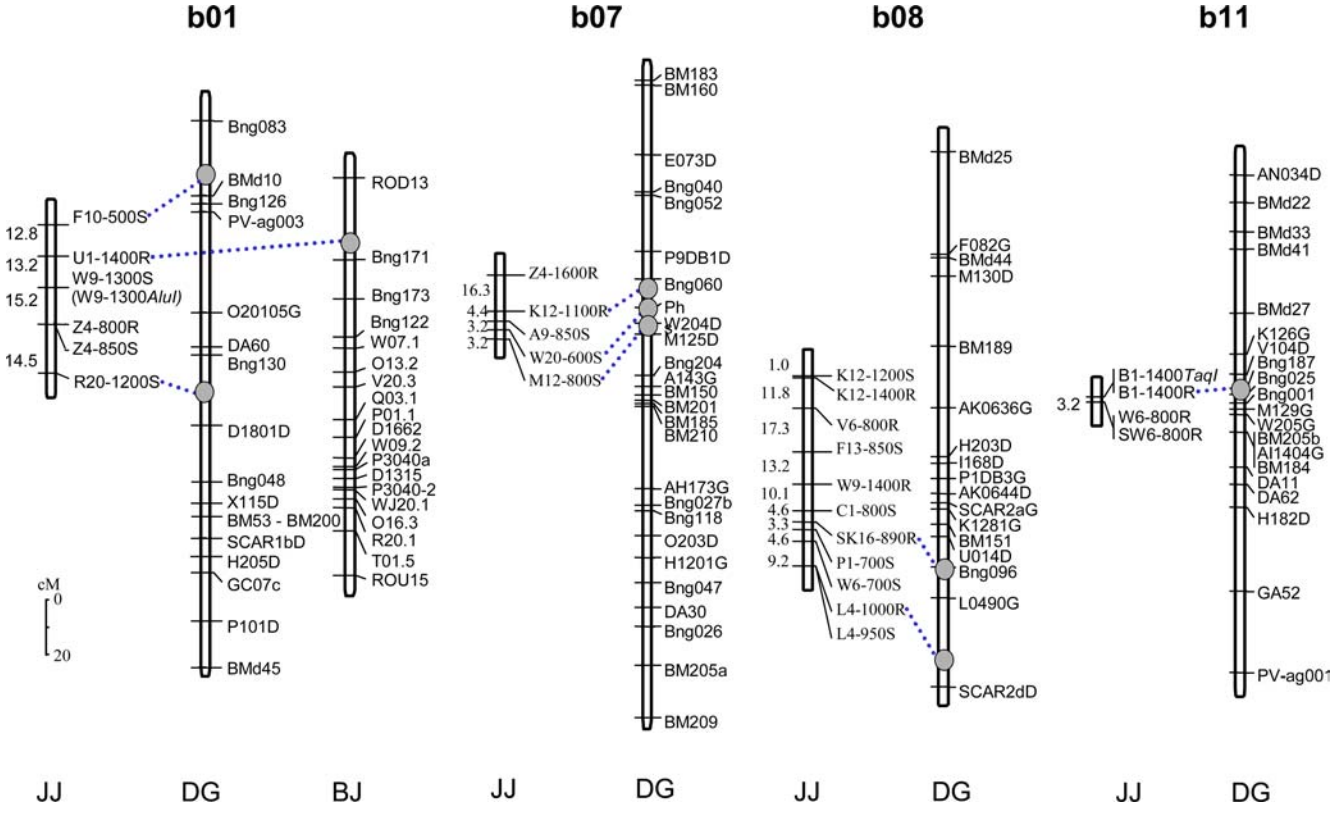

Fig. 3 Linkage relationships of RAPD, SCAR and CAPS markers for Apion resistance in the recombinant inbred line populations Jamapa × J-117 (*JJ*), DOR364 × G19833 (*DG*) and BAT93 × Jalo EEP 558 (*BJ*) for linkage groups b01, b07, b08 and b11. RAPD markers are indicated with designation described in text; SCAR markers indicated by '*S*' prefix; CAPS marker indicated by enzyme used for cleavage

communication) and the RAPD marker L4-1000R and W9-1400S; and chromosome 6 (linkage group b11) contained the RAPD marker B1-1400R. It was not possible to map the corresponding CAPS for the B1-1400R marker or the SCAR for W6-800R in either population. The markers that were most significantly associated with Apion resistance were those that as SCARs, CAPS or RAPDs mapped on chromosome 2 (F10-500S, R20-1200S, U1-1400R, W9-1300S and Z4-800R, especially these latter two) followed by those on chromosome 6 (B1-1400R and W6-800R) and chromosomes 3 and 4. While markers on chromosome 2 (linkage group b01) were significant for association with Apion resistance across all four seasons of evaluation (with 12.7–18.6% of variance explained on average across seasons), markers on the other chromosomes and linkage groups were associated with resistance in fewer seasons (with 4.3–7.3 % of variance explained on average across seasons). Despite the greater number of markers identified for chromosome 3 (linkage group b08), these markers were less significant for *A. godmani* resistance while the smaller number of markers on chromosomes 2 and 6 (linkage groups b01 and b11) seem to be most important for resistance to the insect. In the RAPD analysis, two unlinked clusters of markers were also detected that also were less significant and only important in a few seasons (data not shown). For all the positive markers, the resistant parent J-117 provided the resistant allele.

Discussion

We converted a total of nine RAPD markers associated with Apion resistance into STS markers, one of which was polymorphic as a SCAR and four of which were polymorphic as CAPS, all of these were also associated with resistance and co-localized with the source band (Table 3). Genetic mapping in the DOR364 × G19833 and BAT93 × JaloEEP558 populations was useful in establishing the locations of the markers on linkage groups from two integrated genetic maps for common bean (Freyre et al. 1998; Blair et al. 2003) and on the corresponding chromosomal map for the species (Pedrosa et al. 2003). All the polymorphic markers were mapped to four chromosomes or linkage groups (Fig. 3) of which b01 was the most important and b07, b08 and b11 were less important in terms of Apion resistance. Among the implications of this work was the potential of the new markers to dissect the inheritance of resistance and to help with marker-assisted selection. Previous to this study and based on the analysis by Garza et al. (1996) it was known that inheritance of resistance appeared to be fairly simple, with the hypothesis that two genes, *Agm* and *Agr*, conditioned the best resistance found in the genotype J-117.

From the results of this study, we can expand on this genetic model and postulate that *Agr*, the gene reported to provide higher, more stable levels of resistance (Garza et al. 1996) is on chromosome 2 (also known as linkage group b01) at the locus that was uncovered by the markers F10-500S, R20-1200S, U1-1400R, W9-1300S and Z4-800R and their corresponding CAPS given that these markers detected a highly significant affect on resistance across seasons. Furthermore, we can postulate that the *Agm* gene defined by Garza et al. (1996) as providing intermediate resistance, may be on either chromosome 6 (linkage group b11), chromosome 4 (b07) or chromosome 3 (b08) where marker–phenotype associations in this study were less significant. These loci would then be tagged by a range of RAPD markers and by the SCAR marker for W6-800R or the CAPS marker for C1-800S as well as a SCAR marker, SK16, previously developed by N. Weeden et al. (unpublished results). Since several loci on different linkage groups provide intermediate levels of resistance in the marker-trait association studies presented here we cannot determine the exact location of the *Agm* gene, but we can postulate further that there may be at least two additional modifier genes or QTL that provide resistance to *A. godmani*. Despite the apparently simple inheritance of a two gene model, additional genes involved in Apion resistance, their differential gene effects and their interaction with the environment may explain why there is some level of genotype by environment interaction in the expression of the resistance trait. The possibility of more than two genes being involved in resistance to this insect was suggested by Garza et al. (1996) who also predicted that additional independently segregating genes controlled Apion resistance in another three accessions (Amarillo 169, Pinto 168 and Pinto Texcoco) but that some of these genes were non-allelic and varied in levels of resistance that they provided. Indeed, we observed that the stronger resistance gene on chromosome 2 was less influenced by seasonal variability than the other resistance genes on other chromosomes. The existence of a stable major gene for resistance may explain why correlations between seasons were high for performance of the recombinant inbred lines. These results agree with Beebe et al. (1993) who found that resistance appeared to be stable across geographical areas, seasons and planting systems. While Garza et al. (1996) suggested that Apion resistance genes *Agm* and *Agr* are dominant, we could not confirm the gene effect of each of the loci discovered in this study given that recombinant inbred line population used was highly homozygous.

The mechanism of resistance to *A. godmani* that was previously reported is a unique form of antibiosis not found for other insect pests affecting common bean and consists in a hypersensitive response that causes a concentric series of necrotic rings to form around sites of oviposition in the pod mesocarp encapsulating and killing eggs and first instar larvae (Garza et al. 1996). This encapsulation provides effective protection to the seed by preventing second and third instar larval development and subsequent seed damage. The similarity of the hypersensitive response observed with

oviposition of *A. godmani* in the pod wall of the plant (Garza et al. 2001) to the defense response mounted to fungal or bacterial pathogen attack is interesting. Hypersensitive reactions in plants are controlled by a series of recognition and signal transduction events in which receptor kinases, leucine rich repeat proteins as well as transcription factors play a role (Hammond-Kosack and Jones 1997). In addition, there are structural genes involved in defense response notably enzymes that lead to an oxidative burst and to rapid necrosis such as NADPH oxidase and peroxidases (Lamb and Dixon 1997). In this light it is interesting that we cloned part of a peroxidase gene in our search for genetic markers through the bulked segregant approach and that this gene was linked to the locus on chromosome 2 that provides the highest levels of Apion resistance observed in this study. The peroxidase homolog, may, therefore, be a gene that is important to the hypersensitive response mounted against *A. godmani* and represents as well an interesting candidate gene for *Agr* itself which deserves further investigation. Peroxidases have been implicated in hypersensitive response, general defense responses and cell wall strengthening and have been shown to be upregulated due to wounding, infection or jasmonic acid applications (Bolwell et al. 2002; Moore et al. 2003). Although hypersensitive reactions are rare as a resistance mechanism to insect pests (Fernandes 1990) they are the basis for other resistance to the Hessian fly in wheat (Grover 1995).

The genomic location of the markers identified in this study for Apion resistance genes is interesting because of the potential overlap with location of disease resistance genes found on the same linkage groups and their potential association with candidate genes for insect resistance. Linkage groups b01, b08 and b11 are known to contain clusters of disease resistance genes (reviewed in Kelly et al. 2003) and sequence defined resistance gene analogs (Lopez et al. 2003; Rivkin et al. 1999). A similar trend for co-localization of resistance gene analogs with multiple insect and nematode resistance has been shown for tomato (Kaloshian 2004). In our study, it was perhaps more surprising to find an association of *A. godmani* resistance with the region of the phaseolin locus on chromosome 4 (linkage group b07) where the major seed storage protein of common beans is encoded (Llaca and Gepts 1996). It was not possible to study this further in the Jamapa × J-117 population as both parents had the same Mesoamerican "S" pattern for phaseolin (this laboratory, unpublished results), however, it is tempting to hypothesize that the phaseolin locus itself or linked loci could determine seed protein content which would effect larval development in *A. godmani* since this insect feeds predominantly on immature seeds. The phaseolin locus has been shown to be associated with a range of disease resistances (Kelly et al. 2003). Further crosses and genetic analysis as well as the eventual isolation of disease resistance genes from the positive linkage groups in common bean may be useful for the analysis of *A. godmani* resistance in the common beans.

In the meantime, this research has opened up the possibility of selecting for Apion resistance through genotypic rather than phenotypic analysis. The potential advantages of marker-assisted selection over direct field screening for *A. godmani* resistance include increased efficiency of selection and more accurate introgression of the resistance genes. Insect-based screening of *A. godmani* is difficult because there are years when the insect does not present itself and direct selection in the field is impossible. Furthermore, the insect cannot be mass-reared; so, artificial infestation is not an option and screening is limited to one field season per year since the insect must overwinter before attacking a new bean crop. Marker-assisted selection, on the other hand, could be conducted at any time of the year and would not vary from season to season. The development and use of multiple markers tagging all the resistance genes involved in the trait would need to be implemented to exploit the potential of pyramiding genes and QTL involved in Apion resistance especially for selecting both the *Agm* and *Agr* genes as well as additional quantitative resistance loci simultaneously without the risk of one gene masking the other as has been observed in previous reports (Garza et al. 1996). No evidence exists for biotypes of *A. godmani* that overcome these resistance genes; so, indirect selection using markers could be used to deploy resistance in breeding lines for the entire region affected by the pest and this resistance would be likely to be stable and durable for many years to come.

Marker-assisted selection for resistance using the SCAR markers developed here could also be combined with the selection of multiple disease resistance traits that have also been tagged (Kelly and Miklas 1998). Potential time and savings from marker multiplexing would be worth exploring, as resistance to *A. godmani* is often required in combination with other resistance traits such as for bean golden yellow mosaic virus in breeding lines for Central America (Beebe et al. 1993) and Mexico (Garza et al. 1996). It is important to point out that the best markers for marker-assisted selection would be those that were most significantly associated with Apion resistance. In this regard, while all the markers developed in this study were significantly associated with Apion resistance in one or more seasons, the linked SCAR marker SK16 was not significant in any season and would only be recommended in association with other CAPS markers on linkage group b08. The most promising markers, meanwhile, would be the CAPS markers from linkage groups b01 and b11. These markers along with the identification of the linkage groups involved in resistance are promising for the manipulation and further characterization of the major and minor genes controlling resistance to *A. godmani*.

Acknowledgements We are grateful to N. Weeden for providing the SK16 SCAR, S.P. Singh and H. Terán for population development, S. Beebe and P. Gepts for the seed of reference mapping

populations, W. Pantoja and M.C. Giraldo for technical support and H.F. Buendía for help in data analysis. This research was supported by a Swiss Development Cooperation (SDC) grant to CIAT and INIFAP as part of the PROFRIJOL network as well as by USAID funds for marker development.

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