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DNA marker-assisted selection to pyramid rust resistance genes in "carioca" seeded common bean lines

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Abstract This work reports a gene pyramiding approach assisted by DNA markers used to develop "carioca" seeded common bean (*Phaseolus vulgaris* L.) elite lines harboring three different rust resistance genes. Rust is among the most destructive diseases that attack *P. vulgaris* and cause serious damage worldwide. The rust resistance genes *Ur-5* (from 'Mexico 309'), *Ur-11* (from 'BelMiDak RR-3'), and *Ur-14* (from 'BRS Pioneiro', a "carioca" seeded cultivar derived from the resistance source 'Ouro Negro') were combined in the "carioca" seeded bean cultivar 'Rudá'. Firstly, two different backcross programs were conducted separately to produce progenies harboring individually the *Ur-5* and *Ur-11* genes. Molecular fingerprinting analysis was used to select plants genetically similar to 'Rudá' in the backcross cycles to accelerate the recurrent-background recovery. The obtained progenies were initially intercrossed and then crossed with 'BRSMG Pioneiro' (*Ur-14*). The final F_1 plants derived from these crosses were screened with DNA markers linked to the three rust resistance genes: SI19 (*Ur-5*), SAE19 (*Ur-11*) and OPX11 (*Ur-14*). The plants selected as harboring all the alleles of interest were used to obtain the next generations. The selection based on DNA markers was conducted up to the $F_{4:5}$ generation. We were able to select $F_{4:7}$ progenies showing all the DNA markers associated to the genes of interest and resistant to all specific races of *U. appendiculatus* used for phenotypically detecting each one of the rust resistance genes. Yield evaluations show that these selected lines are as productive as the recurrent parent 'Rudá' and high-performing control cultivars grown in Brazil.

Keywords Disease resistance · Marker-assisted selection · Molecular markers · *Phaseolus vulgaris* · Plant breeding · *Uromyces appendiculatus*

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Introduction

Common bean (*Phaseolus vulgaris* L.) is an economically, nutritionally, and socially important crop, especially in developing countries of Latin America, Eastern and Southern Africa. It has been grown and consumed worldwide in distinct areas and different seasons, mainly by subsistence level farmers with low-technology input as well as by farmers that use high input technologies (Wortmann et al. 1998; Broughton et al. 2003). Dry bean cultivars were grown on approximately 30 million hectares in more than 120 countries in 2012, but their yields are quite low compared to other important grain legumes such as soybean and peas (http://faostat.fao.org). In Brazil, the main producer and consumer country, *P. vulgaris* dry bean is a very popular and relevant crop, representing the major source of dietary protein. It is grown across all edafoclimatic areas of the country, with sowing dates happening almost every month. Its per capita consumption can be as high as 17 kg per year (Feijão 1985-2012). The total growing area in 2012 was 1.78 million ha with a mean productivity of 1,406 kg/ha (Feijão 1985-2012).

One of the several factors that compromising the common bean yield worldwide is the high number of destructive diseases that attack the crop and cause serious damage. Among these diseases is the common bean rust, incited by the basidomycete fungus *Uromyces appendiculatus* F. Strauss (syn. *U. phaseoli* G. Winter), which can cause great yield losses. This fungus is a highly variable and is among the most pathogenically variable of all plant pathogens (Stavely and Pastor-Corrales 1989).

Bean rust is distributed around the world, but it effectively causes major production problems in humid tropical and subtropical areas. Severe epidemics have been reported in Australia, China, the United States, and some areas of Europe. Major losses have occurred in Burundi, Ethiopia, Kenya, Malawi, Rwanda, South Africa, Tanzania, Uganda, and Zimbabwe. In Latin America, the bean rust is also a serious problem, major losses occurred in Argentina, Bolivia, Brazil, Colombia, Costa Rica, Cuba, Dominican Republic, Ecuador, El Salvador, Guatemala, Haiti, Honduras, Jamaica, Mexico, Nicaragua, and Peru (Stavely and Pastor-Corrales 1989; Souza et al. 2008). In Brazil, the disease causes major losses in south, southeast and central areas, including the states of Paraná, Rio Grande do Sul, Santa Catarina, Minas Gerais, São Paulo, and Goiás (Souza et al. 2005). Rust losses worldwide measured in greenhouse and field conditions can vary from 18 to 100 % (Stavely and Pastor-Corrales 1989; Staples 2000). Yield losses higher than 68 % were detected in Brazil (Souza et al. 2008).

The integrated management of the common bean rust is based on three main strategies: application of fungicides, host resistance, and various cultural practices. The use of resistant cultivars is certainly the main component of the integrated bean rust management. The pyramiding of different race-specific resistance genes is an important strategy for developing broad and durable resistance to a large number of *U. appendiculatus* isolates (Souza et al. 2008; Ragagnin et al. 2009).

Gene pyramiding approaches using only conventional breeding methods may not be effective mainly due to the difficulties in selecting genotypes harboring different resistance genes. For this reason, molecular markers are often used to aid gene pyramiding process. With the use of molecular markers, the multiple and/or sequential inoculations can be avoided and selection mistakes caused by confounding phenotypic effect of potential epistatic interactions among the different resistance genes present in the same genotype can also be overcome (Michelmore 1995; Bigirimana and Höfte 2001; Singh et al. 2001; Toenniessen et al. 2003). However, for each resistance allele a specific marker or markers need to be identified. Several RAPD markers associated with genes conferring resistance to rust in common bean have been identified and some of them were converted into SCAR markers to increase the reproducibility of the markers (Souza et al. 2008). Currently, single DNA base differences, referred as single nucleotide polymorphisms (SNPs), and small nucleotide insertions and deletions (InDels), are being detected and used for marker-assisted selection in common bean, in addition to genes and quantitative trait locus (QTL) discovery, assessment of genetic diversity, association analysis, and phylogeny studies. SNP markers are more stable (lower mutation rate) than other DNA marker classes, they are the most abundant form of genetic variation within genomes and can be analyzed using a wide array of high throughput technologies (Souza et al. 2012a).

Molecular markers can also be used to accelerate the recovery of the recurrent parent's genome in backcross breeding programs. Simulation studies and

real data indicate that only three or four backcrosses are necessary to recover the recurrent parent's genome when molecular markers are used (Openshaw et al. 1994; Faleiro et al. 2004; Ragagnin et al. 2009). Separate backcross programs assisted by molecular marker fingerprinting can be used for the individual introgression of resistance genes in modern cultivars. This strategy can be the initial step for pyramiding of rust resistance alleles.

The main goal of the present work was to develop high-yielding "carioca" seeded dry bean advanced lines harboring the rust resistance genes *Ur-5*, *Ur-11* and *Ur-14*. "Carioca" seeded beans are the most consumed in Brazil, representing around 70 % of the internal market.

Materials and methods

Plant genetic material

The Mesoamerican "carioca" seeded common bean cultivar 'Rudá' was used as recurrent parent in all backcrosses. "Carioca" seeded beans are the most consumed in Brazil. 'Rudá' is a high yield cultivar recommended for many growing regions, but it is susceptible to rust. 'Mexico 309' and 'BelMiDak RR-3' (derived from 'PI181996') were used as donor parents. They are Mesoamerican bean lines harboring the genes *Ur-5* and *Ur-11*, respectively, that confer ample resistance to bean rust in Brazil (Souza et al. 2005; 2008) and other parts of the world (Stavely 2000; Steadman et al. 2002). The common bean cultivar 'BRSMG Pioneiro', derived from the cross 'Rudá' × 'Ouro Negro' by molecular marker-assisted backcrosses (Faleiro et al. 2004), was also used as donor parent in the gene pyramiding process. This "carioca" seeded cultivar harbors the gene *Ur-14* from 'Ouro Negro' (Moreira et al. 2006; Souza et al. 2011). It has shown to be resistant to several isolates of *U. appendiculatus* and *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara, the causal agent of bean anthracnose (Faleiro et al. 2004).

Backcrosses and pyramiding process

All crosses were conducted under greenhouse conditions. Two separate backcross (BC) programs were conducted until BC_3 generations using 'Rudá' as recurrent parent and the lines 'Mexico 309' and 'BelMiDak RR-3' as donor parents. At each BC cycle, the plants were screened with the pathogen and the genetic distances between the resistant plants and the recurrent parent were determined using RAPD markers. The plants most similar to 'Rudá' were used for the next BC cycle. The BC_3F_1 plants selected based on the cited criteria were selfed to obtain the BC_3F_2 population that was inoculated with *U. appendiculatus* and screened with molecular markers linked to the genes of interest (Table 1). The resistant BC_3F_2 plants presenting the DNA markers were selfed to obtain $BC_3F_{2:3}$ progenies which were submitted to progeny tests based on inoculation with the pathogen. $BC_3F_{2:3}$ progenies homozygous for resistance to rust were selected for the pyramiding process (Fig. 1). This process was done using a tri-cross design consisted basically of two steps: step 1—pairwise crosses among the $BC_3F_{2:3}$ resistant progenies to obtain single-hybrids; and step 2—crosses of the single-hybrids with the cultivar 'BRSMG Pioneiro' to obtain the triple-hybrids with the three resistance genes of interest. The triple-hybrid plants were screened with the coupling markers SI19 (gene *Ur-5*) and OPX11 (gene *Ur-14*) to confirm cross. Plants harboring the both markers were selfed to obtain the subsequent generations. The F_2, F_3 and F_4 plants derived from the triple-hybrids were also selected with molecular markers specifically linked to each one of the rust resistance genes, which were identified in a preliminary molecular characterization of the bean genetic material to be used for rust resistance gene pyramiding. $F_{4:5}$ seeds from the selected F_4 plants were obtained and used for seed increasing. $F_{4:5}$ plants were also used for progeny tests based on screening with molecular markers aiming to identify homozygous progenies for the rust resistance loci *Ur-5*, *Ur-11*, and *Ur-14* (Fig. 2). $F_{4:6}$ seeds from the selected progenies were sowed in the field for yield evaluation. The corresponding $F_{4:7}$ progenies were also evaluated for grain yield and aspect of grains in other growing season. These $F_{4:7}$ advanced lines (pyramid lines) were also tested for resistance to specific races of *U. appendiculatus* in greenhouse inoculations and screened with DNA markers (Fig. 2).

Inoculation of *U. appendiculatus* and disease evaluation

The Brazilian *U. appendiculatus* mono-pustule isolates used in this work were provided by the fungal

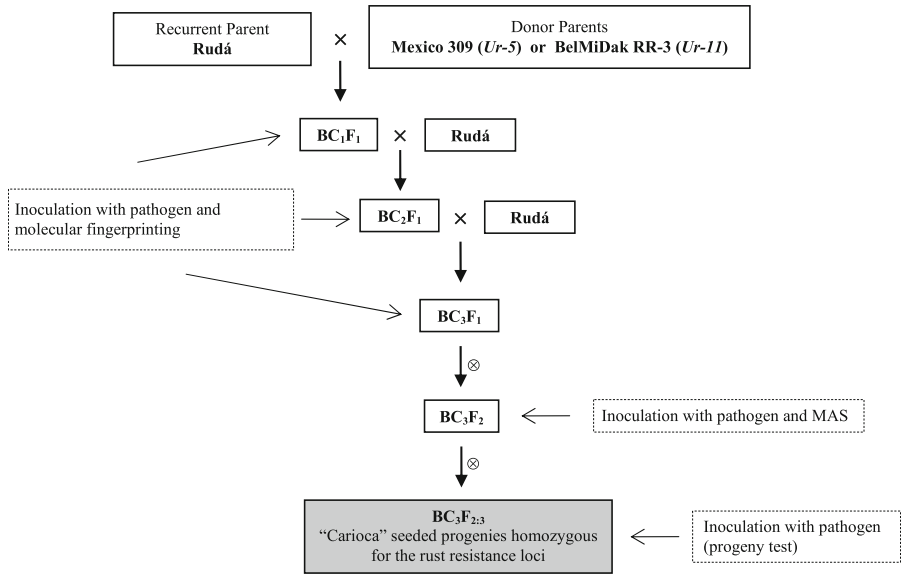

Fig. 1 Diagram representing the breeding strategy used to develop "carioca" seeded common bean progenies harboring individually different rust resistance genes at two separate backcross (BC) programs

collection of the BIOAGRO/UFV Common Bean Breeding Program (Viçosa, MG, Brazil). Artificial inoculations was carried out when the primary leaves of the bean plants reached approximately two-thirds of their full development, about 10 days after sowing under greenhouse conditions (20 ± 5 °C), as described by (Souza et al. 2005). Each one of the BC_nF_1 and BC_3F_2 plants and 12 plants from each tested progeny ($BC_3F_{2:3}$ progenies and $F_{4:7}$ pyramid lines) and control cultivar were inoculated (Figs. 1, 2). Plants derived from the separate backcross programs using 'Mexico 309' (gene *Ur-5*) and 'BelMiDak RR-3' (gene *Ur-11*) as donor parents were inoculated with a mixture of the *U. appendiculatus* races 29-3, 61-3, 63-3 and 63-19. The 16 $F_{4:7}$ pyramid lines and control cultivars were screened with the races 21-3, 29-15 and 53-3, used for specifically detecting the genes *Ur-5*, *Ur-11* and *Ur-14*, respectively.

The inoculum concentration was 2.0×10^4 uredospores/mL of distilled water containing Tween-20 (0.05 %, v:v). The inoculum solution was sprayed on both leaf surfaces using a manual atomizer (De Vilbiss n° 15) adapted to an electric compressor. After inoculation the plants were transferred to a mist chamber (20 ± 1 °C and relative humidity >95 %) where they were kept for approximately 48 h under a 12-h light regime. In order to avoid contamination, plants inoculated with different isolates were kept in separate compartments of the mist chamber. After this period the plants were transferred to a greenhouse (20 ± 5 °C), where they were kept until symptom evaluation, about 15 days after the inoculation.

Six grades of rust reaction were considered in the evaluation of the disease symptoms based on the scale proposed by Stavely et al. (1983). The description of each grade as well as the additional interpretative symbols associated with the rust grading scale is shown on Table 2. In all cases disease reaction was determined visually by at least two evaluators.

DNA marker analysis

The DNA was extracted from the leaf tissue samples according to Doyle and Doyle (1990). In each BC cycle, a screening was done to identify polymorphic RAPD markers (Operon Technologies, Alameda, CA, USA) to be used for molecular fingerprinting analysis aiming to select plants genetically similar to 'Rudá'. The DNA amplification by PCR-RAPD technique was done according to Faleiro et al. (2000). The primers that revealed at least one polymorphic DNA band among the analyzed plants were selected. The polymorphic and monomorphic DNA bands were used to build a matrix based on the presence (1) or absence (0)

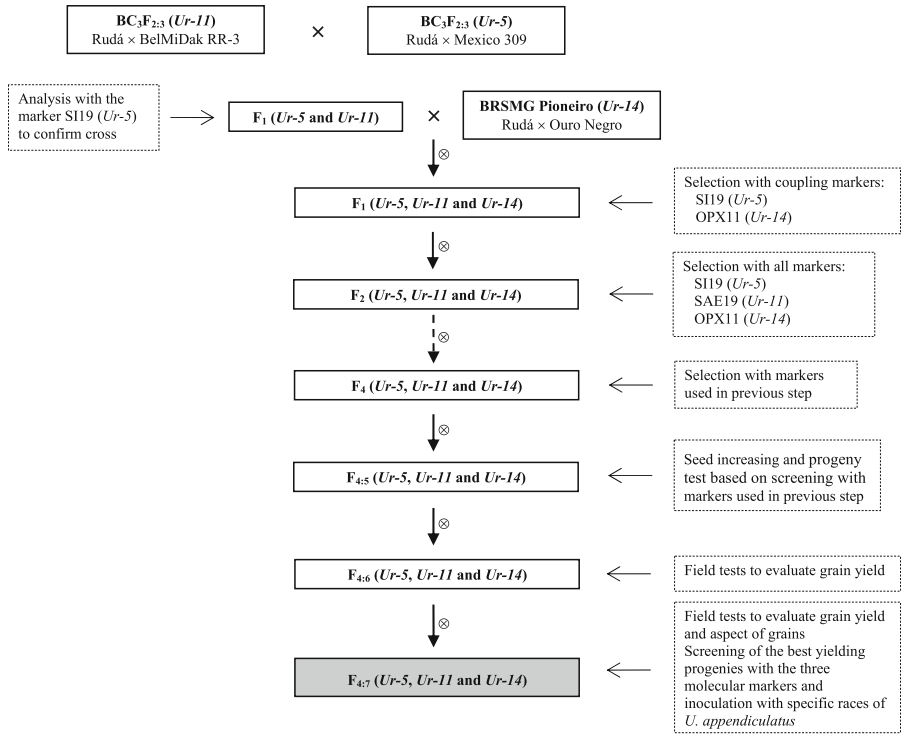

Fig. 2 Diagram representing the breeding strategy used to develop "carioca" seeded common bean elite lines (*pyramid lines*) harboring simultaneously three distinct rust resistance genes

of bands. The genetic similarity values were calculated by the Euclidian method for binary data and used to cluster the plants by the nearest neighbor method with the aid of the statistical genetics software GENES (Cruz 2013).

For the molecular marker-assisted selection, including the progeny test to identify $F_{4:5}$ pyramid progenies homozygous for the rust resistance loci, DNA markers specifically linked to each one of the resistance genes were used. In the assays with the SCAR technique, the DNA amplification reactions were as described by Côrrea et al. (2000). The temperatures of specific annealing for each marker are described in Table 1.

The amplified products were visualized under UV light after electrophoresis on 1.2 % agarose gels containing ethidium bromide (0.2 µg/mL), immersed in a 1X sodium boric acid (SB) buffer (10 mM sodium hydroxide, pH adjusted to 8.5 with boric acid).

Agronomic performance assays

Two field experiments were conducted at experimental station of the Federal University of Viçosa, in Coimbra, MG, Brazil, to evaluate the pyramid lines: one in the autumn of 2007 ($F_{4:6}$ progenies) and other one in the winter/spring of 2007 ($F_{4:7}$ progenies). In the first experiment the only trait evaluated was grain production. Besides this trait, aspect of grains was also evaluated in the second experiment. Both experiments included: pyramid progenies identified as homozygous for all rust resistance loci by the progeny test based on molecular markers (Fig. 2); "carioca" seeded progenies harboring one or two rust resistance genes according to marker analysis or inoculation tests, derived from the BC and pyramid programs; and the control cultivars 'Rudá', 'BRSMG Pioneiro', 'BRSMG Majestoso', 'Pérola', 'BRSMG Talismã', and 'BRSMG Madrepérola'—"carioca" seeded beans high-yielding and widely planted in Brazil, 'Ouro Negro' and 'Mexico 309'—black seeded bean lines resistant to rust, and 'Vermelhinho'—red seeded bean cultivar.

In all experiments a randomized triple 10 × 10 lattice design was used. Each plot consisted of two rows each 2.0 m long, spaced by 0.5 m, with 15 seeds per meter. The equivalent of 350 kg/ha of the

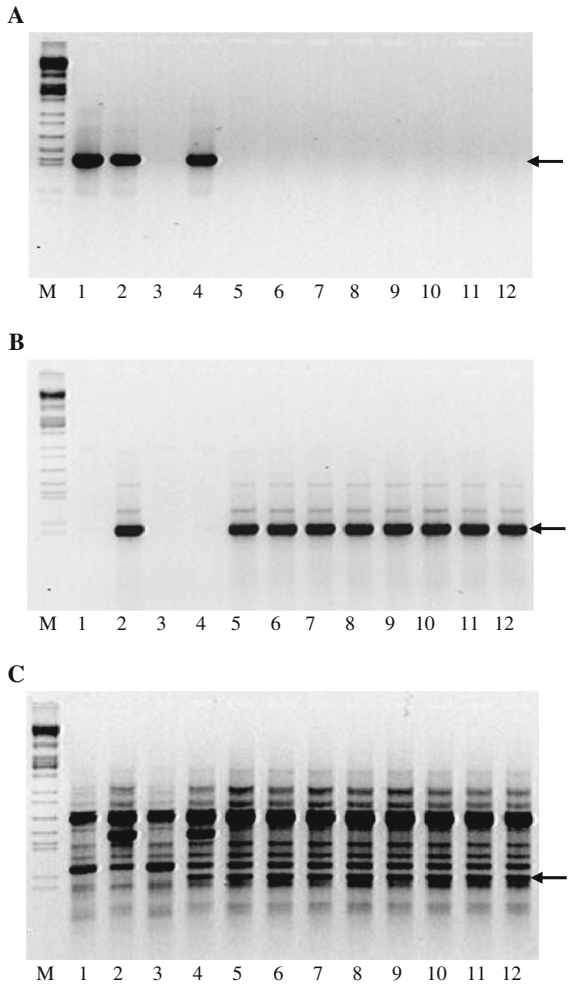

Fig. 3 Electrophoretic analysis on 1.2 % agarose gel of amplification DNA products obtained with the repulsion marker SAE19 (**a**), linked to gene *Ur-11*, and the coupling markers SI19 (**b**) and OPX11 (**c**), linked to genes *Ur-5* and *Ur-14*, respectively (Tables 1 and 4). *Lanes* are as follows: *M* lambda DNA cut with *Eco*RI, *Bam*HI and *Hind*III (size marker), *1* 'Rudá' (susceptible), *2* 'Mexico 309' (*Ur-5*), *3* 'BelMiDak RR-3' (*Ur-11*), *4* 'BRSMG Pioneiro' (*Ur-14*), *5–12* "carioca" seeded F_2 pyramid plants harboring alleles for the rust resistance loci *Ur-5*, *Ur-11*, and *Ur-14*. The *arrows* indicate polymorphic DNA bands associated with the resistance genes

formula 8-28-16 (N, P_2O_5 and K_2O, respectively) was used as fertilizer during sowing, and 150 kg/ha ammonium sulfate was applied 25 days after emergence. Harvest of the whole plot was done manually. The grain yield was determined for each plot and expressed as g/2 m^2 (g/plot). The aspect of grains was evaluated for visual observation using a five-grade scale as reported by Marques-Júnior et al. (1997). In this scale the grade 1 represents the standard "carioca" type wanted by the market (medium-sized light cream-colored grains with light brown stripes) and the grade 5 is the extreme opposite. At least two researchers evaluated the aspect of grains. The Tukey test was used to evaluate the significance of the differences among the mean values obtained for each trait.

Results

Development of pyramid lines assisted by molecular markers

Resistant BC_3F_1 plants derived from the separate backcross programs using 'Mexico 309' (gene *Ur-5*) and 'BelMiDak RR-3' (gene *Ur-11*) as donor parents were selected to be used for molecular fingerprinting analysis. The relative genetic similarities between recurrent genitor 'Rudá' and the resistant BC_3F_1 plants varied from 80.8 to 98.1 % ('Rudá' × 'Mexico 309') and from 82.1 to 98.2 % ('Rudá' × 'BelMiDak RR-3'). The number and the percentage of polymorphic DNA bands used to calculate these genetic similarity values were 39 and 48.7 %, and 39 and 41.0 %, respectively (Table 3).

The resistant BC_3F_1 plants which were genetically closest to 'Rudá' were conduced up to $BC_3F_{2:3}$ generation (Fig. 1). The "carioca" seeded $BC_3F_{2:3}$ progenies homozygous for the rust resistance loci were identified by inoculation with the *U. appendiculatus* races 21-3 ('Rudá' × 'Mexico 309', gene *Ur-5*) and 29-15 ('Rudá' × 'BelMiDak RR-3', gene *Ur-11*). The selected $BC_3F_{2:3}$ progenies were crossed pairwise to start the pyramiding process of resistance genes in the background 'Rudá'. The F_1 plants obtained were crossed with the "carioca" common bean cultivar 'BRSMG Pioneiro' (gene *Ur-14*). The triple-hybrid plants that presented the coupling markers SI19 and OPX11 were selfed and 189 F_2 seeds were obtained. DNA extracted from these F_2 plants was amplified with the molecular markers identified as specifically linked to each one of the rust resistance genes (Table 4), and eight F_2 plants were selected (Fig. 3) based on the marker info. These plants were selfed to obtain the next generations. For the selection of plants harboring all resistance genes to *U. appendiculatus* in subsequent generations, the presence of specific molecular markers (Table 4) was used as the selection

Table 1 DNA markers linked to the common bean rust resistance genes *Ur-5*, *Ur-11* and *Ur-14*

Marker[a]	Primer sequence (5′ → 3′)	Resistance gene	Chr[b]	Source	Product size (bp)	Distance (cM)	AT (°C)	Reference
OPF10	GGAAGCTTGG	*Ur-5*	Pv4	B-190	970	2.1 (C)	35	Haley et al. (1993)
OPAC20	ACGGAAGTGG	*Ur-11*	Pv11	PI181986	490	NR (C)	35	Johnson et al. (1995)
OPX11	GGAGCCTCAG	*Ur-14*	Pv4	Ouro Negro	550	5.8 (C)	35	Faleiro et al. (2000), Souza et al. (2011)
SI19	F-AATGCGGGAGTTCAATAGAAAAACC	*Ur-5*	Pv4	B-190	460	NR (C)	53	Melloto and Kelly (1998)
				Mexico 309		3.3 (C)	50	Souza et al. (2007)
	R-AATGCGGGAGATATTAAAAGGAAAG							
SAE19	F-CAGTCCCTGACAACATAACACC	*Ur-11*	Pv11	BelMiDak RR-3	890	1.0 (R)	58	Queiroz et al. (2004)
	R-CAGTCCCTAAAGTAGTTTGTCCCTA							
SF10	F-GGAAGCTTGGTGAGCAAGGA	*Ur-14*	Pv4	Ouro Negro	1,050	4.3 (C)	65	Corrêa et al. (2000), Souza et al. (2011)
	R-GGAAGCTTGGCTATGATGGT							
SBA08	F-CCACAGCCGACGGAGGAG	*Ur-14*	Pv4	Ouro Negro	560	6.0 (C)	65	Corrêa et al. (2000), Souza et al. (2011)
	R-GCCATGTTTTTTGTCCCC							

NR no recombinants, linkage phase of the molecular marker: coupling (C) and repulsion (R); *AT* annealing temperature

[a] RAPD (OP) and SCAR (S) markers

[b] Chromosome or linkage group based on the integrated common bean genetic and chromosomal map (Pedrosa-Harand et al. 2008)

Table 2 Common bean rust grading scale with the additional interpretative symbols established at the 1983 Bean Rust International Workshop (Stavely et al. 1983)

Grade[a]	Definition	Symbol
1	Immune, having no visible symptoms	I
2	Necrotic or chlorotic spots, without sporulation, and less than 0.3 mm in diameter	HR
2+	Spots, without sporulation, 0.3–1.0 mm diameter	HR
2++	Spots, without sporulation, 1.0–3.0 mm diameter	HR
2+++	Spots, without sporulation, greater than 3.0 mm diameter	HR
3	Uredia less than 0.3 mm diameter	R
4	Uredia 0.3–0.5 mm diameter	MR
5	Uredia 0.5–0.8 mm diameter	MS
6	Uredia larger than 0.8 mm diameter	S

I immune; *HR* hypersensitive or highly resistance; *R* resistance, reactions having any of the grades 2 with grade 3 present or predominant with some grade 4; *MR* moderately resistance, grade 4 predominant and no grade 5 uredia; *MS* moderately susceptible, uredia larger than grade 4, but none larger than grade 5; *S* susceptible, grade 6 uredia (Stavely and Pastor-Corrales 1989)

[a] When several reaction grades are present as evaluation results, they are recorded in order of predominance

criterion (Fig. 2). Following this methodology it was possible to obtain 16 $F_{4:5}$ progenies phenotypic and genotypically similar to the recurrent cultivar 'Rudá' and non-segregating for the DNA markers associated to the rust resistance genes.

Agronomic performance evaluation

The 16 selected $F_{4:5}$ pyramid progenies were multiplied in the field to obtain enough seeds for evaluation of agronomic traits. The corresponding $F_{4:6}$ progenies and 75 "carioca" seeded progenies harboring one or two rust resistance genes derived from the backcross and pyramid programs, in addition to nine control cultivars, were tested for grain yield during the autumn of 2007. The resulting 16 $F_{4:7}$ pyramid lines and the other 84 treatments were also evaluated for grain yield and aspect of grains during the winter/spring of 2007. The *F*-statistic ($P < 0.01$) indicated that there was genetic variability for both evaluated traits among the analyzed common bean genetic material. The mean values for grain yield of the $F_{4:7}$ pyramid lines did not differ statistically in relation to 'Rudá' and other "carioca" seeded control cultivars high yielding and widely grown in Brazil. These lines also shown aspect of grains statistically similar to that presented by recurrent parent 'Rudá' (Table 5).

Rust resistance tests

The 16 $F_{4:7}$ pyramid lines and control cultivars were screened with DNA markers specifically linked to each one of the rust resistance genes (Table 4): the

Table 3 Parameters used to estimate the relative genetic similarity between the recurrent genitor 'Rudá' and BC_2F_1 and BC_3F_1 plants resistant to common bean rust

Parameter	Cross/generation			
	Rudá × Mexico 309 (*Ur-5*)		Rudá × BelMiDak RR-3 (*Ur-11*)	
	BC_2F_1	BC_3F_1	BC_2F_1	BC_3F_1
Proportion of resistant plants	6/22	7/33	5/18	7/22
Number of RAPD primers randomly selected	13	13	13	13
Total number of evaluated DNA bands[a]	93	80	69	95
Number of polymorphic bands	35	39	36	39
Percentage of polymorphic bands	37.6 %	48.7 %	52.2 %	41.0 %
Expected genetic similarity[b]	87.5 %	93.7 %	87.5 %	93.7 %
Average genetic similarity	89.4 %	90.2 %	86.1 %	90.7 %
Range of genetic similarity	86.8–94.3 %	80.8–98.1 %	75.8–97.0 %	82.1–98.2 %

[a] PCR discrete products easily visualized

[b] Estimator: $[1 - (0.5)^{n+1}] \times 100$, where 'n' is the number of backcross cycles

Table 4 Molecular characterization of the common bean genetic material used for rust resistance gene pyramiding using DNA markers linked to the resistant gene

Genetic material	Resistance gene	Molecular marker (resistance gene)[a]						
		$OPX11_{550}$ (*Ur-14*)[b]	$OPF10_{970}$ (*Ur-5*)	$OPAC20_{490}$ (*Ur-11*)	$SF10_{1.050}$ (*Ur-14*)	$SBA08_{560}$ (*Ur-14*)	$SI19_{460}$ (*Ur-5*)[b]	$SAE19_{890}$ (*Ur-11*)[b]
$BC_3F_{2:3}$ plants (Rudá × Mexico 309)	*Ur-5*	0	1	*1*	*1*	*1*	1	1
$BC_3F_{2:3}$ plants (Rudá × BelMiDak RR-3)	*Ur-11*	0	0	1	0	0	0	0
Rudá	–	0	0	0	0	0	0	1
Mexico 309	*Ur-5*	0	1	*1*	*1*	*1*	1	1
BelMiDak RR-3	*Ur-11*	0	0	1	0	0	0	0
BRSMG Pioneiro	*Ur-14*	1	*1*	*1*	1	1	0	1

[a] Presence (1) or absence (0) of DNA marker. Unexpected results (false-positive results) are highlighted in italics

[b] DNA marker selected to assist the introgression of its associated rust resistance gene into cultivar 'Rudá' once it shown to be specifically linked to its tagged gene and polymorphic in relation to 'Rudá'

coupling markers SI19 (*Ur-5*) and OPX11 (*Ur-14*), and the repulsion marker SAE19 (*Ur-11*). In addition, these lines were also screened with specific races of *U. appendiculatus* used for phenotypically detecting each one of the rust resistance genes. The races 21-3, 29-15 and 53-3 of the rust pathogen provided by the fungal collection of the BIOAGRO/UFV can specifically detecting the genes *Ur-5*, *Ur-11* and *Ur-14*, respectively. This is because they elicit hypersensitive or highly resistance reaction (HR, necrotic spots without sporulation) only in plants presenting those genes.

All 16 pyramid lines presented the expected molecular profile based on the DNA marker screening using the rust resistance parents and the recurrent cultivar 'Rudá' as standards (Table 6). In addition to the pyramid lines, only 'Mexico 309' presented the coupling marker SI19 linked to *Ur-5*. DNA marker OPX11, linked in coupling phase to the gene *Ur-14*, was also presented by the 16 pyramid lines and only by the control cultivars 'Ouro Negro' and 'BRSMG Pioneiro', both harboring *Ur-14*. All pyramid lines and only the common bean lines 'BelMiDak RR-3' and 'PI181996', both presenting the gene *Ur-11*, did not present the marker SAE19 linked in repulsion to *Ur-11*.

Out of the 16 $F_{4:7}$ pyramid lines, nine lines were selected as indeed harboring the genes *Ur-5*, *Ur-11* and *Ur-14*, once they presented HR reaction to all tested races of *U. appendiculatus*. The resistance spectra presented by these selected lines (TL-006, TL-009, TL-015, TL-016, TL-026, TL-032, TL-034, TL-035 and TL-037) indicate that they possess the genes *Ur-5*, *Ur-11*, and *Ur-14* in homozygosis (Table 6). This is because no segregation has been observed in the 12 tested plants from each $F_{4:7}$ pyramid line and control cultivar. Lines TL-005, TL-011, TL-012 and TL-031 presented HR reactions to the races 21-3 and 29-15, confirming the presence of the genes *Ur-5* and *Ur-11*, respectively. Lines TL-039 and TL-041 presented HR reactions to the races 21-3 and 53-3, detecting the genes *Ur-5* and *Ur-14*, respectively. The other line (TL-038) shown harboring the genes *Ur-11* and *Ur-14*, once it presented HR reaction respectively to the races 29-15 and 53-3 of *U. appendiculatus* (Table 6).

Except line TL-038, all others presented the gene *Ur-5* based on the rust pathogen inoculation test. Only two lines did not present the gene *Ur-11*: TL-039 and TL-041. The lines TL-005, TL-011, TL-012 and TL-031 shown did not harbor the gene *Ur-14* (Table 6).

Discussion

Resistance to pathogens is one of the main focuses of common bean breeders worldwide. The large number of diseases affecting this crop is a significant cause of the low yield observed in many dry bean growing regions. Among these diseases is the bean rust (Stavely and Pastor-Corrales 1989; Steadman et al. 2002; Souza et al. 2005, 2008, 2011).

Table 5 Agronomic performance of "carioca" seeded pyramid lines and control cultivars in relation to grain yield (autumn and winter/spring of 2007) and aspect of grains (winter/spring of 2007), Coimbra, MG, Brazil

Pyramid line/control cultivar	Market class	Grain yield (g/2 m^2)			Aspect of grains (scale 1–5)[a]
		Autumn 2007	Winter/spring 2007	Joint analysis	
TL-005	Carioca	870.39ab	665.94abcde	768.16ab	2.80cdef
TL-006	Carioca	906.98ab	799.86abc	853.42a	2.93cdef
TL-009	Carioca	927.78ab	626.82bcde	777.30ab	3.13defg
TL-011	Carioca	819.05ab	711.10abcd	765.08ab	2.83cdef
TL-012	Carioca	928.98ab	741.37abcd	835.17a	3.00cdef
TL-015	Carioca	883.53ab	749.76abcd	816.65a	3.03cdefg
TL-016	Carioca	847.52ab	759.17abcd	803.35a	2.70bcde
TL-026	Carioca	783.47ab	740.05abcd	761.76ab	2.73cde
TL-031	Carioca	955.72a	780.22abc	867.97a	3.00cde
TL-032	Carioca	888.08ab	762.50abcd	825.29a	2.83cdef
TL-034	Carioca	905.43ab	752.36abcd	828.89a	2.97cde
TL-035	Carioca	778.49ab	761.24abcd	769.87ab	3.07cdefg
TL-037	Carioca	889.84ab	638.69bcde	764.26ab	3.07cdefg
TL-038	Carioca	930.52ab	759.69abcd	845.11a	2.83cdef
TL-039	Carioca	863.25ab	680.46abcde	771.86ab	3.23defg
TL-041	Carioca	868.40ab	710.47abcd	789.43ab	2.87cdef
Rudá	Carioca	847.62ab	666.24abcde	756.93ab	3.20defg
BRSMG Pioneiro	Carioca	873.31ab	818.21abc	845.76a	2.90cdef
BRSMG Majestoso	Carioca	764.80ab	458.90ef	611.85ab	2.47bc
Pérola	Carioca	817.90ab	615.93cdef	716.92ab	2.07ab
BRSMG Talismã	Carioca	762.29ab	392.09f	577.19ab	2.50bcd
BRSMG Madrepérola	Carioca	803.21ab	873.85a	838.53a	1.50a
Ouro Negro	Black	750.56ab	612.24cdef	681.40ab	5.00h
Mexico 309	Black	678.83b	551.18def	615.00ab	5.00h
Vermelhinho	Red	556.02b	344.40f	450.21c	5.00h

Means followed by the same letter do not differ by the Tukey test at 5 % probability

[a] 1 = standard "carioca" type wanted by the market, medium-sized light cream-colored grains with light brown stripes; and 5 = extreme opposite (Marques-Júnior et al. 1997)

In this work we report a gene pyramiding approach assisted by DNA markers to develop "carioca" seeded high yielding common bean elite lines harboring simultaneously the genes *Ur*-5, from 'Mexico 309'; *Ur-11*, from 'BelMiDak RR-3'; and *Ur-14*, from 'BRSMG Pioneiro', a "carioca" seeded cultivar derived from the cross 'Rudá' × 'Ouro Negro'. The Brazilian black seeded cultivar 'Ouro Negro' is the original source of the gene *Ur-14* (Faleiro et al. 2004; Souza et al. 2011), and the line 'PI181996' is the original source of *Ur-11* (Souza et al. 2002). "Carioca" seeded beans are the most consumed in Brazil, representing around 70 % of the internal market. The developed resistance gene pyramid can potentially present a wide and durable resistance to the rust disease in Brazil and other world areas, e.g. Latin America and Africa. The reason is that it results in a HR type of resistance reaction when bean plants presenting it are challenged with the majority of the *U. appendiculatus* races identified in the above-mentioned locations (Stavely and Pastor-Corrales 1989; Steadman et al. 2002; Souza et al. 2005, 2008).

The concept of molecular marker-assisted breeding was successfully used in this work. The development of two separate backcross programs assisted by RAPD-PCR fingerprinting allowed the individual

Table 6 Rust resistance reaction and molecular characterization of pyramid lines and control cultivars

$F_{4:7}$ pyramid line/Control cultivar	DNA marker (Resistance gene)[a]			Race of *U. appendiculatus* (Resistance gene)[b]			Resistance gene[c]
	$SI19_{460}$ (*Ur-5*)	$SAE19_{890}$ (*Ur-11*)	$OPX11_{550}$ (*Ur-14*)	21-3 (*Ur-5*)	29-15 (*Ur-11*)	53-3 (*Ur-14*)	
TL-005	1	0	1	HR	HR	R	*Ur-5*, *Ur-11*
TL-006	1	0	1	HR	HR	HR	*Ur-5*, *Ur-11*, *Ur-14*
TL-009	1	0	1	HR	HR	HR	*Ur-5*, *Ur-11*, *Ur-14*
TL-011	1	0	1	HR	HR	R	*Ur-5*, *Ur-11*
TL-012	1	0	1	HR	HR	R	*Ur-5*, *Ur-11*
TL-015	1	0	1	HR	HR	HR	*Ur-5*, *Ur-11*, *Ur-14*
TL-016	1	0	1	HR	HR	HR	*Ur-5*, *Ur-11*, *Ur-14*
TL-026	1	0	1	HR	HR	HR	*Ur-5*, *Ur-11*, *Ur-14*
TL-031	1	0	1	HR	HR	R	*Ur-5*, *Ur-11*
TL-032	1	0	1	HR	HR	HR	*Ur-5*, *Ur-11*, *Ur-14*
TL-034	1	0	1	HR	HR	HR	*Ur-5*, *Ur-11*, *Ur-14*
TL-035	1	0	1	HR	HR	HR	*Ur-5*, *Ur-11*, *Ur-14*
TL-037	1	0	1	HR	HR	HR	*Ur-5*, *Ur-11*, *Ur-14*
TL-038	1	0	1	R	HR	HR	*Ur-11*, *Ur-14*
TL-039	1	0	1	HR	R	HR	*Ur-5*, *Ur-14*
TL-041	1	0	1	HR	R	HR	*Ur-5*, *Ur-14*
Mexico 309	1	1	0	HR	S	R	*Ur-5*
BelMiDak RR-3	0	0	0	R	HR	R	*Ur-11*
PI181996	0	0	0	R	I	R	*Ur-11*
Ouro Negro	0	1	1	R	R	HR	*Ur-14*
BRSMG Pioneiro	0	1	1	R	R	HR	*Ur-14*
Rudá	0	1	0	S	S	S	-
US Pinto 111	0	1	0	S	S	S	-

Pyramid lines selected as harboring simultaneously the rust resistance genes *Ur-5*, *Ur-11* and *Ur-14* are highlighted in grey

I immune, no visible symptoms; *HR* hypersensitive or highly resistance, necrotic spots without sporulation; *R* resistance, sporulating lesions (uredias) less than 0.3 mm in diameter; *MR* moderately resistance, uredias with 0.3–0.5 in diameter; *MS* moderately susceptible, uredias with 0.5–0.8 in diameter; *S* susceptible, uredias larger than 0.8 mm in diameter (Table 2)

[a] Presence (1) or absence (0) of DNA marker

[b] Results based on evaluation of 12 plants

[c] Rust resistance genes presented by the pyramid lines and control cultivars detected or confirmed based on reaction elicited by specific races of *U. appendiculatus*

introgression of the *Ur-5* and *Ur-11* genes into cultivar 'Rudá' after only three BC cycles. In addition, molecular markers specifically linked to the three target genes were identified and/or validated during the breeding process (Fig. 1; Tables 1 and 4). These markers were effectively used to aid the pyramiding process and the development of advanced lines harboring simultaneously all resistance genes in homozygosis. The use of the DNA markers reduced the time and cost of pyramiding the rust resistance genes into the commercial "carioca" seeded cultivar 'Rudá'. The efficiency of the markers was checked by inoculation with specific races of the rust pathogen in the end of the breeding process (Table 6). Yield evaluations of the advanced lines demonstrated that no grain yield penalty in relation to recurrent progenitor was observed after the pyramiding process.

Marker-assisted selection strategy applied in this work presented an efficiency estimated at 56.25 %; out of the 16 pyramid lines selected as potentially harboring all the alleles of interest based on DNA markers, nine lines shown HR resistance when tested with specific races of *U. appendiculatus* used for phenotypically detecting each one of the rust resistance genes (Table 6). Considering these same pyramid lines and selection using marker SI19 (*Ur-5*), the efficiency was 93.75 %; one line among 16 did not present HR reaction to the race 21-3 (*Ur-5*). Selection efficiency of the marker SAE19 (*Ur-11*) was 87.50 %, once two lines did not present HR reaction to the race 29-15 (*Ur-11*). Regarding to the marker OPX11 (*Ur-14*), the selection efficiency was 75.00 %, once four lines among 16 did not show HR reaction when tested with the race 53-3 (*Ur-14*) of *U. appendiculatus* (Table 6). These results could be explained by the imperfect linkage between the markers and the rust resistance loci *Ur-5*, *Ur-11* and *Ur-14*, although the frequencies of recombinants are higher than those previously estimated using biparental segregating populations (Table 1). An imperfect linkage exists when there is a certain genetic distance between the marker and the target gene. Being so, there are still a lot of room, opportunity, and need to develop most precise and effective marker-assisted selection tools to be adopted by the common bean breeding programs for cultivar development with multiple and wide disease resistance.

In future research activities of the Embrapa common bean breeding program, the "gene pyramid" formed by the rust resistance genes *Ur-5*, *Ur-11* and *Ur-14* will be transferred from the lines developed in this work to modern cultivars and elite lines from different market classes consumed in Brazil, such as "carioca", black, red, and "rajado" (medium-sized cranberry-sugar bean). In addition, it could also be transferred to white seeded elite lines, cranberry-sugar bean, dark red kidney, light red kidney and calima, targeting the international market. New rust resistance sources are also being tested and added to Embrapa breeding program to widen the basis of the present resistance gene pyramid. To be effective, the gene pyramiding strategy must be a continuous effort. Special focus is being done on the identification of effective rust resistance genes from Andean common bean gene pool. The reason is that combining resistance genes from different gene pools could widen the resistance spectra to a higher number of *U. appendiculatus* races.

Although the gene pyramiding is not fully accepted by the scientific community as a efficient breeding strategy to develop wide and durable resistance to pathogens, there are experimental evidence demonstrating that it confers more effective resistance to a cultivar than that conferred by the sum of the resistance present by its parents (Yoshimura et al. 1995; Huang et al. 1997; Singh et al. 2001). According to Schafer and Roelfs (1985), the probability that a pathogen will overcome a gene pyramid of more than two genes is extremely low. In order for this to happen, independent mutations in the pathogen genome must occur and they should be combined in the same genetic background, or they could occur simultaneously or sequentially in the genome of a specific isolate of the pathogen. Thrall and Burdon (2003) also report epidemiology data that support the use of gene pyramiding as an effective strategy for disease control. According these authors, there is an inverse correlation between pathogen fitness and the number of avirulence genes present in its genome. By studying the pathosystem *Melampsora lini-Linum marginale* in Australia, these authors observed that natural pathogen populations which were able to infect a greater number of host populations were less aggressive than pathogen populations which were able to infect a lower number of host populations. That means that the inactivation of several avirulence genes in the pathogen compromises its adaptability. In other words, it indicates that gene pyramiding can potentially keep

the disease below an economical damage level and also prevent its fast dissemination.

The use of recurrent selection assisted by molecular markers as a breeding strategy to combine different target alleles into a same progeny presenting other desirable agronomic traits and still uses this and other superior progenies to form a promissory new base population has also been adopted by the Embrapa breeding program (Souza et al. 2012b). This strategy could be very useful to develop high agronomic performing and modern common bean cultivars harboring multiple rust resistance genes.

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