Theor Appl Genet (2006) 114:59–66
DOI 10.1007/s00122-006-0410-3

ORIGINAL PAPER

CAPs markers to assist selection for low vicine and convicine contents in faba bean (*Vicia faba* L.)

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Received: 7 April 2006 / Accepted: 6 September 2006 / Published online: 30 September 2006

Abstract The antinutritional factors (ANFs) present in *Vicia* spp. seeds are a major constraint to the wider utilization of these crops as grain legumes. In the case of faba bean (*Vicia faba* L.), a breeding priority is the absence vicine and convicine (v-c); responsible for favism in humans and for the reduced animal performance or low egg production in laying hens. The discovery of a spontaneous mutant allele named *vc-*, which induces a 10–20 fold reduction of v-c contents, may facilitate the process. However, the high cost and difficulty of the chemical detection of v-c seriously restricts the advances in breeding-selection. To identify random amplified polymorphic DNA (RAPD) markers linked to this gene, we have analysed an F_2 population derived from a cross between a line with high v-c content (Vf6) and the *vc-* genotype (line 1268). Quantification of v-c was done by spectrophotometry on the parents and the F_2 population ($n = 136$). By using bulked segregant analysis (BSA), two RAPD markers linked in coupling and repulsion phase to the allele *vc-* were identified and further converted into sequence characterized amplified regions (SCARs). Amplification of SCARS was more consistent, although the initial polymorphism between pools was lost. To recover the polymorphisms several approaches were explored. Restriction digestion with *HhaI* (for SCAR $SCH01_{620}$) and *RsaI* (for SCAR $SCAB12_{850}$) revealed clear differences between the parental lines. The simultaneous use of the two cleavage amplified polymorphism (CAP) markers will allow the correct fingerprinting of faba bean plants and can be efficiently used in breeding selection to track the introgression of the *vc-* allele to develop cultivars with low v-c content and improved nutritional value.

Communicated by D.A. Hoisington.

Electronic supplementary material Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s00122-006-0410-3 and is accessible for authorized users.

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Introduction

The faba bean (*Vicia faba* L.) is one of the oldest crops grown by man providing high-protein seeds for human and animal nutrition. In Europe, it is a traditional crop used as field beans (minor and equina types) for animal feeding and human food and as broad beans (major type) for direct human consumption. Together with peas, faba beans are alternative crops to cereals, maintaining soil fertility and breaking regular cycles of pests and diseases. The world production of faba beans is close to 4.5 millions of tons. Faba bean is the second most important legume crop in Europe, which accounts

for 14% of the world area and about 25% of the world production (FAOSTAT 2004).

Like other beans, faba bean seeds have high nutritional value. They are an excellent source of protein, carbohydrates, minerals, and fiber and supply high value complements in particular lysine and arginine to cereals in animal feeding (Friedman 1996). Legumes seeds also contain several antinutritional factors (ANFs), which negatively affect their digestibility. Together with condensed tannins, vicine and convicine are the ANFs of greatest concern in faba beans (Marquardt and Bell 1988).

Vicine and convicine (v-c) are two pyrimidine glycosides, which are present in the cotyledons of faba bean (Muduuli et al. 1982). They are hydrolyzed by the intestinal microflora (Frohlich and Marquardt 1983) to the highly reactive free radical generating compounds divicine and isouramil (Albano et al. 1984). These compounds have been associated with the aetiology of favism (Mager et al. 1965), an acute form of hemolytic anemia associated with the consumption of faba beans by humans carrying a genetic deficiency in of glucose-6-phosphate dehydrogenase in their red bloods cells. Moreover, dietary v-c has been shown to lower egg and yolk weights and egg production rate in laying hens (Campell et al. 1980) and the animal performance is reduced when faba beans are fed at levels higher than 15%. In broiler chicken, reduction of vicine and convicine contents significantly increases the apparent metabolizable energy value (Vilariño et al. 2006).

Both products can be strongly reduced by soaking the seeds in water or in a weak acid solution (Hegazy and Marquardt 1983) prior to cooking. However, as these glycosides are thermostable, removal or destruction by processing is difficult preventing their extensive use as commercial fodder. The cost and difficulty of the chemical detection of these products seriously restrict the advances in breeding-selection for cultivar development. A rapid spectrophotometric method applied on a two-seed sample is recommended for breeding programs aiming at the reduction of v-c content of faba beans seeds (Sixdenier et al. 1996). However, this is a destructive method that has limitations in early generations of the breeding process when the quantity of seed is restrictive.

After the analysis for v-c content in a large collection of faba bean genetic resources (Duc et al. 1999), the glycoside content showed a continuous variation and no relationship with other seed or plant characteristics (seed size, protein or tannin content, growth vigor, earliness, or disease susceptibility). A spontaneous mutant allele named *vc-* was discovered which induces a 10–20 fold reduction of vicine and convicine contents simultaneously. This mutation has been incorporated in breeding material in order to reduce v-c contents in cultivars registered in Europe (Duc et al. 1989). The *vc-* allele has an additive effect in relation to wild type, and the *vc* gene, was closely linked (10.1 cM) to the gene that controls the white hilum on seeds (Duc et al. 2004). White hilum is a recessive gene with maternal control (Sirks 1931) consequently; normal Mendelian ratios are expressed one generation later than expected, delaying the identification of individuals carrying the desired trait.

Molecular markers linked to desired traits are valuable tools for efficient selection of genotypes of interest. These markers allow precocious screening to be performed directly on DNA extracted from young leaves without waiting for the specific developmental stage at which the trait is expressed (e.g., complete seed ripeness in the present study). This leads to a reduction in both selection time and space, the advantage of which is clearly evident when the trait under selection requires the analysis of large numbers of genotypes or costly and difficult chemical analyses.

PCR-based RAPD (random amplifed polymorphic DNA) markers are extensively applied in many types of genetic studies because of their simplicity and ease of use. A number of problems associated with RAPD assays have been reported such as an occasional lack of reproducibility (He et al. 1994; Jones et al. 1997). To overcome this shortcoming and to improve their utility in marker assisted selection (MAS) applications, RAPDs can be transformed into SCARs (sequence characterized amplified regions) by sequencing the polymorphic DNA fragments and synthesizing two longer primers homologous to each end (Paran and Michelmore 1993). SCARs have the advantage of being allele specific and less sensitive to reaction conditions.

The aim of this study was the identification of RAPD markers linked to low v-c content and their conversion into SCARs for reliable and rapid screening of segregating populations. The availability of these markers will greatly facilitate the production of new faba bean cultivars free of ANFs for human and animal consumption.

Materials and methods

Plant material

Analysis was carried out in a population of 136 F_2 derived from a cross between an asynaptic line (Vf6) and the *vc-* genotype (line 1268). The maternal parent (Vf6) has colored hilum and high vicine-convicine con-

tent while *vc-* shows white hilum and low v-c content. The F_2 plants were selfed to the F_4 generation under insect-proof cages in order to infer their corresponding v-c content and genotype for hilum color.

DNA extraction and PCR analysis

Young leaves from the parental and the F_2 plants were used for DNA isolation according to Torres et al. (1993). RAPD analysis was as described by Williams et al. (1990) with slight modifications (Torres et al. 1993). A total of 800 RAPD primers from Operon Technologies and Sigma-Genosys were surveyed. Amplified products were separated in 2% agarose gels. Electrophoresis was performed at 105 V and maximum amperage for 3 h. Gels were stained with ethidium bromide and photographed under UV light using the software Kodak digital science 1D v 2.0 and 3.5.

Bulk segregant analysis (BSA)

The bulked segregant analysis technique (BSA) (Michelmore et al. 1991) was used to identify RAPD markers linked to low v-c content in faba bean. Molecular analyses were carried out using leaf tissue from the parents and the 136 F_2 plants. Homozygosity of F_2 plants for colored or white hilum to be used in the bulks was determined in their $F_{3:4}$ seeds. Quantification of v-c of the parents and the $F_{2:3}$ seeds were carried out by spectrophotometry at INRA, URLEG, Dijon, France. The analysis was performed in two seeds of both parental lines (Vf6 and 1268), as well as two seeds from each 136 $F_{2:3}$ families from the cross. The average value for v-c content in each family was inferred to the corresponding F_2 plant.

Based on these results, two contrasting bulks per trait were prepared, each containing equal amount of DNA from seven homozygous F_2 individuals with colored hilum and high content in v-c versus white hilum and low v-c content. Primers generating marker polymorphisms between the bulks were subsequently tested individually on DNA from each of the homozygous F_2 plants used in bulks. When the polymorphism was maintained, the primers were screened on the whole F_2 population for linkage analysis.

SCARs/CAPs development

RAPD fragments associated with v-c content were transformed into sequence-characterized amplified region (SCAR) markers (Paran and Michelmore 1993). RAPD fragments were separated on a 1.0% (w/v) low-melting- point agarose gel before being excised and purified by means of the QIAquick gel extraction kit (QIAGEN, Valencia, CA). The purified DNA was cloned into the pGEM-T vector system I (Promega Corporation, USA). Positive (white) colonies were confirmed by PCR. For each RAPD marker, plasmid DNA was purified from 20 transformed *E. coli* colonies. To fingerprint the colonies and corroborate that the inserts corresponded to the expected RAPD marker, the DNA was digested with four endonucleases (*ScrfI, DraI, BglII, Sau96I*). Inserts were sequenced in both forward and reverse orientations using a BigDye terminator cycle sequencing v 3.1 kit (PE Biosystems, Foster City, CA) on an ABI Prism 3100 Genetic analyzer apparatus (Applied Biosystems, Foster City, CA) at the Servicio de secuenciación automática de DNA, SCAI (University of Córdoba, Spain).

The consensus sequence from each RAPD marker was used to design SCAR primers synthesized by Sigma-Genosys (UK). They were first 21–27 nucleotides long, including the ten bases of the RAPD, with similar Tm values for each primer pair and were not susceptible to the formation of dimers and hairpins.

PCR amplifications were carried out with a TGradient thermocycler (Biometra, Goettingen, Germany) in 25 µl reaction volumes. Each PCR reaction contained 50–75 ng of plant genomic DNA, buffer (50 mM KCl, 75 mM Tris–HCl, 20 mM $(NH_4)_2SO_4$), 2 mM $MgCl_2$, 0.6 µM of each dNTP, 0.6 µM of primer ,and 0.6 units of Taq DNA polymerase (Biotools). The thermal profile for PCR was an initial denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 45 s, 65–70°C for 1 min, and 72°C for 1 min 30 s with a final extension at 72°C for 10 min. Amplifications products were electrophoresed in gels composed of a mixture of 1% agarose Seaken in TBE buffer.

To recover polymorphism between monomorphic SCARs, restriction enzyme digestion was performed in order to develop CAPs (cleaved amplified polymorphic sequences) For restriction assays, 10 µl PCR product were incubated overnight at 37°C in a volume of 25 µl with 3 U of a restriction enzyme, and subsequently resolved in a 2.5% agarose gel. Restriction with 12 enzymes was assayed. Finally, *HhaI* and *RsaI* (Fermentas, Life Sciences) were the enzymes used to generate polymorphisms.

Data analysis

ANOVA was performed to study the association of each molecular and morphological marker with the v-c content analysis. Markers with a probability level of $P < 0.001$ based on the F statistic were considered to

show a good association with the v-c content. RAPD markers, v-c content and hilum color were scored in the F_2 plants,$F_{2:3}$, and $F_{3:4}$ seeds, respectively and tested against the expected F_2 segregation ratio using a chi-square goodness of fit. The linkage map was constructed using MAPMAKER v. 3.0 (Lander et al. 1987). A LOD threshold of three and a maximum recombination fraction of 0.5 were employed as linkage criteria to establish the linkage group. Recombination fractions were converted to centimorgans (cM) using the Kosambi mapping function (Kosambi 1944).

Methods for chemistry measurement of vicine and convicine

The reference method was HPLC adapted according to Quemener (1988). HPLC was optimized for separation of v-c at 276 nm. Two seeds per parental line were soaked for 3.5 h in 30 ml water in a 90°C water-bath. Normal HCl (100 µl) was mixed with 10 ml of the resulting solution centrifuged at 13,000 rpm for 15 min and then filtered on Nalgene SFCA filter. The resulting solution was diluted with water (1:5 v/v) prior to HPLC injection. Chromatographic conditions were Licrospher 125–4, 100 RP-18 (5 µm) column with precolumn, MILLI-Q water 1 ml/min as eluant 276 nm wavelength of absorbance measurement. Standards of pure v-c were obtained from Dr. R. Marquardt (University of Manitoba, Canada).

The reference method proposed by Quemener (1988) is a laborious and expensive technique to be applied to large populations. For this reasons, a rapid and economic spectrophotometric method devised by Sixdenier et al. (1996) was applied to the $F_{2:3}$ seeds. The technique is based on a rapid water extraction from a two-seed sample per plant, followed by an absorbance measure at 274 nm which predicts the three phenotypic classes considered in the study (low, intermediate and high v-c content). The extraction procedure of vicine and convicine was the same as previously described without filtration of the Nalgene SFCA filter.

Results

Parental variation and segregation in F_2 and F_3 progenies

Vf6 displayed high contents of vicine and convicine (0.33 and 0.21% of seed dry matter, respectively). In case of line 1268 the corresponding values were markedly lower (0.02 and 0.01%). According to the rapid spectrophotometric method (Sixdenier et al. 1996) used, total v-c content (vicine content + convicine content) in 2 $F_{2:3}$ seeds per family ranged from 0.0255 to 0.2549% and the average was inferred to the corresponding F_2 plant. F_2 individuals displaying mean values higher than 0.15% were considered to have a high v-c content while individuals with values lower than 0.05% were classified as low v-c content. Segregation of the trait in the F_2 population displayed a perfect fit to the 1:2:1 ratio ($\chi^2_{0.05} = 1.41$, $P = 0.49$). Segregation for colored versus white hilum in the $F_{3:4}$ seeds gave a good fit to the 1:2:1 ratio ($\chi^2_{0.05}$ 2.04, $P = 0.36$), confirming the monogenic control of the trait reported by Sirks (1931). The v-c content (% of seed dry matter) in the $F_{2:3}$ genotypes, the phenotypic classes for v-c content and the hilum color segregation scored in $F_{2:3}$ and $F_{3:4}$ seeds, are available as electronic supplementary material (ESM).

We used the BSA to identify DNA markers closely linked to the character under study. The contrasted pools of DNA included seven plants with high v-c content (average = 0.2063) and colored hilum and seven plants with low v-c content (average = 0.0314) and white hilum. From the 800 primers surveyed, 86 revealed polymorphic bands between the bulks. These primers were then tested independently on the DNA of each of the individuals included in the pools. Only five RAPD markers displayed clear and reproducible polymorphisms among the contrasting genotypes and were subsequently screened on the whole F_2 population for linkage analysis. The Chi-square test revealed that only two loci ($OPH01_{620}$ and $OPAB12_{850}$), gave a good fit to a 3:1 ratio. The ANOVA performed to study the association between the two RAPDs, the hilum color and the v-c content revealed a clear association ($P < 0.001$) among these traits. Co-segregating data were used to construct a linkage map (Fig. 1). $OPAB12_{850}$ ($\chi^2_{0.05} = 2.509$, $P = 0.113$) was associated in coupling phase to white hilum and low v-c content while $OPH01_{620}$ ($\chi^2_{0.05} = 0.0392$, $P = 0.843$), was associated in repulsion phase to the same traits (Fig. 2).

Development of SCARs

The development of SCAR markers ($SCH01_{620}$ and $SCAB12_{850}$) from the two RAPD markers ($OPH01_{620}$ and $OPAB12_{850}$) associated with the traits in study was attempted. Based on the cloned sequences, 21 bp primer pairs were synthetized that consisted of the original 10 bases of the RAPD primer sequence plus the next 11 internal bases (Table 1). However, neither of these SCARs was useful due to the occasional emergence of secondary products and more importantly to

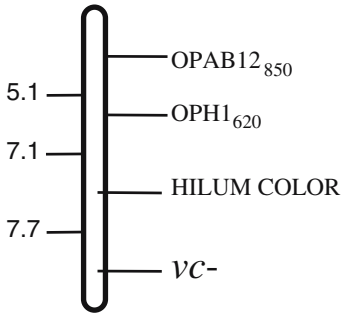

Fig. 1 Linkage map of the Vf6 x *vc*- F_2 populations showing the position of molecular markers $OPAB12_{850}$ and $OPH01_{620}$ and hilum color. Markers names are on the right and the estimated map distances are shown on the left. Recombinant fractions were converted to centimorgans using the mapping function of Kosambi (1944)

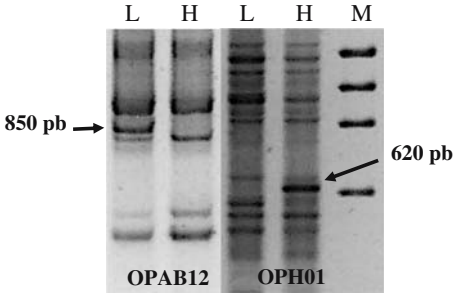

Fig. 2 Amplification products of RAPDs OPH01, and OPAB12. *H* high v-c content bulk, including seven homozygous F_2 individuals with colored hilum and high content in v-c. *L* low v-c content bulk, including seven homozygous F_2 individuals with white hilum and low v-c content. RAPD markers associated with the v-c content are identified by *arrows*. *M* molecular-weight marker (ϕx174/Hae III)

the loss of polymorphism between the parental lines. In order to recover the polymorphisms identified between two parents several methods including optimization of PCR parameters, primer redesign, and restriction enzyme digestion of SCAR products (Zhang and Stommel 2001), were explored.

Modifications of the annealing temperature (ranging from 45 to 65°C) and $MgCl_2$ concentration (from 1.5 to 3 mM) did not succeed in differentiate parental genotypes. A new approach of enlarging the primer sequences to 27 bp was attempted (Table 1). By doing this, the amplification was more consistent revealing intense amplification products of the expected sizes based on the original RAPD bands for each SCAR, although the polymorphism was not recovered (Fig. 3). Finally, the variation in the cloned sequences was further studied through enzymatic digestion of the SCAR products to develop CAP markers. A screening survey of restriction sites using 12 frequent cutter restriction enzymes was used and clear differences between parental lines were observed by digestion with *HhaI* (in case of $SCH01_{620}$) and *RsaI* (for $SCAB12_{850}$) (Fig. 4). *HhaI* did not cut low v-c genotypes while a shorter fragment (572 bp) appears in genotypes with high v-c content. On the contrary, $SCAB12_{850}$ digestion with *RsaI* produced a number of bands among which the 564 and 507 bp products were only observed in the individuals with low v-c content.

Separate survey with each of the CAP markers did not allow discrimination between homozygous and heterozygous F_2 individuals. However, the simultaneous use of these CAP markers allows the correct fingerprinting of faba bean plants in populations segregating for the *VC* gene. Thus, in marker-assisted selection with coupling-repulsion-phase markers, $SCH01_{620}$ can be used in combination with $SCAB12_{850}$ for unambiguous distinction of heterozygous individuals with the same advantages as a codominant marker (Fig. 4). Genotypic segregation of the 2 CAP markers after enzymatic digestion of the corresponding SCAR

Table 1 Primer sequences for SCAR markers derived from RAPD markers linked to locus hilum color and the type of polymorphism observed

Locus[a]	Primer[b]	Sequence (5′ to 3′)[c] (number of pb)	Annealing temperature (°C)	Polymorphism
$SCH01_{620}$	SCH01 F1	GGTCGGAGAAGGTGGTATGTT (21)		
	SCH01 R1	GGTCGGAGAATCATCGATGTG (21)		
$SCH01_{620}$	SCH01 F2	AGAAGAAGGTGGTATGTTGCGTTTTTG (27)	65	Dominant with *Hha*I digestion
	SCH01 R2	GGTCGGAGAATCATCGATGTGAAAATA(27)		
$SCAB12_{850}$	SCAB12 F1	GTACCGAGGGTGCCTGTAAT (21)		
	SCAB12 R1	CCTGTACCGAGGGTGCCTGTA (21)		
$SCAB12_{850}$	SCAB12 F2	CCTGTACCGATATCCCTTGTGTTGCAA (27)	70	Dominant with *Rsa*I digestion
	SCAB12 R2	CCTGTACCGAGGGTGCCTGTAATGCAA (27)		

[a] The subscript number refers to the size in bp of the amplified SCAR product

[b] *F* forward and *R* reverse primer

[c] Underlined nucleotides are derived from the progenitor RAPD primers

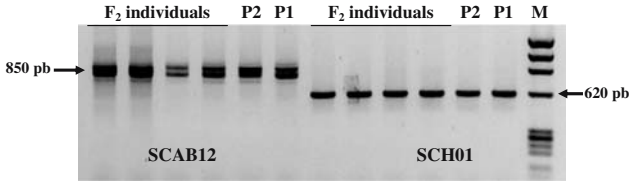

Fig. 3 Agarose gels showing the invariable SCAR fragments (SCH01 and SCAB12) revealed by parental lines Vf6 (P1) and *vc-* (P2) and some F_2 individuals from their progeny

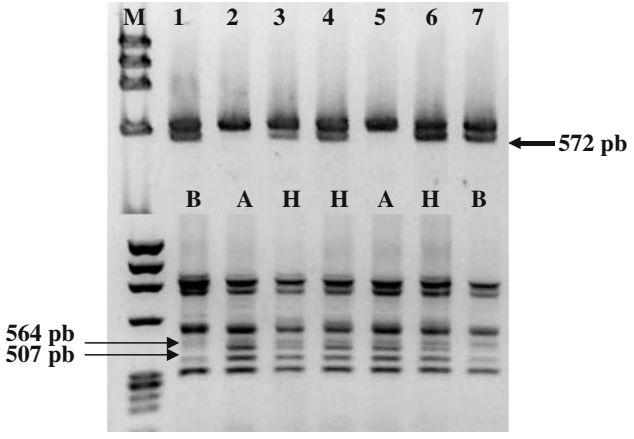

Fig. 4 Product of CAPs markers after enzymatic digestion of the corresponding SCAR fragment. For $SCH01_{620}$ the polymorphims were observed by digestion with *Hha*I (*upper half*) and for $SCAB12_{850}$ by digestion with *Rsa*I (*lower half*). Polymorphic bands and molecular weight are indicated by *arrows*.*Line 1*parental Vf6.*Line 2* parental *vc-*.*Lines 3–7* show the segregation of polymorphims in the F_2 population. The homozygous F_2 individual 7 with colored hilum and high content in v-c (0.242%) is indicated with the letter *B*, homozygous F_2 individual 5 with white hilum and low v-c content (0.039%) with the letter *A* while, heterozygous F_2 individuals (3, 4, 6), with v-c content of 0.125, 0.129 and 0.134%, respectively, are noted with the letter *H*. *M* molecular-weight marker (ϕx174/Hae III)

fragments is provided as electronic supplementary material (ESM).

Discussion

The objective of this work was to identify molecular markers able to distinguish faba bean plants with high and low v-c content for their use in breeding programs. To accomplish this, we used the BSA (Michelmore et al. 1991) a rapid and technically simple method for identifying markers linked to specific genes. By using this approach, two RAPD loci ($OPH01_{620}$ and $OPAB12_{850}$), revealed a clear association with the hilum color and the v-c content. $OPAB12_{850}$ was associated in coupling phase to white hilum and low v-c content while $OPH01_{620}$ was associated in repulsion phase to the same traits (Fig. 2). This strategy has been successfully used in legumes such as faba bean (Avila et al. 2003), pea (Mc Clendon et al. 2002), common bean (Silva et al. 2003) lentil (Ford et al. 1999; Chowdhury et al. 2001), as well as in other crops. In all these cases, BSA allowed rapid mapping of monogenic resistance genes using segregating populations.

Despite the considerable number of primers assayed (800), only 2 RAPDs (0.25%) maintained a clear linkage with the traits. The enormous size (13,000 Mb) and complexity of the faba bean genome (Bennett and Leitch 1995), might have severely limited the discovery of linked markers. Large genome size hampers the precise location and the cloning of important genes, the development of physical maps and makes complete sequencing financially questionable.

RAPD markers do not normally allow discriminating between homozygous and heterozygous individuals. However, the strategy followed in our work, using both coupling and repulsion-linked markers, allow their combined use as a codominant marker (Young and Kelly 1997; Alzate-Marin et al. 1999; Avila et al. 2003), thus greatly increasing the success in the selection of desired genotypes.

There are several problems associated with the occasional lack of reliability between runs of the RAPDs phenotypes (Weeden et al. 1992) that might reduce the value of RAPD for MAS. To overcome these problems, SCARs (Paran and Michelmore 1993), have been widely and successfully used to develop markers for various traits in a number of crops (Cheng et al. 1996; Gill et al. 1998; Zhang and Stommel 2001).

Transformation of RAPD into SCARs frequently results in monomorphic products and loss of the initial polymorphism (Paran and Michelmore 1993; Deng et al. 1997; Hernandez et al. 1999; Chowdhury et al. 2001) (Fig. 3). The loss of polymorphism may occur when the original polymorphisms with the 10-base RAPD primers are due to small mismatches at the priming sites (Paran and Michelmore 1993), resulting in the absence of an amplification product. Several methods to recover the polymorphisms identified between two parents were attempted, including optimization of PCR parameters, primer redesign, and restriction enzyme digestion of SCAR products to develop CAPS markers (Zhang and Stommel 2001). Finally, enzymatic digestion of the SCAR products with *HhaI* (in case of $SCH01_{620}$) and *RsaI* (for $SCAB12_{850}$) allowed the development of coupling-repulsion-phase CAP markers suitable for an unambiguous genotyping for v-c content (Fig. 4).

The application of MAS for the introgression of genes through a backcrossing program is advantageous

for improving cultivars (Tanksley et al. 1989). Using this approach, selection can be performed at early seedling stages of development, and true breeding genotypes identified with relative ease. In this study, 2 RAPD markers linked to hilum color and v-c content were identified and converted into SCAR/CAPs that can be used to perform selective breeding in a more efficient manner. Since white hilum has maternal control, segregations are expressed one generation later than expected, thus delaying the identification of individuals carrying the desired trait. Nevertheless, combined selection for these two molecular markers will improve the efficiency of selection for low v-c content avoiding the cost and difficulties of the chemical determination of these products.

Both RAPD and CAP markers may be applied in high throughput breeding applications. Nevertheless, SCAR/CAPs have clear advantages over RAPDs since they are allele specific and more reliable and stable between laboratories. The 2 CAP markers identified in this study are predicted to be useful in MAS to track the introgression of the *vc-* allele into breeding lines and to develop new faba bean cultivars with low v-c content and improved nutritional value.

Acknowledgments This study was funded by the grants PETRI 95–0682-OP from the Spanish Science and Technology Ministry and by the European Community project EUFABA QLK5-CT2002-02307. The authors thank B. Raffiot, J. Prieto and G. de Lara for their excellent technical assistance. N. Gutierrez acknowledges a fellowship from the Consejería de Innovación Ciencia y Empresa from the Junta de Andalucía. C.M. Avila acknowledges financial support from the Spanish Ministerio de Educación y Ciencia ("Juan de la Cierva program").

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