Plant Breeding
doi:10.1111/j.1439-0523.2008.01584.x

Simple sequence repeats (SSRs) in faba bean: new loci from *Orobanche*-resistant cultivar 'Giza 402'

M. Zeid[1], S. Mitchell[2], W. Link[3], M. Carter[2], A. Nawar[4], T. Fulton[2] and S. Kresovich[2]

[1]Department of Plant Breeding and Genetics, Cornell University, Ithaca, 14853 NY, USA, E-mail: mz67@cornell.edu; [2]Institute for Genomic Diversity, Cornell University, Ithaca, 14853 NY, USA; [3]Department of Crop Sciences, Georg-August University, Göttingen, Germany; [4]Department of Crop Sciences, Alexandria University, Alexandria, Egypt

With 2 figures and 3 tables

Received April 17, 2008/Accepted August 30, 2008
Communicated by R. Varshney

Abstract

Genomic DNA from an inbred line from the *Orobanche crenata* resistant cultivar 'Giza 402' was restricted and enriched for AG-containing simple sequence repeats (SSRs). The overall SSR enrichment rate was 54.6%. Of 73 selected SSR markers that successfully amplified in 'Giza 402' 54 were polymorphic among a group of 10 Egyptian and Spanish faba bean genotypes extensively used in *Orobanche crenata* resistance/susceptibility studies and linkage map development. The polymorphism information content ranged from 0.16 to 0.72 and Unweighted Pair-Group Method using Arithmetic averages cluster analysis based on these loci placed all resistant genotypes descended from the parental line F 402 in the same group. Successful amplification from 28 primer pairs was also observed in five European faba bean genotypes. The SSR loci described here will be extremely useful in studies focusing on *Orobanche crenata* resistance, genetic diversity studies and will provide additional co-dominant loci to complement existing faba bean linkage maps based primarily on dominant markers.

Key words: microsatellites — SSR — faba bean — *Orobanche crenata*

Vicia faba L. (faba bean), a high-protein legume crop, is grown for human consumption in China, Ethiopia, the Nile Valley and Maghreb regions of northern Africa and the Andean States of South America, while in Europe and Australia the crop is grown primarily for livestock feed. Faba bean, a partially allogamous plant, is diploid with a relatively few number of large chromosomes (2n = 2x = 12). The species is genetically isolated, tolerating no exchange of genes with any other species including its close relative *Vicia narbonensis* (Hebblethwaite 1983). Although faba bean has fewer chromosomes than other species within the genus, the large size of the nuclear genome ($1C_{mean}$ = 20 000 Mbp; Bennett and Leitch 2001) poses a challenge for nuclear DNA studies.

Despite the release of new cultivars with higher yield and better adaptation to the prevailing production conditions, parasitism by *Orobanche crenata* Forsk., (*O. crenata*), an achlorophyllous, holoparasitic weed, poses a major constraint to faba bean production. Sauerborn (1991) estimated that over 1 million hectares of faba bean, extending from the Mediterranean basin to western Asia, are either infested or at risk for infestation, with yield losses ranging between 5% and 100% (Sauerborn and Saxena 1986). *Orobanche crenata* deposits large number of seeds that survive in the soil for more than 15 years (Linke and Saxena 1991) forcing farmers to abandon faba bean cultivation. In Italy and Spain, for example, areas of faba bean production have drastically decreased mainly because of the presence of *O. crenata* (Mesa-Garcia and Garcia-Torres 1986).

So far, eight linkage map studies have been reported in faba bean (see review by Torres et al. 2006). However, all were based almost exclusively on dominant markers, primarily random amplified polymorphic DNA (RAPD) including the most current linkage map covering 1636 cM (Arbaoui et al. 2008). One study, devoted to map quantitative trait loci (QTL) for *O. crenata* resistance in faba bean, was conducted by Román et al. 2002. It involved 196 F_2 plants derived from the cross between the lines Vf6 (susceptible) and Vf136 (resistant) and a linkage map comprising 16 linkage groups based on 117 RAPD markers, two isozymes and two seed protein genes, was developed. Three QTL for *O. crenata* resistance were mapped on three different linkage groups in faba bean, and they jointly explained 74% of the phenotypic variance.

The wide use of simple sequence repeat (SSR) markers in molecular genetic studies is generally attributed to their high abundance in the genome, high level of allelic variation, co-dominant inheritance, analytical simplicity and transferability of results across different laboratories (Rafalski and Tingey 1993). Because specific loci are amplified, SSRs are often used as bridge markers for map alignment among different genetic maps (see Feltus et al. 2006, Wu and Huang 2007). In genetic studies of organisms that lack extensive genomic DNA sequence data, use of SSRs has been limited because isolation and development costs are very high compared with other markers such as RAPD and amplified fragment length polymorphism (AFLP). Since the work of Pozarkova et al. (2002) describing the identification of 25 SSR markers in faba bean, to the best of our knowledge no further SSRs were published. Because the mentioned SSRs were chromosome 1-specific, their usefulness was limited to assigning linkage groups to that chromosome. The goals of this study were to develop novel faba bean SSR markers and to test the usefulness of these markers, both in a collection of faba bean germplasm used in *O. crenata*-resistance/susceptibility studies and in accessions from other gene pools.

Materials and Methods

Plant material: Ten faba bean genotypes (five *O. crenata*-resistant and five susceptible) were assayed in this study. Available information and pedigree on the tested genotypes including 'Giza 3' and Vf136, both

Table 1: Faba bean genotypes tested for DNA polymorphism

Genotype	Year of release	Target area or purpose	Pedigree
Orobanche crenata resistant genotypes			
'Giza 402'	1979	Middle & upper Egypt	*cf*. Figure 1
'Giza 429'	1995	Middle Egypt	
'Giza 674'	1995	Middle & upper Egypt	
'Giza 843'	1998	North Delta, Egypt	
Vf 136	–	Research line, Spain	
Orobanche crenata susceptible genotypes			
'Giza 3'	1979	Replacement for 'Giza 1' in upper Egypt	'Giza 1' (Egypt) × NA29 (the Netherlands)
'Giza blanca'	1994	Newly reclaimed areas in Egypt	Selection from Spanish 'Reina blanca'
'Giza 461'	1995	North Nile Delta in Egypt	'Giza 3' (Egypt) × ILB938 (ICARDA/Colombia)
'Rebaya 40'	Late 1960's	Replacement for 'Rebaya 8' in upper Egypt	Landrace
Vf 6	–	Research line, Spain	Asynaptic breeding line program

routinely used as susceptible checks in *O. crenata* studies are shown in Table 1. A schematic presentation of the origin of *O. crenata* resistance in faba bean and the development of commercial Egyptian ('Giza 402', 'Giza 429', 'Giza 674' and 'Giza 843') and Spanish ('Baraca') *O. crenata*-resistant cultivars is summarized in Fig. 1. Spanish lines were provided by Belén Román, Centro de Investigación y Formación Agraria (CIFA), Córdoba, Spain. Two lines, 'Giza 402' (resistant) and 'Giza 3' (susceptible), were produced by inbreeding for at least seven generations, and recombinant inbred lines from a cross between the two lines are currently being established at the Department of Crop Sciences, Göttingen, Germany. Genomic DNA of all plant material was extracted from leaves of a single seedling according to Doyle and Doyle (1990).

SSR development: DNA from 'Giza 402' was simultaneously digested with *Mse*I and ligated to *Mse*I-AFLP adaptor. AFLP fragment production and enrichment employing a 3′-biotinylated $(AG)_{17}$ probe were performed according to Zane et al. (2002). Fragments enriched for the AG repeat were cloned using the TOPO-TA cloning kit (Invitrogen, San Diego, CA, USA), as described in the manufacturer's manual. A total of 396 recombinant clones plated out on LB-agar plates containing 50 μg/ml kanamycin were directly amplified using vector primers and 10 μl of the PCR products were screened on a 1% agarose gel for presence of inserts. PCR products were sequenced on an ABI 3730XL automated DNA sequencer (Applied Biosystems, Foster City, CA, USA), using BigDye™ terminator chemistry (Applied Biosystems). Sequences were edited, aligned and redundant sequences identified using the SEQUENCHER software package (Gene Codes Corporation, Ann Arbor, MI, USA).

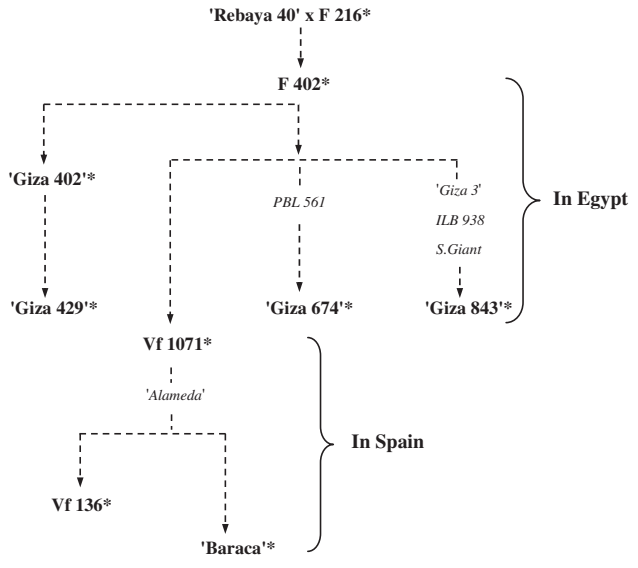

Fig. 1: Pedigree showing the origin of *Orobanche crenata* resistance and pedigrees of resistant genotypes in Egypt and Spain. An asterisk indicates an *O. crenata*-resistant genotype. ('*Alameda*': Spanish cultivar with *O. crenata* tolerance, *PBL 561*: breeding line from ICARDA with Egyptian origin, '*Giza 3*': Egyptian cultivar, *ILB938*: ICARDA accession from Colombia, '*S. Giant*': Spanish accession)

We isolated 400 additional clones to test the efficacy of using a PCR screening step to identify clones containing SSR motifs prior to sequencing. After clones were amplified using vector primers, 2 μl of the PCR products were used as template in another PCR reaction that contained both vector primers and the AG_{17} probe (cf. Rafalski et al. 1996). Only PCR products that amplified additional fragments to the one previously amplified from the vector primers were submitted for sequencing.

Sequences were searched for repeat motifs of a minimum of five units of di-, tri- or tetra nucleotide repeats using the Simple Sequence Repeat Identification Tool available from GRAMENE (http://www.gramene.org/). Primers flanking the repeat motifs were designed using the software Primer 3 (Rozen and Skaletsky 1998). To determine if any of the loci identified were located in or near genes, sequences were queried against the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/BLAST/), at the nucleotide level with BLASTN including non redundant (nr) and genome survey sequence (gss) and at the protein level with BLASTX and TBLASTX using the default settings.

Genotyping and analysis of SSR data: Primer pairs were initially tested on 'Giza 402' for amplification and production of fragments within the expected size range, PCRs were conducted in 20 μl volumes containing 20 ng DNA, 1x PCR buffer [10 mM Tris–HCl pH 9.4, 50 mM $(NH4)_2SO_4$ (US DNA Biotech, Fort Worth, TX, USA), 1.5 mM $MgCl_2$], 2 M betaine, 0.2 mM of each dNTP, 0.2 Units *Taq* DNA polymerase (Promega, Madison, WI, USA) and 10 pmol of each individual primer pair. Cycling protocol consisted of an initial denaturation at 95°C for 4 min, followed by 30 cycles of 95, 50 and 72°C, for 45, 60 and 60 s, respectively and a final elongation time of 7 min using a PTC-225 thermocycler (MJ Research, Waltham, MA, USA). Electrophoretic size separation of SSR loci was performed using both 4% Exclu-sieve™ high resolution agarose gels (The Nest Group, Southborough, MA, USA) and 4% denaturing polyacrylamide gels visualized with silver staining (Bassam et al. 1991). A total of 73 primer pairs were used to check for polymorphism among 10 genotypes involved in *O. crenata*-resistance/susceptibility studies (Table 1). The genotypic data for SSR loci from all 10 genotypes were recorded as a binary matrix of presence/absence of each allele in a particular line. For variable loci, polymorphism information content (PIC) was estimated (Botstein et al. 1980) and genetic similarity between genotypes was calculated according to Nei and Li (1979). An Unweighted Pair-Group Method using Arithmetic averages (UPGMA) based dendrogram showing relationships among 10 genotypes was constructed with NTSYS-pc version 2.20k (Rohlf 2002). To test for the usefulness of the studied loci in a broader genetic background, five further genotypes from the European gene pool (Zeid et al. 2003) were assayed with 30 primers pairs that proved polymorphic among the 10 previously tested

genotypes. Here scoring was based on successful amplification for products separated on 2% agarose gels.

Results

SSR development

From a library enriched for SSR repeat motifs, we initially isolated and sequenced 396 faba bean genomic clones, 216 (54.6%) of which harboured SSR motifs. Addition of a PCR screening step prior to sequencing an additional batch of 400 clones increased sequencing efficiency to 96% (i.e., 96% of clones sequenced contained SSRs). The majority of repeats recovered (AG/CT) were consistent with the use of an $(AG)_{17}$ probe for hybridization-based enrichment. Most of the clones had AG/CT dinucleotide repeats (98.1%) except for a single $(AC/GT)_{17}$. A few contained trinucleotide repeats including: $(AAG/CTT)_5$; (n = 2), an interrupted motif $(CAA/TTG)_{3+6+7}$ (n = 1) and a compound repeat $(AGAT/ATCT)_5$ $(AG/CT)_{13}$ motif (n = 1) and a tetranucleotide (AGAT/ATCT) motif (n = 1). Redundant sequences accounted for 35.2% of the SSR-containing clones. Primers could not be designed for 20.3% of the clones, either because the repeat motif was located very near the cloning site or high A/T content precluded the design of primer(s) with appropriate melting temperatures. Forty-four percent of the SSR-containing clones were suitable for primer design. A total of 110 primer pairs amplified a fragment in 'Giza 402' and fragments from 77.5% of these loci matched the expected size range. Seventy-three primer pairs with best fragment intensity were selected for further analysis.

Amplification of SSR loci

Screening of 73 loci amplified fragments in at least one of the 10 *O. crenata*-resistant/susceptible genotypes (Table 1) and 54 of these appeared to be polymorphic among the tested genotypes. Polymorphisms for 38 loci were resolvable on agarose gels, while 16 polymorphic loci could only be scored on polyacrylamide gels. The ability of the identified primers to amplify products in a broader genetic background was evaluated by testing a sample of 30 primer pairs on five European genotypes namely; 'Scirocco', 'Kristall' (Germany), 'Peleponnes' (Greece), 'Pistache' and 'Victor' (the Netherlands). Successful amplification was detected for 28 primer pairs as indicated in Table 2. The co-dominant nature of 35 SSRs that were polymorphic between parental lines 'Giza 3' and 'Giza 402' was illustrated by observing the banding pattern of their F_1 hybrid (data not shown).

Clustering based on SSR markers

Data from 54 polymorphic loci were used to construct a UPGMA dendrogram showing the genetic relationships among the 10 faba bean genotypes, including both *O. crenata*-susceptible and -resistant genotypes (Fig. 2). This analysis showed clustering of all resistant genotypes along with the susceptible cultivar 'Rebaya 40'. Pairwise similarity coefficients ranged from 0.64 (between 'Giza 429' and 'Giza 674') and 0.27 (between 'Giza 3' and 'Giza 843').

Discussion

In the past, SSR markers were usually developed after screening small insert genomic libraries with repeat-containing probes followed by DNA sequencing of hybridizing clones. The efficiency of DNA hybridization-based methods was later improved by constructing libraries that were highly enriched for selected repeat motifs (Rakoczy-Trojanowska and Bolibok 2004). More recently, similar methods have been developed that do not involve size fractionation of genomic DNA. One such method, FIASCO (Fast Isolation by AFLP of Sequences COntaining repeats) described by Zane et al. (2002), involves a fast digestion–ligation reaction AFLP step followed by denaturation and hybridization of single stranded AFLP products to repeat-containing, biotinylated oligonucleotides followed by reaction with streptavidin-coated magnetic beads. The bead–probe–DNA complexes are then captured with a magnet and the DNA is reamplified (to produce double stranded fragments), cloned and sequenced. The FIASCO technique resulted in a substantial enrichment (54.6%) of repeat motifs compared with traditional hybridization screens of small insert genomic libraries where, in plants, an average of 2.3% of clones contain SSRs (Zane et al. 2002). The method also outperformed a PCR-based technique, degenerate oligonucleotide-primed PCR (DOP-PCR), used to construct chromosome 1-specific SSR-enriched libraries from faba bean (Pozarkova et al. 2002). DOP-PCR followed by magnetic bead-based enrichment resulted in libraries with only 4.9% SSR-containing clones. If chromosome 1 is representative of the remainder of the genome, the poor level of enrichment attained by DOP-PCR might be due to the selective amplification of genomic regions depauperate in SSRs. The FIASCO procedure, based on sampling of a subset of small genomic fragments, should show no such bias.

The AG probe was used because SSRs derived from expressed sequence tags (ESTs) from *Medicago truncatula*, a model legume, showed a higher frequency of AG/CT than the other dinucleotide motifs (Von Stackelberg et al. 2006). Furthermore, the dinucleotide repeat has been shown to be a common motif in different legumes including: common bean (Gaitan-Solis et al. 2002, Blair et al. 2003), groundnut (He et al. 2003) and chickpea (Sethy et al. 2006). The correlation between level of polymorphism and number of repeats has been reported in ryegrass (Jones et al. 2002) and white clover (Kölliker et al. 2001). In this study, this correlation was not observed; sequences with <10 repeats and showing polymorphisms between the 10 genotypes accounted for 55% of all polymorphism detected in dinucleotide repeats. Similar results were reported in common bean (Yu et al. 2000) and peas (Burstin et al. 2001), where dinucleotide repeats as short as five repeats were useful for developing polymorphic SSR markers.

With the complete DNA sequences from all SSR clones queried against the NCBI databases, only five of the query sequences were highly similar to the database sequences (four at the nucleotide level and one on the protein level) (Table 3). These results are consistent with the majority of dinucleotide repeats being located in non-coding regions in eukaryotes (Lawson and Zhang 2006). Most noteworthy, locus VfG 30 contained a stretch of 151 bp that was highly similar to a serine/threonine phosphatase from *Medicago sativa*. Some members of this large gene family are known to play an essential role in signal transduction and actively contribute to the regulation of protein phosphorylation (Schweighofer et al. 2004). Queries were also very useful in identifying sequences highly similar to repetitive sequences and/or transposable elements in faba bean previously identified by Nouzova et al. (1999) and Hill et al. (2005). Taking into account the high

Table 2: Characteristics of polymorphic SSRs detected among 10 faba bean genotypes

Locus[1]	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)	Repeat type & length	Expected size (bp)	PIC[2]
VfG 1[3]	TTTCAGCAAACTAGAACCAATC	GGCATTCAGTTTTTACCTTGTA	(AG)15	224	0.70
VfG 2[3]	AGGGGTCCAAGGGGTTAAAT	ACAGGGAAGCATCAACAATG	(AG)5	145	0.37
VfG 3[3]	TTCTTTGGTCCTCTCTCTATC	GCACTGTTGTTGCTGATACAA	(AG)7 + 14	159	0.62
VfG 4	AAGGGGAGGGCATAACAGAA	AATCCGCAAGGGTCTTCTTT	(AG)5 + 10	218	0.54
VfG 6	CCTAACAGAATATAAATCAGGTTCA	GATGCAAGGTTGCAGATTCT	(AG)8	309	0.35
VfG 7[3]	AGGCATGTGGTGTTTTGATT	GCAATAAAATATGCTTTTCTGGA	(AG)12	284	0.37
VfG 8[3]	ATGGGCAGAGGAGGATAAAA	ATCATCCAGGAGGGAGAAAA	(AG)12	149	0.28
VfG 9[3]	GGTTTTGAATAGAAATGCAA	AAGATGTGTCAATATTGTTTT	(AG)16	154	0.68
VfG 10[3,4]	ACCAAAACGCGCACTTATCA	AAGAGAGAGAAGAGAGCTTC	(AG)5	219	0.53
VfG 11[4]	GCAAAAGGAGAGCAAGGGAA	CGAAAGAGGGGGACATTTTGT	(AG)8	301	0.51
VfG 13[3,4]	GGTTGGGATCTTTTAGGTTGAA	TGGCCTTATATCCGTCCAAT	(AG)10	191	0.57
VfG 14[3]	CAAGCTTGTTGAGAGCCAAA	GAACGAGGCTCACGAAAATA	(AG)10	229	0.16
VfG 15	TCGATAGGGTTTCAGATTGA	GATGTTGACGGTGGTGTTT	(AG)6 + 15	205	0.56
VfG 16	TGCTCCAAAGGAAAACGTT	AGAGAGGGTACTGAGATTA	(GAA)5	109	0.37
VfG 19[3]	AGCGATGGTGCTCATGCTTA	TCTCTCACGGAATCACATCTTT	(AG)9	174	0.72
VfG 21	CTGAGCAAGTTTTCTATGAC	ATATGCCACATGGTAGCTTT	(AG)14	248	0.33
VfG 22	GGCTATTGTCACGAACAAAT	GATTCAGACCCGGATACATT	(AG)15	147	0.66
VfG 24[3]	GTGTCATCGACCACATGGTTA	CAGCTGAGCACGAGACTAA	(AG)9	273	0.27
VfG 27[3]	CCCAAAAAGAGACGAACTGTAT	AGGGTTCATACGTTTGGCTT	(AG)17	206	0.54
VfG 28[3]	AGAGTCCCAAAGAGTGGGTT	CCAAAGGCAAAAATGAGGGCTT	(AG)19	220	0.65
VfG 29[4]	GGACTTTTTATGGAGGTATCA	CCATGCCTGTATTTTGCCAA	(AG)5	235	0.38
VfG 30[3]	GTAAGACGGGGAGCAGAA	TCCAACTTCTATCCTTCTCTTCT	(AG)13	201	0.36
VfG 31[3]	ATAAGAGAGAACGAGGGAGAA	TTATGGTGGGACGTCTTACAT	(AG)7	126	0.50
VfG 33[4]	CAGATATTCATGGTTGAGTAT	GTCAAAACCATTTACTTCACTT	(AG)7	181	0.16
VfG 34[4]	GCACTCGAAGGAATTAATTTT	GAACAGTTGTTTCGTGTCGTA	(AG)12	205	0.70
VfG 36[3]	CATCATCCAGGAGGGAGAA	GGGCAGAGAGGATAAAAAGA	(AG)12	146	0.18
VfG 37[4]	AACGAGAGTGAGGTTACTTC	AACCTCTCTCTCCATGCATA	(AG)6	129	0.33
VfG 38[3]	GACAGCTTGCGTATTTTCAA	GCTCGAAGAAAGTAGAAGAA	(AG)10	178	0.42
VfG 40[4]	GGAGGGAGAAAACGTGAGAT	GCAGAGAGGATAAAAAGAGAAG	(AG)12	100	0.35
VfG 41[3]	AGCCCATGGTTCAAATGCAA	GCAGTCATGCCACTGCTTA	(AG)7 + 10	217	0.67
VfG 42[4]	GAATGGACCGGTTCTGGATT	CCCTAATCCCTTCACTAATACA	(AG)9	213	0.18
VfG 44[4]	GATGTTGTTGGTGTTGTTTA	CAATTAGGAGCAAAATCAGA	(AG)12	262	0.70
VfG 45[4]	GTTATAGAAAGTAAATGAGATAG	AGAACAGTGGTTCTTGCAAA	(AGAT)5(AG)13	331	0.33
VfG 46[4]	GTCCTGGAAAAAAGAAAGAGA	AAAGAAACCTCTCTCTCCAT	(AG)6	173	0.33
VfG 47[4]	CGATTGTTTGCAGAGGAGATA	ACAGAGAGGGACAGAGAGAA	(AG)8	282	0.59
VfG 53[4]	GGTTCATGAAAAGAGGTTAG	CATTTTCCGTTCTCTCTCTA	(AG)9	230	0.59
VfG 55	ATCATCCAGGAGGGAGAAAA	ATGGGCAGAGAGGATAAAAA	(AG)12	148	0.56
VfG 57[4]	AGAGTGAGGTTGCTGCCAT	ACCTCTCTCTCCATGCATAA	(AG)7	126	0.37
VfG 58	TGATGGTGCAACTCACCGAT	CTCTCTCTCACGGAATCACAT	(AG)9	190	0.16
VfG 61[3]	GCCGATATTTTCTTTTGACA	TGAATGCTTCACTAACAA	(AG)10	128	0.47
VfG 62[3]	AACTTCTGACTTCCTCTCA	AGCGGAAATGTAAAATCCTA	(AG)6	170	0.33
VfG 63	GAGAGGCGGTTATGTTGTTA	GATCTCTCTCTCTCTCTCT	(AG)32	190	0.51
VfG 67	GTTCATCAAGCACCAATCTAAAC	TCAATTTGGTTTATCTCTCTCTCT	(AG)10 + 24	169	0.56
VfG 68[4]	AGGCATCAGTTGGTACTTG	CTCTGCATCCCATACTGATA	(AG)8	170	0.16
VfG 69[3]	ATTGGGGAGGATGAAGGTT	TTCCATTTTCCGTTCTCTCT	(AG)9	195	0.61
VfG 77[3]	AGTTGAGGTTTTGAACCCAA	AGGAGGCCTGGTTGTTTTA	(AG)5	191	0.31
VfG 80	GATGTTTATGGGAAAATCTGAT	TCCTTGACAAAAAAACAAAATTG	(AG)8	164	0.35
VfG 81[3]	GTCCTGGAAAAAAGAAAGAGA	AAAGAAACCTCTCTCTCCAT	(AG)7	175	0.53
VfG 82[3]	TTGAACCCAGCCGCCGCTGA	AGGGGACGCTCATTTTGTTG	(AG)5	142	0.37
VfG 84[3]	GATACACGCGACTGCCTCAA	GTGAGTTGTTGGAGAGGATGAT	(AG)9	202	0.16
VfG 86[3]	TCCTGGAAAAAAGAAAGAGA	AAAGAAACCTCTCTCTCCAT	(AG)7	176	0.44
VfG 87[3]	AGGGCCAGCGTGATCCAATA	TGGGTTGGGATCTTTTGGTTG	(AG)10	242	0.61
VfG 88[3]	TAACGAGAGTGAGGTTGCT	ACCTCTCTCTCCATGCATA	(AG)7	131	0.33
VfG 89	TTGTCAAGAGAGGCGGTTA	CACACGATCTCTCTCTCT	(AG)20	150	0.36

[1]Vf, *Vicia faba*; G, 'Giza 402'.
[2]PIC, polymorphism information content.
[3]Amplification detected with at least one of the five European genotypes.
[4]Polymorphism detected only on PAGE and not on agarose gels.

proportion of repetitive DNA in faba bean (more than 85%) according to Flavell et al. (1974), only 20% of the SSRs identified were similar to repetitive sequences and/or transposable elements. These sequences were excluded from further analysis, as positioning of PCR primers in repetitive regions generates spurious or non-specific products (Temnykh et al. 2001). Redundant sequences (35.2%) were much lower than enriched libraries developed for other legumes; 67% in *Arachis hypogaea* (He et al. 2003) and 70% in *Phaseolus vulgaris* (Gaitan-Solis et al. 2002).

Of 54 polymorphic loci observed on 10 faba bean genotypes, the polymorphism of 16 loci were only visible on 4% polyacrylamide gels with silver staining while the rest were detectable on 4% high-resolution agarose gels stained with ethidium bromide (Table 2). It is important to note that although the resolving power of agarose gels is relatively poor

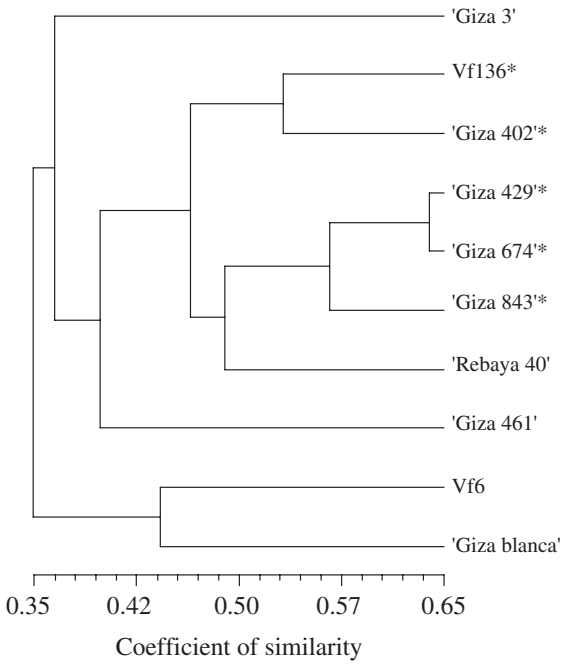

Fig. 2: Unweighted Pair-Group Method using Arithmetic averages dendrogram showing relationships among 10 faba bean genotypes. An asterisk indicates an *Orobanche crenata*-resistant genotype. Dendrogram based on data from 54 SSR markers

(i.e., allele size differences of less than ~4–6 bp could not be distinguished), the ease of using agarose compared with polyacrylamide or capillary electrophoresis, will make these SSRs more accessible to laboratories studying faba bean genetics in developing countries where the crop is a substantial source of human dietary protein.

The SSR markers developed were highly polymorphic with PIC values ranging from 0.16 to 0.72 with an average of 0.44 (Table 2). Successful amplification of 28 SSR markers was achieved when screened on five European cultivars from a larger genetic diversity panel (cf. Zeid et al. 2003). This panel comprised 79 cultivars from Europe, North Africa and from Asia and prior results from AFLP analyses showed both a high genetic similarity among the European lines and fairly substantial divergence from the North African material. Such results indicated that the new SSR markers developed here will be very useful for faba bean research beyond the limits of the actual genotypes that allowed their design and development. Estimates of pairwise genetic similarity clearly revealed that the faba bean genotypes tested here were highly diverse (the maximum similarity coefficient was only 0.64 between 'Giza 429' and 'Giza 674') (Fig. 2). Similar results were observed from previous studies using RAPD (Link et al. 1995) and AFLP (Zeid et al. 2003), where generally low genetic similarity estimates among faba bean genotypes from the same germplasm group were attributed to the partially allogamous nature of the plant. The dendrogram based on Nei and Li (1979) genetic similarity estimates from 54 SSRs (Fig. 2) showed one main cluster that comprised all five *O. crenata*-resistant genotypes, in addition to the susceptible cultivar 'Rebaya 40'. These results are in full agreement with available pedigree data. The line F 402, distributed commercially as 'Giza 402', was developed in Egypt in the 1970's (Nassib et al. 1982) from a cross between 'Rebaya 40' and the *O. crenata* resistant land race F 216 (Fig. 1). Further improvement to 'Giza 402' through direct selection or crossing and recurrent selection have yielded the cultivars 'Giza 429' and both 'Giza 674' and 'Giza 843', respectively. In Spain, the line Vf1071 was selected from 'Giza 402' in *O. crenata* infested fields. Crossing Vf1071 to the Spanish cultivar 'Alameda' lead to the selection of the line Vf136 (Cubero 1991), routinely used as a resistant check in *O. crenata* studies, and eventually the resistant cultivar 'Baraca' was released. We have no pedigree information to explain the clustering of 'Giza blanca' and the breeding line Vf6; however, it is worth noting that 'Giza blanca' was released in Egypt after rounds of direct selection from the Spanish cultivar 'Reina blanca', supporting the evidence of gerplasm exchange between the two countries.

As the sequences flanking of some SSR loci may be highly conserved, SSR primers developed for one species sometimes amplify loci in related species (Röder et al. 1995). Although marker transferability between grass species has been successful (Kuleung et al. 2004, Saha et al. 2004), this has not been the case in legumes, especially not for faba bean. Peakall et al. (1998) investigated the cross-species transferability of 31 soybean SSRs to other legume genera including faba bean and found a low transferability rate (3–13%). On the other hand, with 40% of the SSR markers from *Medicago truncatula* showing amplification in faba bean from the study by Gutierrez et al. (2005), none of the 209 EST-derived SSRs or 33 BAC-derived SSRs tested on different faba bean lines (Vf6, Vf136, Vf27 and 29H) showed polymorphisms. Until appropriate markers from much closer species are identified that cross-amplify polymorphic products in faba bean, it seems inevitable that continued development of faba bean SSRs or similarly useful markers will be needed if genetic improvements are to be accelerated in this crop. The results presented in this study have indicated that with improved SSR-enrichment methods, like FIASCO, the development of SSRs is becoming more successful and affordable than ever before. Because they were developed from an inbred line of the resistant cultivar 'Giza 402', the SSR loci described here should be applicable to studies focusing on resistance to *O. crenata*. These SSRs also amplify in a variety of Egyptian and European faba bean cultivars and should be useful for studies on a wide variety of cultivars from different genetic backgrounds.

Table 3: Significant *E*-values for DNA and protein similarities of SSR sequences for which primer pairs were developed

Primer	*E*-value	Homology	Organism matched GenBank	Accession no.
VFG 3	2e-04	Genomic sequence	*Medicago truncatula*	CT573365
VFG 3	8e-06	Genomic sequence	*Trifolium pretense*	DE240763
VFG 21	1e-09	Genomic sequence	*Medicago truncatula*	CT033764
VFG 30	1e-43	Ser/Thr specific protein phosphatase	*Medicago sativa*	AF196289
VFG 39	8e-04	Genomic sequence	*Medicago truncatula*	CR330936
VFG 45	3e-05	Retrotransposon insertions	*Pisum sativum*	AJ965573

Furthermore, the Spanish lines Vf6 and Vf136 have been employed as parental lines in 75% of the mapping populations used in linkage map development in faba bean (Torres et al. 2006). Consequently, the SSRs developed here have been prescreened for polymorphism in these parental lines and could be easily added to the existing maps based on dominant markers. Preliminary results on the segregating population from the cross between the susceptible 'Giza 3' and the resistant 'Giza 402' lines regarding *O. crenata* resistance have been very promising (data not shown) and future work will concentrate on developing additional SSR markers that would provide better coverage of the faba bean genome before studies for mapping of quantitative trait loci, especially those controlling this parasite are launched.

Acknowledgements

This work was financed by the Centre for Special Studies & Programs, Bibliotheca Alexandrina, Alexandria, Egypt. Project no. 04011169. The support of the Fellowship Program for African Scientists, Institute for Genomic Diversity, Cornell University (2006) is highly acknowledged.

References

Arbaoui, M., W. Link, Z. Satovic, and A. M. Torres, 2008: Quantitative trait loci of frost tolerance and physiologically related traits in faba bean (*Vicia faba* L.). Euphytica. DOI: 10.1007/s10681-008-9654-0.

Bassam, B. J., G. Caetano-Anollés, and P. M. Gresshoff, 1991: Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal. Biochem. **196,** 80—83.

Bennett, M. D., and I. J. Leitch, 2001: Plant DNA C-Values Database. Royal Botanic Gardens, Kew, UK *http://www.kew.org/cval/homepage.html* (accessed on 18 January 2008).

Blair, M. W., F. Pedraza, H. F. Buendia, E. Gaitán-Solís, S. E. Beebe, P. Gepts, and J. Tohme, 2003: Development of a genome-wide anchored microsatellite map for common bean (*Phaseolus vulgaris* L.). Theor. Appl. Genet. **107,** 1362—1374.

Botstein, D., R. L. White, M. Skolnick, and R.W. Davis, 1980: Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am. J. Hum. Genet. **32,** 314—331.

Burstin, J., G. Deniot, J. Potier, C. Winachter, G. Aubert, and A. Baranger, 2001: Microsatellite polymorphism in *Pisum sativum*. Plant Breeding **120,** 311—317.

Cubero, J., 1991: Breeding for resistance to *Orobanche* species: a review. In: K. Wegmann, and L. J. Musselman (eds), Progress in Orobanche Research, 257–277. Ederhard-Karls- Universitaet, Tubingen, FRG.

Doyle, J. J., and J. L. Doyle, 1990: Isolation of plant DNA from fresh tissue. Focus **12,** 13—15.

Feltus, F. A., G. E. Hart, K. F. Schertz, A. M. Casa, S. Kresovich, S. Abraham, P. E. Klein, P. J. Brown, and A. H. Paterson, 2006: Alignment of genetic maps and QTLs between inter- and intra-specific sorghum populations. Theor. Appl. Genet. **112,** 1295—1305.

Flavell, R. B., M. D. Bennett, J. B. Smith, and D. B. Smith, 1974: Genome size and proportion of repeated nucleotide sequence DNA in plants. Biochem. Genet. **12,** 257—269.

Gaitan-Solis, E., M. C. Duque, K. J. Edwards, and J. Tohme, 2002: Microsatellite repeats in common bean (*Phaseolus vulgaris*): isolation, characterisation and cross-species amplification in *Phaseolus* ssp. Crop Sci. **42,** 2128—2136.

Gutierrez, M. V., M. C. Vaz Patto, T. Huguet, J. I. Cubero, M. T. Moreno, and A. M. Torres, 2005: Cross-species amplification of *Medicago truncatula* microsatellites across three major pulse crops. Theor. Appl. Genet. **110,** 1210—1217.

He, G., R. Meng, M. Newman, G. Gao, R. N. Pittman, and C. S. Prakash, 2003: Microsatellites as DNA markers in cultivated peanut (*Arachis hypogaea* L.). BMC Plant Biol. **3,** 3.

Hebblethwaite, P. D., 1983: The Faba Bean (Vicia faba L.). Butterworths, London.

Hill, P., D. Burford, D. M. Martin, and A. J. Flavell, 2005: Retrotransposon populations of *Vicia* species with varying genome size. Mol. Genet. Genomics. **273,** 371—381.

Jones, E. S., M. O. Dupall, J. L. Dumsday, L. J. Hughes, and J. W. Forster, 2002: An SSR-based genetic linkage map for perennial ryegrass (*Lolium perenne* L.). Theor. Appl. Genet. **102,** 405—415.

Kölliker, R., E. S. Jones, M. C. Drayton, M. P. Dupal, and J. W. Forster, 2001: Development and characterisation of simple sequence repeat (SSR) markers for white clover (*Trifolium repens* L.). Theor. Appl. Genet. **102,** 416—424.

Kuleung, C., P. S. Baenziger, and I. Dweikat, 2004: Transferability of SSR markers among wheat, rye, and triticale. Theor. Appl. Genet. **108,** 1147—1150.

Lawson, M. J., and L. Zhang, 2006: Distinct patterns of SSR distribution in the *Arabidopsis thaliana* and rice genomes. Genome Biol. **7,** R14.

Link, W., C. Dixkens, M. Singh, M. Schwall, and A. E. Melchinger, 1995: Genetic diversity in European and Mediterranean faba bean germplasm revealed by RAPD markers. Theor. Appl. Genet. **90,** 27—32.

Linke, K. H., and M. C. Saxena, 1991: Study on variability and longevity of *Orobanche* seed under laboratory conditions. In: K. Wegmann, and L. J. Musselman (eds), Progress in Orobanche Research, 110—114. Ederhard-Karls- Universitaet, Tuebingen, FRG.

Mesa-Garcia, J., and L. Garcia-Torres, 1986: Effect of planting date on parasitism of broad bean (*Vicia faba*) by crenate broomrape (*Orobanche crenata*). Weed Sci. **34,** 544—550.

Nassib, A. M., A. A. Ibrahim, and S. A. Khalil, 1982: Breeding for resistance to *Orobanche*. In: G. Hawtin, and C. Webb (eds), Faba Bean Improvement. 199—206. Marinus Nijhoff, The Hague, the Netherlands

Nei, M., and W. H. Li, 1979: Mathematical models for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA. **76,** 5269—5273.

Nouzova, M., M. Kubalakova, M. Dolezelova, A. Koblizkova, P. Neumann, J. Dolezel, and J. Macas, 1999: Cloning and characterization of new repetitive sequences in field bean (*Vicia faba* L.). Ann. Bot. **83,** 535—541.

Peakall, R., S. Gilmore, W. Keys, M. Morgante, and A. Rafalski, 1998: Cross-species amplification of soybean (*Glycine max*) simple sequence repeats (SSRs) within the genus and other legume genera: implications for the transferability of SSRs in plants. Mol. Biol. Evol. **15,** 1275—1287.

Pozarkova, D., A. Koblizkova, B. Roman, A. M. Torres, S. Lucretti, M. Lysak, J. Dolezel, and J. Macas, 2002: Development and characterization of microsatellite markers from chromosome 1-specific DNA libraries of *Vicia faba*. Biol. Plant. **45,** 337—345.

Rafalski, J. A., and S. V. Tingey, 1993: Genetic diagnostics in plant breeding: RAPDs, microsatellites and machines. Trends Genet. **9,** 275—280.

Rafalski, J. A., J. M. Vogel, M. Morgante, W. Powell, C. Andre, and S. V. Tingey, 1996: Generating and using DNA markers in plants. In: B. Birren, and E. Lai (eds), Nonmammalian Genomic Analysis: A Practical Guide, 75—134. Academic Press, San Diego.

Rakoczy-Trojanowska, M., and H. Bolibok, 2004: Characteristics and a comparison of three classes of microsatellite-based markers and their application in plants. Cell. Mol. Biol. Lett. **9,** 221—238.

Röder, M. S., J. Plaschke, S. U. König, A. Börner, M. E. Sorrells, S. D. Tanksley, and M. W. Ganal, 1995: Abundance, variability and chromosomal location of microsatellites in wheat. Mol. Gen. Genet. **246,** 327—333.

Rohlf, F. J., 2002: NTSYS-pc Numerical Taxonomy and Multivariate Analysis System, Version 2.20k. Exeter Software, Setauket, New York.

Román, B., A. M. Torres, D. Rubiales, J. I. Cubero, and Z. Satovic, 2002: Mapping of quantitative trait loci controlling broomrape (*Orobanche crenata* Forsk.) resistance in faba bean (*Vicia faba* L.). Genome **45,** 1057—1063.

Rozen, S., and H. J. Skaletsky, 1998: Primer3. Code. http://www-genome.wi.mit.edu/genome_software/other/primer3.html.

Saha, M. C., M. A. R. Mian, I. Eujayl, J. C. Zwonitzer, L. Wang, and G. D. May, 2004: Tall fescue EST-SSR markers with transferability across several grass species. Theor. Appl. Genet. **109,** 783—791.

Sauerborn, J., 1991: The economic importance of the phytoparasites *Orobanche* and *Striga*. In: J. K. Ransom, L. J. Musselman, A. D. Worsham, and C. Parker (eds), Proceedings of the 5th International Symposium on Parasitic Weeds, 137—143. CIMMYT, Nairobi, Kenya.

Sauerborn, J., and M. C. Saxena, 1986: A review on agronomy in relation to *Orobanche* problems in faba bean (*Vicia faba* L.). In: S. J. ter Borg (ed.), Biology and Control of Orobanche Proceedings of a Workshop on Biology and Control of Orobanche, 160—165. Agricultural University, Wageningen, the Netherlands.

Schweighofer, A., H. Hirt, and I. Meskiene, 2004: Plant PP2C phosphatases: emerging functions in stress signaling. Trends Plant Sci. **9,** 236—243.

Sethy, N. K., B. Shokeen, K. J. Edwards, and S. Bhatia, 2006: Development of microsatellite markers and analysis of intraspecific genetic variability in chickpea (*Cicer arietinum* L.). Theor. Appl. Genet. **112,** 1416—1428.

Temnykh, S., G. DeClerck, A. Lukashova, L. Lipovich, S. Cartinhour, and S. McCouch, 2001: Computational and experimental analysis of microsatellites in rice (*Oryza sativa* L.): frequency, length variation, transposon associations, and genetic marker potential. Genome Res. **11,** 1441—1452.

Torres, A. M., B. Román, C. M. Avila, Z. Satovic, D. Rubiales, J. C. Sillero, J. I. Cubero, and M. T. Moreno, 2006: Faba bean breeding for resistance against biotic stresses: towards application of marker technology. Euphytica **147,** 67—80.

Von Stackelberg, M., S. A. Rensing, and R. Reski, 2006: Identification of genic moss SSR markers and a comparative analysis of twenty-four algal and plant gene indices reveal species-specific rather than group-specific characteristics of microsatellites. BMC Plant Biol. **6,** 9.

Wu, Y. Q., and Y. Huang, 2007: An SSR genetic map of *Sorghum bicolor* (L.) Moench and its comparison to published genetic map. Genome **50,** 84—89.

Yu, K., S. J. Park, V. Poysa, and P. Gepts, 2000: Integration of simple sequence repeat (SSR) markers into a molecular linkage map of common bean (*Phaseolus vulgaris* L.). J. Hered. **91,** 429–434.

Zane, L., L. Bargelloni, and T. Patarnello, 2002: Strategies for microsatellite isolation: a review. Mol. Ecol. **11,** 1—16.

Zeid, M., C. C. Schön, and W. Link, 2003: Genetic diversity in recent elite faba bean lines using AFLP markers. Theor. Appl. Genet. **107,** 1304—1314.