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Genetic Diversity among Palestinian Faba Bean (*Vicia faba* L.) Ecotypes based on Single Nucleotide Polymorphisms

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Summary

The present study is the first attempt to establish a SNP database for Palestinian faba bean accessions, in order to evaluate the relationships among them and the genetic diversity present. A total of 17 faba bean accessions (13 local and four supplied by ICARDA), were considered using a SNP assay in the coding region of the actin gene. Subsequently, five SNPs were detected, with an average frequency of 1 SNP every 139 bp, showing thereby a higher polymorphism compared with other legume crops. Cluster analysis with UPGMA revealed five groups of faba bean accessions, somehow reflecting their origin. This is a first evaluation of the use of SNP in the characterization of Palestinian faba bean genetic resources for their conservation and future utilization.

Key words. broad beans – genetic fingerprinting – SNPs markers

Introduction

Faba bean (*Vicia faba* L.), sometimes referred to as broad bean, field bean, tic bean, windsor bean or horse bean, is an ancient legume diploid crop (2n = 2x = 12). The oldest seeds of faba bean were found in late 10th millennium b.p. in north-west Syria (Tanno and Willcox 2006) and about at the same time in Jericho and Yiftah'el, suggesting southwestern Asia as the primary center of *Vicia faba* domestication. As no wild *Vicia faba* has ever been found and because it does not cross with other species of the same genus, the wild ancestor of faba bean remains unknown (Hanelt et al. 1972).

Currently, faba bean is one of the most important grain legumes used world-wide for human consumption, animal feeding and other non-food purposes, such as improvement of soil fertility. In Palestine, faba bean is an essential crop grown mainly under rain-fed conditions on an area of approximately 500 ha and with a total production of fresh legumes of about 4000 t. The annual production is low and varies greatly from year to year as well as from location to location (FAO 2010). Moreover, the long history of its cultivation, the wide range of climatic conditions, and the common practice of exchanging seeds among farmers have produced a complex varietal picture in which varieties are often either misidentified or called by different names in different areas. Additionally, the severe decline of its cultivation due to low yield and yield instability (Gresta et al. 2009) increases the risk of losing useful genetic resources for any future breeding programs and variety development. Since autochthonous landraces carry gene pools adapted to different agro-ecological conditions, they may represent an economically valuable opportunity for farmers in marginal areas, and could be useful to plant breeders to develop new varieties (Nadal et al. 2003; Terzopoulos et al. 2008). Therefore, for conservation of plant genetic resources as well as for crop improvement purposes it is critical to be able to discriminate among accessions.

In the last decades, various kinds of molecular markers including restriction fragment length polymorphisms (Van De Ven et al. 1990), random amplified polymorphic DNAs (Torres et al. 1993), amplified fragment length polymorphisms (Zeid et al. 2003; Zong et al. 2009, 2010), inter simple sequence repeats (Terzopoulos and Bebeli 2008), genomic and cDNA microsatellites (Duc et al. 2010), and target region amplification polymorphism (Kwon et al. 2010) have been used to estimate genetic diversity in faba bean. Recently, Single Nucleotide Polymorphisms (SNPs) proved to be one of the most important markers for assessing genetic variation in most organisms (Khlestkina and Salina 2006). Due to the their abundance, the high stability of inheritance, the occurrence in coding regions resulting in changes in the encoded polypeptides, and the high discriminatory ability (Consolandi et al. 2007) they could be used for cultivar certification (Shirasawa et al. 2006).

The present study represents the first attempt to establish a SNPs database for the Palestinian faba bean accessions, in order to evaluate the genetic diversity and the

relationships among the studied genotypes. The results will contribute to the conservation of faba bean genetic resources in the region and to enhance the available knowledge for future projects in related fields.

Material and Methods

Plant materials

A total of 17 faba bean (*Vicia faba*) accessions (Table 1) were investigated in this study. Due to the lack of information, an exploration mission was carried out at the beginning of spring 2013. Thirteen local accessions were collected from different farms across the main agro-ecological zones in West-Bank.

One accession (FTU1) was collected from Tubas in the Eastern slopes zone of the West Bank, located between the Jordan Valley and the Central Highland Regions. The area is characterized by a semi-desert climate with altitudes ranging from 800 m a.s.l. to 200 m b.s.l. and an average annual rainfall between 250 and 300 mm. The land morphology is represented by steep slopes and deep frequently canyon-shaped valleys (= wadi). Agriculture is a marginal activity, limited to the cultivation of field crops (barley and wheat), olive trees, and spring grazing.

The accessions from Jenin, (FCOJ1, FCOJ2, FJ1, FJE3) Ramallah (FCOR1, FR1, FR2) and Hebron (FCOH1, FCOH2, FH1) areas, were collected in the Northern, Median and Southern Central Highland zone, respectively. This zone is characterized by a Mediterranean climate with mountainous and hilly morphology, with elevations ranging from 400 to 1,020 m a.s.l. Average annual rainfall is about 400 mm (minimun about 300 mm in the south and max 600 mm in the north). Agriculture is one of the main economic activities in the area, mainly in rainfed conditions (cereals and grain legumes).

Accessions from Qalqilya (FQ1 and FQ2) were collected in the semi coastal zone, a narrow strip comprised between the cities of Jenin and Tulkarem, with elevations between 100 and 300 m a.s.l. The average annual rainfall ranges between 400 and 700 mm. Agriculture is an important activity in the area, both under irrigated and rainfed management (vegetables, orchards, cereals).

Details on each accession were recorded through interviews with farmers and field observations at the time of collection.

The remaining four accessions (FIC1, FIC2, FIC3, FIC4) were obtained from ICARDA Syria together with a general description of them. They were developed through breeding programs aimed to improve the agronomic features such as grain production and suitability for mechanization.

Table 1. List of faba bean accessions considered in this study.

Code	Source
FCOH1	Hebron
FCOH2	Hebron
FCOJ1	Jenin
FCOJ2	Jenin
FCOR1	Ramallah
FH1	Hebron
FIC1	ICARDA
FIC2	ICARDA
FIC3	ICARDA
FIC4	ICARDA
FJ1	Jenin
FJE3	Jenin
FQ1	Qalqilya
FQ2	Qalqilya
FR1	Ramallah
FR2	Ramallah
FTU1	Tubas

Genomic DNA extraction

For each accession, seeds were germinated, leaf tissue from 20 seedlings (20-days old) was obtained and ground to a fine powder in liquid nitrogen, and genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen Inc.) following the manufacturer's instructions. DNA quality and concentration were checked using 1% agarose gel electrophoresis and absorbance reading (Jenway 6305, UV-Visible Spectrophotometer, UK), respectively.

Choice of target gene

SNP discovery was carried out based on the amplification of DNA fragments using locus-specific primers of the actin gene (*ACT*) of *Vicia faba* (NCBI Ac.No. GQ339767). Since it is highly conserved as an essential ingredient of the cytoskeleton in eukaryotes (Kost et al. 1999), actin was chosen among possible candidates because of its potential for species discrimination (Hightower and Meagher 1986; Goodson and Hawse 2002). In *Vicia faba* actin is present as five isoforms (Janben et al. 1996); in *Arabidopsis* where also several isoforms have been detected, it is suggested that each of them may be critical for successful viability of individual seedlings (McKinney and Meagher 1998).

Polymerase chain reaction (PCR)

PCR amplifications were performed in a total volume of 100 µL containing 10XTaq buffer (Hylabs), 5 µM primer (FP: 5`ATTCTATAATGAGCTTCGTGTTGCC3` & RP: 5`TTCTCTCAGGTGGTGCAACG 3`), 200 mM dNTPs (Fermentas), 25 mM $MgCl_2$ (Hylabs), Taq DNA polymerase (Hy Labs) and 30 ng of genomic DNA. HPLC

water was also added to the PCR reaction mix up to the appropriate volume. DNA amplifications were conducted on a Peltier Thermal Cycler-200 (MJ Research. Inc, Watertown, MA) with an initial denaturation step of 10 min at 95 °C, followed by 35 cycles of denaturation (95 °C for 45 s), annealing (61 °C for 45 s) and extension (72 °C for 60 s), and with a final extension step at 72 °C for 10 min. PCR primer pairs (Invitrogen) were designed to the studied actin gene using Primer-Blast software of NCBI [http://www.ncbi.nlm.nih.gov/tools/primer-blast/].

Before the sequencing step, in order to check the quality of amplification, for each accession 10 µl of PCR product were added of 2 µl of loading buffer and loaded on a 2 % (w/v) agarose gel stained with ethidium bromide and visualized with UV light.

Sequencing, SNP Discovery, and sequence analyses

PCR products obtained from all studied faba bean genotypes were submitted to automated sequencing (Hy Laboratory Ltd., Rehovot, Israel). Chromas lite 2.1 and MEGA 4 softwares were used to detect and characterize the polymorphisms among the sequencing traces obtained.

Genetic diversity and data analyses

Statistics on polymorphism were estimated using DnaSP software version 5 (Librado and Rozas 2009). Nucleotide diversity (π, *i.e.* the average number of nucleotide differences per site; Nei 1987), the number of segregating sites (Θ; Watterson 1975), and their sampling variances were calculated. Using the Tajima's D test, implemented in DnaSP, π and Θ values were compared to test the neutrality of molecular polymorphisms. Relationships among DNA sequences from different lines were investigated calculating the simple matching coefficient (Sneath and Sokal 1973) and by cluster analysis with UPGMA using MEGA4 (Tamura et al. 2007). Branch support of the similarity tree was computed by bootstrap test on 500 replications.

Results and Discussion

Actins in plants are encoded by a multigene family that includes dozens of genes differentially expressed during plant development. For example in *Arabidopsis*, the actin gene family comprises 10 distinct members, eight of which are functional genes and two are pseudogenes (McDowell et al. 1996).

A search in the NCBI database (http://www.ncbi.nlm.nih.gov/) for faba bean sequences provided one partial copy of the actin gene, which allowed the development of an SNP assay in the coding region.

The primers used in this investigation were designed to obtain one specific DNA fragment so that amplifications of the target gene were carried out successfully. The amplified fragment of actin gene was 740 bp long, whereas the length of the sequence subsequently analyzed was 694 bp in order to prevent the low quality of the signal in the electropherograms in the regions close to the annealing sites of primers.

Based on the alignment with the genomic sequence of soybean actin 4 gene (NCBI Ac.No. AF049106.1), the region analyzed in this study represents entirely a coding sequence and corresponds primarily to exon III of the faba bean gene. Five SNPs (Table 2) were detected within the sequence among the 17 accessions, with an average frequency of 1 SNP every 139 bp. Of the five variants, one was a transversion and four were transitions, confirming the general observation that transitions are more common than transversions, probably due to the high rate of spontaneous deamination of 5-methyl cytosine (5mC) to thymidine (C↔T), with (G↔A) on the other strand (Strachan and Read 2003; Morley et al. 2004) (Table 2).

Based on this finding, faba bean seems to have a high level of polymorphism compared with that reported in other crop species (as average of many genes, considering both coding and non-coding regions), such as rapeseed (1 SNP per 600 bp; Fourmann et al. 2002), *Arabidopsis* (1/336 bp; Schmid et al. 2003), soybean (1/273 bp; Zhu et al. 2003) and barley (1/200bp; Rostoks et al. 2005). Moreover, considering only coding regions, the level of polymorphism here observed is close to that of maize, another outcrossing species (1 SNP per 124 bp; Ching et al. 2002) and much higher than that of soybean, an autogamous species (1 per 610 bp; Rafalski 2002).

A detailed description of the nucleotide diversity and neutrality test statistics of the actin gene among the accessions is presented in Table 3. π value for the coding portion of the actin gene observed in the collection of faba bean accessions here considered (0.00250) was lower, although comparable, than the level of polymorphism observed among U.S. elite maize inbreds (0.00630), calculated over genomic sequences of many genes (Ching et al. 2002), but higher than that reported for soybean germplasm, considering only coding regions (0.00054; Zhu et al. 2003). The result of the Tajima's D test indicated that the polymorphisms detected in the actin gene are selectively neutral.

All the 5 SNPs found in the coding region of actin gene are non-synonymous, determining amino acid changes in the encoded protein (Table 2). Three of them introduced the same non-conservative amino acid substitution from Methionine to Threonine at positions 101, 175 and 229. At amino acid position 171 another non-conservative substitution was found where either a Leucine or a Tryptophan is encoded. The last amino acid change was detected at position 129 where either Proline or Leucine is found. Although this substitution does not modify the net charge, since both residues are non-polar and hydrophobic, still it may alter the secondary structure of the peptide (Lanza et al. 2002). Furthermore, it would be premature to speculate about any functional significance of

Table 2. SNPs located in coding regions of actin gene in *Vicia faba* and the coded amino acids.

Accession	Haplotype	Alleles & Positions					Amino acids (AA) & Positions				
		302	386	512	524	686	101	129	171	175	229
FIC1 FIC2 FIC4	1	T	C	G	C	C	M	P	W	T	T
FIC3 FQ1 FTU1 FCOH1 FCOJ1	2	C	T	G	C	T	T	L	W	T	M
FJ1 FJE3 FR2 FH1 FQ2	3	C	T	G	C	C	T	L	W	T	T
FR1 FCOJ2	4	C	T	G	T	C	T	L	W	M	T
FCOH2 FCOR1	5	C	T	T	T	T	T	L	L	M	M
Change of AA class[(1)]							from N-NP to N-P	No change	from N-SP to N-NP	from N-P to N-NP	from N-P to N-NP

[(1)] N-P: neutral-polar; N-NP: neutral-non polar; N-SP: neutral-slightly polar.

Table 3. Nucleotide diversity and neutrality test of the actin gene sequence analyzed in the 17 accessions.

Lenght (bp)	Polymorphic sites (n)	Haplotypes (n)	Hd	k	π	Θ	Tajima's D test
694	5	5	0.816	1,735	0.00250	0.00213	0.55922

Hd = Haplotype diversity
k = Average number of nucleotide differences
π = Nucleotide diversity
Θ = Number of segregating sites

the amino acid substitutions found; predictions on the impact of these variants on protein function could be done only when the other parts of the coding sequence were investigated.

Based on these SNPs, five haplotypes were identified in our panel. Cluster analysis of the 17 nucleotide sequences displays 5 groups supported by 32–86 bootstrap values (Fig. 1).

Haplotype 1 (Hap_1) is the most supported branch since it differs from all other haplotypes at nucleotide positions 302 and 386. It is reported that the genotypes of Hap_1 are improved cultivars bred by ICARDA and this explains their incorporation in the same group. However, 'FIC3', another improved genotype (also from ICARDA), grouped with different local accessions in Hap_2 with which it could be related. Another rather strongly sup-

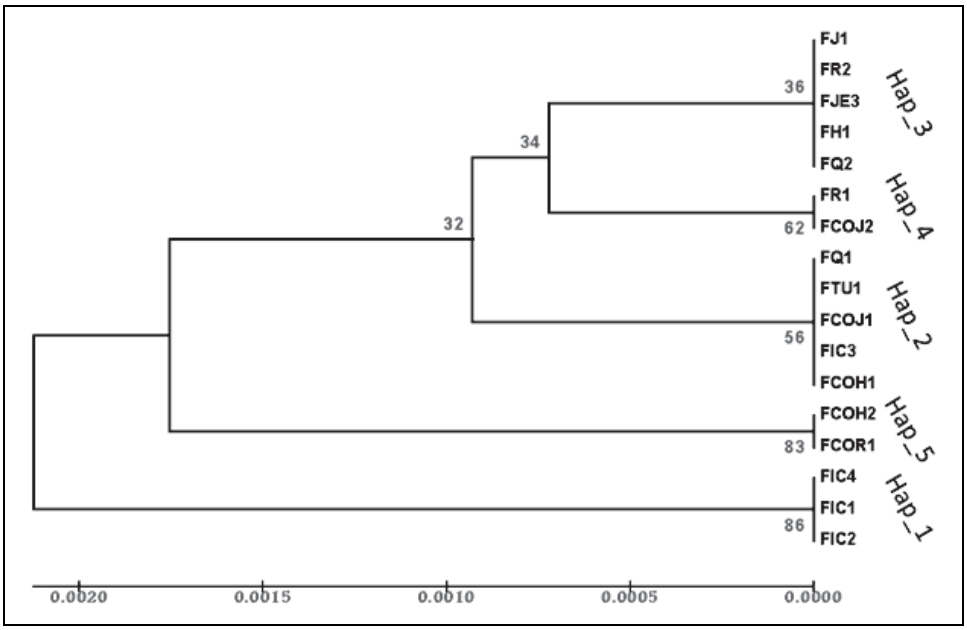


Fig. 1. Phylogenetic tree based on nucleotide sequences of actin gene of the 17 faba bean accessions constructed by UPGMA. Bootstrap values on bifurcating branches are based on 500 random replicates.

ported branch distinguishes Hap_5 that is characterized by the unique SNP at position 512. Haplotypes 2, 3 and 4 show more similarities among themselves. Also Hap_3 like Hap_2 includes accessions collected throughout several localities of West-Bank (Hebron, Jenin, Ramallah and Qalqilya).

In conclusion, our analysis provides a preliminary estimate of genetic diversity and relationships within faba bean germplasm of Palestine, but further research is necessary to support and validate these results. As expected, the analysis of the partial coding region of the actin gene does not allow distinguishing down to the level of single accessions, but just between several haplotypes of a single locus; more genes should be investigated to reach an unambiguous identification of the accessions and to understand the genetic relationships among them. In fact, another gene, phosphogluconate dehydrogenase (PGDH), was examined, but it was not reported since not polymorphic among the accessions here considered. Once a sufficient set of SNP markers were available, time- and cost-efficient assays could be designed for routine use in germplasm conservation and varietal identification.

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