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ARTICLE

Evaluation of SRAP markers for mapping of *Pisum sativum* L.

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Abstract: *Linkage maps have become important tools for genetic studies. With the aim of evaluating the SRAP (sequence-related amplified polymorphism) technique for linkage mapping in Pisum sativum L., a F_2 mapping population derived from an initial cross between cvs. DDR11 and Zav25 was generated. A total of 25 SRAP primer combinations were evaluated in 45 F_2 plants and both parental lines, generating 208 polymorphic bands/markers. The markers were analyzed by the chi-square goodness-of-fit test to check the expected Mendelian segregation ratio. The resulting linkage map consists of 112 genetic markers distributed in 7 linkage groups (LGs), covering a total of 528.8 cM. The length of the LGs ranged from 47.6 to 144.3 cM (mean 75.54 cM), with 9 to 34 markers. The linkage map developed in this study indicates that the SRAP marker system could be applied to mapping studies of pea.*

Key words*: Pea, plant breeding, linkage map, F_2 population, molecular markers.*

INTRODUCTION

Pea (*Pisum sativum* L.) is an autogamous, annual cool-season legume originated from areas in the Middle East, in the East of the Caucasus, Iran and Afghanistan, and West of the Mediterranean basin (Smýkal et al. 2011). Its genome is organized in seven chromosome pairs (2n = 2x = 14), and the haploid size estimated at 4.45 Gb (Smýkal et al. 2012). Peas were an important source of animal and human food for many centuries. The species is rich in protein, slowly digestible starch, soluble sugars, fiber, minerals, and vitamins (Dahl et al. 2012). The global dry pea production averages 10 million tonnes a year. Argentina is one of the top exporting countries, eighth in the global ranking between 2008 and 2011 (FAO 2012). The rising world population will require increased crop production. Moreover, some researchers suggest that the current rate of increase in crop yields will not be enough to meet this demand (Tester and Langridge 2010). Therefore, plant breeding programs are needed to further raise crop yields. In this context, linkage mapping will be useful to maximize the success probability. Genetic linkage maps are powerful tools for genetic research and breeding of plants. The linkage maps are the first step in: 1) the analysis of qualitative and quantitative traits; 2) the introgression of desirable genes and quantitative trait loci (QTLs); 3) positional or map-based cloning of genes responsible for economically important traits (Semagn et al. 2006). Different kinds of markers, such as simple sequence repeats (SSR; Loridon et al. 2005), single nucleotide polymorphisms (SNP; Deulvot et al. 2010), inter simple sequence repeats (ISSR; Mishra et al. 2009), and sequence tagged sites (STS; Barilli et al.

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2010) have been used to develop moderate density linkage maps in pea. Several markers were common in different maps corresponding to different crosses; this allowed an integration of these maps (Loridon et al. 2005, Aubert et al. 2006), as well as the development of consensus linkage maps for the species (Weeden et al. 1998, Bordat et al. 2011).

In this study, we proposed the use of the sequence-related amplified polymorphism (SRAP) technique (Li and Quiros 2001) to generate a number of markers distributed across all pea chromosomes. Since its development, SRAP has been employed in a wide range of plant species for genetic diversity estimation (Cravero et al. 2007, Espósito et al. 2007, Aneja et al. 2012), gene tagging (Martin et al. 2008, Zhang et al. 2010), and map construction (Lin et al. 2005, Sun et al. 2007, Wang et al. 2008, Martin et al. 2013). The aim of the current study was to evaluate the usefulness of SRAP markers in the development of a genetic linkage map of *Pisum sativum* L.

MATERIAL AND METHODS

Plant material

The F_2 mapping population was derived from an initial cross between the cvs. DDR11 and Zav25. The latter is an experimental line obtained from the IICAR-CONICET breeding program (Espósito et al. 2007). For most yield-related traits, such as number of pods and seeds per plot, the values of 'DDR11' are lower than those of 'Zav25'.

Both parents and 45 plants of the F_2 population were sown in an experimental field of Universidad Nacional de Rosario (lat 33° 1' S, long 60° 53' W) in the winter of 2012, in a completely randomized design (inter-row spacing 70 cm, plant spacing 10 cm).

DNA extraction

The genomic DNA of each F_2 plant and both parents was isolated from fresh leaves by the CTAB method described by Doyle and Doyle (1990), with the following modifications: after DNA precipitation, the samples were stored at -20 °C for 30 min, the two washes of the final step were performed with ethanol 70%, and the resulting pellet was resuspended in distilled water.

After RNAase-treatment, each DNA sample was quantified using agarose gel electrophoresis (1% w/v) and comparison of band intensity with the standard λ DNA (76 ng μL^{-1}). The hybrid origin of each plant was checked using two microsatellite markers SSR: PSMPSAA135 and PSMPSAA205 (Tar'an et al. 2005), which were contrasting in both parental lines.

SRAP genotypic analysis

The F_2 population was genotyped using 25 SRAP primer combinations generated from five forward and five reverse primers developed by Li and Quiros (2001) (Table 1). The primers are 17-18 bases long and have a core sequence, which includes 10-11 non-specific bases at the 5´end and sequence CCGG in the forward and AATT in the reverse primer. The core sequence is followed by three selective nucleotides at the 3´ end of each primer. The primers were selected based on the results of Espósito et al. (2007) in a characterization of pea accessions with this type of molecular markers.

Polymerase chain reactions were carried out in a final volume of 20 µL containing 15 ng genomic DNA, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 1X Taq buffer (Invitrogen, California, USA), and 1 U of Taq recombinant polymerase (Invitrogen). Samples were subjected to the following thermal profile: 5 min denaturing at 94 °C and five cycles of three steps: 1 min denaturing at 94 °C, 1 min annealing at 35 °C, and 1 min elongation at 72 °C; for the following 35 cycles, annealing temperature was elevated to 50 °C with a final elongation step of 10 min at 72 °C.

Table 1. Primer sequences used for SRAP (Sequence-related amplified polymorphism) analysis

Forward	Reverse
me1 5´-TGAGTCCAAACCGGATA-3´	em1 5´-GACTGCGTACGAATTAAT-3´
me2 5´-TGAGTCCAAACCGGAGC-3´	em2 5´-GACTGCGTACGAATTTGC-3´
me3 5´-TGAGTCCAAACCGGAAT-3´	em3 5´-GACTGCGTACGAATTGAC-3´
me4 5´-TGAGTCCAAACCGGACC-3´	em4 5´-GACTGCGTACGAATTTGA-3´
me5 5´-TGAGTCCAAACCGGAAG-3´	em5 5´-GACTGCGTACGAATTAAC-3´

The resulting amplicons were separated on 6% (w/v) denaturing polyacrylamide gels and then visualized by silver staining (Bassam et al. 1991). The SRAP fragments were treated as dominant markers. Each marker was labeled according to the primer combination used for its generation plus the estimated amplicon size.

Linkage analysis and linkage map construction

Linkage analyses were performed using JoinMap v4 (van Ooijen 2006). Each segregating marker was tested for deviations from the expected 3:1 segregation ratio using Chi-square tests. Markers with Mendelian segregation ($x^2 \leq x^2_{\alpha=0.1}$) or with minor distortion ($x^2_{\alpha=0.1} < x^2 \leq x^2_{\alpha=0.01}$) were used for the construction of the linkage map. Markers with highly distorted segregation ($x^2 > x^2_{\alpha=0.01}$) were included in a second step of mapping only when their presence did not affect the local marker order. Linkage groups (LGs) were established at a minimum LOD (logarithm of odds) value of 3.0. The marker order for each LG was determined at LOD = 1.0, REC = 0.40 and Jump = 5. Recombination values were converted to genetic distances using the Kosambi (1994) mapping function. Linkage groups were numbered sequentially according to their length in cM. Linkage maps were drawn using MapChart 2.2 software (Voorrips 2002).

RESULTS AND DISCUSSION

Since the development of molecular marker techniques, the number of marker loci identified on genetic maps is increasing at a high rate. The SRAP marker system designed by Li and Quiros (2001) is a simple and efficient technique. It has several advantages over other molecular markers, namely its simplicity and reasonable throughput rate. It also allows easy isolation of bands for sequencing and, most importantly, it targets ORFs (open reading frames). Elsewhere, SRAPs were established as a powerful tool for construction of genetic linkage maps, e.g., of *Brassica* (Li and Quiros 2001, Sun et al. 2007), *Gossypium* (Lin et al. 2005, Yu et al. 2007), *Cucumis melo* L. (Wang et al. 2008), and more recently of *Cynara cardunculus* (Martin et al. 2013). Our study presents the first application of SRAP markers for the construction of a linkage map of *P. sativum* L.

The parental lines to develop the mapping population ('DDR11' and 'Zav25') were selected based on observations of Espósito et al. (2007), who reported that these lines are divergent at the morphological (Euclidean distance = 0.47) and molecular levels (Dice distance = 0.66) and that they were grouped separately by hierarchical cluster analysis.

A total of 25 SRAP primer combinations were used of which 23 pairs were amplified. Most combinations produced clear bands without overlapping, but in some cases the scoring of the markers was somewhat cumbersome, because of the high number of bands and their different intensities, and the presence of minor bands in some plants. The number of fragments amplified by each primer combination ranged from 3 (Me1-Em1; Me1-Em4) to 24 (Me3-Em2), with an average of 9.96. A total of 208 polymorphic bands (PB) were generated. The most polymorphic primer combination was Me2-Em3, with 18 PB (Table 2).

The markers proved efficient for genetic studies in pea, producing an average of 8.32 PB/primer combination. This value is similar to those of the other species, where 3 to 14 bands per primer combination were reported (Li and Quiros 2001, Lin et al. 2003, Sun et al. 2007, Yu et al. 2007, Wang et al. 2008, Martin et al. 2013). Since one primer combination may detect a high number of polymorphic loci,

Table 2. Number of bands and polymorphic bands generated by SRAP primer combinations

Combination	Bands	Polymorphic bands
Me1-Em1	3	3
Me1-Em2	9	9
Me1-Em3	5	5
Me1-Em4	3	2
Me1-Em5	15	14
Me2-Em1	8	8
Me2-Em2	9	9
Me2-Em3	18	18
Me2-Em4	16	14
Me2-Em5	13	9
Me3-Em1	4	1
Me3-Em2	24	16
Me3-Em3	8	8
Me3-Em4	10	8
Me3-Em5	8	7
Me4-Em1	0	0
Me4-Em2	10	7
Me4-Em3	6	6
Me4-Em4	17	12
Me4-Em5	5	5
Me5-Em1	0	0
Me5-Em2	6	3
Me5-Em3	16	16
Me5-Em4	18	14
Me5-Em5	18	14
Total	249	208

Table 3. Characteristics of the linkage groups (LG) generated by SRAP markers

LG	Number of markers	Number of highly distorted markers	Size	Smallest distance between markers	Highest distance between markers	Average distance between markers
				cM (CentiMorgan)		
1	34	6	144.309	0.031	25.392	4.244
2	21	6	87.134	0.778	15.588	4.149
3	18	1	79.119	0.204	16.06	4.395
4	10	1	61.401	1.608	17.801	6.140
5	11	4	54.555	1.973	15.329	4.959
6	9	2	54.674	1.653	14.435	6.074
7	9	2	47.601	2.207	15.1	5.289

this technique can be used to construct ultra-dense genetic maps. Furthermore, SRAP markers can be combined with next-generation techniques to enhance their capacity and effectiveness. Li et al. (2011) combined SRAP with Illumina/Solexa sequencing to directly integrate genetic loci in the *B. rapa* genetic map based on paired-end Solexa sequencing. Results of the SRAP technique obtained in this way may prove invaluable for QTL analysis and map-based cloning.

Chi-square analysis of the 208 loci revealed that 116 loci (~ 55.8%) were consistent with the expected Mendelian 3:1 segregation ratio ($x^2 \leq x^2_{\alpha=0.1}$), the distortion of 37 loci (~ 17.8%) was minor ($x^2_{\alpha=0.1} < x^2 \leq x^2_{\alpha=0.01}$) and 55 loci (~ 26.4%) were highly distorted ($x^2 > x^2_{\alpha=0.01}$).

Initially, the 153 markers with Mendelian or slightly distorted segregation were used for the construction of linkage groups (LG), using a minimum LOD value of 3.0 and the mapping parameters Rec = 0.40, LOD = 1.0, and Jump = 5. Under

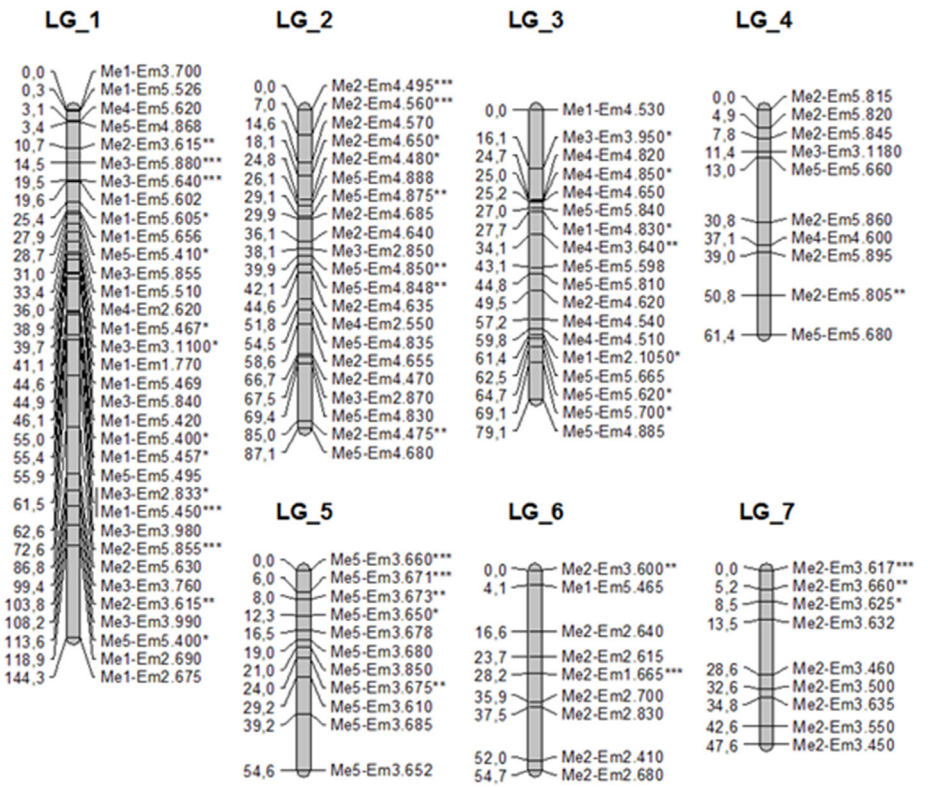

Figure 1. Genetic linkage map of pea. Marker names are shown on the right of each LG (linkage group) and map distances (in cM) on the left. Markers with significant levels of segregation distortion are indicated by asterisks (minor distortion * 0.05 ≥ P > 0.01, highly distorted segregation: **0.01 ≥ P > 0.001, ***P ≤ 0.001).

these conditions, an initial framework map with 62 loci was constructed. Then the framework order was fixed and a second round of mapping performed, including markers with distorted segregation. These markers were only included in the final map if their presence did not alter the surrounding marker order in a given linkage group. By this step, we incorporated 29 Mendelian markers and 22 distorted markers in the previously established linkage groups. This strategy ensures the accuracy and a high coverage of the final map. Similar strategies were successfully used for linkage mapping in different species, e g., of olive (Khadari et al. 2010), globe artichoke and cardoon (Martin et al. 2013), lentil (Verma et al. 2015), poplar (Zhou et al. 2015), and wheat (Li et al. 2015).

The resulting map comprises 112 loci distributed over seven linkage groups (LGs), which is equal to the haploid number of chromosomes in the pea genome (Figure 1). The overall map length was 528.8 cM and the mean inter-marker distance 4.72 cM. The LG length varied from 47.6 cM to 144.3 cM. The number of markers included in each LG ranged from 9 to 34 (Table 3). A total of 22 distorted markers (19%) were included in the genetic map.

Although several linkage maps for pea have been developed using different kinds of markers, this is the first linkage map of this species constructed with SRAP markers. The length of our map (528.8 cM) is shorter than that of previous ones generated with other molecular markers, which covered 1430, 1458, and 1283 cM, respectively (Loridon et al. 2005, Aubert et al. 2006, Barilli et al. 2010). All these genetic maps were constructed using mapping populations with different sizes, derived from different crosses. Both, the size of the mapping population and their origin affect the marker coverage and map length because an increasing divergence between parents generates a greater number of possible recombinations and the possibility of finding recombinant plants is higher when populations are large. Then, the small size of our F_2 population (45 plants) could be the cause of the smaller size of the map obtained in this study (Ferreira et al. 2006). To enhance accuracy and reduce the statistical error, a great number of plants should be evaluated. On the other hand, the large number of unlinked markers with Mendelian or slightly distorted segregation (62) reflects the need to enrich this map with additional markers to cover the entire genome.

CONCLUSIONS

The linkage map generated in this study provided basic information for assistance of future molecular marker application in the local breeding program of *Pisum sativum* L. Since pea has no reference genome, molecular markers that do not require sequence information must be evaluated. In this context, sequence-related amplified polymorphism (SRAP) represents an efficient tool for genetic analysis of pea even though the proposed linkage map was only partly saturated. For the first time, SRAP markers were applied in this study to develop a linkage map of pea. Moreover, since these markers target coding regions of the genome, they can potentially identify markers with inherent biological significance. Additional markers are required to expand the coverage of this map for QTL analysis. The segregating population used to develop this linkage map is currently being phenotyped for yield-related traits to detect QTLs associated to this character.

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