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Short communication. An improved intersubspecific genetic map in *Lens* including functional markers

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Abstract

A previous *Lens* genetic map was improved by adding 31 molecular genetic markers, reaching a total of 190 markers with undistorted segregation. Data were obtained from the segregational analysis of 113 F_2 plants generated from a single hybrid of *Lens culinaris* ssp. *culinaris* × *L. c.* ssp. *orientalis.* The added markers are predominantly codominant (15 SSRs, five CAPSs, four presence-absence polymorphisms, three length polymorphisms, two RAPDs, and two SRAPs). At a LOD score of 3.0, the 190 markers were grouped into eight linkage groups (LG) covering 2,234.4 cM, with an average distance between markers of 12.28 cM. This linkage map has reduced the numbers of linkage groups from ten in the previous map to eight. Most of the added markers must be functional markers since primers were mostly designed to amplify transcribed sequences. Some of the amplicons were sequenced to test if they were functional markers. One of the sequences showed homology with the *Pisum TFL1a* gene, involved in the transition from vegetative to flowering stages. This lentil gene was located in the LG 1 thanks to the presence of a polymorphic microsatellite in the first intron of the gene. Since *L. culinaris* ssp. *orientalis* is the primary source of additional genetic variability for lentil, this improved map could help in the use of such variability in lentil breeding programs.

Additional key words: *Lens culinaris*; *Lens orientalis*; lentil; linkage map; TFL1.

Lentil (*Lens culinaris* Medik.) is a self-pollinated diploid (2n = 14) cool season grain legume normally grown in temperate semi-arid regions, usually in rotation with cereals, contributing to replenish soil nitrogen levels. The cultivated lentil was originated from the wild *Lens culinaris* ssp. *orientalis* Boiss. The lentil cultigene has a relatively low level of genetic polymorphism (Pérez de la Vega *et al*., 2011). Genetic linkage maps are cornerstones in basic genetic analysis as well as in applied plant breeding. In lentil, to date, several intra- and inter-specific genetic maps have been published since 1989, year in which the first map including molecular markers was published (Havey & Muehlbauer, 1989). However, there is not a consensus map in lentil and saturated maps are far from being reached. A review on these topics has been recently published (Pérez de la Vega *et al*., 2011). In order to maximize polymorphism for map construction in lentil, both, recombinant inbred lines and F_2 populations obtained from crosses between the cultivated lentil and the wild *orientalis* subspecies have been used to develop genetic maps in *Lens* (Havey & Muehlbauer, 1989; Eujayl *et al*., 1997, 1998; Durán *et al*., 2004; Hamwieh *et al*., 2005), and as source of resistances (Pérez de la Vega *et al*., 2011). Several QTLs were further located in the intersubspecific map (Fratini *et al*., 2007) previously obtained by Durán *et al.* (2004).

A large list of molecular marker types has been included in lentil genetic maps from RFLPs to SNPs (Pérez de la Vega *et al*., 2011). SRAP markers have been recently used in genetic maps in lentil (Saha *et al*., 2010). The SRAP markers are amplified from open

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Abbreviations used: ALP (amplicon length polymorphism); CAPS (cleaved amplified polymorphic sequences); GLIP (Grain Legumes Integrated Project); P/A (presence-absence); QTL (quantitative trait loci); RAPD (random amplified polymorphic DNA); RFLP (restriction fragment length polymorphism); SNP (single nucleotide polymorphism); SRAP (sequence-related amplified polymorphism).

reading frames and are based in the use of two primers 17 or 18 nucleotides long, where the first 10 or 11 bases are different sequences of no specific constitution, followed by the sequence CCGG in the forward primer or AATT in the reverse primer, and then by three selective nucleotides at the 3' end (Li & Quiros, 2001), in this way regions including exon-intron limits are preferentially amplified. SRAPs were first developed for *Brassica oleracea* and approximately 20% of the SRAP markers were codominant in this species.

The aim of this work was to reach a more saturated intersubspecific lentil map from a previously published map (Durán *et al.*, 2004) by adding new genetic markers among which several functional markers were included.

An F_2 population of 113 individuals derived from the intersubspecific cross of *Lens culinaris* ssp. *culinaris* Medik. cv. 'Lupa' (a microsperma Spanish cultivar) as the female parent and *L. c.* ssp. *orientalis* Boiss (BG 16880) as the pollen donor (Durán *et al.*, 2004; Fratini *et al.*, 2004) was used. Markers and mapping methods are described in Durán *et al*. (2004). The primers used for obtaining each of the new markers included in the map, their annealing temperatures and the corresponding marker sizes are described in Suppl. Table 1 (pdf). For markers derived from previously published papers we also used the procedures described in the Suppl. Table 1 (pdf) with minor modifications. Several new markers were obtained using primers designed within the EU funded Grain Legume Integrated Project (GLIP) to amplify functional markers. GLIP primers were designed from aligned or single DNA sequences of *Pisum sativum* and *Medicago truncatula* to hybridize with exonic sequences including some intron within the amplified sequence.

The genetic map developed by Durán *et al*. (2004) was enhanced without distorted markers, using MAPMAKER v 3.0b software (Lander *et al*., 1987) with the following parameters: LOD score value of 3.0, and maximum distance of 30 cM. Linkage group designation follows the nomenclature used by Durán *et al*. (2004). Only markers fitting the expected Mendelian segregations (3:1 or 1:2:1) were added. PCR amplicons to be sequenced were cloned in the pGEM-T vector and sequenced by the Sanger's method using a Megabace 500 (Amersham) sequencer. Sequences were compared with data from nucleotide and protein databases, aligned by Clustal W, and exon and intron boundaries were determined by similarity with close sequences from other legume species.

A total of 31 new markers [Table 1 and Suppl. Table 1 (pdf)] were added to the genetic map reaching a total of 190 markers with undistorted segregation. Among the new markers, 15 were simple sequence repeats (SSR), five were cleaved amplified polymorphic sequences (CAPS), four were presence-absence polymorphisms of PCR-bands (P/A), three of them were amplicon length polymorphisms (ALP), two were random amplified polymorphic DNA (RAPD) and finally two were sequence-related amplified polymorphisms (SRAPs). Nine of the polymorphisms segregated as dominant and 22 as codominant. The markers were distributed in eight linkage groups (LG) covering 2,234.4 cM, with an average distance between markers of 12.28 cM (Fig. 1 and Table 1). The number of markers and the total distance of each linkage group are indicated in Table 1. In addition, 14 markers were unlinked, and four markers were repeatedly assigned to the LG 5, but since these last four markers were located either in one LG extreme or in the other with distances close to inde-

Table 1. Characteristics of the *Lens* linkage groups

Linkage grup	Length (cM)	Number of markers (%)	Average distance between markers (cM)	New added markers	
				Dominant	Codominant
LG 1	421.0	40 (21.05)	10.79	3	5
LG 2	395.8	25 (13.16)	16.49	0	5
LG 3	384.5	28 (14.74)	14.24	0	1
LG 4	168.7	15 (7.89)	12.05	1	2
LG 5	501.8	55 (28.95)	9.29	3 (1)[1]	5 (3)
LG 6	231.2	18 (9.47)	13.60	1	2
LG 7	93.2	6 (3.16)	18.64	1	2
LG 8	38.2	3 (1.58)	19.10	0	0
Total	2,234.4	190 (100)	12.28	9 (1)	22 (3)

[1] Figures between parentheses indicate markers consistently associated with LG 5 but not precisely located in it.

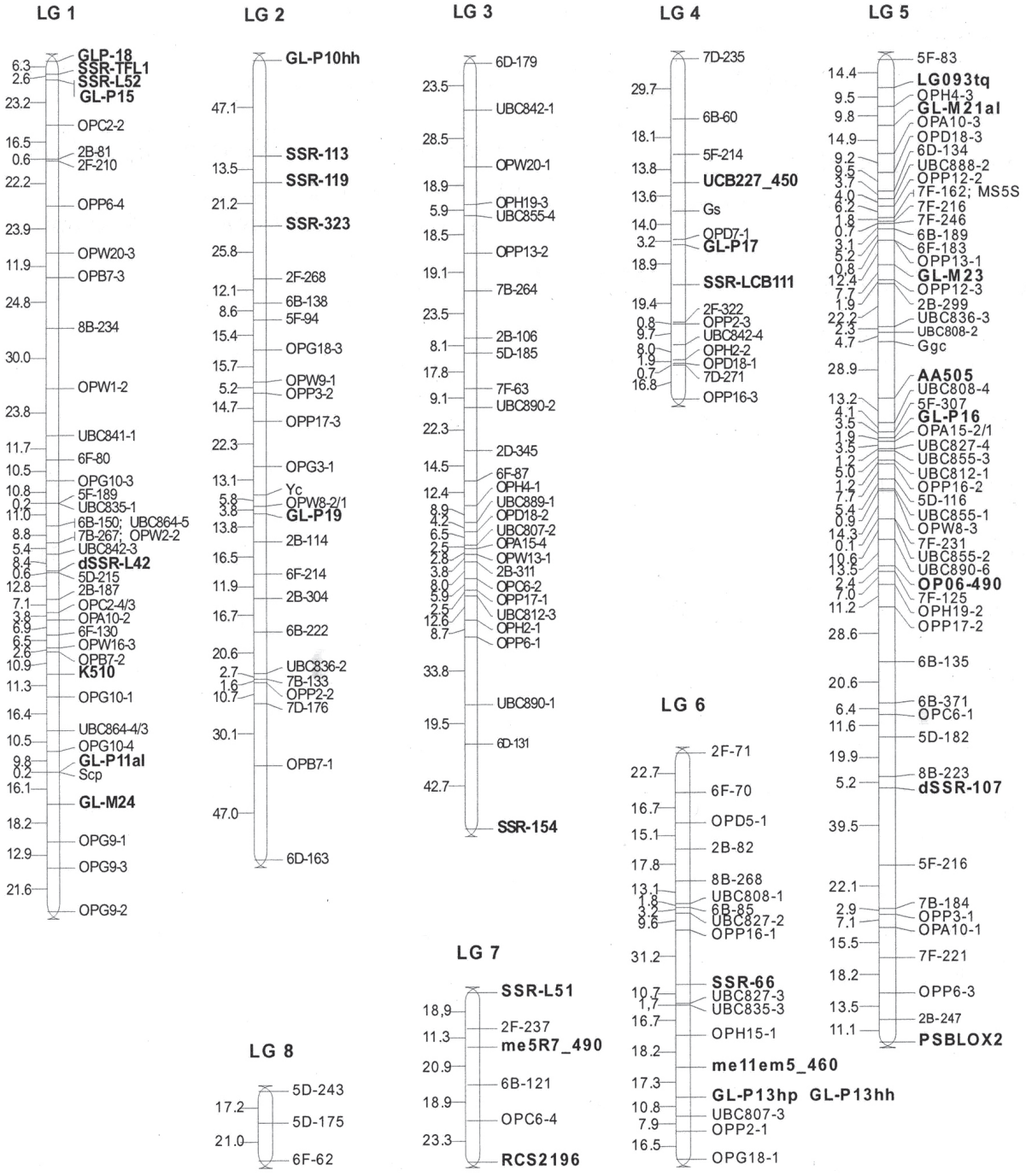

Figure 1. Genetic linkage map of lentil showing the distribution of different markers at LOD 3.0. Genetic distances, on the left side on the maps, are in centiMorgans (Kosambi function). The new markers are indicated in bold. The rest of markers keeps the nomenclature by Durán *et al.* (2004).

pendence between them, they were not mapped [Suppl. Table 1 (pdf)].

The new genetic map has reduced the previous 10 linkage groups (Durán *et al.*, 2004) to eight, since previous LG VII (with two markers) was included in the LG 1 (former LG I) and previous LG IX (with three markers) was integrated in LG 2 (former LG II). The remaining linkage groups conserved the serial order, except LG VIII and X which are now LG 7 and LG 8, respectively (Fig. 1). The average distance between

markers is lower than the 15.87 cM previously described (Durán *et al.,* 2004), while the observed length increased slightly from 2,172.4 cM to 2,234.4 cM.

Some amplicons from one or both parents were cloned and sequenced to check whether the amplified sequences were functional markers and if they corresponded to the expected sequence. Nucleotide sequences were registered in the European Nucleotide Archive under accession numbers HE858065, HE858066, and HE858418 to HE854825. The amplicons related to the unlinked markers GL-M13 and GL-M16, obtained from the GLIP primer pairs mtmt_DEG_05184_02_1 and mtmt_GEN_00599_04_1, respectively, showed similarity with gene sequences coding for vascular proteins in *M. truncatula* (GL-M13), or for an uncharacterized protein observed in *M. truncatula, Glycine max, Vitis vinifera*, and *Ricinus comunis* (GL-M16). CAP marker GL-13hh obtained from GLIP primer pair psat_EST_00164_02_1 generated a band of approximately 1250 bp. The PCR amplified sequence from *L. c.* ssp. *orientalis* had the target site for restriction endonuclease *Hha*I and showed similarity to uncharacterized genes found in several plant species. The same primer pair generated an allelic CAP marker from *L. c.* ssp. *culinaris* (GL-13hp) using the endonuclease *Hap*II [Suppl. Table 1 (pdf)].

A set of two primers (TCGTTGGTAGAGTCATAGGTGAA; CACGACACATAACAAACATACAGTACA) was designed from the pea *TFL1* genes to amplify the corresponding lentil sequence. Amplicons of 1049 bp and 1063 bp were amplified from the *culinaris* and *orientalis* parents, respectively, encompassing almost all the transcribed sequence of the gene (4 exons). The sequence revealed the presence of an $(AT)_n$ microsatellite (n = 5 in *culinaris* and n = 12 in *orientalis*) at the beginning of the first intron, thus a new set of primers was designed (anchored respectively in exons 1 and 2) to amplify a shorter sequence (184 and 198 bp, respectively) including the microsatellite. This functional marker was named as SSR-TFL1 and was included in the map [Suppl. Table 1 (pdf), Fig. 1]. The deduced *Lens* amino acid sequences differed by a single substitution in the first exon (threonine in *culinaris* and alanine in *orientalis*). Genes in the *TERMINAL FLOWER1* (*TFL1*)*/CENTRORADIALIS* family are important key regulatory genes involved in the control of flowering time and floral architecture in several different plant species. In pea, three *TFL1* homologs, designated *PsTFL1a*, *PsTFL1b*, and *PsTFL1c* were identified by Foucher *et al.* (2003). *PsTFL1a* corresponds to the *DETERMINATE* (*DET*) gene and *PsTFL1c* corresponds to the *LATE FLOWERING* (*LF*) gene. *DET (PsTFL1a)* acts to maintain the indeterminacy of the apical meristem during flowering and consistent with this role, its expression is limited to the shoot apex after floral initiation. *LF (PsTFL1c)* delays the induction of flowering by lengthening the vegetative phase, and allelic variation at the *LF* locus is an important component of natural variation for flowering time in pea. Thus, different *TFL1* homologs control two distinct aspects of plant development in pea, whereas a single gene, *TFL1*, performs both functions in *Arabidopsis* (Foucher *et al*., 2003). The corresponding lentil translated sequences were aligned and compared with 24 additional *TFL1* homologs from legume species recorded in databases. The two sequences obtained from lentil were similar to *TFL1a* from pea, and the corresponding dendrogram [Suppl. Fig. 1 (pdf)] obtained from the amino acid sequences grouped the two lentil sequences and pea *TFL1a* in the same cluster, while pea TFL1c was located in a different cluster. Thus, SSR-TFL1 is a functional marker which could be used in the analysis of flowering phenology variation in lentil.

Finally, several bands obtained using primers designed to amplify sequences related to the *Pto-Kin* gene were sequenced. Pto-Kin markers have been related to pathogen resistance associated to serine-threonine protein-kinases (Fayyaz *et al*., 2007). The primers (Fayyaz *et al*., 2007) generated 5 bands ranging from 400 to 800 bp, plus several low-resolution additional bands. The only difference between the parent lines was the absence-presence of the band of 510 bp (marker K510 in the genetic map; Fig. 1). Amplicons larger than 400 bp were cloned and sequenced (K411; K481; K510; K679; and K738, the numbers indicate the size in bp). The subsequent searches in databases revealed that they had similarity with sequences characteristics of retrotransposons (K679), with non-characterized proteins annotated in *M. truncatula* and *G. max* (K510) or no significant similarity with known sequences (K411, K481 and K738). Since retrotransposon families are abundant in species of the Vicieae tribe, in which *Lens* is included, and distributed along the genome, markers related with them are difficult to relate with particular loci. However, K679 (amplified from both *culinaris* and *orientalis*) showed a similarity higher than 70% with an intergenic sequence located about 60,000 bp from the *Rio Grande 72R Pto* locus of *Solanum pimpenillifolium*, raising the question

whether these polymorphic sequences (there were several nucleotide polymorphisms between them) could be a marker related to resistance in lentil. Interspecific genetic maps help in designing gene introgression breeding programs from wild crop relatives using marker assisted selection. Thus, this map improved with functional markers represents a step forward in the use of the genetic variability from the lentil wild progenitor, although more markers are still required to reach a full saturated map.

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