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Detection of a new QTL/gene for growth habit in chickpea CaLG1 using wide and narrow crosses

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Abstract A recombinant inbred line population (RIP-9) derived from an interspecific cross (ILC72 × Cr5-10) was evaluated for growth habit during 2 years (2003 and 2004). This RIP was used to develop a pair of near isogenic lines (NILs) for erect vs prostrate growth habit in chickpea. Molecular characterization of the identified pair of NILs was performed using 52 sequence tagged microsatellite site markers distributed over different chickpea linkage groups (CaLG) of the genetic map. It revealed polymorphic markers in CaLG1 and CaLG3. Starting from a previous data base simple linear regression was applied to detect association between markers and growth habit. The RAPD (random amplified polymorphic DNA) marker $OPAD09_{1053}$ mapped on CaLG1 explained the highest percentage (maximum 15.4 %) of the total phenotypic variation for growth habit and it was used to develop a SCAR (sequence characterized amplified region) marker ($SCAD09_{1053}$). New markers were developed from sequences surrounding $SCAD09_{1053}$ in the physical map. QTL (quantitative trait loci) analysis revealed a new QTL (QTL_{Hg2}) in CaLG1. The Indel marker (deletion/insertion) Indel 3 and the predicted gene Ca_07000 (14,5 Mb of Ca1) and (15,3 Mb of Ca1) had the highest LOD values explaining 24.6 and 23.4 % of the phenotypic variation in years 2003 and 2004, respectively. To confirm these results, another RIP (RIP-5) derived from an intraspecific cross (WR315 × ILC3279) and segregating for erect vs semi-erect growth habit was employed. RIP-5 allowed mapping the gene (*Hg2/hg2*) on CaLG1 that was flnaked by two Indel markers (Indel 1 and Indel 2) in the range of 12,3 and 16,2 Mb. So, *Hg2/hg2* gene corresponds to QTL_{Hg2} region. The annotated genes Ca_07000 and Ca_06999 were homologues to predicted zinc finger genes in *Glycine*

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max and *Pisum sativum*, respectively. Hence, they could be considered as possible candidate genes.

Keywords Linkage analysis · *Cicer* · Erect · Prostrate · Semi-erect · Physical map

Introduction

Chickpea, *Cicer arietinum* L., is an autogamous, diploid species (2n = 2x = 16) and is the second largest cultivated grain legume in the world after dry bean (*Phaseolus vulgaris* L.) considering total production (FAOSTAT 2013). Breeding efforts have substantially contributed to improve chickpea yield in recent years, though chickpea grows in a wide range of cropping systems and the best ideotype could be different for each geographic region and growing conditions (Gaur et al. 2007). Growth habit is one of the morphological traits that play a role in the adaptability to different environments in legumes affecting yield and yield stability (Hughes 1998). In cultivated chickpea, erect and semi-erect (bushy) types are present while prostrate is referred to the growth habit present in annual wild *Cicer* species. Therefore, semi-erect and erect growth habits could be considered desirable traits introgressed in the crop during the domestication process, while prostrate habit is a non-desirable phenotypic trait of the wild ancestor (*Cicer reticulatum* Ladz.). Growth habit is not only related to plant height but also includes differences related to plant structure affecting production. Erect types, in addition to being taller, have more compact canopies and less primary and secondary branches than bushy types. Hence, erect lines have fewer reproductive nodes, and so, a higher number of plants per unit area are recommended compared to semi-erect types (Muehlbauer and Singh 1987; Rubio et al. 2004).

Despite the importance of this trait, there are few studies available in legumes. Previous studies in lentil reported a major gene controlling growth habit (Ladizinsky 1979; Saha et al. 2013). In chickpea, a major gene (*Hg/hg)* has been mapped to chickpea linkage group (CaLG) 3 using interspecific crosses segregating for prostrate vs erect or semi-erect phenotypes (Kazan et al. 1993; Cobos et al. 2009; Aryamanesh et al. 2010). However, there are no studies for populations derived from intraspecific crosses differing for non-prostrate phenotypes (erect or semi-erect) that could be controlled by a different gene from the one (*Hg/hg*) mapped on CaLG3.

The development of near-isogenic lines (NILs) is a useful approach to facilitate mapping and localizing new genomic regions of a trait of interest (Xue et al. 2013). NILs differ only in a small target region of the genome and the genetic background noise is mostly eliminated. Pairs of NILs for growth habit together with the whole chickpea genome sequence recently published (Jain et al. 2013; Varshney et al. 2013) could facilitate the detection of candidate genes underling growth phenotypes.

The objective of this study was to develop pairs of NILs for growth habit in chickpea and use them to search for new markers or candidate genes linked to this important morphological trait.

Materials and methods

Plant material

Two recombinant inbred line populations (RIPs) were used in this study: (i) RIP-9 previously described (Cobos et al. 2006) consists of 104 $F_{6:8}$ RILs (recombinant inbred lines) generated from the interspecific cross *C. arietinum* ILC72 × *C. reticulatum* Cr5-10 and (ii) RIP-5, derived from the intraspecific cross WR315 × ILC3279 and composed of 102 $F_{6:8}$ RILs. Both populations were developed following single seed descent (SSD) method (Johnson and Bernard 1962). ILC72 and ILC3279 are *kabuli* types from the former Soviet Union maintained by the International Center for Agricultural Research in the Dry Area (ICARDA), with erect growth habit. *C. reticulatum* Cr5-10 is a selection from the accession ILWC36 (PI599072) from the United States Department of Agriculture (USDA) with prostrate habit. WR315 is a *desi* landrace from central India maintained by the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), with semi-erect growth habit.

NILs development

RIP-9 was used to develop a pair of NILs for erect vs prostrate growth habit. RILs that showed segregating

plants for this trait were selected and seeds of individual plants were collected and sown in a row. Two non-segregating descendent for both erect and prostrate growth habit were separately harvested and considered as near isogenic lines for this trait.

Phenotypic evaluation

RIPs were sown in the field and evaluated for growth habit in Córdoba (Southern Spain) during two (2002/2003 and 2003/2004) and one cropping season (2002/2003) for RIP-9 and for RIP-5, respectively. RILs were randomly distributed in four blocks and parents were included as reference in each trial in both populations. The unit plot was two rows of 2 m, 10 seeds per meter and 0.7 m between rows. Phenotypic evaluation for growth habit was performed in adult plants using a rating scale of 0 to 2 (0 = prostrate, 1 = semi-erect and 2 = erect).

DNA extraction and marker analysis

For DNA extraction, about 100 mg of young leaf tissue was excised, frozen immediately in liquid nitrogen and stored at −80 °C. DNA was isolated using the DNAzol® method (Invitrogen, USA) following manufacturer's instruction.

To characterize at molecular level the pair of NILs developed in the current study, 52 sequence tagged microsatellite site (STMS) markers distributed through different LGs of the chickpea genetic map were selected (Table 1). PCR amplification was performed as described in previous studies (Winter et al. 1999; Lichtenzveig et al. 2005; Sethy et al. 2006). The PCR products of 28 amplified STMS markers were separated either in 2.5 % agarose (a mixture of agarose 1.25 % SeaKem LE and 1.25 % LM SIEVE, Rockland, ME, USA) in 1× TBE buffer or in 10 % nondenaturing polyacrylamide gels, and stained with ethidium bromide. Twenty-four STMS that required higher resolution were separated by automatic capillary electrophoresis on an ABI3130 Genetic Analyzer (Applied Biosystems/HITACHI, Madrid, Spain) in the Central Research Support Service (SCAI) at the University of Córdoba. The forward primers of these 24 STMS markers were synthesized with fluorescent dyes 6-FAM, HEX or NED (Applied Biosystems, UK) at the 5′ ends. The size of the amplified bands was calculated based on an internal DNA standard (400HD-ROX) with GeneScan software (version 3.x) and the results were interpreted using the Genotyper 3.7 software all from Applied Biosystems.

Linkage analysis

Linkage analysis for RIP-9 included markers previously mapped in this population by Cobos et al. (2006), Palomino et al. (2009) and Millan et al. (2010) together with fourteen STMS markers added in this study. After first linkage analysis, new markers developed in this work using different approaches (described in the next sections) were also included to do the analysis again with all markers. Loci segregation was statistically analyzed for goodness of fit to the expected ratio 1:1 using the Chi square test. Linkage analysis was performed using JoinMap v4.0 with maximum likelihood option (Van Ooijen 2004). Markers were grouped at a minimum LOD score of 3.0 and a maximum recombination fraction of 0.25 as general linkage criteria to establish linkage groups. Kosambi's function was applied to estimate map unit distance (Kosambi 1944).

RIP-5 (intraspecific cross) was used to perform linkage analysis considering growth habit as a qualitative trait (erect vs semi-erect) using the same software and conditions as described above for RIP-9.

SCAR development

Sequence characterized amplified region (SCAR) marker was developed from the RAPD (random amplified polymorphic DNA) fragment $OPAD09_{1053}$ that was mapped on CaLG1 using RIP-9 (Cobos et al. 2006). The selected amplicon was excised from polyacrylamide gel by adding 25 µL of distilled water. The purified DNA was cloned in the pGEM-T vector system I (Promega Corporation, USA). Inserts were sequenced from three different clones using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA). The consensus sequence was used to design SCAR primers with Primer 3 software (http://frodo.wi.mit.edu/primer3/) (Rozen and Skaletsky 1999). Forward primer was 18 nucleotides long (5′-TCGCTTCTCCCCAAAACT) and reverse consisted of 22 nucleotides (5′-TCGCTTCTCCTATCATTGACTC), both including all 10 bases of the RAPD. The SCAR sequence and its translated product were blasted against available DNA and protein sequences in NCBI (http://blast.ncbi.nlm.nih.gov/), *Medicago truncatula*

Table 1 STMS markers distributed across chickpea linkage groups used to characterize a pair of near isogenic lines (NILs) for growth habit [NIL8-6A (prostrate) and NIL18-6B (erect)]

Linkage group	Marker[a]
CaLG1	GA11, STMS12, STMS21, **TA1**, **TA8**, **TA30**, TA113, **TA203**, **TR43, H3H021**
CaLG2	GA16, H1H011, TA59, TA110, TA194, TA200, TR19
CaLG3	GA13, **STMS5**, **STMS10, TA34, TA125, TA142, TS19**
CaLG4	GAA47, STMS11, STMS24, TA2, TA61, TA130, TA186, TR11
CaLG5	GA4, TA5, TA11, TR29, TR59
CaLG6	GA21, STMS2, TA14, TA21, TA80, TA106, TR1
CaLG7	**STMS6**, TA78, TA117, TA18, TA28
CaLG8	GAA46, TS12, TS45

[a] Polymorphic markers are in bold

(http://www.medicago.org/) and chickpea genome (http://mejgenvegetal.uco.es/fgb2/gbrowse/Ca/) databases. Genomic DNA from the prostrate parent (Cr5-10) of RIP-9 was used as template to optimize amplification conditions for SCAR primers. PCR was carried out in a TGradient thermocycler (Biometra, Germany) in 10 µl reaction volumes. Each PCR reaction contained 50 ng of plant genomic DNA, buffer (50 mM KCl, 10 mM Tris–HCl and 0.1 % Triton X-100), 2 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µM of primer and 0.1 unit/10 µL of Taq DNA polymerase (Bioline). The thermal profile for PCR was an initial denaturation at 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min with a final extension at 72 °C for 8 min.

Physical map and predicted gene mapping

Primers of the STMS markers mapped on CaLG1 and CaLG3 of both RIP-9 and RIP-5 were used to amplify in silico in the chickpea genome assembly using In-house script software (Kalendar et al. 2009). In addition, BLASTN analysis was performed against chickpea genome sequence (Varshney et al. 2013) using DNA sequences of two mapped resistance gene analogs (RGAs), [RGA03 (accession no. BF643456) and RGA07 (accession no. AW774607)], two cross genome markers [PsPR59 (accession no TC175113) and PsPR82 (accession no TC172521)], and the SCAR $SCAD09_{1053}$ developed in this study.

In order to saturate the genetic map of the tagged genomic region, sequences of annotated genes were extracted from the chickpea genome sequence (Varshney et al. 2013) and primer pairs were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3/) (Rozen and Skaletsky 1999) (primer sequences are provided in Supplementary file 1). PCR reactions were carried out in 10 µl reaction volume containing 30 ng of plant genomic DNA, 1× buffer (50 mM KCl, 10 mM Tris–HCl, and 0.1 % Triton X-100), 1.5–2.5 mM $MgCl_2$, 0.4 mM of dNTPs, 0.4 µM of each primer and 0.05 units of Taq DNA polymerase (Promega). PCR conditions included an initial DNA denaturation at 94 °C for 5 min and 40 cycles comprising of 94 °C for 30 s, 55–62 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. The PCR products were cleaned up by treating with 1 U each of Exonuclease I (Exo) and shrimp alkaline phosphatase (SAP) and were directly sequenced (Macrogen, Korea). Then, PCR products of selected genes showing single nucleotide polymorphisms (SNPs) were restricted with appropriate endonuclease (Fermentas) following the supplier's instructions and were visualized in 1 % agarose gel. Also chickpea data base (http://cicarmisatdb.icrisat.org/index.html) was used to select five microsatellite markers into the targeted genomic regions of Ca3 and Ca1 (Supplementary file 2).

SNP and Indel (insertion/deletion) detection was also carried out using the re-sequencing information of parental lines by Varshney et al. (2013) and Chen (unpublished data), respectively (Table 2, Supplementary file 1). On the other hand, five SNPs detected by KASPar platform (KBiosciences Competitive Allele-Specific PCR SNP genotyping system) for erect vs prostrate genotypes (non-published data) were physically mapped (Table 2). A derived cleaved amplified polymorphic sequence (dCAPS) marker was developed to map genetically one of the five SNPs. Primers for dCAPS were designed using the software dCAPS Finder (Neff et al. 1998) introducing a single

Table 2 Physical position and possible function of gene specific, SNP and Indel markers located on Ca1 nearby $SCAD09_{1053}$ that were included in RIP-9 and RIP-5 genetic map. Polymorphism detection and PCR product size obtained are described

Marker ID[a]	Physical position (bp)	Putative function	Amplification[e]	Polymorphism detection	Restriction enzyme	Alleles (bp)		
						Erect	Semi-erect	Prostrate
Ca_06993[a]	Ca1: 15444207:15447979	Uncharacterized	NA	–	–	–		–
Ca_06994[a]	Ca1: 15434110:15435930	Predicted probable glucuronoxylan glucuronosyltransferase IRX7-like (LOC101499733)	F1+Rev1	–	–	1,370	1,370	1,370
Ca_06995[b]	Ca1: 15399920:15400621	Uncharacterized	F1+Rev1	Indel	–	280	280	270
Ca_06996[a]	Ca1: 15386956:15391066	Uncharacterized	NA	–	–	–		–
Ca_06997[a]	Ca1: 15377215:15378652	Predicted transcription factor Fer-like iron deficiency-induced transcription factor-like (LOC101498540)	NA	–	–	–		–
Ca_06998[a]	Ca1: 15368187:15373817	Uncharacterized	F1+Rev3	–	–	1,550	1,550	1,550
Ca_06999[a]	Ca1: 15337320:15338210	Predicted zinc finger protein ZAT5-like (LOC101497229)	F1+R2, F2+R1	–	–	1,200 2,200	1,200 2,200	1,200 2,200
Ca_07000[a]	Ca1: 15323486:15324685	Predicted protein Sensitive to proton rhizotoxicity 1-like (LOC101496905)	F1+R1	CAP	TaqI	694 + 381	694 + 381	1,075
Ca_07001[a]	Ca1: 15322204:15322614	Predicted histone H2B-like (LOC101496580)	F1+Rev1	–	–	–		–
Ca_07002[b]	Ca1: 15314930:15321026	Uncharacterized	F1+Rev1	Indel	–	215	215	200
Ca_07003[a]	Ca1: 15310213:15311319	Predicted pentatricopeptide repeat-containing protein At2g44880-like (LOC101497993)	F1+Rev1	–	–	1,100	1,100	1,100
Indel1[c]	Ca1: 12340166:	–	F1+Rev1	Indel	–	320	335	320
Indel2[c]	Ca1: 16187503:	Predicted: *Cicer arietinum* indole-3-acetic acid-amido synthetase GH3.6-like (LOC101492800)	F1+Rev1	Indel	–	415	400	415
Indel[3]	Ca1: 14537581	Intergenic region	F1+Rev1	Indel	–	370	370	410
CKAM0717[d]	Ca1: 15489533:15489633	Intergenic region	F1+Rev1	dCAP	MseI	266	266	239 + 27
CKAM0912[d]	Ca1: 8621759:8613351	Intergenic region	–	–	–	–	–	–

Table 2 continued

Marker ID[a]	Physical position (bp)	Putative function	Amplification[e]	Polymorphism detection	Restriction enzyme	Alleles (bp)		
						Erect	Semi-erect	Prostrate
CKAM1744[d]	Ca1: 21823113:21823213	Predicted: *Cicer arietinum* protein transport protein Sec61 subunit beta-like (LOC101491707)	–	–	–	–	–	–
CKAM0767[d]	Ca1: 23301914:23302014	Predicted: *Cicer arietinum* putative receptor-like protein kinase At1g72540-like (LOC101488466)	–	–	–	–	–	–
CKAM1383[d]	Ca1: 25185532:25189657	Predicted: *Cicer arietinum* protein TIFY 3B-like (LOC101515051)	–	–	–	–	–	–

[a] Clear amplicons of the predicted genes were sequenced directly, and the SNP was detected

[b] SNP detected by resequencing (Varshney et al. 2013)

[c] SNP detected by resequencing parental lines of RIP-5 (Chen, unpublished data)

[d] SNP detected by KASPar platform (unpublished data)

[e] *NA* non-amplicon, F+R primer combination that gave clear amplicon

nucleotide mismatch adjacent to the SNP position to create a restriction site (Table 2, Supplementary File 1). The dCAPS marker was visualized using agarose gel 2 % mixture.

QTL analysis

RIP-9 was used to perform QTL (quantitative trait loci) analysis because the presence of three phenotypes for growth habit (erect, semi-erect and prostrate) could suggest more than one gene controlling this trait, and so, growth habit can be considered as a quantitative trait. The analysis was performed using Map QTL v5 (Van Ooijen 2004). Kruskal–Wallis (Van Ooijen et al. 1993) and Interval mapping (Lander and Botstein 1989; Van Ooijen 1992) were applied. The significance of QTL was empirically determined using a permutation test with 1,000 replications (Churchill and Doerge 1994) and applying 95 % level of significance. The coefficient of determination (R^2) for the marker most tightly linked to a QTL was used to estimate the proportion of the total phenotypic variation explained by the QTL.

Results

Phenotypic evaluation

Three different phenotypes (erect, semi-erect and po-strate) were observed in RIP-9 in the two cropping seasons (2002/2003 and 2003/2004). The RILs with semi-erect and prostrate growth habit constituted 48.2 and 41.8 % of the population respectively, and only 10 % of the population showed erect growth habit. Phenotypic evaluation of RIP-5 showed that the data for growth habit fit the expected segregation ratio 1:1 (erect: semi-erect) for one gene in the $F_{6:8}$ population (Table 3).

NIILs development

Phenotypic evaluation of RIP-9 under field conditions revealed residual heterozygosity in RIL83 that was used to develop a pair of NILs: NIL8-6A (prostrate) and NIL8-6B (erect). Molecular characterization of the obtained pair of NILs showed the same amplification patterns with the STMS markers employed in this study except for six markers located on CaLG1, six on CaLG3 and one on CaLG7 (Table 1). To confirm the association between these polymorphic markers and

Table 3 Growth habit frequency distribution in RIP-9 (erect, semi-erect and prostrate), and RIP-5 (erect and semi-erect) evaluated under different environments

Population	Environment[a]	Growth habit frequency distribution			
		Erect	Semi-erect	Prostrate	Total
RIP-9	2002/2003	10	40	35	85
RIP-9	2003/2004	7	42	36	85
RIP-5	2003/2004	40	51	–	91

[a] Seasons evaluated in Córdoba (Spain)

Table 4 Association of the polymorphic STMS markers with growth habit in twenty RILs with extreme values for this trait in both RIP-9 (10 prostrate/10 erect) and RIP-5 (10 semi-erect/10 erect)

LG	Markers	ILC72 × Cr5-10[1] (RIP-9)				WR315 × ILC3279[2] (RIP-5)			
		10 erect RILs		10 prostrate RILs		10 semi-erect RILs		10 erect RILs	
		a	b	a	b	a	b	a	b
CaLG1	TA30	7	3	2	8	8	2	1	9
	TR43	8	2	2	8	8	2	1	9
	TA1	8	2	2	8	8	2	1	9
	H3H021	7	3	2	8	Monomorphic			
	TA8	8	2	2	8	7	3	1	9
CaLG3	TA125	6	4	4	6	4	6	9	1
	TA34	3	7	4	6	Monomorphic			
	STMS10	5	5	4	6	5	5	9	1
	TA142	8	2	5	4	5	5	4	6
	STMS5	9	1	3	7	6	4	2	8
	TA76	7	3	2	8	7	3	5	5
CaLG7	STMS6	7	3	6	4	Monomorphic			

[1] a and b, alleles present in ILC72 (erect) and Cr5-10 (postrate), respectively

[2] a allele present in the parent WR315 (semierect) and b allele in ILC3279 (erect)

growth habit, twenty RILs with extreme phenotypes from each RIP-9 (10 prostrate and 10 erect) and RIP-5 (10 semi-erect and10 erect) were screened. This analysis validated the association between phenotypes and polymorphic markers located on CaLG1 for both populations (Table 4). Only two (TA76 and STMS5) out of the six polymorphic markers in CaLG3 showed association with this trait in RIP-9. For CaLG7, no association was observed between STMS6 alleles and the phenotypic evaluation (Table 4).

Identification of genomic regions associated with growth habit

Linkage group analysis with the whole RIP-9 population was performed using 32 polymorphic markers previously mapped on CaLG1 and CaLG3 by Cobos et al. (2006), Palomino et al. (2009) and Millan et al. (2010) together with 14 new markers mapped in this study. Ten out of the 19 markers mapped on RIP-9-CaLG1 showed distorted segregation toward the erect parent. Because growth habit was considered as a quantitative trait in RIP-9, simple linear regression analysis was applied to study its association with the mapped markers. The results revealed maximum significant association with the RAPD marker $OPAD09_{1053}$ that was located on CaLG1 and explained the highest percentage of the total phenotypic variation (13.5 and 15.4 % in 2003 and 2004, respectively). Nevertheless, a weak significant association ($P < 0.05$) was found between two markers in CaLG3 (TA142 and STMS5) and this trait.

To confirm the association between markers and growth habit phenotypes, a second population (RIP-5) derived from an intraspecific cross (erect × semi-erect) was evaluated. Eleven markers previously mapped on CaLG1 and CaLG3 of RIP-9 were polymorphic between WR315 and ILC3279, and consequently, they were mapped in RIP-5. All these markers fit the expected ratio 1:1. As mentioned above, growth habit fit the expected segregation ratio (1:1) for one gene in this population. Therefore, it was included in the genetic map as a single gene named *Hg2/hg2* to differentiate it from the gene previously located on CaLG3 (Kazan et al. 1993). As shown in Figs. 1 and 2, CaLG1 and CaLG3 included 5 and 6 STMS markers, respectively and *Hg2/hg2* was located on CaLG1 at a distance of 25.1 cM from the closest STMS marker (TA08).

SCAR marker development

Once the association of CaLG1 with growth habit was confirmed, the RAPD marker $OPAD09_{1053}$, which explained the highest percentage of phenotypic variation, was used to develop a SCAR marker (Fig. 1). The 1,053 bp polymorphic allele (present in prostrate parent and absent in erect parent) was cloned and sequenced. The developed SCAR marker ($SCAD09_{1053}$) was retained as a dominant marker, present in prostrate parent.

Physical map

In order to establish the position of genomic regions related to growth habit, STMS and known sequences mapped on CaLG1 and CaLG3 were physically

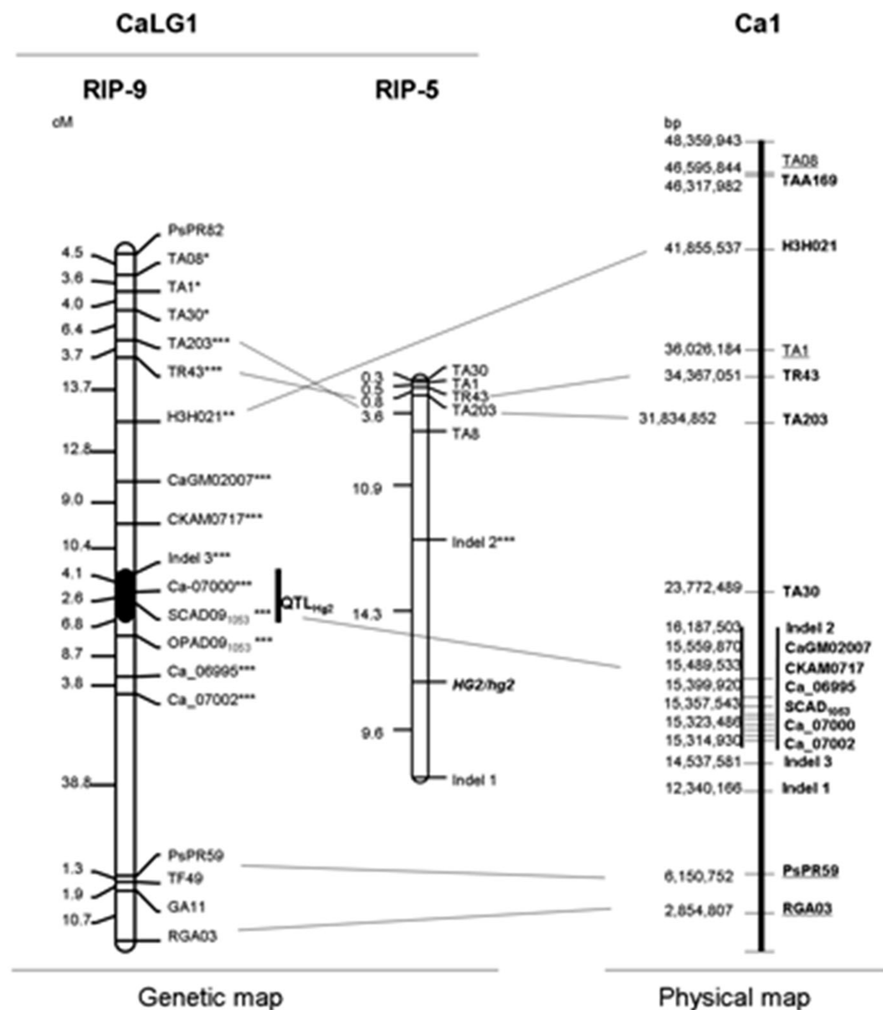

Fig. 1 On the *left*, genetic map of CaLG1 in RIP-9 [*Cicer arietinum* (ILC72) × *C.reticulatum* (Cr5-10)] and RIP-5 [*C.arietinum* (WR315) × *C.arietinum* (ILC3279)]. The quantitative trait loci detected (QTL_{Hg2}) and the mapped gene *HG2/hg2* in LG1-RIP-9 and RIP-5 respectively are in *bold*. Estimated genetic distances are given in cM, markers showing distorted segregation are pointed with an *asterisk*. On the *right*, physical map of CaLG1: The physical position of markers mapped on CaLG1 are shown in base pairs. The markers TA1 and TA08 are subtitled because they were amplified in silico with low confidence (2 mismatches)

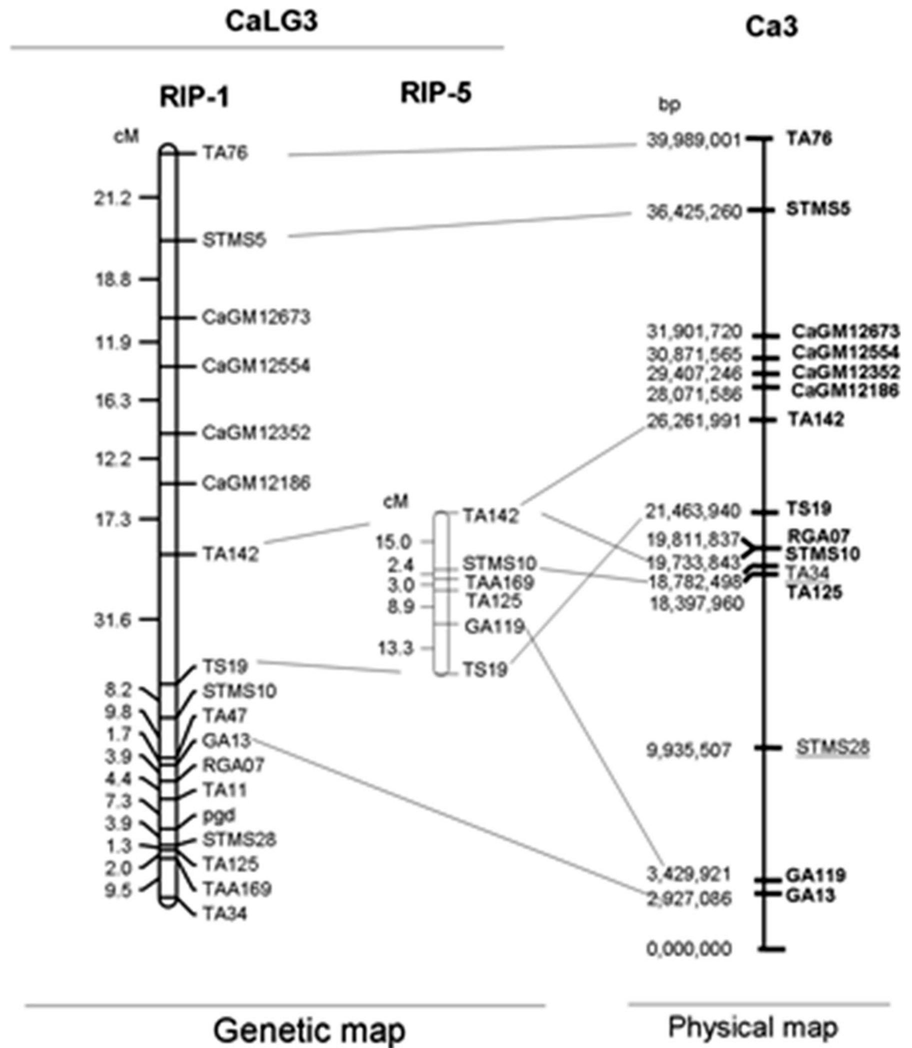

Fig. 2 On the *left*, genetic map of CaLG3 in RIP-9 [*Cicer arietinum* (ILC72) × *C.reticulatum* (Cr5-10)] and RIP-5 [*C.arietinum* (WR315) × *C.arietinum* (ILC3279)]. The quantitative trait loci detected (QTL_{Hg1}) in LG1-RIP-9 is in *bold*. Estimated genetic distances are given in cM, markers showing distorted segregation are pointed with an *asterisk*. On the *right*, physical map of CaLG3, The physical position of markers mapped on CaLG3. The markers TA34 and STMS28 are subtitled because they were amplified in silico with low confidence (2 mismatches)

located in Ca1 and Ca3 pseudomolecules. Four out of the seven STMS markers mapped on CaLG1 and eight out of the 13 mapped on CaLG3 of RIP-9 were amplified in silico in Ca1 and Ca3, respectively without mismatches (Fig. 1 and 2). Four markers, two in Ca1 (TA1, TA8) and two in Ca3 (TA34, STMS28), were amplified but with a lower level of confidence (two mismatches). Consequently, these markers were not considered as reference. Unexpectedly, TAA169, which mapped on CaLG3, was amplified in Ca1 without mismatches instead of in Ca3. The known sequences RGA03 and PsPR59 were mapped on Ca1 and RGA07 on Ca3, supporting the results obtained in the genetic map (Figs. 1 and 2). The sequence of PsPR82 *Pisum* gene mapped on CaLG1 could not be mapped on any pseudomolecule.

Markers distribution through genetic and physical map was co-linear between CaLG1 and Ca1 except for four STMS markers (TA30, TA203, TR43 and H3H021) that showed short distances among them in the genetic map (Fig. 1). In general there was co-linearity of markers between CaLG3 and Ca3 (Fig. 2).

To search for candidate genes related to growth habit, the $SCAD09_{1053}$ nucleotide sequence was used to perform BLASTN analysis that revealed the highest homology (96 %) with the Ca1 intergenic region 15,358,505–15,357,543 Mb in the negative strand (Fig. 1). The genes located in 100,000 bp surrounding $SCAD09_{1053}$ were extracted from the chickpea genome sequence. A total of 11 predicted genes were found in this region (Table 2). To locate these genes in the genetic map, 33 primers were designed and

different approaches were used to detect polymorphisms between parental lines. Only 3 genes (Ca_07002, Ca_06995, and Ca_07000) were polymorphic between erect and prostrate but not for erect vs semi-erect lines (Table 2). Consequently, they were included in the genetic map of RIP-9 (Fig. 1).

Only one out of five Kaspar markers obtained (CKAM0717) was mapped on CaLG1 of RIP-9, because it was located in the interesting region in the physical map (Table 2, Fig. 1). It could not be mapped in RIP-5 as it was monomorphic for parental lines. On the other hand, three Indel markers were developed based on the resequencing data (Chen et al. unpublished data). Indel 1 and 2 were polymorphic for the intraspecific cross parental lines, while Indel 3 was polymorphic for the interspecific cross parents (Table 2). So, Indel 3 was included in CaLG1 of RIP-9. However, Indel 1 and Indel 2 were added to CaLG1 of RIP-5. *Hg2/hg2* gene was flanked by these two markers at a distance of 9.4 and 14.3 cM, respectively (Fig. 1).

In addition, five microsatellite markers were selected from chickpea data base. These markers could be only mapped in RIP-9 and not in RIP-5 population, because they were not polymorphic for its parental lines. Four of them were mapped on CaLG3 between TA142 and STMS5 markers, as it was mentioned above both markers showed light association ($P < 0.05$) with growth habit in RIP-9 (Fig. 2). And one was mapped on CaLG1 because it was in the targeted region (Fig. 1).

QTL analysis

Final version of CaLG1 in RIP-9 comprised a total of 19 markers including $SCAD09_{1053}$, Ca_07000, Ca_07002, Ca_06995, CaGM02007, Indel 3 and CKAM0717. This linkage group covered 146.8 cM of the chickpea genetic map (Fig. 1). After applying IM, a new QTL (QTL_{Hg2}) was detected in CaLG1 being Indel 3 and Ca-07000 gene with the highest LOD values of 5.01 and 4.67. These markers explained a 24.6 and 23.4 % of the total phenotypic variation in years 2003 and 2004, respectively.

As described above, *Hg2/hg2* gene is located between Indel 1 and Indel 2 (12,4–16,3) Mb on Ca1 that includes the QTL_{Hg2} (indicative markers Ca-07000; 15,3 Mb and Indel 3 14,5 Mb).

Discussion

In this study, the first pair of chickpea NILs for erect vs prostrate growth habit was developed. It has been a useful tool to identify a new QTL (QTL_{Hg2} in RIP-9) or a gene (*HG2/hg2* in RIP-5) located on CaLG1 of the chickpea genetic map. Developing pairs of NILs for a given trait provides lines that are almost identical across the whole genome except for a target region. NILs have been widely used in plant breeding for fine mapping as in wheat (Xue et al. 2013), and for expression studies as in wheat and oilseed (Zhu et al. 2012; Ali-Benali et al. 2013). The first pair of NILs developed in chickpea was for single/double pod (Rubio et al. 1998) and was used to map this trait on CaLG6 (Rajesh et al. 2002). As well, pairs of NILs for resistance to different fusarium wilt races were developed by Castro et al. (2010). Recently, another pair of NILs for nodulation was used to describe a candidate gene involved in nodulation dissection pathway (Ali et al. 2014).

Growth habit is an important agronomic trait that evolved during domestication process. It has been described to be controlled by regulatory genes as occurred in rice, where the transition from prostrate to erect growth is controlled by the transcription factor *PROG1* encoding a single Cys2-His2 zinc-finger protein (Jin et al. 2008; Tan et al. 2008). It has been also reported that the two genes for tiller angle control, *TAC1* and *LAZY1,* modulate branch angles and play conserved roles in determining shoot growth angles in *Oryza sativa*, *Zea mays*, *Arabidopsis thaliana* and *Prunus persica* (Yu et al. 2007; Ku et al. 2011; Dardick et al. 2013). In legumes, most of growth habit studies have been focused on determinate vs indeterminate growth, as in common bean and soybean (reviewed by Hughes 1998). Several Arabidopsis TERMINAL FLOWER 1 (*TFL1*) (Bradley et al. 1997) orthologues genes have been described in pea, faba bean, soybean and common bean (Foucher et al. 2003; Avila et al. 2006; Liu et al. 2010; Repinski et al. 2012). However, few studies have been related to key components of plant architecture.

Chickpea has indeterminate growth and phenotypes erect, semi-erect and prostrate have been described (Muehlbauer and Singh 1987). In the current study, two segregating populations for growth habit were used. For RIP-9, this trait was considered as quantitative because segregation for the three different

phenotypes was found (Table 3). The analysis revealed a new significant QTL_{Hg2} in CaLG1 related to erect/prostrate phenotype (Fig. 1). Similarly, a significant QTL for branch elongation rate was located on chromosome 2 of *M. truncatula* that corresponds to CaLG1 (Nayak et al. 2010; Espinoza et al. 2012). In RIP-5, growth habit segregated as a single gene (*Hg2/hg2)* and was also located on CaLG1. As far as we know, there are no previous reports about erect vs semi-erect growth habit inheritance in chickpea. The gene *Hg2/hg2* was flanked by two markers located on 12,3 and 16,2 Mb on Ca1 and in this range was included the QTL_{Hg2}. These results suggest that the gene mapped in RIP-5 (erect vs semi-erect) could be the same QTL detected in RIP-9 (erect vs prostrate).

Other strong QTLs for length and number of primary branches, length of main stem and branch elongation rate were located on chromosome 7 of *M. truncatula* that corresponds to CaLG3 (Nayak et al. 2010; Espinoza et al. 2012). In chickpea, a major gene (*Hg/hg*) mapped on CaLG3 was reported in previous studies using populations derived from interspecific crosses (erect vs prostrate and semi-erect vs prostrate) (Kazan et al. 1993; Cobos et al. 2009; Aryamanesh et al. 2010). In this study, a certain association with markers in CaLG3 was also detected employing a set of 20 lines of RIP-9 with extreme values (Table 4). Also in the whole population a weak significant association ($P < 0.05$) of growth habit was found with two markers in CaLG3 (TA142 and STMS5). However, when the QTL analysis was applied no QTL was detected in CaLG3. As it was described before growth habit was considered as quantitative because segregation for three different phenotypes was found in RIP-9. This result indicate that more than one gen o QTL could be controlled growth habit in this population. Although, this work has been focused mainly on CaLG1, we tried to saturate the broad genomic window delimited by TA142 and STMS5 in CaLG3 with four new markers without positive results. For future studies, more markers should be added to CaLG3 to verify the possible presence of QTL related to growth habit in RIP-9.

In this work, we describe that the RAPD marker $OPAD09_{1053}$, mapped on CaLG1 of RIP-9, had the highest association with growth habit. RAPD markers could not be identified in different genetic backgrounds and the repeatability among laboratories is low. Thus, a robust SCAR marker ($SCAD09_{1053}$) was developed. The successful use of SCAR markers in MAS (marker assisted selection) for targeting resistance for ascochyta blight was previously demonstrated in chickpea (Iruela et al. 2006; Imtiaz et al. 2008). $SCAD09_{1053}$ obtained in this study was a useful tool to perform BLAST analysis against chickpea genome sequence (Varshney et al. 2013) and it was physically mapped on Ca1. Hence, the sequences of flanking genes were extracted to get new gene-specific markers, allowing us to approach candidate genes for growth habit. Ca_07000 predicted gene sensitive to proton rhizotoxicity 1-like was in the QTL_{Hg2} peak (maximum LOD = 4.67) detected in CaLG1. This gene was homologous to a predicted soybean zinc finger gene (LOC100802769) with e^{1-106} value and 78 % of identity. Because the transition from prostrate to erect growth has been reported to be controlled by a zinc-finger protein in rice, Ca_07000 could be a possible candidate gene (Jin et al. 2008; Tan et al. 2008). Another candidate gene could be Ca_06999 (predicted zinc finger ZAT5-like gene) that was close to Ca_07000 and was homologous to a putative zinc finger gene in pea (emb|X87374.1|) with e^{7-170} value and 75 % of identity. However, it was not possible to find any polymorphism between the parental lines used in this study. The SNP detected in Ca_07000 predicted gene sequence only differentiated erect vs prostrate and not erect vs semi-erect lines.

The association between CaLG1 and growth habit in chickpea was detected for the first time. This region together with *Hg1* gene that was previously mapped on CaLG3, suggest that growth habit in chickpea could be controlled by two major genomic regions. Ca_07000 predicted gene could be considered as a good marker and a possible candidate gene for growth habit. Because our results are preliminary, saturation with more markers of QTL_{Hg2} in RIP-9 as well as *Hg2/hg2* locus in RIP-5 using the resequencing data of parental lines WR315 (semierect) and ILC3279 (erect) would be useful for fine mapping in these two populations. In addition to functional analysis that would be necessary to provide insights about the responsible genes for the different growth habit types in chickpea.

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