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Rapid report

The *SINGLE FLOWER (SFL)* gene encodes a MYB transcription factor that regulates the number of flowers produced by the inflorescence of chickpea

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Summary

• Legumes usually have compound inflorescences, where flowers/pods develop from secondary inflorescences (I2), formed laterally at the primary inflorescence (I1). Number of flowers per I2, characteristic of each legume species, has important ecological and evolutionary relevance as it determines diversity in inflorescence architecture; moreover, it is also agronomically important for its potential impact on yield. Nevertheless, the genetic network controlling the number of flowers per I2 is virtually unknown.

• Chickpea (*Cicer arietinum*) typically produces one flower per I2 but *single flower* (*sfl*) mutants produce two (double-pod phenotype). We isolated the *SFL* gene by mapping the *sfl-d* mutation and identifying and characterising a second mutant allele. We analysed the effect of *sfl* on chickpea inflorescence ontogeny with scanning electron microscopy and studied the expression of *SFL* and meristem identity genes by RNA *in situ* hybridisation.

• We show that *SFL* corresponds to *CaRAX1/2a*, which codes a MYB transcription factor specifically expressed in the I2 meristem.

• Our findings reveal *SFL* as a central factor controlling chickpea inflorescence architecture, acting in the I2 meristem to regulate the length of the period for which it remains active, and therefore determining the number of floral meristems that it can produce.

Introduction

Inflorescence architecture is a key trait, ecologically and evolutionary relevant, as it strongly influences pollination and fruit set and determines plant form (Wyatt, 1982; Weberling, 1992; Benlloch *et al.*, 2007); it is an important relevant characteristic in agriculture, because it strongly influences fruit and seed production (Wang & Li, 2008). Inflorescence architecture depends on the identity of the meristems in the inflorescence apex, which determines the position in the inflorescence axes where flowers appear and on the activity of the inflorescence meristems, which controls how many flowers are produced (Prusinkiewicz *et al.*, 2007; Teo *et al.*, 2014; Benlloch *et al.*, 2015).

For simple inflorescences, as in Arabidopsis, flowers are formed at the primary inflorescence (I1) stem; however, in compound inflorescences, as in legumes, flowers appear on secondary inflorescence (I2) stems, formed in lateral positions of the primary inflorescence (Fig. 1a,b; Supporting Information Fig. S1; Benlloch *et al.*, 2015). Development of the legume compound inflorescence depends on the MADS domain transcription factor VEGETATIVE1/MtFULc (VEG1), which specifies I2 meristem identity (Berbel *et al.*, 2012; Cheng *et al.*, 2018), and PROLIFERATING INFLORESCENCE MERISTEM/MtPIM (PIM), a homologue to Arabidopsis APETALA1 (AP1), which

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specifies floral meristem identity (Berbel *et al.*, 2001; Taylor *et al.*, 2002; Benlloch *et al.*, 2006). Legume I2 meristems are generally short-lived meristems that produce some floral meristems before terminating as a stub (Fig. 1e).

The number of flowers produced by the I2 meristem is an important developmental trait for at least two reasons. First, it is a key factor that creates diversity in inflorescence architecture, being the number of flowers per I2 characteristic of each legume species and variety. For instance, while *Pisum sativum* (pea) and *Medicago truncatula* I2s produce one or two flowers, *Medicago sativa* (alfalfa) I2s produce 8–12 flowers and *Vicia cracca* (cow vetch) I2s produce dozens of flowers (Fig. S1; Benlloch *et al.*, 2015). Second, the number of flowers per I2 influences the number of pods produced by the plant, with the potential to positively impact on yield. In fact, in both pea and chickpea, some studies have found a correlation between pod number per I2 and seed yield, or with yield stability, supporting a positive effect of the multipod trait on crop performance (Milbourne & Hardwick, 1968; Sheldrake *et al.*, 1978; French, 1990; Kumar *et al.*, 2000; Rubio *et al.*, 2004; Devi *et al.*, 2018).

Mutations whose only effect is to increase the number of flowers/pods per I2 (multipod phenotype) have been described in several legumes, such as pea, *Cicer arietinum* (chickpea) and, more recently, in *Lens culinaris* (lentil) (White, 1917; Lamprecht, 1947; Singer *et al.*, 1999; Srinivasan *et al.*, 2006; Mishra *et al.*, 2020), indicating the existence of genes that specifically regulate this trait.

Despite the importance of the number of flowers per I2 in legumes, virtually nothing is known about how it is genetically controlled, and no gene specifically related with this trait has been isolated. In chickpea, two loci, *CYMOSE* (*CYM*) and *SINGLE FLOWER* (*SFL*), have been described for which recessive mutations specifically increase the number of flowers in the I2 (Srinivasan *et al.*, 2006). While most chickpea genotypes produce one flower per I2 ('wild-type'; Fig. 1a,c,e) (Prenner, 2012), mutations in the *SFL* gene lead to plants whose only evident phenotype is the production of two flowers/pods per I2 (double-pod trait; Fig. 1b,d,f) (Srinivasan *et al.*, 2006). *SINGLE FLOWER* was recently fine mapped to a 92.6 kb region of chromosome 6 (Ali *et al.*, 2016).

Here, we characterised the *sfl-d* mutant, analysing the ontogeny of its inflorescence and the expression of a floral meristem gene. Then, we identified *SFL* as a homologue of the Arabidopsis *RAX1/2* genes, encoding MYB transcription factors, and investigated how it functions by studying its expression in the chickpea inflorescence apex compared with other inflorescence meristem genes. Our findings revealed that *SFL* plays a central role in the control of inflorescence architecture of chickpea, specifically acting in the I2 meristem to control the time period for which it stays active, and therefore determining the number of floral meristems that it can produce.

Materials and Methods

Plant material

JG62 (syn. ICC 4951), an Indian double-pod chickpea (*C. arietinum*) landrace, maintained by ICRISAT (Hyderabad, India, icrisat.org), was parental in a cross with CA2156 (single pod) to develop pairs of nearly isogenic lines (NILs) used to map *SFL* (Ali

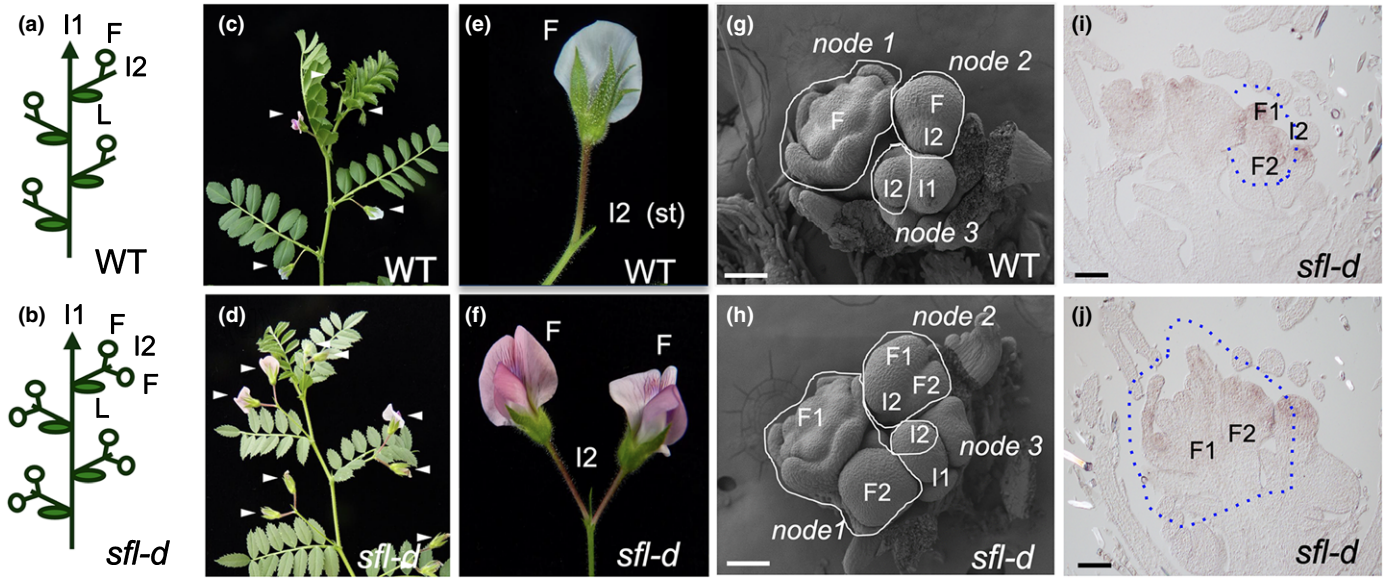


Fig. 1 Double-pod phenotype in chickpea (*Cicer arietinum*) caused by the mutation in the *SINGLE FLOWER* (*SFL*) gene and ontogeny of the inflorescence of the *sfl-d* mutant. (a, b) Diagrams of wild-type (WT) and *sfl-d* chickpea plants. Flowers (F) develop at secondary inflorescences (I2) that are formed in the axil of the leaves (L) of the primary inflorescence (I1) stem. Wild-type I2s (a) produce one flower, whereas *sfl-d* I2s (b) produce two flowers. (c, d) Wild-type and *sfl-d* chickpea plants. Arrowheads mark individual flowers formed at the I2s of the wild-type (c) and two flowers in the *sfl-d* (d) I2s. (e) Close-up of a wild-type I2, in which the stub (st) is marked. (f) Close-up of a *sfl-d* I2. (g) Scanning electron micrograph (SEM) of the inflorescence apex of a wild-type plant. In each I2 node one flower is found. (h) Scanning electron micrograph of the inflorescence apex of a *sfl-d* plant. In the I2 nodes two flowers (at different developmental stages) are found. (i, j) *In situ* hybridisation of *CaPIM* mRNA in inflorescence apices of the *sfl-d* mutant, in which each I2 node bears two flowers at different developmental stages. (g–j) Bar, 100 μm.

et al., 2016). Six double-pod genotypes from USDA (Beltsville, MD, USA, https://npgsweb.ars-grin.gov), AOS1, CA2969, ICC1083, LINE6560, LINE6581 and RPIP12-069-06223 were used to identify a second *sfl* mutant allele.

Single-pod genotypes were kabuli type. CA2156 is a Spanish cultivar, ILC3279 a Russian landrace maintained by ICARDA and BT6-17 is an advanced line from our breeding programme at IFAPA-UCO.

Controlled crosses

Genetic crosses were performed as described previously (Caballo *et al.*, 2018).

Genotyping and sequencing

DNA was isolated using the DNeasy Plant Mini Kit (Qiagen). PCRs were performed using Phusion High-Fidelity DNA polymerase (Thermo Scientific™, Madrid, Spain). PCR products were analysed using nondenaturing polyacrylamide electrophoresis gels. Primers used in this work are listed in Table S1. PCR products were sequenced either directly after purification with SureClean (Bioline, London, UK) or after cloning in pGEMTeasy (Promega).

Bioinformatics analysis

To identify sequence differences between double-pod and single-pod genotypes, the genome of the double-podded accession no. JG62 was re-sequenced using Ion-Torrent, and mapped against the chickpea reference genome (from the single-podded cultivar CDC-Frontier; Varshney *et al.*, 2013). Binary Alignment Map (BAM) files were inspected using Geneious® 8 software to detect polymorphism in relevant regions of the genome between both lines. To infer the extension of the deletion affecting Ca*RAX2-like*, all reads assembled to a region of chromosome 6 spanning 100 kb and containing genes LOC101505360, LOC101505694, LOC101506220 (Ca*RAX2-like*), LOC101506550, LOC101 490413, LOC101490737 and LOC101507108 were extracted and re-assembled using Geneious mapper and the following options: minimum mapping quality = 30; maximum gaps per read = 10%; maximum gap size = 50 000; word length = 17 and Index word length = 14; maximum mismatches per read = 15%; maximum ambiguity = 2.

For phylogenetic trees, sequences of 117 *Arabidopsis thaliana* R2R3-MYB proteins were used to retrieve 1110 R2R3-MYB proteins from 13 plant species using Blastp with an E-value threshold of 1e-40 (Table S2). The *Capsicum annuum BLIND* protein (NP.001311565.1) was also included for subsequent analyses. The sequences of the R2 and R3 domains of these 1228 proteins were aligned in Geneious Prime 2020.2.2 (http://www.geneious.com) software using Mafft (Katoh *et al.*, 2002) with the following options: algorithm = FFT-NSI-i × 1000; scoring matrix = BLOSUM30; gap open penalty = 3; offset value = 0.128. An approximately-maximum-likelihood phylogenetic tree was then derived from this alignment using FastTree v.2.1.12 plugin (Price *et al.*, 2010) on Geneious Prime, with the default settings and a bootstrap support of 1000 rep (Fig. S3). Based on this tree, the complete sequence of a subset of 29 proteins belonging to the S14 subfamily were aligned with Mafft, and the evolutionary history was inferred in Mega X (Kumar *et al.*, 2018) using the maximum-likelihood method, Le and Gascuel model (Le & Gascuel, 2008), and a bootstrap of 1000 replications. Sequences of Arabidopsis MYB35 and MYB80 were used as outgroups. Accessions numbers and information on the sequences used in this analysis are available in Table S2.

Scanning electron microscopy

Inflorescence apices were fixed in FAE (50% ethanol, 3.7% formaldehyde, 5% glacial acetic acid) at 4°C overnight in the dark. Samples were dehydrated with ethanol and critical point dried in liquid CO_2 (CPD300; Leica, Wetzlar, Germany). Dried samples were sputtercoated with argon–platinum plasma at a distance of 6–7 cm and 45 mA intensity for 15 s in a sputtering chamber (Leica Microsystems EM MED020). Scanning electron microscopy (SEM) micrographs were acquired using an AURIGA compact FIB-SEM (Zeiss, http://www.zeiss.com/) at EHT = 1–2 kV.

Quantitative real-time PCR

For expression analysis with quantitative real-time PCR (RT-qPCR) of the wild-type and the *sfl-d* mutant, samples collected from different tissues (roots, stem, leaves, vegetative apices) from plants of the NIL5-1V line (nearly isogenic line 5, single pod), after producing three or four leaves, and from inflorescence apices of NIL5-1V and NIL5-2V (double pod), were used. Total RNA was extracted using the E.Z.N.A® Plant RNA Kit (OMEGA Bio-Tek, Norcross, GA, USA). Next, 1.5 μg of total RNA, previously treated with DNase I (Turbo DNA-free Kit; Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) was retrotranscribed using the First Strand cDNA Synthesis Kit (Invitrogen), and RT-qPCR was carried out using the Evagreen Master Mix (Cultex, Madrid, Spain). PCRs were run and analysed using the Quant Studio3 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). The *C. arietinum UBIQUITIN* (*UBQ*, AJ001901) gene was used as an internal reference (Castro *et al.*, 2012). Calculations of each sample were done according the comparative ΔΔCT method (Livak & Schmittgen, 2001). Expression analyses were performed in three biological replicates of pooled samples, each with three technical replicates. Primers (Table S1) were designed with the Primer Express™ v.3.0 software (Applied Biosystems).

In situ hybridisation

RNA *in situ* hybridisation with digoxigenin-labelled probes was performed on 8-μm longitudinal paraffin sections of chickpea shoot apices as described previously (Ferrándiz *et al.*, 2000). RNA antisense and sense probes were generated using, as substrate, specific fragments of *CaRAX1/2a*, *CaRAX1/2b*, Ca*VEG1*, *CaPIM* or *CaUNI*, amplified by PCR from chickpea inflorescence cDNA and cloned into the pGEM-T Easy vector (Promega). Information on the primers used to generate the cDNA fragments

for probes is provided in Table S1. The sequences of Ca*VEG1* (XM_004491849), *CaPIM* (XM_004509697) and *CaUNI* (XM_004501703), which share 86.4%, 96.2% and 87.9% amino acid identity with *VEG1*, *PIM* and *UNI* (Hofer *et al.*, 1997; Taylor *et al.*, 2002; Berbel *et al.*, 2012) from pea, respectively, were retrieved from the chickpea genome database. For *CaRAX1/2b, CaPIM, CaVEG1* and *CaUNI*, transcription of antisense and sense probes was carried with SP6 or T7 polymerases, after linearising with *Nco*I or *Sal*I, respectively. Transcription of *CaRAX1/2a* AS probe was carried out using T7 polymerase, after linearising with *Sal*I. Signal was viewed as a purple precipitate under a light microscope.

Results

Effect of the *sfl-d* mutation on chickpea development

As previously mentioned, the I2 of wild-type genotypes produce one flower before terminating into a stub (Fig. 1a,c,e). However, in genotypes with homozygous mutations in the *SINGLE FLOWER* (*SFL*) gene, the I2s produce two flowers/pods and a stub (Fig. 1b,d, f) (Srinivasan *et al.*, 2006).

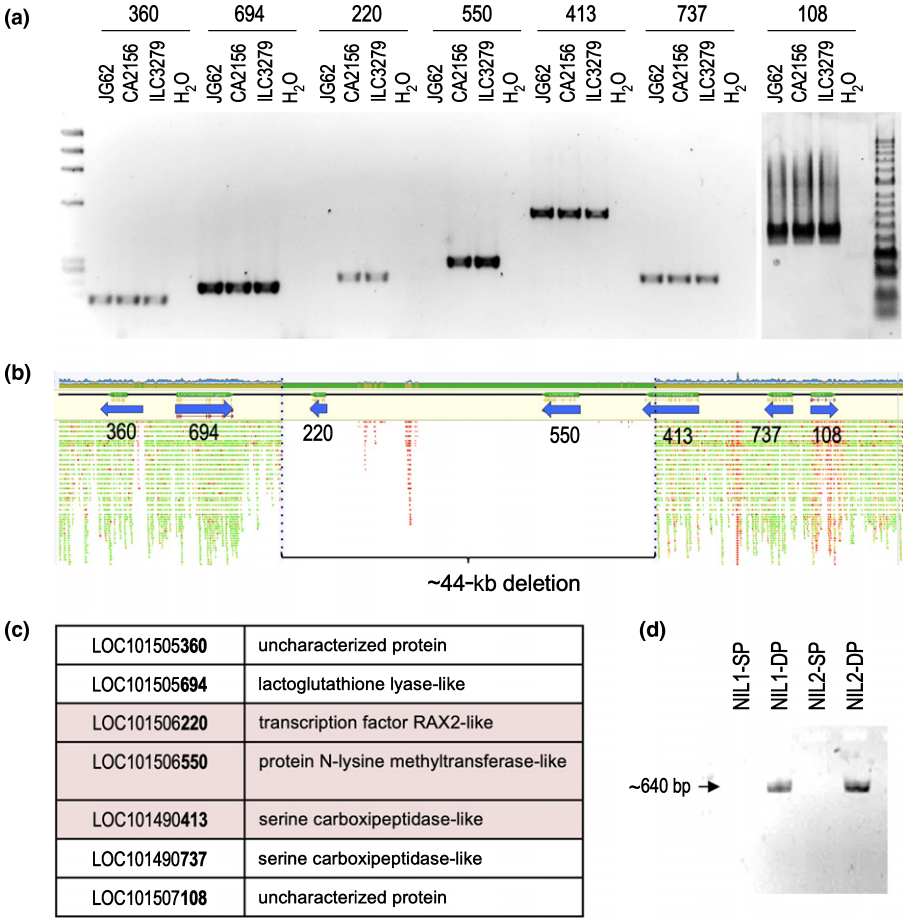
The production of two flowers in double-pod genotypes suggests that the I2 meristem is either larger and divides to produce more flowers, or is active for longer and forms more flowers in a sequential manner. With SEM we analysed inflorescence ontogeny of the pair of nearly isogenic lines NIL5-1V (single pod) and NIL5-2V (double pod), derived from a cross with the *sfl-d* parental JG62. In inflorescence apices of the single-pod plants, each node showed an I2 meristem from which only a floral primordium initiated (Fig. 1g). In inflorescence apices of double-pod plants, I2 meristems seemed to have a similar size to the wild-type but, in each I2 node, two flowers at a different stages of development were observed (Fig. 1h).

In situ hybridisation of double-pod inflorescence apices probed with the floral marker *CaPIM* (a homologue of the floral meristem identity genes *PIM* and *AP1*) showed the presence of two flowers at different developmental stages of the I2 nodes (Fig. 1i,j), confirming the result of the SEM analysis.

Therefore, the two floral meristems produced by the double-pod I2s are initiated sequentially, indicating that these I2 meristems produce more flowers compared with the wild-type I2 meristems because they are active for longer.

Identification of candidates for the *SFL* gene

The *sfl-d* mutation had been previously mapped to a 92.6 kb region of chromosome 6, with seven annotated genes that code for two uncharacterised proteins, four enzymes and a MYB transcription factor, CaRAX2-like (Fig. 2a,b,c) (Ali *et al.*, 2016). When primers



LOC101505**360**	uncharacterized protein
LOC101505**694**	lactoglutathione lyase-like
LOC101506**220**	transcription factor RAX2-like
LOC101506**550**	protein N-lysine methyltransferase-like
LOC101490**413**	serine carboxipeptidase-like
LOC101490**737**	serine carboxipeptidase-like
LOC101507**108**	uncharacterized protein

Fig. 2 Deletion in the 92.6-kb *SFL* mapping interval. (a) PCR amplification with primers for the seven genes in the 92.6-kb *SFL* mapping in DNA from the chickpea (*Cicer arietinum*) double-pod line JG62 and the single-pod lines CA2156 and ILC3279. (b) Mapping of sequencing reads of the JG62 re-sequencing against the genome of the reference single-pod line CDC-Frontier in the 92.6-kb *SFL* mapping interval, showing a deletion affecting three genes. (c) List of the genes contained in the 92.6-kb *SFL* mapping interval. Genes affected by the deletion are highlighted in pink. (d) PCR amplification with primers at the limits of the deletion in two pairs of single-pod (SP) or double-pod (DP) nearly isogenic lines (NIL1, NIL2).

for these genes were tested using PCR in single-pod lines they amplified fragments of the expected size (Fig. 2a). By contrast, in the double-pod line JG62, no amplification of LOC101506550 (*N-lysine methyltransferase-like*) or LOC101506220 (*CaRAX2-like*) genes was observed (Fig. 2a). As the genes encoding CaRAX2-like and *N*-lysine methyltransferase are adjacent, this suggested the existence of a deletion in the 92.6 kb *SFL* mapping interval that affects these genes in the *sfl-d* mutants.

Therefore, we examined the mapping quality of the JG62 (*sfl-d*) re-sequencing against the reference genome in this region of chickpea chromosome 6. We found a region of *c.* 44 kb with an unusually low density of reads (Fig. 2b). We then extracted all the reads mapped to a 100 kb region containing the *SFL* mapping interval (Ali *et al.*, 2016), and performed a new mapping against the same reference but allowing a within-read gap of 50 kb, which allowed us to infer the deletion breakpoints (Figs 2, S2). As expected, the predicted 44-kb deletion included the genes *CaRAX2-like* and *N-lysine methyltransferase-like* genes; it also includes part of the gene LOC101490413 (*serine carboxypeptidase-like*) (Fig. 2b,c). To validate the existence of the deletion in the double-pod genotype JG62, we tested PCR primers from the limits of the deletion in single/double-pod genotypes. In double-pod genotypes, a fragment of the expected size (642 bp) was amplified (Fig. 2d), which was latter sequenced to confirm the deletion borders. Conversely, as expected, no amplification product was obtained in single-pod genotypes.

These results showed the existence of a deletion affecting three genes in the mapping region of the *SFL* gene, only in double-pod genotypes. This points to these genes as the most likely candidates for the double-pod phenotype in *sfl-d* mutants.

Identification and analysis of a new double-pod mutant allele confirms *CaRAX2-like* as the *SFL* gene

To assess whether any of these three genes was in fact responsible for the double-pod phenotype of the *sfl-d* mutant lines, we looked for new *sfl* mutant alleles. We analysed six chickpea double-pod accessions from the USDA collection (Fig. 3a). Two primers pairs designed for the 44-kb deletion and for the *CaRAX2-like* gene were used to test the double-pod USDA genotypes. Three of the six USDA genotypes (ICC1083, RPIP12-069-06223 and CA2969) contained the 44-kb deletion (Fig. 3b), indicating that, in these genotypes, the double-pod mutation was the same as in JG62. However, the other three double-pod genotypes, LINE6560,

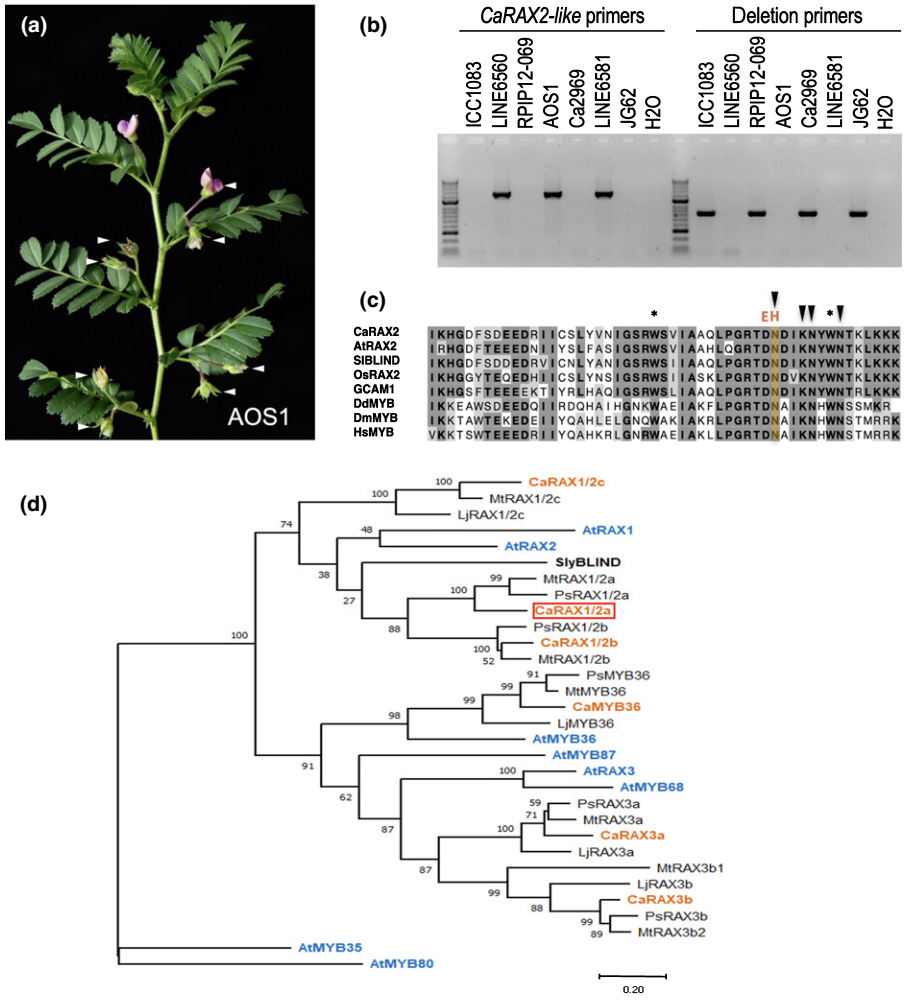


Fig. 3 *CaRAX2-like* is mutated in a new mutant allele of *SFL* and a phylogenetic tree of legume RAX proteins. (a) Double-pod phenotype of a chickpea (*Cicer arietinum*) AOS1 plant. Arrowheads mark the flowers. (b) PCR amplification in the USDA double-pod lines with primer pairs at *CaRAX2-like* or at the limits of the deletion. (c) ClustalW alignment of the R3 repeat of representative R2R3-MYB proteins from plants, microorganisms and animals. At, *Arabidopsis thaliana*; Ca, *Cicer arietinum*; Dd, *Dictyostelium discoideum*; Dm, *Drosophila melanogaster*; GCMA1, *Marchantia polymorpha*; Hs, *Homo sapiens*; Os, *Oriza sativa*; Sl, *Solanum lycopersicum*. Asterisks mark conserved tryptophan residues. Arrowheads mark base-contacting residues of the mouse homologue of HsMYB. (d) Phylogenetic tree of subgroup 14 MYB proteins from Arabidopsis and legumes. Legume proteins have been named after their Arabidopsis homologues. CaRAX2-like/CaRAX1/2a is framed in red. At, *Arabidopsis thaliana*; Ca, *Cicer arietinum* (chickpea); Lj, *Lotus japonicus*; Mt, *Medicago truncatula*; Ps, *Pisum sativum* (pea); Sl, *Solanum lycopersicum*. Accession numbers of the genes in the phylogenetic tree can be found in Supporting Information Table S2.

AOS1 and LINE6581, did not contain the deletion, but showed amplification with the Ca*RAX2-like* primers (Fig. 3b). This suggested that the double-pod phenotype of these three last genotypes could be due to mutations in the *SFL* gene different to that in JG62.

To determine the allelic relationship of the double-pod mutation in the USDA lines with the *sfl-d* double-pod mutation of JG62, we crossed JG62 with the USDA double-pod lines AOS1 and LINE6560. F1 plants from these crosses exhibited a double-pod phenotype (Fig. S3a,b), indicating no complementation. Moreover, F1 plants derived from the cross between the double-pod line AOS1 × single-pod line BT6-17 exhibited a single-pod phenotype (Fig. S3c), confirming that the double-pod mutation in the AOS1 line is recessive. These results indicated that the USDA lines AOS1 and LINE6560 bore mutation(s) in the *SFL* gene allelic to the *sfl-d* mutation in JG62; this new allele was named *sfl-3*.

The three genes affected by the 44-kb deletion present in JG62 and other related double-pod genotypes were sequenced in the USDA lines and aligned against the chickpea reference sequence. In the three double-pod USDA genotypes, both LOC101506550 (*N-lysine methyltransferase-like*) and LOC101490413 (*serine carboxypeptidase-like*) genes had a sequence identical to the reference, while the LOC101506220 (Ca*RAX2-like*) gene had a sequence variant identical in the three lines. In the USDA lines, bases 306 and 307 of the Ca*RAX2-like* coding sequence replaced CA in the reference with AC in the double-pod lines. This changes the amino acids 102 and 103 from aspartate–asparagine in the wild-type reference to glutamate–histidine in the double-pod lines (Fig. 3c).

CaRAX2-like encodes a R2R3-MYB transcription factor with sequence similarity to *REGULATOR OF AXILLARY MERISTEMS* (*RAX*) genes from Arabidopsis (Keller *et al.*, 2006; Müller *et al.*, 2006). Alignment of the sequence of CaRAX2-like from the *sfl-3* mutant lines with other related MYB proteins showed that the mutation in *sfl-3* mutants was located in the R3 repeat of the conserved MYB DNA-binding domain (Fig. 3c; Dubos *et al.*, 2010). The amino acids affected by the *sfl-3* mutation in CaRAX2-like, aspartate–asparagine, are conserved in plant RAX proteins, but are also conserved in MYB proteins from other organisms, from yeast to humans (Fig. 3c). Moreover, in the mouse homologue of the human c-MYB protein, this asparagine residue was shown to directly contact DNA (Ogata *et al.*, 1994; Martin & Paz-Ares, 1997) and therefore is presumably critical for function, strongly suggesting that the *sfl-3* mutation significantly affected the activity of the CaRAX2-like protein.

Phylogenetic analysis confirmed that CaRAX2-like groups with the subgroup 14 of the R2R3-MYB family of transcription factors (Fig. S4a), in Arabidopsis include three RAX proteins, RAX1, 2 and 3, and AtMYB36, AtMYB87 and AtMYB68. (Figs 3d, S4b; Dubos *et al.*, 2010). R2R3-MYB transcription factors, and RAX proteins in particular, are a family and a subgroup, respectively, with a high number of members (Feng *et al.*, 2017; Romani & Moreno, 2021). The subgroup 14 includes six proteins in chickpea, analogous to other legume species analysed in our tree and similar to the number of proteins present in Arabidopsis, indicating conservation in copy number between these species (Fig. 3d). CaRAX2-like belongs to the same clade as Arabidopsis AtRAX1 and AtRAX2 and the tomato Blind protein (Figs 3d, S4b; Schmitz *et al.*, 2002; Dubos *et al.*, 2010), although it groups with a separate legume specific clade. As CaRAX2-like seems similarly close to both Arabidopsis proteins, CaRAX2-like was renamed as CaRAX1/2a (Fig. 3d). In addition, the chickpea genome codes for two homologues to CaRAX1/2a /SFL: CaRAX1/2b and CARX1/2c. These three CaRAX proteins show high amino acid identity in the MYB domain (84–89%) although lower in the rest of the protein (13–30%) (Table S3).

In summary, the fact that two independent allelic mutations in the *CaRAX1/2a* gene, a complete deletion and a probable loss-of-function, associate with the double-pod mutant phenotype strongly indicates that *CaRAX1/2a* corresponds to the *SFL* gene, responsible for the double-pod trait.

CaRAX1/2a/SFL acts in the I2 meristem controlling its activity

SINGLE FLOWER regulates the number of flowers produced by the I2. To learn about where the *CaRAX1/2a/SFL* gene acts, we analysed its expression in different chickpea tissues using RT-qPCR. This analysis showed that, among aerial tissues, the highest expression was in floral apices. High expression was also found in roots (Fig. S5a).

To know in detail where the *CaRAX1/2a/SFL* gene is expressed in inflorescence apices, we performed *in situ* hybridisation with *CaRAX1/2a* on wild-type and *sfl-d* apices. Inflorescence apices of the *sfl-d* mutant showed no signal when hybridised with a probe for *CaRAX1/2a* (Fig. 4a,b), as expected because of the deletion in *sfl-d*. In wild-type inflorescence apices hybridised with *CaRAX1/2a*, the signal was observed in I2 meristems, as confirmed with markers for I2 and floral meristems. Contiguous sections hybridised with probes for *CaRAX1/2a* or *CaVEG1* (orthologue of the I2 meristem gene *VEG1*; Berbel *et al.*, 2012), essentially exhibited the same pattern, showing that *CaRAX1/2a* is expressed throughout the I2 meristem (Fig. 4a). Moreover, *VEG1* expression was not affected in the *sfl* mutant, suggesting that *SFL* does not affect specification of I2 meristem identity.

By contrast, contiguous sections of wild-type inflorescence apices hybridised with probes for *CaRAX1/2a* or *CaPIM* (orthologue of *PIM*, floral meristem gene; Taylor *et al.*, 2002) exhibited a complementary pattern, in which *CaPIM* was detected in the floral meristem and *CaRAX1/2a* was detected in the I2 meristem (Fig. 4b). Expression of *CaRAX1/2a* and *CaPIM* was not completely exclusive, as some overlap of the *CaRAX1/2a* and *CaPIM* signal was found at the boundary between the I2 and the floral meristems (Fig. 4b).

In contrast with *RAX* genes from Arabidopsis and tomato (Müller *et al.*, 2006; Busch *et al.*, 2011), we did not detect the expression of *CaRAX1/2a* in vegetative axillary meristems or leaf axils at the vegetative apex (Fig. 4a,c).

These results indicated that *CaRAX1/2a* was specifically expressed in the I2 meristems, further supporting that *CaRAX1/2a* corresponded to the *SFL* gene, which specifically regulated I2 meristem activity.

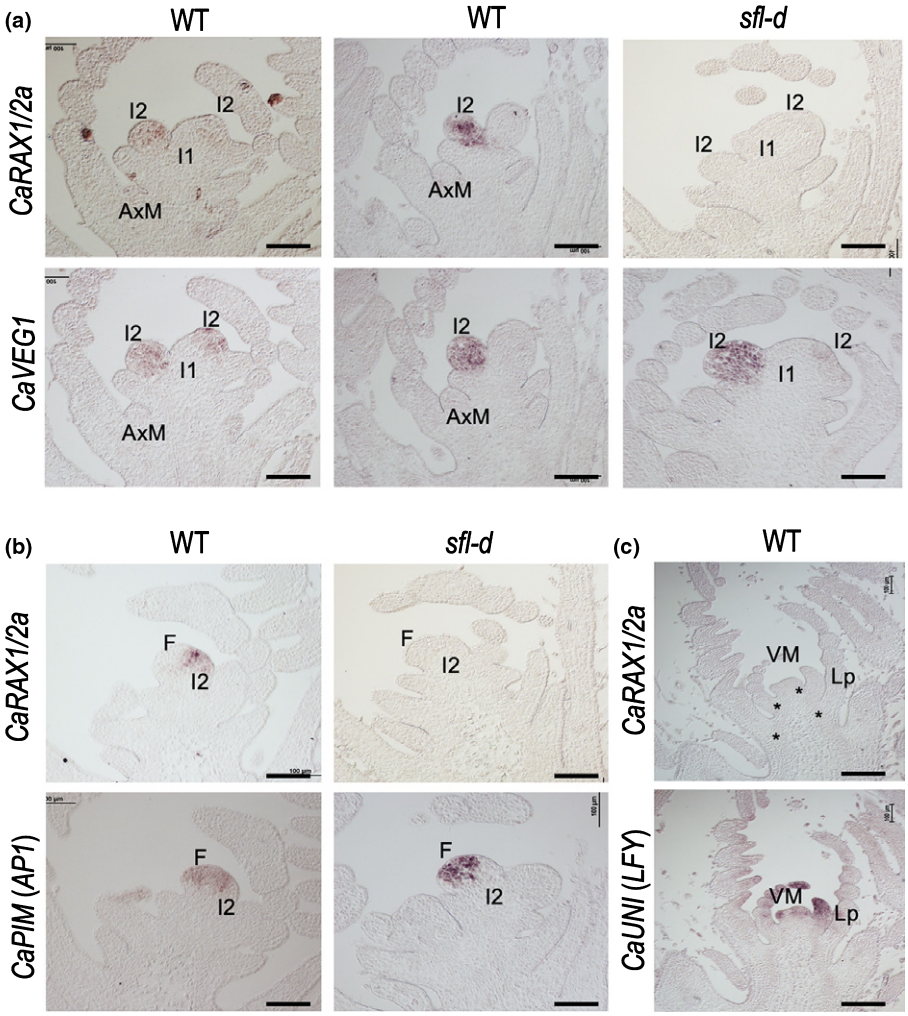


Fig. 4 Expression pattern of the *CaRAX1/2a/SFL* gene in shoot apices of chickpea (*Cicer arietinum*). (a) *In situ* hybridisation of *CaRAX1/2a* mRNA (upper row) and of the secondary inflorescence meristem (I2) marker *CaVEG1* mRNA (lower row) in contiguous sections of inflorescence shoot apices of wild-type (WT) or *sfl-d* plants. AxM, vegetative axillary meristem; I1, primary inflorescence meristem; I2, secondary inflorescence. (b) *In situ* hybridisation of *CaRAX1/2a* mRNA (upper row) and of the floral meristem (F) marker *CaPIM* mRNA (lower row) in contiguous sections of inflorescence shoot apices of wild-type or *sfl-d* plants. (c) *In situ* hybridisation of *CaRAX1/2a* mRNA (upper) and of the leaf (and floral) marker *CaUNI* mRNA (lower) in contiguous sections of vegetative shoot apices of a wild-type plant. Asterisks in the top image mark leaf axils. Lp, leaf primordium; VM, vegetative shoot apical meristem. Bar, 100 μm.

As the homology of *CaRAX1/2b* and *CaRAX1/2c* with *CaRAX1/2a*/SFL could suggest functional redundancy among these genes, we also analysed the expression of these homologues. For both genes we observed a tissue expression pattern similar to that of *CaRAX1/2a/SFL* (Fig. S5a). *In situ* hybridisation showed that the *CaRAX1/2b* homologue was also expressed in the I2 meristems, in domains that partly overlapped with *CaRAX1/2a/SFL* (Fig. S5b). We also observed that the expression of *CaRAX1/2b* or *CaRAX1/2c* did not increase in inflorescence apices of *sfl-d* mutant plants, in which the *CaRAX1/2a*/SFL gene is deleted (Fig. S5c). These results are compatible with the possibility that these genes act redundantly in the control of chickpea inflorescence development.

Discussion

Our results show that the *SFL* gene, responsible for the chickpea double-pod phenotype, corresponds to *CaRAX1/2a*, which encodes a R2R3-MYB transcription factor. Among aerial tissues, the highest expression of *CaRAX1/2a* was observed in inflorescence apices, in agreement with its role in inflorescence development. High expression of *CaRAX1/2a* was observed in roots. Interestingly, expression, and in some cases also function, associated with roots and nodules has also been described for some legume genes that regulate floral development (Zucchero *et al.*, 2001; Couzigou *et al.*, 2012). It will be worth studying the possible function of *CaRAX1/2a* in roots. In accordance with its role in regulating I2 meristem activity, in the inflorescence apex, *CaRAX1/2a* is specifically expressed in the I2 meristem, overlapping with *CaVEG1*, a homologue of legume I2 meristem identity genes (Berbel *et al.*, 2012; Cheng *et al.*, 2018). *CaVEG1* can be placed upstream of *CaRAX1/2a*, as supported by its unchanged expression in the *sfl-d* mutant. Interestingly, that *CaRAX1/2a* is not expressed in the I1 meristem, together with the fact that *sfl* mutations seem to specifically affect I2 activity, suggests that genetic networks that control meristem activity differ for I1 and I2.

CaRAX1/2a expression patterns have similarities, but also marked differences, with that of *RAX1/3* (*REGULATORS OF AXILLARY MERISTEMS1* and *3*) and *Blind* genes, Arabidopsis and tomato homologues, respectively (Keller *et al.*, 2006; Müller *et al.*, 2006; Busch *et al.*, 2011). The Arabidopsis and tomato genes have been shown to be transiently expressed in the axils of vegetative leaves, at axillary meristem initiation and *RAX1* also in stage 1 floral meristems. *CaRAX1/2a* is expressed in the I2 meristems, formed at the axils of inflorescence leaves, but we did not detect its expression

in vegetative axillary meristems or at the axils of vegetative leaves. Moreover, expression of *CaRAX1/2a* was not transient, but was observed in the I2 meristem throughout its development, supporting its role in controlling its activity.

The Arabidopsis *RAX1/2/3* and tomato *Blind* genes regulate axillary meristems, promoting their initiation (Keller *et al.*, 2006; Müller *et al.*, 2006; Busch *et al.*, 2011). The *Marchantia polymorpha GCAM1* (*GEMMA CUP-ASSOCIATED MYB1*) gene, encodes a R2R3-MYB from subgroup 14, as RAX and Blind proteins (Dubos *et al.*, 2010). Overexpression of *GCAM1* in *M. polymorpha* promotes the formation of cell clumps with low differentiation levels and with competence to proliferate, somehow resembling the Arabidopsis and tomato RAX proteins, which promote initiation of meristems (Yasui *et al.*, 2019). The chickpea CaRAX1/2a protein also regulates meristems but, in contrast with Arabidopsis and tomato RAX proteins, CaRAX1/2a acts to limit the proliferative phase of the I2 meristems, suggesting that the chickpea RAX protein interacts in a different way with some central regulatory components of the genetic machinery for meristem functioning. Interestingly, the expression of *GCAM1* in the Arabidopsis *rax1 rax2 rax3* mutant did not promote meristem formation, but inhibited it. However, the expression of a truncated version of the GCAM1 protein in which a N-terminal domain, upstream of the R2R3-MYB domain, not present in Arabidopsis, tomato or chickpea proteins, was deleted notably recovered axillary meristem formation in the triple mutant (Yasui *et al.*, 2019). Therefore, a change in the *M. polymorpha* RAX protein sequence turned it from a negative into a positive meristem regulator, supporting the idea that the different inhibitory activity of *CaRAX1/2a* on meristem regulation might be due to differences in its protein sequence.

What could be the *CaRAX1/2a* contribution to controlling the number of flowers per I2 in nature? The double-pod phenotype, increasing from one to two flowers, is moderate. In addition, although *sfl-d* is a null mutation, not every I2 in a *sfl-d* plant produced two flowers, and the expression of the double-pod phenotype depends on environmental conditions (Kumar *et al.*, 2000). As chickpea *RAX* genes are duplicated, it is possible that redundancy may exist among *CaRAX* genes for regulation of I2 meristem activity, as occurs between Arabidopsis *RAX* genes for regulation of axillary meristems (Müller *et al.*, 2006). Indeed, the tissue expression pattern of *CaRAX1/2a/SFL* and of its homologues, *CaRAX1/2b* and *CaRAX1/2c*, looks similar; *CaRAX1/2b* is expressed in I2 meristems, partly overlapping with *CaRAX1/2a/SFL*, which support this hypothesis. Nevertheless, expression in the inflorescence apex of the *CaRAX1/2a/SFL* homologues apparently did not change to compensate its absence in the *sfl-d* mutant. Thus, the *CaRAX1/2c* expression level is not affected in *the sfl-d* mutant and *CaRAX1/2b* expression seems to show a moderate decrease. This might reflect mutual positive regulation between *CaRAX1/2a/SFL* and *CaRAX1/2b*, but further studies would be required to test this possibility. In addition, the three CaRAX1/2 proteins showed low amino acid identity outside the conserved MYB domain. Therefore, additional studies are required to assess any possible redundancy between the chickpea *RAX* genes. Another gene, *CYM*, has been shown to also repress the production of flowers by the chickpea I2 (Srinivasan *et al.*, 2006). It is likely that the number of flowers at the I2 is determined by the combined action of *CaRAX1/2a* with *CYM* and maybe also other *CaRAX* genes.

Genotypes with a specific increase in the number of flowers in the I2 are also found in other legume species (Murfet, 1985; Mishra *et al.*, 2020), which suggests that the function of *CaRAX1/2a/SFL* could be conserved in other legumes. This would agree with the fact that the function of genes regulating other aspects of inflorescence development is generally conserved in legumes (Benlloch *et al.*, 2015; Cheng *et al.*, 2018; Roque *et al.*, 2018).

SUPERMAN (*SUP*), encoding a C2H2 zinc-finger transcriptional repressor, restricts the proliferation of floral organs in Arabidopsis flowers (Hiratsu *et al.*, 2002). *MtSUPERMAN* (*Mtsup*), its *M. truncatula* orthologue, recently described, restricts the proliferation of floral organs as well, but also regulates I2 meristem activity. *mtsup* mutant plants produced an increased number of abnormal flowers in their I2s, whose stubs were converted into terminal flowers (Rodas *et al.*, 2020). Therefore, although it is not its only role, *MtSUP*, as *CaRAX1/2a*, restricts I2 meristem activity, suggesting that both genes might cooperate in this function.

Further analysis of legume *RAX* genes promises to lead to valuable knowledge for designing useful tools to improve seed yield, but should also help in understanding the basis of form variety among different legume inflorescences to generate morphological diversity.

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Author contributions

CC, AB, TM, JR, JG, RO and FM developed and designed the experiments and interpreted the data; CC, AB, TM and JR performed the experiments; RO carried out the bioinformatics and the phylogenetic analyses; CC and FM wrote the manuscript with the input of all the authors. CC and AB contributed equally to this work.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Legume species with different numbers of flowers in the I2.

Fig. S2 Sequence of chromosome 6 containing the deletion in the *sfl-d* mutant.

Fig. S3 Allelic relationship between double-pod USDA mutants and the JG62 double-pod mutant.

Fig. S4 Phylogenetic tree of the R2R3-MYB proteins from representative eudicot species.

Fig. S5 Expression pattern of the homologue genes *CaRAX1/2b and CaRAX1/2c*.

Table S1 List of primers used in this study.

Table S2 Accession numbers of the genes in the phylogenetic trees in Figs 3(d), S3.

Table S3 Percentage of amino acid identity between chickpea RAX1/2 proteins.

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