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ORIGINAL PAPER

Mapping and identification of a *Cicer arietinum NSP2* gene involved in nodulation pathway

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Abstract
Key message **For the first time the putative *NSP2* gene in chickpea has been identified using pairs of NILs differing for the *Rn1*/*rn1* nodulation gene that was located in LG5 of chickpea genetic map.**
Abstract An intraspecific cross between the mutant non-nodulating genotype PM233, carrying the recessive gene *rn1*, and the wild-type CA2139 was used to develop two pairs of near-isogenic lines (NILs) for nodulation in chickpea. These pairs of NILs were characterized using sequence tagged microsatellite site (STMS) markers distributed across different linkage groups (LGs) of the chickpea genetic map leading to the detection of polymorphic markers located in LG5. Using this information, together with the genome annotation in *Medicago truncatula*, a candidate gene (*NSP2*) known to be involved in nodulation pathway was selected for mapping in chickpea. The full length sequence obtained in chickpea wild-type (*CaNSP2*) was 1,503 bp. Linkage analysis in an F_3 population of 118 plants derived from the cross between the pair of NILS NIL7-2A (nod) × NIL7-2B (non-nod) revealed a co-localization between *CaNSP2* and *Rn1* gene. These data implicate the *CaNSP2* gene as a candidate for identity to *Rn1*, and suggest that it could act in the nodulation signaling transduction pathway similarly to that in other legumes species.

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Introduction

The legume, chickpea (*Cicer arietinum* L.) is a rotation crop that improves soil organic content and fertility by increasing the positive N balance. For example, chickpea improved N levels by 38 % in the chickpea–wheat rotation (Aslam et al. 2003). Legumes form specialized organs called nodules in which the symbiotic *Rhizobium* bacteria are able to fix atmospheric nitrogen. The symbiotic interaction between the chickpea host and *Rhizobium* is controlled by plant signals that activate nodulation (Nod) factors that act as signaling molecules for initiating nodule development (Fisher and Long 1992; Long 1996). In *Medicago truncatula*, genes have been reported that are involved in both the development of nodules (nodulin genes) and the perception/transduction of the Nod factor signal before induction of nodulin genes (Pichon et al. 1992; Asad et al. 1994; Crespi et al. 1994; Cook et al. 1995; Vernoud et al. 1999; Catoira et al. 2000; Oldroyd and Long 2003).

In chickpea, the second most cultivated grain legume in the world (FAOSTAT 2013), there have been no reports of molecular studies of the nodulation pathway. On the other hand, seven nodulation mutants have been described: PM233, PM665, PM679, PM405, PM796 and ICC435M (Davis et al. 1985, 1986; Davis 1988; Singh et al. 1992)

carrying unlinked recessive mutant genes *rn1–rn6*, respectively, and PM638 with one dominant gene (*Rn7*) that is only linked to *rn1* (Paruvangada and Davis 1999). The phenotypes of some of these mutants have been grouped into the following categories: (1) stable non-nodulating (PM233), (2) temperature-dependent non-nodulating (PM665 and PM679) or (3) ineffective nodulating (PM405, PM796 and PM638). To our knowledge, the function of these genes (nodulin or induction genes) is unknown in chickpea.

The extensive conservation of gene order between the model plant *M. truncatula* and chickpea could be useful in identifying orthologous genes facilitating the transfer of genetic information between the two species (Seres et al. 2007). In addition, correspondence between linkage groups (LGs) of the chickpea genetic map with *M. truncatula* chromosomes has been established by synteny analysis (Millan et al. 2010; Nayak et al. 2010; Varshney et al. 2013). Consequently, the localization of the nodulation-related genes from *M. truncatula* on the chickpea genetic map could help with identifying and characterizing these genes in chickpea and, in turn, our understanding of the nodulation signaling pathway in this species.

To facilitate the mapping and identification of a gene of interest, several different approaches could be used. One of these involves the development of near-isogenic lines (NILs). This approach is advantageous for small target regions of the genome that segregates and genetic background noise can be eliminated. Thus, it is easier to localize the genomic region that controls the trait of interest (Oh et al. 2011).

The objective of this research was to develop chickpea NILs for nodulation using the non-nodulating mutant PM233 and use them to locate *rn1* on the chickpea genetic map. In addition, the gene sequence was characterized by synteny analysis using the available information in *M. truncatula*.

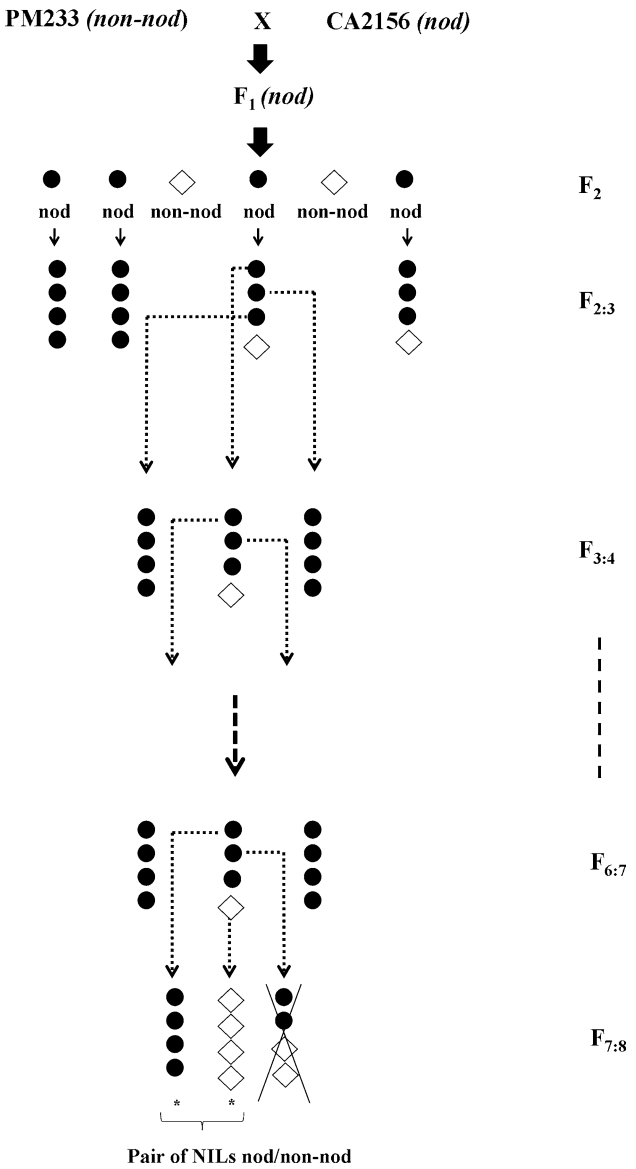


Fig. 1 Scheme for pair of NILs (nod/non-nod) development following residual heterozygosity selection by Pedigree method

Materials and methods

Plant material and *Rhizobium* inoculation

A cross between PM233, a non-nodulating mutant Desi type (Davis et al. 1985), and CA2139, a nodulating Spanish Kabuli type landrace, was used to develop a pair of NILs (Fig. 1). Seeds of non-nod mutant PM233 were supplied by the International Center for Agricultural Research in the Dry Areas (ICARDA) genebank.

To develop pairs of NILs, 28 F_2 plants were inoculated with a suspension of *Mesorhizobium* sp. (isolates ISC7 and ISC11) kindly provided by Dr. F. Temprano (IFAPA, Spain) at a concentration of 4×10^8 rhizobia/g. The seeds were treated with 1 g of the suspension per 100 g of chickpea seeds and 10 % sucrose and then sown in individual pots under controlled conditions (glasshouse). Each $F_{2:3}$ progeny from an F_2 nodulating plant was sown in a single row of a naturally (*Rhizobium*) infected field. $F_{2:3}$ were advanced to $F_{7:8}$ selecting nod plants in rows that segregated for the trait. Finally, residual heterozygosity present in F_7 plants made it possible to select individual nod and non-nod plants to obtain two pairs of NILs following the scheme (Fig. 1) described previously by Rajesh et al. (2002). Descendent of plants homozygous for nod and non-nod was harvested separately from two different $F_{7:8}$ families and was designated as NIL6-1A (nod)/NIL6-1B (non-nod) and NIL7-2A (nod)/NIL7-2B (non-nod).

An F_3 population ($n = 118$), derived by single-seed descent from the segregant F_2 obtained by the cross

between the pair of NILs, NIL7-2A (nod) × NIL7-2B (non-nod), was used to establish the nod/non-nod inheritance ratio and perform linkage analysis. F_2 was sown in a cage in fertile soil. F_3 plants were phenotyped for the presence or absence of nodules, 40 days after sowing in a greenhouse and inoculated with *Mesorhizobium* as described above.

DNA extraction and molecular characterization of NILs

Genomic DNA was extracted from 100 mg of young leaf tissue using the DNAzol method (Invitrogen, Carlsbad, CA, USA). Forty-two sequence tagged microsatellite site (STMS) markers distributed across different LGs of the chickpea genetic map were chosen to detect polymorphisms (Table 1). Marker analysis was performed according to Winter et al. (1999) and Lichtenzveig et al. (2005). PCR products 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 10 % non-denaturing polyacrylamide gels, and stained with ethidium bromide. STMS that required higher resolution was visualized by automatic capillary sequencer (ABI3130 Genetic Analyzer, Applied Biosystems/HITACHI, Madrid, Spain) in the Central Research Support Service (SCAI) at the University of Córdoba. The data points were analyzed using Genotyper 3.7 software from Applied Biosystems for polymorphisms.

Table 1 STMS markers used for genotyping parental lines (CA2139, PM233) and NILs (NIL6-1A/NIL6-1B and NIL7-2A/NIL7-2B)

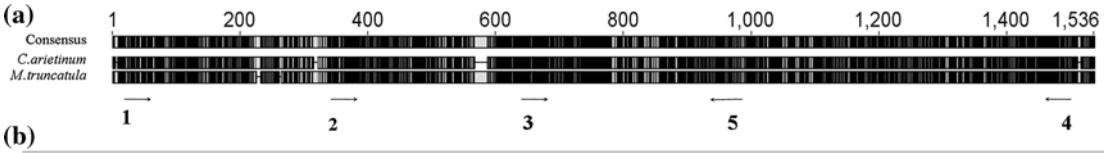
Linkage group	Marker
LG1	GA11, STMS12, STMS21, TA1, TA8, TA30, TA113, TA203
LG2	GA16, H1H011, TA59, TA110, TA194, TA200, TR19
LG3	GA13, STMS5, STMS10, TA34, TA125, TA142, TS19
LG4	GAA47, STMS11, STMS24, TA2, TA61, TA130, TA186, TR11
LG5	GA4, **TA5**[a], **TA11**[a], TR29, **TR59**[a]
LG6	GA21, STMS2, TA14, TA21, TA80, TA106, TR1
LG7	STMS6, TA78, TA117
LG8	GAA46, TS12, TS45

[a] Polymorphic markers in bold

Candidate sequence characterization

Using the genetic information available for *M. truncatula*, a nodulation signaling pathway 2 (*NSP2*) gene (MTR_3g072710) (Oldroyd and Long 2003) was selected to be amplified in chickpea. Using Primer 3 software (http://frodo.wi.mit.edu/primer3/) (Rozen and Skaletsky 1999), the primers were designed at different positions with the aim of amplifying the complete gene sequence in chickpea. Six different primer combinations (Fig. 2) were tested in genomic DNA of each parental line (CA2139 and PM233).

PCR reactions were carried out in 10 µl reaction volume containing 30 ng of plant genomic DNA, 1× buffer



(b)

Primer ID	Name	Position (bp)	Sequence 5′-3′	Tm °C
1	NSP2-L1	1-20	ATGGATTTGATGGACATGGA	58.6
2	NSP2-L2	353-370	TGCACCTATTGATGGCTGG	60.6
3	NSP2-L3	684-700	AGCCATCATCGAAGCCG	60.9
4	NSP2-R1	1484-1503	ACAAGTCCAAAGGGAAGCAG	59.3
5	NSP2- R2	1122-1142	TCCACAAACCCACCAATAACA	61

Fig. 2 **a** Diagram of sequence alignment between *Cicer arietinum* and *Medicago truncatula* of *NSP2* gene. The unique exon in the gene is shown. The location of primers designed in *M. truncatula* is indicated by *arrows*. **b** Sequence of primers designed in *M. truncatula NSP2* gene to be amplified in chickpea genomic DNA (line CA2139)

(50 mM KCl, 10 mM Tris–HCl, and 0.1 % Triton X-100), 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 2 min and 40 cycles comprising 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1.5 min, followed by a final extension at 72 °C for 5 min. PCR products were separated in 10 % non-denaturing polyacrylamide gels and stained with ethidium bromide. High-quality amplicons were excised from polyacrylamide gel by adding 25 μl of distilled water, purified and cloned in the pGEM-T Vector System I (Promega Corporation, Madison, WI, USA). Three different clones were sequenced using vector based primer pairs. The sequence fragments obtained were assembled into a single contig using Geneious Pro v4.7.6 Software (Drummond et al. 2010).

To confirm absence of bands in dominant markers, the universal primers ITSL and ITS4 (Štajner et al. 2002) were used as positive controls. Multiple PCR were performed using the primer combination, NSP2-L2 + NSP2-R1 (Table 1) (0.5 μM) and ITS (0.05 μM) in 10 μl of reaction mix following the same conditions described above for *NSP2* amplification.

Linkage analysis

Linkage analysis was performed using the JoinMap v4.0 regression function (Van Ooijen 2006). A minimum LOD score threshold of 3 and a maximum recombination fraction of 0.25 were applied as general linkage criteria for grouping markers. Kosambi's function (1944) was applied to estimate map distance.

Linkage with the physical map

To compare both genetic and physical map, the primers of three polymorphic STMS markers (TR59, TA5 and TA11) between NILs were amplified in silico from the chickpea genome assembly (Varshney et al. 2013) with in-house script (Kalendar et al. 2009). The amplified fragment of *NSP2* and *LYK9* genes from *M. truncatula* was mapped to the chickpea genome using the BLASTN (NCBI) program, which resulted in two putative genes, annotated as *CaNSP2* and Ca*LYK9*.

Phylogenetic analysis

The evolutionary relationship of the *CaNSP2* sequence obtained in chickpea with related genes from other species available from the GenBank was assessed using the Neighbor-Joining method (Saitou and Nei 1987), based on the deduced amino acid sequences and considering the species *Pinus sylvestris* as the out group. The confidence in the different nodes was calculated using 1,000 bootstrap replications and expressed as a percentage. The evolutionary distances were computed using the Poisson correction method (Zuckercandl and Pauling 1965). All positions containing gaps were eliminated from the dataset (complete deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007).

Results

Two segregating $F_{7:8}$ families were chosen to select pairs of NILs differing for nodulation/non-nodulation. Finally, two pairs of NILs [NIL6-1A (nod)/NIL6-1B (non-nod) and NIL7-2A (nod)/NIL7-2B (non-nod)] were selected.

The molecular characterization of these pairs of NILs showed that NIL6-1A/1B and NIL7-2A/2B have the same amplification patterns using STMS markers. The NILs were monomorphic for all markers across the chickpea genetic map except for three that were located in LG5 (TA5, TA11 and TR59) (Table 1). To analyze the association between polymorphic STMS and the phenotype observed, DNA was amplified from 20 F_3 individuals derived from the cross NIL7-2A × NIL7-2B that differed for nodulation (10 nod/10 non-nod). Results revealed that all non-nod F_3 plants were monomorphic for the same allele of non-nod parent NIL7-2B for markers TA5 (208 bp), TA11 (242 bp) and TR59 (190 bp), indicating their association with non-nodulation. These results suggest that the *Rn1*/*rn1* gene is located in LG5 of the chickpea genetic map.

Chickpea *Rn1*/*rn1* identification

As the nodulation pathway has been well studied in *M. truncatula*, we explored the syntenic relationship between *Medicago* and chickpea. Since LG5 of the chickpea genetic map corresponds to *Medicago* chromosome 3 (Millan et al. 2010; Nayak et al. 2010; Varshney et al. 2013), we investigated chromosome 3 of the model species for nodulation-related genes. Two putative genes for nodulation were found on *Medicago* chromosome 3; the transcription factor *NSP2* (MTR_3g072710) and lysine motif (LysM)-receptor-like kinases *LYK9* (MtD04912) (Oldroyd and Long 2003; Arrighi et al. 2006). Using the information obtained about *Rhizobium* infection process in PM233 (Matthews and Davis 1990), the transcription factor *NSP2* was selected as a candidate gene responsible for the non-nodulating phenotype in this mutant.

To isolate this sequence from the chickpea genome, six different primer combinations designed in *M. truncatula* gene were tested in each of the chickpea parents (PM233 and CA2139) and NILs to amplify the gene sequence (Fig. 2). Only one combination (NSP2-L2 and NSP2-R1)

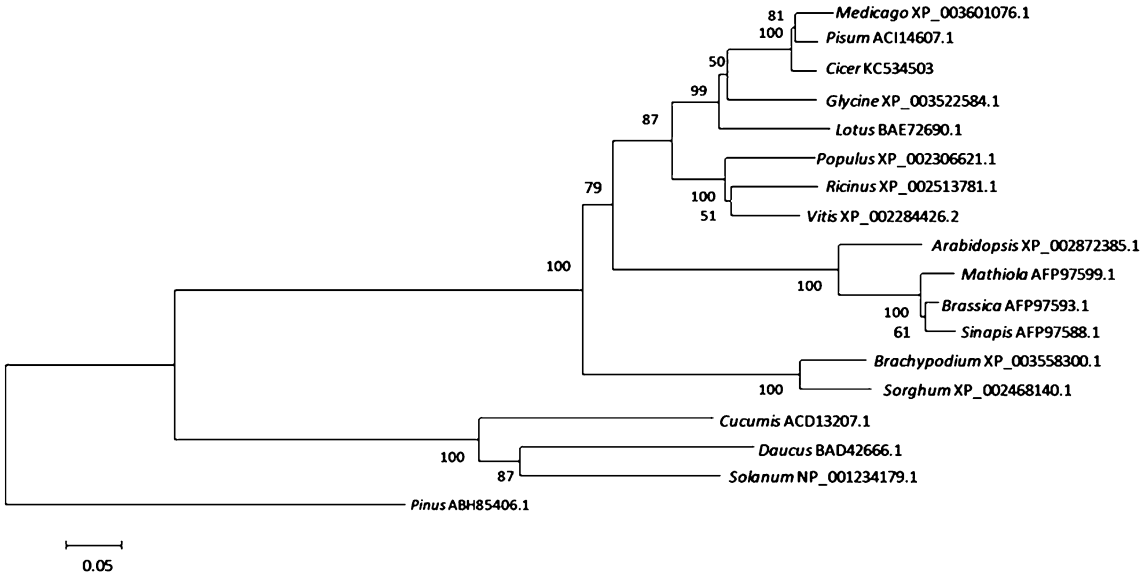


Fig. 3 Phylogenetic tree of 18 amino acid sequences of GRAS family protein in different plant species. Numbers at branch nodes represent bootstrap values out of 1,000 replicates. *Pinus sylvestris* was considered as the out group. Accession numbers appear at the end of genus names

produced a high-quality amplicon. The amplicon was only present in CA2139, NIL6-1A and NIL7-2A (nod), whereas it was absent in PM233, NIL6-1B and NIL7-2B (non-nod). The sequence length obtained in CA2139 was 1,067 bp, covering the gene except for 353 bp at the 5′ end. To obtain the complete gene sequence, BLAST analysis was performed against the draft genome sequence recently obtained from the chickpea variety CDC-Frontier (Varshney et al. 2013). The alignment showed that the homologous sequence was annotated as Ca_26279 gene in the pseudomolecule 5 at the position 1,280,031–1,281,533 in the negative strand. This annotated *NSP2* gene in chickpea consists of a 1,503-bp exon with no introns. The sequence has been deposited in the GenBank (http://www.ncbi.nlm.nih.gov/) database and assigned the accession number KC534503 for chickpea genotype CA2139 and, therefore, the gene in chickpea has been designated as *CaNSP2*.

The sequence of the gene (*CaNSP2*) obtained showed high similarity to that reported for *NSP2* in other legumes based on BLASTN analysis. The predicted coding region indicated that *CaNSP2* has the highest level of identity with the *Pisum sativum* (86 %) and the *M. truncatula* (85 %) homologous genes. The deduced amino acid sequence of *CaNSP2* contains 501 residues, with a molecular weight of 55.270 kDa. The *Pfam* domain search identified 377 (residue 117–493) residues of the sequence that are highly conserved, and belong to the domain of GRAS family transcription factor (PF 03514). As expected, the predicted coding region encodes a protein belonging to the plant-specific GRAS family of putative transcription factors. BLASTX analysis showed that the putative CaNSP2 sequence had highest similarity with *P. sativum* (89 %) and *M. truncatula* (87 %) with most of the variations concentrated at the N-terminal region. The three proteins showed high similarity at these conserved regions, LHRI, LHRII, VHIID, PFYRE and SAW, of the GRAS domain, similar to that demonstrated between *P. sativum* and *M. truncatula* (Kalo et al. 2005).

Phylogenetic associations based on 18 GRAS family protein sequences belonging to 9 different families indicated that all legume species were grouped together. The NSP2 protein sequences of *M. truncatula* and *P. sativum* were the closest to CaNSP2 with *Arabidopsis thaliana* group (Brassicaceae) being distant from the legumes group (Fig. 3).

NSP2 and *Rn1*/*rn1* linkage analysis

Linkage analysis was performed using the F_3 population ($n = 118$) obtained by single-seed descent method. All F_3 individuals were genotyped with three STMS markers as well as the *CaNSP2* gene and phenotyped for presence or absence of nodules (*Rn1*/*rn1* locus). Both the gene marker and the *Rn1*/*rn1* locus showed distorted segregation being coincident with presence of band/nod (89 plants) and absence of band/non-nod (29 plants). That is different from the expected ratio 5:3 ($P < 0.01$) for a F_3 population. As expected, linkage analysis in F_3 revealed that *CaNSP2* and *Rn1*/*rn1* were co-located in LG5 of the chickpea genetic map (Fig. 4). All the individuals presenting nodules had two amplified fragments presents in the PCR products corresponding to 1,067-bp of *NSP2* and 750-bp of ITS, that

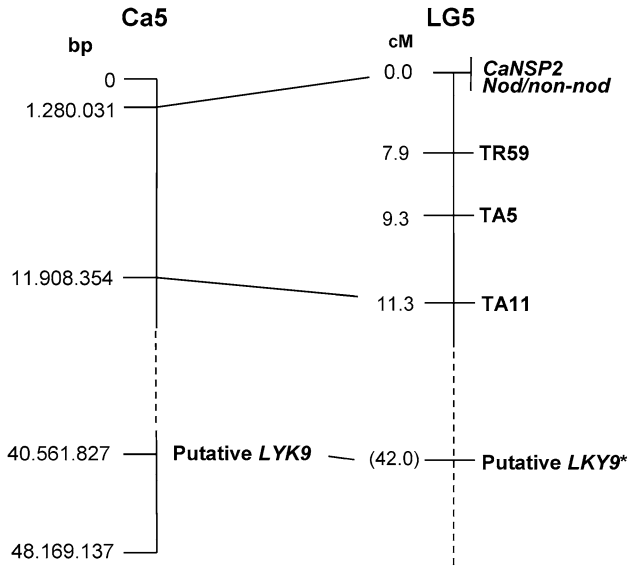


Fig. 4 Comparison between genetic and physical map of chickpea LG5. On the *right*, genetic LG5 including the phenotypic evaluation of nod vs. non-nod in the F_3 population derived from the cross between two near-isogenic lines (NIL7-2A × NIL7-2B). On the *left*, the physical map including CaNSP2, TA11 and putative LYK9 gene located in Ca5 (LG5) showing the distance in base pair (bp). *Asterisk* the estimated position of putative *LYK9* gene in genetic map was calculated considering the physical distance among *CaNSP2*, TA11 and putative *LYK9* as reference

was used as positive control. In contrast, non-nodulating individuals had only one amplified fragment that corresponds to the ITS control (Fig. 5).

Physical map analysis

Out of the three STMS markers, only TA11 could be amplified in silico without any mismatches in primer sequences and was found to be located in the *C. arietinum* pseudomolecule 5 (Ca5). *M. truncatula LYK9* had the highest homology (92 %) with the annotated gene Ca_07575 located from 40,561,827 to 40,568,297 bp in the positive strand of Ca5. The two putative genes *CaNSP2* and *LYK9* were located in opposite sites of the chromosome flanking the STMS marker TA11 (Fig. 4). The physical position of both genes and the TA11 marker was used to estimate the position of the putative *LYK9* locus in chickpea LG5, located 42 cM away from *nod/non-nod* locus (Fig. 4).

Discussion

Pairs of NILs for a given trait have the advantage that they differ for a target region and are almost identical in the rest of the genome. They have been used for fine mapping in rice (Oh et al. 2011) and expression studies in wheat and oilseed rape (Baek and Skinner 2003; Manickavelu et al. 2010). In chickpea, NILs for single/double pods were used to locate the double-podding gene (*s*) in LG6 of the chickpea genetic map (Rajesh et al. 2002). In this study, we report for the first time, pairs of NILs developed for nod vs. non-nod in chickpea. These NILs have proven to be very useful for localizing the *rn1* gene present in the PM233 mutant to LG5 of the chickpea genetic map and characterizing it using genomics and syntenic information from the model species *M. truncatula*.

Previous research on nodulation in chickpea was based on classical genetics (Paruvangada and Davis 1999). The molecular characterization of two pairs of NILs [NIL6-1A (nod)/NIL6-1B (non-nod) and NIL7-2A (nod)/NIL7-2B (non-nod)] using STMS markers allowed us to discover polymorphic markers in LG5, syntenic to *M. truncatula* chromosome 3 (Millan et al. 2010; Nayak et al. 2010; Varshney et al. 2013). In chromosome 3 of *M. truncatula,* two genes *LYK9* and *NSP2* involved in the nodulation pathway have been annotated. Of which *LYK9* is a receptor from the lysine motif (*LysM*)-domain-containing receptor-like kinase family. In *M. truncatula*, Nod factors are perceived for a LysM receptor-like kinase protein of epidermal cells (Arrighi et al. 2006). *NSP2* in *M. truncatula* is an intronless gene, encoding a GRAS family protein with a conserved GRAS domain and a variable N-terminal region (Kalo et al. 2005). Functional studies in *M. truncatula* have shown that

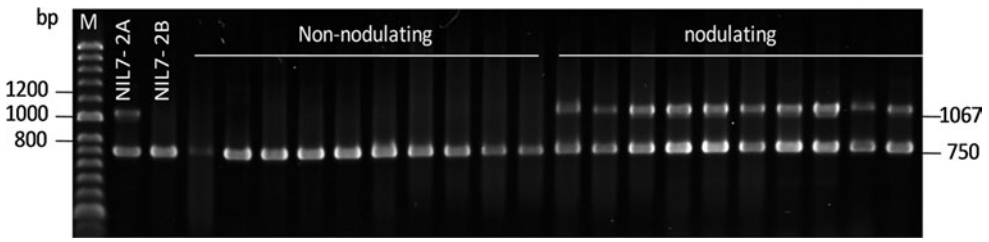


Fig. 5 Agarose gel showing the segregation of the polymorphic marker developed to amplify the locus *CaNSP2* in chickpea using ten F_3 nodulating (1,067 bp) and ten non-nod (non-amplification) individuals. To confirm absence of bands the universal primers ITSL and ITS4 were used as positive controls (750 bp). Lane M: molecular weight marker Hyperladder II with molecular weight in bp

NSP2 is involved in Nod factor signaling (Oldroyd and Long 2003; Kalo et al. 2005). The recessive allele of the *NSP2* gene blocks nodulin gene expression down-stream of calcium spiking, showing a complete absence of infection and cortical cell division following *Mesorhizobium* inoculation (Oldroyd and Long 2003).

In chickpea, the rhizobial infection process was blocked in PM233 mutant at a stage subsequent to root hair adsorption of bacteria, but prior to initiation of infection threads and root cortical cell division (Matthews and Davis 1990). This shows that the *NSP2* gene is possibly homologous to the *rn1* gene in the chickpea. *LYK9* was not selected as candidate because this gene acts as the Nod factor receptor to enable bacterial infection (Arrighi et al. 2006) without showing any differences at the stage of infection, in both PM233 mutant and non-mutants. It has been demonstrated that the *NSP1–NSP2* complex binds directly to the promoters of early nodulin genes (*ENOD11*), and mutations found in the GRAS domain of either *NSP1* or *NSP2,* inhibit nodule production, thus highlighting the significance of the *NSP1–NSP2* complex for nodulation signaling (Hirsch et al. 2009).

The recent sequencing of the chickpea genome (Varshney et al. 2013) allowed us to obtain the complete *NSP2* gene sequence for comparative analysis. Our results showed high homology among chickpea, pea and *M. truncatula* in both at nucleic acid and amino acid level. Phylogenetic analysis of GRAS family proteins formed a separated group for the legumes of which *M. truncatula*, *P. sativu*m and *C. arietinum* showed the highest identity, in support with previous studies in *L. japonicus* (Murakami et al. 2007). These data suggest that the *CaNSP2* putative gene could function similarly to *M. truncatula NSP2*. Nevertheless, functional validation and characterization of *CaNSP2* in PM233 shall be required. Due to the grouping of the *NSP2* gene observed from the phylogenetic tree (Fig. 3), *NSP2* genes could play a different role in legumes and that could vary in other families. GRAS family proteins in other non-nodulating species have different functions and the genetic distance obtained with other plant species supported the functional differences. As reported in *A. thaliana*, the transcription factor SCARECROW is required for the asymmetric cell division of the initial cell in the generation of cortex and endodermis (Sabatini et al. 2003).

Linkage analysis in the F_3 population from study enabled us to map *CaNSP2* and *Rn1*–LG5 of the chickpea genetic map. The distortion of gene marker and phenotype evaluation toward the nodulating parent could be explained by a small attack of wilt at flowering time in the F_2 population causing loss of some non-nod plants. Nodulation has parallels with the processes of systemic acquired resistance (Durrant and Dong 2004). STMS markers chosen for characterization of NILs were distributed across different linkage groups and only the closest markers to the targeted region showed polymorphisms. Thus, this strategy proved to be valuable. The total coincidence of allele distribution in the phenotypic and genotypic evaluation with *CaNSP2* suggested that it is the candidate gene for *rn1* (Davis et al. 1985) that could play a central role in the nodulation signaling pathway in chickpea. However, as mentioned above, characterization of the gene in PM233 mutants is necessary to understand the nature of this mutation and to confirm whether *CaNSP2* function is similar to that reported in legume model plants (*M. truncatula* and *L. japonicus*). The nature of function loss associated with the *NSP2* mutation has only been studied in *M. truncatula*: two mutations, *nsp2-1*and *nsp2-2*, caused by deletions in the gene resulted in the absence of nodules (Kalo et al. 2005), and a third one (*nsp2-3*) results in a change in one amino acid and a reduction in the number of nodules (Catoira et al. 2000).

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Conflict of interest The authors declare that they have no conflict of interest.

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