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DEPARTAMENTO DE GENÉTICA

TESIS DOCTORAL

“Búsqueda de genes candidatos asociados a caracteres de interés
agronómico en garbanzo (*Cicer arietinum* L.)”

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Mayo 2015

TITULO: *Búsqueda de genes asociados a caracteres de interés agronómico del garbanzo (Cicer arietinum L.)*

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La tesis se ha desarrollado de acuerdo con los objetivos propuestos. La metodología empleada ha permitido detectar marcadores asociados a QTLs y proponer genes candidatos relacionados con los caracteres analizados. Durante el desarrollo de su trabajo, la doctoranda ha mostrado una gran iniciativa e interés por desarrollar nuevas técnicas. La calidad de sus resultados está avalado por las dos publicaciones aceptadas en dos revistas con SCI en primer cuartil que constituyen los capítulos I y II del manuscrito, en el tercer capítulo se presenta un trabajo ya enviado a publicar que en la actualidad se encuentra bajo revisión en la revista Theoretical and Applied Genetics. Finalmente, el último capítulo de la tesis se refiere al estudio de un carácter que tiene un gran interés en la Mejora del garbanzo como es la fecha de floración. En este trabajo se han conseguido identificar un gen candidato muy prometedor.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, _16_ de _abril_ de _2015_

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Nota: A fin de establecer una coherencia formal a lo largo del presente documento, se han uniformado las referencias y se han editado los trabajos originales, eliminando de los mismos el apartado de referencias, el cual ha sido agrupado al final del documento.

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Summary

Legumes are an important resource of proteins and carbohydrates, in addition to their ability to perform symbiosis with *Rhizobium* fixing atmospheric nitrogen in the soil. For that reason it is recommended to involve legumes in rotation systems. Chickpea is the second most important grain legume in the world after dry beans (*Phaseolus vulgaris* L.) in production. In spite of its benefits, not enough studies have been done about adaptive traits at molecular level and most of the researches were in classical genetics. Nevertheless, chickpea genome sequence recently published is a powerful tool that helps to start understanding the adaptation mechanisms in this legume.

In this study, pairs of near isogenic lines (NILs) for the traits nodulating/non-nodulating, erect/prostrate growth habit and early/late flowering time were developed. The combination of information reported in the model legume *Medicago truncatula* the chickpea genome sequencing and re-sequencing data together with the pairs of NILs and segregating populations (recombinant inbred populations, RIPs) allowed us to get progress in our study.

For the first time *CaNSP2* gene, related to nodulation pathway, was identified and mapped on linkage group (LG) 5 of the chickpea genetic map.

Growth habit is an important adaptive trait; however the relationship between erect vs semierect was studied for the first time in the present study. A new QTL/gene related to growth habit in inter (erect vs prostrate) and intra (erect vs semierect) specific crosses respectively, was detected in LG1.

Because of its positive effect on the cultivar production and its stability, fine mapping for the simple/double podding trait (*Sfl/sfl*) in LG6 of chickpea genetic map was performed. In the delimited region of *Sfl/sfl* locus, the annotated gene *RAX2* could be a potential candidate controlling double podding in chickpea.

It is necessary to integrate early flowering trait to the desirable chickpea varieties that makes short life cycle. It helps to escape from abiotic

stresses as drought that limits almost 80% of the cultivar production. Therefore, the pairs of NILs, developed for the two major QTLs (QTL_{DF1} and QTL_{DF2} in LG4 and LG3, respectively) in this study, would be good material to perform expression profiles and study the individual effect of each of these QTLs. The annotated gene TEMPRANILLO located in QTL_{DF1} interval could be a good candidate gene for this QTL.

Our study provides new molecular markers for growth habit, simple/double podding and flowering time that could be useful for MAS. In addition, new candidate genes for these traits were suggested.

Resumen

Las leguminosas son una importante fuente de proteínas y carbohidratos, además tienen la capacidad de fijar nitrógeno en el suelo gracias a su relación simbiótica con *Rhizobium* siendo recomendables en la rotación de cultivos. El garbanzo, objeto de estudio en esta tesis, es la segunda leguminosa grano en cuanto a producción mundial. A pesar de las ventajas que supone su cultivo, no se han realizado suficientes estudios sobre caracteres adaptativos a nivel molecular; La reciente publicación de la secuencia completa del genoma de garbanzo es una herramienta muy potente que puede servir de gran ayuda para entender los mecanismos relacionados con caracteres adaptativos en garbanzo.

En este trabajo, se han desarrollado parejas de líneas casi isogénicas (NILs) para los caracteres nodulación/no-nodulación, hábito de crecimiento erecto/postrado y fecha de floración temprana/tardía. La combinación de la información obtenida a partir de la secuencia y resecuenciaciones del genoma de garbanzo junto con las parejas de NILs obtenidas y poblaciones de líneas segregantes disponibles ha permitido identificar por primera vez el gen *CaNSP2*, relacionado con la nodulación y localizarlo en el grupo de ligamiento (GL) 5 del mapa genético de garbanzo.

El hábito de crecimiento es un importante carácter adaptativo. En este trabajo se ha estudiado por primera vez la herencia de los fenotipos erectos y semi-erectos. También se ha detectado un nuevo QTL (quantitative trait loci) en el grupo de ligamiento 1 (GL1) asociado a éste carácter utilizando poblaciones derivadas de cruzamientos inter (erecto vs postrado) e intra (erecto vs semi-erecto) específicos.

Otro carácter que ha sido objeto de estudio en esta tesis, ha sido el carácter simple/doble vaina debido a su efecto positivo y estabilidad en la producción. Se realizó un mapeo fino del GL6 donde se localizaban marcadores asociados a éste carácter. Se han podido desarrollar un conjunto de nuevos marcadores moleculares estrechamente ligados al locus *Sfl/sfl*. Estos marcadores pueden ser muy útiles en programas de mejora asistida por marcadores (MAS) para seleccionar genotipos con doble vaina. En la región genómica donde se encuentra del locus *Sfl/sfl* se localiza el gen *RAX2* que es un posible gen candidato para el carácter doble vaina en garbanzo.

En las variedades de garbanzo es recomendable incluir el carácter floración temprana, ya que ayuda a escapar de estreses abióticos que pueden limitar la producción. En este trabajo, se han desarrollado parejas de NILs, para dos QTLs que controlan este carácter (QTL_{DF1} y QTL_{DF2} en GL4 y GL3, respectivamente). Este material podría ser muy útil para realizar estudios de expresión y poder estudiar el efecto individual de cada uno de los QTL. El gen *TEMPRANILLO* situado en el intervalo de QTL_{DF1} puede ser un serio candidato para fecha de floración en este QTL.

Este trabajo de tesis ha proporcionado nuevos marcadores moleculares para hábito de crecimiento, simple/doble vaina y fecha de floración que pueden ser muy útiles en selección asistida por marcadores y se han propuesto posibles genes candidatos que controlan estos caracteres.

INTRODUCCIÓN GENERAL

INTRODUCCIÓN GENERAL

1. Las leguminosas de grano

La familia de las Leguminosas, con 750 géneros, se considera dentro de las Angiospermas una de las tres mayores familias. Se divide en tres grandes subfamilias: Caesalpinioideae, Mimosoideae y Papilionoideae, siendo en esta última, con más de 10.000 especies, entre las que se encuentran la mayoría de las leguminosas. Una de las características más interesantes de las leguminosas es la fijación del nitrógeno atmosférico en el suelo a través de complejas interacciones con bacterias del género “*Rhizobium*”. Esta característica junto con su elevado contenido en proteína, además de ser una fuente importante de macro y micro-elementos que originan grandes beneficios para la salud (Smýkal et al. 2015), hacen de ellas una familia de gran interés. Debido a la importancia agrícola y ecológica de estos cultivos se pueden considerar los segundos en importancia después de los cereales.

Entre las leguminosas se distinguen las forrajeras y las leguminosas grano. Estas últimas se clasifican según sus requerimientos agroecológicos en: “leguminosas de climas fríos” (cool season) y “leguminosas de climas templados” (warm season). Entre las de climas templados se incluyen especies que pertenecen a dos tribus diferentes: *Viceae* donde se incluyen las habas, guisantes, lentejas y almortas y la *Cicerae* donde se incluye el garbanzo, con 42 especies (9 anuales y 33 perennes) siendo *Cicer arietinum* L. la única especie cultivada de este género y en la que se ha enfocado este trabajo de tesis.

2. Características generales del garbanzo

El garbanzo (*C. arietinum* L.) es una planta anual, autógama y diploide ($2n=2x=16$), con un genoma relativamente pequeño de 740Mbp (Arumuganathan y Earle 1991). Es una de las ocho especies con las que el hombre comenzó el proceso de domesticación sobre el 11.000 a.c. en el

Próximo Oriente. Su centro de origen se sitúa en el sureste de Turquía (Ladizinsky y Adler, 1976) donde se descubrieron dos especies silvestres estrechamente relacionadas con el garbanzo cultivado, *Cicer echinospermum* P.H. Davis y *Cicer reticulatum* Ladiz (van der Maesen 1987). Se considera que el ancestro silvestre del garbanzo cultivado es *C. reticulatum* ya que los cruzamientos entre *C. arietinum* x *C. reticulatum* producen híbridos totalmente fértiles.

Los mejoradores agrupan el garbanzo cultivado en dos grandes grupos: tipo "kabuli" (con flores blancas, semillas grandes y de color crema y cubierta de la semilla fina) y tipo "desi" (de flores púrpura, semillas pequeñas, de color oscuro y angulosas con cubierta de semilla gruesa) (Figura 1). Esta clasificación coincide con la propuesta por (Moreno y Cubero 1978) en razas *macrosperma* y *microsperma* sugerida al analizar diferentes caracteres cualitativos y cuantitativos. Análisis con marcadores moleculares mostró que los genotipos kabuli proceden de los desi (Iruela et al. 2007). Los garbanzos tipo kabuli (similar a *macrosperma*) se cultivan tradicionalmente en la Cuenca Mediterránea, Cercano Oriente, Asia Central y América. Los tipo desi (similar a *microsperma*) se cultivan principalmente en la India, Pakistán, Este de África y más recientemente en Australia. Estos dos tipos también varían para caracteres agronómicos, siendo más frecuentes el hábito de crecimiento erecto, la resistencia a rabia y la tolerancia al frío en los tipo kabuli, mientras que la floración temprana, resistencia a fusarium, tolerancia a elevadas temperaturas y sequía es más frecuente en los tipo desi (Singh 1987). La diferencia en el fondo genético de estos dos grupos de garbanzo cultivado se ha podido diferenciar mediante el análisis de marcadores moleculares (Iruela et al. 2002).



Figura 1. Flores y semillas tipo desi (izquierda) y kabuli (derecha)

Incluir el garbanzo en la dieta diaria es fuente importante de proteínas y carbohidratos además de grasas, vitaminas, fibra y minerales. El contenido proteico del garbanzo es menor que el de otras leguminosas (20-30%), pero es de los más digestibles, el de carbohidratos es bastante elevado (40%) y constituido en gran parte por almidón, el contenido en grasa es de un 3-6% y rico en ácidos grasos insaturados (ácido linoleico y oleico) y la baja presencia de factores antinutritivos (Gil et al. 1999), hacen que este cultivo sea de gran interés desde el punto de vista del consumo humano y de la alimentación animal. Además tiene un efecto positivo en la prevención de algunas enfermedades crónicas como las cardiovasculares (CVD), diabetes tipo 2, algunos tipos de cáncer y problemas digestivos (Jukanti et al. 2012).

El garbanzo, al igual que otras leguminosas, contribuye a mejorar la estructura y fertilidad del suelo debido a su simbiosis con la bacteria *Rhizobium*. Esto explica la importancia del uso de las leguminosas en la rotación de cultivos como es el sistema trigo-garbanzo donde el nivel de nitrógeno se eleva al 38% después de la siembra de garbanzo suponiendo un beneficio para el cultivo siguiente (Aslam et al. 2003).

2.1 Importancia económica del garbanzo

El garbanzo es la segunda leguminosa grano en el mundo, después de las judías, en cuanto a producción (aproximadamente $13 \cdot 10^6$ ton) y área cultivada ($13 \cdot 10^6$ ha), las cuales han sufrido oscilaciones a lo largo del tiempo (Faostat 2014) (Figura 2). $9 \cdot 10^6$ ton (65%). En Europa, España es el

principal productor de garbanzo con 22.000 ton y 35.000 ha, siendo Andalucía la región que mas superficie le dedica a este cultivo (20.240 ha) (MAPA 2014)

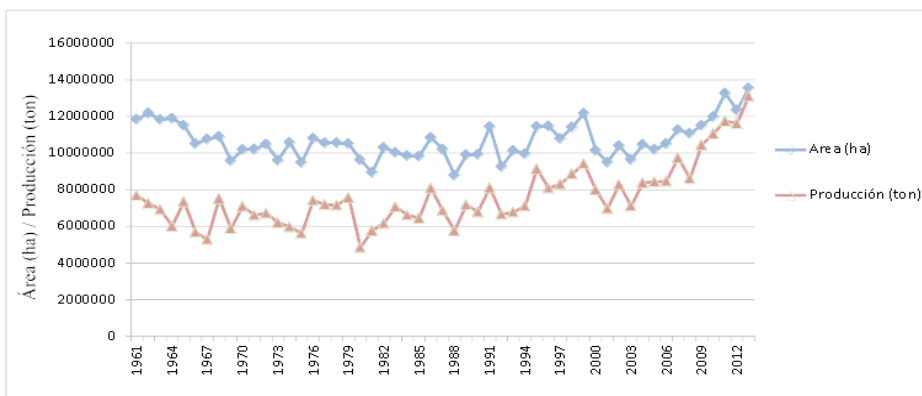


Figura 2. Serie histórica de la superficie cultivada (ha) y la producción (ton) del cultivo de garbanzo en el mundo. Fuente: Faostat 2014

Los principales países exportadores son Australia, Canadá, Irán, México y Turquía, constituyendo un 78% de la producción total exportada. Entre los principales países importadores destacan la India y Pakistán seguida de Europa, la cual importa fundamentalmente desde México. Actualmente España importa casi el doble de lo que produce, concretamente en el año 2010 importó 53.800 ton, siendo el quinto país que más garbanzos importa (FAO 2010).

Aunque el rendimiento medio mundial del garbanzo ha mejorado en los últimos años, desde 504 kg/ha en 1980 a 967 kg/ha en 2013, aún sigue siendo bajo. Este aumento se debe en parte al desarrollo de nuevas variedades resistentes a estreses bióticos y abióticos y a que en muchas regiones el ciclo de cultivo es muy corto no llegando a desarrollar mucha biomasa.

2.2 Mejora del garbanzo

Hay muchos retos que se están abordando en los programas de Mejora de este cultivo debido a los estreses bióticos y abióticos que le afectan. Los estreses bióticos más importantes son los causados por el hongo del suelo *Fusarium oxysporum* Schlechtend: Fr. f. sp. *ciceris*, y el aéreo *Ascochyta rabiei* (Pass.) Lab., que producen la fusariosis y la rabia respectivamente. Ambas enfermedades pueden disminuir dramáticamente las producciones llegando incluso a eliminar totalmente el cultivo (poner el libro de CHen? de referencia). Entre los estreses abióticos la salinidad y la sequía son los más importantes a considerar en garbanzo, junto con las heladas (<-1.5°C) y el “chilling” (entre -1.5°C y 15°C). Así, el obtener genotipos resistentes o tolerantes a enfermedades y a condiciones ambientales extremas y con buenas producciones es uno de los objetivos clave en la mejora del garbanzo.

La producción es un carácter complejo ya que es el resultado del desarrollo completo de la planta y en el cual están implicados varios caracteres como semillas por vaina, vainas por planta, número de vainas por nudo, etc y caracteres adaptativos como tolerancias, resistencias, hábito de crecimiento, fecha de floración, etc.. Debido a su importancia el número de vainas por nudo, hábito de crecimiento y fecha de floración han sido objeto de estudio en este trabajo de tesis.

Aunque gran parte de la Mejora de este cultivo se ha realizado empleando metodologías tradicionales para la Mejora de plantas autógamias, hoy en día, gracias al desarrollo de la genómica se están incluyendo nuevas herramientas que permiten ayudar a aumentar la eficacia en los programas de la Mejora. En garbanzo el avance de las nuevas técnicas biotecnológicas con el desarrollo de mapas genéticos en esta especie y la reciente secuenciación de su genoma está permitiendo conocer más sobre la estructura y función de regiones genómicas implicadas en la regulación de

caracteres de interés agronómico. En este trabajo se han aplicado estas técnicas para conocer más acerca de algunas de estas regiones.

3. Caracteres analizados en este estudio en garbanzo

3.1. Número de vainas por nudo

La mayoría de los genotipos de garbanzo cultivado tienen solamente una flor por nudo y como consecuencia una vaina. Sin embargo, existen mutaciones naturales que producen dos, tres o múltiples flores en cada nudo (Figura 3) (Knights 1987; Singh y Chaturvedi 1998, Gaur y Gour 2002;). La presencia de dos vaina por nudo (doble vaina) es una mutación espontánea y es más frecuente encontrarla en garbanzos tipo desi que en los tipo kabuli. Este carácter (doble vaina por nudo) tiene un gran interés desde el punto de vista agronómico. Se ha descrito que puede aumentar la producción (Singh y van Rheenen 1994; Kumar et al. 2000; Ali et al. 2010). Rubio et al. (1998, 2004) observaron que influye positivamente en la estabilidad de la producción.



Figura 3 Vaina simple (izquierda), doble (centro) y triple (derecha) vaina por nudo.

Srinivasan et al. (2006) demostraron que existen dos genes independientes que controlan el número de flores por nudo en garbanzo (*Sfl* y *Cym*). El gen *Sfl* controla el carácter simple, doble o triple flores (o vainas), siendo el alelo de vaina simple dominante sobre doble o triple vaina, y el de doble vaina dominante sobre el triple. Varios autores han localizado el locus *sfl* en el grupo de

ligamiento (GL) 6 del mapa genético del garbanzo, próximo al marcador microsatélite STMS (sequence tagged microsatellite sites) TA80 o flanqueado por los marcadores TR44 y TR1/TR35 o NCPGR33 y NCPGR47 con intervalos de 19,3 y 3,4 cM, respectivamente (Cho et al. 2002, Rajesh et al. 2002, Radhika et al. 2007,). Estos marcadores pueden ser útiles para utilizarlos en Mejora Asistida por Marcadores (MAS), de hecho, el marcador TA80 se ha utilizado con éxito en un programa de retrocruzamientos asistido por marcadores (MAB, marker assisted backcrossing) para integrar el carácter doble vaina en cultivares de garbanzo (Taran et al. 2013). No obstante, sería necesario desarrollar nuevos marcadores que estén más estrechamente ligados e incluso buscar genes candidatos donde se podría diseñar marcadores diagnósticos para el carácter simple/doble vaina. Por otro lado, teniendo en cuenta que muchos de los marcadores son microsatélites, que por su naturaleza presentan gran diversidad de alelos difícil de asignar al carácter en cuestión y siendo aplicables solo en cruzamientos donde los padres sean polimórficos para el marcador, sería interesante disponer de un alto número de ellos al objeto de poder emplear el más idóneo en cada caso.

3.2. Hábito de crecimiento

El porte o hábito de crecimiento es un carácter de gran importancia en la adaptación del cultivo. El garbanzo cultivado suele presentar porte erecto o semi-erecto y los silvestres suelen ser rastreros (Figura 4). Los tipos erectos son generalmente más altos, con tallos más compactos y mejor adaptados a la mecanización que los tipos semierectos y rastreros. Rubio et al. (2004) observaron que bajo condiciones mediterráneas, el porte semi-erecto influye positivamente en la estabilidad de la producción y el rendimiento. Sin embargo, los estudios sobre el control genético de este carácter se limitaron a emplear poblaciones segregantes derivadas de

cruzamientos interespecíficos [*C. reticulatum* (rastrero) x *C. arietinum* (erecto o semi-erecto)] concretándose que está controlado por un gen (*Hg/hg*) situado en el GL3 del mapa de garbanzo, siendo erecto dominante y rastrero recesivo (Aryamanesh et al. 2010). Para nuestro conocimiento, todavía no se han hecho estudios sobre la relación entre erecto vs semi-erecto, lo que sería interesante para poder comprender cuantos genes controlan este carácter en garbanzo.



Figura 4. Genotipos con hábito de crecimiento erecto y semierecto en garbanzo

3.3. Fecha de floración

Es un carácter esencial en la adaptación del cultivo y complejo ya que se ve afectado por la longitud del día, la temperatura y la edad de la planta, entre otros, suponiendo que diferentes regiones del genoma pueden estar implicadas en el control de éste carácter.

El uso de materiales precoces (asociados con maduración precoz) son de gran interés en ambientes semiáridos, ya que pueden escapar de las sequías terminales y altas temperaturas al final del ciclo, aumentando la producción (Gaur et al. 2007). En ambientes mediterráneos, floración

precoz está positivamente asociada con producción (Siddique et al. 2003, Rubio et al. 2004).

El garbanzo muestra una respuesta diferencial al fotoperiodo y a la temperatura. En la Cuenca Mediterránea la insensibilidad al fotoperiodo podría ser un carácter interesante para evitar los estreses de humedad (Cobos et al. 2009). El control genético de la fecha de floración es bastante discutido, ya que difiere de los genotipos usados como parentales y del ambiente. Gumber y Sarvjeet (1996) en ambientes de ciclo largo y Ambessa et al. (2006) en ambientes de ciclo corto, concluyeron que el control era digénico, siendo precocidad recesivo. Or et al. (1999) y Kumar y van Rheenen (2000) utilizando genotipos precoces, observaron que dicho carácter estaba controlado por un gen mayor. Se han identificado QTLs (quantitative traits loci) asociados a este carácter indicándonos que puede haber varios genes que controlan la fecha de floración. Estos se han localizado en los GL4 (QTL_{DF1}) y GL3 (QTL_{DF2}) del mapa genético del garbanzo. El marcador más estrechamente ligado al QTL_{LG3} fue el microsatélite TA142 mientras que el GAA47 se encuentra en el “*pico*” del QTL_{LG4} (Cobos et al. 2007, 2009).

La evaluación fenotípica de éste carácter no es fácil ya que está muy influenciado por las condiciones ambientales (fecha de siembra, latitud, altitud), por lo tanto, es necesario buscar genes candidatos que ayuden a intentar entender los mecanismos que provocan cambios en la fecha de floración y poder usarlos para diseñar materiales de interés.

3.4 Nodulación

Como ya se ha comentado anteriormente la simbiosis entre las leguminosas y la bacteria *Rhizobium* mejora el contenido orgánico del suelo y su fertilidad. La bacteria se sitúa en los nódulos que forma en la raíz de la planta y fija el nitrógeno atmosférico en el suelo (Figura 5). Para estudiar el proceso de nodulación en garbanzo se han obtenido mutantes mediante mutagénesis artificial por rayos x. Seis de estos mutantes (PM233, PM665,

PM679, PM405, PM796 y ICC435M) llevan genes recesivos independientes (*rn1* hasta *rn6*) (; Davis et al. 1985, 1986, Davis 1988, Singh et al. 1992). Por el contrario, el mutante PM638 lleva un gen dominante *RN7* ligado al gen *rn1* (Paruvangada y Davis 1999). Estas mutaciones originan diferentes deficiencias en cuanto a la formación de nódulos siendo la única mutación estable la del mutante de no-nodulación en la línea PM233. Así pues, tratar de identificar y caracterizar a nivel molecular estos mutantes abre nuevas puertas para comprender la ruta responsable del proceso de nodulación en garbanzo. Este conocimiento nos puede ayudar en un futuro a diseñar plantas más eficientes en la fijación de nitrógeno empleando técnicas de ingeniería genética.

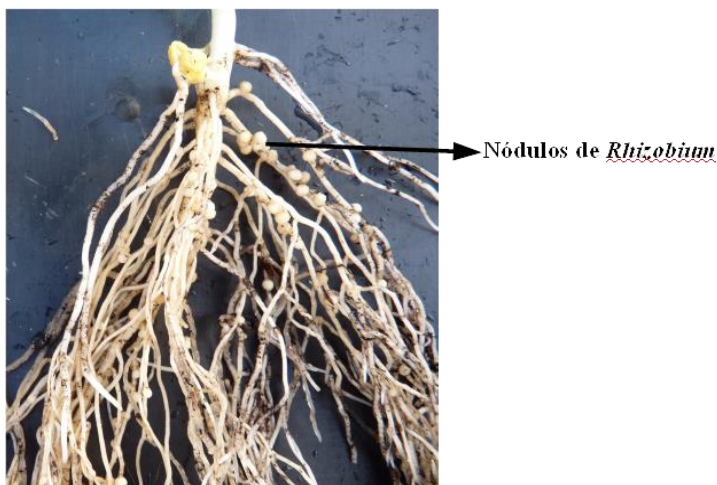


Figura 5. Nódulos de *Rizobium* en la línea JG62 de garbanzo

4. Mejora Asistida por Marcadores (MAS)

La Mejora Clásica ha sido muy eficaz a lo largo de la historia, sin embargo presenta algunas dificultades, fundamentalmente en caracteres cuantitativos, al efecto del ambiente, de la dominancia y a las interacciones epistáticas, incluso a veces a la dificultad de las técnicas de evaluación, además la duración de un programa de mejora es larga (10-15 años). El avance de la genómica en estos últimos años ha permitido disponer de

marcadores moleculares y desarrollar mapas genéticos saturados que han hecho posible plantearse su utilización para seleccionar nuevos materiales de interés, lo que denominamos selección asistida por marcadores (MAS). Entre las ventajas que presenta es que es más rápida, ya que la selección de genotipos se puede hacer en estado de plántula, no está afectada por las condiciones ambientales, y facilita la selección de varios caracteres de forma simultánea. La mejora asistida por marcadores se ha aplicado fundamentalmente en programas de retrocruzamiento (MAB, marker assisted backcrossing) y piramidación de genes de resistencia a enfermedades. Para que sea posible la selección del genotipo deseado con un bajo error es mejor emplear marcadores estrechamente ligados y que flaqueen el gen o el QTL que controle el carácter deseado. Se recomienda que exista una distancia genética menor de 5 cM (o 5% de recombinación) (Collard y Mackill 2008). La mejor manera de evitar la recombinación entre el marcador y el gen o QTL de interés sería el desarrollo de marcadores diagnóstico, es decir, marcadores diseñados en el gen responsable del cambio fenotípico. En garbanzo, se han desarrollado marcadores diagnóstico para resistencia a fusarium que se han empleado en MAS (Ahmad et al. 2014, Madrid et al. 2014, Varshney et al. 2014).

4.1. Mapas genéticos

El desarrollo de mapas genéticos ha sido el método tradicional para detectar marcadores ligados a caracteres cualitativos y cuantitativos de importancia agronómica y poder utilizarlos en los programas de mejora para seleccionar genotipos de interés. El garbanzo, es una planta autógama y con un origen monofilético lo que explica la escasa variabilidad genética que se encuentra entre los genotipos de garbanzo cultivado. Por lo tanto, los primeros mapas genéticos desarrollados en garbanzo se obtuvieron empleando cruzamientos interespecíficos entre la especie cultivada (*C. arietinum*) y la silvestre (*C. reticulatum*) para conseguir un mayor nivel de polimorfismo en los marcadores analizados (Cobos et al. 2009). Gracias al

desarrollo de los marcadores microsátélites (STMS), que amplifican secuencias repetitivas, han permitido detectar mayor polimorfismo incluso entre las líneas cultivadas. Esto ha permitido genotipar poblaciones derivadas de cruzamientos intraespecíficos y se han podido identificar marcadores asociados a resistencias a estreses y a caracteres de interés, como los marcadores STMS TA80 y TA59 asociado a doble vaina y a la resistencia a *F. oxysporum*, respectivamente (Rajesh et al. 2002, Sharma y Muehlbauer 2007). Más recientemente, se están desarrollando plataformas para el genotipado de marcadores SNPs (single nucleotide polymorphisms) que se utilizan para obtener una gran cantidad de marcadores en determinadas regiones del genoma y conseguir mapas con una elevada densidad (Deokar et al. 2014). Además, los marcadores SNPs junto con otro tipo de marcadores como los STMS o los DArTs (diversity arrays technology) están siendo muy útiles para buscar genes candidatos que controlen caracteres como tolerancia a sequía o elevadas temperaturas en garbanzo (Thudi et al. 2014).

4.2. Materiales empleados en el desarrollo de mapas genéticos

Para localizar marcadores asociados a caracteres agronómicos en los mapas genéticos es necesario el desarrollo de **poblaciones de mapeo** que segregan para el carácter/es deseado. Unas de estas poblaciones de gran interés son las poblaciones de líneas recombinantes (RIPs) que están compuestas por líneas homocigóticas obtenidas por autofecundaciones sucesivas por medio del método de descendencia de semilla única (SSD) (Johnson y Bernard 1962). Estas poblaciones tienen la ventaja de que el material está fijado y se pueden evaluar en ambientes diferentes. Las RIPs se han utilizado con mucha frecuencia para la localización de QTLs de importancia agronómica en garbanzo como es la fecha de floración, doble vaina, etc (Cho et al. 2002, Cobos et al. 2007).

Otro material empleado para localizar marcadores son las líneas casi isogénicas (NILs, near isogenic lines), tienen el mismo fondo genético y

solamente difieren en la región genómica relacionada con la característica para la que se han desarrollado, por lo tanto son muy interesantes para buscar genes candidatos mediante mapeo fino y estudios de expresión. Se han empleado parejas de NILs para estudiar el contenido en aceite en soja (Zhu et al. 2012) o la arquitectura de la planta en arroz (Lim et al. 2014). En garbanzo se han desarrollado parejas de NILs para el carácter doble vaina (Rajesh et al 2002) y resistencia a fusarium (Castro et al. 2010). En este trabajo se describe el desarrollo de este tipo de material para otros nuevos caracteres.

4.3. Secuenciación del genoma del garbanzo

Las herramientas biotecnológicas han tenido un desarrollo espectacular en los últimos años ofreciendo una gran cantidad de información disponible. Una de estas herramientas es la secuenciación de genomas y transcriptomas de organismos. En plantas, el genoma de *Arabidopsis thaliana* fue el primero que se secuenció (Initiative 2000) y posteriormente el genoma de una de las especie modelo de leguminosas *Medicago truncatula* (Young et al. 2011). Las secuencias de los genes de estas especies se han empleado como referencia para facilitar la búsqueda de genes de importancia agronómica en garbanzo como los implicados en la resistencia a estreses bióticos (Madrid et al. 2012). Recientemente se ha publicado la secuenciación del transcriptoma y del primer genoma de referencia de garbanzo (Garg et al. 2011, Varshney et al. 2013) que se considera una herramienta muy útil para conseguir información que facilite la comprensión de los mecanismos moleculares relacionados con los caracteres agronómicos más importantes. Los datos de secuenciación y re-secuenciación de diferentes genotipos de garbanzo abren un enorme abanico de posibilidades para detectar nuevos marcadores microsatélites y SNP en regiones concretas del genoma permitiendo tener mapas genético saturados e incluso llegar a genes candidatos relacionados con caracteres de interés

como la tolerancia a estreses abióticos (Deokar et al. 2014, Thudi et al. 2014).

Los mapas genéticos han sido esenciales para detectar marcadores moleculares asociados a caracteres fenotípicos pero las distancias se basan en la cantidad de individuos recombinantes y depende en gran medida del tipo y tamaño de población segregante. La secuenciación de genomas proporciona la posición física de los marcadores situados en los mapas genéticos y por tanto su localización real en el cromosoma. Esta información hace posible centrarse en la región correcta del genoma y extraer nuevos marcadores (STMS, SNPs) para saturar la zona de interés y como consecuencia localizar genes candidatos responsables del carácter estudiado.

OBJETIVOS

El presente trabajo se enmarca dentro de la línea de investigación sobre la Mejora genética del garbanzo que se lleva a cabo en los grupos PAIDI (Plan Andaluz de Investigación, Desarrollo e Innovación) AGR114 y AGR237 de la Junta de Andalucía. Estos grupos están compuestos por miembros del Dpto. de Genética de la Universidad de Córdoba y del Área de Mejora y Biotecnología del IFAPA (Instituto de Formación Agraria y Pesquera). En concreto, el **objetivo general** ha sido la obtención de mapas genéticos de garbanzo de alta densidad en regiones donde se localizan genes de interés y en la medida de lo posible identificar y caracterizar estos genes relacionados con caracteres de interés.

Los **objetivos específicos** han sido:

1. Desarrollar parejas de líneas casi-isogénicas (NILs) para nodulación/no-nodulación, hábito de crecimiento y fecha de floración en garbanzo para utilizarlas en la búsqueda de marcadores y genes candidatos ligados a estos caracteres.
2. Identificar la secuencia del gen *Rn1/rn1* implicado en la nodulación en garbanzo empleando la información disponible en la especie modelo *Medicago truncatula*.
3. Saturar la región del genoma donde se localiza el gen que controla el carácter simple/doble vaina (*Sfl/sfl*) y proponer genes candidatos que sean responsables de este carácter.
4. Analizar genéticamente el carácter porte erecto/semi-erecto.
5. Buscar genes candidatos para fecha de floración situados en la región del QTL_{DFI} en el grupo de ligamiento 4 (LG4) del mapa genético de garbanzo.

CAPÍTULO I

Mapeo e identificación de un gen *NSP2* de *Cicer arietinum* implicado en la ruta de nodulación

Publicado como:

Ali L, Madrid E, Varshney RK, Azam S, Millan T, Rubio J, Gil J (2014) Mapping and identification of a *Cicer arietinum* *NSP2* gene involved in nodulation pathway. Theoretical and Applied Genetics 127 (2) 481-488.

Resumen

En este trabajo se han desarrollado dos pares de líneas casi isogénicas (NILs) para el carácter nodulación en garbanzo a partir de un cruzamiento intraespecífico entre el mutante PM233 (no-nodula y lleva el gen recesivo *rn1*) y el genotipo CA2139 (nodula). Estas NILs fueron genotipadas con marcadores microsatélites (STMS) distribuidos en los diferentes grupos de ligamiento (GL) del mapa genético de garbanzo, observándose la presencia de polimorfismo en los marcadores del GL5. Esta información junto con la proporcionada por el genoma de la especie modelo *Medicago truncatula*, nos permitió seleccionar el gen candidato (*NSP2*) implicado en las rutas de la nodulación y posteriormente mapearlo en garbanzo. La longitud de la secuencia obtenida en el genotipo silvestre (*CaNSP2*) fue de 1.503 bp. El análisis de ligamiento realizado en una población F₃ de 118 individuos derivada de un cruzamiento entre el par de NILs NIL-7-2A (nodula) x NIL7-2B (no-nodula) reveló la co-localización entre *CaNSP2* y el gen *Rn1*. Así pues el gen *CaNSP2* podría ser un gen candidato para identificar genotipos *Rn1*, además se sugiere que podría actuar en la ruta de transducción de las señales de nodulación en garbanzo, similares a las que ocurren en otras leguminosas.

Abstract

An intraspecific cross between the mutant non-nodulating genotype PM233, carrying the recessive gene *nl1*, 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 F₃ population of 118 plants derived from the cross between the pair of NILS NIL7-2A (nod) x NIL7-2B (non-nod) revealed a co-localization between *CaNSP2* and *nl1* gene. These data suggest that the *CaNSP2* gene could act in the nodulation signaling transduction pathway similarly to that in other legumes species.

Key words: chickpea, linkage analysis, molecular markers, non-nodulating

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* to *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: i) stable non-nodulating (PM233), ii) temperature-dependent non-nodulating (PM665 and PM679) or iii) 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 *ml1* on the chickpea genetic map. In addition, the gene sequence was characterized by synteny analysis using the available information in *M. truncatula*.

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 (Figure1). Seeds of non-nod mutant PM233 were supplied by the International Center for Agricultural Research in the Dry Areas (ICARDA) genebank.

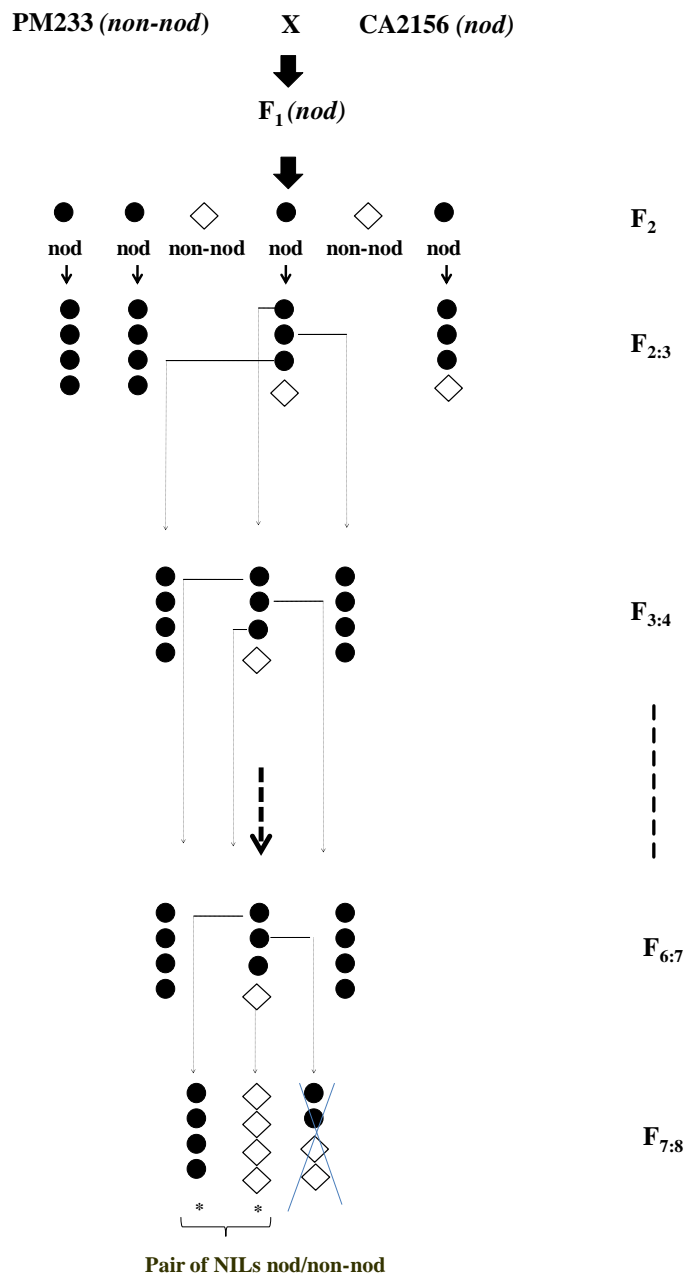


Figure 1. Scheme for pair of NILs (*nod*/*non-nod*) development following residual heterozygosity selection by Pedigree method.

In order to develop pairs of NILs, 28 F₂ 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 x 10⁸ rhizobio/g. The seeds were treated with 1 g of the suspension per 100 chickpea seeds and 10% sucrose and then sown in individual pots under controlled conditions (glasshouse). Each F_{2.3} progeny from an F₂ 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 F₇ 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 were harvested separately from two different F_{7.8} families and were designated as NIL6-1A (nod)/NIL6-1B (non-nod) and NIL7-2A (nod)/NIL7-2B (non-nod).

An F₃ population (n=118), derived by single-seed descent from a cross between the pair of NILs, NIL7-2A (nod) x NIL7-2B (non-nod), was used to establish the nod/non-nod inheritance ratio and perform linkage analysis. This population was sown in a greenhouse and inoculated with *Mesorhizobium* as described above. Plants were phenotyped for the presence or absence of nodules, 40 days after sowing.

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 1x TBE buffer, or 10% non-denaturing

polyacrylamide gels, and stained with ethidium bromide. STMS that required higher resolution were 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*, TA11*, TR29, TR59*
LG6	GA21, STMS2, TA14, TA21, TA80, TA106, TR1
LG7	STMS6, TA78, TA117
LG8	GAA46, TS12, TS45

*Polymorphic markers.

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, 1x buffer (50 mM KCl, 10 mM Tris-HCl, and 0.1% Triton X-100), 2.5 mM MgCl₂, 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 of 94°C for 30s, 50°C for 30s, 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 ITS1 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:

In order 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* were mapped to the chickpea

genome using the BLASTN (NCBI) program, which resulted in two putative genes, annotated as *CaNSP2* and *CaLYK9*.

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 1985). 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 x 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 Lysinemotif (LysM)-receptor-like Kinases *LYK9* (MtD04912) (Oldroyd and Long 2003; Arrighi et al 2006). Using the information obtained about *Rhizobium* infection process in PM233 (Mathews 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 in order to amplify the gene sequence (Fig. 2). Only one combination (NSP2-L2 and NSP2-R1) 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 (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*.

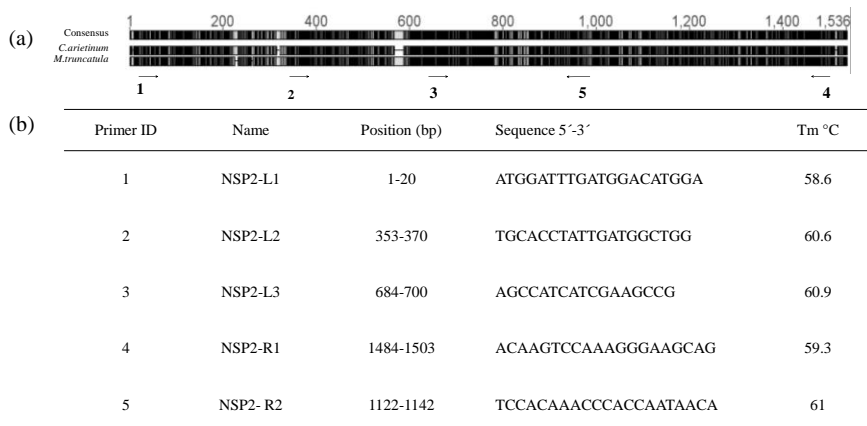


Figure 2. (a) Diagram of sequence alignment between *Cicer arietinum* and *Medicago truncatula* of *NSP₂* gene. The unique exon in the gene is show. The location of primers designed in *M. truncatula* are indicated by arrows. (b) Sequence of primers designed in *M. truncatula* *NSP₂* gene to be amplified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

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 to 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 being (Brassicaceae) distant from the legumes group (Fig. 3).

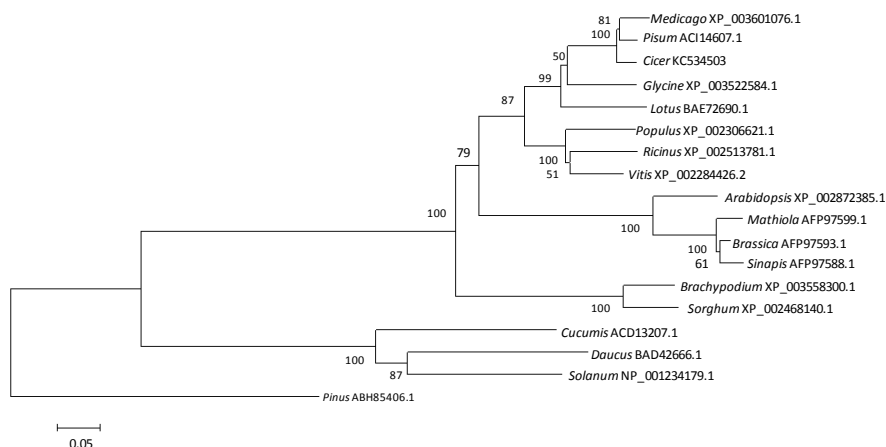


Figure 3. Phylogenetic tree of 18 amino acid sequences of GRAS family protein in different plant species. Numbers at branch nodes represent bootstrap values out of 1,000 replicates. *Pinus sylvestris* was considered as the out group. Accession numbers appear at the end of genus names.

NSP2 and Rn1/rn1 linkage analysis

Linkage analysis was performed using the F₃ population (n=118) obtained by single-seed descent method. All F₃ individuals were genotyped with 3 STMS markers as well 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 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 was used as positive control. In contrast, non-nodulating individuals had only one amplified fragment that corresponds to the ITS control (Fig. 5).

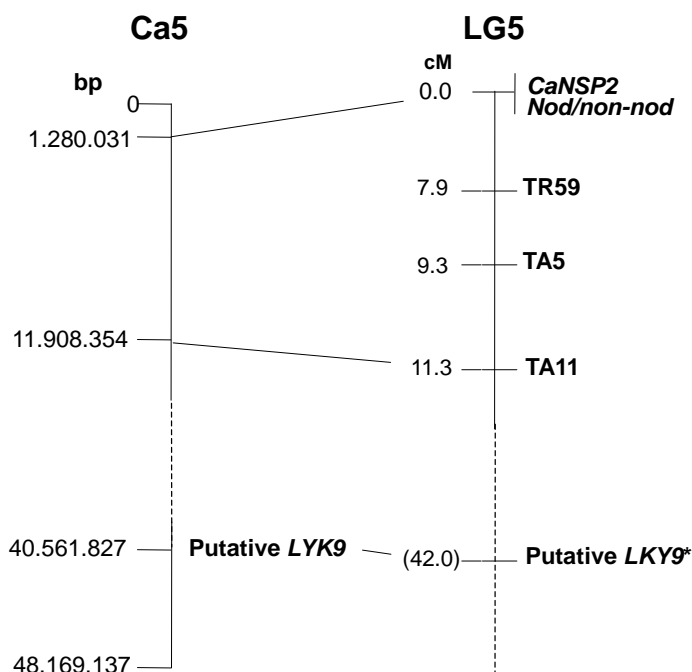


Figure 4. Comparison between genetic and physical map of chickpea LG5. On right, genetic LG5 including the phenotypic evaluation of nod vs non-nod in the F_3 population derived from the cross between two near isogenic lines (NIL7-2A X NIL7-2B). On left, the physical map including *CaNSP2*, *TA11* and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

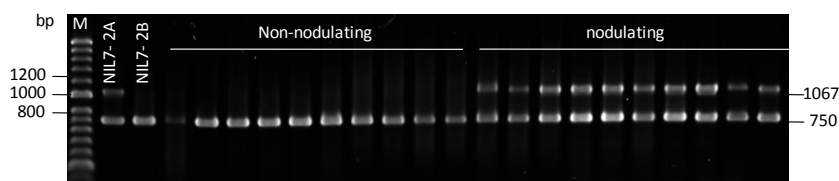


Figure 5. Agarose gel showing the segregation of the polymorphic marker developed to amplify the locus *CaNSP2* in chickpea using 10 F₃ nodulating (1,067 bp) and 10 non-nod (non-amplification) individuals. In order to confirm absence of bands the universal primers ITS1 and ITS4 were used as positive controls (750 bp). Lane M: molecular weight marker Hyperladder II with molecular weight in bp.

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. arietium* pseudomolecule 5 (Ca5). *M. truncatula* *LYK9* had the highest homology (92%) with the annotated gene Ca_07575 located from 40,561,827 bp 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 were 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* has shown that *NSP2* is involved in Nod factor signalling (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 *Mezorrhizobium* 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 (Mathews 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 decoding 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. sativum* 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 F₃ population from study enabled us to map *CaNSP2* and *rn1* to LG5 of the chickpea genetic map. 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).

Acknowledgement

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CAPÍTULO II

Detección de un nuevo QTL/gen para hábito de crecimiento en el CaLG1 de garbanzo utilizando cruzamientos intra e inter específicos

Publicado como:

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Resumen

Durante los años 2003 y 2004 se evaluó para porte la población de líneas recombinantes de garbanzo (RIP-9) derivada de un cruzamiento interespecífico (ILC72 x Cr5-10). Esta población se usó para desarrollar un par de líneas casi isogénicas (NILs) que difieren para porte (erecto vs postrado). Se caracterizaron molecularmente usando 52 STMS (Sequence Tagged Microsatellite Site) distribuidos en diferentes grupos de ligamiento (GL) del mapa genético de garbanzo, presentando polimorfismo los marcadores situados en GL1 y 3. Se consiguió detectar asociación entre los marcadores y el porte mediante análisis de regresión simple. El marcador RAPD (Random Amplified Polymorphic DNA) OPAD09₁₀₅₃ situado en GL1 fue el que explicó el mayor porcentaje de la variación fenotípica total del carácter (15,4%). Este marcador se utilizó para desarrollar el marcador SCAR (Sequence Characterized Amplified Region) SCAD09₁₀₅₃ y además, aprovechando la información del mapa físico, se incluyeron en el mapa genético nuevos marcadores cercanos a este SCAR. El análisis de QTLs (Quantitative Traits Loci) reveló un nuevo QTL (QTL_{Hg2}) en CaGL1. El marcador Indel (delección/inserción) Indel 3 y el gen Ca_07000 (14,5 Mb de Ca1) y (15,3 Mb de Ca1) presentaron los LOD más altos explicando el 24,6 y el 23,4% de la variación fenotípica en los años 2003 y 2004 respectivamente. Para confirmar estos resultados se empleó la población RIP-5 derivada del cruzamiento intraespecífico (WR315 x ILC3279) y que segrega para porte (erecto vs semi-erecto). Esta población nos permitió localizar el gen (*Hg2/hg2*) en CaGL1 y flanquearlo entre dos marcadores Indel (Indel 1 y Indel 2) en un rango de 12,3 y 16,2 Mb. Por lo tanto, el gen *Hg2/hg2* correspondería a la región donde se encuentra el QTL_{Hg2}. Los genes Ca_07000 y Ca_06999, homólogos a genes codificantes de proteínas dedos de zinc de *Glycine max* y *Pisum sativum* respectivamente, podrían considerarse como posibles genes candidatos.

Abstract

A recombinant inbred line population (RIP-9) derived from an interspecific cross (ILC72 × Cr5-10) was evaluated for growth habit during two 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 (STMS) 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 OPAD09₁₀₅₃ 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 (SCAD09₁₀₅₃). New markers were developed from sequences surrounding SCAD09₁₀₅₃ 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 flanked 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 max* and *Pisum sativum*, respectively. Hence, they could be considered as possible candidate genes.

Key words: 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 et al. 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 underlying 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) accession ILWC36 (PI599072) 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 2m, 10 seeds per meter and 0.7m 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 1x TBE buffer or in 10% non-denaturing 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.

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*
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

*Polymorphic markers are in bold.

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). 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 OPAD09₁₀₅₃ that was mapped on CaLG1 using RIP-9 (Cobos et al. 2006). The selected amplicon was excised from polyacrylamide gel by adding 25 μ L 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'-TCGCTTCTCCCCAAACT) 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* (<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 μ l 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 MgCl₂, 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 30s, 65°C for 30s, 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 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 SCAD09₁₀₅₃ developed in this study. In order to saturate the genetic map of the tagged genomic region, sequences of annotated genes were extracted 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, 1x buffer (50 mM KCl, 10 mM Tris-HCl, and 0.1% Triton X-100), 1.5-2.5 mM MgCl₂, 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 30s, 55-62°C for 30s, 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.

SNP detection was also carried out using the re-sequencing information of parental lines (Varshney et al. 2013). 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. A dCAPS (derived cleaved amplified polymorphic sequence) marker was developed to map one of the five SNPs. Primers for dCAPS were designed using the software dCAPS Finder (Neff et al. 1998) introducing a single 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 1000 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.

Table 2. Physical position and possible function of gene specific, SNP and Indel markers located on Ca1 nearby SCAD09₁₀₅₃ that were included in RIP-9 and RIP-5 genetic map. Polymorphism detection and PCR product size obtained are described.

Marker ID*	Physical position (bp)	Putative function	Amplification**	Polymorphism detection	Restriction enzyme	Alleles (bp)		
						Erect	Semi-erect	Prostrate
Ca_06993 ¹	Ca1: 15444207:15447979	Uncharacterized	NA	-	-	-	-	-
Ca_06994 ¹	Ca1: 15434110:15435930	Predicted probable glucuronoxylan glucuronosyltransferase IRX7-like (LOC101499733)	F1+Rev1	-	-	1370	1370	1370
Ca_06995 ²	Ca1: 15399920:15400621	Uncharacterized	F1+Rev1	Indel	-	280	280	270
Ca_06996 ¹	Ca1: 15386956:15391066	Uncharacterized	NA	-	-	-	-	-
Ca_06997 ¹	Ca1: 15377215:15378652	Predicted transcription factor Fer-like iron deficiency-induced transcription factor-like (LOC101498540)	NA	-	-	-	-	-
Ca_06998 ¹	Ca1: 15368187:15373817	Uncharacterized	F1+Rev3	-	-	1550	1550	1550
Ca_06999 ¹	Ca1: 15337320:15338210	Predicted zinc finger protein ZAT5-like (LOC101497229)	F1+R2, F2+R1	-	-	1200 2200	1200 2200	1200 2200
Ca_07000 ¹	Ca1: 15323486:15324685	Predicted protein Sensitive to proton rhizotoxicity 1-like (LOC101496905)	F1+R1	CAP	TaqI	694+381	694+381	1075
Ca_07001 ¹	Ca1: 15322204:15322614	Predicted histone H2B-like (LOC101496580)	F1+Rev1	-	-	-	-	-
Ca_07002 ²	Ca1: 15314930:15321026	Uncharacterized	F1+Rev1	Indel	-	215	215	200
Ca_07003 ¹	Ca1: 15310213:15311319	Predicted pentatricopeptide repeat-containing protein At2g44880-like (LOC101497993)	F1+Rev1	-	-	1100	1100	1100
Indel1 ³	Ca1: 12340166:	-	F1+Rev1	Indel	-	320	335	320
Indel2 ³	Ca1: 16187503:	Predicted: <i>Cicer arietinum</i> indole-3-acetic acid-amido synthetase GH3.6-like (LOC101492800)	F1+Rev1	Indel	-	415	400	415
Indel3 ³	Ca1: 14537581	Intergenic region	F1+Rev1	Indel	-	370	370	410
CKAM0717 ⁴	Ca1: 15489533:15489633	Intergenic region	F1+Rev1	dCAP	MseI	266	266	239+27
CKAM0912 ⁴	Ca1: 8621759:8613351	Intergenic region	-	-	-	-	-	-
CKAM1744 ⁴	Ca1: 21823113:21823213	Predicted: <i>Cicer arietinum</i> protein transport protein Sec61 subunit beta-like (LOC101491707)	-	-	-	-	-	-
CKAM0767 ⁴	Ca1: 23301914:23302014	Predicted: <i>Cicer arietinum</i> putative receptor-like protein kinase At1g72540-like (LOC101488466)	-	-	-	-	-	-
CKAM1383 ⁴	Ca1: 25185532:25189657	Predicted: <i>Cicer arietinum</i> protein TIFY 3B-like (LOC101515051)	-	-	-	-	-	-

*¹ Clear amplicons of the predicted genes were sequenced directly, and the SNP was detected; ²SNP detected by resequencing (Varshney et. al 2013), ³ SNP detected by resequencing parental lines of RIP-5 (Chen, unpublished data), ⁴ SNP detected by KASPar platform (unpublished data).

**NA: non-amplicon ; F+R, primer combination that gave clear amplicon

Results

Phenotypic evaluation

Three different phenotypes (erect, semi-erect and prostrate) 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).

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*	Growth habit frequency distribution			
		<i>Erect</i>	<i>Semi-erect</i>	<i>Prostrate</i>	<i>Total</i>
RIP-9	2002/2003	10	40	35	85
RIP-9	2003/2004	7	42	36	85
RIP-5	2003/2004	40	51	-	91

*Seasons evaluated in Córdoba (Spain)

NILs 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 growth habit, twenty RILs with extreme phenotypes from each RIP-9 (10 prostrate and 10 erect) and RIP-5 (10 semi-erect and 10 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).

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).

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 OPAD09₁₀₅₃ 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 \times 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 Figures 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 OPAD09₁₀₅₃, which explained the highest percentage of phenotypic variation, was used to develop a SCAR marker (Figure 1). The 1053 bp polymorphic allele (present in prostrate parent and absent in erect parent) was cloned and sequenced. The developed SCAR marker (SCAD09₁₀₅₃) 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 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 (Figure 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 (Figures 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 (Figure 1). In general there was co-linearity of markers between CaLG3 and Ca3 (Figure 2).

To search for candidate genes related to growth habit, the SCAD09₁₀₅₃ 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 (Figure 1). The genes located in 100,000 bp surrounding SCAD09₁₀₅₃ 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 (Figure 1).

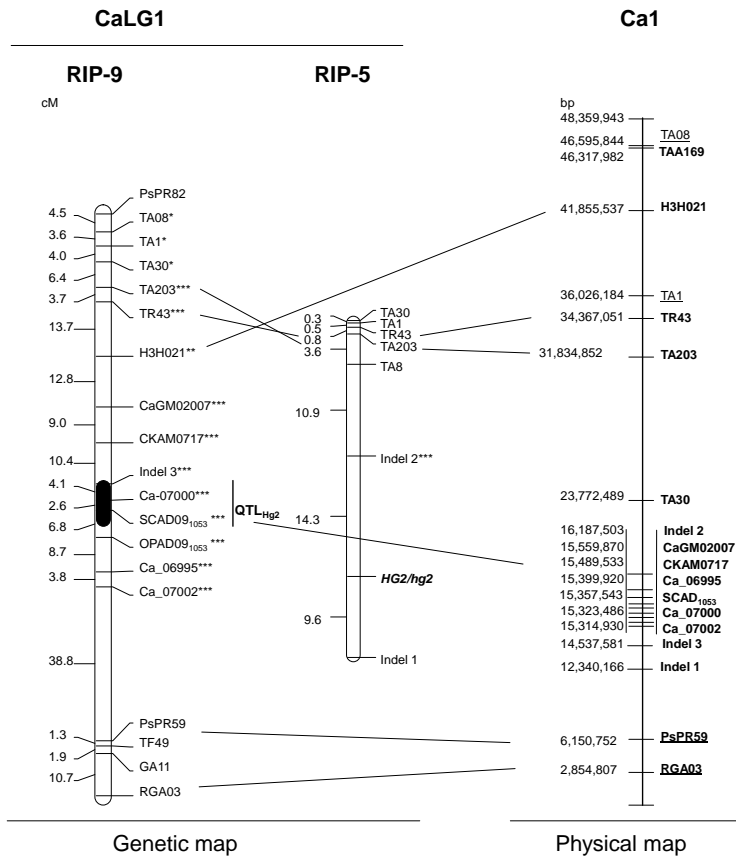


Figure 1. On the left, genetic map of CaLG1 in RIP-9 [*Cicer arietinum* (ILC72) × *C. reticulatum* (Cr5-10)] and RIP-5 [*C. arietinum* (WR315) × *C. arietinum* (ILC3279)]. The quantitative trait loci detected (QTL_{Hg2}) and the mapped gene *HG2/hg2* in LG1-RIP-9 and RIP-5 respectively are in bold. Estimated 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).

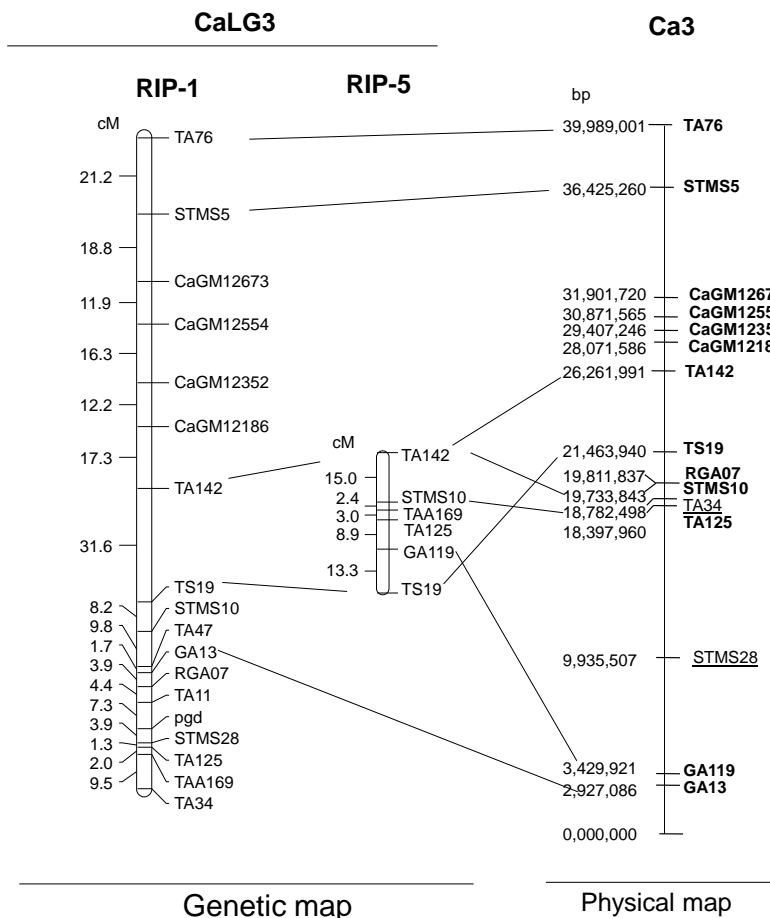


Figure 2. On the left, genetic map of CaLG3 in RIP-9 [*Cicer arietinum* (ILC72) × *C. reticulatum* (Cr5-10)] and RIP-5 [*C. arietinum* (WR315) × *C. arietinum* (ILC3279)]. The quantitative trait loci detected (QTL_{Hg1}) in LG1-RIP-9 is in bold. Estimated genetic distances are given in cM, markers showing 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).

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 (Figure 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 (Figure 2). And one was mapped on CaLG1 because it was in the targeted region (Figure 1).

QTL analysis

Final version of CaLG1 in RIP-9 comprised a total of 19 markers including SCAD09₁₀₅₃, Ca_07000, Ca_07002, Ca_06995, CaGM02007, Indel 3 and CKAM0717. This linkage group covered 146.8 cM of the chickpea genetic map (Figure 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 (Figure 1). Similarly, a significant QTL for branch elongation rate was located on chromosome 2 of *Medicago 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 gene or 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 OPAD09₁₀₅₃, 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 (SCAD09₁₀₅₃) 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). SCAD09₁₀₅₃ 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.

Acknowledgement

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ANEXO I

Primer_ID	Primer Sequence 5'-3'
Ca_06993-Fw1	GGCTGAGAGAATTTGCATATGTG
Ca_06993-Rev1	CATCCCCATAATTCTCGGCA
Ca_06994-Fw1	GGTGTATATACGAGCTACCTTC
Ca_06994-Fw2	GGGTGATGGAATTCTTGAATA
Ca_06994-Rev1	GGTAGGACCTATCAAGCTTCTGACT
Ca_06995-Fw1	TGTA AACGACGGCCAGTG
Ca_06995-Rev1	GGGAGCAACATCGGTGTCTAAAT
Ca_06996-Fw1	GAAGCAAGGATGAAGAAGTATAGGC
Ca_06996-Fw2	CGGTGAAAAACACAGTCTTGA
Ca_06996-Rev2	TACATGAAACCGGCTCCG
Ca_06996-Rev1	TCGAGCTCCAGAAGCTAGCC
Ca_06997-Fw1	GGATAAGCTTTATGCATTGCG
Ca_06997-Rev1	GGATGGCATGAATCAAAGC
Ca_06998-Fw1	GGAGACTCGAAACCCTTCTTCTTC
Ca_06998-Fw2	CACCCTGGTGTGAGAACTGGA
Ca_06998-Fw3	CTGTATACCTGAATGGCGGTGA
Ca_06998-Rev1	TTGATATCCCTCATCTACTGCTCC
Ca_06998-Rev3	GACTGGTTAAGCGACGAGACATC
Ca_06998-Rev2	ACTCTGCACAACAGAGTGGGAT
Ca_07000-Fw1	TGGTCACAGAAGATTTGATGCC
Ca_07000-Fw2	CATGCACCAATCAAGAATTGC
Ca_07000-Rev1	CCAACATGGCCATTAGTTTATC
Ca_07000-Rev2	CCAATCCAATGGTTGTGATTG
Ca_07001-Fw1	CCTGTAGCAGAGAAAGCAGAG
Ca_07001-Rev1	GGTAACAGCCTTAGTTCCTTCAGA
Ca_07002-Fw1	ATCAAAACCCCTCTTGCATCAAGAAGCCAA
Ca_07002-Rev1	CCGACATTAGTACACGTGGTTA
Ca_07003-Fw1	GGGGATTTTGATTGATCGTTATG
Ca_07003-Fw2	GAGAAATCATGCTCGCACAGC
Ca_07003-Rev1	GAAGAAGCAACAGACAGAGCG
Ca_07003-Rev2	CCTCCAAACCTTCTCTCAAATCC
Indel 1-Fw1	ACCCGCACAGCTGTAAGGTCTCA
Indel 1-Rev1	TGTGGTACCAGGGTTGGATTTGGT
Indel 2-Fw1	GGATTGAAGAAGTGTACCATTGGCA
Indel 2-Rev1	CCACCCTCTTCATTTTCATGGTCTACAC
Indel 3-Fw1	TAATGGCCGTAACGTCTCGT
Indel 3-Rev1	GTAGCACGTACATTGAACTTATCGGACT
CKAM0717-Fw1	TGCCCAATCCCATGTCACGGTCAA
CKAM0717-Rev1	CCTTGTTGGTGCCTTTTCATGTAGGC

Supplementary file 1: Primer sequences of the markers developed in this study.

Supplementary file 2: Microsatellite markers screened in RIP-1 parental lines, their physical positions, primer pairs sequences, PCR predicted size and the obtained size.

Marker ID ¹	Repeat	Physical position	Primer pairs sequences (5´-3´)	Predicted size	Obtained size ²	
					Erect	Prostrate
CaGM12352	(TAA)11(CAA)10ttt(TAA)13	Ca3:29407246-29407350	TGGCTGACCAGAGACATGAA/C AAGGAACGAGCTGATCAAA	347	370	390
CaGM12871	(TTA)17tttccttttaataaaaagt aaaaatcggacaaaattaagtgtca acattattaaatattttcaaacttat atttattaa(TAT)6	Ca3: 33828273-33828424	AGAGGCAAACAAGAACCGAA/T GCATATAAGGAATTGACGCC	318	350	400
CaGM12673	(TAT)20(TGT)6	Ca3: 31901720-31901791	CGCGTTCCTTTGGATAATGT/T CCAATTCTCAAAGTTGTAGGC	252	270	320
CaGM12186	(AAT)10aac(AAT)22	Ca3: 28071586-28071684	TGAGTTGCAAGGCACTAATCA/ TTCAAATGTTGCCACCTCAA		220	180
CaGM12554	(TTA)29	Ca3: 30871565-30871651	GGCCCCCTTTCTTCTCTTTA/CC ATTTATAATGCCTGGGACA		300	315
CaGM02007	(AAT)20ag(TAA)24	Ca1: 15559870-15560003	TGGTGTTCGCTCATAGGTGA/A AAAAGTTTGGCGTGAGAAG	305	260	440

¹ Marker ID in chickpea online web (<http://cicarmisatdb.icrisat.org/index.html>)

² PCR product size in both parents ILC72 (erect) and Cr5-10 (prostrate), respectively.

CAPÍTULO III

Mapeo fino del gen para simple/ doble vaina en garbanzo

Enviado:

Ali L, Deokar A, Caballo C, Tar'an B, Gil J, Millan and Rubio J. Fine mapping for simple double podded gene in chickpea. Theoretical and Applied Genetics

Resumen

A partir de cuatro poblaciones de líneas recombinantes (RIP-1, RIP-7, RIP-11 y CPR-01) se ha podido localizar el gen que controla el carácter doble vaina (*Sfl/sfl*) en el mapa genético de garbanzo. En el caso de la población RIP-1, el gen se localizó en el grupo de ligamiento 6 entre los marcadores STMS (SequenceTagged Microsatellite Site) TA120 y TR1 a una distancia de 3,5 y 13,9 cM respectivamente. La disponibilidad de la secuencia del genoma de garbanzo nos ha servido para añadir ocho nuevos marcadores STMS en la región anteriormente mencionada, permitiéndonos localizar el gen *Sfl/sfl* entre los marcadores CAGM27819 y CAGM27777 con un intervalo de separación de 2 cM. Siete de los ocho nuevos marcadores STMS se pudieron genotipar también en las poblaciones RIP-7 y RIP-11 (recíproca), confirmándose la situación del gen *sfl* que se situó entre los marcadores TR44 y CAGM27705 a una distancia de 1,7 y 3,1 cM respectivamente. Además, en la población CPR-01, se desarrolló un mapa de alta densidad con marcadores SNP (Single Nucleotide Polymorphism) y el gen *sfl* se situó de nuevo en la misma región anteriormente descrita, en concreto entre los marcadores TR44 y el SNP scaffold1646p972220 con un intervalo de 5,1 cM. Para refinar la posición de gen en la región en estudio se utilizaron 5 pares de líneas casi isogénicas (NILs) para el carácter doble vaina y 8 líneas recombinantes (RILs) permitiéndonos situar el gen *Sfl/sfl* entre los marcadores SDSNP1 y CAGM27777 en una región de 92.560 bp en la cual se identificaron siete genes. El gen RAX2 (Regulator Axillary Meristem) podría ser un gen candidato para seleccionar plantas con simple o doble vaina. Este trabajo ha proporcionado un conjunto de marcadores fuertemente asociados al gen *Sfl/sfl* de gran utilidad para ser utilizados en selección asistida por marcadores (MAS).

Abstract

Four recombinant inbred populations (RIP-1, RIP-7, RIP-11 and CPR-01) were used to map the simple/double podding gene (*Sfl/sfl*) in chickpea. The gene was mapped on linkage group 6 with a distance of 3.5 and 13.9 cM apart from two STMS (Sequence Tagged Microsatellite Site) markers TA120 and TR1, respectively, in RIP-1. The available genome sequence data were used to add eight new STMS markers in the region between TA120 and TR1. Finally *Sfl/sfl* was flanked by CAGM27819 and CAGM27777 markers within an interval of 2 cM. Seven out of the eight markers were mapped in RIP-7 and its reciprocal RIP-11 confirming the location of *sfl* gene flanked by TR44 and CAGM27705 at 1.7 and 3.1 cM apart, respectively. Furthermore, using a high density of SNP (Single Nucleotide Polymorphism) map of the CPR-01, *sfl* gene was mapped in the same genomic region between TR44 and the SNP scaffold1646p97220 with an interval of 5.1 cM. Five pairs of NILs (Near Isogenic Lines) for the double podding trait and eight recombinant inbred lines (RILs) were used to refine the target region in the chickpea physical map. This allowed to place *Sfl/sfl* gene between the SDSNP1 and CAGM27777 markers within a genomic window of 92,560 bp. Seven annotated genes were extracted from this region. RAX2 (Regulator of Axillary Meristem) predicted gene could be a candidate gene for simple/double podded plants. In addition, this study provides a set of markers flanking and tightly linked to *Sfl/sfl* locus that are useful for marker assisted selection (MAS).

Keywords: chickpea, double pods, candidate gene, 45 physical map, linkage analysis, microsatellite marker

Introduction

Chickpea was one of the first domesticated species in the Middle East during the Neolithic age (Ladizinsky and Adler 1976) and today the crop is cultivated all over the world. It is the second most important pulse crops after common bean considering total production (FAOStat 2013). Despite chickpea production has been gradually raised, world's average yield (0.96 t/ha) is considered low (FAOStat 2013). Thus, chickpea breeding programs are not only focused in the improvement of the resistance and tolerance against biotic and abiotic stresses but also to improve yield and its components (Gaur et al. 2007). There are two different chickpea types: kabuli, with white flowers and cream and medium to large seeds and desi, with pink flowers, small seeds and dark seed coat. Crosses between desi and kabuli types, have been breeding strategy to increase the diversity and to exchange resistant or adaptive genes.

The majority of chickpea produce single flower and consequently single pod per node, but some desi lines carry a mutation that produces two flowers, thus, two pods per node, as described for the first time by Khan and Akhtar in 1934, (in (Muehlbauer and Singh 1987). Pods per node could increase seeds per plant and consequently final yield but could be negatively correlated with seed size. However, most authors reported that double podded increased yield in comparison to single podded genotypes (Singh and Van Rheenen 1994; Ali et al. 2010). Other reports suggested that this mutation does not significantly increase yield but may have a positive effect on yield stability being not linked to genes affecting seed size (Rubio et al 1998; 2004).

Simple/double podding inheritance fits to a single recessive gene assigned as (*s*) or *sfl* (Khan and Akhtar 1934; Ahmad 1964; Patil 1966, D'cruz and Tendulkar 1970; Singh and Van Rheenen 1989). *Sfl/sfl* gene was mapped for first time on linkage group (LG) 6, 4.84 cM apart from the STMS (Sequence Tagged Microsatellite Site) marker TA80 (Rajesh et al.

2002). Other reports suggested that the locus was flanked by STMS TR44 and TR1 (TR35) or NCPGR33 and NCPGR47 with an interval of 19.3 and 3.4 cM, respectively (Cho et al. 2002; Rajesh et al. 2002; Radhika et al. 2007).

The second flower in double podded genotypes sometimes develops irregular pods and forms pods at variable frequency (Knights 1987). This trait has been reported to have unstable penetrance and variable expressivity in different environments, different genetic backgrounds, or reciprocal crosses (Sheldrake et al. 1978; Rubio et al. 1998). The expression of double podding could be superior when the genotype with the '*sfl*' allele is used as the female parent (Yasar et al. 2014).

The marker TA80 was successfully used to introduce double podding using a marker-assisted backcrossing approach (Taran et al. 2013). However, TA80 is not tightly linked to *sfl* gene; as such recombination can occur between the gene and the marker. So the best way to minimize recombination is to have a diagnostic marker. However, in spite of the importance of this trait in chickpea breeding, to date, there is no molecular study focusing on the candidate gene(s) for double podding in chickpea. At present, it is possible to relate the genetic and the physical map positions, thanks to the publication of the two draft genome sequences, each for desi and kabuli chickpea types (Jain et al. 2013; Varshney et al. 2013). This information allowed to design new markers to reduce the genomic window of the target area and to search for candidate genes (Ali et al. 2014; Madrid et al. 2014). In the case of simple/double pod, it is still necessary to refine the position of *Sfl/sfl* gene in the genetic map to narrow down the target area in the physical map. The use of near isogenic lines (NILs) could help to efficiently fine map the gene (Lin et al. 2014) because the difference between NILs is reduced to a small target genomic region and the genetic background noise is mostly eliminated. Thus, in chickpea, pairs of NILs developed for a non-nodulation mutant allowed to localize CaNSP2 gene

that could involve in the nodulation signal transduction pathway (Ali et al. 2014).

The objectives of this research were to refine the genomic region for the double podding gene and to find candidate genes for this trait in chickpea. For this approach, recombinant inbred populations (RIPs) with different genetic backgrounds together with five pairs of NILs developed by Rubio et al (1998) for this trait and the chickpea genome sequencing information were used.

Materials and methods

Plant material

Four RIPs derived from intraspecific crosses of *Cicer arietinum* by single seed descent (SSD) method (Johnson and Bernard 1962) were used in this study. Three of them were developed in Córdoba (Spain) identified as RIP-1 (CA2156 × JG62), RIP-7 (ILC3279 × JG62) and RIP-11 (JG62 × ILC3279) with a population size of 80, 115 and 86 recombinant inbred lines (RILs), respectively. The fourth population identified as CPR-01 (92 RILs) was derived from a cross between ICCV 96029 × CDC Frontier (Tar'an et al. 2007). CPR-01 was genotyped and phenotyped at the Crop Development Center, University of Saskatchewan, Canada.

Each population includes a double-podded parental line either JG62 (also known as ICC 4951) or ICCV 96029; both are desi type. JG62 is a landrace from India and ICCV 96029 is an elite cultivar developed and released by ICRISAT (International Crops Research Institute for the Semi-Arid Tropics) (Kumar and Rao 2001). The other parental lines are single-podded kabuli type; CA2156 is a Spanish local cultivar, ILC72 and ILC3279 are landraces from the former Soviet Union maintained by the International Center for Agricultural Research in the Dry Area (ICARDA), and 'CDC Frontier' is an elite cultivar from Canada (Warkentin et al. 2005).

Five pairs of NILs (NIL-1, NIL-2, NIL-3, NIL-4 and NIL-5) differing for double/simple pod previously developed by Rubio et al (1998) were also used in this study.

Phenotypic evaluation

RIP-1 was sown and evaluated under field conditions in Córdoba (southern Spain) as previously described by Cobos et al. (2005). RIP-7 and RIP-11 were sown and evaluated under field conditions in 2011 and 2014, respectively, in Córdoba (Spain). The RIP derived from ICCV 96029 × CDC Frontier was evaluated in phytotron at the University of Saskatchewan, Canada and field conditions at Saskatoon and Moose Jaw, SK (Canada) in 2012 and 2013. Single/double pod was recorded as a double pod presence vs absence.

Molecular markers and linkage analysis

For RIP-1, RIP-7 and RIP-11, six STMS markers (STMS2, TA22, TA176, TR44, TA120, and TR1) previously mapped on LG6 by (Winter et al. 1999; 2000) were used. Additional ten microsatellite markers were chosen from the chickpea data base (<http://cicarmisatdb.icrisat.org/index.html>). Amplification was carried out in 10 µl reaction volume containing 30 ng of plant genomic DNA, 1 x buffer (50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100), 1.5 mM MgCl₂, 250 µM of each dNTPs, 0.25 µM of each primer and 0.05 units of Taq DNA polymerase (GoTaq® Flexi, Promega). The thermal profile for the PCR was an initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 60°C for 45 s, and 72°C for 45 s with a final extension at 72°C for 8 min. The PCR products of 17 primers 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 x TBE buffer, or in 10% non-denaturing polyacrylamide gels, and stained with ethidium bromide. Linkage analysis was performed using JoinMap v4.0 with the

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-7 and the reciprocal cross RIP-11 were combined as one population to perform the linkage analysis.

High-density genetic map of CPR-01, consisting of 1,336 SNPs (Single Nucleotide Polymorphism) was constructed as previously reported (Deokar et al. 2014). Briefly, CPR-01 population was genotyped using Illumina 1,536 SNPs GoldenGate assay and RAD-Seq (Restriction-site Associated DNA Sequencing) genotyping-by-sequencing (RAD-Seq GBS) approach. Additionally, 59 microsatellite markers were also used for genotyping CPR-01 population. The PCR amplification reaction for microsatellite markers was performed in a 25 µl volume containing 50 ng of genomic DNA, 1U of Taq DNA polymerase, 0.24 mM each of dATP, dCTP, dGTP and dTTP, 1.0 µM of each primer and 1 x buffer with a final concentration of 2.5 mM MgCl₂. The PCR cycling conditions of an initial denaturation step at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 50-58°C (depending on the STMS primers) for 30 s and 72°C for 2 min with a final extension step at 72°C for 5 min was used. The PCR products were separated on a 6% polyacrylamide gel and visualized with silver staining. Linkage map was constructed using QTL IciMapping v3.2 software (<http://www.isbreeding.net>) with RECORD algorithm.

Re-sequencing data of both genotypes JG62 and ILC3279 (kindly supplied by Dr. W. Chen, USDA-ARS Washington State University, USA) allowed us to choose a SNP in the targeted region. A pair of primers was designed for CAPS (Cleaved Amplified Polymorphic Sequence) marker using Primer 3 software (<http://frodo.wi.mit.edu/primer3/>). The forward primer sequence is 5'-GTTTCGCTAGGTTCTTTAGCTATTGT-3' and the reverse primer sequence is 5'-CACCAACGTTACGGACACAT-3'. The

PCR program started with an initial denaturation step at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 60°C for 1 min and 72°C for 1 min with a final extension step at 72°C for 5 min. PCR products were digested by the restriction enzyme MboII following the supplier's instructions and were visualized using 1.2% agarose gel.

Physical map

Primer sequences of the STMS markers mapped on LG6 were used to amplify *in silico* in the chickpea genome assembly of CDC Frontier chickpea line (Varshney et al. 2013) using In-house script software (Kalendar et al. 2009).

Results

Phenotypic evaluation

The phenotypic evaluation of simple versus double podding trait (*Sfl/sfl*) fitted to the expected segregation ratio 1:1 in the four RIPs used in this study. The ratios for single:double podded were 45:33, 54:57, 38:48, and 53:39 with $P>0.05$ in RIP-1, RIP-7, RIP-11 and CPR-01, respectively.

Genetic and physical analysis

Five (STMS2, TA22, TA176, TA120, TR1) out of the six STMS mapped on LG6 were polymorphic in RIP-1 and included in LG6 of the genetic map together with TA80 previously reported by (Cobos et al. 2005). All markers fitted to the expected segregation ratio 1:1. *Sfl/sfl* was mapped on LG6 at a distance of 3.5 and 13.9 cM from the flanking markers TA120 and TR1, respectively. In order to saturate this region, 10 new microsatellite markers were selected from the chickpea whole genome database. Eight out of 10 markers were amplified and were polymorphic between RIP-1 parents (Table 1). Therefore, they were amplified through the whole population and included in LG6 of RIP-1. Linkage analysis revealed that the gene (*Sfl/sfl*)

was flanked by two microsatellite markers CAGM27819 and CAGM27777 at the genetic distance of 2 cM corresponding to 0.354 Mb on the chickpea CDC Frontier physical map (Figure 1). All microsatellite markers were amplified *in silico* in Ca6 with 0 mismatches except TA80 and TA22 primers that were not amplified in any pseudomolecule of chickpea genome. Comparison between genetic and physical markers positions revealed that there was a general co-linearity except for the marker STMS2 that was located at the top of LG6 but in the opposite extreme in Ca6 (Figure 1).

Table 1: List of microsatellite markers used to screen the RIP-1 parental lines, marker physical positions, primer pairs sequences, PCR predicted and obtained sizes.

Marker ID ¹	Repeat	Physical position	Primer pairs sequences (5'-3')	Predicted size	Obtained size ²
CaGM27777	(AC)11(AT)14	28927824-28927873	CCATTTTCTCCGGTTGAA/TGGCAAAGCCTGACCTATTC	332	350/365
CaGM27748	(TAT)12ttattattat(TTA)13	28696928-28697012	TGTTTTTGGTCCCTGCAAAT/CAATTGAGTTGCGACGAGAG	322	366/350
CaGM27705	(TTA)34	28268645-28268746	AGTGGGAATTTGGATGTTCCG/TGCCACCCTGGATTAGTTTT	260	335/320
CaGM27620	(TA)18c(AT)8	27363366-27363418	ATCATATCCCCGGATGCTTT/TTTGGTGGTTGGAGGAGTTC	137	NA ³
CaGM27939	(TTA)31	30257765-30257857	TAAGCCAGACCTGAGGAACG/CCTAGGTTGAATGTGCTTTTG	326	320/340
CaGM28032	(ATA)28	31042946-31043029	TCCGATTCCAATCCAGTTTC/TCACCGCATAACCAACTGAA	271	300/300
CaGM28164	(AAT)29aataataataac(TAA)25	32219443-32219618	AGTTCAAACAAACACACCCA/ATTACCCGTTATGCACTCGC	222	320/270
CaGM27819	(AAT)22	29281343-29281408	TGTAGGAGCAAGGAGTGTGATT/AAAGGTACTTTTCACTCTCC	231	240/225
CaGM27946	(TTA)22	30342506-30342571	GAACGTTAAGGCAGGAGTGG/TGCACGACATGTAACATCCA	312	370/410
CaGM27974	(TTA)19	30591669-30591725	CTTGGTCCAAAACCTGATGA/GGGGACCAAAAACAGGGAGA	219	360/400

¹ Marker ID in chickpea online web (<http://cicamisatdb.icrisat.org/index.html>)

² PCR product size in both parents JG62 and CA2156, respectively.

³ NA: non-amplified

In order to confirm the position of *Sfl/sfl* gene, both RIP-7 and its reciprocal RIP-11 were screened with seven markers in LG6 as anchor points (TR1, CAGM27705, TR44, CAGM27819, TA120, CAGM27939, and CAGM28164). TR44 was not polymorphic in RIP-1 but it was polymorphic between ILC3279 and JG62, so it could be added to both RIP-7 and RIP-11. Linkage analysis of RIP-7/11 (as one population) revealed the location of *Sfl/sfl* gene between the markers TR44 and CAGM27705

with a distance of 1.7 and 3.1 cM, respectively. This result confirmed the previous position of *Sfl/sfl* in RIP-1 (Figure 1).

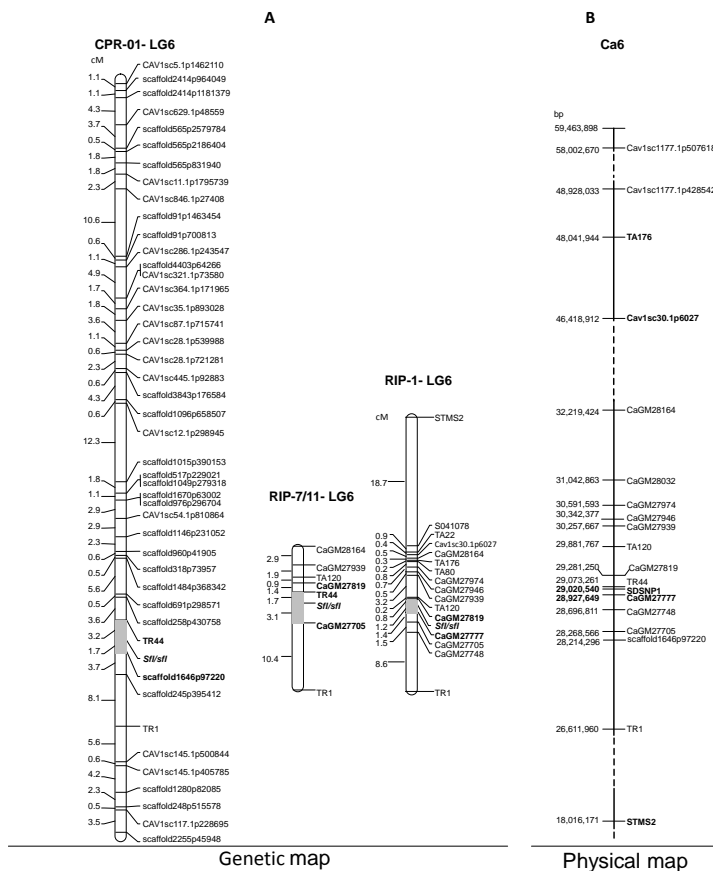


Figure1: On the left, genetic map of LG6 in three RIL populations (CRP-01, RIP-7/11 and RIP-1) derived from intraspecific crosses of *Cicer arietinum*. *Sfl/sfl* gene mapped on LG6 is in bold and the target region is marked by a grey box for each RIP. Estimated genetic distances are given in cM. On the right, physical map of Ca6 where the physical position of markers mapped on LG6 are shown in bp.

The linkage map of CPR-01 containing 1,336 SNPs and 59 microsatellite markers were grouped into eight linkage groups each corresponding to eight chickpea chromosomes. *Sfl/sfl* gene was mapped on LG6 at flanked by microsatellite marker TR44 and a SNP marker scaffold1646p97220 at 3.23 cM and 1.67 cM, respectively, from the gene.

Another microsatellite marker TR1 was also present at a distance of 13.4 cM to the double podding gene. These two microsatellite markers TR44 and TR1/TR35 had been reported to be linked to double podding gene at a distance of 7.8 and 11.5 cM, respectively, in JG62 (Cho et al. 2002). The RAD-GBS and GoldenGate SNP genotyping assays have provided additional SNP markers in the vicinity of the *Sfl/sfl* gene in the CPR-01, which narrow down the flanking marker window of *Sfl/sfl* gene to 4.9 cM (Figure 1). These sequence based markers also provide opportunity to compare genetic map with the chickpea physical map. The genetic distance between double podding gene flanked markers TR44 and scaffold1646p97220 (4.9 cM) corresponds to 0.859 Mb on the chickpea CDC Frontier physical map.

The common flanking markers and the location of the double podded gene across CPR-01 (ICCV 96029 × CDC Frontier), RIP-1 (CA2156 × JG62), RIP-7 (ILC3279 × JG62) and RIP-11 (JG62 × ILC3279) indicated that ICCV 96029 and JG62 may carry same gene for double podding trait (Figure 1).

Trying to further to refine the simple/double podding locus position based on the physical position of markers, 13 SSR markers physically mapped on Ca6 were screened in five pairs of NILs for simple/double pod obtained from a cross with the same parental lines as RIP-1 (CA2156 and JG62) by Rubio et al. (1998). Results showed that markers between CAGM28164 and CAGM27777 in the physical map were polymorphic for all five pairs of NILs. This result indicated that the target region for single/double podding gene was flanked by CAGM28164 and CAGM27777 confirming the results obtained in the genetic maps (Table 2).

Eight RILs (four with single pod and four with double pod), derived from RIP-7/11, were selected as recombinants for some of markers in the target region. After analyzing PCR patterns taking into account their physical position it was possible to delimit the non-recombinant region to

376,450 bp. It was flanked by the two markers TR44 (29,073,261 bp) and CAGM27748 (28,696,811 bp). Additional SNP from resequencing data of ILC3279 and JG62 was detected between TR44 and CAGM27748. From this sequence a CAPS marker (SDSNP1) was developed and used to genotype the eight RILs. Recombinations through the RILs were analyzed showing a delimited region with only 323,729 bp between the markers SDSNP1 and CAGM27748 (29,020,540-28,696,811 bp) (Table 3, Figure 2).

Table2: Single/double podded NILs screening by markers located in Ca6 of chickpea physical map closed to *Sfl/sfl* locus.

		Markers ID and physical position											
		Cav1sc30.1p6027	CaGM28164	CaGM27974	CaGM27946	CaGM27939	TA120	CaGM27819	CaGM27777	CaGM27748	CaGM27705	TR1	STMS2
Pairs of NILs		46,418,912	32,219,443	30,591,669	30,342,506	30,275,765	29,881,767	29,281,343	28,927,824	28,696,928	28,268,645	26,611,960	18,016,171
NIL-1	P	P	P	P	P	P	P	P	P	P	P	P	M
NIL-2	M	M	P	P	P	P	P	P	P	P	P	P	M
NIL-3	M	M	P	P	P	P	P	P	M	M	M	M	M
NIL-4	P	P	P	P	P	P	P	P	P	M	M	M	M
NIL-5	M	M	P	P	P	P	P	P	P	M	M	M	M

Table 3: Simple versus double podding phenotype in 8 selected RILs of RIP 7 and RIP 11, and the score of microsatellites on LG6 showing recombinations.

RIL ID	Simple/double phenotypic trait	CaGM28164	CaGM27939	TA120	CaGM27819	TR44	SDSNP1	CaGM27777	CaGM27748	CaGM27705	TR1
RIP-7-28	simple pod(a)	b	b	b	b	b	a	a	a	a	a
RIP-7-72	simple pod (a)	b	b	b	b	b	b	a	a	a	a
RIP-7-103	simple pod (a)	a	a	a	a	a	a	a	b	b	b
RIP-7-77	double pod (b)	b	b	b	b	b	b	b	a	a	a
RIP-7-41	double pod (b)	b	b	b	b	b	b	b	a	a	a
RIP-7-114	double pod (b)	b	b	b	h	b	b	b	b	a	a
RIP-11-43	double pod (a)	a	a	a	a	a	a	a	a	b	b
RIP-11-96	simple pod (b)	b	b	b	b	b	b	b	b	a	a

Considering recombination events obtained in both pairs of NILs and RILs revealed that the simple/double podding gene should be flanked by the markers SDSNP1 and CAGM27777 in a genomic window of 92,560 bp (Figure 2).

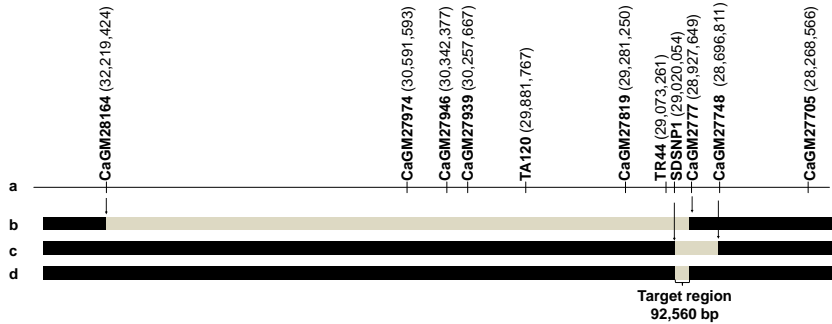


Figure 2: Scheme shows the genomic region that includes single/double podding (*Sfl/sfl*) locus in chickpea. a) The physical positions of markers mapped on LG6, b) NILs recombinations, where black and grey boxes represent monomorphic and polymorphic regions for pair of NILs, respectively, c) RILs recombinations, where black and grey boxes represent recombinant and non-recombinant regions, respectively, d) In grey, the region surrounding *sfl* gene after overlapping NILs and RILs grey boxes showing that the target region is flanked by SDSNP1 and CaGM27777 markers.

Candidate genes

Seven annotated genes could be extracted considering that the two markers SDSNP1 and CAGM27777 are flanking *Sfl/sfl* locus, in a frame of 92,560 bp (28,927,980-29,020,540 bp) in Ca6. These genes with Genebank accession numbers (www.ncbi.nlm.nih.gov/) are: LOC101505360 and LOC101507108 were uncharacterized genes; LOC101490413 and LOC101490737 were predicted serine carboxypeptidase-like 48-like; LOC101506550 was lysine methyltransferase METTL21A-like; LOC101505694 was annotated as lactoylglutathione lyase-like and finally LOC101506220 was a predicted transcription factor RAX2 (Regulator of Axillary Meristem)-like. This gene was annotated in Ca6 at the position 28,962,836:28,964,637 in the negative strand with 1,802 bp of length.

Discussion

Simple/double podding is an important agronomic trait affecting yield and its stability in chickpea (Kumar et al. 2000; Rubio et al. 2004). In our study this trait was mapped on LG6 as previously reported by Cho et al. (2002), Rajesh et al. (2002) and Radhika et al. (2007) where JG62 was used as double podded parent. The comparison of LG6 across the mapping populations employed in this research revealed that the two genotypes JG62 and ICCV 96029 have the same gene of double podding (indicative marker TR44). The available whole genome sequencing information in the reference variety ‘CDC Frontier’ recently published (Varshney et al. 2013) together with re-sequencing data obtained by new DNA sequencing technology tools (RAD-Seq GBS) has been essential to establish the physical position of key markers linked to *Sfl/sfl* gene in the genetic map. Due to the low level of polymorphisms present among cultivated chickpea genotypes (Varshney et al. 2007) a successful approach to saturate a target region is to design microsatellite markers which was useful tool together with SNPs markers developed for desi vs kabuli types (Agarwal et al. 2012; Kujur et al. 2013). In plants, genome sequencing data and next generation sequencing have been used to identify and amplify new microsatellite markers as in rice (Cordeiro et al. 2002) and soybean (Jun et al. 2011). *Sfl/sfl* locus was flanked by two markers with an interval ranged from 4.9 cM (CPR-01) to 2.0 cM (RIP-1) in the studied populations (Figure 1). In other studies this trait was linked to TA80 (4.84 cM) (Rajesh et al. 2002), flanked by two markers (TR44 and TR35) at 7.8 and 11.5 cM, respectively (Cho et al. 2002) or by NCPGR33 and NCPGR47 at an interval of 3,4 cM (Cho et al. 2002; Rajesh et al. 2002; Radhika et al. 2007). TR44 is the most tightly linked marker to *sfl* gene with a distance of 3.2 and 1.7 in CPR-01 and RIP-7/11, respectively. Differences in genetic distances can occur when results of different mapping populations are compared (Iruela et al. 2007).

Different software packages based on different procedures, population size or experimental error in the phenotype score could affect the order or distance between markers. It should be pointed out that phenotypic evaluation of double podded is not an easy task due to its variable expressivity and penetrance through different environments and genetic backgrounds (Kumar et al. 2000).

As mentioned above, *sfl* gene position in the target genomic region could be affected by different factors. In this study the use of both NILs and recombinant lines allowed us to get the physical position of *sfl* gene and flanking markers. NILs have been used in chickpea to map important agronomic traits as simple/double podding (Rajesh et al. 2002) or nodulation signaling pathway2 (*NSP2*) gene (Ali et al. 2014). NILs have been a useful tool in plant breeding to find out new QTLs, fine mapping and detection of candidate genes for oil content in soybean (Zhu et al. 2012) or for plant architecture in rice (Lim et al. 2014). Furthermore, recombinant RILs were used for fine-mapping of a major QTL which regulates pod shattering in soybean (Gao and Zhu 2013).

Chickpea genome sequencing data has been efficiently used to identify candidate genes for nodulation (Ali et al. 2014) and growth habit in chickpea (Ali et al. 2015). In this study it was possible to match genetic and physical map, and to combine this information with the recombination detected in different pairs of NILs reducing the *sfl* gene genomic window to just 92,560 bp.

Seven annotated genes were extracted from the target region, and among them the *RAX2* predicted gene could be a potential candidate for double podded. *RAX2* protein has MYB domain and belongs to R2R3 MYB family proteins (Yanhui et al. 2006). It has been reported that *RAX2* has an accessory effect on side shoot formation during inflorescence development in *Arabidopsis thaliana* (Müller et al. 2006).

In chickpea breeding programs the marker TA80 was successfully used to select double podded plants by MAB (Marker Assisted Backcrossing) (Taran et al. 2013). However, in our study it was demonstrated that the physical position of TA80 is far from the *Sfl/sfl* gene. Our study identified the markers SDSNP1 and CAGM27777 most closely linked with the double podded gene that could be used as flanking markers in plant breeding programs for marker assisted selection (MAS) reducing the probability of recombination events. From the point of view of plant breeding for this trait now it would be possible to choose the most adequate flanking markers in order to reduce selection errors as false positives (Collard and Mackill 2008).

For future studies, more effort should be done to sequence and examine the expression profiles of the candidate gene. The results obtained of the five pairs of NILs revealed that two pairs of them (NIL-3 and NIL-5) differ in a smaller region than the other pairs (Table 2). Hence, these two pairs could be the ideal plant material to be employed for future expression studies using these seven genes located in target region.

In conclusion, our study provides a set of markers closely flanking *Sfl/sfl* gene which could be a useful tool for MAS. Besides, the target region of 92,560 bp with only seven annotated genes opens the possibility to scrutinize the candidate gene controlling simple/double podded phenotype.

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CAPÍTULO IV

Desarrollo de líneas casi isogénicas para fecha de floración en garbanzo y búsqueda de genes candidatos

En preparación:

**Development of near isogenic lines for flowering time for QTL_{DF1} and QTL_{DF2}
and looking for candidate genes for QTL_{DF2} in chickpea**

Resumen

Se han empleado dos poblaciones de líneas recombinantes (RIPs) RIP-10 y RIP-8 para desarrollar parejas de NILs para fecha de floración en garbanzo teniendo en cuenta las dos regiones donde se habían detectado QTLs (quantitative trait loci), el QTL_{DF1} y el QTL_{DF2} en los grupos de ligamiento GL4 y GL3, respectivamente. Para obtener las NILs se seleccionaron dos RILs (recombinant inbred lines) segregantes para fecha de floración en campo, RIL74 (RIP-8) y RIL82 (RIP-10). Las semillas de plantas individuales de estas RILs se sembraron en condiciones de día corto (en cámara y en el invernadero del 2013 y 2014). Los resultados confirmaron que la pareja de plantas NIL82-3/NIL82-4 se puede considerar NILs para QTL_{DF1} , mientras que la pareja NIL74-25/NIL74-32 lo son para el QTL_{DF2} , en este último caso es recomendable confirmar los resultados en futuros estudios.

La búsqueda de genes candidatos para el QTL_{DF1} , se inició comprobando la homología de 13 genes situados en el cromosoma (Chr)1 de *Medicago truncatula* y relacionados con fecha de floración con la secuencia del genoma del garbanzo. Doce de estos genes fueron homólogos a genes situados en el cromosoma 4 (Ca4) del garbanzo siendo cuatro de ellos represores de la inducción de la floración. Además se seleccionaron seis marcadores STMS en el intervalo del QTL_{DF1} . Cuatro de ellos se amplificaron y mapearon en el GL4 de la RIP-1 siendo el STMS GAA47 el que mayor LOD mostró explicando un 25% de la variación fenotípica total del carácter evaluado en campo. El análisis de QTLs con los datos de invernadero reveló que los marcadores CaGM14822 y SNPFT1 eran los más asociados ($LOD=4,9$ $R^2= 24.7\%$). El gen *TEMPRANILLO* (*TEM*) localizado entre GAA47 y CaGM14822 es un serio candidato para el QTL_{DF1} .

Palabras clave: floración, líneas casi isogénicas, *TEMPRANILLO*,
garbanzo, gen candidato

Introducción

El garbanzo es la segunda leguminosa con más producción en el mundo en cuanto a superficie cultivada después de *Phaseolus vulgaris* (Faostat 2013). Por ser originario del medio oriente donde se cultiva tradicionalmente en primavera, el garbanzo necesita días largos para florecer, siendo la fecha de floración un carácter adaptativo importante a diferentes ambientes influyendo en la producción del cultivo (Berger et al. 2004, 2006; Gaur et al. 2008). Sin embargo, al ser tempranamente introducido su cultivo en latitudes más bajas (India o Etiopía) surgieron mutaciones que dieron lugar a genotipos adaptados a condiciones de días más cortos. Genotipos de estas regiones pueden ser utilizados para desarrollar variedades de ciclo corto porque las plantas de floración temprana ayudan a escapar de los estreses abióticos como la sequía que puede limitar más del 80% de la producción global de garbanzo (Kumar y Abbo 2001, Gaur et al. 2008).

La temperatura, el fotoperiodo, las hormonas (giberelinas) y la edad de la planta son factores que afectan a la fecha de floración lo que implica que varios genes y rutas controlan este carácter. En *Arabidopsis thaliana* que necesita días largos para florecer, se han identificado la mayoría de los genes implicados en la inducción de floración a nivel genético y epigenético (Blázquez et al. 2011, Srikanth y Schmid 2011, Song et al. 2013). En las leguminosas la transición del estado vegetativo al reproductivo necesita días largos (LD) como en guisante o días cortos (SD) como en la soja. Los estudios en leguminosas revelan una gran conservación de los genes descritos en *A. thaliana* pero con algunas duplicaciones o pérdida de función durante el proceso evolutivo (Young y Bharti 2012).

En garbanzo, no se han hecho estudios a nivel funcional y lo que se ha publicado respecto al control de la floración son experimentos de genética clásica. Se han descrito cuatro genes no alélicos (*Efl-1*, *Efl-2*, *Efl-3*

y *Efl-4*) que controlan la fecha de floración en diferentes genotipos de garbanzo, siendo los alelos tardíos los dominantes sobre los precoces (Kumar y Abbo 2001, Hegde 2010, Gaur et al. 2014). Dos QTLs mayores (QTL_{DF1} y QTL_{DF2}) se han localizado en los grupos de ligamiento (GL) 4 y 3 del mapa genético de garbanzo respectivamente (Cobos et al. 2007, 2009). También se han detectado QTLs menores en GL1, 2, 5 y 8 (Linchtenzveig et al. 2006, Rehman et al. 2011, Jamalabadi et al. 2013).

Las líneas casi isogénicas (NILs) por ser idénticas en el fondo genético y diferentes solo en la región genómica responsable del carácter de interés, han sido una herramienta útil para buscar genes candidatos a partir de mapeo fino como se ha hecho para el peso de la semilla en el arroz, o para realizar estudios de expresión como el caso del gen candidato represor de la floración en *Capsicum annuum* (Ngu et al. 2014, Borovsky et al. 2015). Por otro lado, la secuenciación del genoma de garbanzo recién publicada (Varshney et al. 2013) junto con los datos de re-secuenciación posteriores son también herramientas de gran ayuda para desarrollar nuevos marcadores polimórficos entre líneas cultivadas en garbanzo y buscar genes candidatos, como ya ha ocurrido para resistencia a rabia y hábito de crecimiento (Madrid et al. 2012, Ali et al. 2015).

El objetivo de este estudio ha sido desarrollar parejas de NILs para los dos mayores QTLs para fecha de floración descritos en garbanzos (QTL_{DF1} y QTL_{DF2}) situados en GL4 y GL3, respectivamente. Además, la búsqueda de genes candidatos para el QTL_{DF1} utilizando una población segregante (RIP) junto con la secuenciación del genoma del garbanzo.

Material y métodos

Material vegetal

Se han utilizado tres poblaciones de líneas recombinantes (RIPs) $F_{6:8}$ derivadas de cruzamientos intraspecíficos que se desarrollaron en el grupo

de Mejora Genética Vegetal (AGR114) de Córdoba siguiendo el método de single seed descent (SSD) (Johnson y Bernard 1962). Estas poblaciones se identificaron como RIP-1 (CA2156 x JG62) y se utilizó para el análisis de ligamiento y QTL. Las poblaciones RIP-8 (ILC3279 x WR315) y RIP-10 (JG62 x ILC72) se evaluaron para desarrollar parejas de NILs para fecha de floración. RIP-1, RIP-8 y RIP-10 constituidas por 80, 113 y 85 líneas recombinantes (RILs) respectivamente. Los parentales ILC3279 e ILC72 son líneas tardías de tipo kabuli, originarios de la antigua Unión Soviética y mantenidos en ICARDA (International Center for Agricultural Research in the Dry Area). WR315 y JG62 son líneas precoces de tipo desi procedentes de la India y mantenidas por ICRISAT (International Crops Research Institute For the Semi-Arid Tropics).

Evaluación fenotípica

RIP-1 y se sembraron en campo en Córdoba (España) y se evaluaron para fecha de floración como se describe en Cobos et al. (2005, 2007), respectivamente. RIP-10 y RIP-8 se evaluaron en 2008, considerando el carácter fecha de floración cuando el 50% de las plantas de cada RIL estaban en flor.

Desarrollo de NILs

Las NILs se obtuvieron a partir de líneas recombinantes de las poblaciones RIP-8 y RIP-10 en las que se observó segregación para fecha de floración según los datos obtenidos en campo. La probabilidad de encontrar una línea segregante $F_{6:8}$ para un gen es de un 3.1%. Una vez detectadas estas líneas segregantes se recolectaron las plantas individualmente y se sembraron sus semillas en cámara de cultivo bajo condiciones de día corto (14 horas día/10 horas noche). Las plantas elegidas como precoces y tardías se sembraron en el invernadero en el otoño de 2013 y 2014 en el IFAPA de Córdoba (España), creciendo bajo condiciones de días cortos. Tanto en la cámara como en el invernadero se recogieron las plantas individualmente y se

sembraron las plantas que tuvieron valores más extremos para precocidad y floración tardía de cada RIL (Tabla 1).

Tabla 1: Evaluación fenotípica de dos RILs segregantes junto con sus parentales creciendo en condiciones de día corto (cámara e invernadero) y patrones de amplificación con los marcadores STMS TA142 y GAA47 que son indicadores de los QTL_{DF2} (GL3) y QTL_{DF1} (GL4), respectivamente.

RIL segregante	Evaluación fenotípica (días para florecer, media)				TA142 (QTL _{DF2})	GAA47 (QTL _{DF1})
	Cámara	Invernadero	Invernadero			
		2013/2014	2014/2015			
<i>2012</i>						
ILC3279	nf	-	140		A	A
WR315	36	-	133		B	B
RIP8/RIL74-25	55	104	133		B	B
RIP8/RIL74-32	72	105	140		A	B
<i>2013</i>						
JG62	57	-	103		A	A
ILC72	117	-	130		B	B
RIP10/RIL82-3	59	96	120		A	A
RIP10/RIL82-4	71	106	132		A	B

nf: no floreció

A y B: Alelos presentes en uno y otro padre

Caracterización molecular

Extracción de ADN

Se recogieron 100 mg aproximadamente de tejido de hojas jóvenes que se congelaron directamente en nitrógeno líquido y se almacenaron a -80°C. El ADN se extrajo utilizando el método de DNAzol (Invitrogen, Carlsbad, CA, USA). Para confirmar la segregación fenotípica, se utilizaron los marcadores STMS (sequence tagged microsatellite site) TA142 y GAA47, ligados al QTL_{DF2} y QTL_{DF1} respectivamente y se amplificaron en las plantas de las RILs segregantes siguiendo el empleado por Winter et al. (1999). Los productos de PCR se separaron en geles de poliacrilamida no

desnaturalizante a una concentración de 10% y se visualizaron con bromuro de etidio.

Mapa físico y genes candidatos

Las secuencias de los cebadores de los marcadores STMS mapeados en GL4 se utilizaron para amplificar *in silico* en el genoma de referencia de garbanzo del cultivar ‘CDC Frontier’ (Varshney et al. 2013) usando In-house script software (Kalendar et al. 2009).

Para buscar genes candidatos para fecha de floración en la región del QTL_{DFI} en GL4, las secuencias de los genes relacionados con este carácter en *Medicago truncatula* en el cromosoma 1, que corresponde al GL4 en garbanzo, se utilizaron para hacer un BLAST contra el genoma de garbanzo citado anteriormente (Tabla 2).

A partir de la base de datos de STMS extraídos de la secuencia del genoma de garbanzo (<http://cicarmisatdb.icrisat.org/index.html>) se seleccionaron STMS situados en el rango del QTL_{DFI} descrito por Cobos et al. (2007). Las secuencias de los cebadores de estos micros están representadas en la Tabla 3. La mezcla de PCR para la amplificación de estos STMS se realizó en un volumen final de 10 µl y contenía 30 ng del ADN genómico, 1 x buffer (50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100), 1.5 mM MgCl₂, 250 µM de cada dNTPs, 0.25 µM de cada cebador y 0.05 units of Taq DNA polimerasa (GoTaq® Flexi, Promega). Las condiciones de amplificación de los STMS empleadas en este trabajo comenzaron con una desnaturalización inicial a 95°C durante 5 min seguida por 35 ciclos de desnaturalización a 95°C durante 30 s, hibridación a 60°C durante 45s, y extensión a 72°C durante 45s con extensión final a 72°C durante 8 min. Los productos de la PCR se separaron por electroforesis en geles de agarosa a una concentración del 2.5% (mezcla de 1.25% de agarosa SeaKem LE y LM SIEVE, Rockland, ME, USA 1.25%) o con geles de poliacrilamida no-desnaturalizante a 10%, y se visualizaron con bromuro de etidio

Tabla 2: Se muestran los genes relacionados con fecha de floración en el cromosoma 1 de *M truncatula* y sus homólogos en *C arietinum*, además de la función que tienen estos genes en *A thaliana*.

Gene symble	Gene ID <i>M truncatula</i> ¹	Gene ID <i>C arietinum</i> ²	identity %	query coverage %	e value	physical position in Ca4 of chickpea	gene annotation in chickpea	gene function in <i>A thaliana</i>
TICb	Ig072160	No similarity	-	-	-	-	-	TIC (time for coffe) está asociado con el reloj circadiano, y la mutación (lees or oro ver-expression) causa defectos en la transición de la fase vegetativa a la fase reproductiva (Shin et al. 2013)
TT8	Ig072320	LOC101506726	78	86	0	2158641:2149010	predicted transcription factor TT8-like	Necesario para el crecimiento del silique (Nesi et al.2000)
EIN4	Ig079790	LOC101508648	89	81	0	4577424:4582008	predicted protein EIN4-like	Tiene papel como receptor de etileno en la senescencia y abscisión de la flor (Chen et al. 2011).
PIF3a	Ig084980	LOC101501589	86	67	0	6505438:6510598	predicted transcription factor PIF3-like	Represor, la mutación acelera la expresión de <i>CO</i> y <i>FT</i> (Oda et al. 2004)
PHYA	Ig085160	LOC101506511	93	54	0	6633985:6627596	predicted phytochrome A-like	Activador (represor de PHYB), receptor de infrarrojo lejano (Chen et al. 2011).
SPL1	Ig086250	LOC101492418	90	81	0	6913271:6921240	predicted squamosa promoter-binding-like protein 12-like	Activador, regulador de la edad de la planta (active LFY, SOCL1, FUI and miR172)(Chen et al. 2011).
GA2ox3	Ig086550	LOC101497057	85	30	0	7018689:7013113	predicted gibberellin 2-beta-dioxygenase 2-like	Represor, desactiva GA (gibberellic acid) y por lo tanto inhibe la expresión de <i>FT</i> (Song et al. 2013).
ICCM0293	Ig087140	LOC101494269	85	99	0	7261066:7253025	predicted histidine kinase CKII-like	Tiene importancia en la floración en arroz (Matsubara et al. 2014)
TEMb	Ig093600	LOC101492303	82	99	0	9472319:9470566	predicted AP2/ERF and B3 domain-containing transcription repressor TEM1-like	Represor de la inducción de la floración en condiciones de días largos (FT) y cortos (GASOX) (Osnato et al. 2012).
GI	Ig098160	LOC101511540	93	100	0	11270960:11280375	predicted protein GIGANTEA-like	Activador, regulado por el reloj circadiano, active <i>CO</i> (Srikanth et a. 2011)
PMB/AGL6	Ig101970	LOC101488623	90	20	5e-61	12965683:12962358	predicted MADS-box transcription factor 17-like	Represor, la mutación produce insensibilidad a la temperatura (Lee et al. 2013)
GA20ox	Ig102070	LOC101490587	48	93	0	13002090:13004384	predicted gibberellin 20 oxidase 2-like	Activador, indirecto de la síntesis de GA (gibberellic acid) (Song et al. 2013).
TICa	Ig104710	LOC101496754	88	63	0	13844072:13836530	predicted protein COFFEEE-like	FOR TIC (time for coffe) está asociado con el reloj circadiano, y la mutación (lees or oro ver-expression) causa defectos en la transición de la fase vegetativa a la fase reproductiva (Shin et al. 2013)

¹ la identidad del gen de Medicago en la base de datos de esta especie (<http://www.medicago-hapmap.org/>).

² el nombre de accesoión del gen de garbanzo en el NCBI (<http://www.ncbi.nlm.nih.gov/>).

Análisis de ligamiento

Para los análisis de ligamiento, en RIP-1 se utilizaron marcadores previamente mapeados por Cobos et al. (2007) junto con los STMS desarrollados en este estudio. El análisis se realizó utilizando el programa JoinMap v4.0 con la opción de “Regression mapping” (Van Ooijen 2006). Los marcadores se agruparon con un LOD mínimo de 3.0 y máxima fracción de recombinación de 0.25 como criterio general para establecer los grupos de ligamiento. La función de Kosambi se empleó para estimar la unidad de distancia en el mapa genético (Kosambi 1943).

Análisis de QTL

Se utilizó el programa Map QTL v5 (Van Ooijen 2004) para hacer el análisis de QTL en RIP-1, donde se aplicó el test de Kruskal-Wallis para analizar la asociación marcador a marcador con la fecha de floración (Van Ooijen et al. 1993) y la opción “Interval mapping” para la localización del QTL (Lander y Botstein 1989, Van Ooijen 1992). La significación del QTL se determinó usando la prueba de permutación con 1000 réplicas (Churchill y Doerge 1994) empleando un nivel de significancia de 99%. Para estimar la proporción de la variación fenotípica total explicada por el QTL se utilizó el coeficiente de determinación (R^2) para el marcador más asociado al QTL.

Resultados

Desarrollo de NILs

En las RIL74 y RIL82 de las poblaciones RIP-8 y RIP-10 respectivamente se observó segregación para fecha de floración según los datos obtenidos en campo. Las plantas de estas RILs se identificaron individualmente y se sembraron sus descendencias en cámara de cultivo bajo condiciones de día corto. En la RIL74 las descendencias de las plantas 25 y 32 (RIL74-25 y RIL74-32) fueron las más precoces y las más tardías, respectivamente.

Mientras que dentro de la RIL82, las plantas 3 y 4 (RIL82-3 y RIL82-4) tuvieron el rango más amplio. Los resultados en la cámara (2013) y el invernadero (otoño de 2013 y 2014) confirmaron que NIL82-3/NIL82-4 (precoz/tardía) son una pareja de NILs para el QTL_{DF1} en el GL4. Sin embargo, para la pareja NIL74-25/NIL74-32 (precoz/tardía) (QTL_{DF2}) los resultados en el invernadero no confirmaron lo obtenido en cámara (Tabla 1).

Mapa físico, genes candidatos para el QTL_{DF1} y análisis de ligamiento

Los marcadores (GAA47, TA130, TA146, TR08, TA72) mapeados por Cobos et al (2007) en el GL4 donde se localiza el QTL_{DF1} se amplificaron *in silico* para obtener su posición en el mapa físico sobre el cromosoma 4 (Ca4) (Figura 1). La extensiva conservación del orden de los genes entre la planta modelo *M. truncatula* y el garbanzo (Seres et al. 2007, Millan et al. 2010, Nayak et al. 2010, Varshney et al. 2013) permitió relacionar cada grupo de ligamiento del mapa genético de garbanzo con sus correspondientes cromosomas en *M. truncatula*, donde el GL4 de garbanzo corresponde al Chr1 de *Medicago*. Trece genes se seleccionaron de la bibliografía de *Medicago* que están relacionados con fecha de floración. El análisis BLAST identificó 12 genes homólogos relacionados con dicho carácter en el GL4 de garbanzo. Once de estos genes se consideran activadores de la inducción de floración en *A. thaliana*, mientras que cuatro están descritos como represores de la iniciación de floración (Tabla 2). Ocho de los genes se localizaron en el mapa físico en el rango del QTL_{DF1} detectado por Cobos et al. (2007). A partir de la secuencia del garbanzo se seleccionaron seis marcadores STMS que estaban cerca de estos genes, cuatro de ellos amplificaron y fueron polimórficos entre los padres de la población RIP-1 (Tabla 3). Los cuatro STMS desarrollados se ajustaron a la segregación esperada 1:1 en la población RIP-1 y se mapearon junto con el marcador GAA47 previamente empleado por Cobos et al. (2007) en GL4 en

la región del QTL_{DF1}, confirmando la correspondencia entre el mapa genético y físico (Figura 1).

Tabla 3: Secuencias de los cebadores de los marcadores de Ca4 del garbanzo, y peso obtenido de los productos de amplificación en los dos parentales de la RIP-1.

¹Marker ID en la página web de garbanzo (<http://cicarmisatdb.icrisat.org/index.html>)

² SNP obtenido de los datos de re-secuenciación de los dos parentales (unpublished data).

Marker ID	La posición física en Ca4 (pb)	Forward seq	Reverse seq	El peso obtenido (pb) ⁶
CaGM14475 ¹	6549599:6549713	TTTCGAAAATGCCAAACCTC	AAACACCAAGCCACAATTC	320/350
CaGM14547 ¹	7300565:7300600	TCTCTCCCGTCCCAAATAC	GCCATCTTACCACTTGCAATTC	180/210
CaGM14806 ¹	9267771:9267830	TTCTCAAACCCTTTCATGGC	GTTGCTGCACAACCCGATAA	NA
CaGM14822 ¹	9383304:9383357	TCCATCCCGTATACCTGAAAA	CATTGTGAGTCGATCATAACAG	380/350
SNPFT1 ²	9526148	TCGCCAAGTTTTGAAGTGATTG	GTCTCAATCACGGTTGCATTGT	390/360
CaGM14849 ¹	9574783:9574842	ATTGACAAAAGTTGGAGGCG	TGCTCTCGACTACCGGTTAC	NA
GISTMS ³	11267271:1126787	GTCTGATCTTCACCGAACTGC	GACCAAAAACGCTGCTGCT	285/300

² STMS desarrollado en la secuencia del gene GIGANTEA (GI) utilizando el software websat (<http://wsmartins.net/websat/>) (Martins et al. 2009).

⁴ el peso del producto de la PCR obtenido en los dos parentales JG62 y CA2156, respectivamente.

⁵ NA: no-amplificado

Análisis de QTL

En el análisis de QTLs con los datos de campo permitió determinar el marcador GAA47 como el más asociado al QTL_{DF1} en los años 2003 y 2004 con un LOD máximo de 2.7 y 4.69 explicando 14.8 y 24.7%, respectivamente. En el caso de los datos invernadero, los marcadores CaGM14822 y SNPFT1 (situados en el mismo punto del grupo de ligamiento) tuvieron el LOD más alto (5,27) explicando el 27.1% de la variación fenotípica. En el QTL detectado, se encontraron ocho genes anotados que podrían estar asociados con fecha de floración en garbanzo. De estos ocho genes hay tres represores de la inducción de floración. Entre ellos, el gen anotado *TEMPRANILLO* (9,5 Mb) está en el intervalo del QTL entre los marcadores CaGM14822/SNPFT1 y GAA47 (el máximo LOD) y tienen el rango (8,3 – 9.6 Mb) (Figura 1). Además, la precocidad en el genotipo JG62 (mutación natural) debe ser resultado de fallo de represor que daría lugar a floración temprana. Por eso, el gen *TEM* puede ser un gen candidato para la precocidad en JG62 y por lo tanto en RIP-1.

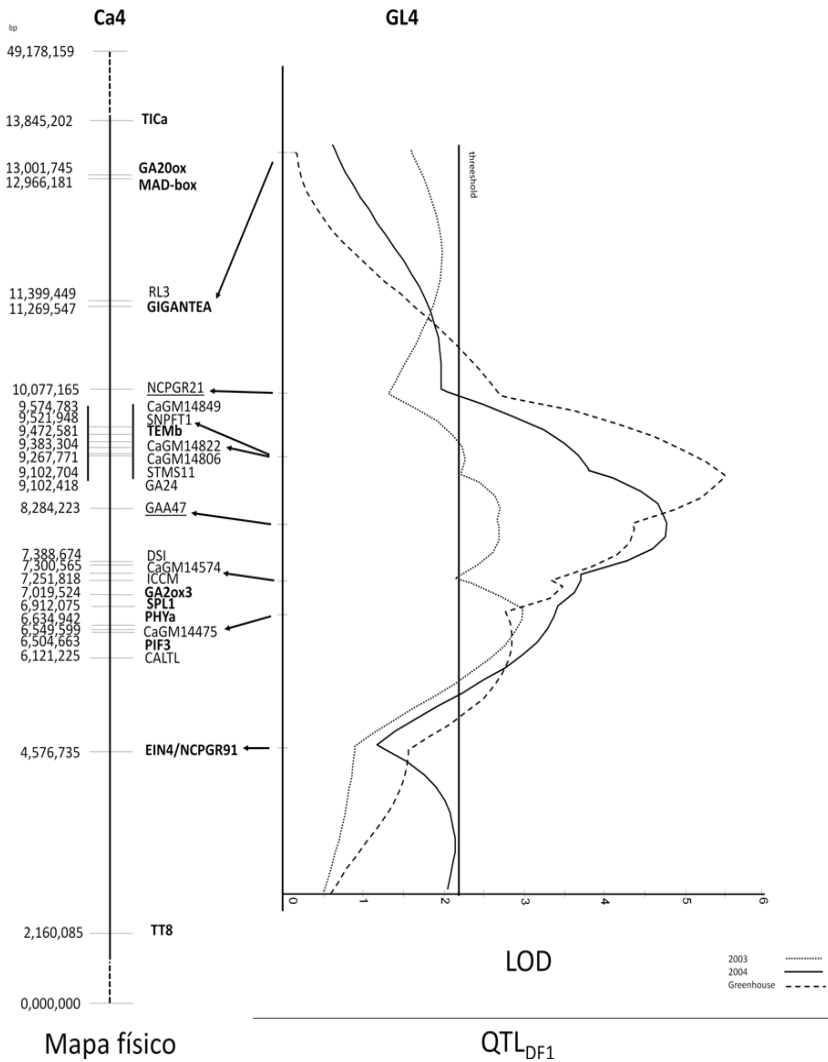


Figure1: A la derecha, el QTLDF1 detectado en el GL4 del mapa de ligamiento de garbanzo (en tres ambientes, 2003, 2004 y en el invernadero) bajo condiciones de día corto. A la izquierda, la posición física de los marcadores mapeados, además de los genes anotados en Ca4 (en negrita) que son homólogos a genes relacionados con floración en *M truncatula*.

Discusión

Las parejas de NILs tienen la ventaja de ser idénticas en el fondo genético excepto en la región genómica relacionada con el carácter de interés, utilizándose para hacer mapeo fino y estudios de expresión, como en soja y arroz (Zhu et al. 2012, Ngu et al. 2014). Rubio et al. (1998) desarrolló las primeras NILs para el carácter simple/doble vaina en garbanzo mediante heterocigosis residual para el carácter en generaciones avanzadas empleando evaluaciones fenotípicas. Sin embargo, Castro et al. (2010) describió las primeras NILs para resistencia a *Fusarium* en garbanzo con la ayuda de marcadores STMS ya que su evaluación fenotípica era más compleja. En nuestro estudio se han utilizado marcadores moleculares estrechamente ligados a dos regiones de interés para fecha de floración junto con evaluación fenotípica, para seleccionar las RILs segregantes, RIL74 para el QTL_{DF2} y RIL82 para el QTL_{DF1}. Esta segregación observada se confirmó a nivel molecular analizando las dos líneas segregantes con los STMS TA142 y GAA47, respectivamente, descritos previamente por Cobos et al. (2007, 2009) como marcadores indicadores para los citados QTLs. El par de NILs obtenidas de la RIL74 (NIL74-25/NIL74-32) sería necesario volver a evaluarlas ya que en el invernadero bajo días cortos no se observaron marcadas diferencias entre ellas sobre todo en la campaña 2013/14. En las últimas evaluaciones realizadas en el invernadero (2014/15) se observó una influencia del sombreado sobre la floración (no todas las plantas recibieron la misma cantidad de luz) siendo esta más precoz en zonas menos sombreadas y resultando que este par de NIL por encontrarse en una zona de mayor sombreado pudieron haberse visto afectadas por este motivo. Incluso el parental precoz WR315, situado en la misma zona floreció mucho más tarde con muy poca diferencia respecto al parental tardío (ILC3279) como se puede ver en la Tabla 1. Así mismo se observó que las descendientes de la NIL74-25 presentaron botones florales 24 días

antes que las de la NIL74-32, pero esto no se tradujo en una mayor diferencia en la floración.

Tradicionalmente las NILs se desarrollan por programas de retrocruzamiento como en maíz y arroz (Mideros et al. 2014 Ngu et al. 2014). En garbanzo la mayoría de las NILs han sido obtenidas a partir de heterocigosis residual en $F_{6,7}$ (Rubio et al. 1998, Castro et al. 2010, Ali et al. 2015) como es el caso de este estudio. El uso de NILs ha sido un método práctico y útil para detectar nuevos QTLs/genes y buscar genes candidatos para nodulación, hábito de crecimiento, doble vaina en garbanzo.

En nuestro estudio, el QTL detectado después de añadir cuatro STMS coincide con el QTL_{DFI} descrito por Cobos et al. (2007) con el marcador GAA47 en 2003 y 2004, confirmando que es un marcador indicativo del QTL_{DFI}. También los marcadores CaGM14822 y SNPFT1 se podrían utilizar junto con el GAA47 para seleccionar plantas de floración precoz que llevan el QTL_{DFI}.

En *Arabidopsis thaliana* se ha estudiado en profundidad la inducción de la floración a nivel genético y epigenético y se han identificado la mayoría de los genes que intervienen en esta ruta como *CONSTANS (CO)*, *FLOWERING LOCUS T (FT)* y *OVEREXPRESSION CO 1 (SOC)* que son los principales activadores, mientras que *TERMINAL FLOWER 1 (TFL)*, *(FLC)* y *TEMPRANILLO 1 (TEM)* son importantes represores de la floración (Shafiq et al. 2014, Wang et al. 2015). En *M truncatula*, planta modelo de las leguminosas, junto con el guisante (planta modelo para días largos) y soja (planta modelo para días cortos), se han identificado genes relacionados con este carácter como *CONSTANS (CO)* y *Flowering time (FT)* (Hecht et al. 2007, Ping et al. 2014). Hasta el momento, solo hay una sola publicación a nivel molecular en garbanzo que identifica genes anotados que podrían estar relacionados con la floración en los cromosomas 1, 2, 3, 4, 5 y 8 basándose en la sintenia con genes

relacionados con este carácter en *A. thaliana* y *M. truncatula* (Weller y Ortega-Martínez, 2015) y que coinciden con regiones genómicas donde previamente se habían localizado QTLs/genes para este carácter (Linchtenzweig et al. 2006, Rehman et al. 2011, Jamalabadi et al. 2013).

La secuenciación del genoma ha sido una herramienta muy eficiente en este trabajo para desarrollar nuevos marcadores STMS que nos han ayudado a delimitar la región donde se localiza el QTL_{DFI}. Además ha sido de gran utilidad para hacer análisis BLAST mediante sintenia de las secuencias de genes relacionados con floración en *M. truncatula* del Chr1, el cual corresponde al Ca4 del garbanzo (Seres et al. 2007, Varshney et al. 2013), y extraer 17 genes homólogos anotados en el genoma de garbanzo. Tres de ellos (GIGANTEA, PHYTOCHROME A y PHYTOCHROME INTERACTING FACTOR) se han situado en Ca4, resultados similares a los obtenidos por Weller y Ortega-Martínez (2015).

El garbanzo es una planta que necesita días largos para florecer como *A. thaliana* en la cual está descrito que *TEMPRANILLO* es un represor de la floración y la mutación en este gen produce plantas de floración temprana en condiciones de días cortos y largos (Osnato et al. 2012). Por lo tanto, considerando su posición en el intervalo donde se localiza el QTL_{DFI} y su función, este gen puede ser un “serio” candidato para fecha de floración en garbanzo, estando presente su alelo para precocidad en la línea JG62. Sería interesante continuar con los estudios de expresión de *TEMPRANILLO* para confirmar su efecto ya que se encuentra en el pico del QTL_{DFI} (entre GAA47 y CaGM14822/SNPFT1 que tienen el máximo LOD).

En conclusión, las dos parejas de NILs desarrolladas en este trabajo para floración pueden ser de gran utilidad para realizar estudios de expresión (después de confirmar la NIL74-25/NIL74-32). Además serían ideales para estudiar el efecto que tienen en la fecha de floración en cada QTL de forma individual. Respecto al QTL_{DFI} (situado en el GL4), el gen

TEM puede ser un serio candidato para explicar las diferencias entre el parental precoz (JG62) y el tardío (CA2156).

DISCUSIÓN GENERAL

Discusión general

Hoy en día, es posible aplicar nuevas herramientas de biotecnología en los programas de Mejora para facilitar y acelerar la obtención de cultivos que incluyan los caracteres deseados. Entre estas técnicas se encuentra la Mejora Asistida por Marcadores (MAS), que ayuda a aumentar la eficiencia de los programas de Mejora, (Morris et al. 2003). El empleo de MAS en garbanzo es reciente. Taran et al. (2013) utilizaron el marcador STMS TA80 para seleccionar plantas de doble vaina en un programa de retrocruzamientos. También se han empleado marcadores moleculares para la selección de genotipos resistentes a rabia (Castro et al. 2013) y resistentes a sequía (Thudi et al 2014). Actualmente en el Grupo de Mejora de Córdoba, donde se ha desarrollado este trabajo de Tesis, se están aplicando marcadores moleculares en un programa de retrocruzamiento para introducir resistencia a fusarium en un cultivar local de garbanzo.

Hasta la última década la mayoría de los mapas genéticos publicados en garbanzo no tenían una elevada densidad de marcadores especialmente en aquellos derivados de cruzamientos intraspecíficos. Este bajo nivel de polimorfismo se debe a que *C. arietinum* tiene una base genética muy estrecha y a que se empleaban métodos que ahora se consideran tradicionales, siendo este un obstáculo para el conocimiento de este cultivo a nivel molecular (Varshney et al. 2007). Esto ha ocurrido para algunos caracteres adaptativos monogénicos (hábito de crecimiento, simple/doble vaina, entre otros) y multigénicos (fecha de floración) donde los estudios publicados estaban enfocados fundamentalmente a genética clásica. Igualmente, el desarrollo del material segregante deseado dependía de la evaluación fenotípica, como por ejemplo en la obtención de NILs para simple/doble vaina (Rajesh et al. 2002). Pero este proceso se ha facilitado con el empleo de marcadores. Así, Castro et al. (2010) utilizaron marcadores moleculares para desarrollar parejas de NILs para resistencia/susceptibilidad a fusarium. En los últimos años, la

secuenciación de genomas en plantas está siendo una herramienta muy útil para hacer mapeo fino y buscar genes candidatos para algunos caracteres como se ha hecho en tomate y patata (Sim et al. 2012, Sharma et al. 2013). En garbanzo, ya se ha publicado su transcriptoma (Garg et al. 2011) y la secuenciación de los genomas completos en dos genotipos, ‘CDC-Frontier’ tipo kabuli (Varshney et al. 2013) y ICC4958 tipo desi (Jain et al. 2013). Ello ha permitido obtener re-secuenciaciones de genotipos que presentan características fenotípicas diferentes al genoma de referencia y así poder identificar marcadores polimórficos, concretamente en el USDA (EEUU) se está haciendo con las líneas de garbanzo JG62 e ILC3279 (Dr. W. Chen, datos sin publicar). Ha habido un gran avance en la aplicación de nuevas tecnologías de secuenciación para desarrollar marcadores SNPs utilizando KAspar, Dart, GBS, entre otros (Thudi et al. 2011, Hiremath et al. 2012, Deokar et al. 2014, Jaganathan et al. 2015). Estas herramientas permiten establecer un puente entre los mapas genéticos disponibles (donde se localizan genes/QTLs para caracteres fenotípicos) y el mapa físico, determinando la posición exacta de los marcadores en el cromosoma y así delimitar regiones de interés. Así se han podido determinar marcadores estrechamente asociados a dos QTLs (QTL_{AR1} y QTL_{AR2}) relacionados con la resistencia a rabia (Madrid et al. 2013; Madrid et al. 2012) o identificar genes para tolerancia a sequía y altas temperaturas (Thudi et al. 2014), y también el factor de transcripción CarERF₁₁₆ que juega un papel importante en la tolerancia a estreses abiótico (Deokar et al. 2015).

El garbanzo tiene la ventaja de tener un tamaño de genoma pequeño (aproximadamente 740 Mb) y presenta una alta homología con la planta modelo *M. truncatula*, ello ha permitido identificar genes candidatos para varios caracteres agronómicos de interés como se describió en Madrid et al. (2012) para resistencia a rabia y para el gen *NSP2* asociado con la nodulación (Capítulo 1 de este trabajo de tesis) (Ali et al. 2014).

Hay que tener en cuenta que un material de gran interés para estudiar y buscar asociación entre regiones del genoma y un carácter en cuestión, son las poblaciones segregantes de líneas homocigóticas o puras, las cuales deben estar cuidadosamente fenotipadas. Tienen la ventaja de que al estar el genotipo fijado, se pueden evaluar en ensayos con repeticiones y en diferentes localidades. Otro material de gran interés son las líneas casi-isogénicas (NILs) que se han empleado con éxito para hacer mapeo fino y buscar genes candidatos en trigo, arroz y maíz (Ali-Benali et al. 2013, Xue et al. 2013, Xie et al. 2014, Benson et al. 2015). Las NILs se pueden desarrollar a partir de programas de retrocruzamiento, pero en nuestro estudio las parejas de NILs se obtuvieron a partir de heterocigosis residual en la $F_{6,7}$. Es cierto que con nuestro método la región recombinante puede ser más amplia que haciendo retrocruzamientos, pero hay que tener en cuenta que el éxito de los cruzamientos en garbanzo es muy bajo y que se obtiene como máximo dos semillas por vaina, por lo que la obtención de NILs por este método requeriría mucho esfuerzo. Las parejas de NILs obtenidas en nuestro grupo proceden de RILs y han sido útiles para detectar nuevos genes/QTLs y hacer mapeo fino para caracteres de interés en esta especie que pueden ser importantes para conseguir mayores rendimientos en garbanzo.

En nuestro estudio, utilizando NILs, se ha mapeado e identificado por primera vez un gen relacionado con la ruta de la inducción de la nodulación (*CaNSP2*) (Ali et al. 2014). Este gen es un factor de transcripción que pertenece a la familia de proteínas GRAS implicadas en el reconocimiento de la señal del factor NOD del *Rhizobium*.

El garbanzo, filogenéticamente es uno de los cultivos más cercanos a *M. truncatula*, manifestándose en el alto grado de sintenia entre ellos (Seres et al. 2007; Millan et al. 2010; Varshney et al. 2013). Por eso, la secuencia del gen *NSP2* en la planta modelo fue útil para diseñar cebadores y amplificar una parte de su homólogo en garbanzo. , Posiblemente el gen

CaNSP2 puede tener una función similar al gen *NSP2* de *M. truncatula* en la inducción de la nodulación.

En nuestro estudio, se desarrolló un marcador en el gen *CaNSP2* que fue dominante (presencia en las plantas que nodulan y ausencia en las que no nodulan). Este marcador se situó en GL5 del mapa genético del garbanzo, coincidente con el gen *rn1* descrito por Davis (1985, 1986). Por lo tanto, este resultado se puede considerar como un pequeño avance para entender la señalización de la nodulación a nivel molecular en garbanzo. Desde el punto de vista de la mejora, sería interesante estudiar la respuesta a la fertilización nitrogenada de las líneas de no-nodulación utilizando las parejas de NILs desarrolladas para nod/no-nod, ya que se sabe que las leguminosas no responden a este tipo de fertilizantes y por lo tanto es un limitante en su producción. Por otro lado un mayor conocimiento de los mecanismos implicados en la fijación nos puede hacer pensar en como manipular estos genes para diseñar plantas más eficientes en la fijación de nitrógeno empleando técnicas de ingeniería genética.

Por lo que sabemos, es la primera vez que se estudia la relación entre porte erecto y semierecto ya que todos los estudios realizados hasta ahora, se han basado en la comparación entre erecto/rastrero, controlado por el gen *Hg1/hg1* situado en el GL3 del mapa genético del garbanzo (Kazan et al 1993, Cobos et al 2009, Aryamanesh et al. 2010). Utilizando dos poblaciones de líneas RILs (RIP-9 y RIP-5) se ha detectado en el GL1 un nuevo QTL (QTL_{Hg2}) el gen *Hg2/hg2*, respectivamente, asociado al hábito de crecimiento. La secuenciación del genoma de garbanzo (Varshney et al. 2013) junto con la re-secuenciación de los parentales (datos no publicados) nos han permitido conocer la posición física de los marcadores asociados al carácter y situarlos en el mapa genético, además de desarrollar nuevos marcadores, especialmente para RIP-5 (cruzamiento intraspecífico) donde el polimorfismo es muy bajo.

Los genes anotados Ca_07000 y Ca_06999 situados en el intervalo de QTL_{Hg2} en el mapa físico podrían ser genes candidatos para el hábito de crecimiento en garbanzo, especialmente porque fueron homólogos a los genes zinc finger predichos en *Glycine max* y *Pisum sativum*, respectivamente. Por lo tanto, el siguiente paso sería saturar la región del QTL con nuevos marcadores haciendo mapeo fino. La pareja de NILs desarrollada en nuestro estudio para porte erecto vs rastrero podría ser un material muy útil para realizar estudios de expresión con el objetivo de estudiar su función en garbanzo.

Además, los marcadores localizados en el pico de QTL_{Hg2} (Ca_07000 e Indel 3) podrían ser aplicados en MAS para este carácter. En condiciones mediterráneas las líneas de porte semi-erecto son más productivas que las erectas (Rubio et al. 2004).

En el presente estudio, el carácter simple/doble vaina se ha mapeado en la región genómica del GL6 utilizando cuatro RIPs diferentes. En las poblaciones RIP-1, RIP-7 y RIP-11, el portador del gen asociado al carácter doble vaina fué el parental JG62, en la otra población RIP CPR-01 fue ICCV96029. Eso quiere decir que ambos parentales podrían llevar el mismo gen (*sfl*). En el capítulo III de esta tesis se describe como se ha saturado la región próxima al locus de simple/doble vaina utilizando cinco parejas de NILs junto con una selección de ocho líneas recombinantes (RILs). La región de interés se delimitó a 92,560 pb flanqueada por los marcadores SDSNP1 y CAGM27777, donde tan sólo hay siete genes anotados. Entre estos, se encuentra el gen *RAX2* que podría ser un candidato potencial para el carácter simple/doble vaina, ya que en *A. thaliana* afecta a la formación del tallo lateral durante el desarrollo de la inflorescencia y se expresa a nivel meristemático (Müller et al. 2006). Estos siete genes se podrían utilizar para hacer estudios de expresión usando las parejas de NILs disponibles. Sería recomendable centrarse en las parejas NIL-3 y NIL-5 ya que el análisis

molecular indicó que la región recombinante en estas NILs es más pequeña. Por el momento, los marcadores desarrollados en este estudio podrán ser muy útiles en MAS, en lugar del microsatélite TA80 (previamente descrito por Rajesh et al. (2002) y que se encuentra más lejano del gen) ya que su ligamiento es mayor y existen menos posibilidades de recombinación entre el marcador y el gen *sfl*.

La fecha de floración es un carácter adaptativo que afecta a la producción en garbanzo. Parte de este trabajo se ha centrado en desarrollar parejas de NILs para los dos QTLs mayores que controlan este carácter, NIL82-3/NIL82-4 y NIL74-25/NIL74-32 para los QTL_{DF1} (GL4) y QTL_{DF2} (GL3) respectivamente. Se han desarrollado nuevos STMS situados en el QTL_{DF1} descrito por Cobos et al. (2007). La secuencia del genoma de garbanzo de referencia también ha sido de gran ayuda para la localización de zonas repetitivas y diseño de cebadores. Además, esta secuencia se utilizó como referencia para hacer BLAST contra los genes relacionados con floración situados en el cromosoma 1 (Chr1) de *M. truncatula* que es el cromosoma homólogo al cromosoma o grupo de ligamiento 4 (Ca4/GL4) de garbanzo (Seres et al. 2007). En total de 13 genes en Chr1 de *M. truncatula*, 12 tuvieron sus homólogos en Ca4 en garbanzo, donde cuatro de los 12 son represores de la transición del estado vegetativo al productivo (Srikanth et al. 2011; Osnato et al. 2012; Song et al. 2013). De estos el gen anotado *TEMPRANILLO* (*TEM*) se situó en el pico del QTL_{DF1}. El garbanzo necesita días largos para su floración al igual que *A. thaliana*, por lo que el gen *TEM* puede ser un potente candidato en QTL_{DF1}.

En resumen, la disponibilidad de información previa sobre el mapa genético de garbanzo y la localización de los marcadores asociados a los caracteres estudiados en este trabajo, además del uso de material apropiado como las parejas de NILs (algunas previamente obtenidas y otras desarrolladas en este estudio), la secuenciación del genoma de esta especie, la información

presente en bases de datos sobre especies modelo, y la aplicación de las técnicas de secuenciación nos ha permitido avanzar en la definición y saturación de regiones genómicas de interés en esta especie cultivada.

CONCLUSIONES

A partir del trabajo realizado se han obtenido las siguientes conclusiones:

Conclusions:

1. *Rn1/rn1* locus, where the recessive mutation impedes nodules formation, was identified as *CaNSP2* gene that could be an important key in nodulation dissection pathway in chickpea.
2. A new genomic region (*QTL_{Hg2}/ HG2* gene) was detected on LG1 related to growth habit. Markers Ca_07000 and Indel3 that showed the maximum LOD in *QTL_{Hg2}* could be used in MAS to distinguish between erect and prostrate genotypes.
3. The *Sfl/sfl* locus position was delimited to 92,560 bp and seven annotated genes were extracted in this genomic area. *RAX2* predicted gene could be a potential candidate gene for simple/double podding trait in chickpea. Expression profiles could be the next step to identify *sfl* gene.
4. Genome sequencing and resequencing data solved the problem related to low molecular diversity among cultivated lines. This information was very useful to develop new microsatellite markers that were used for fine mapping for *sfl* gene and to locate *HG2* related to erect/semierect growth habit, respectively.
5. Two pairs of NILs NIL82-3/NIL82-4 and NIL74-25/NIL74-32 for the QTLs located in *QTL_{DF1}* (LG4) and *QTL_{DF2}* (LG3), respectively were developed. They should be useful to perform expression profiles looking for candidate genes for flowering time in chickpea.
6. *TEMPRANILLO* annotated gene located in the interval of *QTL_{DF1}* could be a serious candidate gene for flowering time in JG62 genotype.
7. Pairs of NILs developed for the studied traits were the basis to detect new QTL/ genes and to perform fine mapping.
8. JG62 genotype is very interesting line for deep molecular studies carrying natural mutations of early flowering, double podding and semi-erect growth habit.

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