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Identification of an STMS marker for the double-podding gene in chickpea

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Abstract Chickpea, a self-pollinating diploid annual with 2n = 2*x* = 16 chromosomes, is an important food legume crop throughout the world and especially in developing countries. A gene that confers double-podding, with the symbol "*s*", is considered important for breeding higher yielding cultivars. Positive effects of "*s*" on yield stability were found by comparing single- and double-podded near-isogenic lines (NILs) derived from a cross of CA-2156 (single-podded) with JG-62 (double-podded). Considering the significant effects on seed yield, the NILs were used to identify molecular markers closely linked to "*s*". Sequence tagged microsatellite site (STMS) markers developed for chickpea, random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers were used to analyze the NILs. Out of 400 RAPD, 100 STMS and 100 ISSR markers, one STMS marker (TA-80) was polymorphic and was used to evaluate a recombinant inbred line population developed from the cross of Surutato-77 (single-podded) × JG-62 (double-podded) for co-segregation of the locus with "*s*". Our results indicated that the marker and "*s*" were 4.84 cM apart. This marker may be used by breeders for marker-assisted selection (MAS) to monitor the incorporation of the double-podding gene into improved germplasm.

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Introduction

Chickpea is the third most important pulse crop in the world and the first in the Mediterranean basin and South Asia (FAO 1996). This legume crop is grown in at least 33 countries including Central and West Asia, southern Europe, Ethiopia, North Africa, North and South America, and Australia (Ladizinsky and Adler 1976; Singh 1997). Although chickpea has high economic value, its world average yield is about 700 kg/ha which is much below its potential of 4,000 kg/ha. One of the main reasons for this is its susceptibility to various biotic and abiotic stresses (Singh et al. 1994), and the development of cultivars tolerant to such stresses would be an ideal approach to overcome this problem. Another direct approach to obtain a higher yield for chickpea is the introgression of various yield-improving traits into cultivated germplasm.

In chickpea, various parameters such as seed mass, seed yield, seed size, etc., contribute to yield (Rao et al. 1994). Double podding is also one such primary trait for yield improvement. This trait is conferred by a single recessive gene that has been assigned the gene symbol "*s*" or "*sfl*" (Khan and Akhtar 1934; Ahmad 1964; Patil 1966; D'Cruz and Tendulkar 1970; Singh and Van Rheenan 1989, 1994). Double-podded cultivars have two flowers per node (Fig. 1) that usually develop into two pods, although the second flower is often irregular and forms pods at variable frequency (Knights 1987). Double-podding can be used to improve the sink capacity of the chickpea plant, which is generally limiting in short-duration environments (Singh and Van Rheenan 1994). Previous results indicated that the double-podding trait could decrease the seed size in chickpea (Singh 1987; Kumar et al. 2000). However, another study using near-isogenic lines reported that "*s*" is not linked to genes affecting seed size and has a positive effect on yield stability (Rubio et al. 1998).

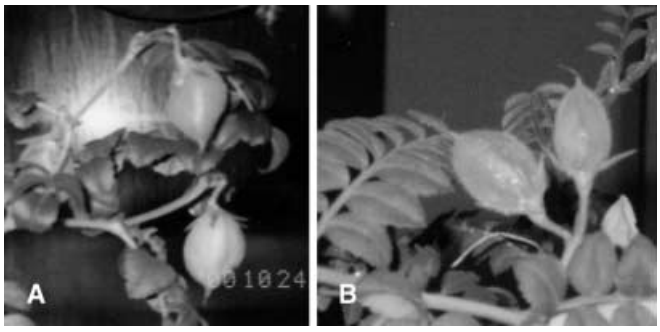

Fig. 1 Representative single-podded (**A**) and double-podded (**B**) plants of chickpea

Considering the agronomic importance and monogenic recessive inheritance of the "*s*" gene, an effort was undertaken to identify DNA markers that are closely linked to this gene which can be used in marker-assisted selection (MAS) to increase chickpea productivity, and also to determine its genomic location.

Materials and methods

Development of mapping population and DNA extraction

The near-isogenic lines (NILs) were developed by crossing CA-2156, a single-podded Spanish Kabuli-type cultivar to JG-62, a double-podded cultivar at the University of Cordoba, Cordoba, Spain (Rubio et al. 1998). From the F_2 to the F_5, single-podded plants in rows that segregated for single- and double-podding were selected. Finally, in segregating F_6 rows, individual single- and double-podded plants were selected and their descendents were sown in a single row in order to eliminate the segregating rows as mentioned in Fig. 2. The plots that were either uniformly single- or double-podded were harvested in separate bulks and designated as NIL4-1V (single-podded) and NIL4-2V (double-podded).

Earlier the donor parent of double-poddedness, JG-62, was also crossed with Surutato-77 (single-podded) and the offspring (F1) was advanced from the F_2 to the F_6 by single seed descent to produce 102 F_6-derived RILs useful for the identification of molecular markers linked to the double-podding character.

DNA was extracted from the leaf tissue of the above-mentioned plant materials according to Doyle and Doyle (1987).

PCR amplification and electrophoresis

One hundred STMS primers reported from the chickpea genome (Huttel et al. 1999; Winter et al. 1999) were synthesized by Gibco-BRL (USA). PCR amplification using these primers was performed in 10 mM of Tris-HCl pH 8.3, 50 mM of KCl, 0.1% TritonX100, 2.5 mM of $MgCl_2$, 0.2 µM of dNTP, 4.5 µM of forward and reverse STMS primers, 1 unit of *Taq* polymerase (Promega, USA) and approximately 20–40 ng of genomic DNA per 20 µl reaction. The amplification was carried out using a Gene Amp PCR system 9700 (Perkin Elmer, USA). After initial denaturation at 94 °C for 2 min, 35 cycles of PCR were carried out. Each cycle comprised 20 s denaturation at 94 °C, 50 s annealing at 55 °C and 2 min extension at 60 °C for 50 s. PCR products were separated on 6% denaturing polyacrylamide gels and stained using a commercially available silver-staining protocol of #Q4132 (Promega, USA). Since STMS markers are locus specific, one band per primer pair was observed and scored in parental lines and in the RIL progeny.

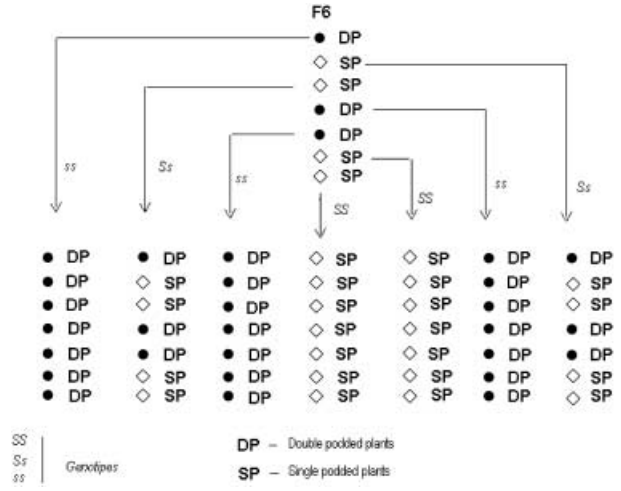

Fig. 2 Scheme for NIL development

Random primers, such as RAPD and ISSR primers (UBC set #9), were obtained from the Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia, Canada. For RAPDs, after initial denaturation of the template at 94 °C for 20 s, the primers were annealed at 36 °C for 1 min followed by elongation at 72 °C for 1 min. This cycle was repeated 40 times.

The ISSR analysis involved 35 cycles of 1 min denaturation at 94 °C, 50 °C annealing for 1 min, and 2 min extension at 72 °C after the initial denaturation at 96 °C for 2 min. The amplified products of RAPD and ISSR primers were run on 2% agarose gels and stained with ethidium bromide.

Linkage analysis

To identify a polymorphic band, NILs were screened with RAPD, STMS and ISSR markers. The use of such NILs along with their original parents for marker analysis would reveal polymorphic markers with more probability of linkage to double-podding. The primers giving polymorphism were then used to evaluate the available RILs. A chi-square test ($P < 0.05$) was performed for the bands polymorphic in the RIL population for goodness of fit to the expected 1:1 ratio. The Kosambi mapping function was used to determine the cM distance between the marker locus and the gene (Kosambi 1994). Linkage analysis was performed at the LOD score of 3.0 using MAPMAKER 3.0 (Lander and Green 1987).

Results

An STMS marker TA-80 is linked to double-podding

We screened 400 RAPD, 100 STMS and 100 ISSR markers between the pair of near-isogenic lines (NILs) along with the parents for polymorphism. One STMS marker (TA-80) was polymorphic between the pair of NILs. Primer sequences were TA-80 F,: CGAATTTTTACATCCGTAATG, and TA-80 R,: AATCAATCCATTTTGCATTC. We then determined the segregation of TA-80 in the $F_{5:6}$ population of 102 RILs. Figure 2 reveals a silver-stained gel showing segregation of TA-80 in the RIL population. Banding patterns for the NILs, CA-2156 and JG-62 (the parents crossed to develop NILs), and Surutato-77 and JG-62 (the parental lines used for developing

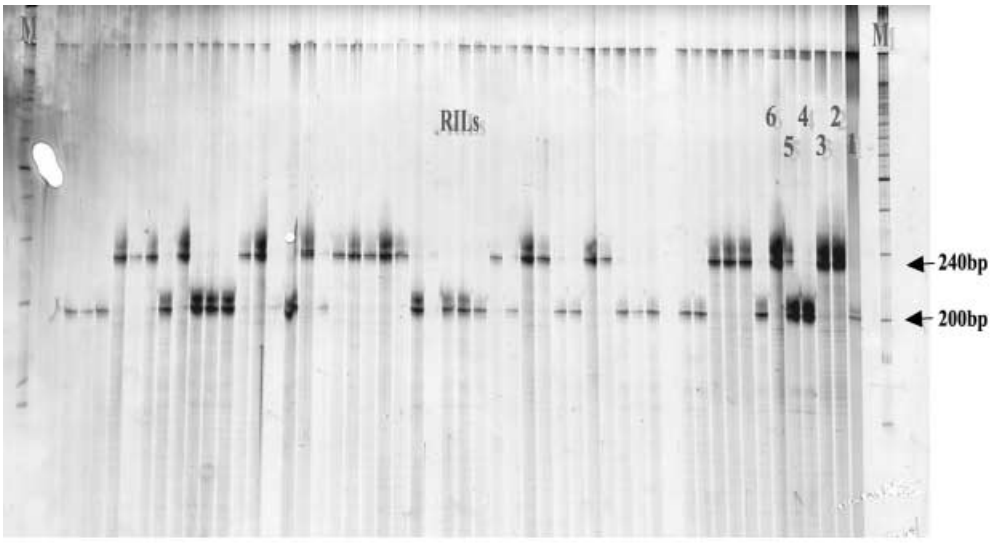

Fig. 3 PCR-amplified STMS pattern on the 6% denaturing polyacrylamide gel. *1* single-podded NIL; *2*-double-podded NIL; *3*- double-podded JG 62; *4* single-podded CA 2156; *5*- single-podded Surutato 77; *6*- double-podded JG 62; *RILs*- recombinant inbred lines; *M*- 50-bp ladder marker

Table 1 Segregation of an STMS (TA-80) marker and "*s*", the gene for double-podding. Mat, maternal; Het, heterozygous; Pat, paternal; X, recombinant; N, number of RILs; Map, map distance in cM; SE, standard error

Locus	Mat	Het	Pat	X	N	Map	SE	Low	High	LOD
TA-80	53	0	49	9	102	4.84	1.69	4.0 (95% confidence)	16.7	17.5
s	56	0	46					3.1 (99% confidence)	19.6	

RILs) (Fig. 3, lanes numbered 1–6), indicate polymorphism for TA-80 depicting a band with a fragment size of 200 bp associated with single-podding, while a band of 240 bp was associated with double-podding.

The $F_{5:6}$ RILs were grown in the greenhouse and the phenotypic expression of the "*s*" gene was monitored after plants started flowering. We observed double-podding in the $F_{4:5}$ RILs also, but did not find a significant difference in the expression of this trait between these two successive generations. Segregation (53:49) for the TA-80 marker fitted the expected 1:1 ratio. Table 1 shows the statistics of the RIL population analysis which indicates that this marker is linked to the double-poddedness gene, "*s*", at a distance of 4.84 cM. Such a close linkage was as expected since the marker TA-80 was polymorphic between the two NILs.

Discussion

The TA-80 marker was earlier mapped to linkage group 6 on the linkage map of *Cicer arietinum* (ICC 4958) and *Cicer reticulatum* (PI 489777), an interspecific cross, which is the most-extensive and comprehensive linkage map for chickpea available to-date (Winter et al. 2000). Hence, the gene controlling the double-podded trait can directly be positioned on linkage group 6 of the linkage map of *Cicer*, even though we studied the linkage between TA-80 and the double-podded trait on an intraspecific cross.

The STMS markers are designed as locus-specific PCR primer pairs based on the sequence information of repeat-flanking regions (Huttel et al. 1999; Winter et al. 1999). These markers have superiority over other molecular markers because they are locus-specific, co-dominant, PCR-based and have a high polymorphic information content and the potential for non-radioactive detection (Mansfield et al. 1994). Our marker TA-80, an STMS marker, can not only be used for identification of the allelic state of the marker locus itself, but also allows it to infer information about the allelic state of the "*s*" locus. It will also be effective to eliminate linkage drag during marker-assisted backcrossing.

Role of double-podding in yield improvement

There have been some reports in the past indicating the positive effect of double-podding on chickpea crop yields (Sheldrake et al. 1978; Singh and Van Rheenan 1989, 1994). On the other hand, Knights (1987) reported that it had no effect on yield in diverse genetic backgrounds. Constitution of the different backgrounds is necessary as it plays a role in the expressivity of the "*s*" allele (Kumar et al. 2000). In order to study the effects of double-podding on yield, it was suggested that true isogenic lines should be developed and evaluated in diverse environments (Knights 1987). Rubio et al. (1998) developed NILs for the double-podding trait and tested it at five locations over 2 years to study the role of this trait

in yield. The results revealed that the double-podded NILs had more yield stability when compared to single-podded lines. Similarly, in another study by Kumar et al. (2000), this trait showed stability for the seed yield though it had unstable penetrance and variable expressivity. These results indicated that the double-podded trait conferred more yield stability than the single-podded trait. Therefore, a tightly linked marker to this gene can be utilized to exploit the agronomic importance of this trait.

In conclusion, we have identified TA-80 as a reliable marker that can be used in marker-assisted selection, which would allow plant breeders to perform indirect selection of the double-podded trait prior to phenotypic expression. This is the first time that a molecular marker linked to double-poddedness, has been identified, and both NILs and RILs were used in this endeavor.

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