

Stability analysis of chickpea large genomic DNA inserts in *Agrobacterium*

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Genetic transformation is a powerful tool for plant genetic studies and serves an important role in complementation analyses. Gene isolation is often followed by complementation analysis to verify function. *Agrobacterium tumefaciens*-mediated transformation (AMT) is a suitable technique to conduct complementation analyses in grain legume species. Development of binary bacterial artificial chromosome (BIBAC) and transformation competent artificial chromosome (TAC) vectors and direct cloning of large genomic inserts (>30 kb) into these vectors have made complementation analysis easier and quicker. The ability to transform large inserts will eliminate the necessity to subclone and verify gene presence in smaller DNA fragments amenable to traditional binary vectors. Use of large insert libraries in successful transformation through AMT in rice (*Oryza sativa*) (<http://www.irri.org/science/abstracts/pdfs/RGIII/Agrobac12.pdf>) and *Bacopa monniera* (Nisha et al. 2003) is well established.

Chickpea (*Cicer arietinum*) is an important cool season legume crop worldwide and standardized protocols and application of genomic tools are essential for chickpea genome analyses. Since direct genetic transformation using *A. tumefaciens* is feasible in chickpea, a bacterial artificial chromosome (BAC) library with an average insert size of 100 kb and 3.5X genome coverage was constructed using the binary vector, pCLDO4541 (V41) (Rajesh et al. 2004). AMT of large DNA inserts directly into plants facilitates the transfer of gene clusters and flanking regulatory elements. Hence, it is recommended that the integrity of large genomic fragments in *A. tumefaciens* be verified prior to plant transformation.

In this study, we investigated the stability of inserts from chickpea BAC clones, 4m10, 4n10, 4o10, 4p10, 15o9, 40c9 and 7n13 ranging in size from 20 kb to 100 kb in *A. tumefaciens* strain *AgI0* by triparental mating. These large DNA inserts (Table 1) represent the genomic region linked to Ascochyta blight resistance with the exception of clone 7n13 (~100 kb) which represents the genomic region linked to Fusarium wilt race 3 resistance (Rajesh et al. 2004). Their sequences are available at http://www.genome.ou.edu/plants_totals.html. These clones have high, medium or low levels of repetitive DNA based on the frequency of repetitive elements; 40c9 and 4n10

are highly repetitive, 15o9 is moderately repetitive and 4m10, 4o10 and 4p10 have a low content of repetitive DNA.

Triparental mating

Single colonies of *AgI0*, pRK2013, a helper plasmid and the BAC clones were streaked and grown on Luria Bertani (LB) medium in the presence of rifampicin (20 µg ml⁻¹), kanamycin (75 µg ml⁻¹) and tetracycline (15 µg ml⁻¹). *AgI0* was grown for two days at 25°C and pRK2013 and BAC clones were grown overnight at 37°C. Mating occurred by mixing a loop sample of *AgI0*, pRK2013 and each BAC clone separately on an LB plate in the absence of selection for two days at 25°C. Control matings were conducted with mixtures of only *AgI0* and pRK2013 and were grown in the absence of selection. After two days, the cell mass was streaked on LB agar containing rifampicin (20 µg ml⁻¹) and tetracycline (15 µg ml⁻¹) to isolate single colonies. Single colonies from each transformation were picked and grown in 10 ml of LB medium for two days at 25°C in the presence of rifampicin (20 µg ml⁻¹) and tetracycline (15 µg ml⁻¹). The BAC plasmids from *Escherichia coli* were isolated in the purified form using the BAC₉₆ Miniprep kit (Millipore, USA). BAC plasmids of each transformation by triparental mating were digested with *NotI* (NEB, USA) overnight at 37°C and then loaded on a 1% agarose gel. The gel was subjected to Pulsed Field Gel Electrophoresis (PFGE) overnight at 6V/cm in 0.5X TBE at 16°C using a 1–12 sec discontinuous ramped switch time for 15 h using the CHEF DRIII system (Bio Rad, USA) (Fig. 1). Stability of the clones in *A. tumefaciens* was assessed by transforming the BAC clones back into *E. coli* - ElectroMAX™ DH10B™ strain and evaluated using PFGE.

Our results show that large inserts up to 100 kb can be stably transformed into *AgI0* by triparental mating (Fig. 1). Although deletions occurred with all the large inserts used in this study when transformed into *AgI0*, individual colonies containing plasmids with complete inserts were obtained. It is reported that tandemly repeated sequences may cause instability of large inserts in *E. coli* (Ishiyama et al. 1989, Song et al. 2003). In our study, despite the

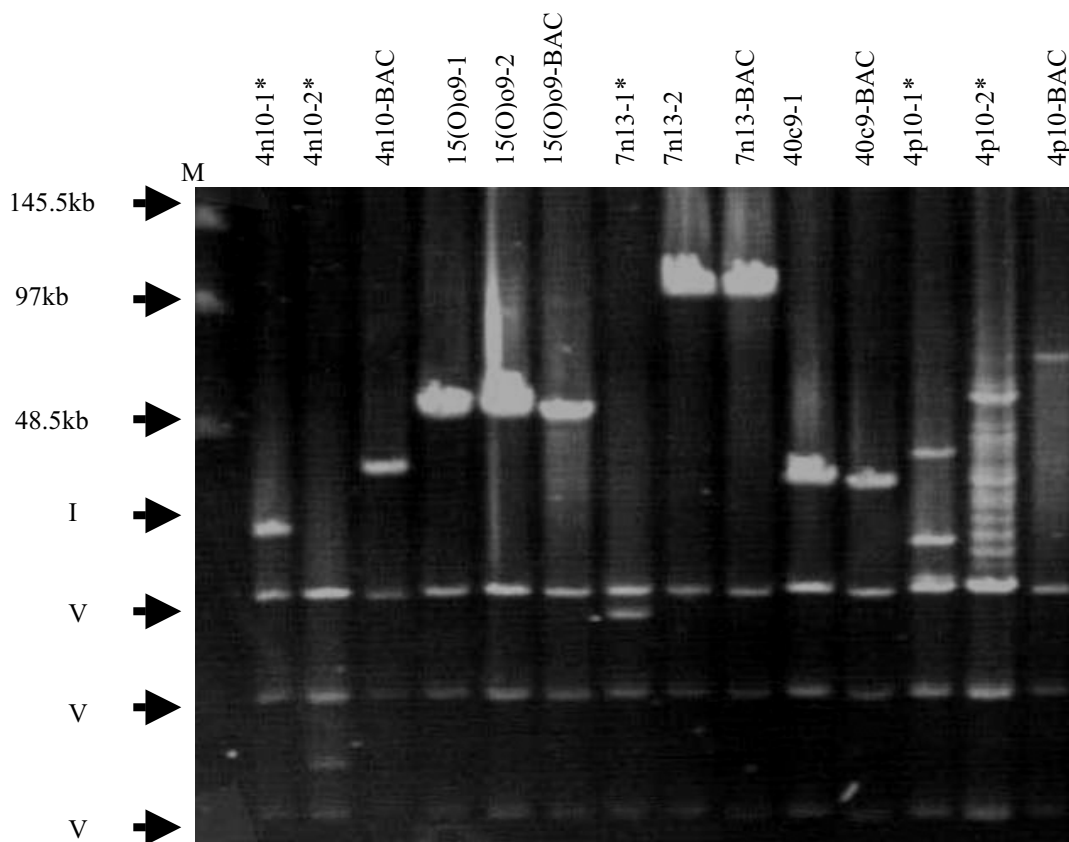


Figure 1. A representative PFGE image of *NotI* digested BAC clones which were transformed back into *E. coli* from *AgI0* compared to the original BAC clones. (Note: V - Vector; I - Insert; * - Unstable BAC clones; 7n13 - The insert size is ~100 kb.)

Table 1. Insert size of original BAC clones based on *NotI* digestion.

BAC clone	Insert size (kb)	BAC clone	Insert size (kb)
4m10	20	15o9	50
4n10	35	40c9	35
4o10	65	7n13	100
4p10	55		

presence of intact plasmids, evidence of deletions from individual clones irrespective of repetitive element frequency was observed in *AgI0*. This result emphasizes the need to verify the presence and integrity of the plasmid prior to transformation.

In summary, transformation of large inserts into chickpea has not been reported in the literature. Our results indicate that chickpea genomic DNA fragments up to 100 kb in size can be stably transformed into *A. tumefaciens* strain *AgI0* through triparental mating and stably maintained in *A. tumefaciens* and *E. coli* thus improving the prospects for successful transformation.

References

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