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BIBAC and TAC clones containing potato genomic DNA fragments larger than 100 kb are not stable in *Agrobacterium*

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Abstract Development of efficient methods to transfer large DNA fragments into plants will greatly facilitate the map-based cloning of genes. The recently developed BIBAC and TAC vectors have shown potential to deliver large DNA fragments into plants via *Agrobacterium*-mediated transformation. Here we report that BIBAC and TAC clones containing potato genomic DNA fragments larger than 100 kb are not stable in *Agrobacterium*. We tested the possible factors that may cause instability, including the insert sizes of the BIBAC and TAC constructs, potato DNA fragments consisting of highly repetitive or largely single-copy DNA sequences, different *Agrobacterium* transformation methods and different *Agrobacterium* strains. The insert sizes of the potato BIBAC and TAC constructs were found to be critical to their stability in *Agrobacterium*. All constructs containing a potato DNA fragment larger than 100 kb were not stable in any of the four tested *Agrobacterium* strains, including two *recA* deficient strains. We developed a transposon-based technique that can be used to efficiently subclone a BAC insert into two to three BIBAC/TAC constructs to circumvent the instability problem.

Keywords BAC · BIBAC · TAC · *Agrobacterium* · Plant transformation

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Introduction

Map-based cloning has become an increasingly effective method to clone plant genes based on their genetic map positions without prior knowledge of their function (Pflieger et al. 2001; Jander et al. 2002). In general, a map-based cloning procedure requires development of a physical map of the genomic region spanning the target gene. After narrowing potential genomic DNA clones containing the target gene to a manageable number, it is usually necessary to complement the plant with a mutated target gene by transformation with the candidate clones. Thus, development of transformable constructs for complementation analysis is a key step in the map-based cloning process.

Agrobacterium-mediated transformation has become one of the most popular techniques for functional complementation and gene verification in plants (Gelvin 2000). Traditional binary vectors used in *Agrobacterium*-mediated transformation can hold relatively small DNA fragments, usually less than 15 kb. Thus, complementation analysis of a large DNA contig resulting from a map-based cloning project would require a daunting amount of subcloning and transformation work. The ability to directly introduce large DNA fragments into plants could greatly reduce the time and effort required to identify the target gene in a map-based cloning project. Large constructs would also ensure inclusion of complete gene clusters or large genes and all flanking regulatory elements in complementation analysis.

The development of the binary bacterial artificial chromosome (BIBAC) and transformation-competent artificial chromosome (TAC) vectors has made significant contributions to *Agrobacterium*-mediated transformation with large DNA fragments (Hamilton 1997; Liu et al. 1999; Shibata and Liu 2000). BIBAC and TAC clones can hold DNA fragments larger than 100 kb and can be directly used for *Agrobacterium*-mediated transformation. Transfer of large DNA fragments into plants via BIBAC and TAC constructs has been documented in tobacco (Hamilton et al. 1996), tomato (Hamilton et al. 1999),

Table 1 Potato BAC clones used in the stability study

BACs	Sizes (kb)	Characteristics	Reference
15K16	110	Derived from centromeric regions, containing highly repetitive sequences	Unpublished results
15D22	115	Derived from centromeric regions, containing highly repetitive sequences	Unpublished results
49N10	180	Containing mainly single-copy sequences	Bradeen et al. 2003
52M2	170	Containing mainly single-copy sequences	Bradeen et al. 2003
157M5	125	Containing mainly single-copy sequences	Bradeen et al. 2003
6M21	120	Containing mainly single-copy sequences	Dong et al. 2000

Arabidopsis thaliana (Liu et al. 1999) and *Brassica napus* (Cui et al. 2000). A number of BIBAC and TAC libraries have been developed recently (Hamilton et al. 1999; Liu et al. 1999, 2000; Wu et al. 2000; Tao et al. 2002). These libraries have greater potential application than traditional BAC libraries because every BIBAC/TAC clone could be used for functional studies.

Toward establishment of an efficient potato transformation system using large insert binary vectors, we developed BIBAC and TAC constructs using BAC clones with different insert sizes and containing different types of DNA sequence. All BIBAC and TAC constructs containing a potato DNA fragment larger than 100 kb were highly unstable in different *Agrobacterium* strains. The possible causes of the instability of BIBAC and TAC constructs in *Agrobacterium* are discussed. To solve this problem we developed a technique to manipulate BAC insert sizes by inserting *Not*I sites using an in vitro transposon approach.

Materials and methods

Materials

Six BAC clones (Table 1) were selected from the *Solanum bulbocastanum* BAC library (Song et al. 2000). These clones contain either mainly single-copy DNA sequences or highly repetitive DNA elements. The BIBAC2 and BIBAC4 vectors were provided by Dr. Carol Hamilton, Plant Science Center, Cornell University (Ithaca, N.Y.). BIBAC2 and BIBAC4 are essentially the same vector and use kanamycin and *bar*, respectively, as the plant selection markers. The TAC vector was provided by Dr. D. Shibata, Mitsui Plant Biotechnology Research Institute (Tsukuba, Japan). Four different *Agrobacterium tumefaciens* strains (LBA4404, GV3101, COR308 and COR309) were used in the stability analysis.

Cloning of BAC inserts into BIBAC and TAC vectors

DNA from overnight cultures of BAC clones and BIBAC and TAC vectors was isolated via the standard alkaline-lysis method (Sambrook et al. 1989). Vector DNAs were digested to completion with *Not*I followed by de-phosphorylation with HK phosphatase (Epicentre, Madison, Wis.). Approximately 100 ng of BAC DNA were digested with *Not*I and incubated at 37 °C for 2 h. The sample was then mixed with 20 ng of de-phosphorylated vector DNA, incubated at 65 °C for 10 min to inactivate the restriction enzyme, and cooled to room temperature. Two units of T4 DNA ligase (USB, Cleveland, Ohio) was added in a total volume of 50 μl and the sample incubated overnight at 16 °C. The ligation product was transformed into ElectroMax DH10B cells (Life technologies, Rockville, Md.) using the Cell-Porator system (Life technologies) under the following electroporation conditions: voltage, 350 V; capacitance, 330 μF; impedance, low ohms; charge rate, fast; voltage boost resistance, 4 K. The cells were immediately resuspended in 1 ml of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast Extract, 10-mM NaCl, 2.5-mM KCl, 10-mM $MgCl_2$, 10-mM $MgSO_4$, and 20-mM glucose, pH 7.0) and incubated at 37 °C with shaking at 225 rpm for 1 h. The samples were then plated on LB plates containing 5% sucrose and kanamycin (50 μg/ml) and the plates were incubated overnight at 37 °C.

Argobacterium transformation

The recombinant BIBAC and TAC plasmids were isolated and introduced into *A. tumefaciens* strains by triparental mating (Ditta et al. 1980) or electroporation using the following conditions: voltage, 330 V; capacitance, 330 μF; impedance, low ohms; charge rate, fast; voltage boost resistance, 4 K. After electroporation the cells were immediately re-suspended in 1 ml of SOC medium, and incubated at 28 °C with shaking at 225 rpm for 2–3 h. *A. tumefaciens* colonies were selected on LB plates containing 50 μg/ml of kanamycin and 100 μg/ml of streptomycin for strains LBA4404 and GV3101, and 50 μg/ml of kanamycin, 100 μg/ml of streptomycin and 5 μg/ml of tetracycline for strains COR308 and COR309. For triparental mating, the transconjugants were selected on 50 μg/l of kanamycin, 100 μg/l of streptomycin and 5 μg/l of tetracycline for strain COR309. After incubation at 28 °C for 2–3 days, colonies were randomly selected for restriction analysis.

Pulsed field gel electrophoresis (PFGE)

BAC clones and *A. tumefaciens* colonies were inoculated into 1.5 ml of LB and incubated at 37 °C with shaking for 16 h. The plasmid DNA was isolated and digested with *Not*I to release the DNA insert. The digested DNA was separated by PFGE on a CHEF-DRII apparatus (Bio-Rad laboratories, Hercules, Calif.) using the following conditions: 1% agarose, 6 V/cm, 0.5× TBE, 14 °C with a linear pulse time from 5 to 15 s for 16–20 h.

In vitro insertion of NotI restriction sites in BAC inserts

The GPS-1 Genome Priming System (New England Biolabs, USA) was used to insert *Not*I restriction sites into BAC inserts. The transposon insertion reaction mixture included 2 μl of 10× GPS buffer (250-mM Tris-HCl, pH 8.0, 20-mM DTT and 20-mM ATP), 1 μl of pGPS1.1 donor DNA (0.02 μg) and 0.1–0.2 μg of BAC plasmid DNA. The volume of the mixture was adjusted to 18 μl with H_2O. One microliter of the TnsABC was added to the mixture and the sample was incubated at 30 °C for 10 min. One microliter of start solution [300 mM of $Mg(CH_3COO)_2$] was added to the mixture and the sample was incubated at 30 °C for 1 h. The mixture was heat-inactivated at 75 °C for 10 min and then introduced into ElectroMax DH10B cells by electroporation. The cells were selected on LB plates containing 20 μg/ml of kanamycin and 12.5 μg/ml of chloramphenicol. The insert sizes of randomly

selected clones were estimated by *Not*I digestion followed by PFGE analysis.

Results

Large-insert BIBAC and TAC clones can be stably maintained in *Escherichia coli*

Six potato BAC clones were selected in this study (Table 1). These six BAC clones have different insert sizes and contain either largely single-copy DNA or highly repetitive DNA sequences. Five of the six potato BAC clones yield a single potato fragment following digestion with *Not*I, indicating that none of these BACs, with the exception of 49N10, contain an internal *Not*I restriction site. Clone 49N10 contains a single *Not*I site within the insert, yielding two potato bands upon *Not*I digestion.

The inserts of the six BAC clones were transferred into BIBAC or TAC vectors. *Not*I digestion of the BIBAC4 clones derived from BACs 15K16 and 15D22 showed that the insert sizes were identical to those of the original BAC clones (Fig. 1). The *Hin*dIII restriction patterns of the BIBAC4 clones were also identical to the original BAC clones with the exception of vector-derived bands, further confirming that the BAC inserts were transferred into the BIBAC4 vector and were stably maintained in *E. coli* (data not shown). Similar results were obtained for all the BIBAC2, BIBAC4 or TAC clones derived from the BAC clones listed in Table 1. No instability was observed for any BIBAC or TAC clones in *E. coli*.

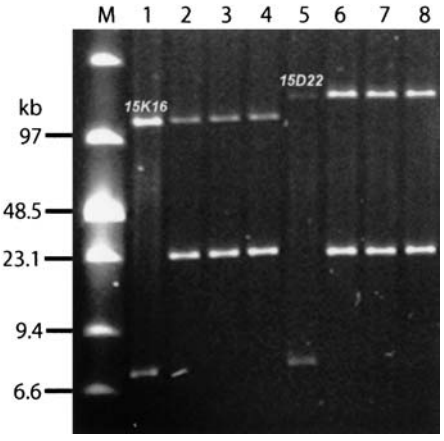

Fig. 1 Stability analysis of BIBAC4 clones in *E. coli*. The inserts of BACs 15K16 and 15D22 were transferred into the BIBAC4 vector. BACs 15K16 (*lane 1*) and 15D22 (*lane 5*) and three randomly selected BIBAC4 clones (*lanes 2–4* and *6–8*, respectively) were digested with *Not*I, and the DNA fragments separated by PFGE. The sizes of the inserts from the BIBAC4 clones were identical to their original BAC clones. The sizes of the BAC and BIBAC vectors are 7.4 kb and 23.5 kb, respectively

Instability of BIBAC and TAC clones in *Agrobacterium*

Two BIBAC4 clones, derived from BACs 15K16 (110 kb) and 15D22 (115 kb) respectively, were used in our first *Agrobacterium* transformation experiment. DNAs from the two BIBAC4 clones were transformed into *A. tumefaciens* strain COR309 by electroporation. In contrast to the results with *E. coli*, all BIBAC4 inserts in COR309 clones were significantly smaller than the original BAC inserts or were missing altogether (Fig. 2A). BIBAC4 constructs were also isolated from several COR309 clones and introduced back into *E. coli* strain DH10B. Subsequent analysis confirmed that significant deletions of these constructs had occurred in COR309 (Fig. 2B).

BACs 15K16 and 15D22 contain highly repetitive DNA elements derived from the centromeric regions (Song and Jiang, unpublished results). To explore the possibility that the repetitive nature of the inserts of these two BACs caused the instability in *Agrobacterium*, we conducted similar experiments using three potato BAC clones which contain largely single-copy DNA sequences. These three BACs, 49N10, 52M2 and 157M5, originate from a BAC contig spanning the late blight resistance gene *RB* (Naess et al. 2000; Bradeen et al. 2003), and are

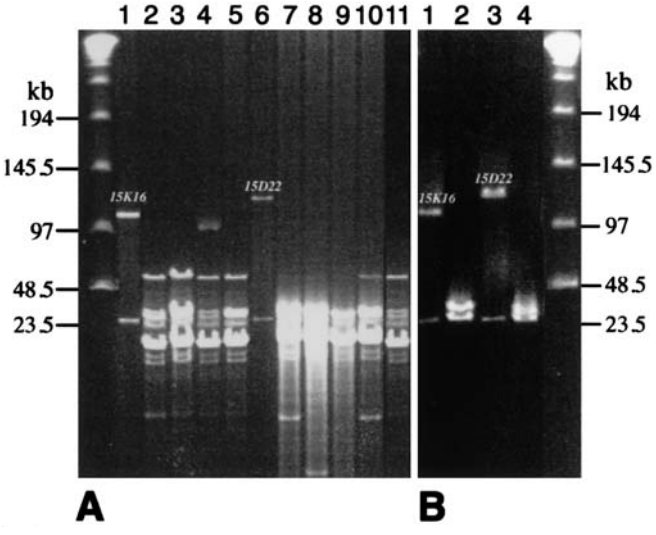

Fig. 2A, B Stability analysis of BIBAC4 clones in *Agrobacterium*. (**A**) The BIBAC4 15K16 and 15D22 were transformed into COR309 by electroporation. DNA was isolated from the original BIBAC4 15K16 (*lane 1*) and 15D22 (*lane 6*) and four randomly selected COR309 clones (*lanes 2–5* and *7–10*, respectively), and digested with *Not*I. *Lane11* is an untransformed COR309 clone. The potato inserts in all COR309 clones were either not observed or significantly deleted. The largest potato DNA fragment observed was approximately 95 kb (*lane 4*). (**B**) The plasmid DNA from two COR309 clones (materials from Fig. 2A, *lanes 2 and 7*, respectively) was transferred back into *E. coli*. DNA was then isolated from *E. coli* clones (*lanes 2 and 4*) and digested with *Not*I. A band that was only slightly larger than the 23.5-kb vector band was observed in *lanes 2 and 4*, confirming that portions of the potato DNA inserts in the COR309 clones were deleted

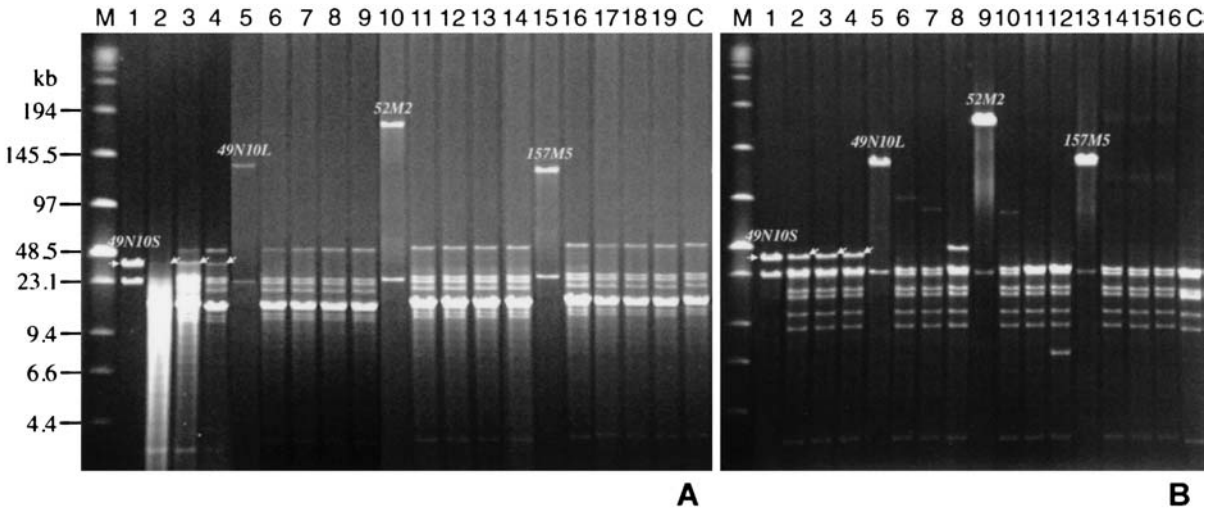

Fig. 3A, B Stability analysis of BIBAC2 clones in *Agrobacterium*. (**A**) BIBAC2 49N10S (*lane 1*), 49N10L (*lane 5*), 52M2 (*lane 10*) and 157M5 (*lane 15*) were transformed into *Agrobacterium* strain COR309 by electroporation. Plasmid DNA from randomly selected COR309 clones of 49N10S (*lanes 2–4*), 49N10L (*lanes 6–9*), 52M2 (*lanes 11–14*) and 157M5 (*lanes 16–19*) was analyzed by PFGE after *Not*I digestion. (**B**) BIBAC2 49N10S (*lane 1*), 49N10L (*lane 5*), 52M2 (*lane 9*) and 157M5 (*lane 13*) were transformed into *Agrobacterium* strain LBA4404 by electroporation. Plasmid DNA from randomly selected LBA4404 clones of 49N10S (*lanes 2–4*), 49N10L (*lanes 6–8*), 52M2 (*lanes 10–12*) and 157M5 (*lanes 14–16*) was analyzed by PFGE after *Not*I digestion. Only the COR309 and LBA4404 clones derived from BIBAC2 49N10S show inserts with the same sizes as the original BIBAC clones (*arrows*). The potato inserts in all COR309 and LBA4404 clones derived from 49N10L, 52M2 and 157M5 were either not observed or partially deleted. A LBA4404 clone in *lane 6* (*B*) showed the largest potato DNA fragment of approximately 95 kb. The two *lane Cs* are untransformed COR309 and LBA4404 clones. *Arrows* point to the unchanged potato DNA fragments from the BIBAC2 49N10S constructs

located in a euchromatic region based on flourescence in situ hybridization on potato meiotic pachytene chromosomes (unpublished results).

*Not*I digestion of clone 49N10 released fragments of approximately 45 kb and 135 kb. These fragments, 49N10S and 49N10L, respectively, were subcloned into the BIBAC2 vector. BIBAC2 constructs from 49N10S, 49N10L, 52M2 and 157M5 were delivered into *A. tumefaciens* strains LBA4404, GV3101, COR308 and COR309 by electroporation. The smallest construct, 49N10S (45 kb), was maintained stably in all four strains (Figs. 3A, B). However, the inserts of the three other BIBAC2 constructs were significantly smaller than the original sizes or were missing altogether in all *Agrobacterium* strains (Figs. 3A, B). The largest potato DNA fragment observed in a LBA4404 clone was approximately 95 kb (Fig. 3B). Most of the rearranged potato inserts of the other three constructs were smaller than 50 kb.

BAC 6M21 (120 kb) was identified by a potato DNA marker GP264 that was mapped to the terminal region on the short arm of potato chromosome 1 (Dong et al. 2000). FISH using BAC 6M21 generated only limited cross-hybridization signals in other chromosomal regions (Dong et al. 2000), indicating that this clone contains mainly single-copy sequences. A BIBAC4 construct was developed from 6M21 and was introduced into COR309. Significant deletions of the BIBAC4 constructs were observed in all COR309 clones analyzed (data not shown).

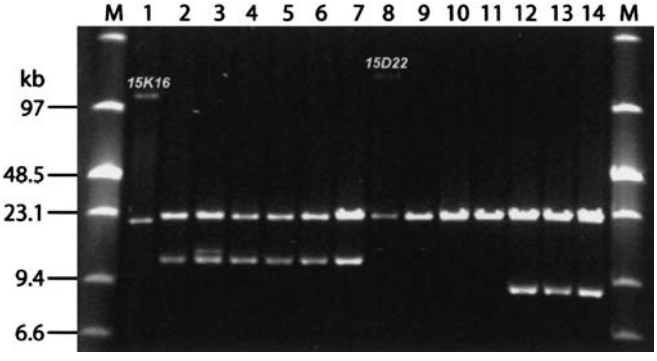

Fig. 4 Stability analysis of BIBAC4 15K16 and 15D22 introduced into *Agrobacterium* strain COR309 by triparental mating. *Lanes 1 and 8* are original 15K16 and 15D22 BIBAC4 clones. The plasmid DNA was isolated from COR309 clones and transferred back into *E. coli*. *Lanes 2–3* and *4–6* are *E. coli* clones derived from two different COR309 clones containing BIBAC4 15K16. *Lanes 9–11* and *12–14* are *E. coli* clones derived from two different COR309 clones containing BIBAC4 15D22. Significant deletion of the potato inserts was observed in all *E. coli* clones

Similar analysis was also conducted using TAC constructs. The inserts of potato BAC 15K16 and 15D22 were transferred into the TAC vector and delivered into COR309 by electroporation. As with the BIBAC constructs, all COR309 clones analyzed showed significant deletions of the TAC constructs (data not shown).

We next explored the possibility that the electroporation process itself caused severe rearrangements of the

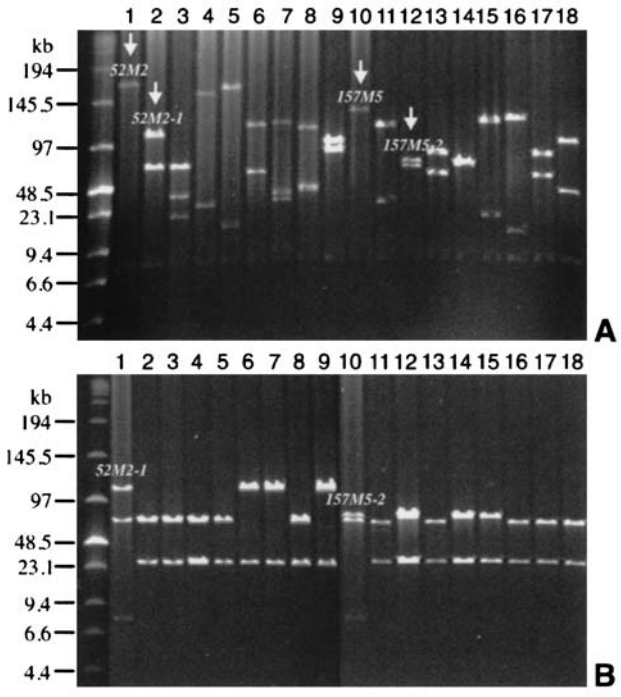

Fig. 5A, B BAC insert size manipulation by inserting *Not*I sites using a transposon-based approach. (**A**) *Not*I digestion and PFGE analysis of eight clones derived from BAC 52M2 (*lanes 2–9*) and eight clones derived from BAC 157M5 (*lanes 11–18*). Most clones had a single *Not*I site insertion, resulting in two DNA fragments. However, clones in *lanes 3 and 7* had two *Not*I site insertions, resulting in three DNA fragments. *Lanes 1 and 10* show the original BACs 52M2 and 157M5. (**B**) Subcloning of four cleaved BAC inserts into the BIBAC2 vector, including two fragments from clone 52M2-1 (*lane 2* in Fig. 5A), which are 70 kb and 110 kb, respectively, and two fragments from clone 157M5-2 (*lane 12* in Fig. 5A), which are 60 kb and 65 kb, respectively. *Lanes 1 and 10* show *Not*I-digested DNA from BACs 52M2-1 and 157M5-2. *Lanes 2–9* and *lanes 11–18* show *Not*I-digested DNA from randomly selected BIBAC2 subclones

larger BIBAC constructs. The traditional triparental mating technique was used to transform *Agrobacterium*. Two BIBAC4 constructs derived from 15K16 and 15D22 were introduced into COR309 by triparental mating. Plasmid DNAs were isolated from COR309 clones and introduced back into *E. coli* strain DH10B. Significant deletions were again found in both BIBAC4 constructs (Fig. 4).

Transposon-based manipulation of BAC insert sizes

The results using different constructs, different *Agrobacterium* strains and different transformation methods suggest that the sizes of the BIBAC and TAC inserts are the critical factor causing instability of the BIBAC/TAC constructs. In these experiments, the largest insert, which the BIBAC vectors could hold and maintain in the currently available *Agrobacterium* strains, was approximately 95 kb (Fig. 3B). As the average insert size of our potato BAC library is 155 kb (Song et al. 2000), the BAC inserts of most of the clones in the library must be fragmented into two or more pieces before they can be stably maintained as BIBAC or TAC constructs for transformation.

The potato genome has relatively few *Not*I restriction sites and more than 99% of the clones in our potato BAC library contain no *Not*I sites (Song et al. 2000). We designed a transposon-based approach to manipulate the insert sizes of potato BAC clones (see Materials and methods). GPS-1 (New England Biolabs, Beverly, Mass.) is a Tn7 transposon-based in vitro system which uses TnsABC transposase to insert a transposon randomly into the DNA target. The transposon in this system contains a single *Not*I site and a kanamycin resistance marker. Thus, a BAC clone containing an inserted Tn7 transposon will grow on LB plates supplemented with both kanamycin and chloramphenicol. Insertion of a single transposon into

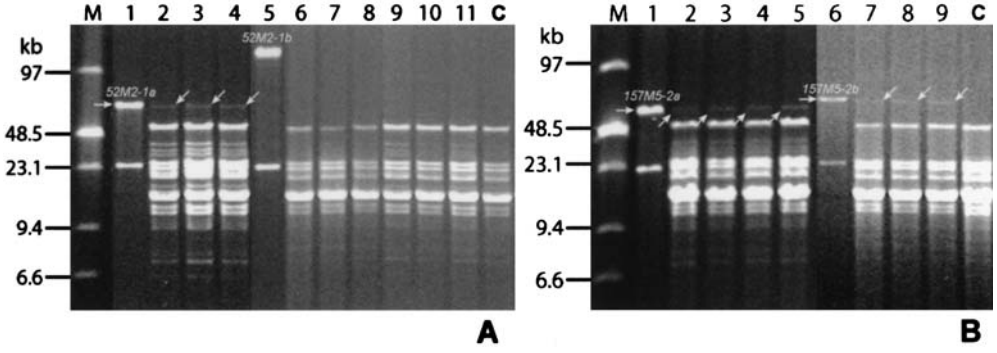

Fig. 6A, B Stability analysis of BIBAC2 subclones in *Agrobacterium* strain COR309. (**A**) BIBAC2 52M2-1a and 52M2-1b, which were subcloned from BAC 52M2, contain inserts of 70 kb and 110 kb, respectively. (**B**) BIBAC2 157M5-2a and 157M5-2b, which were subcloned from BAC 157M5, contain inserts of 60 kb and 65 kb, respectively. The COR309 clones containing 52M2-1a, 157M5-2a and 157M5-2b can all be stably maintained in *Agrobacterium*. However, the potato inserts in all COR309 clones containing 52M2-1b are either smaller than the expected size or are missing altogether. The two *lane Cs* are untransformed COR309 clones. *Arrows* point to the unchanged potato DNA fragments from the BIBAC2 constructs

a BAC clone insert would result in two DNA fragments after *Not*I digestion.

Each in vitro transposon insertion reaction yielded more than 2×10^4 insertion clones. The *Not*I digestion and PFGE analysis of BAC 52M2 and 157M5 clones derived from transposon insertion experiments showed that most of the BACs contain a single transposon, which results in two *Not*I fragments (Fig. 5A). A few BACs contain two transposons, as evidenced by three *Not*I fragments (Fig. 5A). The two *Not*I fragments from a 52M2 clone (lane 2 in Fig. 5A), 70 kb and 110 kb, respectively, and two *Not*I fragments from a 157M5 clone (lane 12 in Fig. 5A), 60 kb and 65 kb, respectively, were subcloned into the BIBAC2 vector (Fig. 5B). The four BIBAC2 constructs were subsequently transformed into COR309 and LBA4404 by electroporation. Constructs 157M5a, 157M5b and 52M2a were stably maintained in both COR309 (Fig. 6) and LBA4404 (data not shown). However, construct 52M2b, containing a 110-kb insert, was not stable in either strain (Fig. 6).

Discussion

In this study, the stability of a number of BIBAC and TAC constructs was tested in four different *Agrobacterium* strains. Instability was associated with all constructs containing a potato DNA fragment larger than 100 kb. Direct association of instability with other factors, including the repetitive nature of the potato DNA inserts, different *Agrobacterium* transformation methods and different *Agrobacterium* strains, was not observed in the study. These results suggest that insert sizes of the BIBAC and TAC constructs are the most critical factor to their stability in *Agrobacterium*. Our empirical results suggest that the upper size limit of stable potato BIBAC and TAC constructs that can be maintained in *Agrobacterium* is approximately 95 kb.

Hamilton et al. (1996) reported that a BIBAC2 clone containing a 150-kb human genomic DNA insert was stably maintained in *Agrobacterium*. The stability of this BIBAC2 clone in *Agrobacterium* was confirmed in our laboratory (data not shown). Liu et al. (1999) tested the stability of 35 Arabidopsis TAC clones in *Agrobacterium* and found that only one clone was not stable. The average insert size of the Arabidopsis TAC library is 80 kb (Liu et al. 1999). A similar stability test was also conducted in a wheat TAC library in which the average insert size is 150 kb (Liu et al. 2000). Six out of 16 clones could not be stably maintained in *Agrobacterium*. It is not known if the stability is associated with insert sizes of the tested clones in these reports.

The mechanism of the instability of the large potato BIBAC and TAC constructs in *Agrobacterium* is unknown. Song et al. (2001) previously reported that BAC clones containing tandemly repeated sequences are not stable in *E. coli*. Such BACs often undergo drastic deletions during routine library maintenance and DNA preparation. In this study, the BIBAC constructs containing either highly repetitive DNA elements or largely single-copy sequences showed similar instability in *Agrobacterium*. We recently found that a BIBAC4 construct containing less than 70 kb of tandemly repetitive DNA of rice is not stable in *Agrobacterium* (unpublished data). The instability of this 70-kb rice BIBAC4 construct and the stability of the 150-kb human BIBAC2 clone suggest that the size of BIBAC/TAC inserts is not the only factor causing instability in *Agrobacterium*. Thus, the relationship between the sizes of BIBAC/TAC constructs and their stability in *Agrobacterium* may vary in different plant species and need to be examined by different research groups.

Recombination is generally the major cause of instability of plasmid clones in *E. coli* (Ishiura et al. 1989). The most important host gene for stabilizing plasmid inserts is *recA*. A defective *recA* allele is an absolute requirement for large insert propagation with a minimal chance of recombination in *E. coli* (Wyman and Wertman 1987). The DH10B strain of *E. coli*, which has been widely used for BAC library construction, carries the stringent *recA1* allele. Other stabilizing mutations in the host recombination pathway have been reported. In particular, defects in one or more of the *recB, recC* or *recD* genes were associated with higher stability of palindromic DNA sequences (Wyman and Wertman 1987). A *recB recC sbcB recJ* quadruple combination of host mutations prevented the deletion of cosmid inserts which were deleted frequently in standard *recA E. coli* hosts (Ishiura et al. 1989).

In contrast to the well-characterized *E. coli* strains, molecular and genetic analysis of insert stability has rarely been done in *Agrobacterium*. Both COR 308 and COR309 are *recA* deficient strains (Hamilton et al. 1996). However, large potato BIBAC and TAC constructs showed similar instability in COR 308 and COR309 as in the two other *Agrobacterium* strains used in this study. Nevertheless, it is reasonable to expect that engineering of new *Agrobacterium* strains with knockouts of more recombination-associated genes may enhance the stability of BIBAC and TAC constructs.

If a large BIBAC/TAC construct is not stable in *Agrobacterium*, it is necessary to subclone the target DNA fragment into smaller constructs. In most monocot species, *Not*I digestion of a typical 100–200 kb BAC will result in several fragments (Woo et al. 1994; Wang et al. 1995; Lijavetzky et al. 1999). These *Not*I fragments can be easily subcloned into BIBAC and TAC vectors. However, many dicot species have fewer *Not*I restriction sites. In our potato BAC library, more than 99% of the BACs lack internal *Not*I sites within the BAC inserts (Song et al. 2000). Similar phenomena have been reported in *Arabidopsis* (Mozo et al. 1998), tomato (Budiman et al. 2000) and soybean (Tomkins et al. 1999). We demonstrated that the transposon-based approach is an effective way to introduce one or more *Not*I restriction sites into BAC inserts for subsequent size manipulation. This technique should be equally applicable to other plasmids,

such as P1 clones (Sternberg et al. 1990) and P1-derived artificial chromosome clones (Ioannou et al. 1994).

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