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Development of a new transformation-competent artificial chromosome (TAC) vector and construction of tomato and rice TAC libraries

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Abstract

Recent research has shown that BIBAC (binary bacterial artificial chromosome) and TAC (transformation-competent artificial chromosome) vector systems are very useful tools for map-based cloning of agronomically important genes in plant species. We have developed a new TAC vector that is suitable for both dicot and monocot transformation. Using this new TAC vector, we constructed large-insert genomic libraries of tomato and rice. The tomato library contains 96,996 clones (28.3-38.5 kb insert size) and has 3.18 haploid genome equivalents. The rice TAC library has 32.7 kb average insert size and has 9.24 haploid genome equivalents. The quality of these two libraries was tested using PCR to verify genome coverage. Individual clones were characterized to confirm insert integrity by Southern analysis, end sequencing and genetic mapping. To investigate the potential application of these TAC libraries in map-based cloning, TAC constructs containing a 45 kb fragment were introduced into the rice genome via *Agrobacterium*-mediated transformation. Molecular analysis indicates that the 45 kb fragment was successfully transferred into the rice genome. Although rearrangements of the introduced DNA were detected, 50% of regenerated plants contained at least one intact copy of the 45 kb clone and associated vector sequences. These libraries provide us with a valuable resource to rapidly isolate important genes in tomato and rice.

Introduction

Rapid advances in plant genomics provide exciting new opportunities for crop improvement. A goal of functional genomics is to determine the biological function of important genes in crop plants, which, in turn, will greatly enhance our ability to utilize these genes in breeding programs. Large-insert genomic libraries are one of the key components in plant genome research and have become one of the most useful resources in the map-based cloning of important genes, genome organization studies, physical mapping of chromosomes and molecular breeding programs.

Cosmid (Collins and Hohn 1978) and yeast artificial chromosome (YAC) vectors (Burke et al. 1987) were initially generated for construction of large-insert genomic libraries. To improve the vector systems for efficient cloning and stable maintenance of large genomic inserts, the bacteriophage P1 (Sternberg 1990), the bacterial artificial chromosome (BAC) (Shizuya et al. 1992) and the P1-derived artificial chromosome (PAC) (Ioannou et al. 1994) were subsequently developed. The P1 library of *Arabidopsis thaliana* (Liu et al. 1995) and the BAC libraries of rice (Wang et al. 1995), soybean (Danesh et al. 1998) and many other agronomically important crop plants have been constructed and extensively used in map-based cloning and plant genomics research. With the

improvements in DNA isolation and cloning techniques, construction of plant large-insert genomic libraries is now becoming a relatively routine procedure in many laboratories.

To facilitate map-based cloning efforts, a new generation of vectors (*e.g.,* BIBAC and TAC) was constructed so that a large genomic fragment could be directly transformed into the plant genome via *Agrobacterium*-mediated transformation (Hamilton et al. 1996; Liu et al. 1999). Both BIBAC and TAC vectors can replicate in *E.coli* and *Agrobacterium tumefaciens* and contain all the features that are theoretically required for transferring large DNA inserts into plant genomes, including the plant transformation markers *NPTII* (neomycin phosphotransferase gene) for resistance to kanamycin and *HPT* (hygromycin phosphotransferase) for resistance to hygromycin. The BIBAC uses an F-plasmid origin of replication, whereas the TAC uses the *E. coli* bacteriophage P1 origin of replication (Pierce et al. 1992). In *Agrobacterium*, the BIBAC and TAC vectors both replicate through the single-copy Ri origin of replication from *Agrobacterium rhizogenes* (Jouanin et al. 1985). Both the BIBAC and TAC vectors contain the right- and left-border (RB and LB) sequences of T-DNA and function as binary vectors for *Agrobacterium*-mediated transformation. The ability to use BIBAC and TAC vectors directly in plant transformation can streamline map-based cloning of genes by eliminating the time and labor expended for subcloning small overlapping DNA fragments from a BAC clone into conventional plant transformation vectors. Since the vector used for plant transformation has large inserts, fewer clones need to be transformed for complementation analysis, thereby saving additional time, labor and resources.

The ability of vectors containing large inserts to transfer DNA into plants through *Agrobacterium*-mediated transformation has been verified. A BIBAC clone containing 150 kb of foreign DNA was successfully transformed into tobacco (Hamilton et al. 1996; Hamilton 1997). In addition, Liu et al. (1999) used the TAC vector to transfer an 80 kb *Arabidopsis* genomic DNA fragment into mutant plants, which complemented a known defect in gravitropism. To date, BIBAC and TAC vectors have been used to construct large-insert libraries of *Lycopersicon esculentum* (cv. Mogeor), *Lycopersicon pennellii* (cv. LA716) (Hamilton et al. 1999), petunia (Bentolila and Hanson 2001), *Arabidopsis* (Liu et al. 1999), wheat (Liu et al. 2000) and rice (*Oryza sativa ssp indica*) (Liu et al. 2002).

Although BIBAC and TAC vectors are functionally similar in the cloning and maintenance of large DNA fragments, they possess different features in respect to plant transformation (Hamilton et al. 1996; Shibata and Liu 2000). For *Agrobacterium*-mediated plant transformation with the BIBAC vector, a virulence helper plasmid is needed in *Agrobacterium* to promote T-DNA transfer into the plant cell, with transformation efficiency dependent on the *Agrobacterium* strain and helper plasmids (Frary et al. 2001). In contrast, the *virG*- and *virE*-carrying helper plasmids used in the BIBAC system for enhancing large T-DNA transfer (Hamilton et al. 1996) were not necessary for efficient transfer of 80 kb or larger TAC inserts (Liu et el. 1999). In addition, the conventional binary vector pCLD04541 was used in the construction of large-insert genomic libraries of soybean and *Lotus japonicus* (Meksem et al. 2000; Men et al. 2001) and *Agrobacterium*-mediated plant transformation of a 20 kb fragment into plants (Bent et al. 1994; Brommonschenkel and Tanksley 1997). Although the pCLD04541 vector was shown to be capable of stable maintenance of large plant DNA fragments in *E. coli* (Tao and Zhang 1998; Meksem et al. 2000; Men et al. 2001), it has not yet been established whether the large-insert clones are stable in *Agrobacterium* and can be efficiently transformed into plants.

In this research, our primary goals were to construct TAC libraries of tomato and rice, and test their application in map-based cloning. The original TAC vector (Liu et al. 1999) was modified into a general TAC vector suitable for efficient transformation of dicot and monocot plants. The new TAC vector was tested in rice transformation by transferring a 45 kb rice genomic DNA fragment into the rice genome. To evaluate the genome coverage and utility of the tomato and rice TAC libraries, pools of DNA from TAC clones were screened by PCR with gene specific primers. For the tomato library, clones distributed across the genome were tested. Clones of the *L. hirsutum Prf* disease resistance gene and a rice gene homologous to the maize nitrate-induced *NOI* gene have been identified and their integrity established. The two libraries constructed in this project will be useful in chromosome walking and offer the particular advantage of allowing direct transformation into plants, a feature that can greatly facilitate the identification of gene function.

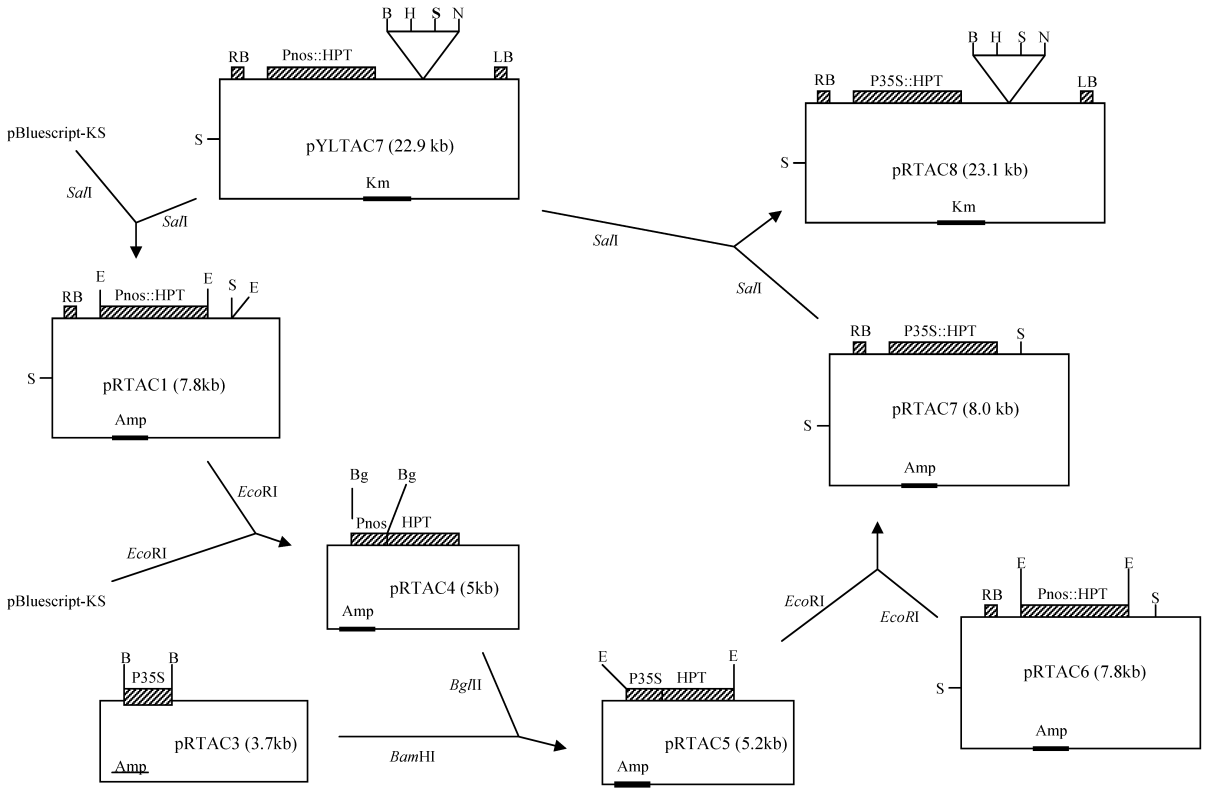

Figure 1. Construction of the new TAC vector (pRTAC8). Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; N, *Not*I; S, *Sal*I; Amp, ampicillin resistance gene; Km, kanamycin resistance gene; LB, left border; RB, right border; Pnos, nos promoter; *HPT,* hygromycin phosphotransferase gene.

Materials and methods

TAC vector construction

The original TAC vector, pYLTAC7 (Figure 1; Liu et al. 1999), was kindly provided by Dr. Y.G. Liu (South China Agricultural University). In this research, a new TAC vector pRTAC8 was constructed by exchanging the Pnos promoter in pYLTAC7 with the CaMV 35S promoter. Standard methods in molecular cloning (Sambrook et al. 1989) were used in the vector construction. Details of the construction of pRTAC8 are described in Figure 1, where several intermediate constructs were made due to the large size (22.9 kb) of pYLTAC7.

To modify the Pnos-*HPT* marker gene in pYLTAC7, the 4.9 kb *Sal*I fragment containing Pnos-*HPT*-3'nos was first cloned into pBluescript KS vector (Stratagene, USA). The Pnos-*HPT*-3'nos fragment was then released and cloned into the KS vector to construct pRTAC4 (Figure 1). To construct pRTAC5, the nos promoter (Pnos) in pRTAC4 was replaced with the 35S promoter (P35S) originating from a 0.8 kb *Bam*H I fragment within pRTAC3. To construct pRTAC6, the 4.9 kb *Sal*I fragment of pYLTAC7 was cloned into the modified pBluescript KS vector in which the *Eco*RI site was removed. The 2.1 kb *Eco*RI fragment (Pnos-*HPT*-3'nos) in pRTAC6 was replaced with the 2.4 kb *Eco*RI fragment (P35S-*HPT*) of pRTAC5 to construct pRTAC7. Finally, a new TAC vector (pRTAC8) was constructed by replacing the 4.9 kb *Sal*I fragment of pYLTAC7 with 5.1 kb *Sal*I fragment of pRTAC7.

Plant material and isolation of high-molecular-weight DNA

The wild species, *Lycopersicon hirsutum* accession LA407 (Kabelka et al. 2002), was used to construct the tomato TAC library. The *indica* rice line C101A51, which contains the rice blast (*Magnaporthe grisea*) resistance gene *Pi2(t)* (Liu et al.

2002), was used to construct the rice TAC library. High molecular weight (HMW) DNA of tomato and rice was prepared as described in the rice BAC library construction (Wang et al. 1995).

TAC library construction

The pRTAC8 vector was isolated using the Qiagen Large Construct Kit (Qiagen, USA). Three ng of pRTAC8 vector DNA was digested with 20 units of *Hin*dIII (Invitrogen, USA), 37 °C, 2 h to completion. The digested vector was separated on 0.8% agarose gel and the vector band was purified with the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, USA). The purified vector DNA was dephosphorylated for 1 h with HK phosphotase (Epicentre Technologies, USA) and further dialysed for 30 min on a Millipore VSWP membrane against TE (10 mM Tris-HCl and 1mM EDTA, pH 8.0).

The TAC library construction procedures were essentially the same as previously described (Wang et al. 1995). Briefly, the HMW genomic DNA of tomato and rice were partially digested with 4-6 units of *Hin*dIII per plug, 37 °C for 30 min. The DNA was size-fractionated using a pulse field gel electrophoresis (PFGE) device (CHEF Mapper II, Bio-Rad, USA). In the construction of tomato and rice TAC libraries, one size selection was conducted with 1.0% low melting point (LMP) agarose gel, switch time 40-60 s, 4.5 V/cm, 14 °C, 20 h. The DNA from two size ranges (100-200 kb and 200-300 kb for tomato; 100-175 kb and 175-250 kb for rice) was used for the construction of two sub-libraries. Gel slices were digested with 1 unit of GELase (Epicentre Technologies, USA) per 100 mg at 45 °C for 1 h. The GELase-digested product was dialyzed on a Millipore VSWP membrane against 1× TE for 2-3 h. The two fractions of partially digested genomic DNA were ligated separately with *Hin*dIII digested and dephosphorylated pRTAC8 vector. Vector DNA (100-200 ng) was ligated with an equal amount of insert DNA in 100 μl volume containing 3-4 units of T4 DNA ligase (Invitrogen, USA). One μl of ligation mix was electroporated into 20 μl of *E. coli* DH10B electro-competent cells (Invitrogen, USA) using a BRL Cell-Porator system (Invitrogen, USA). Transformed *E. coli* cells were plated onto LB agar medium (in 150 mm×15 mm petri-dishes) containing 25 mg l^{-1} kanamycin.

Rice transformation with TAC clones

A 45-kb genomic DNA fragment from the BAC library of *indica* rice cultivar 75-1-127 (Liu et al. 2002) was cloned into the *Not*I site of pRTAC8 to form the constructs pRTAC8-45 kb (I) and pRTAC8-45 kb (II), containing the fragment in different orientations. The stability of TAC clones in *Agrobacterium* was examined by isolating ten clones representing independent transformations of *Agrobacterium* strain LBA4404. One *Agrobacterium* colony was randomly selected from each of the ten transformations for mini-preparation of TAC DNA. The re-isolated TAC DNA was then electro-transformed into *E. coli* strain DH10B. Small scale DNA preparation from *E. coli* colonies representing the ten transformation events were used to verify pRTAC8 clone stability using a *Bam*HI digest.

Constructs pRTAC8-45 kb (I) and pRTAC8-45 kb (II) were used in rice transformation. Rice callus was induced using embryos from mature seeds of *japonica* rice cultivar Taipei 309. Rice transformation was accomplished via *Agrobacterium*-mediated transformation (Hiei et al. 1994; Yin and Wang 2000). For *Agrobacterium*-mediated rice transformation, both constructs were electroporated into *Agrobacterium* strain LBA4404. Plants were regenerated as previously described (Yin and Wang 2000). Leaf tissue was collected from primary transformed rice plants grown in the greenhouse for 2-4 weeks. Leaf DNA was purified by a rice DNA mini-preparation protocol and analyzed for the presence of the *HPT* gene (Chen and Ronald 1999). PCR primers specific to the *HPT* gene were Hpt-F (5'-TACTTCTACACAGCC ATC-3') and Hpt-R (5'-TATGTCCTGCGGCTAAAT-3'). For Southern analysis, rice genomic DNA was purified by CTAB extraction (Saghai-Maroof et al. 1984).

To confirm the integration of *indica* T-DNA into Taipei 309, genomic DNA of 18 transgenic plants was digested with *Eco*RI and *Pac*I. The integrity of the RB region was verified in *Eco*RI digests probed with the 2.4 kb "P35S::HPT::3'nos" fragment of pRTAC8 (Figure 2A, C and D). For the analysis of the T-DNA LB region, genomic DNA of the same plants, digested with *Pac*I, was hybridized with the 2.9 kb SacB/LB probe (Figure 2B, C and D). The 2.9 kb SacB/LB fragment was released from pRTAC8 by *Pml*I/*Not*I digestion.

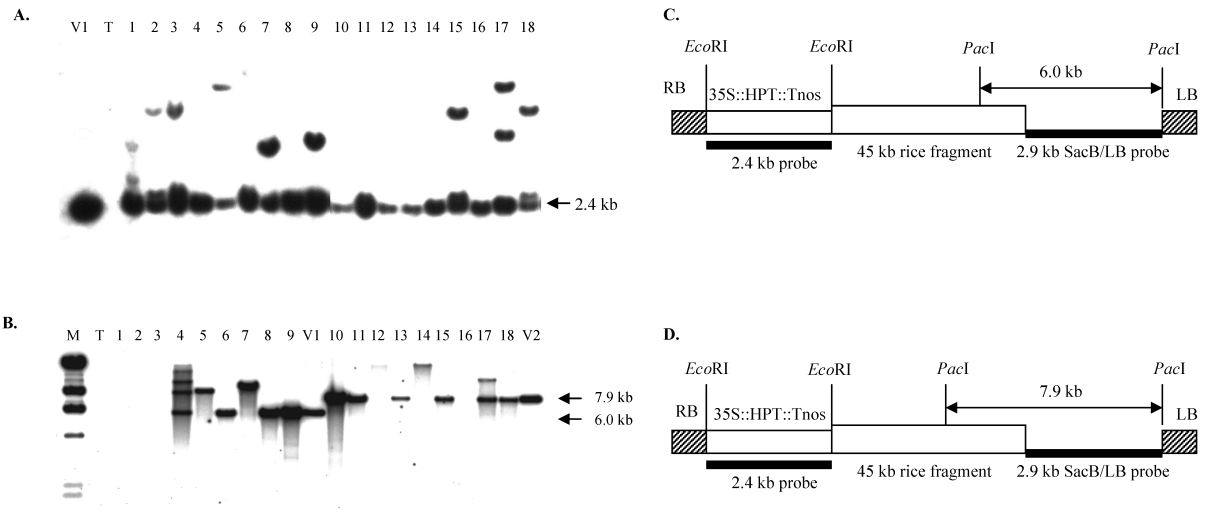

Figure 2. Southern analysis of the T-DNA region in transgenic rice plants transformed with *Agrobacterium* containing TAC clones. A. Southern analysis of the RB/HPT region in rice plants transformed with the vectors pRTAC8-45kb (I) (lane 1 to 9) and pRTAC8-45 kb (II) (lane 10 to 18). Genomic DNA was digested with *Eco*RI and probed with P^{32}-labeled 2.4-kb "35S::HPT::Tnos" fragment. B. Southern analysis of the SacB/LB region in the same set of transgenic lines used in A. C. Schematic representation of the T-DNA region in pRTAC8-45 kb (I). The 2.4-kb "35S::HPT::Tnos probe" and 2.9-kb SacB/LB probe are indicated. D. Schematic representation of the T-DNA region in pRTAC8-45 kb (II). M, λDNA *Hin*dIII; T, Taipei 309; V1, the vector pRTAC8-45kb (I); V2, the vector pRTAC8-45 kb (II).

Characterization and screening of the TAC libraries

For PCR screening of targeted genes, independent TAC colonies were pooled and mixed for TAC DNA isolation. About 5 ml of LB freeze medium (Woo et al. 1994) was used to wash the *E. coli* colonies from each plate (150 mm×15 mm). Approximately 1000-3000 clones for the tomato TAC library and 3000 clones for the rice library were mixed in each pool. Half of the solution containing pooled bacteria was stored at − 80 °C and the other half used for TAC mini-preparation. Each TAC DNA pool was dissolved in 30-50 μl TE and examined on an agarose gel. Each TAC DNA pool was diluted to 1 ng/μl for PCR amplification.

To determine the TAC library coverage, PCR was performed on each pool using primers amplifying genes previously mapped to chromosomes 2, 3, 4, and 5 (Table 1) of tomato. The rice TAC library was screened with PCR primers (Primer 1, 5'-CACTGA ATAACGACTACATC-3'; Primer 2, 5'-ATTGGT GGTTGGGCATCTAG–3'), specific to a rice callus cDNA clone (Genbank accession AU077474). Additional screening of the rice TAC clones was performed using two DNA markers linked to the *Pi2(t)* gene (Liu et al. 2002).

PCR positive pools for tomato and rice genes were used to isolate clones. A single positive pool for the *L. hirsutum Pto* cluster was dilution plated onto 150mm×15mm agar plates, picked into 384-well Genetix plates, and gridded onto a nylon membrane in a duplicate 3×3 array pattern using the Genetix Q Pix robot (Genetix, UK). Overgo probes (Han et al. 2000) generated from the Prf3 PCR sequence (5'-GCAGAAGGGATTTTCTCAAGATGCT-3' and 5'-CGAATGTAAATCAATTTTGGTTTACAAGTTC GAATCC -3') were used to identify specific TAC clones and for Southern verification. A single rice TAC pool, positive for the rice callus cDNA clone (Genbank accession AU077474), was plated and colony lifts were hybridized to identify positive clones.

For preparation of TAC DNA from individual clones, single colonies of the TAC libraries were inoculated into 1.8 ml of LB liquid medium containing 25 mg l^{-1} of kanamycin and incubated at 37 °C with shaking (220 rpm). After culturing overnight (12-16 h), the TAC DNA was prepared using a modified BAC mini-preparation method and dissolved in 30 μl of TE containing 10 mg l^{-1} of *RNa*se A (Wang et al. 1995). Five μl of TAC DNA was digested with *Hin*dIII to verify genomic DNA insertions. To estimate the insert size, 5 μl of the mini-prepared TAC DNA was digested with 1-2 units of *I-Sce* I (Roche, USA) at 37 ºC for at least 6 h. The *I-Sce* I digested TAC DNA was fractionated on a 1.0% PFGE agarose gel

Table 1. PCR screening results with the 55 tomato TAC library pools

Primer	Primer sequence	Annealing (°C)	Chromosome	Amplifies	Positive
Pto5	5'ATCTACCCACAATGAGCATGAGCTG3'	62	5	Pto Kinase domain	5
PtoFen3	5'CACCTTTGACCTCATACGTG3'				
Prf F	5'CCGCGTGATATCTCCTTCAT3'	57	5	Prf LRR domain	3
Prf R	5'TCCACACAAGCAAAAGGGTC3'				
Prf5 F	5'TAAACCTTGTGAAGGGCCAG3'	57	5	5' coding end of Prf	2
Prf5R	5'CCACCCCAAATAGCTTAGGA3'				
Prf5UTR F	5'CTGTTGGAAAATGAACTCAACC3'	57	5	5' UTR of Prf	5
Prf5UTR R	5'CCCACAGGATAGTGAAAGGACT3'				
Prf3 F	5'CAATTTCCCCTCGCAACTAA3'	57	5	3' coding end or Prf	4
Prf3 R	5'AACTCCACTTGACCACGTTG3'				
TG091 F	5'TGCAGAGCTGTAATATTTAGAC3'	60	2	RFLP Probe	3
TG091 R	5'AACTCAACGTTGACTCTGGC3'				
TG599 F	5'TGTTGATCCTTGCTTGCTGT3'	57	3	RFLP Probe	2
TG599 R	5'CCCTTCAACGTGGTATGTT3'				
LEOH10 F	5'TGCCAGATTGACTGTGAAGG 3'	55	4	accession AW443403	5
LEOH10 R	5'GTTCTTGTTACGTCCCAAGG3'				

with a switch time from 5 to 10 s, 4.5V/cm, 1X TAE, 14 °C, and 20 h run time.

To verify clone integrity, a combination of sequence comparison and classical mapping techniques were employed. End sequences of a 33.4 kb tomato clone were compared to sequence data available for the *L. esculentum* and *L. pimpinellifolium Pto* cluster (Genbank accessions AF220602.1 and AF220603.1). Map position of end clone polymorphisms were confirmed in a segregating F_2 population described previously (Kabelka et al. 2002).

Results

Construction of the new vector pRTAC8

The original TAC vector pYLTAC7 was a derivative of the P1 phage vector used in construction of an *Arabidopsis* TAC library (Liu et al. 1999). In pYLTAC7, the plant transformation selectable marker encodes *HPT* driven by the nos promoter. Although the nos promoter can be used for the transformation of many dicot plants, its activity is too weak to be used in monocot transformation (Jeon et al. 2000). To make a general vector suitable for transformation of both dicot and monocot plants, we modified the pYLTAC7 vector by replacing the nos promoter with a CaMV 35S promoter. Since the CaMV35 promoter has a stronger activity and the *HPT* gene is widely used in dicot and monocot plants, the new TAC vector, pRTAC8 (Figure 1), developed in this study can be used in transformation of a variety of monocot and dicot crop plants.

Test transformation of pRTAC8 harboring a 45 kb DNA fragment in rice

To test pRTAC8 for stability in *Agrobacterium*, experiments were performed using a 45-kb rice clone introduced in two orientations, pTRAC8-45 kb (I) and pTRAC8-45 kb (II). As described in Materials and Methods, for each construct, the TAC DNA from ten clones that represented independent transformations into *Agrobacterium* was re-isolated from *E. coli*. The *Bam*HI digestion patterns of the re-transformed TAC clones were the same as the original constructs pTRAC8-45 kb (I) and pTRAC8-45 kb (II), indicating that the 45 Kb fragment in both orientations was stable in *Agrobacterium*.

Next, the two constructs pTRAC8-45 kb (I) and pTRAC8-45 kb (II) were introduced into rice calli via *Agrobacterium*-mediated transformation (Table 2). After two months of selection, hygromycin resistant (Hyg^R) calli were selected from 22.4% of cocultivated rice calli in experiment 1 and 18.7% of those in experiment 2. The Hyg^R calli were cultured for an additional two months and transformed Hyg^R plants were regenerated from 15.7% and 3.4% of cocultivated calli in experiment 1 and 2, respectively. These efficiencies were comparable to rice transformation using conventional binary vectors (data not shown). Since plant regeneration from callus decreases with the age of callus, the lower efficiency

Table 2. Agrobacterium-mediated transformation of rice with pRTAC8 containing a 45 kb rice genomic fragment

Experiment	DNA construct*	Number of calli used	Hyg^R calli obtained	Hyg^R plants obtained
1	pRTAC8-45kb (I)	343 (30 days old)	77 (22.4%)	54 (15.7%)
2	pRTAC8-45kb (II)	864 (60 days old)	162 (18.7%)	30 (3.4%)

* The 45 kb fragment was cloned in different orientations in pRTAC8-45kb (I) and pRTAC8-45 (II).

(3.4%) of regenerated Hyg^R plants in experiment 2 may be due to the use of older callus (60 days old). In experiment 1, vigorous one-month-old calli were used in transformation and a higher efficiency (15.7%) of Hyg^R plants was obtained.

To detect the presence of transgenes in TAC transformed rice plants, one plant from each independent Hyg^R line was selected for genomic DNA purification. PCR assays were performed with primers specific to the *hpt* gene. The results demonstrated that 51 of the 53 Hyg^R plants from Experiment 1 and all 29 Hyg^R plants from experiment 2 contained the *hpt* transgene.

From the PCR positive plants, 9 Hyg^R plants from the transformation with pRTAC8-45 kb (I) (experiment 1 in Table 2) and 9 Hyg^R plants from the transformation with pRTAC8-45 kb (II) (experiment 2) were randomly selected for large-scale extraction of genomic DNA. Southern analysis of the T-DNA RB region (Figure 2A) was performed to confirm the integration of T-DNA RB into the genome of Taipei 309. Results in Figure 2A showed that all the 18 plants contained the 2.4 kb *Eco*RI fragment ("P35S::HPT::3'nos"), indicating the T-DNA RB region of TAC clones was integrated into the rice genome.

As indicated in Figure 2B, a 6.0 kb *Pac*I fragment containing the SacB/LB region was detected in 4 plants (No.4, 6, 8, and 9) transformed with pRTAC8-45 kb (I). The 6.0 kb band was expected according to the restriction map in Figure 2C. In No. 4, 5, and 7 plants (Figure 2B), unexpected bands with different sizes were also found, suggesting DNA rearrangement in the SacB/LB region. For the analysis of the 9 transgenic plants (No.10, 11, 12, 13, 14, 15, 16, 17 and 18) transformed with pRTAC8-45 kb (II), a 7.9 kb *Pac*I fragment in 6 plants (No. 10, 11, 13, 15, 17 and 18) hybridized to the SacB/LB probe (Figure 2B). This was expected according to the restriction map in Figure 2D. In addition, DNA rearrangement in the SacB/LB region occurred in No.14 and 17 plants.

Based on the results of Southern analysis (Figure 2), four of the 18 transgenic plants (No.1, 2, 3 and 16) carried the T-DNA RB region, but did not contain the SacB/LB region. In the remaining 14 transgenic plants, the *hpt* gene and the SacB/LB region flanking the 45 kb rice genomic insert were simultaneously transferred into the genome of Taipei 309. Among the above-mentioned 14 plants, nine plants (No. 4, 6, 8, 9, 10, 11, 13, 15, 17, and 18) carried non-rearranged T-DNA RB and LB regions. Because the T-DNA transfer is initiated at the RB and proceeds toward the LB (Hamilton et al. 1997; Liu et al. 1999; Yin and Wang 2000), it is deduced that a copy of the 45-kb rice genomic sequence within the T-DNA region was transferred and integrated without any rearrangement into the genome of Taipei 309 in 50% (9/18) of the transgenic plants.

Southern analysis of the T-DNA RB and LB regions displayed additional bands that were larger than expected based on the vector maps (Figure 2). This suggested the existence of additional T-DNA copies and rearrangement within the RB or LB region of these T-DNA copies. Additionally, the truncated T-DNA copies in the above-mentioned 4 plants (No.1, 2, 3 and 16 in Figure 2) suggested partial T-DNA transfer. Thus, DNA rearrangement and partial T-DNA transfer occurred in a portion of transgenic plants. Similar results were observed in previous research on *Agrobacterium*-mediated transformation of rice and other plants (Hiei et al. 1994; Yin and Wang 2000; Kumar et al. 2002). Of the nine plants possessing an intact 45 kb genomic sequence, four contained additional rearranged copies and five contained only intact copies.

Construction of tomato and rice TAC libraries

After confirming that pRTAC8 was a suitable vector for transferring a large DNA fragment into the rice genome, TAC libraries of tomato and rice were constructed. The tomato and rice TAC libraries comprise two sub-libraries based on genomic DNA size selection. The tomato sub-library I consists of 23 pools

with an average of 2722 clones per pool (Table 3). Twenty-five sub-library I clones were selected for TAC DNA mini-preparation and *Hin*dIII digestion. Inserts were confirmed in 24 clones (96%). Tomato sub-library II has 32 DNA pools and each pool contains 1075 clones. *Hin*dIII digestion of 20 randomly selected clones from the tomato sub-library II indicated that 100% of clones (20/20) contained genomic inserts. PFGE analysis with these positive clones showed that the tomato sub-library I has an average insert size of 28.3 kb and that sub-library II contains inserts with an average size of 38.5 kb. A total of 96,996 individual clones in the two sub-libraries are about 3.18 times of the tomato genome equivalent (950 Mb, Arumuganathan and Earle 1991) (Table 3). The probability of finding any tomato sequence of interest within the library is 95.8%, which is based on the formula $P = 1\text{-}(1\text{-}L/G)^N$ (Clarke and Carbon 1976). In this formula, L is the average length of the clone inserts, G is the haploid genome size, and N is the clone number of the library.

Rice sub-library I was generated from the 100-175 kb fraction and sub-library II was generated from the 175-250 kb fraction. Ten TAC clones were randomly sampled from each rice sub-library. Mini-preparation and *Hin*dIII digestion showed that 90% (9/10) of the sub-library I and II clones contained inserts. Using PFGE to analyze insert size we estimated that the average insert size of sub-library I and II was 21.2 kb and 32.7, respectively. Forty-five *E. coli* pools of the sub-library II were prepared by mixing approximately 3000 TAC clones in each pool (Table 3). Sub-library I was not used in *E. coli* transformation because sub-library II contained 9.24 equivalents of rice haploid genome (430 Mb, Arumuganathan and Earle 1991). Based on the clone numbers and average insert size of the sub-libraries II and the rice genome size, the probability of finding any rice sequence in the sub-library II is 99.9% (Clarke and Carbon 1976).

Table 3. Characteristics of the TAC libraries of tomato and rice

TAC library	Sub-library	DNA size selection (kb)	Total clone number	Percentage of positive clones (%)	Number of TAC DNA pools	Average size of insert (kb)	Total size of sub-library (Mb)	Haploid genome equivalents
Tomato	1	100-200	62,602	96	23	28.3	1,700	1.79
	2	200-300	34,394	100	32	38.5	1,324	1.39
Rice	1	100-175	N/A	90	N/A	21.2	N/A	N/A
	2	175-250	135,000	90	45	32.7	3,973	9.24

PCR screening of the TAC libraries

To evaluate the potential use of the TAC libraries in map-based cloning of important genes in tomato and rice, a PCR screening method was used to identify pools containing clones of interest from the two libraries. As shown in Table 1, screening with eight primer pairs designed for genes or markers previously mapped in the tomato genome, we identified 2, 3, 4, or 5 positive pools from the 55 pools of the tomato TAC library, respectively. These results agreed with

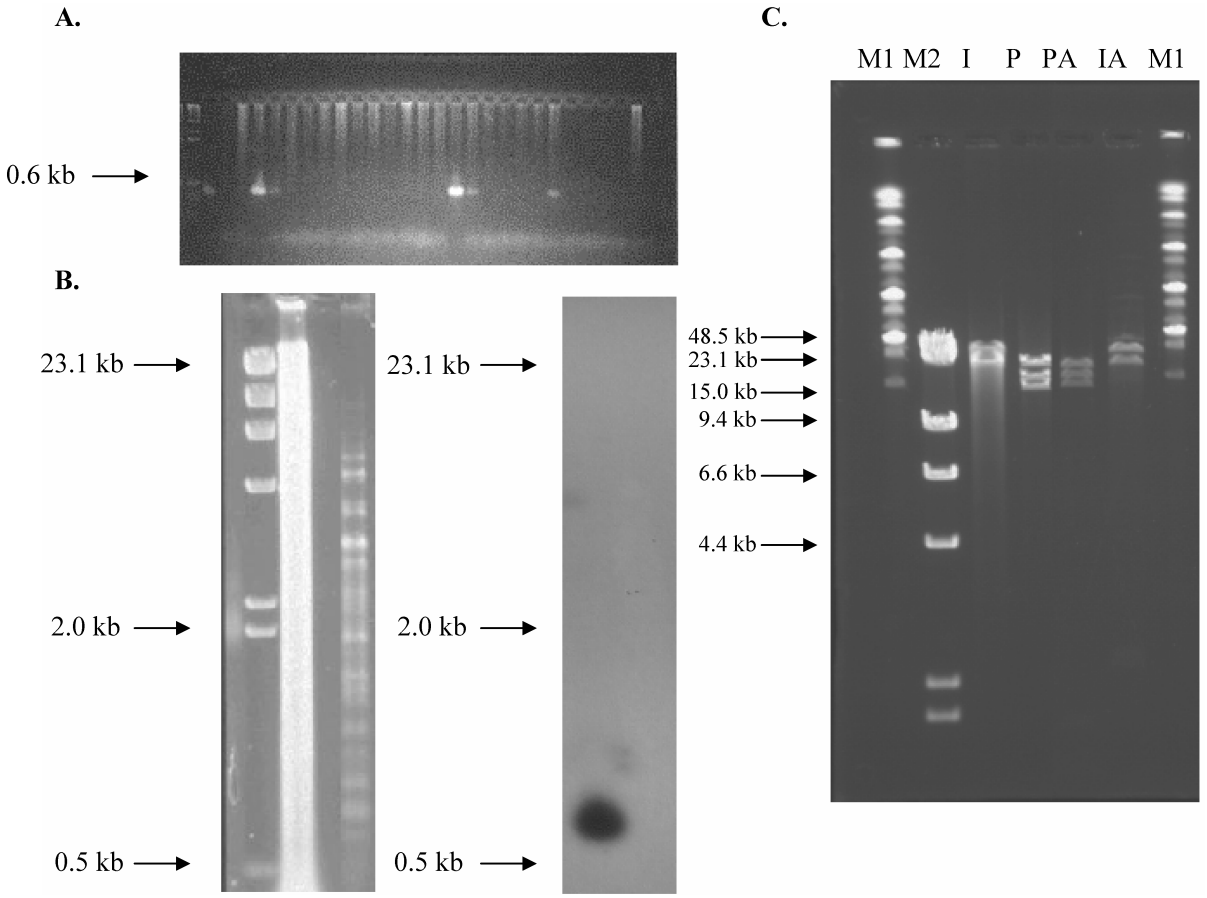

Figure 3. Screening of the tomato TAC library. A. PCR amplification of the TAC pools with the *Prf3* primer pairs (Table 1). Lane 2 contains LA407 genomic DNA. B. Agarose gel and Southern blot of *Bam*HI/*Hind*III digested positive clone with *Prf3* probe. Agarose gel lane 2 contains LA407 genomic DNA. C. PFGE analysis of a positive clone from sub-library I identified with *Prf3* probe. I, *I-sce* I; P, *Pml*I; A, *Asc*I; M1, mid-range PFGE ladder; M2, λDNA *Hin*dIII.

the predicted genome coverage of the tomato TAC library (3.18 genome equivalents). The rice TAC library was screened with PCR primers, which were specific to a rice callus cDNA clone (Genbank accession AU077474). This cDNA is a homologue of the maize nitrate-induced *NOI* gene (Genbank accession AF030385). PCR amplification was obtained with 5 DNA pools. Screening for TAC clones containing two DNA markers linked to the *Pi2(t)* gene (Liu et al. 2002) also yielded 5 and 6 positive pools for each pair of primers (unpublished). These results indicate that the two libraries have high genome coverage and are useful for specific gene identification and map-based cloning in tomato and rice.

One PCR positive pool from sub-library II of rice was used to isolate clones. A single TAC clone was isolated by colony hybridization. TAC DNA of this positive clone was extracted, digested with *I-Sce*I and separated by PFGE. The rice genomic insert in this clone was approximately 60 kb in size, and Southern analysis verified that this clone contained a homologue of the maize *NOI* gene (data not shown).

L. hirsutum is a green-fruited relative of the cultivated tomato, and LA407 is an inbred accession reported to have two genetic loci for bacterial canker resistance (Kabelka et al. 2002). Because resistance to bacterial canker in tomato accession LA407 is linked to markers near the *Pto* cluster (Kabelka et al. 2002), five primer pairs derived from the *Pto* and *Prf* locus were used to screen the tomato TAC library (Table 1, Figure 3A). A single PCR positive pool was used to isolate a clone hybridizing to *Prf3*. PFGE analysis revealed that the size of the genomic insert was 33.4 kb (Standard error 3.1, Figure 3C), and

Southern analysis verified that this clone contained *Prf3* (Figure 3B). End sequences for the 5' (near T-DNA RB) and 3' (near T-DNA LB) ends of the TAC clone possess high homology to *L. pimpinellifolium*'s *Pto* cluster (Genbank accession AF220602.1). The 5' end has 83% homology to bases 156-453 of the 5'UTR of the *Pto* paralog *LpimPth4* (Chang et al. 2002). The 3' end has 96% nucleotide homology to bases 24,427-25,075 of the 3' end of *Prf*. This segment of the *L. hirsutum* accession LA407 *Pto* cluster therefore appears not to be rearranged and contains an insertion estimated to be 8.49 kb with respect to *L. pimpinellifolium*. Polymorphic PCR-based molecular markers were made from the 3' end sequence and placed onto an F_2 population segregating for *L. hirsutum's Pto* cluster. The TAC end sequences co-segregated with *Pto5/PtoFen3* (data not shown).

Discussion

Before the development of BIBAC (Hamilton et al. 1996) and TAC (Liu et al. 1999) vectors, no transformation method was available for routinely introducing DNA fragments larger than 25 kb into the plant nuclear genome. We are interested in the application of these vectors to both monocot and dicot plants for functional analysis of agronomically important genes. We have modified the original pYLTAC7 vector by replacing the nos promoter with the CaMV 35S promoter, which is more suitable for transformation of dicot and monocot plants. We cloned a 45-kb rice genomic fragment into the new TAC vector pRTAC8 and tested the utility of the TAC vector in *Agrobacterium*-mediated transformation of rice. Truncated or rearranged T-DNA copies have often been observed in lines transformed via *Agrobacterium* in multiple plant species (Hiei et al. 1994; Yin and Wang 2000; Kumar et al. 2002). In our study, half of the transgenic plants contained at least one insertion event with unrearranged RB and LB regions. Half of the lines carried hybridization band(s) larger than expected (Figure 2A and B). Therefore, for complementation experiments in map-based cloning projects, more transgenic lines (25-30) for one construct should be produced with the TAC vector than for regular binary vectors when conducting *Agrobacterium*-mediated transformation.

Increasing the insert size of TAC libraries was challenging. Although DNA fragments over 100 kb in size were selected in the construction of TAC libraries of tomato and rice, the average insert sizes are relatively small compared to regular BAC libraries. This may be due to the large size of the TAC vector (23 kb), which limits the ability of *E. coli* to accommodate large inserts.

Although the insert size of TAC clones for map-based cloning is important, other factors should be considered, such as transformation efficiency and insert DNA rearrangement. According to the results of rice transformation in this study, 50% of the transgenic plants contained the intact 45 kb fragment when it was transformed into the rice genome via *Agrobacterium*-mediated transformation. Since large-insert BIBAC and TAC clones have shown to be unstable in *Agrobacterium* (Song et al. 2002; and unpublished) and the transformation efficiency is relative low for larger insert clones, small insert TAC clones (30-50 kb) may be a practical choice in TAC library construction.

Using our new TAC vector, we have constructed TAC libraries of tomato and rice. The average insert sizes of tomato sub-library I and II are 28.3 kb and 38.5 kb respectively. A total of 96,996 individual clones in the two sub-libraries are about 3.18 tomato genome equivalents. Several positive TAC pools containing clones which span the *Pto* cluster in tomato were identified using PCR pooling strategies. These clones are being used for construction of a TAC contig and map-based cloning of a bacterial canker resistance gene in LA407. The rice TAC library II contains an average insert size of 32.7 kb and 9.24 haploid genome equivalents. A 60 kb positive TAC clone was identified from the library when PCR screening was performed with primers specific to a rice gene homologous to the maize nitrate-induced *NOI* gene. These results demonstrate the utility of TAC cloning system in tomato and rice.

The recent publication of the rice draft genome sequences (Goff et al. 2002; Yu et al. 2002) and a large collection of rice genomic resources have made rice one of the most useful plants for genomic research. With the expected completion of the rice genome with high quality sequence data in the near future, rice functional genomics will become an emerging field in plant biology. An established TAC cloning and transformation system will be a useful tool for gene function analysis in rice. Considering the high transformation efficiency of TAC clones with inserts around 45 kb, our rice library with an average insert size of 32.7 kb will be a valuable resource for map-based cloning and mutant complementation. *Agrobacterium*-medi-

ated transformation of tomato is routine in many laboratories. The combination of well defined genetic maps, traits of interest, and an expanding sequence database has made tomato a model for the map-based cloning of genes. The tomato TAC library described in this study will be an additional resource for tomato map-based cloning and gene identification.

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