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ORIGINAL PAPER

Transformation of rice with long DNA-segments consisting of random genomic DNA or centromere-specific DNA

Bao H. Phan · Weiwei Jin · Christopher N. Topp · Cathy X. Zhong · Jiming Jiang · R. Kelly Dawe · Wayne A. Parrott

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Abstract Rice was transformed with either long DNA-segments of random genomic DNA from rice, or centromere-specific DNA sequences from either maize or rice. Despite the repetitive nature of the transgenic DNA sequences, the centromere-specific sequences were inserted largely intact and behave as simple Mendelian units. Between 4 and 5% of bombarded callus clusters were transformed when bombarded with just pCAMBIA 1305.2. Frequency of recovery dropped to 2–3% when BACs with random genomic inserts were co-bombarded with pCAMBIA, and fell to less than 1% when BACs with centromeric DNA inserts and pCAMBIA were co-bombarded. A similar effect was noted on regeneration frequency. Differences in transformation ability, regeneration and behavior of plants transgenic for BACs with random genomic DNA inserts, as compared to those with centromeric DNA inserts, suggests functional differences between these two types of DNA.

Keywords Centromeric DNA · Transformation with long DNA-segments · Microprojectile-mediated transformation

B. H. Phan · C. N. Topp · C. X. Zhong · W. A. Parrott (✉)
Department of Crop and Soil Sciences, University of Georgia, 111 Riverbend Road, Athens, GA 30602, USA
e-mail: wparrott@uga.edu

W. Jin · J. Jiang
Department of Horticulture, University of Wisconsin, Madison, WI 53706, USA

C. X. Zhong · R. K. Dawe
Departments of Plant Biology and Genetics, University of Georgia, Athens, GA 30602, USA

Present Address:
C. N. Topp
Department of Plant Biology, University of Georgia, Athens, GA 30602, USA

Present Address:
C. X. Zhong
Dupont Crop Genetics Research, Wilmington, DE 19880, USA

Introduction

Transformation with long DNA-segments (LDSs), such as the inserts in bacterial artificial chromosome (BAC) vectors, can be a useful tool to help verify the function of genes in the DNA insert (Ercolano et al. 2004). Alternatively, transformation with LDSs can be used to engineer plants with multiple genes at once, as would be the case for gene stacking or the engineering of metabolic pathways. Finally, transformation with LDSs is considered a pre-requisite for developing plant artificial chromosomes.

There have been previous attempts to engineer plants with LDSs. Stable transgenic cell lines have

been obtained following bombardment of yeast artificial chromosomes (YACs) in tomato (Van Eck et al. 1995) and tobacco (Adam et al. 1997; Mullen et al. 1998). The use of a specialized vector for *Agrobacterium*, called binary bacterial artificial chromosomes (BIBAC), permitted the recovery of transgenic tobacco with a 150-kb insert (Hamilton et al. 1996; Hamilton 1997). The use of another specialized *Agrobacterium* vector, transformation-competent artificial chromosomes (TAC), permitted the recovery of *Arabidopsis thaliana* transgenic for inserts 40–80 kb in size (Liu et al. 1999) and of rice transgenic for an 80-kb insert (Liu et al. 2002). However, the number of successful reports on transformation using large BIBAC/TAC constructs in the literature is limited, and Song et al. (2003) reported that BIBAC/TAC clones containing >100 kb potato genomic DNA are not stable in *Agrobacterium*. In a somewhat different approach, BACs modified by adding a *cre* construct and flanking *lox* sites were used to obtain cell lines transgenic for BACs with inserts up to 230 kb in size, and plants with a 150-kb BAC insert, following bombardment into tobacco. The BACs integrated into a previously engineered *lox* site, as determined by the presence of the expected border junctions in transgenic plants (Choi et al. 2000). Most recently, a BAC with a 45-kb insert from sorghum was bombarded into maize, with subsequent discovery of transgenic plants (Song et al. 2004).

There is very little available information on the size, stability, and analysis of LDSs once they have been successfully introduced into plants. Ercolano et al. (2004) were able to infer the presence of both complete and incomplete BAC inserts in potato following microprojectile bombardment. Here, we present extensive analyses to characterize insertions of LDSs consisting of centromeric DNA. We also triple the size of the BAC clones reported to have been delivered into plants without any modification, such as those used to make TAC or BIBAC vectors. Given the large number of BAC libraries in existence, the ability to use them in transformation without having to retrofit them into TAC or BIBAC vectors has the potential to greatly simplify transformation with LDSs.

Materials and methods

DNA constructs

The centromeric DNA inserts used are illustrated in Fig. 1. The BAC 16H10 has been previously described (Nagaki et al. 2003) and contains 95-kb of maize centromeric DNA. The BAC 17p22 (Cheng et al. 2001) contains a 67-kb rice centromeric DNA insert. Both of these DNA inserts include arrays of centromeric satellite DNA and centromere-specific retrotransposons, respectively called *CentC* and CRM for corn, and *CentO* and CRR for rice. To distinguish between DNA size effects and DNA content effects, two BACs containing random (i.e., not selected for any particular DNA content) rice genomic DNA inserts were used as controls, p76A03 (122.5-kb insert) and p7K12 (157.5-kb insert). These two clones were used previously as cytological markers, and are located in the euchromatic regions of rice chromosome 3 (Cheng et al. 2001). In each case, the BAC vector itself added 7.4 kb to the total size of the plasmids. The plant transformation vector, pCAMBIA1305.2 (http://www.cambia.org), which consists of the plant-optimized GUSPlus gene and the *hph* gene for hygromycin resistance, was co-bombarded with the BACs and used to provide the selectable marker.

DNA preparation & microprojectile bombardment

BACs were isolated and purified using the Large-Construct Kit (http://www.qiagen.com).

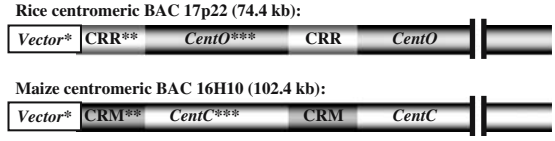

Fig. 1 Centromeric DNA inserts used for rice transformation. The size in parenthesis refers to the size of the insert + the size of the vector backbone. For BAC 17p22: *Vector pBeloBAC11 (7.4 kb); **CRR: Centromeric Retrotransposon Rice; *** *CentO*: *Orzya* centromere-specific repeat element. For BAC 16H10: *Vector pBeloBAC11 (7.4 kb); **CRM: Centromeric Retrotransposon Maize; ***CentC: Corn centromere-specific repeat element

Insert sizes were verified using contour-clamped homogenous electrical field gel electrophoresis (CHEF) analysis and monitored for stability over many generations in *E. coli* DH10B.

Cell culture, microprojectile bombardment & plant regeneration

Rice variety 'Taipei 309' was used throughout these studies, using standard tissue culture techniques. Embryogenic calli from mature seeds were initiated as described by Zhang et al. (1996). Recovery of transformed callus and plants was exactly as described by Chen et al. (1998). Approximately 2–3-month-old rice embryogenic calli were used for co-bombardment with pCAMBIA 1305.2 and BAC DNA. Eight hundred ng of the two plasmids were co-precipitated onto 0.6-m gold particles in a 1:6 molar ratio of pCAMBIA1305.2 : BAC, using the precipitation protocol as described by Hazel et al. (1998). The physical bombardment parameters were also as described by Hazel et al. (1998). The DNA amounts were selected by first testing molar plasmid ratios of 1:1, 1:2, 1:6 and 1:10 (pCAMBIA1305.2:BAC), along with total DNA concentrations of 125, 500, and 800 ng per bombardment.

Sixteen to 20 h after bombardment, tissues were transferred from osmotic conditioning medium (N6 medium supplemented with 0.256 M each of mannitol and sorbitol) onto selection medium (N6 medium supplemented with 50 mg l^{-1} hygromycin B (http://www.calbiochem.com, catalog #400051), ensuring that the bombarded surface was facing

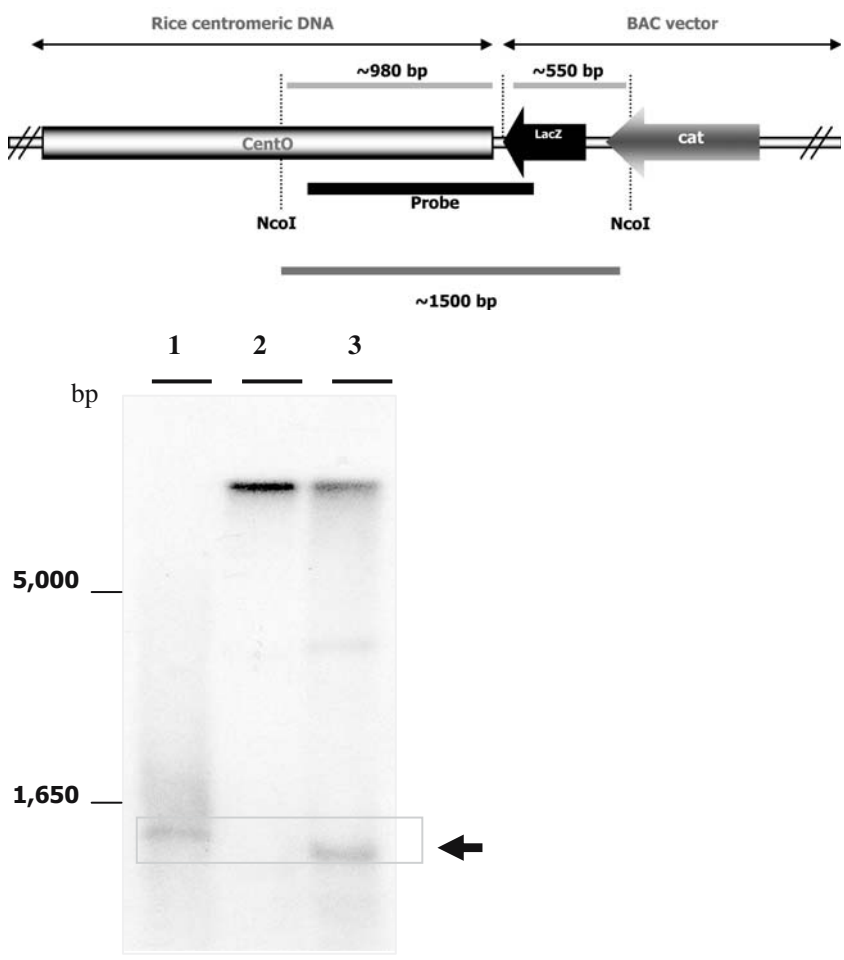

Fig. 2 Southern blot showing integration of an adventitious copy of *CentO* in transgenic rice plants. DNA was digested with *Nco*I to yield an expected 1500-bp fragment. The probe was amplified across the BAC vector-*CentO* junction. The blot shows the high molecular weight DNA band for the endogenous copies of *CentO*, and the expected 1500-bp band found only in transgenic plants. Blot shows an example from plant 14. Lane 1: BAC 17p22, containing a rice centromeric DNA insert; Lane 2: non-transgenic; Lane 3: Transgenic plant 14

upward, and incubated in the dark for 3 weeks. Newly formed callus was then separated from the original bombarded explants and subcultured onto the same medium. Following an additional 3–4 weeks, growing callus was visually identified and transferred to embryo histodifferentiation (pre-regeneration) medium (N6 medium without 2–4-dichlorophenoxyacetic acid (2,4-D)), but with the addition of 2 mg l^{-1} 6-benzylaminopurine (BAP), 1 mg l^{-1} α-naphthaleneacetic acid (NAA), 5 mg l^{-1} abscisic acid (ABA) and 50 mg l^{-1} hygromycin B for 7 days in the dark. Growing callus, which became more compact and opaque, was then subcultured onto embryo germination (regeneration) medium comprising N6 medium without 2,4-D, and supplemented with 3 mg l^{-1} BAP, 0.5 mg l^{-1} NAA, and 50 mg l^{-1} hygromycin B for a period of 3–4 weeks under a 23-h (light/dark) photoperiod with a photon flux of approximately 66–95 μmol m^{-2} s^{-1} at 26°C. Regenerated plantlets were transferred to GA–7 boxes (Magenta Corporation, Chicago, IL) with medium containing half-strength MS salts and vitamins, 15 g l^{-1} sucrose and 3 g l^{-1} GelRite to allow for root development. Rooted plants were potted into soil and grown to maturity in a greenhouse. Multiple plants regenerated from a single explant were considered and treated as one independent transformation event.

Transgenic tissue analysis

The preliminary identification of transgenic callus was done with histochemical assays to detect *gusA* expression as described by Jefferson (1987). Next, initial confirmation of transgenic callus was made by PCR to detect the *SopA* gene of the BAC vector backbone. DNA was extracted from transgenic leaf tissue using the method of Doyle and Doyle (1990). Primers were designed to amplify a 1151-bp fragment of the BAC vector backbone containing *SopA*. The primers were 5′-GCCATTGCACAGTTTAATGATGACA-3′ and 5′-GCGTGGTTTAATCAGAGCATCGAA-3′. The reaction was performed using 40 thermal cycles, each consisting of 45 s at 94°C, 1 min at 58°C and 1.5 min at 72°C. For *hph*, the primers were 5′-CGATGTAGGAGGGCGTGGATA-3′ and 5′-CTTCTGCGGCGATTTGTG-3′, which amplify an 813-bp fragment. The reaction was performed using 35 thermal cycles each consisting of 35 sec at 94°C, 45 s at 60°C and 1 min at 72°C.

For Southern blot analysis, 3–5 μg genomic DNA were digested and separated in 0.9% agarose gels. After separation, the DNA was transferred to nylon membranes (Hybond N, http://www.amershambiosciences.com). Random priming was used to label the probes with

Table 1 Summary of the plants described in this manuscript

Source of High molecular weight DNA	Transformation event	No *SopA* bands on southern	No inserts as determined from FISH	T_1progeny obtained	T_1 segregation	χ^2 (3:1)
BAC 17p22 rice centromeric	19	1	1	Y	30 + /20–	6 (P = 0.014)
	14	5	1	Y	nt*	
	4–1	4–5	4	Y	nt	
	2–02	3	1	N		
	1	1	1	N		
BAC 16H10 maize centromeric	815	1	1	N		
BAC 76A03 random rice DNA	Δ1	1	nt*	Y	45 + /24–	3.52 (P = 0.061)
	6	1		N		
BAC 7K12 Random Rice DNA	2	1		N		0.073 (P = 0.787)
	3a	1	nt	Y	30 + /11–	
	2a	2		Y	nt	

* Not tested

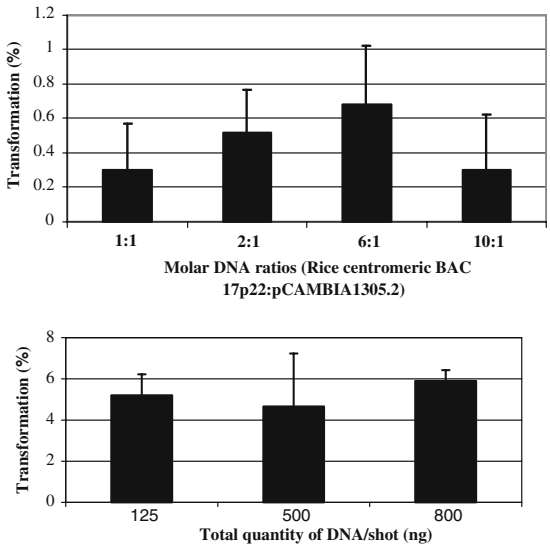

Fig. 3 Optimization of parameters for transformation with BAC DNA. Data from a completely randomized block design with three replications blocked by date were used for single factor ANOVA, (SAS 1990). Each set of calli was bombarded once. The bars represent standard error of the mean. ANOVA revealed no significant differences ($P = 0.05$) due to treatment in the two experiments. *Top*: Transformation frequency as affected by the molar ratio of BAC to selectable marker plasmid (pCAMBIA1305.2) DNA, using 800 ng total DNA per shot. Transformation frequency was calculated as the number of hygromycin-resistant, *sopA*-positive events recovered (as verified by PCR) divided by the number of embryogenic calli bombarded, ×100, with each replication having approximately 100 embryogenic calli. *Bottom:* Transformation frequency as affected by the total amount of DNA used per shot. Transformation frequency was calculated as the number of hygromycin-resistant events recovered (as verified by PCR) divided by the number of embryogenic calli bombarded, ×100, with each replication having 84 embryogenic calli

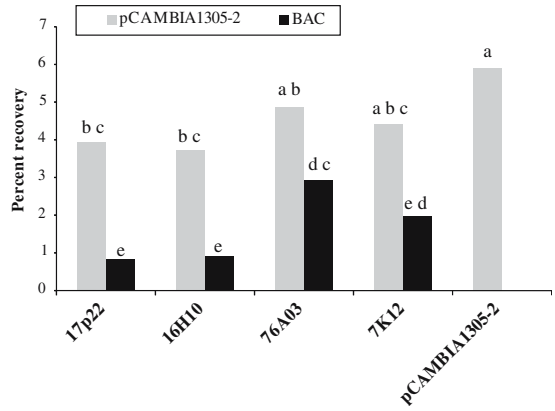

Fig. 4 Frequency of co-transformation as affected by the BAC used, as compared to frequency of transformation when pCAMBIA1305.2 was used alone. Data from a completely randomized block design with three replications blocked by date were used for single factor ANOVA using PROC GLM (SAS 1990). Transformation frequency was calculated as the number of hygromycin-resistant, *SopA*-positive events recovered (as verified by PCR) divided by the number of embryogenic calli bombarded, ×100, with each replication having approximately 100 embryogenic calli. Each set of calli was bombarded once. Means were separated by LSD. Means with the same letters are not significantly different ($P = 0.05$; SAS 1990)

(^{32}P)-dCTP, and hybridizations were done according to the manufacturer's instructions. DNA from plants transgenic for the maize insert was digested with *Bam*HI and probed with *CentC*.

A similar analysis could not be carried out on plants containing the insert from 17p22, since a *CentO* probe would not be able to distinguish between the endogenous copies and the transgenic ones. Thus DNA from T_0 and T_1 plants containing the insert from 17p22 was digested with *Hpa*I and *Xho*I, and probed with *SopA*. To further verify that transgenic *CentO* sequences were present, primers were designed across the BAC backbone–BAC insert junction, using 5′-AGC GGA TAA CAA TTT CAC ACA GGA-3′ as the vector primer and 5′-GGT TCT AAA TCC GAG CAG ATG-3′ as the insert primer. These amplify an ~870-bp fragment. The reaction was performed

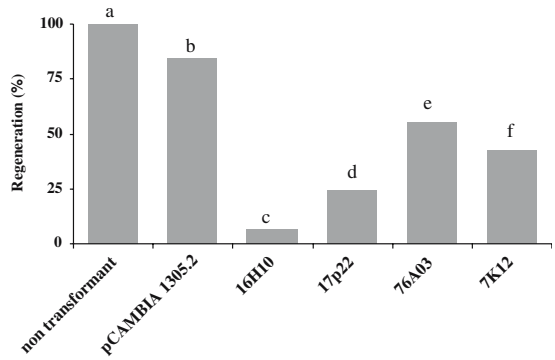

Fig. 5 Frequency of regeneration from callus transgenic for different BAC constructs co-transformed with pCAMBIA1305.2. Data from a completely randomized block design with three replications blocked by date were used for single factor ANOVA using PROC GLM (SAS 1990), with each replication having 15 transgenic calli. Means were separated by LSD. Means with the same letters are not significantly different ($P = 0.05$; SAS 1990)

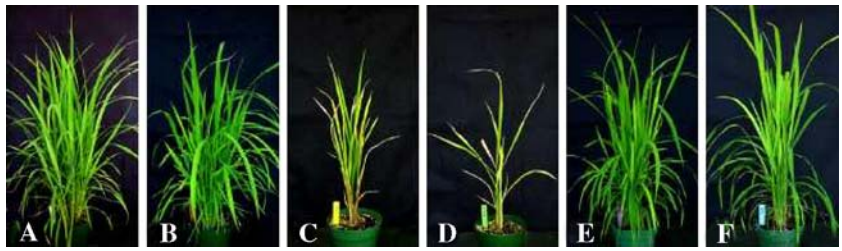

Fig. 6 Three-month old regenerated rice plants: (**A**) non-transgenic; (**B**) transgenic for pCAMBIA 1305.2; (**C**) Plant 14, transgenic for BAC 17p22 (rice centromeric DNA insert); (**D**) Plant 815, transgenic for BAC 16H10 (maize centromeric DNA insert); (**E**) Plant Δ1, transgenic for BAC 76A03 (rice random genomic DNA insert); (**F**) Plant 3a, transgenic for BAC 7K12 (rice random genomic DNA insert)

using 40 thermal cycles each consisting of 45 s at 94°C, 1 min at 58°C and 1 min 30 s at 72°C, using genomic DNA isolated as before and digested with *Nco*I (Fig. 2).

Finally, seven T_1 plants from T_0 plant 19 and five from T_0 plant 14, which were all verified to contain the BAC integrated into their genomes, were analyzed for the presence of *hph* gene as described previously.

FISH and fiber-FISH

Root tips and leaf tissue were harvested from T_1 plants for cytological studies. Plasmid clone pRCS2 (Dong et al. 1998) was used as a fluorescent in situ hybridization (FISH) probe to detect the *CentO* repeat. Maize BAC 16H10 and its BAC vector backbone (pBeloBAC11) were also used in FISH analysis. Chromosome and DNA fiber preparations were as published protocols (Jiang et al. 1995; Jackson et al. 1998). Briefly, the DNA was labeled with either digoxigenin-11-dUTP or biotin-16-dUTP. Probe preparations and signal detection for FISH and fiber-FISH were described previously (Jiang et al. 1995; Jackson et al. 1998). Chromosomes were counterstained by 4′,6-diamidino-2-phenylindole (DAPI) in Vectashield antifade solution (http://www.vectorlabs.com) and were pseudocolored in red. All images were captured digitally using a SenSys CCD (charge coupled device) camera (http://www.roperscientific.com) attached to an Olympus BX60 epifluorescence microscope. The CCD camera was controlled using IPLab Spectrum v3.1 software (http://www.scanalytics.com). Grey scale images were captured for each color channel and then merged. The final images were adjusted with Adobe Photoshop software (http://www.adobe.com). The endogenous centromeres and the transcentromeric DNA sequences are visualized as yellow spots on the chromosomes.

Results and discussion

Although transformation with LDSs has been recognized as an important goal for several years, the parameters for efficient delivery of LDSs into plants and their subsequent analysis have remained largely undefined. Rather than attempting to alter standard cell culture and regeneration protocols, work focused on studying plasmid ratios and amounts, as well as methodology to analyze the resulting plants.

A summary of the plants characterized in this study is provided in Table 1. The treatments that gave the numerically superior results were 800 ng DNA and the 6:1 pCAMBIA1305.2:BAC ratio (Fig. 3). These quantities were chosen for further work, even though the differences between them were not statistically significant.

Consistent with other work using *Agrobacterium*-mediated transformation of LDSs (Shibata and Liu 2000), the transformation frequency was significantly lower with LDSs than when only the visual and the selectable marker genes were used. Furthermore, the number of transgenic events recovered tended to be significantly less when BACs with centromeric inserts were used than when BACs with random DNA inserts were used (Fig. 4). Transformation frequency was between 4 and 5% of bombarded callus clusters when just

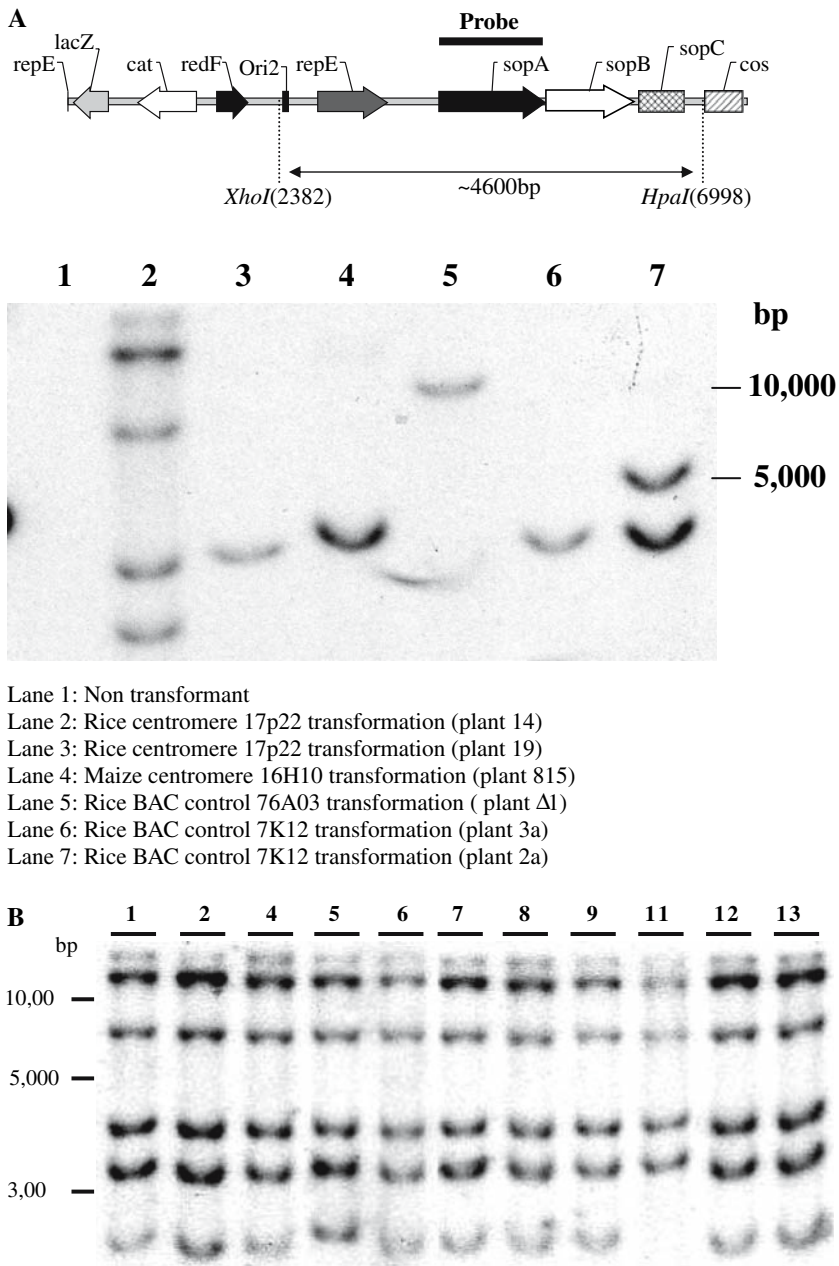

Fig. 7 (**A**) Southern blot analysis of the T_0 progeny. DNA was digested with *Hpa*I and *Xho*I, and probed with *SopA* from the BAC backbone, as diagramed above the blot. (**B**) Southern blot analysis of the positive T_1 generation from plant 14. DNA from PCR-positive T_1 plants was digested with *Hpa*I and *Xho*I, and hybridized to the *SopA*, as shown in Fig. 7A

pCAMBIA 1305.2 was used. Frequency of recovery dropped to 2–3% when BACs with random genomic inserts were co-bombarded with pCAMBIA, and fell to less than 1% when BACs with centromeric DNA inserts and pCAMBIA were co-bombarded.

Once transgenic calli were obtained, the BAC insert used for transformation also affected the ability to regenerate plants from that callus. Over 80% of calli transgenic for pCAMBIA 1305.2 regenerated into plants. In contrast, regeneration frequency was between 40 and 55% for callus clusters transgenic for the BACs with random inserts. The frequency of regeneration was lowest for BACs with the centromeric inserts- about 20% for clusters with the rice centromeric insert, and just over 5% for those with the maize centromeric insert (Fig. 5).

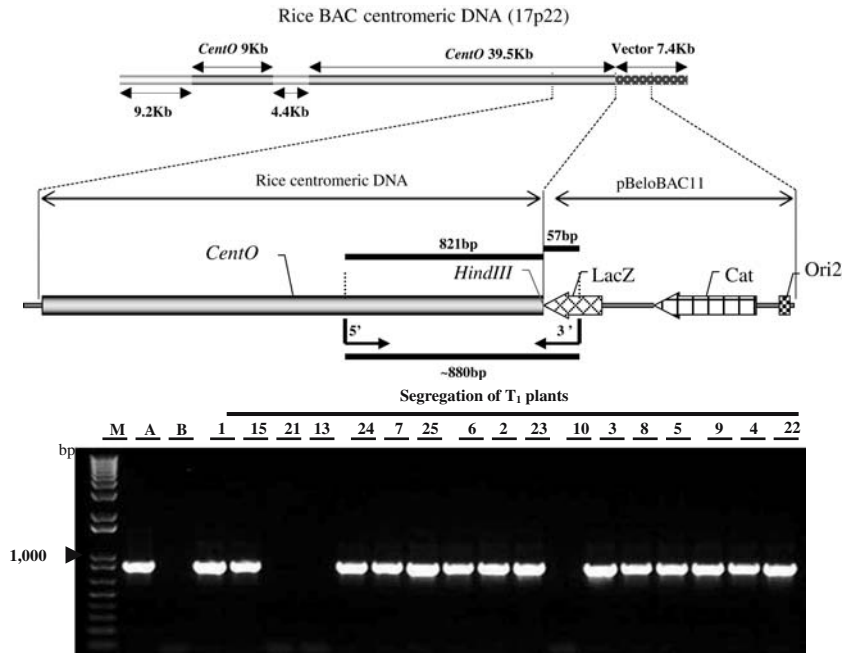

Fig. 8 Presence of transgenic *CentO* sequences in rice. Seventeen T_1 plants derived from T_1 14 (containing BAC 17p22). Primers were designed to amplify an 878-bp fragment of the vector insert junction. Lane A = T_0 plant 14. Lane B = non-transgenic control

As seen in Fig. 6, the phenotype of the transgenic plants revealed striking differences depending on the insert of the BAC used to transform them. Plants transformed with pCAMBIA 1305.2 or with the BACs containing random DNA inserts were essentially indistinguishable from non transgenics. In contrast, plants containing transcentromeric DNA inserts were severely stunted. The stunting effect diminished with age, and was no longer evident after 3 months. Two of the plants with rice transcentromeric DNA inserts (4–1 and 2.02) had extra chromosomes. The phenotype of plantlets transgenic for centromeric DNA was always stunted, regardless of whether the plants were diploid or aneuploid.

The number of bands observed with a probe for the *SopA* gene from the BAC backbone varied from one band for plant 19 to five bands for plant 14 (Fig. 7A, B). For plants transgenic for 16H10 (containing maize centromeric DNA), it was possible to verify the presence of maize centromeric DNA using a probe for the *CentC* repeat, as it is not present in rice. In contrast, the presence of the rice centromeric DNA BAC insert in transgenic rice plants could only be verified by amplifying the BAC-insert DNA junction (Fig. 8). In addition, each of twelve T_1 plants from plant 14 (containing the 17p22 rice centromeric BAC) also contained the *hph* gene.

Although the Southern data are convincing evidence of genomic insertion, these data do not distinguish if the numerous *SopA* bands observed in some lines (Fig. 7) are complex insertions at single sites, or several different insertions at unlinked loci. To address this question, FISH was used on 5 of the total of 6 lines studied. As shown in Fig. 9, the transcentromeric DNA sequences could be clearly distinguished from the endogenous centromeres. The endogenous centromeres are identified by a single *CentO* hybridization site associated with the primary constriction of the chromosomes, whereas the transcentromeric DNA segments are identified by an additional *CentO* hybridization site (Fig. 9). The rice transcentromeric DNA sequences integrated into a single locus in all of the studied lines, except for the plant in Fig. 9C (Plant 4–1, carrying BAC 17p22), which has about 4 insertion sites.

Fiber-FISH performed on plant 815 showed that the transcentromeric DNA sequences could be as long as 130 kb, suggesting that the entire insert of BAC 16H10 has been integrated into the transgenic lines. The fiber-FISH signals contain large gaps because the *CentC* repeat only

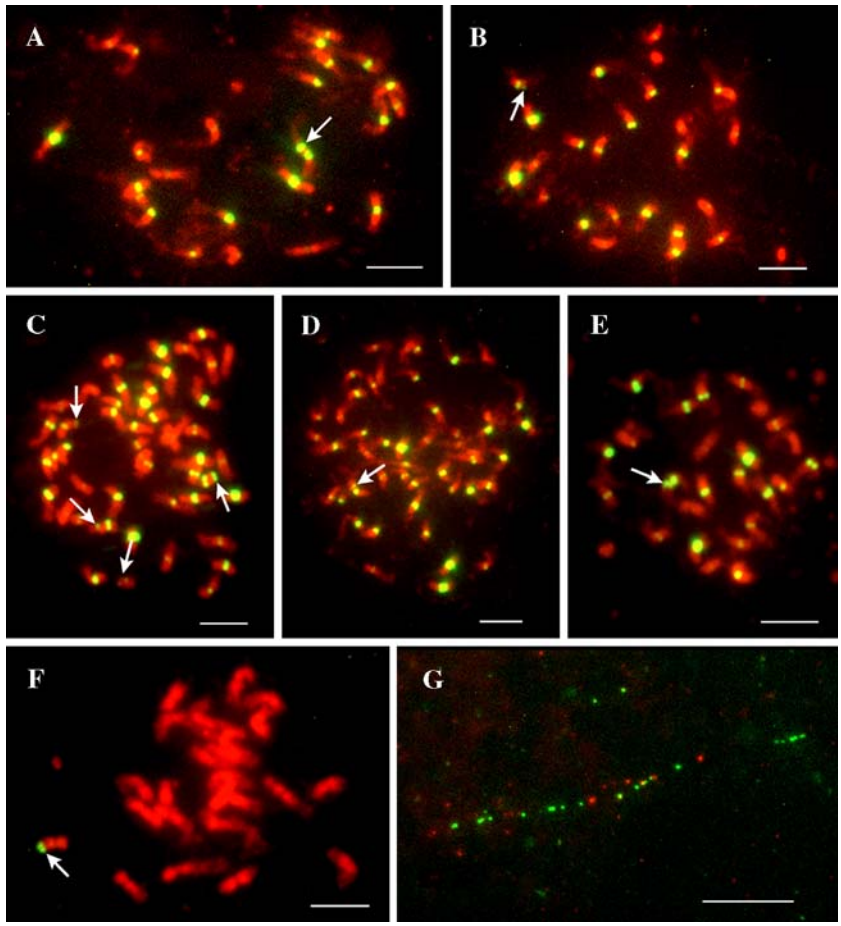

Fig. 9 FISH (**A**–**F**) and fiber-FISH (**G**) of plants transgenic for BACs with centromeric inserts. Plants **A**–**E** have the rice centromeric insert, while **F** and **G** have the maize centromeric insert. Arrows indicate hybridization signals to the transcentromeric DNA. (**A**) plant 14; (**B**) plant 19; (**C**) plant 4–1; (**D**) plant 2-02; (**E**) plant 1; (**F**) plant 815; (**G**) fiber-FISH of plant 815. In fiber-FISH, the BAC vector pBeloBAC11 was labeled in red and the maize centromeric satellite repeat *CentC* was labeled in green. Size bars = 5 μm in **A**–**F**, 10 μm in **G**

accounts for ~22% of the insert of BAC 16H10 (Nagaki et al. 2003). At least two red spots at different locations were consistently observed in the fiber-FISH signals, suggesting that the integrated BAC DNA was rearranged during the transformation.

Half of the T_0 transgenic lines for LDSs were fertile, without any obvious correlation between the nature of the LDS and fertility status. The T_1 plants all had a normal phenotype, even though they contained the transcentromeric DNA inserts. Segregation was examined in one T_1 line each for BAC 17p22, BAC 7K12 and BAC 76A03. Germination of all seeds was upwards of 90%. Segregation fit a 3:1 ratio (Table 1) for all lines except 7K12, from which almost equal numbers of transgenic and nontransgenic progeny were recovered. In the case of BAC17p22 event 14, Southern analysis demonstrated multiple *SopA* bands, though these were inherited as a single unit for the most part. Only one missing *SopA* band was observed in a single progeny plant (Fig. 7B, lane 11). Taken together, these data indicate that, once integrated, large transcentromeric DNA arrays are remarkably stable in rice, despite their highly repetitive nature.

Future work will focus on why centromeric DNA sequences affect transformation efficiency and appear to cause abnormal phenotypes in the early stages of regeneration. It is unlikely that the

BACs caused insertional mutations, given these were independent events and the controls didn't have the same effect, plus the effect was transitory. Further, it is unlikely that the phenotypes are caused by gene over-expression, since there are no genes aside from those encoded by retroelements in either BAC, and which are quite abundant in their respective genomes. Integration of the centromeric DNA sequences would be expected to create a dicentric chromosome, leading to chromosome breakage during anaphase. Although centromeres can be very large (Kaszas and Birchler 1996), recent data suggest that the size necessary for a functional centromere is closer to a few hundred kilobases (Phelps-Durr and Birchler 2004; Nagaki et al. 2004). Thus, it will be interesting to conduct future experiments to study whether the typically poor transformation and regeneration efficiencies (Figs. 4 and 5) and slowed early growth of plants containing rice transcentromeric DNA sequences (Fig. 6) are caused by centromeric function of the transcentromeric DNA sequences in the early stages of transformation and regeneration, but were then silenced later in development. This is consistent with the recent demonstration that when two centromeres are present in maize the second is often inactivated (Han et al. 2006). Such centromere inactivation might also be able to explain why maize centromeric sequences in rice had a greater effect than rice centromeric sequences in rice—presumably, rice is better able to recognize and silence its own centromeric sequences than those of maize.

An eventual goal of this work is to obtain artificial chromosomes for use in plants. Artificial chromosomes are viewed as an enabling technology to produce the next generation of plant transformation vectors (Richards and Dawe 1998; Somerville and Somerville 1999; Brown et al. 2000), as they offer the potential to introduce many genes at once without complications associated with multiple rounds of transformation. So far, artificial chromosome technology has been successful in mammalian and yeast cells (Brown et al. 2000), but not in plants. Progress in the development of plant artificial chromosomes has been limited and our inability to introduce long arrays of centromere repeats into crop plants and our poor understanding of how centromeres are established and maintained. Now that the first limitation has been overcome, research can focus on the functional activation of plant centromere activity.

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