Developing Plant Artificial Chromosome from *Arabidopsis* Genetic Resources for Rapeseed Improvement

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ABSTRACT

Plant artificial chromosomes (PAC) may be used as a new vector to introduce chromosome segments of hundreds Kb to Mb length into plant cell. Two YAC clones were identified to be with the 178 bp *Arabidopsis* centromeric repeat sequences. *Arabidopsis* telomere was cloned from PCR products using telomere repeat primers. A 2000bp fragment of ARS was released from Genomic BAC clone T14A4 by *ClaI* digestion and subcloned into Pbluescript. Plasmid vectors have been constructed to integrate necessary elements of PAC into both right and left YAC arms. Retrofitting YAC clones carrying *Arabidopsis* centromere segment by homologous recombination with the constructed right and left arm vectors were performed and confirmed in yeast. The modified YACs are expected to generate PAC after being transferred into *B. napus* and *Arabidopsis* protoplast by liposome-mediated method.

Key Words: YAC, Centromere, Telomere, *Arabidopsis*, *B. napus*

INTRODUCTION

The successfully construction of yeast artificial chromosomes (YACs) (1) in *S. cerevisiae* provided an important tools both for the study of yeast chromosome function and as large capacity cloning vectors. The use of similar strategy in human cells to produce human artificial chromosome may be expected to provide an important tool for the transgenic of large DNA sequences into human cells (2). Plant artificial chromosomes (PAC) may be used as a new vector to introduce chromosome segments of hundreds Kb to Mb length into plant cell.

Any mitotic stable linear chromosomes, natural or artificial, should bear three basic elements: a centromere, two telomeres and origins of replication. Genome sequencing has produced sufficient genetic resources from which one could mine the necessary elements for PAC construction. There is a highly abundant repetitive DNA sequence family of *Arabidopsis* centromere, Atcon, which is similar to human α-satellite DNA in structure, the best-characterized human DNA associated with the centromeric region (3,4). Sequence information for telomere and ars is also available in Arabidopsis database. We reported primary work on constructing PAC of Arabidopsis for genetic improvement of rapeseed.

MATERIALS AND METHODS

All general DNA manipulation was performed by standard procedures. For purification, DNA was excised from the 1% gel and purified away from the agarose using QIAquick Gel Extraction Kit (Qigen) according to the manufacturer's instructions. For T-A clone, PCR products were legated to pGEM-T Easy Vector (promega) according to the manufacturer's instructions. Plasmid p5RADE2 carrying 2.8 kb *ADE2* fragment was donated by Professor Jingze Lin (National Yang-Ming University, TAIWAN); pRS400 was obtained from Professor John Cannon (University of Missouri-Columbia, USA); pCAMBIA1303 containing GFP5 was obtained from Center for the Application of Molecular Biology to International Agriculture, Australia.

Yeast cells were grown up in the AHC medium to a density OD600 of 1 to 2. The "LiAc/ssDNA/PEG" lithium acetate transformation protocol was performed.

Seedlings of *Brassica napus* and *Arabidopsis* were grown on the MS medium. Cotyledons or leaves from 3-4 weeks old plants were used for protoplast isolation. Leaves were cut into 0.5-1 mm strips with fresh razor blades without wounding. 100-200 leaves were digested in 3 ml cellulase/macerozyme solution for 18hr (*B. napus* cotyledons) or14hr (*Arabidopsis* leaves) without shaking in the dark 25?. Protoplasts were released by shaking gently for 1 min.

RESULTS

Telomeric sequence generation. Telomeric DNA was generated by PCR using telomeric repeats as primers in the absence of template. As the PCR cycles going on, a heterogeneous population of molecules consisting of repeat arrays of various lengths has been produced. Following PCR, each reaction was subjected to agarose gel electrophoresis to purify telomeric DNA >1kb. The purified telomeric DNA was legated to pGEM-T Easy Vector to generate plasmid PGT100.

Centromere isolation and identification. Eight *Arabidopsis* YAC clones near the centromere were selected and collected from the *Arabidopsis* Biological Resource Center. Four pairs of

PCR primers were designed according to the Atcon sequence. Centromeric DNA was amplified by using these YAC clones genomic DNA as template. Two candidate YAC clones, CIC8H8 and CIC6F6 were proved to have the Atcon sequences. These two were chosen to construct PAC. The PCR products were also legated to pGEM-T Easy Vector and the clones were sequenced by GENE Company.

Plasmid Constructions. A 1.9kb ClaI fragment containing the ars3 sequence was digested from one *Arabidopsis* BAC clone T14A4 and subcloned into the pBluescript M13 ClaI site to generate plasmid pBA101. 793bp truncated *URA3*$^{-}$ fragment was produced by PCR using primers ural and urar. After PCR, excising from gel and purifying away from the agarose, the purified DNA was digested by EcoRI and BamHI and then subcloned into pBA101 to generate plasmid pBAU102. 1087bp truncated TRP1^{-} fragment was produced by PCR using primers trpl and trpr. This fragment was also digested by EcoRI and BamHI and then subcloned into pBluescript to generate plasmid pBT103. Both pBAU102 and pBT103 were digested by SalI and BamHI, 2.8kb fragment from pBAU102 and 1.0kb fragment from pBT103 were respectively subcloned into PUC118 to generate plasmid pUAU104 and pUT105. The Arabidopsis telomere fragment was released from PGT100 by ApaI and PstI and subcloned into pBluescript to generate plasmid PBT106. 2.8 kb *ADE2* fragment was released from p5RADE2 by BamHI and subcloned into PBT106 to generate plasmid PBTA107. The 4.1kb fragment generrated by digesting PBTA107 with SacI and KpnI and the 2.8kb fragment generrated by digesting pUAU104 with SalI and KpnI were ligated to pCAMB1303 digested by SacI and SalI to generated plasmid pCR109 (Fig 5a). The 1.3kb fragment generrated by digesting PBT106 with XbaI and KpnI and the 1.0kb fragment generrated by digesting pUT105 with SalI and KpnI were ligated to pRS400 digested by XbaI and SalI to generated plasmid pRL108

YAC Modification and Character. The selected YAC clone was transformed with ApaI-linearized pCR107. The transformants was selected in adenine negative plates. The white transformants that no longer grow on plates lacking uracil but still grow on plates with uracil was transformed with SmaI-linearized pRL108. The transformants were firstly selected in the g418 positive plates and the positive clones that no longer grow on plates lacking tryptophan but still grow on plates with tryptophan were chosen as candidate clones. Bright green fluorescence was observed by fluorescence microscope for the main 395nm excitation and 509nm emission peaks of GFP. The plugs of yeast DNA were separated on 1% PFGE agarose gel with a pulse of 30 seconds for 12 hours followed by a pulse of 60 seconds during 15 hours in 0.5XTBE buffer. A slice containing the YAC is excised and purified by dialyze.

Protoplast Transfection. The protoplasts were spied down and resuspened in MMg solution before PEG transfection. 10 µl commercial liposome and 10 µg purified YAC-DNA were incubated in a total volume of 100 µL sterile water for 10 min at room temperature to allow formation of DNA-lipid complexes. The DNA-containing mixture was added to 0.5 mL of a suspension of protoplasts and mixed gently. 0.5 mL PEG solution was added into the mixture and was incubated for 15 minutes at room temperature. The protoplasts were washed twice by pelleting at 600 rpm for 5 min, and incubate in 3 mL PCM1 in the dark at 25 °C for 48 hr. However, the protoplasts showed only red chlorophyll autofluorescence under fluorescence microscope and are proved to be untransformed.

DISCUSSION

Conventional plant transformation techniques, such as *Agrobacterium*-mediated transformation, PEG-mediated direct gene transfer, and particle bombardment cannot be used for the transfer of intact YACs, either because the technique does not accommodate large pieces of DNA or because it will damage high-molecular-weight DNA. YACs up to 650kb have been introduced into mammalian cells by lipofection, while somewhat smaller YACs have been introduced by microjection. However, neither technique works efficiently in plant transformation for cell wall. So isolating plant protoplast would improve transformation efficiency. We have used commercial liposome with PEG to transform huge PAC into plant protoplasts. Although we didn't get desire transformats by this method, there are still many other methods can be tried such as microjection or fusing yeast spheroplasts to plant protoplasts.

REFERENCES

1. Murray A W, Szostak J W. Construction of artificial chromosomes in yeast, *Nature*, 1983, 305:189-193.
2. Huntington F. Willard. Genomics and Gene Therapy: Artificial Chromosome Coming to Life, Science, 2000, 5495: 1308-1400.

3. Murphy T D, Karpen G H. Centromeres Take Flight: Alpha Satellite and the Quest for the Human Centromere. *Cell*, 1998, 93:317-320.
4. Copenhaver G P, *et al*. Genetic Definition and Sequence Analysis of *Arabidopsis* Centromeres. Science 1999, 286:2468-2474.