Plant Molecular Biology Reporter **24:** 219–227, June 2006

Genetic Resources

Construction and Characterization of a Transformation-Competent Artificial Chromosome (TAC) Library of *Zizania latifolia* (Griseb.)

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Abstract. The tribe Oryzeae consists of 12 genera and 71 species with a world distribution. *Zizania latifolia* (Griseb.) Turcz. ex Stapf is included in this tribe and possesses numerous traits valuable for rice breeding, such as disease and insect resistance, cold and flood tolerance, and high grain quality. The genetics and breeding of *Z. latifolia* are still in their infancy. To facilitate genomic studies of *Zizania*, a genomic DNA library was constructed using a transformation-competent artificial chromosome (TAC) vector system. The TAC library contains 91, 584 TAC clones with an average insert size of approximately 45 kb, covering six haploid *Zizania* genome equivalents. Very low signals after hybridization with chloroplast and mitochondrial genes indicate that the TAC library is predominantly composed of nuclear DNA. The TAC clones were stable in *E. coli* for 100 generations. Clones containing the *dihydrodipicolinate synthase* (*DHPS*) gene were screened by pooled PCR. The positive clones can be used for *Z. latifolia DHPS* gene cloning and functional analysis. The library will be useful in studies of genome structure, gene cloning and evolution of rice.

Key words: dihydrodipicolinate synthase (DHPS), genome library, *Z. latifolia* (Griseb). Turcz.ex Stapf, TAC, transformation-competent artificial chromosome

Abbreviations: CIAP, calf intestinal alkaline phosphatase; DHPS, dihydrodipicolinate synthase; HMW, high molecular weight; PFGE, pulsed field gel electrophoresis; TAC, transformation-competent artificial chromosome.

Introduction

Zizania L. is a wild perennial grass belonging to the tribe *Oryzae*, and is thus related to rice (*Oryza sativa* L.). *Zizania* L. includes *Z. aquatica*, *Z. palustris*, *Z. texana*

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Hitche. and *Z. latifolia* (Griseb.) Turcz. ex Stapf. *Z. latifolia* is distributed throughout China and southeast Asia, and has many traits that make it valuable for rice breeding, such as disease and insect resistance, cold and flood tolerance, and high grain quality. Analysis of nutritional composition indicates that the amino acid content is rational; the first limiting amino acid is threonine; and the content of lysine is higher than that in rice. However, the genetics and breeding of *Z. latifolia* have not been fully explored. *Z. latifolia* is an annual diploid with a chromosome number of 2n=34, and its genome is about twice the size of the rice genome. The comparative map of rice and wild rice (Z. *palustris*) has recently been constructed, and imparts basic information on the composition of the wild rice genome and provides tools to assist in the domestication of this important food source (Hass et al., 2003; Kennard et al., 1999). Colinearity is especially expected between *Z. latifolia* and rice, since they are taxonomically grouped in the same subfamily, Oryzoideae, and tribe, Oryzeae (Gould et al., 1983; Duvall et al., 1993).

At present, potentially beneficial genes of *Z. latifolia* are not being utilized. Therefore, it would be constructive to transfer these genes from *Z. latifolia* into rice. However, *Z. latifolia*, as a related wild species, is sexually incompatible with rice (Liu et al., 1999). One way to accomplish the transfer is to first clone the genes of interest and then transform them into rice. In such cases, the TAC system can meet this requirement by providing many technical advantages, such as the ability to clone large DNA fragments and to directly perform *Agrobacterium*-mediated transformations (Liu et al., 1999, 2000, 2002). The TAC system is highly efficient for the transfer of large DNA fragments into *Arabidopsis* and rice and for the complementation of mutants. In this paper, a *Zizania* genomic DNA library was constructed using the TAC vector system. pYLTAC17 was selected as the vector due to its high success rate in transformation of monocotyledons (Liu et al., 1999, 2000, 2002; Fang et al., 2000; Wang et al., 2002).

Dihydrodipicolinate synthase (DHPS) is the key enzyme of a specific branch of the aspartate pathway leading to lysine biosynthesis in higher plants (Gad Galili et al., 1995; Ghislain et al., 1990; Kumpaisal et al., 1987). *DHPS* genes have been cloned from several plant species (Dereppe et al., 1992; Kaneko et al., 1990; Frisch et al., 1991; Vauterin et al., 1994), but none have been cloned from *Z. latifolia*. In this paper, the *DHPS* gene of *Z. latifolia* was isolated from the TAC library after screening with an amplified fragment from a conserved region of the gene.

Materials and Methods

Biological strains and reagents

Young leaves of Z. *latifolia* (Griseb.) plants were collected from the lakes of the Jiangxi Province, China for use in this study. The *E. coli* strain DH10B was used for construction of the TAC library. The pYLTAC7 vector was kindly provided by Professor Yao Guang Liu. *Hin*dIII restriction enzyme was purchased from TaKaRa (Shiga, Japan) and T4 DNA ligase and CIAP (calf intestinal alkaline phosphatase) were purchased from New England BioLabs (Ipswich, MA).

TAC library construction

High-molecular-weight (HMW) DNA preparation and TAC library construction were carried out according to the procedure described by Liang (Liang et al., 2004).

Partial digestion of HMW DNA using *Hin*dIII and the recovery and separation of size-fractionated DNA were performed as previously described (Liang et al., 2004). Four size fractions (30-50 kb, 50-100 kb, 100-150 kb, 150-200 kb) were recovered from each partial digest and separately ligated into the pYLTAC17 vector digested with *Hin*dIII and dephosphorylated with CIAP.

Ligations were performed in 100 μl reaction solutions containing 80 ng of partially digested HMW DNA, 100 ng of pYLTAC17 vector and 160 units (2.4 Weiss units) of T4 DNA ligase. The reactions were carried out in a thermocycler as previously reported by Lund et al. (1996) with some modifications. Subsequently, the ligation mixtures were desalted and concentrated on a Millipore filter (Type VS 0.025 μm) against sterile distilled water at 4°C for 0.5 h.

The ligation mixtures were used to transform competent *E. coli* DH10B cells by electroporation using a Gibco BRL Electroporator-voltage booster system. Kanamycin resistant colonies were picked and cultured in 96-well plates at 37°C for 16-18h before transferring to 384-well plates. Meanwhile, 12 colonies were collected and pooled into 96-well plates.

TAC library characterization

An estimate of insert-size and library capacity was obtained from *Hin*dIII restriction analysis of 100 randomly selected TAC clones.

Chloroplast (cpDNA) and mitochondrial (mtDNA) DNA contamination in the TAC library were assayed with mixed probes from the sorghum chloroplast gene *psbA* and the maize mitochondrial gene *atp6*. Three 384-well plates were randomly selected for hybridization. TAC clones were transferred from the plates to three pieces of Hybond filter with a 384-replicator, lysed and hybridized with the mixed probe. In addition, 100 TAC clones were selected and digested with *Hin*dIII. After being transferred to the filter, the membrane-bound DNA was hybridized with total *Z. latifolia* genomic DNA.

The stability of the TAC clones was checked by culturing three random clones for approximately 30, 60 and 100 generations. Samples were collected at 0, 30, 60 and 100 generations and analyzed by *Hin*dIII digestion.

TAC library Screening

Specific *DHPS* primers were designed and synthesized based on a conserved region of the *DHPS* gene found in the NCBI GenBank database (www.ncbi.nlm.nih.gov). The forward primer was DHPS-1: 5′ TGTAATAGTGGGAGGAAC AACA3′ and the reverse was DHPS-2: 5′ GAACCCTTGTTCTTTCTGAC3′. Using these primers, an initial screening of the library was carried out using pooled clones. Aliquots (10 μl) from each well of every 96-well plate were removed, pooled together in 5 ml LB medium with 25 $ug \cdot ml^{-1}$ kanamycin, and amplified overnight at 37°C. Plasmid DNA isolated from each overnight culture was resuspended in 50 μl 1× TE buffer (pH 8.0) and subsequently used in the first screen

for the *Z. latifolia* DHPS gene fragment. The PCR reaction was performed in a volume of 20 µl containing 10 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 50 mM KCl, 0.25 mM each dNTP, 0.2 µM each primer, 1 unit of Taq DNA polymerase and 2 µl pooled plasmid DNA. The following cycling parameters were used: 94°C for 2 min; 35 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min; with a final extension step of 72°C for 1 min. A 10 µl sample of each reaction mixture was examined by agarose gel electrophoresis to check for the presence of the *DHPS* gene fragment. When a positive pool was found, the corresponding wells in the original plates were located and the plasmids were isolated and used as templates for a second screen. Each positive well in the second screen contained 12 clones corresponding to the 12 single colonies in the second copy (384-well plates) of the TAC library. The 12 clones from the 384-well plates were used for a third screen from which the positive single colony was obtained.

Results

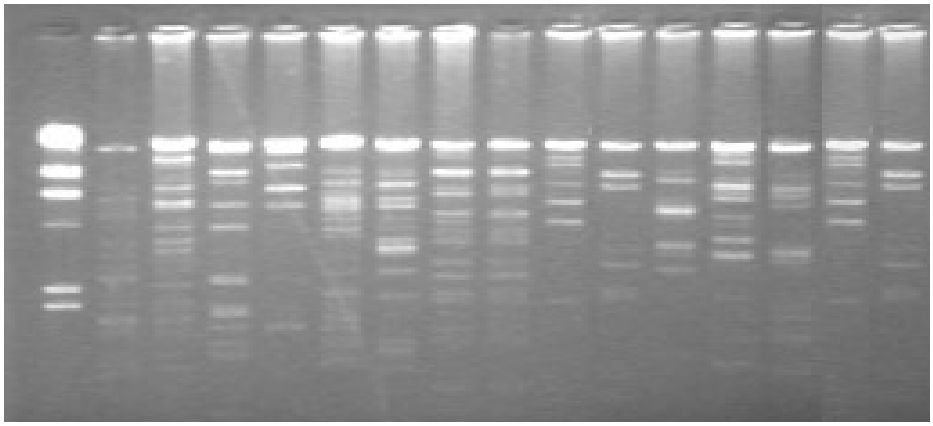
A large amount of high-quality HMW DNA is required to construct a large insert genomic DNA library. To obtain the required amount of DNA, nuclei were isolated from young meristematic tissue. The average size of the purified HMW DNA was greater than 2 Mb and showed little evidence of cell debris contamination. Fragments in the desirable size range of 30-200 kb were obtained by using 0.6 units/ml of *Hin*dIII after 8 min of digestion.

The *Z. latifolia* TAC library contains 91,584 clones and was stored as bulked pools in eighty-one 96-well plates and as single clones in two hundred thirty-nine 384-well plates. Each well of the 96-well plates contained 12 individual clones. To evaluate the potential of the TAC library for gene isolation, genome analysis and genetic transformation, the library was characterized based on insert size distribution, clone representation, stability and levels of cpDNA and mtDNA content.

The plasmids from 100 randomly selected TAC clones were digested with *Hin*dIII to estimate insert size. All of the clones contained an insert (Figure 1), reflecting a low percentage of empty clones. The insert size averaged 45 kb, and ranged from 20-80 kb (Figure 2). Assuming a haploid genome size of 860 Mb, this theoretically represents a 6-fold coverage of the *Z. latifolia* genome. The probability of isolating a unique sequence from this library is >0.99.

To evaluate the degree of chloroplast and mitochondrial DNA contamination, a probe consisting of a mixture of a mitochondrial gene and a chloroplast gene was used to screen the library by filter binding hybridization. Based upon the number (3) of positive clones identified, the frequency of contamination was estimated to be 0.26% (Figure 3). Furthermore, the plasmid DNA from 100 TAC clones was digested, transferred to a filter and hybridized with *Z. latifolia* genomic DNA. The hybridization results indicated that all of the insert DNA fragments produced positive signals, which matched the electrophoretic banding pattern on agrose gel (Figure 4). This indicates that this TAC library is predominantly composed of nuclear DNA.

The stability of TAC clones in *E. coli* was assessed by analyzing *Hin*dIII restriction patterns of four randomly selected TAC clones after 0, 30, 60, and 100,



M 1……15

Figure 1. Restriction analysis of TAC clones. Plasmid DNA of TAC clones was completely digested with *Hin*dIII and separated on 1.0 % agarose gels. Lanes 1-15, TAC clones; M, Molecular weight marker (λ DNA cut with *Hin*dIII).

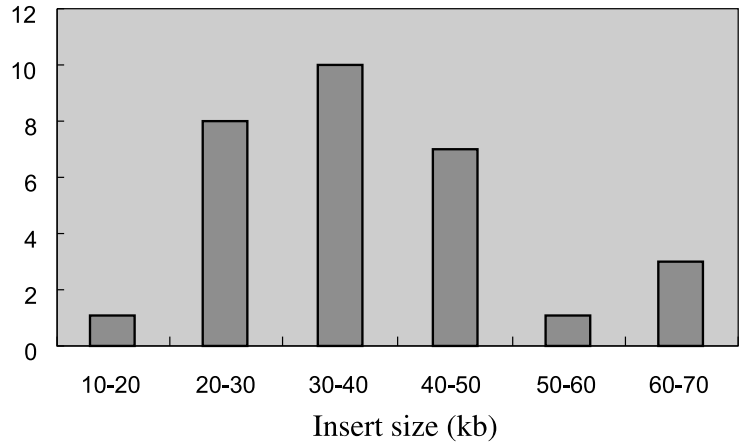


Figure 2. Size distribution of TAC clone inserts. Insert sizes were determined for 60 TAC clones.

generations (Figure 5). No differences were observed, indicating that the TAC clones are stable in *E. coli* for up to 100 generations and are suitable for genetic transformation in functional genomics research.

The feasibility of isolating any gene of interest from this TAC library was evaluated by screening the library for the *DHPS* gene. PCR primers were designed based on a conserved region of known *DHPS* genes in GenBank. A fragment of approximately 750 bp (Figure 6) was amplified, cloned and sequenced (data not shown). The nucleotide and deduced amino acid sequences of the PCR product showed high similarity to *DHPS* genes in rice, wheat and other plants. Seven positive clones of the *DHPS* gene were isolated. Since the library is estimated to cover about six haploid genomes, the number of positive clones is commensurate with the coverage.

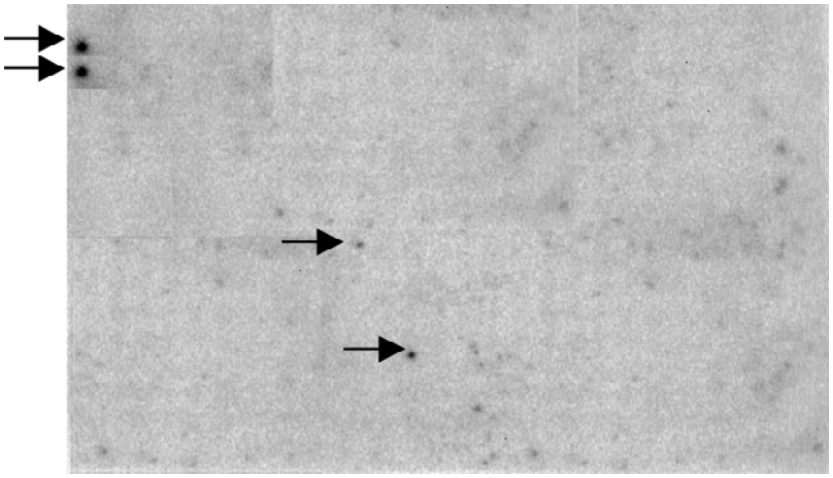


Figure 3. Hybridization analysis of ctDNA and mtDNA contamination in TAC clones. Arrows denote positive controls.

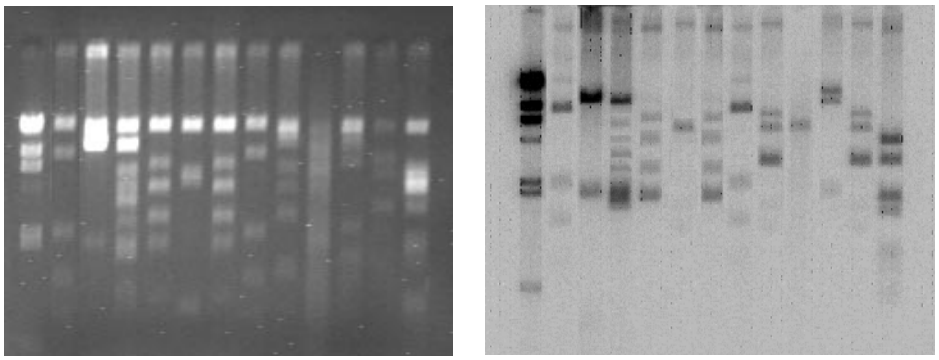
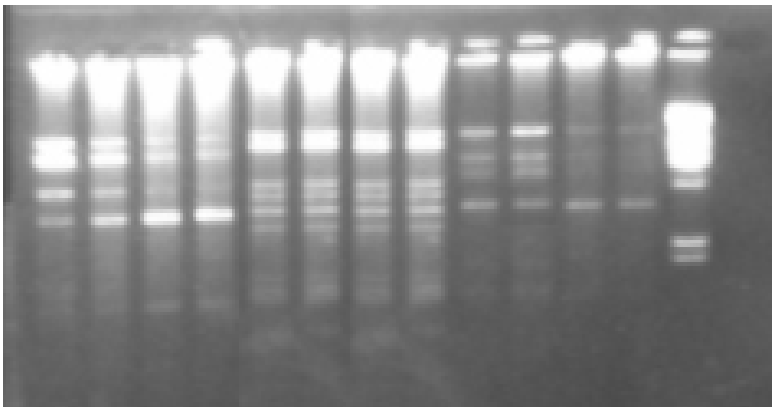


Figure 4. Hybridization analysis of insert DNA fragments from randomly digested clones; A, DNA fragment electrophoretic patterns; B, hybridization signals with DNA from patterns.

Discussion

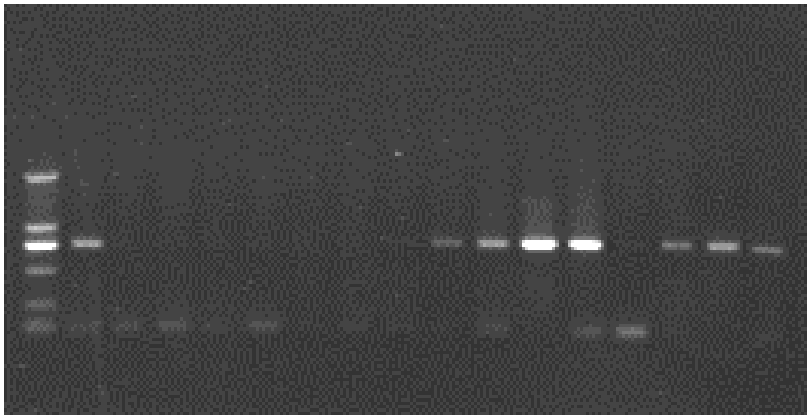
In constructing the genomic library, the selection of different size fractions of DNA was crucial for increasing library capacity. Vector pYLTAC17, which is 22,530 bp, was used to construct the TAC library. We found that the all of the fragment sizes could be ligated into the vector, but that smaller fragments were typically ligated more efficiently than larger fragments. An analysis of ligation transformation efficiencies indicated that the ratio of fragment to vector is an important factor in increasing ligation efficiency. For construction of this library, the ratio of 5:1 was ideal.

Contamination by cytoplasmic DNA can directly affect the practicability of isolating genomic genes and chromosome walking errors (Zhang et al., 1996). Two methods were used to evaluate the degree of contamination. Probes from chloroplast and mitochondrial genes were used to check the degree of contamination



A A A A B B B B C C C C M

Figure 5. Comparison of the *Hin*dIII digestion patterns of 3 TAC clones in generations 0, 30, 60 and 100. A, 0 generation; B, 30 generations; C, 60 generations, D, 100 generations; M, Molecular weight marker (λ DNA cut with *Hin*dIII).



M 1..... 16

Figure 6. Screening of the TAC library with the *DHPS* gene. Lanes 1-16, PCR products of positive TAC clones after the third screen for the *DHPS* gene: Lane 1, PCR product from *Z. latifolia* genomic DNA; Lanes 8-10, 11 and 13-15 are positive clones; M, DNA size marker.

in the library (Shen et al., 2005; Leroy et al., 2005). Among the TAC clones from three randomly selected 384-well plates, only three positive signals were displayed, indicating that the degree of contamination was very low compared to libraries from other species (Adam et al., 2005; Leroy et al., 2005). Another method was adopted to further validate the insert DNA using Z. *latifolia* genomic DNA as the probe. After DNA from random TAC clones was digested with *Hin*d III, hybridization signals and electrophoretic patterns were well-matched, indicating that the insert fragments truly originated from the genomic DNA of *Z. latifolia*.

To date, few genes have been isolated from *Z. latifolia.* However, a comparison with the rice genome shows that *Z. latifolia* has many useful genes. The study of these genes may provide new insight to rice breeders. Among the interesting features, the level of lysine in *Z. latifolia* is higher than that in other crop plants, and the limiting amino acid is threonine rather than lysine (Zhai et al., 2000), as it is in rice. In recent years, many researchers have focused on increasing the lysine content of crop plants. DHPS, as a key enzyme in lysine biosynthesis, has been isolated from other plants. Our newly cloned *DHPS* gene will be transformed into plant mutants to understand the mechanisms of lysine synthesis and metabolism.

Although related to rice, Z. *latifolia* has a chromosome number of $n = 17$, five more than rice ($n = 12$). The construction of this TAC library will aid in performing further comparative analyses between rice and *Z. latifolia,* such as synteny, allelic variation and gene organization studies. *Z. latifolia*, as wild rice, has many important genes, which have been lost in domestic rice. The greatest benefit of the TAC library is the capability to directly perform genetic transformations. Therefore, this library will not only facilitate gene cloning and the study of the structure and organization of genes, but will also be an efficient platform for the elucidation of functional genomics. A greater understanding of *Z. latifolia* could ultimately lead to improvements in the characteristics of domesticated rice.

Acknowledgements

This study was supported by grants from the Provincial Key Science and Technology Project of Jiangxi Province (200638) and the Science and Technology Project of Jiangxi Education Bureau (2006110).

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