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Full Length Research Paper

Full-length enriched multistage cDNA library construction covering floral bud development in *Populus tomentosa*

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Flowering involves expression of a suite of genes associated with floral development. The genome of the Chinese white poplar (*Populus trichocarpa*) was sequenced because of its importance as a model tree for genetic studies as well as being an economically important woody plant. However, information on expressed genes involved in poplar floral bud development is insufficient to allow annotation of genes and use of the genomic information. To isolate and characterize genes involved in flowering of *Populus tomentosa*, floral bud samples were collected at different developmental stages from floral bud initiation to flower maturity, and full-length enriched cDNA libraries from both male and female floral buds were constructed. The results of titer analysis showed that the titer of the female and male primary libraries were 8.00×10^5 and 7.20×10^5 pfu/ml, respectively, and the titer of the amplified libraries were 2.60×10^8 and 2.56×10^8 pfu/ml, respectively. The combination ratio reached 90% and the insert size was 400 to 2000 bp. The results indicated that cDNA libraries were successfully constructed.

Key words: cDNA library, floral bud, flowering, *Populus tomentosa*.

INTRODUCTION

Flowering is an important event in the life cycle of all flowering plants. Current knowledge on the molecular mechanism underlying flower induction and development mostly comes from extensive studies on the model plant *Arabidopsis thaliana* (Tan et al., 2007). A host of genes involved in flowering have been isolated and characterized in *Arabidopsis*. The model tree, poplar, differs markedly from *Arabidopsis* in floral traits. Poplar is a perennial tree with a long juvenile phase and lifespan (Braatne et al., 1996), and it flowers annually or seasonally during the reproductive phase after flowering for the first time (Yuceer et al., 2003). The reiterating developmental cycles between vegetative and reproductive growth periods are also absent in *Arabidopsis* (Boss et al., 2004). The male and female flowers are borne on separate trees from axillary inflorescences, but occasionally, trees are monoecious. Instead of four concentric whorls of organs, poplar flowers have only two whorls, comprising a reduced perianth cup surrounding either the stamens or carpels (Boes and Strauss, 1994; Sheppard, 1997; Rottmann et al., 2000). Clearly, the molecular mechanism regulating floral induction and development in poplar is more complex than that in *Arabidopsis*.

Chinese white poplar (*Populus tomentosa* Carr.) is a Chinese-native tree species in *Populus* section *Leuce* that is fast-growing and produces high-quality wood in the north of China. It currently plays an important role in forest production and forest reclamation, and will be important for biomass and biofuel use in the near future. However, the catkins produced by male and female *P. tomentosa* trees seriously impact on the environment in both urban and rural regions. Pollen from male catkins

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Abbreviations: DMSO, Dimethyl sulfoxide; **LD-PCR,** long-distance polymerase chain reaction; **dscDNA,** double-strand complementary DNA; **pfu,** plaque-forming unit.

are allergenic for some individuals, while hairs produced by female catkins contribute to environmental pollution in rural and urban areas during spring and might be a potential fire risk, or even a vector for the spread of viruses. Therefore, it is important to regulate or control flowering of poplar. Several genes, such as *PTLF*, *PTD*, *PtFT* and *PtAG*, involved in poplar flowering have been identified (Brunner et al., 2000; Rottmann et al., 2000; Sheppard et al., 2000; Böhlenius et al., 2006). The complete *P. trichocarpa* genome has been sequenced (Tuskan et al., 2006). However, the information presently available on a limited number of genes involved in poplar flowering is insufficient to allow annotation of genes and to understand the genetic and molecular mechanisms underlying floral development in *P. tomentosa*.

Construction and screening of a cDNA library is one of the most important methods for gene isolation, and it is also a potential approach to identify novel genes. In this investigation, full-length enriched cDNA libraries were constructed for *P. tomentosa* from male and female floral buds sampled at 15 developmental stages. This study will ultimately allow the isolation of genes expressed during the development of *P. tomentosa* floral buds, and will contribute to elucidation of the molecular mechanism regulating flowering in *P. tomentosa.*

MATERIALS AND METHODS

Floral buds were collected from female and male clones of *P. tomentosa* trees, growing in the Beijing Forestry University Nursery, at 15 developmental stages from floral bud initiation to flower maturity (between June and March of the following year). The samples were frozen immediately in liquid nitrogen and stored at –80°C until use.

Extraction and qualification of total RNAs

The total RNAs from both male and female floral buds were extracted using a CTAB-based method as described previously (Chang et al., 1993). The extracted RNAs were pretreated with RQ1 DNase I (Promega) to remove genomic DNA contaminants. The concentration of total RNAs was measured using a SPEKOL 1300 spectrophotometer (Jena). A 1 µl sample of the extracted RNAs was electrophoresed on 1% agarose gel. Equal quantities of total RNAs from male and female floral buds at each developmental stage were mixed to examine the integrity of the RNAs and samples were electrophoresed as described earlier to measure the total RNA concentration. mRNAs were isolated and purified with the PolyATtract® mRNA Isolation System (Promega).

Construction of SMART cDNA libraries

The purified mRNAs extracted from male and female floral buds were reverse-transcribed into corresponding single-strand cDNAs. Double-strand cDNAs (dscDNAs) were synthesized by long-distance polymerase chain reaction (LD-PCR) with 20 cycles, and 5 µl dscDNAs were analyzed by 1.2% agarose gel electrophoresis. A portion (50 µl) of the dscDNAs was digested with proteinase K and *Sfi* I, and fractions >200 bp in length were recovered using the QIAquick™ Gel Extraction Kit (QIAGEN). Finally, the fractions were ligated into a λ TriplEx2 vector digested by *Sfi* I, and the cDNA ligation products were packaged *in vitro* into the λ phage using the Packagene Lambda DNA Packaging System (Promega). The reclaimed products were electrophoresed on a 1.1% agarose gel to examine cDNA quality.

Titering of primary and amplified libraries

To examine the titer of the primary libraries, the packaged ligation products were diluted by 1:5, 1:10 or 1:20 with buffer. A 1 µl sample of the diluted products was combined with 200 µl *Escherichia coli* XL1-Blue overnight culture, and incubated at 37°C for 15 min. To the infected bacteria, 2 ml melted top agar was added, and the mixture was spread onto the surface of a 90 mm LB/$MgSO_4$ agar plate. The plate was inverted and incubated at 37°C for 12 to 18 h until the phagocytes were visible. The phagocytes were counted and the titer of the primary library was calculated as:

Titer (pfu/ml) = number of plaques × dilution factor

The libraries were amplified according to routine methods. They were stored at 4°C for up to six months or in 7% DMSO (v/v) at -70°C for up to one year. Samples of 0, 5, 10 or 20 µl of 10^4 dilutions were used to test the titer of the amplified libraries following the earlier-mentioned method.

Determining the percentage of recombinant clones

To investigate recombinant efficiency and sizes of inserted cDNA, we randomly selected 12 plaques from the plates of cDNA libraries and placed them in vials containing 20 µl SM for use as PCR templates. The 20 µl PCR reaction mixture was composed of 1 × PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$), 1 µl SM containing phages, 0.4 µl of each 10 µM sequencing primer (5' λTriplEx2: 5'-CTCCGAGATCTGGACGAGC-3'; T7: 5'-TAATACGACTCACTATAGGGC-3'), and 1 U *Taq* DNA polymerase. Thermo cycling was performed at 94°C for 5 min, then 94°C for 20 s, 60°C for 20 s, and 72°C for 1 min for 30 cycles, then 72°C for 7 min and finally kept at 4°C. The PCR products were examined with 1.5% agarose gel electrophoresis.

RESULTS

Isolation and quality of RNAs

The respective integrity of RNAs from male and female floral bud samples was examined by agar gel electrophoresis, as shown in Figure 1. The relative brightness ratio of the two bands for 28S rRNA and 18S rRNA was close to 2:1, which indicated that the total RNAs from male and female floral buds had not been degraded. Spectrophotographic analysis gave OD_{260}/OD_{280} values of 2.04 ± 0.02 and 2.00 ± 0.06 (Table 1), which showed that the RNAs were complete and of high integrity and purity, and met the requirements for cDNA synthesis.

Isolation of mRNAs and synthesis of dscDNA

Integrity of mRNAs was analyzed separately for male and

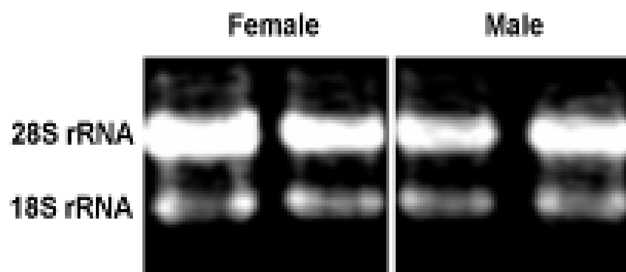

Figure 1. Integrated total RNAs from floral buds of *P. tomentosa*.

Table 1. Quality and estimated concentration of integrated total RNAs extracted from female and male floral buds of *P. tomentosa.*

RNA source	OD_{260} value	OD_{280} value	OD_{260}/OD_{280} value
Female floral buds	2.90 ± 0.01	1.42 ± 0.02	2.04 ± 0.02
Male floral buds	1.96 ± 0.04	0. 98 ± 0.01	2.00 ± 0.06

OD, Optical density.

female floral bud RNAs. Electrophoretic analysis indicated that dispersion of mRNAs ranged from 400 bp to 5.0 kb (Figure 2A). The recovered dscDNAs were represented by an approximately 0.4 to 5.0 kb smear on the 1.1% agarose gel, indicating that the male and female dscDNAs were complete and did not comprise small fragments(Figure 2B).

Construction and quality estimation of cDNA libraries

To ensure the highest-quality library was obtained from the cDNA, three parallel ligations of the cDNA and vector were performed, followed by a separate λ phage packaging reaction for each ligation. The average titer of the primary libraries was 8.00×10^5 and 7.20×10^5 pfu/ml for male and female floral bud libraries, respectively. The primary libraries were used to produce the amplified libraries, which had a titer of 2.60×10^8 and 2.56×10^8 pfu/ml for male and female floral buds, respectively. The results indicated that the titers of both primary and amplified libraries were relatively optimal and met the requirements of a SMART cDNA library. The size of the inserts ranged from 400 bp to 2.0 kb, and the percentage of inserted fragments exceeding 400 bp in length was approximately 100% (Figure 3).

DISCUSSION

Construction and screening of a cDNA library is a common means of identifying transcriptional products of target genes. Generally, the percentage of mRNAs in a cell is only about 5%, although they are essential for protein synthesis. Moreover, many genes show spatio-temporal expression patterns. The mRNAs transcribed during a certain period are used as the starting material to construct the library, and thus represent limited gene expression information. Therefore, a researcher major concern is whether the cDNA library is enriched with most of the genes expressed during the development of the tissue or organ and contains full-length cDNAs. In this study, male and female floral buds were collected over the course of the entire bud development and their total RNAs were mixed equally to construct the cDNA libraries, which ensured that most genes involved in flower development should be represented. The libraries will provide a valuable tool for future investigations of the regulation of flower development in *P. tomentosa.*

Commonly used cDNA synthesis methods rely on the ability of reverse transcriptase (RT) to transcribe mRNA into single-stranded DNA in the first-strand reaction. The occurrence of RT termination before transcription is complete and is a very common problem, particularly for long mRNA sequences. In this study a SMART™ cDNA Library Construction Kit was employed to construct the cDNA libraries, which differentiated it from conventional studies. The SMART protocols are designed to enrich preferentially full-length cDNAs, and eliminate T4 DNA polymerase and adaptor ligation. Therefore, libraries constructed with a SMART cDNA protocol clearly contain a higher percentage of full-length clones and full-length cDNAs with complete 5' ends.

The regulation of flowering is very important in poplar breeding. On one hand, promotion of early flowering is beneficial to shorten breeding cycles and, on the other hand, suppression of flowering will improve environ-mental quality by reducing production of seed hairs and

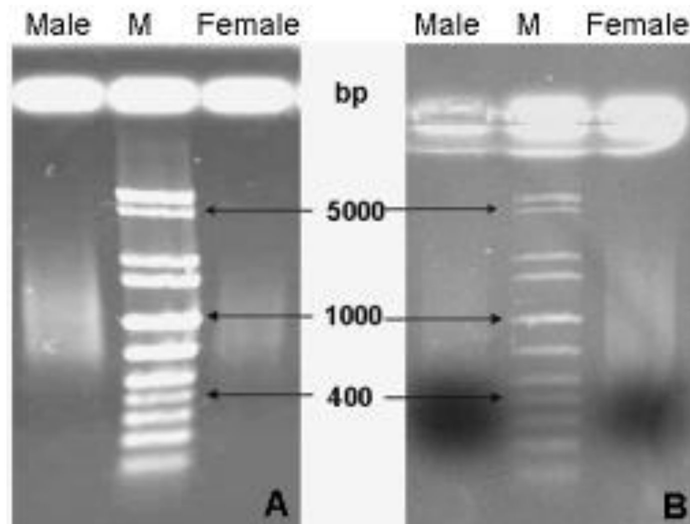

Figure 2. Purification of mRNAs and synthesis of dscDNA. A: Isolation of mRNAs from integrated total RNAs of male and female floral buds. B: synthesis of dscDNA from male and female floral buds.

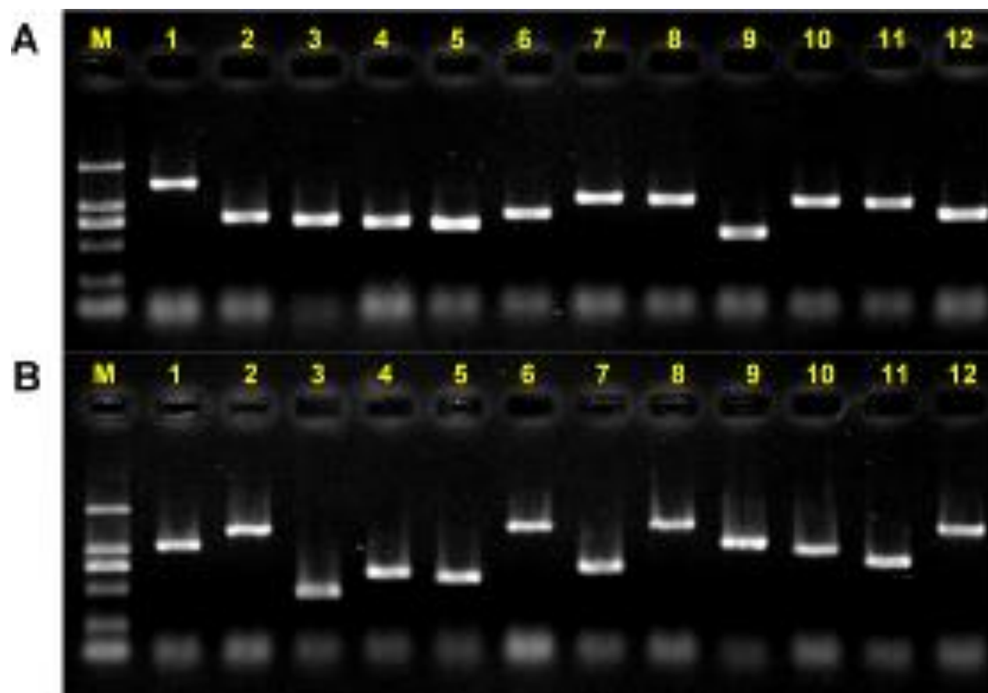

Figure 3. Identification of insert length of cDNA fragment in both female and male floral buds cDNA library. A: PCR products of λ phages from male floral buds cDNA library; B: PCR products of λ phages from female floral buds cDNA library (M, DL2000 marker; 1 to 12, PCR products of different λ phages from male and female floral buds cDNA libraries).

pollen. However, current knowledge of the molecular mechanism regulating poplar flowering is still poor, although the complete poplar genome sequence has been published (Tuskan et al., 2006). Considering the complexity of gene interaction networks and the specificity of gene expression in dioecious poplar, isolation and characterization of genes involved in flowering is essential. The enriched full-length cDNA libraries constructed from multiple samples and covering all floral-bud development stages will provide an

alternative approach to obtain a comprehensive understanding of gene interactions involved in flowering of *P. tomentosa*.

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