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Construction of cDNA library of cotton mutant (Xiangmian-18) library during gland forming stage

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Abstract

Gossypol, a secondary metabolite stored in the glands of cotton, protecting cottonseed from consumption of human and monogastric animal. This ability is unique to the tribe Gossypieae. Although the relationship between gossypol and pigment gland has been studied for a long time, the development mechanism of pigment gland has not been investigated at molecular level. Here we described a simple and efficient method for constructing a normalized cDNA library from a cotton mutant, Xiangmian-18, during its pigments gland forming stage. It combined switching mechanism at 5′-end of RNA transcript (SMART) technique and duplex-specific nuclease (DSN) normalization methods. In a model experiment, double-stranded cDNAs were synthesized from mRNAs, processed by normalization and Sfi I restriction endonuclease, and finally the cDNAs were ligated to pDNR-LIB vector. The ligation mixture was transformed into *E. coli* JM109 by electroporation. Counting the number of colonies, the titer of the original library was 5.86×10^5 cfu/ml in this library. Electrophoresis gel results indicated the fragments ranged from 800 bp to 2 kb, with the average size of 1400 bp. Random picking clones showed that the recombination rate was 94%. The results showed that the cDNA library constructed successfully was a full-length library with high quality, and could be used to screen the genes related to development of pigments gland cottons.
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Keywords: *Gossypium hirsutum* L.; Xiangmian-18; cDNA library; Normalization; Gland

1. Introduction

Gossypol is present in small lysigenous glands of the cotton plant. The compound can protect the plants from predation by insects, viruses, fungus, and bacteria [1–3]. However, it is also toxic to animals, which severely limit the use of cottonseed as food source. The value of cottonseed has been limited by the presence of gossypol [4–6]. In the 1960s, plant breeders developed glandless varieties which were free of pigment glands and gossypol to overcome this problem. However, these varieties lack of glands were easily attacked by a variety of insects compared with the wild type species [7–9]. It is theoretically possible to reduce the gossypol in cottonseed without affecting the amount of gossypol in the whole plant [10–12]. We got a new mutant-type of hybrid upland cotton (*Gossypium hirsutum*), called Xiangmian-18, which is newly bred by tetraploid-dominant glandless cotton *G. barbadense* with crossing gossypol gland *G. hirsutum* through years of selections [13,14]. It had the special property of delayed pigment gland morphogenesis. The glands in the leave appeared to be normal, but the seeds have low gossypol level. So, although its seed is glandless, the foliar gossypol glands in the true leaves can afford

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the whole plant highly protection from the herbivores [15]. In order to popularize the application of cottonseeds, it is necessary to further study the biological properties of the mutant [16–18]. Constructing cDNA library and screening functional genes should be an effective way for this purpose. Here, we used the switching mechanism at 5′-end of RNA transcript (SMART) technique combination to normalized technique to understand the relationship between Gossypol and pigment glands [16–18]. In this study, we constructed a normalized cDNA library during gland formation of Xiangmian-18 seed, which paved the way for the investigation the molecular mechanism of gland formation and the isolation of some of the genes related to gland formation.

2. Materials and methods

2.1. Extraction of mRNA

Cottonseeds of Xiangmian-18 were obtained from National Research Center for Cross-Cotton of China (Changsha, China). Seeds were disinfected in 70% ethanol and 15% H_2O_2 solution, then dipped in sterilized water, and began to bud in the plates containing sterilized filter paper and water. The pigment glands began to develop on the plate at 25 °C after 16–18 h. When the new glands in the hypocotyl could be observed, the seeds were soaked in liquid nitrogen for extraction of mRNA. Isolation of mRNA was carried out according to the protocol of Quick Prep^{TM} Micro mRNA Purification (Amersham Biosciences). The quantity and integrity of mRNA were detected by ultraviolet spectrometer and electrophoresis on a denaturing formaldehyde agarose gel.

2.2. cDNA synthesis

2.2.1. First-strand cDNA synthesis

First-strand cDNA was synthesized according to the protocol of SMART^{TM} cDNA Library Construction Kit (Clontech, USA): 3 μl mRNA sample, SMART IV oligonucleotide and CDS III/3′ PCR primer: (oligonucleotide: 5′-AAGCAGTGG TATCAACGCAGAGTGGCCATTACGGCCGGG-3′; CDS III 3′ PCR primer: 5′-AT TCTAGAGGCCGAGGCGGCCGA-CATG-d(T))-3′ were incubated at 72 °C for 2 min. 5× first-strand buffer, DTT, dNTP mix and powerscript reverse transcriptase were incubated at 42 °C for 1 h.

2.2.2. Amplification of cDNA by long-distance polymerase chain reaction (LD PCR)

Two microliters of the first-strand cDNA, deionized H_2O, advantage 2 PCR buffer, dNTP mix, 5′ PCR primer, CDS III/3′ PCR primer and advantage 2 polymerase mix were added into a new pre-chilled 0.5 ml tube, then amplified by the following program: 95 °C, 20 s; 24 cycles of 95 °C 5 s, 68 °C 6 min. Five microliters of the PCR products were analysis on 1.2% agarose/EtBr gel. The concentration of the double strand (ds) cDNA was roughly estimated by compared with DNA marker.

2.3. cDNA normalization

The reaction mixture was precipitated with the following reagents in a sterile 1.5 ml tube: 4–12 μl duplex-specific nuclease (DSN), 800 ng template, 4 μl hybridization buffer, added sterile water to total volume 16 μl, mixed and spined briefly in a microcentrifuge. The tubes were incubated in a thermal cycler at 98 °C for 2 min, then 68 °C for 5 h [19].

2.3.1. DSN treatment

Shortly before the end of the hybridization procedure, we prepared the following dilutions of the DSN enzyme in two sterile tubes: 1 μl of DSN storage buffer and 1 μl of DSN solution (in storage buffer) were added to the first tube, which was labeled as 1/2 DSN enzyme. The second tube was added 3 μl of DSN storage buffer and 1 μl of DSN solution, labeled as 1/4 DSN. The tubes were placed on ice. Each tube with hybridized cDNA was added 5 μl of the preheated DSN master buffer, incubated at 68 °C for 25 min in the thermal cycler. Finally the tubes were placed on ice.

2.3.2. Analysis of normalization efficiency

cDNA normalization should result in a significant decrease in the concentration of abundant transcripts. Quantitative PCR could be very useful to evaluate the normalization efficiency by comparing the abundance of known cDNAs before and after normalization. In this study, we checked normalization efficiency using PCR with 18S rRNA. 18S rRNA 5′ PCR primer: 5′-TCGTAGTTGGACTTAGGGTGGG-3′, 18S rRNA 3′ PCR primer: 5′-CAAATGCTTTCGCAGTTGTTCG-3′. 3′ PCR primer (10 mM) 1 μl, 5′ PCR primer (10 mM) 1 μl, 10× PCR reaction buffer 2.5 μl, $MgCl_2$ (25 mM) 2 μl, dNTP (2.5 mM) 2.0 μl, ddH_2O 17.25 μl, TaKaRa Ex Taq 0.25 μl, mixed well and PCR reaction condition: 75 °C, 5 min; 94 °C, 30 s; 20×: 94 °C, 10 s; 60 °C, 30 s; 72 °C, 2.5 min. Five microliters PCR products were checked by running 1.2% agarose gel alongside DNA marker.

2.4. Amplification of normalized cDNA

The reaction mixture was precipitated with the following components in the tube: 40.5 μl water, 5 μl 10× advantage 2 PCR buffer, 1 μl 50× dNTP mix, 1.5 μl evrogen PCR primer M_1, 1 μl 50× advantage 2 polymerase mix, then PCR was then carried out according to the following program: 95 °C 3 min; 94 °C 30 s, 55 °C 30 s, 72 °C 2 min for 30 cycles; 72 °C 7 min. PCR products were checked by running 1.2% agarose gel alongside DNA marker. The optimum engine cycle parameters were determined.

2.5. Sfi I digestion and cDNA size fractionation

PCR products were purified with PCR purification kit. The following components were combined in a fresh 0.5 ml tube: 79 μl cDNA, 10 ml 10× Sfi buffer 10 μl Sfi I enzyme, 1 μl 100× BSA, added water to 100 μl total volume. The tube was incubated at 50 °C for 2 h. A gel was prepared containing 1%

concentration of low melting temperature, long linkers (>800 bp) were cut under UV light.

2.6. Ligation cDNA to pDNR-LIB vector

The pDNR-LIB vector was used for cDNA construction. The following components were added in a fresh 0.5 ml tube: 1.0 μl pDNR-LIB, 10× ligation buffer 0.5 μl, ATP (10 mmol) 0.5 μl, T_4 DNA Ligase 0.5 μl, added water to 0.5 μl for ligation reaction, the tube was incubated at 16 °C overnight.

2.7. Transformation of recombinant plasmids into E. coli

The ligation mixture with 100 μl thawed cells (*E. coli* JM109) was transferred to a chilled 0.1 cm cuvette. It was electroporated by discharging, and then immediately removed the cuvette from the chamber. The entire volume was transferred to the prelabeled polypropylene tubes containing 900 μl LB broth. The tube was incubated with shaking (225 rpm) for 1 h at 37 °C. Fifty microliters of aliquot was spread on a pre-warmed 90 mm LB agar plate containing 30 μg/ml of chloramphenicol.

2.8. Quality analysis of cDNA library

To calculate the titer of un-amplification library, if the number of phage was less than 1×10^6 pfu/ml, the linking step was repeated. The library was plated directly on selective medium (containing 30 μg/ml of chloramphenicol). The plates were inverted and incubated at 37 °C overnight. Fifteen isolated clones were selected randomly and amplified by PCR. The mixtures were denatured at 94 °C for 3 min, followed by amplification for 30 cycles: 94 °C, 1 min, 52 °C, 1 min, 72 °C, 3 min. Amplified product (5 μl) was analyzed by 1.2% agarose gel electrophoresis, followed by ethidium bromide staining.

3. Results and discussion

3.1. Observing the gland and extracting mRNA

The new glands of Xianmian-18 began to emerge in germinating seed after 42 h on the hypocotyls near the cotyledon [1], then the glands became bigger and blacker after 4–6 h, the glands developed continuously upward (Fig. 1). So the stage from 38 to 52 h was considered to be the developing gland stage [24]. Seeds were selected randomly from 38 to 52 h to extract mRNA. High-quality mRNA is critical for constructing a cDNA library. In our study, the ratio of OD_{260}/OD_{280} to the mRNA was 1.90, and the concentration was 1.28 μg/μl. It showed that the mRNA obtained from the seeds did not degrade when the purity was high [19].

3.2. Synthesis and modification of cDNA

We used SMART techniques to ensure that amplification of long cDNA fragments. Thus, the average cDNA length was maintained during PCR amplification and the adequate size range of normalized cDNA (0.2–4.0 kb) was attained. The concentration of ds cDNA was 0.28 μg/μl. The size of the cDNA ranged from 200 bp to 4 kb, with an average size of 900 bp, which indicated LD PCR was successfully synthesized (Fig. 2A). A sharp decrease in the representation levels of abundant transcripts was observed in normalized cDNA samples compared with non-normalized samples (Fig. 2B). The effect of normalization was obvious.

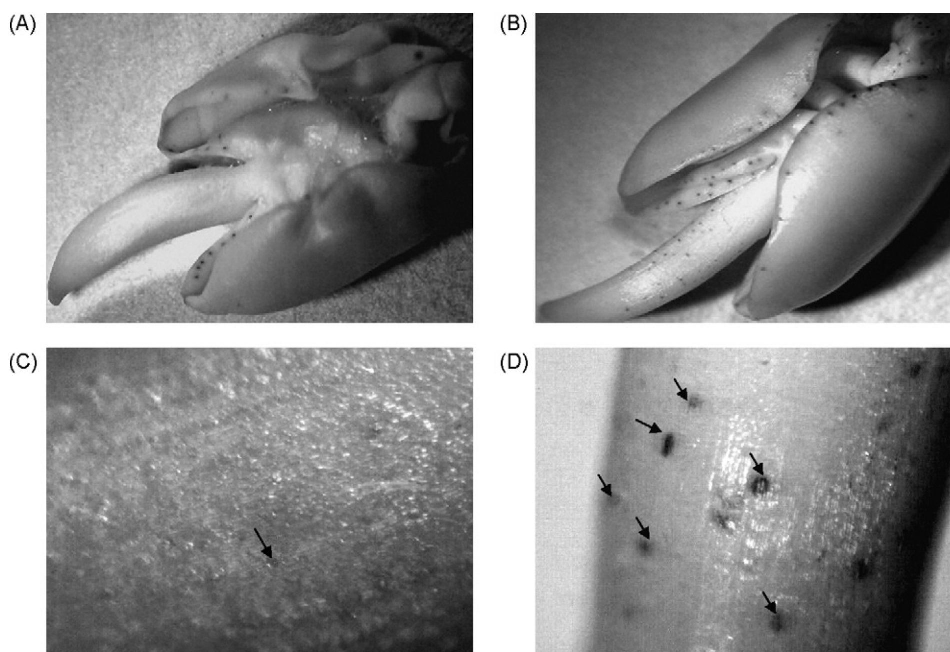

Fig. 1. Seeds and pigment glands of Xiangmian-18. (A) A seed after 38 h germination (40×). (B) A seed after 52 h germination (40×). (C) Rare new pigment glands on the hypocotyl after 38 h germination (100×). An arrow shows a pigment gland. (D) Several pigment glands on the hypocotyl after 52 h germination (100×). Arrows show pigment glands.

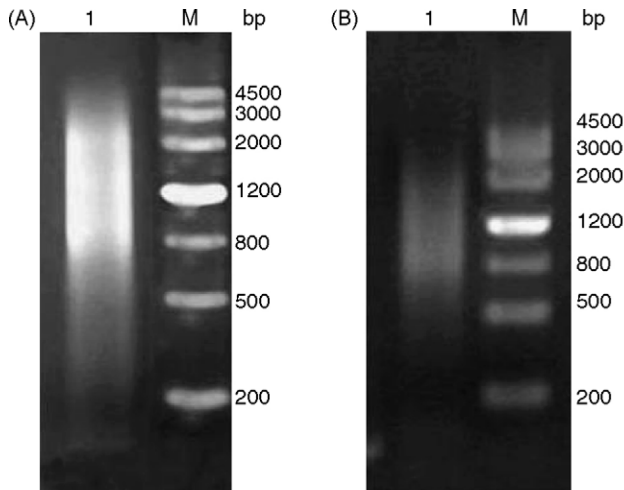

Fig. 2. The amplification of Xiangmian-18 cDNA in 1.2% agarose gel electrophoresis. (A) Amplification of cDNA by LD PCR. Lane 1: products of LD PCR; M: DNA marker. (B) Amplification of cDNA after normalization. Lane 1: products of cDNA after normalization; M: DNA marker.

3.3. Analysis of normalization efficiency

DSN-normalization equalized the abundance of all transcripts in a sample by specifically reducing the proportion of highly abundant transcripts. The efficiency of DSN-normalization was demonstrated by comparison of DSN-normalized and no-normalized SMART amplified Xiangmian-18 cDNA (Fig. 3). In this experiment, efficient normalization was achieved in S1 DSN1/2 tube. In S1 DSN1/4 tube, normalization was not completed. In S1 DSN1 tube, treatment with DSN enzyme was excessive, resulting in cDNA degradation. Agarose gel electrophoresis showed that the bands corresponding to abundant transcripts (18S rRNA), clearly visible in the pattern of the non-normalized cDNA, have a dramatic decrease in the DSN-normalized cDNA sample (Fig. 4). A heavy band represented as 18S rRNA gene in the non-normalized samples, was decreased in the normalized sample after PCR reaction, indicating that the amount of these highly abundant transcripts was sharply reduced following normalization.

3.4. The identification of quality of the cDNA library

The titer of the un-amplified constructed cDNA library was approximately 5.86×10^5 cfu/ml and the percentage of recombinants selected from 150 independent clones was 94%. It showed high recombination efficiency. The results of electrophoresis gel showed that fragments ranged from 800 bp to 2 kb, with an average size of 1400 bp (Fig. 5).

This cDNA library, as a full-length library with high quality, could be used to screen the genes related to the development of cotton pigment glands. The relationship between the pigment gland and the gossypol has been attracted a lot of attentions from botanists and agronomists. However, the molecular mechanism of the gland development is still unknown. In this study, we combined SMART technique into the DSN normalization method. The majority of cDNA clones are not full-length using

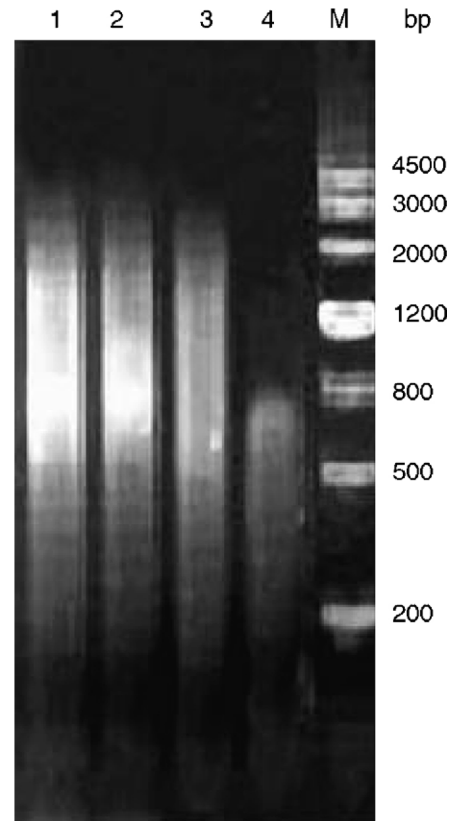

Fig. 3. The comparison of Xiangmian-18 cDNA amplification using different concentration of DSN enzymes. Lane 1: cDNA from control tube (non-normalized cDNA sample); lane 2: cDNA from S1 DSN1/4 tube; lane 3: cDNA from S1 DSN1/2 tube; lane 4: cDNA from S1 DSN1 tube; M: DNA size marker.

conventional cDNA library construction methods [20]. The SMART method can be used effectively for samples enriched with full-length cDNA sequences. The SMART technique can make three reading frames of each insert sequence expressed, which ensure the complete of information [21,22]. We adopt directional cloning into the pDNR vector, providing a rapid and simple method for constructing high-quality cDNA libraries that

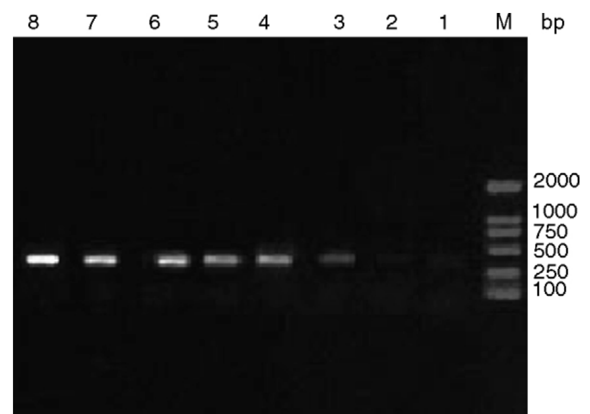

Fig. 4. The comparison of abundant 18S rRNA gene transcripts in normalized and non-normalized Xiangmian-18 cDNA using different PCR cycles. Lanes 1, 3, 5 and 7 show non-normalized samples of 10, 16, 22 and 28 cycles, respectively; lanes 2, 4, 6 and 8 show normalized samples of 10, 16, 22 and 28 cycles, respectively. M: DNA size marker.

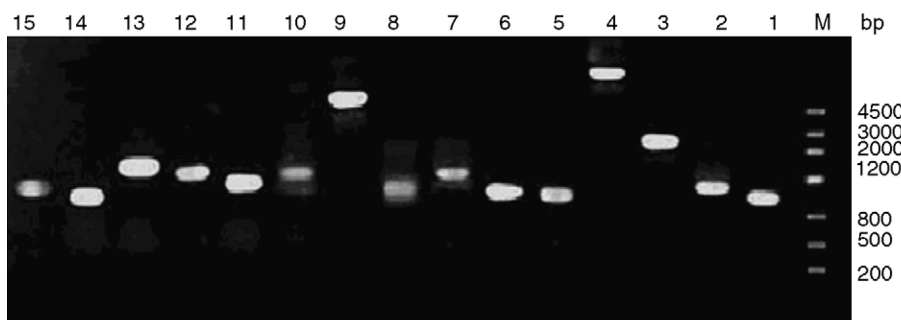

Fig. 5. The PCR production of cDNA clones at random from Xiangmian-18 cDNA library. Lanes 1–15 show the PCR productions of different clones, respectively; M: DNA size marker.

are ready for identification, amplification and analysis of the clones. But there was another problem associated with gene discovery, the researchers might be faced the challenge to make efficient high-throughput analysis of the library, with view to the differential abundance of various transcripts in the cell. For rare mRNA sequence (from any tissue), it was likely to be presented and identified difficultly in a cDNA library. Depending on their expression level, mRNAs could be defined as abundant, intermediate and rare. As a rule, 10–20 abundant genes (several thousands of mRNA copies per cell), several hundreds of genes of medium abundance (several hundreds of mRNA copies per cell), and several thousands of rare genes (from one to several dozens of mRNA copies per cell) were expressed in each cell [23]. Because cDNAs of medium and high abundance were sequenced repeatedly, direct random sequencing of clones from standard cDNA libraries was inefficient for discovering rare transcripts. It often resulted in a huge waste when this type of cDNA library was used for gene screening. Normalized cDNA library was developed to overcome the problem caused by different expression frequencies of genes and it was an efficient tool for gene identification. Normalization performed before cDNA library sequencing decreases the prevalence of clones representing abundant transcripts, which could increase the efficiency of random sequencing dramatically, and was essential for rare gene discovery [23].

4. Conclusion

We constructed cDNA library during pigment gland formation stage, the ultimate goal of which was to collect some full-length cDNA related to gland development [24]. The unique DSN normalization technology [25] and SMART technique would overcome the problem caused by different expression frequencies of genes and it was an efficient tool for gene identification [26,27]. The whole processes were very simple and required very less hand-on-time. In our experiments, we obtained normalized cDNA samples with the size of individual cDNAs ranging from 0.5 to 4.5 kb. The abundant transcripts were removed during DSN treatment. The representation levels of abundant transcripts decreased essentially those of rare transcripts. Normalized cDNA library had a wide range of application because it could significantly increase screening sensitivity and reduce experimental costs. It will be a good probe pool in genetic mapping and large-scale *in situ* hybridization. As an optimized system, it will be used in the large-scale EST sequencing and the cDNA microarray analysis, and will be used to screening the genes related to development of cotton pigment glands. Therefore, the solid foundation had been laid for finding relevant genes and investigating their functions of cotton gland formation.

Acknowledgments

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References

[1] Y. Cai, H. Zhang, Y. Zeng, et al., J. Biosci. 29 (2004) 67–71.
[2] A.A. Agrawal, R. Karban, Entomol. Exp. Appl. 96 (2000) 39–49.
[3] B.T. Akingbemi, P.V. Rao, T.A. Aire, J. Appl. Toxicol. 16 (1996) 375–380.
[4] M. Essenberg, P.B. Grover, E.C. Cover, Phytochemistry 29 (1990) 3107–3113.
[5] P.W. Pare, J.H. Tumlinson, Nature 385 (1997) 30–31.
[6] C.R. Benedict, G.S. Martin, J. Liu, et al., Phytochemistry 65 (2004) 1351–1359.
[7] Y.L. Meng, J.W. Jia, C.J. Liu, et al., J. Nat. Prod. 62 (1999) 248–252.
[8] B.J. Townsend,.A. Poole, C.J. Blake, et al., Plant Physiol. 138 (2005) 516–528.
[9] A. Aharoni, A.P. Giri, S. Deuerlein, et al., J. Plant Cell 15 (2003) 2866–2884.
[10] S.J. Zhu, Y.R. Jiang, Chin. Sci. Bull. 49 (2004) 2470–2476.
[11] S.J. Zhu, N. Reddy, Y.R. Jiang, Plant Breed. 124 (2005) 590–594.
[12] S.J. Zhu, D.F. Ji, Chin. Sci. Bull. 46 (2001) 1168–1173.
[13] Y.H. Xu, J.W. Wang, S. Wang, et al., Plant Physiol. 134 (2004) 507–515.
[14] X.L. Zhang, L. Jin, T.Z. Zhang, Sci. Agric. Sin. 34 (2001) 564–567.
[15] Y.F. Cai, J.C. Mo, Y. Zeng, et al., J. Beijing Forest Univ. 25 (2003) 6–10.
[16] P.A. Hedin, W.L. Parrott, J.N. Jenkins, J. Econ. Entomol. 85 (1992) 359–364.
[17] D. Chen, H. Ye, G. Li, Plant Sci. 155 (2000) 179–185.
[18] Tholl, Dorothea, Curr. Opin. Plant Biol. 9 (2006) 297–304.
[19] B. Li, B.C. Wang, K. Tang, et al., Colloids Surf. B: Biointerf. 49 (2006) 101–105.
[20] Y. Suzuki, S. Sugano, Methods Mol. Biol. 175 (2001) 143–153.
[21] O. Ohara, G. Temple, Nucleic Acids Res. 29 (2001) 22.
[22] W.M. Barnes, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 2216–2220.
[23] D.A. Shagin, D.V. Rebrikov, V.B. Kozhemyako, et al., Genome Res. 12 (2002) 1935–1942.

[24] P.A. Chang, B. Li, X.M. Ni, et al., Colloids Surf. B: Biointerf. 55 (2007) 153–158.
[25] P.A. Zhulidov, E.A. Bogdanova, A.S. Shcheglov, et al., Nucleic Acid Res. 32 (2004) 37.
[26] N.G. Gurskaya, L. Diatchenko, A. Chenchik, et al., Anal. Biochem. 240 (1996) 90–97.
[27] Y.Y. Zhu, E.M. Machleder, A. Chenchik, Siebert, et al., Biotechniques 30 (2001) 892–897.