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Establishment of cDNA-AFLP technology system and stoneless gene difference expression in *Ziziphus jujuba* Mill.

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Abstract An efficient and stable cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis system for Chinese jujube was established and successfully used for the studies of related stoneless gene difference expression in *Ziziphus jujuba* Mill. ‘Wuhejinsixiaozao’ fruit. Several main factors influencing cDNA-AFLP analysis were studied, including the preparation and purification of cDNA, restriction and ligation of cDNA, the preamplification reaction, selective amplification reaction, electrophoresis on denaturing polyacrylamide gels, and silver staining. The results indicated that the total RNA extracted by modified SDS method was pure, complete, and suitable for reverse transcription to cDNA. Restriction digestion of cDNA was performed by using two restriction enzymes, around 150 ng DNA digested with three units of *Eco*RI and *Mse*I enzymes, respectively, and incubated at 37°C for 5 h. The digested cDNA fragment was diluted 5 times and used as templates for preamplification, and the preamplification products were diluted 10 times and used as templates for selective amplification. The selective amplification fragments were subjected to PAGE electrophoresis and silver staining. By cDNA-AFLP analysis, it acquired three transcript-derived fragments (TDFs), DC1, DC5, and DC9, related with stoneless gene of *Z. jujuba* Mill. ‘Wuhejinsixiaozao’ fruit.

Keywords Chinese jujube, cDNA-AFLP, stoneless gene, difference expression

1 Introduction

Biologic responses and developmental programming are controlled by the precise regulation of gene expression. To gain insight into these processes, it is necessary to study the patterns of gene expression. A variety of molecular techniques are now available for identification and also for clone differentially expressed genes. The most recent actions include the PCR-based approaches for selective amplification of cDNAs, such as RNA-fingerprinting by arbitrarily primed polymerase chain reaction (RAP-PCR; Welsh et al., 1992) and differential display (DD/RT-PCR, Liang and Pardee, 1992), collectively referred to as RNA-fingerprinting. However, all methods still suffer problems of repeatability and uncertainty in the identification of specific fragments. The cDNA-AFLP method (Bachem et al., 1996) largely overcomes these limitations and makes a simple and rapid verification of band identity possible. In addition, the systematic screening of nearly all transcripts in a given biologic system using small quantities of starting material is possible. cDNA-AFLP technology is based on selective amplification of a subset of reverse transcription fragments. cDNA is digested with restriction endonucleases, usually a six-base cutter and a four-base cutter, to create a set of double-stranded template cDNA fragments for amplification. Adapters with defined sequences matching the different restriction sites are then ligated to the fragments. PCR amplification of restricted fragments is achieved by using the adapter and restriction site sequence as target sites for primer annealing. The annealing primers, carrying one or several selective nucleotides at the 3-end, extend into the restriction fragments and ensure that only a subset of restriction fragments are amplified. The resulting fragments are then separated by denaturing polyacrylamide gel electrophoresis. By increasing the number of primer combinations, a large number of loci can be screened, whereby the chances to detect polymorphisms are greatly enhanced. As a consequence, genetic variation of strains or closely related species can be revealed, and phonetic relationships can be established.

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Several modifications of the cDNA-AFLP technique for analysis of plants (Bachem et al., 1998; Weiberg et al., 2008; Stölting et al., 2009; Korpelainen and Kostamo, 2010) have been described previously in recent years. Different restriction enzymes, different amounts of template cDNA, T4 DNA ligase, and different numbers of selective nucleotides have been used in order to apply the cDNA-AFLP technique to different plants and to obtain reliable fragment profiles with high information value. In spite of the changes made to the standard cDNA-AFLP protocol described in these articles, the method was still not applicable to Chinese jujube but needed further modifications. Several different parameters were tested until an appropriate protocol was established.

The Chinese jujube is the quasi stone fruit (Wang, 1974), the majority of cultivar's fruit is of stone, and extremely individual cultivar is stoneless, which is a good trait of fresh eating and convenience in processing. *Ziziphus jujuba* Mill. 'Wuhejinsixiaozao' is a stoneless natural mutant of *Ziziphus jujuba* Mill. 'Jinsixiaozao', besides its stoneless fruit, its shape of branches and leaves and its characteristics of fruit and flowers are the same as those of 'Jinsixiaozao' (Qu and Wang, 1993). 'Wuhejinsixiaozao' and 'Jinsixiaozao' were used as materials to analyze the stoneless gene difference expression by cDNA-AFLP technology, revealing the stoneless mechanism of Chinese jujube. It was of great significance that cultivated the stoneless cultivar of Chinese jujube by the genetic engineering.

In the present paper, an improved cDNA-AFLP procedure of Chinese jujube fruit was established, and the different stoneless gene expressions of 'Wuhejinsixiaozao' and 'Jinsixiaozao' fruits were studied.

2 Methods

2.1 Plant materials

'Wuhejinsixiaozao' and 'Jinsixiaozao' fruits were collected from the Breeding Base of Chinese jujube in Cangxian of Hebei Province, China, during the fruit development. The fruits were immediately stored in ice-box, transported to the laboratory, and stored at 80°C until total RNA extraction.

2.2 Total RNA extraction

In reference to the basis of many reports on the isolation of total RNA (Logemann, et al., 1987; Schneiderbauer et al., 1991; Lpez-Gmez and Gmez-Lim, 1992; Ainsworth, 1994; Lewinsohn, et al., 1994; Wan and Wilkins, 1994; Wang and Vodkin, 1994), it was extracted using the improved SDS method. Approximately, 0.25 g fruit added with polyvinyl-polypyrrolidone (PVPP) (1:1) was ground in liquid nitrogen using mortar and pestle. The fine powder was transferred to a 50-mL sterilized centrifuge tube and

6 mL SDS extraction buffer (1 mol·L⁻¹ Tris-borate (pH 7.5), 0.5 mol·L⁻¹ EDTA, 10% SDS, β-mercaptoethanol) was added. The homogenate was mixed by inversion several times. The mixture was placed at 0°C for 5 min; 6 mL chloroform: isoamyl alcohol (49:1) was added into the sample and mixed and then centrifuged at 7000 r·min⁻¹ at 4°C for 20 min. The supernatant was transferred to a new tube, added with equal volume of chloroform: isoamyl alcohol (49:1) and mixed, and then centrifuged at 7000 r·min⁻¹ at 4°C for 20 min, with the supernatant transferred to another tube. Thereafter, the pellet was resuspended in 1 mL 3 mol·L⁻¹ LiCl, dissolved for 12–16 h; and then centrifuged at 7000 r·min⁻¹ at 4°C for 20 min. The supernatant was once again transferred to a 1.5-mL tube, added with equal volume chloroform: isoamyl alcohol (49:1), mixed, and then centrifuged at 10000 r·min⁻¹ at 4°C for 10 min. In another 1.5-mL tube, the supernatant was added with 2.5 times volume ethanol, incubated at 20°C for > 2 h, and centrifuged at 10000 r·min⁻¹ at 4°C for 10 min. The pellet was washed with 70% ethanol (in DEPC-treated H₂O), centrifuged at 10000 r·min⁻¹ at 4°C for 5 min, with liquid discarded and RNA pellet dried in transfer hood for 20 min. The resusped pellet in DEPC-treated H₂O (20 μL) was stored at 80°C.

2.3 cDNA synthesis

For the system of the first strand cDNA synthesis, the total volume is 10 μL, including 5 μL pure RNA, 1.625 μL Oligo(dT)15, 0.125 μL ribonuclease inhibitor (40 U·μL⁻¹), 1 L 10 mmol·μL⁻¹ dNTPs, 2 μL AMV reverse transcription 5×reaction buffer, and 0.25 μL AMV reverse transcriptase (10 U·μL⁻¹).

For the system of the double strand cDNA synthesis, the total volume is 30 μL, including 10 L the first strand mix, 3 μL DNase I 10 × reaction buffer, DNA polymerase (4 U·μL⁻¹), 1.5 L 10 mmol·μL⁻¹ dNTPs, 0.5 μL RNase H (60 U·μL⁻¹), 3 μL T4 DNA ligase 10 × reaction buffer, and 9.8 μL T4 DNA ligase.

The quality of cDNA was examined by means of 0.8% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV fluorescence. The quality and quantity of cDNA were accurately measured with spectrophotometric absorbency at 260 nm and 280 nm, respectively. The DNA was diluted into 100 ng·μL⁻¹ and stored at 20°C.

2.4 Restriction digestion

The restriction digestion as described below was based on the protocol of Bai (2008), with some modifications, including the concentration of cDNA and some reagents, primer concentration, and incubation conditions to improve cDNA fragment profiles. Digestion was performed by mixing with cDNA, 3 U *Eco*RI, 3 U *Mse*I, 1 × buffer (50 mmol·L⁻¹ NaCl, 10 mmol·L⁻¹ Tris-HCl,

10 mmol·L⁻¹ MgCl₂, 1 mmol·L⁻¹ DTT at pH 7.9), and 100 ng·μL⁻¹ BSA in 20 μL volume at 37°C. After digestion, the block was incubated at 70°C for 10 min.

2.5 Adapter ligation

The digestion block was added to a 5 μL mixture solution comprised of 5 pmole *Eco*RI adapter, 50 pmole *Mse*I adapter, 1.5 U T4 DNA ligase, and 1 × ligase buffer (30 mmol·L⁻¹ Tris-HCl, 10 mmol·L⁻¹ MgCl₂, 10 mmol·L⁻¹ DTT, and 10 mmol·L⁻¹ ATP at pH 7.8) at 37°C for overnight. This ligation mixture was diluted with TE buffer stored at 20°C and used as template in the pre-amplification PCR reaction.

2.6 Preamplification

The preamplification reaction contained 0.4 mmol·L⁻¹ dNTPs, 0.5 U Taq polymerase, 1 × buffer (10 mmol·L⁻¹ Tris-HCl, 50 mmol·L⁻¹ KCl, 1.5 mmol·L⁻¹ MgCl₂ of pH 8.3), 30 ng *Eco*RI primer, 30 ng *Mse*I primer, and 5 μL of the diluted adaptor-ligated DNA in a volume of 20 μL. The preamplification thermal cycling included one cycle for 2 min at 95°C, followed by 30 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, and, finally, 72°C for 10 min. After amplification, samples were diluted with TE buffer and used in selective amplifications.

2.7 Selective amplification

Selective amplification reaction contained 0.45 mmol·L⁻¹ dNTPs, 0.75 U Taq polymerase, 1 × buffer (10 mmol·L⁻¹ Tris-HCl, 50 mmol·L⁻¹ KCl, 1.5 mmol·L⁻¹ MgCl₂ of pH 8.3), 40 ng *Eco*RI primer, 40 ng *Mse*I primer, and 5 μL of the diluted pre-amplification mixture in a volume of 20 μL. AFLP reactions were performed with one cycle at 95°C for 2 min, followed by 13 cycles with the following cycle of thermal profile: the annealing temperature was high (65°C) for the first cycle and was reduced by 0.7°C for each of the 12 subsequent cycles. The denaturation and extension steps for each cycle were at 95°C for 50 s and 72°C for 1 min, respectively, followed by 31 cycles for 50 s at 94°C, 40 s at 56°C, 1 min at 72°C, and, finally, one cycle of 10 min at 72°C. All amplification reactions were performed in a thermal MyCycler (BIO-RAD, USA). In all tubes, 10 μL mineral oil was added to prevent water evaporation.

2.8 Gel electrophoresis and silver staining

After selective amplification, PCR products were mixed with 10 μL loading buffer (95% demonized formamide, 10 mmol·L⁻¹ EDTA pH 8.0, 0.25% (w/v) of bromophenol blue, and 0.25% (w/v) of xylene cyanol FF. The resulting mix was heated for 10 min at 95°C and then kept on ice

immediately. The AFLP products were separated on a 6% denaturing polyacrylamide gel. The pre-electrophoresis run was given to the gel at 80 W in 1 × TBE on a vertical gel electrophoresis system about 30–40 min. After loading the denatured PCR products, electrophoresis was performed at 80 W for 2 h. Upon electrophoresis, gels were fixed and stained with silver nitrate. The silver staining procedure included discoloring, rinsing, staining, developing, fixing, and drying (Bassam et al., 1991).

2.9 Primer combinations

cDNA-AFLP fragment detected for each primer combination is shown in Table 1.

Table 1 cDNA-AFLP fragment detected for each primer combination

code	primer combinations
E1M3	E-AA, M-AC
E2M2	E-AT, M-AT
E3M1	E-AC, M-AA
E3M4	E-AC, M-AG
E4M2	E-CA, M- AT
E4M9	E-CA, M-CG
E4M10	E-CA, M-CC
E5M2	E-CT, M-AT
E5M3	E-CT, M-AC
E6M2	E-CC, M-AT
E7M4	E-CG, M-AG
E8M6	E-TA, M-TA
E8M7	E-TA, M-TT
E9M9	E-TT, M- CG
E10M4	E-TC, M-AG
E11M3	E-TG, M-AC
E12M4	E-AG, M- AG

3 Results

3.1 Total RNA quality of Chinese jujube fruit

The quality of total RNA was the crucial prerequisite in cDNA-AFLP analysis. By using routine protocols, we obtained RNA contaminated with polysaccharides, polyphenolics, and other unknown viscous compounds. It was not suitable for cDNA-AFLP analysis. By using the improved procedure described here, we were able to prepare the satisfactory yield of total RNA in high quality from the fruit of Chinese jujube. The resulting total RNA had two clear main bands, 18S and 28S, which meant that total RNA had no degradation. (Fig. 1). The UV spectrophotometric results showed that the OD₂₆₀/OD₂₈₀ ratio of the RNA was 1.8–2.0.

The result of the length of double strand cDNA was

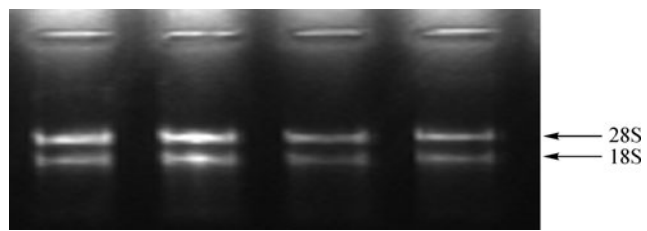


Fig. 1 Agarose gel electrophoresis of total RNA from Chinese jujube fruit by improved SDS method

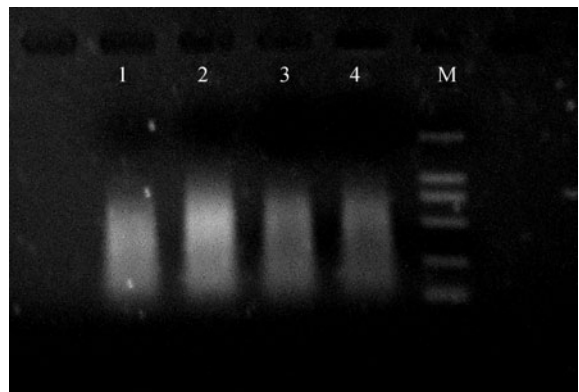


Fig. 2 Agarose gel electrophoresis of the double stand cDNA
Note: M is DL2000 Marker, 1–4 are the double strand cDNA of the Chinese jujube fruit.

100–2000 bp (Fig. 2). It was a satisfactory yield for cDNA-AFLP analysis.

3.2 The modified cDNA-AFLP technology system in Chinese jujube fruit

The result of modified cDNA-AFLP technology system is showed in Table 2. The modified cDNA-AFLP technology system is as follows: Restriction digestion of cDNA was performed by using two restriction enzymes, around 150ng cDNA digested with three units of *Eco*RI and *Mse*I enzymes, respectively, and incubated at 37°C for 5 h. The digested cDNA fragment was diluted five times with TE buffer and used as templates for preamplification. The preamplification products were diluted 10 times with TE buffer and used as templates for selective amplification (Fig. 3). The reproducibility of the technique had been also verified. The AFLP protocol was repeated independently starting from the same cDNA samples. The same expression profile could be observed every time.

elements	gradients
concentration of cDNA	150 ng
time of restriction digestion	5 h
dilution multiple of adaptor-ligated production	5 ×
dilution multiple of pre-amplification production	10 ×



Fig. 3 Amplified results using primer combination E4/M9

3.3 Stoneless gene difference expression

Eighty-one good primer combinations in all 334 combinations generated products ranging in size from about 50 bp to about 800 bp. The cDNA-AFLP procedure yielded an average of 25–30 PCR amplification products per PCR. Some combinations are shown in Table 2. About 2100 transcript-derived fragments (TDFs) could be monitored by modified cDNA-AFLP technology system in this study. Among these, about 328 differentially expressed fragments were identified (some results in Table 1). cDNA-AFLP mainly showed two profile types. On one side, the expression was constitutive way during the fruit development of ‘Wuhejinsixiaozao’ and ‘Jinsixiaozao’. These TDFs of constitutive differential expression may be related with the stoneless genes of Chinese jujube stones. On the other side, the expression was observed in a certain stage of development of ‘Wuhejinsixiaozao’ and ‘Jinsixiaozao’ fruits. By repeating selective amplification three times for removing the false positive clone, there were three TDFs, DC1 (Fig. 4), DC5 (Fig. 5), and DC9 (Fig. 6), which were indented and constitutive expressions during the stone development of ‘Wuhejinsixiaozao’ or ‘Jinsixiaozao’. It can be seen (Figs. 4–6) that DC1 and DC5 denoted by the arrow, was expressed in a constitutive way only in ‘Jinsixiaozao’ stone, but DC9 was constitutively expressed in ‘Wuhejinsixiaozao’ stone. These correct TDFs were cut

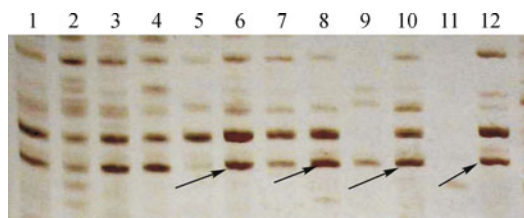


Fig. 4 Specific fragment amplified by using primer combination E4/M9

Note: 1, 3, 5, 7, 9 and 11 are 'Wuhejinsixiaozao' fruit on 8 d, 15 d, 20 d, 30 d, 40 d, and 60 d after blooming, respectively; 2, 4, 6, 8, 10, and 12 are 'Jinsixiaozao' fruit on 8 d, 15 d, 20 d, 30 d, 40 d, and 60 d after blooming, respectively.

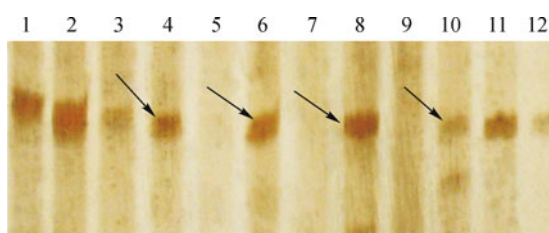


Fig. 5 Specific fragment amplified by using primer combination E4/M10

Note: 1, 3, 5, 7, 9, and 11 are 'Wuhejinsixiaozao' fruit on 8 d, 15 d, 20 d, 30 d, 40 d, and 60 d after blooming, respectively; 2, 4, 6, 8, 10, and 12 are 'Jinsixiaozao' fruit on 8 d, 15 d, 20 d, 30 d, 40 d, and 60 d after blooming, respectively.

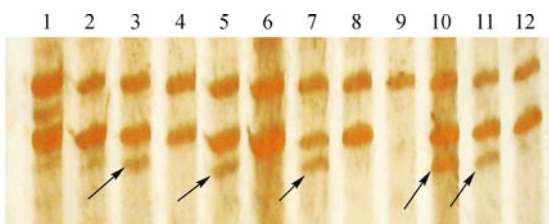


Fig. 6 Specific fragment amplified by using primer combination E10/M4

Note: 1, 3, 5, 7, 9, and 11 are 'Wuhejinsixiaozao' fruit on 8 d, 15 d, 20 d, 30 d, 40 d, and 60 d after blooming, respectively; 2, 4, 6, 8, 10, and 12 are 'Jinsixiaozao' fruit on 8 d, 15 d, 20 d, 30 d, 40 d, and 60 d after blooming, respectively.

out from cDNA-AFLP gel, which had been the second amplification. The results were showed in Fig. 7. Then, these three TDFs were sequenced: DC1 was 185 bp, DC5 was 111 bp, and DC9 was 179 bp. The results were as follows:

DC1:5'-ATCTACGGAGTCAGTTGGTAGATATAA-TATTTAGATTTTTATTTGGAACGAAAAGTGAGTTT-GAATGTACAATAAATAATTGCAACAAAGCAAATG-GAAGAAAAATTCAAATTTTGGTTTTAGCCAA-TACCTTATTCATCAACCGCACAGTCCTACTGTT-GATCGTAATTACTCAGGACTCATCA-3'

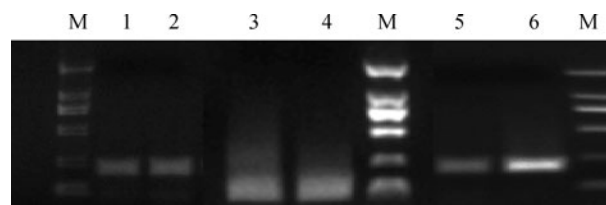


Fig. 7 The result of the second selective amplification

Note: 1–2 are DC1; 3–4 are DC5; 5–6 are DC9; M is the DL2000 DNA Marker.

DC5:5'-CTGATGCATCTATCGCATGCTACTTGAT-CAAGGTAGCTTGGTAGAGTTGCCAGAATCAAGT-CATCAAATCATGTACCAGAGGATTACTCAGGACT-CATCATGACTGCGTAC-3'
DC9:5'-AAATTCCAATGCTCAGAGAATGTTGGAC-TACAGCATGTAAATCATCAGCCTCTTTCTGA-TAATTTGCAAATTCAAATGAACCTTCACTTTCTC-CATTTCCAGAAAAATCAAAACGGAAGGCAC-TAATTCCTTCATTTTCCAATGCAACTGCTATGTT-CACAATTACTCAGGACTCATCC-3'

Using the biologic information sciences tool, similar analysis was carried on to the sequence mainly to contain sequence contents, homophyly retrieval, homologous sequence comparison, multiple sequence alignments, and so on. Our research on jujube fruit stoneless related gene difference expression fragments DC1, DC5, and the DC9 DNA sequence in GenBank was conducted by the similar analysis with BLAST. These three TDFs were all the first report on Chinese jujube. DC1 and DC5 were not found having obviously related homologous sequences in GenBank, and they were perhaps the new genes, whose function was not known and needed to be further confirmed. By Blast in GenBank, DC9 was found having an obviously related homologous sequence, E value 9e-32, with *Ricinus communis* Hydrolytic enzyme that has a degeneration lignin function; therefore, it proved that DC9 was the TDFs related with the stoneless gene of Chinese jujube fruit.

4 Discussion

The ability to isolate good quality total RNA and mRNA free of protein, genomic DNA, and secondary metabolite contamination was crucial for cDNA library construction and molecular analysis, e.g., northern hybridization and reverse transcription-polymerase chain reaction (RT-PCR). To date, most published protocols for RNA isolation have used strong protein denaturants, guanidine/guanidinium salts, for RNase inactivation (Logemann et al., 1987). These included the commercial RNA isolation kit, TRIZOLTM (Gibco-BRL Life Technologies, Gaitherring, MD), routine SDS method, and routine CTAB method. We found that these methods were useful for the isolation of RNA from Chinese jujube younger leaf and fruit, but they

failed to recover RNA from Chinese jujube ripen leaf and fruit. On the base of routine SDS method, we designed the modified SDS method that high RNA can be extracted from Chinese jujube ripen leaf and fruit, which contained lots of polyphenolics, polysaccharide, and other secondary metabolite. Total RNA extraction of Chinese jujube fruit was carried out at 65°C with the buffer and polyvinylpyrrolidone to prevent phenolic oxidation and polysaccharide precipitation. Moreover, this method was followed by precipitating the polysaccharide complex using potassium acetate and overnight precipitation of RNA with LiCl. By the modified SDS method, we had successfully isolated the total RNA of Chinese jujube fruit from younger to ripen. The quality of total RNA was suitable to cDNA-AFLP and other molecular analysis.

Biological responses and developmental programming are controlled by the precise regulation of gene expression. To gain insight into these processes, it is necessary to study the patterns of gene expression. Since its introduction in 1996, cDNA-AFLP has become a preferred tool for the study of differential gene expression in both plant and animal systems. In this paper, we establish a cDNA-AFLP technology system in Chinese jujube for the first time. 150 ng·L⁻¹ cDNA is the most suitable density for cDNA-AFLP technology system in Chinese jujube. If the density of cDNA was lower, we could have obtained the result without any fingerprint. Moreover, if the density of cDNA was higher, we could have not obtained the appropriate fingerprint result also. In our study, we also compare the result of polyacrylamide gel with a different number of selective nucleotides on each primer. We found the primer combinations with two selective nucleotides on *EcoRI*, and the two selective nucleotides on *MseI* were very suitable for cDNA-AFLP technology system in Chinese jujube fruit (Table 1). These combinations can get more fingerprints and detect more enzyme digestion sites than other combinations. The cDNA-AFLP technology system in Chinese jujube by our study is a highly sensitive and reproducible PCR-base technique that can be used without prior knowledge of DNA sequences; it is therefore the favored method for gene expression analysis in Chinese jujube. Moreover, cDNA-AFLP technology system will play an important role in studying the gene regulation on Chinese jujube biologic responses and developmental programming in the next research.

As it is well-known that Chinese jujube was a quasi stone fruit and the stone constituted of stone cells, which are mainly formed by lignin, a secondary metabolism material, precipitation, therefore, the synthesis, transportation, and degradation of lignin had close relations with the stone development. The hydrolytic enzyme was a lignin degradation enzyme (Li et al., 2009), so the hydrolytic enzyme gene expression can reduce lignin precipitation. In this study, we found that DC9, which was only a different expression in 'Wuhejinsixiaozao', had an obviously related homologous sequence with *Ricinus communis*

Hydrolytic enzyme. Therefore, it was proven that DC9 was one of the TDFs related with the stoneless gene of Chinese jujube fruit. In the future, we can make use of the molecular technology to obtain the cDNA span sequence and transfer the clone into vectors for cultivating the new stoneless Chinese jujube and clarifying the Chinese jujube stoneless fruit mechanism.

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