板栗花序与叶的cDNA-AFLP的差异表达分析

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摘 要:为给板栗的分子遗传育种工作提供重要的分子数据参考,作者通过cDNA-AFLP的方法,以同株板栗幼年花序和叶片为实验材料,在转录水平上进行差示分析。结果显示:64对引物组合共产生了2131条PCR产物,对其中的110条克隆测序,发现有28条基因与已知功能基因相关,其中包括参与植物转运、代谢、能量产生相关的蛋白,从而证明这些基因在板栗生长发育中的重要作用。

关键词:板栗;RT-PCR;cDNA-AFLP
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cDNA-AFLP Difference-expression Analysis between Inflorescences and Leaves of Chestnut

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Abstract: In order to provide more references for chestnut molecular breeding, authors used the method of cDNA-AFLP to analyze gene expression profiles between inflorescences and leaves of chestnut. The results showed that: sixty-four combination of AFLP primers produced 2,131 fragments; twenty-eight genes showed significant homolog to known genes according to the sequencing of one hundred and ten genes, including transport facilitation, metabolism and energy-related proteins, which indicate the importance of them.

Key words: Chestnut, RT-PCR, cDNA-AFLP

Chestnut (*Castanea mollissima*) is one of the most commercially valuable fruit trees, with production once only annually. Chestnut has an extended juvenile phase, during which vegetative growth is maintained. This characteristic is recognized as a disadvantage in breeding and in stable annual production. Thus, it will be necessary to understand the genetic mechanism underlying the development of reproductive phase. We had undertaken this study in chestnut to identify the key genes involved in chestnut development[1].

Molecular characterization of plant development has been approached by isolating specific genes through cDNA library screening or subtractive hybridization[2-4]. These techniques have the disadvantage of requiring a large amount of biological material. A relatively simple method for profiling differential gene expression is cDNA-amplified fragment length polymorphism (cDNA-AFLP) [5,6]. This method involves restriction digestion of cDNAs with a combination of two enzymes, one recognising a 6 bp and the other a 4 bp sequence. Adaptors are ligated to the digested material and subsets of cDNA populations are selectively PCR amplified for comparison on polyacrylamide gels. Following electrophoretic fractionation, fragments of specifically up-regulated cDNAs can be excised from the dried polyacrylamide gel, reamplified by PCR and sequenced.

In this investigation, we aimed to use the cDNA-AFLP technique to sample the leaves and inflorescence

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and discover genes commonly expressed between them. Identification of genes expressed in these tissues will form a platform for future functional analysis to determine the key gene controlling the development of chestnut.

1 Materials and methods

1.1 Plant materials

The inflorescences and leaves sample was dissected from developing shoots on *Castanea mollissimas* grown at the city of Yiliang of Yunnan province. The materials used for the construction of dscDNA were dissected in August of 2004. After dissected, the samples were frozen at liquid N_2 immediately and stored at -80℃.

1.2 cDNA-AFLP analysis

Total RNA was extracted by using the method according to Zeng and Yang [7]. Control RNA was prepared by pooling equal amounts of RNA. Double-stranded cDNA was synthesized using a SMARTk cDNA Library Construction Kit (Clontech, USA) according to the manufacturer's instructions. LD-PCR products were purified by a Takara PCR Purification Kit (Takara, Japan) and digested by the EcoRI/HindIII enzyme combination. AFLP reactions were performed according to published methods [5]. The sequences of adaptors and primers for AFLP are shown at Table 1. Selective amplification products were

Table 1 Nucleotide sequences of AFLP primerss

Adaptor	EcoRI-A1	CTC GTA GAC TGC GTA CC
	EcoRI-A2	AAT TGG TAC GCA GTC TAC
	MseI-A1	GAC GAT GAG TCC TGA G
	MseI-A2	TAC TCA GGA CTC AT
Preamplification Primer	EcoRI-P0	GAC TGC GTA CCA ATT CA
	MseI-P0	GAT GAG TCC TGA GTA AC
Selective primer	EcoRI-CT	GAC TGC GTA CCA ATT CAC T
	EcoRI-CA	GAC TGC GTA CCA ATT CAC A
	EcoRI-AC	GAC TGC GTA CCA ATT CAA C
	EcoRI-CC	GAC TGC GTA CCA ATT CAC C
	EcoRI-AG	GAC TGC GTA CCA ATT CAA G
	EcoRI-GC	GAC TGC GTA CCA ATT CAG C
	EcoRI-GG	GAC TGC GTA CCA ATT CAG G
	EcoRI-CG	GAC TGC GTA CCA ATT CAC G
	MseI-AA	GAT GAG TCC TGA GTA ACA A
	MseI-AC	GAT GAG TCC TGA GTA ACA C
	MseI-AG	GAT GAG TCC TGA GTA ACA G
	MseI-AT	GAT GAG TCC TGA GTA ACA T
	MseI-TC	GAT GAG TCC TGA GTA ACT C
	MseI-TA	GAT GAG TCC TGA GTA ACT A
	MseI-TG	GAT GAG TCC TGA GTA ACT G
	MseI-TT	GAT GAG TCC TGA GTA ACT T

separated on a 6% polyacrylamide gel run at 60 W until the xylene cyanole reached the bottom. DNA fragments were visualized by silver-staining according to the Silver Sequencek DNA Sequencing System Technical Manual (Promega, USA).

1.3 Isolation and sequencing of TDFs

The expressed transcript-derived fragment (TDFs) were marked, cut out and incubated in 150 ml TE (10mmol/L Tris, pH 7.5, and 1mmol/L EDTA, pH 8.0) overnight at 37℃. Extracted target bands were used as template for re-amplification using PCR under the same conditions as used for preamplification. Purified PCR products were ligated to the pMD18-T vector (Takara, Japan). The clones were sequenced using ABI3730 (Amersham Pharmacia, USA). Nucleotide' translated sequences were compared with protein sequences of the GenBank non redundant databases and sequences of the expressed sequence tag databases by using the BLAST sequence alignment program[8].

1.4 Expression analysis by reverse transcription polymerase chain reaction (RT-PCR)

First stand cDNA was synthesized from total RNA of *Castanea mollissima* leaves, using oligo $(dT)_{25}$ primer and Superscript Reverse Transcriptase (BD Biosciences Clontech). Two microlitres each of the first strand cDNA's were used as a template for PCR with gene specific primers. The specific primers come from the sequences of Cm2, Cm15, Cm19 and Cm28. The amplification program consisted of 2 min at 94℃ for initial denaturizing, 30 cycles of 30 s at 94℃, 30 s at 55℃, and 30 s at 72℃.

2 Results and Discussions

The cDNA-AFLP procedure employed was very efficient in isolating transcription of genes differentially expressed at the two tissues (leaves and inflorescences) of chestnut. The profiles of developmental tissues of chestnut obtained by this analysis were highly polymorphic. From 64 selected AFLP primer combinations we obtained 2131 amplified fragments. 30 to 50 fragments were observed for each primer combination, with sizes ranging from 50 to 600 bp. Changes in intensity of individual bands did not affect the intensities of other bands in the same lane, implying that the formation of products was not affected by the concentration of the individual substrates in the reaction. When examining tissue-specific fragments, we only scored prominent fragments in order to avoid ambiguity.

There were 484 tissue-specific developmental fragments in the leaves and 466 in inflorescences. The numbers of fragments in common between leaves and inflorescences were 1181, 55.4% of the total number of fragments. Fragments expressed commonly were eluted from gels and re-amplified by PCR using the same primer set as in AFLP. Of the 1181 fragments, one hundred and ten were sequenced and the sequences searched using the Basis Local Alignment Search Tool (BLAST) program[8] in the GenBank public sequence database. Twenty-eight fragments showed significant homology with GenBank data base entries of known function (Table 2). The remaining 36 fragments that did not show significant homology to the annotated genes may represent novel

Table 2 Function analysis of leaves and inflorescences and their expression patterns obtained by cDNA-AFLP

TDF	Size (bp)	Expression[a]		Homology
		L	I	
Cm1	227	+	+	Pathogenesis-related protein Bettv I family
Cm28	186	+	-	Secretory peroxidases.
Cm8	314	+	+	Metallothionein
Cm11	249	+	+	Metallothionein
Cm16	170	+	+	Isopenicillin N synthase
Cm24	105	+	+	Multicopper oxidase
Cm20	234	+	+	Pectinesterase
Cm9	214	+	+	Trehalose-phosphatase
Cm10	170	+	+	Fructose-1,6-bisphosphatase
Cm2	368	+	+	DNA polymerase V
Cm7	189	+	+	Histone acetyltransferase (MYST family)
Cm17	196	+	+	Cytosol aminopeptidase family
Cm26	206	+	-	Glycosyl hydrolase family 20, catalytic domain.
Cm19	187	+	+	Ribosomal protein L1p/L10e family
Cm14	126	+	+	Chlorophyll A-B binding protein
Cm5	117	+	+	Tyrosine kinase
Cm15	194	+	+	Serine/Threonine protein kinases
Cm21	167	+	+	Serine/threonine protein phosphatase 2A
Cm27	231	+	-	Serine hydroxyl methyltransferase..
Cm3	302	+	+	Gamma-glutamyl phosphate reductase
Cm4	253	+	+	SAM dependent carboxyl methyltransferase
Cm6	133	+	+	Fumarate reductase/succinate dehydrogenase
Cm12	361	+	+	Zinc-binding dehydrogenase
Cm13	143	+	+	Formyltetrahydrofolate synthetase (FTHFS)
Cm18	113	+	+	Aldo/keto reductases
Cm22	240	+	+	Glutamate/Leucine/Phenylalanine/Valine dehydrogenase
Cm23	330	+	+	short chain dehydrogenase
Cm25	199	-	+	floral Acetyltransferases, including N-acetylases of ribosomal proteins

Comments: [a] L: leaves of chestnut; I: inflorescences of chestnut '+': means expression.

genes or fragments too short for recognition in database.

The 28 fragments that showed homology to database entries of known function represented such varied functions as cellular production (DNA polymerase V), cell division (Pectinesterase), protein modification and signal transduction (kinase-like proteins), and cellular resisting (Pathogenesis-related protein Bettv I family). Since these functions are critical for cellular when cells switch from undifferentiated cell clusters to mature cell clusters, they may need to express a large set of genes. Another study by microarray analysis will hopefully provide more information on this change in the pattern of gene expression.

To validate the temporal expression patterns, 4 genes- including Cm19, Cm2, Cm15 and Cm28 were used for RT-PCR analysis. The results were comparable to the expression patterns revealed by cDNA-AFLP, Which revealed the reliability of method for cDNA-AFLP in plants. Ribosomal protein, similar to Cm19, has also been implicated in responses to oxidative stress, in addition to protein translation [9]. Peroxidases, similar to Cm28, might be involved in prompt neutralization of H_2O_2, and may thus contribute to the absence of a second phase of oxidative burst in susceptible plant-pathogen interactions described by Lamb and Dixon[10]. Also, the development is involved in the nucleotide synthesis and cell metabolism controlled by Cm2 and Cm15. RT-PCR experiments showed Cm2, Cm15 and Cm19 expressed all indeed and the different expression of Cm28 between leaves and flowers. What roles they play in controlling the development of chestnut still need further study. We have taken some steps to do it and believe that experiment can provide some information for future molecular breeding.

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