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Article

Identification of Differential Gene Expression in Juvenile vs. Mature Leaves of Pear (*Pyrus pyrifolia*) by Using Annealing Control Primer

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The identification of genes specifically expressed at a developmental stage would provide insights into many characteristic features of each of the developmental stages and introduction of molecular markers linked to important agronomic traits. However, the current methods employed to identify genes with specific expression are labor-intensive and suffer from high rates of false positives. Here, we employed a new and accurate reverse transcription-polymerase chain reaction method using annealing control primers (ACP) to identify genes that are preferentially expressed in juvenile or mature leaves of pears (*Pyrus pyrifolia* L.). We identified and sequenced four of these differentially expressed genes (DEGs). Basic Local Alignment Search Tool searches revealed that one was a hypothetical gene and three genes encoded protein with high similarity to the known genes. These DEGs were further characterized using real-time quantitative PCR to assess their expression pattern. This report suggests that the ACP system is a rapid and accurate method for the identification of development-specific genes in woody plants such as pears, of which a few genes are available.

Key words: *annealing control primers, developmental specific expression, differentially expressed genes, phase transition,* Pyrus pyrifolia*, reverse transcription-polymerase chain reaction*

Pears (*Pyrus* spp.) are native to the temperate regions, ranging from Europe, North Africa to East Asia. Pears have been cultivated as economic crop plant for more than 2,000 years [Bell, 1990; Bell *et al*., 1996]. Only few species are cultivated for the production of edible fruits, including the European pears (*Pyrus communis* L.) grown mainly in Europe and North America and the Chinese white pears (*Pyrus pyrifolia* L, *Pyrus bretschneideri* Rehd., and *Pyrus ussuriensis* Maxim.) grown mainly in Eastern Asian countries. In particular, thousands of cultivars of the three Chinese white pear species are grown.

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Abbreviations: ACP, annealing control primers; BLAST, basic local alignment search tool; DEGs, differentially expressed genes; RT-PCR, reverse transcription-polymerase chain reaction

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As do all woody plants, pears have a long term of genetically-determined juvenile phase, during which flowering does not occur and cannot be induced by normal flower-initiating treatments. This juvenile period lasts up to more than 10 years in certain forest trees [Hackett, 1985]. After this phase, woody plants are considered to have attained the adult or sexually mature condition. The change from the juvenile to the mature phase may be associated with the expressional changes of a set of genetic factors, leading to distinguishable physiological and morphological features, including leaf shape, branching, and reproductive capacity [Schaffalitzky de Muckadell, 1959; Sweet and Wells, 1974]. These changes are controlled by a number of endogenous or environmental factors and vary in length among species [Hackett, 1985]. The length of the juvenile period strongly affects the breeding efficiency of woody plants. A long juvenile period is considered to be a major obstacle in the breeding of improved cultivars [Hansche and Beres, 1980; Hansche, 1983; Sherman and Lyrene, 1983]. Thus, several measures have been taken to shorten

the juvenile period in various fruit trees [Hackett, 1985].

A number of reports have suggested the role of genetic factors in controlling the length of the juvenile period in woody plants. For example, a single dominant gene was identified to promote early flowering in *Betula pubescens* [Johnson, 1940]. Comprehensive analysis detected highly significant general combining ability and nonsignificant specific combining ability variances in the juvenile period of 22 apple (*Malus domestica*) and 33 pear (*P. communis*) progenies [Visser *et al*., 1976]. This result suggests that the length of the juvenile period in these species is a quantitative characteristic possessing a number of additive components. Identification of genes preferentially expressed during specific development stages and their functional determination would provide valuable information on the molecular mechanism in phase changes and development of molecular markers.

The transition from juvenile to mature stage is among the most dramatic phase changes that all plants undergo. Most reports on the phase changes in woody plants have been focused on morphological and physiological changes associated with a finer scale. Identification of the genes preferentially expressed in a phase-specific manner is essential to understand the characteristic features of the specific developmental stage on the molecular level in woody plants. However, it is difficult to identify differentially expressed genes (DEGs) at a specific developmental stage, because these genes could be expressed at a very low level in the plant cells. Recently, a number of reports have employed RT-PCR to detect genes that are transcribed at a low level in woody plants [Souleyre *et al*., 2005; Kayal *et al*., 2006]. Differential display methods, which are based on PCR using short arbitrary primers, are simple and fast but have the disadvantage of high rates of false positives. They are also biased toward detecting more abundant transcripts. Suppression subtractive hybridization has been employed as alternative method to identify DEGs in woody plants [Brosche *et al*., 2005]; however, this method is labor-intensive and also prone to false positives.

Here, we describe the identification of DEGs from juvenile and mature leaves of pear plants by using a novel differential display PCR method based on annealing control primers (ACPs) [Hwang *et al*., 2003; Kim *et al*., 2004]. This is an easy technique without false positives, allowing real products to be amplified. We also quantified the expression levels of four selected DEGs. The identification and the subsequent functional determination of DEGs would provide valuable information on the characteristic features of developmental mechanism at the molecular level and the discovery of possible molecular markers in woody plants.

Materials and Methods

First-strand cDNA synthesis. For ACP-based reverse transcription-polymerase chain reaction (RT-PCR) analysis, juvenile and mature leaf samples were harvested from 6-month-old and 5-year-old pear trees (*P. pyrifolia* L.), respectively. Total RNA samples were extracted from these leaves. Fifty microliters of the oligo$(dT)_{25}$ magnetic bead suspension was added to the total RNA samples and incubated at room temperature for 5 min. The hybridized mRNA and oligo$(dT)_{25}$ magnetic beads were washed twice as recommended in the manufacturer's protocol (Dynabeads mRNA purification kit; Dynal, Oslo, Norway). To synthesize the first-strand cDNAs, dT-ACP1 primer was added to the mRNA solution. Reverse transcription was performed for 1.5 h at 42ºC in a final reaction volume of 20 μL containing 0.3 μg of the purified mRNA, 4 μL of 5× reaction buffer (Promega, Madison, WI), 5 μL of dNTPs (each 2 mM), 2 μL of 10 μM dT-ACP1 [5'-CTGTGAATGCTGCGACTA CGATIIIIIT(18)-3'], 0.5 μL of RNasin RNase Inhibitor (40 U/μL; Promega), and 1 μL of Moloney murine leukemia virus reverse transcriptase (200 U/μL; Promega). First-strand cDNAs were diluted by the addition of 80 μL of the ultra-purified water for the GeneFishingTM PCR and stored at −20ºC until use.

ACP-based PCR analysis. DEGs were screened by the ACP-based PCR method (Kim *et al*., 2004) using the GeneFishing DEG kits (Seegene, Seoul, Korea). Briefly, second-strand cDNA synthesis was conducted at 50ºC during one cycle of the first-stage PCR in a final reaction volume of 20 μL containing 3-5 μL (about 50 ng) of the diluted first-strand cDNA, 1 μL of dT-REV (10 μM), 1 μL of 10 μM arbitrary ACP, and 10 μL of 2× Master Mix (Seegene). The PCR protocol for the second-strand synthesis was one cycle at 94ºC for 1 min, followed by 50ºC for 3 min, and 72ºC for 1 min. After the completion of the second-strand DNA synthesis, the second-stage PCR amplification protocol was 40 cycles at 94ºC for 40 s, followed by 65ºC for 40 s, 72ºC for 40 s, and a 5 min final extension at 72ºC. The amplified PCR products were separated on a 2% agarose gel stained with ethidium bromide.

Cloning and sequencing. The differentially expressed bands were extracted from the gel by using the GENCLEAN II Kit (Q-BIO gene, Carlsbad, CA), and directly cloned into a TOPO TA cloning vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The cloned plasmids were sequenced with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

RT-PCR confirmation. The differential expression of

DEG was confirmed by RT-PCR using each gene-specific primer pair. The first-strand cDNA was normalized by the actin gene. The normalized cDNA was used as a template. The PCR reaction was conducted in a final reaction volume of 20 μL containing 2~4 μL (about 50 ng) of diluted first-strand cDNA, 1 μL of forward primer (10 μM), 1 μL of reverse primer (10 μM), and 10 μL of 2× Master Mix (Seegene, Seoul, Korea). The PCR amplification protocol was an initial 3 min denaturation at 94°C, followed by 20~25 cycles at 94°C for 40 s, 60°C for 40 s, 72°C for 40 s, and a 5 min final extension at 72°C. The amplified PCR products were separated on a 2% agarose gel stained with ethidium bromide.

Real-time quantitative RT-PCR. To determine the relative abundance of DEGs, real-time quantitative PCR was performed using mRNA extracted from the juvenile and mature leaves. Using SuperScript II and random primers (Invitrogen), cDNAs were produced according to the manufacturer's instruction. The PCRs were performed in 20 μL of 2× SYBR Green Master mix (Applied Biosystems), with 10 ng of cDNA and 300 nM of each gene-specific primer. Three replicates of each PCR were run in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using a program including a first step (50°C for 2 min and 95°C for 10 min) followed by 40 cycles (95°C for 15 s and 60°C for 1 min). The non-specific products could be detected after the end of the amplification when the PCR assays were submitted to a temperature ramp in order to create the dissociation curve. The dissociation program was 95°C for 15 s, 60°C for 15 s, followed by 20 min of slow ramp from 60 to 95°C. Specific primers for actin gene were used as the internal control for the normalization of the RNA steady state level. The results of relative transcript abundance are presented as mean values of the three assay replicates compared with those of the three controls.

Results

Differentially expressed amplified cDNA products. ACP-based RT-PCR analysis was performed to identify genes specifically or predominantly expressed at the juvenile or adult stage. The first-strand cDNA was synthesized from mRNA of the developmental stage using dT-ACP1 primer (Table 1). Synthesis of the second-strand cDNA was conducted at 50°C annealing temperature during one cycle of PCR using an arbitrary ACP primer. Although the arbitrary ACP primer and dT-REV primer were placed in the same PCR tube, the 3'-end region of dT-REV primer could not anneal to the first-strand cDNAs due to the absence of the complimentary sequence. However, such annealing temperature allowed the 3' end core sequence (10-mer) of the arbitrary ACP to bind to the complementary site of first-strand cDNA. By using a combination of dT-REV (reverse primer) and 40 arbitrary primers (forward primer; dT-FRD), second-strand cDNAs were then amplified during the second-stage PCR at a higher annealing temperature (65°C), which constitutes higher stringent conditions (Fig. 1).

For PCR products exhibiting differential expression levels between juvenile and adult stages were identified (Fig. 2). They were excised from the gel and cloned. The cloned PCR products were denoted as DEG1, 5, 6, and 9. For further analysis, the primary structure of each of their nucleic acid sequence was determined. Sequencing analysis revealed that all DEG clones were less than 1 kb in length with a poly (A) tail, suggesting that they originated from mRNA (Fig. 3). These sequences were translated in three frames to find the possible amino acid sequences of their encoding proteins. These DEGs were then queried against the GenBank database using BLAST for annotation [Altschul *et al*., 1990]. The DEG1, 6 and 9 clones showed significant similarities to known genes encoding aldolase-like protein, defensin precusor, and thaumatin-like protein, respectively. On the other hand, the sequence of DEG5 had the highest similarity to hypothetical gene (Table 2).

Analysis of expression profile of DEGs by quantitative RT-PCR. We have determined the expression pattern of these genes from juvenile and adult leaves by reverse transcription PCR. The determination of their expression level was performed using gene-specific primers employed to amplify transcripts ranging from 274 to 450 bp, and actin as a reference gene for detecting the

Table 1. Primer sequences used in cDNA synthesis and ACP-based PCR

Use	Primer name	Sequence
cDNA synthesis primer	dT-ACP1	CTGTGAATGCTGCGACTACGAIIII$(T)_{18}$
Reverse primer	dT-REV	CTGTGAATGCTGCGACTACGAIIII$(T)_{15}$
Forward primer	dT-FRD31	GTCTACCAGGCATTCGCTTCATIIIICCATCAGCTC
(Arbitrary primer)	dT-FRD38	GTCTACCAGGCATTCGCTTCATIIIIGGAGCGATGT
	dT-FRD47	GTCTACCAGGCATTCGCTTCATIIIITGAGTAAAAG
	dT-FRD56	GTCTACCAGGCATTCGCTTCATIIIICAACTTCAAG

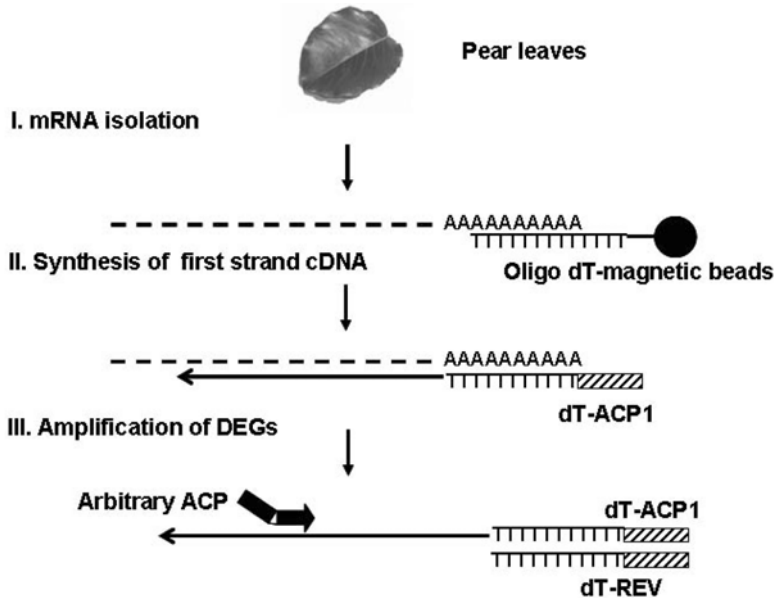

Fig. 1. Schematic illustration of reverse transcription-polymerase chain reaction using annealing control primers. mRNA was isolated by using oligo(dT) magnetic beads from total RNA of juvenile and adult leaves of pear (*P. pyrifolia*). The first strand cDNA was synthesized using dT-ACP1. The DEG clones were amplified by PCR during second-stage PCR, using a combination of dT-REV (reverse primer) and 1 of 40 different arbitrary ACPs (forward primer) and were separated on agarose gels. The dotted and solid lines indicate mRNA and cDNA, respectively.

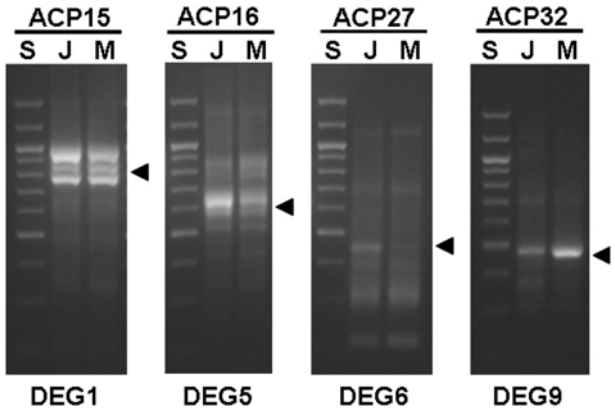

Fig. 2. Ethidium bromide stained 2% agarose gels show differential banding patterns of PCR products obtained from juvenile (J) and mature (M) leaves of pear (*P. pyrifolia*). A specific arbitrary primers used for each PCR reaction are indicated at the top of agarose gels. Arrowheads indicate differential PCR products. The PCR products were excised from the gel for further cloning and sequencing.

quantitative mRNA expression in both juvenile and adult leaves (Table 3).

Included in the RT-PCR analysis were four DEG clones obtained from the ACP-based differential expression assay. We found significant differences in the expression levels of DEG6 and 9 clones between the juvenile and adult leaves, compared to those in DEG1 and 5 (Fig. 4). This result showed that these four DEG transcripts exhibited expression patterns consistent with the results of the ACP-based differential analysis.

To further analyze the relative expression patterns for DEG transcripts in detail, fluorescent-monitored quantitative real-time RT-PCR analysis was employed. Gene-specific primers were used to amplify PCR products with lengths ranging from 274 to 450 bp. The DEG6 showed the highest expression in juvenile leaves, whereas DEG9 showed the highest expression in adult leaves. This result also showed that expression pattern of DEG clones was the same with those of the ACP-based differential and RT-PCR analyses. Taken together, these findings provided experimental evidence for the efficient and accurate method to identify genes with specific expression even in plants, of which little information is available.

Discussion

All plants pass through a series of predictable developmental stages during their lives [Poethig, 2003]. These developmental transitions are controlled by an array of signal transduction pathways that operate independently and interact to produce tremendous variations in the plant morphology encountered in nature [Mouradov *et al.*, 2002]. Coordination of these processes is accomplished by global changes in the gene expression patterns that lead to subsequent metabolic and physiological effects on the developmental procedures. Here, we have attempted to identify genes that were differentially expressed between juvenile and adult leaves. The sequence information of the DEG clones identified in the present report provides

Fig. 3. The sequence of development-specific cDNA fragments, DEG1, DEG5, DEG6 and DEG9, correspond to known genes. DEG1, 820 bp, adolase-like gene; DEG5, 614 bp, hypothetic gene; DEG6, defensin gene; DEG9, thaumatin-like gene. The underlined sequences show poly(A) tail.

Table 2. Sequences similarity and characterization of differentially expressed genes

Clone number	Base pairs sequenced (bp)	Homologous gene	Homology
DEG1	820	Aldolase-like protein	509/613 (83%)
DEG5	614	Hypothetical protein	492/514 (95%)
DEG6	397	Defensin precursor	317/329 (96%)
DEG9	416	Thaumatin-like protein	358/374 (95%)

Table 3. Primer sequences and cycling conditions used in quantitative RT-PCR and real time PCR

Clone name	Primer	Sequence	Product size (bp)
DEG1	Forward	CTTGCTCGCTACGCTTCCAT	450
	Reverse	CTTCACGTTCTCGGGTCTGC	
DEG5	Forward	CTAAGGGACTGTTGGGTTGG	427
	Reverse	AAAGCAGCTCAATAAGACCC	
DEG6	Forward	TTGCCAAACAGAGGGCTTCC	277
	Reverse	ACGAAATAACTCACGACGGT	
DEG9	Forward	GCTTGCCTTGCGTTTGGTGA	274
	Reverse	CCGCGGAATTTATTCTTCTC	
Actin	Forward	GTGCTGGACTCAGGTGATGG	267
	Reverse	GTTCTTCTCAACTGACGAGC	

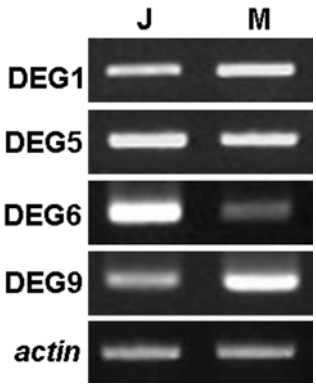

Fig. 4. Differential gene expression between juvenile (J) and mature (M) leaves of pear (*P. pyrifolia*). Comparison of the expression patterns of DEGs by RT-PCR was performed with mRNA from juvenile (J) and mature (M) leaves. The amplified DNA products were separated on 1.5% stained agarose gel and stained with ethidium bromide. The actin gene was used as control to confirm the equal amount of templates cDNAs.

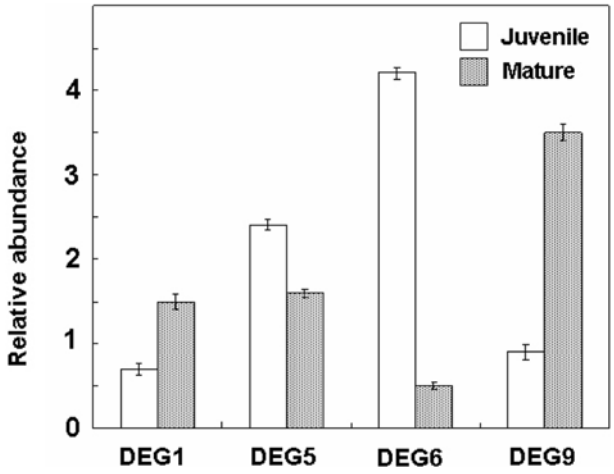

Fig. 5. Relative expression level of DEGs between juvenile (J) and mature (M) leaves of pear (*P. pyrifolia*). Messenger RNA samples from juvenile and mature leaves were reversed transcribed and subjected to quantitative real time RT-PCR using gene-specific primers. All PCRs were conducted in triplicate and normalized for actin mRNA expression. The relative expression level is presented as an n-fold expression difference compared to that of actin mRNA. Data are shown as means and standard deviation of triplicate determination.

resources for genes predominantly expressed in juvenile or adult leaves from *P. pyrifolia*. Although the full application of these genes has yet to be realized, the initial assessment of the comprehensive expression pattern of DEG clones has provided the first insight into the molecular mechanisms of the phase transition in woody plants.

Aldolase has been well known to mediate the reversible conversion of fructose-1,6-bisphosphate into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. The split of six-carbon into two three-carbon molecules allows further conversion of glyceraldehyde-3-phosphate into pyruvate, which is coupled to the formation of ATP and NADPH in glycolysis. The glycolysis is an almost universal central pathway of glucose catabolism for energy production and interacts with many other processes. When aldolase expression was reduced to 25% of that of wild-type, a significant decrease in the enzyme activities of the Calvin cycle, which include fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, and phosphoribulokinase in potato plants [Haake *et al*., 1998]. The reduction of activities of these enzymes resulted in a considerable decease in photosynthetic activity and finally inhibited the potato growth. We can therefore speculate that the up-regulation of aldolase transcripts in mature leaves leads to the increase in photosynthesis and sugar biosynthesis and that mature leaves can produce energy and carbon sources required for fruit production.

Plant defensins, small and basic polypeptides, are stabilized by disulfide-linked cysteines. Although most plant defensins show antifungal activities against a number of pathogenic fungi, some plant defensins, however, do not inhibit fungal growth but rather inhibit α-amylase, which is an insect gut enzyme [Bloch and Richardson, 1991]. The defensin-mediated inhibition of α-amylase activity increases the indigestibility of plant organs as well as plays important role on the anti-insect activities. Thaumatin-like proteins also display the antifungal activity, which is correlated with the plasma membrane permeabilization [Vitali *et al.*, 2006]. Higher plants have developed complex recognition and a number of defense mechanisms to counter the attack by various pathogens. One of the most strongly expressed forms of the plant defense mechanisms is the induction of pathogen-related genes, including defensins and thaumatin-like proteins. Our result showed that two pathogen-related genes were abundantly present in the leaves at different developmental stages. This finding could be explained by the possibility that juvenile and mature leaves of *P. pyrifolia* could be vulnerable to different pathogens recognized by different receptors, subsequently activating the up-regulation of different defense-related genes. Alternatively, upon the same fungal attacks, juvenile and mature leaves of *P. pyrifolia* leaves have developed different perception mechanisms, thereby inducing the expression of different genes with similar defense activities.

In conclusion, we have used a new differential screening method, termed the ACP system, to identify the DEG genes in *P. pyrifolia* leaves. The identification and subsequent characterization of these genes with their

specific expression patterns offer the opportunity to elucidate the molecular mechanisms of development processes of woody plants. The wealth of available information for gene expression pattern is essential for the investigators to obtain a more comprehensive picture of the phase transition in woody plants, which still remain elusive. Determination of the temporal and spatial patterns of the gene expression provides a means to integrate genetic alterations with the dynamic events occurring during the phase transition in woody plants.

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