cDNA-AFLP Analysis of Differentially Expressed Genes During the Flowering Process of Osmanthus fragrans (Post-Print)

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Abstract

Osmanthus is an important traditional renowned fragrant flowering plant in China. Flower color and fragrance constitute two critical ornamental qualities of osmanthus, which are influenced by the flowering process. While previous studies have reported on the composition of color and fragrance substances and their related biosynthetic genes, research on the biosynthetic processes of color and fragrance during flowering and their underlying molecular mechanisms remains scarce. This study established a cDNA-AFLP system applicable to multiple samples using osmanthus petals and young leaves at different flowering stages, and analyzed differential gene expression during the flowering process, obtaining 283 transcript-derived fragments (TDFs) that were specifically and differentially expressed in petals. Among these, 120 TDFs showed no homologous sequences in databases; 12 possessed homologous sequences with unknown functions; and 150 represented sequences with known biological functions, primarily involving secondary metabolism, primary metabolism, and developmental processes. Six TDFs with known functions were subjected to qRT-PCR validation, with the expression patterns of four TDFs exhibiting higher transcriptional levels being largely consistent with AFLP analysis results. These findings establish a foundation for understanding gene expression related to the biosynthesis of color and fragrance in osmanthus during flowering, and provide a reference for further investigation into the molecular mechanisms underlying color and fragrance formation in osmanthus.

Full Text

Preamble

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cDNA-AFLP Analysis of Differentially Expressed Genes During Flowering in Osmanthus fragrans

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Abstract

Osmanthus fragrans is one of China' s ten most famous traditional flowers and serves as an important fragrant plant widely used in landscaping, food processing, essential oil extraction, and natural pigment production. Previous studies have identified the main components of floral scent (terpenoids, esters, alcohols, ketones, and aldehydes) and floral color (carotenoids and flavonoids), as well as related biosynthetic genes such as CCDs and TPSs. However, research on the molecular mechanisms underlying scent and color synthesis during the flowering process remains limited.

Since no reference genome or transcriptome is available for O. fragrans, we established a cDNA-AFLP system suitable for multiple samples to analyze gene expression differences across flowering stages. This technique, originally developed by Bachem et al. (1996) for mRNA fingerprinting, offers cost-effectiveness, high polymorphism, and stability without requiring prior sequence information. We successfully cloned and sequenced 283 transcript-derived fragments (TDFs) showing petal-specific expression and differential regulation during flowering. Database analysis revealed that 120 TDFs had no homologous sequences, 13 had homologous sequences of unknown function, and 150 had known biological functions primarily involved in secondary metabolism, primary metabolism, and developmental processes. Quantitative real-time PCR (qRT-PCR) validation of six functionally annotated TDFs showed that four with higher transcriptional levels exhibited expression patterns consistent with cDNA-AFLP results. These findings provide a foundation for understanding gene expression dynamics during O. fragrans flowering and offer valuable insights into the molecular mechanisms of floral color and scent formation.

Keywords: Osmanthus fragrans, cDNA-AFLP, transcript-derived fragments, floral color, floral scent

Introduction

Osmanthus fragrans is a crucial traditional fragrant flower in China, with floral color and scent representing key ornamental traits that are strongly influenced by the flowering process. While previous research has identified the chemical

components of its color and fragrance and isolated related biosynthetic genes, few studies have investigated the synthesis processes and molecular basis of these traits during flower development. Petals are the primary site for scent and pigment biosynthesis, with synthesis rates varying across developmental stages. In O. fragrans, both scent and color compound accumulation increases significantly during flower opening, peaking at full bloom before declining. However, molecular studies on these dynamic changes remain scarce.

The cDNA-AFLP technique, which applies amplified fragment length polymorphism to mRNA expression analysis, has been successfully used in ornamental plants such as crabapple, Oncidium orchid, and chrysanthemum. Previous cDNA-AFLP studies on O. fragrans compared only two samples, limiting the ability to capture dynamic expression changes. To address this gap, we established a robust cDNA-AFLP system for multiple samples, enabling simultaneous analysis of transcripts from young leaves and petals at six flowering stages of the 'Liuye Jingui' cultivar. This approach allowed us to identify petal-specific, developmentally regulated TDFs and uncover genes associated with color and scent formation during flowering.

Materials and Methods

Plant Materials

Healthy, disease-free 'Liuye Jingui' (O. fragrans 'Liuye jingui') plants growing under uniform light conditions in the Huazhong Agricultural University nursery were used as experimental material. Flower developmental stages followed Zou et al. (2014) with sampling times as shown in [Figure 1: see original paper]: (1) Tight bud stage: unopened flowers with closed petals (time=0 d); (2) Initial flowering stage: semi-open flowers (time=2 d); (3) Full flowering stage: fully expanded flowers at maximum diameter without brown spots (time=4 d and 6 d); (4) Late full flowering stage: petals showing brown spots and some abscission (time=8 d). Young leaves were collected in May, while petals were sampled every 2 days starting from the tight bud stage in October. Except for the initial flowering stage (time=2 d), which was sampled at both 10:00 am and 5:00 pm, all other samples were collected at approximately 10:00 am. Samples were weighed immediately after collection, flash-frozen in liquid nitrogen, and stored at -80°C.

RNA Extraction and cDNA Synthesis

Petal and leaf samples were ground in liquid nitrogen, and total RNA was extracted using the Trizol method (CoWin Biotech Co., Ltd, Beijing, China). RNA integrity and concentration were assessed by 1.0% agarose gel electrophoresis and NanoDrop 2000 spectrophotometry (Thermo Fisher Scientific). mRNA was purified using the Oligotex™-dT30 mRNA purification kit (Takara), and double-stranded cDNA was synthesized following the RevertAid™ Premium First Strand cDNA Synthesis Kit protocol (Fermentas).

Double-Stranded cDNA Digestion

Two restriction enzyme combinations were evaluated: FastDigest® EcoR I/FastDigest® Mse I and FastDigest® Taq I/FastDigest® Ase I. The EcoR I/Mse I digestion protocol consisted of incubation at 37°C for 30 min, 65°C for 5 min, and inactivation at 80°C for 5 min. The Ase I/Taq I protocol involved incubation at 37°C for 30 min and 65°C for 30 min, followed by chloroform extraction for enzyme inactivation.

cDNA-AFLP Analysis

The cDNA-AFLP procedure followed Bachem et al. (1996). Digested cDNA fragments were ligated to adapters using T4 DNA ligase (Fermentas). Preamplification was performed using EcoR00/Mse00 and Ase00/T00 primers, with 5 L of product verified by 1% agarose gel electrophoresis. Suitable preamplification products were diluted 50-fold for selective amplification using 256 primer combinations (16 EcoR primers × 16 Mse primers). Primer sequences are listed in .

Selective amplification reactions (20 L total volume) contained 1 L template, 1 L each of EcoR and Mse selective primers (10 $mol \cdot L^{-1}$), and 10 L 2× DreamTaq PCR Master Mix (Fermentas). The thermal cycling program was: 95°C for 3 min; 12 cycles of 95°C for 30 s, 65°C (decreasing by 0.7°C per cycle) for 30 s, 72°C for 1 min; 25 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 1 min; and final extension at 72°C for 5 min. Amplification products were separated by 6% denaturing polyacrylamide gel electrophoresis to visualize differentially expressed fragments.

Recovery, Cloning, and Sequence Analysis of Differential TDFs

Target fragments were excised from gels using a clean scalpel and transferred to PCR tubes. Gel pieces were washed with double-distilled water and residual liquid was removed. Secondary amplification was performed using the same reaction conditions as selective amplification. Products were purified, ligated into the pEASY-T1 cloning vector (TransGen Biotech), and sequenced. Sequences were subjected to BLAST analysis in NCBI databases and functional annotation using Blast2GO software combined with the Amigo website (http://amigo1.geneontology.org/cgi-bin/amigo/blast.cgi) for Gene Ontology (GO) classification.

3' RACE Cloning of Differential Fragments

Gene-specific primers for 3' RACE were designed based on obtained sequences (). 3' RACE-cDNA synthesis followed the Smart RACE kit protocol (Clontech). Amplified products were purified, ligated into pEASY-T1 vector, and sequenced.

Real-Time qPCR Analysis of Differential Fragments

Real-time qPCR primers were designed based on 3' RACE sequences (). First-strand cDNA was synthesized using the RevertAid™ Premium kit, diluted 20-fold, and analyzed on an ABI 7500 Fast Real-Time PCR System using SYBR® Premix Ex Taq™ (Takara). The O. fragrans β-actin gene served as an internal reference, with leaf expression levels set to 1. Relative expression was calculated using the 2^(-ΔΔCt) method.

Results

Establishment of a Multi-Sample cDNA-AFLP System for Osmanthus fragrans

RNA quality assessment showed that extracting from 200 mg tissue yielded higher concentrations with good purity and integrity ([Figure 2: see original paper]A). Isolated mRNA from petals and leaves showed a diffuse distribution centered around 800-2000 bp, indicating good quality. The resulting double-stranded cDNA also appeared as a diffuse smear with a major band at 500-1000 bp ([Figure 2: see original paper]B). Successful amplification of the β-actin gene produced clear bands of expected size ([Figure 2: see original paper]C), confirming high-quality cDNA suitable for cDNA-AFLP analysis.

Comparison of two restriction enzyme combinations revealed distinct differences. Ase I/Taq I digestion produced concentrated bands around 300 bp ([Figure 3: see original paper]A), while EcoR I/Mse I digestion yielded a uniform smear distribution from 100-1000 bp ([Figure 3: see original paper]B). Preamplification of Ase I/Taq I-digested products showed concentration differences between 25 and 30 cycles, with products mainly at 100-250 bp. In contrast, EcoR I/Mse I preamplification products were evenly distributed across 100-1000 bp, with minimal cycle number effects ([Figure 3: see original paper]).

Using EcoR I/Mse I-digested products, we evaluated 256 selective amplification primer combinations (). Ninety-six combinations (A-grade) produced numerous clear bands, 121 combinations (B-grade) gave moderate band numbers and clarity, and 39 combinations (C-grade) yielded few or faint bands. All A-grade and selected B-grade primers were used for subsequent experiments.

Identification and Functional Analysis of Differentially Expressed TDFs

cDNA-AFLP analysis using leaves as a control (lane 7) revealed three major expression patterns ([Figure 4: see original paper]A): (1) genes continuously expressed in both petals and leaves ([Figure 4: see original paper]B); (2) petal-specific genes without developmental differences ([Figure 4: see original paper]D); and (3) petal-specific genes with differential expression across developmental stages ([Figure 4: see original paper]C, E). We recovered and sequenced 283 TDFs from the third category.

GO functional annotation of these sequences showed that 120 (42%) had no homologous sequences, 13 (5%) had homologous sequences of unknown function, and 150 (53%) had known functions. The annotated TDFs were categorized into ten biological processes: primary metabolism (41 TDFs, 14%), response to stimulus (20, 7%), transport (19, 7%), secondary metabolism (17, 6%), cellular component biogenesis (15, 5%), developmental process (11, 4%), oxidation-reduction (10, 4%), regulation of biological process (8, 3%), signal transduction (5, 2%), and other functions (4, 1%) ([Figure 5: see original paper]).

Expression Pattern Analysis of Selected Differentially Expressed TDFs

Six functionally annotated TDFs (M13E23-11, M13E32-24, M21E14-1, M22E31-11, M34E22-31, and M41E14-13) were selected for qRT-PCR validation ([Figure 6: see original paper]). Four TDFs showed expression patterns consistent with cDNA-AFLP results. M13E32-24 exhibited low expression in leaves and tight bud stage petals, with rapid upregulation after flower opening and peak expression at day 4 full bloom. M21E14-1 showed high expression at day 2 initial flowering (morning) and day 4 full bloom. M22E31-11 expression increased after flower opening, remaining high at day 2 morning and days 4-6 full bloom. M34E22-31 was petal-specific with significantly increased expression after flower opening, maintained throughout the flowering period. M13E23-11 showed consistent patterns except at day 8 late full bloom. M41E14-13 displayed substantial differences from cDNA-AFLP patterns, particularly at late full bloom and in leaves.

Discussion

The choice of restriction enzymes and gene expression abundance are critical factors affecting cDNA-AFLP results. Different species require different enzyme combinations. While Taq /Ase is commonly used in crops like Chinese cabbage and cucumber, EcoR /Mse is preferred in ornamental plants such as chrysanthemum and peony. Our comparison showed that EcoR /Mse produced more uniformly distributed fragments (100-1000 bp) in O. fragrans, making it the superior choice. Validation of six TDFs by qRT-PCR confirmed that four highly expressed fragments showed consistent patterns, demonstrating the reliability and accuracy of cDNA-AFLP for detecting differentially expressed genes. Compared with previous two-sample studies, our multi-sample approach captured dynamic expression changes more comprehensively and improved detection of low-abundance transcripts.

Floral scent and color components in O. fragrans vary throughout flowering and senescence. Our cDNA-AFLP analysis of leaves and petals at different developmental stages identified 283 petal-specific, differentially expressed TDFs. Functional annotation of 150 TDFs revealed involvement in multiple biological processes including secondary metabolism, primary metabolism, development, stimulus response, regulation, and transport. Secondary metabolism directly

participates in scent and pigment synthesis, accounting for 6% of annotated TDFs. Primary metabolism, providing precursors for secondary metabolism, represented the largest category (14%). The remaining 80% were associated with development, stress response, and regulatory processes, indicating that color and scent formation are influenced by multiple biological pathways. Primary metabolism' s role as a provider of intermediate metabolites warrants further investigation. These results provide important references for mining genes related to color and scent formation during flowering and lay a foundation for molecular studies of these traits in O. fragrans.

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Note: Figure translations are in progress. See original paper for figures.