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Analysis of expressed sequence tags from *Petunia* flowers

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Abstract

Petunia cDNA libraries were prepared from whole flower parts (including buds), pollen, and pollen tubes to generate expressed sequence tags (ESTs). A total of 7001 random clones were subjected to unidirectional sequencing, resulting in identification of 611 groups of related sequences and 2410 singletons. Highly conserved 1098 ESTs were functionally assigned. ESTs encoding proteins involved in the calcium-dependent signal pathway and in cell wall metabolism such as pectin degradation and modification were frequently found in the pollen and pollen tube libraries. The 2976 cDNA clones from the bud/flower cDNA libraries were used for the construction of microarrays. The 112 functionally annotated genes were up-regulated in the buds just before opening, including the genes for anthocyanin pigmentation and protein degradation. These ESTs and microarrays will serve the analysis of floral traits of petunias.

Keywords: Anthocyanin; Calmodulin; Microarray; Pectin; Pollen; Pollen tube

1. Introduction

Cultivars of *Petunia* × *hybrida* Vilm. (petunias) are available as a popular bedding plant. *Petunia hybrida* was originally bred as an artificial hybrid between *P. axillaris* (Lam.) Britton, Sterns & Poggenb. and *P. integrifolia* (Hook.) Schinz & Thell. [1]. *P. axillaris* is a species with a white large flower and produces strong scent in the night, whereas *P. integrifolia* is a species with a small reddish purple-color flower lacking scent. Although *P. axillaris* and *P. integrifolia* are found in the same geographic locations in South America, their natural hybrids have not been found in nature [2]. Petunias are presently one of the model plants for plant research including that of several physiological processes concerning floral and reproductive characteristics [3,4]. For example, analysis of the *Petunia* genes for flower pigmentation revealed almost all the structural genes, as well as many regulatory genes in the anthocyanin biosynthetic pathway [5,6].

We have a large number of *Petunia* samples collected from defined natural population in subtropical and temperate South America. All natural taxa of *Petunia* (19 natural *Petunia* species), about nearly 100 commercial cultivars of *P. hybrida*, and many mutant collections including several bud variations are available [7–10]. These *Petunia* collections are expected to be a valuable resource for scientific research and for breeding of garden *Petunia* cultivars. In addition, we used *Petunia* pollen and pollen tubes to characterize enzymes involved in pollen tube growth [11]. To facilitate research using *Petunia* flower parts, there is a growing need to characterize the *Petunia* floral transcriptome. In this study, we carried out partial sequencing of cDNA libraries to generate expressed sequence tags (ESTs) of *Petunia* buds/flowers, pollen and pollen tubes. Production of *Petunia* floral ESTs was then followed by cDNA microarray-based analysis.

2. Materials and methods

2.1. Plant material

Since the most modern petunia cultivars are considered to be offsprings of a crossbred plant, *Petunia* × *hybrida*, flowers of

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this hybrid plant were used as a source of flower ESTs. Seeds of an interspecific hybrid were obtained by crossing an individual of the *P. axillaris* (mother donor, subsp. *axillaris*, accession U157-S1 derived from Cerro, Montevideo, Uruguay) with an individual of *P. integrifolia* (father donor, subsp. *integrifolia*, accession U106-S3 derived from Rio Negro, Uruguay). Respective parental individuals were selected randomly from the individuals raised from seeds collected from respective natural populations. Both species were self-incompatible and of outcrossing nature, but morphology of floral and vegetative organs was very uniform. The hybrid plants were grown in a greenhouse following standard practices for garden petunias. Flowers and flower buds were harvested at developmental stages defined as follows: stage 1, closed bud (less than 20 mm in length); stage 2, enlarged closed bud with slight pigmentation (about 30 mm in length); stage 3, clearly pigmented bud (about 40 mm in length); stage 4, bud just before opening; stage 5, fully opened flower.

For construction of pollen and pollen tube cDNA libraries, flower buds of stage 4 were collected from a self-incompatible individual of *P. axillaris* (subsp. *axillaris*, accession U1-11-14-s6) that has been used in the studies concerning self-incompatibility and identified as homozygous for S_{13}-RNase gene [12]. Anthers were isolated from the flower buds, and pollen grains were separated from dehisced anther using a steel sieve (180-μm mesh). Dried pollen grains were kept at −70 °C until use. The pollen was germinated on a liquid culture medium as previously described [11]. After ungerminated pollens were removed from pollen tubes using a steel sieve (180-μm mesh) as much as possible, we checked the purity of the pollen tube preparation by counting pollen grains and pollen tubes under a microscope. About 10–20% of total grains (pollen grains plus pollen tubes) were found as ungerminated pollen grains in the pollen tube preparation.

2.2. Construction and sequencing of cDNA libraries from flowers and flower buds

The total RNAs were isolated from flowers and buds at each stage (stages 1–5) by using the SDS–phenol extraction method [13]. Aliquots of each RNA sample corresponding to stage 1–5 were mixed. A cDNA population enriched with full-length cDNA clones was prepared by using a commercial service (TOYOBO, Japan) according to the oligo-capping method [14]. The resulting cDNAs were size-fractionated into two groups, namely long cDNA fraction (more than 3.0 kbp in length) and short fraction (1.5–3.0 kbp in length), followed by cloning unidirectionally into a pCMVFL3. pCMVFL3 was prepared by insertion of an EcoRI–DraIII-stuffer–DraIII–NotI fragment of pME18SFL3 (GenBank accession no. AB009864) into EcoRI–NotI site of pCMVSport6 (Invitrogen, USA). After transformation of *Escherichia coli* with the recombinant plasmids, two cDNA libraries were obtained; one enriched with long cDNA inserts and the other enriched with short cDNA inserts. The recombinant colonies were randomly picked, and their inserts were sequenced from the 5' end using a CEQ2000XL DNA sequencer (Beckman Coulter, USA). The chromatograms for sequences were processed to eliminate the low quality sequences. After trimming of vector and adaptor sequences, high quality ESTs with a minimum of 300 bases were selected.

2.3. Construction and sequencing of cDNA libraries from pollen and pollen tubes

The total RNAs were isolated from the pollen or pollen tube by the SDS–phenol method in combination with centrifugation in cesium chloride solution [13]. Poly(A)$^+$RNA was isolated from total RNA using a PolyATract mRNA isolation system (Promega, USA). Double-stranded cDNAs synthesized by using a cDNA synthesis kit (TAKARA BIO, Japan) were ligated with EcoRI/NotI adaptor (GE Healthcare, UK) and then they were inserted into the Lambda ZAP II vector (Stratagene, USA). The resulting phage library was converted to the plasmid form by mass excision according to the manufacturer's instructions. The 5' ends of randomly selected clones were sequenced by an ABI PRISM 377A DNA sequencer (Applied Biosystems, USA).

2.4. Sequence assignment into functional categories

A tBLASTx search was carried out to determine tentative annotations, which served as the primary source of *Petunia* sequence annotation. ESTs were clustered using a BLASTCLUST program and classified into contigs if they had more than 60% similarity. The *Petunia* ESTs that had a match with plant sequences (an expected value less than 1e-50 in the tBLASTx analysis) were classified according to the functional categorization described by the EU Arabidopsis Genome project [15].

2.5. Preparation of cDNA microarrays

The inserts of cDNA clones were PCR-amplified by using following primers; M13-M3-24 (5′-GTAAAACGACGGCCAGTGCCTAGC-3′) and M13-RV-24 (5′-CAGGAAACAGCTATGACCATTAGG-3′). The resulting products were purified and then spotted on a TaKaRa-Habble Slide Glass (TAKARA BIO) [16]. Therefore, the *Petunia* probes on microarrays harbored the sequence corresponding to the multicloning site of pCMVFL3.

2.6. Preparation of fluorescent cDNA targets, hybridization, scanning, and data analysis

We amplified the region corresponding to the multicloning site with flanking short sequence of pCMVFL3 by using a Cy3-conjugated primer (5′-Cy3-ATTAGGCCTATTTAGGTGACACTATAGAAC-3′) and an M13-M3-24 primer under the presence of Cy3-dCTP (GE Healthcare). PCR amplification was performed in 50-μl volumes containing 70 ng of pCMVSport6 plasmid DNA, 1× buffer (minus $MgCl_2$), 2.5 mM $MgCl_2$, 50 μM dATP, dGTP and dTTP, 45.5 μM dCTP, 4.5 μM Cy3-labeled dCTP, 0.5 μM of each primer, and 1 U of *Taq* polymerase (Bioneer Corp., Korea). The resultant PCR product was purified