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Plant Science 170 (2006) 417–425
PLANT SCIENCE
www.elsevier.com/locate/plantsci

Isolation of pollen early genes and analysis of expression pattern during the development of male gametophyte

Semarjit Shary [1], Ravindra Kumar, Sipra Guha-Mukherjee *

School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India

Received 9 October 2004; received in revised form 22 August 2005; accepted 30 August 2005
Available online 19 September 2005

Abstract

The early events of pollen development are critical for the differentiation of male gametophyte. However, little is known about the molecular events that are unique to the early stages of pollen development. This study of gene expression during the pollen development is focused on identifying the early genes in *Nicotiana tabacum* anthers and characterizing the expression and regulation of these genes. A subtraction cDNA library was prepared from the early stages of pollen development (from dyad till uninucleate stage) and 113 unique genes differentially expressed were identified. Searches of the *Nicotiana* and other non-redundant database disclosed known or likely functions for 73 genes while 40 genes were of unknown function. Among those with unknown function 24 found matches in EST database while 16 showed 'no hit' or were novel. Northern blot analysis of 16 randomly picked clones as probes confirmed their expression in early stages of pollen development and no expression or lower expression in later stages. The tissue-specific expression studies showed that among these, 12 genes are anther-specific. To the best of our knowledge, these groups of clones represent the largest collection of 'early genes' isolated so far.

Keywords: Microsporogenesis; Anther; Subtractive hybridization; Tobacco (*Nicotiana tabacum*)

1. Introduction

The morphological aspects of pollen development have been well studied but the knowledge about the underlying molecular processes is still limited. Pollen formation requires coordinated gene expression in the gametophytic cells and the sporophytic tissues surrounding it. Two periods of temporal gene expression were defined in pollen development by Marcarenhas [1]. 'Early genes' are activated soon after meiosis and their expression declines towards pollen maturation. These genes were thought to be important for initial development of pollen while 'late genes' are activated at the time of mitosis and their mRNA accumulates maximally at anthesis. Their transcripts function during pollen maturation and germination. There are relatively few reports of early genes [2–4] whereas late genes have been studied more extensively [5–12]. Additionally, there are also few reports of genes expressed in both periods of male organ development [13–16].

Until the past few years, there were few reports of anther-specific genes but with the advent of techniques like subtractive hybridization and microarrays, there had been major breakthrough in the study of pollen developmental genes. There are also reports describing genes from various plant species specifically expressed in anthers, pollen and pistils [11,17–19]. In a recent study in *Arabidopsis*, Honys et al. showed 13,977 male gametophyte-expressed mRNAs 9.7% of which is male-gametophyte-specific [12]. In another study, Hennig et al. identified 22,000 genes in flower and fruit development in *Arabidopisis* out of which 1886 genes were regulated during reproduction [20]. Honys et al. compared the transcriptome of the sporophyte throughout development with the pollen transcriptome and identified 992 pollen expressed mRNAs, nearly 40% of which were pollen-specific [21]. There are also reports of pollen developmental genes under various conditions like cold and hormonal stress [22,23]. But most of our knowledge about the function of anther-specific genes is restricted to particular developmental phase of pollen grain maturation or pollen tube growth. The main goal of this study was to identify gene programming prior to differentiation of male gametophyte

* Corresponding author at: Garden Estate MA1/6-3C, Gurgaon 122002, Haryana, India. Tel.: +91 95 1245060170; fax: +91 1126165886.
E-mail address: sipra75@rediffmail.com (S. Guha-Mukherjee).
[1] Present address: University of Wisconsin, USDA, Forest Products Laboratory, Madison, WI 53726, USA.

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doi:10.1016/j.plantsci.2005.08.023

through identification and characterization of genes specifically expressed during early stages of microsporogenesis. Studies on such genes may also provide methods for controlling the male fertility in economically important plants [24].

In this work, a subtractive library was prepared taking the early stages of pollen development as tester and later stages as driver. A total of 719 clones were isolated. After screening, 113 unique clones were obtained. We found that a major fraction of these genes (37%) is still not known and comprises novel and unclassified genes. In addition we studied the expression profile of mRNA corresponding to 16 clones and their putative functions. Amongst them, 12 were found to be anther-specific.

2. Material and methods

2.1. Plant material

Nicotiana tabacum var. Xanthi buds of size 6–45 mm in length were harvested from field grown plants. The bud length was measured from base to the tip of the outermost sepal. Anthers were dissected from approximately equal sized buds using fine forceps and immediately frozen in liquid nitrogen. The developmental stage of buds of predetermined length was assessed by light microscopic examination of sporogeneous cells. The buds were classified into seven stages according to the cytological examination and to bud length. Anthers were collected from each stage and used for RNA isolation.

2.2. Northern hybridization

Plant material used for mRNA isolation and further experiments was ground to a fine powder in mortar pestle cooled with liquid nitrogen. Total RNA from various tissues was isolated as described by Chomczynski and Sacchi [25]. Total RNA was extracted from all the seven stages of anthers, corresponding pistils and vegetative tissues—leaf, stem and root. The RNA (20 μg) samples from different tissues were electrophoresed on 1.5% agarose–formaldehyde gels and blot transferred onto hybond N nylon membrane (Amersham Pharmacia Biotech, UK). The membrane was hybridized with [α-^{32}P] dCTP-labelled probes at 65 °C for 18 h and washed twice under highly stringent conditions as described by Sambrook et al. [26]. The washed membrane was then exposed to X-ray film at −80 °C.

2.3. cDNA library construction and subtraction

Tester (bud stage 1–3) and driver (bud stage 5–7) double-stranded cDNA were prepared from 1 μg of total RNA using Clonetech's SMART PCR-synthesis kit. Subtraction was performed using PCR-Select cDNA subtractive hybridization kit according to manufacturer's protocol (Clonetech, USA). Tester cDNA was digested with *RsaI* at 37 °C for 1.5 h and then ligated to adaptors 1 and 2 in separate reactions at 16 °C overnight. After ligation, driver cDNA was added to each of the tester samples, which were subsequently resuspended in the hybridization buffer, heat-denatured, and then allowed to anneal at 68 °C for 8 h. Then, two samples from first hybridization were mixed together and fresh denatured driver cDNA was added to the sample followed by incubation at 68 °C overnight for the second hybridization. PCR amplification with two different nested primers was performed to amplify the differentially expressed cDNAs. The PCR products were inserted into the pGEM-T Easy vector (Promega, USA).

2.4. Differential screening

Seven hundred and nineteen clones were blotted onto hybond N nylon membrane (Amersham Pharmacia Biotech, UK) in duplicates and hybridized with the mRNA probe from both tester and driver under identical conditions according to the manufacturer's instructions.

2.5. Nucleotide sequencing and data analysis

The inserts were sequenced using Sp_6 andT_7 promoter primers which flank the multiple cloning site of pGEMT-easy vector. DNA sequencing was performed with an automatic DNA Sequencer (ABI prizm 3700) at National Center for Plant Genome Research, New Delhi, India according to manufacturer's instructions. DNA and predicted amino acid sequences were searched against DNA and protein databases using the BLAST programme available at NCBI.

3. Results

3.1. Isolation of cDNA expressed during microsporogenesis

Earlier cytological studies have shown that gametogenesis in a flower is synchronized in all the anthers and can be correlated to the bud length [27]. In the present study, anthers of *Nicotiana tabacum* were categorized into seven stages These were: stage 1—dyad, 6 mm; stage 2—tetrad, 8 mm; stage 3—uninucleate, 13 mm; stage 4—late uninucleate 20 mm; stage 5—binucleate, 26 mm; stage 6—late bi nucleate 42 mm, stage 7—mature pollen grain, 45 mm.

To isolate ''early'' genes, subtraction was performed taking RNA from stages 1, 2 and 3 as tester and stages 5, 6 and 7 as driver. Stage 4 (bud length 20 mm) was not taken to avoid any mixing of genes. The final subtracted cDNAs were cloned in pGEMT-Easy vector. A total of 719 clones were obtained. After dot blot, 299 clones were positive and confirmed, among which 176 clones were sequenced. To investigate the possible identity of these clones, the sequences were used to search the DNA database (NCBI). The sequences were also compared with each other to identify identical or overlapping clones. The size of clones ranged from 0.3 to 1.2 kb and was named as ''Pollen early genes'' or ''Peg'' clones.

Clones were defined redundant when they exhibited more than 95% identity over aligned regions or to the same database accession. Analysis revealed a redundancy of 35% as 113 unique clones were obtained. Of the 176 sequences, 88 were represented once, 14 twice, 6 thrice, 2 four times and 4 more than five times