Genes Expressed in the Male Gametophyte of Flowering Plants and Their Isolation

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ABSTRACT

Recombinant cDNA libraries to poly(A)RNA isolated from mature pollen of Zea mays and Tradescantia paludosa have been constructed. Northern blot analyses indicate that several of the clones are unique to pollen and are not expressed in vegetative tissues. The majorit, however, are expressed both in pollen and vegetative tissues. Southern hybridizations show that the pollen specific sequences in corn are present in one or a very few copies in the genome. By using several of the clones as probes, it was found that there are at least two different groups of mRNAs with respect to their synthesis. The mRNAs of the first group represented by the pollen specific clones are synthesized after microspore mitosis and increase in concentration up to maturity. The second group, exemplified by actin mRNA, begins to accumulate soon after meiosis, reaches its maximum by late pollen interphase, and decreases thereafter. Although the actin mRNA and the pollen specific mRNAs studied show very different patterns of initiation of synthesis and accumulation during pollen development, the rates of decline of these mRNAs during the first 60 minutes of germination and pollen tube growth in Tradescantia are similar and reflect the previously observed declines in rates of protein synthesis during this period.

The pollen grains of Tradescantia paludosa (16), corn (17), and tobacco (30) contain a store of presynthesized messenger RNAs (mRNAs) at the time of their release from the anther. These mRNAs have been shown to code in cell-free translation systems for proteins that are similar to the proteins synthesized during pollen germination and tube growth (9, 17). In several pollens that have been studied these presynthesized mRNAs appear to have functions during germination and early pollen tube growth (14).

Analyses have been carried out on the kinetics of hybridization (RNA dot analysis) of radioactively labeled complementary DNAs (cDNAs) made to poly(A)RNA from mature Tradescantia pollen and hybridized to unlabeled poly(A)RNA in excess, from pollen (31). These studies have shown that the mRNAs present in the mature pollen grain are the products of approximately 20,000 different genes. In contrast, the mRNAs present in vegetative shoots are the products of about 30,000 different genes. The pollen-contained sequences are, in general, much more abundant than the mRNAs from the vegetative shoot. Heterologous hybridizations (i.e. [3H]cDNA from pollen or vegetative shoots and poly(A)RNA from vegetative tissue or pollen) have shown that a large fraction (>64%) of the genes expressed in pollen are also expressed in vegetative tissues, whereas no more than 60% of the genes expressed in shoots are similar to those expressed in pollen (31). Similar hybridizations have been carried out with RNA from corn pollen and the results are similar to those obtained with Tradescantia (RP Willing, JP Mascarenhas, unpublished data). To further our knowledge of pollen development it is essential that the genes that are expressed in pollen, especially those unique to pollen, be isolated and characterized in some detail in order to understand their developmentally specific regulation and functions. We report here the construction of two cDNA libraries made to pollen mRNAs from Tradescantia and corn, and the utilization of some of the clones to answer questions about the nature of the pollen expressed genes and their transcription during pollen development and tube growth.

MATERIALS AND METHODS

Plant Material. Tradescantia paludosa L. plants were grown in the greenhouse and pollen was collected and stored as previously described (15). Corn (Zea mays L.) pollen was collected from field grown plants of the cultivar 'Gold Cup' (Harris Seeds, Rochester, NY). Pollen was quick frozen in liquid N2 and stored at −70°C. For later experiments the imbed line of corn W-22 (Illinois Foundation Seeds) was used. Various vegetative and reproductive tissues from Tradescantia and corn plants or seedlings were collected. These were immediately frozen in liquid N2 and stored at −70°C until used for RNA isolation.

Isolation of RNA. Total RNA was isolated from mature pollen and from roots, shoots, and other portions of the plant as described earlier (17, 31). Poly(A)RNA was isolated from total RNA utilizing oligo(dT) cellulose as described (9), and the RNA stored in 70% ethanol at −20°C.

Isolation of Pollen of Different Developmental Stages. Slightly different procedures were used to collect gametophytic material for T. paludosa and corn. Spikelet or bud size were initially used as an approximate index of developmental stage. In the morning, buds of T. paludosa and tassels of corn of the desired size were collected from greenhouse or field grown plants, respectively. In developing corn tassels, the spikelets in the middle of the spike are the most mature and there is a gradation of development that extends in either direction from this point. Generally, each third of the spike contains spikelets in approximately the same stage of development (Dr. Ercole Ottaviano, personal communication). Within each spikelet there are two florets each containing 3 anthers. The two florets are at slightly different stages of development but the anthers in each floret are at the same stage (4). Tassels were dissected from the plant prior to emergence for the early stages and after emergence for the later stages. At intervals along the spike, spikelets were opened with a scalpel and one anther from each floret was squashed and stained using

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Evans's Blue (25) to verify its developmental stage. Anthers of the same developmental stage were pooled in a 6% sucrose solution on ice. In Tradescantia the six anthers from each bud are at the same developmental stage (19). One anther from each bud was stained with methyl green (0.4% w/v) in 6% acetic acid and 3 mM CaCl₂, and the remaining anthers were then pooled on ice in pollen medium I (12).

When 100 to 200 anthers of the same stage had been collected the anthers were ruptured with a loose fitting hand homogenizer, releasing the developing microspores. The suspension was filtered through nylon mesh (40–68 μm for Tradescantia and 68–200 μm for maize) to separate most of the sporophytic material from the gametophytic. The filtrate was centrifuged at low speed (approximately 100g) in a Clay Adams table top centrifuge for 3 min to sediment the pollen material. Figure 1, A to C, is a representative photograph of the results of this isolation procedure on samples of tetrad, microspore mitosis, and early pollen interphase stages of Tradescantia pollen development. Similar results were obtained with corn. Male gametophyte material was collected, frozen in liquid N₂, and stored at −70°C until required for the extraction of RNA.

Pollen of Tradescantia was germinated and grown as described (12). At the end of 30 and 60 min, pollen tubes were sedimented and used for the extraction of RNA.

Construction of cDNA Libraries to Pollen mRNAs. The cDNA libraries made to poly(A)RNA from mature pollen of T. paludosa and corn were constructed in a modified Okayama-Berg type plasmid (1). The dimer-primer vector pBD1 was a gift of D. C. Alexander and B. G. Williams and was identical to pARC7 (1) with the exception that it did not contain the polylinker fragment. The procedures used for cloning were essentially those described by Alexander et al. (1). Each of the libraries consists of several thousand clones initially selected on the basis of colony hybridizations (13) to ³²P-labeled cDNAs made to poly(A)RNA from pollen. The same colony filters were stripped of probe and hybridized to ³²P-labeled cDNA made to poly(A)RNA from vegetative tissues to initially screen for pollen specificity.

Isolation of Plasmids. Small scale plasmid isolation was done by the procedure of Holmes and Quigley (11). Large amounts of plasmid DNA were prepared after amplification in M-9 medium (6, 13) and purification of plasmid DNA by cesium chloride-ethidium bromide centrifugation (8). Radioactive DNA probes for hybridization were prepared by nick translation as described by Maniatis et al. (13), using the buffer system of Bingham and Judd (2) and deoxyctydine 5′-triphosphate [³²P] (New England Nuclear, 3000 Ci/mmol).

Analysis of RNA, Northern, and Dot Blot Hybridizations. Total RNA was electrophoretically separated in a 1.5% agarose gel containing 2.2 M formaldehyde (10) and transferred to nitrocellulose (29) or to Gene Screen, according to the manufacturer's instructions (New England Nuclear; Instruction Manual 1982). The filters were prehybridized for 24 h at 65°C in heat-sealed plastic bags containing 2× SET (1×: 0.15 M NaCl, 0.1 M NaH₂PO₄, 0.1 mM EDTA, 10 mM Tris-HCl [pH 7.0]), and 5× Denhardt’s solution. The prehybridization solution was replaced with an identical solution containing 3–5 × 10⁵ cpm/ml (approximately 10 ng/ml) of [³²P]labeled denatured probe DNA and 10% (w/v) dextran sulfate. Hybridization was for 24 h at 65°C. The filters were then washed in four changes of 30 min each in 2× SET followed by one wash in 0.5× SET, 0.25% SDS for 3 to 4 h at 65°C. The filters were exposed to Kodak XAR-5 film with an intensifying screen at −70°C. The dot blot analyses were carried out by the procedure of Cheley and Anderson (5). With the soybean actin plasmid pSaC3, hybridizations were carried out in 6× SET at 60°C. The filters were washed in 8× SET, 7× SET and finally in 6× SET at 60°C. The Gene Screen filters were stripped of probe for rehybridization as described in the supplier's manual.

Southern Hybridizations. Total DNA (3), isolated from nuclear enriched preparations of maize (W22) embryos dissected from seeds was digested with different restriction endonucleases and electrophoretically separated in a 0.7% agarose gel in a PE running buffer system (7). The DNA was then transferred to Gene Screen Plus and hybridized according to the supplier’s instructions (New England Nuclear).

Hybrid Selection of mRNA and Cell Free Translation. Solid state hybrid selection of mRNA was by the procedure of Paterson and Roberts (21). The hybrid selected mRNA was translated in the reticulocyte cell free system with [³⁵S]methionine and the products analyzed by electrophoresis and autoradiography as described earlier (17).

Restriction endonucleases and other enzymes were purchased from International Biotechnologies Inc., New Haven, CT, or Bethesda Research Laboratories, Gaithersburg, MD.

RESULTS

Description of Clones. About 75 clones from the T. paludosa library and 100 from the corn library were selected for further screening by colony hybridization to [³²P]cDNAs prepared to pollen poly(A)RNAs from the appropriate species. In addition, preliminary screening for sequences specific to pollen was done by sequential hybridization of colony filters to [³²P]cDNAs prepared from pollen, root, and shoot poly(A)RNA. Based on the intensity of the hybridization signals (data not presented) there are several different groups of clones. Some clones are expressed in abundance or in low amounts in pollen but not at all in vegetative tissues. Some are expressed in abundance in both pollen and vegetative tissues. Others are expressed in abundance in pollen but in low amounts in vegetative tissues, and still others
in low amounts in pollen but in abundance in vegetative tissues. The insert size varies from about 50 ntp to about 2000 ntp for the different clones. The clones from *Tradescantia* are labeled with the prefix pTpc, and those from corn with pZmc.

Several of the clones from both *Tradescantia* and corn were selected for further study. The pollen specificity of several clones was confirmed by Northern and dot blot hybridizations to RNAs from pollen and various other tissues. The clone, pTpc70, is an example of a pollen specific clone that hybridizes to pollen RNA of 620 nucleotides (nt), but does not hybridize to RNAs from vegetative shoots or roots (Fig. 2). The clone pZmc26 from corn which hybridizes to a mRNA of 1600 nt, is also pollen specific (Fig. 2). The colony hybridizations indicate that about 20% of the *T. paludosa* clones and about 10% of the corn clones are pollen specific. Most of the clones in the two libraries however, are expressed both in pollen and in vegetative tissues. Examples of such clones are pZmc46 and pTpc46 (Fig. 3). The clones that are expressed in both pollen and vegetative tissues are not necessarily expressed to the same extent in the different tissues. For example, pZmc46 is expressed more abundantly in roots and silks than it is in pollen or shoots, whereas, pTpc46 is expressed most abundantly in pollen, to a lesser extent in shoots, and to a very low extent, if any, in roots (Fig. 3).

The clones represent cDNA copies of mRNAs as shown by hybrid selection of mRNAs and cell free translation. As an example, clone pTpc44 hybrid selects for a mRNA from pollen that codes for a protein of 37kD (Fig. 4).

**Are the Genes Expressed in Pollen, Members of Large or Small Families?** To determine if the genes expressed in corn pollen are present in the genome as members of large families of genes or, as single or a very few genes, Southern hybridizations were carried out to restriction endonuclease digested nuclear DNA. The results with two clones pZmc13 and pZmc26, which are both pollen specific, indicate that the pollen specific genes are present in one or a very few copies in the genome (Figs. 5 and 6). The clone pZmc26 hybridizes to four bands with EcoR1 digested DNA (Fig. 6). If the digestion time is increased, however, i.e. when the digestion is complete, it hybridizes to only two bands (data not shown). Southern analyses with several other
Fig. 6. Southern hybridization of restriction endonuclease digested corn genomic DNA (20 μg per lane) to the pollen specific clone pZmc26. Lane 1, DNA size markers in ntp; Lanes 2 and 3, EcoRI and HindIII digests, respectively.

Fig. 7. Diagrammatic representation of the various stages of microsporogenesis in Tradescantia including the time required for completion of the stages.

clones show the same copy number of the genes in the genome (data not presented).

Stages During Microsporogenesis when the Transcription of the Pollen Sequences Occurs and the Pattern of Accumulation of the mRNAs in the Pollen Grain. The different stages of microsporogenesis in Tradescantia are diagrammatically represented in Figure 7. There is at present no information concerning the stage of pollen development during which the synthesis is initiated of the mRNAs found in the mature pollen grain. Neither is there any information about the pattern of their accumulation during microsporogenesis. To answer these questions two pollen specific clones (pTpc44 and pTpc70) from Tradescantia were used as probes in Northern blot hybridizations to RNA isolated from microspores and pollen at various stages of development beginning with tetrads formed soon after meiosis. Large numbers of pollen grains had to be isolated at each of several developmental stages to obtain sufficient RNA for the Northern analyses. This was accomplished by procedures described in the “Materials and Methods” section. The clones pTpc44 and pTpc70 hybridize to mRNAs of 2000 and 620 nt, respectively, from pollen. The nitrocellulose membrane containing the electrophoretically separated RNAs from the different pollen stages was hybridized simultaneously to both the probes (Fig. 8). The mRNAs complementary to both the pollen specific probes are first detectable after microspore mitosis during early pollen interphase, and they continue to accumulate thereafter, reaching a maximum concentration in the mature pollen grain. The nitrocellulose membrane

Fig. 8. Northern analysis of 5 μg of total RNA from Tradescantia pollen at different stages of development hybridized simultaneously to both pTpc44 and pTpc70 which hybridize to mRNAs from pollen of 2000 and 620 nt, respectively. T, tetrad; MI, microspores during microspore interphase; MM, microspores in microspore mitosis; PT-1 and PT-2, pollen at early and late pollen interphase, respectively; MP, mature pollen.

Fig. 9. Northern analysis of 5 μg of total RNA from Tradescantia pollen at different stages of development hybridized to actin clone pSAc3. The Gene Screen membrane used in Figure 8 was stripped off the two probes pTpc44 and pTpc70 and then hybridized to pSAc3 under reduced conditions of stringency as described under “Materials and Methods.” See Figure 8 for legends to the lanes. Last lane, pBR322 cleaved size markers.

was then stripped of the two probes, pTpc44 and pTpc70, and rehybridized to an actin probe at a lower stringency. The plasmid pSAc3 is a soybean genomic actin clone (24, kindly provided by Richard Meagher), that hybridizes to a mRNA of 1750 nt from
Fig. 10. Northern analysis of 5 μg of total RNA from corn pollen at different stages of development using the pollen specific plasmid pZmc30 as a probe. pZmc30 hybridizes to a mRNA of 1700 nt in size. MM, Microspores undergoing microspore mitosis; PI-1 to PI-4, immature pollen grains at different stages of maturity from early through late pollen interphase, respectively; MP, mature pollen at anthesis.

Fig. 11. Changes in levels of actin mRNA during T. paludosa pollen germination and tube growth. Dot blot analysis of different amounts of total RNA (0.5, 1.0, and 5.0 μg) from ungerminated pollen (0), and pollen grown in culture for 30 and 60 min and hybridized to pSAc3.

T. paludosa pollen. In contrast to the pollen-specific mRNAs, actin mRNA is first seen during microspore interphase prior to microspore mitosis (Fig. 9). It accumulates thereafter, reaching a maximum at late pollen interphase and shows a substantial decrease in the mature pollen grain. A similar pattern of accumulation of mRNAs has been found for several other pollen expressed clones from T. paludosa (data not presented) and also from corn (Fig. 10).

Fate of mRNAs during Pollen Germination. The mature pollen grain of T. paludosa contains approximately 6 × 10⁶ molecules of poly(A)RNA. Within the first 30 min of germination and pollen tube growth in culture there is an almost 50% decrease in poly(A) content of the tube. This decrease continues at a lower rate during the subsequent 30 min of culture (16). It was of interest to determine whether the reduction in the levels of poly(A) during pollen germination and early tube growth represented a degradation of only the poly(A) tail of the mRNA or whether it reflected the turnover of entire mRNA molecules. The changes in specific mRNA levels during in vitro pollen germination and tube growth were determined for the actin mRNA and the two pollen specific mRNAs complementary to the probes pTpc44 and pTpc70. When pollen is placed in a growth medium germination occurs within less than 10 min. There is a dramatic decline in the level of actin mRNA after the first 30 min of tube growth (Fig. 11). The decline in the mRNA level continues during the subsequent 30 min period. The mRNAs complementary to the plasmids pTpc44 and pTpc70 show the same pattern of change during germination and pollen tube growth as the actin mRNA (Figs. 12 and 13). The dot blots for pTpc44 and actin were scanned in a densitometer to arrive at the approximate levels of the mRNAs. The mRNA levels decrease to about 60 and 33% of what they were in the ungerminated pollen after 30 and 60 min, respectively. Similar results were found with several other clones (data not presented).

DISCUSSION

The two cDNA libraries that have been constructed represent a collection of sequences expressed during pollen development. Only a few of the sequences appear to be unique to pollen, whereas the great majority are expressed both in pollen and in...
GENES EXPRESSED IN MALE GAMETOPHYTE

various vegetative tissues. The estimates of pollen specific sequences are about 20 and 10% of the total sequences expressed in pollen of Tragopogon and corn, respectively, based on colony hybridizations to [32P]DNA from pollen and vegetative tissues. Nucleic acid solution hybridizations indicate that greater than 64% of the mRNAs in pollen are also found in vegetative tissues, i.e. less than 36% are pollen specific (31). The lower estimate with colony hybridizations could be because the cloning procedure probably selected for more abundant mRNAs and it is possible that the pollen specific sequences might constitute a larger fraction of the rarer mRNAs. Extensive overlap of sporophytic and gametophytic gene expression was also found by Tanskley et al. (28) in tomato based on isozyme profiles, with as many as 95% of the pollen isozymes also found in one or more of the several vegetative studies. A similar study in corn has shown that about 72% of the isozymes are expressed both in pollen and the sporophyte, whereas only about 6% of the isozymes studied were pollen specific (22).

The Northern analyses showing that the pollen specific sequences are first detectable after microspore cleavage provide direct evidence for the haploid transcription of these mRNAs which was inferred earlier from evidence of genetic segregation of specific enzyme activity in mature pollen grains (18, 20, 23). The results of these analyses also indicate that there are at least two different groups of mRNAs in pollen with respect to their synthesis during pollen development. The first group, represented by the pollen specific clones, are synthesized after microspore mitosis and increase in concentration up to maturity. This pattern of accumulation would seem to suggest a major function for these mRNAs during pollen germination and early tube growth. The second group which would include mRNAs like the actin mRNA, which begin to accumulate soon after meiosis, reach their maximum by late pollen interphase and then decrease substantially. Alcohol dehydrogenase (ADH) is probably another example of a gene that is expressed in a similar fashion to that of actin. The appearance and pattern of increase in ADH enzyme activity during microsporogenesis in corn (27), and that of β-galactosidase in Brassica campestris (26), are what would be expected if their mRNAs were synthesized in a manner similar to that of the actin mRNA seen in Tradescantia.

Although the actin mRNA and the pollen specific mRNAs represented by the clones pTPc44 and pPCP70 show quite different patterns of initiation and accumulation during pollen development, the rates of decline of these mRNAs during the first 60 min of germination and pollen tube growth are very similar. The proteins synthesized during pollen tube growth on premature and newly synthesized mRNAs appear to be the same (16). Hence, these results would indicate that the rate of degradation of the mRNAs during germination and pollen tube growth exceeds that of their synthesis. The reduction in the levels of the three mRNAs correlates well with the previously reported declines in poly(A)(16), suggesting that the entire mRNA molecules turn over, not just the poly(A) tails. The degradation of mRNAs during pollen tube growth appears to be a nonspecific degradation of all mRNAs existing in the pollen grain, and there do not appear to be classes of mRNAs that differ in their rates of turnover. The significance of this rapid decrease in mRNA content at a time when the pollen tube continues to grow at a linear rate is not clear at the present time. The decrease in mRNA content of the pollen tube correlates with the observed rates of protein synthesis. The rate of protein synthesis is at a maximum during the first 15 min of tube growth, decreases thereafter, and is only 20% of the initial rate after 1 h (16).

We are currently isolating genomic clones specific to pollen with the aim of characterizing these genes and understanding their male gametophyte specific expression.

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