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Short Communication

Analysis of the Complexity and Diversity of mRNAs from Pollen and Shoots of *Tradescantia*[1]

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ABSTRACT

The mRNAs of the mature pollen grain of *Tradescantia paludosa* at anesthesia and of vegetative shoots have been compared by analyzing the kinetics of hybridization between homologous and heterologous reactions of cDNA to poly(A)RNA in excess. The mRNAs in pollen can be divided into three abundance classes with complexities of 5.2×10^4, 1.6×10^6, and 2.1×10^7 nucleotides. The three classes are made up of sequences that constitute 15, 60, and 24% of the mRNAs and each sequence is present on an average at 26,000, 3,400, and 100 copies, respectively, per pollen grain. About 20,000 different genes are expressed in pollen as compared to about 30,000 in vegetative shoots. Estimates have been made of pollen mRNA sequences shared with those of shoot tissue and of shoot sequences common to those in pollen.

The role of the male gametophyte is the production of two sperm cells and their transport via a tube, the pollen tube, through the tissues of the style, and into the embryo sac where double fertilization occurs. The male gametophyte when fully developed is a very simple structure consisting of only three cells, a vegetative cell that makes up most of the pollen grain and tube, and two sperm cells that lie within the cytoplasm of the vegetative cell.

The mature pollen grain contains mRNAs that were synthesized during its development and that are utilized during pollen germination and early pollen tube growth (5, 13, 19). The complexity of the mRNA, *i.e.* the number of genes expressed in the pollen grain and the degree of overlap with genes expressed in vegetative tissues, remains, however, largely unknown.

In the present study we have analyzed the kinetics of hybridization of poly(A)RNA to cDNA under conditions of mRNA excess to compare the complexity, number of abundance classes, and extent of shared sequences in the mRNA of mature pollen and young vegetative shoots.

MATERIALS AND METHODS

Plant Material. *Tradescantia paludosa* L. plants were grown in the green house and pollen was collected and stored as described previously (12). The terminal 5 cm of pale green vegetative shoots were excised, the leaf blades discarded, and the shoots with leaf sheaths were immediately frozen in liquid nitrogen and stored at −70°C until used.

Isolation of poly(A)RNA. RNA was isolated from mature pollen as described earlier (5). Because of high nuclease activity in shoots, an initial extraction in guanidine-HCl was found to be necessary (16), followed by extraction with phenol-chloroform-isoamyl alcohol (5). Poly(A)RNA was isolated from total RNA utilizing oligo(dT) cellulose as described (5) and the RNA stored in 70% ethanol at −20°C.

Determination of Poly(A) Tract Length, Poly(A)RNA Length, and Quantitation of Poly(A)RNA. The length of the poly(A) tract was measured by removal of the nonpoly(A) portion of the RNA by digestion with RNase A and RNase T_1, 3′ end labeling of the poly(A) with 3′-deoxyadenosine 5′-triphosphate, [α^{32}P]- with RNA ligase (4) and enumeration of the bands following polyacrylamide gel electrophoresis (1). The size of mRNAs was determined by formamide-sucrose gradient (5 to 20%) sedimentation analysis (3). The RNA in each gradient fraction was precipitated with 2 volumes of ethanol after adding 5 μg *Escherichia coli* tRNA as carrier and sodium acetate to a concentration of 0.2 M. The pelleted RNA in each tube was hybridized to [^{3}H] poly(U) (233 Ci/mmol P) as described earlier (13). The amount of poly(A) per fraction was calculated from a standard curve of known poly(A)54 concentrations run in parallel. To determine poly(A)RNA concentrations for hybridization experiments, the poly(A) content measured by hybridization to [^{3}H]poly(U) was multiplied by 15 (ratio of number average size of poly(A)RNA-to-poly(A) tract length).

Preparation and Sizing of cDNA. [^{3}H]cDNA was prepared from pollen or shoot poly(A)RNA with avian myeloblastosis virus reverse transcriptase as outlined by Maniatis *et al.* (11) using [^{3}H]-dCTP and [^{3}H]-dGTP (ICN; 23 and 16 Ci/mmol, respectively). For sizing, [^{3}H]cDNA was dissolved in 50% formamide, 25 M sodium citrate (pH 3.5) heated to 65°C for 5 min, and analyzed by electrophoresis along with size standards in a 1.5% agarose gel made up in 6 M urea-25 mM sodium citrate buffer pH 3.5 and counting the radioactivity in successive gel slices.

Hybridization of RNA and [^{3}H]cDNA and Analysis of the Data. RNA and cDNA were denatured for 3 min at 110°C and incubated to different RNA Cot values in 30 mM Pipes buffer, pH 6.7, 1 M NaCl, 1 mM EDTA, 0.1% SDS at 68°C. All reactions contained poly(A)RNA at a concentration of 0.0013 M in nucleotides and a poly(A)RNA/cDNA ratio of 500:1. Two μl of the reaction mix was sealed in a 40-μl capillary tube along with 2 to 10 glass beads. The samples were mixed during incubation (20). RNA Cot values for the 1 M NaCl solution were corrected to the standard 0.18 M salt conditions by multiplying by 2.9 (20). Reactions were terminated by freezing in dry ice. The fraction of [^{3}H]cDNA resistant to S-1 nuclease was determined by the DE-81 filter disc assay (7, 14). The discs were treated with 0.5

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[2] Abbreviation Cot,

ml of 90% NCS (Amersham/Searle) and counted in 5 ml of Betafluor (National Diagnostics). A least squares computer program (17) was used to analyze the kinetics of hybridization and to fit the data to a plot. Complexity of poly(A)RNA was determined by comparison to the hybridization kinetics of a known standard, globin mRNA. A mixture of α- and β-globin mRNA (gift of C. Baglioni) hybridized to its own cDNA with a rate constant of 1172 $M^{-1}s^{-1}$ under our experimental conditions. The complexity of mRNA classes was estimated by the relationship: $(C = [Kg \times Cg]/K)$ where C is the complexity in nucleotides, Kg is the rate constant for globin (1172 $M^{-1}s^{-1}$), Cg is the complexity of globin (1200 nucleotides), and K is the experimentally derived rate constant for the hybridization of a given abundance class calculated from the computer program (17). Our cDNAs were longer than 400 nucleotides, hence, no correction factor for length was necessary (20).

RESULTS AND DISCUSSION

Characterization of RNA. RNA isolated from ungerminated pollen was analyzed on a formamide-sucrose gradient and the gradient fractions hybridized to [^{3}H]poly(U) to determine the size of the poly(A)RNA (data not presented). The poly(A)RNA sediments over a range of S values with a peak at 11S. The number average size of the poly(A)RNA was calculated to be 1260 nucleotides, similar to values for other plant materials (6, 8). Poly(A) tracts were isolated from pollen RNA by RNase A and T1 digestion followed by end labeling and enumeration of bands after gel electrophoresis. These results showed the number average size of the poly(A) to be 85 with a range in sizes from about 60 to greater than 120. These values are very similar to those found for cotton cotyledons (6).

cDNA-RNA Hybridization Kinetics. A cDNA probe reflects the overall abundance of RNA transcripts in the cell type from which the poly(A)RNA was originally isolated (2). An estimate of the complexity of the expressed mRNAs can be obtained from the kinetics of hybridizaiton of cDNA-RNA under conditions of mRNA excess (2, 7). In addition, this approach has been used to compare mRNA populations in different animal and plant tissues (8–10).

For the hybridizations, we have used total cellular poly(A)RNA which might be expected to contain both polysomal mRNAs as well as nuclear RNAs that may or may not be translated and that could be expected to have a greater complexity than the polysomal mRNAs (10). This is justified for pollen because the pollen grain at anthesis is dehydrated and no RNA or protein synthesis is occurring. Total cell poly(A)RNA was used from vegetative tissue because cDNA made from total cell poly(A)RNA from cotton was found to hybridize with virtually identical kinetics to either total cell poly(A)RNA or polysomal poly(A)RNA (6).

Complementary DNAs synthesized from pollen poly(A)RNA and from shoot poly(A)RNA were each hybridized to homologous or heterologous poly(A)RNA. The kinetics of these hybridizations are shown in Figure 1 and summarized in Table I. Both homologous hybridizations, *i.e.* pollen cDNA to pollen poly(A)RNA and shoot cDNA to shoot poly(A)RNA, extend over 4 to 5 log units of Cot, indicating that there is a wide range in abundance of the different mRNA classes (2). When hybridization occurs over several orders of magnitude of Cot, the RNAs can be divided into a number of abundance classes and the kinetics of each class can be analyzed by the least squares computer program (17). For pollen there was a reduction in the error term when the number of components was increased from two to three (0.030–0.024), and no change for four components. Thus, three abundance classes best describes the data for pollen. For the shoot data there was virtually no difference in the error term between two and three components, so two components is probably the best description of the data. The assignment of two classes to shoot and three to pollen RNA may be somewhat arbitrary in the sense that each class probably does contain sequences with a spectrum of abundances.

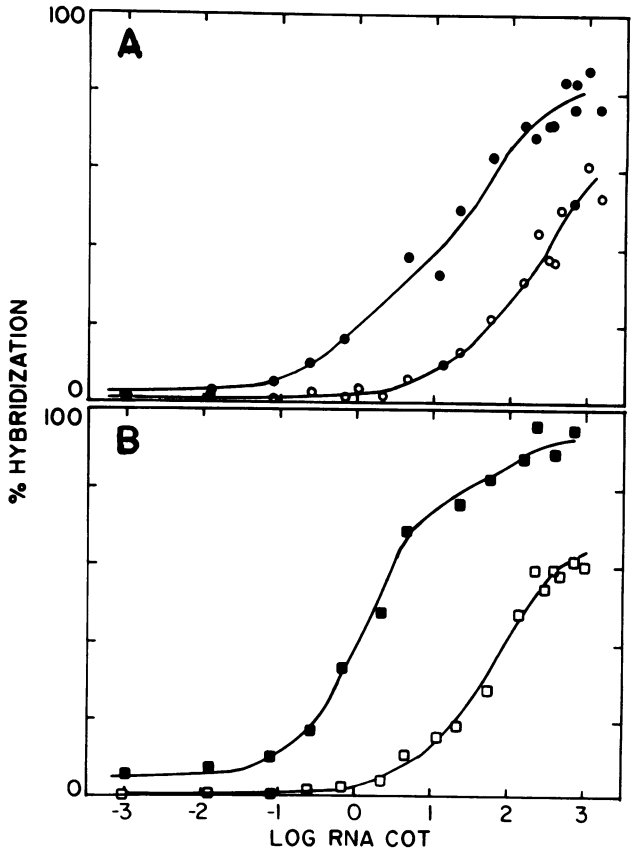

FIG. 1. Hybridization of [^{3}H]cDNAs with various poly(A)RNA fractions. A, cDNA synthesized using poly(A)RNA from shoots was hybridized to shoot RNA (●) or to pollen (○). B, cDNA synthesized to poly(A)RNA from pollen was hybridized to pollen RNA (■), or to shoot RNA (□).

As seen from Figure 1 and Table I, in pollen 15% of the mRNAs is fairly abundant and comprises about 40 diverse sequences each present in 2,600 copies per pollen grain. The major fraction of the mRNAs (60%) is made up of about 1,400 different sequences present in 3,400 copies per grain. The least abundant fraction is relatively small (24%) compared to the vegetative shoot (64%) but consists of 18,000 different sequences each present in about 100 copies per grain. Thus, in pollen about 75% of the mRNAs occur in the two more abundant frequency classes, whereas only about 35% of the shoot mRNAs are abundant. The least abundant shoot mRNAs consist of 29,000 sequences each present in 5 to 10 copies per cell. It is of interest that the least abundant fraction in pollen contains sequences that are much more abundant (100 copies) than in the corresponding fraction in shoots (5–10 copies). What relevance this has to the unique nature of development of the male gametophyte is unclear at present.

The total complexity of pollen poly(A)RNA is 2.3×10^7 nucleotides corresponding to about 20,000 diverse sequences, whereas in shoots it is about 3.4×10^7 nucleotides comprising about 30,000 different sequences. The complexity and number of diverse sequences found in *Tradescantia* shoot tissue is similar to values found in vegetative tissues of other plants (10). There are only about 66% as many diverse sequences in pollen as are present in shoot tissue.

Both heterologous hybridizations, *i.e.* pollen cDNA to shoot

Table I. *Summary of Homologous cDNA-RNA Hybridizations with Pollen and Shoot Poly(A)RNAs*

The homologous hybridization reactions in Figure 1 were fit by computer as described in the text. Values of 5.1 pg (Ref. 13) and 0.13 pg of poly(A)RNA per pollen grain and shoot cell respectively were used.

Tissue	Component	F[a]	Kp[b]	Complexity[c] (nucleotides)	Number of Diverse Messages[d]	Number of Copies per Cell per Sequence[e]
			$M^{-1} \cdot s^{-1}$			
Pollen	1	0.15	26.8	5.2×10^4	44	26,000
	2	0.61	0.86	1.6×10^6	1,400	3,400
	3	0.24	0.066	2.1×10^7	18,000	100
Shoot	1	0.35	4.22	3.3×10^5	280	250–500
	2	0.64	0.041	3.4×10^7	29,000	5–10

[a] Fraction of the total poly(A)RNA, determined by the fraction of reacting cDNA (computer derived) and normalized to 100%.

[b] K pure, the pseudo first order rate constant for a given component, derived by computer.

[c] Number of nucleotides summed for all diverse sequences of given class. See text for calculation from K.

[d] Complexity of abundance class (number average poly(A)RNA length − poly(A)tract length).

[e] $F \times (g$ poly(A)RNA·cell$^{-1}) \times (6 \times 10^{23}$ nucleotides mol$^{-1}) \div$ (Complexity of abundance class $\times$ 339 g mol^{-1} nucleotides).

poly(A)RNA (Fig. 1B) and shoot cDNA to pollen RNA (Fig. 1A) show minimal hybridization by log Cot values below 0.5 to 1.0. This would indicate that the mRNAs present in high or intermediate abundance are different in pollen and shoots. It is, however, possible that these sequences might be present in the corresponding tissue but in very much lower abundance and would not be detected by the type of hybridization that has been carried out.

The hybridization of pollen cDNA to shoot RNA (Fig. 1B) appears to have reached completion by a log Cot of 3 and with about 64% of the pollen cDNA being hybridized to the shoot RNA. In the reciprocal hybridization of shoot cDNA to pollen poly(A)RNA, completion appears to have been reached with about 60% hybridization (Fig. 1A). The simplest, although not necessarily the most correct, explanation for these results is that about 64% of sequences in pollen are similar to those in shoots and about 60% of shoot mRNAs are similar to those from pollen. Since the complexity of pollen RNA is about 66% of that of shoot RNA (Table I), one would expect at a maximum, if all pollen sequences are expressed in shoots, no more than 66% of shoot cDNAs to hybridize to pollen RNA. Thus, the estimate of 60% might be high and partly caused by the conditions of the annealing reactions which are not stringent enough to prevent the cross-hybridization of sequences that are similar but not identical, such as might occur between members of families of genes.

The estimate of pollen sequences that are shared with shoots could very likely be an underestimate because pollen RNA contains a much larger fraction of more abundant sequences than shoot RNA (Table I). Thus, in the heterologous hybridization between pollen cDNA and shoot(A)RNA sequences present in large abundance might saturate complementary shoot RNAs that are much less abundant. This would result in an underestimate of shared sequences because of a deficit of shoot RNAs to fully hybridize with the large number of copies of individual pollen cDNA sequences. The heterologous hybridizations tell us, however, that a minimum of at least 64% of the pollen sequences are expressed in shoots and a maximum of about 60% of shoot mRNAs are also expressed in pollen. We have recently isolated a library of cDNA clones made to pollen poly(A)RNA. Some of these clones are expressed only in pollen, others in both pollen and vegetative tissue. We are presently using these clones to more accurately characterize the common and diverse mRNA sequences with respect to their presence and abundance in pollen and different vegetative tissues of *Tradescantia*.

Based on isozyme profiles, Tanksley *et al.* (18) found that 60% of the isozymes in vegetative tissues were also found in pollen, whereas 18 of 19 pollen isozymes or 95% were also found in one or more of the vegetative tissues studied. Our results cannot be compared directly with the tomato study because we have not hybridized RNAs from roots, leaves, seeds, etc. One might expect the extent of shared sequences between pollen and the entire sporophyte to be higher than for that of pollen and shoot tissue.

Our results lend support to a recent proposal (15) that genetic selection operating during male gametophyte growth in the style could have a positive effect on the sporophyte resulting from this selection if a sizeable number of genes were expressed during both stages. Such positive correlations have been found between pollen tube growth and sporophytic traits (15). The genetic program expressesd during pollen development is extensive (20,000 different genes), and there appears to be a substantial overlap between genes active in gametophytic and vegetative tissue.

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