Identification of Embryoid-Abundant Genes That Are Temporally Expressed during Pollen Embryogenesis in Wheat Anther Cultures

Thomas L. Reynolds* and Sherry L. Kitto

Department of Biology, University of North Carolina at Charlotte, Charlotte, North Carolina 28223 (T.L.R.); and Delaware Agricultural Experiment Station, Department of Plant and Soil Sciences, College of Agricultural Sciences, University of Delaware, Newark, Delaware 19717 (S.L.K.)

ABSTRACT

Uninucleate microspores in anther cultures of bread wheat (Triticum aestivum cv Pavon) are capable of producing haploid pollen embryoids and plants. To gain an understanding of this alternate pathway of pollen development, we constructed a cDNA library to young pollen embryoids, isolated embryoid-specific genes, and analyzed their expression patterns during morphogenesis. Two embryoid-abundant clones, pEMB4 and 94, were expressed very early during culture, suggesting that these genes are associated with development and are not simply expressed as a consequence of differentiation. The accumulation patterns of five cloned mRNAs may indicate the activation of specific genes associated with the major morphological and physiological activities connected with the differentiation of embryoids in vitro. These results suggest that embryoid-abundant gene expression is causally related to this pathway because gene expression is spatially and temporally specific and is not observed when microspores are cultured under noninductive conditions.

The profound changes that occur during male gametogenesis in flowering plants have prompted interest in the study of the molecular mechanisms underlying this developmental process (reviewed in refs. 17 and 18). Much attention has been focused on the complex program of gene expression that occurs during microgametogenesis, and pollen-specific genes that are temporally expressed have been isolated from several plants (1, 6, 13, 24). Analyses of these genes, their promoters, and products are sure to lead to a basic understanding of the developmental biology of normal pollen ontogeny.

Although pollen grains are genetically programmed for terminal differentiation to form pollen tubes and gametes, it has been shown repeatedly that immature pollen or microspores of certain plants are capable of entering an alternate developmental pathway of continued cell division and growth. This phenomenon, known as pollen androgenesis, may proceed through one of two basic pathways. In the first, the pollen grain produces an embryo-like structure that develops in a manner similar to a zygotic embryo but produces a haploid plant. In the second route, the pollen grain gives rise to callus that must be subsequently induced to regenerate plants.

In contrast to the number of studies that have been done concerning the developmental biology of pollen ontogeny, little is known about the physiological, biochemical, or molecular events associated with either pathway of pollen androgenesis. To gain an understanding of this alternate pathway of pollen development, it is essential to identify genes that are developmentally specific for androgenesis. It should then be possible to evaluate the regulation of these genes and their possible role during the production of multicellular embryoids or callus from microspores. We report here the construction of a cDNA library made to mRNA from young pollen embryoids of bread wheat (Triticum aestivum cv Pavon), the isolation of embryoid-specific genes, and the analysis of their expression patterns during morphogenesis.

MATERIALS AND METHODS

Plant Material

Spring wheat plants (Triticum aestivum L. cv. Pavon) were raised from seed (obtained from the National Small Grain Collection, U.S. Department of Agriculture/Agricultural Research Service, University of Idaho Research and Education Center, Aberdeen, ID) and maintained in a growth chamber at 27 ± 2°C with an 18-h photoperiod (120 μmol m⁻² s⁻¹) provided by fluorescent and incandescent lights.

Anther Culture

Microspores of the wheat cv Pavon are most susceptible to androgenic induction at the late uninucleate stage just before the first haploid mitosis (2). Tillers from plants grown in a growth chamber were collected at the late uninucleate microspore stage when the top of the spike was level with the base of the penultimate leaf. Although the original protocol for Pavon anther culture called for pretreating the tillers for 7 d in the dark at 6°C (2), we found this to be unnecessary. Before culture, the tillers were surface sterilized in 20% (v/v) Clorox for 20 min and rinsed several times in sterile water. Anthers were aseptically removed, plated in liquid MN6 medium (7) containing 1 mg L⁻¹ of 2.4-D and 1.5 mg L⁻¹ of

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kinetin, and cultured in the dark at 28°C. To determine stages of embryogenic development, anthers were collected at various times after the initiation of culture, squashed, and stained on a slide with acetoaramine and viewed using bright-field microscopy.

**Microspore and Pollen Embryo Staging**

To obtain uninucleate microspores for nucleic acid isolation, anthers were collected and gently ground on ice using a hand-held homogenizer to release the microspores. The grinding buffer was MN6 medium lacking hormones. The macerate was filtered through Miracloth (Calbiochem), and the filter was rinsed with cold MN6 medium. The filtrate was centrifuged at 100g for 3 min, and the pellet was resuspended in 500 µL of grinding buffer. This suspension was layered on top of a 30% (w/v) sucrose solution and centrifuged at 450g for 5 min at 4°C (25). The band of viable microspores at the top of the sucrose cushion was collected with a pipette, pooled, and examined microscopically to assess purity. A similar procedure was used to obtain bicellular, tricellular, multicellular embryogenic pollen, and pollen embryos, except in these instances, the cellular pellet at the bottom of the tube was collected. Microspores and pollen embryos were frozen in liquid N2 and stored at −70°C.

**RNA Isolation**

Total RNA was isolated from microspores, pollen, pollen embryos, and other tissues of the plant using a guanidine hydrochloride-containing buffer, followed by direct extraction with phenol/chloroform as described by Logemann et al. (16). In a typical isolation, 100 to 300 mg of tissue was homogenized in 1 to 2 volumes of lysis buffer to yield 150 to 200 µg of total RNA. Poly(A)+ RNA was collected from total RNA by affinity chromatography using oligo(dT)-cellulose (Collaborative Research, Inc.) and essentially the same method described by Aviv and Leder (3).

**cDNA Library Construction**

The pollen embryoid cDNA library was constructed by the basic procedures of Gubler and Hoffman (12) using a single-tube synthesis system available commercially as a kit (Invitrogen Corp.). The double-stranded cDNA was ligated to BstXI-restricted nonpalindromic linkers in the presence of T4 ligase overnight. The cDNA was size selected at 500 bp by electrophoresis on a 2% agarose gel, then ligated to BstXI-cut pcDNA II vector, and used to transform competent Escherichia coli (INV1 αF').

**Library Screening**

A differential screening procedure was used to identify genes with enhanced expression in pollen embryos. The library was plated out at low density (<1000 colony-forming units/plate), and duplicate colony lifts were made from each plate using nylon filters. These filters were processed according to standard methods (23). Hybridization probes were synthesized from poly(A)+ RNA in reactions similar to first-strand cDNA synthesis except that unlabeled dCTP was replaced by 100 µCi of [³²P]dCTP. To screen a library, one filter was incubated with hybridization solution containing a cDNA probe (10⁶ cpm mL⁻¹) constructed from mature pollen mRNA. The second filter was hybridized with a similar probe made from mRNA used to make the library being screened. Colonies showing substantially stronger hybridization to the pollen embryo probe over the other were plated at a lower density. Filter replicates were made from these secondary plates and hybridized with the same probes. Single colonies showing detectable hybridization with the library probe and weak or undetectable hybridization with the pollen probe were isolated. Final screening was performed using DNA gel blots of plasmid DNA isolated from specific clones. Duplicate gel blots were hybridized with the same probes used in the previous screening steps.

**Plasmid Isolations**

Plasmids were isolated by the procedure of Holmes and Quigley (14). DNA was digested with HindIII and XbaI to separate cDNA inserts from the vector. Radioactive cDNA probes for hybridization were prepared by the random oligonucleotide-priming method (10).

**Northern and Slot Blot Hybridizations**

Total RNA was electrophoretically separated in a 1.5% agarose gel containing 0.66 M formaldehyde (8) and transferred to GeneScreenPlus (New England Nuclear) by capillary blotting (23). For slot blot hybridizations, RNA was transferred to nylon membranes using a vacuum manifold (8). Northern and slot blot membranes were prehybridized for 12 h at 65°C in heat-sealed plastic bags containing 1% SDS, 1 M NaCl, and 10% dextran sulfate. For hybridization, [³²P]-labeled probe (1–3 × 10⁶ cpm mL⁻¹) was added directly to the prehybridization solution. Filters were hybridized for 24 to 36 h at 65°C and then washed twice in 2x SSC for 5 min, twice in 2x SSC, 1% SDS at 60°C for 30 min, and finally in 0.1x SSC for 30 min at room temperature. The filters were exposed to preflashed Kodak XAR-5 film with an intensifying screen at −70°C. Results were quantified using a Bio-Rad model 620 video densitometer.

**Southern Hybridization**

Total DNA was isolated from wheat leaves as detailed by Dellaporta et al. (9). The DNA was digested with different restriction endonucleases, separated in a 0.8% agarose gel, transferred to GeneScreenPlus, and hybridized as described above for RNA blots.

**RESULTS**

**Pollen Embryogenesis**

By carefully selecting anthers containing uninucleate microspores from vigorously growing plants and by not cold

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**Abbreviations:** SSC, standard sodium citrate; MCPG, multicellular pollen grain.
pretreating the anthers before culture, we were able to routinely obtain 70 to 80% anther induction efficiency and 40% microspore induction frequency (data not shown).

The first structural changes associated with embryogenic induction were observed at the light microscopic level within 2 d after culture. At this time, embryogenic microspores had undergone division, forming MCPG (Fig. 1A). After 7 d of culture, proembryoids, still enclosed in the pollen wall, could be observed in anther-squash preparations (Fig. 1B). With continued cell division, the proembryoids developed into small globular (day 10; Fig. 1C) and bipolar embryos (day 14; Fig. 1D), and after 21 d, young embryos penetrated the anther wall (Fig. 1E). As the embryos matured outside the anther, they appeared similar to zygotic embryos of wheat with a well-developed scutellum, shoot apex, and radicle.

Isolation of Pollen Embryoid-Specific cDNA Clones

To isolate clones corresponding to genes that are differentially expressed during pollen embryogenesis of wheat, we constructed a cDNA library from 21-d-old pollen embryoids (Fig. 1E). The unamplified library consisted of approximately 3.5 × 10⁴ clones, and the average insert size was 800 bp. Nearly 50,000 clones have been screened, and based on the intensity of hybridization signals, it was possible to identify three groups of clones. Group 1 consisted of clones expressed in abundance in both pollen embryoids and mature pollen, group 2 clones were expressed in embryos but in low amounts in pollen, and clones classified as belonging to group 3 were expressed in abundance or low amounts in embryos but not at all in pollen (data not shown).

Specificity of cDNA Clones

Clones that exhibited the strongest preferential hybridization to embryos were further characterized. Because only mature pollen RNA was used to make probes to screen the library, it was possible that the putative embryoid-specific clones we had isolated could be expressed at other stages of pollen development. Expression in vegetative tissues was also possible because our screening procedure would not have detected these patterns either. The developmental specificity of embryoid-abundant mRNA accumulation was assessed in greater detail by reacting RNAs from vegetative tissues, zygotic embryos, and pollen at different stages of development with each cDNA clone in northern blot experiments.

Most of the clones in the library were expressed in both pollen and vegetative tissues; however, Figure 2 shows RNA gel blots that were hybridized to clones displaying various degrees of developmental specificity. For example, pEMB94 (Fig. 2A) had an insert size of 800 bp and hybridized strongly to 1100-base RNA present in 21-d-old pollen embryoids and zygotic embryos of T. aestivum cv Pavon. No RNA was detected in vegetative tissues or in pollen at different stages of development. A second clone, pEMB4, showed a different pattern of hybridization (Fig. 2B). The insert size of this clone was 900 bp, and it hybridized strongly to 1000-base RNA. This gene was not expressed in vegetative tissues, zygotic embryos, pollen tetrads, or binucleate or trinucleate pollen. The signal was seen, however, in uninucleate pollen (at the time of culture) and in pollen embryoids.

Clone pEMB115 exhibited a very simple hybridization pattern because it hybridized exclusively to RNA at 700 bases in pollen embryoids (Fig. 2C). No RNA in any other lanes hybridized to this probe even with very long exposures. Figure 2D shows an example of a clone (pEMB18) that displayed sporophytic specificity. The gene corresponding to this clone was strongly expressed in vegetative tissues, embryos, and embryoids but not in pollen at any stage.

Similar analyses have been performed with four additional clones. Table I shows the results obtained from these and the above experiments. Clones pEMB56 and 98 also appeared to be embryo-specific candidates, whereas clone 27 showed less specificity. Blot hybridization studies demonstrated that none of these clones cross-reacted (data not shown), suggesting that the cloned mRNAs were unique. Clone pEMB36 is one example of a group 1 clone that showed nonspecific expression.

DNA gel blot analyses using T. aestivum cv Pavon DNA digested with several restriction enzymes were performed to determine how many copies of the corresponding gene exist in the genome. The result with pEMB4, shown in Figure 3, suggests that this clone represents a single- or low-copy number gene in wheat. Southern analysis with pEMB94, 115, and 56 showed the same copy number of the genes in the genome (data not shown).

![Figure 1](image-url) Development of pollen embryoids of Triticum aestivum cv Pavon at various stages after culture initiation. A, MCPG, day 2 (×570); B, Proembryoid still enclosed by the pollen wall, day 7 (×570); C, Globular stage, day 10 (×570); D, Bipolar stage, day 14 (×485); E, Young pollen embryoids emerging from the anther, day 21 (×23).
Temporal Expression of Embryogenic Clones

A key to defining mechanisms involved in regulating embryoid-abundant genes is to determine precisely when they are activated. To obtain information concerning the pattern of accumulation of embryoid-specific RNA during pollen embryogenesis, we isolated RNA from potentially embryogenic microspores (time of culture), MCPG (2 d old), proembryoids (7 d old), globular (10 d old), bipolar (14 d old), and young embryoids (21 d old) and probed it with each putative embryoid-specific cDNA clone. Results from the total RNA slot blots (Fig. 4) revealed heterogeneity in mRNA accumulation kinetics among the various clones, suggesting that the corresponding genes were differentially expressed during embryogenesis. RNA complementary to pEMB4 was first detected in uninucleate pollen at the time of culture and continued to accumulate through the mature pollen embryoid stage. Hybridization signals for clone pEMB94 were not detected in uninucleate pollen but appeared in MCPG and maintained constant intensity through the mature pollen embryoid stage (Fig. 4). Embryoid-abundant RNAs represented by pEMB56 and 98 did not appear until the proembryoid stage but then accumulated through the formation of young embryoids after 21 d of culture. It is interesting that pEMB115 only hybridized to embryoid RNA during later stages of development with the strongest signal in 21-d-old embryoids (Fig. 4). In total, our results indicated that these five embryoid-abundant genes were expressed at different times during embryogenesis.

Fate of RNAs during Embryoid “Germination”

Pollen embryoids of T. aestivum cv Pavon can be induced to “germinate” and form whole plants if subcultured onto agar-solidified MN6 medium containing 1 mg mL⁻¹ of kinetin (2). If the embryoids are not subcultured onto germination medium, they fail to develop further and instead produce extensive callus. It was of interest to determine the fate of the embryoid-specific RNA during the germination process. Figure 5 shows that there was a dramatic decline in the RNA

![Image](image_url)

Table 1. Northern Blot Analysis of cDNA Clones

Hybridization signal intensity is indicated by the number of + symbols.

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<th>Leaf</th>
<th>Zygotic Embryos</th>
<th>Pollen Embryoids</th>
<th>Tetads</th>
<th>Uninucleate Microspores</th>
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levels for all five clones during the first 5 d of germination. Hybridization signals continued to decrease and were no longer detectable in any clone except pEMB94 through 8 d of culture, when small green shoot meristems could be observed arising from the embryoids.

**Figure 3.** Southern blot analysis of restriction endonuclease-digested wheat genomic DNA (10 μg/lane) hybridized to embryoid-abundant clone pEMB4. Lane 1, EcoRI; lane 2, HindIII; lane 3, BamHI. Sizes of the markers are given to the left of the autoradiogram.

**Figure 4.** Developmental expression of embryoid-abundant RNAs. Total RNA from various developmental stages of the embryo was blotted onto nylon membranes and hybridized to pollen embryoid cDNAs. The autoradiograms were quantified using a scanning densitometer, and the results are expressed as relative optical density. Embryogenic stages are: 1, uninucleate microspores at the time of culture; 2, MCPP (day 2); 3, proembryo (day 7); 4, globular embryo (day 10); 5, bipolar embryo (day 14); young embryo (day 21).

**Figure 5.** Changes in embryoid-abundant RNA during germination. Total RNA was isolated from wheat pollen embryoids at various times after being transferred to germination medium and blotted onto nylon membranes. Slot blots were hybridized to the indicated pEMB clones. Relative RNA amounts present during germination are represented by the optical density of the hybridization signals on autoradiograms. By day 8 on this medium, embryoids showed well-organized shoot meristems.

**RNA Synthesis in Nonembryogenic Cultures**

To determine whether the observed pattern of RNA synthesis in embryogenic cultures was due to morphogenesis or to some indirect effect of 2,4-D and kinetin, an embryoid-specific clone was used to probe RNA isolated from anther cultures placed in noninductive conditions. In this experiment, anthers containing uninucleate microspores were placed in culture under continuous light, which is known to inhibit the formation of pollen embryos in wheat (26). RNA was isolated from pollen at various times during a 4-week culture period and probed with pEMB94 using northern blot analysis. In contrast to embryogenic cultures, hybridization signals could not be detected at any time, and the uninucleate pollen failed to become embryogenic (data not shown). This supports the idea that the differential gene expression observed in embryogenic cultures is due to androgenesis and not to some indirect effects of tissue culture.

**DISCUSSION**

This study has shown that it is possible to use differential screening of a cDNA library to isolate sequences with enhanced expression in embryogenic microspores and pollen embryoids of wheat. The majority of the clones in this library, however, were expressed in both pollen and various vegetative tissues. Although there are no current estimates concerning the number of pollen embryoid-specific sequences in plants, there are approximately 20,000 diverse genes expressed in zygotic embryos during embryogenesis (11). Most of these genes encode rare mRNAs of unknown function. If
pollen embryoids are similar to zygotic embryos in this regard, cDNAs for these rare mRNAs could be at such low concentrations in our probes that we were unable to produce detectable signals in our screening procedure. This could explain the relatively few clones identified as embryoid specific in our library.

Those cloned mRNAs identified as embryoid-specific in this study represent developmentally regulated genes whose expression is induced during pollen embryogenesis in wheat anther cultures. Two clones, pEMB4 and 94, were expressed very early during culture, suggesting that these genes are associated with morphogenesis and are not simply expressed as a consequence of differentiation. The accumulation patterns of five clones may indicate the activation of specific genes associated with the major morphological and physiological activities connected with the formation and differentiation of pollen embryoids in vitro. Although there is no conclusive evidence that embryoid-abundant gene expression is causally related to this pathway, such a role appears plausible because these genes were spatially and temporally specific and were not expressed in microspores cultured under noninductive conditions.

Studies of the basic mechanisms of pollen androgenesis are rare, and the biochemical or molecular basis for the developmental transformation of microspores into pollen callus or embryoids has not been established for any plant species. In general, there is a requirement for altered synthesis and accumulation of RNA and proteins in responsive microspores, leading to the first sporophytic type divisions. Bhojwani et al. (5) showed that in nonembryogenic tobacco pollen there was a rapid increase in RNA and proteins during culture, whereas both of these macromolecules declined in potentially embryogenic pollen. In this work, Bhojwani et al. (5) proposed that sporophytic gene expression associated with androgenesis only began after the gametophytic program had been suppressed in culture. In contrast, androgenic induction in Hyoscyamus niger involves the synthesis of new mRNA within the first hour of culture and embryogenic divisions 6 to 12 h after that (20, 21). Recently, Pechan et al. (19) used in vitro translation studies to identify mRNAs and proteins that appear to be associated with induction and the early stages of embryogenesis in Brassica microspores. Kyo and Harada (15) showed that certain phosphoproteins appear during the transformation of microspores to embryoids in tobacco and that these phosphoproteins are not observed during normal pollen ontogeny. Our study with wheat supports these observations and demonstrates that these biochemical changes reflect differential expression of developmentally specific genes.

Although our findings do not provide information concerning the mechanisms that activate the synthesis of new mRNA during androgenesis or the functions of the proteins they synthesize, there are some interesting observations that relate to these questions. First, because sporophytic type growth is generally induced in uninculcete microspores in many androgenic plants, including wheat (22), the onset of the first haploid mitosis may represent an important developmental switch. This concept is supported by the finding that new sets of genes are expressed at the time of the first haploid mitosis in corn (4) and wheat (25) and that this characterizes a critical transition stage during microgametogenesis. Furthermore, Pechan et al. (19) noted that some of the proteins synthesized during the embryogenic induction of Brassica microspores were possibly heat-shock proteins. It can be speculated then that the physical shock brought on by anther excision and culture might alter the pattern of gene expression in potentially embryogenic microspores so that during androgenic induction the first haploid mitosis marks a switch in the developmental program of the male gametophyte from one of terminal differentiation to one concerned with continued division and growth. Clone pEMB4 may be an example of a "transition" gene normally only expressed at the time of the first haploid mitosis but, as a consequence of embryogenic induction, remains turned on in developing embryoids. Following induction, other genes become activated, reflecting the dramatic morphological and physiological changes that occur during embryogenesis.

The genes we have identified with enhanced expression in pollen embryoids will be useful tools in future studies on gene expression during pollen androgenesis. Currently, we are using these clones for in situ hybridization studies to follow the distribution of transcripts in potentially embryogenic microspores and embryogenic pollen during development. We are also screening a genomic library to characterize embryoid-specific genes and their regulatory elements.

LITERATURE CITED