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Characterization of a Gene Family Abundantly Expressed in *Oenothera organensis* Pollen That Shows Sequence Similarity to Polygalacturonase

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We have isolated and characterized cDNA clones of a gene family (P2) expressed in *Oenothera organensis* pollen. This family contains approximately six to eight family members and is expressed at high levels only in pollen. The predicted protein sequence from a near full-length cDNA clone shows that the protein products of these genes are at least 38,000 daltons. We identified the protein encoded by one of the cDNAs in this family by using antibodies to β-galactosidase/pollen cDNA fusion proteins. Immunoblot analysis using these antibodies identifies a family of proteins of approximately 40 kilodaltons that is present in mature pollen, indicating that these mRNAs are not stored solely for translation after pollen germination. These proteins accumulate late in pollen development and are not detectable in other parts of the plant. Although not present in unpollinated or self-pollinated styles, the 40-kilodalton to 45-kilodalton antigens are detectable in extracts from cross-pollinated styles, suggesting that the proteins are present in pollen tubes growing through the style during pollination. The proteins are also present in pollen tubes growing in vitro. Both nucleotide and amino acid sequences are similar to the published sequences for cDNAs encoding the enzyme polygalacturonase, which suggests that the P2 gene family may function in depolymerizing pectin during pollen development, germination, and tube growth. Cross-hybridizing RNAs and immunoreactive proteins were detected in pollen from a wide variety of plant species, which indicates that the P2 family of polygalacturonase-like genes are conserved and may be expressed in the pollen from many angiosperms.

INTRODUCTION

Although the male gametophyte of flowering plants has been reduced to the two or three cells of the pollen grain, these cells are highly differentiated and must perform the complex functions of development, pollination, and fertilization. For example, late in its development, the pollen grain accumulates storage materials and becomes able to survive some degree of desiccation. Desiccation may aid in the grain's survival during its journey to the pistil. Once it arrives at the pistil, the pollen grain recognizes whether it is on a compatible stigma and then the pollen tube grows through the tissues of the pistil, often for long distances, to enter the female gametophyte and release the sperm cells for fusion with the egg and central cell.

Although some functions are provided by sporophytic gene products, the developing pollen grain expresses a large number of genes and has considerable influence over its own ontogeny. Gametophytic self-incompatibility, which precludes self-pollination in a number of flowering plants (including *Oenothera organensis*), is controlled by genes expressed by the pollen grain itself (Emerson, 1938). Also, mutations in a number of genes that have a detectable pollen phenotype, such as alcohol dehydrogenase (Freeling, 1976), starch metabolism genes (Demerec, 1924), gametophytic factors (Jimenez and Nelson, 1965), and certain restorers of male fertility (Buchert, 1961), exhibit a gametophytic pattern of inheritance, showing that their functions are expressed post-meiotically by the pollen grain. The mature pollen grains in some species contain stored mRNAs that encode proteins that function during germination and early tube growth; no new transcription is required for these events (Mascarenhas, 1965, 1966).

Approximately 20,000 different genes produce mRNAs present in the mature pollen grain of *Tradescantia paludosa*, as estimated by $r_o t$ analysis (Willing and Mascarenhas, 1984). These analyses also suggest that the majority (>64%) of these sequences in pollen are also present in vegetative tissues. This method of measuring overlap of gene expression has some limitations; for example, related genes cannot be distinguished. Isozyme studies also show extensive overlap between gametophyte and sporophyte gene expression (Tanksley, Zamir, and Rick, 1981; Sari Gorla et al., 1986). Seventy-two percent of the enzymes

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examined in corn and 60% in tomato were present in both sporophyte tissues and in pollen. Only a small percentage (5% to 6%) of the 30 to 34 sporophytic isozymes examined were present only in pollen. Although isozyme studies can examine the products of single genes, they are limited to the relatively few gene products that can be analyzed in this way.

Very little is known about the patterns of expression of specific genes during pollen development. Early studies showed that the 5S rRNA and tRNAs are synthesized early in microsporogenesis (Mascarenhas, 1975). Similar results have been obtained for the 25S and 18S rRNAs; synthesis peaks before pollen mitosis and no synthesis is detectable in the last 2 days of development (Steffenson, 1966; Mascarenhas and Bell, 1970). However, several papers describing total RNA synthesis note a large increase in RNA after the microspore mitosis and just before anthesis (Mascarenhas, 1975). These late RNAs are heterogeneous in size and probably reflect mRNA synthesis late in pollen development. With the advent of the techniques of molecular biology, it has been possible to examine the expression of genes during pollen development in much finer detail. The isolation of cDNA clones corresponding to single- or low-copy genes expressed in mature pollen grains of *T. paludosa*, corn, and tomato has recently been described (Stinson et al., 1987; Hanson et al., 1989; Ursin, Yamaguchi, and McCormick, 1989).

We have chosen to examine pollen gene expression in *O. organensis* because it has a number of desirable characteristics. It is easily grown in the greenhouse, where, under long day lighting conditions, it produces a steady supply of flowers year-round. The flowers are extraordinarily large, allowing easy dissection of individual floral parts. The pollen grains are also large—more than 200 μm (Dickinson and Lawson, 1975)—and approximately 7 mg of pollen can be collected from a single flower. *O. organensis* has also been the classic species in genetic, biochemical, and immunological studies of the role of pollen in pollen-pistil interactions and self-incompatibility (reviewed in DeNettancourt, 1977). These studies have provided valuable information about *O. organensis* pollen development, germination, and growth. Although little information is available in the literature about its molecular biology, *O. organensis* has proven to be a good source of material for tissue culture and has been successfully transformed in our laboratory using standard *Agrobacterium* methodology (C. Scelonge and M.L. Crouch, manuscript in preparation).

In this paper, we report the identification of cDNAs that represent a gene family expressed primarily in pollen and present the results of analyses aimed at determining the possible roles that this gene family may play in pollen development and/or germination. The temporal expression patterns suggest that the products of these genes probably function late during pollen development and/or during germination and tube growth. The nucleotide and predicted amino acid sequences of the cDNAs are similar in sequence to the enzyme polygalacturonase.

RESULTS

Isolation of Pollen-Abundant Clones

Poly(A)+ RNA was isolated from pollen of *O. organensis* collected on the day of anthesis and used to construct a cDNA library in the bacteriophage vector λgt10 as described in Methods. To isolate cDNAs representing genes that are preferentially expressed in pollen, the cDNA library was differentially screened. A portion of the library was plated, and replicate nitrocellulose filters were hybridized with cDNA probes made from RNA from pollen, seedlings, and young flower buds. Several clones that showed significantly higher signals with the pollen probe than with the seedling and floral bud probes were chosen for further characterization. These clones fell into several categories based on their RNA accumulation patterns (S.M. Brown and M.L. Crouch, manuscript in preparation). Pollen clone 2 (P2) was chosen for further analysis because it represents a class of mRNAs that is differentially expressed and very abundant in mature pollen.

P2 cDNA Represents a Medium-Sized Gene Family

To estimate the number of genes in the *Oenothera* genome that are related to the P2 cDNA, a DNA gel blot was prepared from nuclear DNA that had been digested with several restriction endonucleases and probed with the P2 cDNA insert at moderate stringency. The size of the P2 gene family was approximated based on the number of bands in each lane and the intensity of the bands compared with copy standards (described in Methods). Figure 1 shows the results of the genomic DNA gel blot analysis. We estimate that the P2 gene family contains approximately six to eight genes.

To determine whether the majority of the members of the P2 gene family are expressed in mature pollen, a portion of the cDNA library was rescreened to isolate additional cDNA clones. Approximately 1.2% of the sequences in the pollen cDNA library hybridized to the P2 cDNA insert probe. More than 50 additional clones were isolated; the 13 clones with the longest inserts were subcloned and mapped with restriction endonucleases. Figure 2 shows a comparison of the 14 cDNAs. The cDNAs can be grouped into six different classes based on their restriction maps. Four unique clones (P2, P22, P25, and P39) were isolated, each having a different combination of restriction sites. The fifth class includes the five clones (P1, P60, P96, P102, and P107) that have maps identical to the P1 cDNA. All are quite different from the unique clones. Five other clones (P26, P34, P58, P67, and P85) form a

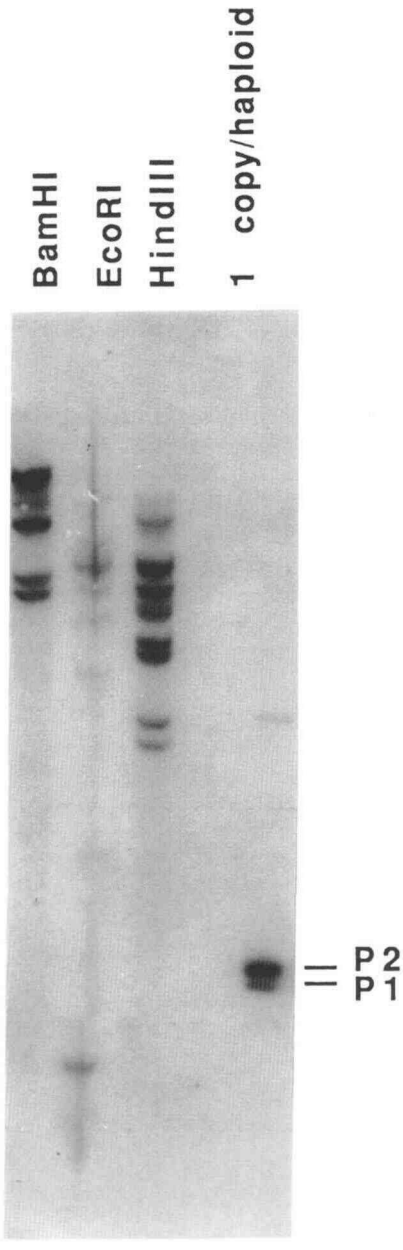

Figure 1. DNA Gel Blot of *O. organensis* Genomic Sequences That Hybridize to the P2 cDNA.

Five micrograms of nuclear DNA digested with the enzyme indicated and the appropriate amount of copy standard were electrophoresed, blotted, and probed with radiolabeled P2 cDNA insert, as described in Methods.

sixth class, each characterized by a restriction map identical to the P26 cDNA that is different from the map of the P1 class or the unique clones. These 14 cDNA clones represent at least six unique mRNAs.

Nucleotide Sequences Show Similarity to Polygalacturonase

The nucleotide sequences of the entire cDNA inserts were obtained for two clones, P2 and P22, along with partial sequence for several others, to obtain information about possible functions for the P2 gene family. The two cDNAs show 87% sequence identity, 89% in the region in the open reading frame and 75% in the 3′-untranslated region (data not shown). The open reading frame in the longer P22 cDNA extends from the first nucleotide to nucleotides 1087 to 1089, where a TAA stop codon presumably ends translation. The other two frames contain numerous stop codons. Because the first methionine codon does not occur until nucleotide 322, this cDNA insert probably does not contain the entire protein coding sequence. The P22 cDNA does encode more than 38,000 daltons of protein, approaching the size of the in vivo proteins detected immunologically (see below).

The amino acid sequence of the open reading frame in the P22 sequence shows significant similarity to the amino

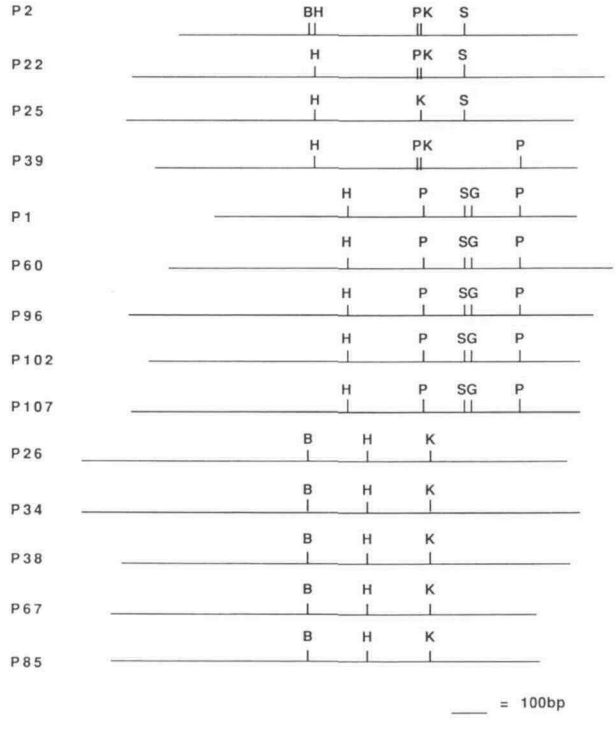

Figure 2. Restriction Endonuclease Maps of Pollen cDNAs Homologous to the P2 cDNA.

The EcoRI inserts of the pollen cDNA clones were digested with a variety of restriction endonucleases to generate the restriction maps shown. Abbreviations for restriction endonucleases are: B, BamHI; G, BglII; H, HincII; K, KpnI; P, PstI; S, SstI.

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PG-TOMATO   68      VDKNGIKVINVLSFGAKGDGKT...      89
                          |||| ||||||| |
P26        -22      CTITNAQLFDITKYGAKGDGAT...      -1


PG-TOMATO
90  YDNIAFEQAWNEACSSRTPVQFVVPKNKNYLLKQITFSGPCRSSISVKIF 139
      || || ||| |  |  ||||| || |  ||| |||||||| |
1   DSTQALTTAWKEACASASPSTILVPKG.NFAVGLITLEGPCKSSIGLQLQ 49
P22

140 GSLEASSKISDYKDRRLWIAFDSVQNLVVGGGGTINGNGQVWWPSSCKIN 189
    | | |    |  ||   || || || | | ||| ||| |   |
50  GTLKAPADPSKIKGLG.WINLNKIDLLTIFGGGVFDGQGKSAWVQNDCHK  98

190 KSLPCRDAPTALTFWNCKNLKVNNLKSKNAQQIHIKFESCTNVVASNLMI 239
     |  ||     | ||   |  | ||   |   |||   || ||    | |
99  NGPICKTLSMNLRLYAVTNSILRDVTTLDSKNFHVNVIGCKNLTFERFKI 148

240 NASAKSPNTDGVHVSNTQYIQISDTIIGTGDDCISIVSGSQNVQATNITC 289
     |   | ||||||||  | | | || | ||||||||  || ||  |||||
149 SAAETSINTDGIHIGRSDGVNIINTEIKTGDDCISLGDGSKNINITNITC 198

290 GPGHGISIGSLGSGNSEAYVSNVTVNEAKIIGAENGVRIKTW.QGGSGQA 338
    ||||||||||||    |  | | | |  | ||||||||||  || |||
199 GPGHGISVGSLGRYKNEESVVGIYVKNCTITGSQNGVRIKTWPKSEPGEA 248

339 SNIKFLNVEMQDVKYPIIIDQNYCDRVEPCIQQFSAVQVKNVVYENIKGT 388
    ||| | || |  |  |||||| ||   |   |  | | |  | | |||||
249 SEMHFQDITMNSVGTPILIDQGYCPYNQCTAEVPSSVKLSKISFKNIKGT 298

389 SATKVAIKFDCSTNFPCEGIIMENINLV.GESGKPSEATCKNVHFNNAEH 437
    | || |||| ||  |||||| | ||||  || | |    | ||     |
299 STTKEAVKLVCSKSFPCNGVELADIDLTYSGKGGPATSVCENIKPTIKGK 348

438 VTPHCTSLEISEDEALLYNY* 457
      |   |
349 QIPAICSGSAAKAA*       362
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Figure 3. Comparison of Predicted Amino Acid Sequences from *O. organanesis* Pollen cDNAs and a Tomato Fruit Polygalacturonase cDNA.

The top panel shows a comparison of the predicted amino acid sequence of the P26 cDNA (which contains 66 nucleotides more 5′ sequence than the P22 cDNA) with the predicted amino acid sequence of a polygalacturonase cDNA (Grierson et al., 1986). Each vertical line represents an identical amino acid or a conservative change. The horizontal line highlights the first 4 amino acids of the mature polygalacturonase protein in tomato fruit. The lower panel shows a continuation of this comparison, now using the predicted amino acid sequence of the P22 cDNA with the homologous region of the predicted amino acid sequence of the same tomato fruit polygalacturonase cDNA (full-length sequence was not obtained for the P26 insert). Each sequence ends with a stop codon (*). For brevity, the nucleic acid sequences are not presented. These sequences are available in Genbank or from the authors.

acid sequence of polygalacturonase from tomato fruit (Grierson et al., 1986). Figure 3 shows the comparison of the predicted amino acid sequences from the pollen and fruit polygalacturonase cDNAs. Overall, 54% of the 382 residues represented by P26 and P22 are identical or substituted with a similar amino acid in the tomato polygalacturonase (PG) sequence. The nucleotide comparison is similar; the cDNAs are identical over 58% of the length of the P22 cDNA. There are striking regions of identical amino acids; for example, in the region from amino acids 285 to 301 in the tomato PG sequence, 16 of 17 are identical to the P22 sequence and the only difference is a conservative change from the isoleucine in PG to the valine in P22. The predicted P22 amino acid sequence, like the tomato PG sequence, contains four glycosylation sites. These sites are not conserved in position, except for the Asn-Ile-Thr (286 to 288 in the tomato sequence), which is present in the highly conserved 285 to 301 region.

P2 RNA Accumulation Patterns

To determine the spatial accumulation pattern of P2 transcripts, an RNA gel blot was prepared from RNA isolated from mature pollen, leaf, stigma, ovary, and seedling tissues and probed with the P2 cDNA insert. Figure 4 shows that the 1.5-kb P2 transcripts are only detectable in pollen, not in the sporophytic tissues examined.

The timing of accumulation of P2 mRNAs during pollen development was also analyzed. In *O. organensis*, the length of the hypanthium is a very good indicator of a variety of events in floral development, including the stages of pollen development (S.M. Brown and M.L. Crouch, manuscript in preparation). The tetrad stage occurs when the hypanthium is approximately 0.1 cm, the free microspore stage extends from 0.15 cm, and the pollen are binucleate in flowers with hypanthia greater than 1 cm. At hypanthia lengths greater than 6 cm, the pollen begin to reach functional maturity (can cause seed set), and full

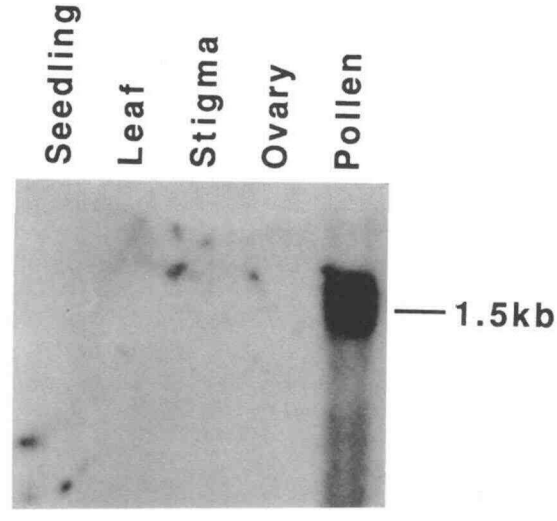

Figure 4. P2 mRNAs Accumulate Only in Pollen.

Five micrograms of total RNA from various organs were electrophoresed, blotted to a nylon membrane, and hybridized with radiolabeled P2 cDNA insert, as described in Methods. The final wash conditions were 1 × SSC, 60°C. The autoradiogram was overexposed to emphasize the absence of the 1.5-kb transcript in other parts of the plant.

maturity (can cause normal rates of seed set) is achieved by pollen in flowers with greater than 9-cm hypanthia. Final hypathium length is an average of 15 cm.

Anthers were collected from flowers at a variety of developmental stages, and the pollen was isolated from the rest of the anther material by filtration and sedimentation through sucrose. RNA was prepared from the isolated pollen and an RNA gel blot was probed with the P2 cDNA insert. Figure 5A shows that P2 transcripts are first detectable at the 1 cm to 2 cm hypanthium stage. At this stage of development, the pollen have just undergone the mitotic division that produces the generative and vegetative cells. The P2 mRNA levels increase throughout the maturation phase following the pollen division and are highest at anthesis.

Quantitative dot blot analyses were performed to determine the kinetics of accumulation of P2 transcripts. Total RNA was immobilized on duplicate filters and hybridized with the P2 cDNA or a clone containing a region of the ribosomal repeat from the radish nuclear genome (Delseny et al., 1983). The radioactivity that hybridized to each dot was quantitated by scintillation counting. Figure 5B shows the results of these analyses. Each point indicates the amount of P2 mRNA relative to rRNA at each stage of *Oenothera* pollen development. The results indicate that the level of P2 transcripts increases at least 200-fold during their induction in pollen development.

RNA from mature pollen was used to determine the absolute amount of P2 transcripts relative to total RNA (data not shown). Total RNA was quantitated by UV absorbance, and serial dilutions were applied in triplicate to nitrocellulose using a commercial slot blotter. Serial dilutions were also made using in vitro synthesized antisense RNAs made from the P2 cDNA insert subcloned into Bluescript. The filters were probed with the P2 cDNA insert and the hybridization signal was quantitated by scanning densitometry. These analyses indicate that the P2 family of mRNAs constitute 0.013% w/w of the total RNA in the mature pollen grain and that the P2 mRNA level does not change significantly in response to temperature.

Protein Identification and Accumulation

An antibody against the polypeptide encoded by the P2 cDNA was produced to identify the protein products of the P2 gene family and to characterize their expression patterns. A 750-bp fragment (from the BamHI site to the 3′ end of the clone) that encodes the carboxy-terminal 213 amino acids of the open reading frame in the P2 cDNA was fused with the truncated β-galactosidase gene in the three members of the pWR590 series of expression vectors (Guo et al., 1982). Only the pWR590-2 construct produced a larger fusion protein when a pollen cDNA fragment was inserted. This result confirms the open reading frame predicted from the P2 DNA sequence. Figure 6

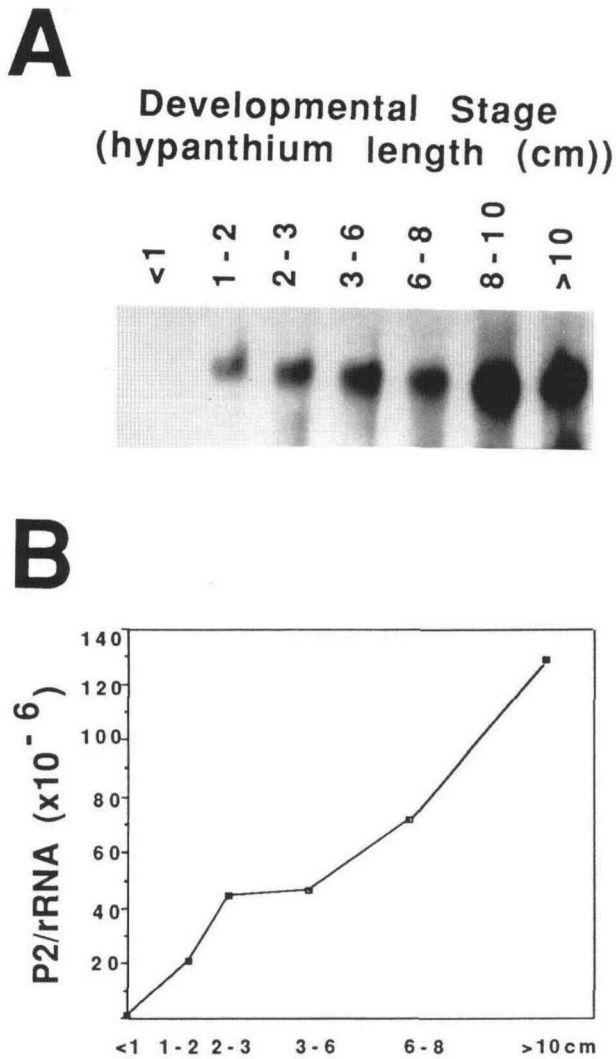

Figure 5. Expression of P2 mRNAs during Pollen Development.

(A) Four micrograms of total RNA from various stages of pollen development (as indicated by the hypanthium length of the floral bud) were electrophoresed, blotted to a nylon membrane, and hybridized with radiolabeled P2 cDNA, as described in Methods. The final wash conditions were 0.1 × SSC, 65°C.

(B) P2 RNA expression was quantitated by RNA dot blots. Two micrograms of denatured total RNA from various stages of pollen development were spotted onto nitrocellulose filters. Replicate filters were hybridized with radiolabeled P2 cDNA. Dilutions (1/1000) of the RNA samples were also spotted and hybridized with a radiolabeled probe for ribosomal RNA. The DNA (pRE12) used to synthesize the probe includes both the 18S and 25S rDNA from radish (Delseny, Cooke, and Penon, 1983). Relative abundance was determined by scintillation counting of the spots. The values for each stage were determined by the relative level of hybridization to the P2 and rRNA probes and corrected to the absolute value of 0.013% P2/rRNA determined for mature pollen.

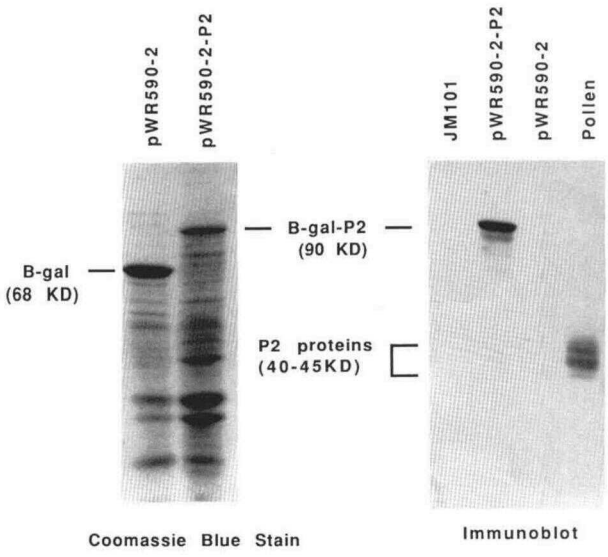

Figure 6. Identification of P2 Family Polypeptides in Pollen.

The left panel shows a Coomassie Blue-stained 8% SDS-polyacrylamide gel of the insoluble proteins from *E. coli* JM101 cells containing the pWR590-2 expression plasmid without an insert or with the 750-bp P2 insert (pWR590-2-P2). The right panel shows an immunoblot of the insoluble proteins from the host *E. coli* cells with no expression plasmids (JM101), containing the expression plasmid without an insert (pWR590-2), with the 750-bp P2 insert (pWR590-2-P2), and the soluble proteins from mature *O. organensis* pollen (pollen). The primary antibody was 1:500 preabsorbed P2 antiserum, the secondary was 1:2500 goat anti-rabbit horseradish peroxidase, and the cross-reacting bands were visualized with the substrate 4-chloro-1-napthol, as described in Methods.

shows the proteins expressed in *Escherichia coli* containing the pWR590-2 expression plasmid with and without the P2 insert. JM101 cells containing the pWR590-2 expression plasmid without insert produce a 68,000-D truncated β-galactosidase protein. The cells containing the pWR590-2-P2 expression plasmid produce a 90,000-D protein. The size of the fusion protein is consistent with the nucleotide sequence of the P2 cDNA plus the 600 amino acids contributed by the truncated β-galactosidase.

The 90-kD β-galactosidase/P2 fusion protein was gel purified and used for immunization. The resulting polyclonal antiserum showed strong cross-reactivity against β-galactosidase and other *E. coli* proteins. To remove the nonspecific antibodies, the antiserum was preabsorbed with proteins isolated from pWR590-2-containing JM101 cells. The predominant protein from these cells is the 68,000-D truncated β-galactosidase protein. The immunoblot in Figure 6 demonstrates that the preabsorbed antiserum reacts only with the β-galactosidase/P2 fusion protein, not with β-galactosidase moiety or with other *E. coli* proteins.

The preabsorbed antiserum was used to identify P2 proteins in extracts from mature pollen grains. As shown in Figure 6, the antiserum reacts with a group of proteins ranging in size from 40,000 D to 45,000 D. Several minor bands (35,000 D to 40,000 D) were also detected when more extract was loaded per lane. The relationships of the various polypeptides detected by the P2 antiserum were not examined.

To determine the timing of the appearance and accumulation of the P2 proteins during pollen development, extracts from immature pollen isolated at various stages of development were examined by immunoblot analysis. Figure 7 shows an immunoblot probed with the preabsorbed antiserum. The P2 antiserum did not detect the cross-reacting polypeptides in the 1-cm- to 3-cm-stage pollen, although P2 transcripts are detectable at this time (described above). The P2 proteins are first detectable at low levels in the 3-cm- to 7-cm-stage pollen, and increase in both the 7-cm- to 10-cm-stage and mature pollen. Interestingly, the various polypeptides do not accumulate coordinately throughout pollen development. The highest

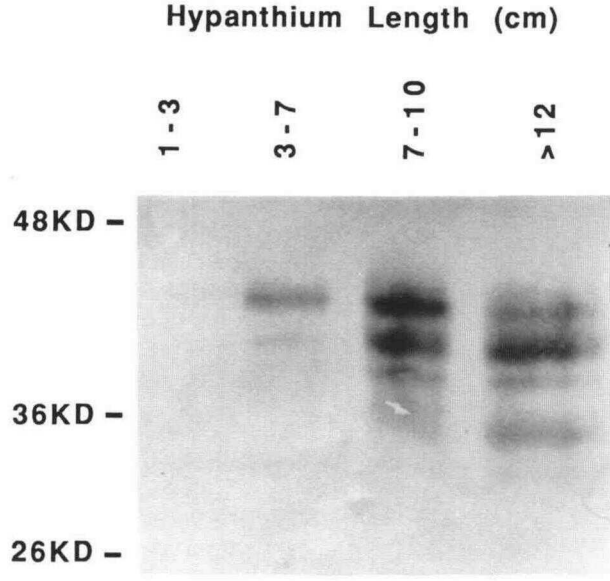

Figure 7. Expression of P2 Family Polypeptides during Pollen Development.

Forty micrograms of crude protein extract from various stages of developing pollen (as indicated by the hypanthium length of the floral bud) were electrophoresed through a 12% SDS-polyacrylamide gel and electroblotted onto nitrocellulose. The primary antibody was 1:1000 preabsorbed P2 antiserum; the secondary antibody and visualization were as described for Figure 6.

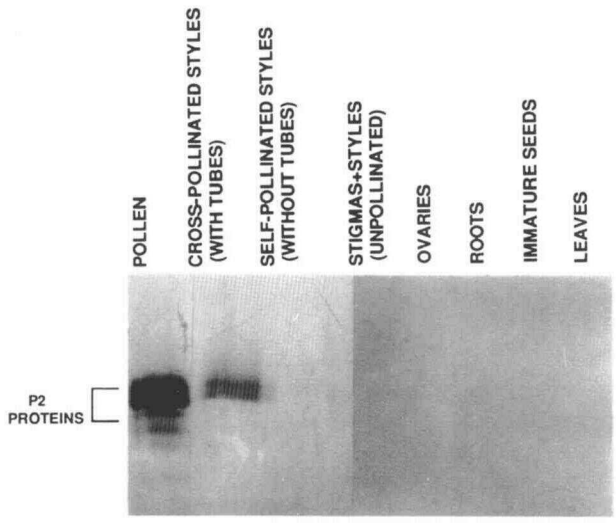

Figure 8. P2 Polypeptides Are Present Only in Pollen and Pollen Tubes.

For each lane, 40 μg of soluble protein from a part of the *Oenothera* plant were loaded onto a 12% SDS-polyacrylamide gel, electrophoresed, and electroblotted onto nitrocellulose. Antibody incubations and visualization were as described in Figure 6.

molecular weight species appears first in the 3-cm- to 7-cm-stage pollen, whereas the smaller polypeptides are not abundant until the later stages.

The antiserum was also used to characterize the presence of P2 proteins in various parts of the plant and during pollination. Figure 8 shows an immunoblot containing proteins extracted from a variety of parts of the plant. *O. organensis* is a self-incompatible plant, and pollen tubes are arrested in the stigmas of self-pollinated pistils and do not enter the styles. P2 proteins were found only in pollen and styles that contain pollen tubes as a result of cross-pollination, indicating that the P2 proteins are present in pollen tubes as well as in pollen grains, but not in other parts of the plant.

To determine the presence of P2 antigens in pollen tubes directly, pollen tubes grown in vitro were also examined. Pollen was germinated on solid culture medium, and a "print" was made by briefly placing a damp piece of nitrocellulose onto the germinating pollen. The pollen antigens bound to the filter were assayed by incubation with the P2 antiserum, followed by detection by gold-conjugated secondary antibodies and silver enhancement. Figure 9 demonstrates the presence of P2 antigens in pollen tubes grown in vitro. The P2 proteins are present throughout the entire length of the pollen tubes and do not appear localized in a specific region. It is not possible to determine by this technique whether the antigens are present in the pollen tube cytoplasm, in the wall, or in both.

P2 Homologs Are Expressed in Pollen in Other Plant Species

Figure 10 shows a blot containing RNA from mature pollen of several different taxa and probed with the P2 cDNA. A homologous transcript is present in all species examined. The sizes of the transcripts in the various species are similar to the 1.5-kb *O. organensis* P2 mRNA, but not identical. There are also some higher molecular weight species present in *A. vittata* and corn that hybridize to the P2 probe.

The P2 antiserum was used to probe immunoblots containing crude extracts of corn pollen proteins. The P2 antibodies detect two protein bands in corn pollen that are approximately 40,000 D and 42,000 D (data not shown). Several minor bands were also detected, including a 38,000-D band similar to the minor bands observed in *O. organensis* and two larger polypeptides (approximately 53,000 D and 100,000 D) that have no detectable counterparts in *O. organensis*.

DISCUSSION

We have demonstrated that a polygalacturonase-like gene family is highly expressed in maturing pollen grains of *O. organensis*. The temporal pattern of expression is not unlike that observed previously for genes expressed pri-

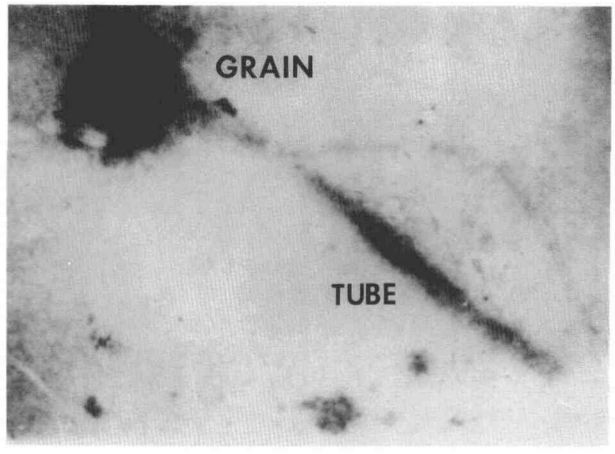

Figure 9. P2 Antigens Are Present in Pollen Germinating in Vitro.

Antigens from pollen germinating in vitro were "printed" onto nitrocellulose and detected by the P2 antiserum. Binding of the primary antibody was visualized by colloidal gold (10 nm) secondary antibodies and silver enhancement. This photograph shows a print of a single pollen grain and its tube. The P2 antigens appear in the grain and throughout the entire length of the tube.

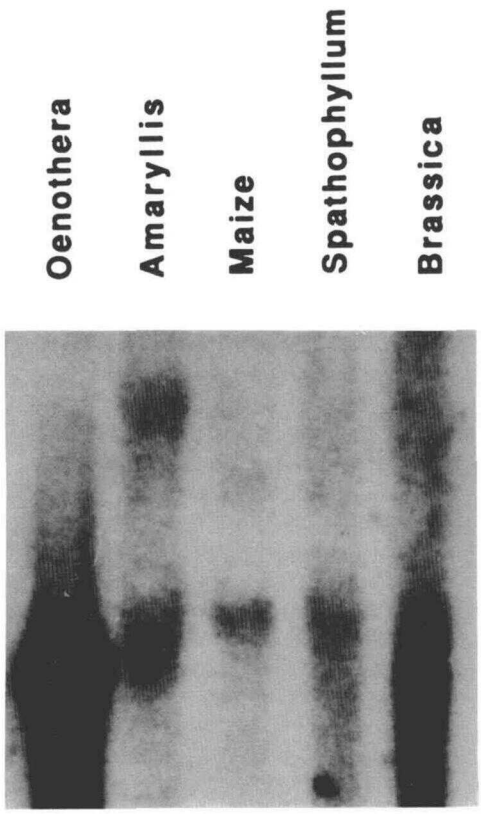

Figure 10. RNA Gel Blot of Homologous RNAs in Other Plant Species.

One microgram of total RNA from mature *Oenothera* pollen and 10 μg of total RNA from mature *Amaryllis vittata*, maize (*Zea mays*), *Spathophyllum clevelandii*, and *Brassica napus* pollen were electrophoresed through a 1% formaldehyde gel and blotted to a nylon membrane. The blot was hybridized with a radiolabeled P2 probe under permissive conditions (hybridization and final wash T_m −36°C). The film shown was exposed for 4 weeks, with intensifying screens, at −70°C.

marily in pollen. Pollen-specific mRNAs from corn and *T. paludosa* begin to accumulate after the pollen mitosis and their levels peak in mature pollen grains (Stinson et al., 1987). This temporal expression pattern was also observed for an unrelated *Oenothera* pollen cDNA (P3) that was isolated in the differential screen that identified the P2 cDNA. However, all pollen-specific genes are not coordinately regulated; a third pollen cDNA (P6) isolated in this screen does not accumulate to detectable levels until just before anthesis, several days after expression of the P2 gene family is first observed (S.M. Brown and M.L. Crouch, manuscript in preparation).

The P2 gene family was estimated to consist of six to eight genes per nucleus, unlike pollen-expressed genes in other species shown to be present in single or very low copy numbers (Stinson et al., 1987; Hanson et al., 1989; Ursin et al., 1989). Six characteristic combinations of restriction sites were found in the cDNAs, suggesting that we have isolated clones representing at least six different transcripts. The simplest interpretation is that the different cDNA clones represent transcripts from different genes. If this is true, most or all of the genes in the P2 family are expressed in mature pollen. Four clones had restriction patterns found only once in the 14 cDNAs; two other cDNA classes were isolated five times each. The isolation of different numbers of clones in each cDNA class suggests that the mRNAs produced from the various gene family members might be present at different levels of abundance in mature pollen.

The protein products of the P2 gene family were identified by a polyclonal antiserum raised against a β-galactosidase/P2 fusion protein synthesized in *E. coli*. The P2 family of polypeptides is first detectable in flowers with 3 cm to 7 cm hypanthium lengths, later than the period when the mRNAs are first detectable. This lag in accumulation may simply reflect the sensitivity of RNA gel blot analyses versus immunoblot analyses. Alternatively, the lag may indicate translational or post-translational regulation. It is interesting that the various polypeptides do not accumulate coordinately. The relationship of the various polypeptides detected by the P2 antiserum is not clear. The different polypeptides may be the products of different members of the P2 gene family. Alternatively, the different polypeptides could be the result of post-transcriptional changes or post-translational changes such as differential protein processing, protein degradation, or variations in glycosylation. Transcripts from P2 homologs were present in pollen from a wide variety of species. *O. organensis* is a dicot with bicellular pollen grains. The other species examined included a dicot with tricellular pollen (*B. napus*), a monocot with tricellular pollen (corn), and a monocot with bicellular pollen (*A. vittata*). The fact that the homologous transcripts could be detected in such diverse taxa suggests that polygalacturonase-like genes are conserved and may be expressed in the pollen from most angiosperm species.

Hanson et al. (1989) noticed that the consensus AATAAA was located an abnormally long distance (180 bases) upstream of the site of polyadenylation in the pollen-specific cDNA Zmc13. The maize alcohol dehydrogenase gene (*ADH1*), which is expressed in pollen as well as in other parts of the plant, also has the AATAAA a long distance from the site of addition (Sachs et al., 1986). The distance between the AATAAA motif and the polyadenylation site in the P22 sequence is 126 bases. However, other cDNAs in the P2 family, such as P1 and P2, do not have this feature; the distance between the consensus AATAAA motif and the site of polyadenylation is 19 and 18 bases, respectively. The distance between the AATAAA motif and the addition site in most plant mRNAs is 27 ± 9 nucleotides (Joshi, 1987).

Complementary DNAs representing the P2 gene family show extensive sequence similarity to the enzyme polygalacturonase (PG: poly(1,4-α-D-galacturonide)glycanohydrolase, EC 3.2.1.15), which has been studied during fruit ripening in tomato. Polygalacturonase cDNA clones have been isolated by several groups, and the sequences of two PG cDNA clones have been determined (Grierson et al., 1986; Sheehy et al., 1987). The similarities of the predicted amino acid sequences from the tomato fruit and the pollen cDNAs appear throughout the lengths, but are especially striking in a few regions.

The products of the P2 gene family have several other similarities to the PG gene products. The mRNA corresponding to the P22 cDNA is 1531 nucleotides long (excluding the polyA tail) as determined by DNA sequence and primer extension analyses (data not shown). This length is similar to the 1.6-kb size of the tomato PG mRNA. The PG mRNA is very abundant when at its maximum level in ripe tomato fruit, where it constitutes 1.2% of the mRNA (Bennett and DellaPenna, 1987). P2 mRNAs are at a similarly high level (0.013% of total RNA) at their peak in mature *O. organensis* pollen. The mature tomato PG protein is predicted to be 42,000 D and appears to be approximately 46,000 D in size on SDS-PAGE, a size similar to that for the P2 family of pollen proteins, which appear to be 40,000 D to 45,000 D on SDS-PAGE.

One major difference between the P2 family and PG gene in tomato is their genome organization. The tomato genome appears to have a single copy of the PG gene (DellaPenna, Alexander, and Bennett, 1986), whereas the *O. organensis* P2 gene family was estimated at approximately six to eight copies by DNA gel blot analysis. It is possible that multiple PG genes also exist in the tomato genome, but were not detected on DNA gel blots with the fruit cDNA probe because they have diverged significantly from the gene that is expressed in fruit.

The role of PG in tomato fruit is thought to be digestion of the middle lamellar region of cell walls, which may contribute to softening of the fruit during the ripening process. Polygalacturonase activity has been described recently in the pollen of a number of grass species, including maize (Pressey and Reger, 1989). It is likely that the P2 family of proteins has polygalacturonase activity, given their extensive similarities to the tomato PG protein. If so, the proteins may function by depolymerizing pectin in the cell walls of the pistil during pollination to allow penetration by the pollen tubes and/or to provide wall precursors for the rapidly growing tube. Another possible function of pollen PG is to act on its own wall to facilitate cell elongation. In plant/pathogen interactions, polygalacturonase activity has been shown to release pectic wall fragments that, when added exogenously, are capable of stimulating physiological changes such as ethylene biosynthesis, proteinase inhibitor I, and phytoalexin activation (Giovannoni et al., 1989). Release of oligosaccharides may also be important in pollen/pistil interactions. The presence of the P2 proteins in pollen tubes is consistent with these hypotheses. We are currently assaying the P2 proteins to determine whether they have polygalacturonase activity and whether they can degrade the pistil cell walls.

METHODS

Plant Material

Oenothera organensis Munz (Emerson) plants (kindly provided by Dr. Adolph Hecht, Washington State University) were grown in a greenhouse using supplemental lighting (mercury vapor or incandescant lamps at 100 to 200 $\mu Em^{-2}sec^{-1}$, 4 AM to 8 AM and 4 PM to 8 PM) to extend the natural daylength to 16 hr. Temperatures during the tissue collection periods usually ranged from 65°F to 82°F. All tissue was collected from a population of plants vegetatively propagated from a single individual.

Mature pollen was collected from *O. organensis* flowers in the evening, just as the flowers opened, using a suction apparatus consisting of a Pasteur pipet attached to a water aspirator. Pollen from the dehisced anthers was trapped within the pipet by 4 layers of cheesecloth. Isolated pollen was immediately frozen in liquid N_2 and stored at −70°C.

Immature pollen at the appropriate stages as determined by hypanthium length (S.M. Brown and M.L. Crouch, manuscript in preparation) was isolated by gently disrupting anthers in 15% sucrose using a loose-fitting sintered glass homogenizer at 0°C. The majority of the anther tissue was removed by filtration through a single layer of cheesecloth. The immature pollen was pelleted away from remaining debris by centrifugation (<1000*g*, 4°C) and washed several times with 15% sucrose. The final pellet was frozen at −70°C until use.

Pollen from *Spathophyllum clevelandii*, *Amaryllis vittata*, and corn (*Zea mays*) was collected from greenhouse-grown plants and stored at −70°C.

RNA Isolation

Total RNA from all tissues was isolated by phenol extraction as reported by Finkelstein et al. (1985). RNA from *Brassica napus* (Topaz) mature pollen was a generous gift of R. Nolan. Poly(A)+ RNA was isolated from total RNA using oligo(dT)-cellulose (Sigma) according to Maniatis, Fritsch, and Sambrook (1982).

Construction of cDNA Library

An *O. organensis* mature pollen cDNA library was constructed by modifying published procedures (Huynh, Young, and Davis, 1985; Gasser et al., 1989). First-strand cDNA was synthesized from poly(A)+ RNA by incubating 20 ng/μL RNA; 20 ng/μL oligo(dT)12–18 (Pharmacia LKB Biotechnology Inc., Piscataway, NJ); 50 mM Tris-HCl, pH 8.3, 40 mM KCl, 8 mM $MgCl_2$, 40 mM DTT, 0.5 mM each of four deoxynucleotide triphosphates, and 0.6 units/μL reverse transcriptase (Seikagaku) for 90 min at 42°C. Homopolymer dG tails were added to the purified products of the first-strand synthesis in a reaction mixture consisting of 90 mM Na-

cacodylate, pH 7.0, 0.9 mM dGTP, 1.8 mM $COCl_2$, and 50 units/25 μL terminal transferase (Boehringer-Mannheim, Indianapolis, IN) at 37°C for 2 hr. The reaction was terminated by the addition of an equal volume of 10 mM Tris-HCl, pH 7.2, 4 mM EDTA, followed by treatment with RNase A (0.25 μg/50 μL) to destroy the mRNA template. The oligo(dC)-primed second-strand synthesis reaction contained 0.6 μg of oligo(dC)12–18 (Pharmacia), 35 mM $MgCl_2$, 0.3 mM each of four deoxynucleotide triphosphates, and 40 units/60 μL Klenow fragment of *E. coli* DNA polymerase I (New England Biolabs, Beverly, MA) and was incubated for 2 hr at 37°C. The duplex cDNA was methylated without further purification using EcoRI methylase (New England Biolabs), and the methylated cDNA was repaired with T4 DNA polymerase (Bethesda Research Laboratories, Gaithersburg, MD) under standard conditions (Maniatis et al., 1982) to produce blunt ends. EcoRI linkers were ligated to the double-stranded, methylated cDNA using T4 DNA ligase (New England Biolabs) under standard conditions (Bethesda Research Laboratories). The products were digested with an excess of EcoRI and the cDNA, then size selected and purified by electrophoresis in low-melting-point agarose (SeaPlaque, FMC). cDNA fragments between 0.5 kb and 12 kb were excised from the gel and purified by extraction and Elutip chromatography (Schleicher & Schuell, Keene, NH).

Approximately equimolar ratios of the size-fractionated cDNA and λgt10 arms (Stratagene, La Jolla, CA) were ligated as above and packaged in vitro using Gigapack Gold (Stratagene) according to the manufacturer's instructions.

Isolation of Pollen-Abundant Clones

^{32}P-labeled cDNA probes from poly(A)+ RNA were prepared using a random priming technique as described by Gasser et al. (1989) and used to perform a differential screen of the pollen library. In the primary screen, the unamplified library was probed (Maniatis et al., 1982) with radiolabeled cDNA prepared from RNA of either mature pollen or 10-day-old seedlings. cDNA clones that hybridized more strongly to the pollen probe than to the seedling probe were subjected to a secondary screen with cDNA probes from either pollen or immature floral bud RNA. The immature buds were collected at least 2 weeks preanthesis and, therefore, included premeiotic anthers. P2 was chosen for further analysis because of its strong hybridization to the pollen probe and lack of hybridization to the seedling or immature floral bud probes. The 1.2-kb EcoRI insert was subcloned into a plasmid vector (Bluescript, Stratagene) for further analysis.

Genomic DNA Gel Blot Analysis

Nuclear DNA was isolated as described by Scofield and Crouch (1987). *Oenothera* genomic DNA was digested with restriction endonucleases, separated by electrophoresis, and transferred to nitrocellulose (Maniatis et al., 1982). Prehybridization was performed in 5 × SSPE (1 × SSPE = 0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA), 1 × PE [50 mM Tris-HCl, pH 7.5, 0.1% sodium pyrophosphate, 5 mM EDTA, 1% SDS, 0.2% PVP (40,000 D), 0.2% Ficoll (40,000 D), 0.2% BSA], 10% dextran sulfate, 50 μg/mL DNA (salmon sperm), and 50 μg/mL tRNA yeast at 65°C for 4 hr to 12 hr. Hybridization was performed at 65°C for 12 hr to 16 hr in the fresh prehybridization solution. Hybridization probes were prepared by random oligonucleotide priming (Feinberg and Vogelstein, 1983; Hodgson and Fisk, 1987) or nick translation (Maniatis et al., 1982). Final wash conditions were 0.1 × SSPE, 0.1% SDS at 65°C.

Copy number standards were prepared by calculating the amount of cDNA insert that would correspond to a single copy gene in the *Oenothera* genome [2C = 3.04 pg, as determined by flow cytometry of nuclei from seedlings (D. Galbraith, personal communication)]. The appropriate amount of each subclone was digested with EcoRI to release the cDNA insert from its plasmid vector, added to sheared salmon sperm DNA, then electrophoresed, blotted, and probed along with the *Oenothera* genomic DNA.

RNA Analysis

Total RNA was initially quantitated spectrophotometrically. If the spectrophotometric A_{260}/A_{280} ratio was less than 1.7 (as it frequently was with *Oenothera* tissues other than pollen), the RNA was also quantified using formaldehyde gel electrophoresis (Maniatis et al., 1982) or a dot assay modified for use with RNA (Sharp, 1985). The dot assay was also used to confirm the spectrophotometric quantitation of poly(A)+ RNA. Concentrations of total RNA samples for dot-blot or RNA gel blot analysis were confirmed using an rDNA clone (pRE12, Delseny et al., 1983) as a hybridization probe for dot-blot analysis.

RNA gel electrophoresis was performed in the presence of formaldehyde essentially as described by Maniatis et al. (1982), and the RNA was transferred to nylon membranes (GeneScreen, Du Pont-New England Nuclear, Boston, MA) according to the manufacturer's instructions. RNA dot-blot analysis was performed as described by Finkelstein et al. (1985). Prehybridization and hybridization conditions were similar to those described for genomic DNA gel blots except that 50% formamide was included and the temperature was 42°C. RNA dot blots were quantitated by excision of the individual dots and liquid scintillation counting.

Nucleotide Sequencing

DNA sequencing was carried out by the dideoxynucleotide method using a kit containing modified T7 DNA polymerase (Sequenase, United States Biochemicals, Cleveland, OH) according to the manufacturer's instructions. Plasmids for double-stranded templates were prepared as described by Maniatis et al. (1982). Preparation of single-stranded templates was performed as recommended by Stratagene. The sequence of both strands was obtained for the majority of the sequences presented. In those regions where both strands were not sequenced, single-strand sequence was obtained from at least two different overlapping subclones. Computer analysis of sequence information was performed using the University of Wisconsin Genetics Computer Group programs (Devereaux, Haeberlil, and Smithies, 1984).

Construction of Expression Plasmid

The P2 expression plasmids were constructed by inserting a portion of the P2 cDNA into each of the three members of the

pWR590 series of expression plasmids constructed by Wu and colleagues (Guo et al., 1982). JM101 cells harboring the fusion constructs were grown 8 hr to 12 hr at 37°C in 5 mL of 1XYT (8 g/L tryptone, 5 g/L yeast extract, 2.5 g/L NaCl) with ampicillin (100 μg/mL). For large preparations, 500 mL of 1XYT/ampicillin was inoculated with 1 mL of the freshly grown cells and cultured for another 8 hr to 12 hr. Isopropyl β-thiogalactopyranoside was not required for induction of the fusion proteins. Freshly grown cells were pelleted by centrifugation, the pellet resuspended in TE plus lysozyme [10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 20 μg/mL lysozyme (Sigma, St. Louis, MO)], and incubated for 20 min to 25 min at 37°C. The cells were then lysed by passage through at least seven cycles of freezing (dry ice/ethanol) and thawing (37°C) or by sonication. The lysate was cleared by centrifugation, and the pellet, which contained the fusion proteins, was resuspended in 2 × Laemmli buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 5% SDS, 10% β-mercaptoethanol, 1 mL/100 mL of culture). The suspension was heated at 85°C to 100°C for 30 min, the insoluble material removed by centrifugation (4°C, 10,000*g*, 10 min), and the resulting supernatant used immediately or stored at −70°C.

To purify the fusion protein, 1 mL of the fusion protein preparation was electrophoresed on a preparative 6.5% polyacrylamide gel. The Coomassie Blue-stained fusion protein band was excised from the gel and frozen at −70°C until use. Acrylamide slices were pulverized and resuspended in 2 ml of TBS (50 mM Tris-HCl, pH 7.5, 200 mM NaCl), and the resultant slurry was injected without further modification for the initial immunization. Subsequent boosts were mixed with an equal volume of Freund's incomplete adjuvant. A total of approximately 150 μg of fusion protein was injected subcutaneously at several sites in the hindquarters of New Zealand White rabbits. The animals were injected at 2-week to 4-week intervals and blood was collected 5 days after each injection.

To remove nonspecific antibodies from the crude antiserum, the antiserum was preabsorbed against protein extracts from JM101 cells containing the pWR590-2 expression plasmid without the P2 insert. Extracts were prepared from these cells as described above and diluted 10-fold in TBS. Nitrocellulose discs (80 mm) were incubated 1 hr to 2 hr in the diluted protein solution and then air dried. The discs were incubated for 1 hr in BLOTTO (5% nonfat dry milk in TBS, 0.01% merthiolate) and then four to eight discs were incubated in 20 mL of crude antiserum (1:500 in BLOTTO) for a total of 24 hr to 48 hr. The removal of nonspecific antibodies was confirmed by immunoblot analysis.

To prepare protein extracts, *O. organensis* pollen, isolated as described above, was homogenized in TBS containing 1 mM phenylmethylsulfonyl fluoride using a tight-fitting sintered-glass homogenizer (Kontes, setting 2 on Tri-R electric motor) at 0°C. The insoluble pollen walls and cellular debris were pelleted (15 min, 4°C, 15,000*g*). The supernatant was collected and used immediately or stored at −70°C. For protein extracts from other *O. organensis* organs [young leaves, stigma/styles, ovaries, roots, and immature seeds (14 days post-fertilization)], material was excised from the plant and immediately frozen in liquid N_2. The frozen tissue was pulverized to a fine powder in a ceramic mortar and pestle in liquid N_2 and then thawed in 5 mL/g of tissue TBS with 1 mM phenylmethylsulfonyl fluoride plus 0.2 g/g of tissue PVP (Sigma) and stirred at 0°C for 5 min. The insoluble cellular material was pelleted by centrifugation (4°C, 15,000*g*) and the supernatant stored at −70°C until use. Protein concentrations were determined using a Bio-Rad assay kit.

Immunoblot Analysis

SDS-PAGE was performed essentially as described (Laemmli, 1970), and the proteins were then electroblotted to nitrocellulose membranes in 20% methanol, 0.19 M glycine, 0.025 M Tris, pH 8.3 (Transblot, Bio-Rad, Richmond, CA). Immunodetection procedures were performed at room temperature and with agitation. The filters were blocked by incubation in BLOTTO for 2 hr to 3 hr and then incubated 8 hr to 12 hr in antiserum (1:500 in BLOTTO). The blots were washed in several changes of TBS with 0.05% Tween 20, incubated for 2 hr with goat anti-rabbit IgGs coupled to horseradish peroxidase (Boehringer-Mannheim, 1:2500 in BLOTTO), and finally washed as before. Antibody binding was visualized using 4-chloro-1-napthol (Sigma).

Antigens from in vitro germinating pollen were blotted to nitrocellulose (pollen printing) by incubation of pollen for 1 hr on agarose plates (Brewbaker and Kwack, 1963) at 20°C, followed by placement of water-dampened nitrocellulose filters atop the pollen tubes germinating on the agarose surface. The filters were removed from the plates after approximately 5 min of contact and air dried. The filters were blocked and probed as described above, except that the primary antibodies were detected by goat anti-rabbit secondary antibodies coupled to 10-nm colloidal gold particles (generously provided by D. Fernandez) and silver enhancement (Intense II, Janssen Life Sciences Products, Olen, Belgium).

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