Mol Gen Genet (1992) 234:379–389

MGG
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Brassica anther-specific genes: characterization and in situ localization of expression

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Received January 21, 1992

Summary. A cDNA library of *Brassica napus* (cv. Westar) was constructed using poly(A)$^+$ RNA isolated from developing anthers of flower buds 2–3 mm in length. Differential hybridization, using cDNA probes complementary to poly(A)$^+$ RNA from developing anthers or seedlings, was used for initial screening. In addition to Southern and Northern blot analyses of selected clones, RNA–PCR assays and in situ hybridization were used to study the temporal and spatial gene regulation in anthers at the transcriptional level. Five independent cDNA clones, showing no cross-hybridization to one another, were characterized, and their expression patterns could be grouped into three distinct categories. Two cDNA clones, BA112 and BA158, are tapetum-specific: the corresponding mRNAs accumulate in young anthers and decline as the tapetum cells degenerate later in anther development. The transcripts represented by BA54 and BA73 accumulate late in anther development and reach a maximum level in mature anthers prior to anthesis; BA54 has been confirmed to be pollen-specific. The third category, represented by BA42, is found to encode a protein sharing 64–67% amino acid similarity with chalcone synthase (CHS) from various plant species; the transcript is localized in the peripheral cells of the vascular bundle, tapetum, and developing microspores.

Key words: *Brassica napus* – Anther-specific – Chalcone synthase – *RNA–PCR* assay – In situ hybridization

Introduction

In angiosperms the formation of pollen, the male gametophyte, is a complex developmental process, involving intricate co-ordination of many cellular events in different cell types in the anther. Over the years, extensive studies have been made on various aspects of the process. More recently the advent of molecular genetic techniques has provided the incentive for much work on the cloning and identification of genes that are expressed during the development of the anther and pollen (Mascarenhas 1990). The molecular genetic studies are mainly directed toward discovering genes that are important for pollen or anther development. The identities of some of the cloned genes have been revealed and these are summarized by Gasser (1991).

One other major impetus for this kind of work comes from the desire to provide alternative methods for controlling plant male fertility in hybrid seed production; hence the initiation of the work described in this paper. *Brassica napus* was chosen as the experimental plant due to its extensive outcrossing, ease of genetic transformation, and the lack of a good method of hybrid seed production for this oilseed crop plant. It has recently been demonstrated that male sterility can be induced in tobacco (Koltunow et al. 1990; Mariani et al. 1990) and *Brassica* (Mariani et al. 1990) using a chimeric gene containing a tobacco tapetal cell transcriptional control sequence (TA29) in conjunction with various cell disruption genes.

The development of oilseed rape male and female gametophytes has been characterized and related to the morphology of the flower bud (Smith and Scarisbrick 1990). Several research groups have been studying gene expression in *Brassica* anther development, and tapetum-, developing microspore- and pollen-specific clones have been identified and characterized (Albani et al. 1990, 1991; Roberts et al. 1991; Scott et al. 1991; Theerakulpisut et al. 1991). Here we report the isolation of five unique *Brassica* anther-specific cDNA clones, which display features that distinguish them from those described in previously published reports. One of these clones shares strong homology with chalcone synthase (CHS) from various plant species.

Materials and methods

Plant material. Plants (*B. napus* cv. Westar) were grown in a walk-in growth chamber with an 18 h photoperiod

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at 24° C. Seedling RNA for differential screening and Northern blot analysis was obtained from 2 week-old plants including roots. Genomic DNA for Southern blot analysis was isolated from either seedlings or mature leaves. For RNA isolation, floral tissues were dissected from flower buds and frozen immediately in a liquid N_2 or a dry ice bath. The frozen tissues were stored at −80° C prior to RNA isolation. Anthers of different developmental stages were collected separately according to their bud sizes. Anthers used for cDNA library construction were dissected from buds of 2–3 mm in length. Bud length was measured from the base to the tip of the outermost sepal.

Genomic DNA and RNA isolations. Genomic DNA was purified using the CTAB method (Saghai-Maroof et al. 1984) followed by standard CsCl gradient centrifugation (Maniatis et al. 1982). Total RNAs of anthers and pistils used for Northern blot analysis (Fig. 3) were purified by the mini-scale hot phenol method (Verwoerd et al. 1989). Total RNAs described in Figs. 2 and 4 were isolated using the Pharmacia RNA extraction kit. Poly$(A)^+$ RNA was purified from total RNA extracted as above followed by two rounds of oligo(dT)-cellulose chromatography using a Pharmacia mRNA purification kit.

cDNA library construction and screening. Double-stranded cDNAs were synthesized from 1 μg poly$(A)^+$ RNA using a Promega riboclone cDNA synthesis kit and size-fractionated by Sepharose CL-6B spin column chromatography (Boehringer Mannheim). cDNAs eluted from the column were ligated to an *Eco*RI adaptor (Promega) and cloned into *Eco*RI-cleaved dephosphorylated λZAP II vector (Stratagene) before being packaged as lambda particles using Gigapack II Gold lambda packaging extracts (Stratagene). The total library contained 5×10^5 independent phage clones.

Phage clones were screened differentially, on duplicate plaque lift membranes (Bio-Rad), with $[^{32}P]$-labeled single-stranded cDNA probes prepared from either the anther poly$(A)^+$ RNA or seedling poly$(A)^+$ RNA using M-MLV reverse transcriptase (BRL). The reaction contained 50 mM TRIS-HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol (DTT), 3 mM $MgCl_2$, 1 mM each of dGTP, dATP, and dTTP, 100 μCi of $[\alpha\text{-}^{32}P]$dCTP (3000 Ci/mmol), 15 μg/ml oligo$(dT)_{12-18}$ or random primer, 20 μg/ml poly$(A)^+$ RNA, 1 unit/μl RNAsin (Promega), and 5 units/ml M-MLV reverse transcriptase. After 1 h incubation at 37° C, the reaction was extended by adding 0.2 mM cold dCTP and extra enzyme (1 unit/ml) for another 30 min at 37° C. The reaction was terminated and RNA template was degraded by incubating at 37° C for 15 min with the addition of 20 units/ml RNAse H. The labelled cDNA probe was then separated from unincorporated nucleotides on a Sephadex G-50 gel filtration spin column (5 prime-3 prime), and activity was quantitated by liquid scintillation counting. Routinely, 10^7–10^8 cpm were obtained from 1 μg poly$(A)^+$ RNA template, and 10^5–10^6 cpm/ml were used in the hybridization solution. Prehybridization, hybridization and washing procedures were performed according to the manufacturer's specifications (Bio-Rad). Film exposure times ranged from 24 to 72 h with Du Pont intensifying screens at −70° C. Once the positive λZAP II clones were identified, the pBluescript SK − plasmids corresponding to the λZAP II clones were rescued in vivo according to the procedures recommended in the Stratagene manual.

Northern and Southern blot analyses. Northern and Southern blot analyses were performed as previously described (Ausubel et al. 1987). Randomly primed $[^{32}P]$-labelled DNA probes were made using cDNA inserts purified from low melting agarose by the Geneclean method (Bio 101) as templates. cDNA inserts were either prepared as *Eco*RI fragments by restriction digestion or as polymerase chain reaction (PCR) fragments using both universal and reverse primers (94° C, 1 min; 37° C, 2 min; 72° C, 3 min; 30 cycles). The PCR method eliminated the need for preparation of plasmid DNA and restriction digestion. The PCR fragments were also suitable for making the riboprobes for in situ hybridization. The universal and reverse primers flanked the T7 and T3 promoter regions at either end of the cDNA insert. Therefore, antisense and sense (for control) probes could be produced in separate reactions using the same PCR fragment as the template. For the Northern blots shown in Fig. 3B and C, the previously hybridized probes were stripped from the blot for re-hybridization. The blot was incubated in 100 ml of 70% formamide, 0.1% SDS, and 0.25 mM Na_2HPO_4 buffer, pH 6.5, at 68° C for 1 h, and washed twice in 0.1 × SSC (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate) at 68° C for 30 min. Overnight autoradiography was performed to confirm complete removal of the probe.

RNA-PCR assay. RNA-PCR reactions were set up as follows using the thermostable DNA polymerase (Replinase; NEN Research Products, Du Pont). A standard 100 μl reaction contained 1 × Replinase buffer (50 mM TRIS-HCl, pH 9.0, 20 mM $(NH_4)_2SO_4$, and 1.5 mM $MgCl_2$), 0.2 mM dNTPs, 1 μM each of PCR primer pairs, 1 μg of total RNA or 25 ng poly$(A)^+$ RNA, 200 units of M-MLV reverse transcriptase (BRL), and 1.25 units of Replinase. The dNTPs and enzymes were added after heating the solution containing all the other reagents at 65° C for 5 min, and cooling on ice for 3 min. The reactions were carried out first at 42° C for 1 h, then PCR amplified for 50 cycles at 94° C for 1 min, 55° C for 1 min and 72° C for 1 min, and at the end of the 50 cycles, 7 min at 72° C allowed the extension of any unfinished termini. Following the discontinuation of production of Replinase by NEN Du Pont, the RNA-PCR kit with *Taq* DNA polymerase (Perkin Elmer Cetus) was used according to the manufacturer's instructions. The data presented in Fig. 4B were produced using Replinase, and the remaining data were obtained using *Taq* DNA polymerase.

The upstream (U) and downstream (D) primers used in the RNA-PCR reactions were as follows: BA42-U, 5′-GGAGCAGACCCTACAGAGTC; BA42-D 5′-CGTAAACCCAGACCCCACTC; BA54-U, 5′-GGACCTGACGGTCCACCC; BA54-D, 5′-CATCATTATGCA-

CTCACAC; BA112-U, 5′-CCTTCGATCTACATTGC-AGC; BA112-D, 5′-CCTGATGGTCTGTTACGTG-TAC; 135-U, 5′-GAAGCTGAGGACTGGGATG; 135-D, 5′-CTGGTAGCCTCTACTCTGAC.

In situ hybridization. In situ hybridization analyses with paraffin-embedded tissue sections were carried out as described by Cox and Goldberg (1988), except that the final wash was done at 55–60° C instead of 45–50° C. Routinely, 15–30 cross-sections of anthers were mounted onto each slide, and subjected to the same hybridization and washing treatments simultaneously. For materials from the same developmental stage, four slides were hybridized to the positive (antisense) probes, and two slides were hybridized to the negative (sense) probes as controls. Autoradiographs were developed following exposure for 2 weeks at 4° C.

Results

Identification of clones with predominant expression in anthers

A differential screening procedure was used to identify and isolate clones for genes with enhanced expression in anthers relative to expression in vegetative organs. Our λZAP II library derived from anthers of 2–3 mm *B. napus* flower buds (as described in the Materials and methods), was plated at low density, and duplicate plaque lifts were made from each plate. A total of about 20 000 clones were screened. One filter was hybridized with a ^{32}P-labeled single-stranded cDNA probe synthesized from the poly(A)$^+$ RNA that had been used to construct the library. The second filter was hybridized with a similar probe made from poly(A)$^+$ RNA from 2 week-old *Brassica* seedlings. The plaques showing substantially stronger hybridization to the anther cDNA probe than to the seedling cDNA probe were picked and plated at 100–300 pfu/plate. Subsequently, single plaques showing only hybridization signals with the anther probe were isolated, and plasmids (pBluescript SK-) were rescued in vivo (see the Materials and methods).

Final screening was done by Southern blot analysis. Plasmid DNAs purified from overnight cell cultures were

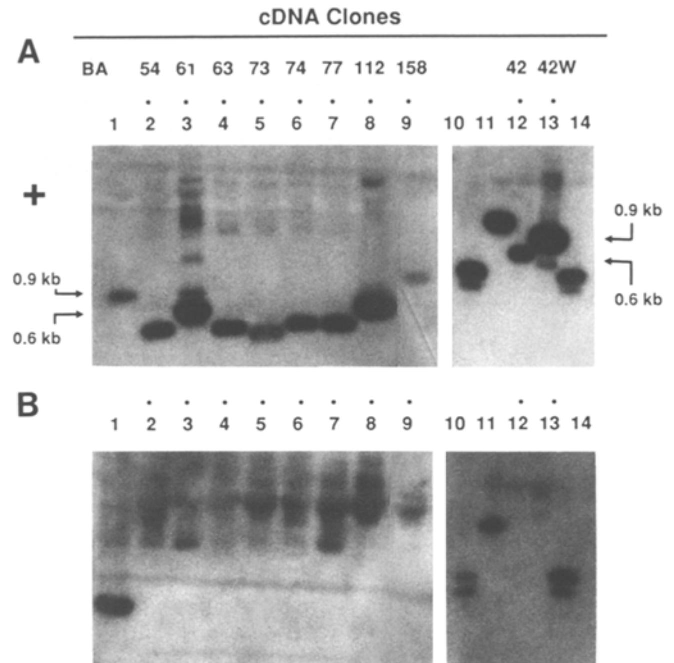

Fig. 1A, B. Southern blots probed with **A** anther or **B** seedling ^{32}P-labeled cDNAs. Positive anther-specific clones are indicated by *dots* above the corresponding lanes. The clones chosen for further characterization are numbered as indicated

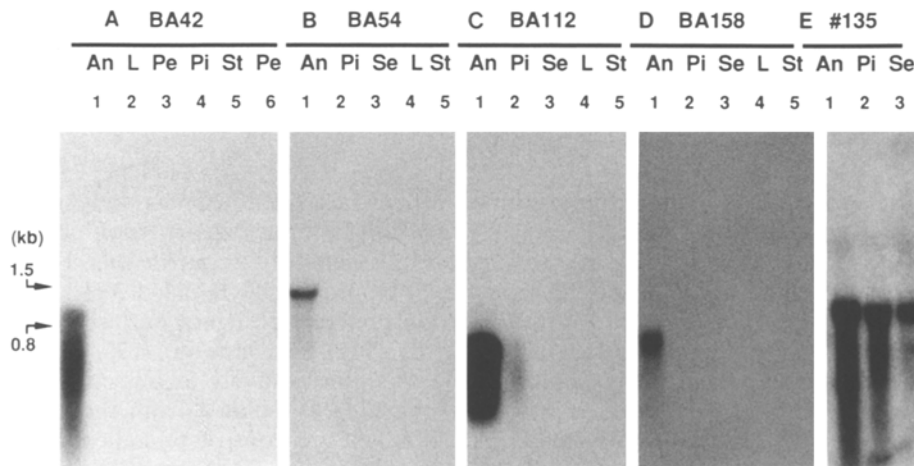

Fig. 2A–E. Presence of anther-specific mRNAs in floral tissues and vegetative organs. Total RNAs of *Brassica* anther (An), pistil (Pi), petal (Pe), leaf (L), stem (St), and seedling (Se) were fractionated on denaturing agarose gels, transferred to nylon membranes and probed with labeled cDNA inserts as described in the Materials and methods. Anther and pistil RNAs were isolated from flower buds of 2–3 mm in length whereas petal RNA was isolated from flowers at anthesis. Seedling RNA was isolated from 2 week-old plants including the roots. Leaf and stem RNAs were isolated from fully expanded young leaves and stems from plants at 4–5 weeks. Ten micrograms of total RNAs were used in each lane except for lane 6 of **A** in which 20 μg were used. The autoradiographs were exposed for 20 h *(A, C, D, E)* and 4 days (**B**) with Du Pont intensifying screens at −70° C

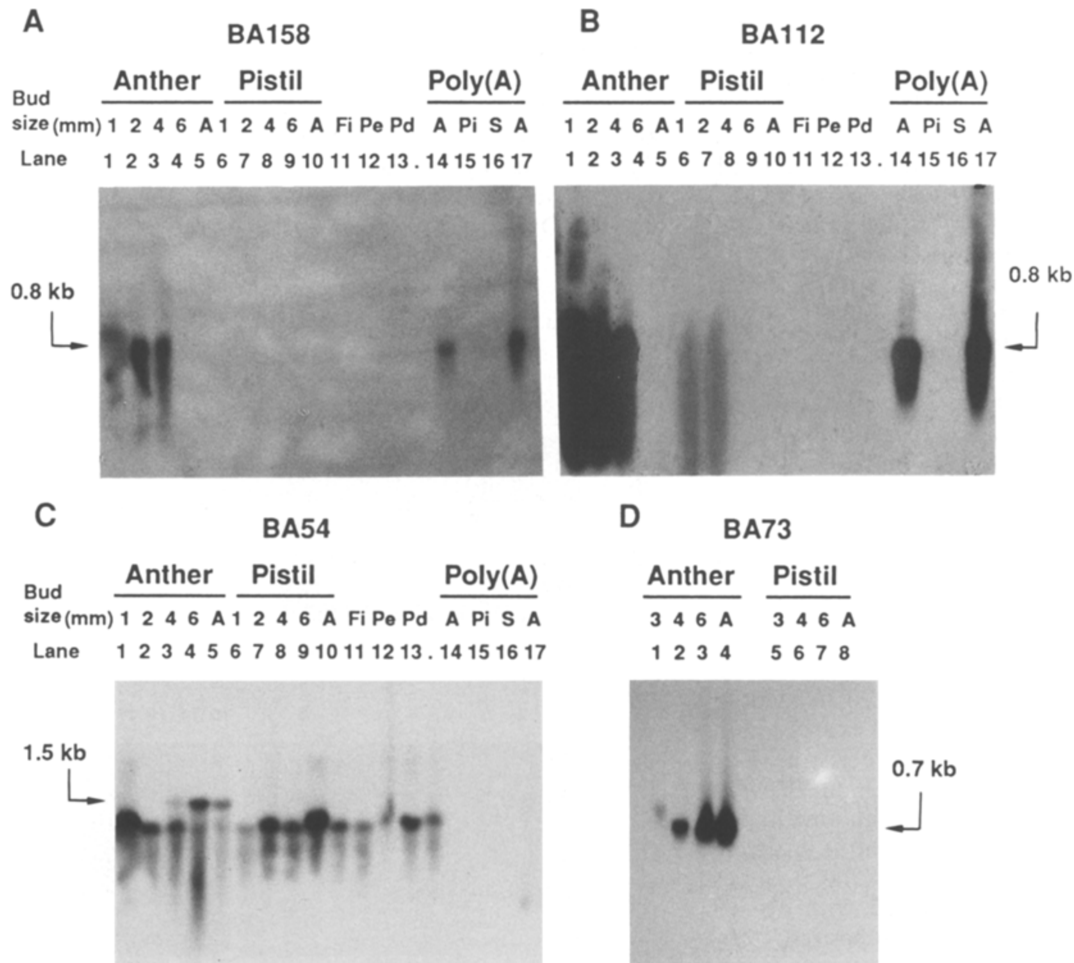

Fig. 3A–D. Northern blots probed with anther-specific cDNA clones. **A** RNA gel blot of total RNAs from the indicated tissues hybridized to randomly primed BA158 probe. Anthers and pistils were from: lanes 1 and 6, 1 mm (1), lanes 2 and 7, 2 mm (2); lanes 3 and 8, 4 mm (4); lanes 4 and 9, 6 mm flower buds (6); lanes 5 and 10, flowers at anthesis (A). Filaments (Fi), petals (Pe), and pedicels (Pd) were from flowers at anthesis. Poly(A)$^+$ RNAs of anthers and pistils were isolated from buds of 2–3 mm. Seedling RNA (S) was isolated as described in Fig. 2. Ten micrograms of total RNAs or 1 μg of poly(A)$^+$ RNA were used in each lane of the gel. Two lanes of anther poly(A)$^+$ RNA were purified from two separate batches (lanes 14 and 17). A lane (.) between lanes 13 and 14 was loaded with immature maize anther RNA. **B** The same blot re-hybridized to randomly primed BA112 probe after stripping of the original probe. **C** The same blot re-hybridized to BA54 riboprobe after stripping. **D** A different RNA gel blot hybridized to randomly primed BA73 probe. Anthers and pistils were isolated from: lanes 1 and 5, 3 mm (3); lanes 2 and 6, 4 mm (4); lanes 3 and 7, 6 mm (6) flower buds; lanes 4 and 8, flowers at anthesis (A). The autoradiographs were exposed for 20 h with Du Pont intensifying screens at −70° C

digested with *Eco*RI and fractionated on agarose gels. Duplicate DNA gel blots were hybridized with either anther or seedling cDNA probes. Ten positive clones, which showed the strongest preferential hybridization to labeled anther cDNAs, were identified and their cDNA sizes are as indicated in Fig. 1. BA42, BA54, BA73, BA112 and BA158 were identified as independent clones that did not show any cross-hybridization with one another (data not shown).

Tissue specificity and developmental regulation

Northern blot analyses were used to determine the tissue specificity of expression of the genes represented by the selected cDNA clones. The cDNA inserts prepared as *Eco*RI fragments or PCR products were used to make randomly primed probes (see the Materials and methods). Total RNAs isolated from various organs or tissues were fractionated on 1.2% denaturing agarose gels. Transcripts represented by clones BA42, BA54, BA112 and BA158 showed highly preferential, if not exclusive, expression in the anther (Fig. 2). Clone no. 135, whose corresponding gene was constitutively expressed in all *Brassica* tissues examined, was isolated from the same library and used as a positive control to indicate the quality and quantity of various RNA samples in the Northern blots and RNA–PCR analyses (Figs. 2 and 4).

Since only RNAs from anthers and pistils of a single developmental stage were used in the screening and preliminary characterization, the possibility was investigated that the isolated clones could be expressed also at other stages. Figure 3 shows Northern blots of anther and pistil total RNAs from different stages of flower

development, and total RNAs from filaments, petals and pedicels of newly opened flowers, hybridized to each of the indicated cDNA probes. A randomly primed probe of BA158 hybridized with transcripts in immature anthers, and the level of messages declined to an undetectable level in the mature anther. No hybridization was observed in other floral tissues (Fig. 3A). Since total RNAs in this gel were extracted from small quantities of tissues using the mini-scale hot phenol method, some degradation in the RNA samples was noticed (Fig. 3A–D). The anther poly(A)$^+$ RNA lanes showed a distinct 0.8 kb band hybridizing to the BA158 probe (Fig. 3A, lanes 14 and 17).

The same blot was re-hybridized to the labeled BA112 probe after the BA158 probe was stripped from the membrane (Fig. 3B: see the Materials and methods). The gene expression pattern was similar to BA158, but the expression level was much higher. The size of the hybridizing band was about 0.8 kb. No detectable signals were found in other floral tissues except for very weak hybridization in the pistils at early stages of development (Fig. 3B, lanes 6 and 7). Although the transcript size of BA112 was similar to BA158, these two clones did not cross-hybridize (data not shown), and no hybridization signal was detected in the pistil sample in the Northern blot probed with BA158, even when the autoradiograph was overexposed (data not shown).

The same blot used for BA158 and BA112 was stripped again and re-hybridized to the riboprobe made from BA54. The gene corresponding to BA54 was expressed at a low level in young anthers, and its message of 1.5 kb progressively increased in signal intensity as the anthers matured (Fig. 3C). This also explained why there were no detectable signals in the anther poly(A)$^+$ RNA lanes, since these poly(A)$^+$ RNA samples were purified from immature anthers of 2–3 mm flower buds in which the BA54 gene was just starting to be expressed (Fig. 3C, lanes 14 and 17). A strong signal of about 1 kb was observed across all of the lanes, which was later shown to be nonspecific. The background signal was always present whenever riboprobes were used, even under highly stringent hybridization [50% formamide, $5 \times$ SSPE ($1 \times$ SSPE is 0.18 M NaCl, 10 mM sodium phosphate, 1 mM EDTA pH 7.4) 52° C] and washing conditions ($0.1 \times$ SSC, 68° C for 1 h; data not shown). The poly(A)$^+$ RNA lanes did not show this background hybridization signal. Another Northern blot was hybridized to the randomly primed BA73 cDNA insert. The expression pattern of BA73 was similar to BA54, in that its transcript level increased through anther development and accumulated to the maximum level in the mature anthers. The transcript size of BA73 was about 0.7 kb (Fig. 3D).

RNA–PCR assay

An RNA–PCR assay was used to determine the abundance of anther-specific transcripts from various tissues sampled at different stages of flower development. Due to the high sensitivity of this technique, very low levels of expression can be easily identified (Rappolee et al. 1988). Partial DNA sequence data were obtained by the dideoxynucleotide termination method (Sanger et al. 1977). Clone-specific primers were synthesized and used in the assays. Only data obtained using highly purified RNAs (Pharmacia RNA extraction kit) are presented in Fig. 4A, C and D, although the RNA samples extracted from limited quantities of tissues using the hot phenol method (Verwoerd et al. 1989) produced similar results (Fig. 4B, lanes 1 versus 2, and 5 versus 6). No detectable products were observed by agarose gel analysis when the reverse transcriptase was not included in the RNA–PCR reactions, or when purified DNA (1 µg of genomic or 5 ng of plasmid DNA) was used as a template in the same PCR program (94° C, 1 min; 55° C, 1 min; 72° C, 1 min; 50 cycles) (data not shown). Both results suggest that the RNA–PCR products observed in Fig. 4 are not due to any DNA contamination. A separate PCR program (94° C, 1 min; 37° C, 2 min; 72° C, 3 min; 30 cycles) was conducted for the genomic (Dg) and plasmid (Dp) DNA templates shown in Fig. 4.

The RNA–PCR fragments of BA42 were only produced when young anther RNAs were used as templates, and no band of the expected size was observed in the assays of mature anther, pistil, petal and other vegetative organs (Fig. 4A). Two small fragments generated from RNA–PCR reactions of all samples except seedling RNAs were observed, which could represent either amplifications from other homologous gene products or nonspecific primer annealing. Using the genomic DNA as a template with the BA42-specific primers, more than one PCR product was generated, indicating that homologous genes are present in the genome (Fig. 4A, lane 11). Similarly, transcripts of BA54 were also anther-specific, since its RNA–PCR product was not detected in pistil or vegetative organs (Fig. 4B). In contrast to the results for BA42, when 1 µg of total RNA was used as the template in each reaction, mRNAs were more abundant in the mature anthers than in the younger anthers (Fig. 4B, lane 3 versus 1). More RNA–PCR product could be generated by using 25 ng of poly(A)$^+$ RNA instead of 1 µg of total RNA (Fig. 4B, lane 2 versus 1).

RNA–PCR fragments corresponding to BA112 were detected only in the RNA samples from young anthers and pistils (Fig. 4C). The transcript of BA112 disappeared in the pistil after flower buds grew to about 4 mm in length, but a relatively abundant level was still present in the same stage of the anther (Fig. 4C, lane 5 versus 2). A larger fragment was generated from the genomic DNA template, indicating that the BA112 primers used are located in two separate exons in the genome (Fig. 4C, lane 12). The gene corresponding to clone no. 135 was constitutively expressed in all tissues and organs examined (Fig. 4D). The RNA–PCR results of BA42, BA54, BA112 and 135 were all consistent with the data from Northern blot analyses (Figs. 2–4).

In situ hybridization analysis

^{35}S-labeled riboprobes of the anther-specific cDNAs were hybridized in situ to anther sections at various

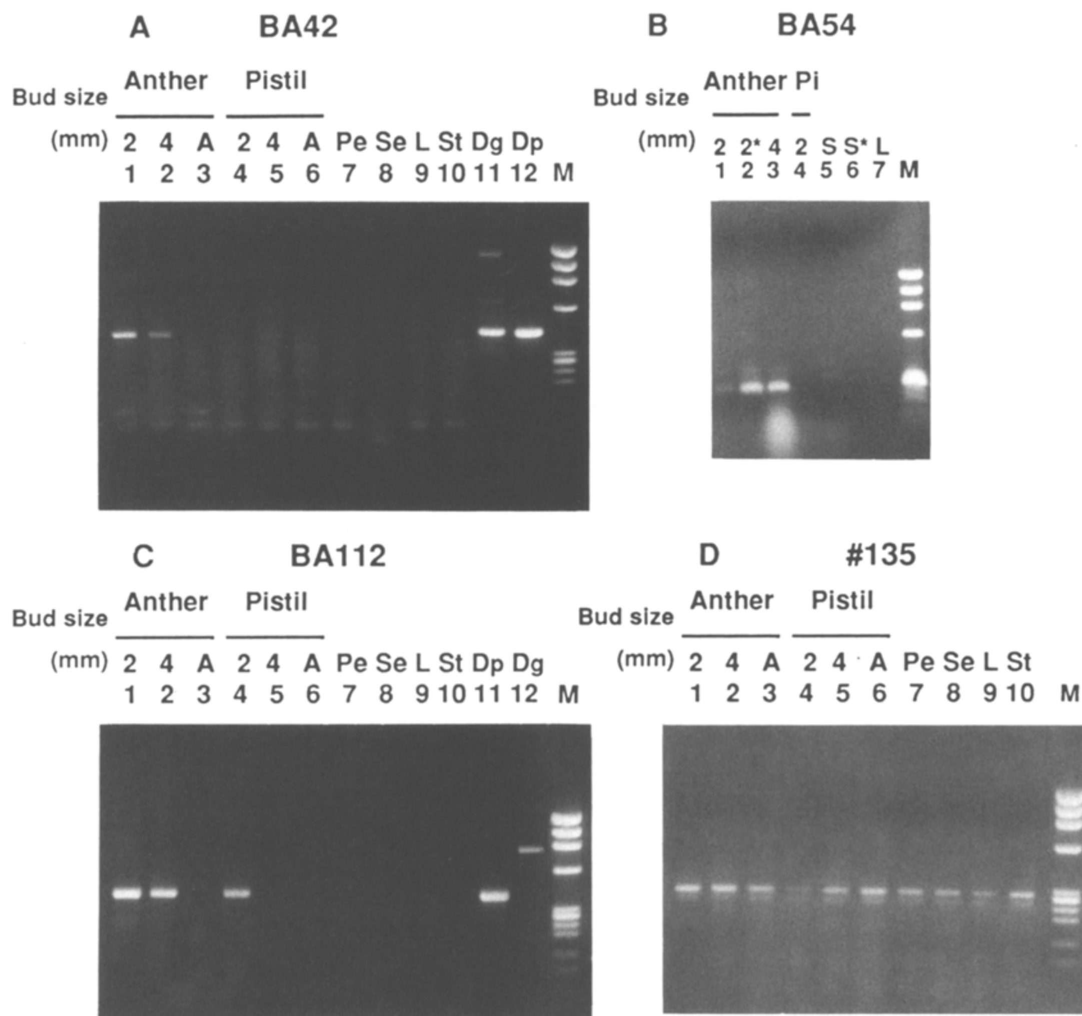

Fig. 4A–D. RNA–PCR assay of gene expression. Clone-specific primers were used in each set of reactions, as indicated. One microgram of total RNA or 25 ng of poly(A)$^+$ RNA (as indicated by *asteriks* in **B,** lanes 2 and 6 only) was used in the RNA–PCR assays. Lane symbols are the same as those in Figs. 2 and 3 with the following exceptions: lane M, mol. wt. markers; lanes Dg and Dp represent genomic (1 μg) and plasmid (5 ng) DNAs, respectively. Polymerase chain reaction (PCR) products were elctrophoresed on 1.5% NuSieve GTG/0.5% SeaKem agarose gels and stained with ethidium bromide

stages of development. Representative autoradiographs are shown in Fig. 5. Only evenly distributed background hybridization throughout the whole sections was detected with the control probes. Since no difference was observed among all controls, only representative photographs of the negative controls are shown in Fig. 5C and D. It should be noted that the dark field micrographs for control sections had to be exposed for 0.8 s in order to obtain reasonable photographs. Under the same conditions, only 0.01–0.04 s exposures were necessary to highlight the silver grains in the dark-field micrographs of antisense probed sections. As a result, the structural details of the anther sections were captured in photographs of the control (Fig. 5C), but not in the antisense probed sections (Fig. 5A, F, and H). The only exceptions were those sections hybridized to the BA158 probe, in which a longer exposure time (0.3 s) was necessary due to the weak expression resulting in a low hybridization signal (Fig. 5E).

The mRNAs homologus to BA112 (Fig. 5A and B) and BA158 (Fig. 5E) were clearly tapetum-specific. No significant signals were observed when the probes of BA112 and BA158 were hybridized to the mature anther sections where the tapetal cells were completely degenerated (data not shown). In contrast, the mRNA represented by BA54 was pollen-specific and was present in both developing and mature pollen grains (Fig. 5F, G and H). No significant hybridization signals were observed in the tapetal cells surrounding the developing pollen in young anther sections probed with BA54 probe (Fig. 5F). The cellular distribution of the mRNA represented by clone BA42 showed a different pattern, being localized in the peripheral cells of the vascular bundle, tapetum, and developing microspores (data not shown).

BA42 showing sequence homology to chalcone synthase (CHS)

The cDNA of BA42 was sequenced to completion using the dideoxynucleotide termination method of Sanger et

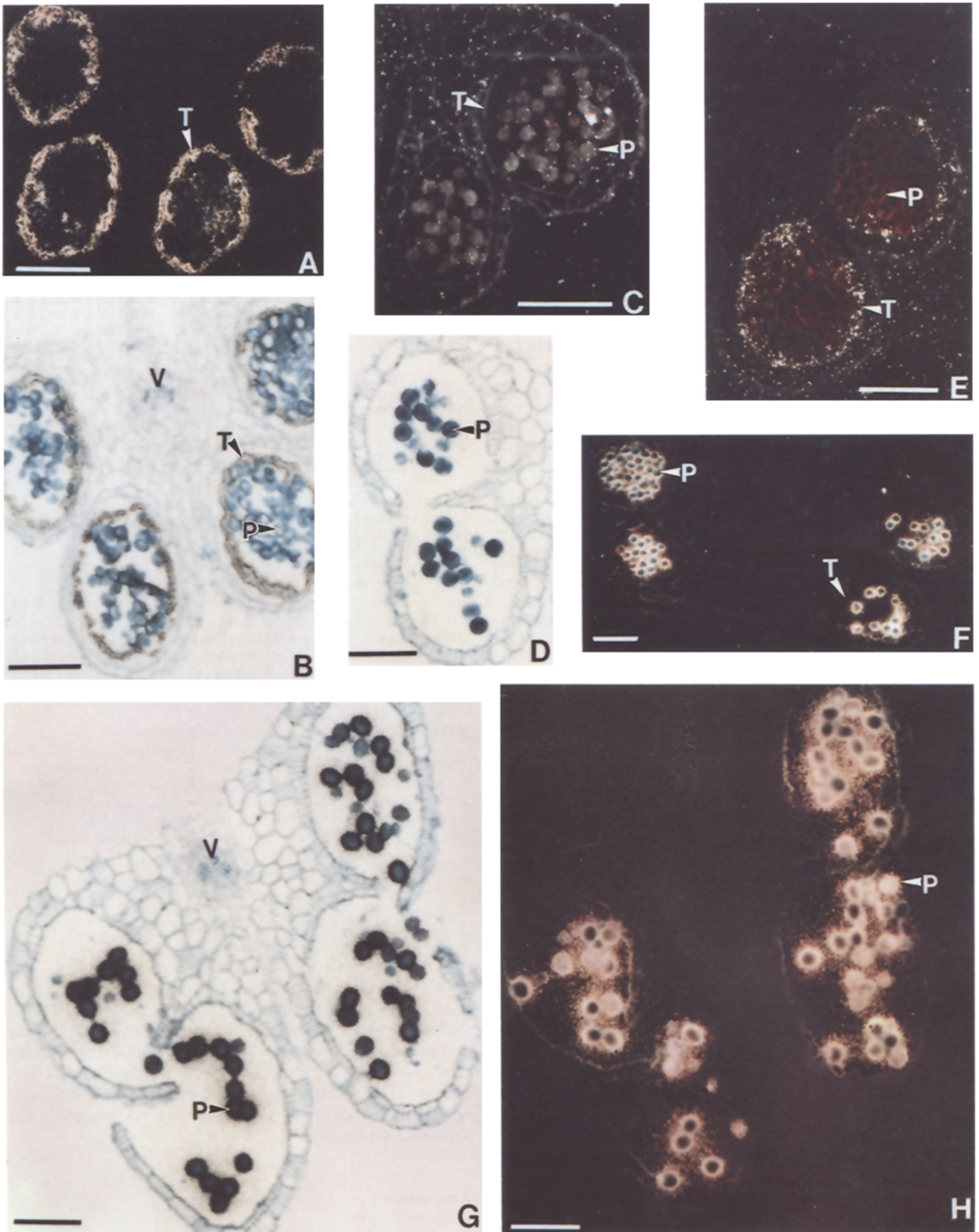

Fig. 5A–H. In situ localization of the BA54, BA112 and BA158 mRNAs in *Brassica* anthers. The black spots in the bright-field and the white spots in the dark-field photographs were silver grains resulting from hyridization of tissue mRNAs with the following probes. **A, B** Hybridization of BA112 antisense riboprobes to anther sections from flower buds of 2 mm. **C** Hybridization of BA112 sense riboprobes (control) to anther sections from 2 mm flower buds. **D** Hybridization of BA54 sense riboprobes (control) to anther sections from 5 mm flower buds. **E** Hybridization of BA158 antisense riboprobes to anther sections from 2 mm flower buds. **F–H** Hybridization of BA54 antisense riboprobes to anther sections from **F** 2 mm, and **G, H** 5 mm flower buds. P, pollen; T, tapetum; V, vascular bundle. Scale bar 0.1 mm

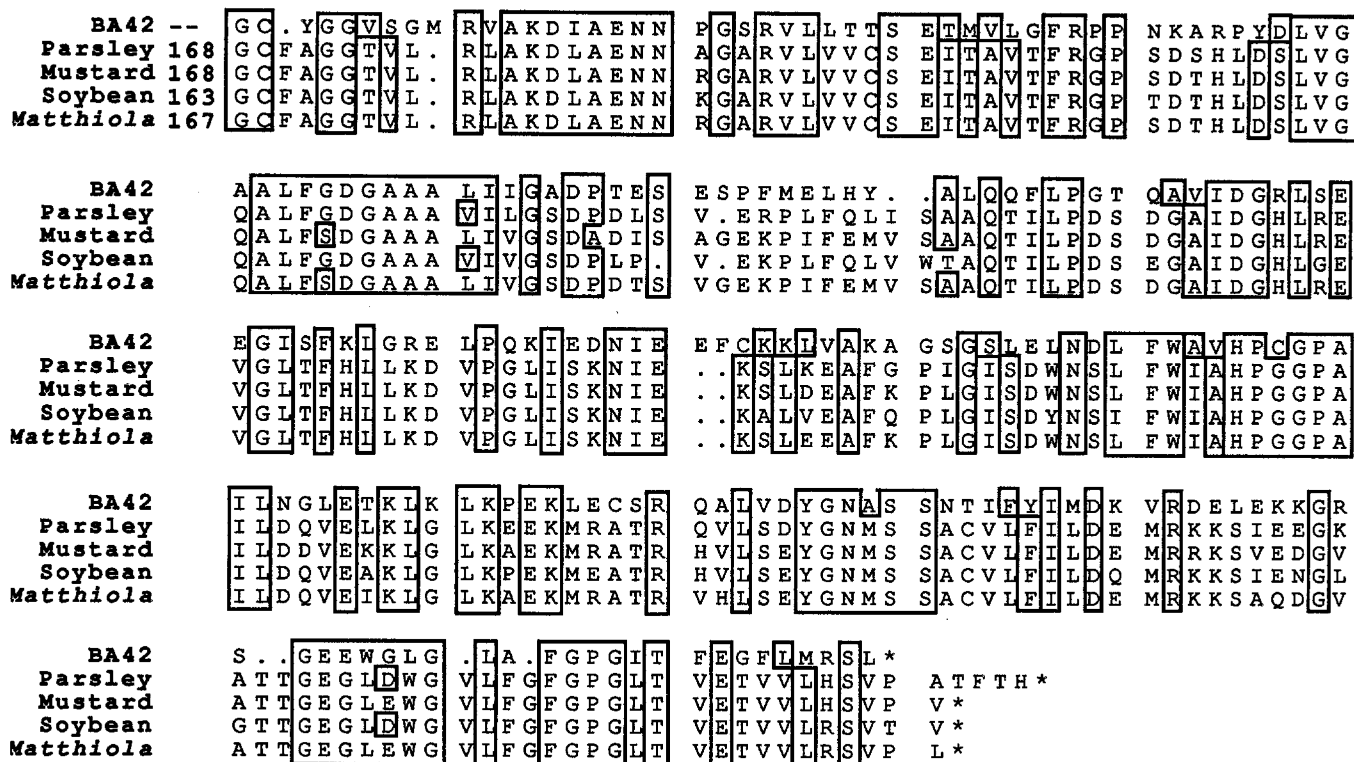

Fig. 6. Comparison of deduced amino acid sequences of BA42 and chalcone synthase (CHS) from parsley, mustard, soybean, and *Matthiola incana*. Amino acid gaps *(dots)* were introduced in the sequences in order to give the best sequence alignment. The position in the first residue of each protein sequence is indicated in the second column. Gly is the first residue deduced from the BA42 cDNA insert. Each sequence ends with a stop codon *(asterisk)*. These sequences are available in GenBank (parsley, v01538; mustard, × 16437; soybean, × 53958; *Matthiola*, × 17577) or from the authors (BA42, unpublished nucleotide sequence data)

al. (1977). The resulting sequence was compiled and analyzed using the computer programs of the University of Wisconsin Genetics Computer Group (Devereux et al. 1984; data not shown). BA42 was not a full-length cDNA clone as evidenced by the lack of a consensus translation initiation site, but unexpectedly, the nucleotide sequence shared significant homology with chalcone synthase (CHS) genes from soybean (52%), *Matthiola incana* (54%), parsley (55%) and mustard (55%) (data not shown). The comparison of the deduced amino acid sequence of BA42 and the CHS sequences is shown in Fig. 6. Although stronger homology was observed among the CHS sequences from the four species examined, the amino acid sequence of BA42 shared significant homology with them: 44% identity with parsley and mustard, 46% with soybean, and 45% with *Matthiola*. If conservative amino acid changes are considered (Pearson and Lipman 1988), the similarity between the BA42 amino acid sequence and CHS sequences increases to 64–67%. When gaps in the amino acid sequences were introduced to give the best alignment, striking regions of identical amino acids were observed throughout the whole sequence (Fig. 6). In these highly conserved regions, an inverted sequence change was found in the BA42 sequence located at 19–22 residues from the C-terminus. The consensus is -G-E-*G-L-E*/*D-W*-G-, while the amino acid order was changed to -G-E-*E-W-G-L*-G- in BA42 (Fig. 6). The cDNA inserts of BA54, BA73, BA112 and BA158 have been completely sequenced.

Table 1. *Brassica* anther-specific cDNA clones

Clone	cDNA (kb)	mRNA (kb)	In situ localization	Homology
1. BA42	0.78	1.0	Tapetum, periphery of VB, microspore	Chalcone synthase
2. BA54	0.45	1.5	Pollen	–
3. BA73	0.4	0.7	nd	–
4. BA112	0.58	0.8	Tapetum	–
5. BA158	1.0	0.8	Tapetum	–

VB, vascular bundle; nd, not determined

None of them shared significant homology with any published sequence in the GenBank (unpublished result). The main features of all five anther-specific cDNA clones: cDNA insert size, transcript size, cellular localizations, and sequence homology with known genes, are summarized in Table 1.

Discussion

Assays to determine tissue specificity

Southern blot, Northern blot, in situ hybridization and RNA–PCR analyses have been used to characterize the expression of several anther-specific genes at the steady-

state mRNA level. Southern blot analysis, often used as the last step of library screening, is the simplest and least sensitive among these methods. The transcripts of low abundance could be under-represented in the cDNA probe made from poly(A)$^+$ RNA, thus leading to difficulties in identifying positive clones representing the rare transcripts. In situ hybridization, in spite of being rather laborious, is the most sensitive technique with the added advantage of localizing transcripts at the cellular or subcellular level. Northern blots are the most commonly used method to determine tissue specificity; however, a major drawback is the significant amounts of RNA required to give detectable signals. In cases where material is difficult to obtain, the usefulness of Northern blots is correspondingly limited. RNA–PCR, also called cDNA–PCR (Kawasaki and Wang 1989), RT–PCR, or mRNA phenotyping (Rappolee et al. 1988), has been reported to be able to detect less than 100 molecules of RNA (Rappolee et al. 1988). It appears to be a good method to determine transcript tissue specificity, requiring only small amounts of material. In addition, multiple samples can be simultanously processed for RNA–PCR reactions.

In the RNA–PCR assays, 1 µg of total RNA was used, which was more than sufficient for the amplification of rare mRNA species (one or few copies per cell) (Kawasaki and Wang 1989). A proper control for RNA–PCR is crucial in order to be able to interpret data correctly. Instead of using an internal standard, we used a constitutively expressed clone (no. 135) as a positive control, and performed all of the assays using the same batch of RNA samples and of reaction mix solution in order to minimize handling variations. Also, each set of assays for different clones could be used as either positive or negative controls against one another due to their varying expression profiles.

The results of Northern blot analyses and RNA–PCR assays were entirely consistent (Figs. 2–4). BA112 mRNA appeared as a smear shadow in the Northern blot, and its low abundance in pistil made it even more difficult to detect. However, a correctly sized band generated in BA112 RNA–PCR assays gave unambiguous confirmation that BA112 mRNA was indeed present in the young but not mature pistil. From our results and those of others, RNA–PCR was undoubtedly superior to Northern blot analysis in ease, speed, sensitivity, and resolution (Rappolee et al. 1988). The only caution is that empirical tests may be needed for each set of primers to minimize nonspecific amplification (Kawasaki and Wang 1989).

Temporal and spatial regulation of the anther-specific genes

In this paper, one anther-predominant and four anther-specific cDNA clones from *Brassica* were characterized. The BA112 gene is expressed at a high level in young anthers as shown in Figs. 2C, 3B, and 4C, and localized in tapetal cells (Fig. 5A and B). The reason for its low abundance in the early stage of the pistil has not yet been determined. The mRNA of BA112 apparently disappears earlier from pistils than from anthers (Figs. 3B and 4C). BA158 mRNA was localized exclusively in tapetal cells (Fig. 5E). Although no attempt was made to quantitate the exact amount of transcripts, under the same experimental conditions using similarly labeled probes in several repetitive analyses, the transcript level of BA158 was always lower than that of BA112 (Figs. 2, 3, and 5). The tapetum-specific expression pattern of BA112 and BA158 was similar to that of the tobacco cDNA clones TA26, TA29 and TA32 (Koltunow et al. 1990). The absence of detectable expression of these tobacco anther genes at stage 6 was reported to coincide with the degeneration process occurring in the tapetum (Koltunow et al. 1990). The same is true for BA112 and BA158, probes of which did not hybridize to the mature anther sections where tapetal cells no longer exist (data not shown).

BA54 and BA73 transcripts were present in developing microspores and levels increase as pollen matures (Figs. 3, 4 and 5). In contrast, recently published results for *B. napus* genomic clones Bp4 (Albani et al. 1990) and Bp19 (Albani et al. 1991), and for five cDNA clones (Roberts et al. 1991; Scott et al. 1991) showed that the expression of these genes occurs early during microspore development, and the levels of the mRNAs decrease significantly during pollen maturation (Albani et al. 1990, 1991; Roberts et al. 1991; Scott et al. 1991), or even become undetectable in the mature pollen grains (Roberts et al. 1991; Scott et al. 1991). Five other cDNAs (Bcp1–5) that are expressed predominantly in pollen have also been isolated from *B. campestris:* Bcp1 shows enhanced expression at the time of pollen maturation and during pollen germination (Theerakulpisut et al. 1991). The exclusively pollen-specific expression of our BA54 gene is rather unique, since the Bp19 transcript is also present at lower levels in the pistil and petal (Albani et al. 1991), and Bcp1 is activated in the tapetal cells in early anther development before showing increased expression in pollen (Theerakulpisut et al. 1991). This pollen-specific localization was also reported for the maize Zmc13 clone (Hanson et al. 1989). On the other hand, five predominantly pollen-expressed genes in tomato, LAT51, LAT52, LAT56, LAT58, and LAT59, are also expressed in the anther wall (Ursin et al. 1989). Furthermore, low levels of mRNA corresponding to LAT52 were detected in petals (Twell et al. 1989). Expression of sunflower cDNA clones SF1, SF2, SF18, and SF19 begins late in anther development, but is localized exclusively in the anther epidermal cell layer (Evrard et al. 1991). The cellular localization of *B. napus* clone BA73 was not determined, but the late accumulation of the transcript was similar to that seen for BA54.

Homology of BA42 to chalcone synthase (CHS)

The functions of several anther-specific gene products have been extensively studied in various plant species (Brown and Crouch 1990; Evrard et al. 1991; Koltunow et al. 1990; Twell et al. 1990; Wing et al. 1989). Tomato

cDNA clones LAT56 and LAT59 show protein sequence similarity to bacterial pectate lyases (Wing et al. 1989). The polypeptides encoded by the cDNA clones of the P2 gene family of *Oenothera organensis* are homologous to tomato polygalacturonase (Brown and Crouch 1990), and *Brassica* Bp19 encodes a protein similar to pectin esterase (Albani et al. 1991). These studies indicate that the gene products may function in pectin degradation during pollen development, germination, and tube growth. Tobacco anther-specific genes were found to code for proteins with potential roles in cell wall structure, lipid transfer, and protein degradation (Koltunow et al. 1990). We found the BA42 clone, which is expressed in more than one cell type in the immature anther, to share 64–67% amino acid homology with chalcone synthase (CHS) from various plant species (Fig. 6). CHS is the first and most abundant enzyme (Kreuzaler et al. 1979) in the flavonoid biosynthesis pathway, which gives rise to compounds involved in flower pigmentation, protection against stress, and induction of nodulation. In general, expression of CHS and other anthocyanin biosynthesis genes is flower specific, light dependent, and developmentally controlled (van Tunen et al. 1988; Koes et al. 1989). Expression in other tissues can be induced, however, by UV light, wounding, or fungal attack (Dixon 1986; Koes et al. 1989; Lamb et al. 1989).

The strong homology of the polypeptide encoded by BA42 with CHS suggests the possibility of a similar catalytic function. Both BA42 and petunia CHS genes show maximum expression during early stages of flower development (Koes et al. 1990). However, expression of the petunia CHS gene was also seen in corolla and petal tube (Koes et al. 1990), whereas BA42 is anther-specific. The in situ hybridization studies suggest that the BA42 gene transcript is present in tapetum, periphery cells of the vascular bundle, and the developing microspore (data not shown), and the result of the RNA-PCR assay indicates that the BA42 message does not accumulate in the mature pollen (Fig. 4A). Studies on the homologous genes of chalcone flavanone isomerase (CHI), the next enzyme after CHS in the flavonoid pathway, have revealed that one gene is specifically expressed in immature anthers, whereas the other gene is expressed both in more mature anthers and in corolla and petal tube (van Tunen et al. 1989, 1990). Several genomic DNA fragments were shown to hybridize to the BA42 probe (data not shown), and several amplified products were detected from the RNA-PCR assay using genomic DNA as a template with BA42-specific primers, thus indicating the existence of homologous genes. These other CHS genes might be responsible for anthocyanin synthesis in other pigmented floral tissues.

In summary, our data together with those of other studies demonstrate that many anther-specific genes are regulated temporally and spatially. We are now in the process of isolating the corresponding genomic clones. This study will reveal whether there are consensus sequences for the promoter and flanking regions as reported by Albani et al. (1991). BA42 represents a new, divergent member of the CHS gene family. The differential expression of BA42, in relation to other members in the gene family, will be further studied. The more complete understanding of the expression and regulation of these genes, and the elucidation of the physiological and biochemical significance of the gene products, will greatly enhance our overall knowledge of anther and pollen development, and eventually enable manipulation of gene expression to achieve the desired effects on anther development.

Acknowledgement. We thank C. Jeff Mauvais for planting and maintaining *Brassica* plants in the growth chamber for the tissue collection. We also thank Janet Rice and Christine I. Wandelt for their comments on this manuscript. We are especially indebted to Joan T. Odell for her support throughout the whole project, and critical reading of this manuscript.

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Communicated by E. Meyerowitz