The Identification of Candidate Genes for a Reverse Genetic Analysis of Development and Function in the Arabidopsis Gynoecium[1]

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The screening for mutants and their subsequent molecular analysis has permitted the identification of a number of genes of Arabidopsis involved in the development and functions of the gynoecium. However, these processes remain far from completely understood. It is clear that in many cases, genetic redundancy and other factors can limit the efficiency of classical mutant screening. We have taken the alternative approach of a reverse genetic analysis of gene function in the Arabidopsis gynoecium. A high-throughput fluorescent differential display screen performed between two Arabidopsis floral homeotic mutants has permitted the identification of a number of genes that are specifically or preferentially expressed in the gynoecium. Here, we present the results of this screen and a detailed characterization of the expression profiles of the genes identified. Our expression analysis makes novel use of several Arabidopsis floral homeotic mutants to provide floral organ-specific gene expression profiles. The results of these studies permit the efficient targeting of effort into a functional analysis of gynoecium-expressed genes.

The gynoecium is the fourth and innermost whorl of a typical bisexual flower. It is composed of the female reproductive organs, or carpels, and encloses the ovules, which develop into seeds after fertilization. The gynoecium may be composed of simple, unfused carpels, although in most species it is syncarpic, i.e. composed of several carpels fused together. The gynoecium functions to protect the ovules and to allow the operation of pollen-pistil incompatibility mechanisms. After fertilization, it develops into a fruit that participates in seed dissemination.

In Arabidopsis, the gynoecium is a complex syncarpic structure. This first develops as an open-ended tube from a primordial dome in the center of the floral meristem. A vertical septum then forms internally from either side of the gynoecial tube, and the two halves of this septum fuse to divide the structure into two loculi. Placental tissues develop in the zones where the vertical septum and gynoecial wall meet to generate two rows of ovule primordia within each loculus. Each ovule consists of a seven-celled embryo sac of the *Polygonum* type (Fahn, 1975), together with a small nucellus and two covering integuments. Cell division occurring at the distal end of the gynoecial cylinder forms the style and stigma tissues. The stigma consists of a pappillate epidermal cell layer with a modified external wall and cuticle. This tissue receives and permits the germination of compatible pollen grains. After the penetration of the stigma by pollen tubes, a transmitting tissue in the style and vertical septum functions to guide the pollen tubes toward the ovules where fertilization takes place. After fertilization, the Arabidopsis gynoecium develops into a two-chambered, capsular fruit, termed a silique. This structure opens at maturity to release its seeds by rupture along four zones of dehiscence in the silique wall situated on either side of the vertical septum. Detailed descriptions of gynoecium development in Arabidopsis are given by Bowman (1994) and Sessions (1997).

Relatively few genes have so far been identified that play important roles in the functional processes of the Arabidopsis gynoecium such as pollen reception, pollen tube guidance, and fertilization (for review, see Wilhelmi and Preuss, 1999; Faure and Dumas, 2001). By contrast, mutant screening and subsequent molecular analysis has been very successful in the identification of genes that control gynoecium development (for review, see Bowman et al., 1999, 2001; Ferrandiz et al., 1999) and ovule de-

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velopment (for review, see Schneitz et al., 1998). Despite the success of these mutagenesis-based approaches, some of the genes now known to influence gynoecium development had to be identified by the alternative reverse genetic approach. In these cases, genetic redundancy between similar genes led to a lack of mutant phenotypes in single-mutant plants. Examples of such genetic redundancy are to be found in two groups of MADS box genes controlling flower development: the genes *SHATTERPROOF1* and -2, which are necessary for dehiscence zone development (Liljegren et al., 2000), and the genes *SEPALLATA1*, -2, and -3, which are necessary for the specification of floral organ identity (Pelaz et al., 2000; Honma and Goto, 2001).

To identify further genes that play important roles in the development and functions of the gynoecium, we have taken a reverse genetic approach by firstly performing a differential expression screen between two floral homeotic mutants to identify genes that are up-regulated in the Arabidopsis gynoecium. The availability of large collections of T-DNA and transposon insertion lines (Bouché and Bouchez, 2001), together with efficient techniques for the suppression of gene activity based on posttranscriptional gene silencing (PTGS; Wesley et al., 2001), then makes possible the reverse genetic analysis of the genes of interest and of closely related sequences with which these may show redundant interactions. Here, we present the first stage in a reverse genetic analysis: the results of a differential screen to identify novel genes that are up-regulated in the Arabidopsis gynoecium. We describe in detail the homologies and expression patterns of the genes identified, and for certain identified genes, we discuss possible functions.

RESULTS

The Identification of Genes Up-Regulated in the Arabidopsis Gynoecium by Fluorescent Differential Display (FDD) Analysis

To identify genes that were specifically up-regulated in tissues of the gynoecium, we performed a differential screen of gene expression between the inflorescences of two floral homeotic mutants of Arabidopsis, *pistillata-1* (*pi-1*) and *agamous-1* (*ag-1*). Flowers of the *pi-1* mutant are composed of carpel and sepal organs, whereas those of the *ag-1* mutant are composed of sepals and petals. Genes specifically up-regulated in *pi-1* inflorescences were, therefore, expected to be also up-regulated in the wild-type (wt) Arabidopsis gynoecium. Our studies focused mainly on early flower developmental stages to identify genes involved in early gynoecium development. A total of 360 PCR primer combinations were used to amplify an estimated 18,000 reverse transcriptase (RT)-PCR products from inflorescences of *pi-1* and *ag-1* mutants (data not shown), which included flower buds at up to stage 10 of flower development (Bowman, 1994). Key events in the gynoecium at stage 10, which precedes female meiosis, include the elongation of the developing ovules, the closure of the end of the gynoecial cylinder, and the fusion of the two halves of the vertical septum (Bowman, 1994). From the PCR products generated from early flower bud developmental stages, 20 *pi-1*-up-regulated bands were observed on FDD gels (approximately 0.06 RT-PCR products per primer combination). Of these, 17 RT-PCR products were successfully purified and cloned in plasmid cloning vectors. In addition, a second FDD analysis on *ag-1* and *pi-1* material including later bud stages was performed. For this analysis, 60 PCR primer combinations were used together with RNA samples derived from inflorescences containing all flower bud developmental stages up to the mature flower stage 13 (Bowman, 1994). From these later samples, gene expression differences between *pi-1* and *ag-1* mutants were much more numerous than at early bud developmental stages. Forty-five clearly *pi-1*-up-regulated bands (approximately 0.75 RT-PCR products per primer combination) were observed on FDD gels using these samples. Of these, 12 *pi-1*-up-regulated bands were cloned in plasmid vectors for further analysis.

Sequencing of the total number of 29 RT-PCR products cloned after FDD analysis demonstrated the presence of 22 distinct sequences, the other seven having been amplified by more than one combination of PCR primer. One of these 22 unique PCR products proved, by searching of the complete Arabidopsis genome sequence database and by Southern blotting (data not shown), to show no homology to Arabidopsis DNA. Two others were derived from the Arabidopsis chloroplast genome and a further two represented nuclear rRNA genes. The remaining 18 PCR products represented the 3′ ends of known or predicted protein-encoding genes from the Arabidopsis nuclear genome. Further analysis of four of these by northern blotting (data not shown) failed to provide clear evidence of up-regulation in *pi-1* over *ag-1* mutant inflorescences. The remaining 14 PCR products represent partial cDNA sequences that were confirmed by subsequent analyses to be up-regulated in inflorescences of the *pi-1* mutant. These cDNAs were termed *Pup* (for *pistillata-up-regulated*) *1* to *14* and are described in Table I, which includes details of the stages of development from which they were identified during FDD analysis. For each of the 14 *Pup* cDNAs, full-length gene-coding regions predicted from the complete Arabidopsis genome sequence were amplified using RT-PCR from wt inflorescence RNA. These putatively full-length cDNAs were cloned and partially sequenced to demonstrate their authenticity. In several cases, where full-length cDNAs had not previously been characterized, sequencing of the full-length amplified cDNAs was performed. These novel full-length cDNA sequence data have been deposited with the EMBL database

Table I. *Details of the cDNA sequences Pup1–14, identified as up-regulated in the Arabidopsis gynoecium, and of their associated genes*
Details of the full-length cDNAs referred to can be found at http://signal.salk.edu/SSP/index.html.

cDNA	Published Name	Unique Gene Identifier No.	Homology or Classification	Full-Length cDNA	Stages of Development Used in FDD Analysis	Main Tissues of Expression in Flowers and Siliques
Pup1	*ARGONAUTE9 (AGO9)*	At5g21150	*ARGONAUTE* family genes	EMBL accession no. AJ544236	Before stage 11	Ovules and anther sporogenous tissues
Pup2		At5g53870	Phytocyanins	EMBL accession no. AJ544237	All stages	Embryo sac
Pup3		At2g34870	Pro-rich proteins	Ceres 29605	All stages	Epidermis of vertical septum
Pup4		At5g24420	Phosphogluconolactonases	Ceres 13806	Before stage 11	Ovary and ovules
Pup5		At1g72290	Kunitz protease inhibitors	Ceres 106020	All stages	Transmitting tissue
Pup6		At5g14700	Cinnamyl coA reductases	Ceres 17229	Before stage 11	Seed integuments
Pup7	*Thi2.1*	At1g72260	Thionins	Ceres 26029	All stages	Integuments of ovules and seeds
Pup8		At5g23960	δ-Cadinene synthases	EMBL accession no. AJ544238	All stages	Mesocarp of silique wall
Pup9	*VSP1*	At5g24780	Vegetative storage proteins	Ceres 32606	Before stage 11	Ovary wall
Pup10	*MBP2*	At1g52030	Myrosinase-binding proteins and other lectins	SSP R12767	Before stage 11	Inner epidermis of ovary; ovule integuments; tapetum
Pup11		At3g16470	Myrosinase-binding proteins and other lectins	Ceres 30003	Before stage 11	Inner epidermis of ovary; ovule integuments; vasculature of gynoecium and stamens
Pup12		At2g39330	Myrosinase-binding proteins and other lectins	Ceres 39069	Before stage 11	Ovary wall; vasculature of gyneocium, stamens, and petals
Pup13		At1g69870	Peptide transporters	Ceres 22243	All stages	Transmitting tissue; vasculature of floral organs; anther wall
Pup14		At1g52400	β-Glucosidases	Ceres 17229	Before stage 11	Inner epidermis of ovary; ovule integuments; vasculature of gynoecium and stamens

(Table I). The homologies shown by the genes identified in this study are discussed in detail below, together with descriptions of their expression profiles.

Expression Profiles of Genes Up-Regulated in the Arabidopsis Gynoecium

The expression profiles of the 14 *Pup* cDNAs identified from *pi-1* flowers by FDD analysis were investigated by a combination of northern blotting and in situ hybridization. As floral organs of Arabidopsis cannot easily be dissected in adequate quantities for northern blotting, we used RNA samples from entire inflorescence tissues of wt plants and of three floral homeotic mutants whose flowers contain different combinations of floral organs. The floral homeotic mutants used in these analyses were *pi-1* (flowers containing sepals and carpels), *ag-1* (flowers containing sepals and petals), and *superman-1* (*sup-1*; flowers containing sepals, petals, and stamens, with occasionally a vestigial gynoecium). The results of northern blotting for the 14 sequences analyzed, shown in Figure 1, demonstrate stronger hybridization signals for all of the cDNAs presented from *pi-1* than from *ag-1* flower RNA, thereby validating the results of the FDD screen. The detailed tissue-specific expression patterns of the 14 cDNAs were also investigated by non-radioisotopic in situ hybridizations to wt Arabidopsis flower bud, flower, and silique tissues, the results of which are presented in Figure 2. In situ hybridizations indicate that the majority of the *Pup* cDNA sequences show expression patterns largely confined to the gynoecium. In all cases, sense-strand cDNA control probes were used (data not shown) to verify the signals apparent with antisense cDNA probes. The detailed expression patterns shown by the 14 *Pup* cDNAs, as deduced from comparison of northern and in situ hybridization data, are as follows.

The *Pup1* cDNA corresponds to the gene *ARGONAUTE9* (*AGO9*) of the *ARGONAUTE* gene family. This family consists of 10 genes in Arabidopsis, three of which, *ARGONAUTE* (*AGO1*), *ZWILLE* (*ZLL*, also known as *PINHEAD*), and *ARGONAUTE4* (*AGO4*), have been functionally characterized. Mutations in *AGO1* (Bohmert et al., 1998) and *ZLL* (Moussian et al., 1998; Lynn et al., 1999) lead to developmental perturbations in both vegetative and reproductive tissues. Additionally, *AGO1* has been found to be necessary for PTGS (Fagard et al., 2000). *AGO4* is required for the methylation-dependent silencing of the *SUPERMAN* gene in a *clark kent* epigenetic mutant and for the accumulation of certain classes of small interfering RNAs (Zilberman et al., 2003). Northern hybridization (Fig. 1) shows *Pup1/AGO9* to be expressed in wt and *pi-1* mutant inflorescences and unexpressed in *ag-1* inflorescences and in wt vegetative tissues, suggesting the up-regulation of *Pup1/AGO9* in the gynoecium. A *Pup1/AGO9* hybridization signal on northern blots is also revealed in *sup-1* inflorescence RNA, suggesting this gene to

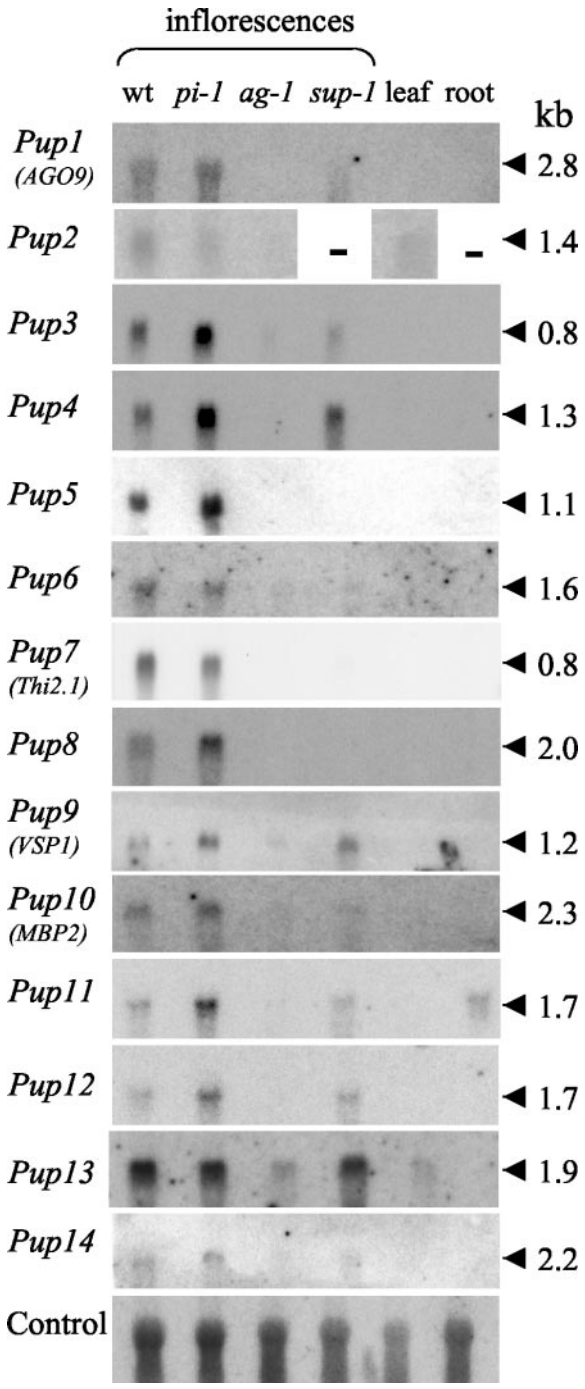


Figure 1. Northern blots probed with full-length predicted coding regions of the cDNAs *Pup1* to *Pup14*. The blots contain total RNA samples (or polyadenylated RNA samples in the case of *Pup2*) extracted from Arabidopsis plants of the Landsberg *erecta* (wt) genetic background and three mutant lines *pi-1*, *ag-1*, and *sup-1*. A control hybridization to demonstrate RNA loading in all tracks is shown using a cDNA probe corresponding to the gene At2g27040, encoding a close homolog of the *Pup1/AGO9* cDNA, which is expressed in all Arabidopsis tissues so far tested. Molecular sizes of hybridizing transcripts, calculated from tracks of RNA size markers, are shown on the right.

be also expressed in stamens (Fig. 1). In situ hybridization shows that *Pup1/AGO9* is expressed principally in the ovule (Fig. 2, A and B). The *Pup1/AGO9* gene appears to have a uniform level of expression throughout each ovule up to stage 12 (Fig. 2A) and lower and less uniform expression in ovule tissues at stage 13 (Fig. 2B) and later. A *Pup1/AGO9 in situ* hybridization signal is also apparent in the sporogenous tissue contained in the loculi of the anthers at around stage 8 of flower development (Fig. 2C), which is before pollen meiosis. This result is in accordance with the *Pup1/AGO9* signal detected on northern blots of *sup-1* inflorescence RNA (Fig. 1). At this earlier stage of development, *Pup1/AGO9* expression is also apparent in two zones of the gynoecial tube corresponding to the placentae and ovule primordia (Fig. 2C), demonstrating that expression of this gene in female tissues also commences early in development. *AGO9* is the first member of the *ARGONAUTE* family for which expression specifically in reproductive tissues has been demonstrated.

The *Pup2* cDNA encodes a phytocyanin protein and is homologous to a number of early nodulin (*ENOD*) genes expressed in the developing root nodules of species of the Leguminosae (Greene et al., 1998). Northern blotting of total RNA samples failed to detect expression of the *Pup2* gene (data not shown). However, northern blotting of polyadenylated RNA samples demonstrated weak *Pup2* expression specifically in wt and *pi-1* flowers and in leaves (Fig. 1). In situ hybridization (Fig. 2, D and E) shows the precise location of *Pup2* expression in flowers to be the embryo sac, with the signal being more apparent toward the chalazal pole, opposite to the micropyle where pollen tubes enter (Fig. 2D). In situ hybridization to leaf tissue (data not shown) failed to reveal any *Pup2* expression. *Pup2* expression in leaves, which was apparent on northern blots, may therefore be below the limit of detection of in situ analysis, or be at a uniform level in all leaf cells and therefore difficult to locate. Database searching indicates the *Pup2* cDNA to be a member of a gene family containing approximately 35 genes in Arabidopsis. However, no other Arabidopsis-predicted protein shows particularly close similarity to that predicted from the *Pup2* cDNA, the closest being the protein predicted from the gene At4g27520 (40.4% amino acid identity).

The *Pup3* cDNA encodes a short, Pro- or Hyp-rich peptide. Hydropathy predictions (Kyte and Doolittle, 1982) for this predicted peptide (data not shown) indicate a high probability of an N-terminal signal sequence with a consensus peptide cleavage site, allowing the possibility that the mature peptide may be secreted from the cell. Northern blotting demonstrates expression of the *Pup3* gene to be entirely flower specific and mostly confined to the gynoecium, because hybridization signals are very weak from *ag-1* and *sup-1* mutants by comparison with

those from wt plants and *pi-1* mutants (Fig. 1). In situ hybridizations (Fig. 2, F and G) demonstrate expression of *Pup3* in the gynoecium to be confined to the epidermal layers of the vertical septum. Expression of this gene commences in the latter half of stage 12 of flower development in the central region of the epidermis of the vertical septum (Fig. 2F). *Pup3* expression then spreads to all epidermal cells of the vertical septum in fully mature flowers at stage 13 (Fig. 2G). This gene is additionally, but more weakly, expressed in the adaxial epidermis of the petal and the epidermis of the stamen filaments at the fully mature stage 13 of flower development (Fig. 2G). Although several distinct gene families encoding Pro and Hyp-rich proteins are known to be expressed in plant reproductive tissues (Sommer-Knudsen et al., 1997), the protein encoded by *Pup3* belongs to a novel family containing only three members in Arabidopsis, the other two being the genes At1g30875 and At3g02670.

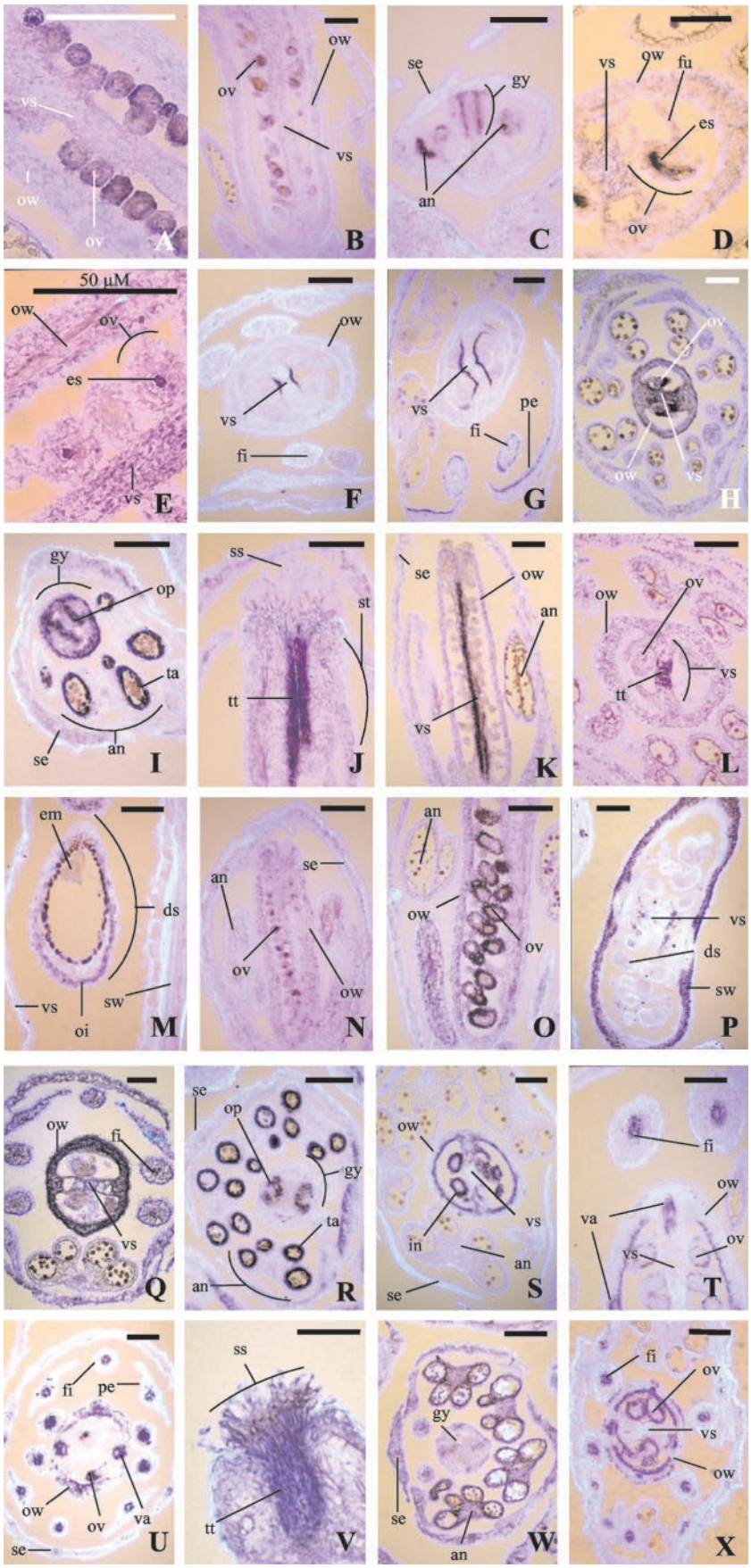
The *Pup4* gene putatively encodes a phosphogluconolactonase, the second enzyme in the oxidative pentose phosphate pathway. This enzyme is responsible for the conversion of D-glucono-∂-lactone-6-phosphate into 6-phosphogluconic acid. Northern blotting (Fig. 1) shows the *Pup4* mRNA to be present uniquely in inflorescence tissues, with expression specifically in wt, *pi-1*, and *sup-1* inflorescences, suggesting expression only in the gynoecium and stamens. In situ hybridization (Fig. 2H) confirms the northern-blot results and demonstrates expression of the *Pup4* gene in all parts of the gynoecium, with strongest expression in the developing ovules. A strong *Pup4* signal is also apparent in the tapetum of the anthers at around stages 9 to 10 of flower development (Fig. 2I). At this earlier stage of development, gynoecial expression of *Pup4* seems to be particularly strong in the ovary wall and in two internal zones corresponding to the placentae and developing ovule primordia (Fig. 2I). The specific expression of a phosphogluconolactonase in rapidly developing reproductive tissues is indicative of high activity in the pentose phosphate pathway. This pathway provides NADPH in non-photosynthetic tissues, generates Rib-5-phosphase for nucleic acid synthesis and contributes to the production of shikimic acid, which is a precursor of aromatic ring compounds (Goodwin and Mercer, 1983; Miclet et al., 2001). Database searching indicates the *Pup4* gene to belong to a family of five genes in Arabidopsis. *Pup4*, therefore, seems to be a differentially regulated member of the phosphogluconolactonase gene family that functions uniquely in tissues within the third and fourth floral whorls.

Pup5 encodes a protein that shows 57.2% amino acid sequence identity to a heat shock-induced kunitz proteinase inhibitor from cauliflower (*Brassica oleracea*; Annamalai and Yanagihara, 1999). The *Pup5* mRNA appears to be entirely specific to gynoecial tissues, giving signals only in *pi-1* and wt inflorescence tissues on northern blotting (Fig. 1). In situ hybridization reveals this gene to be expressed in the transmitting tissue of the style (Fig. 2J) and vertical septum (Fig. 2, K and L), the route taken by pollen tubes toward the ovules.

The *Pup6* cDNA encodes a putative cinnamoyl CoA reductase, the enzyme which catalyzes the first committed step in the production of lignin by the conversion of cinnamoyl CoAs to their respective cinnamaldehydes (Lauvergeat et al., 2001). Northern analysis (Fig. 1) indicates the *Pup6* cDNA to be entirely flower specific and, from its much higher expression in wt and *pi-1* inflorescences as compared with *ag-1* and *sup-1* inflorescences, to be largely gynoecium-specific. Although the *Pup6* mRNA was identified from relatively early stages of flower development, no strong expression signal could be found in young flower buds by in situ hybridization (data not shown). By contrast, the *Pup6* cDNA was found to be expressed in the outer integument of the developing seeds (Fig. 2M). Its expression seems to be strongest in the subepidermal cell layer. A natural coloration of the tissue, possibly due to an accumulation of phenolic compounds (Western et al., 2000), obscures any possible *Pup6* expression in the inner cell layer of the inner epidermis (Fig. 2M).

Pup7 corresponds to the published *AthTH1* cDNA encoding a thionin (Epple et al., 1995). The corresponding gene, *Thi2.1* (Bohlmann et al., 1998), has been previously shown by northern blotting (Epple et al., 1995) and by *GUS* reporter gene studies (Vignutelli et al., 1998) to be expressed constitutively in flowers and siliques. In addition to its constitutive expression in reproductive tissues, *Thi2.1* expression can be induced in vegetative tissues by wounding and infection (Bohlmann, et al., 1998; Vignutelli et al., 1998). Northern blotting (Fig. 1) performed in the current study confirms the findings of Epple et al. (1995), showing signals only in wt and *pi-1* inflorescence tissue, suggesting up-regulation in the gynoecium. In situ hybridization demonstrates the exact location of *Pup7/Thi2.1* expression to be the integuments of the ovule, a result that was not apparent from the study of the promoter activity of this gene (Vignutelli et al., 1998), which appeared to show a rather more generalized expression in the ovary. Expression of *Pup7/Thi2.1* commences at stage 11 (Fig. 2N), during the formation of inner and outer integument primordia, and then increases throughout stages 12 and 13 to a maximum level in the integuments of the mature ovule at late stage 13 (Fig. 2O).

The *Pup8* cDNA encodes a putative δ-cadinene synthase, the enzyme that catalyzes the conversion of farnesyl diphosphate into δ-cadinene, a committed step in the production of sesquiterpene phytoalexins (Benedict et al., 2001). Northern blotting (Fig. 1) indicates *Pup8* expression to be entirely flower-specific. Furthermore, the expression of this gene is limited to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wt and *pi-1* mutant inflorescences, with no detectable expression in *ag-1* or *sup-1* inflorescences, suggesting an entirely gynoecium-specific expression profile. In situ hybridization (Fig. 2P) demonstrates expression of the *Pup8* gene only in post-fertilization stages of development. Its expression is limited to the wall of the silique and seems to be particularly abundant in the mesocarp cell layers. No *Pup8* hybridization signals are apparent in the ovary wall before stage 14 of flower development (data not shown).

The *Pup9* cDNA is identical to the previously identified *vegetative storage protein1* (*VSP1*)cDNA, encoding a vegetative storage protein (Utsugi et al., 1998). *VSP1* is located at a distance of 6 kb from its close homolog *VSP2* on Arabidopsis chromosome 5. Vegetative storage proteins were first identified by their accumulation in the vacuoles of leaf mesophyll cells of soybean (*Glycine max*) plants that had been depodded and thereby deprived of a nutrient sink (Wittenbach, 1983). It is thought that VSPs represent a protein reserve in the mature or developing plant and are termed vegetative to distinguish them from the storage proteins that accumulate in seeds. The promoter activity of the *Pup9/VSP1* gene in Arabidopsis has been previously investigated using a β-glucuronidase reporter gene strategy (Utsugi et al., 1998). The *Pup9/VSP1* promoter was found to be active in gynoecium tissues, although no promoter activity in the stamens or elsewhere in the flower was noted. Northern blotting in the present work indicates *Pup9/VSP1* expression only in inflorescence tissues (Fig. 1). Hybridization signals are strongest from *pi-1* mutant inflorescences, with considerable expression also from wt and *sup-1* mutant inflorescences. A slight hybridization signal is also apparent in *ag-1* mutant inflorescence tissue. Taken together, these data suggest a strong *Pup9/VSP1* expression in the gynoecium and in stamens and a weak *Pup9/VSP1* expression elsewhere in the flower. In situ hybridization of *Pup9/VSP1* confirms the findings of northern analysis, showing a high level of expression in the ovary wall and the inner and outer epidermal cell layers of the ovary (Fig. 2Q). The high levels of *Pup9/VSP1* expression apparent in *sup-1* inflorescences by northern blotting (Fig. 1) are in agreement with in situ hybridization signals detected in the vasculature of the stamen filaments (Fig. 2Q). Although *Pup9/VSP1* seems less highly expressed in filaments that in the gynoecium, a considerable level of expression in *sup-1* mutants would be apparent on northern blots, because *sup-1* flowers contain large numbers of stamens. In addition to high levels of *Pup9/VSP1* in the gynoecium and stamen filaments, a general low level of hybridization of *Pup9/VSP1* to all floral tissues is apparent (Fig. 2Q). This observation is in agreement with the low level of *Pup9/VSP1* expression in *ag-1* inflorescences observed on northern blots (Fig. 1).

The three cDNAs *Pup10*, *Pup11*, and *Pup12* are homologous to genes encoding various classes of lectins, including myrosinase-binding proteins (MBPs), β-glucosidase-binding proteins (Rask et al.,

Figure 2. Non-radioisotopic in situ hybridizations using antisense-strand riboprobes corresponding to full-length predicted gene-coding regions of cDNAs *Pup1* to *Pup14*. Hybridizations were performed on tissues of flower buds, mature flowers, and siliques of Arabidopsis plants of the Landsberg *erecta* genetic background. Probe hybridization signals corresponds to dark blue/violet staining, whereas counterstaining of cellulose-containing material shows as bright fluorescence. A, Longitudinal section (LS) of ovary showing *Pup1/AGO9* signals in ovules at early stage 12. B, LS of flower bud showing *Pup1/AGO9* signals in ovules at stage 13. C, LS of flower bud showing *Pup1/AGO9* signals in placentae/ovule primordia and in anther loculi at stage 8. D, Transverse section (TS) of ovary showing a *Pup2* signal in the embryo sac at stage 13. E, LS of ovary showing *Pup2* signals in embryo sacs at stage 13. F, TS of flower bud showing *Pup3* signals in epidermal cells of the vertical septum at late stage 12. G, TS of flower bud showing *Pup3* signals in epidermal layers of the vertical septum, petals (abaxial surface), and filaments at stage 13. H, TS of flower bud showing *Pup4* signals in the ovary and ovules at stages 11 to 12. I, TS (oblique) of flower bud showing *Pup4* signals in the gynoecium wall, placentae/ovule primordia, and tapetum at stages 9 to 10. J, LS of upper gynoecium showing a *Pup5* signal in the stylar transmitting tissue at stage 13. K, LS of flower bud showing a *Pup5* signal in the vertical septum at stage 13. L, TS of flower bud showing a *Pup5* signal in the transmitting tissue of the vertical septum at stage 13. M, LS of silique showing a *Pup6* signal in the outer integument of the seed, approximately 3 d after fertilization. N, LS of flower bud showing *Pup7/AtTH1* signals in ovules at early stage 12. O, LS of ovary showing *Pup7/Thi2.1* signals in ovule integuments at stages 12–13. P, TS (oblique) of silique showing a *Pup8* signal in mesocarp cell layers, 1 to 2 d after fertilization. Q, TS of flower bud showing *Pup9/VSP1* signals in the ovary wall and stamen filaments at stage 13. R, TS of flower bud showing *Pup10* signals in the placentae/ovule primordia of the gynoecium and in the tapetum at stages 9 to 10. S, TS of flower bud showing *Pup10* signals in the inner epidermis of the ovary and in the integuments at stage 13. T, TS (oblique) flower bud showing *Pup11* signals in the inner epidermis of the ovary, in the integuments, and in the vasculature of the gynoecium and filaments at stage 13. U, TS of flower bud showing *Pup12* signals in the ovules, in the valves of the ovary and in the vasculature of the gynoecium, filaments, and petals at early stage 12. V, LS of upper gynoecium showing general *Pup13* signals, although stronger in the stigmatic epidermis and stylar transmitting tissue at stage 13. W, TS of flower bud showing general *Pup13* signals, although stronger in the anther walls and vascular tissue of floral organs at stage 10. X, TS of flower bud showing *Pup14* signals in the epidermal cell layers of the ovary, in the ovule integuments, and in the vasculature of the gynoecium and filaments at stage 13. an, Anther; ds, developing seed; em, embryo; es, embryo sac; fi, filament of stamen; fu, funiculus; gy, gynoecium; in, integuments; oi, outer integument; op, ovule primordia; ov, ovule; ow, ovary wall; pe, petal; se, sepal; ss, stigmatic surface; st, style; sw, silique wall; ta, tapetum; tt, transmitting tissue; va, vasculature; vs, vertical septum. All scale bars = 100 μM, except where otherwise indicated.

2000), and jacalin lectin from the seeds of *Artocarpus integrifolia* (Young et al., 1991). The *Pup10* cDNA has been previously characterized (Capella et al., 2001) and termed *MPB2* on the grounds of similarity between its predicted protein and a characterized MBP from canola (*Brassica napus*; Taipalensuu et al., 1997). The *Pup2/MBP2*-predicted protein shows 47.2% amino acid similarity to this MBP from canola and is the most similar predicted protein to the canola MBP that exists in the Arabidopsis predicted proteome. However, no evidence as yet exists to indicate that the *Pup10*-predicted protein binds to myrosinases either in vitro or in vivo. Because many other classes of lectins share close similarity to MBPs and because database searching indicates *Pup10*, *Pup11*, and *Pup12* to belong to a family of approximately 50 genes in Arabidopsis, the designation of *Pup10* as encoding an MBP must await further evidence.

MBPs bind to myrosinases, which are glycosylated enzymes that show a thioglucosidase activity and are implicated in defense against insects, especially in the Brassicaceae and closely related families. Myrosinases catalyze the release of thiocyanates and other toxic compounds from glucosylated precursors, termed glucosinolates. Myrosinase enzymes are known to be constitutively present in specialized myrosinase cells, particularly in the seed and seedling, and are also inducible in the mature plant by wounding (Rask et al., 2000). MPBs are capable of binding to myrosinases and colocalize with these in some plant tissues (Geshi et al., 1998). The role of MPBs is not known, although it has been suggested that they may be capable of modulating myrosinase activity (Andreasson et al., 1999) or may show direct toxicity toward insects (Rask et al., 2000). Although the *Pup10 and Pup11*-predicted peptides show reasonable levels of similarity to a characterized MBP from canola (Taipalensuu et al., 1997), the putative lectin predicted from the *Pup11* cDNA is considerably less similar to this protein of known myrosinase-binding activity (40.4% amino acid identity).

Northern blotting of the *Pup10*, *Pup11*, and *Pup12* cDNAs (Fig. 1) demonstrates these to be expressed in wt and *pi-1* inflorescences and to be expressed very lowly or to be unexpressed in *ag-1* inflorescences, suggesting up-regulation in the gynoecium. All three of these genes additionally show hybridization to *sup-1* inflorescence RNA, suggesting expression in the stamens. None of the genes *Pup10*, *Pup11*, or *Pup12* is constitutively expressed in leaf tissue, and of the three of them, only *Pup11* was found to be expressed in roots (Fig. 1).

In situ hybridization demonstrates that *Pup10* is expressed at stages 9 to 10 of flower development in two regions internal to the gynoecial cylinder corresponding to the developing placentae and ovule primordia (Fig. 2R). By the mature stage 13, *Pup10* is expressed specifically in the ovule integuments, in the funiculus, and in the internal epidermis of the ovary (Fig. 2S). In addition to its expression in female tissues, *Pup10* is expressed in the tapetum of the anthers at stages 9 to 10 of flower development, during and immediately following pollen meiosis (Fig. 2R). Tapetal expression of *Pup10* (*MBP2*) is also apparent in the in situ analysis performed by Capella et al. (2001), although the precise location of the expression of this gene in the inner epidermis and ovule integuments, clearly demonstrated in the present work, is not clear from the in situ hybridization data presented by Capella et al. (2001).

Pup11, like *Pup10*, shows expression in the integuments and in the inner epidermis of the ovary (Fig. 2T). However, this gene is additionally expressed in the four vascular strands of the gynoecium and in the vasculature of the stamen filaments in flowers buds at stage 13 of development (Fig. 2T). *Pup11*, again unlike *Pup10*, is not expressed in the tapetum (data not shown).

Pup12 is principally expressed in the vasculature of the immature floral organs (Fig. 2U). Expression of this gene is apparent in the four vascular bundles of the gynoecium, in the vasculature of the stamen filaments, and in the mid-vein of the petals (Fig. 2U). *Pup12* is also expressed in the developing ovules at stages 11 and 12 (Fig. 2U). However, the ovule expression of *Pup12* ceases at more mature developmental stages such that it is not expressed in the integuments of the mature ovule, as are *Pup10* and *Pup11* (data not shown). *Pup12* is more generally expressed in the tissues of the immature ovary wall (Fig. 2U) than are *Pup10* and *Pup11*. Again unlike *Pup10* and *Pup11*, this gene does not show specific expression in the inner epidermis of the mature ovary. Like *Pup11* but unlike *Pup10*, *Pup12* is not expressed in the tapetum (data not shown). The *Pup10*, *Pup11*, and *Pup12* cDNAs therefore show precise and distinct cellular patterns of expression that do not correspond to the presence of specialized myrosinase cells. Although from their sequence homologies, the proteins encoded by these genes may be predicted to show probable lectin activities, any carbohydrate-containing molecules to which they may bind in vivo have yet to be identified.

The *Pup13* cDNA encodes a putative peptide transporter protein. The peptide transporter of known activity that exhibits closest similarity to the *Pup13*-predicted protein (36.4% amino acid sequence identity) is encoded by the *HvPTR1* gene of barley (*Hordeum vulgare*; West et al., 1998). This gene is expressed specifically in the barley embryo during seed germination and is thought to be involved in the mobilization of protein reserves. Database searching indicates that the *Pup13* gene is a member of a very extensive Arabidopsis gene family containing over 50 members. Northern blotting indicates the *Pup13* gene to be expressed in all tissues tested other than roots (Fig. 1). Its expression is highest in the inflorescences of wt plants and of *pi-1* and *sup-1* mutants, with lower

expression in *ag-1* mutant inflorescences and in wt leaves. These results suggest the up-regulation of *Pup13* expression in the gyneocium and stamens, with lower levels of expression in the perianth organs and the aboveground vegetative organs. In situ hybridization to mature flowers indicates some *Pup13* expression in all flower tissues, with higher signals in the stigmatic epidermis (Fig. 2V) and the transmitting tissue of the style (Fig. 2V) and vertical septum (data not shown). At an earlier stage of flower development, higher levels of *Pup13* expression are also apparent in the anther wall layers (Fig. 2W), explaining the high expression levels of this gene apparent on northern blotting of RNA from *sup-1* mutant inflorescences (Fig. 1). In flower buds at stage 10, *Pup13* is not expressed at a detectable level in the tapetum or sporogenous tissues (Fig. 2W). *Pup13* is, however, highly expressed in the vasculature of the gynoecium, stamen filaments, and sepals at this same developmental stage (Fig. 2W).

The *Pup14* cDNA encodes a putative β-glucosidase. β-Glucosidases are known to play a large variety of roles in plants by liberating many different classes of molecules from inactive glucosylated precursor forms. Databases searching indicates the protein of known function most closely resembling the *Pup14*-predicted protein (71.2% amino acid identity) to be a β-glucosidase that specifically degrades zeatin-*o*-glucosides in canola seeds (Falk and Rask, 1995). Another enzyme showing a similar activity related to hormone metabolism is involved in the release of active cytokinin during the development of maize (*Zea mays*) embryos (Brzobohaty et al., 1993). Several other β-glucosidases of unknown functions and substrate specificities have previously been found to be differentially regulated in response to environmental conditions such as phosphate starvation (Malboobi and Lefebvre, 1997) or to be developmentally up-regulated in reproductive tissues such as the Arabidopsis tapetum (Rubinelli et al., 1998). β-Glucosidases show high levels of sequence similarity to thioglucosidases (myrosinases). One amino acid position within the active sites of these two classes of enzymes is occupied invariably by a Glu residue in β-glucosidases and by a Gln residue in myrosinases (Rask et al., 2000). This amino acid residue corresponds to position 207 in the *Pup14*-predicted peptide (data not shown), where the presence of Glu indicates a probable β-glucosidase activity.

Northern blotting (Fig. 1) indicates *Pup14* to be entirely flower-specifically expressed. It is expressed in wt, *pi-1*, and *sup-1* mutant inflorescences and also shows a very slight hybridization to *ag-1* inflorescence RNA, suggesting up-regulation of this gene in the gynoecium and in stamens, with some expression elsewhere in the flower. In situ hybridization confirms the findings of northern blotting and demonstrates expression of *Pup14* in various tissues of the mature flower bud. The *Pup14* gene is strongly expressed in the integuments of the ovules and in the inner epidermis of the ovary and less strongly expressed in the outer epidermis of the ovary (Fig. 2X). In addition, it is strongly expressed in the four vascular strands of the gynoecium and in the vasculature of the stamen filaments and petals (Fig. 2X). The *Pup14* expression pattern is therefore complex, involving several distinct tissues. This expression pattern resembles very closely that of the gene *Pup11*, also characterized in the present study, which shows similarity to myrosinase-binding and β-glucosidase-binding proteins and other lectins.

DISCUSSION

The FDD Analysis of Floral Homeotic Mutants Proves an Efficient Method for the Identification of Novel Gynoecium-Specific Genes

We have identified 14 genes that are up-regulated in tissues of the Arabidopsis gynoecium by the comparison of the genes expressed in the inflorescences of two Arabidopsis floral homeotic mutants, *pi-1* and *ag-1*, using the technique of FDD. Eight of the 14 sequences described here were identified from early stages of flower development. However, in situ hybridization demonstrated all of these cDNAs to be also expressed at later developmental stages, suggesting that our intention to clone genes specifically expressed early in gynoecium development did not work as well as intended.

Most of the sequences identified in the present study were at least moderately highly expressed. All except one of them, *Pup2*, could be detected by northern blotting of total rather than polyadenylated RNA. In addition, none of the low-expressed genes already known to control gynoecium development (Bowman et al., 1999; Ferrandiz et al., 1999) was re-identified in the present study. This suggests that FDD may be more efficient for the detection of moderately or highly expressed rather than low-expressed mRNAs.

Comparison of *pi-1* and *ag-1* inflorescences containing flower buds up to developmental stage 10 in the present study yielded approximately 0.06 *pi-1* mutant-up-regulated RT-PCR products per PCR primer combination. Inflorescences additionally containing buds up to developmental stage 13, however, yielded the much higher number of approximately 0.75 *pi-1* mutant-up-regulated RT-PCR products per PCR primer combination. This suggests a large (approximately 12.5-fold) increase in the expression of moderately and highly abundant mRNAs during the latter phases of gynoecium and ovule development. Only a small proportion of the differential genes detected by FDD at later stages of flower development were cloned in the present study. Having already been identified in FDD analysis, these other differential mRNAs should, however, be amenable to cloning at a later time.

Despite the potential drawback of limited sensitivity, our results indicate the FDD technique to repre-

sent a substantial improvement over conventional differential display using radioactive detection (Liang and Pardee, 1992). An earlier study of gynoecium-specific gene expression in Arabidopsis using a similar mutant-based strategy in combination with conventional differential display succeeded in identifying only two gynoecium-expressed genes (Yung et al., 1999), one of which had been previously characterized. Although FDD may continue to provide the most efficient available means of differential screening that is of applicability to any eukaryotic organism, it is probable that microarray-based methods, due to their potentially greater convenience and sensitivity, will supersede the use of FDD in organisms for which complete genome sequences and/or large expressed sequence tag collections are available.

A Member of the *ARGONAUTE* Gene Family Functions in Developing Ovules and Sporogenous Tissue

The principal aim of this work has been to identify target genes for a reverse genetic functional analysis of processes occurring in the gynoecium. Our studies have identified the *Pup1* cDNA as a gene with potential functions in ovules and the sporogenous tissues of the anther. *Pup1* is a previously unstudied member of the *ARGONAUTE* gene family, designated after a homology search of the Arabidopsis genome by Morel et al. (2002) as *AGO9*. *ARGONAUTE* genes encode proteins containing PAZ and PIWI domains (Cerutti et al., 2000) and are known from plants, fungi, and animals (Fagard et al., 2000). These genes are variously involved in development and in a group of related processes termed PTGS in plants, RNA interference in animals, and quelling in fungi. The effect of PTGS can be seen in the silencing of reporter genes in transgenic plants by the specific destruction of their transcripts (for review, see Vaucheret and Fagard, 2001). Plants that are defective in PTGS due to mutations in the *AGO1* gene have been shown to be less resistant to viruses (Morel et al., 2002), and it is hypothesized that the natural function of PTGS and related processes is in virus resistance.

In the present work, we show *Pup1/AGO9* to be a transcribed gene that is expressed specifically in the ovule and in the sporogenous tissues of the anther loculus. In both of these tissues *Pup1/AGO9* expression commences before meiosis. Both ovules and sporogenous tissues are in phases of rapid development and, in addition, both of these tissues might represent control points for the prevention of the vertical transmission of viruses. Several mutant alleles with differential effects on development and PTGS are known for the previously investigated *AGO1* gene. In the *ago1-27* allele, a mutation converting the predicted Ala-992 residue to Val causes a complete loss of PTGS, but has only a slight effect on plant development (Morel et al., 2002). This Ala residue is not conserved with the wt Pup1/AGO9-predicted peptide, which may suggest that *Pup1/AGO9* is not involved in PTGS. Further studies, however, will be required to determine whether *Pup1/AGO9* functions in ovule and pollen development, in PTGS for virus resistance, or in both of these processes.

FDD Identifies Several Classes of Genes with Potential Roles in the Development or Essential Functions of the Gynoecium

In addition to *Pup1/AGO9*, our studies have identified a number of other genes that present homologies and expression patterns suggesting potential roles either in the development of the gynoecium or in its essential processes. These genes also represent interesting candidates for reverse genetic functional analysis. The *Pup2* gene is expressed specifically in the Arabidopsis embryo sac and is homologous to various early nodulin (*ENOD*) genes including *ENOD5* from pea (*Pisum sativum*; Scheres et al., 1990), *ENOD55* from soybean (de Blank et al., 1993), and *ENOD16* and *ENOD20* from *Medicago truncata* (Greene et al., 1998). These *ENOD* genes encode proteins of the phytocyanin class and, like other classes of *ENOD* genes, are expressed early in the development of nitrogen-fixing root nodules in species of the Leguminosae. The phytocyanins include a group of chloroplast copper-binding proteins termed plastocyanins that function as photosynthetic electron transporters. However, the known ENOD phytocyanins lack the ligands necessary for binding copper that are present in plastocyanins (Greene et al., 1998). The cellular or intracellular locations of the plastocyanin-like ENOD proteins and their functions during the process of nodulation are unknown. Multiple sequence alignment with plastocyanin proteins (data not shown) indicates that the *Pup2*-predicted phytocyanin also lacks three out of four of the residues that coordinate copper in plastocyanins (for review, see Greene et al., 1998), strongly suggesting that this predicted protein would be unable to bind copper.

The *Pup3* cDNA encodes a short Pro- or Hyp-rich peptide that presents a very specific expression pattern in the epidermis of the vertical septum during the later stages of flower development. Pro- and Hyp-rich proteins are frequently secreted from the cell and often accumulate in the cell wall. This may be the case for the *Pup3*-predicted peptide, which shows a putative N-terminal signal peptide sequence and consensus cleavage site. Hyp-rich peptides are involved in many processes in plant reproductive tissues, including some cell-signaling processes, reviewed by Sommer-Knudsen et al. (1997). Because *Pup3* is expressed late in flower development, it may not be involved in developmental processes that

would alter the pattern of cell division and differentiation in the vertical septum. *Pup3* is, however, expressed in a cell layer that must be traversed by growing pollen tubes immediately before fertilization.

Pup14 encodes a β-glucosidase with greatest similarity to an enzyme involved in the release of active cytokinin in canola seeds. Our studies show *Pup14*, by contrast, to be expressed in several different tissues of the developing Arabidopsis gynoecium. Our studies have serendipitously identified, along with *Pup14*, three genes encoding proteins that are similar to lectins known to bind to myrosinases, β-glucosidases, and other glycoproteins. The β-glucosidase from canola to which *Pup14* shows close similarity was originally copurified together with two low M_r proteins (Falk and Rask, 1995) that may be examples of β-glucosidase-binding proteins from *B. napus*. One of the putative lectin-encoding cDNAs identified in the present study, *Pup11*, shows a pattern of expression very similar to that of the *Pup14*-predicted β-glucosidase. Further studies will be necessary to determine the substrate specificity of the predicted *Pup14* β-glucosidase, its possible in vivo functions, and any potential interaction with the protein encoded by the *Pup11* cDNA.

Many Classes of Defense-Related Genes Are Up-Regulated in the Gynoecium

One of the primary functions of the carpel appears to be the protection of the ovules and seeds that are contained within it. In addition to providing a physical barrier to insects and microbial pathogens, the carpel tissues and the integuments of the ovules and seeds of Arabidopsis have been shown in the present study to be the location of expression of many genes that are specifically related to plant defense. *Pup5* encodes a putative proteinase inhibitor expressed in the transmitting tissue of the style and vertical septum. Proteinase inhibitors from the Brassicaceae have been shown to be active against enzymes of both fungal and animal origin (Lorito et al., 1994), whereas another class of Ser proteinase inhibitor expressed in the stigmas of *Nicotiana alata* (Atkinson et al., 1993) has activity against insect proteinases (Heath et al., 1997). Because the *Pup5*-encoded putative proteinase inhibitor is very precisely expressed along the transmitting tissue, a potential route of entry route for fungal hyphae, the natural function of *Pup5* may be principally in antifungal defense.

Pup6 encodes a putative cinnamyl CoA reductase, expressed principally in the subepidermal cell layer of the outer seed integument, potentially involved in the production of lignin. Lignins of various structures and chemical compositions are known to play a variety of structural and defensive roles in plants tissues (Nicholson and Hammerschmidt, 1992). The epidermal cell layer of the Arabidopsis outer seed integument is specialized for the production of mucilage and sheds its outer cell wall on wetting (Western et al., 2000; Windsor et al., 2000). The expression of *Pup6* in the subepidermal layer, therefore, suggests the presence of a lignified barrier below the outer mucilage-secreting cell layer. *Pup7/Thi2.1* encodes a thionin that, like *Pup6*, is also specific to the integuments. Because thionins are implicated in plant defense, it is likely that *Pup7/Thi2.1* also plays a role in the protection of the seeds.

Pup8 encodes a putative δ-cadinene synthase that is specific to the mesocarp cell layers of the silique wall. *Pup8* can therefore be predicted to contribute to the production of sesquiterpene phytoalexins in the silique wall. These compounds are secondary plant products that play a defensive role against fungal pathogens (Harborne, 1993).

In addition to the above-mentioned genes that are likely to be primarily involved in plant defense, defensive roles cannot be excluded for some or all of the genes *Pup10*, *Pup11*, and *Pup12*, encoding putative lectins that may bind to myrosinases, β-glucosidases, or other carbohydrate-containing molecules. A defensive role is also possible for *Pup1/AGO9*, which may be involved in PTGS for viral resistance, in addition to a possible function in ovule and pollen development.

The Continued Reverse Genetic Analysis of Gene Function in the Gynoecium

The data presented here mainly relate to the position and timing of specific gene expression in the gynoecium and other Arabidopsis floral tissues. This information, together with the complete Arabidopsis genome sequence and efficient methods for insertional mutagenesis and gene knock-outs through RNA interference, facilitates the next stage in our analyses. The present work will enable the efficient targeting of effort into a reverse genetic analysis of gene function in the Arabidopsis gynoecium.

MATERIALS AND METHODS

Plant Material

Seed stocks of wt and mutant lines of Arabidopsis were originally obtained from the Nottingham Arabidopsis Seed Centre Seed Bank (Nottingham, UK). Both the wt plants and the mutants analyzed, *pi-1*, *ag-1* (Bowman et al., 1989), and *sup-1* (Bowman et al., 1992), were obtained in the Landsberg *erecta* genetic background. Arabidopsis plants were grown in peat-based potting compost in a growth chamber at 22°C with an 8-h photoperiod per 24 h. Flowering was induced by transfer to a greenhouse equipped with supplementary lighting to increase the photoperiod to ≥14 h.

FDD Analysis

Differential screening to identify mRNAs that were up-regulated in inflorescences of *pi-1*, compared with *ag-1*, mutants of Arabidopsis was performed by the semi-automated method of FDD, as described by Kuno et al. (2000). This method is based on the RT-PCR amplification of the 3′ extremities of mRNAs derived from different tissue samples. Reverse tran-

scriptase PCR amplifications for FDD analysis employed three different fluorescent oligo(dT) primers, respectively containing dA, dC, and dG residues immediately 3′ to an oligo(dT) sequence. These 3′ residues were included to anneal with the last base of mRNAs, adjacent to their poly(A) tails, thereby selecting three subgroups of mRNAs. The three fluorescent 3′ primers were separately used in conjunction with 120 randomly chosen 10-mer primers (Operon Technologies, Alameda, CA). The mixtures of products resulting from PCR amplifications were analyzed on an automated DNA sequencer (SQ5500T, Hitachi, Tokyo). The molecular cloning of differentially expressed RT-PCR products identified by FDD analysis was performed using a Southern blotting-based system as described by Scutt et al. (2002).

Sequence Analysis and PCR Amplification of Full-Length cDNAs

The cloned RT-PCR products identified by FDD analysis, representing the 3′ extremities of transcribed sequences, were sequenced and their respective genes were located in the complete Arabidopsis genome sequence by BLAST searching (Altschul et al., 1990). Full-length cDNAs corresponding to the identified genes were then amplified by RT-PCR (McPherson et al., 1995) from inflorescence RNA of either the Landsberg or Columbia ecotypes of Arabidopsis using primers designed from the 5′ and 3′ extremities of their respective predicted protein-coding regions. In cases where full-length cDNAs had not previously been identified, these were fully sequenced to confirm or correct the coding sequence predictions accompanying the complete Arabidopsis genome sequence.

Northern-Blot Analysis

Plants used for northern-blot analysis were of the Landsberg *erecta* genetic background, with or without additional mutations in floral homeotic genes, as indicated. RNA was extracted from plant tissues either by a method based on hot SDS and phenol (Scutt, 1997) or using Trisol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Where required, polyadenylated RNA samples were purified using a PolyATract RNA extraction kit (Promega, Madison, WI). Inflorescence tissues included all stages of flower bud and flower development up to stage 14, the fertilization stage of wt flowers (Bowman, 1994). Inflorescence tissues from three homeotic mutant lines were also included in northern analyses. Of these, the *pi-1* and *ag-1* mutations showed high penetrance, giving uniform mutant phenotypes. The *sup-1* mutation, by contrast, showed low penetrance, giving highly plastic phenotypic effects. For this reason, RNA used in northern blotting from *sup-1* mutants was extracted only from plants that showed an extreme *sup-1* phenotype. This phenotype corresponded to a greatly increased number of stamens and a gynoecium that was reduced to a thin filament-like structure or was absent (Bowman et al., 1992). Total RNA samples of 10 μg or polyadenylated RNA samples of 4 μg were analyzed on formaldehyde-containing agarose gels (Sambrook et al., 1989) and capillary blotted onto Hybond-N membranes (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions. Northern hybridizations with radiolabeled full-length cDNA probes were carried out as described by Scutt et al. (1997) and washed to high stringency in solutions containing 0.1× SSPE (Sambrook et al., 1989) and 0.1% (w/v) SDS at 65°C. This washing stringency has been demonstrated (data not shown) to discriminate between homologous sequences of 4 kb sharing an average of >95% nucleic acid sequence similarity.

In Situ Hybridization

In situ hybridizations were performed on flower bud, mature flower, and silique material of plants of the Landsberg *erecta* genetic background. Riboprobes of full-length gynoecium-expressed cDNAs were labeled by incorporation of digoxygenin-conjugated ribonucleotides during in vitro transcription reactions. These reactions employed templates of linearized plasmids containing T3, T7, or SP6 RNA polymerase promoter sites flanking the full-length, gynoecium-expressed cDNAs. Riboprobes corresponding to antisense cDNA strands were used to detect the presence of homologous mRNAs, whereas the corresponding sense strands were generated using alternative RNA polymerases for use as negative controls. Tissue fixation, embedding in Paraplast Extra (Sherwood Medical, St. Louis), sectioning, and in situ hybridization was carried out as described by Bradley et al. (1993). Sections were counterstained with 0.1% (w/v) Calcofluor White MR2 (Sigma-Aldrich, San Luis Obispo, CA) to visualize cellulose-containing material under UV fluorescence microscopy. The sections were then dried and rendered permanent in Entellan reagent (Merck, Darmstadt, Germany). Photomicrographs were taken on daylight color reversal film using mixed UV and visible illumination on a fluorescence microscope (Optiphot-2, Nikon, Tokyo).

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LITERATURE CITED

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol **215:** 403–410

Andreasson E, Taipalensuu J, Rask L, Meijer J (1999) Age-dependent wound induction of a myrosinase-associated protein from oilseed rape (*Brassica napus*). Plant Mol Biol **41:** 171–180

Annamalai P, Yanagihara S (1999) Identification and characterization of a heat-stress induced gene in cabbage encodes a kunitz type protease inhibitor. J Plant Physiol **155:** 226–233

Atkinson AH, Heath RL, Simpson RJ, Clarke AE, Anderson MA (1993) Proteinase inhibitors in *Nicotiana alata* stigmas are derived from a precursor protein which is processed into five homologous inhibitors. Plant Cell **5:** 203–213

Benedict CR, Lu JL, Pettigrew DW, Liu JG, Stipanovic RD, Williams HJ (2001) The cyclization of farnesyl diphosphate and nerolidyl diphosphate by a purified recombinant δ-cadinene synthase. Plant Physiol **125:** 1754–1765

Bohlmann H, Vignutelli A, Hilpert B, Miersch O, Wasternack C, Apel K (1998) Wounding and chemicals induce expression of the *Arabidopsis thaliana* gene *Thi2.1*, encoding a fungal defense thionin, via the octadecanoid pathway. FEBS Lett **437:** 281–286

Bohmert K, Camus I, Bellini C, Bouchez D, Caboche M, Benning C (1998) *AGO1* defines a novel locus of *Arabidopsis* controlling leaf development. EMBO J **17:** 170–180

Bouché N, Bouchez D (2001) *Arabidopsis* gene knockout: phenotypes wanted. Curr Opin Plant Biol **4:** 111–117

Bowman J (1994) *Arabidopsis*: An Atlas of Morphology and Development. Springer-Verlag, New York

Bowman JL, Baum SF, Eshed Y, Putterill J, Alvarez J (1999) Molecular genetics of gynoecium development in *Arabidopsis*. Curr Topics Dev Biol **45:** 155–205

Bowman JL, Eshed Y, Baum S, Emery JF, Floyd SK, Alvarez J, Hawker NP, Lee J-Y, Siegfried KR, Khodosh R et al. (2001) The story of *crabs claw* (or how we learned to love the mutagen). Flowering Newslett **31:** 3–11

Bowman JL, Sakai H, Jack T, Weigel D, Mayer U, Meyerowitz EM (1992) *SUPERMAN*, a regulator of floral homeotic genes in *Arabidopsis*. Development **114:** 599–615

Bowman JL, Smyth DR, Meyerowitz EM (1989) Genes directing flower development in *Arabidopsis*. Plant Cell **1:** 37–52

Bradley D, Carpenter R, Sommer H, Hartley N, Coen E (1993) Complementary floral homeotic phenotypes result from opposite orientations of a transposon at the *PLENA* locus of *Antirrhinum*. Cell **72:** 85–95

Brzobohaty B, Moore I, Kristoffersen P, Bako L, Campos N, Schell J, Palme K (1993) Release of active cytokinin by a β-glucosidase localized to the maize root meristem. Science **262:** 1051–1054

Capella AN, Menossi M, Arruda P, Benedetti CE (2001) *COI1* affects myrosinase activity and controls the expression of two flower-specific myrosinase-binding protein homologues in *Arabidopsis*. Planta **213:** 691–699

Cerutti L, Mian N, Bateman A (2000) Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the PIWI domain. Trends Biochem Sci **25:** 481–482

de Blank C, Mylona P, Yang WC, Katinakis P, Bisseling T, Franssen H (1993) Characterization of the soybean early nodulin cDNA clone *Gm-ENOD55*. Plant Mol Biol **22:** 1167–1171

Epple P, Apel K, Bohlmann H (1995) An *Arabidopsis thaliana* thionin gene is inducible via a signal-transduction pathway different from that for pathogenesis-related proteins. Plant Physiol **109:** 813–820

Fagard M, Boutet S, Morel JB, Bellini C, Vaucheret H (2000) *AGO1, QDE-2,* and *RDZ-1* are related proteins required for post-transcriptional gene silencing in plants, quelling in fungi, and RNA interference in animals. Proc Natl Acad Sci USA **97:** 11650–11654

Fahn A (1975) Plant Anatomy, Ed 2. Pergamon Press, Oxford

Falk A, Rask L (1995) Expression of a zeatin-o-glucoside-degrading β-glucosidase in *Brassica napus*. Plant Physiol **108:** 1369–1377

Faure J-E, Dumas C (2001) Fertilization in flowering plants: new approaches for an old story. Plant Physiol **125:** 102–104

Ferrandiz C, Pelaz S, Yanofsky MF (1999) Control of carpel and fruit development in *Arabidopsis*. Annu Rev Biochem **68:** 321–354

Geshi N, Andreasson E, Meijer J, Rask L, Brandt A (1998) Co-localization of myrosinase- and myrosinase-binding proteins in grains of myrosin cells in cotyledon of *Brassica napus* seedlings. Plant Physiol Biochem **36:** 583–590

Goodwin TW, Mercer EI (1983) Introduction to Plant Biochemistry, Ed 2. Pergamon, Oxford

Greene EA, Erard M, Dedieu A, Barker DG (1998) *MtENOD16* and *20* are members of a family of phytocyanin-related early nodulins. Plant Mol Biol **36:** 775–783

Harborne JB (1993) Introduction to Ecological Biochemistry, Ed 4. Academic Press, London

Heath RL, McDonald G, Christeller JT, Lee M, Bateman K, West J, van Heeswijck R, Anderson MA (1997) Proteinase inhibitors from *Nicotiana alata* enhance plant resistance to insect pests. J Insect Physiol **43:** 833–842

Honma T, Goto K (2001) Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. Nature **409:** 525–529

Kuno N, Muramatsu T, Hamazato F, Furuya M (2000) Identification by large-scale screening of phytochrome-regulated genes in etiolated seedlings of *Arabidopsis* using a fluorescent differential display technique. Plant Physiol **122:** 15–24

Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol **157:** 105–132

Lauvergeat V, Lacomme C, Lacombe E, Lasserre E, Roby D, Grima-Pettenati J (2001) Two cinnamoyl-coA reductase (CCR) genes from *Arabidopsis thaliana* are differentially expressed during development and in response to infection with pathogenic bacteria. Phytochemistry **57:** 1187–1195

Liang P, Pardee AB (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science **257:** 967–971

Liljegren SJ, Ditta GS, Eshed HY, Savidge B, Bowman JL, Yanofsky MF (2000) *SHATTERPROOF* MADS-box genes control seed dispersal in *Arabidopsis*. Nature **404:** 766–770

Lorito M, Broadway RM, Hayes CK, Woo SL, Williams DL, Harman GE (1994) Proteinase inhibitors from plants as a novel class of fungicides. Mol Plant-Microbe Interact **4:** 525–527

Lynn K, Fernandez A, Aida M, Sedbrook J, Tasaka M, Masson P, Barton MK (1999) The *PINHEAD/ZWILLE* gene acts pleiotropically in *Arabidopsis* development and has overlapping functions with the *ARGONAUTE1* gene. Development **126:** 469–481

Malboobi MA, Lefebvre DD (1997) A phosphate-starvation inducible β-glucosidase gene (*PSR3.2*) isolated from *Arabidopsis thaliana* is a member of a distinct subfamily of the BGA family. Plant Mol Biol **34:** 57–68

McPherson MJ, Hames BD, Taylor GR (1995) PCR 2: A Practical Approach. Oxford University Press, Oxford

Miclet E, Stoven V, Michels PAM, Opperdoes FR, Lallemand JY, Duffieux F (2001) NMR spectroscopic analysis of the first two steps of the pentose-phosphate pathway elucidates the role of 6-phosphogluconolactonase. J Biol Chem **276:** 34840–34846

Morel JB, Godon C, Mourrain P, Beclin C, Boutet S, Feuerbach F, Proux F, Vaucheret H (2002) Fertile hypomorphic *argonaute1* (*ago1*) mutants impaired in post-transcriptional gene silencing and virus resistance. Plant Cell **14:** 629–639

Moussian B, Schoof H, Haecker A, Jurgens G, Laux T (1998) Role of the *ZWILLE* gene in the regulation of central shoot meristem cell fate during *Arabidopsis* embryogenesis. EMBO J **17:** 1799–1809

Nicholson RL, Hammerschmidt R (1992) Phenolic-compounds and their role in disease resistance. Annu Rev Phytopathol **30:** 369–389

Pelaz S, Ditta GS, Baumann E, Wisman E, Yanofsky MF (2000) B and C floral organ identity functions require *SEPALLATA* MADS-box genes. Nature **405:** 200–203

Rask L, Andreasson E, Ekbom B, Eriksson S, Pontoppidan B, Meijer J (2000) Myrosinase: gene family evolution and herbivore defense in Brassicaceae. Plant Mol Biol **42:** 93–113

Rubinelli P, Hu Y, Ma H (1998) Identification, sequence analysis and expression studies of novel anther-specific genes of *Arabidopsis thaliana*. Plant Mol Biol **37:** 607–619

Sambrook J, Fritsh EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Scheres B, Vanengelen F, Vanderknaap E, Vandewiel C, Vankammen A, Bisseling T (1990) Sequential induction of nodulin gene-expression in the developing pea nodule. Plant Cell **2:** 687–700

Schneitz K, Balasubramanian S, Schiefthaler U (1998) Organogenesis in plants: the molecular and genetic control of ovule development. Trends Plant Sci **3:** 468–472

Scutt CP (1997) Differential screening. *In* E Hansen, G Harper, eds, Differentially-Expressed Genes in Plants: A Bench Manual. Taylor and Francis, London, pp 1–22

Scutt CP, Jenkins T, Furuya M, Gilmartin PM (2002) Male specific genes from dioecious white campion identified by fluorescent differential display. Plant Cell Physiol **43:** 563–572

Scutt CP, Li Y, Robertson SE, Willis ME, Gilmartin PM (1997) Sex determination in dioecious *Silene latifolia*: effects of the y chromosome and the parasitic smut fungus (*Ustilago violacea*) on gene expression during flower development. Plant Physiol **114:** 969–979

Sessions RA (1997) *Arabidopsis* (Brassicaceae) flower development and gynoecium patterning in wild type and *ETTIN* mutants. Am J Bot **84:** 1179–1191

Sommer-Knudsen J, Clarke AE, Bacic A (1997) Proline- and hydroxyproline-rich gene products in the sexual tissues of flowers. Sex Plant Reprod **10:** 253–260

Taipalensuu J, Eriksson S, Rask L (1997) The myrosinase-binding protein from *Brassica napus* seeds possesses lectin activity and has a highly similar vegetatively-expressed, wound-inducible counterpart. Eur J Biochem **250:** 680–688

Utsugi S, Sakamoto W, Murata M, Motoyoshi F (1998) *Arabidopsis thaliana* vegetative storage protein (VSP) genes: gene organization and tissue-specific expression. Plant Mol Biol **38:** 565–576

Vaucheret H, Fagard M (2001) Transcriptional gene silencing in plants: targets, inducers and regulators. Trends Genet **17:** 29–35

Vignutelli A, Wasternack C, Apel K, Bohlmann H (1998) Systemic and local induction of an *Arabidopsis* thionin gene by wounding and pathogens. Plant J **14:** 285–295

Wesley SV, Helliwell CA, Smith NA, Wang MB, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA et al. (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J **27:** 581–590

West CE, Waterworth WM, Stephens SM, Smith CP, Bray CM (1998) Cloning and functional characterisation of a peptide transporter expressed in the scutellum of barley grain during the early stages of germination. Plant J **15:** 221–229

Western TL, Skinner DJ, Haughn GW (2000) Differentiation of mucilage secretory cells of the *Arabidopsis* outer seed coat. Plant Physiol **122:** 345–355

Wilhelmi LK, Preuss D (1999) The mating game: pollination and fertilization in flowering plants. Curr Opin Plant Biol **2:** 18–22

Windsor JB, Symonds VV, Mendenhall J, Llotd AM (2000) *Arabidopsis* seed coat development: morphological differentiation of the outer integument. Plant J **22:** 483–493

Wittenbach VA (1983) Effect of pod removal on leaf photosynthesis and soluble protein composition of field-grown soybeans. Plant Physiol **73:** 121–124

Young NM, Johnston RAZ, Watson DC (1991) The amino acid sequences of jacalin and the *Maclura pomifera* aglutinin. FEBS Lett **282:** 382–386

Yung MH, Schaffer R, Putterill J (1999) Identification of genes expressed during early *Arabidopsis* carpel development by mRNA differential display: characterisation of *ATCEL2*, a novel endo-1,4-β-d-glucanase gene. Plant J **17:** 203–208

Zilberman D, Cao X, Jacobsen SE (2003) *ARGONAUTE4* control of locus-specific siRNA accumulation and DNA and histone methylation. Science **299:** 716–719