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Male and Female Flowers of the Dioecious Plant Sorrel Show Different Patterns of MADS Box Gene Expression

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Male and female flowers of the dioecious plant sorrel (*Rumex acetosa*) each produce three whorls of developed floral organs: two similar whorls of three perianth segments and either six stamens (in the male) or a gynoecium consisting of a fertile carpel and two sterile carpels (in the female). In the developing male flower, there is no significant proliferation of cells in the center of the flower, in the position normally occupied by the carpels of a hermaphrodite plant. In the female flower, small stamen primordia are formed. To determine whether the organ differences are associated with differences in the expression of organ identity genes, cDNA clones representing the putative homologs of B and C function MADS box genes were isolated and used in an in situ hybridization analysis. The expression of *RAD1* and *RAD2* (two different *DEFICIENS* homologs) in males and females was confined to the stamen whorl; the lack of expression in the second, inner perianth whorl correlated with the sepaloid nature of the inner whorl of perianth segments. Expression of *RAP1* (a *PLENA* homolog) occurred in the carpel and stamen whorls in very young flower primordia from both males and females. However, as soon as the inappropriate set of organs ceased to develop, *RAP1* expression became undetectable in those organs. The absence of expression of *RAP1* may be the cause of the arrest in organ development or may be a consequence.

INTRODUCTION

In dioecious plants, the male and female flowers are borne on separate individuals (male and female plants). These plants constitute only 4% of flowering plant species. Sorrel (*Rumex acetosa*; Polygonaceae) is one of only 13 plant species in which dioecy is determined by identifiable sex chromosomes (Parker, 1990). Among this small group, there are two main types of sex determination systems. In the active Y system, found, for example, in white campion (*Silene latifolia*), males are XY and females XX, with the Y chromosome acting as a maleness enhancer as well as suppressing the development of the gynoecium. The second system of sex determination, such as the one found in sorrel, has an X-to-autosome balance, with sex determined by the balance of X chromosomes to autosomes.

Sorrel has an aneuploid sex system in which 2n is 12 plus XX in females and 12 plus XY_1Y_2 in males. This system, depicted in Figure 1, was first described by Kihara and Ohno (1923). When the ratio between X chromosomes and autosome sets is 1.0 or higher, the resulting individuals are female, whereas X-to-autosome ratios of 0.5 and lower result in males. Ratios between 0.5 and 1.0 can produce an intermediate, hermaphrodite phenotype. The primary sex determination is therefore independent of the presence or absence of the Y chromosomes. Production of the male floral parts is also independent of the Y chromosomes because near tetraploids in which 2n is 24 plus XXX (with a ratio of 0.75) are hermaphrodites. The two Y chromosomes, however, are required for the successful progress of meiosis in pollen mother cells (Parker and Clark, 1991).

Sex determination in sorrel is influenced not only by the X-to-autosome ratio but also by the number and types of autosomes. Analysis of near-triploids that are tetrasomic for particular autosomes has enabled the autosomes to be classified as male promoting, female promoting, or sex neutral (Yamamoto, 1938). For example, triploids in which 2n is 18 plus XXY_1Y_2 are hermaphrodites, whereas triploids in which 2n is 19 plus XXY_1Y_2, which includes an extra copy of chromosome 2, flower as females. Parts of chromosomes also affect sex determination. In plants that carry a translocation resulting in disomy for a quarter of the X chromosome, in addition to XY_1Y_2, hermaphrodite flowers are formed that are female fertile but have shriveled anthers (Parker, 1990). Plants lacking this X chromosome segment are male. The extra X chromatin is therefore able to give a functionally female phenotype,

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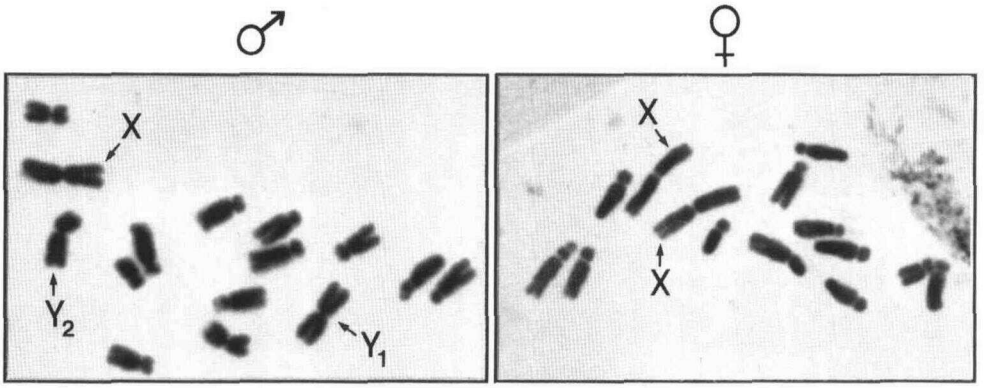

Figure 1. The Chromosomes of Sorrel.

Mitotic chromosome spreads from root tip cells of male and female sorrel plants. The X and Y chromosomes are indicated with arrows.

although it is insufficient to suppress the development of an androecium. These observations point to the fact that a number of genes, carried on autosomes and sex chromosomes, interact in a complex way to determine sex. Genes on the X chromosome and autosomes determine the onset of the cascade of events that results in development of maleness and femaleness. It is only after the establishment of maleness that genes on the Y chromosomes are involved in pollen development.

The large genus *Rumex* contains ~200 species, most of which occur in north temperate regions. The majority of species are hermaphrodites, but dioecy has evolved at least three times. The basic Polygonaceae flower type consists of four whorls of organs. In the hermaphrodite *Rumex* species, such as *R. obtusifolius*, the two outer whorls belong to the perianth, and one stamen whorl follows, with the tripartite ovary occupying the central whorl. In *R. acetosa*, both male and female flowers have only three whorls of developed organs: the male flowers consist of a whorl of stamens and two whorls of perianth segments; the female flower consists of three fused carpels with a single ovule and two whorls of perianth segments—one of which later encircles the fruit. In both male and female flowers, the organs in the perianth whorls are very similar and sepaloid; there is no clear distinction between sepals and petals as there is with most plant species. In addition to mutants carrying hermaphrodite flowers, occasional aberrant flowers are found on otherwise normal plants.

Almost nothing is known about the molecular mechanisms that determine sex in dioecious plants or the mechanisms that control the organ differences between the sexes. A family of regulatory genes, the MADS box homeotic genes (Schwarz-Sommer et al., 1990), has been shown to be instrumental in determining the identity of floral organs in the hermaphrodite angiosperm flower. Three sets of genes (the A, B, and C function genes) are, in most cases, each expressed in two adjacent whorls of the four whorls of the floral primordium and together may result in determination of the four types of organ; this is the ABC model of Coen and Meyerowitz (1991).

A possible mechanism for differentiation of the male and female flowers involves the differential expression of homeotic genes. A switch between the carpel whorl and the stamen whorl is all that is required for the homeotic-like interconversion of male and female flowers. In Antirrhinum, mutation of the B function *DEFICIENS* gene effectively results in the conversion of a hermaphrodite flower to a female flower (the organs in whorls 2 and 3 are converted to sepals and carpels) (Schwarz-Sommer et al., 1990). Although the male and female sorrel flowers differ from homeotic mutants in that organs in one whorl are suppressed, rather than the transformation of organs in a pair of adjacent whorls, we propose that differences in the expression patterns of the B or C function genes could account for the organ differences that distinguish between the two sexes.

The initial goal of our research was to isolate genes known to be involved in initiating floral organ development in hermaphrodite plants and to focus on the MADS box genes and their expression patterns. If differences in MADS box gene expression could be found between the sexes, then the pathway could be traced back to the primary gene or genes determining sex.

RESULTS

Morphology of Developing Male and Female Flowers

Scanning electron and light microscopy studies have been conducted with male and female inflorescences at a range of developmental stages to describe the differences between the flowers during development in the two sexes. Both male and female inflorescences are racemose, with several branches, and the youngest flowers are at the top of each branch. In both the male and the female, the flowers are borne in clusters (of up to 10 flowers) from each node on the inflorescence. Mature male and female inflorescences and single flowers are shown in Figures 2A to 2E. In Figure 2F, a male flower is shown in

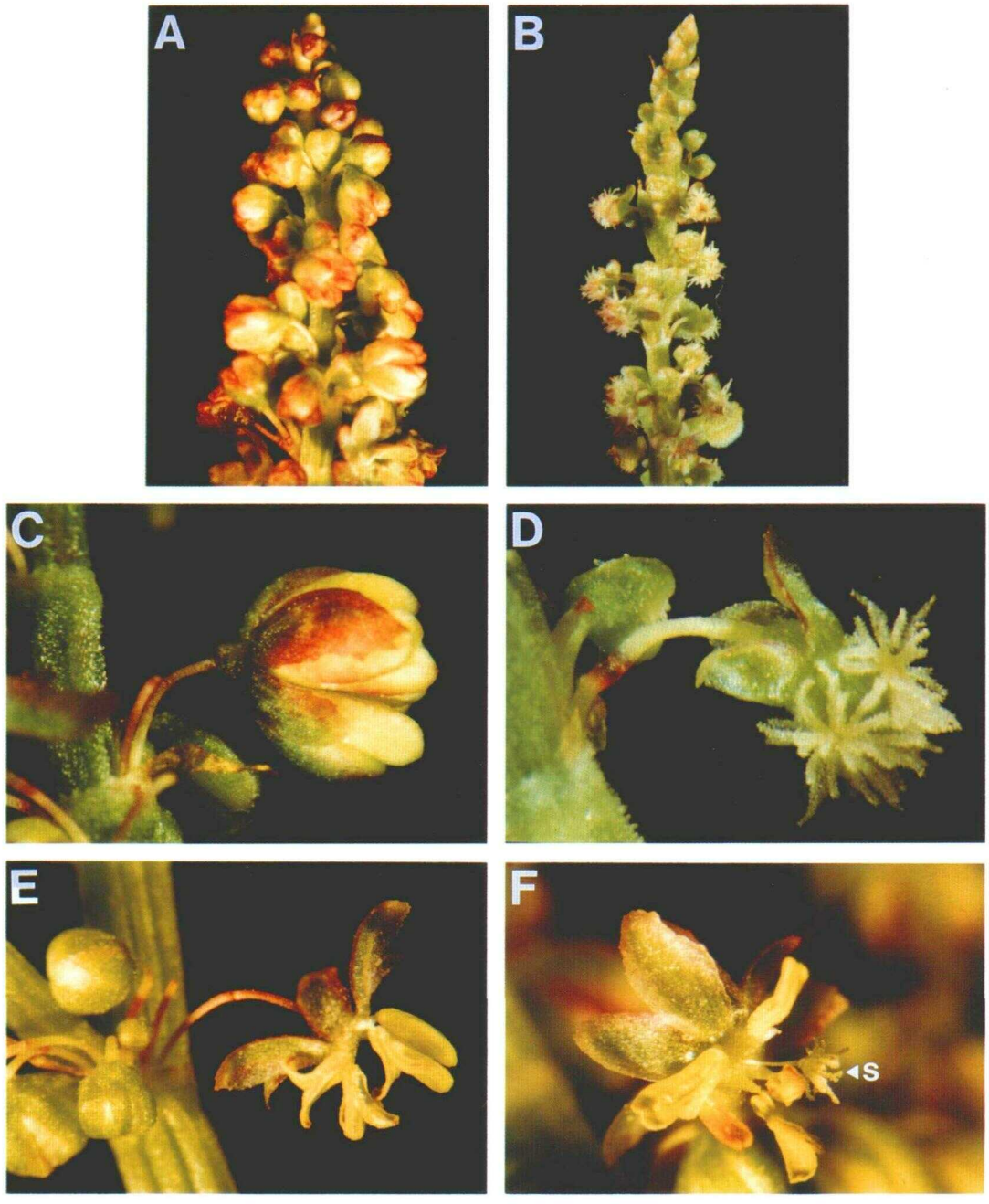

Figure 2. Flower Morphology in Sorrel.

(A) Male inflorescence.
(B) Female inflorescence.
(C) Mature male flower prior to anthesis.
(D) Mature female flower.
(E) Mature male flower after anthesis.
(F) Mutant male flower in which one stamen has been replaced by a style (arrowhead).

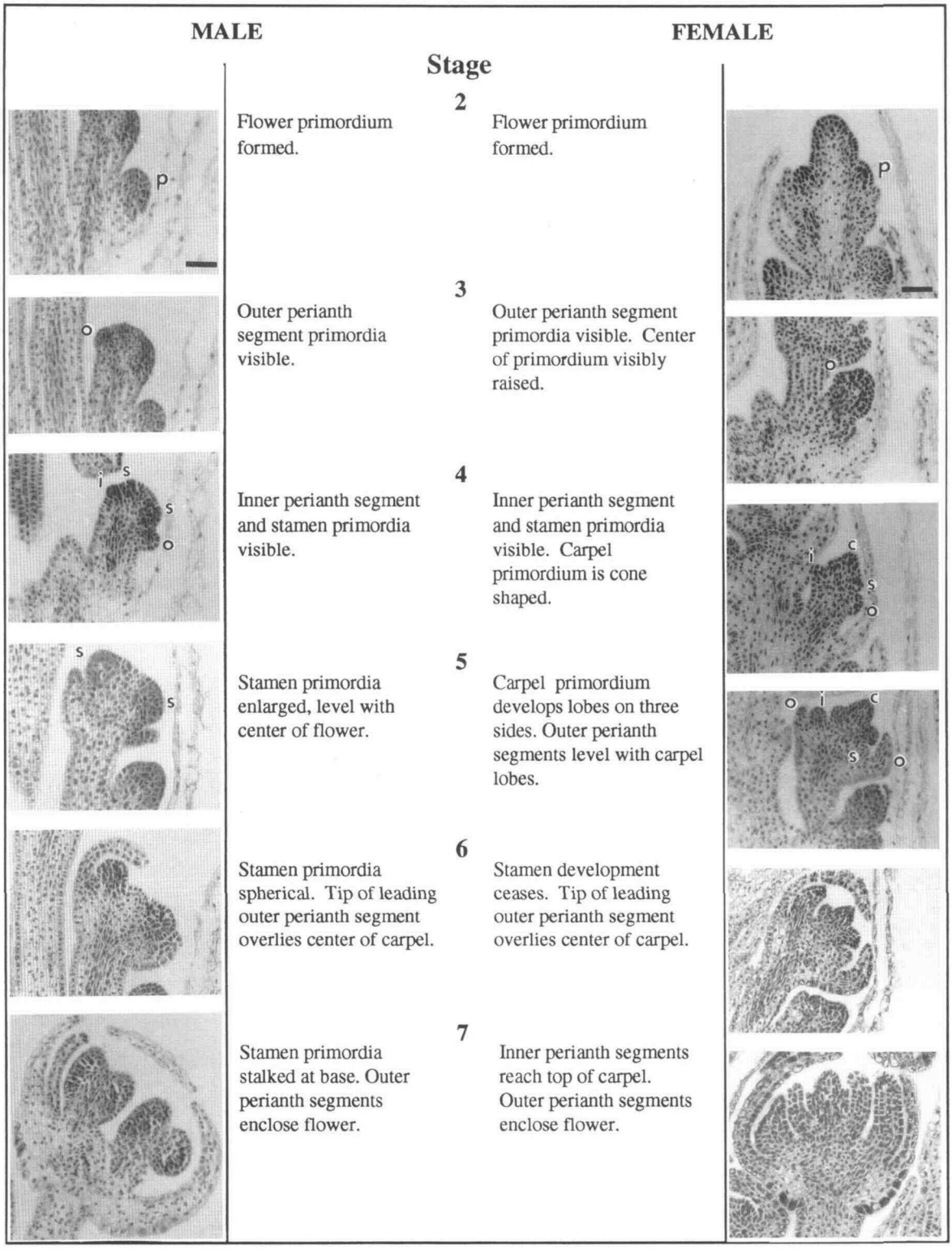

Figure 3. Light Microscopy Used To Summarize Development in Male and Female Sorrel Flowers.

Flower primordia at the stages described are numbered. The scale is the same; bars = 50 μm. c, carpel; i, inner perianth segment; o, outer perianth segment; p, flower primordium; s, stamen.

which one stamen has been replaced by a sterile carpel and stigma, illustrating that the developmental program for a single organ can be aberrant within an otherwise normal whorl of organs.

To characterize the morphological changes that occur during the development of the flower, we have adopted a scheme similar to the one used in Arabidopsis (Smyth et al., 1990). In this model, the development of the flower is divided into identifiable stages. Light microscopy of the six stages identified are shown in Figure 3, and scanning electron micrographs of late stage 5 flowers are shown in Figure 4.

Flower Development in the Male

The male flower primordia are formed on the flanks of the inflorescence and are subtended by a bract. The stage at which the hemispherical primordium is first visible is termed stage 2. In stage 3, the outer three perianth segment primordia form on the flanks of the primordium. In stage 4, the inner three perianth segment primordia and the six stamen primordia become visible. The stamen primordia and the two whorls of perianth segment primordia continue to develop until, in stage 5, the stamen primordia appear as hemispherical structures on the flanks of the developing flower and are level with the center of the flower. In stage 6, the stamen primordia are further developed, but no development of a carpel in the center of the flower has occurred. The lack of carpel development, which was not seen to be accompanied by tissue degeneration or obvious signs of cell death, is clear in the scanning electron micrograph of a stage 5 flower shown in Figure 4. The tip of the leading outer perianth segment now lies over the center of the flower. In stage 7, the perianth segments enclose the flower, with the inner and outer perianth segments (which are alternate) overlapping on their sides. The stamen primordia have constrictions at their bases, which is the first sign of differentiation of the anther filaments. Stages later than stage 7 are not defined. The diameter of the male flower bud immediately prior to opening is ~2 mm. After opening, flower diameter is ~4 mm.

In the mature male flower (shown in Figures 2C and 2E), the six anthers are enclosed by the two whorls of three perianth segments that open and become wide spreading after anthesis. The perianth segments persist after the dehisced anthers have been shed. A feature of both male and female flowers is the accumulation of anthocyanins in some of the organs: anthers and perianth segments in the male; stigmata in the female.

Flower Development in the Female

Female flower primordia are formed on the flanks of the inflorescence and are visible as hemispherical structures in stage 2. The clusters of primordia are each subtended by a bract. In stage 3, the three outer perianth segment primordia are visible. Female floral primordia can be distinguished from those in the male at this early stage, because the carpel primordium has developed to give the flower primordium a clearly raised center. In stage 4, the central carpel develops further to give the flower primordium a distinct conical appearance, and the stamen primordia and the inner perianth segment primordia become visible. The stamen primordia develop further and, in stage 5 flowers, reach their maximum size. The stamen primordia persist in later stages and do not appear to degenerate. During stage 5, the leading outer perianth segment is level with three lobes that have developed on the flanks of the carpel primordium. These lobes will form the outer walls of the

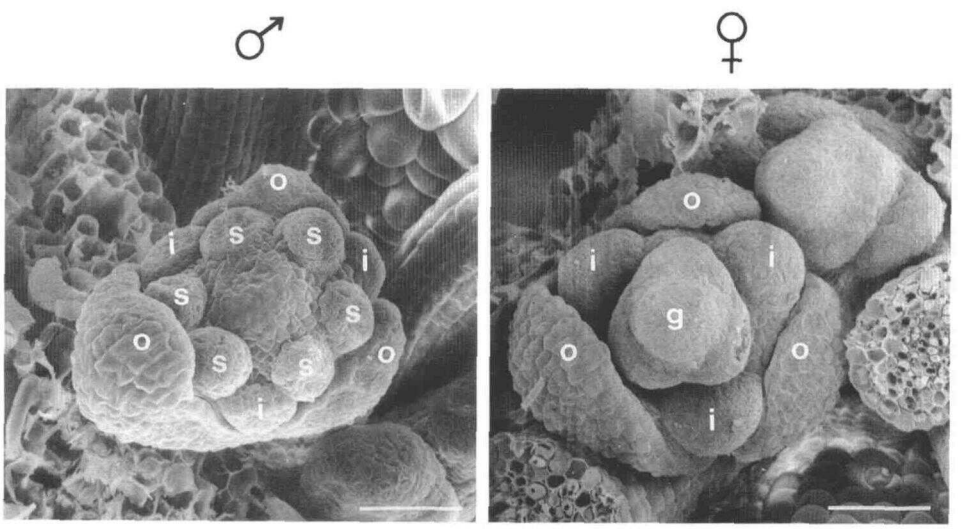

Figure 4. Scanning Electron Microscopy of Stage 5 Male and Female Flower Primordia.

Bars = 50 μm. g, gynoecium; i, inner perianth segment; o, outer perianth segment; s, stamen.

ovary. Scanning electron microscopy shows this structure clearly in Figure 4. In stage 6 buds, stamen development has ceased, the tip of the leading outer perianth segment overlies the center of the flower, and the lobes on the carpel extend and begin to envelop the carpel itself. In stage 7 buds, these lobes have elongated to reach the top of the carpel, and the inner perianth segments are also level with the tip of the carpel. A second set of lateral lobes has developed on the flanks of the carpel below the apex, and these form the innermost walls of the ovary. Stages later than stage 7 are not defined. The diameter of the female flower bud just prior to maturity is ~1 mm.

In the mature female flower (shown in Figure 2D), the three outer perianth segments are reflexed back toward the pedicel; the three inner perianth segments enclose the gynoecium and the three (separate) styles, which grow out from the tip of the ovary. The unusual three-whorled type of gynoecium development in which there is a single carpel that contains the unilocular ovary and two sets of sterile carpels that form the ovary walls is rare and confined to the Polygonaceae and Balsaminaceae.

MADS Box Genes in Sorrel

Poly(A)$^+$ RNA isolated from developing bud RNA from small male and female inflorescences (2 to 6 mm in length after bract removal) was used in the construction of cDNA libraries. These libraries were screened at low stringency with probes for floral homeotic genes from Antirrhinum (*DEFICIENS* [Schwarz-Sommer et al., 1990] and *PLENA* [Bradley et al., 1993]). We isolated 244 hybridizing clones (88 with *DEFICIENS* and 156 with *PLENA*) by screening 2×10^6 independent recombinants; 32 of these, which appeared to vary in hybridization intensity, were plaque purified. RNA gel blot analysis was used to determine whether the clones represented genes that were expressed in flowers. From these, seven different full-length MADS box clones, ranging in length from 0.9 to 1.2 kb, were sequenced.

Comparison of the deduced amino acid sequences with MADS box protein sequences from other plants indicated possible homologies. This analysis suggests that three of the clones may represent putative homologs of B and C function MADS box genes. These cloned genes were assigned the names *RAD1* and *RAD2* (for *R. acetosa DEFICIENS*-like) and *RAP1* (*R. acetosa PLENA*-like), based on sequence similarity. The sequences of *RAD1*, *RAD2*, and *RAP1* and their derived proteins are shown in Figure 5. They have the typical structure expected of MADS box genes and proteins, with a highly conserved MADS domain (encoding 58 amino acids) located toward the 5′ end, followed by a region, which includes the K-box, which is less conserved between different MADS box genes.

The multiple alignment of the derived amino acid sequences from these clones with the Antirrhinum and Arabidopsis B and C function protein homologs is shown in Figure 6. *RAP1* most likely represents the C function (*PLENA*/*AGAMOUS*) homolog, which shows a high degree of amino acid similarity with *PLENA* (94.8% within the MADS box and 49.4% downstream of the MADS box). As in *PLENA* and *AGAMOUS*, the protein deduced from *RAP1* contains an extension N-terminal to the MADS box, which in this case is of 22 amino acids. The size of the RAP1 protein is 253 amino acids; the PLENA and AGAMOUS proteins are 239 and 285 amino acids, respectively.

RAD1 and *RAD2* both appear to represent *DEFICIENS* and *APETALA3* homologs, and the deduced proteins show 86.2 and 82.8% amino acid similarity within the MADS box and 48.4 and 42.4% downstream of the MADS domain when compared with DEFICIENS. The deduced proteins from *RAD1* and *RAD2* are 220 and 195 amino acids, respectively, and in relation to each other are 81% similar in the MADS domain and 54.5% similar in the regions downstream of it. The derived proteins from *RAD1* and *RAD2* show only ~60% similarity within the MADS box and 15% downstream of it when compared with the RAP1 protein.

The remaining five full-length clones did not include putative *GLOBOSA* and *PISTILLATA* homologs; the cDNAs were most similar to *SQUAMOSA* and some of the Arabidopsis *AGL* (for *AGAMOUS*-like) genes (data not shown). These other clones were not considered further because their expression is less likely to be related to sexual dimorphism. *SQUAMOSA*, for example, appears to be involved with the determination of shoot meristem identity (Huijser et al., 1992).

Genomic DNA gel blots were hybridized with 3′ gene-specific probes derived from the three clones so that the conserved MADS domain would be absent. Figure 7 shows the hybridization patterns resulting from probing blots carrying HindIII-digested male and female sorrel DNA (each from a single plant). *RAD1* and *RAP1* gave relatively simple patterns, indicating the presence of a small number of copies (one to two) of each gene in the sorrel genome. The pattern given by *RAD2* is more complex and suggests that a family of related sequences is present in the sorrel genome. Comparison of the hybridization patterns resulting from the three different clones shows that all three clones hybridized to different fragments, indicating that the clones represent different genes. This is particularly significant with respect to the two putative *DEFICIENS* homologs, *RAD1* and *RAD2*, which show an overall level of sequence similarity of 68.6% (81% within the MADS box and 54.8% in the region downstream). Differences in the hybridization patterns between males and females were evident (and strikingly so in the case of *RAD2*), but blots with DNA from several males and several females showed that the differences were simply restriction fragment length polymorphisms that were not related to the sex of the plant (or to the sex chromosomes) (data not shown).

Expression of MADS Box Genes

To examine the patterns of expression of the sorrel MADS box genes, the 3′ subclones of *RAD1*, *RAD2*, and *RAP1* were used

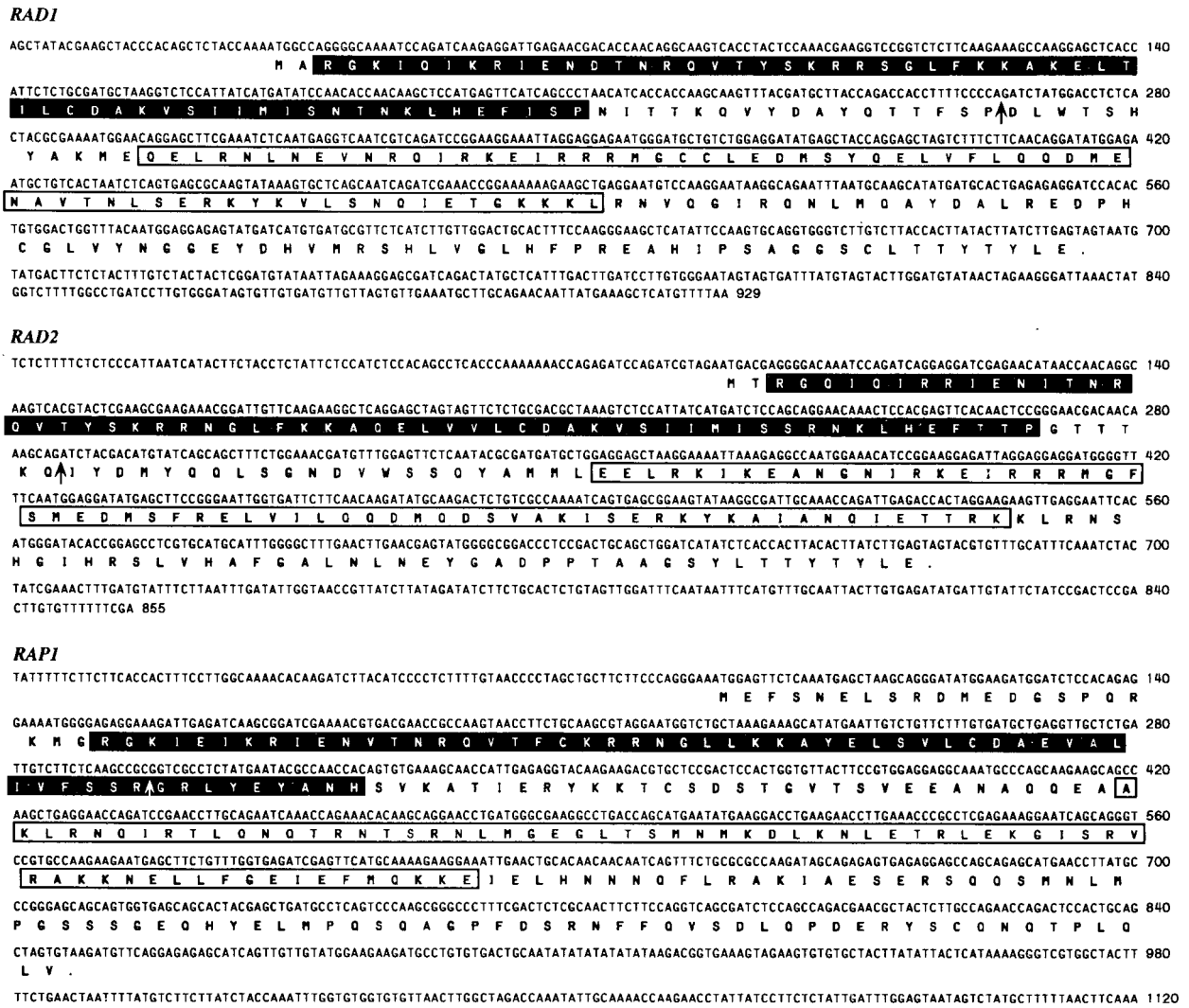

Figure 5. Nucleotide and Deduced Amino Acid Sequences of *RAD1*, *RAD2*, and *RAP1*.

Nucleotide sequences of the *RAD1*, *RAD2*, and *RAP1* cDNA inserts and their deduced amino acid sequences are shown. The MADS domains are boxed in black, and the K-boxes are represented by open boxes. The 5′ extremities of the subclones used for the generation of antisense probes used in in situ analysis and probes used in RNA and DNA gel blot analyses are indicated with arrows. The sorrel MADS box sequences have been assigned the following EMBL accession numbers: *RAD1*, X89113; *RAD2*, X89108; *RAP1*, X89107.

to probe RNA gel blots of RNA isolated from developing male and female inflorescences and RNA from mature leaves and roots, as shown in Figure 8. All three probes gave the same basic pattern of expression: transcripts were present in developing flowers from plants of both sexes but were not detectable in RNA samples from leaves and roots (from male or female plants). Based on signal intensity on the RNA gel blots, the *RAD2* gene(s) appears to be expressed at much lower levels than either the *RAD1* or *RAP1* gene. Overall, the *RAP1* gene is expressed the most strongly.

To examine the patterns of expression of these inflorescence-specific MADS box genes within the developing inflorescence, digoxigenin-labeled antisense RNA probes were generated from these 3′ gene-specific clones and were used in in situ hybridization experiments with developing male and female inflorescence tissue. These in situ hybridization patterns are shown in Figures 9 and 10.

RAD1

Expression of B function genes in a hermaphrodite plant would be expected in the whorls giving rise to the petals and stamens; these would be whorls 2 and 3, respectively. The gene

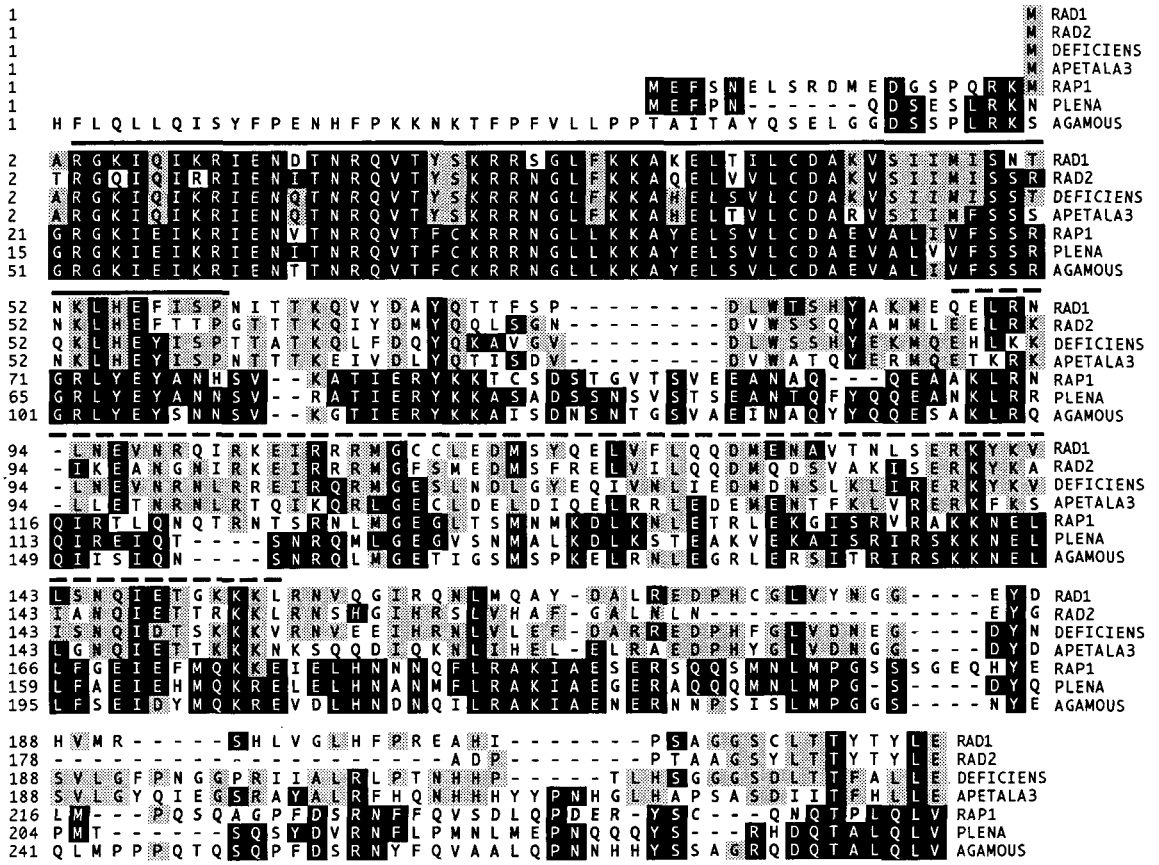

Figure 6. Amino Acid Alignment.

Multiple alignment of the amino acid sequences derived from the *RAD1*, *RAD2*, and *RAP1* cDNA clones with the amino acid sequences encoded by *PLENA* and *DEFICIENS* from Antirrhinum and *APETALA3* and *AGAMOUS* from Arabidopsis. Gaps have been introduced to maximize the alignment. The MADS domain is overlined. The K-box regions are overlined with a dashed line. Amino acids identical to those in the consensus based on DEFICIENS are shaded in gray. Amino acids identical to those in the consensus based on PLENA are shaded in black.

represented by *RAD1*, which is the putative B function gene that is more similar to *DEFICIENS* and *APETALA3* than is *RAD2*, is expressed in floral primordia from both male and female inflorescences. Inflorescences that were hybridized with *RAD1* are shown in Figures 9A and 9B.

In the male flower, expression of the *RAD1* gene was not seen in the youngest primordia (stage 2) and first became detectable in stage 3 primordia, when the outer perianth segments began to develop. Expression is localized to the shoulder region of the primordium (Figure 9E). At this early stage in development, it is difficult to define the whorls showing *RAD1* expression, although it is clear that there is no significant expression (that is, above background levels) in the central whorl (whorl 4) that would give rise to the carpel. This absence of *RAD1* expression in the central whorl persists through the development of the male flower. In stage 4 flower primordia, the expression of *RAD1* in the stamen primordia is clear (Figure 9F). This expression persists as the flower develops and is shown in stage 6 and stage 7 flowers in Figures 9G to 9I.

In male flower primordia of all stages, no hybridization with *RAD1* was observed in the perianth segment primordia. Expression in the outer sepal whorl could not be predicted from the ABC model (Coen and Meyerowitz, 1991). Because the perianth whorls each contain three members, the members of each whorl being arranged opposite each other, the sections used in the in situ hybridization experiments did not allow the sectioned perianth segments to be identified easily. The exception was when both perianth segments on one side were sectioned in the overlap region. Sections of this type are shown in Figure 9H, and it is clear that *RAD1* is not expressed in the inner perianth whorl either. Therefore, *RAD1* expression is confined to a single whorl, whorl 3, which gives rise to the stamens.

In the female, *RAD1* expression was first detectable in stage 3 flower primordia on the shoulders of each primordium, where

there is clearly no expression in the center of the primordium (Figure 10A). Analysis of older flower primordia showed that *RAD1* expression is confined to whorl 3, which gives rise to the stamen primordia that are arrested. As with the male, there is no evidence of *RAD1* expression in either perianth whorls (Figures 10F and 10G; sections include an inner and outer perianth member on one side of the primordium). *RAD1* expression in the stamen primordia is strong up to stage 5 (Figures 10A to 10E) and then becomes weaker (Figures 10F and 10G). In late stage primordia, where the ovary walls have formed, *RAD1* expression was barely detectable (Figure 10H).

RAD2

The genes represented by *RAD2* show an expression pattern similar to *RAD1*, although with much lower levels of transcript accumulation, as indicated by the RNA gel blot hybridizations. As was the case for *RAD1*, no expression was detected in the outer whorls, giving rise to the perianth segments in male or female flower primordia of any age. Expression was confined to a single whorl, whorl 3, in flowers from both sexes. In the male, expression was detected in the stamen primordia through their development (Figure 9C). In the female, expression was seen in the stamen primordia before their development stopped (Figure 9D).

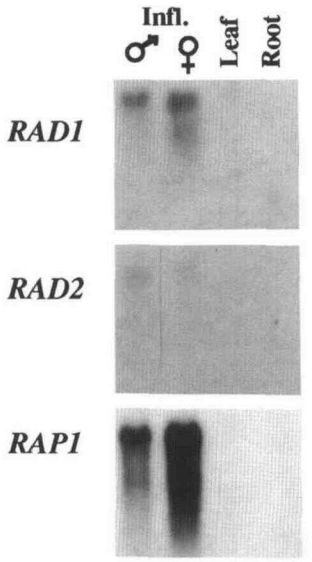

Figure 8. RNA Gel Blot Analysis of *RAD1*, *RAD2*, and *RAP1* mRNA Accumulation in Sorrel Organs.

Twenty micrograms of total RNA from vegetative organs (leaves and roots) and inflorescences (Infl.) from male and female plants was electrophoresed, blotted onto nylon membranes, and hybridized with the *RAD1*, *RAD2*, and *RAP1* cDNA inserts.

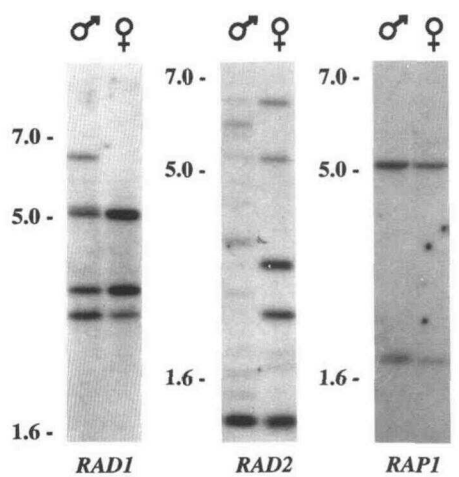

Figure 7. Genomic DNA Gel Blot Analysis of *RAD1*, *RAD2*, and *RAP1* Genes in Sorrel.

Ten micrograms of DNA from a male and a female plant was digested with HindIII, electrophoresed, blotted onto nylon membranes, and hybridized with the *RAD1*, *RAD2*, and *RAP1* cDNA inserts. The positions of DNA length markers are shown at left in kilobases.

RAP1

The putative *PLENA* and *AGAMOUS* (C function) homolog, *RAP1*, is expressed in floral primordia from both male and female inflorescences. Inflorescences hybridized with *RAP1* are shown in Figures 9A and 9B. In Arabidopsis and Antirrhinum, the C function gene is expressed in the stamen and carpel whorls (Yanofsky et al., 1990; Bradley et al., 1993).

In the male flower, expression of the *RAP1* gene is first evident in stage 3 primordia, in which the outer perianth segments begin to develop. The expression is localized in the central region of the primordium, and from analysis of the adjacent sections hybridized with *RAD1* and *RAP1*, it can be seen that the region of *RAP1* hybridization overlaps the expression of *RAD1* in the stamen primordial region (Figure 9E). In stage 4 primordia, it is clear that there is *RAP1* expression in the stamen primordia and the area between the stamen primordia (Figure 9F). In male floral primordia older than stage 4, the expression of *RAP1* disappears from the center of the flower (that is, the region that would give rise to the carpel in a hermaphrodite or female; Figure 9F). It is at this stage (stage 5 or 6) that the stamen primordia develop further, whereas development in the center of the flower stops. This expression

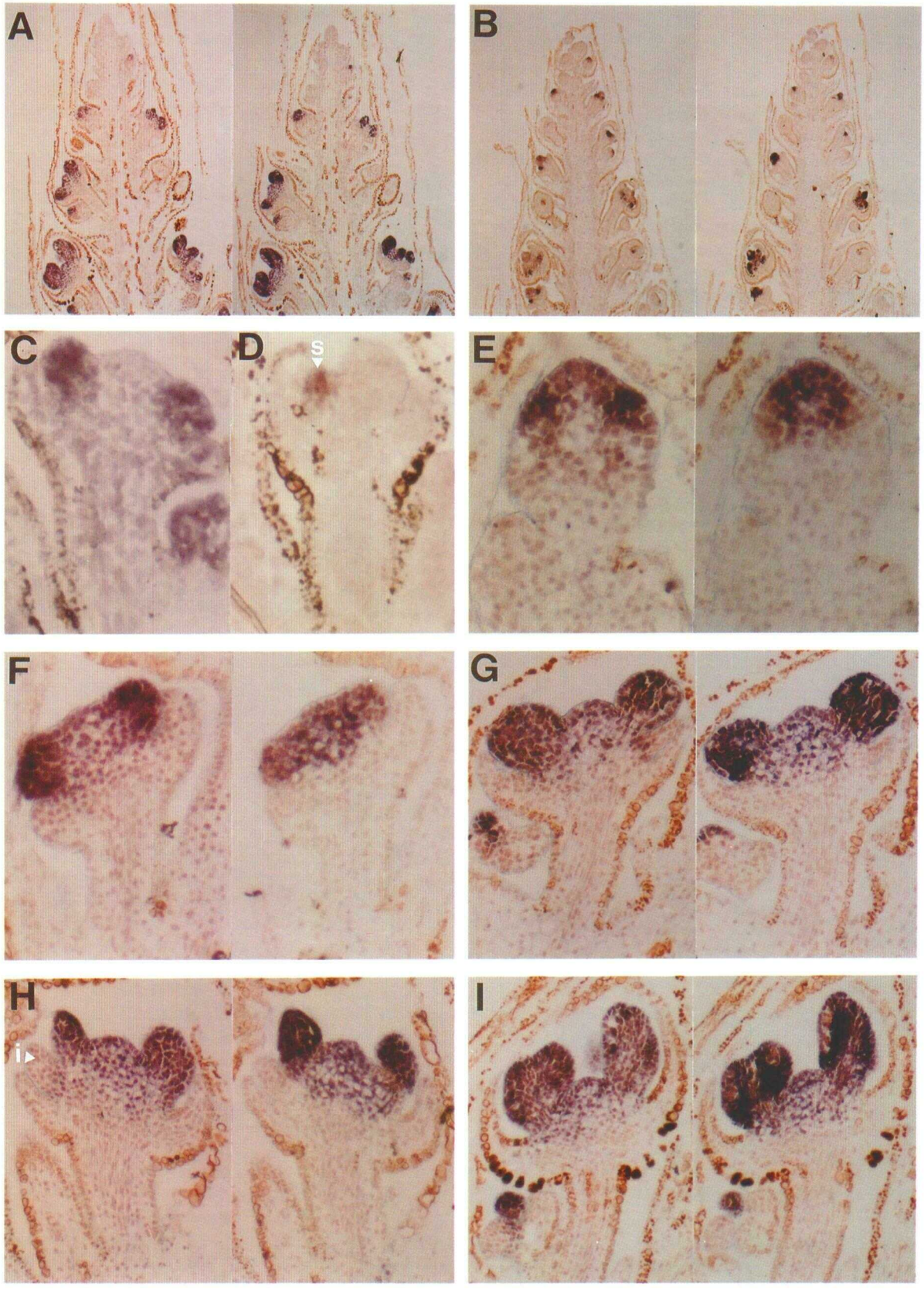

Figure 9. In Situ Localization of *RAD1*, *RAD2*, and *RAP1* mRNAs in Developing Male and Female Sorrel Flowers.

pattern is shown in a stage 6 flower in Figure 9G. The stamen-specific expression of *RAP1* continues as the primordia develop further and can be seen in late stage 6 and stage 7 primordia in Figures 9H and 9I. As predicted from the ABC model for the expression of a C function gene, no *RAP1* expression was observed in either whorl of perianth segment primordia from male flowers at any stage.

In the development of the female flower, the expression of *RAP1* begins in stage 3 primordia and is located in the central two whorls (stamen and carpel). Examination of adjacent stage 3 sections probed with *RAD1* and *RAP1* shows that, as in the male, *RAP1* expression includes the region that gives rise to the stamen primordia (Figure 10A). *RAP1* expression is retained in these two whorls in stage 4 flowers (Figure 10B) but is lost from the stamen primordia (Figures 10C and 10D). It is at this developmental stage that the stamen primordia cease to develop further. *RAP1* expression continues to be strong in the gynoecium through stages 5, 6, and 7 (Figures 10E to 10G) but is not distributed uniformly throughout the gynoecium tissue (Figure 10F). In flower primordia in which the two sets of sterile carpels have fused to become the inner and outer ovary walls, there is much reduced expression in the ovary tissue, whereas expression is retained in the inner wall (Figure 10H). As with the male, there is no evidence of *RAP1* expression in either perianth whorls in flower primordia of any age.

DISCUSSION

Structural Differences between Male and Female Sorrel Flowers

In the vegetative phase, plants of the two different sexes of sorrel are indistinguishable from one another. However, on reaching the reproductive phase, the differences between the male and female flowers are striking. The mature flowers differ greatly in size; the male flower bud before opening is 2 mm in diameter compared with the 1 mm of the female bud. The first differences between male and female are manifested very early during the development of the flowers. In stage 3 flower primordia, in which the outer perianth segment primordia are visible, the female flower primordia have a conical appearance relative to the much flatter male flower primordium. In white campion, differences between male and female flowers are apparent only after initiation of stamen and petal primordia (Grant et al., 1994).

The crucial difference between the sorrel system and all other dioecious plants that have been studied to date is the relative lack of development of the inappropriate sex organs: the carpels in the male and the stamens in the female. In the developing male flower, there is no significant proliferation of cells in the center of the flower in the position normally occupied by the carpels of a hermaphrodite plant. In the female flower, groups of cells in the third whorl proliferate to form small stamen primordia (stage 4). These develop no further and are soon obscured by the developing carpels. In flowers of both sexes, the arrest in development does not appear to be accompanied by cell death and tissue degeneration. This contrasts with the dioecious plant white campion, in which a rudimentary gynoecium of 3 mm is formed in male flowers (which reach 10 to 30 mm in length) and persists to maturity (Grant et al., 1994). In the female flower, rudimentary stamens are produced but abort and degenerate such that they are absent from the mature flower (Grant et al., 1994). In other dioecious systems, the arrest in the development of the inappropriate sex organs occurs much later, such as in *Actinidia deliciosa* and *Asparagus officinalis* (Bracale et al., 1991).

B and C Function MADS Box Genes in Sorrel

We isolated putative sorrel homologs of B and C function MADS box genes. Although we do not have homeotic mutants that are necessary to confirm their identities, the levels of amino acid similarity are high enough (80 to 90% within the MADS

Figure 9. (continued).

Developing inflorescences from male and female plants were sectioned and hybridized with antisense RNA probes of *RAD1*, *RAD2*, and *RAP1*. With the exception of **(C)** and **(D)**, which were probed with *RAD2*, each pair represents two adjacent (or near adjacent) sections of inflorescences or flower primordia probed with *RAD1* (at left) or *RAP1* (at right).

(A) Male inflorescence probed with *RAD1* or *RAP1*.

(B) Female inflorescence probed with *RAD1* or *RAP1*.

(C) Stage 6 male flower primordium probed with *RAD2*.

(D) Stage 4 female flower primordium probed with *RAD2*.

(E) Stage 3 male flower primordium probed with *RAD1* or *RAP1*.

(F) Stage 4 male flower primordium probed with *RAD1* or *RAP1*.

(G) Early stage 6 male flower primordium probed with *RAD1* or *RAP1*.

(H) Late stage 6 male flower primordium probed with *RAD1* or *RAP1*.

(I) Stage 7 male flower primordium probed with *RAD1* or *RAP1*.

i, inner perianth segment; s, stamen.

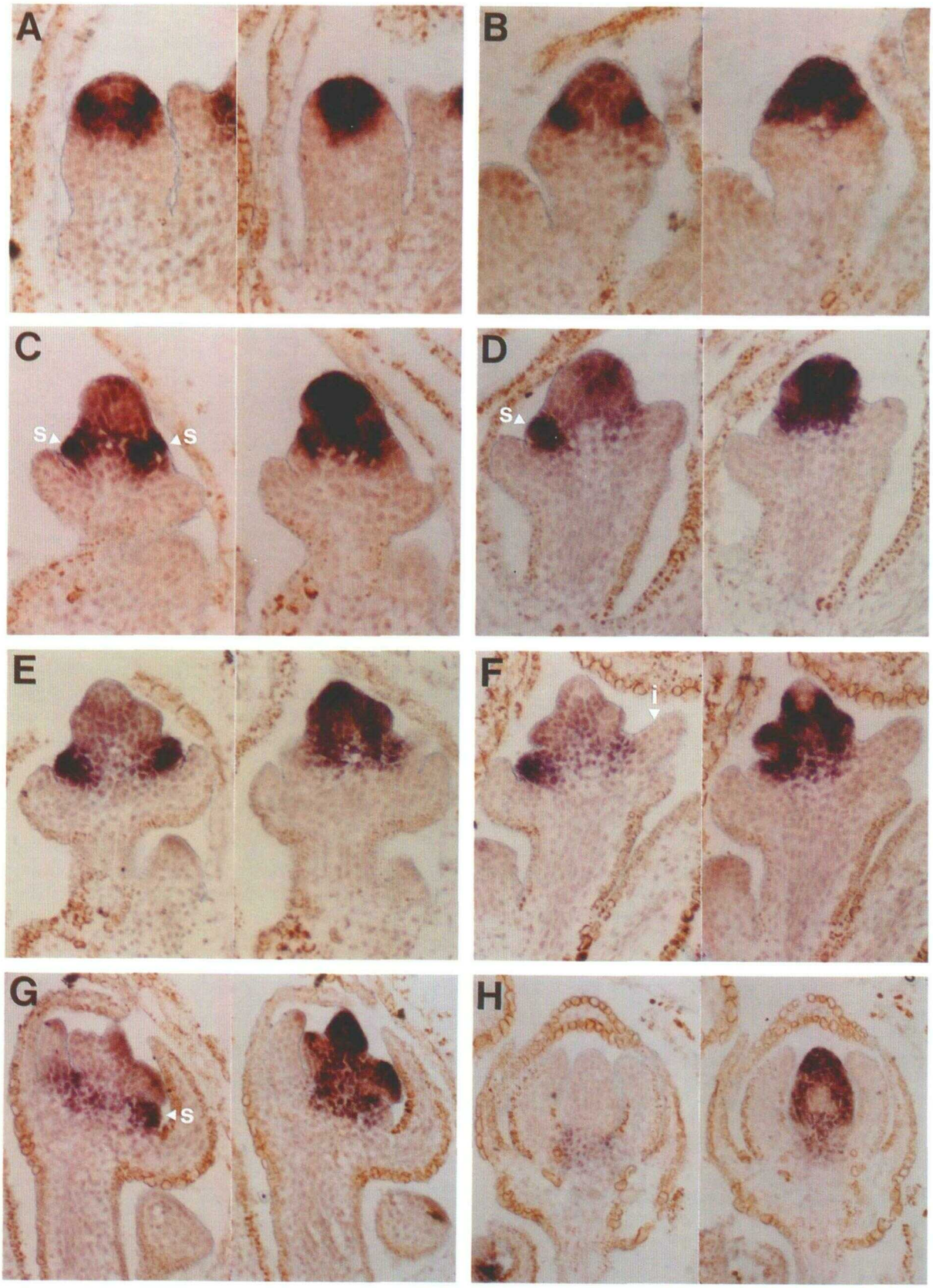

Figure 10. In Situ Localization of *RAD1* and *RAP1* mRNAs in Developing Female Sorrel Flowers.

domain and ~50% downstream of the MADS domain) to suggest strongly that *RAD1* and *RAD2* are *DEFICIENS* and *APETALA3* homologs and that *RAP1* is the *PLENA* and *AGAMOUS* homolog. These sequence similarities are, in part, borne out by the expression patterns. The existence of more than one *DEFICIENS/APETALA3* homolog has not previously been demonstrated in any plant species. *RAD1* and *RAD2* differ in that there appear to be several *RAD2*-like sequences in the sorrel genome, whereas there are only one or two *RAD1* genes.

No homolog of the second B function gene (*GLOBOSA* in Antirrhinum and *PISTILLATA* in Arabidopsis; Tröbner et al., 1992; Goto and Meyerowitz, 1994) was found among the 32 sorrel MADS box clones that were examined. It nevertheless seems likely that there is a homolog in sorrel because it has been proposed that the protein products of the two different B function genes form a heterodimer that binds to the promoters of the target genes (Tröbner et al., 1992).

The genes represented by all three clones are expressed only in male and female flowers and have no detectable levels of expression in vegetative tissues such as leaves and roots, as determined by RNA gel blot hybridization analysis. The expression of *RAD2* is very much weaker than *RAD1* expression in both male and female flowers.

In situ hybridizations using gene-specific probes have revealed patterns of gene expression different from those seen with cognate genes in other plant systems. The expression of both putative B function genes (*RAD1* and *RAD2*) is confined to a single whorl, whorl 3, the stamen whorl, in both male and female flowers. In a hermaphrodite flower, such as Antirrhinum or Arabidopsis, the B function genes are expressed in the petal and stamen whorls (Sommer et al., 1990; Jack et al., 1992; Schwarz-Sommer et al., 1992; Tröbner et al., 1992; Bradley et al., 1993; Goto and Meyerowitz, 1994). Neither of the putative *DEFICIENS*-like B function homologs from sorrel are expressed in the whorl giving rise to the petals (whorl 2). In view of this observation, we propose that the lack of expression causes the petals to become sepaloid and that sorrel displays a "mutant" phenotype (relative to plants whose flowers contain typical sepals and petals). To determine whether this is unique to *R. acetosa*, we need to extend the in situ analysis to other hermaphrodite species of *Rumex*. It is possible that all of the species in the genus have two whorls of sepals (and no petals) and the expression pattern of the *DEFICIENS* homologs is restricted to one whorl in all of them. Furthermore, it is possible that this phenomenon may extend to the entire Polygonaceae family, because its members all have two similar whorls of perianth segments.

An analogous situation exists in petunia: in the homeotic *green petals* (*gp*) mutant, the whorl 2 organs have the same shape and color as the sepals in whorl 1, and petaloid cells occur on the stamen filaments (van der Krol and Chua, 1993). Analysis of the expression of the petunia MADS box genes shows that in the wild type, the *DEFICIENS* homolog *pMADS1* is expressed in the petal and stamen whorls (Angenent et al., 1995), whereas in the *gp* mutant, expression of *pMADS1* mRNA is not detectable in any whorl (van der Krol and Chua, 1993; Angenent et al., 1995). The two *GLOBOSA* homologs found in petunia, *fbp1* and *pMADS2*, show the wild-type spatial pattern of gene expression in the *gp* mutant, being expressed in whorls 2 and 3, although the levels of mRNA are reduced. It is proposed that the *pMADS1* gene product up-regulates the expression of *fbp1* and *pMADS2* (Angenent et al., 1995). Interestingly, although *pMADS1* mRNA accumulates in whorls 2 and 3 and its presence is required for petal determination (van der Krol and Chua, 1993), no function has been assigned to *pMADS1* in whorl 3 (Angenent et al., 1995). The petunia and sorrel systems serve to demonstrate that there is variation among plant species in the properties of the organ identity genes and in the way in which they are expressed. Not all plants follow the precise patterns described for Arabidopsis and Antirrhinum.

The expression of the putative B function genes in the sorrel male is coincident with the initiation and development of the stamens. *RAP1*, the putative C function gene, is expressed in the young male flower primordia in whorls 3 and 4, the stamen, and the carpel whorls, respectively. The expression in the carpel whorl is, however, transient, and as soon as the stamen primordia begin to enlarge significantly, in stage 5 flowers, expression in the center of the flower becomes undetectable. Because in normal flowers the ABC model predicts that the

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Figure 10. (continued).

Developing inflorescences from female plants were sectioned and hybridized with antisense RNA probes of *RAD1* and *RAP1*. Each pair represents two adjacent (or near adjacent) sections of inflorescences or flower primordia probed with *RAD1* (at left) or *RAP1* (at right).

(A) Early stage 3 female flower primordium probed with *RAD1* or *RAP1*.

(B) Later stage 3 female flower primordium probed with *RAD1* or *RAP1*.

(C) Early stage 4 female flower primordium probed with *RAD1* or *RAP1*.

(D) Later stage 4 female flower primordium probed with *RAD1* or *RAP1*.

(E) Stage 5 female flower primordium probed with *RAD1* or *RAP1*.

(F) Stage 6 female flower primordium probed with *RAD1* or *RAP1*. Note the lack of expression in the inner perianth segment primordium.

(G) Stage 7 female flower primordium probed with *RAD1* or *RAP1*.

(H) Female flower at a later stage than stage 7 probed with *RAD1* or *RAP1*.

i, inner perianth segment; s, stamen.

C function alone is needed for carpel development (Coen and Meyerowitz, 1991), the lack of continued carpel development could be due to the inactivation of the C function gene. Alternatively, repression of C function gene expression may be a consequence of the fact that carpel development is prevented by the activity of other genes. A gene similar to the sex-determining *TASSELSEED2* gene of maize, which results in the abortion of the androecium tissue in the tassel (DeLong et al., 1993), might operate in causing the arrest of carpel development in the male, which itself (or by cell death) results in the inactivation of *RAP1* expression. However, obvious signs of cell death were not observed during the development of male or female flowers.

In female flowers, the expression of the putative B function genes correlates with the formation of the stamen primordia that abort after a short period of development. *RAP1*, the putative C function gene, is expressed in the young female flower primordia up to stage 4 in whorls 3 and 4, the stamen and carpel whorls. In stages later than this, in which the stamen primordia are visible as hemispherical structures, the expression is retained in the carpel whorl but is lost from the stamen primordia themselves. The inactivation of *RAP1* expression is coincident with the cessation in the further development of the stamen primordia. The ABC model predicts that both the B and C functions are needed for stamen development (Coen and Meyerowitz, 1991). As is the case with carpel development in the male, the lack of expression of this gene in the female may be a consequence of the cessation in stamen development or a cause of it. However, the latter seems likely, because the B function genes *RAD1* and *RAD2* are still expressed in the stamen primordia after *RAP1* transcripts become undetectable. Therefore, in the female at least, the arrest in development is not caused by cell death. Additional experiments are needed to investigate this phenomenon in both males and females. Is the mechanism of arrest the same in flowers from both sexes and is arrest simply a result of the switching off of the C function gene, or is there a more extensive inactivation of gene activity in these tissues?

Two complications arise when considering *RAP1* and its possible role in suppressing organ development in sorrel. First, in wild-type flowers of petunia, Arabidopsis, and Antirrhinum, the expression of the A, B, and C function genes persists until organogenesis is basically completed (Weigel and Meyerowitz, 1994). There are no examples of mutants in which there is temporally reduced expression of a specific gene, as is the case for *RAP1* in sorrel males and females. All A, B, and C function mutants examined to date in other species contain complete loss-of-function mutations. However, it is difficult to correlate C function gene mRNA accumulation with function because in *agamous* mutants, *AGAMOUS* RNA is present in whorls 3 and 4 (Gustafson-Browne et al., 1994). In sorrel, the transient expression of the C function gene is insufficient to allow growth of the stamens in the female and carpel in the male, but we are unable to state unequivocally whether there has been a determination event. However, this observation might argue that the C function is required for the initial determination of an organ, and its continued presence is required for continued organ development. Second, in addition to organ conversion in whorls 3 and 4, the complete loss of C function, as occurs in the *plena* and *agamous* mutants, also leads to a lack of determinacy in the center of the flower and to the development of "flowers within flowers" (Coen and Meyerowitz, 1991). In sorrel, the suppression of floral meristem growth appears to have been separated from the organ identity function; transient expression of *RAP1* is sufficient to suppress further growth in the center of the flower but is not sufficient for full organ development. A fuller understanding of the mechanisms underlying these events will be possible only when the target genes that are regulated by the C function product are analyzed.

This situation in sorrel contrasts with that of white campion, in which the expression of the B and C function MADS box genes has also been investigated in the development of male and female flowers (Hardenack et al., 1994). The white campion C function gene (*SLM1*) shows no differences in expression between male and female flowers from flower initiation to meiosis. In female flowers, the stamens arrest following meiosis and undergo cell death with the concomitant loss of *SLM1* expression. *SLM1* expression continues in the rudimentary gynoecium present in the female flower (which does not degenerate) until the later stages of development. The expression pattern of the white campion B function genes (*SLM2* and *SLM3*) differs between males and females in that the expression is more central in the male flower, which is associated with the formation of a smaller fourth whorl, and as in sorrel, it was impossible to attribute cause and effect. However, the inappropriate sex organs in white campion develop to a much greater degree before they abort (Grant et al., 1994) than is the case in sorrel. The molecular mechanisms that operate to create the organ differences must act much earlier in the sorrel flower primordia.

These data describe a molecular analysis of the early gene events that occur in the development of male and female flowers in a dioecious plant of the X-to-autosome balance type. Additional work is required to unravel the mechanism of determination in sorrel, but the differential patterns of MADS box gene expression that have been described should aid us in our endeavor. Although we have not assessed the chromosomal locations of the sorrel MADS box genes, the dosage compensation system of sex determination must involve interactions between X chromosome–borne genes and their products and specific autosomes. The primary gene determining sex could, for example, be an X chromosome–located regulator of the sorrel *RAP1* gene.

METHODS

Plant Material

Plants from natural populations of *Rumex acetosa* in Wye, Kent, were used as the experimental material.

Light Microscopy

Developing male and female inflorescences were dissected to remove bracts, fixed in 4% formaldehyde containing 0.1% Tween 20 and 0.1% Triton X-100, dehydrated through an ethanol series, wax embedded, and sectioned. Sections were stained in 0.64% hematoxylin (in 16% glycerol, 16% ethanol, 1.3% aluminum ammonium sulfate) and counterstained in 1% eosin Y.

Chromosome Squashes

Mitotic chromosome analyses were made on roots as described by Wilby and Parker (1987).

Scanning Electron Microscopy

Inflorescences were partially dissected to remove the bracts and were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0. After two washes in 0.1 M phosphate buffer, samples were dehydrated through an acetone series and subjected to critical point drying (Polaron, Watford, UK). Specimens were mounted, coated with gold, and examined with an S430 scanning electron microscope (Hitachi, Woringham, UK) with Hexland stage (Oxford Instruments, Witney, UK).

Nucleic Acid Extraction

Total RNA was isolated from inflorescences, leaves, stems, and roots, and poly $(A)^+$ RNA selection from total inflorescence RNA was performed as described by Ainsworth (1994).

For the isolation of DNA from leaves, 5 g fresh weight of healthy leaves was harvested, snap frozen in liquid nitrogen, and ground to a fine powder with a mortar and pestle. The frozen powder was transferred to a tube containing 15 mL of a buffer containing 100 mM Tris, pH 8.0, 50 mM EDTA, pH 8.0, 500 mM NaCl, 10 mM β-mercaptoethanol. SDS (1.2 mL of 20% SDS [w/v]) was added and mixed thoroughly by vigorous shaking, and the samples were incubated at 65°C for 10 min. Five milliliters of 5 M potassium acetate was added, and the samples were incubated on ice for 20 min. After centrifugation at 10,000*g* for 20 min, the supernatant was filtered through Miracloth (Calbiochem, La Jolla, CA), and the DNA was precipitated with isopropanol. The DNA was spooled out with a flamed Pasteur pipette. DNA pellets were dissolved in Tris-EDTA buffer, treated with RNase, phenol–chloroform extracted, and reprecipitated with ethanol.

cDNA Cloning

Poly$(A)^+$ RNA (4 μg) isolated from pooled developing male and female inflorescences (2 to 5 mm in length) was used for cDNA synthesis (cDNA synthesis kit 27-9260-01; Pharmacia Biotech, Uppsala, Sweden). After the addition of EcoRI-NotI adapters, cDNAs were cloned into the EcoRI sites of λEX*lox* (Novagen, Madison, WI) or λZapII (Stratagene). Libraries of 10^7 and 2×10^6 recombinants, respectively, were generated. Recombinants (800,000) from each library were screened at low stringency (6 × SSC [1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate], 0.5% [w/v] SDS, 0.1% [w/v] Ficoll, 0.1% [w/v] PVP, 0.1% [w/v] BSA, 100 μg/mL denatured, autoclaved salmon sperm DNA at 55°C for 16 hr) with ^{32}P-labeled cDNA inserts from clones of the Antirrhinum MADS box genes *DEFICIENS* (Schwarz-Sommer et al., 1990) and *PLENA* (Bradley et al., 1993). Plaque lifts were given two 20-min posthybridization washes in 5 × SSC, 1% SDS at 55°C before autoradiography at −70°C with intensifying screens.

From the 244 positively hybridizing plaques, 32 that showed a range of hybridization intensity were selected for further study. These were plaque purified, and pBluescript SK− or pEX*lox* versions were generated by in vivo excision.

DNA Sequencing

Nucleotide sequencing was performed by the dideoxy chain termination method with supercoiled plasmid DNA (Sanger et al., 1977) using T7 polymerase (Pharmacia Biotech), T3 and T7, and pBluescript SK and KS commercial sequencing primers (Stratagene) and specifically synthesized oligonucleotides. (An Applied Biosystems [Foster City, CA] Model No. 391A sequencer was used.) All sequences were confirmed on both DNA strands. Computer assembly of sequence data was performed using the DNASTAR (Madison, WI) programs EditSeq and SeqMan. Multiple alignments and analysis of the deduced proteins were accomplished using the DNASTAR programs MegAlign and Protean. Sequence similarities were calculated using the following formula: similarity = 100 × the sum of the number of matches/([length − gap residues] − [gap residues]).

Gel Blot Analysis

DNA and RNA gel blot hybridization analyses were performed as described by Ainsworth et al. (1993). Twenty micrograms of total RNA was used per lane. Subcloned 3′ fragments of the sorrel MADS box clones (see below) were used to make ^{32}P-labeled probes.

In Situ Hybridization

Developing male and female inflorescences were dissected to remove bracts, fixed in 4% formaldehyde containing 0.1% Tween 20 and 0.1% Triton X-100, dehydrated through an ethanol series, wax embedded, and sectioned. The 3′ ends of the sorrel MADS box clones were subcloned into pBluescript KS+ and SK+ to provide templates for T7 polymerase generation of antisense and sense RNA probes in which the conserved MADS domain was not present. The fragments subcloned are as follows: *RAD1* (for *R. acetosa DEFICIENS*-like), 0.65-kb BglII-NotI fragment; *RAD2*, 0.56-kb BglII-NotI fragment; *RAP1* (for *R. acetosa PLENA*-like), 0.82-kb SstII fragment (the NotI sites are present in the adapters used in cDNA cloning). In situ hybridization using digoxigenin-labeled probes was performed as described by Coen et al. (1990).

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