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Genetics of sex determination in flowering plants

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Although most flowering plant species are hermaphrodites, there are still a large number of species with unisexual flowers. Classical geneticists identified the genes that distinguish the sexes in many species, but, until recently, nothing was known about the molecular mechanisms involved. Knowledge of the genes that control floral organ identity in a broad range of angiosperms has now allowed detailed comparative expression studies. Intriguingly, the sex-related differences indicate that the mechanism of sex determination is not the same in all species. In addition, genes that affect the sexual expression of flowers have been cloned from maize, and the sequences of these indicate that they are involved in hormone metabolism.

Approximately 10% of angiosperm species develop unisexual flowers¹. This attribute may have evolved to promote outcrossing, and in some cases appears to allow efficient allocation of resources to optimize reproduction^{2,3}. Unisexually flowering species appear in many different plant families, and several are agronomically important, including maize, cucumber (*Cucumis sativus*), spinach

(*Spinacia oleracea*), asparagus (*Asparagus officinalis*) and yam (*Dioscorea* spp.). Their distribution throughout the plant kingdom suggests that unisexual species have arisen independently from one another in many lineages – they are usually more closely related to hermaphrodite species within their own genus or family than they are to each other.

Species with unisexual flowers can be divided into two main classes: monoecious species (such as maize) develop male and female flowers on one plant; whereas dioecious species [such as hemp (*Cannabis sativa*)] bear male and female flowers on different individuals (Fig. 1b). Intermediate phenotypes can also be found; in andromonoecious or gynomonoecious species, for instance, individual plants carry male or female flowers, respectively, as well as hermaphrodite flowers.

The basic architecture of a hermaphrodite flower consists of whorls of floral organs arranged concentrically around the axis of the stem. The two outer whorls usually carry the non-reproductive organs, the sepals and petals, and the inner whorls contain the reproductive organs, the stamens and gynoecium (Fig. 1a). In the model dicotyledons *Antirrhinum majus* and *Arabidopsis*, four sets of floral organs are arranged in whorls – sepals, petals, stamens and carpels, from the first to the fourth whorl, respectively^{4,5}.

Flowers of the majority of monoecious and dioecious species cannot be distinguished from hermaphrodite flowers during their early development, because the primordia of both stamens

and carpels are initiated in the unisexual flower (Fig. 1a). Abortion or arrest of the carpel primordia in the male flower and the stamen primordia in the female flower occurs later, the time of arrest varying between species. Some species, such as spinach⁶, hemp⁷ and *Mercurialis annua*⁸ seem to bypass the hermaphrodite stage; their flowers develop organ primordia for carpels or stamens, but not for both.

The genetics of sex determination in flowering plants has been a popular topic of research since the beginning of this century^{9–11}. Recent progress in cloning floral homeotic genes, which regulate the identity of the floral organs (see Ref. 12), has generated molecular markers with which to compare the developmental programs of male, female and hermaphrodite flowers in several species. In addition, in maize, the cloning of sex-determining genes (genes that, when mutated, lead to a change in the sex of mutant flowers) has allowed direct examination of the control of gender^{13,14}. In this review, we focus our attention on these recent findings and provide a brief overview of the genetics underlying plant sex determination and the effects of hormones on flower gender (see also Refs 15–18).

Genetics of sex determination

Simple genetic mechanisms differentiate the sexes in some species, such as the wild cucumber (*Ecballium elaterium*), in which the allelic constitution at a single locus determines whether a plant is to be a dioecious male, a dioecious female, or monoecious¹⁹. Other species have several unlinked loci that in combination determine sex. For example, seven loci influence the sexual differentiation of cucumber, with the major effects being controlled by two loci²⁰. In several dioecious species, the sex-determining

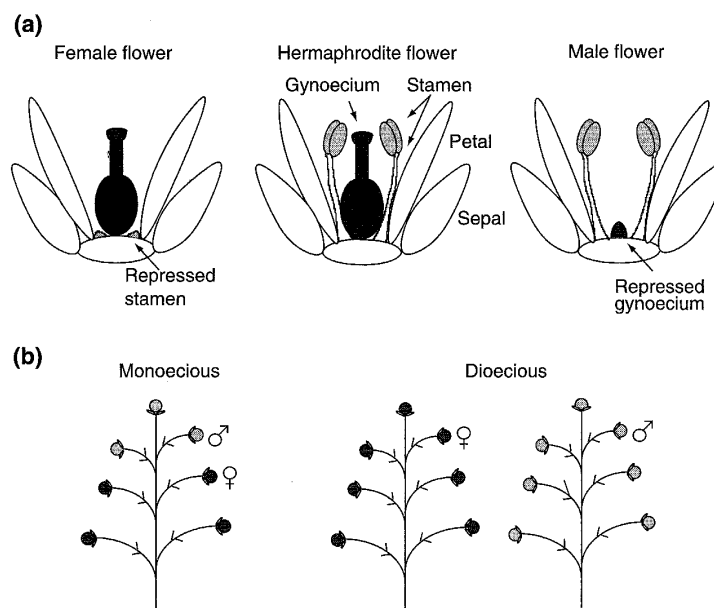


Fig. 1. Sexual characteristics of flowering plants. (a) Basic architecture of female, hermaphrodite and male flowers. The gynoecium can be composed of several carpels. (b) Diagram of monoecious and dioecious plants. A monoecious species bears both male (light circles) and female (dark circles) flowers on the same individual; a dioecious species carries male and female flowers on different individuals.

genes are compiled into special linkage groups, forming sex chromosomes. Males are most commonly the heterogametic sex, with a male-specific and a female-specific sex chromosome (XY), and females are homogametic with two female-specific sex chromosomes (XX). An exception is the wild strawberry (*Fragaria elatior*), in which the female is the heterogametic sex (see Ref. 21).

In most cases, the evidence for sex chromosomes depends on genetic analysis^{21–23}. However, thirteen plant species have morphologically distinguishable sex chromosomes. Among these, two types of sex determination systems are found²⁴: the active Y-system, which resembles the mammalian sex determination scheme; and the X-to-autosome ratio system (an autosome is a chromosome that is not a sex chromosome), which has similarity to the mechanism operating in *Drosophila* and *Caenorhabditis elegans*. The active Y-system can be found in white campion (*Silene latifolia*, also known as *Melandrium album* or *Silene alba*). In polyploid white campion, the presence of a single Y-chromosome is sufficient to determine male characteristics²¹; its Y-chromosome carries genes that are required for stamen development, and the suppression of gynoecium development.

The X-to-autosome ratio system determines sex in sorrel (*Rumex acetosa*)²⁵: sorrel plants are female if the ratio between X chromosomes and autosome sets is 1.0 or higher (i.e. AA, XX); a ratio of 0.5 or lower (as in the normal male genotype AA, XYY) results in a male phenotype; and a ratio between 0.5 and 1.0 [as in a triploid with the chromosome constitution AAA, XXYY (Ref. 26)] leads to an intermediate phenotype with the individuals having hermaphrodite flowers. In the sorrel X-to-autosome ratio system, the

Y-chromosome does not carry the primary genes for gender differentiation, but the presence of two Y-chromosomes in males is essential for the proper completion of meiosis²⁵.

In all 13 species with morphologically distinct sex chromosomes, the male-specific chromosomes are larger than the other chromosomes in the complement²⁴. The DNA isolated from the sex chromosomes of sorrel²⁷ or white campion¹⁸ is rich in dispersed repetitive DNA, including sequences homologous to retrotransposons. These observations support the theoretical model that once chromosome pairing is lost in the male-specific sex chromosomes, they tend to be sinks for 'junk' DNA that might otherwise be removed by recombination in meiosis²⁸.

Using a method of genomic subtraction, known as RDA (representational difference analysis), designed to identify restriction fragment length polymorphisms²⁹, low copy number sequences linked to the white campion Y-chromosome have been isolated³⁰. These have proven to be useful markers with which to begin to map sex-determining loci on the Y-chromosome. A collection of gender mutants of white campion were generated by X-ray or gamma-irradiation of pollen^{30,31}. Hermaphrodite mutants were identified in which repression of gynoecium development no longer occurred. Asexual mutants in which the development of the gynoecium was repressed, but stamen development was inhibited, were also found. Some mutants of both classes were found to have large deletions in their Y-chromosomes. A correlation between the loss of DNA markers and deletions in the Y-chromosomes of these mutants has defined a region of the Y-chromosome that carries genes for both gynoecium suppression and early stamen development³⁰. Using RDA-derived markers to examine the sex chromosomes of a large collection of gender mutants may make it possible to clone the genes involved and so determine their biochemical function.

Sex-related differences in the expression of homeotic genes

The homeotic genes have been prominently studied in hermaphrodite flower development. A homeotic mutation is defined by the production of an organ in an inappropriate place. A null mutation in the *Arabidopsis* gene *AG* (*AGAMOUS*), for example, leads to the replacement of stamens and carpels by sepals and petals³². A model, based on studies in *Antirrhinum* and *Arabidopsis*, proposes that the identity of the floral organs depends on the combination of homeotic genes that are expressed in organ primordia⁴. Three sets of genes (A-, B- and C-function genes) are involved. Each whorl expresses a subset of the genes, leading to the specification of four types of organ: sepal whorls express A-function genes; petal whorls express A- and B-function genes; stamen whorls express B- and C-function genes; and carpel whorls express C-function genes. Many homeotic genes, including the *Arabidopsis* A-function gene *AP1* (*APETALA*), the B-function genes *AP3* and *PI* (*PISTILLATA*) and the C-function gene *AG*, fall into the category of MADS-box genes⁵. The gene products of MADS-box genes share a common protein motif at the amino terminal end (the MADS-box) – this domain has been shown to be required for DNA binding³³.

The MADS-box homeotic genes tend to be highly conserved among the angiosperms^{33,34}. This makes it possible to identify genes in dioecious and monoecious species that potentially have a common evolutionary origin, and related

function to the homeotic genes of *Arabidopsis*, simply by their sequence homology and tissue specificity. Comparison of expression patterns of these genes in males and females of species with unisexual flowers have provided clues to the developmental differences that lead to sexual dimorphism.

Comparisons of mRNA expression patterns of MADS-box genes have been carried out in two dioecious dicotyledons^{35,36} and in the monoecious monocotyledon maize^{37,38}. One of these studies focused on white campion, in which both male and female floral meristems undergo a hermaphrodite phase early in their development³⁵. The development of the carpel primordia in male flowers is arrested immediately after their initiation, whereas the stamen primordia in female flowers are not aborted until tapetum initiation in the anthers³⁹. The MADS-box genes corresponding to the A-, B- and C-function genes were found to be expressed in developing male and female flowers, as they are in hermaphrodite flowers. However, the spatial expression pattern of the petal- and stamen-specific genes, *SLM2* (*Silene latifolia* MADS') (a *PI* homolog) and *SLM3* (an *AP3* homolog), differed between male and female flowers. In both sexes, the expression of these genes was confined to the regions that give rise to petal and stamen primordia. However, *SLM2* and *SLM3* transcripts were expressed in a more central region of the male flower meristem than in the equivalent female meristems (Fig. 2a). Their expression patterns indicated that the fourth whorl is smaller in male flowers than female flowers, and that the third whorl is the same size in both sexes. This was confirmed in morphological comparisons of early stages of male and female flower development³⁹. The gynoecium primordium that forms in a male flower is much smaller than the corresponding primordium in a female flower. A hermaphrodite mutant flower resembles a female flower in the size of all the floral whorls. These results suggest that genes suppressing gynoecium development are responsible for limiting the growth and division of cells in the fourth whorl. Recently, genes affecting the size of the carpel whorl, such as *CLA1* (*CLAVATA1*) (Ref. 40) and *SUP* (*SUPERMAN*) (Ref. 41), have been isolated from *Arabidopsis*. It remains to be determined whether homologous genes that lead to gender dimorphism are differentially regulated in male and female white campion.

A similar study was also carried out in sorrel³⁶. The flowers of this species consist of two outer whorls of sepaloid perianth organs with either an inner whorl of three stamens in male flowers or a gynoecium in female flowers. There is no proliferation of cells in the center of male flowers (internal to the stamen primordia) and stamen primordia are barely visible in developing female flowers. In each sex, one whorl appears to be repressed extremely early in development; here, expression of a stamen- and carpel-specific MADS-box gene, *RAP1* (*Rumex acetosa* *PLENA*-like'), revealed differences between the sexes (Fig. 2b). The sequence of *RAP1* is homologous to *AG*, which specifies the identity of the stamen and carpel organs in *Arabidopsis*. In the male and female floral meristems of very young sorrel, *RAP1* expression was observed briefly in both stamen and carpel whorls, indicating that even the male flowers, which lack morphologically detectable carpel primordia, show the initial signs of a hermaphrodite stage. However, the expression of *RAP1* became undetectable in the inappropriate primordia as soon as these primordia were arrested in their development. The disappearance of *RAP1* expression in the repressed primordia contrasts with the

continued expression of the *AGAMOUS*-homolog *SLM1* in repressed sex organs of white campion. The sorrel sex determination mechanism leads to the early repression of a gene potentially important for organ identity, but the white campion mechanism functions without much influence on the expression of the related gene.

Two sorrel genes with homology to the *AP3* gene of *Arabidopsis* were also found, *RAD1* and *RAD2* (*Rumex acetosa DEFICIENS-like*) (Ref. 36). They were both expressed in the stamen whorls of male and female flowers. The *RAD* gene expression persisted in the stamen primordia of both sexes after *RAP1* transcripts disappeared in repressed sex organ primordia. The *RAD* expression patterns did not reveal a difference in the size of the fourth (carpel) whorl between the sexes. Hence, the mechanisms of sexual differentiation in white campion and sorrel have different effects on the expression of several homologous MADS-box genes. This is further evidence that the mechanisms are fundamentally different.

Although the role of MADS-box genes in the floral organ development of monocotyledons has not been clearly defined, several MADS-box-containing sequences have been identified⁴², including two genes with high homology to *AG* [*ZAG1* (*Zea AGAMOUS*) (Ref. 38) and *ZMM2* (*Zea mays MADS*) (Ref. 42)]. The expression patterns of these two genes have been compared in male and female maize flowers³⁷. In wild-type maize, male flowers (florets) are found in a terminal inflorescence (tassel), and female florets are produced in the lateral inflorescences (ears). During early flower development, many initials form on the inflorescences of both sexes, each giving rise to a pair of spikelets⁴³. A single spikelet produces a pair of floral meristems. In the tassel, both meristems develop to full maturity, resulting in an upper and lower floret. In contrast, in the ear, the lower floret aborts during early development (Fig. 3a). Floral meristems of both the ear and the tassel initiate stamen and carpel primordia, but one type of sex organ primordium is selectively aborted shortly after its initiation^{43,44}. Like *AG* in *Arabidopsis*, both *ZAG1* and *ZMM2* are expressed in carpels and stamens of male and female maize florets, but *ZMM2* is expressed more highly in stamens of male florets than in carpels of female florets³⁷. The *ZAG1* gene has the opposite tendency. Recently a loss-of-function insertion mutant of *ZAG1* has been described³⁷. The mutation only affected the development of the gynoecium in female florets. In the florets of wild-type maize ears, three carpel primordia form; two elongate and fuse to form a silk, and the third is reduced in its development. In the ear florets of the *ZAG1*-defective mutants, an extra silk was produced from two primordia, and this replaced the third carpel. This mutation can be considered a defect in dorsal-ventral polarity. Mutations in both *ZAG1* and the sequence-homologous *AG* of *Arabidopsis* leads to indeterminate growth in the fourth whorl. However, loss-of-function mutations in the *AG* gene result in the conversion of the carpels and the stamens to nonreproductive floral organ types (sepaloid and petaloid, respectively). The difference in conversion effects may reflect a fundamental difference in the developmental programs of flowers of grasses and dicotyledons. Alternatively, the *ZMM2* gene may compensate for the loss of *ZAG1* and provide information for correct carpel and stamen identity. The sex-specific difference between male and female florets revealed by this study was in the relative levels of *ZMM2* and *ZAG1* transcripts, and

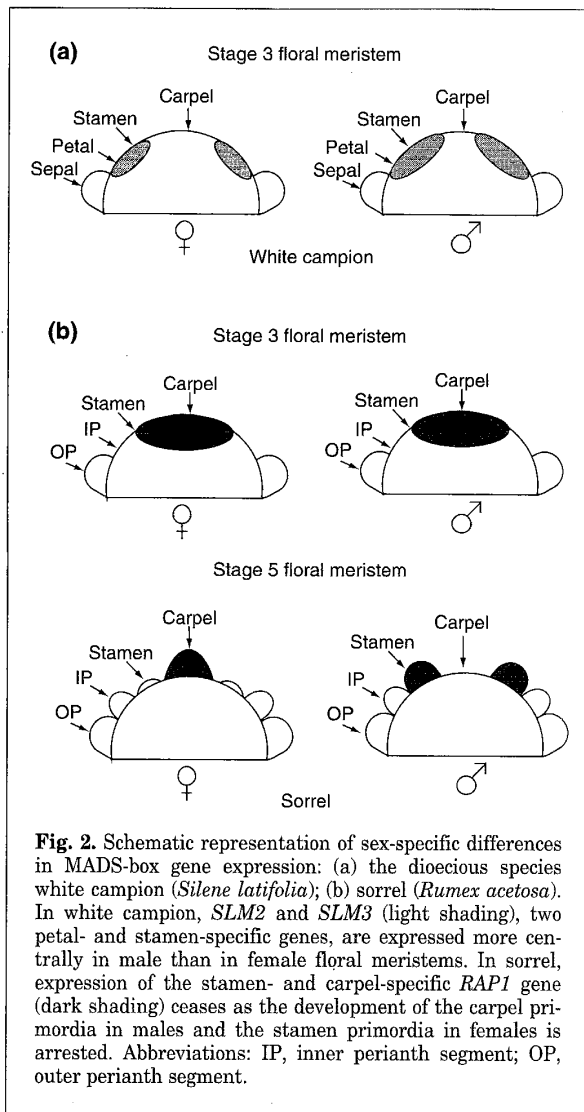


Fig. 2. Schematic representation of sex-specific differences in MADS-box gene expression: (a) the dioecious species white campion (*Silene latifolia*); (b) sorrel (*Rumex acetosa*). In white campion, *SLM2* and *SLM3* (light shading), two petal- and stamen-specific genes, are expressed more centrally in male than in female floral meristems. In sorrel, expression of the stamen- and carpel-specific *RAP1* gene (dark shading) ceases as the development of the carpel primordia in males and the stamen primordia in females is arrested. Abbreviations: IP, inner perianth segment; OP, outer perianth segment.

the limitation of the phenotype of the *ZAG1* mutant to female reproductive organs. This suggests a division of function for *ZMM2* and *ZAG1*, with *ZAG1* having more influence on the development of female reproductive organs and *ZMM2* having more effect on male reproductive organs.

The effects of sex determination on MADS-box gene expression reveal that the mechanisms of sex determination are different in the three species examined. An intriguing question arises: could homeotic MADS-box genes play an active role in sex determination? It is conceivable that a female flower could form by a homeotic conversion of stamens to carpels. For example, a mutation in the *Arabidopsis* gene *PI* (necessary for petal and stamen development), leads to a female flower, because petals are converted into sepals and stamens are converted into carpels. Homeotic genes could be directly involved in sex determination in some unisexual species, particularly those in which floral meristems seem to bypass the hermaphrodite stage, such as hemp, *Mercurialis* or spinach. However, in the species that have been examined, sexual differentiation

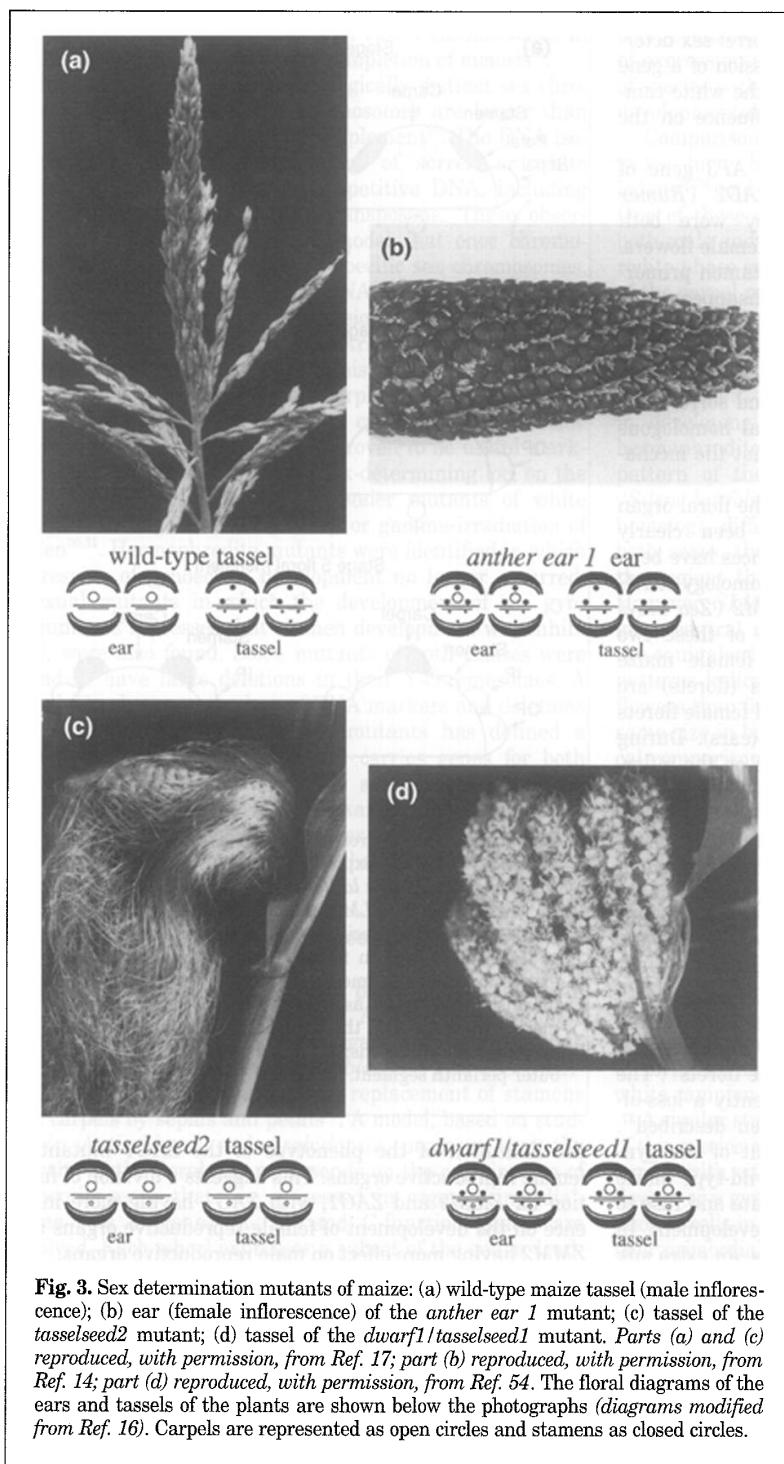


Fig. 3. Sex determination mutants of maize: (a) wild-type maize tassel (male inflorescence); (b) ear (female inflorescence) of the *anther ear 1* mutant; (c) tassel of the *tasselseed2* mutant; (d) tassel of the *dwarf1/tasselseed1* mutant. Parts (a) and (c) reproduced, with permission, from Ref. 17; part (b) reproduced, with permission, from Ref. 14; part (d) reproduced, with permission, from Ref. 54. The floral diagrams of the ears and tassels of the plants are shown below the photographs (diagrams modified from Ref. 16). Carpels are represented as open circles and stamens as closed circles.

involves repression of primordium development, instead of a change in the organ identity of some primordia.

Hormones and sex expression

Sex expression is affected by plant hormones in many monoecious and dioecious species. In addition, environmental

factors, such as temperature and day length, can trigger the reversion of sex in several species⁴⁵, possibly caused by changes in hormone levels. Sex-related differences in the levels of particular endogenous hormones have also been observed. For example, male and female *Mercurialis* plants differ in their complement of endogenous cytokinins to such an extent that males have no *trans*-zeatin and females have no *trans*-zeatin mononucleotide⁴⁶.

The effect of a specific hormone on sex varies between species. For example, application of gibberellin to female cucumber flowers is masculinizing⁴⁷, whereas application of gibberellin to male flowers of maize can be feminizing⁴⁸. The examples of sex reversion by hormone application suggest that in many species unisexual floral meristems are sexually bipotent, and sex-determining genes may trigger the switch between alternative developmental programs by changing the levels or ratios of endogenous hormones.

Sex determination in maize

Maize is the first plant for which sex-determining genes have been isolated, and genetic, biochemical and molecular data indicate that hormones play an essential role in this process. A number of different loci are known to influence flower sex in maize^{16,49}, but here the focus is on the masculinizing *Ts2* (*Tasselseed2*) gene and on the feminizing *An1* (*Anther ear1*) and *D* (*Dwarf*) genes.

Mutations in the *D* genes or in the *An1* gene affect the sexual development of the ear florets. Pistil development is normal, but stamen development in both florets is derepressed, leading to a hermaphrodite upper floret and a male lower floret (see Fig. 3b). Mutations in these genes also cause a delay in flowering and have a pronounced influence on the plant stature, causing a dwarfed phenotype or, in the case of the *an1* mutation, a semidwarfed phenotype. From genetic and biochemical studies it has become evident that *d* mutants are deficient in gibberellin biosynthesis. Exogenous application of gibberellin to *d* mutants restores the wild-type phenotype⁵⁰.

The *An1* gene has recently been cloned via transposon tagging¹⁴ and found to encode a protein involved in the synthesis of *ent*-kaurene, the first tetracyclic intermediate in the pathway for gibberellin biosynthesis. Reversion of the mutant phenotype is possible by application of *ent*-kaurene. The phenotypes of these mutants suggest that gibberellin

is required for the abortion of stamens. In wild-type plants, gibberellin levels are much lower in tassels than in ears⁵¹. In contrast, gibberellin seems to be important for male fertility in most dicotyledonous species. For example, gibberellin-deficient mutants in *Arabidopsis* are male-sterile⁵², and application of gibberellins to cucumber⁴⁷ and hemp⁵³ is masculinizing rather than feminizing.

A loss-of-function mutation in the *Ts2* gene leads to the formation of female florets in the tassel⁴⁴ (Fig. 3c). The pistil of the tassel floret reaches full maturity, whereas its stamen primordia abort. A *ts2*-defective mutation, therefore, results in the complete conversion of gender in the tassel floret. Even though the sex expression of the ear florets is not affected by this mutation, there is an effect on the structure of the ear inflorescence. In contrast to the wild-type, the lower floret of each pair is not aborted, resulting in an uneven distribution of kernels in the ear. The gene *Ts2*, which has been cloned through transposon tagging, is expressed in the carpel primordium of the male floral meristem prior to the abortion of the female tissue¹³. The TS2 protein is thought to induce, directly or indirectly, cell death in the carpel primordia. The predicted TS2 protein sequence shows significant homology to steroid-specific dehydrogenases from bacteria¹³, suggesting that TS2 could be acting on steroid-like compounds, such as brassinosteroids, produced in florets. The function of sequence-related steroid dehydrogenases suggests that it could inactivate a hormone needed for pistil development. Alternatively, it could lead to the production of a toxin that kills the cells in which it is formed.

The phenotype of *ts2/d1* double mutants is additive. Hermaphrodite florets are produced in tassels and ears because of stamen development in ear florets and loss of pistil abortion in tassel florets⁵⁴. This suggests that *Ts2* acts in a separate pathway from the *D* genes⁴⁹. As *ts1/d1* and *ts2/d2* double mutants are described as having similar phenotypes⁵⁴, the ear of a *ts1/d1* double mutant is shown in Fig. 3d for illustration.

Conclusions

A comparison of the expression patterns of genes containing the MADS-box in male and female flowers indicates that sex-related differences in the expression of homeotic genes do not account for sexual differentiation. Instead, the development of one type of reproductive organ (either stamens or carpels) is repressed through mechanisms that remain to be characterized. The emerging picture is that hormones or genes regulating hormone metabolism might prove to be key players in several species. In maize, removal of a steroid-like substance by the TS2 gene product could be necessary for the development of male flowers, and the production of gibberellins is necessary for female flowers. As hormone levels and the application of hormones affect sex expression in other species, sex determination may be regulated through hormone metabolism in many monoecious and dioecious plants. Now that the first sex-determining genes have been cloned and characterized in maize, and studies in other monoecious and dioecious species are progressing, the next few years promise to deliver exciting results.

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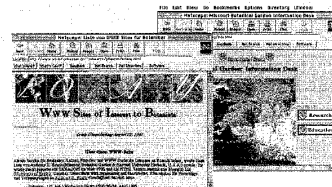
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