ANNUAL REVIEWS

Annual Review of Plant Biology

One Hundred Ways to Invent the Sexes: Theoretical and Observed Paths to Dioecy in Plants

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Annu. Rev. Plant Biol. 2018. 69:553–75

The *Annual Review of Plant Biology* is online at plant.annualreviews.org

https://doi.org/10.1146/annurev-arplant-042817-040615

Keywords

dioecy, sex determination, sex chromosomes, flower development, genomics

Abstract

Dioecy, the presence of male and female flowers on separate individuals, is both widespread and uncommon within flowering plants, with only a few percent of dioecious species spread across most major phylogenetic taxa. It is therefore safe to assume that dioecy evolved independently in these different groups, which allows us to ask questions regarding the molecular and developmental mechanisms underlying these independent transitions to dioecy. We start this review by examining the problem from the standpoint of a genetic engineer trying to develop dioecy, discuss various potential solutions, and compare them to models proposed in the past and based on genetic and evolutionary considerations. Next, we present recent information regarding candidate sex determinants in three species, acquired using newly established genomic approaches. Although such specific information is still scarce, it is slowly becoming apparent that various genes or pathways can be altered to evolve dioecy.

Contents

INTRODUCTION

Dioecy, the stable arrangement of a population into separate sexes, is a situation familiar to us, as the vast majority of the human population is divided into males and females based on inheritance of the Y chromosome. Many botanists, however, do not typically encounter dioecy. Most model plants and crops are hermaphroditic, such as Arabidopsis (*A. thaliana*) and rice (*Oryza sativa*), or monoecious (each individual bears both female and male flowers), such as maize (*Zea mays*). Only a small but relevant minority (approximately 5%) of plant species are dioecious (76). Of these, a significant number are cultivated, such as spinach (*Spinacia oleracea*), asparagus (*Asparagus* species), hops (*Humulus* species), cannabis (*Cannabis* species), kiwifruit (*Actinidia* species), pistachio (*Pistacia* species), date palm (*Phoenix dactylifera*), papaya (*Carica papaya*), and fig (*Ficus carica*). Additionally, nondioecious cultivated varieties of persimmon (*Diospyros kaki*), grape (*Vitis vinifera*), and strawberry (*Fragaria* species) are derived from close dioecious relatives (4, 56, 74). Thus, dioecy is infrequent but not rare. Different from the unique origin of sex determination in mammals, in which a single ancestral event yielded the dominant sex determination system (10), dioecy has arisen independently in many angiosperm phyla (76). This raises the questions of what selective forces favor dioecy emergence and persistence, what molecular and chromosomal mechanisms are coopted to determine sex, and finally, what is the long-term evolutionary outcome.

Dioecy: population in which each individual bears either male or female flowers but not both

In the past 10 years, plant genomics and functional discovery have accelerated, elucidating genome evolution and the biochemical and regulatory basis of plant traits, including the molecular basis of dioecy in plants. In light of these findings, this review considers potential paths to dioecy, comparing them with emerging evidence. Specifically, we focus on the molecular mechanisms underlying the transition into dioecy and the specific genetic factors that might play the role of sex

determinants. We present theoretical paths and the presumed steps required for the establishment of dioecy from hermaphroditism before we compare them to observed scenarios in specific plant species. We reflect on paths into and out of dioecy and how frequently this type of transition is observed. We have elected not to discuss the evolution or structure of sex chromosomes because this has been thoroughly reviewed recently (21, 64).

Polyploidy: a state of having more than two sets of the basic genomes

ADVANTAGES AND DISADVANTAGES OF DIOECY

About 150 years ago, Darwin (26) noted both obvious and potential advantages to dioecy. An obvious advantage is hybrid vigor. Separate sexes enforce outcrossing, favoring the maintenance of high heterozygosity and associated heterosis. A potential advantage may derive from specialization (31). Dedicated males and females must be more efficient at their sexual tasks than hermaphrodites to be at least as competitive (19), and it is plausible that males and females of successful dioecious species outperform hermaphrodites. For example, males could dedicate all their resources to produce and efficiently disperse high-quality pollen grains, resulting in more successful fertilization (19, 93). Male efficiency may be particularly important in wind-pollinated species, potentially explaining why wind pollination is more frequent among dioecious (31%) than nondioecious (6%) species (76). Additionally, females may invest more energy into seed production than hermaphrodites do, thus providing more resources to their progeny and enhancing their survival. Sex-dependent niche specialization could make dioecious species more competitive by enabling better utilization of varying environments (33). Although these advantages appear plausible, their evolutionary role in sex determination is still being debated (13).

There are at least four disadvantages to dioecy. First, males do not bear progeny. They are unproductive in the absence of available females but still compete with them for resources. Second, mating between sessile individuals requires that much pollen be wasted in the attempt to reach distant ovaries (52). This disadvantage is not unique to dioecy, but affects all outcrossing systems. Third, bottleneck events could result in populations that are depleted of one of the two sexes and, barring sex reversion (32), destined to local extinction. Further, evolutionary transitions such as polyploidy would require that two individuals of opposite sexes undergo them at the same time and location. Fourth, the relatively low number of dioecious species combined with the fact that they are present in most plant families suggests that dioecy may come at the cost of more limited evolutionary and speciation potential or increased risks of extinction, a possible cause for the overabundance of wind-pollinated dioecious species (13). These disadvantages may have prevented widespread adoption of this reproductive system by flowering plants. Nevertheless, combinations of certain genotypes and environments have been sufficiently advantageous to engender dioecious taxa such as those in the Ebenaceae that might have persisted for well over 50 million years (14, 15).

Related advantages and disadvantages apply to plant domestication and breeding as well. Dioecy is an effective system to produce hybrid seed and potentially increase yield through heterosis. However, if the harvested product is seed or fruit, the presence of males in the field is wasteful and must be limited to the minimum needed for efficient seed set. If dedicated females were indeed more productive than hermaphrodites, superior yield might be achieved through dioecy as long as the male-to-female ratio can be managed effectively in the field. This could be achieved by engineering conditional lethality on the Y chromosome to be triggered by a simple seed treatment (96) or by an interaction with a gene expressed by the female inbred (16, 37).

THEORETICAL PATHS TO DIOECY

The frequent and independent emergence of dioecy in flowering plants raises the question of what pathways, genes, and connected mechanisms have engendered it. For comparison, we can

also ask the question of how we could engineer dioecy through biotechnology using emergent molecular tools. The two problems are likely to share solutions and present unique challenges. A conclusion of this review is that we should expect multiple solutions, a recurring theme in dioecy across all taxa (10). It would be presumptuous and prolix to attempt to cover more than a few. Instead, selected examples leading to the development of an XY (heterogametic male) system are used. Analogous considerations apply to ZW by simply changing the sex relation.

Haplotype: a series of two or more alleles linked physically, i.e., positioned relatively close to each other on the same DNA molecule

Null mutation: a severe mutation that results in the complete loss of function of a gene and its product

Gynodioecy: population consisting of a mixture of females and individuals that bear hermaphroditic flowers or are monoecious

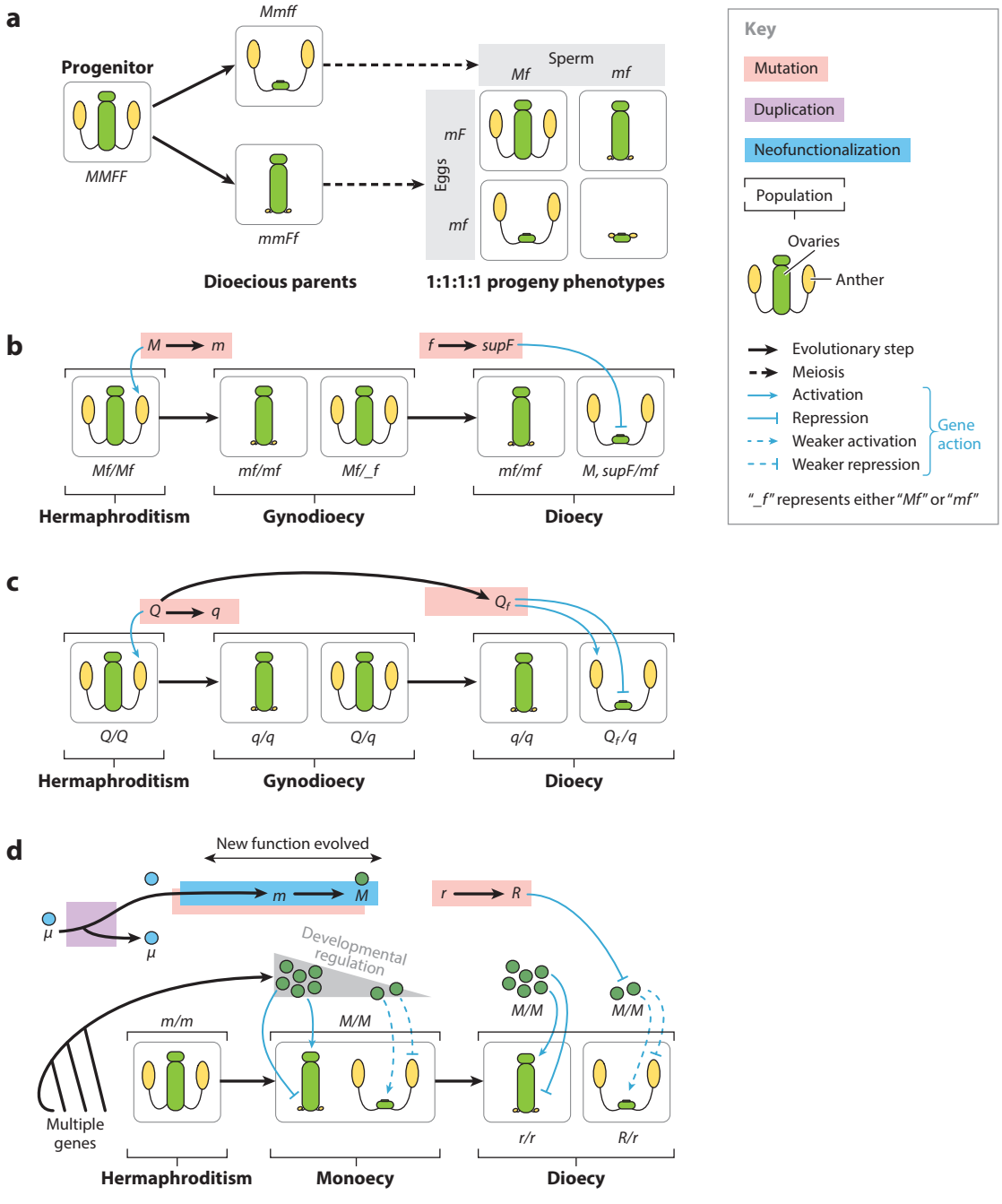
Androdioecy: population consisting of a mixture of males and individuals that bear hermaphroditic flowers or are monoecious

One or Two Sex Determinants?

Lewis (52) reviewed numerous cases of recessive mutations at different genes that, in combination, could produce dioecy. For example, genes required for male (M) and female (F) function could be mutated, producing recessive alleles: $M \rightarrow m$ and $F \rightarrow f$. He noted, however, that stable dioecy required dominant functions in the heterogametic sex (**Figure 1*a***). To explain how this could be achieved, he proposed that formation of a heterogametic male would entail dominant alleles at two loci: one a newly evolved dominant suppressor of female organs (*supF*), the other a dominant activator of maleness (M) (**Figure 1*b***). Stability of the male heterogametic system requires that M and *supF* be inherited as a linked unit (a haplotype) (**Figure 1*b***). Stabilization by a chromosomal inversion spanning M and *supF* would engender a proto-Y chromosome, which would pair with a proto-X carrying m and f (**Figure 2*a***).

Although the two-factor model is a plausible one, it may not be the only path to dioecy, as exemplified by mammals, in which the *SRY* gene acts early to promote male development instead of female development (10). How would a single-factor system for dioecy work? Let us consider the XY heterogametic system, although analogous considerations would apply to the ZW. From a genetic point of view, such a system would be simple: If we call the responsible gene Q, let us define males as Qq and females as qq. Their cross will result in a 1:1 sex ratio. The details, however, are not obvious. What is Q and how would the effective alleles arise? By definition, the dominant allele must both activate maleness and suppress femininity. Therefore, any gene necessary for male development could contribute the starting material to this evolutionary step. Two independent events must take place (**Figure 1*c***): One entails a loss-of-function mutation ($Q \rightarrow q$) forming a recessive allele. The other involves a gain of function ($Q \rightarrow Q_f$), where Q_f is a suppressor of femininity. In biotechnology, such a gene could be constructed by fusing Q, the male factor, with an altered female regulator that exerts a dominant negative activity (41) on female development. The q allele could be a spontaneous or edited null mutation. Although natural formation of an allele equivalent to Q_f may seem improbable, from an evolutionary perspective it is not so: New gene formation and acquisition of critical new functions are frequent (45). This path is plausible particularly if it is premised by a gene duplication event, creating an additional copy of the Q gene that can acquire new function without jeopardizing the original one. As illustrated in **Figure 1*c***, Q_f/q individuals are male and q/q individuals are female. In this scenario, the Q_f allele alone can engender proto-Y formation because of positive selection for linkage with male-favoring genes (**Figure 2*b***).

The systems described above are just two of many possible scenarios, at least in theory. Which one(s) are more likely according to the population genetics point of view? Charlesworth & Charlesworth (19) addressed this problem using a computational approach. They examined conditions upon which the alleles for male or female sterility would spread in a large population to achieve gynodioecy or androdioecy, respectively. They further considered how an intermediate condition such as gynodioecy could evolve upon emergence of an allele such as *supF* (**Figure 1*c***). The authors demonstrated that dioecy is favored only when recombination between M and *supF* is completely suppressed (**Figure 2**), providing quantitative proof that the scenario in **Figure 1*b***



(Caption appears on following page)

Figure 1 (*Figure appears on preceding page*)

Theoretical models for the emergence of dioecy. The figure displays key examples but does not attempt to be inclusive of all possible paths. (*a*) Unstable dioecy. Two dominant, sex-determining *F* and *M* alleles at two different, unlinked loci can result in dioecious individuals. However, this form of dioecy is unstable unless the dominant alleles are linked. The Punnett square on the right illustrates how crosses between unisexual parents with unlinked *M* and *F* loci yield four equally represented phenotypic classes: hermaphrodite, male, female, and sterile individuals (52). In addition to negative selection on the recessive alleles in the sterile individuals, crosses involving hermaphrodites are possible, further skewing future progenies toward hermaphrodites. (*b*) Development of dioecy in a two-factor, two-mutation system. A male-sterility mutation affecting *M* leads to gynodioecy. A newly formed suppressor of femininity (*supF*), when linked to *M*, converts this intermediary state to dioecy. Changing the order of the mutation events results in intermediate androdioecy (19, 52). (*c*) Single-factor dioecy. Two independent mutations in gene *Q* produce two alleles, one recessive (*q*) and the other dominant (Q_f). Depending on the order of occurrence, either gynodioecy (shown here) or androdioecy could be the intermediate state. (*d*) Dosage-sensitive sex determination leading to monoecy and dioecy. *M*, a factor that represses anthers and promotes carpels, can arise through multiple mechanisms, such as duplication of an ancestral gene μ followed by neofunctionalization of one copy (illustrated here), mutation and neofunctionalization of a dispensable gene, or neogenesis (45). High *M* results in females, and low *M* results in males. *M* concentration responds to developmental cues, which are in turn controlled by multiple genes, engendering monoecy. Subsequently, a newly developed factor *R* represses *M*. Starting with an *R/r* male and an *r/r* female, a stable dioecy system is produced.

was more probable than alternative ones (19). The rigorous quantitative treatment of this problem established the model in **Figure 1*b*** as a premier hypothesis for the evolution of dioecy.

A lingering question is which intermediate sexual states lead to dioecy and which states result from it. The hypothesis of a path to dioecy involving gynodioecy is supported by the higher prevalence of gynodioecious species than androdioecious species. Still, it remains to be proven that gynodioecy is an intermediate state to dioecy. Renner (77) commented that the two-factor model (**Figure 1*b***) might not be sufficient to explain common paths to dioecy. Emerging evidence in plants (see below) and studies in nonplant systems suggest that more than one prophecy will be proven correct, as more than one path and genetic system can lead to dioecy.

WHAT HAS NATURE TAUGHT US SO FAR?

Transitions into Dioecy

Dioecy has evolved independently many times (35, 64, 76). From a macroevolutionary point of view, the evidence supporting this transition is obvious and compelling: Dioecious taxa occur at the tips of phylogenetic branches rooted in hermaphroditic species (64, 76). It is more challenging to determine how dioecy evolved. As discussed above, researchers have proposed several potential evolutionary pathways to dioecy (10, 35). The best known is the two-factor model (**Figure 1*b***) (19, 52), whose evolution should entail a dimorphic pathway (**Figure 1**) (35), and in which dioecy evolves from hermaphroditism via an intermediate sexual system, gynodioecy or androdioecy. Genetic evidence for two factors controlling dioecy has been found in some plant species (47, 90, 91). For example, in *Silene* (formerly *Melandrium)*, deletions of the Y chromosome localized both *M* (male inducer) and *supF* (female suppressor) (**Figure 2*c***) (47, 91). The recent progress in the identification of putative sex determinants in *Asparagus officinalis* (asparagus) and *Actinidia* spp. (kiwifruit) are discussed below and additional examples in other species such as *Silene* (47) and *C. papaya* (papaya) (88, 90) are soon likely to emerge.

Monoecy: population of individuals bearing both male and female flowers

In the alternative monomorphic pathway (35), in which dioecy evolves from hermaphroditism through a population including monoecious individuals (22), a single factor may be sufficient (**Figure 1*c*,*d***). This is the case, for example, when genetic sex determination evolved from environmental sex determination (ESD) in vertebrates (75; see 20 for review). The transition is hypothesized to start from monoecy, in which regulatory states suppressing male and female

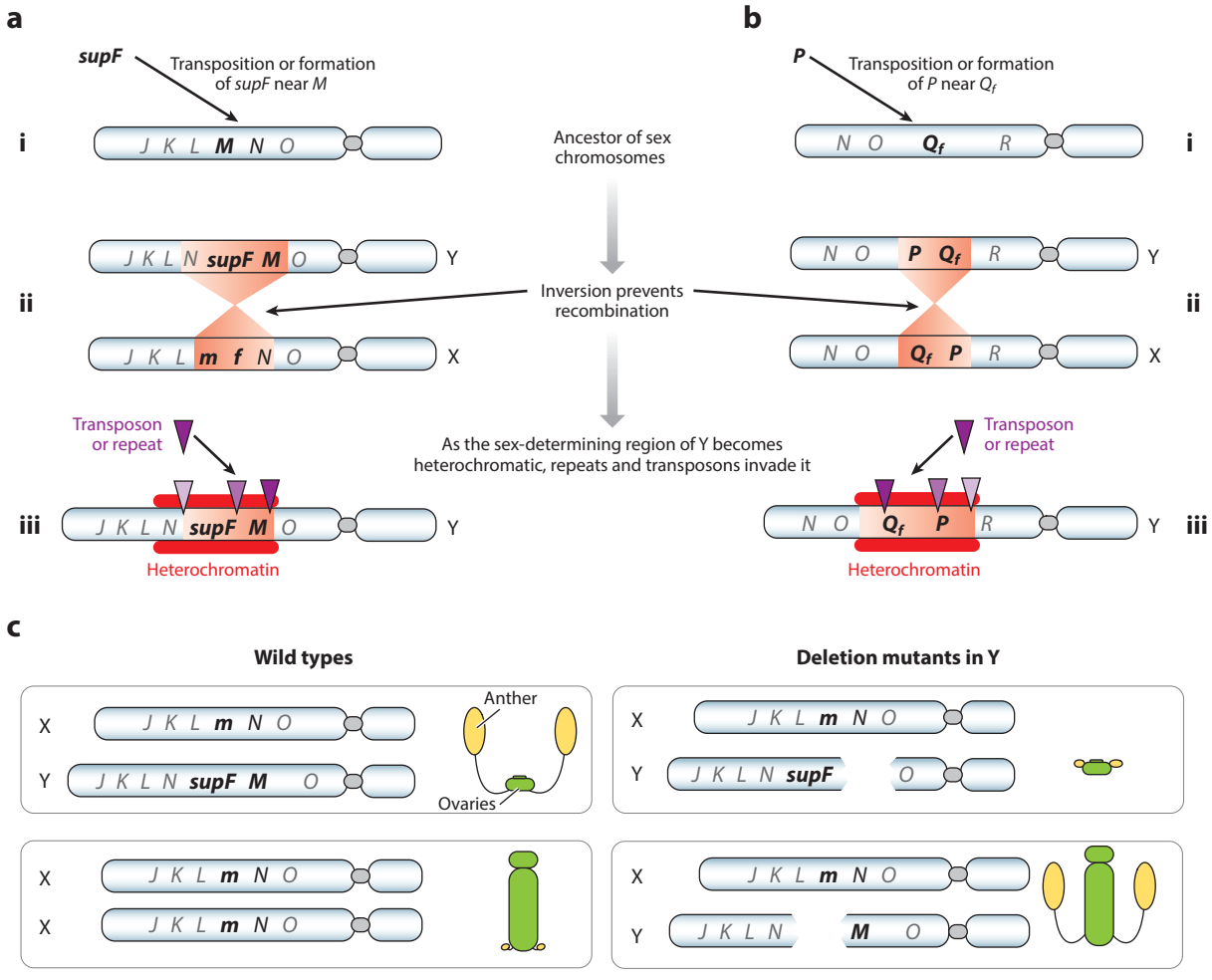


Figure 2

Formation and evolution of the Y chromosome. (*a*) Two-factor model displaying the relevant sex determinants (in *bold*). (*i*) *M* and *supF* alleles must be co-inherited for stable dioecy. A *supF* allele that has transposed or formed next to *M* is subject to positive selection. (*ii*) A chromosomal inversion carrying both *supF* and *M* prevents recombination with the homologous region on the X chromosome. This isolates the male-determining, dominant haplotype on the proto-Y from the homologous one on the X chromosome. (*iii*) Heterochromatinization. Whereas the corresponding region on X recombines in females, the sex-determining region on Y (*red*) does not. The nonrecombining region undergoes heterochromatinization because inserted transposons cannot be purged by recombination. (*b*) Single-factor model. (*i*) Dominant genes that improve male function (*P*) are predicted to appear next to the sex-determining locus through transposition or neogenesis (45). (*ii*) Positive selection for maintaining and reinforcing linkage favors the establishment of rearrangements that prevent recombination in a manner similar to the two-factor model leading to heterochromatinization (*iii*). (*c*) Experimental evidence for two-factor system sex karyotypes and respective sex phenotypes in *Silene latifolia* (47). (*Left*) Wild-type male and female with the respective sex chromosomes. (*Right*) The phenotype of induced deletions affecting Y provides evidence for an anther promoter (*M*; *top*) and *supF* (*bottom*). Abbreviations: *M*, dominant activator of maleness; *P*, pollen fertility enhancer; *supF*, suppressor of femininity.

functions already exist, and the path to male or female flowers is determined environmentally or developmentally (ESD). If a change in the regulatory system causes a disequilibrium in the male-to-female ratio (20), a genetic mutation promoting a switch to the opposite direction could be positively selected and fixed heterozygously (in the heteromorphic sex), resulting in the establishment of a single-factor sex-determination system **Figure 1*c*,*d***). We discuss the specific cases of *Diospyros lotus* (diploid persimmon) and *Cucumis sativus* (cucumber) below.

k-mer: an arbitrary sequence of length k

Recent Advances from Genomic Approaches

In the last few years, much progress has been made toward understanding the genetic factors and molecular mechanisms that regulate sex in dioecious plant species. These advances were enabled by the application of modern genomic approaches. Therefore, we expect continued progress and an increasing opportunity for in-depth comparisons between the independently evolved angiosperm dioecious systems. Here, we focus on four systems for which substantial information has recently become available. For most of these species, the path to the discovery of putative sex determinants was similar and involved a combination of simple yet powerful genomic methods that have become available only in the last few years. First, in an XY system, Y-specific sequences are identified and cataloged through whole-genome shotgun or transcriptome sequencing of sets of males and females (pools). A genomic reference is not required for this step. Instead, a reference-blind approach catalogs all k-mers (arbitrary sequences of length k) found in the male and female sequence pools. Subsequently, comparison of the relative frequency of each k-mer allows rapid identification of male-specific sequences. These sequences can then be assembled into longer ones (contigs or scaffolds), ultimately resulting in a draft reference for the male-specific Y chromosome (MSY) region of the species of interest. A similar approach on ZW (female heterogametic) systems would focus on identifying W-specific sequences.

Second, when reference genome sequences are available, sophisticated genetic and physical mapping can be used. For example, comparing varieties or accessions that are dioecious with varieties or induced mutants that have reverted back to hermaphroditism (or another sexual system) could provide the ideal system to identify the sex determinants. For example, in species such as papaya (*C. papaya*) or grapevine (*Vitis*), some commercial cultivars are hermaphroditic but carry Y chromosomes similar to those of male individuals (74, 88), consistent with disruption or recombination of the *supF* factors. Similarly, a male *Ginkgo biloba* tree can produce viable seed on one localized branch while the rest of the tree remains male (67). This localized sex conversion provides the perfect system to investigate sex determination in this species.

Third, at critical development times when male and female flower fates are determined, candidate functional differences are investigated. This step can be performed at the transcriptome, proteome, DNA methylation, chromatin marks, and small RNA (smRNA) level, among others. Irrespective of the technique used, the goal is to identify either qualitative (e.g., presence or absence of a specific protein) or quantitative (differential accumulation of a specific protein) differences between flowers from two sexes. The differences observed can then be narrowed to a few candidate factors on the basis of whether they can be associated with the previously identified Y-specific sequences. Finally, candidates can be assessed by a variety of functional assays, either directly in the species at hand or in a model system.

There are three species for which such genomic approaches have yielded insightful information on sex determination mechanisms in the last few years: *Diospyros lotus* (diploid persimmon), *Actinidia* species (kiwifruit), and *A. officinalis* (garden asparagus). We also discuss the monoecious genus *Cucumis*, of which cucumber and melon have been well characterized. The main results are summarized in **Table 1**.

Table 1 Summary of the candidate sex determinants identified so far

Species	Y-linked gene model	Candidate Y-encoded sex determinant
Diospyros lotus (diploid persimmon)	Single-factor model (*OGI*)	*OGI* miRNA to Homeobox transcription factor *MEGI* (4)
Actinidia spp. (kiwifruit)	Two-factor model	*SupF*: type-C cytokinin response regulator *M*: not identified yet
Asparagus officinalis (asparagus)	Two-factor model	*M*: defective in tapetal development and function 1 (TDF1) *SupF*: SOFF (unknown function) (64, 84)
Cucumis sativus (cucumber)	Not applicable because there is no Y chromosome	*G* locus alone could favor formation of a proto-Y chromosome (10, 17)

Abbreviation: miRNA, microRNA.

Diploid Persimmon

The diploid persimmon, or date plum (*D. lotus*), is dioecious. *D. lotus* flowers start by producing both types of sexual organs, which are then repressed differentially in male and female individuals. In female flowers, anthers are visible but smaller than those in male flowers. They can sometimes bear pollen grains, but they are nonfunctional. Similarly, stigma development starts in male flowers but arrests early in development. Male and female inflorescence architecture is also different: Female inflorescences bear single apical flowers, whereas male inflorescences are trifurcated. Lateral flower primordia are visible on either side of the female inflorescence, but their development is arrested very early (5).

Using a combination of genomic approaches outlined above (5), researchers identified and named two genes, *OGI* and *MeGI* (5). The *OGI* sequence is Y specific and fully associated with maleness in all *Diospyros* species tested. The sequence context surrounding the *OGI* sequence is consistent with that of a MSY region (5), characterized by high percentages of repeated sequences and the presence of predominantly male-specific sequences. The *OGI* gene encodes an apparent pseudogene, homologous to the barley (*Hordeum vulgare*) homeobox transcription factor *six-rowed spike 1* (*Vrs1*) (49) and the maize (*Z. mays*) *grassy tillers 1* (*gt1*) (92). In *D. lotus*, *OGI* transcripts are processed to produce 21-nucleotide (nt) smRNAs that target both the *OGI* and the *MeGI* sequences, resulting in lower levels of *MeGI* transcripts in the male developing buds and flowers than in the female (5). *MeGI* is not sex specific. Its exact role remains to be deciphered, but its overexpression in *Arabidopsis thaliana* can result in flowers in which male function is repressed, with shorter anthers and nonfunctional pollen grains. Single-flower inflorescences are produced in *Nicotiana tabacum*, reminiscent of the single female flowers of *D. lotus* (5).

Two aspects of the *OGI-MeGI* interaction are of particular interest. First, the action of *MeGI* is dosage sensitive, as suggested by the observation that transgenic phenotypes ranged from very severe to undetectable, depending on the concentration of correctly spliced *MeGI* transcripts (5). Second, whereas *MeGI* homologs are found in many plant species (7), the *OGI-MeGI* interaction is specific to *Diospyros* and is believed to originate from a gene duplication event. The *OGI-MeGI* system depends on the interaction between these two genes, but only *OGI* is located on the MSY region (5).

The biological function of the *MeGI* gene products can, to some extent, be extrapolated from those of its homolog, *Vrs1*. In barley, mutations in the *Vrs1* gene affect flower development in different ways. Knockout mutations of *Vrs1* result in the conversion of two-rowed barley to six-rowed barley, through the development of the two lateral spikelets, which are not normally repressed by *Vrs1* expression (49). The principal role of *Vrs1* in barley is to suppress pistil development in a dosage-dependent manner (80). The effect on the lateral flower primordia is also reminiscent of flower development in persimmon, in which female inflorescences bear single flowers and male inflorescences are trifurcated. Other aspects of flower development are affected by mutation in *Vrs1*. Pollen viability, for example, can be affected by certain alleles of *Vrs1* (50). Tight temporal and spatial regulation of *MeGI* expression by *OGI* may affect both male and female function in persimmon flowers. In this case, the theoretical scenario presented in **Figure 1*c*** best fits the observations in *D. lotus*, with *OGI* playing the role of the Q_f allele.

Asparagus

Garden asparagus (*A. officinalis*) is dioecious, with XX females and males carrying homomorphic X and Y chromosomes (27). Maleness has been linked to the *M* locus and is expected to be dominant, with *Mm* males and *mm* females. *MM* supermales can be produced, through either anther culture or selfing of hermaphroditic individuals (86). Similar to persimmon flower development, male and female asparagus flowers do not differ in the number or organization of flower organs but exhibit different developmental patterns, resulting in sexual differentiation (51). Specifically, male flowers initiate pistil development but arrest early, leaving only rudimentary pistils (51). Female flowers carry stamens, but the anthers are collapsed, the tapetum degenerates early (51), and no functional pollen grains are produced (86).

As described above, advancements in genomics have allowed rapid progress in the identification of candidate sex determinants, especially in non-model species. The case of asparagus is particularly striking, as three independent reports have recently investigated sex determinants in this species and identified potential candidate genes underlying the *M* locus. In 2015, Harkess et al. (38) examined differential gene expression between male, female, and supermale flowers in *A. officinalis*. They determined that more genes were biased for male-specific expression, as can be expected from an XY system and sex-determining loci located on the MSY region (38). In 2017, Tsugama et al. (86) revisited these data and their own transcriptomics data to identify a MYB transcription factor, *AoMYB35*, expressed early in anther development. *AoMYB35* is a homolog of the *A. thaliana* gene *TDF1* (DEFECTIVE IN TAPETAL DEVELOPMENT AND FUNCTION 1), which is necessary for microspore maturation (86). Tsugama et al. (86) reported that they were unable to amplify *AoMYB35* from females in *A. officinalis* and concluded that it is Y encoded in this species. Together with its expression pattern and known role in flower development, *AoMYB35* is a promising candidate sex determinant underlying the *M* locus. The same candidate male activator was identified independently by Murase et al. (66), whose approach also involved comparing gene expression in male and female buds. In this case, the researchers identified an R2R3-MYB transcription factor called Male Specific Expression 1 (*MSE1*) that exhibits male-specific expression. Its closest homolog in *A. thaliana* is again *TDF1*. Although this factor is present in females, the male and female alleles are different, with the female allele being most likely nonfunctional (66). Taken together, these results are consistent with this gene's role in male flower development in asparagus, but whether it is a sex-determining factor remains to be robustly established.

Harkess et al. (39) recently reported the sequencing of the asparagus genome, focusing on the structure of the young Y chromosome and the identification of potential sex determinants in this species. They assembled a Y-specific nonrecombining region that contained only 12 genes,

including *TDF1*. The authors then undertook a functional genomics approach to identify the sex determinant in this species. Toward this end, they generated a mutant population by irradiating the XY males, screening the resulting population for individuals producing female flowers, consistent with loss of the male-promoting factor. Three such mutants carried a deletion of the entire Y-specific region, which did not narrow the number of candidate sex determinants but did result in male-to-female conversion, supporting the idea that this region encompasses the sex-determining factor(s). One additional mutant produced all hermaphrodite flowers and was determined to carry a small deletion encompassing only one gene, the function of which is unknown (39). Finally, a spontaneous mutant carrying hermaphrodite flowers as well as a frameshift mutation was identified. This finding provided independent support for a sex-determining role and warranted naming this gene *SOFF* (Suppressor Of Female Function) on the basis of its presumed function (39). Although functional confirmation is still needed, it is probable that *TDF1* is indeed a male-promoting factor and the strong evidence presented above suggests that *SOFF* acts as a female-suppressing factor. If so, *SOFF* and *TDF1* together provide the first example of two identified genes that fit the two-factor model presented in **Figure 1*c*** and proposed many years ago (19, 52).

Kiwifruit

Most *Actinidia* species (kiwifruit) are dioecious (53), and male individuals are heterogametic irrespective of the ploidy level of the specific cultivar or species. The X and Y chromosomes are homomorphic, suggesting another case of recent and incipient sex chromosomes characterized by a fairly small MSY region (30, 42). Using a combination of genomic and genetic approaches, Akagi et al. (97) identified male-specific sequences and a candidate sex-determining gene. *Shy Girl* (*SyGI*), a Y-encoded type-C cytokinin response regulator, was identified as a potential candidate because of its male specificity and expression pattern. Transgenic expression of *SyGI* in *A. thaliana* and *N. tabacum* resulted in varying levels of suppression of carpel development, consistent with *SyGI*'s role as a potential suppressor of female function (*supF*). Homology analysis revealed that it probably originated from a recent duplication event specific to *Actinidia*. Although protein sequence comparisons and transgenic experiments in *A. thaliana* and *N. tabacum* suggest that the two homologs are functionally similar, they exhibit strikingly different expression patterns. *SyGI* is expressed mainly in flower organs, whereas its duplicate is expressed in leaves but not flowers. Consistent with *SyGI*'s inferred function as a cytokinin signaling regulator, exogenous application of synthetic cytokinin exhibited an inhibitory effect on male function development in kiwifruit. Because the expression of *SyGI* is strictly limited to female organs, the authors hypothesized that *SyGI* is one component of a two-factor system regulating sex in kiwifruit (97). The second factor, a hypothetical male-promoting factor (*M*), remains to be identified.

Sex Determination in *Cucumis* spp.

Cucumis is not dioecious and does not carry sex chromosomes, but regulation of flower sex types is well characterized in this system and thus of relevance to mechanisms of sex determination. Cultivated cucumber (*Cucumis sativus*) and melon (*C. melo*) are typically monoecious species, but varieties can be gynoecious (female), gynomonoecious (female and hermaphroditic flowers on the same plant), hermaphroditic, andromonoecious (male and hermaphroditic flowers on the same plant), or androecious (male) (**Figure 3*d***). Wild species of the Cucurbitaceae can be dioecious (44). In addition, sex in cucurbits is sensitive to physical and chemical treatments: Long days, heat, gibberellins, and ethylene inhibitors favor male flowers, whereas short days, cold, auxins, and ethylene favor female flowers (59). Induced changes in sexuality are convenient in breeding

and seed production because they enable seed production from a gynoecious variety by artificial induction of male flowers. Researchers have identified multiple genes contributing to sex, first by performing a genetic analysis of varieties that display different sexualities (59), then molecularly by mapping loci of interest, and last by characterizing additional mutants. The difficulty of producing transgenic plants has complicated the analysis, which nonetheless has uncovered an extensive regulatory network based on ethylene synthesis and its regulation (**Figure 3*a***). All but one of the characterized genes encode ethylene synthesis enzymes that favor femininity. The exception is WIP1, a Cys2-His2 zinc-finger transcription factor of the WIP protein subfamily (6), which represses femininity by binding at least some of the ethylene genes' promoters (23) and may have other actions (17). Ethylene in turn suppresses *WIP1*. Presumably, this sets up bimodal, metastable regulatory states that result in monoecy (**Figure 3*b***).

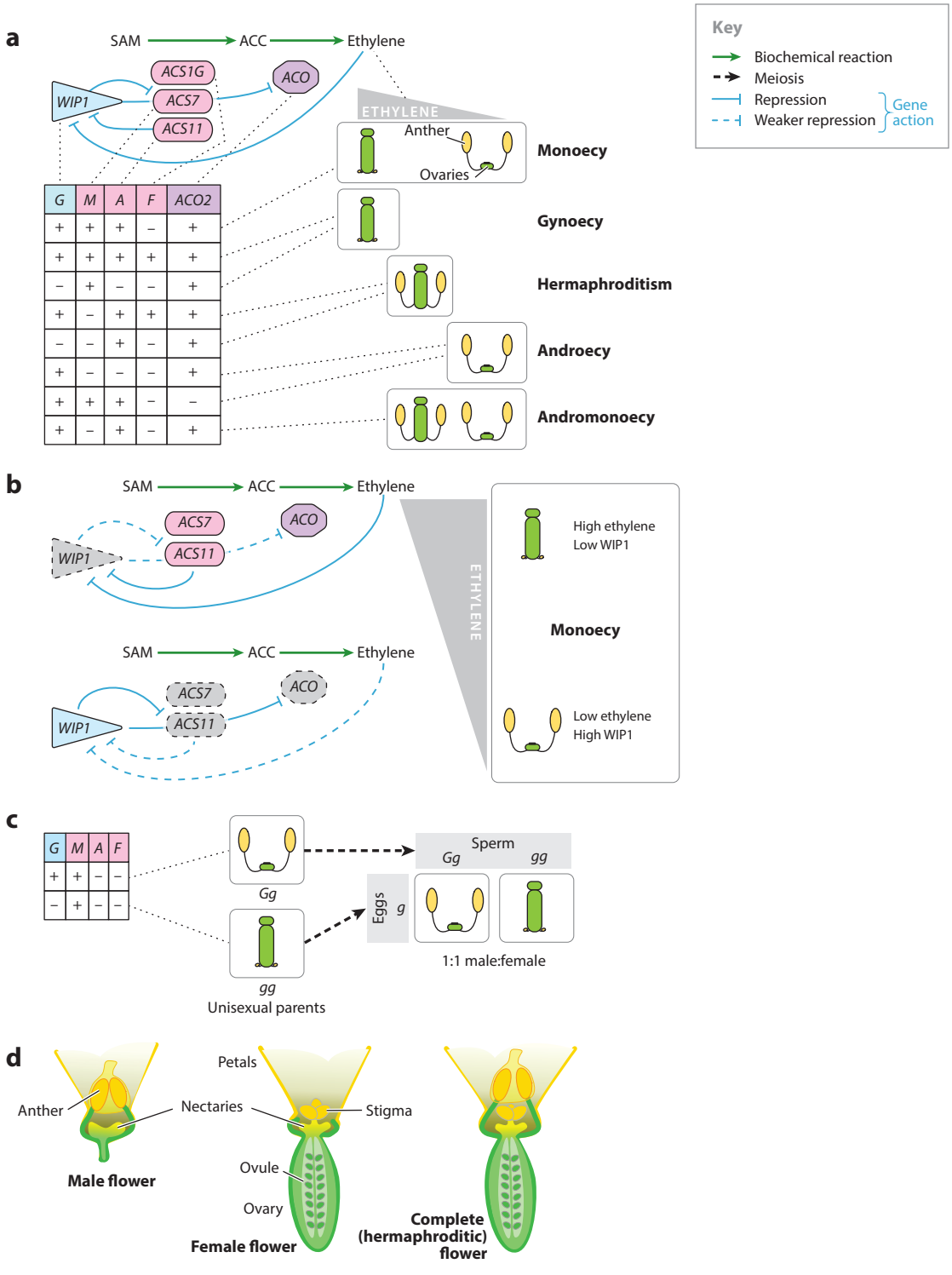
The multiple loci of *Cucumis* sex determination that encode enzymes of the ethylene pathway reveal the central role of this hormone. When the amount and the location of ethylene are modulated, different sexual states can be specified. This system provides an interesting platform from which to investigate the origin of dioecy. For example, once *ACS11* has been knocked out in all genotypes, a *WIP1/wip1* heterozygote crossed to *wip1/wip1* results in 1:1 sex ratios and engenders an artificial monogenic dioecious system (**Figure 3*c***) (17, 77), exemplifying a potential path to dioecy.

Dosage-Dependent Regulation in Dioecy

Many genes are necessary for male or female development, such as those that encode tissue-specific enzymes, structural proteins, and regulators. One or more of these could be recruited to be a sex determinant, as variation in the genes encoding them could affect flower sex characteristics. Genetic systems leading to dioecy such as those described in **Figure 1*b*,*c*** are both plausible and consistent with the emerging evidence presented above. Although flower sex switches appear as qualitative traits (a flower is male, female, or hermaphrodite), the underlying regulation is often quantitative. For example, a critical threshold in the concentration of a factor may trigger a sex change. Dosage sensitivity is a common feature of regulatory systems (89) and is likely to play a significant role in plant sex determination. For example, a simple, alternative single-factor model for dosage-dependent sex determination is illustrated in **Figure 1*d***.

Although it is easier to understand simple qualitative (on-off) switches, dosage-dependent regulation is more likely to occur, as indicated by the following evidence. First, plants display flexible sex determination. Monoecious species (with male and female flowers in different positions of the same body) demonstrate how the same genotype can produce both types of sexually specialized flowers. The best explanation for this phenotype is that flower organ primordia respond to quantitative changes in developmentally sensitive factors. Consistent with this explanation, multiple genes involved in hormonal regulation are necessary for developmental sex determination in monoecious maize and cucurbits (17, 54), implicating hormonal gradients in sex traits. Dosage dependence is suggested by the additive feminization action of the cucumber *F* locus (*F/F* is more feminine than *F/f*). *F* appears to consist of a duplicate of ACS, an ethylene synthesis gene (48, 85, 95). Second, sex in monoecious and dioecious species can be switched by stress and hormonal treatments (32). In dioecious *Cannabis*, ethylene inhibitors can induce females to pollinate. The genetically female pollen is used to fertilize normal females, resulting in feminized seed, i.e., consisting of female individuals only.

Dosage of the X chromosome to the autosomes is a well-characterized sex determinant in animals, including *Drosophila melanogaster* and *Caenorhabditis elegans* (73). However, the dosage-dependent effects of sex chromosomes can be found in plants as well: *Rumex* and *Humulus* regulate sex by a genetically similar system (62, 70, 72). In sex determination systems that fit



(Caption appears on following page)

Figure 3 (*Figure appears on preceding page*)

Sex determination loci and their action in cucumber (*Cucumis sativus*). (*a*) Interaction between ethylene synthesis genes and the WIP1 transcription factor. The allelic status of these dominant genes has a strong effect on sex traits (+, active; −, inactive). Paralogous genes *M* (55, 94), *A* (17), and *F* (85, 95) encode ACC synthetase (ACS) enzymes, which convert SAM to ACC. A newly discovered locus (*ACO2*) (23) encodes an ACC oxidase, which converts ACC to ethylene. Locus *F* represents a gain-of-function allele resulting from duplication of a DNA segment carrying ACS1G, whose role in *F* function appears likely but has not been strictly demonstrated (85, 95). WIP1 is a repressor of femininity that suppresses ACS and ACO2 functions (61). It is in turn repressed by ethylene. (*b*) A monoecy model postulates two semistable states involving a conflict between WIP1 on one side and ACS, ACO2, and their product, ethylene, on the other. The system kinetics might enable only two stable states and thus explain monoecy (23). (*c*) Synthesis of dioecy. Male and female differ only at the *G* locus. The male is heterozygous and the female is recessive. In the absence of the *G* allele there is sufficient ethylene to form only female flowers (gynoecy) (17). (*d*) Schematic structure of *Cucumis* flower types. Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; SAM, *S*-adenosyl-methionine.

the X:autosomes model, it is challenging to distinguish between mechanisms that count the X:cytoplasm ratio and those that count the ratio of X-encoded numerators to autosomally encoded denominators (28) (i.e., [X] versus [X:A]). In either case, however, regulation is dosage dependent. A comparison of female and male flowers in *Rumex acetosa* led to the discovery of a candidate sex determination gene encoding a protein related to the pore-forming toxin aerolysin (60). Aerolysin expression is high in female flower primordia and lower in males, and it suppresses male fertility in transgenic tobacco. Its characteristics fit the role of a dosage-dependent sex determinant.

GENE DUPLICATION, POLYPLOIDY, AND THE APPEARANCE OF DIOECY

Whole-Genome Duplication

Polyploidization or whole-genome duplication might play a role in establishing a dioecious sexual system (8, 34, 63). The hypothesis that polyploidy is associated with sexual dimorphisms or dioecy was proposed long ago on the basis of phylogenetic or empirical information and has been investigated in detail in *Lycium* and *Fragaria* (for a review, see 8). For instance, diploid *Fragaria* species are predominantly hermaphrodites. Although some sexual differentiation can occur in diploids, such as in gynodioecious *Fragaria vesca* ssp. *bracteata*, its descendant octoallopolyploid and subdioecious *F. chiloensis* and *F. virginiana* display either different or highly diverged sex determinants, implying that substantial sex evolution occurred during or after polyploidization (9, 68). Emergence of dioecy in this taxon is consistent with the conclusion by Glick et al. (34, p. 1,231) that sexual evolution is associated with polyploidization events "more often than expected by chance."

Innovation Through Gene Duplication

What could drive this association? The formation of gene duplicates can result in the evolution of dominant mutations and new protein function, such as those needed for the establishment of the dominant *supF* function (57, 69), either via neofunctionalization of the encoded protein (29, 40) or through *cis*-evolution resulting in novel expression patterns (1). *Cis*-evolution is thought to occur rapidly after gene duplication to alleviate the consequences of increased dosage (78). There is evidence that gene duplication plays a role in the evolution of all three dioecious systems described above. In diploid persimmon, the sex determination genes *OGI* and *MeGI* are the product of a *Diospyros*-specific gene duplication event (5). Similarly, findings from a phylogenetic analysis in *Actinidia* are consistent with the hypothesis that *SyGI* resulted from a recent duplication event and transposition to the Y-specific locus (97). In this case, the data suggest that the two paralogs

retained the same function but acquired specific expression patterns instead. Finally, the presence of a paralog of the sex-determining gene *SOFF* in asparagus suggests that it also arose from a duplication event, followed by evolution of the female-suppressing function on the copy associated with the MSY (39).

BREAKDOWN OF DIOECY

Although dioecy is expected to break down frequently (25, 34), this transition is poorly documented. Empirical studies have proposed possible scenarios that would result in a move from dioecy to another sexual system (46, 76), but the exact mechanism is difficult to investigate without prior knowledge of the genetic sex determinants in the dioecious system. Evidence that a phylogenetic basal dioecious ancestral taxon has hermaphroditic descendants is difficult to gather (82). Often, a criterion used for reversal is the appearance of individuals carrying complete flowers in a dioecious population (35). This, however, is not surprising because the loss of a single gene in a dioecious species can produce hermaphrodites (**Figure 2*c***) (47). Furthermore, their appearance in the population does not demonstrate the potential of the hermaphrodites to form a new species.

The Effect of Domestication

Artificial selection, which occurs, for example, during domestication, can also drive the transition switch from dioecy to hermaphroditic or self-fertile sexual systems, as outcrossing systems are generally less desirable for cultivated crops (79). In papaya (*C. papaya*), Y^h, a Y chromosome variant that contributes to the expression of hermaphroditism (90), exhibits dramatic reduction in genetic diversity. This is indicative of a selective sweep, presumably due to artificial selection during domestication (87, 88). A similar scenario has also been proposed in grapevine (*Vitis vinifera*) (84). Cultivated grape is typically hermaphroditic, whereas its direct ancestor is dioecious. This change is controlled by a single locus, at which the *H* allele is predicted to be a variant of the male (*M*) allele on the Y chromosome (74). Although population genetic approaches have not detected any clear selective sweep on the *H* allele, a population bottleneck during domestication may have been involved in the establishment of cultivated hermaphrodite grapes (11, 12, 84). In both cases, the simplest explanation for the switch to hermaphroditism is the disruption or recombination of the *supF* locus of the two-factor system, followed by artificial selection. If so, comparison of Y chromosome sequences from domesticated varieties and their ancestors could be a powerful strategy to identify *supF* genes.

Effect of Polyploidy

Although polyploidization and whole-genome duplication (24, 71) can engender dosage variation, limited experimental evidence for the impact of this phenomenon on dioecy is available. Polyploidization can be associated with the breakdown of dioecy (8). For example, polyploidy can subvert the optimal dosage of sex chromosomes (2, 3, 72), as is the case for *Rumex*. In polyploid or aneuploid plants of *R. acetosa*, for instance, in a heterozygous tetraploid, the ratio of X:A, where A is autosome sets, can easily distort from 1:2 (XXYY+4A), which is the same as the heterozygous diploid (XY+2A), to 1:4 (XYYY+4A) or 3:4 (XXXY+4A). Matching sex to chromosome ratios, Ono (70) found the following formula to predict sex: $n = \text{X/A}$. Plants with $n \geq 1$ are female, and plants with $n \leq 0.5$ are male. Intermediate values result in intersexual plants, which bear hermaphrodite and either male or female flowers. Although originally formulated differently with a factor of 0.6, the dosage equation is the same as that for *D. melanogaster* (62, 70).

Polyploidization can also affect sex determination indirectly. Nearly a century ago, Muller (65) proposed that dioecy constitutes a barrier to polyploidization because the dosage of sex chromosomes and their inheritance would compromise both sex determination and sex ratios. Muller's arguments were reasonable, and indeed, there are a few examples of plant polyploid species with dioecious origins. Nonetheless, the frequent occurrence of dioecious polyploids indicates that the assumptions on which they are based are not general (reviewed in 36, 58). We now know that sex transitions can accompany ploidy changes and have been observed in any pattern, including maintenance and formation of dioecy (reviewed in 8, 15). A strategy for meshing polyploidy and dioecy is to fix the simplex (single copy) Y genotype (such as YXXX or YXXXXX), which yields a 50:50 ratio of gametes with a single copy of Y and ensures equal sex ratios. This seems to be the case in most hexaploid kiwifruit (*Actinidia deliciosa*) (81, 83). Another strategy is to transition to a more flexible sexual system (see below). Finally, few genera transitioned from dioecy to hermaphroditism upon polyploidization (8, 35).

The Case for Epigenetic Regulation in Hexaploid Persimmon

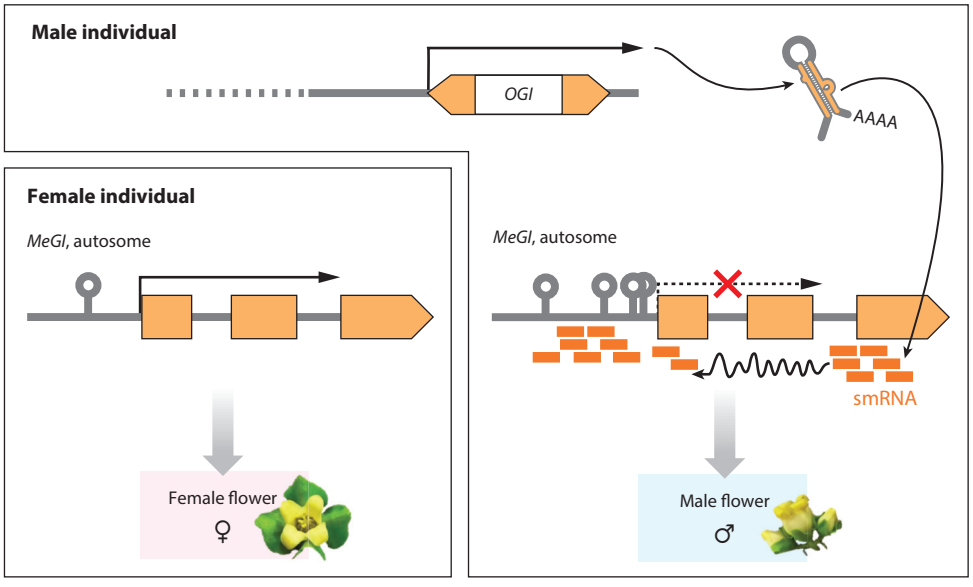
Recent progress on hexaploid persimmon, a species closely related to the dioecious diploid species *D. lotus*, provides concrete clues to the relationship between polyploidization and the acquisition of a more plastic sex determination system. Hexaploid persimmon, *Diospyros kaki*, is gynodioecious and exists in two types: trees that carry exclusively female flowers and trees that carry both male and female flowers (monoecious trees). Dioecy or possibly monoecy could be ancestral to the Ebenaceae because dioecy can be found in all four genera, *Lyssocarpa*, *Royena*, *Euclea*, and *Diospyros* (15). Therefore, gynodioecy in *D. kaki* must have evolved from a dioecious system, and hence the mechanisms for sex determination in *D. kaki* and *D. lotus* have common origins. Consistently, the male-determining gene *OGI* is fully associated with the formation of male flowers in *D. kaki*: Monoecious trees always carry one or two copies of the Y-specific sequences (XXXXXY or XXXXYY).

The expression of the *OGI* target transcription factor *MeGI* is differentially expressed in male versus female flowers in *D. kaki*, as originally found in diploid persimmon (**Figure 4*a***) (5). The presence of smRNA targeting the *MeGI* promoter in *D. kaki* male flowers suggests that the mechanisms downstream of *MeGI* are conserved between the two species as well (4). The major difference between the two species lies in the role of the *OGI* pseudogene. Whereas *OGI* expression can be detected at critical stages early in *D. lotus* flower bud development (June/July) and flower development (subsequent April), no *OGI* expression in *D. kaki* male flowers has been detected to date (4). One likely explanation for the lack of detectable *OGI* expression is the presence of a SINE-like transposable element named *Kali* in the middle of the *OGI* promoter (4). The *Kali* element is targeted by 24-nt smRNAs and cytosine methylation, both of which probably contribute to the silencing of *OGI* transcription. Although this explains the lack of *OGI* expression, it also raises the question of what is responsible for the differential expression of *MeGI* in male versus female developing buds and flowers.

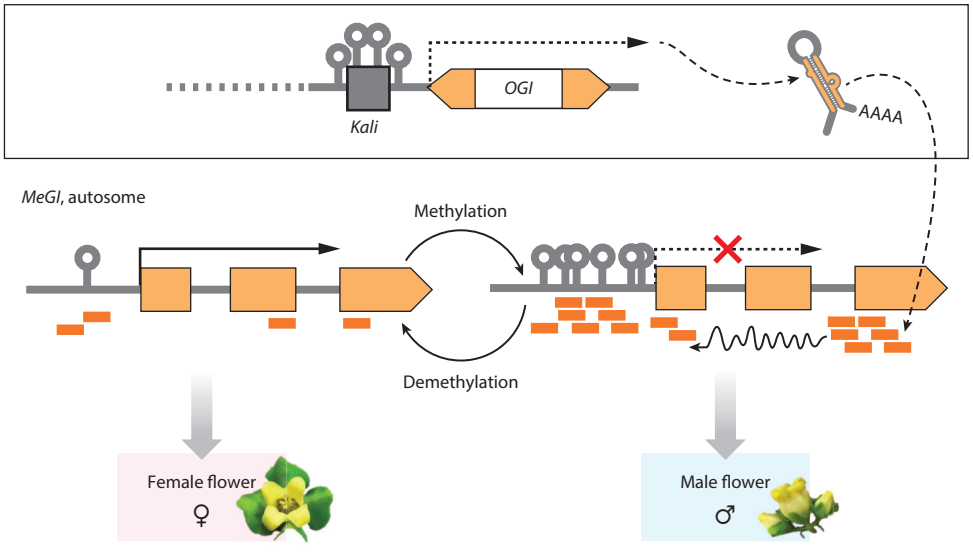
Investigating the methylation of the *MeGI* promoter region provided some clues to this question. Indeed, *MeGI* promoter methylation gradually increased throughout the development of male buds and male flowers, whereas it remained low in female bud and flower development whether they came from female trees or monoecious trees. This difference in methylation was also visible in *D. lotus*, but it was more pronounced in *D. kaki*. It is also consistent with the observed pattern of year-to-year flower development in *D. kaki*. Indeed, buds produced on flower-carrying branches eventually develop into branches themselves after a few months of dormancy, and the pattern of flower formation on those new branches is far from random. Male branches have a

strong (but not absolute) tendency to produce male branches, whereas female branches are less predictable and their sexual fate is subject to a positional effect. Developing buds at the apex of female branches develop most often into female branches, whereas developing buds that are more proximal can produce with approximately equal probability either male or female branches. Methylation of the developing buds can predict with perfect accuracy the sex of the future flower

a Diploid *Diospyros lotus*



b Monoecious hexaploid *Diospyros kaki*



(*Caption appears on following page*)

Figure 4 (*Figure appears on preceding page*)

Models for sex determination in diploid and hexaploid persimmon. (*a*) Sex determination in diploid *Diospyros lotus* follows a one-factor model in which *MeGI* expression dosage is critical to determining flower sexuality, as presented in the dosage model (**Figure 1*d***). High levels of *MeGI* expression promote female functions and repress male functions; low levels of *MeGI* expression result in the opposite. The first role is consistent with the observation that *Arabidopsis thaliana* plants overexpressing *MeGI* are feminized (5). We currently lack information about whether reduced *MeGI* expression is sufficient to suppress female function. The Y-encoded pseudogene *OGI* regulates *MeGI* expression via the production of small RNAs (smRNAs). (*b*) In hexaploid *Diospyros kaki*, the expression of *OGI* is repressed by the presence of *Kali*, a SINE-like element. The Y-encoded factor that regulates the expression of *MeGI* is located on the Y chromosome and is either *OGI* or another factor that remains to be determined. Both male and female flowers can be produced on genetically male (carrying at least one copy of the Y chromosome) individuals. The expression of *MeGI* is controlled by the level of its promoter methylation. Switches from one state to the other allow for the reversal from male to female branches and vice versa. Persimmon flower drawings copyright American Society of Plant Biologists; used with permission from Reference 4.

branch, even in the case of sex reversal, i.e., when a male parental branch produces a female branch or vice versa. Taken together, these results suggest that flower sex in monoecious individuals is determined via *MeGI* expression level tweaked by methylation of the *MeGI* promoter. Methylation is maintained most of the time but occasionally it is deposited de novo (resulting in a switch to male branches) or removed (resulting in a switch to female branches). Interesting questions about this system remain to be addressed, not the least of which is whether spikes of *OGI* expression are responsible for the occasional de novo deposition of methylation marks on the *MeGI* promoter.

The *D. kaki* example is an interesting variation of the situation that occurs in *D. lotus*, where sex is fully controlled genetically via the presence of *OGI* (**Figure 4*b***). Instead, although probably controlled by the same molecular mechanism, sex in *D. kaki* can be steered one way or the other through epigenetic control over the *MeGI* promoter. This observation provides further support for the hypothesis that *MeGI* regulation might be sufficient for the regulation of both male and female organs in *Diospyros*, as proposed in **Figure 1*d***. Some hexaploid persimmon cultivars carry multiple Y alleles (4), but the SINE-like element, *Kali*, which is responsible for the silencing of *OGI*, is perfectly conserved in all copies and all cultivars examined so far (4). This suggests a strong bottleneck or possibly adaptive selection for the presence of the *Kali* element early after the hexaploidization event. The acquisition of this epigenetic layer to the regulation of *MeGI* is a key mechanism to allow sexual plasticity and transition out of dioecy. A related mechanism might be at play in *Silene latifolia*, in which treatment with the DNA demethylation agent 5-azacytidine converts male flowers to hermaphrodite ones (43). Epigenetic regulation of dioecy was also hypothesized to occur in *Populus balsamifera*, in which DNA methylation of a candidate sex determination gene was associated with sex (18).

SUMMARY POINTS

1. Most plants carry hermaphrodite flowers, but a small subset of species spread fairly evenly across the phylogenetic tree are dioecious, with individuals carrying either male or female flowers. This pattern provides a unique opportunity to ask whether similar events have led to the transition to dioecy and whether similar developmental and molecular mechanisms result in single-sex flowers.

2. Many theoretical scenarios can lead to the transition from hermaphroditism to dioecy. For example, the two-factor model involves two sex determinants linked on the Y chromosome, one repressing female function (*supF* gene) and the other promoting male function (*M*). An alternative one-factor model proposes the evolution of a single sex determinant that is also Y encoded and carries both sex-determining functions.
3. With the recent availability of powerful genomic tools, questions regarding the identity of these sex determinants in various dioecious species can be addressed more rapidly, and in the last three years, strong candidate sex determinants in four different taxa have emerged: dioecious diploid persimmon, kiwifruit, and asparagus, and monoecious hexaploid persimmon and cucumber.
4. Angiosperm phylogeny supports the independent formation of multiple dioecious taxa. The emerging evidence of molecular mechanisms of dioecy in persimmon, asparagus, and kiwifruit supports the hypothesis that different mechanisms resulting from either monogenic or digenic control are possible. Specifically, data from persimmon favor a single-factor model, whereas data from kiwifruit and asparagus favor the two-factor model.
5. Although the end solution is different, i.e., different gene functions and different developmental stages are targeted, it appears that the original trigger for the transition to dioecy in all three species characterized so far involved a gene duplication event, capacitating the evolutionary search for new gene function through regulatory or structural innovation, buffering *cis*- or *trans*-evolution of one of the paralogs into a sex determinant while potentially retaining the function of the other copy.
6. Transitions out of dioecy are also expected to occur via multiple paths, including domestication and whole-genome duplication leading to polyploidy.
7. Our increased ability to fully sequence and assemble the MSY regions, and their corresponding X sequences, combined with cheaper and more accessible functional genomic approaches, will allow for more thorough scans for potential sex determinants in a variety of dioecious species. We expect better and more general conclusions regarding the path to dioecy.
8. Plant polyploids can manage sex determination efficiently, demonstrating that Muller's predictions were premature. This is not to say that for a dioecious plant species transition through ploidy is seamless. Rather, a flexible strategy is sometimes necessary, as demonstrated in persimmon, in which males become monoecious by epigenetic sex switching.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

This work was supported by PRESTO, Japan Science and Technology Agency (to T.A.); by a Grant-in-Aid for Young Scientists (A) (no. 26712005 to T.A.) and a Grant-in-Aid for Challenging Exploratory Research (no. 15K14654 to T.A. and no. 26660025 to R.T.) from the Japan Society

for the Promotion of Science; and by the National Science Foundation IOS award under grant no. 1457230 (to I.M.H. and L.C.).

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5. This article compares anonymous sequences (k-mers) between male and female sibs to find Y-encoded genes that are candidate sex determinants.

17. Quantitative regulation of ethylene synthesis may engender dioecy.

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