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Pathways to sex determination in plants: how many roads lead to Rome?

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The presence of thousands of independent origins of dioecy in angiosperms provides a unique opportunity to address the parallel evolution of the molecular pathways underlying unisexual flowers. Recent progress towards identifying sex determination genes has identified hormone response pathways, mainly associated with cytokinin and ethylene response pathways, as having been recruited multiple times independently to control unisexuality. Moreover, transcriptomics has begun to identify commonalities among intermediate sections of signal transduction pathways. These recent advances set the stage for development of a comparative evolutionary development research program to identify the shared and unique aspects of the genetic pathways of unisexual flower development in angiosperms.

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In angiosperms, dioecy is found in only ~6% of all species [1], and the associated complex phenotypes may have independently evolved thousands of times [2]. Over the last decade, impressive progress has been made towards understanding sex determination and identification of the master regulators of sex determination in several species [3••,4••,5–7,8••,9•], yet there is no current consensus regarding commonalities, or lack thereof, among the molecular pathways controlling sex determination across different taxonomic groups remains unexplored. In animals there is a growing understanding that molecular pathways and signaling cascades share many of the same basic components and structure, with the top-most components, including the master regulators of sex determination, being the most evolutionarily labile [10,11]. However, the ultimate reason for these commonalities may not lie in the common origin of gonochory (the term used to refer to dioecy in animals), but instead in the common molecular pathways that control the development of tissues and organs that protect, nurture, and generate gametes. Support of this hypothesis, in fact, may reside in a taxonomic group such as the angiosperms in which dioecy has evolved multiple times independently, yet share many aspects of the anatomy and development of plant gametophytes and gametes.

Intermediate breeding systems when dioecy has evolved from hermaphroditism in plants have most commonly been either gynodioecy (females and hermaphrodites in populations) or monoecy (all individuals produce both male and female unisexual flowers) [12], both of which exhibit unisexual flowers. A large number of genes are involved in the development of the androecium and gynoecium, and mutations in any of these genes could potentially lead to losses of function resulting in the cessation of male or female organ development [13–15]. One theory regarding the evolution of sex-chromosomes involves two genes: one with an allele that results in male sterility and a second with an allele that results in female sterility [16], and has been supported by recent evidence from kiwifruit [5] and asparagus [6], although there also is support for a single gene system controlling sex determination [4••], and the genetic origins of dioecy remain contentious [17–19]. These one or two genes become the ultimate master regulators of sex determination in dioecious plants (Box 1). Signals from

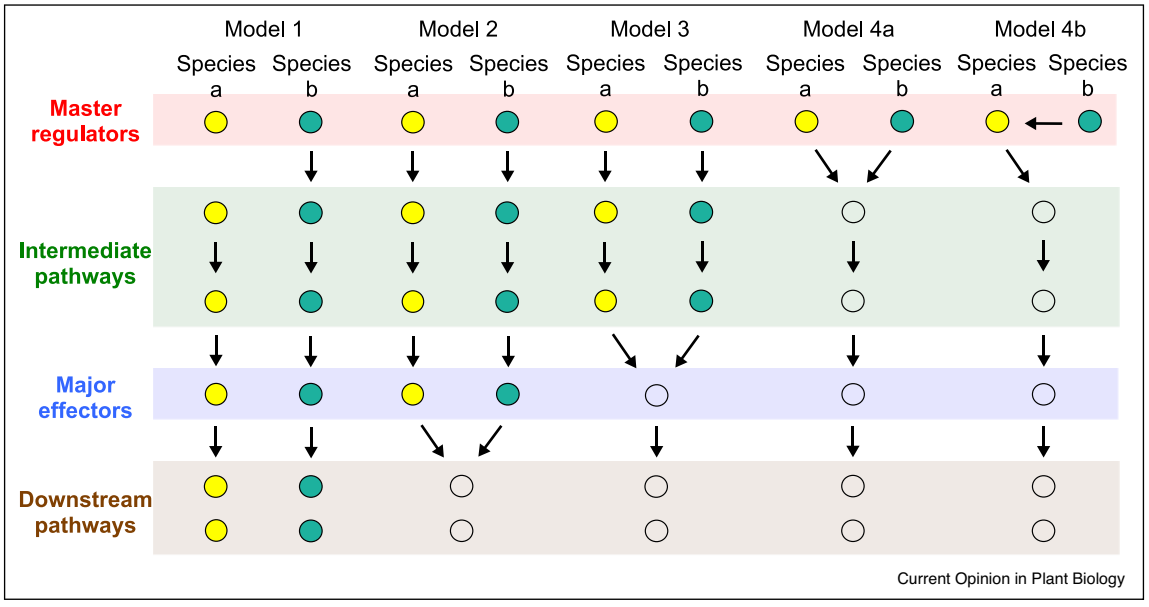
Box 1 Models of the comparative evolution of pathways controlling sex determination in different plant species.

The molecular pathways resulting in sex determination can be thought of as containing 4 modules from master regulators to the downstream pathways. Here we represent five semi-discrete hypotheses (models) of the comparative evolution of sex determination pathways. First, sex determination in different species may result from completely different expression interaction pathways (Model 1). This extreme case represents the evolution of fully independent sex determination pathways of two dioecious species where master regulators, intermediate pathways, major effectors, and downstream pathways all differ. A second possibility, Model 2, depicts the condition wherein differences between the two dioecious species result from evolutionary divergence of the expression of the MIKC-type MADS box genes and all genes influencing their expression, but the expression pathways below the major effectors remain the same. Models 1 and 2 may be more commonly observed when comparing distantly related and independently evolved dioecious species with different dioecious flower types (type I and type II flowers). A third hypothesis, model 3, describes a situation where the expression of MIKC-type MADS box genes and the downstream pathways are the same across species, but both master regulators and intermediate pathways that regulate those MADS-box genes differ. We may expect pathways consistent with model 3 when comparing two distantly related dioecious species with the same category of flower type. Model 4a, which is supported in animals [11], results when only the master regulatory genes differ between the two species, but intermediate pathways, major effectors, and downstream pathways are the same across species. In this case, the two different master regulators could be either homologs or unrelated genes that replace one another as regulators of the intermediate pathways. Finally, model 4b represents the case where species a and b share all of the same genes and expression pathways, but a gene in species b has taken over the role of the master regulator so that the homolog of the master regulator in species a becomes part of the intermediate pathway in species b.

these master regulators will be transmitted by genes in the intermediate pathway, which reinforce the bipartition of sex signals [20], to the major effectors controlling floral development, the MIKC-type MADS box transcriptional factors, especially the B, C, and D class genes which initiate the development of the gynoecium and/or androecium [21]. Importantly, the MIKC-type MADS box transcriptional factors have been hypothesized to be a key to understanding differences in development between Type I and Type II unisexual flowers [22,23]. Unisexuality in Type I flowers, which are found in species such as *Asparagus officinalis* and *Silene latifolia*, is exhibited after the development of carpels or stamens, and is hypothesized to result from differential regulation of genes downstream of the MIKC-type MADS box genes or differential regulation of MIKC-type MADS box genes late in flower primordium development (Box 1) [24,25,26••]. In contrast, unisexuality in Type II flowers, which are found in species such as *Populus trichocarpa and Spinacia oleracea*, is exhibited as early abortion of carpels or stamens, and is hypothesized to result from the differential regulation of the MIKC-type MADS box genes in the early flower primordium stage [20,22] (Figure 1).

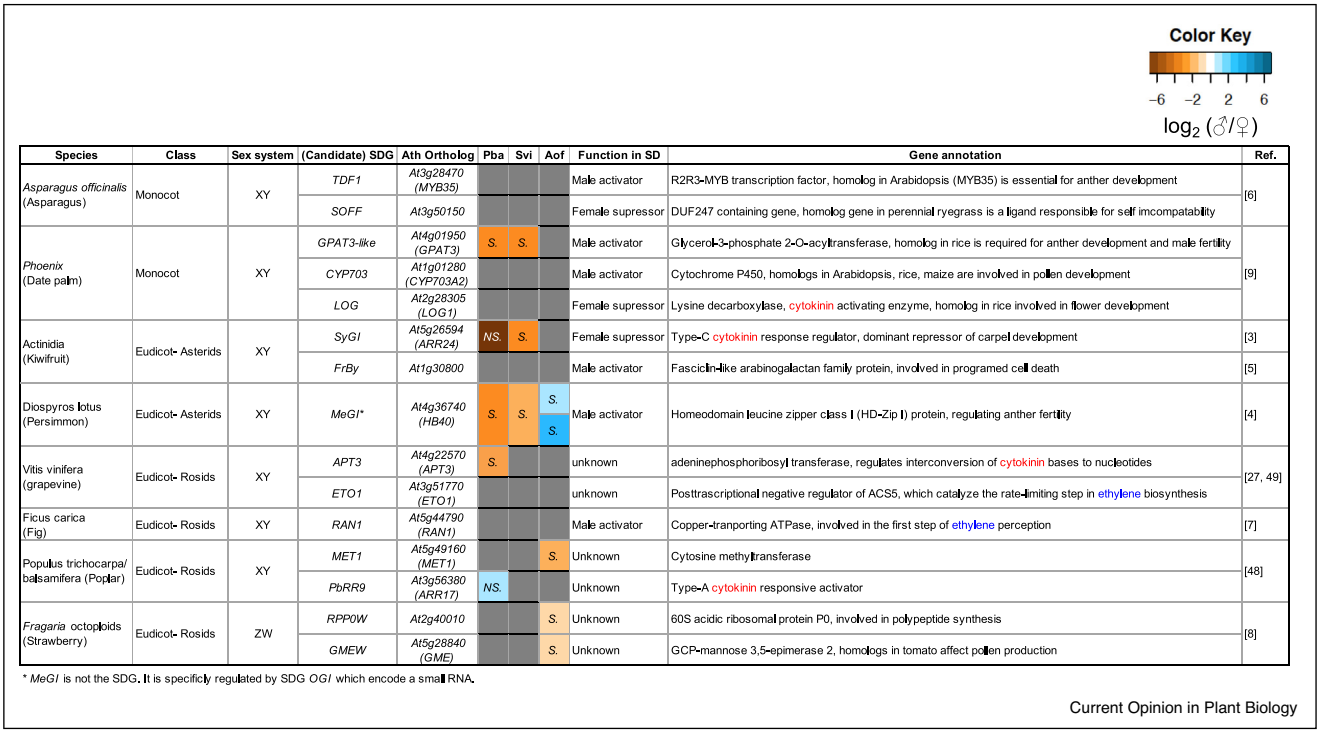
We are now at a critical juncture regarding our understanding of sex determination in plants because the master regulators and patterns of sex-biased gene expression have been studied in a sufficient number of plant species to allow coherent comparative hypotheses to be constructed and tested. For example, commonalities in the patterns in developmental genetic models of the control of sex determination can be used to test among

Figure 1



Hypothesis regarding the comparative evolution of sex determination pathways in plants.

Figure 2



Species	Class	Sex system	(Candidate) SDG	Ath Ortholog	Pba	Svi	Aof	Function in SD	Gene annotation	Ref.
Asparagus officinalis (Asparagus)	Monocot	XY	*TDF1*	*At3g28470 (MYB35)*				Male activator	R2R3-MYB transcription factor, homolog in Arabidopsis (MYB35) is essential for anther development	[6]
			SOFF	*At3g50150*				Female supressor	DUF247 containing gene, homolog gene in perennial ryegrass is a ligand responsible for self imcompatability	
Phoenix (Date palm)	Monocot	XY	*GPAT3-like*	*At4g01950 (GPAT3)*	S.	S.		Male activator	Glycerol-3-phosphate 2-O-acyltransferase, homolog in rice is required for anther development and male fertility	[9]
			CYP703	*At1g01280 (CYP703A2)*				Male activator	Cytochrome P450, homologs in Arabidopsis, rice, maize are involved in pollen development	
			LOG	*At2g28305 (LOG1)*				Female supressor	Lysine decarboxylase, cytokinin activating enzyme, homolog in rice involved in flower development	
Actinidia (Kiwifruit)	Eudicot- Asterids	XY	*SyGI*	*At5g26594 (ARR24)*	NS.	S.		Female supressor	Type-C cytokinin response regulator, dominant repressor of carpel development	[3]
			FrBy	*At1g30800*				Male activator	Fasciclin-like arabinogalactan family protein, involved in programed cell death	[5]
Diospyros lotus (Persimmon)	Eudicot- Asterids	XY	*MeGI**	*At4g36740 (HB40)*	S.	S.	S. S.	Male activator	Homeodomain leucine zipper class I (HD-Zip I) protein, regulating anther fertility	[4]
Vitis vinifera (grapevine)	Eudicot- Rosids	XY	*APT3*	*At4g22570 (APT3)*	S.			unknown	adeninephosphoribosyl transferase, regulates interconversion of cytokinin bases to nucleotides	[27, 49]
			ETO1	*At3g51770 (ETO1)*				unknown	Posttrascriptional negative regulator of ACS5, which catalyze the rate-limiting step in ethylene biosynthesis	
Ficus carica (Fig)	Eudicot- Rosids	XY	*RAN1*	*At5g44790 (RAN1)*				Male activator	Copper-tranporting ATPase, involved in the first step of ethylene perception	[7]
Populus trichocarpa/ balsamifera (Poplar)	Eudicot- Rosids	XY	*MET1*	*At5g49160 (MET1)*			S.	Unknown	Cytosine methyltransferase	[48]
			PbRR9	*At3g56380 (ARR17)*	NS.			Unknown	Type-A cytokinin responsive activator	
Fragaria octoploids (Strawberry)	Eudicot- Rosids	ZW	*RPP0W*	*At2g40010*			S.	Unknown	60S acidic ribosomal protein P0, involved in polypeptide synthesis	[8]
			GMEW	*At5g28840 (GME)*			S.	Unknown	GCP-mannose 3,5-epimerase 2, homologs in tomato affect pollen production	

* *MeGI* is not the SDG. It is specificly regulated by SDG *OGI* which encode a small RNA.

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Sex determination genes and strong candidates identified in angiosperms, their function and expression in the same and other dioecious species. Many of these genes influence hormone response pathways including cytokinin and ethylene. Three columns indicate whether these sex determination genes (SDGs) or strong candidates exhibit sex biased expression in *Populus balsamifera* (Pba), *Salix viminalis* (Svi), or *Asparagus officinalis* (Aof). Each colored box in these columns represents one homolog of the corresponding SDG, and the color represents the strength and direction of sex biased expression. 'S' indicates significant and 'NS' indicates not significant. These three species are all well studied and represent a phylogenetic hierarchy with *Populus* and *Salix* being closely related and *Asparagus* being more distantly related, and are the focus of our comparative analysis in Figure 3. The five *P. balsamifera* genes (colored boxes) from top to bottom in the table are *Potri.005G202200*, *Potri.002G253000*, *Potri.007G029500*, *Potri.014G017200* and *Potri.019G058900*. The three *S. viminalis* genes (colored box) from top to bottom in the table are *Sa940v51016366m*, *Sa940v51007211m* and *Sa940v51021910m*. The five *A. officinalis* genes (colored box) from top to bottom in the table are *AsparagusV1_03.2711*, *AsparagusV1_08.1167*, *AsparagusV1_07.3493*, *Asoff_XLOC_037178* and *AsparagusV1_03.553*. [3••,4••,5,6,7,8••,9•,27,44•,47,48].

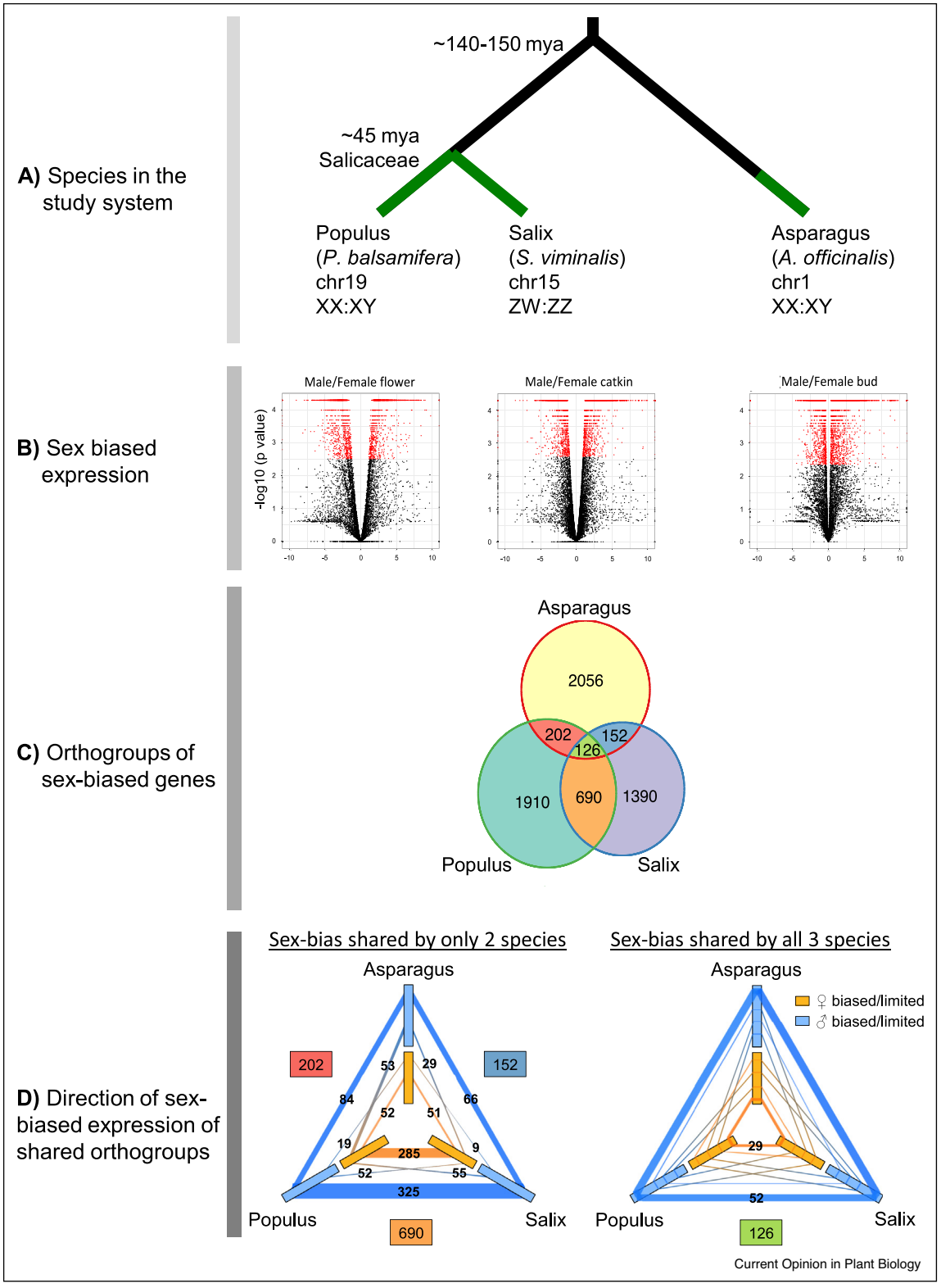
models concerning cross-species similarities and differences in the sex determination pathways of dioecious plants (Figure 1; Box 1). Moreover, the number of shared genes in sex determination pathways can be predicted by factors such as phylogenetic relatedness and the type of floral development (Type I versus Type II).

Our current understanding of sex determination pathways

Sex determination genes (SDGs) or strong candidates have now been identified in eight angiosperm species, representing a variety of monocots and eudicots (Figure 2). Consistent with the multiple origins of dioecy, each identified (or candidate) SDG is unique (Figure 2), representing different mechanisms that ultimately block pollen or egg development. Upon close inspection, however, intriguing functional similarities exist that are rooted in the synergistic regulation of phytohormomes, such as cytokinins and ethylene that stimulate the differentiation and development of floral organs [20]. For instance, date palm, kiwifruit, grape, and poplar all contain SDGs (or candidate SDGs) in cytokinin-related pathways (Figure 2), and in grape and fig candidate SDGs are associated with the ethylene signaling pathway (*ETO1* in grape [27], *RAN1* in fig [7]). Additional support for the importance of phytohormone-related genes can be found from the control of floral unisexuality in monoecious plants. For instance, adjustment in the ratio of gibberellic and jasmonic acid controls the sex of the flowers in *Zea mays* [28,29], and the *androecy* gene in cucurbits limits ethylene biosynthesis and induces female floral development [30].

To determine whether the same top-level genes may be important for sex determination across a broad taxonomic scale, we assessed expression homologs of these 15 genes in a monocot, *A. officinalis*, and two related eudicots,

Figure 3



Comparative transcriptomics of *Populus balsamifera*, *Salix viminalis* and *Asparagus officinalis* reveals ancient homology in sex-biased expression. **(a)** *Populus balsamifera* and *S. viminalis* belong to Salicaceae and diverged ~45 mya. Salicaceae, whereas Asparagus diverged ~140–150 mya from *Populus* and *Salix*. Male and female flower/catkin of the three species were shown. **(b)** Volcano plot of sex-biased expression of the reproductive tissues of the three species using the GSNAP-Cufflinks-Cuffdiff pipeline (https://github.com/guanqiaofeng/Comparative_transcriptome_project). *A.*

Populus balsamifera and *Salix viminalis* (Figures 2 and 3). Expression of homologs of eight of these 15 genes was detected in at least one species, with homologs of seven genes exhibiting sex biased expression in at least one species. Although these homologs may not be the master regulators of sex in these species, they may still play key roles in sex determination pathways. Notably, expression of some of these genes was not detected in the species in which they control sex determination (e.g. *TDF1* and *SOF* in *Asparagus*), indicating that differences in sampled tissues or developmental time periods may influence the ability to identify genes controlling sex determination. Thus, more studies that compare patterns of transcription across floral developmental series are warranted [26••,31,32], but nevertheless, it will remain challenging to sample homologous tissues and timepoints for widely divergent species with radical differences in floral ontogeny and morphology.

The extent to which intermediate pathways are commonly shared across species with different origins of dioecy remains an open question. One promising strategy is to compare co-expression networks across floral developmental series for males and females. Using this technique, Yang *et al.* [26••] identified a suite of 18 genes with co-expression connections to *MeGI*, which is directly regulated by the SDG *OGI in* persimmon (Figure 2). DAP-seq was further applied to identify a subset that were directly targeted by the *MeGI* transcription factor [26••]. Similar experimental and analytical techniques are being used to elucidate the genetic control of ethylene signal transduction resulting in the development of unisexual instead of monoecious individuals in cucurbits [32]. Other intermediate level genes have been identified through careful studies of genes interacting with MADS-box genes. For instance, in dioecious papaya, *CpHUA1* is differentially methylated between sexes and shows high expression in carpels [33]. Its ortholog in *Arabidopsis*, *AtHUA1*, interacts with the MADS-box C class gene *agamous* (*AG*) [34], suggesting that *CpHUA1* may function as an upstream regulator for the major effector MIKC-type MADS-box genes in papaya [33], and placing it near the bottom of the intermediate regulatory pathway.

Downstream of the intermediate genes, the major effectors (Box 1), which consist of the MIKC-type MADS box genes, as are known to be relatively conserved across angiosperms [18]. The timing of differential regulation of the MIKC-type MADS box genes, however, may result in different developmental types of flowers (I or II; [22]). Under the floral quartet model, stamens are defined by B, C and E class genes, carpels are defined by C and E class genes, and ovules are defined by C, D and E class genes [21]. Consistent with this model, class B genes exhibit male-biased or male-limited expression; this includes stamen-limited expression of *PTD* in *P. trichocarpa* [35], male-biased expression of *AODEF* in asparagus [25], male-biased expression of *AP3* and *PI* in kiwifruit [26••] and *Mercurialis annua* [36]. Class C genes, however, differ in their direction of sex bias across species, indicating that major effectors have evolved different mechanisms across angiosperms. For instance, in grapevine, the C class gene *VvMADS5* is expressed in female but not male flowers, suggesting a role in gynoecium development [24], but in kiwifruit *AG* has male-biased expression, suggesting a role in androecium development [26••]. Finally, consistent with its ovule-specific expression, the papaya D class gene *CpSTK* is only expressed in female and hermaphrodite flowers, but not in male flowers [37] and the *M. annua* D class genes AGL1/AGL3 have strong sex-biased expression in female flowers [36].

Comparative genomics for identifying conserved sex regulation pathways

Comparative transcriptomics offers a strategy to identify common patterns in sex-biased gene expression among dioecious taxa with different levels of divergence. To introduce the potential of an explicit comparative analysis of differential gene expression across species, we re-analyzed published transcriptome data on sex biased gene expression in three dioecious species (Figure 3), *A. officinalis* [38], *P. balsamifera* [39•], and *S. viminalis* [40]. These three species represent both XY (*Populus* Chr 19, *Asparagus* Chr 01) and ZW (*Salix* Chr 15) types of sex chromosome heteromorphism (Figure 3a) and represent different levels of phylogenetic divergence: *P. balsamifera* and *S. viminalis* are eudicots from the same family (Salicaceae), likely share a common origin of dioecy, and may share the same SDGs [41,42,43,44•], whereas *A. officinalis* is a monocot and represents an independent origin of dioecy that diverged from the Salicaceae ~140–150 million years ago (Figure 3a).

Comparisons of expression among ortholog groups (orthogroups) showed that *Populus* and *Salix* shared higher numbers of sex-biased orthogroups and more genes with sex bias in the same direction (Figure 3; e.g. 690 sex-biased orthogroups shared between *Populus*

officinalis female and male flower buds were collected during the initial floral differentiation [38]. *P. balsamifera* female and male flowers were collected from catkins 3 days after catkin bud burst and before the flowers opened [39•]. *S. viminalis* female and male catkins were fully developed when collected [40]. We chose these three species because all three, or their close relatives, have annotated genome assemblies, enabling the application of the same reference-guided RNA-seq analysis pipeline across species, and transcriptome data has been generated in recent years, to provide greater comparability across data sets. All three species have been previously shown to have sex biased expression [39•,40,49]. **(c)** Orthogroups of sex-biased genes of the three species based on OrthoFinder v2.2.6 [50]. **(d)** Sex biased orthogroups shared by two species (left; indicated by connected lines) and by all three species (right; indicated by triangles). Whether transcripts were female or male biased is indicated by blue and orange colors and may have switched among species.

and *Salix*, among which 610 [285 + 325] have the same direction of sex bias). Still, most differentially expressed orthogroups were species-specific (65%, 59%, and 81%in *P. balsamifera*, *S. viminalis*, and *A. officinalis*, respectively; Figure 3c), even *Populus* and *Salix*, which may share an origin of dioecy. Nonetheless, 126 orthogroups exhibited sex-biased expression in all three species (Figure 3c). These 126 orthogroups are candidates for conserved components of sex regulation pathways across angiosperms, and because of their deep functional homology, likely represent shared genes in downstream pathways, such as those influencing androecium or gynoecium development (Box 1). It is important to recognize that not all genes with sex-biased expression will influence the development of unisexual flowers, as secondary sexual characteristics may also evolve to influence mating success [45]. Although genes influencing secondary sexual characteristics may exhibit sex-biased expression in non-floral tissues, sex biased expression is generally much less common in leaves than flowers [39•,40]. Moreover, it is unlikely that the 126 shared orthogroups influence shared secondary sexual characteristics because of the widely independent origins of dioecy in Asparagus and the Salicaceae and their highly divergent reproductive ecologies.

Bottom up or top down evolution of sex determination pathways

Patterns of the evolution of sex regulation in animals support a bottom up model of the evolution of sex determination pathways, where the downstream regulatory pathways are more conserved, and the upstream regulatory pathways are more variable [11,46]. Taking into account the current data, it is unclear whether the same evolutionary patterns are exhibited in plants. The observation of different SDGs in different dioecious plants (Figure 2) suggest high variation in the master regulators of sex determination, but homologs to SDGs also tended to be sex biased in species with independent origins of dioecy (Figure 2), providing evidence that minor adjustments in similar developmental pathways may be all that is necessary for the independent evolution of dioecy. Downstream pathways, however, also show some commonalities, with 126 orthogroups exhibiting sex biased expression across *Populus*, *Salix*, *and Asparagus* (Figure 3; Box 1). Differences among expression patterns in downstream pathways remain much more common than similarities, though, suggesting that simplistic bottom-up or top-down characterization of the patterns of the evolution in pathways controlling unisexuality may not be supported by the evidence. With a better understanding for the similarities and differences in the sex determination pathways among different dioecious species, a clearer and more comprehensive picture of the evolution of sex determination pathways in plants will begin to come into focus.

Conflict of interest statement

Nothing declared.

CRediT authorship contribution statement

Guanqiao Feng: Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing. **Brian J Sanderson:** Conceptualization, Writing - review & editing. **Ken Keefover-Ring:** Funding acquisition. **Jianquan Liu:** Conceptualization, Funding acquisition. **Tao Ma:** . **Tongming Yin:** Funding acquisition. **Lawrence B Smart:** Funding acquisition. **Stephen P DiFazio:** Funding acquisition, Writing - review & editing. **Matthew S Olson:** Conceptualization, Funding acquisition, Writing - review & editing, Supervision.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.pbi.2020.01.004.

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