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Review

The genomics of plant sex chromosomes

Boris Vyskot*, Roman Hobza

Department of Plant Developmental Genetics, Institute of Biophysics, Academy of Sciences of the Czech Republic, 61265 Brno, Czech Republic

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ABSTRACT

Around six percent of flowering species are dioecious, with separate female and male individuals. Sex determination is mostly based on genetics, but morphologically distinct sex chromosomes have only evolved in a few species. Of these, heteromorphic sex chromosomes have been most clearly described in the two model species – *Silene latifolia* and *Rumex acetosa*. In both species, the sex chromosomes are the largest chromosomes in the genome. They are hence easily distinguished, can be physically separated and analyzed. This review discusses some recent experimental data on selected model dioecious species, with a focus on *S. latifolia*. Phylogenetic analyses show that dioecy in plants originated independently and repeatedly even within individual genera. A cogent question is whether there is genetic degeneration of the non-recombining part of the plant Y chromosome, as in mammals, and, if so, whether reduced levels of gene expression in the heterogametic sex are equalized by dosage compensation. Current data provide no clear conclusion. We speculate that although some transcriptome analyses indicate the first signs of degeneration, especially in *S. latifolia*, the evolutionary processes forming plant sex chromosomes in plants may, to some extent, differ from those in animals.

Contents

1. Introduction

Genetic recombination is a key process in the variation achieved during meiosis when paternal and maternal chromosomes are combined and exchanged. Random combination of male and female gametes completes the variation necessary for evolution. However, there are exceptions demonstrating other mechanisms for ensuring genetic variation. For example, bdelloid rotifers reproduce asexually and are propagated by parthenogenesis without meiosis. Their genome is totally restructured during anhydrobiosis, including the integration of foreign DNA sequences from adjacent organisms (horizontal gene transfer). Mechanisms like this appear to be functionally equivalent to genetic exchange and allow a large divergence and speciation [1].

The flowers of angiosperms are largely bisexual, i.e., they contain both pistils and stamens. These develop differently from most animals. Briefly, they do not possess a true germline, and sexual organs are formed in flowers after transition from the vegetative to the generative state from somatic axillary meristems late in development. This enables them to partially maintain the environmentally induced epigenetic changes occurring during development. The products of meiosis are not gametes, as in animals, but haploid spores which require gene expression for differentiation. Finally, there are two fertilization events between sperms produced by the male gametophyte (the pollen tube) and the embryo sac: one fertilization event leads to the formation of a zygote, and the other

* Corresponding author. Tel.: +420 541240500.
E-mail address: vyskot@ibp.cz (B. Vyskot).

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leads to (usually triploid) endosperm, which is critical for embryo nutrition and viability. Plant bisexuality may result in inbreeding depression, and dioecy (the separation of the sexes into different individuals) may have evolved in many plant species in response to selection to avoid inbreeding.

The separation of male and female organs in different flowers occurs either as dioecy (pistillate or staminate flowers in different individuals) or monoecy (pistillate or staminate flowers on the same individual). Either way, unisexuality in flowers is achieved in floral development as an arrest of sex organ formation (either the pistil or the stamen). In dioecious species, sex is most often determined by genotype, but environmental, hormonal, and epigenetic cues are also used in determination [2–4]. Sexual differentiation includes not only floral differentiation and the formation of male and female gametes. It is also responsible for gender dimorphism, and, in some groups of organisms, dosage compensation of X-linked genes and genomic imprinting. Sex-determining genes are often clustered in heteromorphic sex chromosomes, which are more common in animals, and less relevant in plants. Heteromorphic sex chromosomes are defined by being distinguishable under a microscope [3–5].

Similar to animal species, sex determination systems in plants can be classified with respect to whether each sex forms different gametes: in X/Y systems males (XY) are heterogametic and females (XX) are homogametic (e.g., *Silene latifolia*, *Rumex acetosa*, and *Carica papaya*). By contrast, in Z/W systems males (ZZ) are homogametic and females (ZW) are heterogametic (e.g., *Populus trichocarpa*, *Fragaria chiloensis*, *Silene ottites*). In X/Y species, there are two basic systems of sex determination, the mammalian type with the dominant Y chromosome (e.g., *S. latifolia*) and the *Drosophila* type with the critical X/A ratio (e.g., *R. acetosa*).

2. Evolution of sex chromosomes

According to evolutionary theories [6,7], the sex chromosomes originated from an ordinary pair of chromosomes (autosomes), usually in lineages derived from hermaphrodite plants. For dioecy to evolve from hermaphroditism, two mutations are needed, a male-sterility (usually the first to occur) and a female-sterility mutation. These loci had to be linked at one chromosome pair (the sex chromosomes) for the stability of the sexes. Later, selection for alleles advantageous to males and disadvantageous to females is hypothesized to bring about further genetic differences between the X and Y chromosomes and sometimes suppression of recombination between them in further regions [8]. In discussing sex determination mechanisms and specific features of sex chromosome evolution (especially degeneration of non-recombining regions of Y or W), one should bear in mind the variety of sex-determining mechanisms (genetic, environmental, hormonal, and epigenetic), sex determination genes and pathways. Further, ageing and degeneration of Y (or W) sex chromosomes make generalizations difficult [9].

The early steps in Y-chromosome evolution have been revealed through experimental interspecific hybrids created between dioecious and non-dioecious species [2]. In the genus *Silene*, hybrids between *S. latifolia* and the closely related *S. viscosa* have given insights into the dominance of Y-linked alleles [10]. In these hybrids, there is only one sex chromosome (X) inherited from the *S. latifolia* seed parent. Its counterpart (an autosome) originates from the *S. viscosa* pollen donor. Despite the absence of the Y chromosome, the hybrid plants should form bisexual flowers. Indeed, in these hybrids, anthers developed far beyond the early bilobal stage characteristic of XX *S. latifolia* female plants. The *S. viscosa* genome can thus replace the Y-linked sex determination gene(s) whose absence, or lack of function, in females abolishes early stamen development.

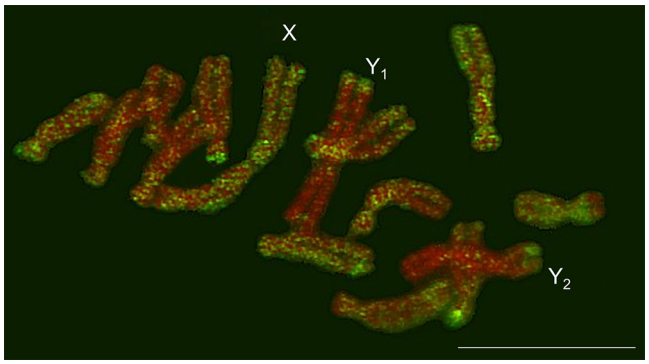

Fig. 1. Immunostaining of male mitotic metaphase chromosomes of *R. acetosa*. Chromosomes were mild denaturated with formaldehyde and stained with anti-acetyl-lysine5-H4 histone antibody (green) and counterstained with DAPI (red). The sex chromosomes are indicated. The bar indicates 10 μm.

3. Case studies: recent advances through genomic studies

Here we provide some recent genomic data on the most commonly studied dioecious species (Table 1). The following section is focused on the classical model of dioecy, *S. latifolia*.

Various types of reproductive systems occur in *Rumex*: hermaphroditism, polygamy, gynodioecy, monoecy and dioecy. Phylogenetic analysis of ITS rDNA sequences suggest that dioecy appeared in *Rumex* between 15–16 million years ago [11]. Two different sex-chromosomal systems and sex-determining mechanisms have been described in dioecious *Rumex* species: XX/XY with an active Y chromosome (e.g., *R. acetosella*) and XX/XY_1Y_2 with sex determination based on the X/A ratio (e.g., *R. acetosa*). There is one exceptional species, *R. hastatulus*, which has two chromosomal "races": the Texas race possesses an XX/XY system, while the North Carolina race has an XX/XY_1Y_2 system. In this species, the X/A ratio controls sex determination, but the presence of the Y chromosome is necessary for male fertility [12]. The two Y chromosomes of *R. acetosa* are large, full of repetitive satellites, and cytologically heterochromatic [13,14]. Their epigenetic analysis reveals that they are depleted in acetylated H4 histones, which is a clear marker of heterochromatin (Fig. 1). In somatic cells of some male tissues, these Y chromosomes form heterochromatic interphase bodies that are typically located in the nuclear periphery [15]. *R. acetosa* is an ideal subject for cytogenetic studies: its genome is huge (C ~ 7.0 Gb), and it is divided into seven pairs of acrocentric autosomes and larger metacentric sex chromosomes (XX in females and XY_1Y_2 in males). In *R. acetosa*, similar repetitive sequences in both Y chromosomes suggest that they might originate from one Y chromosome that underwent centromere fission and gave rise to a pair of metacentric chromosomes possessing identical chromosomal arms (isochromosomes). These isochromosomes have been subsequently modified by deletions. One view suggests evolution of the $XX–XY_1Y_2$ system through autosomal translocation to the original X chromosome. A phylogenetic study indicates that all dioecious *Rumex* species evolved from a common hermaphroditic ancestor [11]. The switch from a sex-determining mechanism based on the active role of the Y chromosome to a mechanism based on the X/A ratio occurred at least twice [16]. The dynamics of microsatellite expansion vary between closely related *Rumex* species [17] and the abundance of microsatellites within the individual genomes differs with higher frequency in the neighbourhood of transposable elements. This fact suggests that microsatellites are probably targets for transposon insertions and that microsatellite expansion is an

Table 1
List of angiosperm dioecious species, and their basic characteristics, as described in the text.

Family	Species	Sex determination	Sex chromosomes	Notes and references
Caryophyllaceae	*Silene latifolia*, white campion	Dominant Y, heterogametic males XY	Heteromorphic	First described dominant Y sex determination, [2,44]
	S. diclinis	Dominant Y, heterogametic males XXneoY1Y2	Heteromorphic	[45]
	S. colpophylla	Heterogametic males XY	Homomorphic	[46]
	S. otites, Spanish catchfly	Heterogametic females ZW	Homomorphic	[47]
Polygonaceae	*Rumex acetosa*, common sorrel	X/A ratio, heterogametic males XY1Y2	Heteromorphic	First described sex chromo-somes in plants, [11,48]
	R. hastatulus, heartwing dock	X/A ratio, heterogametic males XY or XY1Y2	Heteromorphic	[19]
Caricaceae	*Carica papaya*, papaya tree	Dominant Y, heterogametic males XY	Homomorphic	Draft genome sequence, [4]
Cannabinaceae	*Cannabis sativa*, hemp	X/A ratio, heterogametic males XY	Heteromorphic	[27,28]
	Humulus lupulus, hop	X/A ratio, heterogametic males XY	Heteromorphic	[26]
Cucurbitaceae	*Bryonia dioica*, white bryony	Dominant Y, heterogametic males XY	Homomorphic	First genetic evidence of dioecy, [29]
	Coccinia grandis, ivy gourd	Dominant Y, heterogametic males XY	Heteromorphic	Large Y chromosome, [30]
Vitaceae	*Vitis vinifera*, grapevine	Dominant Y, heterogametic males XY	Homomorphic	Draft genome sequence, [31,32]
Salicaceae	*Populus trichocarpa*, black cottonwood	Heterogametic XY males or ZW females?	Homomorphic	Draft genome sequence, [34–36]
Euphorbiaceae	*Mercurialis annua*, annual mercury	Dominant Y, heterogametic males XY	Homomorphic	Association of ploidy levels and sex expression, [37,38]
Asparagaceae	*Asparagus officinalis*, asparagus	Dominant Y, heterogametic males XY	Homomorphic	[39,40]
Ebenaceae	*Diospyros lotus*, Caucasian persimmon	Dominant Y, heterogametic males XY	Homomorphic	First sex-determining candi-date gene in plants, [41]

early event that shapes Y chromosome evolution with the accumulation of transposons and chromosome shrinkage occurring later. Comprehensive analysis of male and female genomic DNA datasets has shown not only the accumulation, but also the depletion of some repetitive DNA within non-recombining regions of the Y chromosomes (Fig. 2a, c, and e) [18]. *R. hastatulus* is a species with two distinct systems of sex chromosomes—the neo-Y sex chromosome system (XX/XY_1Y_2) was derived from an ancestral XX/XY system. SNPs inheritance from parental to F1 generation (X alleles transmitted from fathers to daughters and Y alleles transmitted from fathers to sons) has been recently analyzed. Segregation-based experiments using RNA-Seq have identified hundreds of sex linked genes in *R. hastatulus*. This study describes two types of Y-linked genes. First, genes shared by both *R. hastatulus* races are old sex-linked genes. This set includes hemizygous genes suggesting that a gene loss has occurred as a consequence of Y chromosome degeneration [19]. Seventy percent of sex-linked genes from the XY system have been calculated to be present in the XY_1Y_2 system, and 40% of genes in the XY_1Y_2 system are shared with the XY system. Detailed RNA-Seq-based analysis reveals that 488 "old" sex-linked genes are shared between the XY and XY_1Y_2 races, while 607 "young" genes are unique to the XY_1Y_2 system. This further suggests that the XY_1Y_2 system has acquired many new sex-linked genes since the fusion event. The younger Y linked genes have a significantly reduced pattern of genetic degeneration and this confirms the evolution of XX/XY_1Y_2 via fusion of the X chromosome and a former autosome about 600,000 years ago [20]. How much expression variation of $X–Y_1$ or $X–Y_2$ allelic pairs correlates with the degeneration (loss of function) of individual Y_1Y_2 alleles needs to be confirmed by further proteomic and reverse genetic (functional) analyses.

The papaya model has been recently reviewed [4]. Although it does not belong to the two dozen known species with clearly heteromorphic sex chromosomes, it is a leading dioecious model with structurally differentiated sex chromosomes [3]. Papaya is a favourite model because of its small nuclear genome (C ~ 0.7 Gb) and its importance as a crop. Since males of papaya are heterogametic and females are homogametic, sex determination can be classified as an XY type, with a dominant Y chromosome. A large amount of DNA polymorphism near the sex locus initially led to the discovery of the Y chromosome in papaya. There is a small 8.1 Mb region, called the MSY (male-specific region Y), which harbours the primary sex-determining genes. It has been proposed that recombination suppression in the sex-determining region of the Y chromosome is a result of proximity of the centromere to this region [21]. As shown in other dioecious species with heteromorphic sex chromosomes, even in papaya with evolutionarily young and homomorphic sex chromosomes, organelle DNA accumulates in the sex chromosomes in the absence of recombination [22]. Sex chromosomes in papaya contain 21 specific repeats that are absent on autosomes and with no homology to other plant sequences suggesting a recent origin [23]. Sequencing of sex chromosomes in this species reveals two large-scale inversions along with numerous chromosomal rearrangements. Moreover, sequencing data confirm an accumulation of transposable elements in the initial stages of sex-chromosome evolution after recombination arrest, leading to the physical expansion of the sex-determining regions [24]. Papaya diverged from its close relative, *Vasconcellea parviflora*, about 27 million years ago. Both species share a homologous pair of X/Y chromosomes. Surprisingly, the extent of non-recombining region is much larger in *S. latifolia* with younger sex chromosomes [25]. *S. latifolia*, according to cytogenetic data, also accumulated more transposons and other satellites than papaya. It is a question, whether higher repetitive DNA content is merely a consequence of the large non-recombining region in *S. latifolia* or whether other processes play a role in the spread of repeats in evolving sex chromosomes. On the other hand, heterochromatization (cytological data) of the Y chromosome has been described only in papaya,

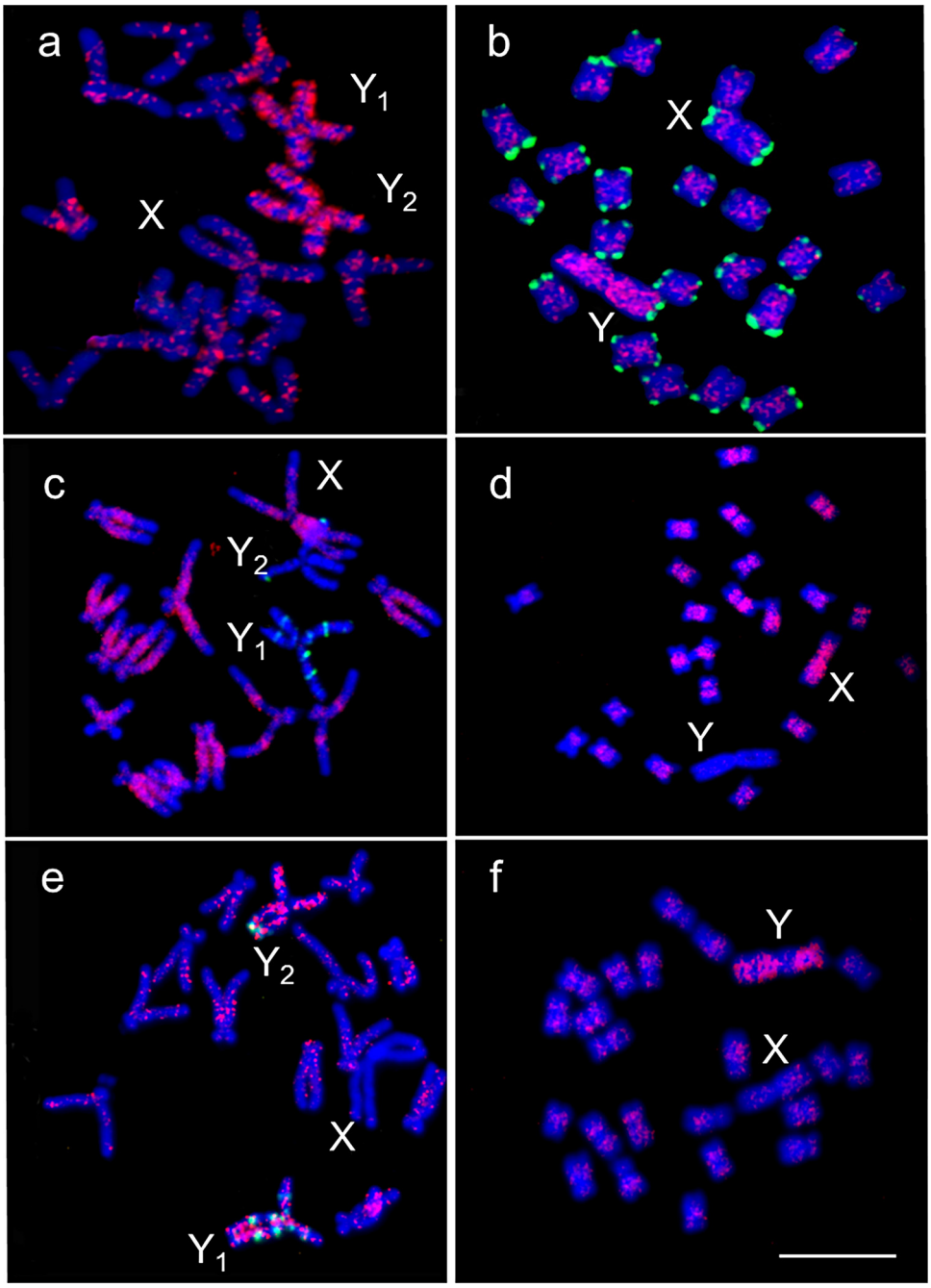

Fig. 2. FISH mapping of microsatellites and retroelements on *R. acetosa* (a, c, e) and *S. latifolia* (b, d, f) male metaphase chromosomes. The hybridization signals are in red, the chromosomes are counterstained with DAPI (blue). The sex chromosomes are indicated. (a) *R. acetosa* $(TA)_n$ microsatellite, (b) *S. latifolia* $(CAA)_n$ microsatellite, (c) *R. acetosa Maximus/SIRE* retroelement, (d) *S. latifolia Athila* CL3 retroelement, (e) *R. acetosa CRM* retroelement, (f) *S. latifolia Athila* CL10 retroelement. The bar indicates 10 μm and is shared by all figures.

not in *S. latifolia*. This may suggest that epigenetic processes play a role even in early stages of sex chromosome evolution and repetitive content leading to large Y chromosomes in some dioecious species reflects only the extent of the non-recombining region. The correlation between the non-recombining regions of the Y/W chromosomes (relative proportion to the rest of the chromosome) and the content of repetitive elements needs to be confirmed by further experiments. It has been suggested that the relation between the degree of heteromorphism and the age of sex chromosomes is just a myth [9].

In spite of its agronomical importance, relatively little is known about the sex determination mechanism in *Humulus*. The genus *Humulus* comprises only three species: *H. lupulus*, *H. japonicus*, and *H. yunnanensis*, the most important crop being the first of these. Males of *H. lupulus* are heterogametic (with either a simple XY or multiple sex chromosome systems), whereas females are homogametic ($2n = 18 + XX$). It has been suggested that sex expression is determined by the X/A ratio. The obvious sex-related differences are found in the plant at the stage of inflorescence initiation [26]. Cytogenetic studies on *Humulus* reveal that the sex chromosomes have evolved from a pair of autosomes via ancient translocation and/or inversion.

Cannabis sativa is another of the few dioecious species possessing heteromorphic sex chromosomes, albeit a difference in size

between the X and Y chromosome being rather small (reviewed in [27]). Males are heterogametic (XY), and females homogametic (XX). The sex-determining system is of the *Drosophila* type with the ratio of Xs to autosomes being decisive. In contrast to other dioecious models with heteromorphic sex chromosomes (e.g., *S. latifolia* and *R. acetosa*), the Y-chromosome of *C. sativa* probably does not contain the genes necessary for male fertility. Several genes that are involved in sex determination and/or sexual dimorphism were identified using cDNA AFLP [28].

Most *Cucurbitaceae* species have unisexual flowers. Of the 800 species in this family, 460 are monoecious, and 340 are dioecious. The best studied genus containing dioecious species is *Bryonia*. Genetic crosses between the dioecious *B. dioica* and the monoecious *B. alba* provided the first clear evidence for Mendelian inheritance of sexual phenotypes (dioecy) and made *B. dioica* the first organism for which the XY sex-determination was experimentally proved [29]. The *Coccinia grandis* Y chromosome appears to be completely heterochromatinised with twice the size of any of the other chromosomes [30].

Although *Vitis vinifera* cultivars form bisexual flowers, almost all wild *Vitis* relatives are dioecious. In the genus *Vitis*, sex determination is putatively controlled by one major locus with three alleles: male *M*, hermaphrodite *H*, and female *F*, with an allelic dominance *M>H>F*. Hermaphrodite alleles appear to derive from male alleles of wild grapevines. It has been shown that sex is controlled by a single genomic region. Combination of a high-resolution genetic map and markers derived from the BAC library screening points to a physical map of a 143 kb sex-specific region spanning less than 1% of the sex chromosome [31]. Although sex chromosomes in *V. vinifera* resemble a very early evolutionary stage, dioecy is at least as old as the separation of the *Vitis* and *Muscadinia* subgenera, about 18 million years. It has been proposed that this trait makes this species an ideal model to address the question why some dioecious plants rapidly developed specific sex-chromosomes, while others did not [32].

Members of the genus *Populus* (poplars, cottonwoods and aspens), along with *Salix* species that present sister genera, are composed exclusively of dioecious species. A single ancient origin of dioecy around 65 million years ago in this genus has been proposed [33]. A peritelomeric region on chromosome 19 was identified as a non-recombining region, with ZZ/ZW sex determination, and <5% of the maternal W chromosome (706 kb) subjected to recombination suppression [34]. This observation is contradicted by a new study indicating that the *TOZ19* gene is present in males and absent in females in aspens [35]. A more extensive study has now confirmed the XX/XY system in poplar, suggesting that assembly problems are sufficient to explain the incorrect distribution of the sex-associated markers found in previous studies [36]. This study further indicates that divergence of *P. trichocarpa* (poplar) and *P. tremuloides* (aspen) predates divergence times of X and Y haplotype sequences (6–7 million years). Importantly, this observation is not consistent with the hypothesis of a single origin of dioecy in this group. The labile nature of sex-determining regions is well documented in other species and it now seems likely that there has been at least one "turnover" in sex-determination mechanism since the divergence of poplars and aspens.

The genus *Mercurialis* is mostly dioecious, with monoecy having evolved from dioecy at least twice. In a clade of annual species, dioecy has given way to monoecy in loose association with polyploidy: thus, tetraploid populations of *M. annua* are monoecious, and hexaploid populations are often androdioecious, with the frequent co-occurrence of males and hermaphrodites [37]. A closely related species, *M. canariensis*, is tetraploid but fully dioecious, and some Moroccan populations of hexaploid *M. annua* have become almost fully dioecious, so that dioecy was probably lost at a couple of times and was subsequently regained. The sex determination of *M. annua* has been recently re-evaluated: rather than having a 3-locus system, sex is determined at a single locus with XY determination [38].

In *Asparagus officinalis*, an M-locus controlling sexual dimorphism was identified on chromosome 5. Although a partial physical map of this region was constructed based on BAC library screening with M-locus specific DNA markers, no candidate gene for sex determination has yet been identified [39]. Comparison of male and female genomes revealed that most of the repeat groups had similar abundance in males and females. This suggests that asparagus sex chromosomes are in an early stage of evolution, a conclusion supported by the fact that YY plants are viable [40].

Diospyros lotus (Caucasian persimmon) is a dioecious plant with dominant male (XY) sex determination. The *D. lotus* was known to the ancient Greeks as "the fruit of the gods"(*Dios pyros*). Dioecy is very ancient in the genus *Diospyros* and dates back to the origin of *Ebenacea* family (35–65 million years ago). Massive DNA sequencing (32 females and 25 males) revealed a male-specific region with a total length of ~1 Mb. Sixty-two genes differentially expressed in males and females according to RNA-Seq analysis, seven genes were MSY-linked. One gene with male-specific expression in developing buds named *OGI* (class I homeodomain transcription factor) displays male-specific conservation among *Diospyros* species. *OGI* encodes a small RNA-regulating transcription of *MeGI*, an autosomally linked gene with a female-biased bud and flower-specific expression [41]. In females, *MeGI* shows elevated expression, followed by repression of pollen formation. In males, *OGI* prevents the accumulation of *MeGI*. Detailed transcriptional, functional, and evolutionary analyses of *OGI-MeGI* complex makes *D. lotus* the most advanced model in terms of our understanding sex determination. The question remains whether a single locus is sufficient for sex-determination resembling SRY in humans or an additional Y-linked locus has to be identified, according to the two-mutation model for the evolution of dioecy [7].

There are many other dioecious models, but knowledge of their structure and evolution remains limited. *Actinidia chinensis* represents an XX/XY system with a sex-determining region located in a recombination-suppressed subtelomeric locus within the linkage group 17 and showing characteristics of early stages of sex chromosome evolution [42]. *Phoenix dactylifera* has a smaller Y than X chromosome (which appears to be exceptional in plants) and probably possesses one of the most ancient sex chromosomes in flowering plants so far studied. Its separate male flowers were identified in fossil sediments dating from the middle Eocene period [43]. Other emerging dioecious species include mainly *Acnida tamariscina* (XY), *Datisca cannabiba* (ZW), *Dioscorea tokoro* (XY), *Viscum fischeri* (XY, multiple sex chromosomes), *Fragaria virginiana* (ZW), and others (reviewed in [5]).

4. Silene as a model system

The genus *Silene* includes about 700 species, most of which are hermaphroditic or gynodioecious. However, there are two groups of dioecious species that have proved invaluable for the study of sex-determining mechanisms. These dioecious species belong to two different *Silene* groups: section Melandrium (e.g., *S. latifolia*, *S. dioica*, *S. diclinis*) and subsection Otites (e.g., *S. otites*, *S. colpophylla*). The former possesses large heteromorphic sex chromosomes. Their X-chromosomes have been mapped with reasonable resolution: except for a recent rearrangement in *S. dioica*, the gene order appears to be the same in all three species. This points to a monophyletic origin of this sex chromosome and its relative short evolution (less than 10 million years) [49]. Analysis of amino acid replacements (vs. synonymous substitutions) has shown that Y-linked alleles appear to be largely functional in all three species and indications of degenerative processes are negligible.

S. latifolia is one of the best studied plants possessing heteromorphic sex chromosomes. In contrast to the human Y chromosome, which is relatively small, the Y chromosome in *S. latifolia* is the largest chromosome in the entire genome. Chromosomes of *S. latifolia* (formerly *Melandrium album*) were described nearly a century ago, in 1923. The nuclear genome of *S. latifolia* is arranged in 11 autosomal pairs and one pair of sex chromosomes. Its *C* value is rather high (~2.8 Gb). The X chromosome is approximately 1.4 times larger than the average size of autosomes, being 1.4 times smaller than the Y chromosome [50].

The sex chromosomes of the dioecious species from the subsection Otites are homomorphic. A comparative mapping study of *S. colpophylla*, which is also a male heterogametic species like *S. latifolia*, indicates that its sex chromosomes have evolved from a different pair of autosomes that those of *S. latifolia* [46]. The results of this study indicate that the sex-determination system in *S. colpophylla* evolved independently from that of *S. latifolia*. Other analyses have been done on *S. otites*, a close relative of *S. colpophylla*. These have revealed that its sex-determining system is based on female heterogamety, which is unique among the *Silene* species so far studied [47]. An analysis of ancestral states indicates that the most recent common ancestor of *S. otites* and *S. colpophylla* was also dioecious and a switch from an XX/XY sex determination to a ZZ/ZW system (or vice versa) is implied in the subsection Otites. *Silene* species (together with *Populus*) therefore possess two different types of heterogamety within one plant genus.

In principle, there are two options for identifying sex-linked sequences. Indirect methods are based on the generation of DNA sequences used as genetic markers that detect sex-linkage (e.g., RAPD, AFLP). This approach is frequently accompanied by the establishment of genetic crosses that support observed data. Recently, high-throughput sequencing methods (RNA-Seq) have been employed to increase number of known ESTs (genes) in *S. latifolia*. The combination of segregation analysis of individual SNPs with massive sequencing of transcripts was independently used to identify sex-linked genes [51–53]. Hundreds to thousands of genes (sex-linked contigs) have been described as localized on *S. latifolia* sex chromosomes in these studies along with expression levels of identified genes. The question arises, how many genes are missing in the list due to, e.g., low levels of expression in examined tissues. Another important issue concerns quantification of expression (transcription) of individual genes to study Y degeneration and dosage compensation. Since RNA-Seq data provide only a very rough view of what is transcribed without any direct connection to translation (*de facto* expression), new methods as, e.g., Ribo-Seq are needed to confirm RNA-Seq data.

To decrease the complexity of the studied material (due to the large genomes) and get directly focused on the chromosome of interest, direct methods are frequently used. One of the main advantages of direct methods is rapid identification of sex-specific sequences, typically starting with separation of individual chromosomes. Historically, manual microdissection was followed by laser microdissection and flow sorting [54]. The manual dissection of sex chromosomes was successfully applied experiments leading to the isolation of the first active gene from a plant Y chromosome [55].

Functional studies of Y-linked sequences have largely led to analyses of Y deletion mutants. Large-scale deletions (disruptions) of the Y chromosome were introduced by means of either X-ray or gamma-irradiation [56,57] and by heavy-ion beam irradiation in a ring cyclotron [58]. With increasing knowledge of sex-linked genes, analysis of individual deletion mutants should assist in identifying candidate genes in flower development. Since anonymous DNA markers have been replaced by ESTs (expressed sequence tags), analysis of deletion mutants of known phenotypes is beginning to be a feasible technique for mapping regions and subsequently genes responsible for sex expression (male promoting, male fertility, female suppression). Moreover, local deletions are currently generated through targeted genome manipulation using, e.g., TALE and/or CRISPR/Cas9 technologies and the number of candidate genes "coding for" individual phenotypes can be significantly decreased.

The structural features of the sex chromosomes in *S. latifolia* have been studied over many years using a combination of various cytogenetic and genomic approaches [2]. The first cytogenetic map of the *S. latifolia* genome was constructed using FISH analysis of selected low copy, sex-linked BAC clones [50]. Although the *S. latifolia* Y chromosome is not heterochromatinised, it has accumulated a significant number of tandemly arranged DNA repeats. It has been demonstrated that many microsatellites have accumulated on the Y chromosome (with a greater abundance on the q arm, which stopped recombination relatively recently compared to the p arm). This suggests that microsatellite spread predates other structural changes that occurred during the Y chromosome evolution (Fig. 2b) [59].

A global survey of all the major types of transposable elements in *S. latifolia* revealed that most of the Copia elements had accumulated on the Y chromosome. Surprisingly, one type of Gypsy elements, which is similar to Ogre elements known from legumes, was almost absent on the Y chromosome (with the exception of the pseudoautosomal region), but otherwise uniformly distributed in all chromosomes [60]. It was suggested that the absence of one Ogre family on the Y chromosome may be caused by 24-nucleotide small RNA-mediated silencing leading to exclusively female-specific spreading [61]. The spread of Ogre elements has been confirmed to be relatively recent [62]. Ogre retrotransposons appeared before sex chromosomes evolved but were mobilized soon after the formation of the Y chromosome. This result is supported by identical chromosomal distribution of this element in *S. latifolia* close relatives possessing sex chromosomes in the *Melandrium* section. These data suggest that the appearance of sex chromosomes is quickly followed by complex genome response with transposons playing a dominant role. Identification of another retroelement underrepresented on the Y chromosome in *S. latifolia* provides supporting data for rather complex process shaping genome structure after sex chromosome establishment (Fig. 2d and f) [63].

S. latifolia represents an intermediate step between early stages of sex chromosome establishment and genetic degeneration accompanied by either strong heterochromatinisation or loss of non-functional DNA. Analysis of genomic libraries constructed from individual sex chromosomes showed that there is a preferential accumulation of chloroplast sequences on the Y chromosome [64]. A subsequent comprehensive study revealed that the majority of the chloroplast DNA, corresponding to the single copy regions, was localized near the centromere of the Y chromosome, while the inverted repeat region was also present in other loci (Fig. 3) [18]. It has been demonstrated that repetitive DNA also accumulate in the vicinity and within the Y alleles of sex-linked genes [51]. Although accumulation of various transposons within the Y chromosome has been confirmed in many other plant species (e.g., *Rumex*, papaya, and *Asparagus*) accurate quantification is needed. Due to the size differences between the X and Y chromosomes in *S. latifolia*, chromosome sorting was recently employed to begin sequencing of individual sex chromosomes. The combination of a draft sequence of *S. latifolia* sex chromosomes and detailed analysis of transposon insertions (dating using LTR divergence) will provide comprehensive data on the extent and timescale of transposon participation in sex chromosome formation.

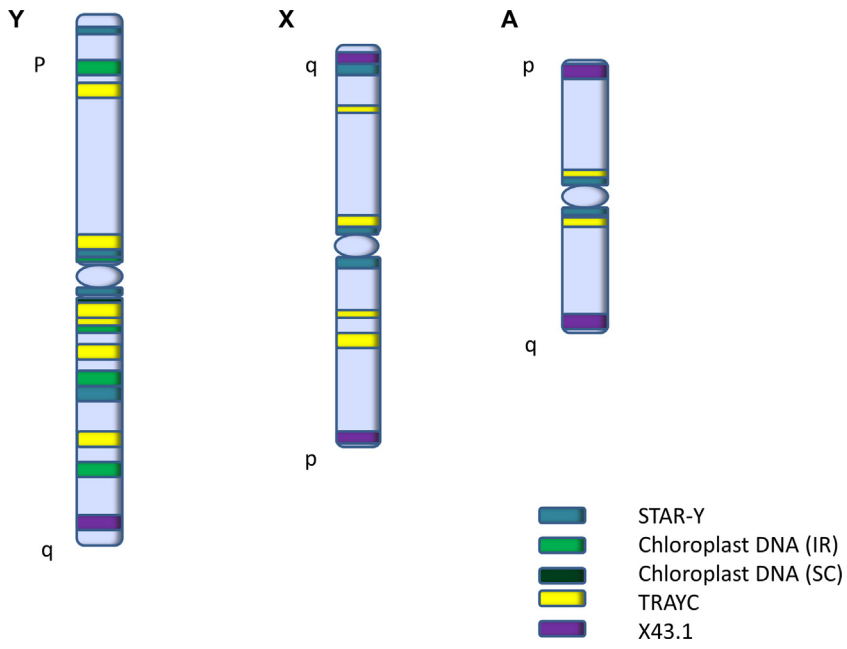

Fig. 3. Frequency and distribution of various DNA repeats in the sex chromosomes and an average autosome of the model dioecious species *S. latifolia*. STAR-Y, TRAYC, and X43.1 are tandem repeats isolated from the *S. latifolia* nuclear genome, and chloroplast DNA (IR and SC) are sequences from the inverted repeat and the single copy region of chloroplast genome, respectively. Compiled from [77,78].

5. Y-chromosome degeneration and dosage compensation?

Y-linked *S. latifolia* genes show indications of genetic degeneration and are probably not fully functional [51–53,65]. Conversely, due to the differentiation of the male gametophyte (haploid pollen tube, either AX or AY) and its required gene expression, many deleterious Y-linked mutations have probably been removed by purifying selection [52]. The necessary biological functions of the Y-chromosome in *S. latifolia* could be summarized as follows:

[*] The gametophyte (i.e., male pollen tube and female embryo sac) is a phase of the life-cycle unique to plants. It is a haploid "checkpoint" in development and the only period where the (male) AY-individual can survive (as pollen grain and pollen tube) without the X. The Y chromosome carries genes that are important for male gametophyte development, and its proper function suggests that a large part of the Y-linked genes cannot be excessively degenerated.

[*] Many dioecious species show female-biased sex ratios (up to 70% female individuals). Most sex-biased species are probably the result of differential mortality in adult individuals (e.g., because of different demands on their resource status by sexual reproduction). In *S. latifolia*, and perhaps in other species, an observed female bias reflects the different viability of AAXX vs. AAXY embryos and the function of corresponding triploid endosperm tissues. The endosperm consists of AAXX/AX or AAXX/AY genomes in females and males, respectively. This may indicate that the Y chromosome is not fully competent to support the nutritional functions of the endosperm (and the embryo). In reality, it cannot be excluded that the female bias is a result of a sex-linked meiotic drive system, or a lower viability of AY-pollen tubes (Fig. 4) [66].

[*] Genomic imprinting is a well-described epigenetic phenomenon, in flowering plants documented in triploid endosperm. Imprinting is responsible for the proper function of this feeding tissue and its malfunction leads to the abortion of both endosperm and embryo. As depicted in Fig. 4, the *S. latifolia* endosperm consists of maternal (AAXX) and paternal (AX or AY) contributions. Obviously, both genetic and epigenetic modifications of the Y chromosome can influence its function in the endosperm and may be responsible for the lower frequency of male individuals in populations (the female bias).

[*] In studying the behaviour of the Y chromosome in the female gametophyte (the embryo sac), we isolated two types of *S. latifolia* hermaphrodites: one with an aberrant Y (a genetic deletion leading to the loss of its ability to pass on to next generations) and the other with an epigenetically modified Y (experimentally hypomethylated by 5-azacytidine). In the crossed plants, we checked whether the epigenetically modified Y could pass through the female germinal pathway. However, in the progeny, only female plants occurred. Hence, the Y chromosome was insufficient for the embryo sac development. We conclude that embryo sacs with the AY constitution are not viable and that the female gametophyte cannot survive without the X chromosome [67].

[*] Androgenesis is a powerful tool for enabling the formation of haploid sporophytes from in vitro cultivated pollen grains. The cultivation of *S. latifolia* anthers yielded only female haploid and dihaploid plants (AX and AAXX, resp.) rather than males (AY and AAYY). A detailed molecular analysis of in vitro plantlets revealed no Y-markers. Hence, it is clear that abortion of putative AY embryoids occurred in very early development. Taken together, complete sporophyte development strictly requires at least one X chromosome. The Y chromosome is not sufficient, suggesting that it has lost some important genes [68,69].

[*] Sex determination in *S. latifolia* is under genetic control and realized by the expression of at least three (groups of) Y-linked genes [2]. However, their expression appears to be regulated by epigenetic mechanisms. An epigenetic activation of pistil development by a 5-azacytidine treatment in male plants possessing the AAXY karyotype led to hermaphrodite flowers. This trait was inherited only from pollen donor (holandric heritable) [70]. The data indicate that the arrest of pistil formation in AAXY plants was overcome by inactivation of a Y-linked gynoecium suppressor. This demonstrates that the basic (original) floral phenotype of *S. latifolia* is bisexual and this can be achieved by an artificially hypomethylated AAXY genotype. Conversely, the epigenetic activation of AAXX plants did not lead to any change in female floral phenotype. This could indicate that the AAXX plants do not contain all the genetic information necessary for the basic bisexual phenotype, which may be connected with an evolutionary loss of corresponding proto-sex-chromosome genes now linked to the Y-chromosome.

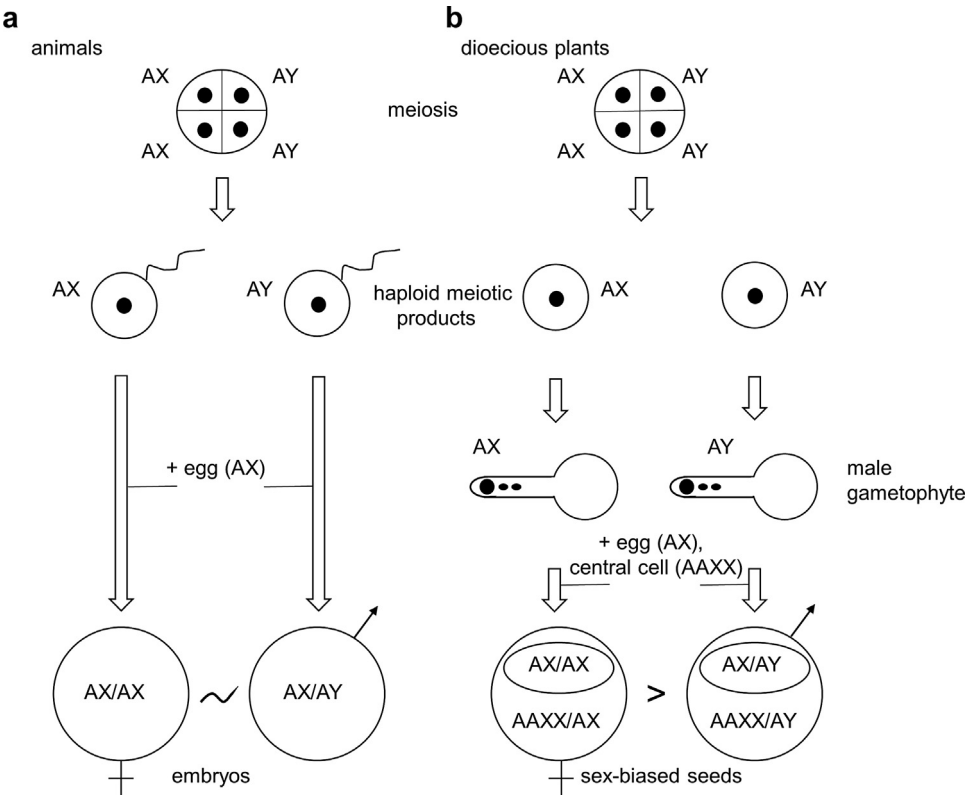

Fig. 4. Comparison of animal (a) and dioecious plant (b) male development in species possessing the X/Y sex chromosome system. Meiosis produces haploid cells (AX and AY), which are the gametes (sperm) in animals, but are only spores (pollen) in plants. Formation of the male gametophyte (pollen tubes AX and AY) requires massive gene expression, thus preventing a large degeneration of the Y chromosome. Fertilization is simple in animals yielding female and male (diploid) individuals in nearly the same frequency, while in plants there is a double fertilization yielding diploid embryos with triploid endosperm. In many dioecious plants there is female bias that might sometimes be a result of a partial degeneration of the Y chromosome, which is not fully functional in the AY pollen tubes or the AAXX/AY endosperm. "A" stands for one set of autosomes.

[*] The Y chromosome in *S. latifolia* is invaded by accumulation of many types of DNA sequence repeats (satellites, microsatellites, plastid sequences, transposons [60]). Chiefly as a result of these sequences, the Y chromosome in *S. latifolia* is the largest in the nuclear genome. However, there is yet no direct evidence for a causal association between accumulation of DNA repeats and genetic degeneration. Moreover, the Y chromosome has a global euchromatic character, like autosomes [71], and several dozen X-linked genes have been identified that still have functional Y-alleles [52,53,65]. Clearly, substantial degeneration of the Y chromosome with global X-dosage compensation has thus not occurred in *S. latifolia*. In any event, the numbers of X-linked transcriptome reads seem to be equalized in AAXX and AAXY individuals: the transcript levels in males appear to be increased, which indicates a positive dosage compensation (as known in *Drosophila*) [65]. Some cytogenetic experiments have shown DNA hypermethylation and late replication in one of the two Xs in female somatic cells, which could indicate a negative type of dosage compensation (the mammalian type) [72]. Other data show that Y-linked genes evolve faster at the protein level than their X-homologues and that their expression is lower [51]. Dosage compensation could certainly evolve, not only at a global level of the whole X chromosome, but also as a regulatory mechanism operating at the level of an X-linked locus lacking its Y-partner. Nevertheless, there is still no clear evidence for this idea: the gene *SlWUS1* (a homolog of the *Arabidopsis* gene *WUSCHEL*) is X-linked and has no Y-linked allele, and yet it is not dosage compensated [73]. The question of dosage compensation in *S. latifolia* will be resolved when more X-linked genes with functional identification are available for careful quantitative analysis.

[*] An important question is whether the sex chromosomes in *S. latifolia* actively influence somatic development as early as at pre-flowering stages, with differential expression in males and females. So far, only a few quantitative markers for sexual dimorphism in dioecious plants have been found in dioecious plants in general [74]. In *S. latifolia*, a large functional screening of EST sequences led to the unambiguous conclusion that some genes are sex-specifically activated (either male or female) at early stages of their vegetative development [75].

[*] Many species of the genus *Silene* are interfertile, despite the sex differences [10]. For instance, crosses between the dioecious species *S. latifolia* (white campion) and *S. dioica* (red campion) are especially common in natural populations. The Y chromosome of *S. latifolia* is fully functional in these interspecific hybrid plants, which suggests that its evolutionary divergence is not very large (these two *Silene* species separated about one million years ago [47,49]).

6. Current research strategies

Although the advent of sequencing techniques has shed light on many aspects of sex chromosome structure and evolution in plants, there is still limited information about the various biological consequences for dioecious plants.

First, the role of individual genes localized on sex chromosomes is not commonly confirmed by reverse genetics. Due to the advent of targeted genome manipulation, there are a variety of methods that could be optimized and employed to directly study the role of candidate genes in plant sex development.

Second, the Y chromosome has a low-complexity genomic region with large DNA stretches composed of tandemly arranged sequences. These regions present a complicated template for de novo assembly. These difficulties can be partially overcome by two genomics approaches: the construction and physical assembly

of deep coverage BAC libraries for individual species in parallel with direct sequencing experiments; and flow sorting of sex chromosomes, with subsequent sequencing of a unique chromosomal template by various next generation sequencing platforms. Similar strategies have been successfully employed in sequencing projects focused on plants with huge genomes such as wheat [76]. Inaccurate data processing and/or assembly of sequenced genomes can lead to very erroneous conclusions, as documented in poplar.

Third, although the amount of sequencing data (both genomic and transcriptomic) is progressively increasing, there is almost no proteomic confirmation of the results obtained. There is as yet no evidence that the degeneration revealed by lower expression of individual Y alleles affects gene functionality at the protein level.

Fourth, more information about sex-linked genes from different dioecious models is needed along with detailed genetic and physical maps. Only identification of sex-determining genes in different species can precisely date the age of sex chromosomes systems.

Fifth, it should be tested whether dosage compensation exists in different dioecious species at various stages of sex chromosome evolution. If so, the question arises as to whether individual genes or loci are affected (compensated), or whether there is large-scale dosage compensation within X (Z) chromosomes.

Sixth, transposable elements can play both direct (genetic) and indirect (epigenetic) roles in the Y-chromosome degeneration. Transposon insertion (gene mutation/disruption) has mostly negative consequences on gene expression. Accumulation of repetitive sequences can also trigger local/global rearrangements. On the other hand, introduction of transposons in the vicinity of gene(s) can affect gene expression resulting in new patterns of transcription regulation. Indirect effects show mainly epigenetic changes. Transposon insertion is frequently regulated by the genome itself (e.g., using RNAi machinery), resulting in the DNA methylation of target sequences. Methylation spreads into regions surrounding transposon and locally affects the expression of linked genes. Moreover, large-scale responses to repetitive DNA accumulation results in changes in chromatin status (heterochromatisation). It is still an open question as to whether the accumulation of transposons is the cause or consequence of sex chromosome degeneration, and to what extent it contributes to the evolution of sex chromosomes generally.

Although our understanding of plant sex chromosomes remains fragmented, rapid developments in sequencing and reverse genetics in combination with standard approaches promise to yield interesting results.

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