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Plant sex chromosome evolution

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Abstract

It is now well established that plants have an important place in studies of sex chromosome evolution because of the repeated independent evolution of separate sexes and sex chromosomes. There has been considerable recent progress in studying plant sex chromosomes. In this review, I focus on how these recent studies have helped clarify or answer several important questions about sex chromosome evolution, and I shall also try to clarify some common misconceptions. I also outline future work that will be needed to make further progress, including testing some important ideas by genetic, molecular, and developmental approaches. Systems with different ages can clearly help show the time course of events during changes from an ancestral co-sexual state (hermaphroditism or monoecy), and I will also explain how different questions can be studied in lineages whose dioecy or sex chromosomes evolved at different times in the past.

Key words: Hermaphrodite, papaya, recombination suppression, *Silene*, transposable element, Y chromosome.

Introduction: the evolution of separate sexes and sex chromosomes

The study of plant sex chromosome involves non-model organisms, and the results have no obvious immediate applied benefits for crops in most countries, apart from the usefulness of genetic markers for sexing plants before flowering, so as to increase the proportion of females (the desired sex for fruit crops such as grapevines, kiwi fruit, date palms, hops, holly trees, strawberries, and *Cannabis sativa*), or of males in asparagus or papaya (where a hermaphroditic form of male that fruits well is often desired). Nevertheless, there is wide interest in understanding sex chromosome evolution, which involves strange phenomena such as selection for suppression of genetic recombination (which is widespread throughout the genomes of most organisms, other than in parts of the sex chromosomes), and the resulting 'genetic degeneration' involving loss of function, or complete loss, of many genes on the non-recombining chromosome; this chromosome is the Y in XY systems, and the W in ZW systems such as that of birds and Lepidoptera, and a few plants. The intrinsic interest in understanding such strange phenomena has led to studies in a diversity of dioecious plants, even though separate sexes are rare among flowering plants, and sex chromosomes are not present in all dioecious plants, though they are common in liverworts (Westergaard, 1958; Renner and Ricklefs, 1995; Ming *et al*., 2011). As will be explained below, plants appear to have sex-determining systems of different ages, whereas the best studied animal systems, in mammals, birds, and insects, are ancient. Thus plants can be used to study the time course of events during sex chromosome evolution.

As will become clear, understanding the evolution of sex chromosomes is impossible without first understanding the evolution of separate sexes, because the genetic changes involved in the latter lead with high probability to the chromosomes involved losing recombination, and it is loss of recombination that leads to the special properties of sex chromosomes. Studies of species that have recently evolved separate sexes are ideal for testing the idea that hermaphrodites are

selected to 'compromise' between male and female functions, an important test case for the existence of trade-offs between different functions. If this idea is correct, then changing from being hermaphrodite to becoming purely female or male ought to trigger a burst of adaptation, as females optimize their female functions, and males optimize their male functions. Testing for such adaptive changes on sex chromosomes, and testing whether they are involved in recombination suppression between Y and X chromosomes, therefore has wide implications.

Sexes and mating types

Although this review is confined to the evolution of separate sexes and sex chromosomes, and will not deal with mating types, a brief mention of the differences between the two is first needed, for clarity, as mating type loci are often referred to as sex-determining regions (Fraser *et al*., 2004) or sex chromosomes (Fraser and Heitman, 2004). One similarity is that these loci control mating functions. A more striking similarity is that loss of recombination may occur in genome regions surrounding mating type loci, for example in green algae (Ferris *et al*., 2002, 2010); it is currently unclear whether loci exist with alleles that benefit one mating type, while other alleles benefit the other (i.e. trade-offs, such as those at genes that interact with the sex-determining region). However, the causes of selection for loss of recombination in some mating type regions, such as some inbreeding or automictic fungi, differ from those thought to operate in sex-determining regions (Hood and Antonovics, 2000; Jacobson, 2005; Menkis *et al*., 2008; Abbate and Hood, 2010; Metin *et al*., 2010; Ellison *et al*., 2011).

Nevertheless, even the fundamental differences between sexes and mating types are not yet fully clear. The long-established criterion for concluding that a species has male and female sexes is based on the species being anisogamous, with large female gametes and small, motile male gametes. However, the haploid individuals in the 'dioecious' species in the brown alga genus *Ectocarpus* produce either many highly motile gametes called zoospores, or fewer zoospores that quickly settle on the substrate and emit pheromone that attracts the motile gametes, which fertilize them (Silberfeld *et al*., 2010). Although the zoospores might be called male and female, there is no size dimorphism, and the differences between the two kinds are distinct from those in most familiar species (the immotile zoospores are capable of asexual reproduction, while the motile ones are not). Phylogenies based on DNA sequences have changed biologists' conclusions about the relationships of many taxa, and brown and red algae are now placed in distinct groups, separate from plants (reviewed in Cock *et al*., 2010). These species might therefore have a form of anisogamy different from other dioecious species. Alternatively, these types might be classified as two mating types that also have some other differences. In this article, I shall restrict myself to species with the familiar gamete size differences (anisogamy) that are widely used to define the two sexes.

Genetic and environmental control of plant sex determination

Even restricting the term 'sex' to mean the gender of individuals, the terminology of plant sex types is still confusing, and some definitions are needed. The term 'unisexuality' applies to several distinct situations observed in plants, covering species with environmental sex determination (e.g. Policansky, 1981; Zimmerman, 1991; Pannell, 1997*a*) and monoecious species, as well as dioecious species. Here, I shall use 'sex-determining gene' only for dioecious species with genetic sex determination. In monoecious species and in environmental sex determination, genes are, of course, involved in the sex-determining developmental pathway, but there are no sex-determining loci, since all individuals are either monoecious, and capable of developing flowers of either sex, or can develop as either sex, depending on the environment experienced in a given flowering season. In contrast, many dioecious species have a genetic polymorphism involving sex-determining genes, or 'primary sex-determining genes', which control whether a plant (or animal) as a whole develops as a male or female (e.g. genes suppressing female functions on a Y chromosome, and loss of function of male fertility factors on the X).

The confusing usage of the term sex determination has led to the idea that candidates for the sex-determining genes of dioecious species might be identifiable from studies of flower development and/or the sex-determining developmental pathway of monoecious species. While it is true that genes in the sex-determining developmental pathway of monoecious species may be involved in dioecious species, the same is true of hermaphroditic species. Indeed genes involved in flower development are often studied as candidate sex-determining genes, and a few have proved to be sex linked (Matsunaga *et al*., 1996, 2003, 2005; Guttman and Charlesworth, 1998; Moore *et al*., 2003; Nishiyama *et al*., 2010). However, mutations at many loci in plant genomes can cause male sterility (Ohnishi, 1985; Klekowski, 1988), and the same is likely for female sterility, since large numbers of genes are expressed during flower development (e.g. Wellmer *et al*., 2006). The males and females in different dioecious species might therefore evolve via sterility mutations at many different loci, restricted only by pleiotropic effects that preclude some loci from being involved in naturally evolved unisexuality. Thus we should not expect different dioecious species to have the same sex-determining genes, so there may be nothing that should be termed 'the plant sex-determining system'. This is unlike the situation in the Eutherian and marsupial mammals (Wallis *et al*., 2007), or in insects, where different species appear to share a common sex determination pathway, even though different taxa have different master sex-determining genes (e.g. Pomiankowski *et al*., 2004; Hasselmann *et al*., 2008).

The possibility of locating the sex-determining regions in dioecious plant genomes by genetic mapping (see below) may make it possible to identify the sex-determining genes in individual species and test whether they are indeed different in different taxa, which would support independent evolution

of dioecy in different plant taxa. However, these genes may often be located within large non-recombining genome regions. Even if it is possible to sequence the region, which, as explained below, may be very difficult, there may be too many candidates for the approach to be very helpful. Below, I outline some alternative approaches that can help to test whether dioecy evolved independently in different angiosperms, and provide rough dates for such events.

Single and multiple gene sex determination

Before discussing the age of plant sex chromosomes, I briefly outline reasoning that suggests that dioecy in flowering plants must often have evolved through at least two mutations, a male-sterility mutation (creating females) and one or more female-sterility mutations (creating males). First, dioecy in flowering plants seems often to have evolved from either hermaphroditism or monoecy, or, using the combined term, from co-sexuality. It follows directly that, during the evolution of dioecy, either females or males must first have arisen, and become established in the population (creating a situation with both co-sexuals and unisexuals, either gynodioecy or androdioecy), and then mutations must have spread among the co-sexuals, making them become the complementary unisexual form. Theory predicts that the first mutation most probably led to gynodioecy, with females invading a population of partially self-fertilizing unisexuals in a situation where inbreeding depression made the females' high outcrossing rate advantageous, and this advantage is augmented by females' seed output being increased by re-allocating some resources used by the ancestral co-sexuals for male functions (Charlesworth and Charlesworth, 1978). Population genetic modelling of sterility mutations shows that female-sterility mutations, creating androdioecy, are much less likely to invade partially selfing co-sexual populations than are male-sterility mutations. However, they can be advantageous in gynodioecious populations, where the presence of females favours increased pollen output, even if it is accompanied by reduced female fertility. Overall, therefore, a co-sexual population can, under suitable conditions, evolve dioecy; when dioecy is reached, the females will carry the initial male-sterility mutation, while the males carry one or more male-promoting mutations (Fig. 1).

It is easily seen that suppressed recombination between X and Y chromosomes must have evolved to prevent the sex-determining genes recombining, and the two-mutation model for evolution of dioecy provides a reason why such recombination is disadvantageous. If the male-promoting mutations also reduce female fertility, they are also female-sterility mutations. Such 'trade-offs' between male and female functions may often arise, for example if an allele increasing resources devoted to male functions reduces those available for female functions; thus alleles of some genes cannot simultaneously be best in both sexes. Such trade-offs are central to models explaining the maintenance of hermaphroditism against invasion by mutations that lead

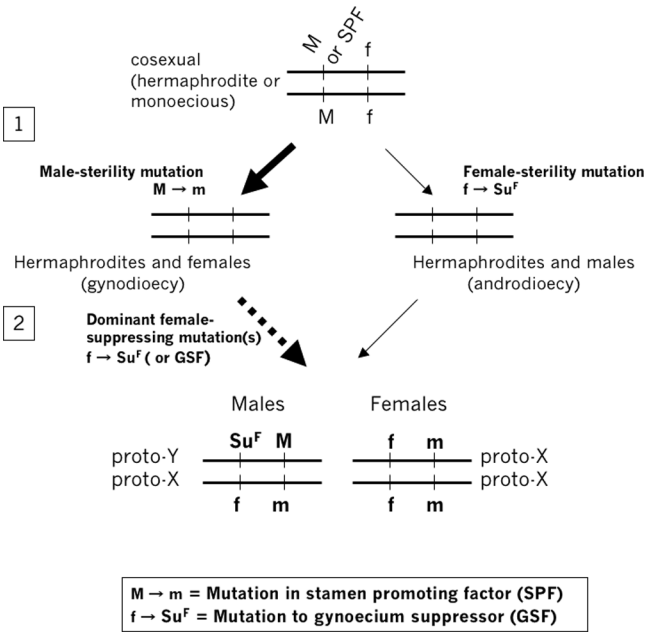

Fig. 1. Diagram showing the required minimum of two sterility mutations for a co-sexual plant to evolve into a population with unisexual males and females. The mutation that causes the appearance of females must affect a stamen-promoting factor (often denoted by SPF); here, the male-sterility mutation is symbolized by $M \rightarrow m$. The mutation creating males must be a dominant gynoecium-suppressing mutation (often denoted by GSF; symbolized here by $f \rightarrow Su^F$. The loci with the sterility mutations may recombine, and so the chromosomes are not yet a fully evolved sex chromosome pair, but are 'proto-sex chromosomes'.

to separate sexed individuals (Charnov *et al.*, 1976). In the context of the evolution of dioecy from co-sexuality, if a male-promoting mutation severely reduces female fertility, it can increase in frequency only if the locus is closely linked to the male-sterility locus, because recombination creates females with low fertility; without recombination (or with rare recombination), the male-advantage/female-disadvantage mutation can be confined to males. In this model (Fig. 2A), an evolving X chromosome carries a recessive male-sterility mutation (and the Y carries its dominant non-sterility allele), while the Y carries a dominant or partly dominant mutation that benefits male function with a trade-off that makes it a female suppressor (while the X carries its recessive allele). It is intuitively easy to understand that, under this model, both mutations can establish polymorphisms in the population, and that natural selection then favours reduced recombination between the two loci (Bull, 1983). A single sex-determining gene creates no such situation, as pointed out by J.B.S. Haldane: 'If sex were determined by a single factor, it is very difficult to see what advantage there could be in its being linked with other factors' (Haldane, 1922).

Single-factor sex-determining systems are not, however, impossible. They can certainly evolve from pre-existing

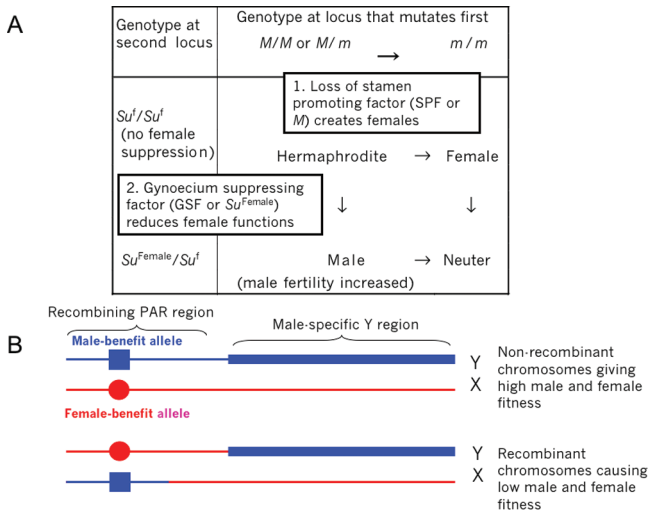

Fig. 2. Diagram showing situations during the evolution of dioecy when two mutations will segregate (i.e. although the mutations can increase in the population, they do not spread throughout all individuals and become fixed in the population), and lead to selection for reduced recombination. (A) Two sterility mutations causing female and male phenotypes (as in Fig. 1) are prevented from going to fixation because each causes sterility of the other sex. (B) A sexually antaogonistic mutation that benefits males, but lowers the fitness of females, may also establish a polymorphism in the population at a locus in the recombining region closely linked to the male-determining region of the Y.

sex-determining systems. For example, a gene can take control of sex development, and can sometimes replace an established system (reviewed in Bull, 1983; vanDoorn and Kirkpatrick, 2007; Blaser *et al.*, 2012). Such 'turnover events' may have occurred in plants where a system with male heterogamety (an XY system) has changed to female heterogamety (a ZW system, with ZZ males and ZW females), which may have occurred in the genus *Silene*, where *S. latifolia* and its close relatives have XY systems, and *S. otites* is probably ZW (Westergaard, 1958), and in the Salicaceae (see below). Change can also occur to an XO system, where the male-determining factor(s) and genes necessary for male functions, and functions needed by the haploid pollen stage, have moved from the Y to the autosomes, so that the Y can be lost; this system may not have evolved in plants (reviewed in Ming *et al.*, 2011). Single-factor sex-determining systems might also evolve from environmental sex determination. If the environment initially had two states leading to male and female development, and then gradually changed so that most individuals developed as one sex, say males, a genetic change to make some individuals develop as females, regardless of the environment, could then be advantageous; selection on the sex ratio could prevent this mutation becoming fixed, leading to a population with both sexes, provided that the environment did not change back so that it no longer triggered male development (Quinn *et al.*, 2011).

Sex linkage, partial sex linkage, and the evolution of suppressed recombination in sex-determining regions

We have seen why recombination becomes suppressed in sex-determining regions. There are several possibilities for how this could occur. The ancestors of the sex-determining genes, that underwent the mutations so that separate sexes evolved, might simply be in a non-recombining genome region, such as near a centromere. This seems unsatisfactory, because it does not explain why sex-determining genes should often happen to be located in such regions. However, it is important to note that many of the plants so far studied have large pericentromeric regions with limited crossing-over, that contain a large proportion of all genes (King *et al.*, 2002; Tomato Genome Consortium, 2012). If this is true of the genomes of dioecious plants (which is not currently known, as it requires both genetic and physical maps of their genomes), it will be necessary to find out whether the locations of sex-determining genes are in such regions to an unexpected extent (relative to other genes). Such data are so far lacking, but physical maps have recently been made for dioecious *Populus* (Yin *et al.*, 2008) and papaya species, and it is thought that the papaya sex-determining region may be in a pericentromeric region of low recombination (Wang *et al.*, 2012). However, no map is yet available for the corresponding chromosome region in a female, so it is unclear whether the fully sex-linked genes recombine in the XX genotype.

Given that the sex-determining loci of many animals, as well as a wide diversity of plants, are in non-recombining genome regions, it seems more likely that these genome regions initially recombined, and that selection has acted to suppress recombination. This is supported by the fact that, in *S. latifolia*, the fully sex-linked genes do recombine in females. Non-dioecious outgroup species may also help uncover the ancestral recombination situation. For instance, the non-dioecious species *S. vulgaris* appears suitable as an outgroup for *S. latifolia* and its close dioecious relatives (Marais *et al.*, 2011). All *S. latifolia* fully sex-linked genes map to a single *S. vulgaris* linkage group, with an order similar to the X chromosome map (Filatov, 2005; Bergero *et al.*, 2013).

Recombination suppression may involve inversions, for instance in mammals (Lemaitre *et al.*, 2009). In papaya, the non-recombining region of the Y region arrangement differs from the X region by two large inversions that appear to define recombination suppression events (Wang *et al.*, 2012), suggesting that they caused the recombination suppression.

Other kinds of chromosomal rearrangements, including translocations, can also suppress recombination. Translocations involving sex chromosomes are known in several species, and could be advantageous because they cause linkage between autosomal and sex-linked genes (Charlesworth and Charlesworth, 1980). It is, however, currently unclear whether sex chromosomes are involved in such events unexpectedly often, which might suggest such selection (Charlesworth and Wall, 1998). Even with no selection, rearrangements can generate new non-recombining genome regions. In *Drosophila*, males have no crossing-over, and so translocations involving

sex chromosomes and autosomes immediately stop recombination in a chromosomal arm that becomes attached to either the X or the Y, forming a newly non-recombining neo-Y chromosome (e.g. Yi and Charlesworth, 2000; Bachtrog, 2003). In species with crossing over in both sexes (which probably includes dioecious plant species), such rearrangements will not create a non-recombining region (even a neo-Y chromosome will probably continue recombining), though crossing over may be reduced in regions near the breakpoints, as has been documented in mice (Davisson and Akeson, 1993; Franchini *et al*., 2010). Such events are usually detectable cytologically, as in *Rumex acetosa* (Smith, 1969; Shibata *et al*., 2000) and *Humulus lupulus* (Kihara and Hirayoshi, 1932). If the region was added to a recombining region of the sex chromosomes, it may become part of both homologues, which could be detected by comparison with related species.

To test between the different mechanisms, many new empirical data are needed. Because dioecy has probably evolved independently in many lineages, often recently, plants may offer opportunities for such tests. The possibility that sex chromosome/autosome translocations are involved will require comparative genetic mapping combined with cytogenetic studies of dioecious and closely related non-dioecious species, to test whether dioecious species undergo an excess of such events. Another non-selective possibility (discussed below) is that accumulation of repetitive sequences in a newly non-recombining region, or a region adjacent to a rearrangement, creates excess repetitive sequences in the adjacent region, causing micro-heteromorphism and physically preventing recombination. To know whether this occurs will require identifying the boundary region and physically mapping it, for example using bacterial artificial chromosome (BAC) sequences. The two inversions mentioned above as differentiating the Y region arrangement from the X region argue against this possibility for papaya.

Discovering fully and partially sex-linked genes in dioecious plants is not simple. With even occasional recombination (partial sex linkage), an allele that is male linked in one family within a species can be female linked in another. Co-segregation with gender merely shows that these variants are linked to the sex-determining locus, and are in the *cis* configuration in a particular family, but is not conclusive evidence for lack of recombination (because detecting rare recombination requires large families). Complete sex linkage cannot therefore be determined using just a single family and just the heterogametic sex (if the same genes recombine much more often in the homogametic sex, the evidence becomes much stronger, but such data are often not obtained, because suitable variants are difficult to find). To distinguish between partial and complete sex linkage, it is therefore also necessary to check that variants exist that are truly specific to the heterogametic sex; that is, for the case of an XY system, variants found only in males, indicating Y linkage. Such variants provide evidence for complete linkage disequilibrium between the variant alleles and gender, implying the presence of a non-recombining region.

Fully sex-linked markers have now been identified in many plants, and show a clear predominance of male heterogamety in plants, as the two-gene model above predicts (reviewed in Ming *et al*., 2011). This prediction (Charlesworth and Charlesworth, 1978) arises because male-sterility mutations (like most loss-of-function mutations) are expected generally to be recessive, rather than dominant.

Genetic map data can also identify changes in which sex is heterogametic. For instance, in the Salicaeae, genetic markers suggest a ZW system in *S. viminalis* (Semerikov *et al*., 2003), as does *P. trichocarpa*, in one *Populus* subgenus (Semerikov *et al*., 2003; Yin *et al*., 2008), but members of other *Populus* subgenera, including *P. tremuloides*, *P. alba*, and *P. tomentosa*, apparently have XY systems (Paolucci *et al*., 2008; Hou *et al*., 2009; Pakull *et al*., 2009; Tuskan *et al*., 2012). The sex-determining region maps on a homologous linkage group in both these species, but is located in the centromeric region of the *P. tremuloides* chromosome, versus near one end of the *P. trichocarpa* chromosome (Tuskan *et al*., 2012). It is currently unclear whether these differences reflect independently evolved sex-determining systems in these subgenera (or re-evolution of dioecy after its breakdown), versus a turnover event.

The ages of plant sex chromosomes: strata and sizes of non-recombining regions

Once genes have been identified on a sex chromosome pair, it is possible to estimate the time since recombination stopped. I will discuss this for the case of an XY system, but the same principles apply to ZW systems. An important consequence of the Y chromosome losing the ability to recombine with the X is the accumulation of neutral substitutions in DNA sequences, providing a molecular clock for estimating the age of sex chromosome systems. Once recombination stops, the Y-linked alleles start to diverge in sequence from those which are X-linked, and the divergence can be used to delimit the non-recombining region and determine its extent and age (see below). Among dioecious plants, such regions are currently well characterized only in papaya (Liu *et al*., 2004; Wang *et al*., 2012) and *S. latifolia* (Bergero *et al*., 2007), although cytogenetic data showing sex chromosome differences ranging from undetectable to extreme heteromorphism strongly suggest that other dioecious plants have sex chromosomes whose ages may be very different (because chromosome rearrangements tend to accumulate with time, as will repetitive sequences; see below).

Until sequence data became available, the age of a sex chromosome or sex determination system could be estimated only from the degree of heteromorphism, or from the age of the group of species in which it is found. For instance, almost all species in the family Salicaceae are dioecious, and the same is true in the Caricaceae and Actinidiaceae (which includes the crop plants *Carica papaya* and *Actinidia* species), suggesting that separate sexes is the ancestral condition in these families as a whole.

Genetic results from *Populus* and *Salix* species suggest that they have not evolved large non-recombining regions with sex chromosome-like properties (Yin *et al*., 2008), yet

dioecy probably did not evolve very recently in the Salicaceae, given the number of species in this family, which includes 55 genera. Species in this family may therefore have old-established sex-determining regions that have not evolved non-recombining regions, like those of the ratite birds, whose sex chromosomes are only slightly heteromorphic, and are probably largely pseudoautosomal (Pigozzi, 2011), despite being homologous with the highly heteromorphic Z and W chromosomes of neognathous birds (Nishida-Umehara *et al.*, 2007). The small non-recombining region in *C. papaya*, only ~10% of one chromosome (Liu *et al.*, 2004), may, however, simply reflect a recent origin, with not enough time for this region to expand. In *Vitis vinifera*, male-specific variants were found in one of the 11 genes identified in the sex-determining region, and male–female sequence differences were shared across several related species (Fechter *et al.*, 2012). If confirmed, this would strongly suggest that recombination is absent, and that, despite the small physical size of the region (only ~143 kb), genetic sex determination did not evolve very recently. Clearly, much more work is needed to estimate the age of this system.

A dearth of fully sex-linked genetic markers in several other plants suggests that they may have very small non-recombining sex-determining regions, or no such regions. These include *Asparagus officinalis* (Telgmann-Rauber *et al.*, 2007), *Spinacia oleracea* (Khattak *et al.*, 2006; Onodera *et al.*, 2011), and kiwifruit, *Actinidia* species (Fraser *et al.*, 2009), which may be in the early stages of early sex chromosome evolution, because hermaphroditic flowers or monoecious plants are occasionally produced, and may possibly not yet have evolved full recombination suppression between the regions where the sex-determining genes are located. In strawberries, which have a ZW system, there is indeed evidence that recombination occurs in the region, in some species (Spigler *et al.*, 2008; Goldberg *et al.*, 2010). Species that still have a proto-Y chromosome, with recombination between male- and female-determining genes, are ideal for testing the two-gene hypothesis for the evolution of dioecy. In some species, the sex-determining locus apparently has three alleles, a female-determining allele, a male-determining allele, and also an allele leading to hermaphroditism or monoecy (reviewed by Westergaard, 1958; see also Onodera *et al.*, 2011). Such situations support the two-gene hypothesis, although they could be either evolving proto-sex chromosomes or cases of breakdown of dioecy. *Carica papaya* hermaphrodites are heterozygous for a Y chromosome variant, Y^h, that is much more similar in sequence to the male-determining Y than to the X (Yu *et al.*, 2008), suggesting that, in this case, a female suppressor was recently deleted from a fully non-recombining Y chromosome, causing reversion to hermaphroditism (Westergaard, 1958; Lardon *et al.*, 1999).

Other dioecious plants probably have older systems, including species with heteromorphic sex chromosomes such as *Rumex* species (reviewed in Navajas-Pérez *et al.*, 2005) and hops, where one linkage group was identified as the Y chromosome, with multiple markers closely linked to the male sex-determining factor (Seefelder *et al.*, 1999). However, genetic maps with large numbers of markers are needed to confirm these conclusions. In several dioecious plants, markers linked to the sex-determining region have been found, and are useful for determining the sex of seedlings and immature plants, but the age of the system is unknown. Examples include *Ginkgo biloba* (Liao *et al.*, 2009), the date palm *Phoenix dactylifera*, which may have a long history of dioecy (see Al-Dous *et al.*, 2011; Al-Mahmoud *et al.*, 2012), and the grass *Buchloe dactyloides* (Zhou *et al.*, 2011). As will be explained in the next section, markers within protein-coding genes are particularly valuable, as their sequences can be analysed. New methods for finding such markers, such as RNA-Seq, have yielded co-dominant genic markers in *S. latifolia* (Bergero and Charlesworth, 2011; Chibalina and Filatov, 2011; Muyle *et al.*, 2012), and should allow studies of further species, and a better understanding of the time course of sex chromosome evolution.

X–Y divergence and evolutionary strata

The most reliable way to estimate the age of a sex chromosome system is to study X–Y divergence. The time of the evolution of dioecy itself can, of course, not be estimated (unless the sex-determining gene or genes are known and both X- and Y-linked copies still exist and can be sequenced to estimate their divergence). However, fully sex-linked genes can be used to estimate the time when recombination stopped, and, by obtaining estimates from many genes, sequence divergence can be accurately estimated, allowing one to compare values for different regions of the non-recombining region, or for different species. Divergence values estimated for silent sites allow one to use a molecular clock with rates estimated from related plants, and thereby estimate the times in years when sex chromosomes stopped recombining, assuming a similar neutral mutation rate for different genes and lineage; such results should be compatible with the dating obtained from the first approach mentioned above.

However, such age estimates are rough. More work on plant molecular clocks is clearly needed, both to improve their calibration (e.g. using fossils to date the split of relevant taxa, or using mutation rates estimated using sequences from plants separated by a few generations in experiments), and to assess the extent to which neutral substitution rates vary over time. For nuclear gene sequences, substitutions rates per synonymous site per year are estimated to be $\sim 1–2 \times 10^{-8}$ (Gaut, 1998). However, rates for some plant genes are much slower, particularly for plants with long generation times (Gaut *et al.*, 1996), and the estimated divergence dates between species used for such calibrations are also subject to wide differences (Beilstein *et al.*, 2010; Huang *et al.*, 2012).

Despite these difficulties, it is now well established from detailed information obtained by such estimates in several species, using multiple X–Y gene pairs, that recombination between fully sex-linked regions of several sex chromosome systems stopped at different times in the past. This conclusion remains true even when the X–Y silent site divergence values are adjusted using divergence from suitable related outgroup species to take account of possible mutation rate differences of different genes (Fig. 3). Sex chromosome regions with

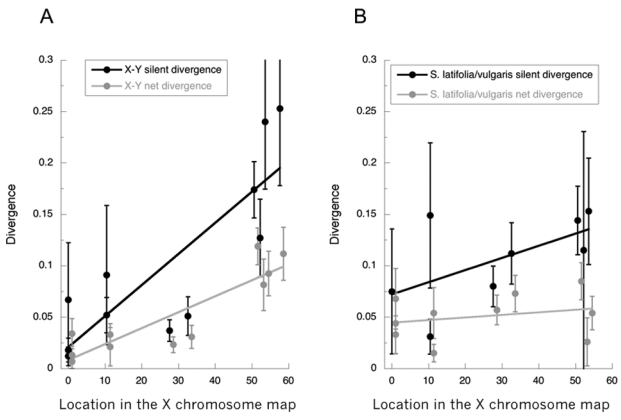

Fig. 3. Evolutionary strata with greater DNA sequence divergence between the Y and the X chromosomes of *Silene latifolia* with increasing genetic map distance from the pseudoautosomal region (PAR; at the left-hand end of the X chromosome map). Error bars show 95% confidence intervals of Jukes–Cantor corrected values. (A) Divergence estimates for the currently mapped genes using silent sites, and also (because silent site estimates are based on quite small numbers of nucleotide sites, and have high variance) using all site types (these are lower, because these sequences include coding regions, in which changes are selectively disadvantageous). The increase in both divergence measures with distance from the PAR is significant (R^2 values are 0.71 for silent sites and 0.79 for all sites). (B) Divergence between *S. latifolia* X and homologous *S. vulgaris* genes. The increase in both divergence measures with distance from the PAR is not significant (R^2 values are 0.34 for silent sites and 0.07 for all sites).

different silent site sequence divergence are called 'evolutionary strata', and such strata have been discovered in both mammals (Lahn and Page, 1999) and birds (Lawson-Handley *et al.*, 2004; Nam and Ellegren, 2008), and in the plants *S. latifolia* (Bergero *et al.*, 2007) and *C. papaya*. In both these plants, the strata are much younger than the youngest strata of mammalian or modern bird sex chromosomes, supporting the belief that these plants are in early stages of sex chromosome evolution; the oldest *S. latifolia* stratum suggests an age of 5–10 miilion years (Nicolas *et al.*, 2005), and in papaya rather less (Wang *et al.*, 2012).

In the Caricaceae, dioecy is widespread, but recombination suppression may have evolved independently in different lineages. *Vasconcellea monoica*, the sole monoecious species in the genus, may be a suitable outgroup for studying *C. papaya*, because sequences of fully sex-linked genes in this monoecious species are roughly equally diverged from papaya X- and Y-linked homologues, showing that *C. papaya* split from the *Vasconcellea* lineage before recombination was suppressed in the sex-determining region (Gschwend *et al.*, 2012; Wang *et al.*, 2012). It is not yet known whether papaya fully sex-linked genes are also fully sex linked in dioecious *Vasconcellea* species; that is, have male-specific variants (the estimated divergence times between two dioecious lineages, based on silent site divergence, is just under 30 million years; see Wu *et al.*, 2010; Carvalho and Renner, 2012). So far, only genes in the papaya region that stopped recombining most recently have been sequenced in dioecious *Vasconcellea* species, and it is not known whether these are fully sex linked in these species. Genes have now been identified that, in papaya, stopped recombining longer ago (see below), and it will be interesting to test them for sex linkage in *Vasconcellea*; the alternative is that dioecious *Vasconcellea* species have a different sex-determining region. In *Silene*, dioecy seems to have evolved in two distinct lineages, one including *S. latifolia* and its close dioecious relatives, and the other including *S. otites* and close relatives (Desfeux *et al.*, 1996), and in the *S. otites* relative *S. colpophylla*, three *S. latifolia* fully sex-linked genes are not sex linked. More *S. latifolia* fully sex-linked genes should be tested, to find out whether this second lineage has simply not evolved suppressed recombination, or has a distinct sex-determining region, on a chromosome not homologous to the *S. latifolia* X and Y.

Clearly, much further work in plants is needed to find out which species have evolved non-recombining regions, and to relate this information to the age of the system, so as to understand whether some sex-determining regions have failed to evolve suppressed recombination, even though others of similar age have done so. Given the existence of strata, it will clearly be necessary to obtain information on many genes, since genes in recently formed, young strata will underestimate the time in the past when dioecy evolved. If enough fully sex-linked loci are sequenced, the largest X–Y divergence values should often estimate the time of the origin of dioecy, which should be compatible with the dating obtained from the first approach mentioned above. Older plant sex chromosome systems, such as *Rumex* and members of the *Curcubitaceae*, will also be very interesting for studying Y chromosome degeneration and gene loss (see below). Note, however, that if a region has been deleted from the Y after recombination stopped with the X (which could occur after non-essential genes in the region have been lost from the Y), these genes are no longer available, so one will underestimate the time of the origin of dioecy.

Adaptation of sex-linked and other genes

As explained in the Introduction, the evolution of separate sexes from an initially co-sexual state might be expected to provoke evolutionary changes, as each sex adapts to the release of constraints imposed by the other sex function. This view assumes that some plant functions in co-sexual or monoecious species are unable to evolve to the optimum for one sex or the other, due to trade-offs. As discussed above, re-allocation of limited reproductive resources from one sex function to the other is thought to occur when dioecy first evolves, and necessarily involves trade-offs.

The trade-off hypothesis seems reasonable, given the sometimes striking differences between the flowers and inflorescences and growth of the two sexes in dioecious plants (Steven *et al.*, 2007; Delph and Herlihy, 2012), and even in monoecious plants (Pannell, 1997*b*; Elze and Pannell, 2011),

and an experiment using *Mercurialis annua* plants from subdioecious source populations showed that plants can respond to a situation with a greatly reduced frequency of males by evolving greater allocation to male structures (Dorken and Pannell, 2009), proving that these populations have genetic variation that allows re-allocation of resources. It is, however, difficult to estimate how often selection will involve trade-offs between the sexes. Some non-flowering characteristics in dioecious plants have probably also evolved sexual dimorphism (Cox, 1981; Bierzychudek and Eckhart, 1988; Thomas and LaFrankie, 1993; Delph *et al.*, 1998; Antos and Allen, 1999; Eppley, 2001; Espirito-Santo *et al.*, 2003; Sun *et al.*, 2009). Such traits may also involve trade-offs between the sexes; for example, females' need to conserve resources for support of seeds and fruits might favour fewer flowers than optimal for males' pollen output.

If genes with such properties exist, they could contribute to the evolution of suppressed recombination, because male-benefit mutations in genes closely linked to the non-recombining male-specific region of the Y chromosome (or 'MSY' in Fig. 2B) may increase in frequency but be prevented from spreading throughout the whole population, if the deleterious effects in females are strong (Jordan and Charlesworth, 2012). If such a 'sexually antagonistic' polymorphism arises at a selected locus in the recombining, or pseudoautosomal regions (labelled 'PAR' in Fig. 2B), recombination with the sex-determining region will be disfavoured. Given some genetic variation in recombination rates in the population, reduced or suppressed recombination may evolve, as in the case of the sterility mutations explained above.

If many adaptive changes occur when a chromosome in a co-sexual species evolves into a sex chromosome, this would suggest that trade-offs are common; that is, that these changes were maladaptive in co-sexual ancestors. Adaptive changes can be detected using molecular evolutionary analyses of gene sequences. For instance, one can test whether there is an unexpected abundance of non-synonymous substitutions in Y-linked coding sequences, compared with X-linked sequences, using an outgroup species to indicate which sequence has changed during the evolution of dioecy (Fig. 4A). Such an excess of non-synonymous changes might be due to selection for changed amino acid sequences of Y-linked alleles, suggesting adaptation. However, as explained in the next section, a proportion of these changes could be due to an inability of natural selection to eliminate deleterious non-synonymous mutations that occur on the Y chromosome. To distinguish between these two alternatives, one can test whether these changes are unexpectedly common in genes likely to be involved in functions specific to males, relative to genes encoding proteins with no sex specificity in their functions. Such tests suggest adaptive changes in the neo-Y chromosome of the insect *Drosophila miranda*. In this species, one arm of the Y was added to a pre-existing degenerated Y chromosome, so that recombination on this former autosomal genome region stopped, since males have no genetic recombination in this species (see Bachtrog *et al.*, 2009), and evidence was found for considerable adaptive changes in this neo-Y region (Zhou and Bachtrog, 2012).

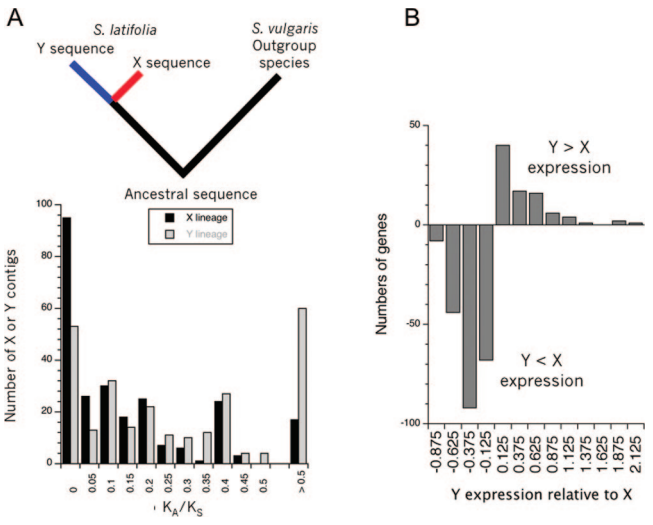

Fig. 4. Evidence suggesting deterioration of *S. latifolia* Y-linked gene functions. The lower part of (A) plots estimates of the relative numbers of non-synonymous to synonymous substitutions in the lineages leading to the *S. latifolia* Y and X chromosomes since the split from the outgroup species, *S. vulgaris*; the lineages are diagrammed in the top of (A). (B) Summary of expression estimates for Y- versus X-linked alleles at loci where the Y has retained a functional copy, showing the overall tendency for lower Y than X expression.

As just mentioned, tests for adaptive changes in Y-linked genes must exclude the possibility that the observed changes are all simply deleterious. Before describing gene expression differences between Y-linked genes and their X-linked alleles, and adaptive changes in expression levels, the next section therefore outlines the reasons for expecting Y-linked genes to undergo deleterious changes that do not occur in the X.

Deleterious consequences of suppressed recombination: genetic deterioration and gene loss

The bad effects of suppressed recombination are better understood than its evolutionary causes, and have been reviewed in recent years (Charlesworth *et al.*, 2005; Bachtrog, 2008; Kaiser and Charlesworth, 2010), so only a brief outline is necessary here. In genome regions with suppressed recombination, selection acting on either advantageous or disadvantageous mutations in the region affects all linked variants. This effectively restricts the size of the population, compared with the rest of the species' genome, because some genotypes fail to reproduce. The effective population size can be considerably reduced for a Y chromosome that still carries many functional sequences. Low effective population sizes compared with the rest of the species' genome are detectable from unexpectedly low genetic diversity for silent sites (synonymous sites in coding sequences, plus intronic and other non-coding sites); these variants are generally weakly selected, or neutral, and their levels of diversity will depend on the

mutation rate and the effective population size. The expected low diversity is clearly seen in the *S. latifolia* Y chromosome and, to a predictably lesser extent, the X chromosome (Qiu *et al*., 2010).

The reduced effective population size allows deleterious mutations in functional sequences to increase in frequency in the population, or become fixed, causing deterioration of the sequences. In plants, however, few deleterious changes may occur in Y-linked alleles, because a high proportion of genes are expressed in the haploid pollen (e.g. Honys and Twell, 2003), and, if these genes have important functions during pollination, they may experience strong purifying selection against sequence changes that impair their functions.

However, surprisingly, empirical evidence that Y-linked genes do deteriorate is starting to emerge in plants. Using an outgroup species to distinguish between substitutions in *S. latifolia* Y- versus X-linked alleles, a clear excess of Y-linked substitutions was seen for non-synonymous (amino acid changing) variants, while the X and Y lineages have similar numbers of synonymous (probably near neutral) substitutions (Bergero and Charlesworth, 2011; Chibalina and Filatov, 2011), suggesting that even this quite recently evolved non-recombining Y chromosome has already deteriorated detectably. Some non-synonymous substitutions could, however, be adaptive, and this possibility has not yet been tested by approaches such as that outlined in the previous section. Moreover, in *S. latifolia*, expression of the Y-linked allele of many genes is lower than that of the X-linked allele (Fig. 4B). Again, however, it cannot be ruled out that higher X than Y expression in males reflects adaptive up-regulation in response to low functionality of Y-linked alleles (dosage compensation; see the next section). It may be possible to test this possibility using outgroup species to show whether the X has been up-regulated in males. If the expression of the Y has decreased, relative to non-dioecious relatives, adaptation can still not be definitively excluded, as lower expression might be selected in males, though a widespread trend in this direction seems unlikely.

Genes that are not preserved by selection may be lost from the Y. This is called 'genetic degeneration'. Current knowledge about gene losses from plant Y chromosomes is scanty. Analyses of expression data suggest that the *S. latifolia* Y may have lost ~20% of the genes originally present (Bergero and Charlesworth, 2011; Chibalina and Filatov, 2011). However, such experiments cannot distinguish between gene loss and low expression of Y alleles, and further tests of genomic DNA are needed. It will be interesting to test whether regions that stopped recombining most recently tend to include most of the X–Y gene pairs that can be identified, which would suggest that older regions have lost Y copies. Complete sequences of the Y-linked regions and their X-like counterparts can potentially show what proportion of genes present in the X have been lost from the Y. Such an analysis requires the assumption that the X still carries the ancestral set of genes; this seems reasonable for recently evolved sex chromosomes, but should nevertheless be tested, given that there is evidence for gene movements off the *Drosophila* X chromosome (Vibranovski *et al*., 2009; Schrider *et al*., 2011), and some evidence of its invasion by genes with female-specific functions (Zhang *et al*., 2010; Han, 2012).

Obtaining physical maps of plant sex chromosomes is, however, still very laborious, and seems feasible only for species with small non-recombining regions. The papaya sex chromosomal regions have recently been analysed, and yielded no clear signal of an excess of gene losses from the Y (Gschwend *et al*., 2012; Wang *et al*., 2012), partly because several papaya Y region genes are in the most recent stratum, which stopped recombining only a few million years ago. This may be too little time for extensive degeneration and gene loss, particularly in plants whose generation times are several years. The *S. latifolia* Y chromosome, currently thought to be little degenerated (see above), is often compared with the neo-Y of *D. miranda*, which formed ~1 million years ago, but whose genes have degenerated much more than has so far been found in plants, with stop codons inactivating some genes, and gene losses (Bachtrog, 2008). In terms of numbers of generations, however, this neo-Y may not be much more evolutionarily recent than the cessation of recombination in some plants, for example the older *S. latifolia* or papaya sex chromosome strata. A more important factor limiting gene loss from sex chromosomes of plants such as papaya is probably the small size of the non-recombining regions, in terms of the total number of genes, so that the forces causing genetic degeneration are weaker than in the *D. miranda* neo-Y. In addition, as mentioned above, selection to maintain pollen function may act on plant sex-linked genes.

Gene expression differences and dosage compensation

Whether sex differences exist in gene expression is a major gap in our knowledge of plant biology, potentially of considerable relevance for dioecious crops. Sex differences are found in animal somatic tissues, even in species with no sex hormones, such as *Drosophila* (Meisel, 2011), and in developmental stages of mice too early for gonadal hormone influences to occur, suggesting that genetic differences between the sexes induce different expression of at least some genes (Dewing *et al*., 2003). In plants, it is of particular interest to study leaves before flowering, to ask whether the sexes differ physiologically in ways that might suggest trade-offs between their slightly different sets of functions. Some evidence for sex differences has recently been published in a *Populus* species (Jiang *et al*., 2012), which appears to have only a small sex-linked region (see above). Comparison with *S. latifolia* will be interesting, as its sex chromosomes have a large fully sex-linked region, so that more genes with sex differences in expression might be expected. Many such genes were indeed detected in *S. latifolia*, and were found to be particularly abundant on the sex chromosomes (Zemp *et al*., 2012). More work is needed to extend and confirm these findings, as an RNA-Seq experiment showed no sex difference in expression in this species (Chibalina and Filatov, 2011).

As explained above, situations generating sexually antagonistic mutations in plants are currently unknown, though

situations with sexual dimorphism might potentially generate strong selection differences between the two sexes (this remains to be tested). If sex differences in expression, and trade-offs, are common, sexual antagonism, and trade-offs, would seem likely to occur, at least sometimes. Currently, the few known empirical examples demonstrating the existence of sexual antagonism involve fish species with sexual selection, where bright colours can be advantageous in males, provided that predation is not too intense, but are disadvantageous in females, whose eggs are usually fully fertilized (e.g. Lindholm and Breden, 2002; Lampert *et al*., 2010).

A form of adaptation involving selection that must often generate sexual antagonism is the evolution of dosage compensation, which acts when genes on the Y chromosome in XY systems (or the W in ZW systems) lose functions, so that males (in XY systems) are equivalent to loss-of-function mutants for those genes. In the insect, *Drosophila*, it has long been understood that males produce a higher amount of gene product from their single gene than do females mutant for the same X-linked gene (Bridges, 1922; Lucchesi, 1978), and gene expression estimates show that the single X in *Drosophila* males yields mRNA levels similar to those from the two X chromosomes of females. Deletions of *Drosophila* genomic regions reduce transcript abundance, though abundance often exceeds half normal values (McAnally and Yampolsky, 2010). Thus, although increased transcription need not imply that evolutionary change was involved, the high X-linked gene expression in males suggests that selection was responsible. Control of transcription is probably rarely sex specific, so genetic changes that increase transcription in males may often also increase it in females, which may sometimes be disadvantageous (Charlesworth, 1996). The strength of such sexual antagonism effects remains to be tested.

The ideal genes for studying whether dosage compensation has evolved are those that have been lost from the Y, but are still present on the X chromosome, which will have the largest difference in dosage. Using 124 putative 'X-only' genes of *S. latifolia*, their expression levels in males and females did not suggest dosage compensation (Chibalina and Filatov, 2011); although some of these genes may be present on the Y, and have escaped detection in this RNA-Seq analysis, such genes probably have low expression of their Y-linked alleles, and might still be expected to evolve dosage compensation. The mean expression level of 'X-only' genes in four males studied was similar to that for a large set of non-sex-linked genes (which showed no sex difference in expression), whereas, in females, the expression of 'X-only' genes was higher, albeit much less than the 2-fold difference expected without any dosage compensation, perhaps because of another compensation mechanism, as mentioned above. However, Muyle *et al*. (2012) concluded that dosage compensation does occur in *S. latifolia*, so further study is needed. It will be interesting to study outgroup species for any genes that appear to have evolved dosage compensation, to find out whether the X has been up-regulated in males. The availability of non-dioecious outgroup species in genera with dioecious members may make plants excellent for studying the early stages, and the time course, of the evolution of dosage compensation, and many dioecious plants have smaller chromosome numbers than mammals, making it more reasonable to assume that the different chromosomes may not differ very greatly in expression levels due to carrying genes with very different functions.

Accumulation of repetitive sequences, and evolution of sex chromosome heteromorphism

A reduced effective population size not only allows deleterious substitutions to occur, it also allows accumulation of transposable element (TE) insertions, which are mostly deleterious (Charlesworth *et al*., 1994; Hollister and Gaut, 2009). TE insertions may therefore accumulate in non-recombining Y chromosome regions, or the loss of gene functions in these regions (see below) may reduce selection against such insertions. The idea that such insertions could be one of the earliest consequences of stopping recombination is potentially testable by studying plant species with young sex chromosomes whose Y chromosomes still retain functional copies of most genes carried by the X.

The accumulation of TEs and other repetitive sequences is probably a major contributor to the tendency for plant Y chromosomes to be larger than their X homologues. A higher abundance of AT-rich satellite repeats has been detected by *in situ* hybridization on the two Y chromosomes (see below) of *R. acetosa* and its close dioecious relatives (Shibata *et al*., 1999; Mariotti *et al*., 2008), but it remains unclear whether the *S. latifolia* Y has more such insertions per megabase than other parts of this species' genome (i.e. a higher TE density). In papaya, the Y region is larger than the homologous X region, and this is due largely to a higher TE density (Gschwend *et al*., 2012; Wang *et al*., 2012). The *S. latifolia* Y is too large for complete sequencing, and other approaches have not detected a higher abundance of TEs (Macas *et al*., 2008), probably because of high overall TE abundance in the *S. latifolia* genome. By studying a sample of *S. latifolia* Y chromosomes by a method known as transposon display, it is nevertheless clear that TE insertions are present at the predicted higher frequencies at sites on the Y than elsewhere in the genome, consistent with this predicted effect of a low effective population size (Bergero *et al*., 2008*b*).

TE accumulation may occur on the X as well as the Y chromosome. In dioecious plants so far studied genetically, recombination occurs in both sexes, as in mammals. The X chromosome thus has a lower recombination frequency than the autosomes, because X chromosomes recombine only in females (note that this is the opposite of the recombination rate difference in *Drosophila* species, where crossing-over does not occur in males, so that the X recombines more, on average, than the autosomes, which spend half their time in males, versus only one-third for the X chromosome). Its lower recombination frequency appears to have led to the mammalian X chromosome having a higher repetitive content than the genome-wide average (though less than the Y), and the modern bird Z similarly has a higher than average repetitive content (Bellott *et al*., 2010). Little is yet known about the

repetitive content of plant X chromosomes, but comparison of the small X-like region in *C. papaya* finds an unexpectedly high abundance of TEs, though much less than in the Y region (Gschwend *et al.*, 2012; Wang *et al.*, 2012).

TE insertions probably contribute to the greater degree of rearrangement of Y than X chromosomes observed in mammals (Repping *et al.*, 2006) and in the plants papaya (Gschwend *et al.*, 2012; Wang *et al.*, 2012) and *S. latifolia* (Hobza *et al.*, 2007; Zluvova *et al.*, 2007; Bergero *et al.*, 2008*a*). Rearrangements occur due to ectopic pairing and crossing-over between repeated sequences in different locations on the Y (e.g. Tenaillon *et al.*, 2012). Such events can change chromosome morphology, leading to visible heteromorphism in sex chromosomes that are large enough to be studied cytologically. Ectopic recombination events may also cause deletion of regions where the Y genes have degenerated, although tests will need to be done to distinguish this from adaptive movements off the X and Y chromosomes (Gschwend *et al.*, 2012; Wang *et al.*, 2012). This will require genetic mapping in outgroup species. TE insertions may also be the cause of some genetic degeneration, as insertions in genes, or near them, can cause loss of function or reduced expression levels (Hollister and Gaut, 2009).

When additions occur onto the sex chromosomes (e.g. fusions of autosomes with X or Y chromosomes) this may enlarge the PAR. If there is some restriction of recombination close to the rearrangement breakpoint, these regions might start to accumulate repetitive sequences, similar to the accumulation observed on X chromosomes. This might affect the recombining region outside the PAR boundary. Some TEs tend to move preferentially to genome locations near the source element (Wicker *et al.*, 2007), and TE heterozygosity is known to reduce recombination in maize (Dooner and Martinez-Férez, 1997; Fu *et al.*, 2002; Dooner and He, 2008). Thus, if insertions are abundant in the youngest fully sex-linked stratum, or in the adjacent PAR boundary region, the closest recombining region may preferentially acquire new (heterozygous) insertions, and this may directly inhibit recombination and lead to the non-recombining region encroaching into the PAR (Fig. 5). However, as already mentioned, recombination suppression need not inevitably evolve. It is currently unclear whether all plants with large recombining (pseudoautosomal) regions of their sex chromosomes are always young sex chromosomes, and studies of plant sex chromosomes of a range of ages will be needed to find out whether some plants that have been dioecious for a long time have retained small non-recombining regions. If so, this might argue against the hypothesis just outlined.

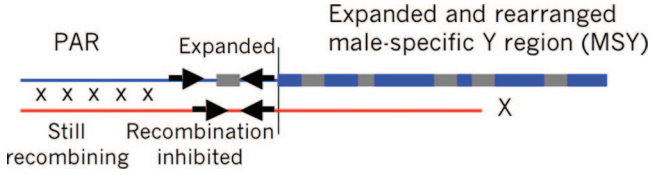

Fig. 5. Diagram suggesting a possible mechanism that could lead to the progressive evolution of suppressed recombination in a formerly recombining PAR of a sex chromosome.

Conclusions

Clearly, there is much that is still not well understood about sex chromosomes, and much genetic work needs to be done in the future. Empirical tests for the existence of sexual antagonism and estimates of its strength will be important in order to understand whether male-benefit/female-disadvantage genes are the main reason why Y chromosomes stop recombining with the homologous X. Such work will benefit from developmental approaches that can help us understand pleiotropy during development that may lead to such trade-offs between male and female functions. It will also be valuable to have detailed data to show whether PAR boundaries changed between related dioecious plants. If recombining regions of plant sex chromosome pairs are shrinking, and their genes are becoming parts of non-recombining regions, the newly non-recombining regions will be ideal for studying the earliest changes after recombination stops. Older plant sex chromosomes should also be studied, in order to get an idea of the time course of changes such as accumulation of repetitive sequences, genetic degeneration, and deletion of Y-linked regions, and to study the stages in sex chromosome evolution when turnover events occur that replace one sex-determining system with another. Only when the sex-determining genes are known will we know the age of a sex chromosome system with certainty, but minimum ages can be estimated from X–Y gene pairs across the chromosome. Even establishing the sex-determining system of a dioecious species still represents considerable work, and identifying its sex-determining gene(s) is much more difficult. Fine genetic mapping can identify the genome region involved, which often clarifies whether males or females are the heterogametic sex for the region. Finding clusters of genes related to floral development in the region can provide support for a candidate region, but, ideally, more detailed studies are needed, including finding genes that are expressed only in males at the time when sex is determined during development. Among such genes, Y-linked loci will be good candidates for the sex-determining gene, and can be further tested by developmental experiments. This approach has recently succeeded in discovering a fish sex-determining gene that is distinct from those previously known in other fish, and other vertebrates (Yano *et al.*, 2012).

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