Dioecious Plants. A Key to the Early Events of Sex Chromosome Evolution[1]

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Around 200 BC, the Nordic tribes devised rune symbols to represent the forces of nature. Among these symbols were X-GEBA, the rune of love and sexuality, and Y-FEOH, the rune of success. It was believed that by picking the right rune, the wearer could harness the power the rune represented. Hazard or deep intuition? It turns out that in biology, X and Y symbols define sexual fates and reproduction success.

Sex determination systems based on heteromorphic X and Y sex chromosomes are particularly interesting to study from both a developmental and an evolutionary perspective. There are many parallels between the sex determination systems, as well as the organization of sex chromosomes, in different species, even between animals and plants.

Two main systems of chromosomal sex determination, XY and X:A (autosomal chromosome) ratio, apparently have evolved many times. Mammals, for example, have the XY system, with a dominant (active) Y chromosome containing the key sex determination function(s), whereas *Drosophila melanogaster* has an X:A system, where the ratio of X:A chromosomes determines sex by an X chromosome counting system, the Y chromosome being largely dispensable (Hodgkin, 1992).

Dioecy is a widespread condition in flowering plants, despite their recent evolutionary origin: 6% of the 240,000 angiosperm species are dioecious and 7% of 13,000 genera of angiosperms include dioecious species, suggesting that it has arisen many times during flowering plant evolution (Renner and Ricklefs, 1995). Dioecy is correlated with perennial climbing growth, wind, or water pollination and has a preponderance in tropical flora. Model species with a chromosomal sex determination are white campion (*Silene latifolia*; XY system), hop (*Humulus lupulus*; X:A system), and sorrel species (*Rumex* spp.) which include both XY-like and X:A systems (Figs. 1 and 2).

Plant sex determination has been recently and extensively covered (Ainsworth et al., 1998); therefore, we will mainly concentrate on the contribution of this very particular group of plants to the universal question of sex chromosome evolution.

A SURPRISING DIVERSITY OF SEX DETERMINATION MECHANISMS

In animals, sex determination processes usually involve a similar basic strategy: a primary (genetic) signal, a master regulator that responds to the signal, and a double-switch gene selecting between two alternative sexual programs (Nöthiger and Steinmann-Zwicky, 1987). The molecular mechanisms vary extensively, and can differ within genera or even within a species. For example, *Sxl,* the master regulatory gene in certain *Drosophila* spp., is present in other flies, all of which have separate sexes and sexual dimorphism, but *Sxl* does not appear to control sex determination in the latter (Wray and Abouheif, 1998).

The corresponding mechanisms have not been characterized at the molecular level in dioecious plants so far, but it is clear that sexual dimorphism is a late developmental decision during the life cycle of the plant, mainly restricted to flower organogenesis or reproductive organ differentiation. It is interesting that in plant species with clearly identified sex chromosomes such as hop, sorrel, or white campion (Fig. 1), the sexual dimorphism is expressed at very early stages of flower development, namely at stages of organ initiation or specification (Farbos et al., 1997; Ainsworth et al., 1998). In other words, sex determination processes in these species act in a male or female whorl-specific manner at or just downstream but independent of the ABC flower regulatory network (Ainsworth et al., 1998, and refs. therein; Scutt et al., 1999). Therefore, understanding flower development in model species such as Arabidopsis is essential in addressing the question of how sex determination might work in unisexual plants.

X AND Y SEX CHROMOSOMES: ALWAYS THE SAME TUNE?

The evolution of heteromorphic sex chromosome systems in widely differing species suggests that sim-

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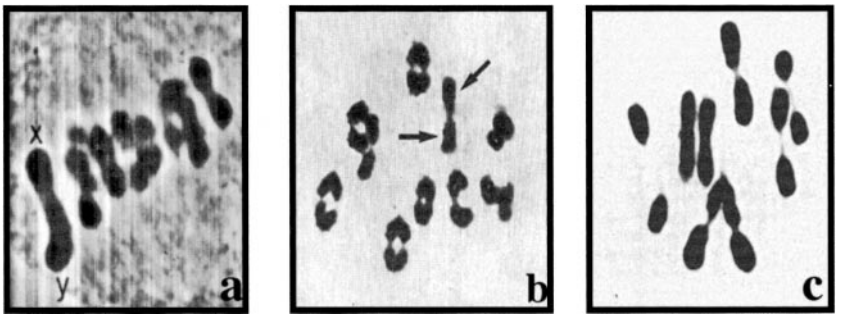

Figure 1. Historical pictures of meiotic preparations showing terminal pairing of X and Y chromosomes in male flowers of: a, white campion ($2n = 24$, XY, metaphase I; van Nigtevecht, 1966); b, hop ($2n = 20$, XY, early anaphase I, arrows; Moutchen et al., 1973); and c, sorrel (*Rumex acetosa*; $2n = 15$, XY1Y2, metaphase I, with sex-trivalent XY1Y2 in convergent orientation; Parker and Clark, 1991).

ilar forces have been at work in every case. The outcome is the accumulation on the sex chromosomes of key genetic components (molecular switches) determining sexual dimorphism and, following a controlled arrest of recombination along most regions of X and Y, the concentration of sex-related genes on sex chromosomes, Y chromosome genetic isolation and erosion, X chromosome dosage compensation, etc. (Charlesworth, 1992; Ellis, 1998; Charlesworth and Guttman, 1999; Mitchell, 2000).

Taken together, the above can be summarized as follows: (a) Sex determination exhibits similarities (such as male heterogamety, extensive sexual dimorphism, and X chromosome dosage compensation) that have arisen by convergent evolution. Sex determination is probably the most typical case where evolution can produce a variety of solutions to the same basic problems in development (Hodgkin, 1992); and (b) Plants are key players in the study of the evolution of sex determination because they offer a unique opportunity in giving access to the very early stages of X and Y chromosome history.

Because this is a critical matter in developmental and evolutionary biology, we have chosen to illustrate this latter point by using sorrel and white campion as examples in the more general context of sex determination. Sorrel will be briefly introduced, whereas a more detailed analysis of white campion is envisaged in regard to the similarities of this plant and mammalian XY system (Westergaard, 1958; van Nigtevecht, 1966; Ciupercescu et al., 1990).

SORREL: A "MULTIPLE" SEX DETERMINATION SYSTEM

Sorrel (Fig. 2, a and b) has a multiple sex chromosome system with two X chromosomes in females ($2n = 14$, XX) and one X plus two Y chromosomes in males ($2n = 15$, XY_1Y_2). In this species, sex determination is controlled by activities of genes located both on the X chromosome and on the autosomes (Ainsworth et al., 1998). The male flower phenotype is not dependent on the presence of the Y chromosomes, but they are necessary for the production of fertile pollen. The two Y chromosomes are highly heterochromatic, which can be demonstrated by simple staining. Recent studies have revealed that the Y chromosomes have accumulated numerous repetitive DNA sequences, with at least one family being unique to the Y (Shibata et al., 1999). The Y chromosomes maintain their condensed status also in interphase, thus forming two peripheral bodies in male nuclei. Immunostaining experiments demonstrated that the Y bodies display a characteristic epigenetic modification: depletion of H4 histone acetylation (Lengerova and Vyskot, 2001). Thus, the sorrel Y chromosomes represent an example of constitutive heterochromatin, which is not true of the white campion Y chromosome (Grant et al., 1994; Scutt et al., 1997). From this difference, we infer that the sex

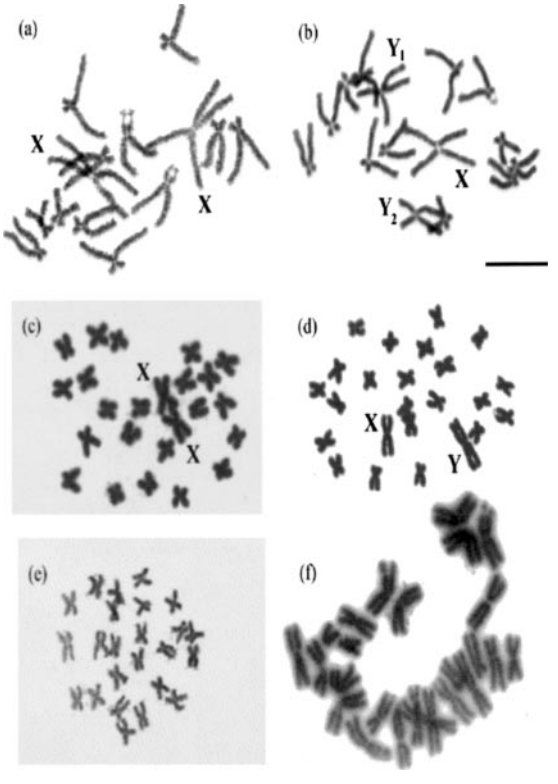

Figure 2. Root tip metaphases of dioecious sorrel female (a) and male (b). Metaphases from permanent hairy root cultures of dioecious white campion female (c) and male (d), and related gynodioecious *Silene vulgaris* (e) and hermaphrodite *Silene chalcedonica* (f). Sex chromosomes are indicated. Bar = 10 microns.

chromosomes of sorrel are older than those of white campion.

THE GENUS *SILENE*: A BOTANIST'S POINT OF CONTENTION

White campion (previously *Melandrium album)*, belongs to the genus *Silene*. The genus contains more than 700 species in 44 sections, and possesses a range of reproduction modes, from hermaphroditism through gynodioecy and occasional monoecy to stable dioecy (Chater and Walters, 1964; Desfeux and Lejeune, 1996). It now includes the previously separate genera *Melandrium* and *Lychnis*. Even though their chromosome number is the same ($2n = 24$; Fig. 2, c–f; Degraeve, 1980), there is high heterogeneity in genome size, indicating an accumulation of numerous DNA repeats in some species (Siroky et al., 2001). For example, flow cytometric analysis revealed a small genome size in gynodioecious *S. vulgaris* (section Inflatae, 2.25 pg of DNA per diploid nucleus [pg/2C]) and *Silene pendula* (section Erectorefractae, 2.35 pg/2C), but large values in dioecious white campion (section Elisanthe, 5.73 pg/2C) and hermaphrodite *S. chalcedonica* (previously *Lychnis chalcedonica*, section Lychnidiformes, 6.59 pg/2C). Moreover, a great variation in number and localization of rDNA loci (two – seven nuclear organizing regions) was found among these species (Siroky et al., 2001). All these data clearly demonstrate that nuclear genomes of *Silene* spp. are highly diversified.

Recently constructed molecular phylogenies based on rDNA intergenic spacer sequences suggest that in the last 20 to 25 million years, separate sexes evolved at least twice within the genus (the white campion versus *Silene otites* branches), in agreement with more classical classification criteria (Degraeve, 1980; Desfeux and Lejeune, 1996). However, the form of sex determination in different sections of the genus remains controversial and the support for some branches in the intergenic spacer sequences clustering is weak. Additional comparative sequence data are required to improve the resolution of phylogenetic relationships between key dioecious (white campion and *S. otites*) and hermaphrodite species (*Silene conica* and *Silene gallica*), as well as to estimate the age of the sex chromosomes across the tree by evaluating the time since recombination between defined X and Y loci or regions has ceased (see below).

These are critical questions to be answered if we want to understand why, among the many dioecious species, only a few have evolved sex chromosomes, a handful of which possess an XY sex determination system (Charlesworth and Guttman, 1999). The fact that the genus *Silene* contains species that evolved X and Y chromosomes makes it an attractive system in the study of breeding system evolution in general and sex chromosome evolution in particular.

THE XY SEX CHROMOSOME SYSTEM

The two sexes share a common gene pool while performing many different biological functions. In the case of mammals and white campion, the presence or absence of the Y determines which reproductive organs, male or female, will develop. Thus, the Y is dominant and active with regard to sex determination.

From Genic to Chromosomal Systems of Sex Determination

All sex chromosomes are believed to be derived from pairs of autosomes. Proto-X and -Y chromosomes are considered to contain a simple diallelic system of sex determination. The case of the *SUPERMAN* (*SUP*) gene, which, when mutated, causes imperfect unisexuality in Arabidopsis, could illustrate how primitive genic sex determination systems might arise. Although certain *sup* alleles (*sup-1*) enhance maleness in agreement with the gene name, others, including epialleles exhibiting gene hypermethylation (*clk* alleles; Jacobsen and Meyerowitz, 1997; Rohde et al., 1999, and refs. therein), produce a rather distinct "super-woman" phenotype. The result is a contrasting series of SUPm (male enhancing) and a SUPf (female enhancing) states (Fig. 3a). Such states need to be subsequently enforced by the action of other, genetically linked, sexually antagonistic genes and modifier genes differentially acting on such alleles (Charlesworth, 1992; Rice, 1992).

The situation in white campion and other dioecious species with established sex chromosome systems indicates that more than one locus is involved in sex determination, as shown by both crosses between dioecious plants and related monoecious or hermaphrodite species (reviewed by Westergaard, 1958), or by mutagenesis (Lardon et al., 1999 a, 1999b). In white campion, sex determination is controlled by at least three loci (Lardon et al., 1999b). The Y chromosome contains two of these loci: a female suppression function, negatively controlling cell proliferation during carpel initiation, and a male promoting function controlling the specification of male gametophytic cell fate. That these are independent pathways for male and female developmental arrest is reflected by the fact that changes in sex expression generate either hermaphrodite or asexual (neuter, both male and female sterile) mutants. The genetic analysis of gamma ray-induced mutants has enabled us to distinguish two loci with female suppression properties: a Y-linked locus (called *GSF-Y*) and an autosomal locus (called *GSF-A*). In this context, *GSF-A* appeared as a potential enhancer of the *GSF-Y* locus. Phenocopies of such mutations were induced chemically when genetically male plants were treated with 5-azacytidine, a DNA demethylation agent (Fig. 3c), or trichostatin A, a potent inhibitor of histone deacetylases (Ja-

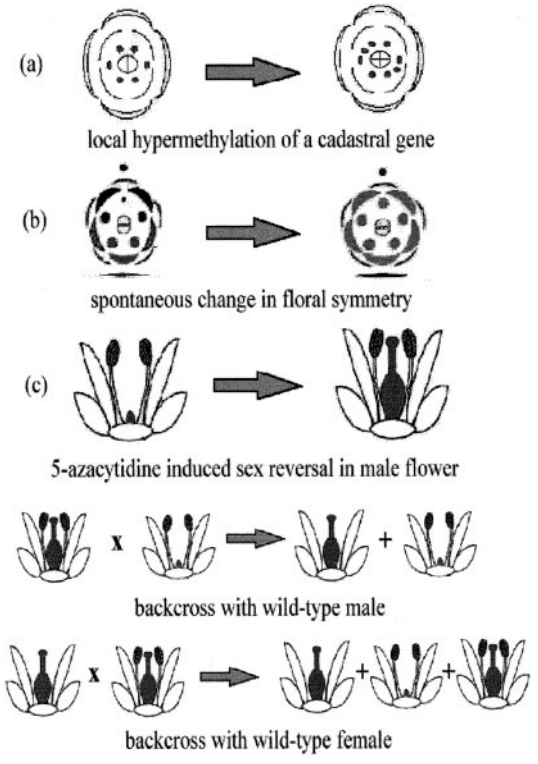

Figure 3. Epigenetic control of flower development. DNA methylation changes often lead to modification in floral patterns. a, Hypermethylation of the *SUPERMAN* gene displays an increase in number of anthers and/or carpels in Arabidopsis (Jacobsen and Meyerowitz, 1997). b, Epigenetic inactivation of a *CYCLOIDEA* homolog is responsible for variation in floral symmetry in *Linaria vulgaris* (Cubas et al., 1999). c, A global CpG hypomethylation of nuclear genome of white campion by 5-azacytidine induces a sex reversal from male to androhermaphrodite phenotype. This epimutation was likely located on the Y chromosome because it displayed holandric inheritance and the genes controlling carpel suppression are Y linked (Janousek et al., 1996). 5-Azacytidine is expected to activate genes, whereas in this case an inactivation of female suppressor genes located on the Y chromosome was observed. One plausible explanation is that a large and global CpG hypomethylation induced by 5-azacytidine could disturb nDNA methylation patterns in such a way that some gene regions were (CpNpG) hypermethylated and thus inactivated, as demonstrated at the Arabidopsis *SUP* locus (Jacobsen et al., 2000; Lindroth et al., 2001).

nousek et al., 1996; J. Hodurkova and B. Vyskot, unpublished data). We conclude that sex expression control in white campion can be added to the list of flower developmental processes that are regulated epigenetically (Finnegan et al., 2000; Jacobsen et al., 2000; Fig. 3).

What is the nature of evolutionary processes that turn proto-X and -Y chromosomes into heteromorphic sex chromosomes? Key events appear to be the suppression of meiotic recombination between proto-sex chromosome regions in the heterogametic sex, Y degeneration, and X chromosome dosage compensation (Charlesworth, 1992; Ellis, 1998).

Arrest of X-Y Recombination: A Critical Event in the Evolution of Sex Chromosomes

The reduction and subsequent suppression of recombination between the sex determination loci and the male-advantage/female-disadvantage genes linked to them is most likely selected for to avoid production of neuters or hermaphrodites (Rice, 1987; Charlesworth and Guttman, 1999). From this point onwards, the differentiation of sex chromosomes can begin, with an increasing functional differentiation between the initial homologs resulting in morphologically and genetically distinct sex chromosomes. Sex chromosome differentiation appears to be a continuing process.

The mechanism that underlies the lack of recombination between sex chromosomes in males may be of great importance in the evolution of the Y chromosome because it could determine the nature and timing of subsequent genetic events. Recombination can be suppressed by: (a) chromosomal inversions, or (b) more specific control functions restricting the pairing of defined pairs of chromosomes (recombination modifiers; Nei, 1969; also see Charlesworth and Charlesworth, 1980). Models proposed by these authors predict that recombination suppression can occur with sex-specific selection.

The strata model in the human XY system is illustrating the first cited mechanism. Lahn and Page (1999) have proposed a model in which human Y chromosome evolution involved four inversion events, each suppressing X-Y recombination separately and without disturbing gene order on the X. These events spanned along a time scale of 240 to 300 million years of animal evolution. The results show that in humans, the arrest of XY recombination has occurred progressively. In *Drosophila* spp., on the contrary, the breakdown of recombination has apparently taken place quite suddenly (Clark, 1988), and might correspond to the second mechanism.

These situations need to be evaluated experimentally in *Silene* spp. by looking, on the one hand, for the existence of strata and, on the other hand, for sterile mutants defective in meiotic pairing. In the first case, for example, a molecular characterization of sex-linked loci is required. The first active Y-linked genes described have a very similar X chromosomal copy and the relative age of individual X-Y gene pairs has been measured by nucleotide divergence (Delichère et al., 1999; Filatov et al., 2000; Atanassov et al., 2001). The results indicate, as for human XY-linked genes, that the two loci characterized so far identify Y chromosome regions that have ceased recombining at different times during the evolution of sex chromosomes, namely 5 and 15 million years on the 20- to 25-million year scale since the last common hermaphrodite ancestor. They reveal distinct events in the evolutionary history of the sex chromosomes and stimulate further studies in this direction.

Y Organization Indicates Functional Coherence

Recombination suppression and concomitant evolution of dosage compensation are believed to be necessary to avoid exchanging the accumulating dysfunctional genes from the Y onto the X (Clark, 1988). Because there is only one Y for four autosomes and three X chromosomes in a population, the Y is much more sensitive to genetic drift (random fluctuations of gene frequencies; Nei, 1970). Events such as sequence translocations, insertions, inversions, or amplification, associated or not with (retro) transposition are documented in the human Y chromosome (Schwartz et al., 1998, and refs. therein). Therefore, the evolution of the Y chromosome reflects these basic properties. Contrary to ordinary chromosomes containing random assortments of genes, the gene content in the large non-recombining Y region of the human Y appears as a functionally coherent exception (Lahn and Page 1997), in that there is tendency to accumulate male-benefit genes by selectively retaining and amplifying male fertility factors or genes that enhance male reproduction fitness. These are linked to the sex determination loci. In other words, the Y becomes an increasingly specialized male chromosome and this specialization parallels the deterioration of the genetic content of the large non-recombining Y region (Mitchell, 2000).

The gene content of the Y chromosome of white campion shows that 15 to 20 million years of XY evolution is sufficient to achieve a "functional coherence" of Y chromosome organization: In addition to the genes involved in sex determination (see above) and sex ratio bias that localize on the p arm (Lardon et al., 1999b), the Y chromosome carries several loci involved in stamen differentiation and microsporogenesis (Donnison et al., 1996; J. Zluvova and S. Georgiev, unpublished data) that are concentrated on the q arm, together with several of the cloned Y-linked genes that have X homologs (see also Fig. 4). The working hypothesis is that the p arm contains the block of sex determination genes and is rather gene poor, whereas the q arm concentrates several male-specific functions and contains regions of homology with the X.

Evidence for Y Decay and X Chromosome Dosage Compensation in *Silene* Spp. Remains Ambiguous

At present, experimental evidence for X chromosome inactivation based on methylation and H4 acetylation patterns in white campion female cells remains ambiguous (Vyskot et al., 1993; Siroky et al., 1999). Furthermore, evidence for Y degeneration in white campion is relatively weak. It is based on YY seedling lethality (Westergaard, 1958), on the large size of the Y relative to the X or autosomes in several related hermaphrodite species suggesting that the Y has been accumulating DNA (Figs. 2 and 4), and on the identification of the first X-linked gene shown to have a degenerated counterpart on the Y (Guttman and Charlesworth, 1998). On the other hand, the sex chromosomes in white campion were reported to have the same length as the longest autosomal pair in certain non-dioecious *Silene* spp. belonging to the Lychnidiformes section (Degraeve, 1980), there is important repetitive sequence similarity between the sex chromosomes and autosomes of white campion (Scutt et al., 1997), and the Y chromosome is largely non-heterochromatic with all *Silene* spp. chromosomes, Y included, possessing strikingly gene-dense regions near their ends (as shown by early DNA-replicating patterns and fluorescence in situ hybridization signal profiles following hybridization with a total cDNA library; Vyskot et al., 1999).

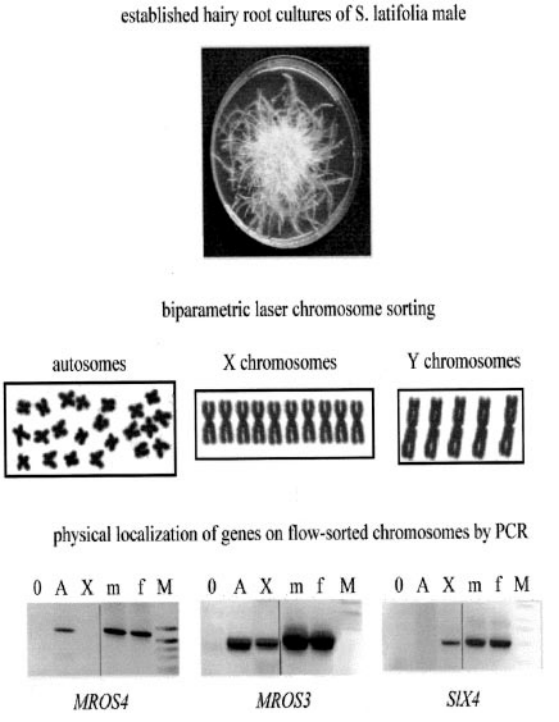

Figure 4. Immortal hairy root cultures of white campion serve as a permanent source of synchronized metaphases. Due to their large size, the sex chromosomes are a suitable material for laser flow sorting. Because the X chromosome is nearly twice as large as compared with the average autosome, it can easily be purified and used for physical localization of genes by PCR and construction of chromosome-specific libraries (Kejnovsky et al., 2001). The purity of the sorted X and autosomes is high (95%) as estimated by both fluorescence in situ hybridization and PCR reconstruction experiments. The physical mapping is illustrated by the autosomal localization of *MROS4*, the autosomal and X location of *MROS3* (Matsunaga et al., 1996), and the unique position of *SlX4* on the X chromosome (Atanassov et al., 2001). 0, Control, no DNA template; A, autosomes; X, X chromosomes; m, male genomic DNA; f, female genomic DNA; M, marker (pBR322/*Alu*I).

At the population genetics level, a reduction in the effective population size of Y-linked genes is observed when estimating sequence divergence at nonsynonymous and silent sites in pairs of active genes on the sex chromosomes, showing that some selective

processes do affect this plant Y chromosome (Filatov et al., 2000). Further work on additional loci is needed to evaluate more precisely which genetic processes contribute most to coding sequence evolution on sex chromosomes and to the genetic erosion of Y-linked alleles (Charlesworth and Charlesworth, 2000). Taken together, evidence is accumulating in support of a recent origin for the XY chromosome system within the genus *Silene*.

CONCLUSIONS: THE MANY REASONS TO SUPPORT RESEARCH ON PLANT SEX CHROMOSOME SYSTEMS

Male genomes consist of an association of three nuclear subgenomes: the autosomes, the X and the Y. Each component, although living under the same roof, has distinct evolutionary constraints and fates. In this respect, the *Silene* genus is an example of how the evolution of an XY system contributes to morphological change and speciation.

The Y chromosome differs from all other chromosomes not only in that it is the only chromosome that does not recombine along the majority of its length, but also in being present only in the male sex in a permanent haploid condition (Y genetic isolation), in having a common ancestry and persistent meiotic relationship with the X, and the tendency of its genes to degenerate during evolution (Y genetic erosion). The Y becomes a specialized male chromosome, which essentially behaves like a single recombination unit. The lesson we can learn from white campion in this context is that a functional coherence of the Y can be achieved relatively early during Y evolution, which might be an essential condition for the maintenance of an XY system. Concerning Y decay and X chromosome dosage compensation, there is so far no solid evidence that either of these two stages has been reached.

Therefore, we anticipate that white campion will help to elucidate the evolutionary forces that shape the genetic content of a Y chromosome during the early stages of its evolution. Sex chromosomes in animals go back 300 million years and Y chromosomes are genetically eroded (Ellis, 1998; Mitchell, 2000). Because similar constraints operate in all sex chromosome systems, the *Silene* genus with its estimated 20- to 25-million year ancestry appears to contain the most recently evolved XY system known so far in Eukaryotes, therefore holding the key to at least one major question: How did all this start?

In brief, in white campion, the first active genes on the Y have been identified, a large collection of Y deletion mutants is available, and a powerful chromosome technology is being established (Fig. 4). Such tools can be transposed to other species within the *Silene* genus. We now have the opportunity to perform a comparative analysis with chosen members of the *Silene* genus that do not have heteromorphic sex chromosomes to characterize in depth such early evolutionary stages, to test different hypotheses, and hopefully to clone the sex determination master genes of white campion in the (near) future.

More generally, dioecious plants with XY sex determination systems are typical flowering plants with a modular and sequential developmental strategy and, at the same time, resemble animals in their sexual reproduction strategy. Therefore, the full understanding of the evolution of sex chromosomes can only be achieved by integrating the molecular aspects of sex determination from dioecious plants.

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