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Accumulation of chloroplast DNA sequences on the Y chromosome of *Silene latifolia*

Eduard Kejnovsky[1], Zdenek Kubat[1], Roman Hobza[1], Martina Lengerova[1], Shusei Sato[2], Satoshi Tabata[2], Kiichi Fukui[3], Sachihiro Matsunaga[3] & Boris Vyskot[1]
[1]*Laboratory of Plant Developmental Genetics, Institute of Biophysics, Academy of Sciences of the Czech Republic, CZ-612 65, Brno, Czech Republic (Phone: +420-541517203; Fax: +420-541240500; E-mail: kejnovsk@ibp.cz);* [2]*Kazusa DNA Research Institute, Kisarazu, 292-0818, Chiba, Japan;* [3]*Department of Biotechnology, Graduate School of Engineering, Osaka University, Suita, 565-0871, Osaka, Japan*

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Abstract

Silene latifolia is a model dioecious plant with heteromorphic sex chromosomes. The Y chromosome is the largest in this species. Theoretical models propose an accumulation of repetitive DNA sequences in non-recombining parts of the Y chromosome. In this study, we isolated a BAC7H5 clone preferentially hybridizing to the Y chromosome of *S. latifolia*. Sequence analysis revealed that this BAC7H5 contains part of the chloroplast genome, indicating that these chloroplast sequences have accumulated on the Y chromosome and also may contribute to its large size. We constructed Y chromosome- and X chromosome-specific libraries and screened them to find Y- and/or X-linked copies of chloroplast sequences. Sequence analysis revealed higher divergence of a non-genic region of the chloroplast sequences located on the Y chromosome while genic regions tested showed only very low (max 0.9%) divergence from their chloroplast homologues.

Introduction

It is generally accepted that the evolution of structurally distinct sex chromosomes from a pair of autosomes involves a stepwise restriction of recombination between proto-Y and proto-X chromosomes. Genomic regions with little or no recombination are subject to several processes, given a sufficiently high rate of deleterious mutations (Charlesworth & Charlesworth, 2000). These processes could lead to Y chromosome degeneration, resulting in both accumulation of repetitive DNA sequences on the Y chromosome and loss of many functionally important genes from the Y chromosome, except for genes with male-specific functions. In addition, autosomal genes can be moved to Y chromosomes by duplicative transfer and subsequently acquire male-specific expression, as demonstrated in humans (Lahn & Page, 1999) and *Silene latifolia* (Matsunaga et al., 2003). According to mathematical models, repetitive DNA sequences, both transposable elements and tandem repeats, should accumulate in non-recombining parts of the Y chromosome (Charlesworth, 1991; Charlesworth et al., 1994).

While the phenomenon of accumulation of repetitive DNA sequences on the Y chromosome is common in animals (Steineman & Steineman, 1992, 1998; Erlandsson et al., 2000; Junakovic et al., 1998), there are only a few examples of accumulated repetitive DNA sequences on plant Y chromosomes. These examples include non-LTR retrotransposons in hemp, *Cannabis sativa* (Sakamoto et al., 2000); tandem repeats in sorrel, *Rumex acetosa* (Shibata et al., 1999; Shibata, 2000); unique repetitive DNA sequences in liverwort, *Marchantia polymorpha* (Okada et al., 2001); and accumulated retroelements in the male-specific

region of the primitive Y chromosome of papaya (Liu et al., 2004). An accumulation of repetitive DNA sequences on the Y chromosome in *Silene latifolia* has been also found (Grant et al., 1994).

Silene latifolia, the best studied model dioecious plant, represents a unique system in which the Y chromosome is not only larger than the X chromosome, but is also larger than any autosome (for review, see Vyskot & Hobza, 2004), and recombines with the X chromosome only in a small part of its q arm (Lengerova et al., 2003). While the sex chromosomes of mammals and *Drosophila* are ancient, originating about 300 million years ago, the sex chromosomes of *S. latifolia* are estimated to be only 15–20 million years old (Charlesworth & Guttman, 1999). Therefore, *S. latifolia* enables us to study early stages of sex chromosome evolution in which suppression of recombination in non-recombining parts of the Y chromosome could result in accumulation of repetitive sequences, accounting for the large size of the Y chromosome.

In addition to the nuclear genome, plants have mitochondrial and chloroplast genomes. The genomes of these endosymbiotic organelles have been genetically eroded during evolution. This erosion consists of the loss of many genes and their transfer to the nucleus which, over time, concentrates genetic material in the nucleus (for reviews see Martin & Herrmann 1998; Timmis et al., 2004). These sequences, known as 'promiscuous DNA' (that is, DNA sequences that migrate between cellular genomes in evolution), have been known since the early 1980s (Ellis, 1982; Lewin, 1984). Interestingly, genome-sequencing projects revealed that the fragments of organelle DNA are a normal attribute of plant chromosomes, demonstrating the frequency of organelle-to-nucleus transfer. For example, the nuclear genome of *Arabidopsis thaliana* contains a large (~620 kb) insert of mitochondrial DNA (mtDNA) on chromosome 2 (Lin et al., 1999; Stupar et al., 2001) and 17 insertions of chloroplast DNA (cpDNA) with a total length of 11 kb (The Arabidopsis Genome Initiative, 2000).

Here we demonstrate that chloroplast DNA sequences have accumulated on the Y chromosome of the model dioecious plant, *S. latifolia*. Chloroplast sequences located on the Y chromosome were isolated from a Y chromosome-specific library. They showed high divergence from cpDNA in non-genic region (19%) and degeneration at the functional level in genic regions containing stop codons in contrast to X-linked sequences lacking stop codons.

Materials and methods

Chloroplast DNA isolation, PCR and cloning

S. latifolia Poiret plant material came from the seed collection of the Institute of Biophysics, Brno, Czech Republic. Young leaves of female *S. latifolia* plants were used as a source of chloroplast DNA. Chloroplasts were isolated according to Triboush et al. (1998). To amplify the four regions of chloroplast DNA indicated in Figure 2 we used PCR with following primer pairs:

F1: 5′-GGTTTGTTGGGGTGTAATGG-3′, R1: 5′-GGTTTCAAGGAGCCCAAAAT-3′, product length 523 bp;
F2: 5′-TTGGGGGTTGCTAACTCAAC-3′, R2: 5′-GATAATCCTTTCCCGCATCA-3′, product length 404 bp;
F3: 5′-TGCGAATTAGGAAGGATTGG-3′, R3: 5′-AACCTGTTCCCACGCAATAG-3′, product length 774 bp;
F4: 5′-AGATGCCTCGTCTTTGCATT-3′, R4: 5′-TGAGGCCAGAATGGATTTTC-3′, product length 355 bp.

The reactions were performed in a volume of 50 μl, and the final concentration of reagents was: 0.2 mM dNTP, 0.2 μM primers, 1×buffer (TopBio) containing 1.5 mM $MgCl_2$, and 0.6 U of Taq polymerase (TopBio). Initial denaturation was followed by 25 cycles of 50 s at 94°C, 1 min at 55°C, and 1 min at 72°C, with a final extension for 7 min. The PTC-200 thermal cycler (MJ Research) was used. PCR products were purified using the Qiagen PCR purification kit, cloned using the pGEM-T Easy cloning system (Invitrogen), and sequenced by VBC-Genomics (Vienna, Austria).

Fluorescence in situ *hybridization*

In order to synchronize germinating seeds, the DNA polymerase inhibitor aphidicolin was added for 12 h, and mitoses were then accumulated with oryzalin as described by Siroky et al. (2001). BAC

DNA was isolated using the Qiagen Plasmid Midi kit with a modification for low-copy plasmids. DNA was labeled with FluorolinkCy3 dUTP (Amersham Pharmacia Biotech) (red labeling) in combination with the Nick Translation Mix (Roche). Similarly, the X43.1 tandem repeat (208 bp monomer, isolated by Buzek et al., 1997), and 25 S rDNA cloned in pBluescript II SK + (2.5 kb long *Eco*RI fragment of 25 S rDNA isolated by Kiss et al., 1989), were amplified by PCR and labeled with SpectrumGreen dUTP (green labeling). Slides were treated as described in Lengerova et al. (2004) with slight modifications. Slide denaturation was performed in 7:3 (v/v) formamide: 2×SSC for 2 min at 72°C. Slides were immediately dehydrated through 50, 70, and 100% ethanol (−20°C), and air dried. The probe was denatured at 70°C for 10 min, and 5–200 ng of the denatured probe was added at room temperature and hybridized for 18 h at 37°C. Slides were analyzed using an Olympus Provis microscope, and image analysis was performed using ISIS software (Metasystems).

DNA sequencing

The nucleotide sequence of the BAC7H5 insert was determined using the bridging shotgun method described previously (Sato et al., 1997, 2001). Briefly, the BAC7H5 DNA was sonicated, followed by size-fractionation using agarose gel electrophoresis. Fractions of approximately 1.0 and 2.5 kb were cloned into M13mp18, and designated as libraries of element clones and bridge clones, respectively. The element clones were propagated on microtiter dishes, and single-stranded DNA was prepared for the sequencing reaction. For the bridging clones, each insert was amplified by PCR, and used as a template.

Sequencing was performed using cycle-sequencing kits (Dye-terminator Cycle Sequencing kit of Applied Biosystems, USA) with DNA sequences type 377XL (Applied Biosystems, USA) according to the protocol recommended by the manufacturer. The single-pass sequences deduced for one strand of element clones and those for both ends of bridge clones were assembled using Phred–Phrap programs (Phil Green, Univ. Washington, Seattle, USA). A total of 544 reads was assembled into two contigs. The remaining gap was covered by extending the termini of each contig using the primer extension method.

Construction of BAC, DOP-Y and DOP-X libraries and their screening

An *S. latifolia* BAC library was constructed as described by Lengerova et al. (2004). The DOP-Y and DOP-X libraries were constructed as follows. First, the Y or X chromosomes were microdissected, collected, and DOP-PCR-amplified as described by Hobza et al. (2004). Ten individual Y or X chromosomes were used for the DOP-PCR reaction. The DOP-PCR products were purified using the Qiagen PCR purification kit, and cloned into pGEM-T Easy (Promega). The transformants (10,000 clones from each Y/X ligation) were picked into 384-well plates using the GeneTAC G3 robot. DOP-Y and DOP-X, as well as the BAC libraries, were screened by colony hybridization. Clones were first robotically spotted onto the nylon membranes (10,000 clones on one membrane, 30×30 cm, in DOP libraries, and 960 BAC clones on one membrane, 13×9 cm), bacteria were grown for 5 h at 37°C, and then treated as described by Lengerova et al. (2004). Membranes were then used for hybridization with the AlkPhos Direct hybridization kit (Amersham Pharmacia Biotech); detection was performed using the CDP-Star detection reagent. When DOP libraries were screened with BAC7H5 as a probe, a *Hin*dIII fragment (7.5 kb) corresponding to the BAC vector pBeloBAC11 was removed to prevent its hybridization with the homologous region of the plasmid vector pGEM-T Easy used in the DOP-Y/X library construction.

Results

Selection of the BAC clone 7H5 hybridizing preferentially to the Y chromosome of Silene latifolia

In order to prepare FISH markers specific for sex chromosomes we constructed a sample BAC library of *S. latifolia* (Lengerova et al., 2004). This library was screened with male and female genomic DNA probes, as well as with a complex DOP-Y probe (DOP-PCR-amplified laser-microdissected Y chromosome). Clones hybridizing more strongly

Figure 1. Fluorescence *in situ* hybridization using red-labeled BAC7H5 and green-labeled 25 S rDNA (a) or X43.1 tandem repeat (d–f) probes on the *S. latifolia* metaphase chromosomes. Pictures (b–f) show the sex chromosomes with a representative hybridization pattern of BAC7H5. (g–l) Distribution of the FISH signal of BAC7H5 along the sex chromosomes from pictures (a–f). The axes representing the length of the chromosome and the intensity of the signal are in arbitrary units. Bright red spots outside the chromosomes in (a) probably reflect hybridization of the BAC7H5 probe to chloroplasts. 25 S rDNA hybridizing to five autosomal pairs (a) was used as a positive control of FISH sensitivity. Bar = 10 μm.

with male than female genomic DNA were selected. One of these BAC clones, BAC7H5, hybridized in FISH experiments strongly to the Y chromosome with significantly weaker hybridization to the X chromosome and autosomes (Figure 1). Signals always accumulated more strongly on the long arm of the Y chromosome containing the pseudoautosomal region, as confirmed by two-color FISH (Figure 1d–f) using the q arm-specific X43.1 repetitive sequence (Buzek et al., 1987; Lengerova et al., 2003). In all FISH experiments with BAC7H5, in addition to the chromosomal signals, bright spots were present outside the chromosomes irrespective of the amount of probe used. These spots may represent root cell plastids as was confirmed by FISH on crude fraction of isolated chloroplasts (not shown). To quantify the FISH signals we measured the signal intensity of all chromosomes in 20 metaphases using image analysis software. On the Y chromosome, the intensity of the signal was 3.3 times higher than the average. In these calculations we used the chromosome length determined by Lengerova et al. (2004).

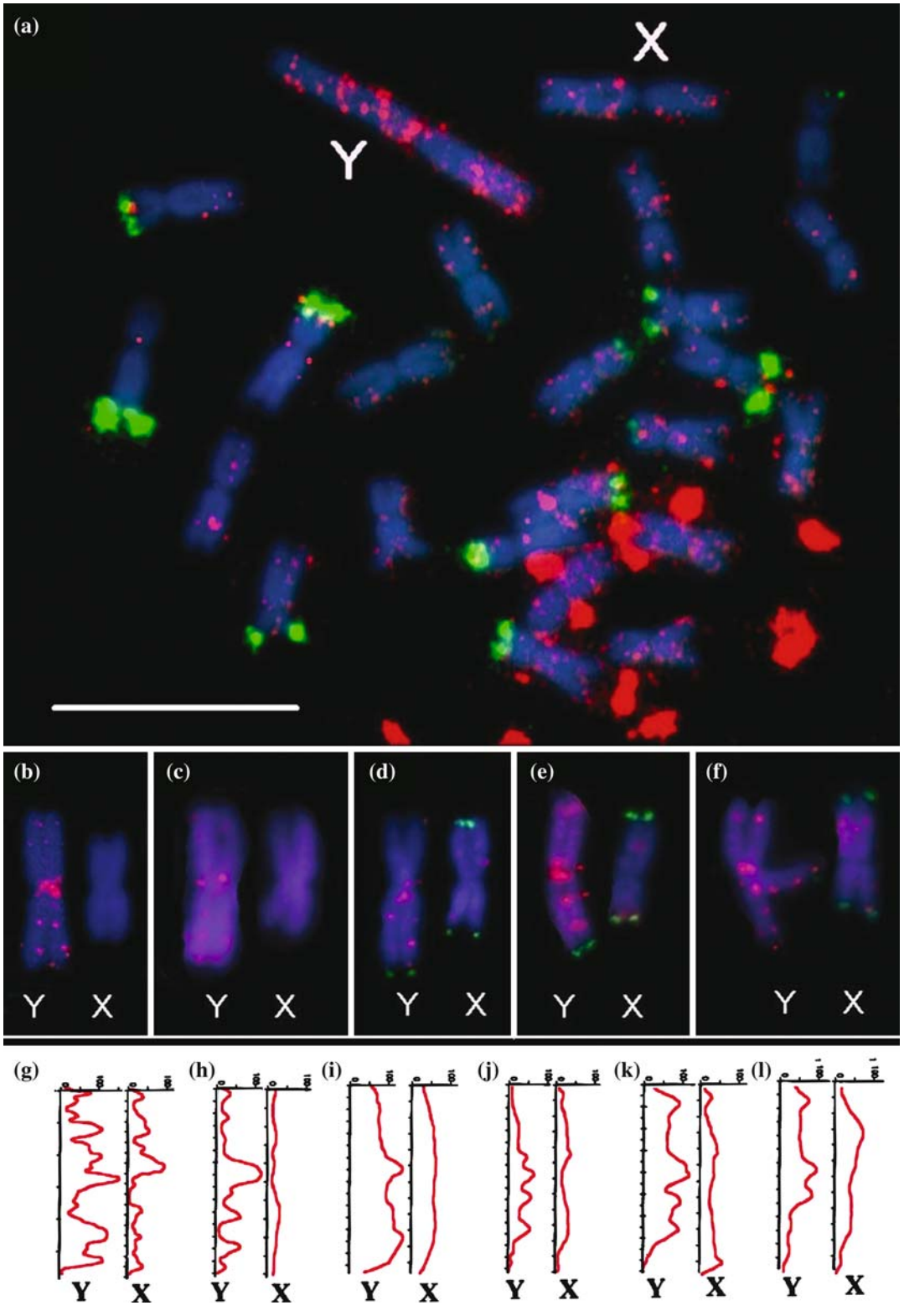
Sequencing of BAC7H5 shows homology to chloroplast DNA

BAC7H5 was completely sequenced and the insert length was determined to be 47,249 bp (Acc. No. AB189069). Database searches revealed its homology to the chloroplast genome of some species, with the highest homology to spinach chloroplast DNA (cpDNA) (*Spinacia oleracea* chloroplast, complete genome, Acc. no. NC002202). The BAC7H5 insert contains part of the chloroplast genome stretching from the *rps12* gene at one end to the *rpoB* gene at the other end with the same order of genes as that of spinach cpDNA (Figure 2).

To answer the question whether the BAC insert originates from chloroplast or nuclear genome we designed primer pairs to amplify four regions from isolated cpDNA of *S. latifolia*. Three regions corresponded to parts of the *rps16*, *rpoB*, and *trnK* genes, and one region represented a non-genic region located upstream of the *rps16* gene (Figure 2). These regions were amplified by PCR, cloned, and sequenced (Acc. nos. AY707939–41 and AY707959). The total length of amplified sequences was 2,056 bp. Sequence comparison showed that regions amplified from chloroplast DNA were completely identical with the sequence of BAC7H5, indicating that the insert of BAC7H5 originates directly from the chloroplast genome.

Isolation and analysis of chloroplast sequences from Y and X chromosomes

We have constructed the Y chromosome- and X chromosome-specific libraries using DOP PCR (degenerate oligonucleotide-primed PCR) on microdissected sex chromosomes. Screening of the Y chromosome-specific library resulted in the isolation of five clones containing chloroplast sequences, while 12 clones were isolated from the X chromosome library. All these clones were sequenced (Acc. nos. AY707942–58), and the sequences aligned with BAC7H5. These clones were homologous to three genic and one non-genic region. Two Y-linked clones and eleven X-linked clones were homologous to the same part of the *ycf2* gene. One Y-linked and one X-linked clone were homologous to the same part of the *rpoB* gene, one Y-linked clone was homologous to the *matK* gene, and one Y-linked clone covered the non-genic region (Table 1). All clones always covered homologous regions of corresponding genes, which is obviously a result of the homology of the primers used in DOP PCR (Telenius et al. 1992) to several parts of the chloroplast genome. All Y/X-linked clones homologous to the *ycf2*, *rpoB* and *matK* genes showed very low divergence (max 0.9%) from the chloroplast DNA sequence; five of eleven X-linked clones representing part of the *ycf2* gene were even identical with the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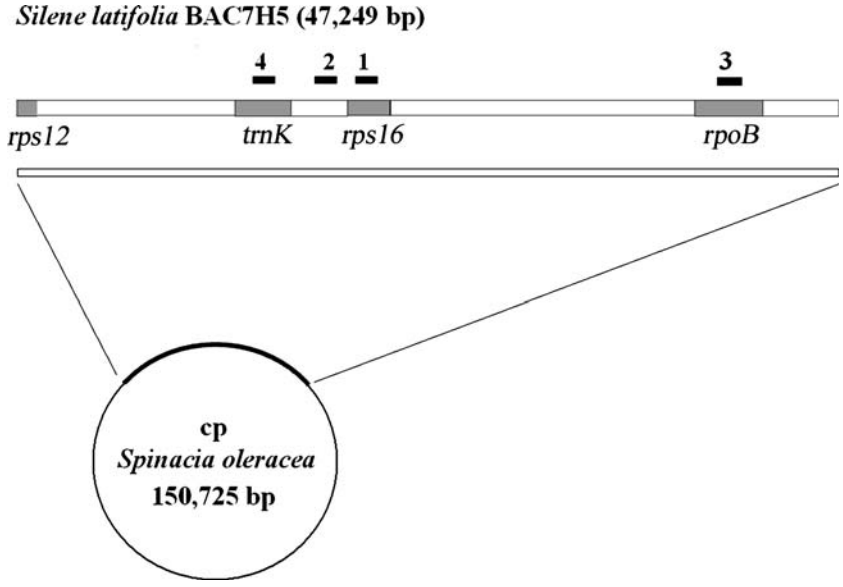

Figure 2. The homology of BAC7H5 to the *Spinacia oleracea* chloroplast genome that exhibited the highest identity to the BAC7H5 sequence in the database search. Four regions (marked 1–4) of the BAC7H5 insert which were amplified on the cpDNA template are schematically marked with respect to certain genes.

Table 1. Properties of clones homologous to cpDNA that were isolated from DOP-Y (CTY clones) or DOP-X (CTX clones) sex chromosome-specific libraries

Clone	Sex chromosome of origin	Sequence characteristics (length)	Divergence from chloroplast (%)	Presence of STOP codons
CTY12	Y	*ycf2* gene (812 bp)	0.5	+
CTY15	Y		0.0	–
CTX4	X		0.6	–
CTX5	X		0.0	–
CTX7	X		0.2	–
CTX8	X		0.0	–
CTX9	X		0.2	–
CTX10	X		0.0	–
CTX11	X		0.0	–
CTX12	X		0.2	–
CTX13	X		0.4	–
CTX14	X		0.9	–
CTX15	X		0.3	–
CTY9	Y	*rpoB* gene (573 bp)	0.7	+
CTX3	X		0.5	–
CTY7	Y	*maturase K* gene (354 bp)	0.6	+
CTY16	Y	non-genic (516 bp)	19.0	ND

ND, not determined.

chloroplast sequence. On the other hand, the only non-genic region studied was more divergent, with 19% divergence. Mutations, mostly nucleotide substitutions, with only a few insertions or deletions, were evenly distributed throughout all sequenced regions of the Y/X-linked clones, as

demonstrated in the Y-linked intergenic region (clone CTY16) exhibiting the highest divergence from cpDNA (Figure 3).

We searched for stop codons to investigate the potential activity of chloroplast genes located on the sex chromosomes. No clone originating from the segment of the X chromosome (CTX clones), that included parts of the *ycf2* or *rpoB* genes, contained stop codons. In the *ycf2* genic region, three clones (CTX7, 9, and 13) with non-zero divergence from chloroplast sequence contained only synonymous mutations, while four other clones (CTX4, 12, 14, 15) contained non-synonymous mutations. In the *rpoB* genic region two synonymous mutations were detected in clone CTX3. In contrast to the X-linked clones, clones derived from the genic regions of the Y chromosome contained either sequences without stop codons (CTY15, *ycf2* genic region), or sequences with stop codons resulting from point mutations in the *matK* gene (CTY7), or frameshift mutations in the *ycf2* (CTY12) and *rpoB* genes (CTY9). Taken together, the only one genic region studied here which is located on the Y chromosome exhibits a higher level of divergence from its chloroplast homologue than all the genic regions tested. Degeneration of genic regions on the Y chromosome was higher than on the X chromosome, especially at the functional level as a consequence of the presence of stop codons, while the sequence divergence was low (Table 1).

Discussion

In this report we describe the accumulation of chloroplast sequences on the Y chromosome in *S. latifolia*. Although the transport of organellar DNA sequences to the nucleus is common, so far, there has been a question whether organelle DNA

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BAC7H5: 43299 CCTTCGGGAACGCGGACACAGGTGGTGCATGGCTGTCGTCAGCTCGTGCCGTAAGGTGTT
CTY16:    518 --------G--CT-----------C----------------------T---G--A----

BAC7H5: 43359 GGGTTAAGTCCCGCAACGAGCGCAACCCTCGTGTTTAGTTGCCAACGTTGAGTTTGGAAC
CTY16:    458 --------------------------------ACC----------T-A--T----G--C--

BAC7H5: 43419 CCTGAACAGACTGCCGGTGATAAGCCGGAGGAAGGTGAGGATGACGTCAAGTCATCATGC
CTY16:    398 TT-A-GGTA--C-------------------------G--------------C-----G

BAC7H5: 43479 CCCTTATGCCCTGGGCGACACACGTGCTACAATGGCCGGGACAAAGGGTCGCGATCCCGC
CTY16:    338 ------C--G------T-------------------G-T----GT---CA--A-G-A---

BAC7H5: 43539 GAGGGTGAGCTAACCCCAAAAACCCGTCCTCAGTTCGGATTGCAGGCTGCAACTCGCCTG
CTY16:    278 ---T---------T-T-C----G----*--------------TTCT----------AGA-

BAC7H5: 43599 CATGAAGCCGGAATCGCTAGTAATCGCCGGTCAGCCATACGGCGGTGAATTCGTTCCCGG
CTY16:    218 -------G-------------------G-A----*---G-C---------A-------A-

BAC7H5: 43659 GCCTTGTACACACCGCCCGTCACACTATGGGAGCTGGCCATGCCCGAAGTCGTTACCTTA
CTY16:    158 -------------------------C-----------ATTCA-------G----G-GC--

BAC7H5: 43719 AC-CGCAAG-GAGGGGGATGCCGAAGGCAGGGCTAGTGACTGGAGTGAAGTCGTAACAAG
CTY16:     98 --T------A----CA-GC-A-C-C--TG--TT---C------G----------------

BAC7H5: 43779 GTAGCCGTACTGGAAGGTGCGGCTGGATCACCTCCTTT 43814
CTY16:     38 ---------GG----CC--------------------- 1
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Figure 3. Alignment of nucleotide sequences of clone CTY16 from DOP-Y library with the sequence of BAC7H5, showing a high degree of degeneration in the non-genic region between the *rps7* and *rps12* genes. Missing nucleotides are represented by asterisks; nucleotides that differ from those of BAC7H5 are shaded.

is integrated into preferred chromosomes, chromosomal regions, or sequence contexts. Very recently it has been shown that human Y chromosome is more susceptible to colonization by mitochondrial DNA (Ricchetti et al., 2004). What could account for the accumulation of cpDNA on the Y chromosome in *S. latifolia*?

Regardless of which mechanism drives this process, endosymbiotic gene transfer is ubiquitous and occurs at frequencies that were previously unimagined (Timmis et al., 2004). A process of sequence elimination must counterbalance these insertions to prevent the swamping of plant genomes with organellar sequences. We propose that these processes could involve chromosome recombination. Non-recombining parts of the Y chromosome could represent a quiescent part of the genome, where duplication of cpDNA sequences is not opposed by selection for elimination.

Our experiments demonstrate very high identity (99.1–100%) of genic chloroplast sequences located on the Y/X chromosomes to the chloroplast genome. This is consistent with recently published data which show that although organelle sequences in the nuclear genome vary in age, most are very similar to their organelle counterparts, often having >95% sequence identity (Timmis et al., 2004). Yuan et al. (2002) aligned sequences of a 239 kb region of rice chromosome 10 with the rice chloroplast sequence and discovered a large (32,974 bp) chloroplast insert. The sequence of the chloroplast insert was nearly identical (99.7% identity) to the corresponding region of the rice chloroplast genome, with only 92 bp differing out of 32,974 bp. The high degree of identity of chloroplast sequences located in the nucleus with cpDNA suggests that these sequences were inserted into the nucleus recently and have not had time to accumulate more mutations. It indicates that there is probably a high turnover of organelle sequences in the nucleus. In contrast to the genic sequences, the only non-genic sequence we have isolated from the Y chromosome is more divergent. This could reflect a higher degree of divergence in the non-recombining parts of the Y chromosome. In addition, nucleotide alterations were distributed throughout the chloroplast insertion in both coding and non-coding regions, suggesting a lack of preferential accumulation of mutations within the insertion. Most of the genic sequences located on the Y chromosome (three of four) contain stop codons and so cannot represent functional copies of the genes. Sequence divergence of genic regions containing stop codons was comparable to those lacking stop codons, indicating the recent appearance of stop codons in the Y-linked chloroplast genes studied here.

We suggest that the non-recombining Y chromosome represents an unusual part of the genome in which processes of sequence elimination are suppressed, thus allowing accumulation of chloroplast DNA sequences and their divergence. The accumulation of chloroplast DNA sequences could also thus contribute to the large size of the Y chromosome in *S. latifolia*.

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Electronic supplementary material

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