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Homologous recombination and retention of a single form of most genes shape the highly chimeric mitochondrial genome of a cybrid plant

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Summary

• The structure and evolution of angiosperm mitochondrial genomes are driven by extremely high rates of recombination and rearrangement. An excellent experimental system for studying these events is offered by cybrid plants, in which parental mitochondria usually fuse and their genomes recombine. Little is known about the extent, nature, and consequences of mitochondrial recombination in these plants.

• We conducted the first study in which the organellar genomes of a cybrid – between *Nicotiana tabacum* and *Hyoscyamus niger* – were sequenced and compared to those of its parents.

• This cybrid mitochondrial genome is highly recombinant, reflecting at least 30 crossovers and five gene conversions between its parental genomes. It is also surprisingly large (41 and 64% larger than the parental genomes), yet contains single alleles for 90% of mitochondrial genes.

• Recombination produced a remarkably chimeric cybrid mitochondrial genome and occurred entirely via homologous mechanisms involving the double-strand break repair and/or break-induced replication pathways. Retention of a single form of most genes could be advantageous to minimize intracellular incompatibilities and/or reflect neutral forces that preferentially eliminate duplicated regions. We discuss the relevance of these findings to the surprisingly frequent occurrence of horizontal gene – and genome – transfer in angiosperm mitochondrial DNAs.

Keywords

chimeric; cybrid; homologous recombination; mitochondria; mtDNA; Solanaceae

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Introduction

A hallmark of angiosperm mitochondrial DNAs (mtDNAs) is their high rate of recombination and rearrangement, which is reflected in a number of unusual properties. Most angiosperm mtDNAs exhibit extraordinarily high rates of reciprocal, intra- and intermolecular recombination between large direct and inverted repeats, along with less frequent recombination across smaller repeats. On an organismal time-scale, this leads to genomes consisting of a plethora of subgenomic and multimeric structures (Palmer & Shields, 1984; Backert *et al.*, 1997; Marechal & Brisson, 2010), while on an evolutionary time-scale, it results in a highly scrambled gene order between closely related species and sometimes even within a species (Palmer & Herbon, 1988; Darracq *et al.*, 2010; Sloan *et al.*, 2012). Low-frequency recombination between short repeats is linked to the phenomenon of substoichiometric shifting of alternative configurations of the genome (Mackenzie, 2005; Arrieta-Montiel *et al.*, 2009). Of great functional and economic importance are those rearrangements that create functionally novel, chimeric genes involved in cytoplasmic male sterility (Kubo *et al.*, 2011). Finally, angiosperm mtDNAs incorporate foreign sequences remarkably often, from chloroplast and nuclear genomes of the same plant via intracellular gene transfer (Stern & Lonsdale, 1982; Knoop *et al.*, 1996), and from other plants via horizontal gene transfer (Sanchez-Puerta *et al.*, 2008; Rice *et al.*, 2013; Xi *et al.*, 2013).

Substantial progress has been made in understanding certain aspects of plant mitochondrial recombination, particularly through the use of *Arabidopsis* nuclear mutants that affect mitochondrial recombination and repair (Shedge *et al.*, 2007; Arrieta-Montiel *et al.*, 2009; Davila *et al.*, 2011; Miller-Messmer *et al.*, 2012). A major impediment to even greater understanding is the predominantly uniparental (usually maternal) inheritance of mitochondria and their genomes (Greiner & Bock, 2013), with the only known exception being two *Pelargonium* species for which predominantly biparental inheritance (in contrast to occasional paternal leakage; McCauley, 2013) has been shown (Weihe *et al.*, 2009; Apitz *et al.*, 2013). Fortunately, well-developed procedures are available in plants for creating parasexual hybrids (cybrids in particular) that overcome the sexual roadblock to studying mtDNA recombination at potentially the whole-genome level.

Somatic hybrids result from the fusion of protoplasts from two plant species (or varieties) followed by regeneration of hybrid plants containing genomes from both parents. Cybrids (cytoplasmic hybrids) are those somatic hybrids in which the nuclear genome is engineered to derive from one parent, whereas chloroplasts and mitochondria (and their genomes) follow entirely different, non-engineered pathways owing to fundamental biological differences: chloroplasts normally don't fuse with one another during plant growth and development, whereas mitochondria regularly do, sometimes massively (Arimura *et al.*, 2004; Sheahan *et al.*, 2005). Following protoplast fusion, plastids almost invariably sort out quickly (Morgan & Maliga, 1987; Earle *et al.*, 1992), such that the chloroplast population of the cybrid plant is entirely of one parental type or the other (Belliard *et al.*, 1979; Aviv *et al.*, 1984a,b). By contrast, the mitochondrial genomes of somatic hybrid plants are usually recombinant (Belliard *et al.*, 1979; Vedel *et al.*, 1986; Temple *et al.*, 1992;.

The evidence for this recombination is currently limited to detection of novel mitochondrial restriction fragments by electrophoresis of purified mtDNA, Southern blot hybridization, PCR amplification, or, only rarely, sequencing PCR fragments (Belliard *et al.*, 1979; Galun *et al.*, 1982; Nagy *et al.*, 1983; Aviv *et al.*, 1984b; Scotti *et al.*, 2004; Morgan & Maliga, 1987). Novel fragments may be formed by interparental recombination (Vedel *et al.*, 1986; Rothenberg & Hanson 1987; Temple *et al.*, 1992; Akagi *et al.*, 1995) or by selective amplification of pre-existing substoichiometric sequence arrangements (Bellaoui *et al.*, 1998; Lossl *et al.*, 1999; Rasmussen *et al.*, 2000). In only a few cases have these novel fragments actually been shown, via cloning and DNA sequencing, to be the product of recombination between the fusion parents (Vedel *et al.*, 1986; Temple *et al.*, 1992; Akagi *et al.*, 1995; Shikanai *et al.*, 1998; Scotti *et al.*, 2004). In only one case has the mitochondrial genome of a cybrid plant been sequenced (Wang *et al.*, 2012), but the unfortunate lack of a genome sequence for one of the fusion parents precludes meaningful analysis of the recombination history of this cybrid genome.

In sum, little is known about the overall extent of mitochondrial recombination in cybrids, the mechanisms involved, and the constraints and consequences operating on mtDNA recombination in the context of nuclear-cytoplasmic interactions and incompatibilities. This knowledge gap makes cybrids an important subject for genome-level studies of mtDNA recombination and evolutionary dynamics, as well as nuclear-cytoplasmic coordination of gene expression and development (Levin, 2003; Greiner & Bock, 2013). To help fill this gap, we carried out a whole-genome sequencing study of a cybrid plant – the product of fusion between protoplasts from two distantly related members of the Solanaceae, *Nicotiana tabacum* (Nt; tobacco) and *Hyoscyamus niger* (Hn; henbane) (Fig. 1). These plants last shared common ancestry approximately 24 million years ago (Sarkinen *et al.*, 2013). We carefully compared the cybrid mitochondrial genome to those of both parents (one of them also sequenced as part of this study) in order to address the following questions concerning plant mtDNA recombination: (1) How many recombination events (crossovers and gene conversions) occurred between the parental genomes to form the cybrid genome? (2) What is the balance between homologous and illegitimate recombination in generating these events? (3) Overall, what fraction of the cybrid genome is derived from one parent vs the other? (4) How much duplication – in the sense of retention of homologous regions (including genes) from both parents – is created by whole-genome recombination? (5) Does the cybrid contain chimeric and likely functional genes, created by homologous recombination between cognate parental genes? (6) Is there any bias towards retention of mitochondrial genes from Nt, whose nuclear genome predominates in the cybrid, presumably in order to preserve nuclear-cytoplasmic compatibilities?

Materials and Methods

Genome sequencing, assembly and validation

Seeds of *Hyoscyamus niger* L. collection #A04750027 were obtained from the Nijmegen Botanical Garden (the Netherlands). Total DNA was extracted from leaves of individual plants of Hn and the cybrid Nt(+Hn) (Zubko *et al.*, 1996) using a cetyl-trimethyl-ammonium-bromide DNA-extraction protocol (Doyle & Doyle, 1987) and sequenced at the

Beijing Genomics Institute using Illumina Hiseq2000 sequencing technology (Illumina Inc.). This produced 70 million clean paired-end reads of 100 bp for each plant. Paired-end reads were assembled using the Velvet assembler 1.2.03 (Daniel Zerbino, European Bioinformatics Institute, Cambridge, UK) on the Mason large-memory computer cluster at Indiana University-Bloomington (IN, USA). To optimize parameters for the assembly (hash value, coverage cutoff, expected coverage), the program Velvet Optimiser 2.2 was used (Simon Gladman, CSIRO and Monash University, Australia). We conducted several independent Velvet assembly runs without scaffolding and using different assembly parameters each time. Taking advantage of the differences in read depths between nuclear (read depth <5) and organelle contigs, putative chloroplast (read depth >150) and mitochondrial (read depth between 20 and 150) contigs were separated. All chloroplast contigs from the set of Velvet runs of the cybrid were combined, as were all mitochondrial contigs of Hn and of the cybrid. The published cpDNA sequence of *H. niger* (Sanchez-Puerta & Abbona, 2014) is available in GenBank (KF248009). Genome assemblies and read-pair mapping patterns were visually inspected using Consed v26 (Gordon & Green, 2013). Mitochondrial contigs were manually connected into larger contigs using Sequencher 5.2 (Gene Codes Corporation, USA) based on consistent paired-end reads visualized in Consed v26. The DNA libraries had a mean size, estimated by Consed v26, of 768 bp (SD = 266 bp) for the cybrid mtDNA and 888 bp (SD = 200 bp) for Hn mtDNA. The resulting organellar genome assemblies were compared against all Illumina reads to identify errors until no errors remained, i.e., until the high-quality read depth and paired-end read depth were as expected at each base and the high quality mismatches were very low or zero.

To test whether the cybrid mitochondria maintained intact parental mtDNAs at low stoichiometries, all Illumina paired-end reads of the cybrid were uniquely mapped to the Nt or Hn mitochondrial genomes, in two separate analyses, using Consed v26 (Gordon & Green, 2013). This led to uneven depths across the parental mtDNAs of cybrid sequence reads and consistent paired-end reads, including regions not covered by any reads, as well as regions with inconsistent reads. Thus, the entire parental mitochondrial genomes are not present in the cybrid mitochondria and the highly chimeric nature of the cybrid mtDNA is not an artifact of the assembly.

All raw sequence data are available from the NCBI Sequence Read Archive sequence database as accession number SRP044148. The Annotated Hn and cybrid mitochondrial genomes were deposited in the GenBank data libraries under accession numbers KM207685 and KM207678-KM207684.

Genome annotation

Mitochondrial contigs were annotated using Mitofy (Alverson *et al.*, 2010), the Blast tool (Altschul *et al.*, 1990), and tRNAscan (Lowe & Eddy, 1997). Graphical genome maps were generated using OGDraw software (Lohse *et al.*, 2007). The cybrid cpDNA was not annotated because it is identical to that of *H. niger* (Sanchez-Puerta & Abbona, 2014). Non-synonymous sites of RNA editing were predicted using PREP-Mt (Mower, 2005) with a cutoff value of 0.5.

Analysis of repeated sequences

Repeats larger than the DNA library size (~800 bp) were identified on the basis of the total read depth and consistent paired-end read depth calculated by Consed v26 (Gordon & Green, 2013). To determine repeat boundaries, we examined the reads flanking the region with an increased total read depth, in which we found reads that shared only part of their sequence, indicating the limits of a repeat region. Repeats shorter than 800 bp were detected in Consed by inspecting inconsistent and consistent reads and by pairwise Blastn analyses (using the default settings) of each genome with itself.

Comparisons between genomes

Chloroplast genomes were compared with Blastn using the default settings. Pairwise analyses of mitochondrial genomes were conducted using the Blast2seq tool and visualized with Genome Workbench. Putative interparental recombination events in the cybrid mtDNA were inferred by visual inspection of the Blast results and then tested by two computer programs: SBP (Single BreakPoint Recombination) and GeneConv. The statistical test SBP as implemented in the HyPhy software package (Kosakovsky Pond *et al.*, 2005) was used to detect crossovers in the cybrid mtDNA by comparing it to the parental mitochondrial sequences. For those cases of putative recombination without crossing over, aligned mitochondrial regions of the cybrid and both parents were analyzed for evidence of gene conversion by the program GeneConv (Sawyer, 1989). Putative gene conversion events were identified visually using the criterion that three or more polymorphisms were shared by the cybrid and one parent within a region of the cybrid genome that was otherwise clearly derived from the other parent. Of the eight putative conversion events identified in this manner, GeneConv detected a gene conversion event in five cases with a p-value <0.01 in the global analyses with no mismatches. Genes were aligned and coding differences between Nt and Hn were scored as synonymous or non-synonymous substitutions using MacClade 4.07 (Maddison & Maddison, 2000).

cox1 transcript analysis

Total RNA was isolated using the RNeasy Plant Mini kit (QIAGEN). RNA was treated with DNase (Invitrogen) and then reverse-transcribed using the SuperScript III First Strand Synthesis System (Invitrogen) with random hexamers. *cox1*-specific cDNA products were amplified and sequenced using primers (5’-GGAGCAGTTGATTTAGC-3’ and 5’-GAGCAATGTCTAGCCC-3’) designed to not contain any predicted editing sites. RNA editing sites were determined by comparison of cDNA sequences to genomic sequences.

Results

The *Hyoscyamus niger* mitochondrial genome and comparison of parental organelle genomes

We sequenced and assembled the mitochondrial genome of Hn to enable genomic comparisons among both parents and the cybrid. The organellar genomes of Nt were sequenced previously (Shinozaki *et al.*, 1986; Sugiyama *et al.*, 2005), as was the chloroplast DNA (cpDNA) of Hn (Sanchez-Puerta & Abbona, 2014). The Hn cpDNA is highly similar

to that of Nt in size and in sequence (98.4% identity excluding gaps), and the two genomes are identical in gene order and gene and intron content (Table 1).

The mitochondrial genome of Hn assembled into a single linear molecule of 501,401 bp in length (Fig. 2b). This compares to a circular assembly of 430,597 bp (shown linearized in Fig. 2a) for the mtDNA of Nt, even though *in vivo* it forms a multipartite structure composed of subgenomic circular and branched molecules (Dale *et al.*, 1983; Oldenburg & Bendich, 1996; Sugiyama *et al.*, 2005). PCR across the ends of the Hn linear assembly did not yield any amplification product, but, based on paired-end reads, the ends recombine at a detectable frequency with other regions of the genome, forming alternative genome arrangements. Many additional arrangements of the Hn genome also occur *in vivo*, as the result of frequent recombination between the 15 largest (>250 bp) and best-matched (>90% identity) repeats present in the genome. This recombination was inferred for the four 'large' repeats (2.6–12.5 kb in length; Fig. 2b and Supporting Information S1A) by the presence in the assembly of alternative, recombination-diagnostic (Palmer & Shields, 1984) arrangements of unique sequences flanking the ends of these repeats and for the 11 'intermediate' (250–800 bp) repeats by detecting inconsistent paired-end reads. The Hn genome contains >190 'small' (100-250 bp), mostly imperfect repeats. Nt mtDNA contains a similar number and amount of large repeats (Fig. 2a), but only one intermediate repeat and 30 small repeats (Fig. S1B). Except for a few 100-bp repeats shared by both genomes, the Hn and Nt genomes do not contain any of the same repeats.

The Hn mtDNA contains the same 37 intact protein genes (not counting duplicates) and 23 group II introns (Table 1) as found in Nt mtDNA (Sugiyama *et al.*, 2005). In addition, Hn contains a single group I intron (in *cox1*), whereas Nt contains no group I introns. Three of the 37 protein genes (*ccmC*, *nad4L* and *sdh4*) are identical in Nt and Hn, while the rest have an identity >97.9% (Table 2). The two genomes share 99% of RNA editing sites (449) with only six species-specific editing sites.

The amount and synteny of DNA sequences shared by the Hn and Nt mitochondrial genomes were assessed by pairwise Blast analysis. Despite their relatively similar sizes (501 and 431 kb), but in accord with observations made for other comparably related angiosperm pairs (Handa, 2003; Liu *et al.*, 2013; Jo *et al.*, 2014), many regions in both genomes are not present in the other genome, and those that are shared are highly rearranged (Figs 2, S1C). Excluding repeats, the two genomes have 203 kb of DNA in common; this represents 45% and 52% of the Hn and Nt genomes, respectively (Fig. 2). Unique and shared sequences are highly interspersed in these two genomes (Fig. 2). Shared regions include, but are by no means limited to, all mitochondrial genes and their flanking sequences and are highly similar, with an average sequence identity of 98.6% (excluding gaps).

A Blast search was performed to identify the sources of the 55% of the Hn genome that is not homologous to Nt mtDNA. Only *c*. 5% of the Hn mitochondrial genome is homologous to sequences in other plant mitochondrial genomes (but not in Nt), ~1.5% is homologous to plastid DNA not present in Nt mtDNA, <0.1% is homologous to nuclear DNA not present in Nt mtDNA, and the rest (~48%) did not show similarity to any sequence in the NCBI databases.

Cybrid Nt(+Hn) organellar genomes

The green cybrid line Nt(+Hn) FH-4 chosen for this study was produced by fusion of mesophyll protoplasts (with no chemical treatment or irradiation) from two Solanaceae species (Fig. 1): an albino plastid genome mutant of Nt and wild-type Hn (Zubko *et al.*, 1996).This cybrid line is characterized by corolla-containing flowers, regular morphology, and phenotypic similarity to Nt (Zubko *et al.*, 1996), accompanied by slight pigment deficiency and retarded growth during early development (Zubko *et al.*, 2001). Its flowers (Fig. 1) are deficient in fertile pollen grains (a cytoplasmic-male-sterility phenotype) but are capable of seed production after backcrossing (Zubko *et al.*, 1996). The line FH-4 was backcrossed four times with wild type Nt, which resulted in a nuclear genome containing the Nt chromosome number (2N = 48, compared to 2N = 34 in Hn) and consisting predominantly of Nt sequences (Zubko *et al.*, 1996) (hence the 'Nt(+Hn)' designation of this cybrid). After recurrent backcrosses, sorting out of different mitochondrial types should be complete such that the mitochondrial genome is stable and largely homogeneous within each cybrid plant (Morgan & Maliga, 1987; Earle, 1995). Southern blot experiments showed that the FH-4 cybrid appears to have the chloroplast genome of Hn, but a recombinant mitochondrial genome (Zubko *et al.*, 1996, 2001).

As expected, the cybrid chloroplast genome is derived from Hn; indeed, the two chloroplast genomes are identical with no substitutions, indels or rearrangements (Table 1). The cybrid mitochondrial genome assembled into seven contigs totaling 705,036 bp in size (Fig. 3). The size of the cybrid mtDNA is intermediate in comparison to the parental mtDNAs (501 and 431 kb) and the sum of the parental genomes (~932 kb). All mitochondrial contigs, except for the smallest one, are related to each other through large and intermediate repeats (Fig. 3). In addition, based on paired-end reads, the ends of all contigs appear to recombine with regions in other contigs, producing alternative genome structures. In light of previous observations (Oldenburg & Bendich, 1996; Grewe *et al.*, 2009; Marechal & Brisson, 2010; Hecht *et al.*, 2011; Mower *et al.*, 2012b; Sloan, 2013; Gualberto *et al.*, 2014;), the cybrid Nt(+Hn) mtDNA, like other angiosperm mitochondrial genomes, probably consists *in vivo* of a dynamically-changing population of alternative structures, such as overlapping linear and branched molecules.

Repeats larger than 800 bp (library size) were identified by an increase in read depth in preliminary assemblies. The final assembly of the cybrid mitochondrial contigs shows a relatively even distribution of total reads (average read depth is 107; Fig. S2). Twenty-two repeats are larger than 500 bp and seven are 250–500 bp in length, while >80 are 100–250 bp in length (Fig. 4a). Repeats amount to almost 33% of the cybrid mtDNA (Table 1) and originated in three ways: (1) by duplication (the majority of repeats), i.e., by keeping two copies of a region present only once in a parental donor; (2) by retention of both copies of repeats already present in one or the other parental genome, and (3) by retention of homologous, single-copy regions from both parents, thereby creating 'heterologous repeats.'

Recombination events and the origin of the cybrid mitochondrial genome

The cybrid mtDNA is highly chimeric (Fig. 3), as a result of at least 35 intergenomic recombination events between the parental mitochondrial genomes (Table 3). Consistent

with its 'intermediate' size (see previous section), the cybrid mtDNA does not contain the entire parental mitochondrial genomes, only a subset of each of them. The cybrid mtDNA contains longer tracts of homology to Nt mtDNA than to Hn mtDNA (Fig. 3, see 'Blast hits', and Fig. 4b,c).

No novel sequences were identified in the cybrid mtDNA, 405.9 kb (57.6%) of which came from Nt and 299.1 kb (42.4%) from Hn (Fig. 3). Sequence identity between each cybrid region and the corresponding parental region is extremely high (99.8-100%, with an average identity of 99.95%). The parental plants of the cybrid (*N. tabacum* R100a1 mutant and an ecotype of *H. niger* from Marburg Botanical Garden) are not the same as the Nt and Hn plants (*H. niger* A04750027 and *N. tabacum* cultivar Bright Yellow 4) whose sequenced organelle genomes were used for comparison to the cybrid genomes. However, the extremely high level of nucleotide identity between the cybrid mtDNA regions and the cognate region(s) in the sequenced Nt and/or Hn mtDNAs shows that the two different varieties of Nt and of Hn are virtually identical in mtDNA sequence. Furthermore, the chloroplast genomes of the cybrid and the sequenced strain of Hn are identical (see previous section).

Thirty cases of crossing-over were readily identified because parental-unique sequences were found at each side of the inferred crossover (Fig. 3). Five cases of gene conversion without crossing-over were detected at the $P<0.01$ level by GeneConv (Table 3). We did not find evidence for illegitimate recombination, i.e. non-homologous end joining (NHEJ). Eight of the 35 recombination events took place within gene sequences, with the rest occurring in intergenic regions (Table 3; Fig. 3). On average, there was one intergenomic recombination event every 20.1 kb of the resulting cybrid genome. The sites of recombination are not evenly distributed along the cybrid mtDNA: the closest pair of adjacent sites is only 0.6 kb apart, while the farthest is 64 kb apart (Fig. 3).

All detectable recombination events occurred in areas where parental mitochondrial sequences overlapped, i.e., were homologous (Fig. 3, see 'Blast hits'). Overlapping homologous sequences from the fusion parents became repeats when the parental mitochondria fused, enabling homologous recombination to occur between their genomes. The homologous regions that engaged in the 35 intergenomic recombination events that were detected in the cybrid mitochondrial genome ranged between 100 bp and 9 kb in length, with an identity of at least >94% and usually much higher (Table 3). For 27 of the 30 crossover events, a single product was found at a detectable stoichiometry in the cybrid mtDNA, whereas in the other three cases two reciprocal recombination products were detected, and at similar stoichiometries (Table 3, events # 1, 15, and 17).

Gene content in the cybrid mitochondrial genome

Counting each gene only once, the cybrid mitochondrial genome contains 37 protein genes, three rRNA genes, and 23 tRNA genes (Table 1). Intron content is identical to Hn and includes 23 group II introns and one group I intron. The cybrid genome contains two copies of 15 genes and three copies of one gene. Six of the two-copy cases (*atp1*, *atp9*, *trnK*, *trnN*, *trnQ*, *trnS*) result from retention of either entire genes or partial genes in the form of intact

chimeric genes (e.g. see *atp1* in Table 2) from both parents. All other multi-copy cases result from duplication or triplication of genes inherited from only a single parent (Table 2; Fig. 3).

The cybrid genome possesses a total of 45 and 26 genes that were inherited from Nt and Hn, respectively. It also contains nine genes that are chimeric as a result of intergenic recombination (seven protein genes and one rRNA gene (Table 2), plus one tRNA gene (not tabulated)). Counting each gene only once, the cybrid mtDNA contains 21 protein, two rRNA, and 11 tRNA genes that were inherited exclusively from Nt, as compared to only eight protein and eight tRNA genes from Hn (Table 2). Of the 16 genes inherited exclusively from Hn, eight tRNA genes and one protein gene are identical to those in Nt and probably don't cause any nuclear-cytoplasmic incompatibility. By contrast, the other seven mitochondrial genes inherited only from Hn possess non-synonymous differences in coding sequences and, in two cases, differences in intron content and/or editing sites (Table 2).

Of special note, the cybrid *cox1* gene, inherited from Hn, has a group I intron, which likely requires a nuclear factor for its splicing because its homolog in yeast mitochondria does not self-splice (Dujardin *et al.* 1982; Herbert *et al.* 1988). We showed that the *cox1* gene is spliced and edited in the cybrid, which indicates that the cybrid possesses the necessary machinery for proper processing of the *cox1* primary transcript (Fig. S3). Note that the Hn *cox1* gene lacks any species-specific editing sites, whereas the Nt homolog contains two such sites.

Discussion

Generating new nuclear-cytoplasmic combinations in cybridization experiments is an important avenue for modeling evolution of the cytoplasm (Greiner & Bock 2013). It has been known for over three decades that somatic hybridization often results in novel mitochondrial genome arrangements (Belliard *et al.,* 1979; Pelletier *et al.,* 1985; Vedel *et al.,* 1986). Yet this is the first study in which complete mitochondrial sequences from a cybrid and both of its parents are compared, allowing the first detailed examination of the extent and nature of recombination between two plant mitochondrial genomes.

The cybrid mitochondrial genome is unexpectedly large and highly chimeric

The cybrid mtDNA is ~705 kb in length, which is 227 kb less than the sum of its parental mtDNAs (932 kb) but considerably larger than each alone (431 and 501 kb). It is also remarkably chimeric as a result of at least 30 crossover and five gene conversion events between its parental genomes. Based on cytological studies of plant mitochondrial fusion (Arimura *et al.,* 2004; Sheahan *et al.,* 2005), we assume that during the formation of the cybrid, mitochondria from both parents fused massively, bringing both parental genomes into a single mitochondrion and thus enabling extensive recombination of the two genomes, accompanied (or followed) by loss of some parental sequences (Fig. 5). Some 57.6% and 42.4% of the cybrid mtDNA came from Nt and Hn, respectively; this corresponds to the loss of ~55 kb worth of Nt mitochondrial sequences and ~202 kb of the Hn mtDNA (Fig. 5).

The size of a hybrid mitochondrial genome relative to its parental genomes has been previously estimated in 12 *Brassica* somatic hybrids. In these cases, the hybrid mtDNA was

either intermediate in size to its parental mtDNAs or at most 11% larger than the larger parental mtDNA (Temple *et al.*, 1992; Wang *et al.*, 2012). Strikingly, the Solanaceae cybrid mtDNA in this study is fully 41% and 64% larger than the Hn and Nt genomes, respectively. Mitochondrial genome size in somatic hybrids or cybrids is influenced by several factors, including: (1) cell type selected for protoplast isolation (particular cells may differ in the amount of mitochondria they contain and their ability to undergo massive mitochondrial fusion); (2) protoplast pre-treatments (chemical treatment or irradiation of protoplasts before fusion could inactivate or eliminate mitochondria from one parent); (3) nuclear genome composition (nuclear-mitochondrial interactions may impose a bias towards mitochondrial sequence retention or elimination); (4) recombination rate and efficiency between the parental mtDNAs depending on their overall synteny and similarity; and (5) culture, fusion and selection procedures (Earle *et al.*, 1992; Sheahan *et al.*, 2005; Preuten *et al.*, 2010; Greiner & Bock, 2013;). Genome sequencing of many more cybrid mtDNAs, of diverse parentage and method of generation, is necessary to determine whether this Solanaceae cybrid genome is indeed unusually large, whether other cybrid genomes might be proportionately even larger, and whether any general patterns emerge with respect to cybrid genome size relative to parental genome sizes.

Mitochondrial rearrangements occurred only by homologous recombination Homologous recombination in plant mitochondria is restricted to sequences of low divergence. The mismatch repair system does not allow recombination between sequences that are highly divergent (Shedge *et al.*, 2007; Arrieta-Montiel *et al.*, 2009), acting as a safeguard to prevent deleterious recombination between non-homologous regions. Homologous recombination in plant mitochondria is mediated by nuclear-encoded *recA* homologs (Miller-Messmer *et al.*, 2012). These events are classified as either crossovers, in which both strands of each homologous duplex are reciprocally broken and joined, so that alleles flanking the break site are exchanged; or non-crossovers, i.e., gene conversions, in which there is no reciprocal exchange and instead a non-reciprocal transfer of sequence information occurs from one homologous DNA segment to the other, with the donor sequence unchanged (Nieto Feliner & Rosello, 2012). The minimal pairing sequence of bacterial *recA* can be as small as 25 bp, but efficiency greatly increases with lengths larger than 200 bp (Gualberto *et al.*, 2014). Knowing the minimum repeat length necessary for recombination in the mitochondrial genome is important for understanding how they rearrange over time and for identifying the mechanism(s) of these recombination events.

We posit that in the cybrid under study, homologous recombination took place across repeats that originated when homologous sequences from both parents were incorporated in the cybrid mitochondrial genome. The homologous regions that engaged in the 35 intergenomic recombination events that were detected in the cybrid mitochondrial genome ranged between 100 bp and 9 kb in length. Three of the five gene-conversion events detected extended for a minimum of 1kb (Table 3). These are longer than previous reports on plant mitochondria, which identified a converted region of no more than a few hundred nucleotides long (Hao & Palmer, 2009; Hao *et al.*, 2010; Mower *et al.*, 2010; Sloan *et al.*, 2010). However, it is difficult to distinguish a long gene conversion from multiple crossover events because multiple crossovers mimic gene conversion (Santoyo *et al.*, 2005).

Only a single recombinant product was detected for 27 of the observed crossover events, including all but one of the 19 crossovers that involve large (>1 kb) repeats (Table 3). This is surprising in the sense that in non-cybrid angiosperm mitochondrial genomes, repeats of this size are expected to mediate high frequency, reciprocal homologous recombination (Kuhn & Gualberto, 2012). This finding raises the possibility that recombination in the cybrid occurred in a predominantly non-reciprocal manner, presumably via the break-induced replication pathway (Marechal & Brisson 2010; Shedge *et al.*, 2007). Alternatively, most or all crossovers may have occurred reciprocally, probably via the classic double-strand break repair pathway (Marechal & Brisson, 2010; Kuhn & Gualberto, 2012), but with only one of the recombination products retained at a detectable level in the cybrid.

Non-homologous end joining (NHEJ) refers to the ligation of two nonhomologous DNA ends, which may show regions of microhomology (Deriano & Roth, 2013). This process is suppressed by the nuclear gene MSH1 in *Arabidopsis* (Davila *et al.*, 2011). No evidence for non-homologous (illegitimate) recombination was found in the cybrid Nt(+Hn) mitochondria. The absence of illegitimate recombination implies that homologous sequences were incorporated by recombination into this cybrid genome far more efficiently than non-homologous sequences.

Biased sequence loss in the cybrid mitochondrial genome

The cybrid mitochondrial genome shows two pronounced biases in sequence loss (Fig. 5). The first is related to parental origin: the cybrid genome has lost fully 40% (202 kb) of sequences from Hn but only 13% (55.1 kb) of Nt sequences. This could be the result of an unequal contribution of mitochondrial genome copies during protoplast fusion, i.e., the Nt protoplast may have contributed many more mtDNA copies than did Hn, such that the initial recombination events produced a cybrid genome that was biased toward Nt from the outset. Alternatively, the Nt-derived nuclear genome of the cybrid may have preferentially selected for retention of Nt mitochondrial sequences (especially genes) owing to nuclear-cytoplasmic incompatibilities with certain Hn sequences. This would be consistent with similar observations made in other cybrids (Wachocki *et al.*, 1991; Yamaoka *et al.*, 2000; Dey *et al.*, 2000) and in introgressed populations (Burton *et al.*, 2006).

The other bias in sequence loss involves duplicated vs unique sequences. The cybrid mtDNA has lost one copy of 94% (191 kb) of the 203 kb of sequences that are shared by the parental genomes and which presumably were initially present as ‘heterologous’ duplications in the cybrid. By contrast, only 11% (58.6 kb) of the 526 kb of the sequences that are unique to the parental genomes were lost in the cybrid (Fig. 5). This bias may reflect non-adaptive molecular mechanisms of sequence elimination that preferentially remove duplicated sequences. These mechanisms include reciprocal and non-reciprocal homologous crossovers between repeated sequences, gene conversion between homeoalleles, and illegitimate recombination (Shaked *et al.*, 2001; Bennetzen *et al.*, 2005; Shedge *et al.*, 2007; Grover & Wendel, 2010; Davila *et al.*, 2011; Leitch & Leitch, 2013). Alternatively, adaptive forces may be at work, involving deleterious effects of keeping potentially conflicting copies of coding regions derived from each parent. Relevant here is the striking pattern of allele retention in the cybrid mtDNA, for which 57 of the 63 mitochondrial genes (including 35 of

37 protein-coding genes) were found in a single form, derived from either a single parent or chimeric. In other words, 57 alleles of 63 genes were lost by the cybrid mitochondria.

Previous studies on somatic hybrids and cybrids showed a similar pattern of single-allele retention, although only one or a few genes per hybrid were analyzed in most of these studies (Temple *et al.,* 1992; Bonnema *et al.,* 1995; Motomura *et al.,* 1996; Kanno *et al.,* 1997; Moriguchi *et al.,* 1997; Cardi *et al.,* 1999; Lossl *et al.,* 1999; Rasmussen *et al.* 2000; Bastia *et al.,* 2001; Scotti *et al.* 2004). Also, a genomic study (Wang et al., 2012) on a *Brassica* cybrid showed that its mitochondrial genome contained only 59 genes, very similar to the gene content in each parental mtDNA (Tanaka *et al.,* 2012), although the parental origin of the cybrid genes was not determined.

We propose that the co-existence of different alleles in the mtDNA of somatic hybrids and cybrids may be deleterious, as they might impair genetic homeostasis or global gene expression, resulting in an improved fitness of those hybrids that more efficiently eliminate these extra alleles. During this process of gene elimination, non-coding duplicated regions flanking the genes may also be deleted, resulting in an overall loss of duplicated regions.

This selective process probably also affects plant mtDNAs that acquire foreign mitochondrial genes by horizontal gene transfer (HGT). In the majority of known cases, a single allele of each mitochondrial gene - either the native, the foreign, or a chimeric form - has been retained (Bergthorsson *et al.,* 2003; Barkman *et al.,* 2007; Hao *et al.,* 2010; Mower *et al.,* 2010; Xi *et al.,* 2013). The few angiosperms that maintain both foreign and native alleles appear to overcome the putative selective disadvantage of keeping two distinct alleles by silencing and/or rapid pseudogenization of one allele or the other, almost always the foreign one (Mower *et al.,* 2004, 2010; Rice *et al.* 2013).

Further studies are necessary to establish whether the biased elimination of repeated sequences and genes seen in the Nt(+Hn) cybrid is due primarily to a selective advantage of having single alleles or to largely neutral molecular mechanisms that remove duplicated sequences from cybrid mitochondrial genomes.

Nuclear-cytoplasmic incompatibility

Nuclear-cytoplasmic incompatibilities are expected when the nuclear and cytoplasmic genomes have a different origin (Greiner & Bock, 2013). Some organelle genes in angiosperms are subjected to C-to-U RNA editing, which restores the conserved amino acid sequence (Freyer *et al.,* 1997). This editing is controlled by the nuclear genome, opening the door to nuclear-cytoplasmic incompatibilities (Schmitz-Linneweber *et al.,* 2005). The Nt-derived nuclear-encoded editing machinery in the cybrid Nt(+Hn) faces the challenge of editing three Hn-specific sites of chloroplast RNA editing, one in each of three genes (*ndhA*, *ndhD*, and *rpoB*) in which the cybrid (this study) and Hn (Sanchez-Puerta & Abbona, 2014) cpDNAs are predicted to undergo RNA editing at sites at which Nt cpDNA encodes a T and does not require editing. Those edits restore what would otherwise be non-synonymous changes at highly conserved amino acids. The Hn-specific editing sites in the cybrid cpDNA are presumably recognized and edited by the Nt nuclear-encoded machinery, although perhaps with reduced efficiency, given that the cybrid Nt(+Hn) is pale green in early

development but photosynthetically functional (Zubko *et al.,* 2001). Indeed, a study on *N. tabacum (+Atropa belladonna*) cybrids showed experimentally that the same three editing sites that are specific to Hn-derived sequences present in our cybrid were successfully edited by the Nt nuclear–encoded machinery in *Atropa* chloroplast transcripts (Schmitz-Linneweber *et al.,* 2005). It is believed that the Nt nuclear genome has additional editing factors and thus is capable of heterologous editing (Schmitz-Linneweber *et al.,* 2005). In an extreme case of nuclear-cytoplasmic incompatibility, cybrids *Atropa belladonna* (+Nt) with the Nt plastid genome manifested an albino phenotype (Kushnir *et al.,* 1991). These cybrids failed to edit all or some of the five Nt-specific editing sites in four chloroplast genes (Schmitz-Linneweber *et al.,* 2005).

Nuclear-mitochondrial incompatibilities are also expected in the cybrid under study given the observed differences in intron content, editing sites, and non-synonymous sites in those mitochondrial genes inherited exclusively from Hn. That the group I intron acquired from Hn was successfully spliced in the cybrid is surprising in the context of the best-studied cognate of this intron, from yeast, whose splicing requires nuclear factors (Dujardin *et al.,* 1982; Herbert *et al.,* 1988). On the other hand, this intron is well-established to have been horizontally transferred hundreds if not thousands of times among angiosperm mitochondrial genomes (Cho *et al.,* 1998; Sanchez-Puerta *et al.,* 2008, 2011), which suggests that such factor(s) were widely present in plants before the evolutionary spread of this intron (presumably because it/they play some other, important role). Other differences in Hn-derived mitochondrial genes and/or newly-formed ORFs could be related to the cytoplasmic male sterility and other phenotypic characteristics observed in the cybrid Nt(+Hn) (Zubko *et al.,* 2001). The results from this study set the stage for in-depth investigation of the expression of these genes and ORFs.

Cybrids as a model system for studying HGT in plant mitochondrial genomes

Whereas mitochondrial-to-mitochondrial HGT occurs relatively often in angiosperms, HGT is unknown in their chloroplast genomes (Mower *et al.,* 2012a; Rice *et al.,* 2013; Xi *et al.,* 2013). These evolutionary findings are consistent with evidence from both cell biology (Arimura *et al.,* 2004; Sheahan *et al.,* 2005) and genetics (Belliard *et al.,* 1979; Earle *et al.,* 1992; Morgan & Maliga, 1987; Temple *et al.* 1992) that mitochondria readily fuse with one another but not chloroplasts. These observations, plus those specific to the case of extraordinarily massive HGT in the *Amborella* mitochondrial genome (Rice *et al.,* 2013), led Rice *et al.* (2013) to propose that the entry of foreign mtDNA into angiosperm mitochondrial genomes is driven principally, if not entirely, by the ease of mitochondrial fusion. Therefore, the fusion-driven creation of a novel, chimeric mitochondrial genome in cybrids is probably quite analogous to the early stage of the creation of a novel mitochondrial genome when HGT occurs in plant mitochondria. That is, soon after the fixation of an HGT event, the mitochondrial genome probably resembles those of cybrids in containing substantial portion of both ‘parental’ genomes (i.e., the HGT donor and recipient genomes). Over evolutionary time, selection for nuclear-mitochondrial compatibilities, in conjunction with neutral deletional forces that in most plant mtDNAs result in high rates of DNA loss, likely leads to loss of most of the foreign DNA and especially (see previous section) the foreign genes.

Nuclear vs mitochondrial allopolyploidy

The potential to combine the entirety of two distinct plant mitochondrial genomes through cybrid formation makes this process somewhat analogous to the well-studied and evolutionarily important process of allopolyploidization, in which sexually hybrid plants contain two copies of each parental nuclear genome (Ozkan *et al.*, 2001; Adams *et al.*, 2003). As in cybrid mitochondrial genomes, the doubled nuclear genome in allopolyploids is rapidly reduced by elimination of significant portions of both parental nuclear genomes. In particular, allopolyploids undergo rapid loss of non-coding regions that are shared by the parental species, presumably to suppress homeologous pairing at meiosis (Shaked *et al.*, 2001; Leitch & Bennet, 2004; Doyle *et al.*, 2008). However, in contrast to the single-copy fate of most genes in cybrid mitochondria, nuclear allopolyploids generally retain both parental alleles, although often silencing one of them (D'Arcy & Zhang, 1992; Ozkan *et al.*, 2001; Adams *et al.*, 2003). Another key difference is that recombination between homeologous chromosomes in nuclear allopolyploids is usually quite limited, whereas, as shown in this study, recombination between parental mitochondrial genomes in cybrids can be extensive, creating a highly novel and intensely chimeric genome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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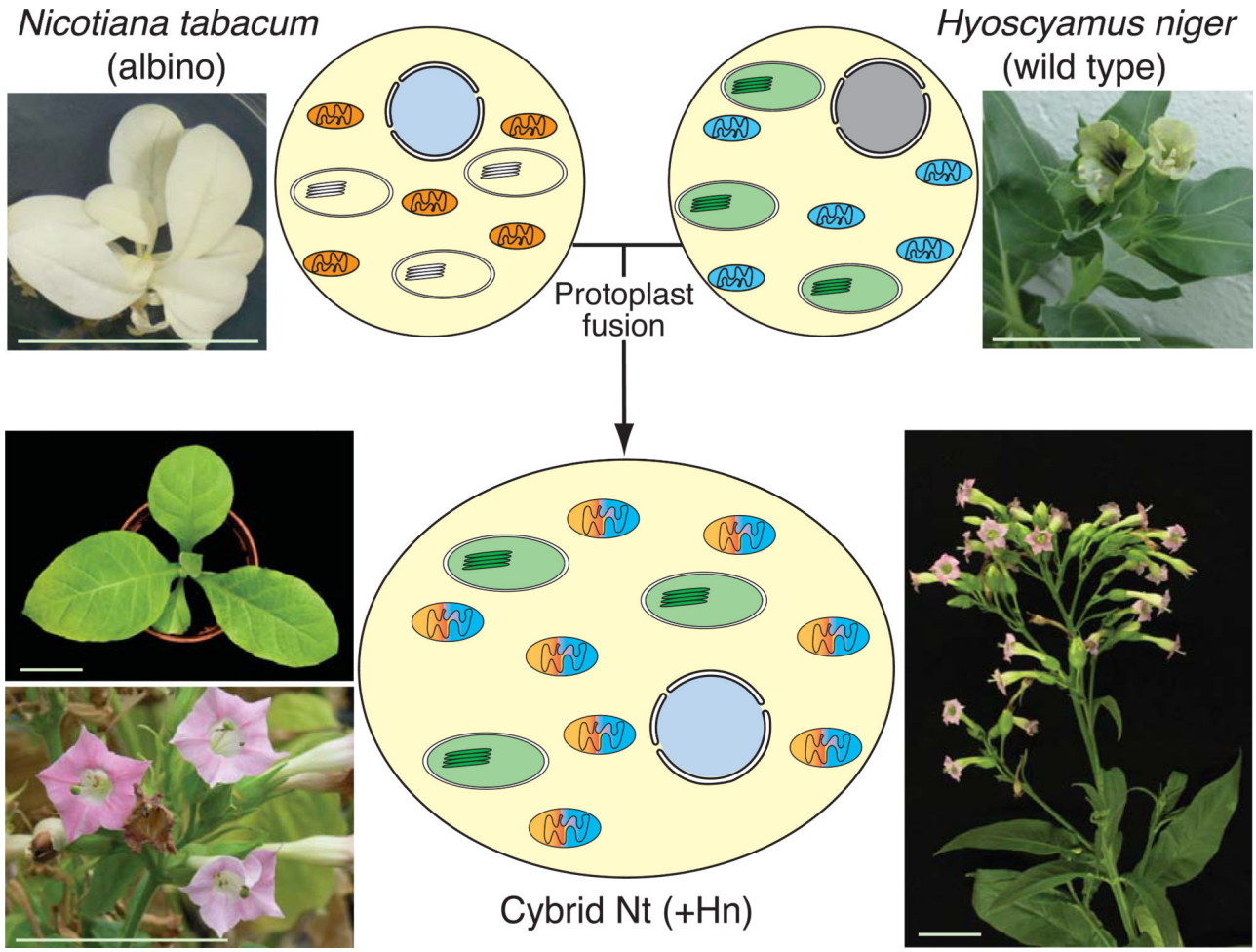

Fig. 1.

Production of a Solanaceae cybrid plant. Schematic production of a cybrid plant by chemical fusion of protoplasts from an albino mutant line of *Nicotiana tabacum* (Nt) and a wild type line of *Hyoscyamus niger* (Hn; Zubko *et al*., 1996). Green hybrid plants were backcrossed at least four times to wild type Nt (Zubko *et al*., 1996, 2001). The resulting cybrid plant has green chloroplasts derived from Hn, recombinant mitochondria derived from both parents, and a nuclear genome consisting predominantly of Nt sequences (Zubko *et al*., 1996). Bars: 5 cm, except top-left panel, 3 cm.

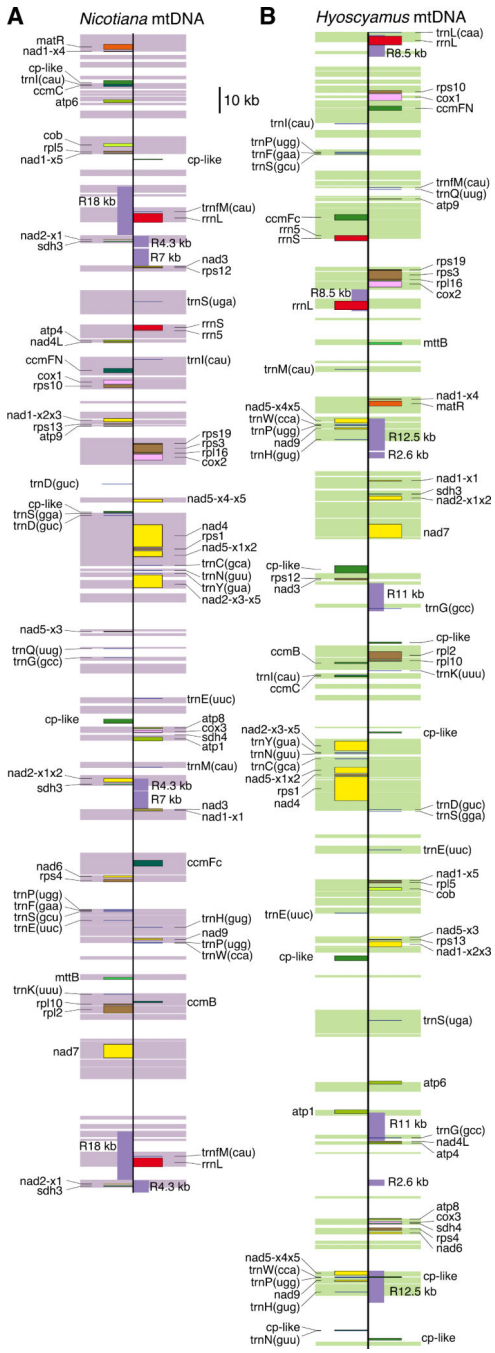

Fig. 2.

Parental mitochondrial genomes of the cybrid. Linearized, scale maps of the 430,597 and 501,401 bp mitochondrial genomes of *Nicotiana tabacum* (a) and *Hyoscyamus niger* (b), respectively. The wide violet (a) and green (b) shadings indicate regions present in the other mitochondrial genome, i.e., these represent the 203 kb of DNA shared between the two genomes. Shown are all full-length genes, repeats ≥1 kb in length (purple boxes labeled 'R', followed by the repeat lengths in kb), and chloroplast-derived regions >400 bp in length

(green boxes labeled ‘cp-like’). Genes marked on different sides of the central black vertical lines are transcribed from opposite strands of the genome.

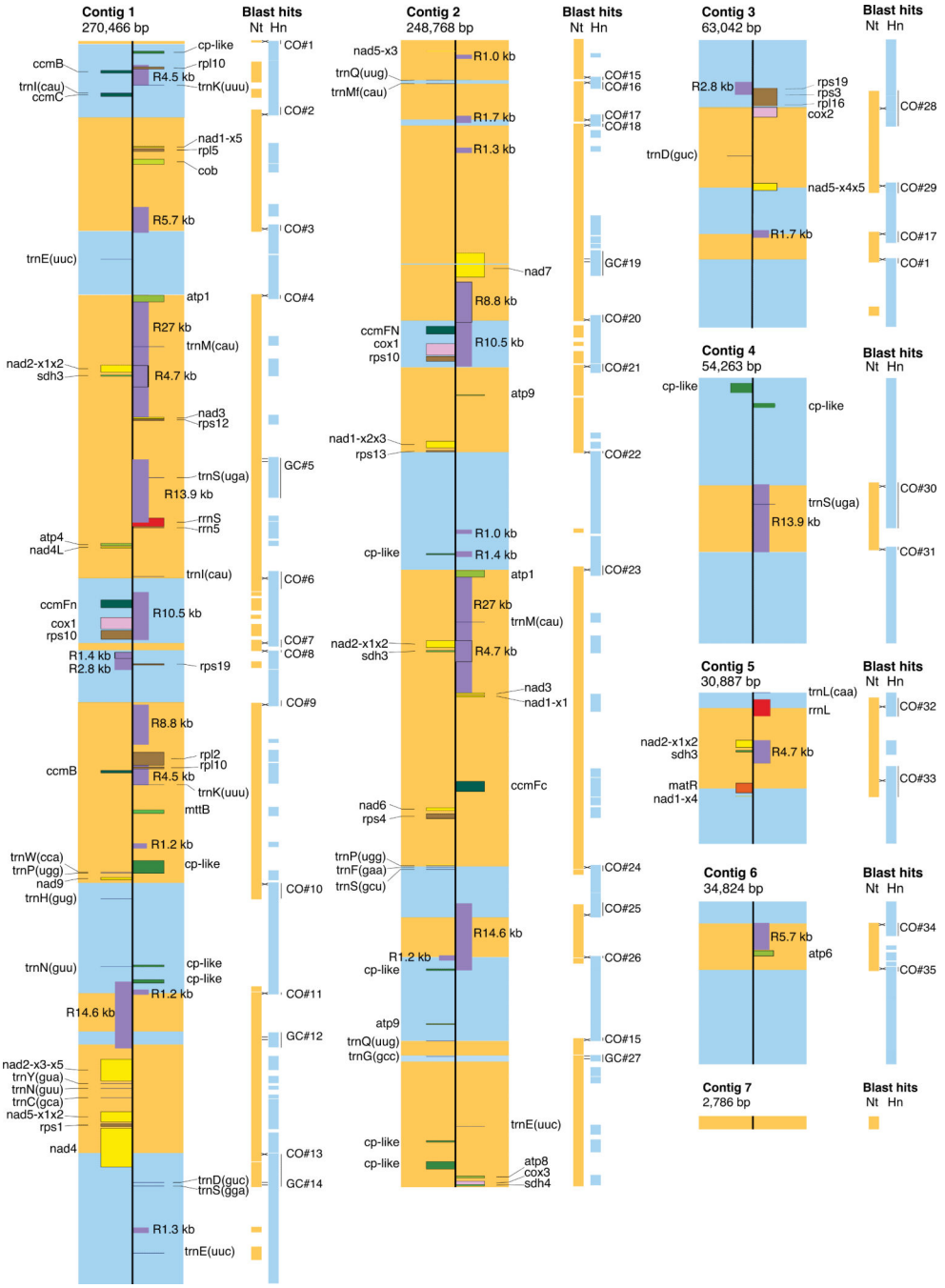

Fig. 3.

Mitochondrial genome of the cybrid Nt(+Hn). Scale maps of the seven contigs that comprise the 705,036 bp mitochondrial genome of the cybrid. The wide orange and blue boxes located on the cybrid genome indicate regions inferred to derive from either the *Nicotiana tabacum* (Nt) or *Hyoscyamus niger* (Hn) parents, respectively. The thinner, orange and blue boxes to the right of the cybrid contig maps indicate regions shared (as defined by Blast hits) with the mitochondrial genomes of Nt or Hn, respectively. Numbers to the right of the blast hits designate the 35 intergenomic homologous recombination events detected in the cybrid

(also see Table 3). Crossing and parallel lines mark inferred sites of crossover (CO) and gene conversion (GC) events, respectively. The cybrid contig maps show full-length genes, repeats ≥1 kb in length (purple boxes labeled ‘R’, followed by the repeat lengths in kb), and chloroplast-derived regions >400 bp in length (green boxes labeled ‘cp-like’). Genes marked on different sides of the central vertical lines are transcribed from opposite strands of the genome.

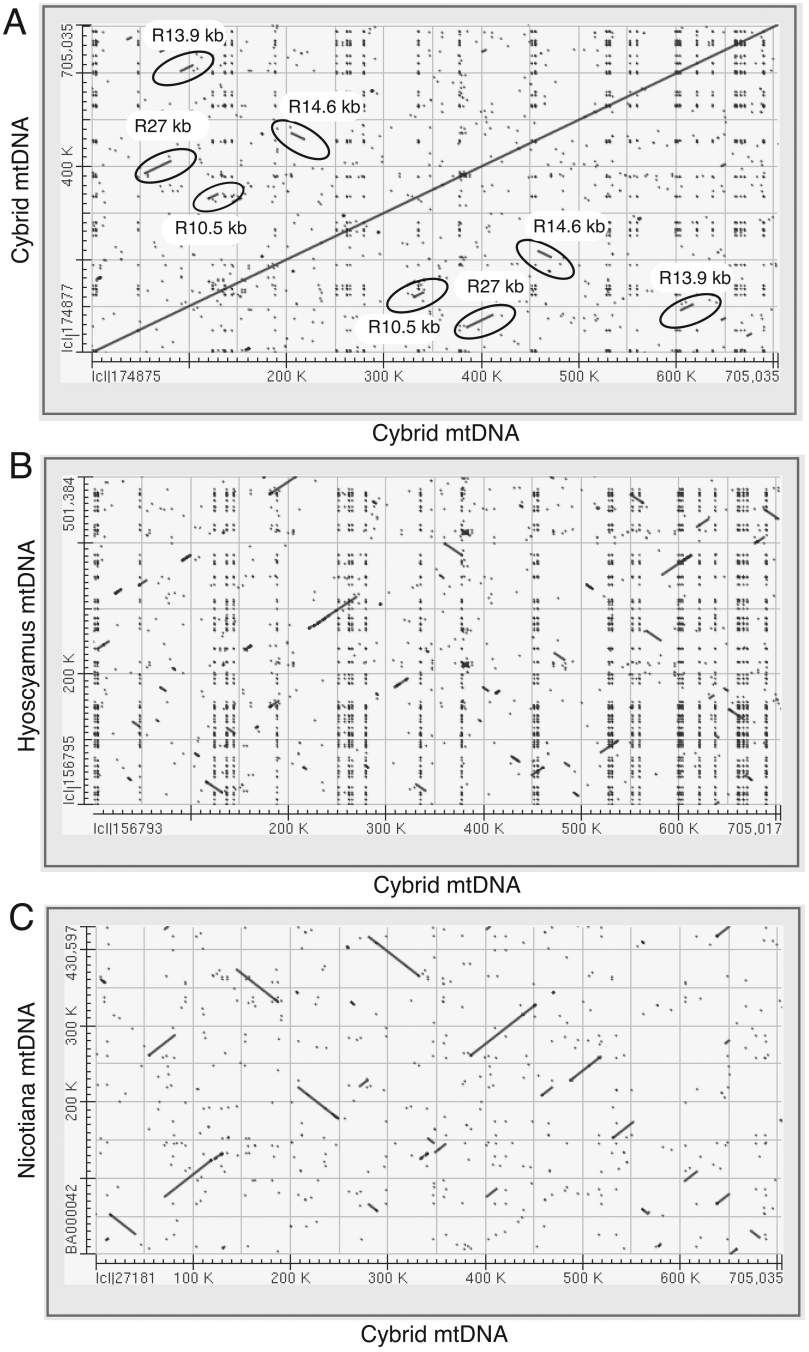

Fig. 4.

Dot-plot comparisons of mitochondrial genomes. (a) Self-self dot-plot analysis of the mitochondrial genome of the cybrid conducted to visualize repeats (ovals surround the largest repeats, which are labeled by 'R', followed by their lengths in kb). (b, c) Dot-plot comparisons of the mitochondrial genomes of the cybrid and *Hyoscyamus niger* (b), and the cybrid and *Nicotiana tabacum* (c), conducted to visualize regions of shared synteny. The cybrid mtDNA is represented by a concatenation of the seven contigs 1–7.

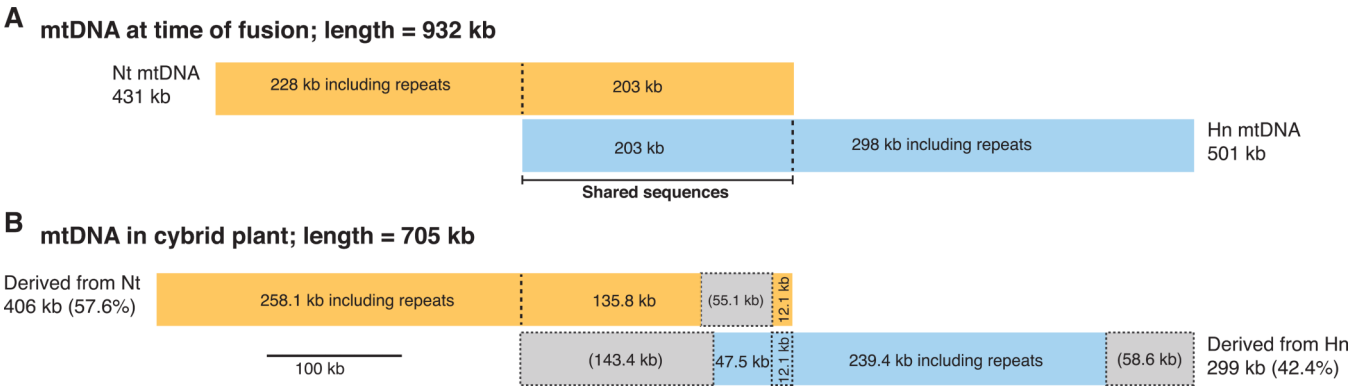

Fig. 5.

Cybrid mitochondrial genome composition. Summary status of the cybrid mitochondrial genome with respect to its parental composition either (a) soon after protoplast fusion, at which point the single fusion cell probably possessed a mitochondrial network that contained the entirety of both parental mitochondrial genomes, or (b) as determined from the mtDNA sequence of the mature cybrid plant investigated in this study. In both parts, the cybrid genome comprises the sum of the orange *Nicotiana tabacum* (Nt)-derived, and blue, *Hyoscyamus niger* (Hn)-derived, sequences. The gray regions in (b) indicate the aggregate length of parental-shared or parental-unique regions that are absent from the genome of the cybrid plant.

Table 1

Comparison of the mitochondrial and plastid genomes of the cybrid and its parents

Genome features	*Hyoscyamus niger*	*Nicotiana tabacum*	Cybrid
Mitochondrial genome			
Genome length in bp	501,401	430,597	705,036
Protein genes[a]	37 (38)	37 (40)	37 (48)
rRNA genes[a]	3 (4)	3 (4)	3 (3)
tRNA genes[a]	22 (27)[c]	22 (23)[c]	23 (29)
Group II introns	23	23	23
cis-splicing	17	17	17
trans-splicing	6	6	6
Group I introns	1	0	1
Repeats in kb (% of genome)[b]	97.4 (19.4%)	68.6 (15.9%)	229.5 (32.5%)
Short repeats in kb (100-250 bp)	20.4 (4.1%)	4.5 (1%)	12.9 (1.8%)
GC content	45.2%	45.0%	44.8%
Chloroplast-derived sequences	2.3%	2.2%	1.8%
Chloroplast genome			
Genome length in bp	155,720	155,943	155,720
Protein genes[a]	80 (85)	80 (85)	80 (85)
rRNA + tRNA genes[a]	34 (45)	34 (45)	34 (45)
Introns (all Group II)	21	21	21
GC content	37.6%	37.9%	37.6%

[a] First value excludes duplicates; value in parentheses includes them.

[b] Total length of repeats, including large, intermediate, and short repeats.

[c] *Hyoscyamus niger* (Hn) and *Nicotiana tabacum* (Nt) share 21 of the 22 tRNA genes.

Table 2

Features of protein and rRNA genes in parental (*Nicotiana tabacum* (Nt) and *Hyoscyamus niger* (Hn)) and cybrid (Cyb) mitochondrial genomes

Gene name[a]		Length (bp)[b]	Hn/Nt differences[c]	Hn/Nt differences in coding regions		Hn/Nt differences in predicted editing sites	# intact genes			Origin of cybrid genes
				Synonymous	Non-synonymous		Hn	Nt	Cyb	
atp1		1530	25	19	6	0	1	1	2	chimeric & Nt
atp4		597	5	1	4	0	1	1	1	Nt
atp6		711	3	2	1	0	1	1	1	Nt
atp8		471	4	2	2	0	1	1	1	Nt
atp9		234	2	0	2	0	1	1	2	Nt & Hn
ccmB		621	2	1	1	0	1	1	2	Nt
ccmC		753	0	0	0	0	1	1	1	Hn
ccmFc		2271	14	2	0	0	1	1	1	Nt
ccmFn		1740	37	7	30	2	1	1	2	Hn
cob		1182	3	2	1	0	1	1	1	Nt
cox1		2551	11	9	2	2	1	1	2	Hn
cox2		2143	8	2	4	1	1	1	1	chimeric
cox3		798	2	1	1	0	1	1	1	Nt
matR		1977	5	2	3	0	1	1	1	chimeric
mttB		840	1	0	1	0	1	1	1	Nt
nad1[d]	x1	387	0	0	0	0	1	1	1	Nt
	x2-3	1485	12	1	0	0	1	1	1	Nt
	x4	59	0	0	0	0	1	1	1	Hn
	x5	259	0	0	0	0	1	1	1	Nt
nad2	x1-2	1575	6	0	0	0	1	2	2	Nt
	x3-5	4796	24	1	0	0	1	1	1	Nt
nad3		357	4	2	2	0	1	2	2	Nt
nad4		8512	28	1	0	0	1	1	1	chimeric
nad4L		303	0	0	0	0	1	1	1	Nt
nad5	x1-2	2290	8	1	1	0	1	1	1	Nt
	x3	22	0	0	0	0	1	1	1	Nt
	x4-5	1633	20	1	0	0	2	1	1	chimeric

Gene name[a]	Length (bp)[b]	Hn/Nt differences[c]	Hn/Nt differences in coding regions		Hn/Nt differences in predicted editing sites	# intact genes			Origin of cybrid genes
			Synonymous	Non-synonymous		Hn	Nt	Cyb	
nad6	687	3	2	1	0	1	1	1	Nt
nad7	5294	37	0	0	0	1	1	1	chimeric
nad9	573	2	0	2	0	2	1	1	Nt
rpl2	2898	36	4	4	0	1	1	1	Nt
rpl5	555	9	4	5	1	1	1	1	Nt
rpl10	480	4	2	2	0	1	1	2	Hn
rpl16	516	3	1	2	0	1	1	1	Hn
rps1	651	3	2	1	0	1	1	1	Nt
rps3	3469	27	4	6	0	1	1	1	Hn
rps4	1050	6	5	1	0	1	1	1	Nt
rps10	1138	4	1	2	0	1	1	2	Hn
rps12	378	2	1	1	0	1	1	1	Nt
rps13	351	3	1	2	0	1	1	1	Nt
rps19	285	3	1	2	0	1	1	2	Hn
rrn5	119	1	NA	NA	ND	1	1	1	Nt
rrn18	1902	4	NA	NA	ND	1	1	1	Nt
rrn26	3432	15	NA	NA	ND	2	2	1	chimeric
sdh3	327	3	1	2	0	1	3	3	Nt
sdh4	378	0	0	0	0	1	1	1	Nt

NA, not available; ND, not determined. **[Author, please check inserted text 'NA, not available; ND, not determined.' is correct. (or should it read 'not applicable'?]**

[a] Intron-containing genes are in bold face.

[b] Gene lengths are from the cybrid mtDNA.

[c] These are nucleotide differences, excluding gaps.

[d] *nad1* is chimeric in the sense that the four separately-transcribed portions of this *trans*-spliced gene are derived from different parents.

Table 3

Recombination events between parental mitochondrial genomes (*Nicotiana tabacum* (Nt) and *Hyoscyamus niger* (Hn)) in the cybrid Nt(+Hn)

Event #	Contig #	Recombining regions		Crossover (CO) or gene conversion (GC) event	Location of recombination		SBP test support[a]	Length of gene conversion tract	# of recombination products[b]
		# bp	Identity		Genic	Breakpoint[a]			
1[b]	1	536	99%	CO	no	417-587	NS		2[b]
2	1	1824	95%	CO	no	16,493	100%		1
3	1	909	99%	CO	no	41,000-41,150	NS		1
4	1	398	99%	CO	*atp1*	55,160-55,330	NS		1
5	1	8987	98%	GC[c]	no	90,730-91,400	NA	18-371	1
6	1	4107	96%	CO	no	117,050	100%		1
7	1	1597	98%	CO	no	131,635	100%		1
8	1	226	99%	CO	no	144,226	96%		1
9	1	862	98%	CO	no	144,100-144,350	NS		1
10	1	3815	99%	CO	no	184,360	100%		1
11	1	199	99%	CO	no	207,461	86%		1
12	1	3385	97%	GC[c]	no	215,950-219,100	NA	2866-3150	1
13	1	7042	97%	CO	*nad4*	245,122	100%		1
14	1	7042	97%	GC[c]	no	248,650-249,088	NA	151-344	1
15[b]	2	634	99%	CO	*trnQ*	8,592-8,794	NS		2[b]
16	2	1721	97%	CO	no	9,245	100%		1
17[b]	2	1691	98%	CO	no	17,420	100%		2[b]
18	2	678	99%	CO	no	18,357-18,600	NS		1
19	2	5716	99%	GC[c]	*nad7*	48,068-49,444	NA	168-1376	1
20	2	1669	96%	CO	no	60,844	100%		1
21	2	1852	98%	CO	no	72,300	100%		1
22	2	103	100%	CO	no	89,332-89,435	NS		1
23	2	2183	94%	CO	no	114,942	100%		1
24	2	1003	99%	CO	no	179,018-179,289	NS		1
25	2	3385	99%	CO	no	190,346	100%		1
26	2	199	99%	CO	no	198,710-198,813	NS		1
15[b]	2	634	99%	CO	no	216,921-216,928	NS		2[b]
27	2	1524	99%	GC[c]	no	220,198-221,579	NA	1020-1381	1
28	3	3488	99%	CO	*cox2*	12,725	100%		1
29	3	1861	99%	CO	*nad5*	32,629	100%		1
17[b]	3	1691	98%	CO	no	42,718	100%		2[b]
1[b]	3	536	99%	CO	no	48,170-48,240	NS		2[b]

Event #	Contig #	Recombining regions		Crossover (CO) or gene conversion (GC) event	Location of recombination		SBP test support[a]	Length of gene conversion tract	# of recombination products[b]
		# bp	Identity		Genic	Breakpoint[a]			
30	4	9000	99%	CO	no	22,140	100%		1
31	4	100	99%	CO	no	35,525-35,624	NS		1
32	5	3563	99%	CO	*rrnL*	2,021	100%		1
33	5	6374	99%	CO	*matR*	18,482	100%		1
34	6	2866	99%	CO	no	4,726	95%		1
35	6	921	99%	CO	no	14,413	99%		1

NA, not available. **[Author, please check inserted text 'NA, not available' is correct (or should it read 'not applicable'?]**

[a]Breakpoint locations were calculated by the SBP (Single BreakPoint Recombination) program when they were significant as determined by this program. In those cases in which the SBP test was not significant (NS) but a crossover was nonetheless apparent because parental-unique sequences were found at each side of the putative crossover, the region of the breakpoint was estimated based on the length of the cybrid region that is identical to both parents and which is flanked by regions containing shared polymorphisms with one parent on one end and the other parent on the other end.

[b]For only three of the 35 recombination events were two recombination products detected. These events (#'s 1, 15, and 17) therefore appear twice in this table.

[c]The GeneConv program was significant ($P<0.01$) for these gene conversion events.