REVIEWS PAPER

Heteroplasmy and stoichiometric complexity of plant mitochondrial genomes—though this be madness, yet there’s method in’t

Magdalena Woloszynska*

Laboratory of Molecular Cell Biology, Faculty of Biotechnology, University of Wroclaw, ul. Przybyszewskiego 63/77, 51-148 Wroclaw, Poland

* E-mail: Magdalena.Woloszynska@ibmb.uni.wroc.pl

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Abstract

Mitochondrial heteroplasmy is defined as the coexistence of divergent mitochondrial genotypes in a cell. The ratio of the alternative genomes may be variable, but in plants, the usually prevalent main genome is accompanied by sublimons—substoichiometric mitochondrial DNA (mtDNA) molecules. Plant mitochondrial heteroplasmy was originally viewed as being associated with pathological mutations or was found in non-natural plant populations. Currently, it is considered to be a common situation in plants. Recent years have changed the previous view on the role of homologous recombination, small-scale mutations, and paternal leakage of mtDNA in the generation of heteroplasmy. Newly developed sensitive techniques have allowed the precise estimation of mtDNA stoichiometry. Mechanisms of maintenance and transmission of heteroplasmatic genomes, including DNA recombination and replication, as well as mitochondrial fusion and fission, have been studied. This review describes the high level of plant mitochondrial genome complexity—the ‘madness’ resulting from the heteroplasmic state and explains the method hidden in this madness. Heteroplasmy is described as the evolutionary strategy of uniparentally inherited plant mitochondrial genomes which do not undergo sexual recombination. In order to compensate for this deficiency, alternative types of mtDNA are substoichiometrically accumulated as a reservoir of genetic variability and may undergo accelerated evolution. Occasionally, sublimons are selected and amplified in the process called substoichiometric shifting, to take over the role of the main genome. Alternative mitochondrial genomes may recombine, yielding new mtDNA variants, or segregate during plant growth resulting in plants with mosaic phenotypes. Two opposite roles of mitochondrial heteroplasmy with respect to acceleration or counteracting of mutation accumulation are also discussed. Finally, nuclear control of heteroplasmy and substoichiometric shifting is described.

Key words: Evolution of mtDNA, heteroplasmy, mtDNA, paternal leakage of mtDNA, plant mitochondrial genome, recombination, replication, sublimons.

Structural, stoichiometric and functional variety of DNA molecules in plant mitochondria

Mitochondrial genomes of higher plants are unusually large (208–2500 kb; Ward et al., 1981; Palmer and Herbon, 1987) compared with their animal (usually between 16–20 kb; Boore, 1999) and fungal (17–100 kb; Zimmer et al., 1984; Cummings et al., 1990) counterparts; therefore uncovering their structure has remained a challenge for a long time.
Restriction maps of the analysed genomes predicted a circular master chromosome enclosing the whole genome sequence and subgenomic circles generated from the master molecule via recombinations occurring between direct large repeats (reviewed by Janska and Woloszynska, 1997). Alternatively, for some plant species, two or three independently replicating chromosomes were proposed (Janska and Mackenzie, 1993; Janska et al., 1998). However, large circular molecules have been observed only sporadically. Instead, long linear (up to 80%) and smaller circular (12%) or branched DNA molecules have been described (Oldenburg and Bendich, 1996; Backert et al., 1997; Manchekar et al., 2006). It was proposed by Manchekar et al. (2006) that the seeming contradiction between physical mapping and pictures produced by electron microscopy would be explained if the DNA molecules in mitochondria of higher plants were linear, but circularly permuted as previously reported for the lower plant Marchantia polymorpha (Oldenburg and Bendich, 2001).

Although the exact structure and organization of plant mtDNA remains elusive, it is certain that plant mitochondria contain a spectrum of variable DNA molecules undergoing active homologous recombination. Besides the large size, recombination activity is the most distinctive feature of these genomes. It is considered as an important and active force which determines structure, organization and evolution of plant mtDNA (Kanazawa and Shimamoto, 1999). For a long time, the occurrence of recombination in plant mitochondria has been supported only by the presence of repeated sequences surrounded by genomic environments that could have been generated by recombination. Large repeats (above 1 kb) are usually found within the given genome in four recombination forms—recombination between one pair of reciprocal forms results in the generation of two remaining forms or vice versa (Fig. 1A). Since all recombination forms are found in similar stoichiometry, large repeats are believed to recombine frequently and reversibly to sustain interconversions between DNA molecules. Large repeats are also suggested as initiation sites of recombination-dependent replication of DNA (Zaegel et al., 2006). Short repeated sequences of six to several hundred base pairs are not always represented by all four forms within the genome and those forms which are detected differ significantly in stoichiometry (Fig. 1B, C, D) (Kanazawa et al., 1994; Bellaoui et al., 1998; Woloszynska et al., 2001; Woloszynska and Trojanowski, 2009). Consequently, short repeats were assumed to recombine sporadically and irreversibly, producing new and stable DNA arrangements which do not interconvert with the main genome. Since plant mitochondrial genomes are rich in short repeated sequences, recombinations of these repeats may significantly influence the evolution of plant mtDNA (Grabau et al., 1992; Moeykens et al., 1995; Bellaoui et al., 1998; Kanazawa and Shimamoto, 1999; Woloszynska et al., 2001; Satoh et al., 2006; Woloszynska and Trojanowski, 2009). As many as 22 pairs of identical repeats were found in mtDNA of Arabidopsis and, with the exception of the two largest ones, as many as 20 repeats seem to recombine infrequently (Unseld et al., 1997). The role of short repeats in the evolution of plant mitochondrial genomes is illustrated by comparative mtDNA analyses performed for various cultivars of the same or related species. Rearrangements observed between the mitochondrial genomes of these plants were revealed to result from recombinations involving short repeats (Grabau et al., 1992; Moeykens et al., 1995; Kanazawa and Shimamoto, 1999; Satoh et al., 2006). Some short repeats have been found in several copies in mitochondrial genomes of various plant species and these are called hot-spots for recombination (Hanlon and Grabau, 1997; Kato et al., 1998; Scotti et al., 2004).

Advanced electrophoresis techniques and electron microscopy allowed molecular structures to be detected which provided further proofs of mtDNA recombination in higher plants (Backert and Borner, 2000; Manchekar et al., 2006). Branched molecules and complex networks observed in the mitochondria of soybean and Chenopodium album were similar to the recombination and replication intermediates found in yeast and those produced during the recombinational replication of the T4 phage DNA. These observations suggested that plant mtDNA may undergo not only recombination but also the T4-like recombinational replication (Backert and Borner, 2000). Finally, the most convincing evidence for recombination appeared
when recombination-related proteins of predicted or proved mitochondrial localization were found. In the moss Physcomitrella patens, an orthologue of bacterial RecA, with a possible function in the repair of mtDNA, was described (Odahara et al., 2007). Edmondson et al. (2005) identified the mitochondrially targeted homologue of bacterial single-stranded DNA binding protein (mtSSB) in Arabidopsis thaliana. The first report of strand-invasion activity in plant mitochondria with a biochemical characteristic resembling bacterial RecA was provided by Manchekar et al. (2006).

A variety of sizes and structures is followed by stoichiometric and functional differences detected between mtDNA molecules in plants. Plant mitochondria are commonly heteroplasmic, i.e. in addition to an abundant main genome they also contain substoichiometric DNA molecules. Coexisting types of mtDNA are often named mitotypes or mitochondrial haplotypes. According to the commonly used definition, a haplotype is a set of closely linked genetic markers present on one chromosome which tend to be inherited together. Regarding the complex organization of the plant mitochondrial genome (Oldenburg and Bendich, 1996; Backert et al., 1997; Manchekar et al., 2006) I propose that the term mitotype or mitochondrial haplotype is defined as a set of genetic information located in mtDNA molecules which can operate as the main genome and are inherited together. Since heteroplasy was also detected in plastid genomes, it should be emphasized that, in this review, the term ‘heteroplasy’ will refer exclusively to mitochondrial DNA. On the other hand, the phrase ‘plastid heteroplasy’ will be used with regard to plastid genomes.

While the genetic information enclosed within the main genome determines the plant phenotype, sublimons usually remain functionally silent. Substoichiometric molecules are 10-, 100- or even 1000-fold less accumulated compared with the main genome (Laser et al., 1997; Arrieta-Montiel et al., 2001; Lilly et al., 2001; Ballestros et al., 2009; Feng et al., 2009; Woloszynska and Trojanowska, 2009). In some cases, the copy number of the sublimons is so low that only a small fraction of plant cells contains sublimons (Arrieta-Montiel et al., 2001). The pool of these sparse molecules is not quantitatively uniform but it may contain more and less abundant subpopulations (Woloszynska and Trojanowska, 2009). This issue will be discussed in detail in later sections. Stoichiometric differences may also exist within the main mitochondrial genome. When the real-time PCR technique was applied to compare the copy numbers of six mitochondrial genes in four related Phaseolus vulgaris lines, relatively high differences were detected within each line (Woloszynska et al., 2006). The copy numbers of the analysed genes varied maximally by a factor of 10.

The composition and stoichiometry of DNA molecules within plant mitochondria is further complicated by mitochondrial plasmids. It has been shown that several plasmids may coexist within one cultivar and different cultivars may vary with respect to content and relative quantity of these DNA molecules (Kanazawa et al., 1992). Moreover, the same plasmid may exist in two lines of one plant species either as an element integrated with the mitochondrial genome or as a low-copy number extrachromosomal replicon (McDermott et al., 2008).

**Heteroplasy occurrence: from rare phenomenon to normal situation**

In the late 1980s and 1990s, plant heteroplasy was detected mainly in mitochondrial mutants (Small et al., 1987; Gu et al., 1993; Wintz, 1994; Sakamoto et al., 1996; Janska et al., 1998), in hybrids (Belloumi et al., 1998), tissue cultures (Kanazawa et al., 1994), and plants regenerated from tissue culture (Vitart et al., 1992; Hartmann et al., 1994). In the former case, the heteroplasmic pool of DNA molecules consisted of the wild-type and deleteriously mutated mtDNA. Mutations caused severe phenotypic abnormalities: cytoplasmic male sterility (CMS) in various plant species (Small et al., 1987; Janska et al., 1998), non-chromosomal stripe mutants (NCS) in maize (Gu et al., 1993; Wintz, 1994) and the chloroplast mutator mutant (CHM) in Arabidopsis (Martinez-Zapater et al., 1992; Sakamoto et al., 1996). The hybridization process often resulted in heteroplasmic genomes containing predominant maternal and substoichiometric paternal sequences. Consequently, heteroplasy was viewed as present only in the pathological or non-natural plant populations. However, more recent analyses of cultivated species (Woloszynska et al., 2001; Hattori et al., 2002; Garcia-Diaz et al., 2003) and the wide-range screenings of natural plant populations (Arrieta-Montiel et al., 2001; Stadler and Delph, 2002; Welch et al., 2006) showed that heteroplasy is a common state of plant mitochondria. Alternative genome types often differ only in sequence arrangement and none of them contains mutations deleterious or significantly affecting phenotype (Albert et al., 2003; Welch et al., 2006). Thus, heteroplasy is currently considered as a normal physiological situation in plants.

As already mentioned, heteroplasy has also been described for plastid genomes. Although, the phenomenon differs in many aspects in the two organelles. Interestingly, both plastid and mitochondrial heteroplasy, once believed to be rare, are now recognized as quite common (Frey et al., 2005).

Even though there is no doubt that mitochondrial heteroplasy is widespread, all studies aimed at proving its occurrence should be interpreted with a great deal of criticism. Mitochondrial DNA fragments are commonly transferred into the nuclear genomes of many organisms (Bensasson et al., 2001; Timmis et al., 2004) and, in the case of some plant species, the integrated sequences are very long (Stupar et al., 2001). Moreover, nuclear sequences of mitochondrial origin may be detected using the same sensitive PCR techniques as those applied for heteroplasy identification. The potential risk of data misinterpretation is high because total DNA is usually used as a template for PCR. Therefore it is always advisable to use purified mtDNA instead of total DNA. This issue was addressed in more detail in our previous review (Kmiec et al., 2006).
Origin of plant heteroplasmy: the changed picture

DNA mutations which may distinguish main genome and substoichiometric molecules

Recombinations occurring via short repeats have always been considered as the most common mutations and the reason for heteroplasmy in plant mitochondria (Vitart et al., 1992; Hartmann et al., 1994; Kanazawa et al., 1994; Bellaoui et al., 1998; Janska et al., 1998; Arrieta-Montiel et al., 2001; Albert et al., 2003; Woloszynska and Trojanowski, 2009). In some heteroplastic plants, the mitochondrial main genome contains two parental forms of the recombination repeat, while substoichiometric molecules include two (Fig. 1B) (Vitart et al., 1992; Albert et al., 2003; Woloszynska and Trojanowski, 2009) or only one (Fig. 1C) (Hartmann et al., 1994; Kanazawa et al., 1994; Bellaoui et al., 1998) of the resulting products. This situation can be explained by relatively recent recombination occurring between short repeats located in the main genome which, in some cases, is followed by the loss of one product molecule due to its replication deficiency or selective elimination during mtDNA segregation. However, the substoichiometric mitotype may contain DNA configurations which are not accompanied by parental recombination forms present within the main genome (Figs 1D, 2) (Janska et al., 1998; Arrieta-Montiel et al., 2001; Woloszynska and Trojanowski, 2009). Some sublimons may indicate substantial similarity to the main genome molecules, but they may also carry several kilobases long nucleotide sequences that do not indicate any homology to the main genome (Fig. 2). These situations cannot be explained by a single recombination event but rather by a sequence of rearrangements occurring independently in the main genome and sublimons over a long evolutionary span. The rearrangements may involve recombinations but also insertions/deletions, DNA transfer between organelles (Arrieta-Montiel et al., 2001), horizontal DNA transfer (Woloszynska et al., 2004), and sequence transposition (Chaw et al., 2008). The DNA sequences progenitor to the resulting sublimon can be lost from mitochondria (Fig. 2). Reconstruction of this scenario is possible only if the common progenitor and/or molecular intermediates of the chain of rearrangements are identified in mitochondrial genomes of phylogenetically related species (Arrieta-Montiel et al., 2001).

For many years, most of the known cases of plant heteroplasmy were associated with recombination. This was consistent with the knowledge of the time postulating extremely low rates of substitutions in plant mitochondrial genomes. However, this view can no longer be upheld. Very high rates of mtDNA sequence variation have been reported for Pelargonium (Parkinson et al., 2005), Plantago (Cho et al., 2004), Silene (Stadler and Delph, 2002; Houliston and Olson, 2006) and, most recently, in Cycas (Chaw et al., 2008). Studies of substitution rates in mtDNA in the genus Silene resulted in the detection of substantial variation in evolutionary rates, not only among species but also among lineages within species (Sloan et al., 2008) and even among mitochondrial genes (Barr et al., 2007). The role of point mutations and small-scale indels in the generation of plant heteroplasmy has been documented in several plant species: Olea europaea (Garcia-Diaz et al., 2003), Triticum, Aegilops, and hybrid triticale (Tsukamoto et al., 2000; Hattori et al., 2002), Silene species (Stadler and Delph, 2002; McCauley et al., 2005; Houliston and Olson, 2006) and two conifers (black and red spruce) (Jaramillo-Correa and Bousquet, 2005). Interestingly, heteroplasmy resulting from nucleotide substitutions and indels was relatively frequent in natural plant populations. Particularly abundant mitochondrial sequence diversity was observed in natural populations of Silene acaulis (Stadler and Delph, 2002). DNA sequence alterations responsible for heteroplasmy were found in both coding (Tsukamoto et al., 2000; Hattori et al., 2002; Stadler and Delph, 2002; McCauley et al., 2005; Houliston and Olson, 2006) and non-coding (Tsukamoto et al., 2000; Hattori et al., 2002; Garcia-Diaz et al., 2003) regions of mitochondrial genomes. In the majority of these studies, only one or two mitochondrial genes or genome fragments were searched for heteroplasmy (Hattori et al., 2002; Stadler and Delph, 2002; Garcia-Diaz et al., 2003; McCauley et al.,
2005). Consequently, the estimation of the scale of sequence heteroplasmy in the whole mitochondrial genome was difficult. A more comprehensive survey was performed by Tsukamoto et al. (2000) who analysed nucleus-cytoplasm hybrids of wheat and Aegilops. From the 15 mtDNA searched regions, six regions encompassing 11 genes appeared heteroplasmic.

The events leading to coexistence of diverging mitotypes

The coexistence of alternative mitotypes can be achieved in two ways: either by mutation occurring within a homoplasmic genome and the subsequent concomitance of the wild-type and mutated DNA molecules (Fig. 3, pathway 1) or by paternal leakage of mtDNA followed by the presence of both maternal and paternal mitotypes within progeny (Fig. 3, pathway 2). The former mechanism was originally considered to be the main or even the exclusive source of heteroplasmy in plants. It was supported by the fact that heteroplasmy was found in self-pollinating species. Paternal transmission of mtDNA in such species, even if it occurred, would not produce heteroplasmy due to the homogeneity of pollen and ovule mitochondrial genomes. The first report, on the coexistence of maternal and paternal mitochondrial sequences, concerned triticale—a crop plant obtained by crossing wheat and rye (Laser et al., 1997). However, in this case, paternal leakage was not the only possible reason for heteroplasmy, because the paternal-like substoichiometric sequence was detected in maternal wheat mitochondria. Paternal mtDNA transmission during heteroplasmy generation was undoubtedly proven by studies focused on plants produced via hybridization (Tsukamoto et al., 2000; Hattori et al., 2002; Aksyonova et al., 2005). Hybrids of wheat and Aegilops (Tsukamoto et al., 2000; Hattori et al., 2002) as well as wheat and barley (Aksyonova et al., 2005) contained a mixture of mitotypes found in both parents. In these cases, however, paternal sequences were not detected in the maternal parent, clearly indicating leakage of the paternal mtDNA. Interestingly, in the wheat–Aegilops hybrids, either the maternal or the paternal mitotype prevailed depending on the Aegilops line used as the cytoplasm donor for hybridization (Hattori et al., 2002). Hybridization between plants representing diverse species may facilitate paternal leakage, since this phenomenon was found to be most frequent between plants differing in the cytoplasm type (Svab and Maliga, 2007). However, conclusive evidence for paternal transmission of mtDNA and heteroplasmy generation was also obtained for intraspecific crosses of Silene vulgaris plants (McCauley et al., 2005).

Contrary to the above-described artificial hybridizations or greenhouse controlled crosses, a paternal leakage followed by heteroplasmy generation is much more difficult to prove in natural populations. Paternal or even maternal parents of plants analysed during such studies, are usually unknown. Consequently, the arguments must be based on indirect evidence indicating that heteroplasmy, generated via paternal transmission of mtDNA, is the most tenable explanation to the observed pattern of mtDNA variability and geographic distribution of the mitotypes identified. Based on similar reasoning, occasional leakage of paternal mtDNA was proposed to occur during natural hybridizations between two conifers, black spruce (Picea mariana) and red spruce (P. rubens) (Jaramillo-Correa and Bousquet, 2005). Paternal transmission of mitochondrial genomes was presumably followed by transient heteroplasmy which promoted the recombination of parental genomes yielding a new intermediate mitotype. A frequent paternal leakage is also one of the possible factors considered as contributing to high levels of heteroplasmy found in natural populations of Silene vulgaris (Welch et al., 2006).

Let's talk about numbers: stoichiometry of mtDNA molecules in heteroplasmic genomes

In heteroplasmic genomes, the accumulation of sublimons is usually much lower than the quantity of the main genome molecules, however, only sporadically precise stoichiometry is known. The substoichiometric copy of the orf25 gene was quantified in wheat using competitive PCR and found to represent 0.1% of the total orf25 gene copies (Laser et al., 1997). MtDNA molecules representing the wild-type cucumber genome were detected as sublimons in the mitochondria of mosaic plants at the level of 0.2% (Lilly et al., 2001). In the fertile revertant line of Phaseolus vulgaris, the pvs sequence, responsible for cytoplasmic male sterility, was determined by real-time PCR to be 1000-2000-fold less abundant than the main genome. This result corresponds to 1 copy of pvs per 100 cells (Arrieta-Montiel et al., 2001). In further studies concerning heteroplasm in Phaseolus vulgaris, two classes of sublimons differing in quantity were found (Woloszynska and Trojanowski, 2009). Relatively
high copious sublimons were about two orders of magnitude less abundant compared with the main genome. The quantity of the remaining sublimons was five to six orders of magnitude below the main genome. Interestingly, high- and low-abundant sublimons coexisted within the same *Phaseolus vulgaris* line and the same substoichiometric sequence could be found either as a relatively high- or low-copy number sublimon in two different bean lines.

Much more balanced stoichiometry of alternative mitotypes was detected in triticale, a hybrid plant produced when female wheat is fertilized with pollen from rye. Accumulations of the maternal (85%), paternal (12%) and novel (3%) copies of *orf25* were all quite high (Laser et al., 1997).

Heteroplasmy is commonly described as the coexistence of two types of mitochondrial genomes. In plants, the situation can clearly be more complicated—heteroplasmic genomes of wheat, *Aegilops* and their hybrids may contain two, three or four mtDNA variants but even those of the lowest representation correspond to at least 1% of total mtDNA copies (Hattori et al., 2002). Some genome types contain such evenly distributed mitotypes that it is impossible to indicate which variant is the main mitochondrial genome (Hattori et al., 2002).

**Maintenance and transmission of heteroplasmic genomes**

Although heteroplasmy is a universal phenomenon which was also described in fungi and animals (reviewed by Barr et al., 2005), plants are distinguished from the other taxa in that they may inherit sublimons over many generations (Laser et al., 1997; Arrieta-Montiel et al., 2001). How this complex population of DNA molecules is maintained within the plant and transmitted from generation to generation is still poorly known.

To explain the maintenance of heteroplasmy, authors have proposed replicative and recombination mechanisms (Kanazawa et al., 1994; Bellaoui et al., 1998) or selection favouring heteroplasmic cells (Welch et al., 2006). Since sublimons are mostly generated by recombination across short repeats located in the main genome, it would be quite easy to imagine that these molecules, at least those accompanied by parental recombination forms, were maintained by continuous *de novo* recombination (Fig. 4, arrow 1). Such sublimons could further interconvert with the main genome molecules via reverse recombination (Fig. 4, arrow 2) or non-reciprocal recombinations (Fig. 4, arrows 3). Until recently, there was only one report claiming that heteroplasmy is maintained via continuous recombination and frequent interconversion of the predominant and substoichiometric mtDNA molecules (Bellaoui et al., 1998). However, opinion which prevailed over long time was that recombinations of short repeats were rare and irreversible, resulting in stable rearrangements (Kubo and Newton, 2008) and that sublimons did not interconvert with the main genome. This point of view was supported by reports describing sublimons which were not accompanied by parental recombination forms located in the main genome and could not be generated *de novo* (Figs 1D, 4, arrows 4 and 5) (Kanazawa et al., 1994; Janska et al., 1998). Thus, it was obvious that an exclusively replicative, non-requiring recombination mechanism has to be active to maintain sublimons.

As mentioned in the previous section, two quantitative classes of sublimons were recently reported in *Phaseolus vulgaris* (Wołoszynska and Trojanowski, 2009). Quantitative differences between sublimons were explained to result from the molecular mechanisms underlying their maintenance. The substoichiometric sequences which were present in the absence of parental recombination forms, were, most probably, maintained exclusively by the replicative mechanism. Sublimons accompanied by parental recombination forms present in the main genome, were shown to be maintained by recombination. Frequency of recombination was high enough to account for the quantity of sublimons three to four orders of magnitude higher than in the case of the replicative mechanism. Moreover, this class of sublimons was not only actively produced by recombination but also underwent frequent interconversion with sequences present in the main genome (Wołoszynska and Trojanowski, 2009).

Mechanisms allowing successful inheritance of heteroplasmic populations of DNA molecules are still unclear. Nucleoids, the chromatin-like structures, found in mung bean mitochondria were proposed to serve as organizational units of DNA transmission and centres of genome maintenance (Dai et al., 2005). However, in the light of other reports, it is unlikely that all nucleoids contain the whole complement of the mitochondrial genome. It was found that different mitochondria hold various amounts of DNA, mtDNA content per mitochondrion can be lower than the genomic equivalent and some organelles do not
contain any DNA (Lonsdale et al., 1988; Takanashi et al., 2006). These observations concerning the intracellular distribution of plant mitochondrial genomes are consistent with the molecular data regarding DNA stoichiometry within the heteroplasmic population. Plant cells contain hundreds of discrete mitochondria (600 in tobacco mesophyll protoplasts; Sheahan et al., 2004) that may greatly vary in DNA content (from 0 kbp to 1.2 Mbp in the rice root cells; Takanashi et al., 2006). Unequal DNA loading of individual mitochondria could be reflected to some extent by stoichiometric differences ranging from two to six orders of magnitude between the main genome and sublimons measured by PCR. Despite the complicated organization of their genomes, plant mitochondria may still properly function and inherit DNA because they undergo frequent fusion and fission associated with an exchange of mtDNA molecules between organelles (Arimura et al., 2004; Sheahan et al., 2005). This characteristic of the plant mitochondrial behavior gave rise to the concept of ‘dynamic syncytium’ (Lonsdale et al., 1988) or ‘discontinuous whole’ (Logan, 2006). According to this hypothesis, all mitochondria of the given plant cell normally exist as separate organelles that must transiently fuse to exchange their genetic information. Although usually morphologically disconnected, plant mitochondria are genetically dependent of each other (Logan, 2006).

To sustain transmission of the complete mitochondrial genome to the next generations, the integrity of mtDNA has to be most particularly guarded in meristems and egg cells. It can be achieved by an increased copy number of mtDNA and the special structure of the mitochondrial genome or the mitochondrion itself. Meristem cells contain a large quantity of organelar DNA, which, in many plant species, is synthesized preferentially before multiple cell divisions begin in the RAM (root apical meristem) (Kuroiwa and Fujie, 1992; Suzuki et al., 1992, 1995; Fujie et al., 1993). In the SAM (shoot apical meristem) and young leaf primordium of the Arabidopsis seedling, mtDNA is actively synthesized and individual mitochondria contain six times more DNA than those in the mature leaf (Fujie et al., 1994). Compared to differentiated tissues, higher levels of sublimons are found in meristems and non-differentiated tissues (Kanazawa et al., 1994; Arrieta-Montiel et al., 2001; Albert et al., 2003). Moreover, organization of mtDNA in the meristematic cells is different from the differentiated cells (Suzuki et al., 1996). It was proposed that plants possess a ‘transmitted form’ of mtDNA residing in meristems and containing the whole genetic complement on a single replicative unit to ensure the transmission of the entire genome to subsequent generations (Arrieta-Montiel et al., 2001). Successful inheritance of heteroplasmic genomes may also be facilitated by extensive fusion of mitochondria resulting in the appearance of giant mitochondria which have been observed in egg cells of Pelargonium zonale (Kuroiwa and Kuroiwa, 1992) as well as in the SAM and meristematic leaf primordium (LP) of Arabidopsis thaliana (Segui-Simarro et al., 2008). The cells of the SAM and the meristematic LP of Arabidopsis contain two structurally distinct types of mitochondria: small, round/tubular mitochondria located in the cell periphery and a single, very large, tentaculate/cage-like mitochondrion closely apposed to the nucleus in the cell centre (Segui-Simarro et al., 2008). In Arabidopsis the large mitochondria were found only in SAM and LP meristematic cells, whereas in all other cell types studied only small and dispersed mitochondria presenting round, oval, or sausage-like morphology were observed. The large mitochondrion of SAM cells undergoes architectural changes during the cell cycle which provide an efficient means for the intermixing of mtDNA and for the equal partitioning of the intermixed DNA to the two daughter cells. In the G1 and G1-S interphase stages, the large mitochondrion is centred on one pole of the nucleus with a single central sheet-like domain. Many individual round and tubular mitochondria are seen dispersed in the peripheral cytoplasm. During G2, both types of mitochondria double their volume and the large mitochondrion develops a clamp-like morphology by forming a second sheet-like domain opposite the first one. During mitosis, the majority of small mitochondria fuse with the large mitochondrion which now represents about 80% of the total mitochondrial volume and is converted into the cage-like organelle. This structure is composed of two sheet-like domains connected by large mitochondrial tubules undergoing cycles of fission and fusion. The cage-like organelle surrounds first the mitotic spindle and then the entire cytokinetic apparatus. This stage of mitochondrial cell-dependent architectural changes is particularly important for the stable inheritance of the plant mitochondrial genomes. Approximately 80% of mtDNA is already located within the same compartment and is available for intermixing and, possibly, recombination. The remaining mitochondria stay in dynamic fusion/fission equilibrium with the cage-like organelle and their DNA is also, at least transiently, available for intermixing. Following the phase of intermixing, mtDNA is redistributed into individual mitochondria. As the cytokinesis proceeds, small discrete mitochondria arise again by fission and appear in the cell cortical region, while the large mitochondrion divides into two independent mitochondria presenting tentaculate morphology of the G1 interphase cells (Segui-Simarro et al., 2008).

**Heteroplasmy: the madness or the method?**

Heteroplasmy can be viewed as a condition which complicates an already complex organization of plant mitochondrial genomes. Firstly, the number of mitotypes coexisting within heteroplasmic genomes may reach as many as four. Secondly, the copy number of substoichiometric sequences can be either several times or several orders of magnitude lower compared to the main genome and may reach extremely low levels. Third, the accumulation of sublimons may vary substantially between and within species as well as between different sublimons present in the same organism. Finally, mechanisms resulting in the generation of plant heteroplasmy are diverse (recombinations, point mutations, indels, and paternal leakage). Consequently, heteroplasmic plant genomes achieve a very high level of complexity in
terms of mitotypes diversity, stoichiometry, and origin of individual mtDNA molecules. This complexity, as well as the effort made by plants to maintain heteroplasm in many following generations, can either be viewed as a madness or explained as a mitochondrial strategy which, in some way, may benefit the plant. Thus, there is the question: Is there any method in the heteroplasmic madness?

The mitotypes coexisting in many of the known plant mitochondrial genomes differ only by minor rearrangements or by DNA sequence alterations which do not result in any severe phenotypic consequences. These examples of heteroplasm do not provide a simple and direct explanation of how this phenomenon can be useful for the plant. Such cases may rather suggest that heteroplasm is the consequence of a pointless and wasteful accumulation of DNA replication or recombination by-products. Heteroplasm appears to be a costly and useless madness based on the above-mentioned information. On the other hand, if potential beneficial consequences are considered and not only the costs and difficulties raised by heteroplasm, the method hidden in this madness becomes obvious. The universal and common presence of heteroplasm in eukaryotic organisms, as well as the universal character of molecular mechanisms responsible for the generation of this state, suggest that it might be a useful phenomenon. Detection of heteroplasm in plastids, another uniparentally inherited organelle, indicates that it could be a strategy developed by genomes that do not undergo sexual recombination, in order to compensate for this deficiency by the generation of genetic diversity. However, heteroplasm should not only be interpreted as a simple accumulation of alternative mitotypes. This condition also provides tools to increase and exploit the mitochondrial genetic diversity via stoichiometric changes within mtDNA molecules, the generation of clonal cell lines differing in mtDNA composition, or the creation of new mtDNA variants by recombination of coexisting mitotypes (Fig. 5). Finally, since the composition and stoichiometry of mitochondrial genomes is controlled by nuclear genes, heteroplasm may also be useful in establishing favourable co-operation between the two genomes. These issues will be addressed in the following sections.

**Substoichiometric shifting: the way of rapid evolution and phenotype switch**

The heteroplasmic state of the mitochondrial genome allows evolution to be speeded-up via the so-called substoichiometric shifting (SSS), which results in the sudden transition to new mitochondrial organization taking place between two consecutive generations. Occasionally, in heteroplasmic plant mitochondria, sublimons are amplified and take on the role of the main genome, while the mtDNA molecules of the former main genome are suppressed to substoichiometric levels (Fig. 5) (Small et al., 1989; Janska et al., 1998). SSS has frequently been observed during in vitro culture and the regeneration process—the main genome of regenerated plants was composed of sublimons already present in parental plants (Vitart et al., 1992; Hartmann et al., 1994; Bartoszewski et al., 2004). Alternatively, quantitative shifts in the composition of tobacco mtDNA occurring within the in vitro culture can be reversed during the regeneration process (Kanazawa et al., 1994). The extent of the quantitative change depended on the degree of differentiation of cells.

Substoichiometric shifting was also shown to occur spontaneously within cultivated (Janska et al., 1998) and natural (Arrieta-Montiel et al., 2001) plant populations. Even if the novel main genome does not present any obvious differences in genetic information and results in only a limited phenotypic effect it may not be neutral from a selection point of view. Relatively simple rearrangements, which keep the genetic information unchanged, may alter the expression pattern of mitochondrial genes and induce a subtle but important effect. As described for *Nicotiana sylvestris*, a very simple rearrangement of mtDNA, resulting from a single recombination event within the progenitor genome, is associated with a higher stem height and late flowering, traits that may be advantageous for the plant (Albert et al., 2003). Consequently, SSS occurring between seemingly similar mtDNA variants may still be significant. However, before the shift occurs, the substoichiometric variant may evolve into a form carrying truly diverse genetic information. Evolution may also be accelerated at this stage, because some sublimons seem to be exceptionally prone to mutations. Since their copy number is usually very low, the mutations occurring within the individual sublimon will be propagated much faster than in the case of the main genome molecules. This is due the fact that even a single sublimon represents a significant fraction of all the substoichiometric molecules (Leaver et al., 1988). Moreover, sublimons may easily accumulate mutations because they are usually functionally silent and do not undergo selective...
pressure (Leaver et al., 1988). Such an accelerated evolution of substoichiometric molecules may finally lead to the generation of an alternative mitotype characterized by dramatically changed organization and genetic information. Therefore, the most spectacular substoichiometric shifts are associated with the clear change in the plant phenotype. The resulting new mitochondrial genomes may condition serious defects in mitochondrial metabolism as in the case of NCS maize mutants but they may also encode phenotypic traits which clearly benefit the plant. This is the case of substoichiometric shifting, responsible for the transition between the hermaphrodite and the CMS condition and back again (Janska et al., 1998; Abdelnoor et al., 2006). With only one known exception (Aksyonova et al., 2005), SSS has never been reported to result in the transition from hetero- to homoplasmy. Since after SSS, plants remain heteroplasmic, following plant generations may switch between the two phenotypic conditions, for example, male sterile and fertile (Janska et al., 1998). These examples of substoichiometric shifting allow it to be hypothesized that SSS operates as an adaptive evolutionary mechanism linking alterations of heteroplasmy with phenotypic plasticity (Abdelnoor et al., 2006). Similar ideas concerning the role of plastid heteroplasmy viewed as a fine-tuned adaptation to environmental conditions were also proposed (Frey et al., 2005).

Various molecular mechanisms for SSS were considered: (i) increased homologous recombination, which continuously generates recombination products in somatic tissues, (ii) favoured replication of one of the mitotypes, (iii) increased homologous recombination which creates a pool of sequences that, by strand invasion, prime the asymmetric replication of mtDNA chimeras (Zaegel et al., 2006). Kanazawa et al. (1994) showed that alterations of the tobacco mitochondrial genome occurring during the de-differentiation stage of tissue culture most probably resulted from the DNA recombination while reversion accompanying the differentiation phase could be driven only by the selective amplification of mtDNA molecules. The role of selective amplification during SSS is also supported by the observation that some sublimons undergoing quantitative shifting are not accompanied by parental recombination forms. Consequently, increased copy number of these molecules can not result simply from increased homologous recombination (Janska et al., 1998). Whether the selective amplification relies only on favoured replication or is facilitated in any way by recombination, remains unclear.

Selection of mtDNA molecules in heteroplasmic genomes operating during SSS and gene expression

An interesting and still unexplained feature of substoichiometric shifting is the selection of mtDNA molecules which undergo quantitative changes. In wheat–Aegilops nucleus–cytoplasm hybrids, both maternal and paternal mitotypes were present, but from the three maternal variants detected in heteroplasmic Aegilops, only one was found in the hybrid (Hattori et al., 2002). The complete selection of one mitotype is striking, indicating some selective advantage of this mtDNA variant. It is possible that this particular mitotype gains the selective advantage only in combination with the paternal nuclear genome. Differential amplification of the heteroplasmic mtDNA copies in this case might be under control of nucleus-cytoplasm interaction.

Rigorous selection operating during substoichiometric shifting was also reported in common bean where, from two sequence variants differing by single nucleotide A/C polymorphism, only one variant was selected during SSS (Woloszynska and Trojanowski, 2009). The mtDNA molecules with the C polymorphic variant were selected, although they were a minority compared to the A variant. Moreover, selection was so rigorous that, following the shift, the second polymorphic variant A was not detected even by the highly sensitive real-time PCR assay.

Functional selection of mtDNA molecules was observed in heteroplasmic plants during gene expression. In a fertile common bean line containing substoichiometric amounts of the male sterility-inducing pvs sequence, the transcripts originating from this sequence were not detected (Janska et al., 1998). In hybrids shown to experience the paternal transmission of mtDNA, only maternal gene copies were transcribed, although maternal gene variants were not always predominant (Laser et al., 1997; Hattori et al., 2002). Moreover, only the maternal pattern of editing was found in wheat–Aegilops hybrids (Hattori et al., 2002).

Content and stoichiometry of mtDNA may differ between individual cells in differentiated tissues

The role of heteroplasmy in plant evolution may only be fulfilled if it is maintained in germ cells and if quantitative and qualitative changes of mtDNA are transmitted to or occur in these cells. Thus, heteroplasmy is clearly beneficial in germ cells, because it allows preserving and even increasing a precious genetic diversity of mitochondrial genomes. But is heteroplasmy also so useful and widely maintained in all somatic cells? Considering that sublimons are low in copy number and usually silent in terms of gene expression, they can be easily lost and there is no pressure to save them in each somatic cell. Moreover, once lost, sublimons will not reappear in the cell, because mitochondria do not exchange their DNA with the neighbouring cells. Indeed, only 1 copy of the substoichiometric pvs sequence per 100 cells was quantified in the fertile revertant line of Phaseolus vulgaris (Arrieta-Montiel et al., 2001). Intra-individual genetic drift within a heteroplasmic population of mtDNA occurring during plant growth could potentially lead to an unequal load of mitochondrial variants in different plant tissues or organs (McCaulay et al., 2005). As a consequence of mitotypes segregation occurring in the somatic cells, mosaic plants would appear that present the spatial distribution of tissue sectors or
organs with different phenotypic traits or at least various severities of these traits. However, variable distribution of heteroplasmic genomes in cells of differentiated tissues has only sporadically been reported. The most well-known examples of cell-to-cell variation in mtDNA stoichiometry are the NCS mutants of maize, which carry deletions of parts of the essential mitochondrial genes (Yamato and Newton, 1999). The segregation of mutated from normal mitochondrial genomes occurs during the development of the heteroplasmic NCS plants. The segregation results in clonal lineages of defective cells containing exclusively or predominantly mutated mtDNA. These cells are represented by sectors of collapsed kernels on ears and pale green, yellow or brown necrotic stripes on the leaves (Fig. 5). The type of striping depends on the gene carrying the mutation, while the severity of the plant phenotype is related to the contribution of the mutated genome. In the NCS6 plants, having a partially deleted mitochondrial cox2 gene, yellow stripes were found to contain only mutated mtDNA molecules while green sectors were heteroplasmic (Fig. 5) (Gu et al., 1993). PCR analysis of single cells of NCS6 plants revealed two types of cells: those homoplasmic for the deleted mtDNA and those that were heteroplasmic (Wintz, 1994). Similarly, in the NCS2 plants carrying partial deletion of the nad4 gene, pale green tissues were nearly homoplasmic for mutation while dark green tissues were heteroplasmic (Yamato and Newton, 1999).

Intra-individual segregation of two mitotypes—one conditioning development of the male flower function and the other inhibiting it—was proposed to explain the spatial distribution of female and hermaphrodite flowers found on the same Silene vulgaris plants (Andersson, 1999). Segregation of mtDNA variants was also suggested as the mechanism underlying differences in the offspring sex ratios produced by both flower types. According to this hypothesis, the proportion of the mitochondrial types could change progressively during plant growth, leading to different sexual phenotypes in different parts of the plant, as determined by the most common mitochondrial genotype.

Recombination of alternative mitotypes in heteroplasmic mitochondria

The coexistence of alternative genomes within plant mitochondria raises the possibility of recombination between divergent mitotypes (Fig. 5). Similar to paternal leakage, the first convincing evidence for intermitotype recombination was provided by the analysis of heteroplasmic mtDNA molecules detected in hybrid plants. Mitochondria of triticale were found to contain the maternal (wheat), the paternal (rye), and the novel copies of the gene orf25 (Laser et al., 1997). The novel type of orf25 might have been generated by recombination between the parental gene copies. Interalteral recombination was also proposed to explain the origin of various configurations of the mitochondrial rpl5-rps14 region found in the somatic hybrids of Solanum tuberosum and S. commersonii (Scotti et al., 2004).

In this case, multiple recombination events across two repeated sequences were assumed.

Intra- and intergenic recombination occurring between alternative mitotypes in heteroplasmic mitochondria was also proposed to explain the variation of mtDNA surveyed at one (Stadler and Delph, 2002), two (McCauley et al., 2005), three (Houlston and Olson, 2006) or four (Jaramillo-Correa and Bousquet, 2005) gene loci of natural populations of Silene vulgaris (Houlston and Olson, 2006; McCauley et al., 2005), Silene acaulis (Stadler and Delph, 2002), and Picea species (Jaramillo-Correa and Bousquet, 2005). Sequence analysis and statistical tests for DNA recombination indicated that polymorphic patterns found in mitochondrial haplotypes resulted from recombination and/or gene conversion rather than from recurrent mutations.

Heteroplasmy: the condition accelerating or counteracting mutations?

Heteroplasmy is considered to accelerate mutations accumulation in substoichiometric molecules because of their low copy number and because they are usually functionally silent. However, some sublimons might not be so prone to accumulate mutations. Those substoichiometric molecules which are maintained by constant recombination and interconvert with the main genome, may be cleared of the potential mutations by the gene conversion process. Moreover, not all sublimons are necessarily silent in terms of gene expression. The NCS maize mutants are maintained heteroplasmically and the appearance of homoplasmic plants is extremely rare because kernels that are homoplasmic for the mitochondrial deletions are usually aborted (Baker and Newton, 1995). Heteroplasmic sectors of the NCS plants containing both mutated and wild-type mtDNA, remain green while sectors with homoplasmic cells including only mutated mitochondrial genomes are defective (Gu et al., 1993). These facts point out that, in the NCS maize, heteroplasmy counteracts mutation: undeleted copies of mitochondrial genes located in the substoichiometric wild-type mtDNA molecules are expressed and can, at least partially, compensate for the consequences of deletions in the main mutated genome. Indeed, normal transcripts of the deleted genes have been found in the NCS mutants and their levels reflected the severity of the mutant trait (Marienfeld et al., 1993). It is possible that whether the gene located in the sublimon molecule is expressed or not depends on the mtDNA stoichiometry. It is clear that in all analysed maize NCS mutants the copy number of the wild-type genome is relatively high in the green sectors. Compared to substoichiometric sequences found in other plant species, detection of the wild-type sequences in maize mutants was relatively easily achieved via conventional PCR or Southern-blot hybridization (Gu et al., 1993; Wintz, 1994; Yamato and Newton, 1999).

According to the above-mentioned scenario, heteroplasmy allows counteracting mitochondrial mutations only if it is a permanent condition and continuously compensates.
for mutation consequences. Another possible mechanism requires only transient heteroplasmy following paternal leakage of plant mtDNA and the subsequent recombination between coexisting mitotypes (Barr et al., 2005). Since data reporting biparental reproduction and recombination between maternal and paternal mtDNAs has accumulated within last years, it is possible that these events occur at a high enough frequency to counter mutation accumulation.

Nuclear control of heteroplasmic genomes

Heteroplasmic populations of mtDNA molecules are usually maintained in following generations and are stable among siblings. Crosses between plants, differing in nuclear backgrounds, commonly result in substoichiometric shifting. Consequently, there must be an interaction between particular nuclear genes and mitotypes that aim to guard specific mtDNA configurations and eliminate or reduce the copy number of others.

One of the best known examples of nuclear genes controlling the mitochondrial genome is Fr in common bean. Introduction of the Fr gene into cytoplasmically male-sterile plants results in pollen fertility restoration caused by the rearrangement of mtDNA which is identical to the rearrangement found upon spontaneous reversion to fertility (Mackenzie and Chase, 1990). Both reversion and restoration are effects of the copy number reduction of autonomously replicating subgenomic molecules containing the CMS inducing pvs sequence (Mackenzie and Chase, 1990; Janska et al., 1998). Nuclear genes restoring pollen fertility via changes in mtDNA were also described for other plant species (reviewed by Chase, 2006). Interestingly, mitochondrial genes causing CMS in plants and also their respective nuclear restorers are different in each species. Li et al. (2008) identified seven haplotypes of orfH79 resulting in CMS in wild rice and found that different haplotypes have different nuclear restorers.

Nuclear control of mtDNA can be viewed as a mechanism allowing the uniparentally inherited mitochondrial genome to be affected by another parent. Psm is a nuclear locus which controls the sorting of mtDNA variants from the heteroplasmic population in cucumber. Transmission of the mitochondrial genome in this plant species is normally paternal but via the Psm locus, the maternal genome affects the sorting of mtDNA (Havey et al., 2004).

The availability of Arabidopsis thaliana mutants allowed the characterization of the three nuclear genes participating in the control of the stoichiometry of alternative mtDNA forms generated by recombination: OSB1 (Zaegel et al., 2006), MSH1 and RECA3 (Shedge et al., 2007). OSB1 (organellar single-stranded DNA binding protein 1) is required for correct stoichiometric DNA transmission. OSB1 T-DNA mutants were shown to accumulate products of homologous mtDNA recombination and to develop phenotypes of leaf variegation and distortion. The rearrangements occurred in two steps: first, homologous recombination products accumulated, next one of the products became predominant in subsequent generations. After the second step, the process was no longer reversible. The transition from partial and reversible mtDNA shifting to irreversible shifting correlated with the loss of reciprocal recombination products (Zaegel et al., 2006). Comparison of msh1 and recA3 mutants showed that both were characterized by the asymmetric accumulation of recombinant DNA molecules (Shedge et al., 2007). The two genes act in distinct but overlapping pathways and their simultaneous loss resulted in a higher degree of genome instability. High expression of the respective proteins was found in flowers correlating with the timing of SSS. Shedge et al. (2007) proposed the model linking MSH1 and RECA3 with mtDNA replication and non-reciprocal recombination between short repeats that resulted in altered stoichiometry. According to this model, normal functions of RECA3 and MSH1 are to prevent the crossing over at short repeats by directing recombination intermediates exclusively into gene conversion events. RECA3 and MSH1 are components of the surveillance mechanism that allows crossing over and recombination-dependent replication to be initiated only at long repeats. Substoichiometric shifting associated with RECA3 and/or MSH1 dysfunction is the consequence of the aberrant event, which occurs when the asymmetric recombinant mtDNA molecule appears and replicates efficiently, changing the stoichiometry in the genome.

In most cases, only individual nuclear genes controlling heteroplasmic genomes were described, but sporadically much more complicated interactions were reported. The nuclear control of wheat mtDNA reorganization was shown to be a complex process that involved many genes located on almost every chromosome (Hartmann et al., 2000). Rearrangements were controlled predominantly by a major nuclear gene and, to a lesser extent, by many minor genes. The control was qualitative and quantitative. The nuclear P2 phenotype of maize characterized by recessive mutations, resulted in the general destabilization of the mitochondrial genome caused by the large-scale changes: accumulation of arrangements previously maintained at low copy number and decrease in the copy number of normal regions (Kuzmin et al., 2005). Moreover, the unique feature of the maize P2 phenotype were multiple differences in mtDNA molecules composition observed among siblings and between parents and progeny. To explain the observed destabilization of mitochondrial genomes, authors have suggested that one recessive nuclear allele disrupts the negative control of replication of some mitochondrial subgenomes, while the other allele (or alleles) deregulates the stable inheritance of subgenomes.

Concluding remarks and further perspectives

Despite the recent progress in the understanding of plant heteroplasmy, there are still many fundamental problems concerning this phenomenon that need to be explained, such as: the mechanism(s) of sublimon maintenance,
transmission, and substoichiometric shifting. This situation results, at least in some considerable degree, from methodological difficulties. Very minute amounts of substoichiometric molecules, sometimes present only in a fraction of plant cells, may easily remain undetected even by relatively sensitive conventional PCR. Obviously, detection and quantification of sublimons became much easier and more precise when quantitative real-time PCR was applied. Study on heteroplasmy was also hampered by another methodological barrier: the inability to transform plant mitochondria. Consequently, research was restricted to available natural heteroplasmic systems or those produced by hybridization experiments. Moreover, many cases of heteroplasmy resulting from paternal leakage, nucleotide substitutions, and small-scale mutations were overlooked due to the general assumption that plant mtDNA is strictly maternally inherited and evolves very slowly at the sequence level. New techniques and attention paid to previously neglected sources of mtDNA variability should result in the further accumulation of data concerning plant heteroplasmy.

One of the most intriguing issues, which have not been satisfactorily addressed, concerns stoichiometric changes in heteroplasmic genomes dependent on plant tissue, organ, and age or stimulated by environmental factors. As long as these problems are unexplained, the true nature of plant heteroplasmy and its role in plant physiology and evolution will remain elusive.

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