The loss of DNA from chloroplasts as leaves mature: fact or artefact?

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Abstract

In this review, the controversy regarding the preservation or degradation of chloroplast DNA (cpDNA) as chloroplasts develop their photosynthetic capacity and leaves reach maturity is addressed. A constant amount of cpDNA during maturity might be expected in order to support photosynthesis over the lifespan of the leaf. Nevertheless, a decline in cpDNA during leaf development was found for all seven plant species investigated. Initial measurements showed that Arabidopsis was similar to the other seven. The controversy arose with two recent studies concluding that the amount of cpDNA remains constant as Arabidopsis leaves mature. These authors proposed that the observation of Arabidopsis chloroplasts with undetectable levels of DNA was an artefact, although the most recent data support the original findings. If the amount of cpDNA remains constant, then Arabidopsis is atypical and would not serve as a good model for chloroplast development. It is shown that the apparently contradictory data may be attributed to methodology and the choice of leaves to be compared. Thus, it is concluded that the controversy can be resolved, Arabidopsis can serve as a representative model, and cpDNA degradation is a common event in chloroplast development.

Key words: Chloroplast, degradation, DNA, leaf development.

Introduction

Since the cytological discovery of DNA-containing ‘bodies’ in the chloroplast of Chlamydomonas in 1962 (Ris and Plaut, 1962), the course of research on chloroplast DNA (cpDNA) has been marked by three major controversies. The first involved the identification of the true cpDNA among the DNAs obtained from chloroplast preparations. It was not until 1972 that the use of DNase to remove contaminating nuclear DNA from the surface of extracted chloroplasts allowed cpDNA to be isolated in pure form (Kolodner and Tewari, 1972). The second controversy involved the size and linear or circular form of DNA molecules within plastids. The use of in-gel procedures to avoid shearing the DNA combined with the analysis of moving pictures of ethidium-stained cpDNA showed that the commonly depicted genome-sized circle represented neither most of the cpDNA, the template for replication, nor the segregating genetic unit (the chromosome) in plastids (Bendich, 2004; Oldenburg and Bendich, 2004, 2009). The third and current controversy involves the degree to which DNA is retained as chloroplasts develop their photosynthetic capacity and leaves reach the period of maturity. This third controversy is the subject of the present article.

Plastid DNA during development

Unlike eukaryotic nuclei, chloroplasts can contain many copies of their genome. In meristematic cells, cpDNA replication outpaces chloroplast division, leading to a net increase in DNA per chloroplast during early seedling development (Fujie et al., 1994; Kuroiwa, 1991). For example, the increase is 7.5-fold and about 4-fold for wheat and spinach, respectively (Lawrence and Possingham, 1986; Miyamura et al., 1986). From 3–7 d after germination, cpDNA increases in the first true Arabidopsis leaf from ~40 to ~600 genomes per chloroplast (Fujie et al., 1994). The increase occurs when the leaf is still <0.5 mm in length, leaves that are so small that it would be impractical to
obtain sufficient leaf material to detect the increase using the Southern blotting procedure.

As the leaf cells continue to expand, the amount of cpDNA declines. For spinach, an approximately 6-fold decrease in genomes per chloroplast was attributed to a constant amount of cpDNA per cell partitioned among an increasing number of chloroplasts per cell without cpDNA replication (Scott and Possingham, 1980). The decline in cpDNA during leaf expansion for barley and oat, however, involves cpDNA degradation in addition to dilution, since the rate of cpDNA decline exceeds the rate of chloroplast division (Baumgartner et al., 1989; Hashimoto and Possingham, 1989). Degradation of cpDNA in developing juvenile rice leaves was proposed to act as a signal for the initiation of senescence (Sodmergen et al., 1991). Note that the above studies focused on immature plants, and the fate of cpDNA throughout the lifespan of the plant was not addressed.

The controversy over constant or decreasing amounts of cpDNA during development

A constant amount of cpDNA during maturity might be expected in order to support photosynthesis over the lifespan of the leaf. Nevertheless, undetectable levels of cpDNA were found in many chloroplasts isolated from mature Arabidopsis thaliana leaves, as determined by staining with 4′,6-diamidino-2-phenylindole (DAPI) (Rowan et al., 2004). The amount of DNA per chloroplast declined on average 2–7-fold long before the initiation of senescence. We made a mistake in the Abstract of Rowan et al. (2004) when we said that the amount of cpDNA declines until ‘most of the leaves contain little or no DNA’. We meant to say that the amount of cpDNA declines until most of the chloroplasts contain little or no DNA. This mistake may have contributed to the controversy that arose with two later studies concluding that the amount of cpDNA remains constant as leaves mature (Li et al., 2006; Zoschke et al., 2007). In both studies, only one method was used to measure the cpDNA and neither used the same method as we did in our original study. These data are now analysed in an attempt to resolve this controversy.

Li et al. (2006) investigated the amount of cpDNA in Arabidopsis and tobacco by visual inspection of blot-hybridization signals. They concluded that cpDNA remained constant throughout development in both species, but this conclusion was not supported by all of their data. By our visual inspection, there is an apparent 2–3-fold reduction in hybridization signal between the ‘young’ and ‘mature’ leaf of a 36-d-old Arabidopsis plant (Lanes 2 and 3 in Fig. 3b of Li et al., 2006). They provided no control data for Arabidopsis to indicate equal loading of all samples or to reveal the extent of the restriction digestion, since their images did not include the well of the gel. We also analysed cpDNA by blot hybridization (including the proper controls) and found that the hybridization signal for mature tissues was 2–5-fold less than that of young tissues (Rowan et al., 2009). For tobacco, hybridization of the chloroplast probe with ‘promiscuous’ cpDNA in the nucleus served as a loading control, and all signals from chloroplast-derived cpDNA appeared to be constant by our visual inspection.

Even if we now suppose that all of their experiments included the proper controls and did, in fact, show a constant hybridization signal for both species, the following alternative interpretations are offered. Li et al. (2006) claim that their leaf material ‘was produced exactly (our emphasis) as described by Rowan et al. (2004)’. They provided the age of the plant, but described the leaves only as ‘young’ or ‘mature’. Without knowing the size of the leaf or which leaf was examined (and such information was not provided) it cannot be determined whether these leaves are comparable to ours. For tobacco, the position of the leaf along the stem was described, but the age of the plants was not given. Thus, it cannot be assessed whether their tobacco leaves were at a similar developmental stage as ours, which did show a modest decrease in DNA per chloroplast for the largest leaf (Shaver et al., 2006).

Arabidopsis leaves undergo several rounds of endoreduplication during leaf development (Galbraith et al., 1991; Zoschke et al., 2007; Rowan et al., 2009), and this further confounds evaluation of the blot-hybridization data. The DNA obtained from older leaves represents a smaller number of cells than the same mass of DNA obtained from young leaves, due to the increase in nuclear ploidy level (genome copies per cell). Thus, constant hybridization signals for cpDNA during development actually mean that the fraction of cellular DNA represented by cpDNA is higher for the older leaves. Since cpDNA replication ceases after cells leave the meristem (Fujie et al., 1994), it seems unlikely that cpDNA could possibly increase as leaves age. Tobacco leaves, however, do not undergo endoreduplication during development (our unpublished observations) and constant hybridization signals would reflect a constant amount of cpDNA during development.

As discussed above, cpDNA first increases and then decreases during leaf development and maturity. For Arabidopsis, the increase occurs very early during development of the first leaf, when the leaf is less than 1.5 mm long (about 15% expanded; Fujie et al., 1994; Rowan et al., 2004). For tobacco, the increase occurs until the leaf is 30% expanded (146 cm$^2$; Shaver et al., 2006). Figure 1 is a schematic representation of the changes in the average DNA amount per plastid as a function of leaf development for maize, Arabidopsis, and tobacco. Limited sampling of developmental stages could lead to the erroneous conclusion that cpDNA remains constant throughout development. For example, a young leaf before cpDNA replication and a mature leaf in which cpDNA is in decline can have about the same amount of cpDNA, on average (compare positions I and III for maize and Arabidopsis or positions II and IV for tobacco). A constant amount of cpDNA would also be observed if the period of decline had already passed (positions IV and V for each plant).

Zoschke et al. (2007) concluded that the amount of cpDNA remains constant throughout development in Arabidopsis using data from real-time quantitative PCR.
(qPCR) and nuclear ploidy. The ratio of cpDNA copies to nuclear DNA copies was assessed for each developmental stage using qPCR. This ratio was then multiplied by the average ploidy of nuclei obtained from leaves of the same developmental stage to calculate the number of chloroplast genomes for an ‘average’ cell. We also found that the amount of DNA per ‘average’ cell did not vary during development. Why, then, does the amount of cpDNA increase more gradually and the decline is less severe. The Roman numerals indicate stages of leaf development. I–III represent expanding leaves (LDS <1, see text), and IV and V represent expanded leaves (LDS >1).

In summary, the data of Li et al. (2006) and Zoschke et al. (2007) may not conflict with those of Rowan et al. (2004) and Shaver et al. (2006). Differences in the selection of developmental stages and the methods used may explain the different conclusions reached in these studies.

Alternative interpretations of the cpDNA decline

Li et al. (2006) suggested that the undetectable levels of cpDNA observed by Rowan et al. (2004) could have arisen as an artefact of the isolation process because the DNA used to remove extraorganellar DNA preferentially degraded the DNA of chloroplasts from mature leaves. While DNase can artefactually reduce the amount of DNA per chloroplast for tobacco and *Medicago truncatula* (Shaver et al., 2006), our original data (Rowan et al., 2004) did show, in fact, that DNase has no effect on the *Arabidopsis* chloroplasts we studied. We have since adopted a high-salt protocol for isolating chloroplasts that avoids the use of DNase and still observe a developmental decline in cpDNA amount (Rowan et al., 2007). Thus, it is unlikely that the observation of chloroplasts with undetectable levels of DNA in *Arabidopsis* resulted from DNase treatment during isolation.

Zoschke et al. (2007) also suggested that our observation of chloroplasts with undetectable DNA resulted from an artefact of the isolation process. Chloroplasts from older leaves are expected to contain larger starch grains than those from younger leaves and might therefore be more likely to rupture during isolation. Since the DAPI-DNA staining of chloroplasts within leaf sections also becomes less intense during leaf development (Rowan et al., 2009),
the cpDNA decline occurs in vivo and is not an artefact of chloroplast isolation.

Additional hypotheses for how the original data from Rowan et al. (2004) might not be due to degradation of cpDNA during development have to be considered. In chloroplasts of mature leaves, the autofluorescence of chlorophyll could quench the DAPI signal or DNA might be present as small fragments incapable of generating a signal. Neither of these hypotheses is supported by our data, which showed that variation in DNA content among plastids as measured by qPCR corresponds to variation in fluorescence from DAPI (using fluorescence microscopy) or the more sensitive fluorophore SYTO 42 (using flow cytometry) (Rowan et al., 2009). The possibility also has to be considered that the decline we observed for Arabidopsis is due to dilution of a constant amount of cpDNA by chloroplast division, as observed for some other plant species. However, a decline in average DNA amount per plastid was found when chloroplast numbers are not increasing, indicating that dilution is not responsible for the reduced DNA content of mature chloroplasts (Rowan et al., 2009). The amount of cpDNA per plastid declined 2–3-fold on average, which is similar to the 2–7-fold decline in average DNA amount per plastid for the broader developmental range of tissues examined in our original report (Rowan et al., 2004, 2009).

Viability in the absence of DNA

Cells and organelles may survive despite the complete loss of genetic material. As red blood cells develop from their haematopoietic precursors, they can lose their nuclear DNA in mammals, but not in birds or amphibians. The mean lifespan for red blood cells in humans is ~50 d (Cohen et al., 2008). In the mesozoan Dicyema japonicum, mtDNA copy number increases during early embryogenesis, then decreases as larvae develop (Awata et al., 2005). In mature larvae, mtDNA was detected in germ cells using in situ hybridization, but ‘no signals were detected in peripheral cells, reflecting an extremely low copy number or the complete absence of mtDNA’. Mitochondria of adult somatic cells were similarly found to retain little or no mtDNA. Thus, organelles and cells may function for extended periods without DNA.

Avoiding controversy in the future

There is a general lack of consistency in how leaf ‘age’ is reported. Descriptions of leaf development range from ‘young’ and ‘mature’ to measurements of leaf size. Research on all aspects of leaf development will be improved by the establishment of a standard method for reporting the age of leaves. Leaf size alone is not sufficient to determine the age of the leaf because different leaves may reach different maximum sizes. The plastochron index and leaf plastochron index can be useful for comparing the development of individual plants or leaves of a species (Erickson and Michelin, 1957; Larson and Isebrands, 1971). These methods give a good estimate of leaf development relative to a reference leaf. Since the reference leaf size is arbitrarily assigned, however, it is difficult to describe the true developmental status of the leaf, especially if leaves on an individual plant reach different maximum sizes or if leaf development is compared among plant species with different maximum leaf sizes. For monocot leaves, development is perhaps best expressed as a function of distance from the basal meristem. This method is not suitable for dicot leaves because the location of the meristematic tissue is less distinct.

We propose defining leaf age as a function of leaf expansion and time. Leaves are assigned a ‘Leaf Development Stage’ (LDS) using the following equation:

\[
LDS = \frac{(S/MS) + D}{S/MS}
\]

where \(S\) is the size (area or length) of the leaf, \(MS\) is the maximum size that particular leaf reaches in the growth conditions specified, and \(D\) is the number of days after the leaf has reached full expansion.

LDS calculations for Arabidopsis are illustrated. A 1 mm Arabidopsis first rosette leaf would have an LDS of 0.1 because \(S = 1\), \(MS = 10\), and \(D = 0\) because the leaf has not reached full expansion. A 1 mm fifth rosette leaf would have an LDS of 0.07 because \(S = 1\), \(MS = 15\), and \(D = 0\). A 10 mm first rosette leaf on a 30-d-old plant would have an LDS of 11 because \(S = 10\), \(MS = 10\), and \(D = 0\), since maximum expansion is reached at about day 20 (Rowan et al., 2007). This is a useful and convenient method for expressing the age of a leaf because LDS values less than 1 represent leaves that are still expanding, while LDS values greater than 1 represent fully expanded leaves. (A leaf with an LDS of 10 should not be considered to be 100-fold more developed than a leaf with an LDS of 0.1. The LDS values scale linearly with development until maximum expansion is reached (LDS=1). LDS values greater than 1 indicate the passage of time since the completion of leaf expansion and do not scale linearly with development.) Figure 2 shows how LDS is useful to compare developmental status between two leaves that reach different maximum sizes.

In addition to a common system for defining leaf age, future controversy may also be avoided by exercising caution when comparing data obtained from averaging methods to data obtained using methods that measure variation among individuals. It should be made clear whether cpDNA amount refers to an average amount or to an amount per chloroplast, per cell, or a fraction of total cellular DNA.

Conclusion

Like the two previous controversies in cpDNA research, the controversy over whether the amount of cpDNA declines or remains constant during development can be resolved. Differences in the leaf material and methods employed in different studies may explain the conflicting results. The alternative explanations for the original finding that DNA per chloroplast declines during development (Rowan et al.,
M. truncatula et al. retain cpDNA during leaf development (Oldenburg 2004). The amount of cpDNA: plants grown in constant darkness (Oldenburg 1991, 1992). Chloroplast biogenesis also affect the persistence of cpDNA. Yet, even single-celled algae shed cpDNA. Chloroplasts of Chlamydomonas reinhardtii lose DNA during the vegetative phase of their life cycle (Coleman, 1979; Woodcock et al., 2006; Shaver et al., 2008). For maize, genes that affect chloroplast biogenesis also affect the persistence of cpDNA (Oldenburg et al., 2006).

A reluctance to accept cpDNA loss without compromising plant fitness may prolong the third and current controversy. Yet, even single-celled algae shed cpDNA. Chloroplasts of Acetabularia lose DNA during the vegetative phase of their life cycle (Coleman, 1979; Woodcock and Bogorad, 1970), and Chlamydomonas reinhardtii cpDNA is degraded in response to phosphorous limitation without substantially affecting viability (Yehudai-Resheff et al., 2007) and in the zygote during mating (Nishimura et al., 2002). For mature leaves of multicellular plants, the loss of cpDNA would not affect reproduction because gametes arise from the shoot apical meristem, not the leaf. Despite the large body of existing evidence, the third controversy will only be laid to rest with the realization that cpDNA degradation is a common event in chloroplast development.

**Fig. 2.** The leaf development stage (LDS) method for expressing the age of leaves. The value for LDS expresses the developmental stage of a leaf as a function of leaf expansion and time (see text). The LDS values over time are shown for two different leaves. LDS values less than 1 represent expanding leaves, and values greater than 1 reflect the time since maximum expansion. The leaf shown in (A) reaches a maximum size of 10 mm. The leaf shown in (B) reaches a maximum size of 25 mm. Leaf size is measured as the length of the leaf blade. At Time 1, both leaves are 6 mm long, but they are not at the same developmental stage. At Time 2, both leaves have just reached full expansion. At Time 3, both leaves have been fully expanded for 4 d.

References


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