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The demise of chloroplast DNA in *Arabidopsis*

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Abstract Although it might be expected that chloroplast DNA (cpDNA) would be stably maintained in mature leaves, we report the surprising observation that cpDNA levels decline during plastid development in *Arabidopsis thaliana* (Col.) until most of the leaves contain little or no DNA long before the onset of senescence. We measured the cpDNA content in developing cotyledons, rosette leaves, and cauline leaves. The amount of cpDNA per chloroplast decreases as the chloroplasts develop, reaching undetectable levels in mature leaves. In young cauline leaves, most individual molecules of cpDNA are found in complex, branched forms. In expanded cauline leaves, cpDNA is present in smaller branched forms only at the base of the leaf and is virtually absent in the distal part of the leaf. We conclude that photosynthetic activity may persist long after the demise of the cpDNA.

Keywords Chloroplast · DAPI · DNA · Fluorescence microscopy · Nucleoid

Introduction

There can be many copies of the plastid genome per plastid. The average chloroplast DNA (cpDNA) content in plastids changes during leaf development, as revealed by DNA hybridization analysis (Lamppa and Bendich 1979) and staining with 4',6-diamidino-2-phenylindole (DAPI; Kuroiwa 1991). DAPI staining is localized to regions within the plastid known as nucleoids, which vary in size, number, and distribution during the early stages of leaf development (Kuroiwa et al. 1981). During the development of chloroplasts from proplastids in

wheat, for example, the cpDNA content increases by 7.5-fold per plastid and 34-fold per leaf cell, since plastid number also increases (Miyamura et al. 1986). The reason for the amplification of plastid genomes may be to provide an increased dosage of rRNA genes for the ribosomes needed to meet the demand for protein synthetic capacity during leaf development (Bendich 1987).

The replication of cpDNA is restricted to meristematic cells (Kuroiwa 1991; Fujie et al. 1994). As cells leave the meristem and expand, the increasing number of chloroplasts leads to a decrease in the cpDNA per chloroplast. This decrease in chloroplast “ploidy” can occur either by dilution of a constant amount of cpDNA per cell, as in pea (Lamppa et al. 1980) and spinach (Scott and Possingham 1980), or by dilution accompanied by cpDNA degradation, as in barley (Baumgartner et al. 1989) and rice (Sodmergen et al. 1991). For barley, data were obtained from DNA hybridization measurements using isolated and pooled chloroplasts, so that the possibility that some chloroplasts might have contained no DNA was not assessed. For rice, DAPI fluorescence data were obtained for mesophyll cells in the upper half of the blade of the second leaf; and almost all of the individual chloroplasts had lost all detectable cpDNA by the seventh day after seeds were sown. The degradation of cpDNA in rice was proposed as a signal for the start of leaf senescence.

In this study, we survey chloroplasts from three types of leaves of *Arabidopsis* plants during early and later stages of leaf development. We find that, as the leaves mature, cpDNA is absent from most of the chloroplasts long before the onset of senescence. The photosynthetic process in *Arabidopsis* can evidently be unaffected by the demise of cpDNA.

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Materials and methods

Growth conditions

Seeds of *A. thaliana* (Col.) were sown in a sterile agarose medium containing MS salts (Murashige and Skoog

1962) and grown at 20°C on 16 h light, 8 h dark cycles. The cotyledons were harvested at 3, 5, and 10 days after imbibition. The first rosette leaf was harvested at 7 and 9 days. Seeds were also sown in soil and grown in a greenhouse; and the first rosette leaf was harvested at 38 days. The first cauline leaf was harvested at 27 and 30 days. The cauline leaf at 30 days was divided into a basal 1-cm section and a distal 2-cm section.

Chloroplast isolation

Tissue from plants grown in soil was soaked in 0.5% sarkosyl solution for 3–5 min, rinsed thoroughly, and ground using a mortar and pestle in grinding buffer (GB; Oldenburg and Bendich 2004). The homogenate was filtered through Miracloth and centrifuged in a microcentrifuge at 12,000 *g* for 20 s. The pellet was resuspended and washed twice in dilution buffer (DB; Oldenburg and Bendich 2004). The chloroplasts were treated with deoxyribonuclease (DNase) for 1–2 h at 0°C, washed in DB and loaded onto step gradients (30:70 Percoll in DB) and centrifuged at 4°C for 10 min at 12,000 *g* in a microcentrifuge. The chloroplasts were taken from the 30:70 Percoll interface and washed twice in DB.

Preparation of chloroplast DNA for visualization by fluorescence microscopy

Chloroplasts were embedded in agarose and lysed overnight at 48°C in 0.47 M EDTA, pH 8, 1% sarkosyl with 200 µg/ml proteinase K. Agarose-embedded cpDNA was stained with 0.1 µg/ml ethidium bromide and visualized as described by Oldenburg and Bendich (2004). The chloroplasts were diluted sufficiently so that each microscopic field contained the DNA from an individual chloroplast. Representative images are shown in Fig. 6.

Fluorescence and light microscopy of chloroplasts

The chloroplasts were adjusted to 0.8–1.6% glutaraldehyde, 1% β-mercaptoethanol, and 2 µg/ml DAPI and were examined microscopically. Images of DAPI-stained chloroplasts were obtained using a Nikon Microphoto FX epi-fluorescence microscope outfitted with a Qimaging Retiga 1300 digital camera. Open *lab* image-capture software was used to analyze the fluorescence intensity. DNA-DAPI fluorescence was measured for images taken from microscopic fields using a filter with an emission maximum of 420 nm; and chlorophyll autofluorescence was measured similarly using a green light G-1B filter (emission maximum: 590 nm). Fluorescence was analyzed on a scale of 0–1,023 grayscale units. The value of relative fluorescence (Rfl) was calculated by multiplying the fluorescence intensity by the

area of fluorescence and dividing by 1,000. Light microscopy was used to determine the size of the chloroplasts.

Treatment of chloroplasts with DNase for correction of fluorescence measurements

Chloroplasts were fixed in 0.8% glutaraldehyde for 1–2 h and washed four times with DB before adding 1 mg/ml DNase and incubating on ice for ca. 24 h. DNase-treated and non-DNase-treated chloroplasts were stained with DAPI and visualized as described above.

Controlling effects of DNase treatment during chloroplast isolation procedure

Plants were grown in soil in a greenhouse until they had reached the same stage of development as those used for the experiments with the first cauline leaf. Whole cauline leaves less than 8 mm long (young leaves) and whole rosette leaves 3 cm or longer (mature leaves) were harvested. Chloroplasts from both samples of leaves were isolated as described above, with and without DNase treatment. The chloroplasts were stained with DAPI and the DNA content was assessed by microscopic examination. DNA was considered to be absent in chloroplasts with no visible blue–white fluorescence and present in trace amounts in chloroplasts that had faint blue–white fluorescence. Intense fluorescence was taken to indicate abundant DNA.

Results

Correction of DAPI fluorescence measurements

The fluorescence of DAPI-stained DNA is blue–white in appearance. However, some chloroplasts had a reddish tinge with little or no blue–white color. The reddish color was most likely due to some chlorophyll autofluorescence passing through the DAPI filter that could interfere with the quantitation of DNA-DAPI fluorescence. Thus, it was necessary to correct the total fluorescence measurement to accurately determine the intensity of the DNA-DAPI fluorescence.

To do this, we treated chloroplasts with and without DNase, stained them with DAPI, and examined them by fluorescence microscopy. Non-DNase-treated chloroplasts had a visible blue–white DAPI fluorescence, whereas DNase-treated chloroplasts had no visible DAPI fluorescence, but had a faint reddish color. Rfl values (see Materials and methods) were determined for images taken using the DAPI filter. Since the cpDNA had been eliminated, the only fluorescence signal was that due to crossover chlorophyll autofluorescence. We then measured the chlorophyll autofluorescence using a green light (G-1B) filter. Figure 1 shows a linear

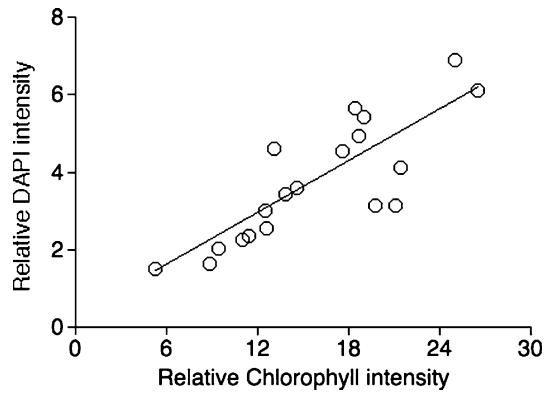


Fig. 1 Comparison of DAPI and chlorophyll relative fluorescence values for DNase-treated chloroplasts. There is a positive correlation between the chlorophyll intensity and DAPI intensity, indicating that the DAPI relative fluorescence values are attributable to crossover from chlorophyll autofluorescence. The equation of the line is $y = 0.279 + 0.223x$. $R = 0.81$

relationship between the Rfl from chlorophyll autofluorescence and the crossover Rfl values measured using the DAPI filter. An equation was derived and used to subtract the crossover Rfl, to obtain the DAPI Rfl value attributable to the cpDNA. For example, a chloroplast with a chlorophyll Rfl of 15 contributed a crossover Rfl of 3.6.

Changes in DAPI fluorescence in chloroplasts of the cotyledons

DNA-DAPI fluorescence values ranged considerably among the chloroplasts in all three samples. At 3 days after imbibition, the cotyledons were about 1 mm in length (Fig. 2a) and the Rfl of the chloroplasts ranged from 1.8 to 26.0. Two days later (Fig. 2b), the cotyledons reached approximately 2 mm in length. The mean Rfl (Fig. 2, arrows) increased, as did the range of Rfl values. After 10 days, the cotyledons grew to a maximum of 3 mm in length (Fig. 2c). The range and mean of Rfl values had fallen to what they had been at 3 days. Two chloroplasts had Rfl values less than 1.0, meaning they contained almost no detectable cpDNA.

Changes in DAPI fluorescence during development of the first rosette leaf

Seven days after imbibition, the first rosette leaves were approximately 1 mm in length (Fig. 3a). Rfl values at this stage showed the widest range of the three stages sampled. Two days later, the first rosette leaves had increased in length to about 1.5 mm (Fig. 3b). The mean Rfl had increased marginally, but the range of Rfl values decreased. By 38 days after imbibition, the first rosette leaves were fully expanded and approximately 1 cm in length. The range of Rfl values had narrowed (Fig. 3c) and the mean Rfl value was much lower. Now, 69% of

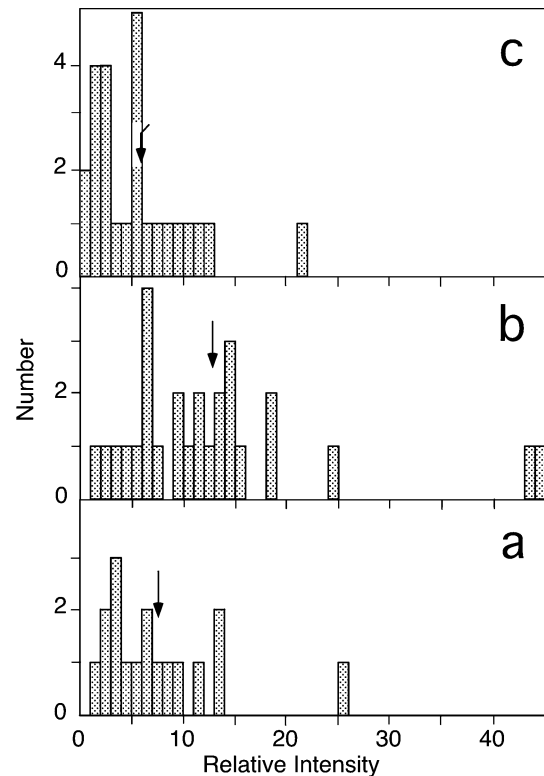


Fig. 2a-c The frequency of relative fluorescence values of DAPI-stained chloroplasts isolated at three different times during growth of the cotyledons. Time after imbibition: **a** 3 days, **b** 5 days, **c** 10 days. The means (arrows) are 7.6 ± 1.4 , 13 ± 2.0 , and 6.0 ± 1.3 , respectively. The mean of **b** is significantly different from the means of **a** and **c** ($P < 0.05$). The arrangement of the three panels represents the form of a developing plant

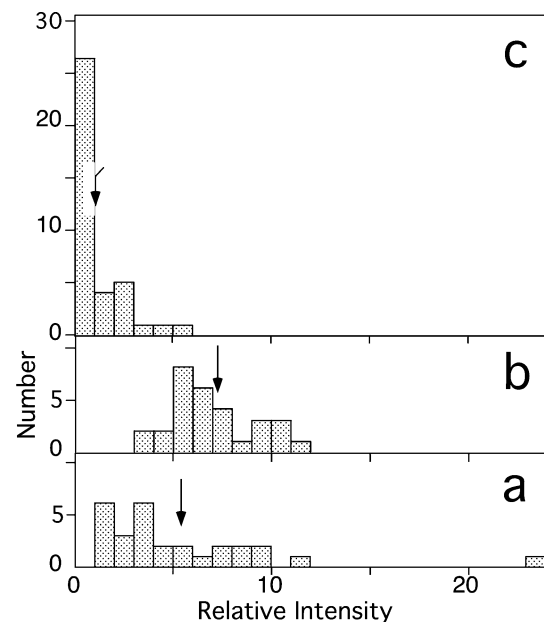


Fig. 3a-c The frequency of relative fluorescence values of DAPI-stained chloroplasts isolated at three different times during growth of the first rosette leaf. **a** 7 days, **b** 9 days, **c** 38 days. The means (arrows) are 5.4 ± 0.9 , 7.0 ± 0.4 , and 1.1 ± 0.2 , respectively. The mean of **c** is significantly different from **a** and **b** ($P < 0.005$)

the chloroplasts had little or no measurable cpDNA ($R_{fl} < 1$) and, of these, 58% had no detectable DAPI fluorescence.

Changes in DAPI fluorescence in chloroplasts of the first cauline leaf

Young cauline leaves (27 days after imbibition) were approximately 8 mm in length (Fig. 4a). Their DAPI-stained chloroplasts showed the highest mean and widest range of R_{fl} values of three samples of cauline leaf tissue. Three days later, the cauline leaves increased to 3 cm in length. R_{fl} values of the chloroplasts sampled from the basal 1-cm section of this leaf decreased sharply (Fig. 4b). In the upper portion of the leaf, the mean R_{fl} was marginally lower (Fig. 4c), but 45% of the chloroplasts were found to have R_{fl} values less than 1, including 21% with no detectable DAPI fluorescence.

DAPI fluorescence in chloroplasts isolated with and without DNase treatment

To test for the possibility that plastids isolated at different stages of development might be differentially permeable to the DNase used during isolation, we isolated chloroplasts from young and mature leaves with and without adding DNase during isolation (see Materials and methods). There was no difference in DNA content between samples of chloroplasts isolated with

Table 1 DNA contents of chloroplasts isolated with and without DNase treatment

Leaf	DNase	Chloroplasts examined	DNA detected		
			None	Trace	Abundant
Young	Yes	20	1	2	17
Young	No	19	1	2	16
Mature	Yes	24	7	12	5
Mature	No	24	7	13	4

and without DNase treatment (Table 1). Specifically, in mature leaves, the fraction of chloroplasts that showed little or no DNA was essentially the same with and without DNase treatment, indicating that the reduced DNA content was not due to increased permeability to DNase.

Changes in size and chlorophyll content of chloroplasts with leaf age

The size of individual chloroplasts in the cotyledons increased during the period between 3 and 5 days after imbibition (stages 1, 2 in Fig. 5a): the mean size increased from $27.0 \pm 3.2 \mu\text{m}^2$ at 3 days to $36.0 \pm 3.1 \mu\text{m}^2$ at 5 days. The mean chlorophyll R_{fl} also increased from 4.9 ± 1.1 to 12.0 ± 1.7 . From 5 to 10 days after imbibition (stages 2, 3), the mean chlorophyll R_{fl} further

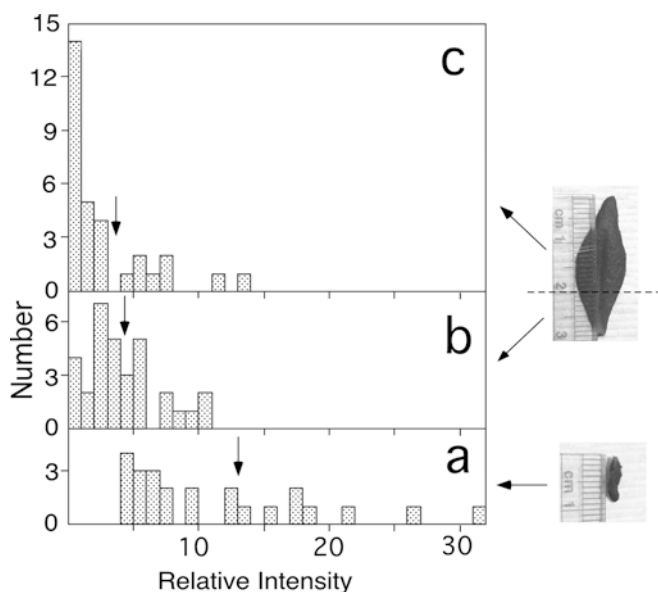


Fig. 4a-c The frequency of relative fluorescence values of DAPI-stained chloroplasts isolated from different tissues of the first cauline leaf at two different times. **a** Whole leaf at 27 days. **b** Basal 1-cm portion and **c** upper portion of the leaf at 30 days after imbibition. The means (arrows) are 13 ± 1.9 , 4.2 ± 0.5 , and 3.4 ± 0.8 , respectively. The mean of **a** is significantly different from **b** and **c** ($P < 0.005$)

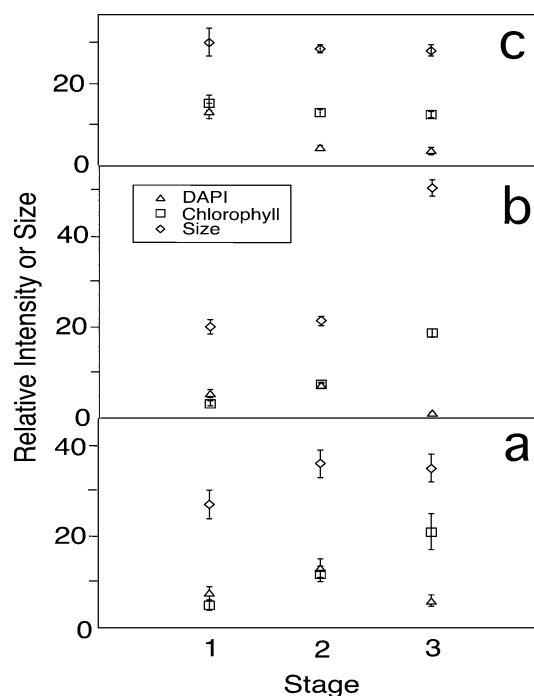


Fig. 5a-c Comparison of DAPI intensity, chlorophyll autofluorescence, and size of chloroplasts among different developmental stages of different leaves. **a** Cotyledons, **b** first rosette leaf, **c** first cauline leaf. Developmental stages are not equivalent in different leaf types (see text). Sizes (μm^2) are means \pm standard error

increased to 21.0 ± 4.0 , although the mean size of the chloroplasts did not increase significantly during this period. At 15 days, signs of senescence (leaf yellowing) were evident. In the rosette leaves, the mean size of the chloroplasts did not increase between day 7 and day 9 (stages 1, 2 in Fig. 5b). A dramatic increase in size did occur between day 9 and day 38 (stage 3), from $21.0 \pm 1.1 \mu\text{m}^2$ to $50.0 \pm 1.9 \mu\text{m}^2$. The mean chlorophyll Rfl increased steadily from stage 1 to stage 3. The onset of senescence for the rosette leaf was not observed until day 45. For cauline leaves, the mean size and chlorophyll content of the chloroplasts were not significantly different among the three tissues during the three stages of leaf development (Fig. 5c). It was not until day 57 (27 days after stage 3) that the first signs of cauline leaf senescence were evident.

Changes in structure of chloroplast DNA in the first cauline leaf

The structure of the DNA molecules from individual chloroplasts is shown in Fig. 6. DNA from the chloroplasts of the young cauline leaf was found either in large, complex, branched forms (Fig. 6b) or in a group of smaller forms with (Fig. 6a) or without (Fig. 6c) large forms. Some of the DNA from the chloroplasts isolated from the basal portion of the fully expanded cauline leaf was also found in smaller branched forms, but the size of these complex forms was typically smaller; and a larger fraction of the DNA was present in simpler forms (Fig. 6d,e). Very large structures, such as the one depicted in Fig. 6b, were not found in the base of this leaf. No DNA structures of any size were found in the chloroplasts sampled from the upper portion of this leaf. Since the proportion of chloroplasts with measurable cpDNA was lowest for the upper portion of the cauline leaf, we expected fewer fields of cpDNA for the same dilution of chloroplasts. This is a likely reason why we found no cpDNA structures from the upper portion of the leaf. We also expected the structures to be smaller and more difficult to find (anything less than 10 kb being undetectable). The branched nature of the forms is likely due to a recombination-dependent DNA replication mechanism, as proposed previously for analogous structures in maize (Oldenburg and Bendich 2004).

We conclude that there is a drastic reduction in the molecular size of cpDNA structures during chloroplast development in *Arabidopsis* leaves. In many chloroplasts, the DNA falls to undetectable levels well before the onset of senescence.

Discussion

Although DNA surely is essential during the early stages of leaf development, it is evident from our results that chloroplast genes are no longer required during the later stages of leaf development in *Arabidopsis* and maize

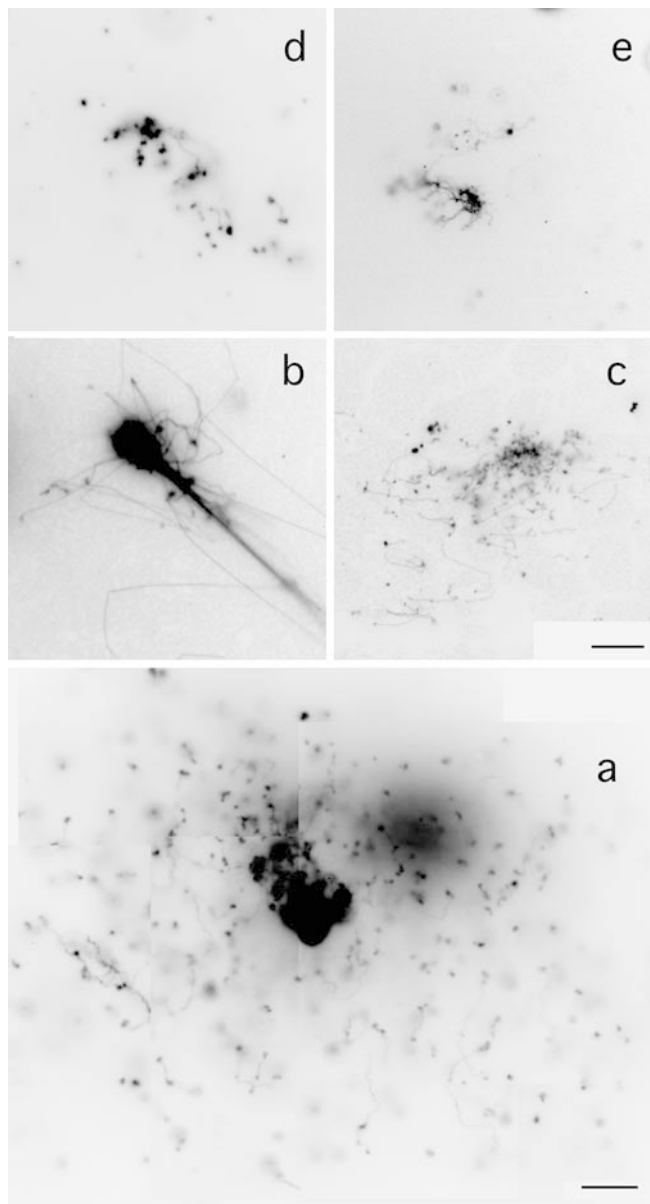


Fig. 6a–e Comparison of the structural forms of cpDNA present in different tissues of the first cauline leaf. **a,b,c** Representative images of DNA from chloroplasts isolated from the whole leaf 27 days after imbibition. **d,e** Representative images of DNA from chloroplasts isolated from the basal portion of the cauline leaf 30 days after imbibition. Images show DNA from individual chloroplasts. There were no small molecules in the entire microscopic field from which the image in **b** was taken. Bars 10 μm

(Oldenburg and Bendich, unpublished data). Woodcock and Bogorad (1970) reported that DNA was absent in 65–80% of the chloroplasts from *Acetabularia*. Subsequent work showed that the DNA per chloroplast decreases as the *Acetabularia* cell develops and that most chloroplasts at the vegetative stage lack DNA (Coleman 1979; Lüttke and Bonotto 1981). Since cpDNA is present in the chloroplasts of reproductive caps and developing cysts, it appears that some chloroplasts containing DNA can migrate to the cap to ensure transmission of the chloroplast genome to the next generation. The

DNA-less chloroplasts were thought to arise from biased segregation of DNA during chloroplast division, rather than from active degradation of cpDNA (Lüttke and Bonotto 1981).

For *Arabidopsis*, we find that DNA is maintained in some chloroplasts in the oldest rosette leaf tissue. These chloroplasts may have been present in meristemoids, which are precursor cells in stomatal development and are capable of further division (Nadeau and Sack 2002). Thus in *Arabidopsis*, cpDNA may be retained in cells that may yet divide, but is degraded in most cells of the mature leaf not destined to divide.

Since cells in the mature leaf of *Arabidopsis* do not develop into gametes, their chloroplast genes will not be passed to progeny plants and the degradation of cpDNA molecules may provide the plant with some benefit. Sears and VanWinkle-Swift (1994) proposed that one parent's cpDNA can be degraded and its nucleotides recycled as nutrients, which inadvertently leads to the uniparental inheritance of chloroplast genes in *Chlamydomonas reinhardtii*. Baumgartner et al. (1989) demonstrated the degradation of cpDNA during leaf development in barley, but did not discuss the adaptive significance of cpDNA turnover.

A lack of cpDNA in most chloroplasts of mature leaves that are (presumably) highly active in photosynthesis means either that the photosynthetic apparatus is extremely stable or that extremely stable mRNA molecules are translated to replenish proteins that turn over. The D1 protein (the *psbA* gene product) does turn over in the light, but the stability of its mRNA is high and increases during chloroplast development (Kim et al. 1993). Thus, the DNA in mature *Arabidopsis* chloroplasts might no longer be needed to support photosynthesis. Its degradation would then serve either no function for the plant or a function not yet identified, or it would provide nutrients to the plant.

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