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Constancy of organellar genome copy numbers during leaf development and senescence in higher plants

Abstract In higher plants, plastid and mitochondrial genomes occur at high copy numbers per cell. Several recent publications have suggested that, in higher plants like *Arabidopsis* and maize, chloroplast DNA is virtually absent in mature and old leaves. This conclusion was mainly based on DAPI staining of isolated chloroplasts. If correct, the finding that chloroplasts in mature leaves lack DNA would change dramatically our understanding of gene expression, mRNA stability and protein stability in chloroplasts. In view of the wide implications that the disposal of chloroplast DNA during leaf development would have, we have reinvestigated the age dependency of genome copy numbers in chloroplasts and, in addition, tested for possible changes in mitochondrial genome copy number during plant development. Analyzing chloroplast and mitochondrial DNA amounts in *Arabidopsis* and tobacco plants, we find that organellar genome copy numbers remain remarkably constant during leaf development and are present in essentially unchanged numbers even in the senescing leaves. We conclude that, during leaf development, organellar gene expression in higher plants is not significantly regulated at the level of genome copy number and we discuss possible explanations for the failure to detect DNA in isolated chloroplasts stained with DAPI.

Keywords *Arabidopsis thaliana* · *Nicotiana tabacum* Plastid · Mitochondrion · Genome copy number · Leaf development

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Introduction

In plant cells, the genetic information is distributed among three compartments: the nucleus, the mitochondria and the plastids. Each of these cellular compartments harbors a genome and, consequently, expresses heritable traits. Whereas traits encoded in the nuclear genome are usually inherited according to the Mendelian rules, plastid and mitochondrial genomes are often transmitted uniparentally. In higher plant species, maternal transmission is the predominantly found mode of organelle inheritance, whereas biparental inheritance or predominantly paternal inheritance are rather rare (Birky 1995; Hagemann 2002).

Compared to the cell's nuclear genome, the genomes of higher plant plastids and mitochondria contain relatively little genetic information. The plastid genome (plastome; ptDNA) of a typical higher plant is 120–160 kb in size and contains approximately 120 genes, densely arranged on a circular genetic map (Sugiura 1992; Wakasugi et al. 2001). Identical copies of this genome are present in all differentiation forms of plastids (plastid types): the undifferentiated proplastids of meristematic tissues, the green chloroplasts in photosynthetically active cells, the carotenoid-accumulating chromoplasts of flowers and fruits, the starch-storing amyloplasts in roots and several other plastid types specialized in the storage of proteins or lipids.

Although higher plant mitochondrial genomes (chondriomes; mtDNA) have an even lower coding capacity than plastid genomes and typically harbor only approximately 60 genes (Unseld et al. 1997; Knoop 2004) they are generally bigger than plastid genomes. The chondriomes of higher plants are also much larger in size (200–2,400 kb) and more complex in their genome organization than those of protists (5.7–76 kb), animals (14–42 kb) and fungi (18–176 kb; Backert et al. 1997; Gray et al. 1998). The great size difference between the highly compact mitochondrial genomes of animals and those of higher plants is mostly due to the presence of large intergenic spacers, introns and duplicated

sequences in plant mitochondrial genomes (Unseld et al. 1997; Backert et al. 1997; Knoop 2004).

A salient feature of plant organellar genomes is their enormously high ploidy level, i.e., their vast copy number per cell (Kuroiwa 1982; Bendich 1987; Kuroiwa 1991; Coleman and Nerozzi 1999). A single leaf mesophyll cell, for example, may contain 50–100 chloroplasts, each of which harbors 100 or more identical copies of the plastid genome (Bendich 1987). Thus leaf cells in higher plants can easily reach ploidy degrees of 10,000 plastid genomes per cell. In both plastids and mitochondria, multiple genome copies are compacted in distinct structures, which, by analogy to prokaryotic cells, are referred to as nucleoids (Kuroiwa 1982, 1991).

In theory, the enormous copy number at which organellar genomes can be present in plant cells offers a potential for regulating gene expression by adjusting copy numbers to changing demands for expression of organellar genomes. This possibility seems attractive, because most plastome-encoded gene products are involved in photosynthesis and most chondriome-encoded proteins participate in respiration. Thus, by regulating the organellar genome copy number, two key metabolic processes of the cell could be regulated by simultaneously changing the gene dosage of a large set of genes involved. In fact, cultivation of unicellular algae under different growth conditions has been shown to result in significant changes in both plastid and mitochondrial genome copy numbers suggesting that organellar genome copy numbers indeed can be regulated (Kroymann et al. 1995; Eberhard et al. 2002). Also, it is well established that, in higher plants, non-green plastids contain significantly fewer plastid genomes than chloroplasts (e.g., Aguettaz et al. 1987; Miyamura et al. 1990; Baumgartner et al. 1988). For example, in spinach, chloroplast-containing cells harbor more than five times the amount of plastid DNA present in amyloplast-containing cells (Aguettaz et al. 1987).

Several recent publications have suggested that, in higher plants like *Arabidopsis thalina* and maize (*Zea mays*), the copy number of the plastid genome changes dramatically during leaf development and with leaf age (Rowan et al. 2004; Oldenburg and Bendich 2004; Bendich 2004). These findings are remarkable for two reasons: first, as the vast majority of plastids in leaves are chloroplasts, the data imply a plastid differentiation-independent level of regulation in genome copy number. Second, the described extent of this regulation is enormous in that chloroplast DNA was found to be virtually absent from mature and old leaves (Rowan et al. 2004; Oldenburg and Bendich 2004). These data challenge our current understanding of how gene expression in chloroplasts is regulated. Notably, in the absence of a genome, plastome-encoded proteins would have to be extremely stable and have essentially no turnover. This, however, contrasts the high turnover rate reported for at least some chloroplast-encoded photosynthesis genes, with the D1 protein of photosystem II being the prime example (Yamamoto 2001; Bailey et al. 2002; Nixon

et al. 2005). Alternatively, if plastid protein biosynthesis was to persist in the absence of a genome in mature leaves, an extreme longevity of plastid RNAs would be required.

In view of the wide implications that a decay of chloroplast DNA in mature leaves would have for our understanding of plastid gene expression, we decided to reinvestigate the dependency of chloroplast genome copy number on leaf age and developmental stage.

Materials and methods

Plant material

Leaf material of *A. thaliana* plants (ecotype Columbia) was produced exactly as described by Rowan et al. (2004). Both plants grown in sterile culture on synthetic agar-solidified medium (containing MS salts) and soil-grown plants were investigated. Rosette leaves were harvested from 28-day-old plants. When plants were 36 days old, young and mature rosette leaves were harvested and the cauline leaves were divided into basal and distal sections. In addition to the materials analyzed by Rowan et al. (2004), senescent leaves from soil-grown plants were also included. A senescent leaf was defined as a yellow leaf taken from the bottom of the rosette. Tobacco plants (*Nicotiana tabacum*, cv. Petit Havana) were grown under greenhouse conditions and leaves were harvested from mature plants at the indicated stages. A series of leaves were collected ranging from very young and young leaves (taken from the top of 15 cm high plants) to old leaves taken from the bottom of mature plants at the onset of flowering. In addition, senescent leaves (leaves that had turned yellow) were harvested from flowering plants.

Generation of hybridization probes

A cloned 5,471 bp *Sal*I restriction fragment from *N. tabaccum* chloroplast DNA was used as a probe to detect the chloroplast genomes of tobacco and *Arabidopsis* (Fig. 1). A probe for detection of the tobacco mitochondrial genome was generated by excising a 2,375 bp *Xho*I/*Nhe*I fragment from a cloned tobacco mitochondrial DNA fragment (corresponding to nucleotide positions 86,892–89,237 in the tobacco mitochondrial genome; Sugiyama et al. 2005). The 1,014 bp fragment used as a probe to detect the *Arabidopsis* mitochondrial genome (Fig. 1) was produced by excising a *Bam*HI/*Hin*dIII fragment from a tobacco mtDNA clone. The probe corresponds to nucleotide positions 308,622–309,636 in the tobacco mitochondrial genome and to nucleotide positions 51,238–52,028 in the *Arabidopsis* mitochondrial genome (Sugiyama et al. 2005; Unseld et al. 1997). All probes were purified by agarose gel electrophoresis following extraction of the DNA fragments of interest from excised gel slices using the

Nicotiana tabacum

Arabidopsis thaliana
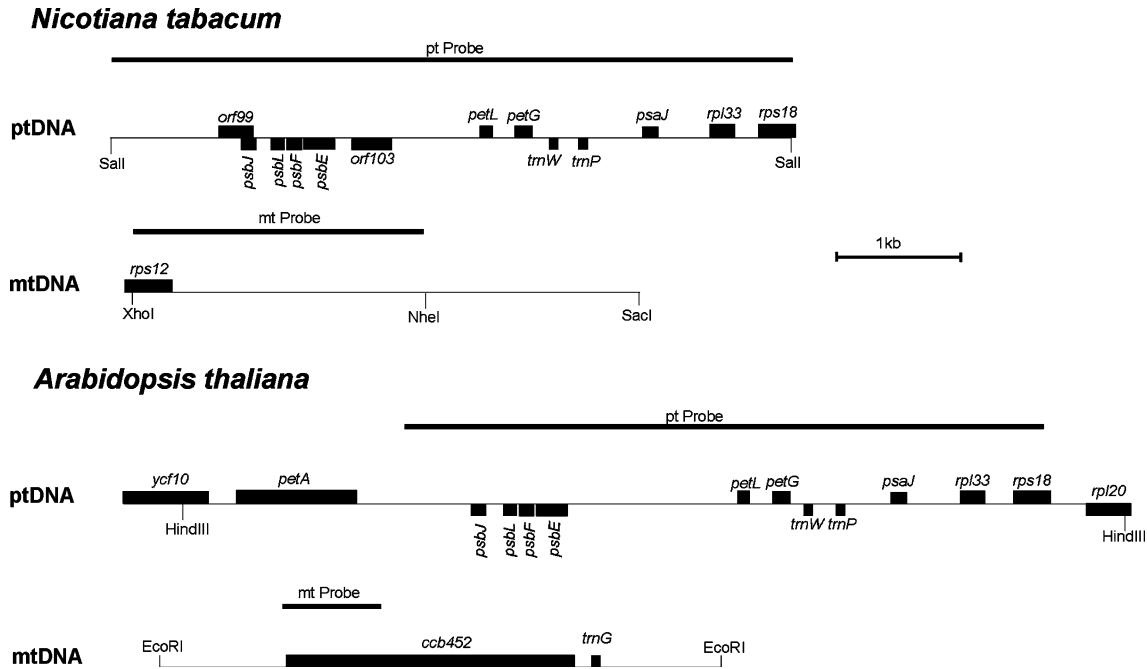
Fig. 1 Physical maps of the regions in the plastid and mitochondrial genomes of tobacco and *Arabidopsis* detected by DNA gel blot analyses. Genes above the lines are transcribed from the left to the right, genes below the lines are transcribed in the opposite direction. Hybridization probes are indicated above the maps. Restriction sites used for Southern blot analyses and/or generation of hybridization probes are also shown

GFX™ PCR (DNA and Gel Band Purification) kit (Amersham, Buckinghamshire, UK).

Isolation of nucleic acids and DNA gel blot analyses

Total plant nucleic acids were isolated from fresh leaf tissue by a cetyltrimethylammoniumbromide (CTAB)-based method (Doyle and Doyle 1990). After RNase A digestion and ethanol precipitation, DNA samples were digested with restriction enzymes, separated by gel electrophoresis on 0.8% agarose gels and transferred onto Hybond nylon membranes (Amersham) by capillary blotting. For the detection of chloroplast DNA, 5 µg total DNA was used in restriction digests. For detection of the mitochondrial genome, 15 µg DNA was digested. Hybridizations were performed at 65°C in Rapid-Hyb buffer (Amersham) following the manufacturer's protocol.

Results and discussion

Analysis of chloroplast genome copy numbers during plant development

Among the techniques available for comparative organelle genome quantitation, Southern blot hybridization of restriction enzyme-digested total cellular DNA is clearly the most reliable and least artifact-prone method. It is independent of organelle purification, offers high specificity of detection and, unlike q-PCR, is also insensitive to impurities and trace contaminations in DNA preparations. We, therefore, decided to use DNA gel blot assays for comparatively assaying organellar DNA content during leaf development.
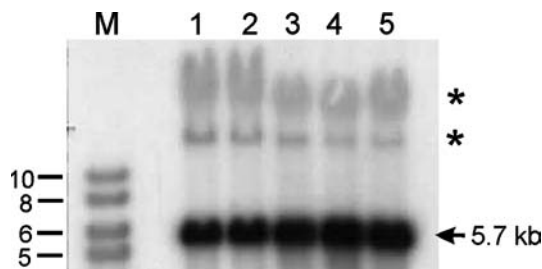
In order to make sure that identical leaf material was used as in earlier studies, we grew *Arabidopsis* plants under exactly the same conditions and harvested leaf material at identical time points and developmental stages as reported (Rowan et al. 2004). To cover all stages of leaf development, we decided to additionally analyze senescent leaves. Senescent leaves were not included in the previous studies, presumably because plastid genomes were found to be absent already from much earlier developmental stages (Rowan et al. 2004). Also, the isolation of intact chloroplasts from senescent leaves is technically difficult (see Conclusions).

Besides *Arabidopsis*, which had been analyzed previously and reported to lack plastid DNA in mature leaves, we were also interested in following the fate of plastid genomes during leaf development in tobacco (*N. tabacum*) plants. Tobacco is of equal importance to plastid research as *Arabidopsis*, because tobacco is the standard model plant for in vivo studies of plastid gene expression employing chloroplast transformation (e.g., Allison and Maliga 1995; Staub and Maliga 1994; Hermann and Bock 1999; Eibl et al. 1999; Suzuki et al. 2003; reviewed in Bock 2001; Maliga 2004). In addition to plastid genomes, we also wanted to analyze

mitochondrial genomes in both model species and detect possible developmental changes in copy number.

The specific probes used for the detection of organellar genomes and the relevant regions of the plastid and mitochondrial genomes from *A. thaliana* and tobacco (*N. tabacum*) are shown in Fig. 1. To exclude artifacts, all assays were performed by probing restriction enzyme-digested total cellular DNA samples. The results for chloroplasts are displayed in Figs. 2 and 3. For both tobacco and *Arabidopsis*, series of leaf samples ranging from very young leaves to senescent leaves were comparatively analyzed. In addition, for *Arabidopsis*, material grown under sterile conditions on synthetic medium was compared with plants grown in soil. DNA gel blot analyses with chloroplast probes revealed that, in both tobacco and *Arabidopsis*, the amount of chloroplast DNA remained remarkably constant during leaf development and was largely independent of leaf age. Even in senescent leaves, chloroplast DNA was present in essentially unchanged amounts strongly contradicting the results reported earlier (Rowan et al. 2004; Oldenburg and Bendich 2004).

In order to have an internal control for equal loading and to provide additional evidence that the ratio of plastid to nuclear DNA remains unchanged during leaf development, we overexposed the blots in order to visualize promiscuous plastid DNA. It has long been known that large pieces of plastid DNA are found integrated into the nuclear genome (Timmis and Scott 1983; Scott and Timmis 1984; Ayliffe and Timmis 1992; Ayliffe et al. 1998). These sequences dubbed as 'promiscuous DNA', are believed to be inactive in the nucleus and serve no apparent function. Recent transgenic experiments in tobacco have demonstrated that DNA-mediated gene transfer from the plastid to the nucleus is

a still ongoing process occurring at a surprisingly high frequency (Huang et al. 2003; Stegemann et al. 2003; for review see Timmis et al. 2004; Bock 2005). Promiscuous DNA of plastid origin is particularly easily detectable in tobacco, because, compared to *Arabidopsis*, tobacco has a much bigger nuclear genome and therefore, harbors much more promiscuous DNA (Ayliffe and Timmis 1992; Ayliffe et al. 1998; Noutsos et al. 2005; Huang et al. 2005). For example, analysis of the *rbcL* region of the tobacco plastid genome revealed that there is a minimum of 15 nuclear plastid DNA insertions that contain at least a proportion of the *rbcL* gene (Ayliffe and Timmis 1992). The restriction enzyme used to determine the plastid genome content in tobacco leaves, *Sal*I, was chosen because it is sensitive to CpG methylation and hence, does not digest nuclear DNA (which is heavily CpG methylated). Therefore, promiscuous DNA becomes visible as separate bands migrating in the high-molecular weight range of the gel. Indeed, Southern blot hybridization of *Sal*I-digested total cellular DNA detected cross-hybridizing promiscuous DNA which was largely undigested consistent with CpG methylation of these sequences and their location in the nuclear genome (Fig. 2). Analysis of the relative intensities of the plastid and nuclear hybridization signals revealed no significant differences between leaves of different ages and developmental stages confirming that chloroplast genome number remains constant and strikingly contrasting the previous reports that chloroplasts in mature leaves would dispose of their genomes (Rowan et al. 2004; Oldenburg and Bendich 2004).

As mentioned above, promiscuous plastid DNA is not readily detectable in *Arabidopsis*, presumably because there is more stringent selection against it to maintain the small genome size. In fact, when the *Arabidopsis* and rice nuclear genomes were searched for the presence of promiscuous DNA fragments that were sufficiently large to trace back their evolutionary origin, 88 pieces of plastid DNA were found in the rice genome but none in the *Arabidopsis* genome (Noutsos et al. 2005).

Fig. 2 Analysis of plastid genome abundance in tobacco leaves of different developmental stages. Equal amounts of extracted total cellular DNA were digested with *Sal*I, separated in a 0.8% agarose gel, blotted and hybridized to a radiolabeled restriction fragment derived from cloned tobacco plastid DNA (Fig. 1). The probe detects the expected 5.7 kb fragment in all samples (*arrow*). Cross-hybridizing promiscuous DNA of plastid origin confirms equal loading and is indicated by *asterisks*. Fragment sizes of the molecular weight marker (*M*) are given in kilobases. *Lane 1* very young leaf (first and second leaves from the top), *lane 2* young leaf (third leaf from the top), *lane 3* mature leaf (seventh leaf from the bottom, fully expanded leaf), *lane 4* old leaf (fourth leaf from the bottom), *lane 5* senescent leaf (yellow leaf taken from a flowering plant)

Analysis of mitochondrial genome copy numbers during plant development

Having established that chloroplast genome copy numbers do not undergo significant changes during leaf development, we were next interested in determining possible changes in mitochondrial genome copy numbers. To this end, we investigated tobacco and *Arabidopsis* leaf samples representing the same developmental stages as in our analyses of plastid genome copy number. Likewise, soil-grown *Arabidopsis* plants were compared with plants grown in sterile culture. Employing hybridization probes that specifically detect the mitochondrial genomes of tobacco and *Arabidopsis*, respectively (Fig. 1), equal amounts of restriction
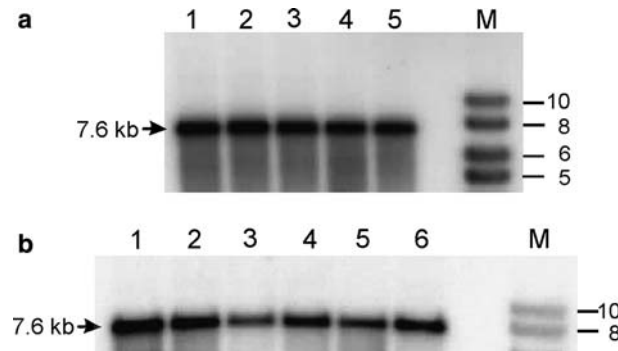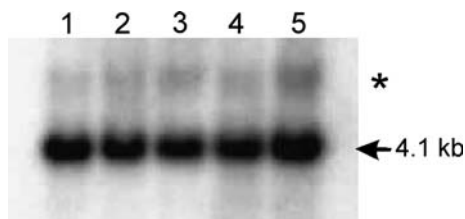
Fig. 3 Comparative analysis of plastid genome abundance in *Arabidopsis* leaves representing different developmental stages. **a** Analysis of plants grown in sterile culture on sucrose-containing synthetic medium. *Lane 1* rosette leaf from a 28-day-old plant, *lane 2* young rosette leaf from a 36-day-old plant, *lane 3* mature rosette leaf from a 36-day-old plant, *lane 4* cauline leaf from a 36-day-old plant (basal part), *lane 5* cauline leaf from a 36-day-old plant (distal part). **b** Analysis of soil-grown plants. *Lane 1* rosette leaf from a 28-day old plant, *lane 2* young rosette leaf from a 36-day-old plant, *lane 3* mature rosette leaf from a 36-day-old plant, *lane 4* cauline leaf from a 36-day-old plant (basal part), *lane 5* cauline leaf from a 36-day-old plant (distal part), *lane 6* senescent rosette leaf. Equal amounts of extracted total cellular DNA were digested with *Hin*dIII, separated in a 0.8% agarose gel, blotted and hybridized to the radiolabeled *Sal*I restriction fragment derived from cloned tobacco plastid DNA and shown in Fig. 1. The probe detects the expected 7.6 kb fragment in all samples (*arrow*). Fragment sizes of the molecular weight marker (*M*) are given in kilobases

enzyme-digested total DNA were assayed for the presence of mitochondrial genomes.

The results for tobacco plants are presented in Fig. 4. Hybridization with a probe derived from the *rps12* region of the mitochondrial genome detects a strong signal of the expected size (Sugiyama et al. 2005). Signal intensity is comparable for all samples analyzed suggesting that the copy number of the chondriome stays about the same during leaf development in tobacco.

The nuclear genome of higher plants not only harbors promiscuous DNA of plastid origin, but also contains large pieces of mitochondrial sequences (Sun and Callis 1993; Major et al. 1993; Timmis et al. 2004; Bock 2005). Therefore, similar to the detection of plastid genomes, we could also use cross-hybridizing promiscuous DNA as an internal standard and loading control for the mitochondrial DNA gel blot analysis. These data confirmed that the ratio of nuclear to mitochondrial DNA does not undergo significant changes during leaf development and is largely independent of leaf age (Fig. 4).

Similar analyses with *Arabidopsis* plants using a probe for the *ccb452* region of the mitochondrial genome confirmed these results and revealed no difference between soil-grown plants and plants grown in sterile culture on synthetic sucrose-containing medium (Fig. 5). Moreover, senescent leaves were found to contain essentially unaltered amounts of mitochondrial DNA (Fig. 5) suggesting that not even leaf senescence results in preferential decay of organellar genomes.

Together, our results reveal no indication of organellar genome copy number being regulated during leaf
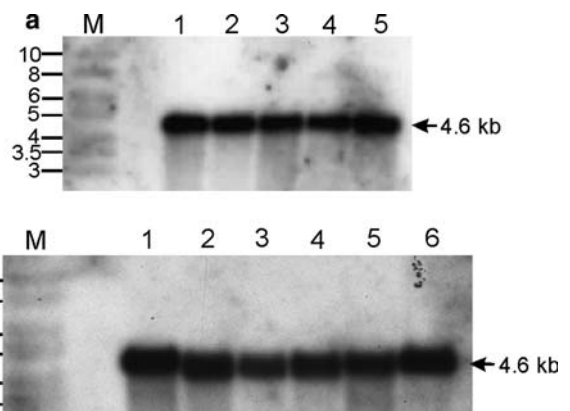

Fig. 4 Assay for the presence of mitochondrial genomes in tobacco leaves of different developmental stages. Equal amounts of extracted total cellular DNA were digested with *Xho*I and *Sac*I, separated in a 0.8% agarose gel, blotted and hybridized to a radiolabeled restriction fragment obtained by *Xho*I/*Nhe*I digestion of a cloned tobacco mitochondrial DNA fragment (Fig. 1). The probe detects the expected 4.1 kb fragment in all samples (*arrow*). Cross-hybridizing promiscuous DNA of mitochondrial origin confirms equal loading and is indicated by asterisks. Leaf ages and developmental stages are as in Fig. 2

Fig. 5 Comparative analysis of mitochondrial genome abundance in *Arabidopsis* leaves representing different developmental stages. **a** Analysis of plants grown in sterile culture on sucrose-containing synthetic medium. **b** Analysis of soil-grown plants. Equal amounts of extracted total cellular DNA were digested with *Eco*RI, separated in a 0.8% agarose gel, blotted and hybridized to the radiolabeled restriction fragment shown in Fig. 1. The probe detects the expected 4.6 kb fragment in all samples (*arrow*). Leaf ages and developmental stages are as in Fig. 3. Fragment sizes of the molecular weight marker (*M*) are given in kilobases

development. Instead, the relative ratios of nuclear to organellar DNA remain remarkably constant. Thus, although there may be a reduced need for the synthesis of plastid genome-encoded proteins (and possibly also of mitochondrial proteins) in aging leaves, the activity of organellar gene expression is not adjusted to changing metabolic demands at the level of genome copy number.

Conclusions

In this work, we have demonstrated that the copy numbers of chloroplast and mitochondrial genomes of higher plants are largely independent of leaf age. Both genomes are already present in high numbers in very young leaves and persist in essentially unchanged amounts even in senescent leaf material. This finding demonstrates that, during leaf development, neither chloroplast nor mitochondrial gene expression is significantly regulated at the level of the genome copy number. Instead, our data suggest that transcriptional and post-transcriptional processes are the predominant, if not the only, levels at which gene expression is regulated. Earlier work has shown that, in both plastids and mitochondria, post-transcriptional processes may make a much greater contribution to gene regulation than control at the level of transcription (Gillham et al. 1994; Rochaix 1992; Eibl et al. 1999; Zerges 2000; Giegé et al. 2000). In light of this, the decay of organellar DNA relatively early in leaf development, as reported recently (Rowan et al. 2004; Oldenburg and Bendich 2004), would have dramatic implications on our understanding of gene expression, RNA stability and protein stability in plant cell organelles: transcripts and/or proteins would have to be extremely stable to compensate for the absence of the genome in mature and old leaves. Our data demonstrate that this assumption does not need to be made.

Our finding that the copy numbers of organellar genomes in higher plants do not undergo significant changes during leaf development is in sharp contrast to the recently published data obtained from DAPI staining of isolated chloroplasts, which suggest that the plastid genome is no longer present in mature leaves (Rowan et al. 2004; Oldenburg and Bendich 2004). Nonetheless, we believe that our data reported here are correct for a number of reasons. First, the extraction of total cellular DNA is a simple, unbiased and highly reliable method. It is inconceivable that extracting total DNA from leaves of different ages introduces a bias in the relative amounts of organellar versus nuclear DNA. As will be explained below, this is clearly not the case for organelle isolation. Second, restriction fragment analysis by Southern blotting is a highly specific and reproducible method for the direct detection of DNA and quantitative comparisons between samples. Unlike q-PCR, the results are insensitive to trace contaminations, not artifact prone and largely independent of impurities (which can inhibit PCR in different samples

to different extents). Finally, hybridization to promiscuous DNA (Figs. 2, 4) provides an internal standard for the relative ratios of organellar to nuclear DNA and excludes incorrect DNA quantitation or unequal loading of DNA samples as a possible source of error.

The obvious discrepancies between our results and the previously reported failure to detect plastid DNA in mature leaves (Rowan et al. 2004; Oldenburg and Bendich 2004) raise the pressing question of how to explain these diametrically opposed findings. We believe that the explanation lies in the isolation of chloroplasts from leaves at different developmental stages and the subsequent treatment of the chloroplasts with DNase. In our hands, DNase treatment of chloroplasts isolated from mature leaves resulted in complete degradation of the chloroplast DNA whereas, untreated control samples still had intact plastid genomes as evidenced by restriction enzyme analysis (which yielded the expected fragment pattern on ethidium bromide-stained agarose gels; Sabine Kahlau and R. Bock, unpublished results). This is consistent with the earlier findings that isolated chloroplasts are not impermeable to exogenously added enzymes (Atchison et al. 1976). In fact, addition of restriction enzymes to chloroplast preparations produces identical chloroplast genome fragmentation patterns as does cleavage of purified chloroplast DNA (Atchison et al. 1976). Thus, it is conceivable that also DNases can enter chloroplasts purified by standard organelle isolation procedures, which would explain the virtual absence of plastid DNA from DNase I-treated chloroplast preparations. However, two questions remain: (1) how do exogenously added enzymes enter the chloroplast and (2) how can the difference between young and mature leaves observed in the previous work (Rowan et al. 2004; Oldenburg and Bendich 2004) be explained? Chloroplasts in mature and old leaves (sink leaves) contain large starch grains. By contrast, in young leaves (source leaves), starch grains are either absent or at least much smaller than in sink leaves. All standard chloroplast isolation procedures involve centrifugation steps and, when mature leaf tissue is used for the isolation, a large white pellet of starch is usually observed at the bottom of the tube after centrifugation. This indicates that large starch grains penetrate the double membrane of the organelle when centrifugation forces act. It is conceivable that, upon rupturing the membranes, the starch grains punch holes in the chloroplast envelope, which later serve as entry sites for DNase molecules. As chloroplasts in young leaves have little, if any, starch grains, they are largely protected from this mechanical damage and thus, the chloroplast DNA remains detectable by DAPI staining even after DNase I treatment. We, therefore, propose that the reported absence of chloroplast genomes from mature leaf material is an experimental artifact caused by the DNase treatment of isolated chloroplasts.

In sum, our data establish that neither chloroplast gene expression nor mitochondrial gene expression in leaves of higher plants are developmentally regulated to

any significant extent and underscore the pivotal role of transcriptional and post-transcriptional processes in the regulation of organellar genes.

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