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Plastid division and morphology in the genus *Peperomia*

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Abstract

We have investigated several factors determining plastid size and number in *Peperomia*, a genus in the *Piperaceae* family whose species naturally display great interspecific variation in chloroplast size and number per cell. Using microscopic techniques, we show that chloroplast size and number are differently regulated in the palisade parenchyma and the spongy parenchyma, suggesting that chloroplast division in these cell types is controlled in different ways. Microscopic studies of iodine-stained root cells revealed a correlation between amyloplast size in root cells and chloroplast size in palisade parenchyma cells. However, despite substantial variation in chloroplast number in leaf mesophyll cells, amyloplast number in root cells was very similar in all species. The results suggest that organelle size and number are regulated in a tissue-specific manner rather than in dependency on the plastid type. We also demonstrate that plastid size determines the size but not the number of starch grains in root amyloplasts.

Additional key words: amyloplast, chloroplast, plastid number, plastid size.

Introduction

Plastids divide by binary fission and, during the past years, the cellular mechanisms underlying the division process have begun to emerge (Pyke and Leech 1992, 1994, Osteryoung *et al*. 1998, Marrison *et al*. 1999, Colletti *et al*. 2000, Glynn *et al* 2008, 2009). Ultrastructurally, the division apparatus is visible as a ring-like structure constricting the organelle and termed the plastid-dividing (PD) ring. In addition to the ring structures, proteins associated with the inner envelope membrane are also required for plastid division, but how all the components of the division apparatus interact to co-ordinate the division process in a concerted manner is not yet fully understood (for review see, *e.g*., Aldridge *et al*. 2005).

The genus *Peperomia* belongs to the *Piperaceae* family and comprises approximately 1600 mostly tropical species (Wanke *et al*. 2006). A well-known species in the genus is *Peperomia metallica* which was reported to contain giant chloroplasts in its palisade parenchyma cells (Schürhoff 1908, Neumann 1973). Because of this unique feature, *P. metallica* chloroplasts have been a preferred model of electrophysiological studies (Bulche *et al*. 1972). The large size of the chloroplasts is compensated by a small chloroplast number, which is in the range of only 2 to 6 per palisade cell (Bartels 1965).

While the giant chloroplasts in the palisade parenchyma of *P. metallica* have been intensively investigated, little is known about plastid size, number and morphology in other tissues and organs of *P. metallica* (such as root amyloplasts) and about plastids in other *Peperomia* species. Furthermore, whether plastid size and number is controlled in a tissue-specific or plastid type-specific manner, is not yet understood. In this study, we have used different *Peperomia* species to analyze plastid morphology, the relationships between cell size and chloroplast size and the regulation of plastid number in different tissues.

Materials and methods

Four *Peperomia* species, *P. metallica* Linden & Rodigas, *P. peduncularis* Sodiro, *P. argyreia* (Hook.f.) E. Morren and *P. serpens* (Sw.) Loudon, were used to study plastid morphology. Plants were obtained from the Botanical Gardens in Freiburg and Berlin. For comparison, tomato plants (*Solanum lycopersicum* Mill. cv. IPA-6) were also

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analyzed. Plants were grown either in soil in a growth chamber or under aseptic condition in *Magenta* boxes containing Murashige and Skoog (1962; MS) medium. The standard growth regime was 16-h photoperiod, irradiance of 25 µmol m^{-2} s^{-1} and day/night temperature of 25/20 °C.

Amyloplasts were firstly counted in thin cross sections of root tissues harvested during day cycle. To confirm these data, the same samples were then stained with iodine solution (2 % potassium iodide, 1 % iodine) by injecting a small volume of the solution between the glass slide and the cover slip, followed by washing with water. After complete removal of the iodine solution, the samples were used for microscopic studies.

In order to study chloroplasts in different cell types, thin cross sections of 50 to 100 µm were prepared from young leaves using a *Leica* vibratome (*Leica Microsystems*, Wetzlar, Germany). Samples were analyzed by *Olympus BX41* high resolution light microscope (*Olympus*, Melville, NY, USA) connected to an *Olympus U-CMAD3* microscopy camera. A *Leica TCS SP2* spectral laser-scanning confocal microscope (*Leica Microsystems*) was used to analyze chlorophyll autofluorescence (excitation 488 nm, emission 600 nm) in leaf cells.

To measure chloroplast size in leaf mesophyll cells, photographs were taken from leaf cross sections and 100 - 330 chloroplasts were randomly selected and their diameter was measured. Light microscopy (mainly live observations) of thin leaf cross sections was also employed to determine chloroplast numbers in leaf palisade and spongy parenchyma cells. Chloroplasts and amyloplasts were counted under microscope in at least 100 cells per species. Finally, around 50 cells were randomly selected, their length and width were measured and the cell area was calculated. All microscopic analyses were done in at least three biological replicas.

The data were analyzed statistically using the *SPSS* software. Analysis of variance was used to test the statistical significance. The significance of differences among means was tested using Duncan's test at $P = 0.01$.

Results and discussion

We firstly investigated chloroplast size and numbers in four different species of *Peperomia* (Fig. 1), and found that one of the species, *Peperomia peduncularis*, also harbor giant chloroplasts in palisade parenchyma cells (average size 16.65 µm; Fig. 1*D*,*I*), as has been reported previously for *P. metallica* (Schürhoff 1908, Fig. 1*E*,*J*). Interestingly, palisade cells from *P. serpens* contained normal chloroplasts that were ordinarily comparable in size and number per cell to chloroplasts in tomato (Fig. 1*A*,*B*,*F*,*G*, Table 1). Another *Peperomia* species, *P. argyreia*, represents an interesting intermediate (Fig. 1*C*,*H*, Table 1) with the chloroplast number in palisade cells similarly low as in the two species with giant chloroplasts, *P. peduncularis* and *P. metallica*

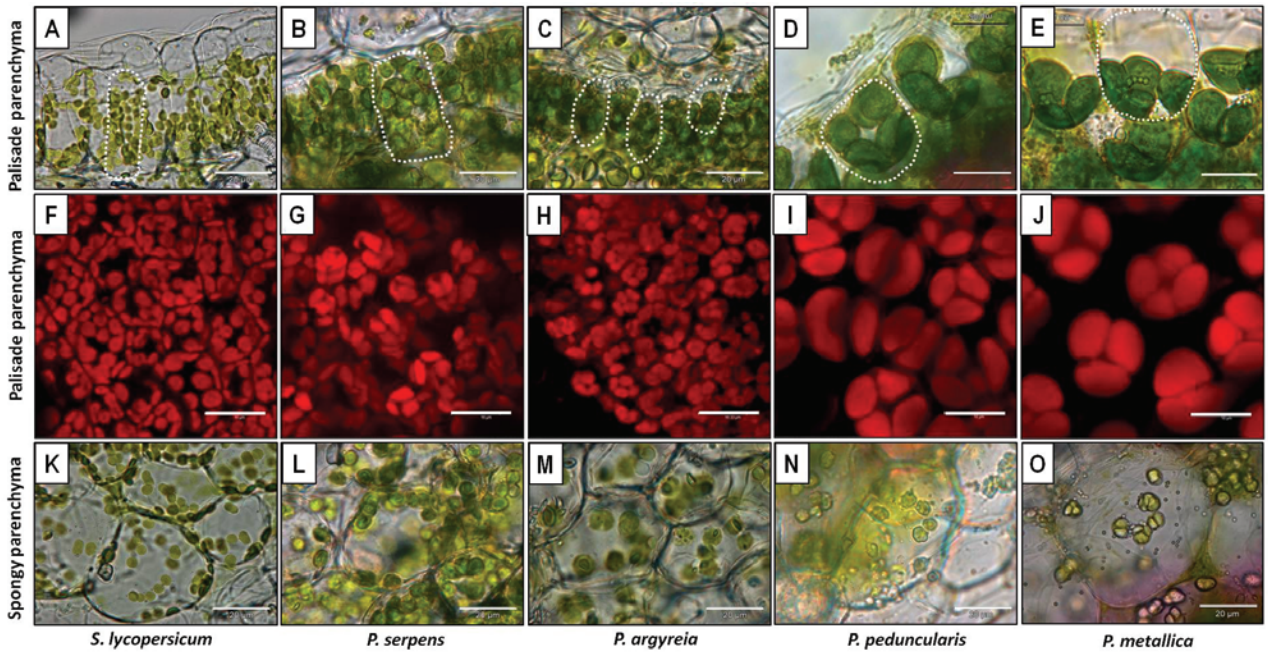

Fig. 1. Visualization of chloroplasts in palisade (*A-J*) and spongy mesophyll cells (*K-O*) of tomato, *P. serpens*, *P. argyreia*, *P. peduncularis* and *P. metallica* using high-resolution light microscopy (*A-E*, *K-O*) or confocal laser-scanning microscopy based on chlorophyll autofluorescence (*F-J*). While *P. argyreia* has the same low number of chloroplasts per cell as *P. peduncularis* and *P. metallica*, the chloroplasts are much smaller (and comparable in size with tomato chloroplasts) which is due to the smaller cell size in *P. argyreia*. For clarity, the cell walls of single palisade cells are marked by *white dotted lines*. *Scale bars* 20 µm.

Table 1. Cell area [μm^2], and chloroplast (CP) and amyloplast (AP) sizes (diameter) [μm] and numbers in different cells of tomato, *P. serpens*, *P. argyreia*, *P. peduncularis* and *P. metallica*. Means ± SE. Values indicated by different letters in the superscript are significantly different at $P < 0.01$, n.a. - not analyzed.

Parameter	Tomato	*P. serpens*	*P. argyreia*	*P. peduncularis*	*P. metallica*
Cell area in palisade par.	883.63±29.08^b	1142.33±65.43^c	195.63±9.64^a	975.62±34.89^{bc}	987.19±50.12^{bc}
Cell area in root cells	1139.33±64.33^a	n.a.	3641.36±175.08^c	n.a.	2997.11±152.18^b
CP size in palisade par.	6.27±1.14^a	7.32±1.04^b	6.50±1.60^a	16.65±4.50^c	16.55±4.74^c
CP size in spongy par.	5.30±0.65^a	6.05±0.77^b	5.42±0.74^a	5.93±1.17^b	5.90±1.22^b
AP size in root cells	4.25±0.64^a	n.a.	5.51±0.73^b	n.a.	11.81±2.50^c
CP number in palisade par.	28.59±9.54^c	16.77±3.33^b	2.90±1.00^a	2.62±0.80^a	2.24±0.61^a
CP number in spongy par.	19.13±6.30^e	17.13±3.90^d	8.85±1.72^c	7.39±2.00^b	6.52±2.24^a
AP number in root cells	16.42±1.84^a	n.a.	17.43±1.82^a	n.a.	16.86±2.42^a
CP area/cell area in palisade par.	0.89	0.62	0.50	0.58	0.49
AP area/cell area in root cells	0.20	n.a.	0.11	n.a.	0.62

(Table 1). This striking finding is readily explained by the fact that *P. argyreia* has much smaller palisade cells than the other species (Table 1). It thus seems reasonable to assume that *P. argyreia* compensates for its smaller palisade cells by a reduction in either chloroplast number or chloroplast size.

Interestingly, when we investigated chloroplast size in spongy parenchyma cells, we found no significant correlation with chloroplast size in the palisade parenchyma. In fact, chloroplasts in spongy parenchyma cells had approximately similar sizes in all *Peperomia* species (Fig. 1, Table 1) suggesting that chloroplast size, and perhaps division, are differently regulated in different leaf cell types. Nonetheless, how and when the diffe-rences in chloroplast division between palisade and spongy parenchyma are established during the course of tissue differentiation, remains to be investigated.

A comparison of chloroplast numbers per cell in the four *Peperomia* species investigated here reveal an interesting aspect: *P. argyreia*, although having similar-size palisade chloroplasts as tomato and *P. serpens*, shows the same uncoupling of chloroplast numbers in spongy and palisade parenchyma cells as the species with giant chloroplasts, *P. peduncularis* and *P. metallica* (Table 1). This may suggest that a mechanistically similar mode of regulating plastid division operates in *P. argyreia*, *P. peduncularis* and *P. metallica* and that the potential of *P. argyreia* to develop giant chloroplasts in palisade cells is masked by the small cell size in the palisade parenchyma (Fig. 1). The data also show that the low number of palisade cell chloroplasts in *P. peduncularis* and *P. metallica* is compensated for by a more or less proportional increase in organelle size thus keeping the volume that the plastid compartment occupies per cell fairly constant. However, why *P. metallica* and *P. peduncularis* but not other two species have evolved larger chloroplasts in their palisade parenchyma, remains unexplained.

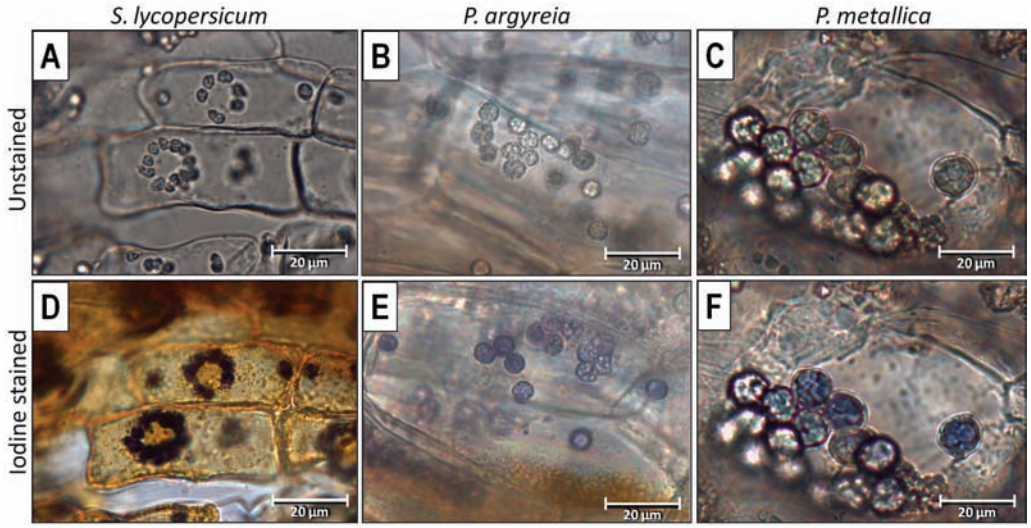

Fig. 2. High-resolution light microscopy images from root cells of *S. lycopersicum* (*A*,*D*), *P. argyreia* (*B*,*E*) and *P. metallica* (*C*,*F*). Amyloplasts in unstained root cells were analyzed using light microscopy (*A-C*) and data were subsequently confirmed by iodine staining of starch grains (*D-F*). *Scale bars* 20 μm.

With high-resolution light microscopy, amyloplasts could be visualized in thin root sections (~100 µm) without any treatment or staining technique (Fig. 2*A*-*C*). To demonstrate that these organelles are indeed amyloplasts, iodine staining was performed. As expected, the analyzed organelles were stained with iodine indicating that they are indeed starch-storing amyloplasts (Fig. 2*D*-*F*).

Interestingly, a correlation was observed between chloroplast size in the palisade cells and amyloplast size in root cells: *P. metallica* with giant chloroplasts in the palisade parenchyma cells also had big amyloplasts in roots, whereas *P. argyreia* with normal-sized chloroplasts in palisade cells also had amyloplasts of normal size, similar to those in tomato root cells (Table 1). The correlation between chloroplast size in the palisade parenchyma and amyloplast size in roots becomes obvious when chloroplast sizes in spongy parenchyma and palisade parenchyma cells are compared with amyloplast sizes (Table 1). While all species have chloroplasts of similar size in their spongy parenchyma cells, the large size of chloroplasts in *P. metallica* and *P. peduncularis* palisade cells correlates well with the large sizes of the amyloplasts in root cells of these two species.

We have known from the mutant studies that chloroplast area per cell area stays constant, presumably to maintain proper plastid function (Pyke and Leech 1994, Maple *et al*. 2007). Similar results were observed in our study using *Peperomia* species with natural variation in plastid size and number (Table 1). However, this ratio was significantly different between tomato and *Peperomia* species (Table 1), indicating that the chloroplast area per cell can vary among different plant genera. Interestingly, in root cells, amyloplast area per cell area was not similar along with species (Table 1). In contrast, irrespective of the amyloplast size, amyloplast number appeared to be constant (Table 1, Fig. 2), indicating that amyloplast number may play a significant role in root cells and thus, the plastid division regulatory machine tries to keep amyloplast number fairly constant.
Yet another interesting finding was that amyloplast size correlated with the size but not the number of starch grains. The giant amyloplasts do not harbor many small starch grains, but rather contain few big starch grains (Fig. 3*A*,*B*). This may suggest that the number of starch grains per amyloplast is kept constant and that the (genetically determined) size of the amyloplast determines the size of the starch grains, presumably by setting the upper limit for starch grain growth.

The investigation of the division of giant plastids has been restricted to mutants impaired in plastid division. Analysis of a tomato mutant provided evidence that, at least in some tissues, division occurs by fragmentation of giant plastids (Forth and Pyke 2006). In contrast, the naturally occurring giant plastids in palisade cells of *Peperomia metallica* and *P. peduncularis* seem to follow the normal division mechanism by binary fission. We frequently observed division intermediates as evidenced by the presence of constrictions resembling

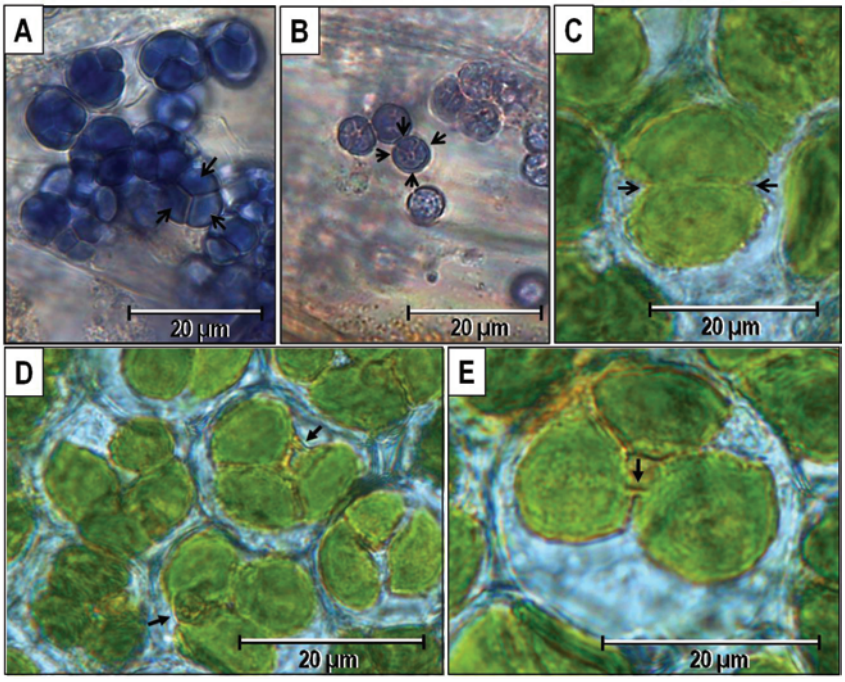

Fig. 3. Iodine-stained amyloplasts in root cells of *P. metallica* (*A*) and *P. argyreia* (*B*), an actively dividing giant chloroplast in a single palisade cell of *P. metallica* (*C*), and chloroplast networks in *P. metallica* palisade cells (*D*,*E*). *Arrows* point to starch grains of an amyloplast in parts (*A*,*B*), constriction of the dividing giant chloroplast in part (*C*), and examples of plasmatic connections between chloroplasts (stromules) in parts (*D*,*E*).

PD rings (Fig. 3*C*). We also noticed an increase in the average number of chloroplasts per palisade cell during development from young leaves to fully-expanded mature leaves indicating that giant chloroplasts in palisade cells still undergo division.

An interesting feature of chloroplasts that was discovered relatively recently is the presence of so-called stromules: stroma-filled tubular protrusions that emanate from chloroplasts as well as non-green plastid types (Köhler *et al*. 1997, Köhler and Hanson 2000, Gray *et al*. 2001, Pyke and Howells 2002, Waters *et al*. 2004, Natesan *et al*. 2005). Stromules can sometimes connect neighbouring plastids and, in this way, mediate the exchange between plastids of at least some proteins, like GFP and GFP fusion proteins (Köhler *et al*. 1997, Kwok and Hanson 2004). In most of the studies, stromules have been investigated mainly by visualizing them *via* GFP labelling and confocal laser-scanning microscopy. As GFP overexpression is known to sometimes produce structural artifacts, this has raised questions about the significance of stromules *in vivo*. In 2005, for the first time, Gunning (2005) observed stromules using light microscope without the use of GFP. Therefore, we were also interested in using the giant chloroplasts present in some *Peperomia* species to test if stromules can be observed in the absence of GFP expression or tissue fixation for electron microscopy. When we investigated giant chloroplasts in palisade cells by high-resolution light microscopy, we indeed observed frequently neighbouring chloroplasts being interconnected by tubular structure (Fig. 3*D*,*E*). This proves unambiguously that stromules are a genuine morphological feature of higher plant chloroplasts and provides light-microscopic confirmation for stromules forming plasmatic connections between chloroplasts. It should be noted that increased temperature during prolonged investigation of samples under microscope could be one of the reasons for frequent observation of such connections (*e.g*. Buchner *et al*. 2007, Holzinger *et al*. 2007).

So far, most studies on the factors controlling plastid size and number have been carried out using mutants with alterations in chloroplast number (Pyke and Leech 1994, Pyke 1999, Aldridge *et al*. 2005, Maple *et al*. 2007, Holzinger *et al*. 2008). In this work, we have established correlations between cell size, plastid size and plastid number using naturally occurring variation in *Peperomia* species. We provide further evidences that plastid size and number are regulated in a tissue-specific manner. Although such relationship has been reported previously by studying chloroplasts in guard cells and in cells close to vascular tissue that are smaller than those of mesophyll cells (*e.g*., Chen *et al*. 2009), our results indicate that this regulation is largely independent of the plastid type. Tissues containing the same plastid type (*e.g*., palisade and spongy mesophyll cells both containing chloroplasts), can exhibit significant differences in plastid number and size. In turn, tissues harboring very different plastid types (*e.g*., palisade cells and root cells) can have apparently similar modes of regulating plastid size. As it is well-established that chloroplast size and number are inversely correlated and largely determined by the rate of organelle division (Pyke and Leech 1994, Pyke 1997, 1999, Aldridge *et al*. 2005), it seems reasonable to conclude that the differences in plastid size and number per cell are caused by tissue-specific differences in the activity of the plastid division machinery.

Investigation of an intermediate species, *P. argyreia*, containing similar-sized palisade chloroplasts as tomato but the same chloroplast numbers in spongy and palisade parenchyma cells as the species with giant chloroplasts, provided further evidence for the cell size to have an important effect on the controlling of the plastid size (Fig. 1, Table 1). Similar results have been reported by Pyke and Leech (1992, 1994) using mutant *Arabidopsis* plants.

Another interesting correlation revealed by our comparative analysis of plastid size and number in *Peperomia* species is that amyloplast size determines the size but not the number of starch grains: big amyloplasts in *Peperomia* species with giant chloroplasts in palisade cells contain few huge starch grains rather than many small starch grains (Fig. 3*A*). Also, with light microscopy studies of giant chloroplasts in palisade cells of *P. metallica*, the formation of chloroplast networks connected *via* stromules was further confirmed (Fig. 3*D*,*E*), indicating the relevance of these structures *in vivo*.

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