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Temperature-sensitive formation of chloroplast protrusions and stromules in mesophyll cells of *Arabidopsis thaliana*

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Summary. In leaf mesophyll cells of transgenic *Arabidopsis thaliana* plants expressing GFP in the chloroplast, stromules (stroma-filled tubules) with a length of up to 20 μm and a diameter of about 400–600 nm are observed in cells with spaces between the chloroplasts. They appear extremely dynamic, occasionally branched or polymorphic. In order to investigate the effect of temperature on chloroplasts, we have constructed a special temperature-controlled chamber for usage with a light microscope (LM-TCC). This LM-TCC enables presetting of the temperature for investigation directly at the microscope stage with an accuracy of ±0.1 °C in a temperature range of 0 °C to +60 °C. With the LM-TCC a temperature-dependent appearance of chloroplast protrusions has been found. These structures have a considerably smaller length-to-diameter ratio than typical stromules and reach a length of 3–5 μm. At 5–15 °C (low temperatures), almost no chloroplast protrusions are observed, but they appear with increasing temperatures. At 35–45 °C (high temperatures), numerous chloroplast protrusions with a beaklike appearance extend from a single chloroplast. Interaction of stromules with other organelles has also been investigated by transmission electron microscopy. At 20 °C, transverse sections of stromules are frequently observed with a diameter of about 450 nm. A close membrane-to-membrane contact of stromules with the nucleus and mitochondria has been visualised. Golgi stacks and microbodies are found in the spatial vicinity of stromules. At 5 °C, virtually no chloroplast protrusions or stromules are observed. At 35 °C, chloroplast protrusions are present as broader thylakoid-free stroma-filled areas, resulting in an irregular chloroplast appearance.

Keywords: *Arabidopsis thaliana*; Photosynthesis; Stromule; Temperature; Ultrastructure.

Abbreviations: CLSM confocal laser scanning microscope; LM-TCC light microscope temperature-controlled chamber; TEM transmission electron microscope.

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Introduction

Temperature is one of the critical factors in plant life. Low- and high-temperature regimes might be especially harmful to plants. Cold adaptation in *Arabidopsis thaliana* was studied by means of chlorophyll fluorescence imaging in whole plants (Gray et al. 2003). Photoinhibition occurred in cold-shifted *A. thaliana* plants, but cold acclimation resulted in an increased tolerance to photoinhibition. Changes in leaf ultrastructure of *A. thaliana* have been observed upon cold shift (Ristitc and Ashworth 1993); however, net photosynthesis is only slightly lower in cold-shifted leaves (Talts et al. 2004). Swelling of chloroplasts, causing large areas with thylakoid-free zones, might also be a consequence of chilling effects (Niki et al. 1978, Ristic and Ashworth 1993, Stefanowska et al. 2002). These alterations have generally been considered as a form of injury but they might also reflect the ability of chloroplasts to accommodate excess water entering cells during thawing in frost-pretreated cold-acclimated cells (Stefanowska et al. 2002).

The effect of higher temperatures in plants is primarily on photosynthetic functions (Weis and Berry 1988). Heat-induced changes of chlorophyll fluorescence and damage of the photosynthetic apparatus were reported decades ago (Schreiber and Berry 1977). Dynamics of heat tolerance have been observed on the whole-organ level by visual assessment in alpine plants (Buchner and Neuner 2001, 2003). Upon temperature stress, photoinhibition of photosystem II, rearrangement of membranes and production of heat shock proteins may occur (Braun et al. 2002). Heat tolerance exhibits diurnal changes and may be as high as 45–65 °C for short periods of times in vascular plants

(Larcher et al. 1997). In modified *A. thaliana* plants that had altered Rubisco activase or thylakoid membrane fluidity, a temperature increase from 25 to 40 °C led to reversible inhibition of photosynthesis (Kim and Portis 2005). The physiological observations on temperature effects suggest that structural investigations on chloroplasts are worthwhile.

The outer surface of chloroplasts has the ability to form thin stroma-filled tubules, named stromules by Köhler and Hanson (2000). Stromules have been defined by those authors as less than 800 nm wide in order to distinguish them from irregularly shaped chloroplasts. Stromules may reach lengths of several tens of micrometers and therefore often have a high length-to-diameter ratio. However, stromules are dynamic structures that can be observed to grow out or retract into the main plastid body in vivo. In early stages of their formation as well as during the retraction phase, their length-to-diameter ratio is much lower. Their occurrence in different tissues and plastid types has been summarised (Gray et al. 2001, Kwok and Hanson 2004a, Natesan et al. 2005). Stromule formation is tissue-specific (Köhler and Hanson 2000), depending upon plastid size, plastid differentiation status, and the density of plastids within the cell (Pyke and Howells 2002, Waters et al. 2004). Cytoskeletal elements have been reported to control morphology and movement of stromules (Kwok and Hanson 2003, 2004b). Some of the postulated functions of stromules are to provide connections between plastids that may allow the exchange of macromolecules (Köhler et al. 1997, Kwok and Hanson 2004c), to reduce the diffusion distance between plastids and other oganelles, and to increase the chloroplast surface area.

Stromules are present in all plastid types but are more common and extensive in nongreen plastids such as chromoplasts and leucoplasts. Despite several recent investigations dealing with this topic, stromule function and conditions causing their occurrence remain unresolved.

None of the published studies has considered in detail the temperature conditions under which stromules have been observed. Microscopic preparations may experience rather high temperatures during examination, especially in the classical arrangement of slide and coverslip with only a small volume of liquid. Dynamic structures such as stromules may be especially sensitive to temperature changes. We therefore studied the effect of temperature on stromule formation with a newly developed temperature-controlled chamber directly at the confocal laser scanning microscope (CLSM) and investigated their structure and the spatial relationship to other organelles at selected temperatures by transmission electron microscopy (TEM).

Material and methods

Plant material

Roots of *Arabidopsis thaliana* ecotype Columbia plants were transformed by *Agrobacterium tumefaciens* pCIB542/A136 carrying the pCT37 vector containing a chimeric gene composed of the double 35S promoter and AMV translation signals linked to an *A. thaliana* chloroplast *recA* transit sequence and an mGFP4 coding region that was further modified by introduction of the S65T mutation. Transformed roots were regenerated into shoots (Valvekens et al. 1988). Transgenic tobacco and petunia plants carrying the same vector have previously been described (Köhler et al. 1997). Transgenic *A. thaliana* plants were grown at 16 h light (400 μmol of photons per m^2 per s) and 8 h darkness at 20 °C.

Confocal laser scanning microscopy

Arabidopsis thaliana rosette leaf sections of 3- to 4-week-old plants were prepared manually with a razor blade. The sections were investigated with a Zeiss Pascal CLSM, 63× (numerical aperture, 1.4) objective lens. Excitation was generated with an argon laser at 488 nm. Long pass 560 nm filtered emission and band pass 505–530 nm filtered emission were recorded simultaneously.

Temperature experiments

Connected to the microscope is a temperature-controlled chamber (LM-TCC) developed together with BK-Elektronik (Natters, Austria). The chamber consists of an aluminum block containing a round chamber with a diameter of 13 mm and a height of 10 mm, holding approximately 1 ml of water during the temperature treatment. A coverslip is used as the bottom of the chamber. The aluminum block is insulated against the microscope with fiberglass elements. Temperature is generated with a Peltier element-based temperature generator connected to the aluminum block. The temperature generator itself is connected to a water-cooled circuit and the unit is operated with a special PID (proportional integral derivative) controller (Drews Elektronic GmbH, Kamp-Lintfort, Federal Republic of Germany). The temperature was constantly monitored inside the chamber in close vicinity to the leaf sections with a thermocouple sensor (Type T; solder junction diameter, 0.2 mm; Thermo-Est, Vienna, Austria).

Determination of a "shape index" and statistical analysis

Stromules and chloroplast protrusions were examined according to their length-to-radius ratio following the equation $q = e/r$. The "shape index" q was determined, where e is the elongation and r is the radius of the structure (see Fig. 1a). On the basis of the frequency distribution ($n = 200$) of shape indices, two different clusters could be determined. The mean values of these were statistically tested by t test comparison with the SPSS 13.0 software (SPSS Inc., Chicago, Ill.).

For the percentages of chloroplasts with protrusions, the number of chloroplasts with protrusions relative to the total number of chloroplasts per cell was calculated ($n = 8$, giving a total amount of approximately 40–50 counted chloroplasts) for each temperature step in a representative experiment. Statistical analysis of the mean values was carried out by analysis of variance and Bonferroni test with the SPSS software.

Transmission electron microscopy

Rosette leaves were cut with a razor blade into pieces (about 3 by 3 mm), exposed to 5, 20, or 35 °C for 2 h in darkness, and then fixed with 2.5% glutaraldeyde for 2 h in 50 mM sodium cacodylate buffer, pH 7.0. After a rinsing step, samples were postfixed in buffered 1% OsO_4 for 4 h. After a

further rinsing step, samples were dehydrated in increasing concentrations of ethanol, each 15 min, and infiltrated with Spurr embedding resin (Serva, Heidelberg, Federal Republic of Germany). Polymerisation was carried out for 8 h at 70 °C. Ultrathin sections were counterstained in aqueous uranyl acetate and Reynolds lead citrate and examined with a Zeiss EM 902 transmission electron microscope (TEM) at 80 kV or a Libra 120 transmission electron microscope at 120 kV. Images were captured digitally with a 2K camera or on Kodak negative material, digitised and further processed by the Adobe Photoshop software.

Results

Stromule observation at the CLSM

The GFP expressed in the transgenic *A. thaliana* plants is targeted to the stroma fraction by the *recA* transit sequence, as shown by prior fractionation experiments performed with tobacco transgenic plants (Köhler et al. 1997). In leaf mesophyll cells of transgenic *A. thaliana*, stromules are occasionally branched or appear polymorphic when observed at the increasing temperature of 20 °C (see Fig. 2a–c). In this tissue, stromules are up to approximately 20 μm long and 400–600 nm wide; however, stromules are not seen in every mesophyll cell but appear most prominent when spaces between chloroplasts exist within cells. When chloroplasts are densely arranged, almost no stromules are observed. The calculated shape index for stromules sensu stricto has a value of 7.0 ± 1.3 (mean with standard deviation) (Fig. 1b).

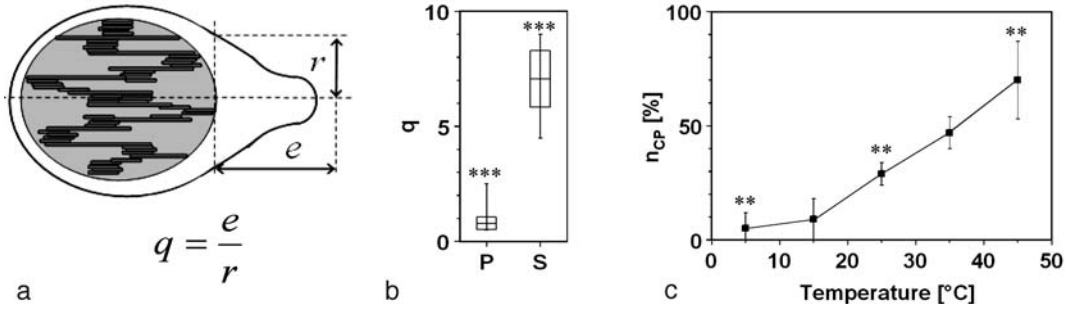

Fig. 1 a–c. Description of stromules and chloroplast protrusions and the influence of temperature on their formation in *A. thaliana*. **a** Determination of the shape index (q) for a schematic chloroplast with a thylakoid-containing central zone (grey) and a stroma-containing jacket (white). **b** Shape index (mean value with standard deviation; minimum and maximum indicated by a vertical line) of chloroplast protrusions (*P*) and stromules (*S*) is significantly different (***P $<$ 0.001). **c** Percentages of chloroplasts with chloroplast protrusions (n_{CP} [%]) at increasing temperature steps. Statistically significantly different values (**P $<$ 0.01) were obtained at the temperature steps 5, 25, and 45 °C

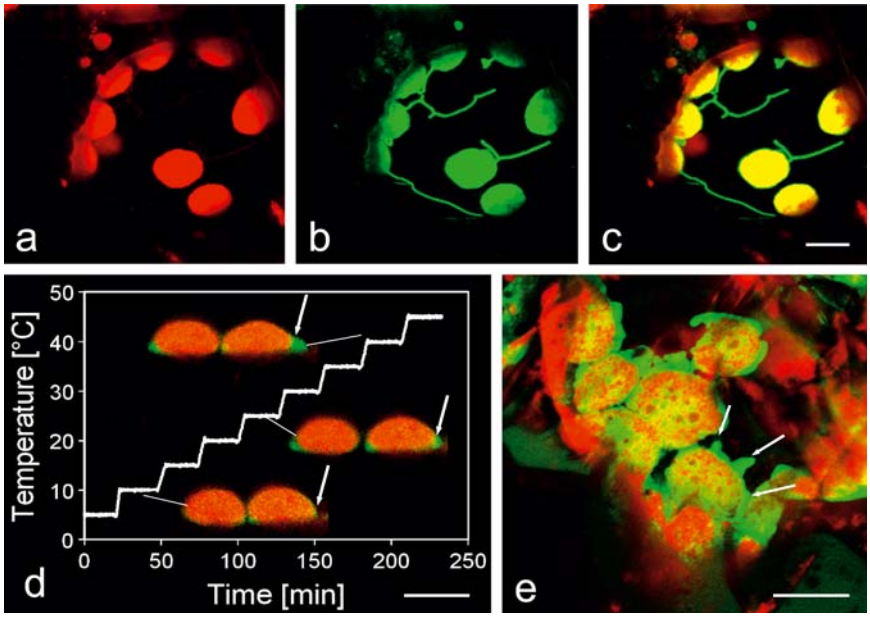

Fig. 2 a–e. Confocal laser scanning microscopic images of *A. thaliana* rosette leaf mesophyll cells and temperature experiments carried out with the LM-TCC. **a–c** 20 °C, mesophyll cell with fully developed stromules. **a** Chlorophyll autofluorescence demonstrated by a 560 nm long pass filter; **b** GFP fluorescence demonstrated by a 505–530 nm band pass filter; **c** merge of **a** and **b**. **d** Temperature experiment carried out with the LM-TCC, each temperature step applied for 25 min, very little temperature fluctuation is monitored in close vicinity of the leaf section. The same chloroplasts have been recorded throughout the experiments, side views of chloroplasts at three temperature steps (10, 25, 40 °C) are included in the graph. Chloroplast protrusions are absent at 10 °C but emerge at 25 °C and become more abundant at 40 °C (arrows). **e** Mesophyll cell at 35 °C, numerous chloroplast protrusions with beaklike appearance emerge from the chloroplasts (arrows). Bars: 10 μm

Temperature experiments

The LM-TCC enables presetting of the temperature for investigation directly at the microscope stage with an accuracy of ±0.1 °C in close vicinity of the leaf sections in a temperature range of 0 °C to +60 °C. Leaf sections were exposed to different temperature regimes. For the experiments, temperatures between 5 °C and 45 °C (Figs. 1c and 2d) were chosen and applied in steps of 5 °C, each temperature step was maintained for 25 min. Multiple ($n = 12$) temperature-increasing experiments revealed that the appearance of chloroplast protrusions is temperature-dependent (Fig. 1c). At low temperatures (5–15 °C) virtually no chloroplast protrusions or stromules were observed, but they emerged with increasing temperatures and were already clearly visible at 25 °C (Fig. 2d). The mean percentage of chloroplasts with protrusions at 5 °C is significantly different from that at 25 °C ($P < 0.01$). With elevated temperatures, protrusions increase in length (Fig. 2d). The temperature-induced chloroplast protrusions have a beaklike appearance and may reach a length of approximately 3–5 μm and a width of 3–5 μm. The shape index of chloroplast protrusions is 0.8 ± 0.3 (Fig. 1b). This value is significantly different ($P < 0.001$) from that of stromules seen in cells with spaces between the chloroplasts (Fig. 2c). The number of chloroplast protrusions per chloroplast is increased with elevated temperatures; up to 5 individual protrusions were counted on the same chloroplast in the plane of focus (Fig. 2e). Especially at higher temperatures (35–45 °C), chloroplast protrusions are covering wide ranges of the chloroplast edges (Fig. 2e). The mean percentage of chloroplasts with protrusions at the highest temperature (45 °C) is significantly different from that at 25 °C ($P < 0.01$).

TEM investigations

In *A. thaliana* plants exposed for 2 h at 5 °C, the general cell architecture of rosette leaf mesophyll cells appears to

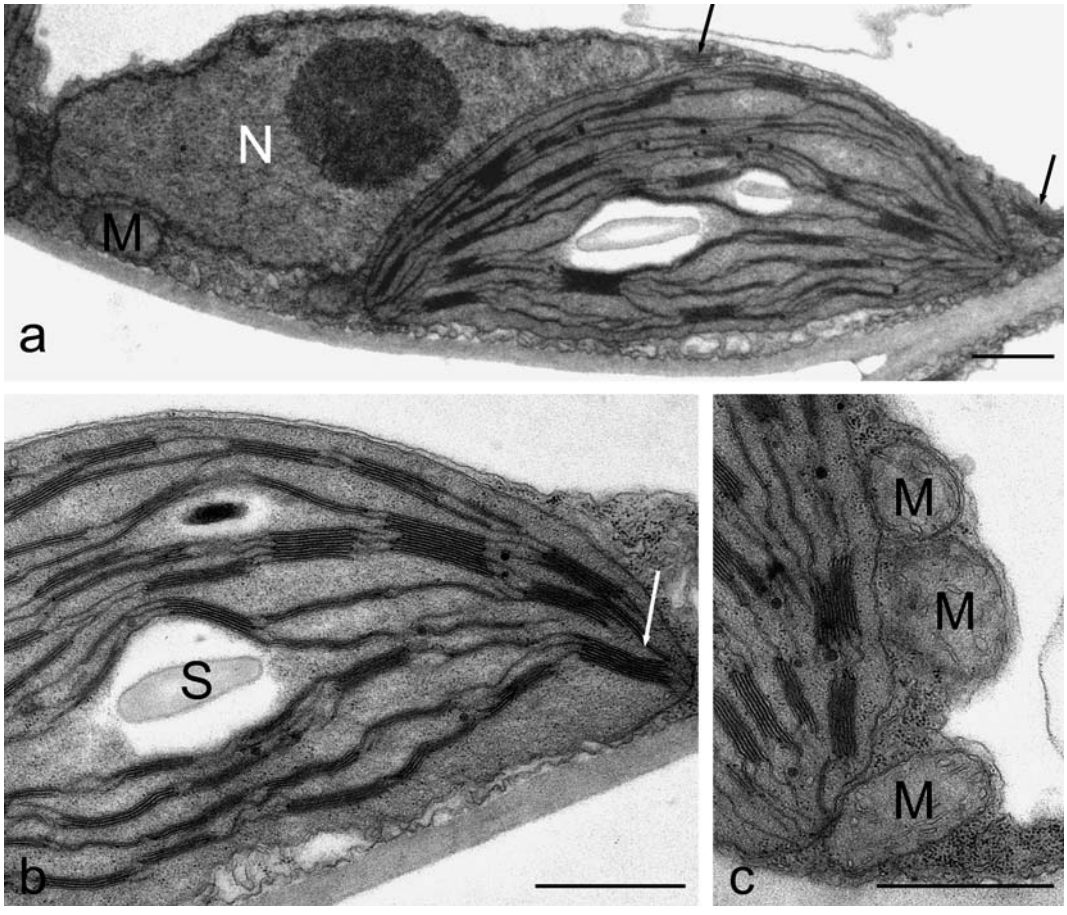

Fig. 3 a–c. Transmission electron micrographs of *A. thaliana* rosette leaf mesophyll cells exposed to 5 °C for 2 h. **a** Overview of cortically arranged chloroplast, nucleus (*N*), mitochondria (*M*), and Golgi stacks (arrows). **b** Detail of the chloroplast with starch grain (*S*), thylakoid membranes reaching toward the tip of the chloroplast (arrow). **c** Detail of the chloroplast with mitochondria (*M*) in close vicinity. Bars: 1 μm

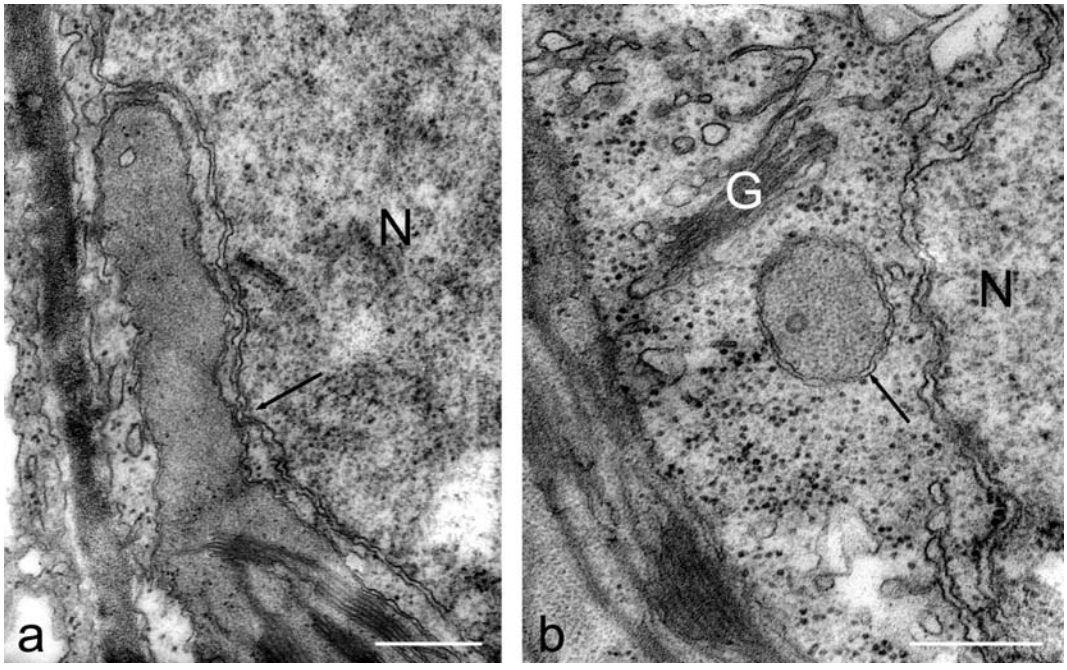

Fig. 4 a, b. Details of chloroplast stromules of *A. thaliana* rosette leaf mesophyll cells at 20 °C. **a** Longitudinal section of stromule emerging from the chloroplast in close vicinity to the nucleus. Membranes of the nucleus and the stromule are in very close contact at some points (arrow). **b** Transverse section of stromule with double-layered membrane (arrow) in close vicinity to the nucleus (*N*) and a Golgi stack (*G*). Bars: 0.5 μm

be intact with numerous cortical chloroplasts, nucleus, and other organelles like mitochondria and Golgi stacks (Fig. 3a–c). Chloroplasts show the typical architecture with thylakoid membranes arranged in grana stacks and stroma thylakoids (Fig. 3b), starch grains are observed, but no stromules are visible. The thylakoid membranes expand towards the longitudinal ends of the chloroplasts (Fig. 3b, c). Mitochondria are found in close vicinity to chloroplasts (Fig. 3c).

Sections through the longitudinal axis of stromules (Fig. 4a) are observed occasionally in samples fixed at the regular growth temperature of 20 °C. Transverse sections of stromules with a diameter of about 450 nm (Fig. 4b) are observed more frequently. Stromules are clearly distinguishable from other organelles by their chloroplast-typical double membrane. Stromules show close membrane-to-membrane contact with nuclei (Fig. 4a) and mitochondria, but no fusions have been seen. Golgi stacks are found in the spatial vicinity of stromules (Fig. 4b).

At 35 °C, broader areas of the chloroplast appear to contain only stroma and representing chloroplast protrusions (Fig. 5a). At the longitudinal ends of the chloroplasts, chloroplast protrusions are observed especially frequently (Fig. 5b). On tangentially sectioned chloroplasts, chloroplast protrusions are also observed, frequently appearing to be in close contact with mitochondria (Fig. 5c).

Discussion

In this study, data are presented on the temperature-dependent formation of chloroplast protrusions and stromules in *A. thaliana* mesophyll cells. Stromules are present in cells in which spaces between the chloroplasts occur, but chloroplast protrusions emerge from previously stromule-free chloroplasts exposed to increasing temperatures in the LM-TCC. There is good coincidence between the CLSM and TEM observations on the occurrence of stromules and chloroplast protrusions as being temperature-dependent in their formation. Whereas at low temperatures chloroplast protrusions are virtually absent, they appear with increasing temperature. Moreover, the number of individual protrusions per chloroplast increases. The appearance of protrusions is short and beaklike, and they may emerge from broader stroma-containing wings. With the determination of a shape index, we have provided a mathematical method to distinguish between stromules and chloroplast protrusions. A statistically significant difference between stromules and protrusions was observed; however, an interchange between these groups might still be possible. Temperature-induced chloroplast protrusions are likely to correspond to a phenomenon described as the "mobile jacket" of chloroplasts, which may occur under stress conditions as reported several decades ago (Spencer

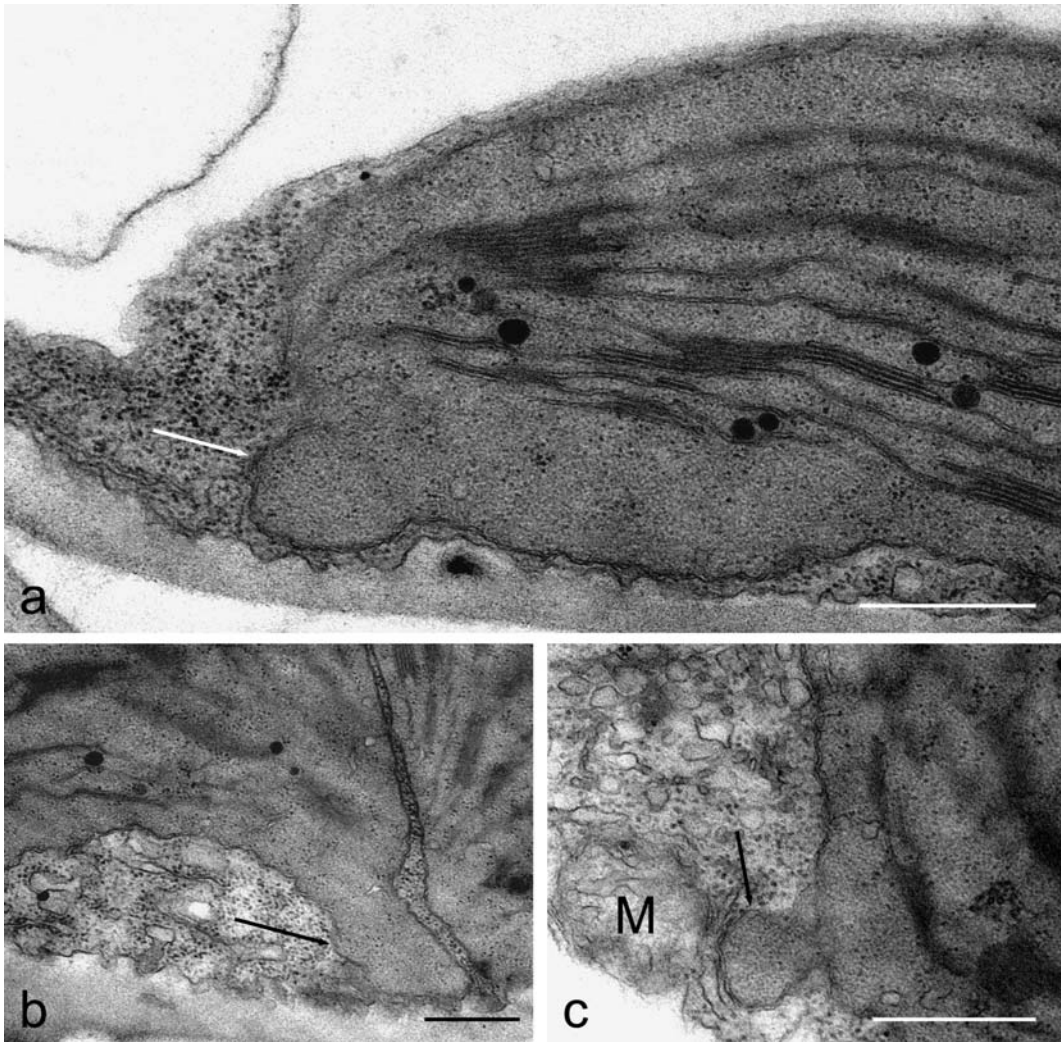

Fig. 5 a–c. Transmission electron micrographs of *A. thaliana* rosette leaf mesophyll cells exposed to 35 °C for 2 h. **a** Chloroplast with marked thylakoid-free area (arrow); **b** chloroplast with chloroplast protrusion (arrow); **c** surface section of a chloroplast with connected chloroplast protuberance (arrow) in close vicinity of a mitochondrion (*M*). Bars: 0.5 μm

and Wildman 1964). The mobile jacket of chloroplasts and the shorter protrusions we have observed may actually be an early stage of stromule formation. Whether a protrusion goes on to become a stromule of more typical long and thin appearance could depend on the space available and the unknown factors that cause stromule growth. Within densely packed mesophyll cells, there is little space available for stromules to extend; also, there may be density-dependent mechanisms that determine the length to which a stromule can extend.

Previously the occurrence of stromules has been reported as strongly tissue-specific (Köhler and Hanson 2000, Waters et al. 2004). In general, chloroplasts of mesophyll cells were described to have only few stromules. In transgenic Arabidopsis leaves, stromules have been observed with different stroma-targeted GFP fusion proteins (Tirlapur et al. 1999). Here we were able to report that, especially at higher temperatures, an increased number of chloroplast protrusions were found to emerge from individual chloroplasts, but not stromules sensu stricto.

A detailed description of stromule behaviour concerning outgrowth, retraction, anchoring, stretching, branching and bridging has been made by video microscopy (Gunning 2005). In that study, maximal values for the outgrowth of 0.23 μm/s and for the retraction of −1.16 μm/s have been described for *Iris unguicularis*. As the information was captured from video sequences generated with differential interference contrast illumination for time periods of >10 min, one could speculate that temperature may have affected the stromule dynamics. In general, the temperature effects that might occur in microscopic preparations of live samples when ob-

served without a temperature-controlled chamber remain unstudied.

Long stromules and chloroplast protrusions were observed even when leaf tissue was dark-incubated at temperatures of 20 °C and 35 °C prior to fixation for TEM. Stromules are also readily observed by CLSM in dark-grown hypocotyls and roots (Kwok and Hanson 2003), demonstrating that stromule formation does not require light and is thus not directly related to light reaction processes of photosynthesis. As a consequence of temperature increase, photosynthesis is reversibly inhibited in Arabidopsis plants in which Rubisco activase or thylakoid membrane fluidity has been modified (Kim and Portis 2005). Growth temperature changes the fatty acid composition of Arabidopsis leaves; however, this appears to be a long-term effect (Falcone et al. 2004). There is an about 60 h period before differences become obvious and membrane fluidity might have changed. This corresponds with the occurrence of newly synthesised lipids, and not to an alteration in existing lipids. Therefore, in our investigations, where temperature experiments were conducted with an exposure time of 25 min per temperature step, we were likely able to exclude changes in the membrane composition as a reason for altered stromule appearance and morphology.

TEM observations on stromules of chloroplasts are sparse; most likely they have been overlooked or were thought to be artefacts. TEM sections are thin (about 70 nm), and therefore sections along the longitudinal axis of stromules are rarely observed. There are some early reports of "amoeboid plastids" (for references see Kwok and Hanson 2004a), and "chloroplast proliferations" have been observed in the arctic-alpine plant *Ranunculus glacialis* (Lütz and Moser 1977, Lütz 1987). These chloroplast proliferations are distinct from stromules, a conclusion that can be drawn from their shape. More likely they are related to the temperature-induced chloroplast protrusions as described in this study. Heat-induced changes in *R. glacialis* occur at 42–44 °C, resulting in disorders of the photosynthetic apparatus with swollen chloroplasts (Larcher et al. 1997). A report on "chloroplast protuberances" in rice leaves has been obtained with high-pressure freeze fixation (Bourett et al. 1999). Again these structures appear to be rather massive in comparison with the thin stromules seen in vivo. While Bourett et al. (1999) commented that high-pressure freeze fixation was likely to be required to visualise chloroplast protuberances, we have been able to clearly demonstrate their existence in chemically fixed tissue in our study. Stromules are readily distinguishable from other organelles by their chloroplast-typical double membrane in combination with the stroma texture.

Bourett et al. (1999) also reported an irregular surface of the chloroplast protuberances, which was also detected in stromules and chloroplast protrusions in this report. High-pressure-frozen tissue is generally accepted to be better preserved than chemically fixed tissue. In chloroplasts of higher plants, several disadvantages after high-pressure freeze fixation are unavoidable. Membrane lipids of the thylakoid systems are usually washed out during the freeze-substitution procedure and the residue appears white, whereas the stroma portions are usually heavily stained and the outer membranes of the chloroplast are either not visible or less clear than after chemical fixation (Wesley-Smith 2001).

Here we were able to demonstrate a close membrane-to-membrane contact of stromules and the nuclear envelope at the TEM level. A synapse-like interaction between chloroplast and Golgi body is described in rye leaves and there is the suggestion that, for example, plastoquinone is synthesised in Golgi stacks and transported to the chloroplast (Selga and Selga 2000). Interaction of stromules with the nucleus have been reported on the confocal level in various tissues of *A. thaliana* and *Nicotiana tabacum* (Kwok and Hanson 2004c).

Stromules might facilitate interactions with other organelles and increase the plastid–cytoplasm contact area. They might also be involved in signal transduction or metabolite exchange (Kwok and Hanson 2004a). An enhanced demand for all of these proposed functions with increasing temperatures is likely. Therefore, understanding the behaviour of stromules may implicate them in the plant's cell response to environmental parameters such as temperature.

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