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NEW IDEAS IN CELL BIOLOGY

The myth of interconnected plastids and related phenomena

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Abstract Studies spread over nearly two and a half centuries have identified the primary plastid in autotrophic algae and plants as a pleomorphic, multifunctional organelle comprising of a double-membrane envelope enclosing an organization of internal membranes submerged in a watery stroma. All plastid units have been observed extending and retracting thin stroma-filled tubules named stromules sporadically. Observations on living plant cells often convey the impression that stromules connect two or more independent plastids with each other. When photo-bleaching techniques were used to suggest that macromolecules such as the green fluorescent protein could flow between already interconnected plastids, for many people this impression changed to conviction. However, it was noticed only recently that the concept of protein flow between plastids rests solely on the words "interconnected plastids" for which details have never been provided. We have critically reviewed botanical literature dating back to the 1880s for understanding this term and the phenomena that have become associated with it. We find that while meticulously detailed ontogenic studies spanning nearly 150 years have established the plastid as a singular unit organelle, there is no experimental support for the idea that interconnected plastids exist under normal conditions of growth and development. In this review, while we consider several possibilities that might allow a single elongated plastid to be misinterpreted as two or more interconnected plastids, our final conclusion is that the concept of direct protein flow between plastids is based on an unfounded assumption.

Keywords Interconnected plastids · Stromules · Leucoplasts · Etioplasts · Fluorescent proteins · Photo-convertible proteins

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The primary plastid in Viridiplantae: a discrete, double membrane bound unit organelle

Plastids, the defining organelles of the Viridiplantae, are postulated as having originated from a symbiotic interaction between an eukaryotic host and a free-living photosynthetically active prokaryote (Keeling 2013). One of the first mentions of plastids in scientific literature can be traced back to the letters from the 1670s, sent by Leeuwenhoek to the Royal Society of London, Britain. In one of them (Letter 11[6] dated September 7, 1674), he describes the ribbon-like plastids found in the green alga Spirogyra (Leeuwenhoek A van 1674). In the late nineteenth century, as the quality of light microscopes improved, chloroplasts drew attention first in the plant cell due to their green pigmentation. Accordingly, different botanists have independently described single cup-shaped or multiple spheroidal "chlorophyll grains" in cells of mosses, horsetails, ferns and vascular plants (Schmidt 1870; Schimper 1882, 1883, 1885; Haberlandt 1888; Senn 1908; Reinhard 1933). The detailed observations from different taxa made at the turn of the nineteenth century form the basis for our understanding of the structure and functions of these "chromo bodies".

From the early observations, it became evident that a colourless "mobile jacket" or "peristromium" (accepted today as the plastid stroma enclosed within the double membrane

envelope) surrounds a number of dense granules or "chlorophyll grains", known presently as grana (reviewed by Senn 1908; Haberlandt 1914). Furthermore, it was found that "chlorophyll grains" do not form de novo out of unstructured cytoplasm but instead always originate from ancestral grains propagated through division and resulting in two independent daughter chlorophyll grains (Senn 1908). Another important discovery was that the chlorophyll grains, the later described "starch formers" and the so called "pigment bodies" found in flowers and some roots (known today as chloroplasts, leucoplasts/amyloplasts and chromoplasts, respectively), are not different kinds of organelles, but instead originate from the same non-pigmented round particles found in all shoot meristems (today known as pro-plastids; summarised by Schimper 1882; 1885). Recognizing that these different types could change from one form into another, Schimper (1882) introduced the general term "plastid" (derived from the Greek word plastikos, meaning—something that can be moulded) for all forms of this organelle. Schimper (1882) acknowledged that the term plastid had originally been introduced into biology by Ernst Haeckel (1866) but was interpreted differently as the lowest level of organization in biological hierarchy. The reader is directed to Gunning et al. (2007), Wise (2007) and Biswal et al. (2013) for excellent, chronologically arranged information on research on plastids.

After the invention of the electron microscope, many of the basic features of plastid structure, founded on light microscopic observations, were confirmed. Despite the great diversity in their shapes, it was recognized that all primary plastids are singular, double membrane-bound organelles that contain an organization of internal membranes bathing in stromal fluid (Block et al. 2007) (Fig. 1a). It was also established that compared to the relatively rigid grana formed by thylakoid stacks in chloroplasts, the internal membranes are arranged into loose longitudinal arrays in leucoplasts or into prolamellar bodies in etioplasts (Gunning 1965; Wise 2007). Chromoplasts and gerontoplasts display variable internal membranes remodelled from the basic chloroplast organization (Wise 2007).

Cinematic observations created an impression of plastid connectivity

Cine-photomicrography of living plant cells provided fresh insights on plastids. Wildman et al. (1962) observed the natural behaviour of mitochondria and chloroplasts and suggested their possible inter-relatedness. They also described the stroma-filled jacket around chloroplasts in spinach and observed long protrusions extending into the cytoplasm. Green (1964) carried out detailed studies on the growth and division of chloroplasts in *Nitella* and commented on connections between chloroplasts. Although the continuous observations recorded in this study do not show tubules being extended and connecting independent plastids, there is mention of nearly "invisible" and highly elastic connections that are speculated to be incompletely cleaved portions of the plastid (Green 1964). Whereas these studies provided a fascinating view of the dynamic nature of the plastid, they did not dispute the unitary nature of the organelle.

"Interconnected plastids"—challenging the autonomous nature of the plastid unit

In 1997, observations on living plants expressing a green fluorescent protein targeted to the stroma challenged the autonomous and discrete nature of the plastid (Köhler et al. 1997). Contrary to the knowledge accumulated until then, it was suggested that plastids are able to exchange protein molecules through thin, stroma-filled interconnections. Subsequently, Köhler and Hanson (2000) named plastidic extensions that appear less than 0.8 μm in diameter as "stromules" and larger parts of the plastid as the "body" (Fig. 1b). Under light microscopy, stromules appear to extend towards other plastids and sometimes give the impression of connecting them (Köhler et al. 1997). However, light microscopy lacks the resolution required to discriminate whether stromules actually contact and connect with each other or merely appear to do so. Therefore, a demonstration of interplastid connectivity was carried out by selective photobleaching techniques to show that GFP could flow between already interconnected plastids (Köhler et al. 1997; Fig. 1c). Through their seminal publication, Köhler et al. (1997) introduced two new concepts about plastids: one, that all independent plastids are able to extend stromules, and two, that somehow plastids interconnected plastids are formed. It was insinuated that interconnected plastids must have formed via stromules.

One of the most detailed observations of plastid behaviour and stromule extension and retraction using video rate differential interference contrast on epidermal peels of *Iris unguicularis* was provided by Gunning (2005). While time-lapse DIC images do not resolve between actual fusion and "appearing to fuse", Gunning (2005) provided succinct examples where stromules appeared to form bridges between two or more plastids.

Of the two ideas introduced by the observations of Köhler et al. (1997), the presence of stromules is now well-accepted as they have been observed in almost all plastid types in plants and green algae (Menzel 1994; Langeveld et al. 2000; Arimura et al. 2001; Gray et al. 2001; Pyke and Howells 2002; Gunning 2005; Hanson and Sattarzadeh 2011; Shaw and Gray 2011; Mueller et al. 2014). A detailed description of stromules from different plastid types has been provided

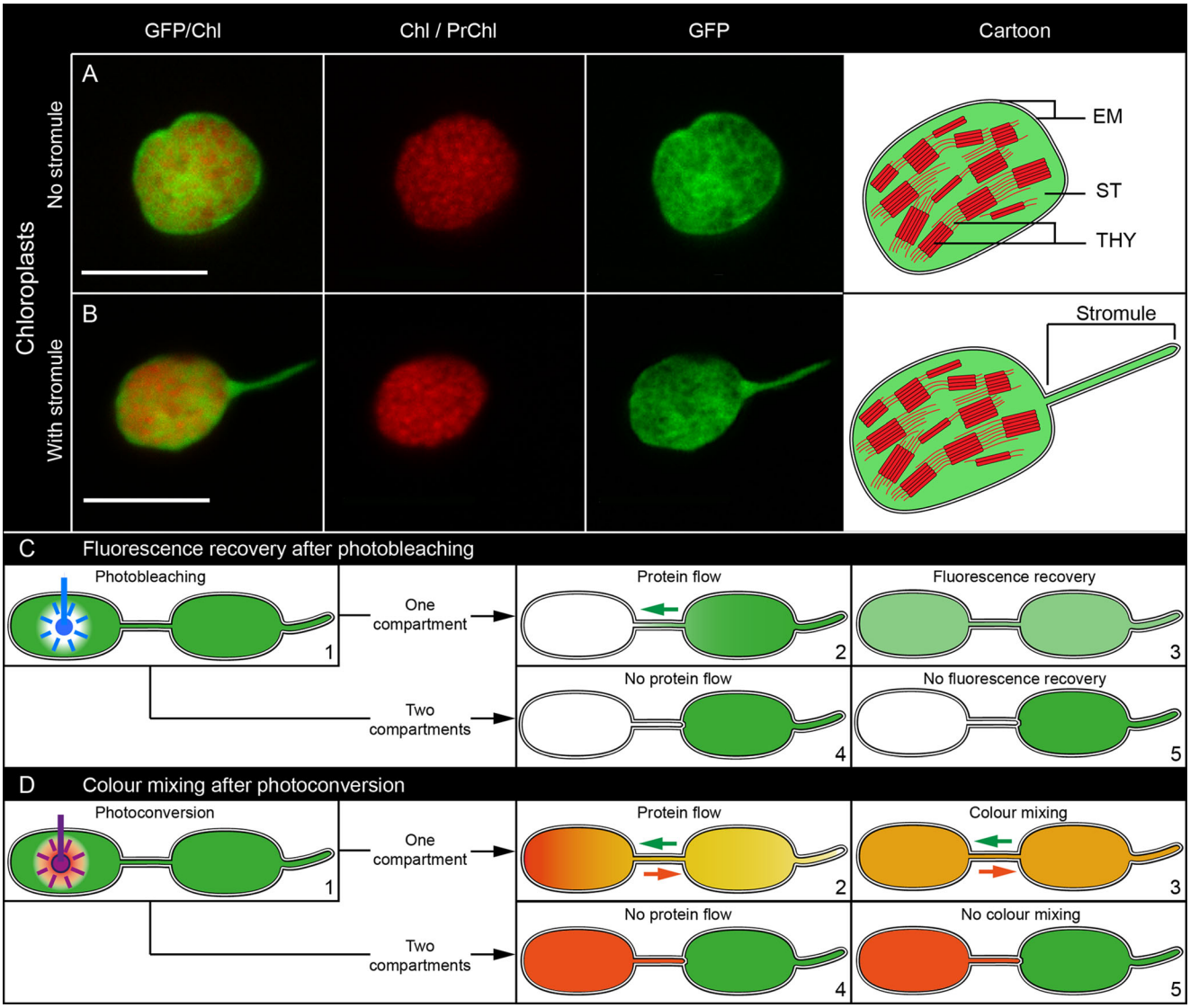


Fig. 1 Basic features and fluorescence based techniques involved in the concept of interconnected plastids. **a** Merged and single fluorescent image of a chloroplast with red chlorophyll fluorescence and a green stroma targeted tpFNR:eGFP followed by a cartoon labelling the double membrane envelope (*EM*), stroma (*ST*) and internal thylakoid membranes (*THY*) with appressed regions (*red blocks*) depicting the grana. **b** Merged and single fluorescent images of a chloroplast similar to **a** but extending a tubular stroma filled region called the stromule. **c** Diagrammatic depiction of fluorescence recovery after photobleaching (FRAP) used to demonstrate flow of proteins between compartments. *Panel 1* shows that GFP is photo-bleached in a region using high intensity laser. The recovery of green fluorescence due to protein flow from an unbleached region suggests interconnectivity (*panels 2, 3*). Protein flow (*panel 4*) and fluorescence recovery (*panel 5*) would not occur when two compartments are not connected. Already interconnected plastids (*panel 1*) were used to prove connectivity between two regions. It was assumed that the two areas (bleached and unbleached, *panel 1*) represented two independent plastids that were connected by a stromule (Köhler et al. 1997). **d** Diagrammatic depiction of colour mixing after photoconversion involves a green to red photoconvertible protein and has also been used to demonstrate flow (*panel 2*) and mixing (*panel 3*) of red and green proteins between compartments. If one compartment is involved then the colours merge quickly to provide an intermediate colour (*panel 3*), whereas two unconnected compartments maintain their separate colour identity (*panels 4, 5*). Whereas Schattat et al. (2012a, b) have used unconnected plastids to investigate how they fuse together, Hanson and Sattarzadeh (2013) have used the same technique to prove that protein flows between two already interconnected plastids. While flow and redistribution of protein within a compartment cannot be disputed the connection of two independent plastids by a stromule for protein exchange has not been demonstrated unequivocally. Size bars in a, b=5 μm

(Köhler et al. 2000). However, the idea of "interconnected" plastids, which forms the sole basis for suggesting that proteins in the range of 27 to 500 kDa (Köhler et al. 1997; Kwok and Hanson 2004a; Table 1) can be exchanged between plastids via stromules, has not been clarified to date.

Questioning the idea of interconnected plastids and the start of a debate

Based on the reports and reviews published since 1997, Schattat et al. created a green to red photo-convertible

Table 1 Primary publications using already interconnected plastids for demonstrating protein flow between plastids

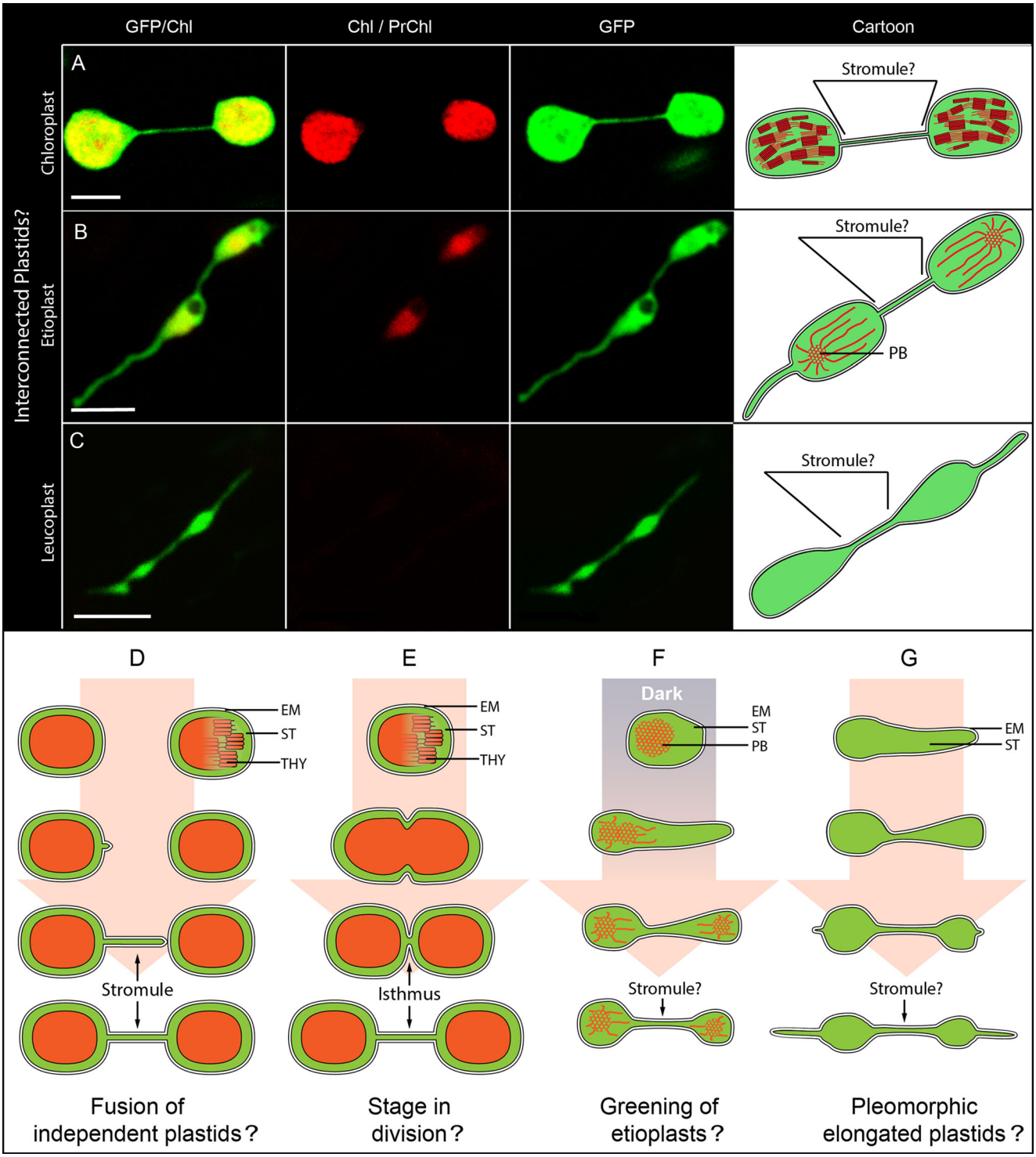
Plant	Cell/tissue type	Plastid type	Description—conclusions	Reference
Tobacco	Root	Leucoplasts	Protein flow within a stromule and between already interconnected plastids	Köhler et al. (1997)
Arabidopsis	Hypocotyl guard cells, liquid culture	Chloroplasts, etioplasts	Protein flow between already connected plastid pairs	Tirlapur et al. (1999)
Tobacco	Cell suspensions	Leucoplasts	Protein flow within a long stromule. Find that most plastids are not interconnected	Köhler and Hanson (2000)
Tobacco	Cell suspensions	Leucoplasts	Established speed and dynamics of protein flow within stromules	Köhler et al. (2000)
Tobacco	Dark-grown hypocotyl	Etioplasts	Protein flow between connected plastids not affected by cytoskeletal inhibitors	Kwok and Hanson (2003)
Tobacco	Dark-grown hypocotyl	Etioplasts	Rubisco and aspartate aminotransferase flow between interconnected plastids	Kwok and Hanson (2004a)
Arabidopsis	Dark-grown hypocotyl	Etioplasts	Protein flow between plastid bodies through a stromule	Hanson and Sattarzadeh (2013)

fluorescent protein targeted to the stroma to understand how exactly stromules extended by independent plastids might fuse to form interconnected plastids for facilitating protein traffic between plastids (Schattat et al. 2012a, b; Fig. 1d). Instead of uncovering a mechanism for plastid fusion via stromules, Schattat et al. (2012a, b) found that these tubular extensions still maintain the boundary of the unit plastid. Thus, even when stromules from different plastids seem to connect, they do not actually fuse. Nor as suggested by earlier studies do they exchange fluorescent or other proteins larger than 25 kDa. While the possibility of non-vesicular protein and lipid exchange between independent plastids still remained, the observations of Schattat et al. (2012a, b) strongly suggested that stromule-aided fusion of plastids (Fig. 2) does not take place. The findings upheld the conclusions of numerous earlier researchers and maintained the plastid as a discrete unit.

Nevertheless, a commentary entitled "Trafficking of proteins through plastid stromules" categorically announced that new observations "demonstrates trafficking of fluorescent protein between plastids" (Hanson and Sattarzadeh 2013). Through this recent publication, the earlier fluorescence recovery after photo-bleaching (FRAP)-based observations of 1997 were upheld even when using a photo-conversion-based approach. Once again, it was concluded that plastids are able to exchange fluorescent protein via stromules. It is notable that as in the first publication by Köhler et al. (1997), the new findings also do not explain how two plastids became connected for exchanging proteins. Since this most recent work of Hanson and Sattarzadeh (2013) utilized the same materials and techniques (Fig. 1d) used by Schattat et al. (2012a, b), it was perplexing how two independent groups had arrived at completely opposite conclusions and created a controversy.

A careful perusal of the recent commentary by Hanson and Sattarzadeh (2013) and the response by Mathur et al (2013) as well as earlier publications on plastids and stromules has traced the difference in opinion to the different interpretations that have been applied to the words "independent plastid" and "interconnected plastids".

Fig. 2 Bulbous regions connected by a tubular stroma-filled stretch have often conveyed the impression of interconnected plastids. Labelling the transiently occurring areas (marked by *question mark*) as a stromule is debatable as this result of plastid stretching clearly differs from the free ending stromules extended by independent plastids. **a** Apparently interconnected chloroplasts in *Arabidopsis* with the merged fluorescence of GFP and chlorophyll, followed by single fluorescent images of chlorophyll fluorescence (*red*), and of stroma targeted GFP (*green*). A diagrammatic depiction suggests that the stroma-filled region between the two thylakoid-grana containing chlorophyll regions might be considered a stromule. However, this interpretation is debatable as in a dividing plastid this region is called the isthmus. **b** A pleomorphic etioplast in *Arabidopsis* with two red fluorescing protochlorophyllide (PChl) containing pro-lamellar bodies. The diagrammatic depiction questions that the stroma-filled region between the two PChl-containing areas be considered a stromule. If the etioplast will divide subsequently, then the region would be considered as the isthmus. Alternatively following greening, the region would probably disappear as the thylakoid-grana become organized. **c** A single leucoplast where the loose internal membrane organization allows the constant morphing of the plastid into bulged and stretched regions. The diagrammatic depiction questions the feasibility of labelling a transient stroma-filled regions in such an elongated plastid as a stromule. **d**–**g** Diagrammatic depiction of some processes that may convey the impression of interconnected plastids. **d** A stromule that extends, contacts and connects two independent plastids. Note that this process has not been observed so far. **e** A plastid in the process of division where the stroma-filled isthmus may suggest a stromule. **f** Elongated greening etioplasts with a stretched stroma-filled region and **g** pleomorphic leucoplasts that may be misinterpreted as interconnected plastids. However, a plastid remains a continuous unit compartment until it has divided into two daughter plastids and they have separated completely from each other. (*Arrows in the background* of **d**–**g** indicate the order of suggested events; *pink colour* represents light while *blue-grey* depicts darkness; intra-organelle membranes are shown in *orange*; stroma as *green*; membrane envelope shown as *black-white* boundary). Size bars in **a**–**c**=5 μm



What exactly are "interconnected plastids"?

Historically, the membrane envelope defines the independent plastid unit, and the publication by Köhler et al. (1997) appears to be the first to introduce the term "interconnected plastids". This publication juxtaposed observations of free ending stromules from independent chloroplasts, ostensibly interacting with each other, alongside photo-bleaching experiments that demonstrated the flow of GFP in already interconnected plastids. The impression thus created for the reader was that the already interconnected plastids must have formed through a fusion of free ending stromules from independent plastids (diagrammatic depiction, Fig. 2d). These interconnected plastids appeared as two or more bulged regions

connected by a stroma-filled region. The bulged regions were considered to be the main plastid body containing the bulk of internal membranes and the stroma-filled region was considered the stromule. Without knowing the antecedent subcellular events that might have resulted in such morphology, these assumed interconnected plastids were used for the GFP photo-bleaching experiments (Köhler et al. 1997; Tirlapur et al. 1999; Kwok and Hanson 2003, 2004a, b). As shown in the diagrammatic representation in Fig. 1c(1), the photobleaching of any part of a plastid contained within its double membrane envelope is followed by a quick recovery of fluorescence at the bleached spot (Fig. 1c(2,3). This is precisely what Köhler et al. (1997) were able to show in their interconnected plastids.

Somehow, it remained unnoticed that a plastid, irrespective of its shape, constitutes a single unit as long as it is delimited by one continuous membrane envelope. Moreover, even if two independent plastids manage to become interconnected somehow (Fig. 2), after fusion they would clearly represent one continuous compartment. Flow of proteins from one side to the other within a single compartment is to be expected.

Notably, the circular reasoning of Köhler et al. (1997) in using already interconnected plastids (Fig. 1c) for demonstrating the interconnectivity of plastids was not questioned. Neither have the mechanisms that might be involved in creating interconnected plastids been elucidated to date. No reports of attempts made to estimate the extent to which the formerly independent plastids would keep their individuality and get disconnected again after interconnecting have been found either.

Nonetheless, since 1997, while declaiming the general ability of unit plastids to form an intracellular network (Köhler and Hanson 2000; Natesan et al. 2005; Hanson and Sattarzadeh 2011, 2013) and suggesting that interplastid fusion might be a very rare event of little physiological significance (Natesan et al. 2005; Hanson and Sattarzadeh 2013), the main idea that protein molecules are able to flow through interconnected plastids has been firmly maintained in primary publications, authoritative reviews (Table 1 and 2) and text books (Buchanan et al. 2000; Hanson and Köhler 2006). In trying to find justification for the continued use of the term interconnected plastids, we re-scrutinized the publications.

Which interconnected plastids were actually used for demonstrating protein flow between plastids?

In our reappraisal of earlier publications, it is apparent that whereas tubules extending from chloroplasts clearly provided evidence for the occurrence of stromules (Figs. 2 and 3 in Köhler et al. 1997), the actual photo-bleaching experiments aimed at demonstrating protein flow between plastids were carried out on leucoplasts from roots of tobacco plants (Fig. 4a–c in Köhler et al. 1997). It is noteworthy that while leucoplasts do produce thin stroma-filled extensions sporadically, their general appearance is also tubular. Their internal membranes are not organized in the typical thylakoid-grana manner characteristic of the chloroplast body. Moreover, tubular leucoplasts can display two or more bulged regions that might be misconstrued as the main plastid body similar to that observed in chloroplasts (Fig. 2a, c). Indeed in their authoritative review, Köhler and Hanson (2000) point to the remarkable shape variability and the difficulty in distinguishing between the plastid body and the stromule in non-green plastids.

Whereas the first primary report presents tubular leucoplasts as interconnected plastids, a perusal of literature after 1997 shows that with one exception that used chloroplasts (Tirlapur et al. 1999), similar experiments were carried out using other elongated and highly flexible plastids (Table 1). While there are more than 100 publications identifying or discussing stromules after 1997, it is notable that only seven (Table 1), six of which are from the same group, have actually suggested protein flow between plastids. All subsequent publications including key reviews on the subject (Table 2 and references in paper cited therein) have neither questioned nor explained the notion of interconnected plastids but cited the primary publications (Table 1) to support their findings and speculations. Whereas a general feeling exists in the field that interconnected plastid formation is a very rare event, it is not clear how this rare event happened often enough to provide a sufficient number of interconnected plastids for conducting photo-bleaching and photo-conversion-aided experiments on protein flow between plastids (Table 1; Fig. 1c, d). Some other possibilities are suggested in the next sections to explain how researchers might mistakenly conclude plastid interconnectivity.

The rapid greening of etioplasts might suggest interconnected plastids

One of the criterion that is commonly used in fluorescence microscopy for considering an organelle as a chloroplast is the localized auto-fluorescence emitted by chlorophyll molecules maintained on thylakoids (Fig. 1, Fig. 2a). Indeed in their commentary, Hanson and Sattarzadeh (2013) state that two plastid bodies both containing "chlorophyll (and therefore thylakoid membranes) and well separated from one another should be considered to be individual chloroplasts connected by a stromule".

In this context, etioplasts can be very misleading since they often become elongated, are pleomorphic and may display more than one bulged region containing pro-lamellar bodies (Fig. 2b, e). Following exposure to light, the disassembly of pro-lamellar bodies and the organization of thylakoids (Gunning 1965, 2001;

Table 2 A few key publications that accepted and further propagated the idea without explaining interconnected plastids

Publication type	Reference
Primary—Speculates on proteins flow between plastids through stromules as a favoured mechanism to explain transient expression related observations on chloroplasts.	Knoblauch et al. (1999)
Review—Does not question the notion of interconnected plastids but actually take it a step further by reinterpreting the meticulous ontogenic studies of Haberlandt	Gray et al. (2001)
Primary—Suggests that stromule-mediated interplastidic migration of macromolecules may be a tool for improving the yield of transplastomic products	Van Bel et al. (2001)
Review—States, "that GFP, a 27 kDa globular protein, was able to move through stromules interconnecting two different plastids". Does point out that the movement of macromolecules cannot be a primary function of stromules because most plastids are not interconnected	Natesan et al. (2005)
Primary—Used the concept to suggest transport of tomato LeHxk4 between independent plastids	Kandel-Kfir et al. (2006)
Review—Maintains plastids can be connected by stromules for protein exchange. Suggests additional functions for stromules	Hanson and Sattarzadeh (2008)
Review—States "most plastids are not connected by stromules at any one time, although over the course of a day, it is possible that many plastids within a cell establish transient contacts with one another through stromules." Also states that macromolecule transfer is not likely a major function of stromules	Hanson and Sattarzadeh (2011)
Review—Suggests that light induced electro-chemical signals can be transduced from chloroplasts to the plasma membrane by chloroplast stromules. States "a body of evidence, accumulated over a period of one hundred years or so, which indicates that plastids are interconnected with other plastids, with the nucleus and plasma membrane by the stromules that form a cellular network of extended chloroplast envelope membranes"	Szechyńska-Hebda and Karpiński (2013)
Primary—Accepts the concept of protein flow between interconnected plastids and investigates the possibility that DNA and ribosomes are also exchanged in the same manner. Finds no evidence for such a transfer route	Newell et al. (2012)

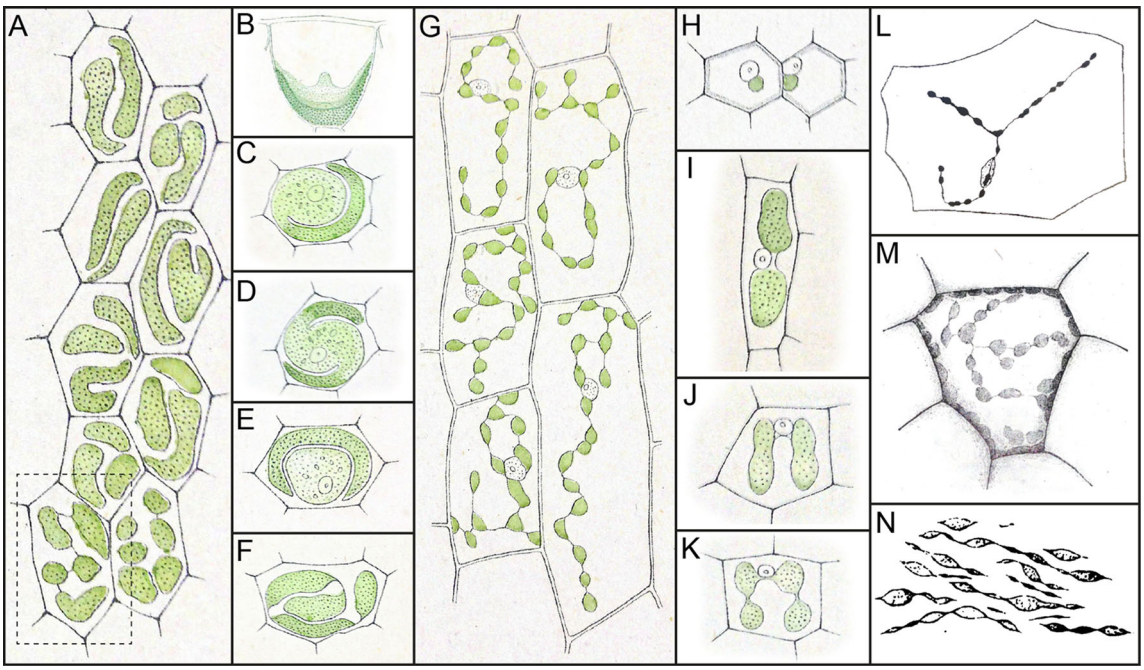


Fig. 3 Historical reports of chlorophyll chains in non-flowering plants. Several plastid related studies published around the turn of the nineteenth century reported the formation of so called chlorophyll chains as a result of incomplete division in non-flowering plants. **a**–**k** Drawings reproduced from Haberlandt (1888) depicting chlorophyll grains in *S. martensii* leaf cells (**a**–**f**) and *S. kraussiana* stem parenchyma cells (**g**–**k**). **a** *S. martensii* leaf cells show a gradual transition from cells exhibiting a single or few chlorophyll grains to cells with multiple chlorophyll grains, most often connected by thin threads of colourless plastid matter. **b**–**f** Progression from the apex to the leaf base shows that development of the chlorophyll chain results from the progressive constriction and separation of chlorophyll grains. **g** Similar to *S. martensii* leaf cells, *S. kraussiana* stem parenchyma cells show chains consisting of several chlorophyll grains connected by thin colourless strands. **h**–**k** By following the developmental gradient from meristem cells onwards, Haberlandt found that the incomplete division of daughter plastids was responsible for the development of these plastid chains. **l** A drawing of a *Selaginella* sp. stem parenchyma cell reproduced from Reinhard (1933) reconfirms Haberlandt's observations. **m** Chlorophyll grains in prothallium cells of the fern *Aneimia phyllitis* (reproduced from Senn 1908) and **n** chromo-bodies from the Rhodophycean *Monospora pedicellata* (reproduced from Berthold 1886) provide evidence of similar plastid chains in other taxa

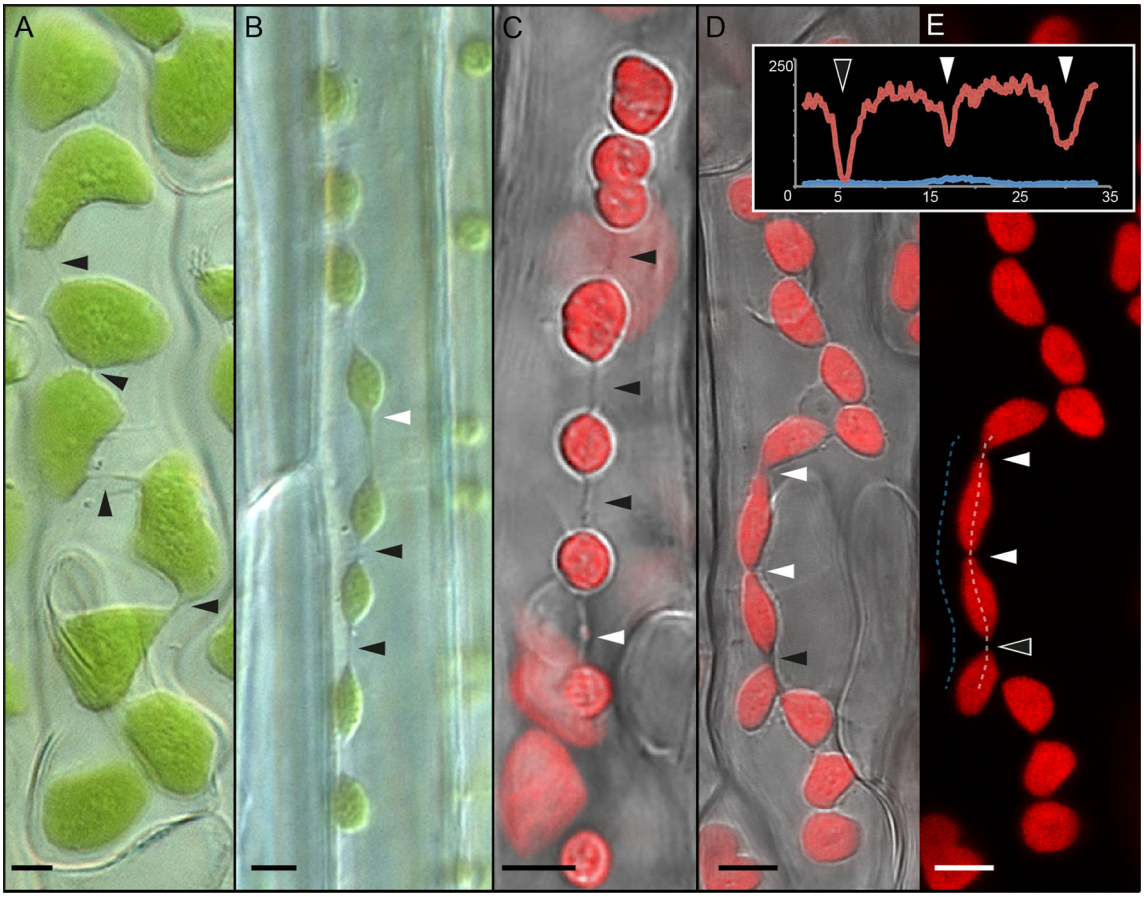


Fig. 4 DIC- and CLSM-based images on chlorophyll chains in *S. kraussiana*. **a**, **b** Differential interference contrast (DIC) images of chlorophyll chains in *S. kraussiana*. **a** A cell of the upper epidermis close to the leaf base shows large green thylakoids (corresponding to chlorophyll grains; Haberlandt 1888) connected by thin potentially stroma-filled tubules (*black arrow heads*). **b** A parenchyma cell of an older stem exhibiting part of a straight chain of small green thylakoids stacks also connected by thin threads (*black arrow heads*); in some cases, the thylakoids extend into the connecting thread (*white arrow head*). Cells in **a** and **b** closely resemble the drawings of Haberlandt 1888 (compare **a** and **b** with rectangle in Fig. 3a and Fig. 3g). **c** Confocal laser scanning microscopy (CLSM)-based images of *S. kraussiana* lower leaf epidermis cells close to the leaf base (*grey*=transmitted light; *red*=chlorophyll auto-fluorescence). Imaging with CLSM confirms the observations made by Haberlandt (1888). Clearly, chlorophyll-free connecting threads are visible between the thylakoids (*black arrow heads*). In some threads, very small thylakoid-filled areas are also present (*white arrow head*). **d**, **e** Observation of thylakoid division was an important indication for Haberlandt (1888) that the origin of the chains lay in division-related activity. In **d** and **e**, both fully separated thylakoids (visible by the gap in chlorophyll auto-fluorescence, *black arrow head*) and late stages of thylakoid division (visible by a thin stretch of chlorophyll auto-fluorescence connecting two daughter thylakoid stacks, *white arrow heads*) are visible. For better interpretation, a fluorescence intensity plot along the chlorophyll chain (*red*) as well as along the background (*blue*) is shown in the *inset* (*x*-axis=distance in pixel; *y*-axis=grey scale value of the red channel showing the chlorophyll auto-fluorescence). Size bars: **a**–**d**= 10 μm; **f**, **g**=1 μm

Mackender 1978; Wise 2007) as well as the greening process involving conversion of proto-chlorophyllide (Pchlide) into chlorophyllide (Chlide) proceeds very rapidly (Meskauskiene et al. 2001; Solymosi and Schoefs 2010). During the greening process, many of the flexible etioplasts can exhibit more than one region of chlorophyll fluorescence (Weier et al. 1970; Mostowska 1985; Murakami et al. 1985; Reinbothe et al. 1999; Grzyb et al. 2013). Moreover like chlorophyll, its precursor proto-chlorophyllide also fluoresces red under blue light illumination (Fig. 2b; Meskauskiene et al. 2001) and can suggest two chloroplasts.

Seedlings grown in the dark have often been used for observing stromules and a large number of interconnected plastids (Table 1; Newell et al. 2012; Gray et al. 2012). If auto-fluorescence is used as a major criterion for inferring chloroplasts and the stretched portion between two auto-fluorescent regions is considered to be a stromule (Fig. 2b, f), it is possible to mistake greening etioplasts as interconnected plastids. However, this interpretation does not mean that two independent chloroplasts came together and fused.

As we did not find a clear explanation for interconnected plastids in the post-1997 literature, we searched botanical literature dating back to the 1880s in the belief that earlier biologists might have faced a similar question before they concluded in favour of the unitary nature of the plastid.

"Chlorophyll chains" in *Selaginella kraussiana* suggest interconnected plastids

Plastid-related literature from the late nineteenth century reveals that one of the most intensively studied questions at this time related to the propagation of "chlorophyll grains" (chloroplasts; German words used were chlorophyllkörper as well as chlorophyllkerne). Whereas a large variety of plant species were investigated, an ontogenic study carried out by Haberlandt (1888) on chains of chlorophyll-containing bodies in the spike moss *S. kraussiana* and other *Selaginella* species merits special mention. The figures presented in Haberlandt's (1888) publication closely resemble the image evoked by the term interconnected plastids (Fig. 2 vs Fig. 3). Through meticulous observations, Haberlandt pieced together the sequence of events and concluded that the beaded chlorophyll bodies (chlorophyllkörper—interpreted as chloroplasts) are formed through successive bi-partitioning of a single chlorophyll body (originating in the meristematic zone). During division, Haberlandt notes, the chlorophyll containing body stretches and gets constricted in the middle and then the narrow middle part becomes colourless and forms a plasma blade (plasmalamellae–lamellae). The plasma–lamella is stretched further to form a thin strand when the two halves are pulled apart. In one of the best examples of a plant where one may point to the presence of interconnected plastids, Haberlandt concluded that the beaded plastid actually represents an arrested or very late stage in plastid division. This early work again reinforces the view that not every chlorophyll-containing thylakoid region necessarily constitutes an individual chloroplast. As observed in *S. kraussiana*, a single elongated chloroplast might possess multiple bulged regions with thylakoid systems (Fig. 3).

Haberlandt also reported that similar but shorter chains could also be found in cells of moss stems and in cells in fern prothalli (Haberlandt 1888). Reinhard (1933), after studying the timing of chloroplast division by long-term observations, also confirmed the existence of long chlorophyll chains in *Selaginella martensii* (Fig. 3l) and described chlorophyll chains in *Equisetum arvense* prothallium cells in exquisite detail. By following the division process of individual chloroplasts over several hours to a few days, Reinhard (1933) concluded that while in most cases two individual daughter chloroplasts were formed as a result of division, in a few cells the daughter plastids remained connected by a thin but clearly visible thread of "plastid matter". Another early work, in which interconnected "chlorophyll grains" were specifically mentioned and depicted in drawings, is the monograph "Die Gestallt- und Lageveränderung der Pflanzen-Chromatophoren" by Gustav Senn (1908). In his monograph, among other aspects of plant chromatophore shape and dynamics, Senn reviews the existence of chlorophyll chains and adds his own observations of such chains in prothallium cells of the fern Anemia (Fig. 3m). This work was originally inspired by descriptions from Schmidt (1870) who observed such chains in *Mohria caffrorum* prothallium cells, describing them as tree like and exhibiting several branches. Similar chains are described in *Monospora pedicellata* (Rhodophycee) (Berthold 1886). Taking all these independent observations together, "chlorophyll chains" appear to be a common phenomenon in non-flowering plants but are considered to result from incomplete division processes (Reinhard 1933) rather than independent plastids becoming linked together again.

Whereas the detailed descriptions and drawings presented by earlier researchers form the very basis for our present knowledge on plastids, modern biologists have recourse to new kinds of microscopes and techniques that create an opportunity for older works to be reinterpreted. In this context, a review by Gray et al. (2001) re-interpreted the findings of Haberlandt (1888) to lend support for the discovery of stromules. By considering the single, beaded plastids observed in *S. kraussiana* as chains of independent chloroplasts, Gray et al. (2001) interpreted the intervening stroma-filled regions as stromules. Whereas Gray et al. (2001) clearly mention Haberlandt's original conclusion of considering the single beaded plastids as being at a late stage of division, in effect their fresh interpretation created interconnected plastids.

A re-inspection of fresh *S. kraussiana* (Fig. 4) confirmed the presence of single bulbous green regions harbouring the thylakoid membranes connected by thin, nearly colourless stretches as described originally by Haberlandt (1888) and Reinhard (1933). In modern terminology, such thin tubular stretches between two regions of a dividing plastid would be labelled as the isthmus (Fig. 2e) that under certain conditions may stretch considerably (Pyke 2009; Fig. 1b, c in Osteryoung and Pyke 2014). Confocal fluorescence microscopy (Fig. 4c–e) confirms that these continuous chains consist of multiple chlorophyll-harbouring loci with occasional extension of chlorophyll fluorescence into the translucent intervening region (Fig. 4d, e). While the beaded plastids may twist and rearrange the meticulous observations of earlier workers on such plastids did not point to any tubules that interconnect independent chains. We conclude that while earlier researchers might have observed clear, stroma-filled extensions from plastids, they did not consider them as a means of creating interconnected plastids.

Plastids in higher plants in different stages of division might be misinterpreted as interconnected plastids

Since plastid divisions in a single cell or in different cells of a tissue such as the cotyledon and hypocotyl are not synchronous, it is possible to find plastids in different stages of division in young plants of *Arabidopsis* and tobacco. The

separation of thylakoid stacks usually takes place much earlier than the completion of division of a mother plastid into two daughter plastids (Osteryoung and Pyke 2014). Notably, prior to their final separation, the daughter compartments constitute a single plastidic unit since they still share a single, continuous envelope. While instances of delayed and impaired division can be encountered in nearly every young plant, in *Arabidopsis thaliana*, the angiosperm model, specific mutants impaired in plastid division have been isolated. These mutants furnish succinct proof for the occurrence of multiple regions with grana and chlorophyll auto-fluorescence in one plastid.

Arabidopsis mutants such as *arc5* and *arc6* are impaired in different aspects of plastid division (Pyke 2009; Osteryoung and Pyke 2014) and routinely exhibit phenotypes reminiscent of interconnected plastids. In *arc5* mutants, the plastid constriction is initiated but not taken to normal completion. Consequently, the thylakoids often fail to separate in light grown plastids in *arc5* seedlings and result in a significantly high frequency of constricted, dumbbell-shaped plastids as compared to the wild type (Pyke et al. 1994; Robertson et al. 1996). Observations of plastids in dark-grown arc5 hypocotyl cells reveal plastids with bulbous regions that match the image of interconnected plastids. Similar observations are made on chloroplasts in *arc6* mutant plants, which do not enter the plastid constriction stage and are consequently much larger than wild-type plastids (Pyke et al. 1994). Interestingly, mesophyll chloroplasts in *arc6* exhibit multiple chlorophyll-harbouring thylakoids and grana but are considered as single giant plastids due to the presence of a single double-membrane envelope (Holzinger et al. 2008; Osteryoung and Pyke 2014). While being only a remote possibility, the misinterpretation of single plastids in the process of division might allow them to be considered interconnected plastids.

One of the best examples from a higher plant that comes close to providing images suggestive of interconnected plastids is the *clumped chloroplasts 1* (*clmp 1*) mutant of *Arabidopsis* (Yang et al. 2011). Chloroplasts in this mutant are retarded in the process of separation and exhibit numerous points of connectivity in their membrane envelope. However, ontogenic considerations of the clumped chloroplasts mutant and very thorough high resolution electron microscopy has allowed Yang et al. (2011) to conclude that while the constriction of dividing plastids progresses normally their separation from each other is impaired. Thus, while *clmp1* provides a wonderful tool for understanding the distribution of plastids after their division, present evidence indicates that the phenotype is not due to previously independent plastids reconnecting together.

Plastids in senescing and necrotic leaves have been shown to fuse with each other and engulf other organelles during their breakdown (Barton 1966; Hurkman 1979; Thomson and Whatley 1980). Whereas Esau (1944) provides detailed descriptions of coalescing plastids as part of the pathologic changes in leaves of beet plants affected by mosaic disease, we have been unable to find accounts of similar occurrences under normal conditions of plant growth and development.

The earlier discussion strongly suggests that interconnected plastids do not actually exist and might merely be the result of interpreting a single elongated double membrane-bound plastid as two or more plastids. This line of reasoning raises two important questions.

Should any stroma-filled region between two (or more) bulged domains in an elongated plastid still be called a stromule?

Köhler and Hanson (2000) coined the term "stromule" to prevent confusion with other tubular structures in the cell. Stromules have been observed in response to a large number of abiotic (Gray et al. 2012) and biotic stresses (Shalla 1964; Fester et al. 2001; Hans et al. 2004; Lohse et al. 2005; Caplan et al. 2008; Krenz et al. 2012) and in response to changes in cellular sugars (Schattat and Klösgen 2011; Schattat et al. 2012a). Whereas the fluid stroma in a plastid is free to flow into the shape created by the flexible plastid envelope, the internal membranes of a plastid do not swim around freely. Thus, many transient situations occur when a tubular region of a plastid may be only stroma filled and makes one wonder if all reports on stromules have actually dealt with these extensions or have unknowingly misinterpreted elongated plastids as stromules. Whereas Köhler and Hanson (2000) provide clear guidelines on what should be considered as stromules, here we clearly state that stroma-filled tubular regions that stretch between transiently bulged areas of elongated plastids should not be equated with stromules. We recommend that the term stromule, as introduced by Köhler and Hanson (2000), be maintained but its use be limited strictly for describing free ending extensions from independent plastids.

If interconnected plastids do not exist could protein-exchange between plastids still occur through stromules?

Our appraisal suggests that interconnected plastids do not exist. In that case, the direct trafficking of proteins between independent plastids, as suggested by FRAP experiments (Köhler et al. 1997) and most recently through selective photo-conversion (Hanson and Sattarzadeh 2013), does not exist either. Notably, the title "Trafficking of proteins through plastid stromules" for the commentary by Hanson and Sattarzadeh (2013) does not specify a destination for the traffic and should not be considered as trafficking of proteins between previously independent plastid units. The work clearly shows proteins flow within (through) stromules for which

there is adequate and undisputed evidence (Gray et al. 2001; Kwok and Hanson 2004a, b, c, d; Hanson and Sattarzadeh 2011). However, after reading the different publications, it becomes very clear that there is not a single demonstration, so far, of proteins flowing from one independent plastid to another via the stromule route unless one changes the definition of a unit plastid and calls a single elongated, pleomorphic plastid as two or more interconnected plastids. Whether proteins actually flow from one plastid unit to another at all by other, as yet undiscovered ways remains un-addressed.

Conclusions

Through an appraisal of extant literature and careful reasoning, we conclude that in healthy developing plant tissues there is presently no experimental evidence to support the idea that the fusion of two or more independent, physiologically normal plastid units creates interconnected plastids. Despite its pleomorphy, each plastid remains as a singular unit within the spatial confines defined by the double membrane boundary. Further, since an assumption of plastid interconnectivity had been the sole basis for suggesting that proteins are able to traffic between plastids, we submit that such protein flow remains to be demonstrated in unequivocal terms. However, as our understanding of plastid interactions grows exponentially, we are confident that the attempts to understand plastid extensions will prompt further discovery and perhaps even a refutation of our present literature-based conclusions.

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