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Published in:
Protoplasma

DOI:
10.1007/s00709-015-0935-x

Publication date:
2017

Citation for published version (APA):
Primavesi, L. F., Wu, H., Mudd, E. A., Day, A., & Jones, H. D. (2017). Visualisation of plastid degradation in sperm cells of wheat pollen. *Protoplasma*, *254*(1), 229-237. https://doi.org/10.1007/s00709-015-0935-x

Visualisation of plastid degradation in sperm cells of wheat pollen

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Abstract

Like most angiosperms, wheat (*Triticum aestivum*) shows maternal inheritance of plastids. It is thought that this takes place by cytoplasmic stripping at fertilisation rather than the absence of plastids in sperm cells. To determine the fate of plastids during sperm cell development, plastid-targeted green fluorescent protein was used to visualise these organelles in nuclear transgenic wheat lines. Fewer than thirty small 1–2-µm plastids were visible in early uninucleate pollen cells. These dramatically increased to several hundred larger (4 µm) plastids during pollen maturation and went through distinct morphological changes.

Only small plastids were visible in generative cells ($n = 25$) and young sperm cells ($n = 9$). In mature sperm cells, these green fluorescent protein (GFP)-tagged plastids were absent. This is consistent with maternal inheritance of plastids resulting from their degradation in mature sperm cells in wheat.

Keywords

Wheat
Pollen
GFP
Plastid
Sperm cell
Plastid networks
Maternal inheritance

Handling Editor: Benedikt Kost

Electronic supplementary material

The online version of this article (doi: 10.1007/s00709-015-0935-x) contains supplementary material, which is available to authorized users.

Introduction

All plastids present in a plant arise, by division, from those originally inherited from the egg and/or sperm cell at fertilisation. Some angiosperm species inherit plastids from both male and female gametes; however, others obtain them from only the egg cell with no contribution of plastids from the paternal parent (Corriveau and Coleman 1988; Mogensen 1996; Nagata 2010). Transplastomic technologies (Svab et al. 1990), where the transgene is inserted into the plastid genome rather than the nuclear genome, exploits this concept to reduce the risk of pollen-mediated transfer of recombinant DNA from crop to crop or from crop to closely related weeds (Daniell et al. 1998; Ruf et al. 2007; Svab and Maliga 2007).

Previous studies on the inheritance of variegation (Briggle 1966; Pao and Li 1946) have shown that wheat (*Triticum aestivum*) plastids are maternally inherited. Mature wheat pollen is tricellular consisting of a vegetative cell containing starch-filled amyloplasts and two sperm cells.

During pollen development, the single-celled haploid microspore undergoes mitosis to produce two new cells: the vegetative and generative cells (pollen mitosis I). The generative cell later divides again to form two sperm cells (pollen mitosis II). One sperm cell goes on to fertilise the egg cell and form the new zygote, the other sperm cell fuses with the central cell to eventually form the endosperm.

Several mechanisms have been identified to explain plastid maternal inheritance (Birky 1995; Mogensen 1996) which may result in various patterns of plastid distribution and persistence through pollen mitoses, generative/sperm cell development and subsequent fertilisation (Hagemann and Schroder 1989). For example, paternal plastids may be unequally distributed during the first pollen mitosis such that they are excluded from the generative cell (*Lycopersicon*-type inheritance). Plastids may be selectively degenerated during maturation of the generative cell (*Solanum* type) so that they are not present at the second mitosis and are thus absent from the subsequent sperm cells. Plastids may be excluded from one sperm cell (sperm dimorphism) or both at the second pollen mitosis. Plastids may also be excluded from the zygote during fertilisation.

In wheat, it is thought that plastid maternal inheritance occurs by cytoplasmic stripping at fertilisation (*Triticum* type) where the cytoplasm (and hence organelles) of the sperm cell stay outside the egg cell at fertilisation (Bock 2007; Greiner et al. 2015; Hagemann and Schroder 1989). This conclusion was based on earlier observations using electron microscopy where plastids were visible in the generative cell (Hu et al. 1979) and the sperm cells (Hagemann et al. 1985) yet they were not transmitted to the zygote (Briggle 1966; Pao and Li 1946). Thus, it was assumed that the sperm cell nucleus was somehow separated from the other cellular contents during the process of fertilisation. Cytoplasmic stripping has also been observed in barley (Mogensen 1988) where sperm cell cytoplasmic contents have been detected on the exterior of the egg by electron microscopy, although no direct evidence currently exists for wheat.

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In this study, we tagged plastids with green fluorescent protein (GFP) to examine their distribution and behaviour in wheat pollen and particularly in developing and mature sperm cells with the aim of understanding the

mechanism of maternal inheritance of plastids in *T. aestivum*.

Materials and methods

Plant material

Homozygous transgenic wheat (*Triticum aestivum* var. Cadenza) lines expressing plastid-targeted GFP (N-terminal transit sequence from maize ferredoxin III) and non-targeted GFP (no transit sequence) were created and tested as described in Primavesi et al. (2008). Pollen from seven independent transgenic lines was examined. Plants were grown in a glasshouse with 20 °C day / 15 °C night temperatures with 16 h day length. Natural light was supplemented with Son-T lamps when below 300 $\mu mol.m^{-2}.s^{-1}$ but not above 400 $\mu mol.m^{-2}.s^{-1}$.

Preparation of samples

Pollen samples were harvested, stored briefly in a humid environment and examined almost immediately after harvest. For fresh mature pollen, anthers were harvested on the day when the ear had just reached anthesis. Selected anthers were yellow, still retained within the floret and freely shed pollen but showed no signs of dehydration. Immature pollen was obtained by selecting green anthers at different heights within the florets. Three anthers from each floret examined were placed in a drop of raffinose medium (Primavesi et al. 2008) on a glass slide and chopped quickly into approximately 1-mm lengths to release the pollen grains. Most anther debris was quickly removed with forceps before the addition of the coverslip. Incubation of the pollen in raffinose medium eventually caused the pollen grains to burst in a manner that expelled the cell contents including the generative cell or sperm cells into the medium. These cells then persisted in a viable state for several hours before eventually bursting. Pollen was germinated on microscope slides prepared with a thin layer of raffinose medium containing 0.7 % agar.

Microscopy

Prepared tissue samples were viewed with a Zeiss 780 LSM inverted confocal laser scanning microscope equipped with Argon 488 nm and HeNe 633 nm lasers. and Zen2010 software. For CLSM, cells were imaged with ×40/1.0 C Apo or ×63/1.2 C Apo objectives and recorded at 1024 × 1024 pixels per image. GFP fluorescence was collected in the range 510–

532 nm and false-coloured green. Overlays were captured at the same time as single images. Images in TIFF format were cropped using Adobe Photoshop CS5 version 12.1. To estimate of the number of plastids within generative and sperm cells, the cells were rapidly optically sectioned in increments of 1 μm. The stack of images was then projected into one single image and the number of GFP-labelled spots counted. Mitchondria were visualised with Mitotracker Deep Red (Life Technologies) according to the manufacturer's instructions. Fluorescence was collected in the range 647–686 nm and false-coloured red. DAPI (Thermo Fisher) was used to stain nuclei in sperm cells according to the manufacturer's instructions. Fluorescence was collected in the range 410–585 nm and false-coloured blue.

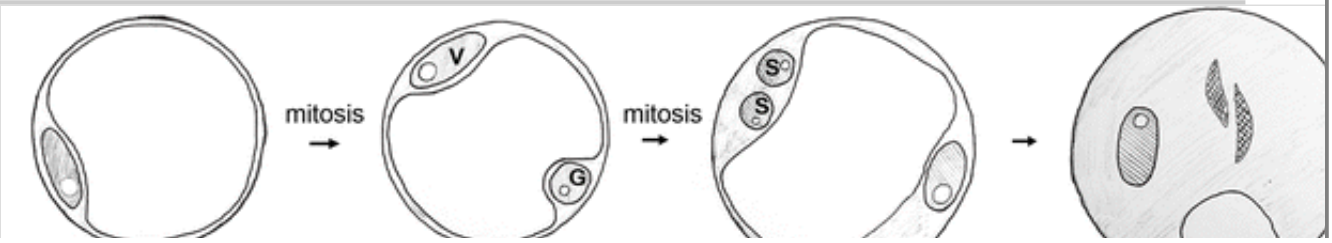
Results

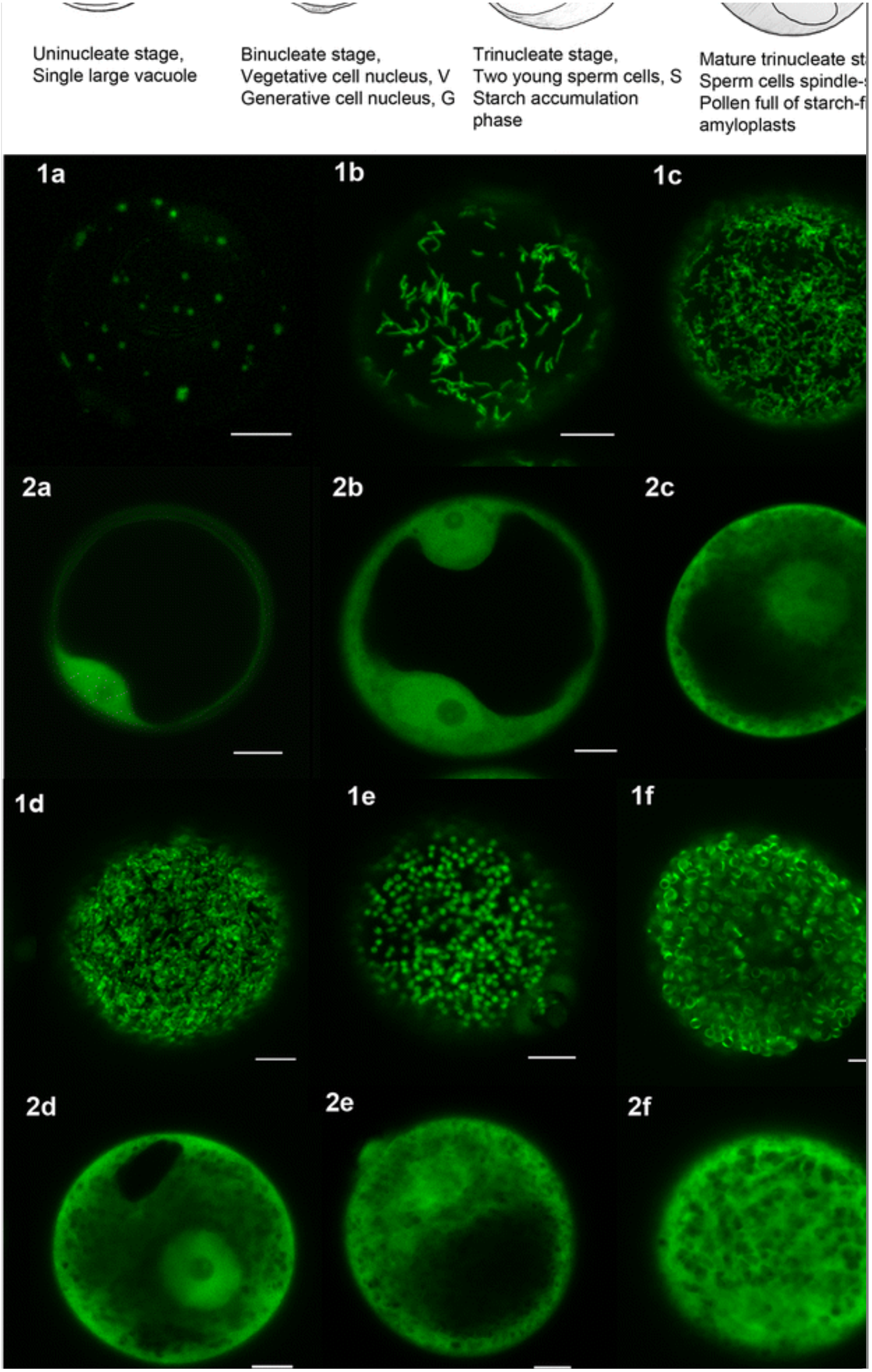
Wheat pollen plastids show highly variable morphology during pollen development

Discrete GFP-tagged plastids (Primavesi et al. 2008) were easily visible by confocal laser scanning microscopy in mature viable pollen grains in the plastid-targeted lines. Figure 1 shows the morphological changes in pollen cell plastids through pollen development starting from the uninucleate stage and ending at the completely mature stage which has many starch-filled amyloplasts.

Fig. 1

Plastids in developing pollen *1a–f* GFP-labelled plastids. *2a–f* Untargeted GFP (vacuole often clearly delineated). *1a* and *2a* uninucleate stage, plastids visible as small GFP labelled dots. *1b*,*c* and *2b*, *c* binucleate stage (vegetative nucleus and generative cell nucleus clearly visible in *2b*). Plastids now visible as elongated structures in increased numbers. *1d*, *e* and *2d*, *e* trinucleate stage. Plastids form an indistinct network-like structure (*1c*, *d*) before becoming clearly visible as discrete dots again (*1e*). *1f* and *2f* mature trinucleate stage, starch filled pollen. Plastids present as discrete expanded structures. *Scale bars* represent 10 μm



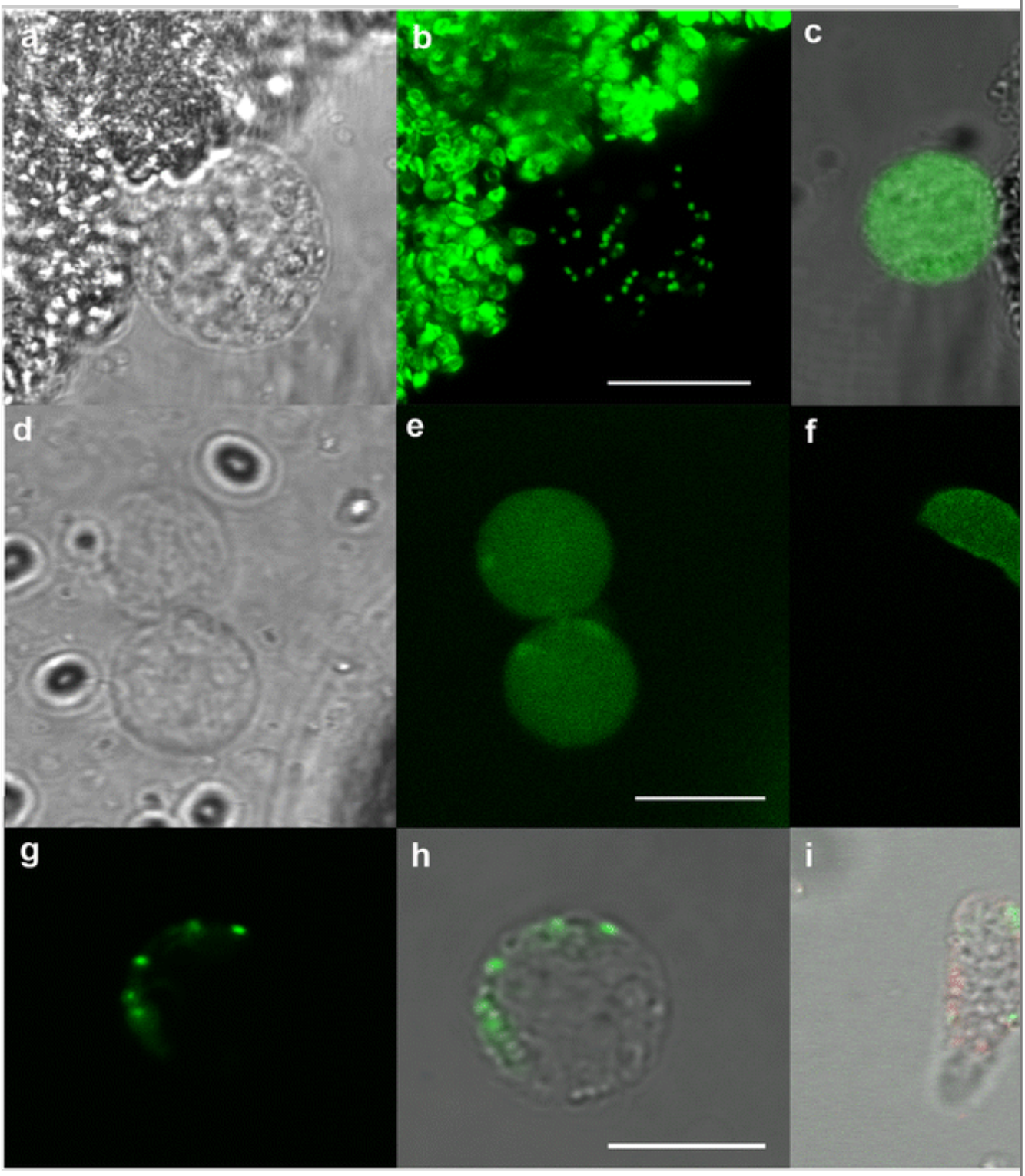

Uninucleate stage,
Single large vacuole
Binucleate stage,
Vegetative cell nucleus, V
Generative cell nucleus, G
Trinucleate stage,
Two young sperm cells, S
Starch accumulation phase
Mature trinucleate st
Sperm cells spindle-
Pollen full of starch-f
amyloplasts
1a
1b
1c
2a
2b
2c
1d
1e
1f
2d
2e
2f

At the uninucleate stage (single microspore cell with large vacuole), there are relatively few, small plastids distributed throughout the cytoplasm (around 26 plastids, 1–2 μm, Fig. 1a). As the pollen cell develops, the microspore cell undergoes mitosis to form the vegetative cell and the smaller generative cell. At this stage, the plastids have increased in number and become thin elongated structures about 3–4-μm long (Fig. 1b). Optical sections deeper into the pollen grain showed elongated plastids surrounding dark areas corresponding to the vegetative nucleus and the central vacuole (not shown). By the late binucleate stage, the plastids appear to form an extensive network throughout the cytoplasm (Fig. 1c). It was not clear if they were actually connected to each other because they were also highly dynamic and subject to rapid photobleaching. After the binucleate stage, the generative cell undergoes mitosis to produce two sperm cells, the trinucleate stage. At this point, the channels of the 'plastid network' appear to thicken (Fig. 1d) and then become discrete spherical plastids (Fig. 1e). As the pollen grain finally matures, the spheres expand, filling up with starch, to a maximum size of 4 μm. There were several hundred plastids distributed throughout the cytoplasm which itself surrounded a large vacuole (Fig. 1f). At this point, GFP was often visible as a band confined around the middle of the plastid. At all stages, untargeted GFP fluorescence patterns are shown for comparison (Fig. 1 panels 2a–f). We observed the same changes in the morphologies and numbers of plastids in pollen using three different plastid-targeted transit peptides (rubisco SSU, ferredoxin III, ftsZ) (data not shown). Progression from the unicellular stage shown in Fig. 1a to mature pollen in Fig. 2f took around 72 h. During this relatively short time period, large changes in the morphologies and abundance of GFP-tagged plastids were observed. These included familiar circular forms (Fig. 1a, e–f) as well as novel forms (Fig. 1b–d) not previously described in pollen. Continuous monitoring of GFP-tagged plastids during the pollen development allowed us to uncover these dynamic features of pollen plastids, which would not be easily revealed by classical electron microscopic approaches.

Fig. 2

Plastids in generative and sperm cells (**a**) bright field and (**b**) GFP-labelled plastids in a generative cell. **c** Overlay of bright field and GFP fluorescence from non-targeted GFP generative cell. Two young sperm cells (**d**) bright

field, (**e**) GFP fluorescence and a mature sperm cell (**f**) with untargeted GFP. An immature sperm cell with targeted GFP in plastids (**g**) GFP fluorescence and (**h**) merged bright field. **i** Maturing sperm cell showing mitochondria (*red fluorescence*), GFP plastids and a circular GFP-labelled structure (*arrow*). *Scale bars* represent 10 μm



GFP-labelled plastids are present in generative cells and immature sperm cells but undetectable in mature sperm cells

Wheat pollen was obtained at different stages of development, namely the generative cell stage (binucleate), two sperm cell stage (trinucleate) and mature sperm cell stage (where sperm cells adopt a characteristic spindle-shaped structure).

The generative and sperm cells were ejected when the pollen grains burst open and survived independently for some time. The type of cell released was identified on the basis of its size, shape and the number ejected from a single burst pollen grain. Initial observations to train the eye were carried out on untargeted GFP lines where the different cell types could be easily located and identified because the entire cytoplasm was highlighted by GFP. Generative cells were easily identified by their size (c. 25-μm diameter) and the fact that there was only one per burst pollen grain. Generative cells contained, on average, 25 plastids (Table 1 and Fig. 2a–b). Generative cells, young and mature sperm cells with untargeted GFP showed a uniform GFP fluorescence (Fig. 2c–f). Young sperm cells were much smaller (c. 8–12-μm diameter) and were ejected in loosely connected pairs (Fig. 2d–e). Interestingly, when freshly ejected their three dimensional structure resembled haemocytes in being a disc-like structure rather than a sphere. Young sperm cells contained nine plastids on average (Table 1 and Fig. 2g–h) and the plastids were highly dynamic and in continuous motion.

Table 1

Numbers of observed plastids in generative and sperm cells. Mean number of GFP-labelled plastids observed in generative cells and young and mature sperm cells. Standard deviation shown

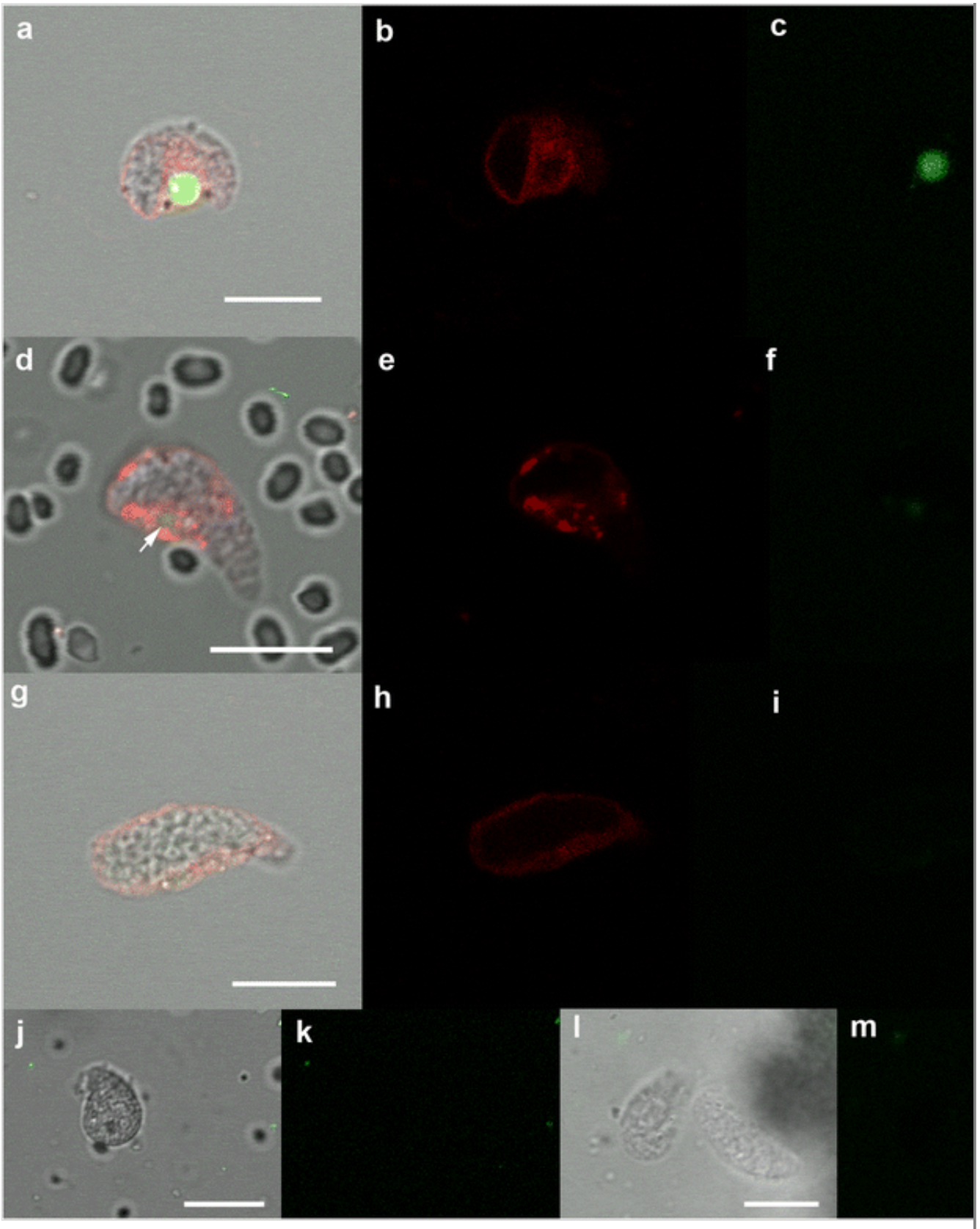
Cell type	Mean number of plastids	Sample size, *n*
Generative	25 ± 7.0	29
Immature sperm	9 ± 2.9	17
Mature sperm	Not detected	101

In mature spindle-shaped sperm cells (Fig. 3i, k, m), despite examining many cells (Table 1), no plastid–like structures like those observed in younger sperm cells were observed. Prior to the mature stage, several intermediate stages were detected in maturing sperm cells (Figs. 2i and 3c,

f), where a single unidentified structure containing GFP was also observed. This structure was detected with plastids present, (Fig. 2i) but in later stages, it remained even in the absence of detectable plastids and contained much fainter GFP fluorescence (Fig. 3i). Compared to earlier-stage plastids, this structure was larger, static and circular in cross section. In contrast, for transgenic lines containing the untargeted GFP, fluorescence was retained in mature sperm cells (Fig. 2f). To confirm that the GFP-labelled structure in Figs. 2i and 3i was not simply the nucleus, DAPI staining of mature sperm cells was performed. As expected, the DAPI-stained nucleus is much larger and appears to completely fill the cell (Supplementary Fig 1.)

Fig. 3

Plastids throughout sperm cell maturation. **a**, **d** Overlay images of maturing sperm cells, (**d**) *arrow points* to faint circular GFP structure. **b**, **e** Corresponding fluorescence from Mitotracker, and (**c**, **f**) GFP. (**g**–**i**) Overlay, Mitotracker and GFP fluorescence from a mature sperm cell. **j**, **k** and **l**, **m** Two more examples of mature sperm cells showing overlay and GFP channels. *Scale bars* represent 10 μm



Mitotracker staining was performed to confirm that GFP was found only in plastids and not unexpectedly being targeted to both plastids and mitochondria in these cells. Mitotracker cannot enter intact mature wheat pollen grains and the addition of permeabilisation reagents such as DMSO irreversibly disrupts the structure of internal membranes (Pierson 1988 and our own observations). However, Mitotracker was used to confirm

specific targeting of GFP to plastids by staining mitochondria in germinated pollen tubes (Supplementary Fig 2). In addition, simultaneous staining with Mitotracker to label mitochondria showed that GFP and Mitotracker labelled distinct and separate structures in sperm cells (Fig. 3b, e, h).

Discussion

By using plastid-targeted GFP it was possible to examine living plastids in wheat pollen vegetative cells and observe the extensive morphological changes that they undergo throughout pollen development. We observed that through pollen development the number of plastids in the vegetative cell increases from a low number (approx. 30–40) of small plastids (1–2 μm) to several hundred plastids (around 4-μm diameter) at maturity. The plastids pass through a distinct morphological stage where they form thin elongated tube-like structures (binucleate stage). Such elongated plastids have previously been observed in young green wheat leaves during an early step in chloroplast division (Leech et al. 1981) and also in *Arabidopsis thaliana* pollen plastids (Fujiwara et al. 2010). After this stage, the plastids appear to form a very dynamic rapidly moving and complex network. In this study it was not possible to determine whether this complex network provided a mechanism to join the contents of multiple plastids. Elegant evidence from (Schattat et al. 2012) showed that plastids connected by stromules do not exchange stromal contents. Apparent complex networks of plastids have been observed in a number of different species and cells (Pyke 2010; Osteryoung and Pyke 2014). The functional significance of these networks is not fully understood (Schattat et al. 2015) although wheat pollen has well-defined developmental stages and might be a particularly good system to study these networks. It is not clear what is happening to the plastids at this stage or why they adopt these varied shapes although amoeboid or pleomorphic amyloplasts have been observed in the root tips of *Phaseolus* (Newcomb 1967), in epithelial cells of resin ducts in pine needles (Dvorak and Stokrova 1993) and in *Alnus* actinorhizal root nodules (Gardner et al. 1989) which are all non-photosynthetic cells. GFP-tagged plastids appear as spherical structures at early and late stages of pollen development (Fig. 1a, e). Spherical GFP-tagged plastids were also observed in mature wheat (Fig. 1f and Primavesi et al. 2008) and *Arabidopsis* pollen (Matsushima et al. 2011). In contrast, mitochondria appear more oval in mature *Arabidopsis* pollen (Matsushima

et al. 2011).

We were also able to detect small GFP-labelled plastids present in the reproductive cells. Generative cells contained an average of 25 very small plastids (1–2 μm). This is similar to meristem cells which contain 10–20 proplastids per cell (Cran and Possingham 1972; Lyndon and Robertson 1976). Young sperm cells contained an average of nine very small plastids per cell. Previously, no overall count of the actual plastid numbers in these cells has been obtained from electron microscopy (Hagemann et al. 1985). Since the number of plastids decreases from generative cell to sperm cell, it is likely that the generative cell plastids are simply distributed to the two sperm cells such that each sperm cell ends up with roughly half the plastids.

At no point during development and maturation did the generative/sperm cell plastids increase in size as they do in the vegetative pollen cell when they accumulate starch, nor did the numbers significantly increase and nor was there any obvious change in their morphology. Our observations are consistent with division of plastids being arrested in the generative cell and their number per cell reducing further following formation of the two sperm cells. Once inside the sperm cell, it is possible that the plastids simply remain as proplastids and are protected in some way from the signals which cause the vegetative cell plastids to elongate, divide and accumulate starch. It was only when the sperm cell had undergone maturation and become spindle-shaped and the pollen grain had reached the correct state for pollination that any change was observed in the sperm cell plastids. At this stage, the typical small dynamic GFP-labelled plastid was never observed in sperm cells. This loss of GFP-tagged plastids was observed in several independent transgenic lines. In contrast, sperm cells with untargeted GFP continued to show GFP fluorescence right through development. No dimorphism with regard to plastid number or presence was observed in young or mature sperm cells. An earlier electron microscopic study on barley pollen showed limited or no differences in the content of mitochondria in sperm pairs (Mogensen and Rusche 1985).

It had been assumed from earlier evidence in cereals (Hagemann and Schroder 1989; Schroder and Hagemann 1986) that the cytoplasmic stripping mode of maternal inheritance must occur in *T. aestivum* (*Triticum* type). This assumes that plastids are present in sperm cells and are then

somehow excluded from the egg cell at fertilisation when the sperm cell cytoplasm is left on the outside of the egg cell while the sperm nucleus goes on to fuse with that of the egg. Our study provides evidence that paternal plastids are clearly present in young sperm cells but at a point very close to sperm cell maturity (very close to the point of pollen release) the plastids are degraded. We conclude, that the detection of plastids in pollen sperm cells by electron microscopy is not sufficient to deduce their continued presence in mature sperm. This requires monitoring the fate of plastids during sperm development, which is more easily achieved by visualising GFP-tagged plastids (this work) than by electron microscopic analysis. It is also possible that environmental factors play a role in the maintenance of plastids in sperm cells. This was addressed by placing tillers with developing pollen continuously at 15 or 5 °C for a week followed by 23 °C. The cold temperatures used did not appear to affect the fate of plastids in sperm cells, which were degraded in mature pollen (results not shown). Cytoplasmic stripping for organelles other than plastids is not ruled out by this study, but we provide evidence that the plastids are degraded prior to fertilisation.

In this study, we observe the disappearance of intact GFP-labelled plastids. The fate of these plastids might be related to the observation that during the sperm maturation process, a single small area of GFP fluorescence was often observed and which later disappeared from mature sperm. The fluorescence in this structure was contained in a static circular/spherical structure unlike the variable shape of the dynamic plastids in earlier pollen stages. The fluorescence was sometimes quite bright, but as the sperm matured, it became greatly diminished. We speculate that the structure is a candidate for an organelle involved in the terminal degradation of plastids, possibly a lytic vacuole. Several pathways have been described for the destruction of plastids including stromal and thylakoid proteins (Chiba et al. 2003; Michaeli et al. 2014; Wang and Blumwald 2014). The presence of chloroplasts within the vacuole has been observed by electron microscopy studies on senescing leaves of wheat (Wittenbach et al. 1982) and *Phaseolus vulgaris* (Minamikawa et al. 2001). Plastid degradation is supported by the observation that plastid nucleoids are detected by DAPI staining in wheat generative cells (Miyamura et al. 1987) but not in sperm cells (Corriveau and Coleman 1988). Two further observations are consistent with destruction of plastids and their genomes during pollen development. First, wheat plants regenerated from pollen by anther culture

show a very high level of albinism and large deletions in their plastid DNA (Day and Ellis 1984). Second, escape of plastid DNA to the nucleus is elevated in the sperm cells of tobacco pollen (Sheppard et al. 2008).

This study provides evidence that plastids do not persist in mature wheat sperm cells, and that they may be selectively degraded in the germ line during the final stages of sperm cell maturation. We propose the plastid inheritance pattern in wheat is actually more like the *Solanum* type where plastids are not present in the sperm cells (Greiner et al. 2015; Hagemann and Schroder 1989), although in the case of wheat, they are present in the generative cell and destroyed during maturation of sperm cells.

Acknowledgments

Rothamsted Research receives support from the Biotechnological and Biological Sciences Research Council (BBSRC) of the UK as part of the 20:20 Wheat® Programme.
Work was supported by grant number GM114215. We thank Caroline Sparks for her invaluable help and also Richard Parkinson and Fiona Gilzean for their assistance in growing the wheat plants.

Electronic supplementary material

Below is the link to the electronic supplementary material.

ESM 1

(DOCX 1977 kb)

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