RESEARCH ARTICLE

Chloroplast behaviour and interactions with other organelles in *Arabidopsis thaliana* pavement cells

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ABSTRACT

Chloroplasts are a characteristic feature of green plants. Mesophyll cells possess the majority of chloroplasts and it is widely believed that, with the exception of guard cells, the epidermal layer in most higher plants does not contain chloroplasts. However, recent observations on *Arabidopsis thaliana* have shown a population of chloroplasts in pavement cells that are smaller than mesophyll chloroplasts and have a high stroma to grana ratio. Here, using stable transgenic lines expressing fluorescent proteins targeted to the plastid stroma, plasma membrane, endoplasmic reticulum, tonoplast, nucleus, mitochondria, peroxisomes, F-actin and microtubules, we characterize the spatiotemporal relationships between the pavement cell chloroplasts (PCCs) and their subcellular environment. Observations on the PCCs suggest a source–sink relationship between the epidermal and the mesophyll layers, and experiments with the *Arabidopsis* mutants *gabra2* (*gl2*) and *immutans* (*im*), which show altered epidermal plastid development, underscored their developmental plasticity. Our findings lay down the foundation for further investigations aimed at understanding the precise role and contributions of PCCs in plant interactions with the environment.

KEY WORDS: Chloroplasts, Stromules, Epidermis, Pavement cells, Organelle interactions

INTRODUCTION

Plastids are multi-functional, pleomorphic organelles of purported endo-symbiotic origin that display a characteristic double membrane envelope in plants and green algae (Wise, 2007). Observations on the basic features of plastids date back to the mid-seventeenth century (reviewed by Gunning et al., 2007). All plastid types are believed to originate as colourless pro-plastids in meristems (Schimper, 1883, 1885) and their subsequent differentiation takes place according to tissue and developmental requirements (Jarvis and López-Juez, 2013; Paila et al., 2015; Liebers et al., 2017). Early studies by Schimper (1883, 1885) established the inter-convertibility of plastids from one type to another. Presently, on the basis of pigmentation, chloroplasts are distinguished from other plastids by the presence of chlorophyll, chromoplasts by the predominance of other pigments and leucoplasts by the absence of all pigmentation.

In higher plants, the majority of chloroplasts are found in the leaf mesophyll tissue and, at the ultra-structural level, display the classical thylakoid membrane system of stacked thylakoid grana connected by intergranal lamellae. However, chloroplasts in the bundle sheath cells of C4 plants are often agranal (Bisalputra et al., 1969; Woo et al., 1970), suggesting that well-defined grana are not a requirement for identification as a chloroplast. Although the majority of photosynthesis occurs in the mesophyll, the guard cells of most plant species are recognized as having chloroplasts that possess an active electron transport chain, are capable of fixing carbon dioxide, and play an important role in stomatal opening and closing (Lawson, 2008; Lawson et al., 2014).

In addition to guard cells, the aerial epidermis consists of trichomes and large pavement cells. It is widely believed that chloroplasts are absent from the pavement cells of *Arabidopsis thaliana* and most other higher plants (Brunkard et al., 2015; MacDonald, 2003; Smith, 2005; Bowes and Mauseth, 2008; Solomon et al., 2010; Vaughan, 2013). However, the presence of chloroplasts in pavement cells of *Arabidopsis* has been reported (Pyke and Leech, 1994; Robertson et al., 1996; Vitha et al., 2001; Joo et al., 2005; Pyke, 2009) and was reinforced by recent observations (Barton et al., 2016).

Pavement cell chloroplasts (PCCs) in *Arabidopsis* are comparable in size to guard cell chloroplasts but are approximately half the size of mesophyll chloroplasts (MCCs). In the jigsaw-shaped pavement cells in expanded cotyledons and leaves of *Arabidopsis*, nine to 15 PCCs are found per cell, while a single mesophyll cell may contain over 120 chloroplasts (Pyke and Leech, 1992, 1994; Barton et al., 2016). PCCs are photosynthetically active and show clear grana, but their chlorophyll autofluorescence signal is low compared to MCCs (Barton et al., 2016). A high stroma to thylakoid ratio in PCCs means, upon expressing plastid-targeted fluorescent proteins, they are easier to image than are MCCs, so these plastids are sometimes used for visual studies of plastid responses (Kwok and Hanson, 2004a; Higa et al., 2014; Caplan et al., 2015; Brunkard et al., 2015). However, whether this small population shows responses that are representative of all chloroplasts is uncertain and much remains unknown about the spatiotemporal behaviour of PCCs and their relationship with other cellular components and compartments.

Here, using a range of fluorescent protein probes targeted to the chloroplasts and other subcellular structures, we have investigated PCCs and their surroundings in *Arabidopsis thaliana* and contrasted the responses of PCCs to light and sucrose with that of MCCs. The creation of new transgenic plant resources, and characterization of PCCs from a cell biological, physiological and developmental perspective lays the foundation for further investigations on the actual contribution of these small chloroplasts during plant interactions with the environment.

RESULTS

**Identifying the PCCs and creating resources for their characterization**

The presence of PCCs in *Arabidopsis* was best appreciated when the pavement cells were viewed from a lateral perspective. As shown in
Fig. 1, this view was easily achieved for pavement cells lying at the edges of cotyledons and leaves. It allowed direct observation of the subcellular environment around the PCCs without interference from the fluorescence signal of underlying mesophyll layers. The PCCs are small when compared to the MCCs; however, even from a lateral view, it can appear that some small chloroplasts are in the mesophyll plane when the pavement and mesophyll cells are closely interlocked. In order to confirm that the smaller chloroplasts were located exclusively in the pavement cells, the Arabidopsis crooked (crk) and wrm (wrm) mutants were used. In these mutants, epidermal cells in the hypocotyl, cotyledons, and young leaves become abnormally elongated upon detachment from each other along the long axis (Fig. 1A; Mathur, 2005). Similar to in wild-type plants, two chloroplast populations were visible in crk and wrm mutants, one consisting of small chloroplasts and the other of large chloroplasts. The pavement cells that had curved out of the general epidermal plane in crk and wrm plants clearly exhibited small PCCs, while cells in the layers beneath displayed only the larger MCCs (Fig. 1B; Movie 1). In wild-type Arabidopsis, uncertainty about the exact location of chloroplasts in the epidermal or the mesophyll layer can thus be resolved by observing their relative sizes.

A number of double transgenic and mutant Arabidopsis lines expressing different targeted fluorescent probes were created for understanding PCC behaviour in relation to their subcellular environment (Table 1). The following general observations were made on the interactions between the highlighted organelles and the PCCs.

**PCCs and their surroundings**

In 10-day-old cotyledons of soil grown tpFNR:GFP-RFP:ER plants (Table 1), the average cell depth for guard cells was estimated at 12.4±0.6 μm, for pavement cells at 38.3±3.0 μm and for mesophyll cells at 53.8±4.6 μm (mean±95% CI, n=104 guard cells, 149 pavement cells, 173 mesophyll cells). In comparison, the pavement cells in petioles of the first leaves and cotyledons were relatively elongated and thin with depths ranging from 12 to 17 μm. Observations on PCCs in many plants taken at different times of the day established that PCCs are not always located in the same position with regard to the upper cell boundary. While observations taken after an 8-h dark period showed that up to 90% of the PCCs were located near the upper surface (Fig. 1C), they were localized mainly in the lower boundary of the cell if the plants had been exposed for a few hours to light intensity of ~120 μmol m–2 s–1. Owing to their localization near the lower surface of the pavement cell these chloroplasts appeared to lie on top of the MCC layer (Fig. 1D). In both locations, the PCCs extended and retracted stromules (stroma-filled tubules) sporadically. These observations on the relative sizes and positions of PCCs and MCCs were reinforced by using a plant line expressing GFP targeted to the plasma membrane (Fig. 2A).

Similar to the situation for the majority of organelles in plant cells (reviewed by Vick and Nebenführ, 2012; Geitmann and Nebenführ, 2015) and in agreement with Kwok and Hanson (2004a), time-lapse imaging of the double-transgenic tpFNR:mEosFP-GFP:mTalin line (Table 1) showed PCC alignment and movement along F-actin strands (Fig. 2B,C; Movie 2) and the F-actin could be traced as a loose cage around the PCCs (Fig. 2B). Compared to the dynamic association between PCCs and F-actin, their spatiotemporal relationship with microtubules, investigated using a tpFNR: mEosFP-GFP:MAP4 line (Table 1), was less clear. While a collapsed z-stack of confocal images conveyed the impression that the PCCs were embedded between cortical microtubules (Fig. 2D), in most optical sections the PCCs were found in a distinct plane. PCCs with extended stromules did not show a consistent relationship with microtubules, as stromules were often extended parallel to the cortical microtubule array, but could also extend perpendicular to it (Fig. 2E). In the instances that alignment with neighbouring cortical microtubules was observed (Fig. 2E) it was not sustained and did not occur along a particular microtubule. Often a stromule extended across multiple microtubules and, while retracting, could pause at several locations.

In contrast to the microtubules, time-lapse observations in the tpFNR:mEosFP-GFP:Vac line (Table 1) highlighted the tonoplast and revealed the PCCs were almost surrounded by, and could be tugged on, by the movement of thin vacuolar membrane tubules (Fig. 2F; Movie 4). For PCCs located on the cell periphery (Fig. 2G), the vacuole was closely appressed to the plastid on the side interior to the cell. In all cases, time-lapse imaging suggested that the vacuolar membrane flowed loosely over the chloroplast surface (Fig. 2H; Movie 3). Whereas a top-down view (Fig. 2H) also suggested a very close association between PCCs and the vacuolar membranes, we were perplexed by the presence of many areas that looked very similar in size to the PCCs but did not show the typical chloroplast fluorescence (Fig. 2I). We found the observations with the vacuole probe reminiscent of earlier observations on chloroplasts made using lines co-expressing tpFNR:GFP and an ER-targeted RFP (Schattat et al., 2011). As reported previously (Schattat et al., 2011), the PCCs were embedded within an ER cage surrounding the entire chloroplast (Fig. 2K) and both organelles moved in concert. Stromules extended sporadically from the PCCs and aligned closely with the ER mesh or extended along ER-lined channels (Fig. 2L, Fig. 2M, arrowheads). In order to understand the PCC–vacuole–ER relationship better, we developed a double-
transgenic line co-expressing the vacuole-targeted GFP and the ER-targeted RFP. Using this line, it was found that the vacuole presses against and outlines other organelles in a similar manner. Lateral views of PCCs in the GFP:Vac-RFP:ER line (Table 1) showed that the ER-cage was located between the vacuolar membrane and the PCCs (Fig. 2J) and that ER bodies could be amongst the organelles appearing similar in size to PCCs (Fig. 2J).

The vacuolar tubules also engulfed the nucleus and allowed us to observe nucleus-associated PCCs (Fig. 2N). A line co-expressing the tpFNR:GFP and a nucleus-targeted RFP was developed and showed that in 15±3% (mean±s.d.; n=80 cells from four seedlings) of cotyledon pavement cells in 7-day-old seedlings, anywhere between three and 12 PCCs could be found in the perinuclear region (Fig. 2O). The nuclear association was often transient and a variable number of PCCs were observed reaching the perinuclear region or moving away from it. In the remaining cells, the PCCs were dispersed and showed no clear nuclear association. Upon observing stromules extended by the perinuclear PCCs we were unable to find a clear indication as to whether the stromules extended towards or away from the nucleus (Movie 5).

Each probe described here requires a more-detailed assessment for understanding the details of the response of the PCC to different environmental stimuli. However, the PCCs are clearly present in a narrow cytoplasmic sleeve appressed to the pavement cell boundary, comprising the plasma membrane and the subtending cytoskeleton, by the dynamic vacuole. The narrow cytoplasmic sleeve with PCCs is also populated by small organelles like mitochondria and peroxisomes, and the relationship of PCCs to these organelles was investigated next.

### Assessing PCCs interactivity with mitochondria and peroxisomes

Published biochemical (reviewed by Raghavendra and Padmasree, 2003; Hodges et al., 2016) as well as microscopy-based findings (Frederick and Newcomb, 1969; Sage and Sage, 2009; Gao et al., 2016; Jaiparg et al., 2016) suggest close interactivity between chloroplasts, peroxisomes and mitochondria. Stromules extended from plastids have been specifically implicated in such interactions (Kwok and Hanson, 2004a) and a relationship between stromule extension and the cell size has been proposed (Waters et al., 2004). Between nine and 15 small chloroplasts are found in a pavement cell compared to up to 120 chloroplasts of nearly twice their size in mesophyll cells (Pyke and Leech, 1992, 1994); thus, the pavement cells provide a much larger total cell volume to total chloroplast volume ratio as compared to the mesophyll cell. Based on published literature (Waters et al., 2004) there seemed to be a high likelihood of observing stromules and their interactions with small organelles in these cells. We created a double transgenic line tpFNR:YFP-mitoGFP (Logan and Leaver, 2000) to investigate the interactivity between mitochondria and PCCs (Table 1; Fig. 3A) and a tpFNR:mEosFP-GFP-PTS1 line (Table 1; Fig. 3B) for observing the interactions of PCCs with peroxisomes.

Both mitochondria and peroxisomes appear to come into close proximity with the PCCs (Movie 6). Time-lapse image series showed that associations with PCCs could range from fleeting single-frame encounters to sustained encounters of over 10 s. However, small organelles that were permanently associated with PCCs were not observed. Mitochondria and peroxisomes both show punctate or elongated morphologies. Elongated organelles were very dynamic, and the region in contact with the plastid could change over time. Fig. 3C summarizes the variations in organelle shape and position that were seen. In order to determine whether these organelles associated more frequently with stromules or with the plastid body, the total number of mitochondria or peroxisomes that came into close apposition with PCCs producing stromules over time was counted and averaged. There was no difference in the average number of mitochondria or peroxisomes in close juxtaposition to the PCCs main body or to the extended stromule (Fig. 3D). In the PCCs considered in these time-lapse image sets, the average perimeter that was available for association with other organelles did not differ between the plastid bodies and the stromules (Fig. S1). Having observed the general subcellular environs of the PCCs, we focused next on the implications of their position relative to the MCCs.

### Table 1. Transgenic lines and mutants of *Arabidopsis* used for assessing the subcellular environment around pavement cell chloroplasts

<table>
<thead>
<tr>
<th>Line</th>
<th>Highlighted subcellular compartment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Single transgenics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tpFNR:GFP</td>
<td>Plastid stroma green</td>
<td>Marques et al., 2003, 2004</td>
</tr>
<tr>
<td>tpFNR:YFP</td>
<td>Plastid stroma yellow; replaced GFP by YFP</td>
<td>Schattat et al., 2011</td>
</tr>
<tr>
<td>tpFNR:mEosFP</td>
<td>Plastid stroma green-to-red photo-convertible</td>
<td>Schattat et al., 2012b</td>
</tr>
<tr>
<td>35S-NPC4:GFP</td>
<td>Plasma membrane green</td>
<td>Gaude et al., 2008</td>
</tr>
<tr>
<td><strong>Double transgenics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tpFNR:GFP×RFP:ER</td>
<td>Plastid stroma green, ER lumen red</td>
<td>Schattat et al. 2011; Sinclair et al., 2009</td>
</tr>
<tr>
<td>tpFNR:YFP×T-Mito:GFP</td>
<td>Plastid stroma yellow, mitochondrial matrix green</td>
<td>Marques et al., 2004; Dhanoa et al., 2010</td>
</tr>
<tr>
<td>tpFNR:mEosFP×GFP:MAP4</td>
<td>Plastid stroma green-to-red photo-convertible, microtubules green</td>
<td>Schattat et al., 2012b; Logan and Leaver, 2000</td>
</tr>
<tr>
<td>tpFNR:mEosFP×GFP:talin</td>
<td>Plastid stroma green-to-red photo-convertible, actin cytoskeleton green</td>
<td>Schattat et al., 2012b; Kost et al., 1998</td>
</tr>
<tr>
<td>GFP:Vac-T-RFP:ER</td>
<td>Tonoplast green, ER lumen red</td>
<td>Cutler et al., 2000; Sinclair et al., 2009</td>
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<tr>
<td><strong>Mutant backgrounds</strong></td>
<td></td>
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<tr>
<td>Gla2 (gl2)</td>
<td>Plastid stroma green</td>
<td>Wetzel et al., 1994; Marques et al., 2004</td>
</tr>
<tr>
<td>Crooked (crk)</td>
<td>Plastid stroma green</td>
<td>Rérie et al., 1994; Marques et al., 2004</td>
</tr>
<tr>
<td>Wurm (wrm)</td>
<td>Plastid stroma green</td>
<td>Mathur, 2005</td>
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<tr>
<td>Immutans (im)</td>
<td>Plastid stroma green</td>
<td>Mathur, 2005</td>
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*Note:* Only mutants of *Arabidopsis* used for assessing the subcellular environment around pavement cell chloroplasts are included in this table.
PCCs positioning over the MCCs creates a physiological relationship between the two layers

PCCs have photosynthetic capability; however, the number and thickness of grana and the chlorophyll content in PCCs is low compared to that in MCCs (Barton et al., 2016). In *Arabidopsis*, the general frequency at which PCCs form stromules is known to increase during the day (Schattat et al., 2012a; Brunkard et al., 2015) but whether this is representative of the response of MCCs has yet to be studied. Observations taken separately on the two cell layers in 6-week-old short-day grown plants showed that the stromule frequency of PCCs increased in response to light exposure and continued to rise through the 8 h light cycle. By contrast the MCCs had a consistently low stromule formation frequency that showed no apparent change throughout the day (Fig. 4A).

Exogenous sugar feeding is known to increase the frequency of stromule formation (Schattat and Klösgen, 2011), and was used to...
determine whether stromule formation responses from PCCs and MCCs differed under other conditions. Intact 21-day-old Arabidopsis plants that were floated on 40 mM sucrose in the dark showed an increase in stromule frequency for both PCCs and MCCs (Fig. 4B). Control treatments indicated that neither the immersion in water nor the prolonged darkness influenced the basal stromule levels. PCC stromule frequency was consistently higher than that of MCCs, but for both populations the increase began within the first hour, and continued to rise throughout the 5 h of treatment (Fig. 4B). However, the stromule formation response from MCCs was almost entirely inhibited when the plants were exposed to light during the sucrose treatment, while the PCC response was unchanged from that in the dark conditions (Fig. 4C,D).

During our light and sugar experiments, chlorophyll autofluorescence confirmed that the plastids of all pavement cells observed were indeed chloroplasts. However, reports of leucoplasts in pavement cells of Arabidopsis do exist, so we further investigated under what conditions leucoplasts occur, and how the behaviour and appearance of epidermal leucoplasts differ from PCCs.

A developmental perspective – exploring PCC and plastid interconvertibility

Leucoplasts, by definition (Schimper, 1883, 1885), do not contain chlorophyll and are stroma rich. When using the stroma-targeted tpFNR-GFP probe (Marques et al., 2004; Schattat et al., 2011), leucoplasts had uniformly green fluorescence (emission 510–520 nm), while the green stromal fluorescence in chloroplasts surrounded the chlorophyll autofluorescence of the grana (emission 650–750 nm). This allowed us to monitor the presence of both plastid types in pavement cells during development. We first investigated whether chloroplasts are the default plastid type in the epidermis of Arabidopsis.

Observations on Arabidopsis leaves in the tpFNR:GFP line (Table 1) revealed that, unlike in the chloroplast-containing guard and pavement cells, trichome plastids are leucoplasts (Fig. 5A). As trichomes are initiated from protodermal cells in expanding leaves (Szymanski et al., 2000; Schellmann and Hülskamp, 2005), if the chloroplast represents the standard plastid type in epidermal cells, the chlorophyll must be lost from trichome plastids as the cell differentiates. In the glabra2 (gl2) mutant of Arabidopsis the trichome differentiation is initiated, but the majority of trichomes do not remain committed to the trichome fate and either mature into small spikes (Fig. 5B,C) or collapse into large misshapen cells very similar to pavement cells (Fig. 5D; Rédei et al., 1994). In a gl2 GFP: mTalin line where the green fluorescence provides cellular context to the chlorophyll auto-fluorescence, the aberrant trichomes have chloroplasts rather than leucoplasts (Fig. 5E,F). Therefore, guard cells, pavement cells and trichome cells that have not completely differentiated all contain chloroplasts in Arabidopsis.

We next looked for conditions under which epidermal leucoplasts might be observed. Leucoplasts were not seen in cotyledon pavement cells of healthy 7- to 12-day-old tpFNR:GFP plants grown either in the soil or on agar-solidified medium, nor were they seen in green leaves of older plants. Pavement cell leucoplasts were only found in regions of these plants with mechanical damage or in plants that showed high anthocyanin accumulation indicative of stress. Chloroplasts remained the predominant population in older cotyledons aged between 14 and 28 days. Seldom, uniformly green fluorescent leucoplasts and senescing chloroplasts with a distinct separation of GFP and chlorophyll fluorescence but an intact boundary could be seen in these cotyledons (Fig. 6A). In chlorotic tissue of 6-week-old plants, the chlorophyll signal was minimal, but still present in most epidermal plastids. Similar to in senescing cotyledons, leucoplasts could be seen (Fig. 6B), but were extremely infrequent. It is therefore possible to see pavement cell leucoplasts under some conditions, but the occurrence is rare. Since we were unable to consistently observe leucoplasts in pavement cells in young wild-type tissue, we used the immutans (im) mutant of Arabidopsis (Rédei, 1967; Wetzel et al., 1994) for a thorough comparison of PCCs and leucoplast appearance and behaviour.

The im mutant is variegated with bright green chloroplas-containing regions randomly interspersed with white sectors that contain leucoplasts as well as intermediate plastid forms (Fig. 6C,D) (Rédei, 1967; Wetzel et al., 1994). This mutant thus provided an ideal tool to investigate the different appearances of leucoplasts and chloroplasts within the same tissue. Introduction of the tpFNR:GFP probe into im plants allowed easy identification of leucoplasts by their complete lack of chlorophyll autofluorescence (Fig. 6E). The shape of the various plastid types was clearly different, with leucoplasts displaying an elongated and irregular form as compared to the consistent, round to oval shape of chloroplasts (e.g. Fig. 1C). PCCs in green sectors, apart from the occasional extension of stromules, maintained their lens-like shape over time, whereas leucoplasts exhibited a dynamic morphology. In rare cases, individual leucoplasts did maintain a round morphology over time. Observations on the im leaf epidermis provided another interesting insight (Fig. 6F) on plastids. No chlorophyll signal was visible in any epidermal cell type in the white sectors and even within a pavement or guard cell, the leucoplast size varied considerably. Similarly, chloroplasts of different sizes were visible in the pavement cells of green sectors. While the presence of chlorophyll in green sectors reinforced our earlier observations on wild-type plants, it became apparent from the im mutant that plastid size cannot be considered as a consistent criterion for identification of either leucoplasts or chloroplasts within a tissue.
Based on our investigations on the gl2 and im mutants it is clear that there is tremendous condition-dependent inter-convertibility between the chloroplasts and leucoplasts. In wild-type Arabidopsis, whereas pavement cells in young cotyledons and leaves possess small chloroplasts, cells of older tissues may have an increased number of leucoplasts.

**DISCUSSION**

The presence of chloroplasts in epidermal pavement cells of Arabidopsis had already been established (Pyke, 2009; Barton et al., 2016). The present work aimed to describe fresh resources and the basic subcellular relationships for PCCs to facilitate further study on this under-researched subpopulation of chloroplasts.

Based on the probes that have been used, a general picture of the PCCs in Arabidopsis has emerged. They are found in a thin cytoplasmic sleeve delimited on its exterior by the cell boundary consisting of the plasma membrane and a reinforcing cytoskeletal mesh. PCCs and stromules exhibit clear alignments with actin filaments. Interactions with the actin cytoskeleton have been suggested to play a role in both stromule extension and whole plastid movement (Kwok and Hanson, 2004a; Kadota et al., 2009). Indeed, the coordinated movement of the perinuclear plastids by the actin cytoskeleton has been reported to play a role in nuclear movement during the blue-light avoidance response (Higa et al., 2014). Despite this, we saw clustering of PCCs around nuclei in only a small proportion of pavement cells, suggesting that nuclear movement either does not require the presence of PCC or requires a mass migration of PCCs to the nucleus before it can occur. The actin cytoskeleton also supports the cortical ER and the vacuolar membrane appears to press cortical PCCs against the cell periphery and almost completely encloses more cell-central PCCs. Although we saw that rapid vacuolar movement was often accompanied by the appearance of ‘tugging’ on PCCs, the vacuole appears primarily to move freely over the plastid surface. This is in accordance with the idea of a shared association with the actin cytoskeleton but does not suggest a role for the vacuole in directed PCC or stromule movement. In contrast, as reported by Schattat et al. (2011) and reconfirmed here, the PCCs and extended stromules are firmly enmeshed in the cortical ER and plastid behaviour appears to directly correlate with ER re-arrangement. Unlike the previous organelles discussed, no clear spatial association of the microtubules with PCCs could be seen. Microtubules form a tight array of largely parallel filaments that traverse the cell periphery (reviewed in Dixit and Cyr, 2004), and PCCs seemed to sit below the plane of this array, with stromules extending either parallel or perpendicular to it. The potential for a microtubule effect on PCCs should, however, be investigated further, as the ER has been implicated in stromule extension (Schattat et al., 2011) and an effect of microtubules on ER rearrangement has been reported previously (Hamada et al., 2014). The relationship between the outer membranes of the PCCs, the ER and the vacuole creates a very dynamic field for membrane contacts and exchange of metabolites and ions. Ongoing investigations on the membrane contacts between the PCCs and the surrounding endomembranes facilitated by the double transgenic lines reported here are providing interesting insights that will be reported elsewhere.

All plastids are known to produce stromules (reviewed by Gray et al., 2001; Hanson and Sattarzadeh, 2011; Schattat et al., 2015) and these extensions have been suggested to be interaction...
platforms for organelles such as nuclei, mitochondria and peroxisomes (Kwok and Hanson, 2004a,b). Our present observations strongly suggest that these organelles associate only occasionally and rather transiently, and that mitochondria and peroxisomes show no preference for association with the stromule over the plastid body. On this basis, we do not consider that there is a specific subpopulation of mitochondria and peroxisomes that are targeted particularly to the PCC body or stromule and maintain a sustained presence around them. However, although the individual perimeters of the stromule and plastid body were of comparable area, extension of a stromule does increase the total plastid perimeter. Although no preference is shown for association with a stromule, its

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Fig. 5. Images showing leucoplasts and chloroplasts in and around trichome cells in Arabidopsis expressing tpFNR:GFP and in the gl2 mutant epidermal cells. (A) A flattened z-stack of 10 confocal images showing the base of a trichome, the epidermis and the mesophyll layers in an Arabidopsis (Columbia ecotype) plant expressing tpFNR:GFP. Panels 1, 2, 3 and 4 show GFP (green) and chlorophyll autofluorescence (red), a bright-field image and a merged image, respectively. Note the presence of chlorophyll fluorescence in the pavement cells of the epidermal layer but its complete absence in the trichome cell (panel 2). As shown by the stroma-targeted tpFNR:GFP probe, mature trichomes in Arabidopsis possess leucoplasts only (panel 1; green; arrowhead). (B) A scanning electron microscope image of a stubby trichome (*) that has not completed its differentiation in the gl2 mutant. (C) A short, collapsed trichome in gl2 similar to that shown in B has plastids that exhibit chlorophyll autofluorescence (arrowheads, red) that is comparable to the fluorescence of chloroplasts in the sub-epidermal layers. (D) A scanning electron microscope picture of a cell on a gl2 leaf (*) that appears to have entered the trichome differentiation pathway but did not progress to branching and maturation stages. Its collapse and enlargement suggest its continuation or reversion into a pavement cell fate. (E) Image of a gl2 trichome similar to that shown in D that has not completed its differentiation showing plastids with a chlorophyll signal (arrowheads, red) alongside a GFP:mTalin F-actin fluorescence (green). (F) A gl2 mutant line expressing a plasma-membrane targeted GFP probe showing chlorophyll-containing plastids (arrowheads, red) in a putatively aborted trichome. Scale bars: 50 µm (A,B); 25 µm (C–F).

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Fig. 6. Images from Arabidopsis wild-type plants and mutants allow clear differentiation between leucoplast and chloroplasts. (A) A group of physiologically compromised swollen PCCs in a senescent cotyledon exhibit the clear separation of their GFP-highlighted stroma and the chlorophyll contents. (B) An image from a pavement cell in a senescing 16-day-old cotyledon of an Arabidopsis plant expressing tpFNR:GFP showing several green leucoplasts (e.g. arrowhead) alongside a plastid exhibiting a green–red mix (panel 4), suggesting the presence of chlorophyll (red; panel 2). (C) Image of young plants of the Arabidopsis variegated mutant im showing the characteristic green (g) and white (w) sectors containing chloroplasts and leucoplasts, respectively. (D) Image of a leaf of the im mutant transformed with stroma-targeted tpFNR:GFP showing green fluorescent sectors that contain mostly leucoplasts and regions with chloroplasts whose fluorescence is false coloured blue. (E) A compressed confocal image stack of a portion of a leaf petiole in im shows lens-shaped chloroplasts (false coloured blue) and variously shaped and elongated green fluorescent leucoplasts that clearly lack chlorophyll. Arrowheads, chloroplasts; *, leucoplast. (F) Confocal image of a variegated leaf from an im mutant showing chlorophyll fluorescence (blue) against a bright-field background. Chloroplasts are absent in cells on the upper left that represent a white leucoplast rich sector whereas the right side of the image shows mesophyll chloroplasts. Chloroplasts that are intermediate in size between guard cell chloroplasts (boxed areas) and mesophyll chloroplasts are visible in the pavement cells in the lower left leaf sector. Chloroplasts in guard cells (similar to the three in boxed areas) appear in a typical curved pattern as compared to the scattered chloroplasts in neighbouring pavement cells. Scale bars: 5 µm (A, B); 1 mm (C); 100 µm (D); 20 µm (E); 25 µm (F).
extension may increase the total number of organelles with which a plastid can interact. Accumulation of plastids has been observed in the perinuclear region of hypocotyl cells and dark-adapted leaves of *Arabidopsis* (Kwok and Hanson, 2004b; Higa et al., 2014), and in pathogen-challenged cells of tobacco (Caplan et al., 2008, 2015; Krenz et al., 2012), however our present observations do not suggest the presence of a sustained PCC population around the nuclei under standard growth conditions. In addition, we have been unable to support the idea that stromules extended by the PCCs in the perinuclear region display a specific configuration in relation to the nucleus. Whether the perinuclear aggregation of chloroplasts occurs in mesophyll cells in *Arabidopsis*, as well as whether this phenomenon is specific to stressed plants or plays a role in normal growth and development remain to be investigated.

A major insight provided from this work involves the inter-relationship between chloroplasts and the differences in plastid behaviour between the pavement and mesophyll cells. In a model that considered PCCs to be leucoplasts, it was proposed that the epidermal layer acts as a sink for sugars produced by the mesophyll (Brunkard et al., 2015). In principle, all chloroplasts are capable of photosynthesis, but their output of photosynthates might differ considerably. The sugar output from PCCs is unknown, but given their small size and number (Pyke and Leech, 1994; Barton et al., 2016), it is likely small compared to that of the underlying MCCs. Therefore, recognizing the plastids in pavement cells as chloroplasts does not negate a potential source–sink relationship between these two tissues. In order for continuous export of photosynthates from MCCs and efficient photosynthesis, mesophyll cells must shuttle sugars to sink tissues (Ainsworth and Bush, 2011). The pavement cells, being close in proximity and potentially low in sugar production, could provide a readily accessible sink. Experiments performed by Schattat and Klösgen (2011) showed that sugar induces stromule formation, so an accumulation of sugar in sink tissues may be accompanied by an increased stromule frequency. In observations throughout the photosynthetic period, we found that PCC stromules increased in number throughout the day but that MCCs maintained a consistently low stromule frequency. This observation, alongside the sugar responsiveness of stromules, suggests that while MCCs mainly exported their photosynthates, the PCCs responded to the consequent increase in levels of cytosolic sugar within the pavement cells. The idea was further tested by inducing stromules by exogenously feeding with 40 mM sucrose (Schattat and Klösgen, 2011; Schattat et al., 2011). The stromule formation frequency increased significantly in PCCs regardless of whether light was given during treatment. Interestingly, an increase in the stromule frequency of MCCs occurred in the dark, but not when plants were exposed to light during treatment. This supports a model whereby mesophyll chloroplasts are capable of sugar-induced stromule induction but, while photosynthesis is active, continuous export of sugars from the cytosol prevents sufficient accumulation for induction. We therefore propose that pavement cells act as a sink for sugars from the mesophyll during the daylight period. Investigations are underway to explore the physiological sugar-regulated inter-chloroplastic relationship further. Another important implication of these results is that PCC behaviour is not representative of the majority of leaf chloroplasts under all conditions. Therefore, care should be taken when choosing an experimental tissue, and before applying a model based on PCCs to all chloroplasts, the individual PCC and MCC responses should be compared.

Our results also highlight the potential effect of growth and treatment conditions on stromule responses. Previous studies in *Arabidopsis* and tobacco on stromule responses throughout the day–night cycle showed an increase in stromule frequency within the first few hours of light exposure and then consistent stromule frequency throughout the day (Schattat et al., 2012a; Brunkard et al., 2015). This is in contrast to our observations of a gradual increase in stromules over the course of the entire day. Interestingly, both previous studies grew their plants under a 12-h-light–12-h-dark photoperiod, while we used an 8-h-light–16-h-dark cycle. If the diurnal changes in cellular sugar status were responsible for the stromule response then photoperiod would certainly have an effect, as plant sugar metabolism adjusts to compensate for a longer night (Suplice et al., 2014). Photoperiod, as well as the time of day that tissue is collected, should therefore be taken into consideration when comparing observations of stromule frequency between experiments. Similarly, our observations of increased stromules in the mesophyll in response to sugar treatment appear to conflict with the statement by Brunkard et al. (2015) that mesophyll chloroplasts are not sugar responsive. However, as demonstrated here, mesophyll stromules are only induced by sucrose treatment in the dark. Brunkard et al. (2015) do not specify the lighting conditions for their treatment, but if they carried out their treatments in the light, then both observations are in accordance. It is therefore apparent that many factors, including photoperiod, time of observation and light exposure during treatment, influence plastid behaviour and that thorough reporting of all conditions is necessary to allow for comparison between studies.

The suggested link between PCC behaviour, photosynthesis and sugar regulation also led us to explore the developmental aspects of chloroplast formation. The present view traces all plastids to a population of pro-plastids, which are colourless and so by definition, are leucoplasts. Further development of these colourless plastids into chlorophyll-containing chloroplasts occurs in a tissue- and cell-specific manner and requires input from nuclear-encoded proteins (Jarvis and Lopez-Juez, 2013; Liebers et al., 2017). The linear route of leucoplasts and etioplasts greening into chloroplasts is thus well supported. Studies on plastids in the L1 layer of the shoot apical meristem and the epidermis of developing embryos suggest that this conversion occurs early in the epidermal cells, as either stacked grana or chlorophyll autofluorescence was seen in the plastids of these tissues (Tejos et al., 2010; Charuvi et al., 2012). Our observations with the gl2 mutant support the idea that chloroplasts are the standard plastid type in epidermal cells, as the presence of chloroplasts in the under-developed trichomes suggests the presence of PCCs in the protodermal cells from which trichomes originate. It follows that loss of chlorophyll is responsible for the presence of leucoplasts in wild-type trichomes, and that chloroplast-to-leucoplast conversion is part of normal development. We found that this phenomenon also occurs in senescent cells and tissues, and in these cases is dependent upon a change in the cell physiology. As senescence is accompanied by breakdown of chlorophyll (Hörtsteiner, 2006) and the chlorophyll signal is relatively low in PCCs (Barton et al., 2016), they are likely to be among the first plastids in which chlorophyll loss is visible during senescence. Based on our observations, it is clear that investigations of PCCs in *Arabidopsis* must always take the growing conditions, tissue health and developmental stage of the plant into consideration.

The use of the im mutant (Rédei, 1967; Wetzel et al., 1994) allowed us to reliably observe leucoplasts in pavement cells and indicated another facet of the leucoplast–chloroplast relationship. The two plastid types show considerable differences in behaviour but we determined that neither shape nor size was sufficient on its own to distinguish between chloroplasts and leucoplasts. This mutant also provides an interesting tool for future research to test, in
a horizontal context, our hypothesis that differences in the stromule response can be indicative of a source–sink relationship between cells. The white sectors with leucoplasts and the green sectors with photosynthetic chloroplasts lie side by side on a leaf in this mutant. Since the white sectors are non-photosynthetic, they represent sink tissues that should show a stromule response to accumulation of photosynthates from juxtaposed green regions.

Conclusions

Here, we have provided a cell biological characterization of the small subpopulation of chloroplasts in epidermal pavement cells of Arabidopsis. Plastid responses to changes in the cellular milieu and the phenomenon of plastid inter-convertibility have been highlighted through our observations. Our basic characterization of chloroplasts in the pavement cells of Arabidopsis triggers several interesting questions and new approaches to understand their functional significance and their relationship with chloroplast populations in contiguous and subtending cells.

MATERIALS AND METHODS

Plant material and growth conditions

Single and double transgenic lines were created through Agrobacterium-mediated floral dip transformation (Clough and Bent, 1998) or through crossing. A complete list of plant lines used is provided in Table 1. The four different conditions for plant growth were as follows: (1) growth in sealed tissue culture boxes for 7–12 days or 21 days on sterilized Sunshine mix LA4 (SunGro Horticulture, USA) under a long-day light regime (16-h-light–8-h-dark) for experiments on stromule frequency in response to sucrose; (2) growth for 7–12 days in petri dishes containing Murashige and Skoog basal medium (Murashige and Skoog, 1962; Sigma M404) with B5 vitamins and 3% sucrose under a long-day light regime for observations of the interactions between PCCs and other organelles; (3) growth in pots for 6 to 7 weeks on Sunshine mix LA4 in a controlled chamber under a short-day light regime (8-h-light–16-h-dark) for observations on diurnal plastid responses and leucoplast conversion in leaves. Diurnal stromule observations were taken on healthy leaves, while senescence observations were taken on older chlorotic leaves. All plants were grown under 120 μmol m⁻² s⁻¹ light and at a temperature of 21°C.

Confocal microscopy

All fluorescence imaging was performed on whole plants, with the exception of imaging of leaves over 3 weeks of age, which were detached and imaged immediately. The plants were mounted in water and imaged using a Leica TCS-SP5 confocal microscopy system equipped with a 488 nm Ar laser and a 543 nm HeNe Laser. GFP fluorescence was acquired at 500–520 nm, RFP fluorescence at 570–620 nm, YFP fluorescence at 530–550 nm and chlorophyll autofluorescence at 660–750 nm. mEosFP was photo-converted using a mercury lamp and the Leica fluorescence filter set D (excitation 355 to 425 nm). The entire field of view was exposed to the conversion light for 10 to 15 s. All confocal images were acquired at a resolution of 1024×512 or 1024×1024 pixels. Three-dimensional (x,y,z) stacks were collected with a step-size of 0.99 μm and successive frames in x, y,t time lapse series were 1.935 s apart.

Images were processed using proprietary Leica (LSM SPII) software, ImageJ (Schneider et al., 2012) and Adobe Photoshop CS6. Quick time 7 and Videator v2 (Stone Design Corp., New Mexico) were used for assembling and annotating time-lapse movies.

Scanning electron microscopy

Scanning electron microscopy (SEM) was carried out on unfixed, uncoated samples taken directly from the greenhouse and observed using a Hitachi Tabletop TM-1000 system (Hitachi High-Technologies Corp., Tokyo).

Calculation of cell depths

Cell depths were calculated in the tpFNR:GFP-RFP-ER background to allow visualization of the plastid populations and the cortical ER surrounding the cell borders. z-stacks were taken with a step size of 0.4 μm from the upper epidermal surface to the bottom of the mesophyll cells in the cotyledons of eight individual 10-day-old seedlings. In ImageJ (Schneider et al., 2012), orthogonal views allowed the calculation of cell depth of between 15 and 20 cells of each type (pavement, guard or mesophyll) per stack based on the number of steps required to traverse the cell.

Analysis of PCCs and small organelles

Time-lapse images were collected for 41 to 321 frames. One to three PCCs per image set had stromules that remained in focus for the frames analyzed. Collection time for each frame was 1.935 s. A total of n=38 and n=10 PCCs were followed for analyzing mitochondria and peroxisome encounters with PCCs respectively. The number of mitochondria or peroxisomes closely appressed to the PCC body or stromule was counted manually for each frame of the image sets with the ImageJ Cell Counter plugin [Kurt De Vos (University of Sheffield; https://imagej.nih.gov/ij/plugins/cell-counter.html, 2001)]. The total number of close encounters between PCCs and either mitochondria or peroxisomes was summed and averaged over the total number of frames analyzed. 95% confidence intervals (CI) for the estimated means were calculated for the data. The perimeter of the body and stromule of PCCs was measured in the first frame of the time-lapse image sets used for counting the average number of mitochondria juxtaposed to PCCs and the mean perimeters were reported with 95% confidence intervals.

Stromule frequency: treatment, image acquisition, calculation and statistical analysis

Treatment of seedlings with 40 mM sucrose or water controls was performed by carefully excising the entire plant from the soil and floating the plants in solution for 3 h in either a dark chamber or under normal growth lighting. For consistency, plants were always taken at the end of the night cycle to begin treatment. For each condition under which stromule frequency was measured, n=16 z-stacks were taken, with a step size of 0.99 μm, through the epidermis and the upper region of the mesophyll on the adaxial surface of the most expanded leaves. Standard deviation y-projections were obtained using the function in ImageJ (Schneider et al., 2012) and the number of plastids exhibiting or lacking stromules was counted manually for both the PCC and MCCs populations in each image. The calculated stromule frequencies were transformed with the arcsin (y/Freq) function according to Schattat and Klösgen (2011). 95% confidence intervals (CI) for the estimated mean and statistical significance (two-way ANOVA with post hoc Bonferroni test) were assessed using the transformed data. The mean and CI were back-transformed for representation in the figures.

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Competing interests

The authors declare no competing or financial interests.

Author contributions


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