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**Epidermal pavement cells of *Arabidopsis thaliana* have chloroplasts**

Plastids are multi-functional, pleomorphic organelles of purported endo-symbiotic origin, that in plants and green algae display a characteristic double membrane envelope (Wise, 2007). Whereas all plastids originate from colour-less pro-plastids a simple pigmentation-based classification distinguishes chloroplasts from other plastids by the presence of chlorophyll, chromoplasts by the predominance of other pigments, and leucoplasts by the absence of all pigmentation (Schimper, 1883; 1885). Plastids are able to inter-convert according to tissue and developmental requirements. (Schimper, 1883; 1885).

In higher plants the majority of chloroplasts are found in the leaf mesophyll tissue. The presence of chloroplasts in the epidermis of some higher plant species, including tobacco, is also generally accepted (Shaw and MacLachlan, 1954; Dupree et al., 1991; Brunkard et al., 2015). However, several modern textbooks and primary publications categorically state that the epidermis of higher plants contains chloroplasts only in the guard cells, while pavement and trichome cells have leucoplasts (MacDonald, 2003; Smith, 2005; Bowes and Mauseth, 2008; Solomon et al., 2010; Vaughan, 2013). In the model plant *Arabidopsis thaliana* observations of leucoplasts in the unicellular trichomes are consistent, but there is considerable ambiguity regarding the presence or absence of chloroplasts in pavement cells (Table 1).

Whereas several publications show clear chloroplasts in the pavement cells of Arabidopsis a precise, observation-based statement that contradicts the common text book knowledge has been made by Pyke (2009): “In a leaf, the chloroplasts in the epidermal cells covering the leaf surface are significantly smaller and poorly developed compared with mesophyll chloroplasts, but do contain low levels of chlorophyll and should be considered as chloroplasts”. However, a degree of uncertainty is maintained since other investigators, who have also observed chlorophyll fluorescence in pavement cells, have either dismissed it as an artifact or have described such chloroplasts as not being fully developed (Haseloff et al., 1997; Chiang et al., 2012; Higa et al., 2014). The

uncertainity on the issue is apparent in a recent publication that uses the purported absence of chloroplasts in the pavement cells to explain the differences in plastid behaviour that they observe between cotyledon pavement and mesophyll cells in response to chemically induced redox stress (Brunkard et al., 2015).

Recognizing the correct plastid type in a tissue creates an association with specific attributes. The name influences our comprehension of its internal biochemistry, its response and susceptibility to environmental stimuli such as redox imbalances and its overall behaviour and interactions with other cytoplasmic components and compartments. For example, photosynthesis in chloroplasts suggests a primary source of sugars, whereas leucoplasts are recognized as sink plastids that receive already synthesized sugar molecules. For models that rely on identifying a plastid type to explain plastid behavior, a changed label can suggest a different but perhaps experimentally unsubstantiated interpretation.

Here, after recognizing the present ambiguity on the subject, we address the issue of the presence or absence of chloroplasts in epidermal pavement cells in Arabidopsis by presenting a few representative images and observations (Figure 1). The observations, undertaken independently in several different labs, routinely detect chlorophyll autofluorescence (emission peak 485nm) using epi-fluorescent microscopy (B-3A long pass filter set) as well as confocal laser scanning microscopy (excitation 488nm; emission collected 650-750nm) in pavement cell plastids. The observations remain consistent for plants in different stages of development, grown on soil or on sucrose-containing medium under varying light conditions (Figure 1A,B). As reported earlier by Pyke and Leech (1994), the number of chloroplasts in a pavement cell is nearly a tenth (10 ± 3) of that observed for mesophyll cells. In comparison to the clustered chloroplasts in mesophyll cells, pavement cell chloroplasts appear very dispersed, often located near the edges of the zigsaw puzzle-shaped cells. The average size of pavement cell chloroplasts is approximately half the size of a mesophyll chloroplast but slightly larger than guard cell chloroplasts. The average chlorophyll-a

fluorescence values of pavement cell chloroplasts lies between that of guard cell and mesophyll cell chloroplasts. Observations of low chlorophyll content are matched by ultrastructural details that show a small number of clearly defined grana (Fig. 1C-E). Although pulse amplitude modulated fluorescence (PAM) measurements provide lower values in comparison to mesophyll chloroplasts ($\Phi_{max}$ = 0.62 compared to 0.76 for palisade parenchyma chloroplasts) they clearly demonstrate that the pavement cell chloroplasts do have an active photosystem (PS) II.

Whereas each observation presented here supports earlier publications (referenced in Table 1) suggesting the presence of chloroplasts in Arabidopsis pavement cells there is some basis for their perceived absence too. One reason appears to lie in their low numbers and sparse distribution in pavement cells. Further, like chloroplasts in the mesophyll, pavement cell chloroplasts also exhibit light avoidance responses (Higa et al. 2014) and relocate to the lower lateral regions of the cells in tissue exposed to light. This location places them very close to the mesophyll layer so that when imaged from above, as is the usual practice, they are easily confused with mesophyll chloroplasts. Their location in the lower region of pavement cells also removes them from the focal plane for guard cell chloroplasts and conveys an impression of their absence. However, a comparison of chloroplast size and in cases where a stroma-targetted probe has been used clearly demonstrates their presence (Fig.1B). Another prevelent practice during multi-channel confocal imaging that might lead to a similar conclusion is the minimization of fluorescence detection levels for chlorophyll in order to obtain clear images of the strongly autofluorescent mesophyll chloroplasts. Since pavement cell chloroplasts display considerably lower autofluorescence there is a strong likelihood that their fluorescent signal falls below the detection range. As shown in Fig. 1A imaging a tissue from a lateral perspective in addition to the top-down view (Fig. 1B) allows all autofluorescent plastids to be detected and helps dispel the illusion of absence.

Another factor requiring consideration in the context of pavement cell chloroplasts is the intrinsic property of plastids to inter-convert from one kind to

another. Chlorophyll, the distinguishing feature of a chloroplast, is lost quite rapidly in senescing as well as wounded tissue. This would allow a plastid to be classified as a leucoplast. As cotyledons of varying ages have been used in some studies (Chiang et al., 2012; Brunkard et al., 2015), we observed this tissue carefully and found that pavement cells in older cotyledons and leaves do contain a mixture of chloroplasts and leucoplasts. Whether observations made on tissues with a mixture of plastid types should be presented as being representative and used to promote the view that pavement cells in Arabidopsis plants have only leucoplasts remains questionable.

It appears that considering plastids in pavement cells in *Arabidopsis thaliana* to be leucoplasts is largely due to limited information to the contrary, rather than evidence in favour of this conclusion. We believe that this letter will help to remove the existing ambiguity on the subject. While Arabidopsis becomes another lab plant, like tobacco (Dupree et al., 1991), where chloroplasts in pavement cells can be observed, it is noteworthy that independent surveys by Moore (1887) and Stohr (1879) had already indicated that between 85 and 95% of dicotyledonous species contain chlorophyll in the lower epidermis, while at least half of the 120 species investigated by Moore (1887) had chloroplasts in the upper epidermis. Recognition of small chloroplasts with a high stroma to grana ratio in the pavement cells should open out new avenues for research on their actual contribution to the general upkeep and functioning of the aerial plant epidermis.

Table1. Non-comprehensive list of publications reflecting on the status of chloroplasts in pavement cells in *Arabidopsis thaliana*.

| **Suggested** | **Basis** | **Reference** |
|---|---|---|
| Absent | Chlorophyll auto-fluorescence in guard cells | Brunkard et al., 2015 |
| Absent | Chlorophyll containing plastids not observed | Haseloff et al., 1997 |
| Absent | Reported as being non-green. Chlorophyll signal not shown | Haswell and Meyerowitz 2006 |
| Absent | Chlorophyll autofluorescence in guard cells | Chiang et al., 2012 |
| Absent | Reported. Chlorophyll signal not shown | Bergmann et al., 2004 |
| Absent | Expression of 35S-PAC-GFP construct only in guard cells. Chlorophyll signal not shown | Meurer et al., 1998 |
| Absent | Stated in discussion, no citation. | Kagawa and Wada 2000 |
| Ambiguous | Chlorophyll fluorescence; internal structure in embryo. | Tejos et al., 2010 |
| Ambiguous | Chlorophyll auto-fluorescence in leaf primordia. Indicate loss of chlorophyll later. | Charuvi et al., 2012 |
| Ambiguous | Chlorophyll auto-fluorescence | Higa et al., 2014 |
| Present | Citation | Pyke and Page 1998 |
| Present | Internal thylakoid ultrastructure observed | Robertson et al., 1996 |
| Present | Chlorophyll auto-fluorescence | Kojo et al., 2009 |
| Present | Chlorophyll auto-fluorescence | Fujiwara et al., 2015 |
| Present | Chlorophyll auto-fluorescence | Holzinger et al., 2008 |
| Present | Pale chloroplasts reported. | Pyke and Leech 1994 |
| Present | Chlorophyll auto-fluorescence | Joo et al., 2005 |
| Present | Acknowledged as chloroplasts. | Vitha et al., 2001 |

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