Rice bundle sheath cell shape is regulated by the timing of light exposure during leaf development

Plackett, Andrew; Hibberd, Julian M

DOI:
10.1111/pce.14902

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (Harvard):
Plackett, A & Hibberd, JM 2024, 'Rice bundle sheath cell shape is regulated by the timing of light exposure during leaf development', *Plant, Cell and Environment*. https://doi.org/10.1111/pce.14902

Received: 23 October 2023 | Revised: 14 February 2024 | Accepted: 16 March 2024

DOI: 10.1111/pce.14902

ORIGINAL ARTICLE

Plant, Cell & Environment | WILEY

Rice bundle sheath cell shape is regulated by the timing of light exposure during leaf development

Andrew R. G. Plackett | Julian M. Hibberd

Department of Plant Sciences, University of Cambridge, Cambridge, UK

Correspondence
Andrew R. G. Plackett and Julian M. Hibberd, Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, UK.
Email: A.R.G.Plackett@bham.ac.uk and jmh65@cam.ac.uk

Present address
Andrew R. G. Plackett, School of Biosciences, University of Birmingham, Birmingham B15 2TT, Edgbaston, UK..

Funding information
Bill and Melinda Gates Foundation; Biotechnology and Biological Sciences Research Council

Abstract

Plant leaves contain multiple cell types which achieve distinct characteristics whilst still coordinating development within the leaf. The bundle sheath possesses larger individual cells and lower chloroplast content than the adjacent mesophyll, but how this morphology is achieved remains unknown. To identify regulatory mechanisms determining bundle sheath cell morphology we tested the effects of perturbing environmental (light) and endogenous signals (hormones) during leaf development of *Oryza sativa* (rice). Total chloroplast area in bundle sheath cells was found to increase with cell size as in the mesophyll but did not maintain a 'set-point' relationship, with the longest bundle sheath cells demonstrating the lowest chloroplast content. Application of exogenous cytokinin and gibberellin significantly altered the relationship between cell size and chloroplast biosynthesis in the bundle sheath, increasing chloroplast content of the longest cells. Delayed exposure to light reduced the mean length of bundle sheath cells but increased corresponding leaf length, whereas premature light reduced final leaf length but did not affect bundle sheath cells. This suggests that the plant hormones cytokinin and gibberellin are regulators of the bundle sheath cell-chloroplast relationship and that final bundle sheath length may potentially be affected by light-mediated control of exit from the cell cycle.

KEYWORDS
chloroplasts, hormones, plant leaves

1 | INTRODUCTION

The leaves of land plants have evolved to maximise their photosynthetic efficiency (Gago et al., 2019; Ren et al., 2019), which has involved individual cell types developing morphological specialisations. In most plants the leaf mesophyll represents the tissue in which the majority of carbon fixation occurs (Borsuk and Brodersen, 2019; Vogelman et al., 1996) whilst cells of the bundle sheath, a layer of cells surrounding each vascular bundle, are typically larger and less 'green', with a lower chloroplast content than the mesophyll (Khoshravesh et al., 2016, 2020; Kinsman and Pyke, 1998; McKown & Dengler, 2007; Muhaidat et al., 2011; Sage et al., 2013; Wang

Abbreviations: BAP, 6-benzylaminopurine; BS, bundle sheath; C_3, 3-carbon; C_4, 4-carbon; CK, cytokinin; GA, gibberellic acid; M, mesophyll; ML, mediolateral; NAA, 1-Napthaleneacetic acid; PD, proximodistal; RuBisCO, ribulose bisphosphate carboxylase oxygenase.

Andrew R. G. Plackett and Julian M. Hibberd are co-corresponding authors.

et al., 2017b; Williams et al., 1989). Evidence indicates that the bundle sheath plays important roles in sulphur metabolism, glucosinolate biosynthesis, photosynthate storage and transport, water transport, and responses to high light stress (Aubry et al., 2014; Hua et al., 2021; Leegood, 2007; Miyake, 2016; Shatil-Cohen et al., 2011; Xiong et al., 2021). The establishment of distinct cell morphologies during leaf development is of fundamental importance to plant biology but the mechanisms responsible remain unclear.

One striking example of a cell type specific morphology is associated with the bundle sheath that is critical to C_4 photosynthesis, a photosynthetic adaptation found in many plant groups and thought to have evolved in response to falling atmospheric CO_2 concentration and increasing aridity (Christin et al., 2008; Ehleringer et al., 1997; Sage et al., 2011). From around 30 million years ago plant species evolved the C_4 pathway which enhances the efficiency of the core photosynthetic enzyme Ribulose Bisphosphate Carboxylase Oxygenase (RuBisCO) by concentrating CO_2 in photosynthetic cells (Christin et al., 2013; Sage et al., 2011). This trait has evolved independently at least 67 times in the flowering plants (Sage et al., 2011; Sage, 2016), with the transport of CO_2 mediated by synthesis of C_4 acids, and is thus known as the C_4 pathway. In most cases the mesophyll becomes specialised for carbon capture whilst the bundle sheath undertakes carbon fixation (Edwards et al., 2004). C_4 bundle sheath cells have become specialised for this role, typically with increased size or surface area (Christin et al., 2013; Ermakova et al., 2020; Lundgren et al., 2019; McKown & Dengler, 2007), increased plasmodesmata connections (Danila et al., 2016, 2019; Schreier et al., 2024) and an increased chloroplast content typically greater than the corresponding mesophyll (Khoshravesh et al., 2016; McKown & Dengler, 2007; Wang et al., 2017b). Determining how the bundle sheath and mesophyll cell types achieve their respective cell morphologies is thus of fundamental importance to understanding the evolution of such plant adaptations to environmental change.

The divergent morphologies of the bundle sheath and mesophyll become apparent during leaf development. Cell size is determined by the balance between cell division and cell expansion (Jones et al., 2019; Sablowski, 2016) and the final proportion of each cell that is occupied by chloroplasts ('chloroplast content') is influenced by cell size and, within those developing cells, the regulation of chloroplast biogenesis and chloroplast division (Jarvis and López-Juez, 2013). Cell and chloroplast development are closely coordinated, with mature mesophyll cells of the dicotyledonous model *Arabidopsis thaliana* maintaining similar relative chloroplast contents despite variation in individual cell size (Pyke & Leech, 1992; Pyke, 1999). Whether this also occurs in bundle sheath cells has not been reported. The mechanism through which cell and chloroplast development are coordinated to achieve this constant 'set-point' chloroplast content per cell is not known, nor is it known how different cell types apparently achieve and maintain different chloroplast contents.

In *A. thaliana*, leaf development first progresses through coordinated organ-wide cell proliferation (combined division and expansion) followed by cell expansion alone to determine final organ size (Beemster et al., 2005; Donnelly et al., 1999). Several phytohormones (auxin, cytokinin [CK] and gibberellin [GA]) regulate cell division and expansion (Achard et al., 2009; Claeys et al., 2012; Holst et al., 2011; Hu et al., 2003; Jiang et al., 2012; Schruff et al., 2006). Light is also a key environmental regulator of leaf development, with evidence that a light-derived chloroplast-dependent signal promotes the exit of *A. thaliana* epidermal pavement cells from the proliferation phase (Andriankaja et al., 2012). However, the regulation of plant cell development by light can differ between cell types, as demonstrated during seedling de-etiolation where exposure to light causes differing growth responses between the shoot apex, cotyledons and hypocotyl (Whitelam & Halliday, 2007). These same light and hormone signals also regulate chloroplast biogenesis and division (Cackett et al., 2022; Richter et al., 2013), acting through a network of transcription factors including members of the *GOLDEN2-LIKE* (*GLK*) and *GATA* families (Chiang et al., 2012; Lee et al., 2021; Naito et al., 2007; Wang et al., 2017b; Zubo et al., 2018), and could potentially contribute to coordinating cell and chloroplast development.

In contrast, in monocotyledonous plants each leaf develops from a persistent basal zone of cell proliferation from which cells emerge, cease dividing and then expand as they mature from base to tip (Conklin et al., 2019). In the monocotyledonous model rice (*Oryza sativa*) each leaf primordium initiates as a ring around the margin of the shoot apex (Itoh et al., 2005). Recent modelling studies have identified homologies between dicotyledonous and monocotyledonous leaves (Richardson et al., 2021) and there is evidence that cell and chloroplast development in rice are regulated by the same hormonal signals (Aya et al., 2014; Ding et al., 2017; Ikeda et al., 2001; Jiang et al., 2012; Matsukura et al., 1998; Yang et al., 2002) via a gene network partially-conserved with *A. thaliana* (Wang et al., 2017a). However, the shoot architecture of rice (where each leaf primordium emerges from within the sheath of the preceding leaf) means that the exposure of developing rice leaves to light is delayed compared with typical dicotyledonous leaf development, and the light environment might therefore be expected to play additional or different regulatory roles. It has previously been shown that final rice leaf anatomy is responsive to the level and timing of light perceived during development (Murchie et al., 2005; van Campen et al., 2016) but to our knowledge this response has not been characterised at the cellular level.

To test whether bundle sheath cell characteristics of rice, including increased size and low chloroplast content compared with the mesophyll, can be explained by differential responses to light exposure or to hormonal signals during development, we first characterised the relationship between bundle sheath cell area and chloroplast content at maturity. Subsequently, experimental perturbation of leaf development by manipulating hormone and light inputs were used to compare their effect on bundle sheath and mesophyll cells. Although we did not find evidence for a constant relationship between chloroplast content and cell area in the bundle sheath—previously defined in mesophyll cells as a 'set point'—bundle sheath chloroplast biogenesis did increase in response to increasing cell size.

Exogenous treatment with the hormones CK and GA significantly altered this relationship such that larger cells contained more chloroplast material at maturity, identifying these hormones as candidates for a coordinating signal between cell and chloroplast development. Our findings suggest that chloroplast biogenesis is responsive to cell size in the bundle sheath but that this mechanism is somehow repressed compared with the mesophyll. Cell size was not affected by hormone treatments for either cell type, whereas manipulating the timing of light exposure significantly changed bundle sheath cell size and shape, influencing their length and lateral expansion. Delayed and premature exposure of developing rice leaves to light increased and reduced final leaf length, respectively, but these responses could not be explained entirely through altered cell expansion.

2 | MATERIALS AND METHODS

2.1 | Plant material and growth conditions

All experiments were performed using rice (*Oryza sativa*) cv. Kitaake. Seed was imbibed in sterile water and germinated on filter paper in darkness at 32°C for 3 days before transplanting to soil. Germinating seeds were sown into a 1:1 mix of Erin topsoil (LBS Horticulture) and washed sand, supplemented with 1x Everris Peters Excel Cal-Mag Grower fertilizer solution (LBS Horticulture) with additional 0.35% chelated iron (wt/vol) (Garden Direct). Seeds were sown into 7 cm pots and kept covered with transparent propagator lids for 4 days after sowing. All plants were grown in a controlled environment under a photoperiod of 12 h light (photon flux density of 300 µmol m^{-2} s^{-1}) and 12 h darkness, a temperature of 28°C (day) and 25°C (night) and a constant relative humidity of 60%.

2.2 | Exogenous hormone treatment

Exogenous hormone treatments comprised 5 µM 1-napthaleneacetic acid (NAA; Merck Life Science UK Ltd.) (0.1% vol/vol Tween20), 5 µM 6-benzylaminopurine (BAP; Merck Life Science UK Ltd.) (0.1% vol/vol Tween20), 100 µM gibberellin A3 (GA_3; Melford Laboratories Ltd.) (0.1% vol/vol ethanol, 0.1% vol/vol Tween20) and mock solution (0.1% vol/vol ethanol; 0.1% vol/vol Tween20). Concentrations were selected to exceed the concentrations known to elicit rice growth responses at the gross anatomical level (Ding et al., 2017; Singh et al., 2015; Ueguchi-Tanaka et al., 2005) or where a cellular response to direct application was verified (Yang et al., 2017). Hormone treatments were applied to whole plants as a foliar spray, taking care to supply an equal quantity of hormone solution (five sprays per plant). Hormone solutions were stored at 4°C and brought to room temperature before use. Plants were treated every 2–3 days from 4 days after sowing. Measurements of leaf 4 length were taken at 15 days after sowing, measuring six plants per treatment. On the same day leaf 4 blade tissue was harvested from the same plants (5 mm either side of the mid-point) for single cell isolation. Leaf tissue was imaged from three plants per treatment, and from five mesophyll and five bundle sheath cells per plant.

2.3 | Delayed light exposure

Imbibed seed were sown in a darkroom under green light at a density of three seeds per pot. All leaf tissues were harvested 8 days after sowing from plants grown under three different light regimes (light-grown, dark-grown and delayed-light). Light-grown control plants were exposed to light from germination onwards, with incubation under a transparent propagator lid for the first 4 days after sowing. Plants under dark-grown and delayed-light treatments were sown alongside these but initially kept in complete darkness. Delayed-light plants were kept in darkness for 6 days after sowing, with 2 days exposure to standard light-grown conditions before harvesting. Dark-grown plants were kept entirely in darkness for 8 days and only exposed to standard light-grown conditions immediately before tissue harvesting. Tissues for analysis were all harvested from leaf 2. Leaf 2 blade length was measured from six plants per treatment and tissue was harvested for single cell isolation on the same day, taking 5 mm either side of the blade mid-point. Tissue was imaged from six plants per treatment, and from five mesophyll and five bundle sheath cells per plant. In an experiment performed to determine dark-grown leaf 2 growth responses to delayed light exposure, 48 plants were grown under dark-grown treatment as above. At 7 days after sowing 24 plants were exposed to standard light-grown conditions and leaf 2 blade length was measured at 0, 0.5, 1, 2, 3, 4, 6 and 24 h after light exposure. Length was measured from three light-induced and three corresponding plants not exposed to light at each timepoint. For consistency, three independent plants were measured per timepoint under each treatment.

2.4 | Premature light exposure

Imbibed seeds were sown at a density of three seeds per pot and exposed to standard light-grown conditions from seed sowing onwards. Premature exposure of leaf 4 to light was achieved through the manual removal of the surrounding leaf 3. On the day that the tip of the leaf 4 primordium emerged from within the sheath of leaf 3 (6–8 days after sowing), leaf 3 was removed by initially tearing the blade in half lengthways (along the proximodistal axis) using two pairs of watchmakers' forceps and then extending the tear down through the sheath to near the plant base. As a separate treatment, removal of the leaf 3 blade was achieved by cutting with scissors at or just above the leaf 3 ligule. One replicate of each treatment (control, leaf 4 exposure, leaf 3 blade removed) was applied per pot on the day of leaf 4 emergence, and leaf 4 length was subsequently measured every 24 h until leaf growth had ceased in all plants. Leaf 4 length was measured from 20 replicates per treatment. For leaf 4 cell measurements, leaf 4 tissue was harvested 3 days after premature

exposure and simultaneously from control plants within the same population, taking 5 mm of blade tissue above the ligule. Leaf tissue was imaged from three plants per treatment, and from 10 mesophyll, bundle sheath and epidermal cells per plant.

2.5 | Leaf tissue embedding and sectioning

Harvested tissue was cut into 2-mm-wide lengths along the leaf proximodistal axis under 100% acetone on ice and fixed in 100% acetone overnight at 4°C. Fixed tissues were embedded in Steedmans wax for sectioning following the protocol of Hua and Hibberd (2019). Embedded tissues were cut into 10-µm-thick paradermal sections using a Leica RM2035 Jung BioCut rotary microtome (Leica Microsystems (UK) Ltd.) and stretched over water on microscope slides at room temperature. Surrounding wax was removed from the sectioned tissue by incubation in 100% acetone for 1 min, after which remaining acetone was allowed to evaporate from the side surface.

2.6 | Single cell isolation

Harvested leaf tissue was cut into 2-mm-wide strips along the leaf proximodistal axis in a drop of water and immediately immersed in room temperature 4% wt/vol paraformaldehyde in 1x phosphate buffered saline (PBS) buffer (pH 6.9) (Merck Life Science UK Ltd.). Tissue was immediately placed in darkness and fixed at 4°C overnight, after which fixed tissue was stored in 1x PBS solution (pH 6.9) at 4°C. Cell walls were digested following the protocol of Khoshravesh and Sage (2018) by incubating in 0.2 M sodium-EDTA solution (pH 9.0) 55°C for 2 h, then incubating with 2% wt/vol *Aspegillus niger* pectinase (Merck Life Science UK Ltd.) dissolved in digestion buffer (0.15 M sodium hydrogen phosphate, 0.04 M citric acid; pH 5.3) at 45°C for 2 h. Digestion was stopped by incubating the samples in empty digestion buffer twice at room temperature for 30 min each. Individual cells were imaged within 24 h of cell wall digestion to prevent distortion due to cell swelling. Cells were separated by placing digested tissue on a microscope slide in 30 µL of empty digestion buffer and then physically disrupting the tissue using the base of a clean plastic 1.5 mL microcentrifuge tube. The resulting homogenate was covered with a coverslip and immediately imaged.

2.7 | Cell imaging

Sectioned tissues and isolated cells were imaged by brightfield microscopy using an Olympus BX51 microscope (Olympus UK and Ireland, Southend-on-Sea, UK; RRID:SCR_018949), recording each cell at 4–5 separate focal depths to accurately measure the morphology of individual chloroplasts throughout the cell volume. Images were captured using an MP3.3-RTV-R-CLR-10-C Micro-Publisher camera and QCapture Pro 7 software (Teledyne Photometrics). Cells were imaged with an Olympus PLN ×20 objective lens, numerical aperture 0.40, field depth 1.72 µm, resolving power 0.84 µm, field number 22 mm (Olympus UK and Ireland, Southend-on-Sea, UK). Cell and chloroplast measurements were taken from scaled images using FIJI (Schindelin et al., 2012; RRID:SCR_002285), using the multiple images captured per cell to increase the accuracy of measurement at the whole-cell level. Whole plant photographs were taken using a Cybershot DSC-HX7V digital camera (Sony). Figures were prepared using Photoshop 23.0.2 (Adobe; RRID:SCR_014199). Photographic images included in these were individually adjusted for brightness and contrast.

2.8 | Data analysis

Statistical analyses were performed in RStudio version 1.2.5033 (RStudio Team, 2020; RRID:SCR_000432) using R version 3.6.3 (R Core Team, 2020). Analysis of variance (ANOVA) and regression analyses were undertaken using the car software package (Fox & Weisberg, 2019; RRID:SCR_022137). Relative chloroplast content was calculated by dividing total chloroplast area by the corresponding cell area. The range of cell lengths within a cell type + treatment combination was standardised by creating a Z-score (each value minus the population mean, divided by the population standard deviation). As an estimate of chloroplast size the largest chloroplast visible per cell was chosen as the least-biased method of comparison. Datasets were tested for normal distributions using the Shapiro–Wilk test (Shapiro & Wilk, 1965) and variances were compared using Levene's test (Levene, 1960). Where the assumptions of ANOVA were met pairwise comparisons were made using two-tailed pairwise *t*-tests, otherwise they were made using two-tailed Mann–Whitney tests. Where multiple pairwise comparisons were made within an experiment, the *p*-values obtained were corrected post hoc to minimise false discoveries (Benjamini & Hochberg, 1995). All cell measurements, transformations and statistical test outputs are given in Datasets S1–S4. All plots were prepared with the ggplot2 software package (Wickham, 2016; RRID:SCR_014601).

3 | RESULTS

3.1 | Chloroplast biogenesis responds the size of mesophyll and bundle sheath cells but a set point is not maintained in the bundle sheath

To better understand developmental processes associated with differences in chloroplast content between rice mesophyll and bundle sheath cells we characterised the morphology of these cell types at maturity. Paradermal sections through embedded rice leaf tissue demonstrated that mature bundle sheath and mesophyll cells each develop in files parallel to the leaf vein (the leaf proximodistal axis) but the two cell types have different geometries, with the long and short axes of the bundle sheath and mesophyll cells forming parallel to the vein, respectively (Figure 1a). To image individual cells

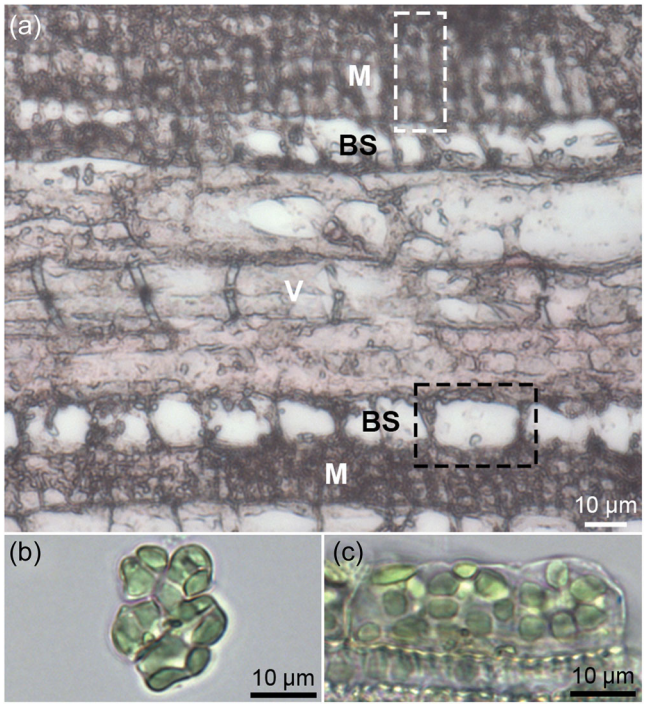

FIGURE 1 Geometry of bundle sheath and mesophyll cells within the rice leaf. (a) Brightfield microscopy image of a paradermal section through the rice leaf blade, showing a vein (V), adjacent bundle sheath cell file (BS) and mesophyll cell file (M). Individual cells are highlighted with a dashed box (BS, black; M, white) Cells are stained with Eosin Y. (b, c) Representative brightfield microscopy images of an individual rice mesophyll cell (b) and bundle sheath cell (c) in the longitudinal plane, isolated from leaf tissue by cell wall digestion for cell measurement. Both cell types are shown oriented with respect to the original leaf proximodistal axis (a) to allow comparison of measurements between cell types. Chloroplasts are visible at multiple focal planes within each cell type, with far greater overlap between chloroplasts in the mesophyll. Analysis of chloroplast characters within each cell type was conducted using images taken at multiple focal planes within each cell (see Section 2). [Color figure can be viewed at wileyonlinelibrary.com]

in sufficient detail for anatomical measurements it was necessary to separate them by cell wall digestion (Khoshravesh & Sage, 2018). We determined that under our digestion conditions rice mesophyll cells fully separated from surrounding leaf tissues retained their complex shape, with a distinct long and short axis visible (Figure 1b). Fully-isolated bundle sheath cells were visibly swollen and misshapen, so morphological measurements were restricted to bundle sheath cells still attached to the vein where they appeared morphologically normal (Figure 1c). Because of the disassociated nature of the digested leaf tissue and the cylindrical shape of leaf veins it was not possible to determine whether isolated cells remained in their original orientation after digestion- for example, bundle sheath cells could be in either the paradermal or longitudinal leaf planes. To minimise any potential bias arising from this ambiguity multiple cells of both cell types were imaged per leaf. Digestion and disruption of the leaf tissue also made it impossible to distinguish whether the bundle sheath cells imaged were associated with the midvein, lateral veins or rank 1 intermediate veins within the rice leaf, which are established sequentially during leaf development (Nelson & Dengler, 1997; Sedelnikova et al., 2018). To avoid further potential bias in bundle sheath morphology caused by possible differences between vein classes we imaged individual bundle sheath cells from multiple different veins per sample. To establish a common frame of reference between isolated cells of both cell types we defined 'cell length' as corresponding to the cell axis originally parallel to the vein before digestion (i.e., the long axis of bundle sheath cells and short axis of mesophyll cells, Figure 1a), 'cell width' as the axis originally at right-angles to the vein (the leaf mediolateral axis, Figure 1a) and 'cell area' as the cross-sectional area visible through these two axes (Figure 1b,c). This approach established a consistent basis for comparison of morphology (and thus developmental processes) between these two cell types.

As expected, the area of cell occupied by chloroplasts (hereafter relative chloroplast content) was significantly lower for bundle sheath than mesophyll cells ($p < 0.05$; Figure 2a). Although no statistically significant difference was detected between the total area of chloroplast per mesophyll or bundle sheath cell ($p > 0.05$, Figure 2b) both the number and size of chloroplasts was lower in bundle sheath cells ($p < 0.05$, Figure 2c,d). We note that the two-dimensional method of image analysis employed here could underestimate chloroplast area in mesophyll cells, where chloroplasts can overlap (Figure 1b). Bundle sheath cell area and cell length parallel to the leaf proximodistal axis (along the vein) were greater than mesophyll cells ($p < 0.05$), whereas cell width (parallel to the leaf mediolateral axis) was smaller ($p < 0.05$; Dataset S1). Analysis of variance indicated that individual cell measurements were statistically independent of the plant from which they were isolated ($p > 0.05$; Dataset S1) and so each cell could be treated as an independent replicate. Bundle sheath cells demonstrated greater variance in cell area and length than mesophyll cells ($p < 0.05$, Levene's test), but variance in cell width was similar ($p = 0.055$). Similarly, except for size, chloroplast characters of bundle sheath cells (including relative chloroplast content) had greater variance than those of mesophyll cells ($p < 0.05$). These results confirmed that individual cells within the bundle sheath are more variable in their morphology than mesophyll cells, but the relative chloroplast content of the bundle sheath is consistently reduced compared with mesophyll cells. Our results suggest that both increased cell size and reduced chloroplast biogenesis (smaller, fewer chloroplasts) in bundle sheath cells contribute to the lower relative chloroplast content compared with the mesophyll.

To investigate the relationship between cell and chloroplast development in more detail, regression analysis was used (Figure 2e–h). For mesophyll cells, no significant relationship was detected between relative chloroplast content and cell area ($p = 0.4710$; Figure 2e). In contrast, for the bundle sheath a significant negative relationship was apparent ($p = 0.0031$; Figure 2f). In both cell types, relative chloroplast content was found to be dependent on the individual plant from which cells were isolated but this factor was independent of the relationship with cell area (Figure S1a,b,

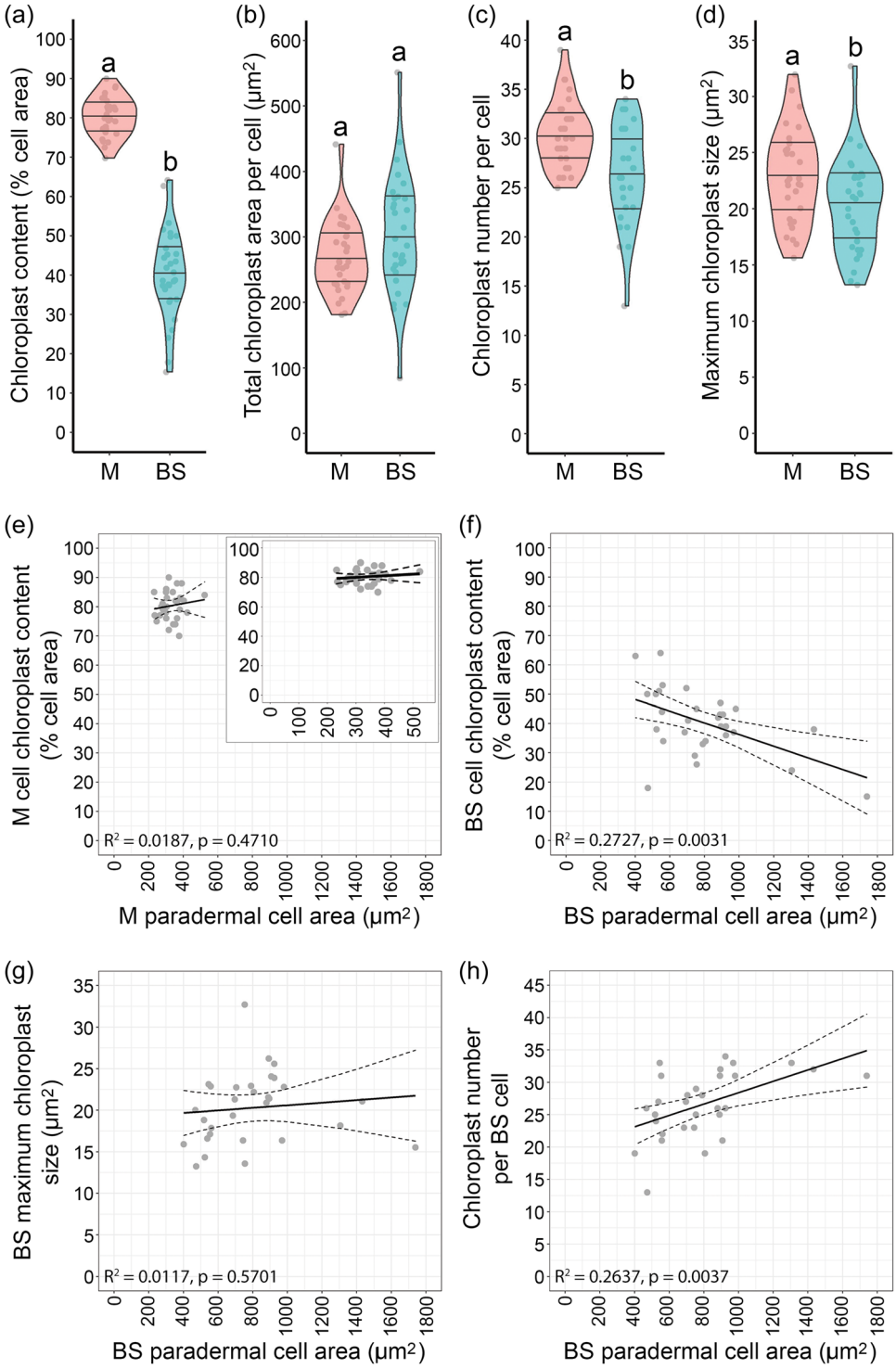

FIGURE 2 Chloroplast biogenesis is reduced in rice bundle sheath compared with mesophyll cells. (a–d) Comparison of relative chloroplast content (a), total chloroplast area (b), chloroplast numbers (c) and maximum chloroplast size (d) per cell between mature mesophyll (M) and bundle sheath (BS) cells isolated from leaf 4. Violin plots represent data distribution density, with their width corresponding to frequency of datapoints (grey circles) at that value. Median value and upper and lower quartiles are represented by the middle, upper and lower horizontal lines, respectively. Different letters denote statistically significant differences ($p < 0.05$) between BS and M cells within each plot. Comparisons of relative chloroplast content and chloroplast size were made using transformed datasets to meet the assumptions of statistical tests (see Dataset S1). (e–h) Linear regression analyses of relative chloroplast content against longitudinal cell area for M (e) and BS cells (f), and of BS cell chloroplast size (g) and numbers (h) against cell area, respectively. Graphs show the line of best fit and 95% confidence interval of the linear model fitted to the data (grey circles). Insert in (e) shows same data with x-axis re-scaled to better visualise the distribution of cell areas captured. $n = 30$ (five cells of each type measured from six individual plants, see Figure S1). [Color figure can be viewed at wileyonlinelibrary.com]

Dataset S1). To investigate whether the apparent negative relationship in the bundle sheath was an artefact caused by swelling of cells during cell wall digestion, we tested the relationship between relative chloroplast content and cell length. Because the bundle sheath cells measured were still physically connected to the vascular bundle via their cell wall and frequently bounded by adjacent bundle sheath cells also connected to the vein (Figure 1c) the potential for them to swell along this specific axis due to osmotic pressure is constrained, whereas lateral cell swelling (increasing cell width) could influence cell area measurements. We confirmed a significant negative relationship between bundle sheath relative chloroplast content and cell length ($p = 0.0010$; Dataset S1). Given the differences in variance observed between mesophyll and bundle sheath cell areas, we tested whether the difference in significance between cell types was caused by these differences in variance by transforming both bundle sheath and mesophyll cell areas to a standardised Z-scale within each cell type to equalise variances (see Section 2). Re-analysis using this transformed scale confirmed that the relationships in chloroplast content and standardised cell area was significantly different between the two cell types ($p = 0.005$; Figure S1c), and that the plant from which cells were isolated remained a significant independent factor (Figure S1d). When tested separately, a significant negative relationship was still found between bundle sheath chloroplast content and standardised cell area ($p = 0.0012$) but no significant relationship was found for the mesophyll ($p = 0.4001$; Dataset S1). A difference in the cell-chloroplast relationships between the rice mesophyll and bundle sheath was thus confirmed.

To understand the mechanism underlying this difference we tested the effect of cell size on separate chloroplast characteristics in each cell type. No statistically significant relationship was detected between bundle sheath cell area and chloroplast size ($p = 0.5701$; Figure 2g) but positive relationships were found between cell area and the number of chloroplasts ($p = 0.0037$, Figure 2h) and between cell area and total chloroplast area per cell ($p = 0.0003$; Dataset S1). Similar positive relationships were also detected for mesophyll cells ($p < 0.05$; Figure S1e, Dataset S1), as was a marginal positive relationship between mesophyll cell area and chloroplast size (Figure S1f). When regression was performed on a standardised Z-scale, the relationships between cell area and total chloroplast area, chloroplast number or chloroplast size were not significantly affected by cell type ($p > 0.05$; Dataset S1). Mindful of the limitations of two-dimensional cell analysis, we tested the effect of this on our interpretation of changing chloroplast number with bundle sheath cell size. Because published transverse sections of rice bundle sheath cells are approximately circular in cross-section (Wang et al., 2017b) we modelled individual bundle sheath cells as cylinders using cell length and width measurements (Figure S2a–d). The number of chloroplasts per cell still showed a positive relationship with estimated cell volume ($p = 0.0012$; Figure S2e) supporting the robustness of this relationship. These results indicate that chloroplast biogenesis in the bundle sheath is responsive to increasing cell size, but this response is not sufficiently strong to achieve a constant 'set-point' chloroplast content in this cell type. Our finding that chloroplast number changes with cell size in the rice mesophyll whilst relative chloroplast content remains stable size is similar to findings from wheat (Ellis and Leech, 1985) and supports the notion that mesophyll chloroplast biogenesis is regulated in a cell size-dependent manner. We conclude that mechanisms coordinating cell and chloroplast development are active in both mesophyll and bundle sheath cells, but propose that they are differentially regulated between the two cell types.

3.2 | Cytokinin and gibberellin increase chloroplast biogenesis in the bundle sheath in a cell size-dependent manner

The ability of CK, auxin and GA to affect cell and chloroplast development in rice was tested by exogenous application of each hormone during development of leaf 4. Exogenous treatments were applied to whole plants, using concentrations in excess of the minimum required to elicit developmental responses (see Section 2) to ensure penetration of the treatment to the enclosed primordium. The length of leaf 4 at maturity was not altered by treatment with CK or auxin ($p > 0.05$) but in GA-treated plants leaf blade and leaf sheath length was increased compared with mock-treated controls ($p < 0.05$; Figure 3a). The proportion of blade to sheath was significantly reduced by GA treatment ($p < 0.05$; Dataset S2). None of the hormones had a detectable impact on the longitudinal area of mesophyll or bundle sheath cells ($p > 0.05$; Figure 3b), nor on cell length (Dataset S2). As GA application generated a longer leaf blade but cell length was not changed, we infer that cell division in the leaf blade was prolonged by this treatment.

The effect of exogenous hormone treatments on the chloroplast characteristics of the bundle sheath and mesophyll were tested. Hormone treatments did not alter relative chloroplast content (Figure 3c), the number of chloroplasts per cell (Figure 3d) nor maximum chloroplast size (Figure 3e) compared with the mock treatments. In the bundle sheath, a significant negative relationship between cell area and chloroplast content was detected under mock treatment ($p < 0.05$; Figure 3f) but under auxin, CK and GA treatment this relationship was no longer statistically significant ($p > 0.05$; Figure 3f). Comparison of each separate hormone treatment against the mock confirmed that the relationship between cell area and relative chloroplast content was significantly altered by each hormone ($p < 0.05$) to become more positive (Figure S3a–c, Dataset S2). Bundle sheath cells under mock and hormone treatments all showed a significant positive relationship between cell area and total chloroplast area ($p < 0.05$) but this relationship became stronger (i.e. the p-value decreased) under each hormone treatment (Figure 3g). Separate comparisons against the mock treatment confirmed that CK and GA (but not auxin) each had a statistically significant effect on the relationship between cell area and total chloroplast area ($p < 0.05$), making this relationship more positive (Figure S3d–f). The effect of GA remained robust when potential outlier datapoints were excluded (Figure S3h, Dataset S2).

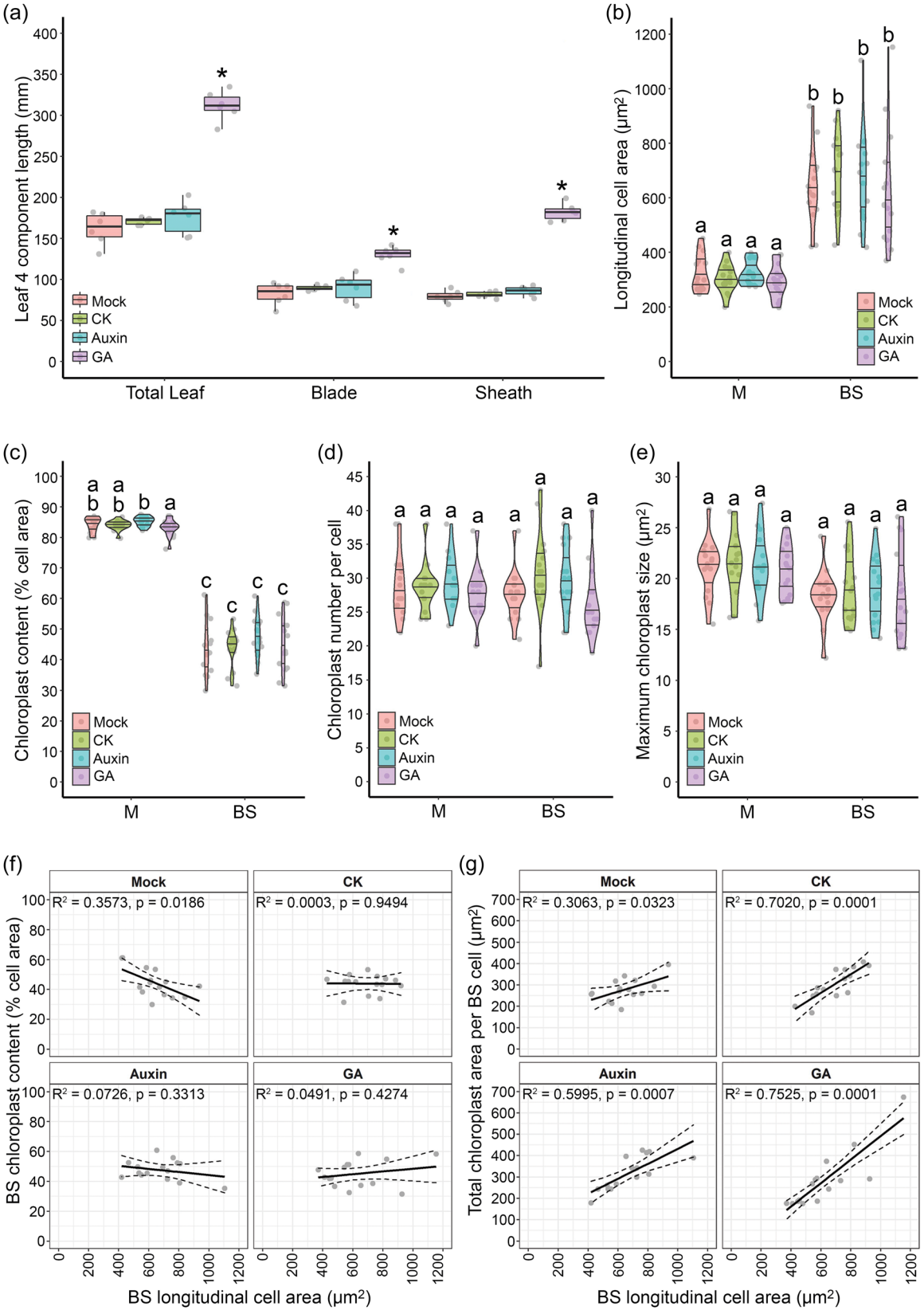

FIGURE 3 (See caption on next page).

The results demonstrate that the relationship between cell size and chloroplast content in the rice bundle sheath is capable of responding to the hormones CK and GA, resulting in more chloroplast biogenesis occurring in larger cells.

In contrast to the bundle sheath, no significant relationship was found between mesophyll cell area and chloroplast content under mock or any hormone treatment ($p < 0.05$; Figure S4a), and no individual hormone treatment had a significant effect on this relationship compared with the mock (Dataset S2). To directly test the effects of hormone treatments between the mesophyll and bundle sheath, regression was performed against standardised cell areas. A significant three-way interaction effect between cell area, hormone treatment and cell type ($p < 0.05$) was detected when the relationships between standardised cell area and total chloroplast area per cell and between standardised cell area and chloroplast number were compared between mock and each separate hormone treatment (Figure S4b,c). These data indicate a significant difference in the response of these two relationships to hormone treatments between the bundle sheath and mesophyll. Additional regression analysis within each cell type (Dataset S2) confirmed significant positive effects of CK and GA on the relationship between standardised bundle sheath area and total chloroplast area ($p < 0.05$) but found a significant negative effect of all three hormone treatments on this relationship in the mesophyll ($p < 0.05$; Figure S4b). A significant positive effect of GA was detected on the relationship between bundle sheath standardised cell area and chloroplast number ($p = 0.0329$) whereas no corresponding significant effect was found in the mesophyll ($p = 0.4846$; Figure S4c). CK also had a significant positive effect on this relationship specifically in the bundle sheath ($p = 0.0292$) if the plant from which cells originated is included as a factor (Dataset S2). CK had a further significant negative effect on the relationship between standardised area and chloroplast size in the mesophyll ($p = 0.0262$) that was not seen in the bundle sheath ($p = 0.5296$; Dataset S2). Our analysis thus shows that both cell types responded to exogenous hormone treatments but that chloroplast-cell relationships responded differently between the bundle sheath and mesophyll.

3.3 | Darkness reduces bundle sheath length

The effect of light exposure on cell and chloroplast development was tested by experimentally changing the timing of their exposure to light. Prolonged absence of light represents an abiotic stress in rice development (Gad et al., 2021), and to avoid conflating stress-related growth responses with direct light responses the effect of delaying light exposure was tested in leaf 2. This leaf is already established in the embryo, is not dependent on apical meristem activity (Itoh et al., 2005), and could successfully complete development by 7 days after germination in darkness (Figure 4a; Figure S5a). Cell morphology was compared in leaf 2 eight days after germination, isolated from control plants grown in the light or in darkness, plus a third treatment in which exposure to light was delayed until 6 days after germination (see Section 2). Mature blade length was significantly increased in both dark-grown and delayed-light plants compared with light-grown controls ($p < 0.05$; Figure 4b). Green chloroplasts were visible in bundle sheath and mesophyll cells of light- but not dark-grown plants (Figure 4c,d and 4f,g), whereas in delayed-light plants chloroplasts from both cell types had only partially greened (Figure 4e and 4h). In contrast to increasing leaf length, the mean areas of mesophyll and bundle sheath cells were both significantly reduced in the dark-grown leaves compared with light-grown controls ($p < 0.05$; Figure 4i). Interestingly, mesophyll cell area under delayed light was similar to dark-grown cells ($p > 0.05$) but bundle sheath cell area was intermediate between dark-grown and light-grown controls ($p > 0.05$; Figure 4i). Thus, bundle sheath and mesophyll cell development in leaf 2 responded differently to delayed light.

Dissection of the bundle sheath growth response to delayed light suggests that both cell expansion and cell division are light-regulated in this tissue. Length and width of mesophyll cells were reduced under both the dark-grown and delayed light treatments compared with light-grown controls ($p < 0.05$) (Figure 4j,k). However, while bundle sheath cell length was also reduced under both dark treatment and delayed exposure to light ($p < 0.05$; Figure 4j), cell width under delayed light treatment was similar to light-grown controls ($p > 0.05$; Figure 4k). The reduced length of bundle sheath

FIGURE 3 Exogenous hormone treatments alter the rice bundle sheath cell-chloroplast relationships. (a) Comparison of mature leaf 4 length characteristics between mock (0.1% EtOH vol/vol), 5 μM BAP, 5 μM NAA and 100 μM GA_3 treatments (as shown). Boxplots represent the 25th −75th percentile (box), median value (mid-line) further datapoints within 1.5x of the interquartile distance (whiskers). Asterisks denote significant difference ($p < 0.05$) from mock treatment. $n = 6$. (b–e) Comparison of bundle sheath (BS) and mesophyll (M) cell longitudinal area (b), relative chloroplast content (c), number of chloroplasts per cell (d) and maximum chloroplast size (e) from mature leaf 4 between mock (0.1% EtOH vol/vol), 5 μM BAP, 5 μM NAA and 100 μM GA_3 treatments (as shown). Violin plots represent data distribution density, with their width corresponding to frequency of datapoints (grey circles) at that value. Median value and upper and lower quartiles are represented by the middle, upper and lower horizontal lines, respectively. Different letters denote a significant difference ($p < 0.05$) between cell type + hormone treatment combinations within each plot. Comparisons within all characters except chloroplast size were made using transformed datasets to meet the assumptions of statistical tests (Dataset S2). (f, g) Regression analyses of relative chloroplast content (f) and total chloroplast area per cell (g) against cell area in BS cells under mock (0.1% EtOH vol/vol), 5 μM BAP, 5 μM NAA and 100 μM GA_3 treatments (as shown), showing the line of best fit and 95% confidence interval of the model fitted to the data (grey circles). All p-values and R^2 values are from regression analyses of the data against cell area within each treatment, denoting the significance of cell area as an explanatory variate and the explanatory power of the fitted model, respectively. $n = 15$ (5 cells measured from three individual plants). [Color figure can be viewed at wileyonlinelibrary.com]

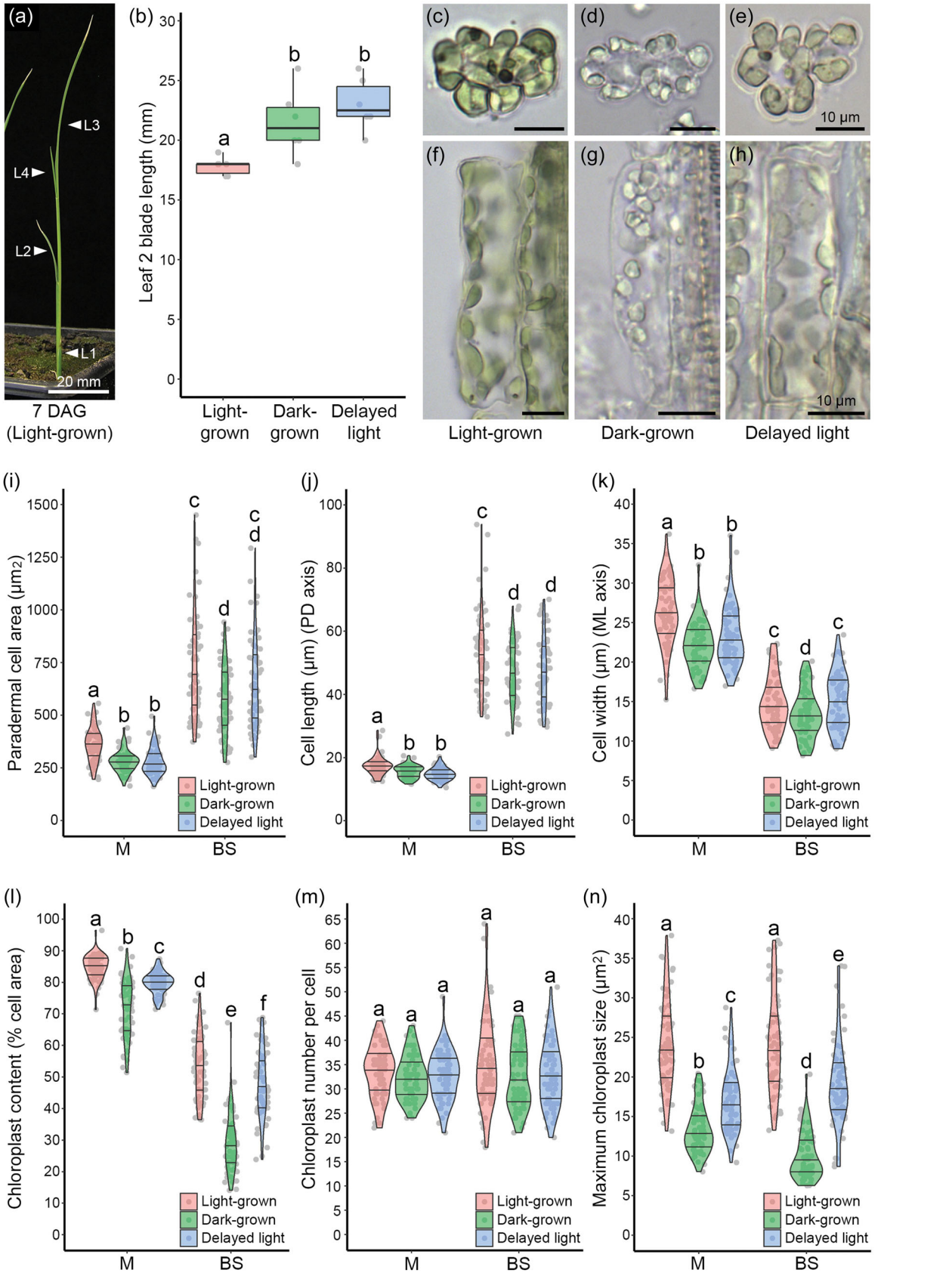

FIGURE 4 (See caption on next page).

cells in the dark-grown and delayed light treatments was associated with a changed distribution caused by the absence of the longest cells (Figure 4j): in light-grown controls maximum bundle sheath cell length was 2.9 times that of the shortest cell, but under dark-grown and delayed-light treatments this was reduced to 2.5 and 2.4, respectively (Dataset S3). Variance in bundle sheath cell lengths was similar between all three treatments ($p > 0.05$, Levene's test), and while in light-grown controls the longest cells meant that the data were not normally distributed ($p = 0.007$, Shapiro–Wilk test), dark-grown and delayed-light populations were normally distributed ($p = 0.228$ and $p = 0.067$, respectively). Chloroplast content of both mesophyll and bundle sheath cells from leaves subjected to delayed light was intermediate between light-grown and dark-grown controls and significantly different from both ($p < 0.05$; Figure 4l). The number of chloroplasts per cell was not affected by delayed light or the absence of light ($p > 0.05$; Figure 4m). However, under delayed light treatment chloroplasts were intermediate in size between those from both dark-grown and light-grown controls ($p < 0.05$; Figure 4n). Our findings demonstrate that bundle sheath and mesophyll cells have overlapping but distinct responses to delayed light exposure, with light specifically triggering lateral expansion of bundle sheath cells. The reduced length observed in both cell types is seemingly inconsistent with the finding that delayed light also increases the length of leaves from which these cells were isolated. We propose that this could be explained by prolonged cell division.

3.4 | Premature light reduces leaf but not bundle sheath length

The effect of premature light exposure on leaf development was tested using leaf 4, which arises from the shoot apex after germination (Itoh et al., 2005) and develops inside the sheath of leaf 3, where it necessarily experiences a reduced light intensity. Under our growth conditions leaf 4 first emerged from the leaf 3 sheath 6–8 days after germination (Figure 5a). In contrast leaf 2 emerged almost immediately after germination, making it unsuitable for these experiments.

A premature light treatment was applied to the leaf 4 primordium by mechanical removal of leaf 3 (Figure 5b). For technical reasons leaf 3 could only be removed when the tip of leaf 4 had emerged. Because isotope tracing experiments demonstrate that photosynthate from leaf 3 is exported to newly-developing leaves (Tanaka, 1961), to distinguish between direct effects of light exposure on leaf 4 growth and indirect effects caused by a reduction in photosynthate supply, an additional control treatment was included whereby the blade of leaf 3 was removed whilst leaf 4 remained within the leaf 3 sheath (Figure 5c). The subsequent length of leaf 4 was recorded (Figure 5d–f). In some cases mature leaf 4 exhibited visible damage, apparently arising from the mechanical removal of leaf 3 (Figure S5b,c). To avoid conflating this with the effects of light exposure such plants were excluded from our analysis. Similarly, a dark-grown control was not included due to abiotic stress and skotomorphogenic responses masking direct light-dependent responses.

When exposed prematurely to light, leaf 4 was significantly shorter than controls ($p < 0.05$). Removal of the leaf 3 blade alone ('L3 control') did not reduce final leaf length ($p > 0.05$) but instead caused slower growth ($p < 0.05$, Figure 5g). Premature light treatment did not cause early termination of leaf 4 growth but rather slower growth over a similar time period (Figure 5g). Under both control treatments the leaf 4 blade fully emerged from the leaf 3 sheath 3 days after L3 removal, by which time the blade had ceased growing in all treatments (Figure S5d,e). Under both premature light and L3 control treatments the leaf 4 sheath reached its mature length 1 day later than the control (Figure S5f). Under premature light the final lengths of both the leaf 4 blade and sheath were reduced compared with controls ($p < 0.05$), with the greatest reduction in the sheath, as evidenced by a significant reduction in the sheath as a proportion of total leaf length ($p < 0.05$; Figure S5e–g). This differential effect corresponds both with the sheath being developmentally younger than the blade and, because it is at the base of the leaf, potentially being more shaded by the leaf 3 sheath surrounding it. Thus,

FIGURE 4 Delayed light exposure increases final rice leaf length but reduces the length of photosynthetic cells. (a) WT rice plant (cultivar Kitaake) 7 days after germination (DAG) under light-grown conditions. The relative position of the four visible leaves (L1-L4) are indicated (arrowheads). (b) Comparison of leaf 2 blade length between 8-day-old light-grown, dark-grown and delayed light treatments (as shown). Boxplots represent the 25th–75th percentile (box), median value (mid-line) and further datapoints within 1.5x of the interquartile distance (whiskers). Different letters denote statistically significant differences ($p < 0.05$) between light treatments. $n = 6$. (c–h) Experimental light treatments caused visible changes in chloroplast morphology of both BS (c–e) and M cells (f–h). Compared to cells from 8-day-old control plants exposed to light from germination onwards ('Light-grown'; c, f), cells from 8-day-old plants not exposed to light ('Dark-grown') contained etioplasts visibly lacking chlorophyll (d, g). Cells from 8-day-old plants exposed to delayed light at 6 DAG ('Delayed light') exhibited visible greening compared to dark-grown controls but not to the extent of light-grown controls (e, h). (i–n) Comparison of cell longitudinal area (i), length (j), width (k), relative chloroplast content (l), chloroplast number per cell (m) and maximum chloroplast size (n) of mesophyll (M) and bundle sheath (BS) cells isolated from the 8-day-old leaf 2 blade between light-grown, dark-grown and delayed light treatments (as shown). Violin plots represent data distribution density, with their width corresponding to frequency of datapoints at that value. Median value and upper and lower quartiles are represented by the middle, upper and lower horizontal lines, respectively. Different letters denote a significant difference ($p < 0.05$) between cell type + light treatment combinations within each plot. Comparisons within all characters except leaf length were made using transformed datasets to meet the assumptions of statistical tests (see Dataset S3). $n = 60$ (10 cells of each type measured from six individual plants per treatment). [Color figure can be viewed at wileyonlinelibrary.com]

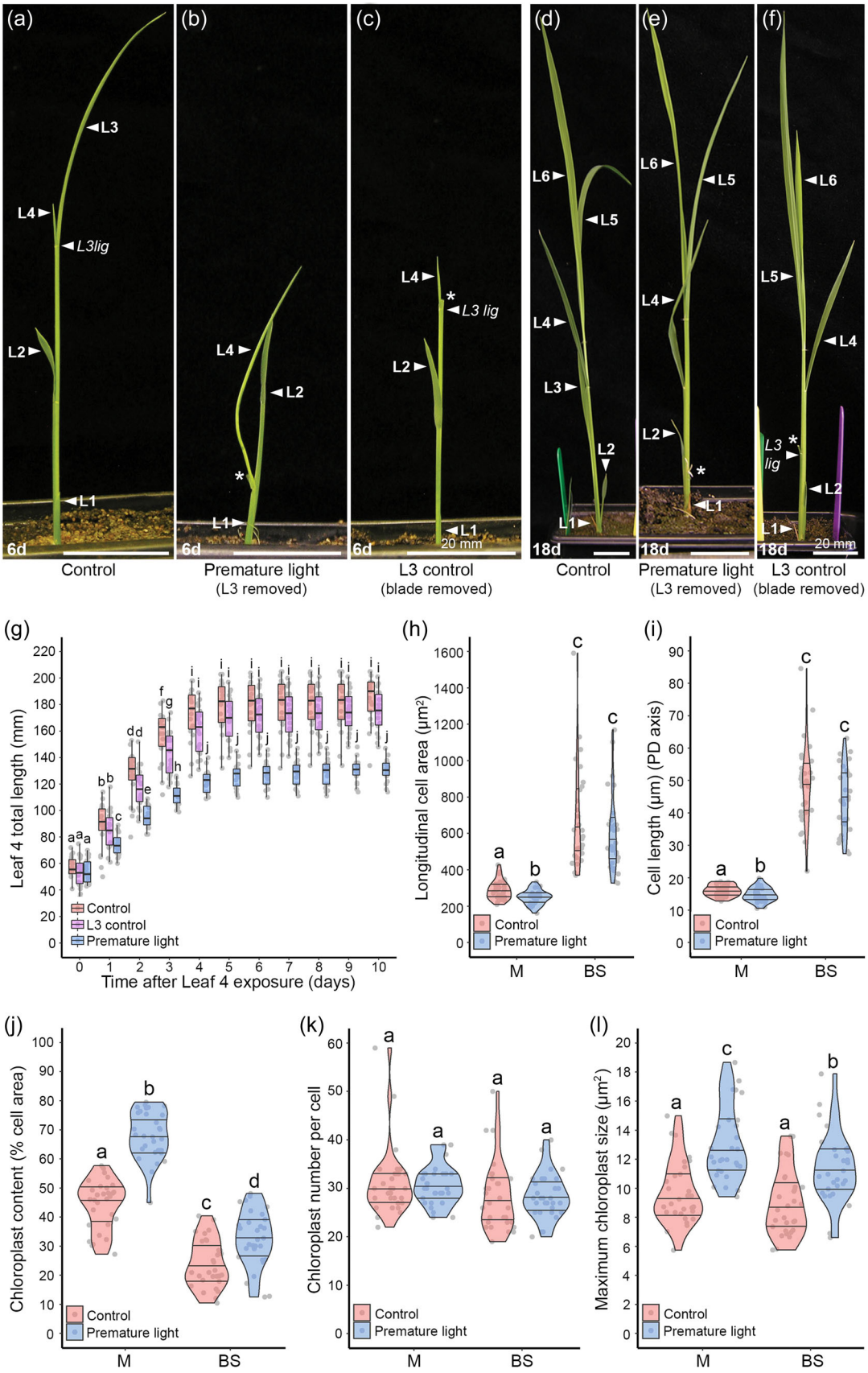

FIGURE 5 (See caption on next page).

premature light treatment was likely more extreme for this tissue. These results thus identify an effect of light exposure that negatively regulates leaf 4 growth that is separable from effects promoting the rate of leaf 4 growth attributable to the nutritive role of leaf 3 (Tanaka, 1961).

To provide insight into how premature light exposure causes reduced leaf length, the leaf 4 primordium was prematurely exposed (Figure 5c) and cell morphology at the base of the blade compared with those of control plants after 3 days. In control plants the sampled blade tissue was still surrounded by the leaf 3 sheath at harvesting but cell expansion was already complete (Figure S6a,b). This stage was selected to avoid any effects of light on mesophyll cell development being masked due to chloroplast crowding. Premature light significantly reduced the area and length of mesophyll cells ($p < 0.05$; Figure 4h,i) but no statistically significant effects on bundle sheath cell area or length were detected ($p > 0.05$). As a control, epidermal cells were also tested, and these exhibited a significant reduction ($p < 0.05$) in area and length when prematurely exposed to light similar to the mesophyll (Figure S6c–e). Chloroplast content was significantly increased in prematurely exposed mesophyll and bundle sheath tissue compared with cells from control plants ($p < 0.05$, Figure 5j) demonstrating that both cell types had perceived the premature light signal. This was not associated with an increase in the number of chloroplasts ($p > 0.05$, Figure 5k) but instead an increase in chloroplast size was detected ($p < 0.05$, Figure 5l). These results suggest that cell development is affected by premature light exposure but that cell types responded differently. Cell expansion is inhibited in the epidermis and mesophyll layers, consistent with the reduced leaf growth and final length observed under premature light, but not in the bundle sheath.

4 | DISCUSSION

4.1 | Chloroplast biogenesis in rice bundle sheath cells responds to changing cell size

Mechanisms that co-ordinate chloroplast and cell development to achieve 'set-point' chloroplast content, and how different set-point values are achieved in different cell types during leaf development, are unclear. We investigated this phenomenon by comparing two cell types within the rice leaf (mesophyll and bundle sheath) that possess different chloroplast contents at maturity (Wang et al., 2017b). This same cell type divergence has been observed in many other species (Khoshravesh et al., 2016, 2020; Kinsman and Pyke, 1998; McKown & Dengler, 2007; Williams et al., 1989). We show that chloroplast content in the bundle sheath is cell size-dependent: as cell area increases so does total chloroplast area per cell. However, chloroplast biogenesis does not increase sufficiently to keep pace with cell expansion, creating a negative relationship between relative chloroplast content and cell size. Chloroplast numbers but not size increase with increasing cell size, a response also observed in the rice mesophyll, where we confirmed that total chloroplast area and chloroplast number both responded to cell size, supporting active set-point control in this cell type. These findings are consistent with evidence of changing chloroplast numbers in the mesophyll cells of both *A. thaliana* and wheat (Ellis and Leech, 1985; Pyke & Leech, 1991), but in *A. thaliana* altered chloroplast numbers have been shown to be an outcome rather than a driver of increased chloroplast content (Miyagishima et al., 2006; Okazaki et al., 2009; Pyke & Leech, 1992; Pyke et al., 1994; Schmitz et al., 2009). We conjecture that the same mechanism coordinating cell and chloroplast development is active in both bundle sheath and mesophyll cells and that the reduced relative chloroplast content of the bundle sheath could arise through differential regulation of a conserved cell size-dependent network.

Prior publications and our analysis use two-dimensional imaging of three-dimensional cells, and so information relating to cell depth and volume was not captured. Because of this, two-dimensional imaging is likely to under-report the number of chloroplasts in mesophyll cells where they are more closely packed, and uniformly over-estimate the relative chloroplast content of cells by area compared with 3-dimensional imaging (Lee et al., 2023). Furthermore, changes in plant cell area or volume may not accurately reflect changes in the quantity of cytoplasm due to the presence and expansion of the internal vacuole, which may not be a linear relationship (Kaiser & Scheuring, 2020). It is not known what physical

FIGURE 5 Premature exposure of leaf 4 primordia to light reduces both final leaf 4 length and expansion of mesophyll but not bundle sheath cells. (a–f) Premature light exposure treatments, when applied to leaf 4 at emergence (a, control; b, leaf 3 removed prematurely exposing leaf 4 primordium; c, leaf 3 blade only removed) and subsequent whole plant phenotypes at leaf 4 maturity (d, control; e, leaf 3 removed; f, leaf 3 blade only removed). The positions of leaves 1–6 (L1–L6) and the ligule of leaf 3 (L3 lig) are indicated by arrowheads. Asterisks denote the point at which leaf 3 has been cut. (g) Comparison of leaf 4 length between control, leaf 3 control and premature light treatments over time from the point of leaf 4 exposure. Boxplots represent the 25th–75th percentile (box), median value (mid-line) and further datapoints within 1.5x of the interquartile distance (whiskers) over raw data (grey circles). Different letters denote statistically significant differences ($p < 0.05$) between light treatment + time combinations. All raw data and *p*-values are given in Dataset S4. $n = 20$. (h–l) Comparison of the longitudinal cell area (h), cell length (i), relative chloroplast content (j), chloroplast number (k) and chloroplast size (l) of mesophyll (M) and bundle sheath (BS) cells between control and early light treatments. Violin plots represent data distribution density, with their width corresponding to frequency of datapoints at that value. Median value and upper and lower quartiles are represented by the middle, upper and lower horizontal lines, respectively. Cell type + treatment combinations denoted with different letters within each plot are significantly different from each other ($p < 0.05$). Comparisons within all characters were made using transformed datasets to meet the assumptions of statistical tests (see Dataset S4). $n = 30$ (10 cells of each type measured from three individual plants per treatment). [Color figure can be viewed at wileyonlinelibrary.com]

change chloroplast biogenesis is sensing and responding to during cell expansion, and so this and the technical limitations described above must be borne in mind when considering correlative results. Despite this, geometric extrapolation of bundle sheath cell volumes from our two-dimensional data still supports a positive relationship between cell size and chloroplast numbers, suggesting that the relationship detected here is robust.

4.2 | Bundle sheath cell-chloroplast relationships are responsive to exogenous phytohormones

The hormones auxin, CK and GA have previously been identified as regulators of cell division (auxin, CK, GA; Achard et al., 2009; Claeys et al., 2012; Hu et al., 2003; Kieber & Schaller, 2018; Schruff et al., 2006), cell expansion (auxin, GA; Ikeda et al., 2001; Löfke et al., 2015) and chloroplast division (CK, GA; Cortleven & Schmülling, 2015; Jiang et al., 2012), and as such are strong candidates to co-ordinate these processes. We found that the relationship between total chloroplast area and cell size in the bundle sheath was significantly enhanced by CK and GA treatment but mean cell size was unaffected, indicating that chloroplast biogenesis was increased in a cell size-dependent manner. For GA this could be explained by increasing chloroplast number in larger cells, consistent with its previously proposed function. Hormone treatments in this study did not affect the mesophyll set-point relationship, but we detected contrasting positive and negative effects on the relationship between cell size and chloroplast biogenesis between bundle sheath and mesophyll cells, respectively. These differential responses might relate to different endogenous hormone concentrations between the two cell types, the high chloroplast content already present within mesophyll cells or potentially greater exposure of mesophyll cells to exogenous treatments. Given their small magnitude and the potential for measurement bias from overlapping chloroplasts in crowded cells discussed above, we cannot exclude the possibility that the mesophyll hormone responses identified could represent stochastic variation that the two-dimensional analysis is not sufficiently sensitive to exclude. Our analysis nevertheless demonstrates that cell size-dependent regulation of chloroplast biogenesis in the bundle sheath can be altered by CK and GA, and although the treatments applied here were not at concentrations physiologically normal for plant cells, our results suggest that endogenous hormone signalling could play a role in establishing the set-point in different cell types during leaf development.

4.3 | The timing of light exposure differentially regulates development of different leaf cell types

Our results support the notion that timing of light exposure during rice leaf development is a significant environmental regulator of cell morphologies. Experimentally delaying and accelerating the exposure to light caused leaves to be longer and shorter at maturity, respectively. Transverse cell expansion in response to high light has previously been reported, but not quantified (Murchie et al., 2005). Within the leaf we detected a combination of overlapping and differential responses to light exposure between bundle sheath and mesophyll cell development. When exposed to light prematurely, the expansion of mesophyll cells parallel to the leaf proximodistal axis was reduced, whereas bundle sheath cell length was unaffected. Conversely, we determined that bundle sheath cells remained capable of lateral expansion in response to light if exposure was delayed, whereas mesophyll cells were not. It is possible that the observed differential cellular responses reflect reduced light penetrating to the bundle sheath. However, chloroplast retrograde signalling is known to mediate some aspects of light-dependent responses in *A. thaliana* de-etiolation and leaf development (Andriankaja et al., 2012; Chan et al., 2016) and as greening chloroplasts were observed in both cell types these data argue against this possibility. Of course, interception of light by surrounding cell layers could alter the light level and quality perceived by bundle sheath and mesophyll. The specific light environment experienced by the early rice leaf primordium developing within older leaves has not been quantified but some light is likely to be present, and so changes in both light level and light composition may both be important environmental signals. It has been shown that exposure to different light wavelengths triggers distinct whole-plant rice transcriptional responses (Lakshmanan et al., 2015) and that rice bundle sheath cells exhibit greater responsiveness to high light stress than the mesophyll (Xiong et al., 2021). Evidence is thus mounting that rice bundle sheath and mesophyll cell development are either differentially responsive or differentially sensitive to their local light environment.

It has been suggested that cell type-specific differential responses to light may have contributed to the evolution of the C_4 bundle sheath, because bundle sheath and mesophyll cells in maize exhibit differential transcriptional responses to blue and red light (Hendron & Kelly, 2020). A relative of maize, our results in rice suggest that these differences may predate the evolution of C_4 photosynthesis in the grass lineage. Through manipulating the timing of light exposure we were able to reduce the length of mature rice bundle sheath cells such that they more closely resembled bundle sheath cell shape in Kranz anatomy (Khoshravesh et al., 2020; McKown & Dengler, 2007; Muhaidat et al., 2007). It is thus possible that changing the underlying responses of bundle sheath cell development to light could have contributed to the cell shape adaptations of the C_4 bundle sheath.

Lastly, analysis of light responses generated two apparent contradictions: when light exposure was delayed both cell types were shorter at maturity whereas leaf length increased, and when light exposure was premature bundle sheath cell lengths were unaffected despite leaf length being reduced. These inconsistencies cannot be reconciled from our existing data, but we propose that if light exposure promotes exit from the cell cycle cell division would continue for longer in the dark-grown leaves. This is consistent with light signalling promoting exit of *A. thaliana* leaf epidermal cells from cell division (Andriankaja et al., 2012), and an RNA-seq study of rice

leaf 5 development found that cell cycle genes are significantly downregulated during the leaf's transition into the light (van Campen et al., 2016). Interestingly, changes in hormone signalling were not identified in association with the light transition. Hormonal differences exist between light-grown and dark-grown plants (Deepika et al., 2020), and removal of leaf 3 could also conceivably alter hormone fluxes, but a role for hormones in the observed leaf cell responses to delayed or premature light cannot be extrapolated conclusively. In future this may be resolved through measuring hormone signalling in individual cells, recently demonstrated in *A. thaliana* using a GA biosensor (Shi et al., 2022). A transgenic cell division reporter in *A. thaliana* shows that cell division persists in vasculature after the mesophyll and epidermis have exited the cell cycle (Donnelly et al., 1999), which if also found to be true in rice could explain the observed different mesophyll and bundle sheath growth responses to premature light. Light-dependent regulation of cell division could thus represent a conserved function between both dicotyledon and monocotyledon leaf development despite their different patterns of development.

ACKNOWLEDGEMENTS

We are grateful to Dr. Roxana Khoshravesh for the single cell isolation protocol and for discussions on optimisation in rice. We are grateful to staff at the University of Cambridge Plant Growth Facility and Susan Stanley for support in growing plant material. This work was supported by BBSRC Grant BBP0031171 to JMH, and the C_4 Rice project grant from The Bill and Melinda Gates Foundation to the University of Oxford (2015–2019). For the purpose of open access, the authors have applied a Creative Commons Attribution (CC BY) license to any Author Accepted Manuscript version arising from this submission.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article. All data supporting the findings of this study are available within the paper and within its Supporting Information published online.

ORCID

Andrew R. G. Plackett http://orcid.org/0000-0002-2321-7849
Julian M. Hibberd http://orcid.org/0000-0003-0662-7958

REFERENCES

Achard, P., Gusti, A., Cheminant, S., Alioua, M., Dhondt, S., Coppens, F. et al. (2009) Gibberellin signaling controls cell proliferation rate in arabidopsis. *Current Biology*, 19, 1188–1193.

Andriankaja, M., Dhondt, S., De Bodt, S., Vanhaeren, H., Coppens, F., De Milde, L. et al. (2012) Exit from proliferation during leaf development in *Arabidopsis thaliana*: a not-so-gradual process. *Developmental Cell*, 22, 64–78.

Aubry, S., Smith-Unna, R.D., Boursnell, C.M., Kopriva, S. & Hibberd, J.M. (2014) Transcript residency on ribosomes reveals a key role for the arabidopsis thalianabundle sheath in sulfur and glucosinolate metabolism. *The Plant Journal*, 78, 659–673.

Aya, K., Hobo, T., Sato-Izawa, K., Ueguchi-Tanaka, M., Kitano, H. & Matsuoka, M. (2014) A novel AP2-type transcription factor, SMALL ORGAN SIZE1, controls organ size downstream of an auxin signaling pathway. *Plant and Cell Physiology*, 55, 897–912.

Beemster, G.T.S., De Veylder, L., Vercruysse, S., West, G., Rombaut, D., Van Hummelen, P. et al. (2005) Genome-wide analysis of gene expression profiles associated with cell cycle transitions in growing organs of arabidopsis. *Plant Physiology*, 138, 734–743.

Benjamini, Y. & Hochberg, Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B: Statistical Methodology*, 57, 289–300.

Borsuk, A.M. & Brodersen, C.R. (2019) The spatial distribution of chlorophyll in leaves. *Plant Physiology*, 180, 1406–1417.

Cackett, L., Luginbuehl, L.H., Schreier, T.B., Lopez-Juez, E. & Hibberd, J.M. (2022) Chloroplast development in green plant tissues: the interplay between light, hormone, and transcriptional regulation. *New Phytologist*, 233, 2000–2016.

van Campen, J.C., Yaapar, M.N., Narawatthana, S., Lehmeier, C., Wanchana, S., Thakur, V. et al. (2016) Combined chlorophyll fluorescence and transcriptomic analysis identifies the P3/P4 transition as a key stage in rice leaf photosynthetic development. *Plant Physiology*, 170, 1655–1674.

Chan, K.X., Phua, S.Y., Crisp, P., McQuinn, R. & Pogson, B.J. (2016) Learning the languages of the chloroplast: retrograde signaling and beyond. *Annual Review of Plant Biology*, 67, 25–53.

Chiang, Y.H., Zubo, Y.O., Tapken, W., Kim, H.J., Lavanway, A.M., Howard, L. et al. (2012) Functional characterization of the GATA transcription factors GNC and CGA1 reveals their key role in chloroplast development, growth and division in arabidopsis. *Plant Physiology*, 160, 332–348.

Christin, P.A., Besnard, G., Samaritani, E., Duvall, M.R., Hodkinson, T.R., Savolainen, V. et al. (2008) Oligocene CO_2 decline promoted C_4 photosynthesis in grasses. *Current Biology*, 18, 37–43.

Christin, P.A., Osborne, C.P., Chatelet, D.S., Columbus, J.T., Besnard, G., Hodkinson, T.R. et al. (2013) Anatomical enablers and the evolution of C_4 photosynthesis in grasses. *Proceedings of the National Academy of Sciences*, 110, 1381–1386.

Claeys, H., Skirycz, A., Maleux, K. & Inzé, D. (2012) DELLA signaling mediates stress-induced cell differentiation in arabidopsis leaves through modulation of anaphase-promoting complex/cyclosome activity. *Plant Physiology*, 159, 739–747.

Conklin, P.A., Strable, J., Li, S. & Scanlon, M.J. (2019) On the mechanisms of development in monocot and eudicot leaves. *New Phytologist*, 221, 706–724.

Cortleven, A. & Schmülling, T. (2015) Regulation of chloroplast development and function by cytokinin. *Journal of Experimental Botany*, 66, 4999–5013.

Danila, F.R., Quick, W.P., White, R.G., von Caemmerer, S. & Furbank, R.T. (2019) Response of plasmodesmata formation in leaves of C4 grasses to growth irradiance. *Plant, Cell & Environment*, 42, 2482–2494.

Danila, F.R., Quick, W.P., White, R.G., Furbank, R.T. & von Caemmerer, S. (2016) The metabolite pathway between bundle sheath and mesophyll: quantification of plasmodesmata in leaves of C_3 and C_4 monocots. *The Plant Cell*, 28, 1461–1471.

Deepika, A., Ankit, S., Sagar, S. & Singh, A. (2020) Dark-induced hormonal regulation of plant growth and development. *Frontiers in Plant Science*, 11, 581666.

Ding, W., Tong, H., Zheng, W., Ye, J., Pan, Z., Zhang, B. et al. (2017) Isolation, characterization and transcriptome analysis of a cytokinin receptor mutant *Osckt1* in rice. *Frontiers in Plant Science*, 8, 88.

Donnelly, P.M., Bonetta, D., Tsukaya, H., Dengler, R.E. & Dengler, N.G. (1999) Cell cycling and cell enlargement in developing leaves of *Arabidopsis*. *Developmental Biology*, 215, 407–419.

Edwards, G.E., Franceschi, V.R. & Voznesenskaya, E.V. (2004) Single-cell C_4 photosynthesis versus the dual-cell (Kranz) paradigm. *Annual Review of Plant Biology*, 55, 173–196.

Ehleringer, J.R., Cerling, T.E. & Helliker, B.R. (1997) C_4 photosynthesis, atmospheric CO_2, and climate. *Oecologia*, 112, 285–299.

Ellis, J.R. & Leech, R.M. (1985) Cell size and chloroplast size in relation to chloroplast replication in light-grown wheat leaves. *Planta*, 165, 120–125.

Ermakova, M., Danila, F.R., Furbank, R.T. & von Caemmerer, S. (2020) On the road to C_4 rice: advances and perspectives. *The Plant Journal*, 101, 940–950.

Fox, J. & Weisberg, S. (2019) *An R Companion to Applied Regression*, Third edition. Sage.

Gad, A.G., Habiba, Zheng, X. & Miao, Y. (2021) Low light/darkness as stressors of multifactor-induced senescence in rice plants. *International Journal of Molecular Sciences*, 22, 3936.

Gago, J., Carriquí, M., Nadal, M., Clemente-Moreno, M.J., Coopman, R.E., Fernie, A.R. et al. (2019) Photosynthesis optimized across land plant phylogeny. *Trends in Plant Science*, 24, 947–958.

Hendron, R.W. & Kelly, S. (2020) Subdivision of light signaling networks contributes to partitioning of C4 photosynthesis. *Plant Physiology*, 182, 1297–1309.

Holst, K., Schmülling, T. & Werner, T. (2011) Enhanced cytokinin degradation in leaf primordia of transgenic *Arabidopsis* plants reduces leaf size and shoot organ primordia formation. *Journal of Plant Physiology*, 168, 1328–1334.

Hu, Y., Xie, Q. & Chua, N.H. (2003) The arabidopsis auxin-inducible gene *ARGOS* controls lateral organ size. *The Plant Cell*, 15, 1951–1961.

Hua, L. & Hibberd, J.M. (2019) An optimized protocol for isolation of high-quality RNA through laser capture microdissection of leaf material. *Plant Direct*, 3, e00156.

Hua, L., Stevenson, S.R., Reyna-Llorens, I., Xiong, H., Kopriva, S. & Hibberd, J.M. (2021) The bundle sheath of rice is conditioned to play an active role in water transport as well as sulfur assimilation and jasmonic acid synthesis. *The Plant Journal*, 107, 268–286.

Ikeda, A., Ueguchi-Tanaka, M., Sonoda, Y., Kitano, H., Koshioka, M., Futsuhara, Y. et al. (2001) *slender* rice, a constitutive gibberellin response mutant, is caused by a null mutation of the *SLR1* gene, an ortholog of the height-regulating gene *GAI/RGA/RHT/D8*. *The Plant Cell*, 13, 999–1010.

Itoh, J.I., Nonomura, K.I., Ikeda, K., Yamaki, S., Inukai, Y., Yamagishi, H. et al. (2005) Rice plant development: from zygote to spikelet. *Plant and Cell Physiology*, 46, 23–47.

Jarvis, P. & López-Juez, E. (2013) Biogenesis and homeostasis of chloroplasts and other plastids. *Nature Reviews Molecular Cell Biology*, 14, 787–802.

Jiang, X., Li, H., Wang, T., Peng, C., Wang, H., Wu, H. et al. (2012) Gibberellin indirectly promotes chloroplast biogenesis as a means to maintain the chloroplast population of expanded cells. *The Plant Journal*, 72, 768–780.

Jones, A.R., Band, L.R. & Murray, J.A.H. (2019) Double or nothing? Cell division and cell size control. *Trends in Plant Science*, 24, 1083–1093.

Kaiser, S. & Scheuring, D. (2020) To lead or to follow: contribution of the plant vacuole to cell growth. *Frontiers in Plant Science*, 11, 553.

Khoshravesh, R. & Sage, T.L. (2018) Creating leaf cell suspensions for characterization of mesophyll and bundle sheath cellular features. In: Covshoff, S., ed. *Photosynthesis. Methods in Molecular Biology*, 1770. New York: Humana Press. pp. 253–261.

Khoshravesh, R., Stata, M., Busch, F.A., Saladié, M., Castelli, J.M., Dakin, N. et al. (2020) The evolutionary origin of C_4 photosynthesis in the grass subtribe Neurachninae. *Plant Physiology*, 182, 566–583.

Khoshravesh, R., Stinson, C.R., Stata, M., Busch, F.A., Sage, R.F., Ludwig, M. et al. (2016) C_3-C_4 intermediacy in grasses: organelle enrichment and distribution, glycine decarboxylase expression, and the rise of C_2 photosynthesis. *Journal of Experimental Botany*, 67, 3065–3078.

Kieber, J.J. & Schaller, G.E. (2018) Cytokinin signaling in plant development. *Development*, 145, dev149344.

Kinsman, E.A. & Pyke, K.A. (1998) Bundle Sheath cells and cell-specific plastid development in *Arabidopsis* leaves. *Development*, 125, 1815–1822.

Lakshmanan, M., Lim, S.H., Mohanty, B., Kim, J.K., Ha, S.H. & Lee, D.Y. (2015) Unraveling the light-specific metabolic and regulatory signatures of rice through combined in silico modeling and multiomics analysis. *Plant Physiology*, 169, 3002–3020.

Lee, D.Y., Hua, L., Khoshravesh, R., Giuliani, R., Kumar, I., Cousins, A. et al. (2021) Engineering chloroplast development in rice through cell-specific control of endogenous genetic circuits. *Plant Biotechnology Journal*, 19, 2291–2303.

Lee, M.S., Boyd, R.A., Boateng, K.A. & Ort, D.R. (2023) Exploring 3D leaf anatomical traits for C4 photosynthesis: chloroplast and plasmodesmata pit field size in maize and sugarcane. *New Phytologist*, 239, 506–517.

Leegood, R.C. (2007) Roles of the bundle sheath cells in leaves of C_3 plants. *Journal of Experimental Botany*, 59, 1663–1673.

Levene, H. (1960) Robust tests for equality of variances. In: Olkin, I., ed. *Contributions to probability and statistics: Essays in honor of Harold Hotelling*. Stanford University Press. pp. 278–292.

Löfke, C., Dünser, K., Scheuring, D. & Kleine-Vehn, J. (2015) Auxin regulates SNARE-dependent vacuolar morphology restricting cell size. *eLife*, 4, e05868.

Lundgren, M.R., Dunning, L.T., Olofsson, J.K., Moreno-Villena, J.J., Bouvier, J.W., Sage, T.L. et al. (2019) C_4 anatomy can evolve via a single developmental change. *Ecology Letters*, 22, 302–312.

Matsukura, C., Itoh, S., Nemoto, K., Tanimoto, E. & Yamaguchi, J. (1998) Promotion of leaf sheath growth by gibberellic acid in a dwarf mutant of rice. *Planta*, 205, 145–152.

McKown, A.D. & Dengler, N.G. (2007) Key innovations in the evolution of Kranz anatomy and C_4 vein pattern in *Flaveria* (Asteraceae). *American Journal of Botany*, 94, 382–399.

Miyagishima, S., Froehlich, J.E. & Osteryoung, K.W. (2006) PDV1 and PDV2 mediate recruitment of the dynamin-related protein ARC5 to the plastid division site. *The Plant Cell*, 18, 2517–2530.

Miyake, H. (2016) Starch accumulation in the bundle sheaths of C3 plants: a possible pre-condition for C4 photosynthesis. *Plant and Cell Physiology*, 57, 890–896.

Muhaidat, R., Sage, R.F. & Dengler, N.G. (2007) Diversity of Kranz anatomy and biochemistry in C4eudicots. *American Journal of Botany*, 94, 362–381.

Muhaidat, R., Sage, T.L., Frohlich, M.W., Dengler, N.G. & Sage, R.F. (2011) Characterization of C_3–C_4 intermediate species in the genus *Heliotropium* L. (Boraginaceae): anatomy, ultrastructure and enzyme activity. *Plant, Cell & Environment*, 34, 1723–1736.

Murchie, E.H., Hubbart, S., Peng, S. & Horton, P. (2005) Acclimation of photosynthesis to high irradiance in rice: gene expression and interactions with leaf development. *Journal of Experimental Botany*, 56, 449–460.

Naito, T., Kiba, T., Koizumi, N., Yamashino, T. & Mizuno, T. (2007) Characterization of a unique GATA family gene that responds to both light and cytokinin in *Arabidopsis thaliana*. *Bioscience, Biotechnology, and Biochemistry*, 71, 1557–1560.

Nelson, T. & Dengler, N. (1997) Leaf vascular pattern formation. *The Plant Cell*, 9, 1121–1135.

Okazaki, K., Kabeya, Y., Suzuki, K., Mori, T., Ichikawa, T., Matsui, M. et al. (2009) The PLASTID DIVISION1 and 2 components of the chloroplast division machinery determine the rate of chloroplast division in land plant cell differentiation. *The Plant Cell*, 21, 1769–1780.

Pyke, K.A. (1999) Plastid division and development. *The Plant Cell*, 11, 549–556.

Pyke, K.A. & Leech, R.M. (1991) Rapid image analysis screening procedure for identifying chloroplast number mutants in mesophyll cells of *Arabidopsis thaliana* (L.) Heynh. *Plant Physiology*, 96, 1193–1195.

Pyke, K.A. & Leech, R.M. (1992) Chloroplast division and expansion is radically altered by nuclear mutations in *Arabidopsis thaliana*. *Plant Physiology*, 99, 1005–1008.

Pyke, K.A., Rutherford, S.M., Robertson, E.J. & Leech, R.M. (1994) *arc6*, a fertile *Arabidopsis* mutant with only two mesophyll cell chloroplasts. *Plant Physiology*, 106, 1169–1177.

R Core Team. (2020) R: A language and environment for statistical computing. (R Foundation for Statistical Computing). https://www.R-project.org/

Ren, T., Weraduwage, S.M. & Sharkey, T.D. (2019) Prospects for enhancing leaf photosynthetic capacity by manipulating mesophyll cell morphology. *Journal of Experimental Botany*, 70, 1153–1165.

Richardson, A.E., Cheng, J., Johnston, R., Kennaway, R., Conlon, B.R., Rebocho, A.B. et al. (2021) Evolution of the grass leaf by primordium extension and petiole-lamina remodeling. *Science*, 374, 1377–1381.

Richter, R., Behringer, C., Zourelidou, M. & Schwechheimer, C. (2013) Convergence of auxin and gibberellin signaling on the regulation of the GATA transcription factorsGNCandGNLinArabidopsis thaliana. *Proceedings of the National Academy of Sciences*, 110, 13192–13197.

RStudio Team. (2020) RStudio: Integrated Development for R. RStudio, PBC, Boston, MA. http://www.rstudio.com/

Sablowski, R. (2016) Coordination of plant cell growth and division: collective control or mutual agreement? *Current Opinion in Plant Biology*, 34, 54–60.

Sage, R.F. (2016) A portrait of the C_4 photosynthetic family on the 50th anniversary of its discovery: species number, evolutionary lineages and hall of fame. *Journal of Experimental Botany*, 67, 4039–4056.

Sage, R.F., Christin, P.A. & Edwards, E.J. (2011) The C4 plant lineages of planet earth. *Journal of Experimental Botany*, 62, 3155–3169.

Sage, T.L., Busch, F.A., Johnson, D.C., Friesen, P.C., Stinson, C.R., Stata, M. et al. (2013) Initial events during the evolution of C4 photosynthesis in C3 species of Flaveria. *Plant Physiology*, 163, 1266–1276.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T. et al. (2012) Fiji: an open-source platform for biological-image analysis. *Nature Methods*, 9, 676–682.

Schmitz, A.J., Glynn, J.M., Olson, B.J.S.C., Stokes, K.D. & Osteryoung, K.W. (2009) *Arabidopsis* FtsZ2-1 and FtsZ2-2 are functionally redundant, but FtsZ-based plastid division is not essential for chloroplast partitioning or plant growth and development. *Molecular Plant*, 2, 1211–1222.

Schreier, T.B., Müller, K.H., Eicke, S., Faulkner, C., Zeeman, S.C. & Hibberd, J.M. (2024) Plasmodesmal connectivity in C_4 *Gynandropsis gynandra* is induced by light and dependent on photosynthesis. *New Phytologist*, 241, 298–313.

Schruff, M.C., Spielman, M., Tiwari, S., Adams, S., Fenby, N. & Scott, R.J. (2006) The *AUXIN RESPONSE FACTOR 2* gene of *Arabidopsis* links auxin signalling, cell division, and the size of seeds and other organs. *Development*, 133, 251–261.

Sedelnikova, O.V., Hughes, T.E. & Langdale, J.A. (2018) Understanding the genetic basis of C_4 Kranz anatomy with a view to engineering C_3 crops. *Annual Review of Genetics*, 52, 249–270.

Shapiro, S.S. & Wilk, M.B. (1965) An analysis of variance test for normality (complete samples). *Biometrika*, 52, 591–611.

Shatil-Cohen, A., Attia, Z. & Moshelion, M. (2011) Bundle-sheath cell regulation of xylem-mesophyll water transport via aquaporins under drought stress: a target of xylem-borne ABA? *The Plant Journal*, 67, 72–80.

Shi, B., Felipo-Benavent, A., Cerutti, G., Galvan-Ampudia, C., Jilli, L. & Brunoud, G. et al. (2022) A quantitative gibberellin signalling biosensor reveals a role for gibberellins in internode specification at the shoot apical meristem. *BioRXiv*. https://doi.org/10.1101/2021.06.11.448154

Singh, P., Mohanta, T.K. & Sinha, A.K. (2015) Unraveling the intricate nexus of molecular mechanisms governing rice root development: OsMPK3/6 and auxin-cytokinin interplay. *PLoS One*, 10, e0123620.

Tanaka, A. (1961) Studies on the nutrio-physiology of leaves of rice plant. *Journal of the Faculty of Agriculture, Hokkaido University*, 51, 449–550.

Ueguchi-Tanaka, M., Ashikari, M., Nakajima, M., Itoh, H., Katoh, E., Kobayashi, M. et al. (2005) GIBBERELLIN INSENSITIVE DWARF1 encodes a soluble receptor for gibberellin. *Nature*, 437, 693–698.

Vogelman, T.C., Nishio, J.N. & Smith, W.K. (1996) Leaves and light capture: light propagation and gradients of carbon fixation within leaves. *Trends in Plant Science*, 1, 65–70.

Wang, P., Hendron, R.W. & Kelly, S. (2017a) Transcriptional control of photosynthetic capacity: conservation and divergence from arabidopsis to rice. *New Phytologist*, 216, 32–45.

Wang, P., Khoshravesh, R., Karki, S., Tapia, R., Balahadia, C.P., Bandyopadhyay, A. et al. (2017b) Re-creation of a key step in the evolutionary switch from C_3 to C_4 leaf anatomy. *Current Biology*, 27, 3278–3287.

Whitelam, G. & Halliday, K. (2007) *Light and Plant Development*. Oxford: Blackwell.

Wickham, H. (2016) *ggplot2: Elegant Graphics for Data Analysis*. New York: Springer-Verlag.

Williams, M.L., Farrar, J.F. & Pollock, C.J. (1989) Cell specialization within the parenchymatous bundle sheath of barley. *Plant, Cell & Environment*, 12, 909–918.

Xiong, H., Hua, L., Reyna-Llorens, I., Shi, Y., Chen, K.M., Smirnoff, N. et al. (2021) Photosynthesis-independent production of reactive oxygen species in the rice bundle sheath during high light is mediated by NADPH oxidase. *Proceedings of the National Academy of Sciences*, 118, e2022702118.

Yang, J. (2002) Correlation of cytokinin levels in the endosperms and roots with cell number and cell division activity during endosperm development in rice. *Annals of Botany*, 90, 369–377.

Yang, J., Yuan, Z., Meng, Q., Huang, G., Périn, C., Bureau, C. et al. (2017) Dynamic regulation of auxin response during rice development revealed by newly established hormone biosensor markers. *Frontiers in Plant Science*, 8, 256.

Zubo, Y.O., Blakley, I.C., Franco-Zorrilla, J.M., Yamburenko, M.V., Solano, R., Kieber, J.J. et al. (2018) Coordination of chloroplast development through the action of the GNC and GLK transcription factor families. *Plant Physiology*, 178, 130–147.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Plackett, A.R.G. & Hibberd, J.M. (2024) Rice bundle sheath cell shape is regulated by the timing of light exposure during leaf development. *Plant, Cell & Environment*, 1–17. https://doi.org/10.1111/pce.14902