Masters, N., Lopez Garcia, M., Oulton, R., & Whitney, H. (2018). Characterization of chloroplast iridescence in Selaginella erythropus. *Journal of the Royal Society Interface*, *15*, [20180559]. https://doi.org/10.1098/rsif.2018.0559

Peer reviewed version

Link to published version (if available):
10.1098/rsif.2018.0559

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Characterisation of chloroplast iridescence in *Selaginella erythropus*

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Abstract

Iridescence in shade-dwelling plants has previously been described in only a few plant groups, and even fewer where the structural colour is produced by intracellular structures. In contrast to other *Selaginella* species, this work reports the first example in the genus of structural colour originating from modified chloroplasts. Characterisation of these structures determines that they form 1-dimensional photonic multilayers. The *Selaginella* bizonoplasts present an analogous structure to recently reported *Begonia* iridoplasts, however unlike *Begonia* species that produce iridoplasts, this *Selaginella* species was not previously described as iridescent. This therefore raises the possibility of widespread but unobserved and uncharacterised photonic structures in plants.

Introduction

Chloroplasts are green - most of the time. The presence of thylakoids, grana, constituent proteins, and green pigments are largely consistent between chloroplasts, particularly across land plants. There are however exceptions to this rule such as in the chloroplasts of certain algae and marine bacteria[1]. Another exception is when chloroplasts produce structural colour. This mechanism of colour generation is caused neither by pigmentation nor fluorescence, but by structures on the sub-micron scale. As light interacts with these structures select bandwidths

of the electromagnetic spectrum are either reflected or transmitted. As a result, extremely vivid colours can be produced, most typically exemplified by the wings of butterflies[2] or the elytra of beetles.[3,4] Recently it has also been demonstrated that structural colour can play a role in photosynthetic systems[5,6,7].

Initial investigations by David Lee into plant species such as *Begonia*, *Phyllagathus* and *Trichromanes*[8,9] discovered periodic thylakoid membrane arrangements, and suggested that these structures were responsible for the blue colouration seen on the leaves of these deep-shade adapted understory plants. More recent investigations into many species of *Begonia* have characterised these iridescent chloroplasts (termed iridoplasts), and established the blue colour is the result of the highly periodic thylakoid arrangements forming a multilayer photonic structure[6,10]. Further to this, the photonic phenomenon of slow-light created by the multilayer has been suggested to enhance the absorption of green wavelengths and play a role in increasing quantum yield in low light conditions by 5-10%[6].

The presence of these highly periodic thylakoid arrangements is also present in another tropical understory plant, *Selaginella erythropus*[11]. The chloroplasts in this lycophyte are large and fill the majority of the volume of the epidermal cells. The chloroplast size reflects an adaptation to increase light capture in the deep-shade environments of the tropical understory. These bizonoplasts are so named due to the two distinct regions of thylakoid membranes and grana within the same chloroplast. The lower region, away from the microphyll surface, demonstrates a standard arrangement of grana, however the upper region, closest to the microphyll surface, has a highly ordered structure similar to the periodic photonic crystal organisation of thylakoid membranes originally investigated by David Lee and recently characterised in *Begonia*[8,9,6]. Previous work has investigated the development of these bizonoplasts[11,12,13] but reports a distinct lack of structural colour. The *Selaginella* genus is already known for producing a range of photonic structures such as in the multilayers of the

microphyll cell wall[14,15] and opal structures in the spores[16]. This work looks at the potential of a third type of photonic structure in *Selaginella* by characterising the regular periodic structures in the chloroplasts of *Selaginella erythropus*.

Material and Methods

Growth conditions

S. erythropus plants were initially purchased from Siamgreenculture[17] and cultivated within the department of Biological Sciences at the University of Bristol while being grown in humidity controlled environments. Humidity was kept at 85%, temperature at 25°C, and lighting was provided by a mixture of cool and warm white fluorescent bulbs (Sylvania F36W 840/830). The incident light levels had a photosynthetic photon fluence rate (PPFR) of 2.88 $\mu mol.m^{-2}.s^{-1}$ over the waveband 400-700 nm with associated spectra demonstrated in Figure S1. Lighting was measured on a calibrated Flame-S-UV-VIS Spectrometer (Ocean Optics, USA) and a calibrated LI-COR Quantum Sensor (LI-250A). The microphylls measured in all experiments were well developed, beyond five leaves from the stem apex. The leaves were photographed with a Nikon D3200 (Fig. 1a), and low magnification microscopy with a VHX-1000E Digital Microscope (Keyence) (Fig S8).

Transmission Electron Microscopy

We measured chloroplasts ultrastructure from transmission electron microscopy (TEM) images. A general protocol was followed for the preparation of samples as previously reported[6]. Imaging was performed using a Tecnai T12 microscope (FEI) and analysis was conducted using Fiji[18].

Cryo-Scanning Electron Microscopy

Microphylls were removed and quickly frozen in a liquid nitrogen slush close to the freezing point of liquid nitrogen (-210°C). Fracturing, sputter coating with platinum for two minutes,

and transfer to a Quanta 400 scanning electron microscope (FEI) were under a vacuum at -145°C. Samples were sublimed for 3 minutes at -95°C before sputter coating.

Epi-Illumination Microscopy

We measured reflective properties of individual cells with a custom-made white light epi-illumination reflectance microscope. White light illumination (Thorlabs OSL-1) was collimated and focussed onto the sample with a high-numerical aperture (0.75) lens (Zeiss 63x). The reflected light was then collected into an optical fibre (Thorlabs M92L01) in a confocal configuration or passed to a camera sensor. This allows easy switching between spectral measurements and imaging. Fourier image spectroscopy is also possible in this setup which allows for spectral characterisation across the entire numerical aperture of the lens, and hence spectral reflectance measurements as a function of angle[19].

Transfer Matrix Method Model

Inputting the details of the layer by layer structure found in the upper region into a Transfer Matrix Model[20] allowed the modelling of light interactions with the layered thylakoid structure of the bizonoplast. The inputs required are the refractive index of the layers and the dimensions of the structure. Refractive index values were obtained from established literature values for the thylakoid membranes[21], while structural dimensions were from TEM observations. The refractive index values contain both real and imaginary components to account for the strong optical dispersion of thylakoid membranes (Figure S7). Refractive index values for the stroma and lumen were from published values[21]. These values assume a non-dispersive medium with indices of 1.35 for both the stroma and lumen.

Results and Discussion

Plant development and structural morphology

S. erythropus develops with a distinctive arrangement of microphylls. Along the stems there are two different microphyll morphologies: those towards the centre of the stems which are

smaller and overlap the larger microphylls towards the edge. The blue colouration of the microphylls can be visually observed apart from in those of the first two or three recently developed microphylls (Figure 1a, S8). The colour is dependent on the angle of observation and the lighting environment during development. Plants grown in medium to bright light conditions exhibit limited structural colour, whereas low light conditions (5 $\mu mol.m^{-2}.s^{-1}$) result in observable blue colouration. Transmission Electron Microscopy (TEM) demonstrated the distinct bi-zonal arrangement of the singular chloroplast in each epidermal cell, as previously reported[11]. The layered thylakoid structure towards the dorsal side of the microphyll consists of periodic arrangements of 2-3 thylakoid membranes closely packed and a spacing in the stroma before the arrangement repeats itself up to ~20 times. Measurements of the dimensions were calculated from transmission electron microscopy (TEM) images (Figure 2). Thylakoid membranes (L_T) show a thickness of 4.28 ± 0.55 nm (n=29) and a lumen thickness (L_L) of 5.20 ± 0.68 nm (n=40), and the spacing of the stroma (L_S) between these stacked thylakoids is 81.52 ± 5.68 nm (n=56). The overall periodic length as measured by TEM for a combined thylakoid stack and associated stroma (Λ) is 130 ± 7 nm (n=74) (Figure 3a). Cryo-Scanning Electron Microscopy (Cryo-SEM) was conducted in order to compare the size of the period spacing observed under TEM. The measurements performed demonstrated a periodic spacing of 156 ± 10 nm which is larger than that obtained by TEM observations (Figures S9, S10). Cryo-preservation however is a more robust morphological technique which limits the shrinking which can occur during the dehydration steps involved in chemical fixation[22], but lacks the resolution of TEM which can distinguish single thylakoid membranes and help understand the ultrastructure. Our cryo-SEM measurements demonstrate a period which is 20% longer than when measured by TEM. This compares with previous work which uses atomic force microscopy to measure thylakoid membrane thicknesses of 19.5 nm[23]. A stack of three of these membranes (58.5 nm) would constitute a larger grana size by 19.6% when compared to our

TEM measurements (48.9 nm) (Figure S2). The similarity in size contrast between the two techniques gives us confidence to say that a period of 156 ± 10 nm as measured by cryo-SEM is more indicative of the natural spacing contributing to the structural colour production.

From previous reports we can see how the development of the structural region in bizonoplasts is highly plastic with the lighting environment influencing the formation of a periodic upper zone of thylakoid stacks. When grown in high light (450-500 $\mu mol.m^{-2}.s^{-1}$) there is a complete shift in chloroplast morphology to produce multiple chloroplasts with no sign of a dimorphic ultrastructure[12]. In contrast, low light (15-30 $\mu mol.m^{-2}.s^{-1}$) environments, indicative of the typical light regime experienced, encourage the development of the characteristic dimorphic ultrastructure[12,13]. The intensity experienced in the high light growth conditions previously reported (450-500 $\mu mol.m^{-2}.s^{-1}$) would seem exceedingly high in comparison to the light levels typically experienced in the tropical understory[24], however effective growth appears unaffected. This would suggest that light level acclimatisation of the plant plays a central role in chloroplast development within this species. This is also supported by recent work which looks at how mature bizonoplasts react when exposed to high light without prior high light acclimatisation. When exposed, despite the high light being considerably lower (75 $\mu mol.m^{-2}.s^{-1}$) than that used by Sheue *et al.*[12], the plants demonstrated signs of light stress with bleaching, growth inhibition, and chloroplast disintegration[13]. These findings further support that suggestion that bizonoplasts are likely to be an adaption to very low-light environments similar to that observed in *Begonia*[6] – hence to simulate an accurate developmental environment experiments were conducted under low-light conditions. In fact, it is this particularly plastic development of chloroplast morphology which has likely led to the dismissal of structural colour present in *Selaginella erythropus*. In our experience, optimum growth conditions (with respect to rate of new microphyll production) agrees with that of Ghaffar *et al.*[13] at a light level of 15 $\mu mol.m^{-2}.s^{-1}$, however light levels below this (1-5 $\mu mol.m^{-}$

$^{2}.s^{-1}$) still obtain substantial microphyll production while the development of blue colouration is more obvious.

Structural colour characterisation and modelling

Epi-illumination microscopy allows for the reflected light to be observed. By capturing images with the epidermis and the chloroplasts at different focal planes we can see that the blue colouration originates from the chloroplast structure within the cell (Figures 1g, 1h) which is in contrast to structural colour originating from the cell wall in other *Selaginella* species (Figures 1b-c, 1e-f, S11). An optical model informed through our morphological studies and literature values for cellular refractive indices was constructed. The use of a Transfer Matrix Method (TMM) model predicts a peak in reflectance in the blue region of the visible spectrum (Figure 3b), alongside an angular dependence with the dominant wavelengths of reflection decreasing with angle (Figure S6). To confirm whether the blue colouration of *S. erythropus* is a result of this mechanism we performed both normal incidence and angular dependence measurements of the reflection. The wavelength for the measured peak of reflectance at normal incidence is 450 nm, positioned well within the blue region of the visible light spectrum (Figure 3b, S3, S4), while angular measurements demonstrate a slight shift towards lower wavelengths with angle (Figure S5). Experimental uncertainties required in the optical model will however produce differences between the bizonoplast dimensions predicted by theory and experimental values. Fine tuning of the parameters allowed for a fit where the peak in reflectance of the optical model and experimental measurements aligned - these values suggest a period of 164 nm which is obtained from a homogeneous expansion of the lattice by 5.1%. This expansion predicted by the model could be produced by small differences in the real refractive index of the membranes and those found in nature. Other sources of error could be due to a non-planar surface morphology of the leaf or inhomogeneous contraction of dimensions in TEM sample

preparation. In general, and considering the complexity of the real system, the agreement is outstanding.

Contrary to previous reports[11,13], our observations and measurements of the reflected light from individual chloroplasts has enabled us to demonstrate that blue structural colour is produced by the bizonoplasts. This emphasises the need for suitable techniques to characterize structural colour in intracellular organelles; structural colour can be subtle and inconspicuous to the human eye with no necessity to be visually detectable in the leaf.

Our model and reflectance measurements demonstrate that the presence of the photonic multilayer structure increases reflectance in the blue region of the spectrum around 450 nm. The effect of this is a decrease in the transmittance and/or absorbance in this region of the spectrum. This influence of the multilayer structure on the photonic behaviour of the bizonoplast appears counter intuitive – a deep-shade plant, presumably adapted to harvesting as much light as possible should in theory want to limit the reflection of useful wavelengths of light (i.e. those absorbed by the photosynthetic pigments). Despite this, structural colour in deep-shade plants is now understood to be widespread; such other examples include iridoplasts in many *Begonia* species[8,6], as well as in *Phyllagathis rotundifolia*[25] and *Trichomanes elegans*[9]. In addition, there are examples of multilayers in the cuticle of *Teratophyllum rotundifoliatum*[27], *Selaginella willdenowwii*, and *Selaginella uncinata*[26,14,15] which similarly produce blue structural colour.

Blue iridescence and the influence it might have on photosynthesis in deep-shade plants has been discussed with respect to *Selaginella willdenowii*. Lee *et al.* proposed that the waxy cuticle, which is the origin of the structural colour in this species, acts as an anti-reflective coating (similar to the coatings found on camera lenses)[25]. This anti-reflective coating would lead to greater absorption of longer, red, wavelengths of light which are more prevalent than shorter wavelengths in the deep-shade tropical understory. To test this, Thomas *et al.*

characterised the optical behaviour of *S. willdenowii*, and modelled the layered cuticle. The results of this demonstrated the layered cuticle does not effectively enhance red light transmission into the microsphylls. It also appears to be a poor evolutionary strategy when far simpler and more efficient anti-reflective systems could have developed[15]. The possible adaptive advantage of this system is still unknown and further investigation should be conducted, however recent work has demonstrated that iridescence can disrupt the visual systems of insects[28] and hence could play a role in the prevention of herbivory. In contrast to the striking iridescence of *S. willdenowii*, the subtle colouration produced by the chloroplast structure of *S. erythropus* hints perhaps more directly at a photosynthetic function, as has been demonstrated in the equally deep-shade adapted, *Begonia pavonina*.

Jacobs *et al*. characterised and modelled the iridoplasts from *B. pavonina* as one-dimensional photonic crystals, demonstrating a higher photosynthetic efficiency by increasing the quantum yield by 5-10%[6]. The reason for this increase in yield is suggested to be due to the periodic spacing of the grana stacks which locally increases the intensity of green wavelengths of light. The photonic environment set up by a periodically spaced arrangement of contrasting refractive indices leads to a reduction in the group velocity of wavelengths at the photonic band edge[29]. In this case, the increased reflection in blue wavelengths, means that light at slightly longer green wavelengths exhibits a localised higher electric field at the stacked thylakoid regions. It should not be surprising that this effect could also be present in the periodic stacked thylakoid regions of *S. erythropus* since the species inhabits similar deep-shade habitats rich in green wavelengths of light while also containing the photonic environment suitable for structurally produced colour in the blue spectrum. Future work with *S. erythropus* will look to examine if the photosynthetic performance in this upper stacked region is modified.

Perhaps what is most interesting is the range of similarities and difference exhibited by iridescent chloroplasts in very different plant groups – the parent clade of *Selaginella*,

Lycopodiophyta, diverged from all other vascular plants 376 - 407 Mya[30-32]. This apparent convergent evolution to arrive at very similar chloroplast structures suggests a selective pressure which comes from the environments which *Begonias* and *Selaginella erythropus* inhabit. The chloroplasts in these taxonomically distinct plants have developed thylakoid arrangements which are so alike that they produce very similar structural colour effects. In fact, the structural colour seen in *Begonia* and *S. erythropus* is quite different to other forms of structural colour in plants: due to the colour being produced within the cell, the colour is not as vivid as that seen in other examples of structural colour which is produced by the cuticle. This subtle point is why it is easy to overlook examples of intracellular structural colour – for example many species of *Begonia*, despite at first appearance looking green with no hint of blue colouration do contain iridoplasts which when observed under reflection microscopy produce blue structural colour[6]. There are reports of structures similar to the iridoplasts of *Begonia* and bizonoplasts of *S. erythropus* both in other species of *Selaginella* and in a range of species from the brown algae, ferns and flowering plants[11]. While iridescence has not been reported in all of these species, our results suggest that this does not preclude them from having similar photonic properties.

Conclusion

The bizonoplasts in *Selaginella erythropus* present a unique chloroplast structure with two distinct regions. This structure acts as a photonic one-dimensional multilayer resulting in an increased reflection in the blue region of the visible spectrum. We have demonstrated this through an optical model based on electron microscopy observations as well as direct measurements of the reflected light. The subtle nature of the blue reflection means that it is not easily visually observable, and examples from the literature suggest that this overlooked phenomenon could be more widespread than currently realised. Blue structural colour as demonstrated in this system is a growing area of research in plants with numerous examples

now present in diverse plant species from deep-shade environments. However, despite the expanding literature there is still limited understanding of the evolutionary benefits of structural colour in these environments. Furthermore *S. erythropus* is one of only a few characterised systems in which this blue structural colour is produced by modifications to the chloroplast ultrastructure. While it may initially appear counter-intuitive nature to reflect away photosynthetically active radiation, it should not be too surprising that it could be advantageous to manipulate the photonic environment of the chloroplast to enhance photosynthetic efficiency to adapt to different light scenarios. It is well known that chloroplasts typically have very plastic behaviour to ensure photosynthesis is managed in the most efficient manner – expanding this to envision systems which use photonic multilayers could be another example of such adaptation to difficult environmental conditions which now requires further investigation.

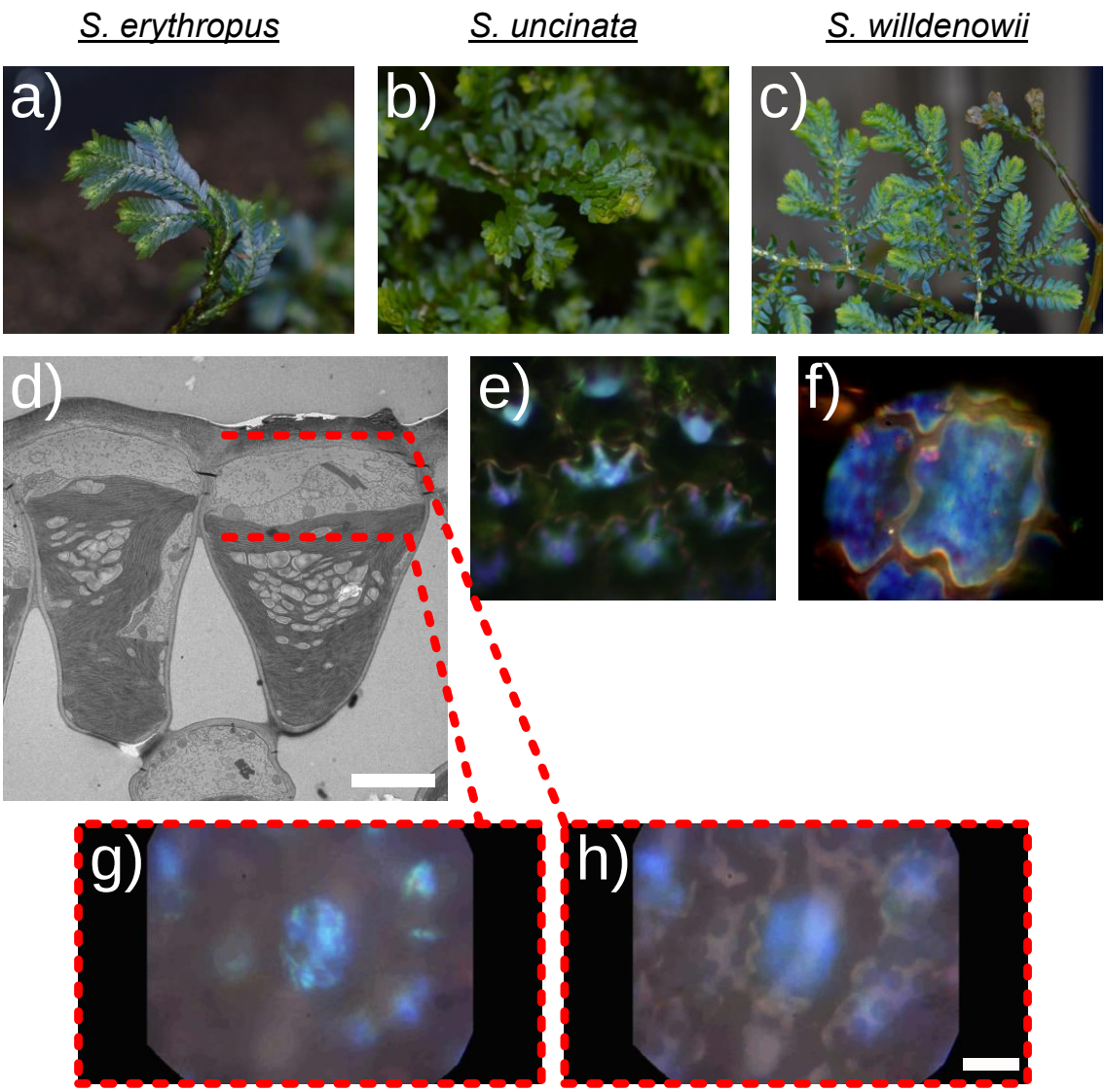
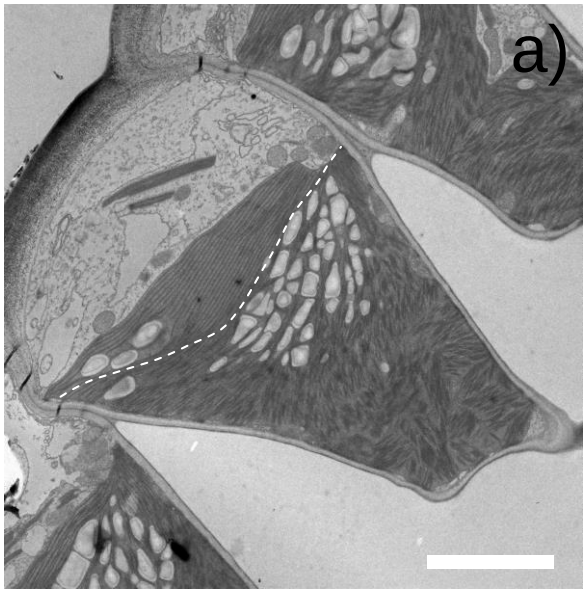
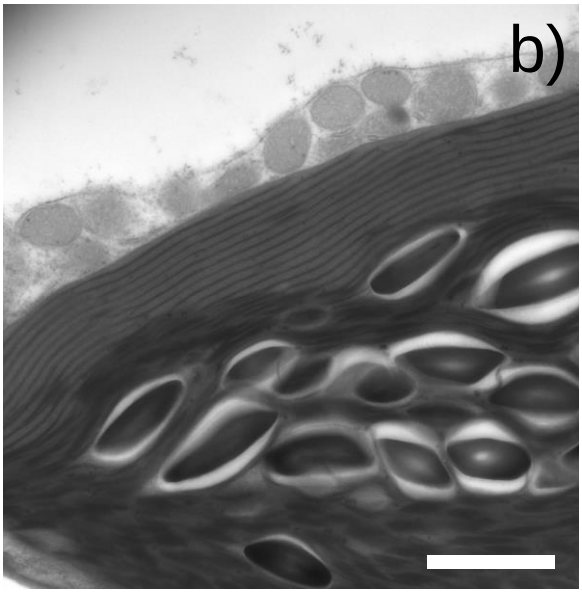
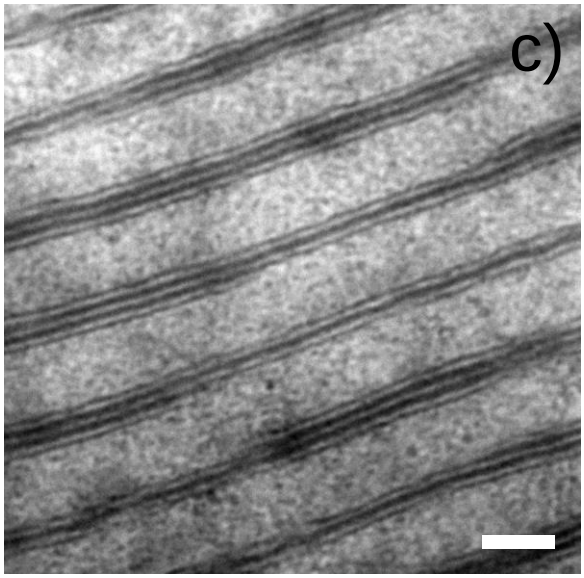


Figure 1: a-c) *Photographs of S. erythropus, S. uncinata, and S. willdenowii demonstrating the blue colouration present in the Selaginella genus.* ***d)*** *TEM image of two epidermal cells and a demonstration of the approximate vertical positions where d) and e) observations were performed. Scale bar = 5µm;* ***e + f)*** *Reflection microscopy images demonstrating the reflection from the cell walls of S. uncinata and S. willdenowii respectively;* ***g) + h)*** *Reflection microscopy images focused at the source of the blue colouration (d) and at the cell wall (e). Scale bar = 10µm.*







	Dimension (nm)
Thylakoid membrane (TEM)	4.28 ± 0.55 (n=29)
Lumen (TEM)	5.20 ± 0.68 (n=40)
Stroma gap (TEM)	81.52 ± 5.68 (n=56)
Repeating period (TEM)	130 ± 6.72 (n=74)
Repeating period (Cryo-SEM)	156 ± 10 (n=11)

Figure 2: a) *TEM image of an epidermal cell of S. erythropus with the majority of the cell volume being the bi-zonal chloroplast. The white dashed line differentiates the upper periodically stacked thylakoid region from the lower typical thylakoid structure. Scale bar = 5µm.* ***b)*** *TEM image of the location of the layered thylakoid region alongside starch grains. Scale bar = 0.5µm.* ***c)*** *A higher magnification image of a layered thylakoid region. The dark regions are the thylakoid membranes. Scale bar = 100nm;* ***Table)*** *Structure dimensions as determined by TEM and Cryo-SEM.*

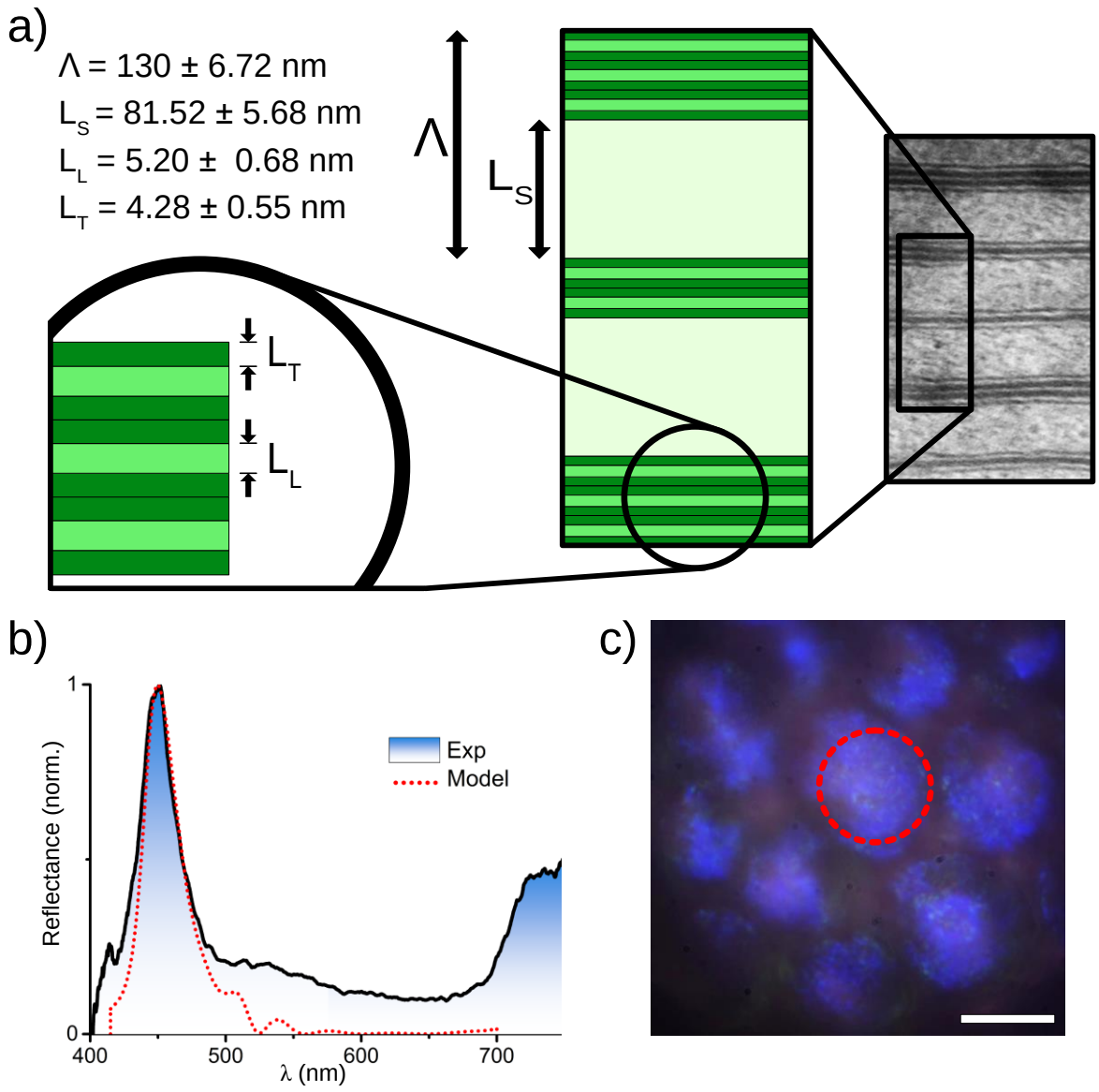


Figure 3: a) *Sketches showing the parameters used in the optical model when defining the photonic structure. Λ, full repeating period; L_S, stroma spacing between stacked thylakoids; L_L, lumen thickness; L_T, thylakoid membrane thickness.* ***b)*** *Calculated reflectance from optical model and typical reflectance as measured from area highlighted in c).* ***c)*** *Reflection image from epi-illumination microscope (scale bar = 10µm).*

Author Contributions

N.M., M.L.-G., R.O., and H.M.W. conceived experiments. N.M. and M.L.-G. carried out optical analysis of bizonoplasts. N.M. conducted electron microscopy studies. M.L.-G and R.O. designed and ran optical models, and N.M., M.L.-G., R.O., and H.M.W. wrote the manuscript which all authors commented on.

Acknowledgements

We would like to thank Alanna Kelly for the maintenance of the plants and her horticultural expertise, and O.-Phart Phrathep for assistance in sample preparation. We would also like to thank and acknowledge the Wolfson Bioimaging Facility (Univeristy of Bristol), particularly J. Mantell for assistance with imaging. Thank you to Lifeng Chen for assistance in the maintenance of and with queries relating to the reflection spectroscopy setup. Thank you to the anonymous referees for their insightful comments on an earlier version of this manuscript.

Data Accessibility

The custom code used for implementation of the transfer matrix method was developed as a MATLAB script and is available upon request from M.L.-G. (martin.lopez@inl.int). Data can be accessed at https://data.bris.ac.uk/data/.

Funding

N.M is funded by EPSRC PhD studentship 1643709. M.L.G acknowledges the project BIOMPHO2 (POCI-01-0145-FEDER-031739) co-funded by FCT and COMPETE2020. R.O acknowledges EPSRC grant EP/N003381/1. H.M.W acknowledges funding from ERC project number 260920.

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